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Inhaled Silica Dust Increases Nitric Oxide and Cytokine Production Associated with Collagen Synthesis and Fibrosis in Rats

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The association of silica with pulmonary inflammation and fibrosis is well documented. However, the mediators involved in the pathogenesis of silica-induced pulmonary fibrosis are not completely defined. In parallel studies, which will be summarized in this presentation, intratracheal instillation of silica resulted in nitric oxide production and pulmonary inflammation within 24 hours. The objective of the current study was to determine the involvement of nitric oxide and cytokines in the pathogenesis of inflammation and fibrosis associated with an occupationally-relevant inhaled exposure to silica. Male Fischer rats were exposed by inhalation to 2 mg/m^3 of freshly-generated silica for 2 weeks, 2 months, and 6 months at a rate of 5 days/week, 8 hour per day. Cellular differentials, chemiluminescence, and mRNA levels of inducible nitric oxide synthase (iNOS), transforming growth factor β (TGFβ), and platelet-derived growth factor (PDGF) were all measured in cells obtained by bronchoalveolar lavage. Tissue histopathology and hydroxyproline content were examined for evidence of pulmonary fibrosis and collagen production, respectively. Cellular differentials showed that red blood cells (RBC) and leukocytes were both increased after 2 and 6 months of exposure, indicating pulmonary damage and inflammation. In addition, both total and NO-dependent chemiluminescence were increased after 2 and 6 months of exposure, suggesting that oxygen radicals and nitric oxide were being produced by the bronchoalveolar lavage cells. Northern analysis revealed that mRNA levels for iNOS was increased after 2 months, but returned to normal levels after 6 months. Pathological examination revealed multiple granulomatous lesions after 6 months characterized by focal, subpleural epithelial proliferation, and early stages of fibrosis. At 6 months, there was a small but significant increase in the hydroxyproline content suggesting collagen synthesis. TGFβ mRNA level were elevated after 6 months, but PDGF mRNA was not different than controls. The results suggest that exposure to 2 mg/m^3 of silica results in pulmonary damage and inflammation after 2 months of exposure. After 6 months, there is evidence of early pulmonary fibrosis characterized by granulomatous lesions, increased hydroxyproline content, and increased TGFβ synthesis. DEY, R.D.; STANLEY, C.; BLACKFORD, J.A., JR.; HARNESS, J.; DURHAM, J.; CASTRANOVA, V.; HUBBS, A.: INHALED SILICA DUST INCREASES NITRIC OXIDE AND CYTOKINE PRODUCTION ASSOCIATED WITH COLLAGEN SYNTHESIS AND FIBROSIS IN RATS. APPL. OCCUP. ENVIRON. HYG. 11(7):914–918; 1996.

The pulmonary response to inhaled silica, silicosis, is characterized by early alveolar inflammation[1] followed by interstitial fibrosis.[2] Macrophages play an important role in the pathogenesis of silicosis.[3] During inflammation, macrophages produce oxidants and release lysosomal enzymes that contribute to alveolar wall damage.[4] Macrophages also mediate inflammatory cell migration and activation in the alveoli through the release various cytokines, including interleukin 1 (IL-1)[5] and tumor necrosis factor α (TNFα),[6] as well as arachidonic acid metabolites.[7,8] Analysis of bronchoalveolar lavage cell profiles show that silica causes the recruitment of neutrophils into the alveolar spaces, possibly playing a role in inflammation.[6,9]

Pulmonary fibrosis often occurs after prolonged or severe inflammation and continued alveolar damage.[10] Alveolar macrophages may also play a role in the pathogenesis of pulmonary fibrosis by releasing cytokines such as platelet-derived growth factor (PDGF),[11] which is a competence factor for fibroblasts,[12] and transforming growth factor β (TGFβ),[13] which simulates fibroblast proliferation and collagen synthesis.

Recent studies show that macrophages produce the free radical gas nitric oxide (NO·).[14] NO· produced by macrophages is bacteriocidal, disrupting oxidative metabolism.[15] However, macrophages also produce NO· in response to ozone inhalation[16] suggesting that NO· may be involved in other pulmonary responses.

