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Enhanced nitric oxide and reactive oxygen species production and damage after inhalation of silica

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Porter, Dale W., Lyndell Millecchia, Victor A. Robinson, Ann Hubbs, Patsy Willard, Donna Pack, Dawn Ramsey, Jeff McLaurin, Amir Khan, Douglas Landsittel, Alexander Teass, and Vincent Castranova. Enhanced nitric oxide and reactive oxygen species production and damage after inhalation of silica. *Am J Physiol Lung Cell Mol Physiol* 283: L485–L493, 2002. First published March 15, 2002; 10.1152/ajplung.00427.2001.—In previous reports from this study, measurements of pulmonary inflammation, bronchoalveolar lavage cell cytokine production and nuclear factor-κB activation, cytotoxic damage, and fibrosis were detailed. In this study, we investigated the temporal relationship between silica inhalation, nitric oxide (NO), and reactive oxygen species (ROS) production, and damage mediated by these radicals in the rat. Rats were exposed to a silica aerosol (15 mg/m^3 silica, 6 h/day, 5 days/wk) for 116 days. We report time-dependent changes in *1*) activation of alveolar macrophages and concomitant production of NO and ROS, *2*) immunohistochemical localization of inducible NO synthase and the NO-induced damage product nitrotyrosine, *3*) bronchoalveolar lavage fluid NO_x and superoxide dismutase concentrations, and *4*) lung lipid peroxidation levels. The major observations made in this study are as follows: *1*) NO and ROS production and resultant damage increased during silica exposure, and *2*) the sites of inducible NO synthase activation and NO-mediated damage are associated anatomically with pathological lesions in the lungs.

silicosis; fibrosis; oxidant injury; nitrotyrosine

SILICA INHALATION IN HUMANS has been linked to the pulmonary disease silicosis, which is generally characterized by a severe decline in respiratory function and premature death (3). Because silicotic lungs are known to be in a state of oxidative stress (35), it has been proposed that oxidant-mediated lung damage may participate in the development of silica-induced pulmonary disease (21).

Alveolar macrophages (AM) become activated after exposure to silica, and this activation results in the production of reactive oxygen species (ROS), which can be measured by chemiluminescence (8). The production of ROS by AM contributes to the cytotoxic damage to the lung, indicated by increased lactate dehydrogenase activity and total protein in the bronchoalveolar lavage (BAL) fluid of silica-exposed rats (15).

Other studies have suggested that nitric oxide (NO) may also participate in silica-induced pulmonary inflammation, damage, and fibrosis. mRNA levels for inducible NO synthase (iNOS), an inducible enzyme that produces NO, are increased in BAL cells after intratracheal instillation of silica in rats (4, 17). Furthermore, pharmacological evidence suggests that NO production by AM is increased after intratracheal instillation (4) or inhalation (8) of silica.

A recent workshop on poorly soluble particles concluded that a significant gap in our knowledge exists concerning the temporal sequence of molecular, cellular, and histopathological changes that occur after exposure to poorly soluble particles, such as silica (18). Indeed, although providing substantial mechanistic information concerning silica-induced production of NO and ROS, previous studies did not establish the detailed temporal relationship between silica inhalation, NO and ROS production, and damage mediated by these radicals, and the development of pulmonary fibrosis. Thus we initiated a study with the comprehensive goal to investigate the temporal relationship between silica inhalation, NO and ROS production, and the resultant pulmonary damage in the rat model. Specifically, we report the time course of *1*) AM activation and production of NO and ROS, *2*) BAL fluid NO_x and superoxide dismutase (SOD) levels, and *3*) immunohistochemical localization of iNOS and nitrotyrosine (NT) in silica-exposed rat lungs.

METHODS

Silica chemical analyses. The silica used in this study was MIN-U-SIL 5 (US Silica, Berkeley Springs, WV). The bulk silica was analyzed for inorganic contaminants and desorbable organic carbon compounds, and aerosolized silica sam-

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ples were analyzed for trace inorganic elements and elemental and organic carbon. The results of these analyses have been reported elsewhere (27).

Silica aerosol exposure of rats. Pathogen-free male Fischer 344 rats (strain CDF, 75–100 g; Charles River, Raleigh, NC) were housed in an American Association for Accreditation of Laboratory Animal Care-accredited animal facility in individual cages in two 5-m^3 Hinners-type inhalation chambers. One chamber was used for filtered air exposures (control) and the other for exposure to 15 mg/m^3 silica. Exposures were conducted for 6 h/day, 5 days/wk for a total of 116 exposure days. Water was available ad libitum, and food was available at all times except during exposures. The rats were subjected to a 12:12-h light-dark schedule and were exposed during the dark cycle to coincide with their most active period.

The silica aerosol concentration was monitored using two independent methods: an RAS-2 particle sensor allowed real-time monitoring, and gravimetric determinations were made at hourly intervals during each day of exposure. The gravimetric determinations indicated that the silica aerosol concentration was 14.9–15.5 mg silica/m^3 during the study (27). Silica particle size averaged ≤2 μm as determined with an Anderson eight-stage cascade impactor, and the mass median aerodynamic diameter of the silica particles was 1.47–1.86 μm (27). Further details of the silica chemical composition analyses and the silica aerosol particle characterization, generation, and exposure system have been described elsewhere (27).