We have demonstrated that the intratracheal instillation of silica increases NO· production in macrophages and neutrophils, and also increase the cellular mRNA level of inducible nitric oxide synthase (iNOS), the macrophage enzyme responsible for NO· synthesis.[17] The purpose of this study was to examine the production of NO· and TGFβ in lungs from rats exposed by inhalation to occupationally relevant levels of silica. It was hypothesized that NO· production would be increased during the early inflammatory stage of the silica response and that TGFβ production would increase later and in association with fibrosis.

Methods

Specific pathogen-free male Fischer rats (175–225 gm) were obtained from Hilltop Labs (Scottdale, Pennsylvania). All pro-

cedures were approved by the WVU-ACUC under protocol #9210-01.

The goal of this study was to determine pulmonary responses to the inhalation of occupationally relevant levels of silica using rats as a test animal. After 2 weeks, 2 months, and 6 months of exposure, lungs from exposed and control rats were used to evaluate the following: pathological changes in histological sections, bronchoalveolar lavage cell (BALC) profiles, iNOS and TGFβ mRNA levels, NO·-dependent chemiluminescence, and collagen content. Details for all of these procedures are described in a previous publication[17] and will be briefly described here.

For the inhalation exposure, rats were divided into two groups of 144 each and placed in Hazelton 2000 exposure chambers. Food and water were available *ad libitum*. Rats in one chamber were exposed for 8 hours/day, 5 days/week to filtered air containing 2 mg/m^3 silica. Silica for the exposures (IOTA Standard quartz, 95% between 75 and 300 μm, Unimin Corporation) was fractured in a Jet-O-Mizer mill (Fluid Energy Processing and Equipment Co.) to an average size of 1 μm. The aerosolized fresh dust was passed through a TSI Model 3054 aerosol neutralizer before being injected into the Hazelton exposure chamber. Dust concentration in the chamber was monitored by a RAM-1 real time dust monitor. The other chamber received filtered air only. Rats were removed from the chambers after 2 weeks, 2 months, and 6 months.

For histological analysis, rats were perfused through the pulmonary artery with 4 percent paraformaldehyde. This approach preserves *in situ* location of inflammatory cells in the alveolar spaces. Perfusion fixation was performed using 20 cm H_2O for 3 hours. Then the lungs were rinsed, embedded in paraffin, sectioned at 6 μm, and stained with Masson's trichrome.

Bronchoalveolar lavage was performed through a tracheal cannula with 10 pulmonary lavages of 8 ml each using ice cold Ca^{2+}, Mg^{2+} free phosphate buffered medium. The bronchoalveolar lavage cells (BALC) were centrifuged and resuspended twice in HEPES-buffered medium. Cell counts and differentials were determined using an electronic cell counter equipped with a cell sizing attachment (Coulter Model ZBI with a Channelizer 256, Coulter Electronics, Inc., Hialeah, Florida).[18]

For RNA isolation, lavaged cells from each rat were washed and centrifuged (500 g × 10 min) with buffered medium. Total cellular RNA was extracted by a modification of the acidic guanidinium thiocyanate-phenol-chloroform method.[19] All solutions used for RNA analysis were prepared with 1 percent diethyl pyrocarbonate (DEPC; Sigma) treated, double distilled water that was autoclaved for 20 minutes to inactivate the DEPC. The RNA was quantified in a spectrophotometer at 260 nm.

cDNA probes for iNOS mRNA were generated from a plasmid CL-BS containing a 4100 bp cDNA fragment for mouse iNOS kindly provided by Dr. S.H. Snyder (Johns Hopkins University, Maryland).[20] DNA templates were amplified by conventional polymerase chain reaction (PCR) using a GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer/Cetus, Norwalk, Connecticut) using 20-mer synthetic oligonucleotide primers. The iNOS sense primer sequence was 5′-TGACCATCGTTGACCACCAC (pos 2655) and the anti-sense primer sequence was 5′-CTCCCCTCCCAGT TCCTCCA (pos 3474) to create a ssDNA probe of 819 bp. The plasmid pBR322 containing a 1700 bp cDNA fragment for mouse TGF-b1 was kindly provided by Genentech (San Francisco, California). The TGF-b sense primer sequence was 5′-GCGACTCCTGCTGCTTTCTC (pos 191) and the anti-sense primer sequence was 5′-TCCGTCTCCTTGGTTC AGCC (pos 972) to create a probe of 801 bp. Very high efficiency single stranded DNA (ssDNA) hybridization probes were generated by PCR[21] on these templates using anti-sense primer and ^{32}P-α-labeled dCTP (ICN Biochemicals, Costa Mesa, California). Probes were purified on a G-25 quick spin column (Boehringer, Indianapolis, Indiana).

Northern analysis was performed by electrophoresis of 20 μg of total RNA on a denaturing gel and subsequently vacuum transferred onto a Nytran membrane. The membrane was washed, dried, and cross-linked using ultraviolet light and stained with 0.02 percent methylene blue to visualize ribosomal RNA. Then, the membrane was washed to remove the methylene blue, prehybridized, and finally hybridized with the iNOS or TGFβ probes. The membrane was washed at low stringency and opposed to Kodak XAR film with one intensifying screen and exposed at −80°C for various periods of time to ensure linearity.

Quantitation of the blots were performed by a PC-based Optimas imaging system (Bioscan, Edmonds, Washington). Optical density of rRNA bands stained by methylene blue and of the autoradiographic signals for iNOS and TGFβ was determined as the inverse log of the integrated gray value (ILIGV). The ILIGV of the autoradiographic signal was divided by the rRNA ILIGV to assess the total amount of RNA transferred onto the membrane. The optimal amount of RNA is 10 to 20 μg of total RNA. The use of rRNA bands to normalize Northern blots has been described previously using oligoprobes.[22]

Chemiluminescence generated by AM from individual rats was measured in BALC resuspended in buffer to a final concentration of 1 × 10^6 AM/ml. From each cell suspension, 0.75 ml was used to determine chemiluminescence under four separate conditions: (1) resting cell, with no treatment, to serve a baseline control; (2) resting cells treated with 1 mM Nω-nitro-L-arginine methyl ester (L-NAME) to determine baseline L-NAME-inhibitable chemiluminescence; (3) cells treated with 2 mg/ml unopsonized zymosan to stimulate chemiluminescence only in AM but not in neutrophils; and (4) cells treated with 2 mg/ml unopsonized zymosan and 1 mM L-NAME to determine the percentage of iNOS-dependent (L-NAME-inhibitable) chemiluminescence in AM following stimulation with unopsonized zymosan. Luminol (8 μg%) was used to emit light measured using a Berthold LB953 luminometer expressed as the integral of cpm/time. Zymosan-induced total chemiluminescence was determined as the response to zymosan minus the resting level of chemiluminescence. iNOS-dependent chemiluminescence was determined by subtracting zymosan-dependent total chemiluminescence in the presence of the iNOS inhibitor, L-NAME, from the samples without inhibitor.

Collagen content was determined by extracting and measuring hydroxyproline.[23] Lung tissue was hydrolyzed in 6N HCL for 16 hours at 116°C to release hydroxyproline. A

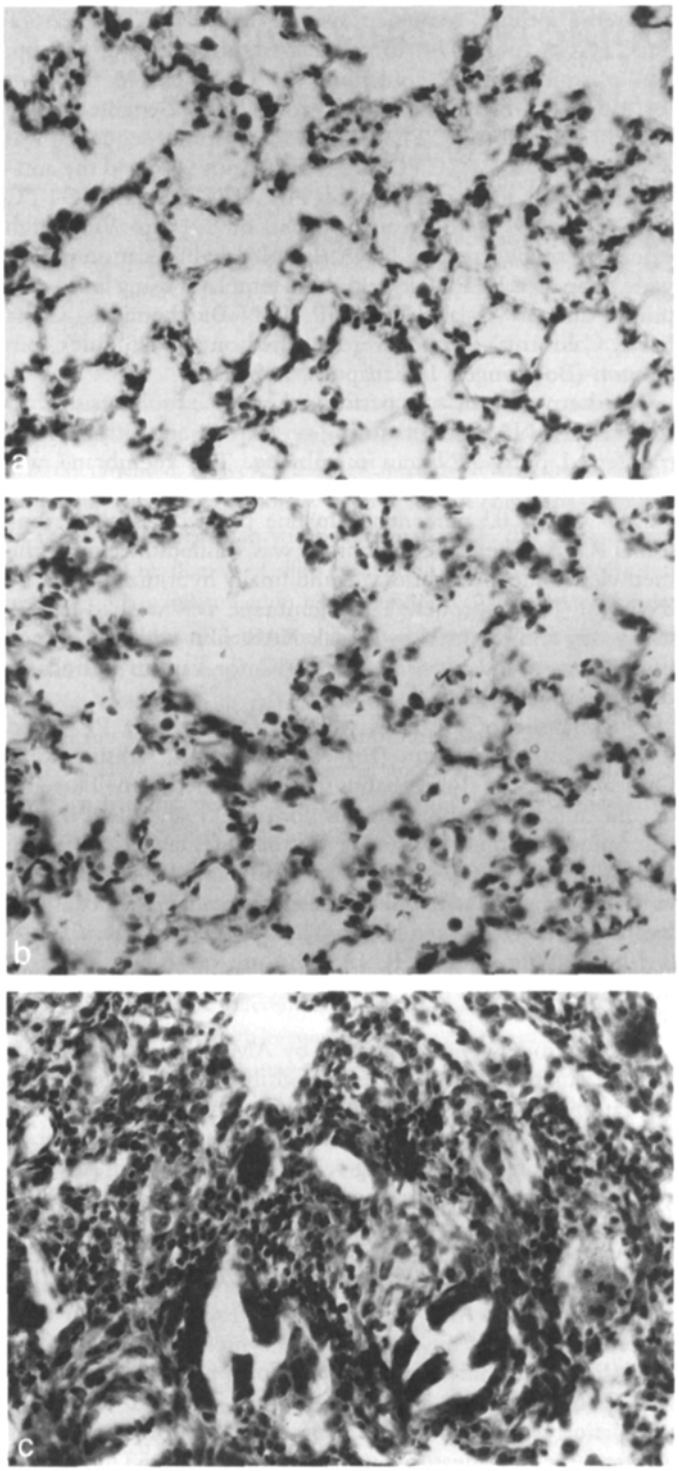

FIGURE 1. Light micrographs of lung sections from (a) 2-week control rat, (b) from rats exposed to 2 mg/m^3 silica for 2 weeks, and (c) 6 (months). Lungs of rats exposed for 6 months had focal lesions characterized by epithelial cell proliferations and collagen deposits (Masson's trichrome; original magnification ×300).

colorimetric reaction was then produced by oxidation with chloramine-T. The reaction was stopped by the addition of sodium thiosulfate to form a complex detected and quantified as a chromaphore having an absorbance at 560 nm. Standard

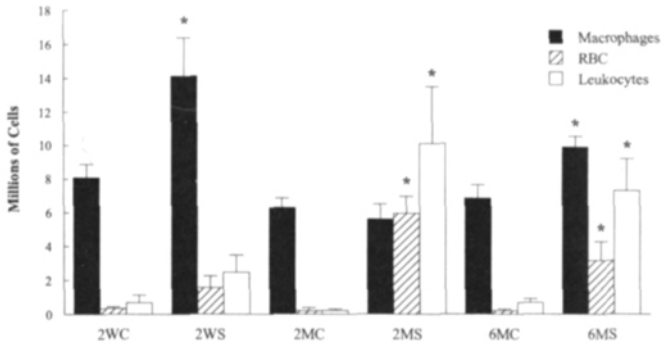

FIGURE 2. Differential cell counts of bronchoalveolar lavage cells from control (C) and silica-treated (S) rats exposed for 2 weeks (2W), 2 months (2M), or 6 months (6M). Means ± SE; N = 4–7 rat per group; • = significant at $p < 0.05$ compared to control at the same time point.

curves were constructed for each assay from standards containing known amounts of hydroxyproline. Mean values of the ILIGV of autoradiograph signals, iNOS-dependent chemiluminescence, collagen content, and cellular differentials were compared by one-way analysis of variance using the Macintosh-based software Statview II (Abacus Concepts, Berkely, California). Significance was set at $p < 0.05$. When significant F values were obtained, individual means were compared using the least significant difference test.

Results

In control rats at all time points, alveolar walls were intact and alveolar spaces were free of exudate and cells except for occasional macrophages (Figure 1a). Some early interstitial inflammation believed to be associated with viral infection was noted in the 2-week controls. This condition was resolved by 2 months. Connective tissue was observed only around airways and blood vessels. Examination of sections from rats exposed to silica for 2 weeks showed limited infiltration of neutrophils into the alveolar spaces (Figure 1b) and some evidence of perivascular cellular infiltrations. The cellular infiltration con-

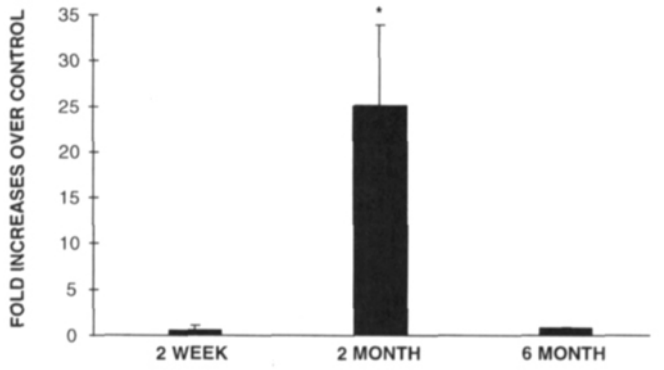

FIGURE 3. Northern blot analysis of iNOS mRNA in bronchoalveolar lavage cells from control (C) and silica-treated (S) rats exposed for 2 weeks, 2 months, or 6 months. N = 2–4 rats per group; means ± SE; * = significant at $p < 0.05$ compared to control at the same time point.

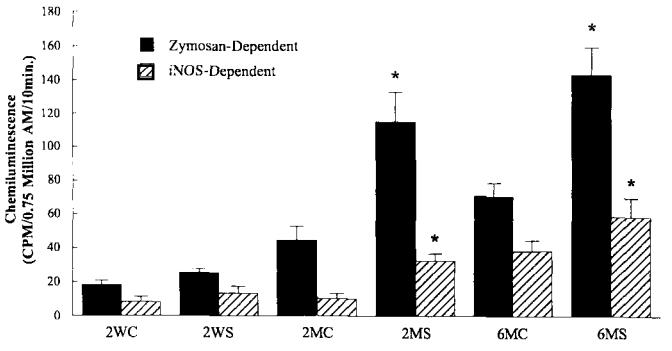

FIGURE 4. Chemiluminescence from bronchoalveolar lavage cells of control (C) and silica-treated (S) rats exposed for 2 weeks (2W), 2 months (2M), or 6 months (6M). N = 3–4 rats per group; means ± SE; * = significant at $p < 0.05$ compared to control at the same time point.

tinued 2 months. After 6 months of exposure to silica, multiple granulomatous lesions were observed and were associated with focal, subpleural epithelial proliferations and the appearance of collagen (Figure 1c).

The BALC profiles (Figure 2) showed a significant increase in macrophages after 2 weeks of exposure, but no changes in neutrophils or RBCs. However, after 2 months of exposure, neutrophils and RBCs were significantly increased and macrophages had returned to normal. After 6 months, macrophage numbers had increased again and both RBC and neutrophil counts remained elevated.

Northern analysis showed that iNOS mRNA was not statistically different after 2 weeks, increased after 2 months, and then returned to control values after 6 months of exposure (Figure 3). Zymosan-stimulated chemiluminescence was increased after both 2 months and 6 months of exposure (Figure 4). The iNOS-dependent chemiluminescence was also increased at both 2 and 6 months.

TGFβ mRNA was significantly increased only after 6 months of exposure to silica (Figure 5). There was also a small

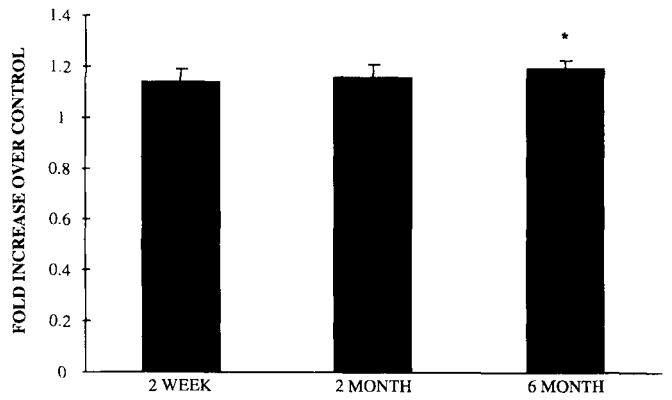

FIGURE 5. Northern blot analysis of TGFβ mRNA in bronchoalveolar lavage cells from control (C) and silica-treated (S) rats exposed for 2 weeks, 2 months, or 6 months. N = 3–4 rats per group; means ± SE; * = significant at $p < 0.05$ compared to control at the same time point.

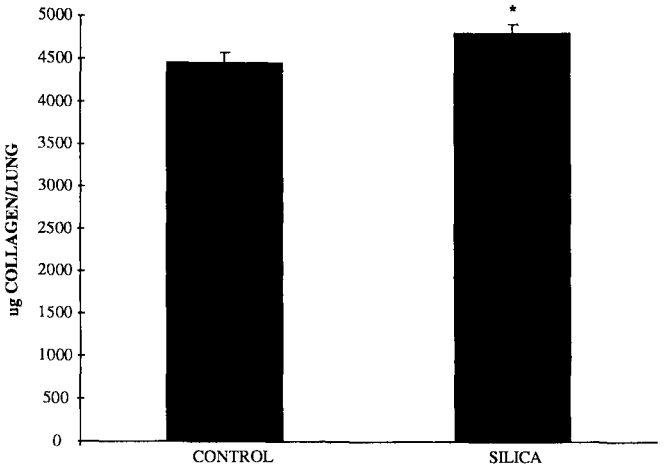

FIGURE 6. Collagen content of lung tissue from control (C) and silica-treated (S) rats exposed for 6 months. N = 6 rats per group; means ± SE; * = significant at $p < 0.05$ compared to control.

but statistically significant increase in collagen content after 6 months of silica exposure (Figure 6).

Discussion

The findings demonstrate that significant pulmonary inflammation and fibrosis occur in rats exposed to occupationally relevant levels of silica. The alveolar inflammation was characterized both histologically and in cell differentials of BALC by neutrophil and red blood cell infiltration prominent after 2 months of exposure. NO· production measured as L-NAME-inhibitable chemiluminescence was increased after 2 months of exposure and iNOS mRNA levels were also increased. This suggests that silica exposure induces expression of the iNOS gene. The findings are consistent with our previous study showing that intratracheal instillation of silica increases NO· production and iNOS mRNA expression.[17] Other pulmonary irritants such as ozone also cause increased iNOS synthesis and NO· production.[16] The association of NO· production very early in the pulmonary response suggests that it may be important in mediating the inflammatory response. NO· is an oxidant that can directly damage the alveoli or combine with superoxide anion to produce an even more cytotoxic molecule, peroxynitrite.[24,25]

After 6 months of exposure, there was evidence of focal type II cell proliferation and fibrosis similar to the lesions reported after intratracheal silica instillation.[13] This response is associated with increased collagen content in the lung and an increase in the expression of TGFβ mRNA. TGFβ mRNA is a cytokine known to simulate fibroblast proliferation and collagen synthesis.[26,27]

Recommendation

The findings from this study suggest that the inhalation of silica at concentrations that could occur in the mining and sandblasting industry may present a health concern.[28–30] It is recommended that employees who work in environments containing airborne silica should wear appropriate protection to avoid inhalation of dust.

Conclusion

The data in this study demonstrate that inhalation of 2 mg/m^3 silica produces an early inflammatory response associated with the production of the free radical gas NO· after 2 months of exposure. Continued exposure causes the appearance of focal fibrotic lesions associated with increased synthesis of TGFβ mRNA suggesting increased production of TGFβ, a cytokine know to stimulate collagen production.

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