The silica lung burden of rats ranged from 0.42 ± 0.05 mg SiO_2/lung after 1 day of exposure to 6.27 ± 0.15 mg SiO_2/lung after 116 days of exposure (27). On the basis of the volumetric model described by Oberdorster and colleagues (26), the percentage of AM volume occupied by silica in this study was below overload levels (27). Furthermore, the silica burden-exposure duration relationship exhibited equilibrium between 79 and 116 days of exposure, suggesting that the silica-exposed animals were not in pulmonary overload (27).

BAL. Rats were euthanized with an injection of pentobarbital sodium (≥100 mg/kg body wt ip) after 5, 10, 16, 20, 30, 41, 79, and 116 days of exposure. BAL with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (pH 7.4) with 5.5 mM D-glucose added and the isolation of acellular first BAL fluid and BAL cells were conducted as described previously (27). BAL cells were resuspended in HEPES-buffered medium (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5.5 mM D-glucose, pH 7.4) and counted using an electronic cell counter equipped with a cell sizer (Multisizer II, Coulter Electronics, Hialeah, FL) as described previously (6).

BAL fluid SOD activity. BAL fluid SOD activities were determined by monitoring the reduction of cytochrome *c* at 550 nm as previously described (34, 35) with a Cobas Fara II Analyzer (Roche Diagnostic Systems, Montclair, NJ).

BAL fluid NO_x concentrations. We define NO_x as the total of nitrite (NO_2^-) plus nitrate (NO_3^-) in a sample. To determine BAL NO_x, it was necessary to reduce NO_3^- to NO_2^- in the BAL sample. To reduce NO_3^-, a previously described NO_3^- reduction assay (14) was modified. The NO_3^- reduction reaction consisted of 50 mM HEPES, 5 mM flavin adenine dinucleotide, 0.1 mM reduced nicotinamide adenine dinucleotide phosphate, 0.2 unit of nitrate reductase (Roche Molecular Biochemicals, Indianapolis, IN), and BAL fluid in a total volume of 1.0 ml. This reaction was incubated with gentle mixing at 37°C for 30 min. At the end of the incubation, the reaction was diluted with water, and NO_2^- was determined by flow injection analysis colorimetry at 540 nm using the Griess reaction (Quick-Chem 8000, Lachat Instruments, Milwaukee, WI).

AM zymosan-stimulated and NO-dependent chemiluminescence. AM chemiluminescence was determined in a total volume of 0.25 ml of HEPES-buffered medium. Resting AM chemiluminescence was determined by incubating 1.0×10^6 AM/ml at 37°C for 20 min, adding 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) to a final concentration of 0.08 μg/ml, and then measuring chemiluminescence. To determine zymosan-stimulated chemiluminescence, unopsonized zymosan (2 mg/ml) was added immediately before the measurement of chemiluminescence. All chemiluminescence measurements were made with an automated luminometer (Berthold Autolumat LB 953, EG & G, Gaithersburg, MD) at 390–620 nm for 15 min. The integral of counts per minute vs. time was calculated. Zymosan-stimulated chemiluminescence was calculated as counts per minute in the zymosan-stimulated assay minus counts per minute in the resting assay. NO-dependent chemiluminescence was determined by subtracting zymosan-stimulated chemiluminescence from cells preincubated with 1 mM nitro-L-arginine methyl ester from zymosan-stimulated chemiluminescence from cells without nitro-L-arginine methyl ester. The use of unopsonized zymosan in the chemiluminescence assay allowed only AM chemiluminescence to be measured, because unopsonized zymosan stimulates AM chemiluminescence (7) but not polymorphonuclear (PMN) chemiluminescence (1, 16).

Lung lipid peroxidation. Rats, separate from those used for BAL, were euthanized with an injection of pentobarbital sodium (≥100 mg/kg body wt ip) after 10, 20, 41, 79, and 116 days of exposure. Lungs were removed en bloc, washed with ice-cold 0.9% (wt/vol) NaCl, blotted dry, and weighed. Lung tissue was processed, and lipid peroxidation was measured using a colorimetric assay at 586 nm (BIOXYTECH LPO-586, Oxis International, Portland, OR) following the protocol provided by the manufacturer.

Immunohistochemistry. Silica- and air-exposed control rats, separate from those used for BAL, were euthanized with an injection of pentobarbital sodium (≥100 mg/kg body wt ip) after 10, 20, 41, 79, and 116 days of exposure. The left lobe was inflated transpleurally with 2–3 ml of formalin, processed within 24 h, and embedded in paraffin. Paraffin sections were cut at 5 μm, deparaffinized in xylene, and rehydrated. Slides were placed in citrate buffer (pH 6.0) and microwaved (32). After endogenous peroxidase was blocked in a 1:1 mixture of 3% H_2O_2 and methanol, slides were placed in 10% bovine serum albumin for 30 min at room temperature and then incubated overnight at 4°C in the primary antibody [monoclonal anti-iNOS, 1:50 dilution (N32020, Transduction Laboratories, Lexington, KY); polyclonal anti-NT, 1:100 dilution (06-284, Upstate Biotechnology, Lake Placid, NY)]. A kit (K0609, LSAB-2 kit, Dako, Carpenteria, CA) for rat specimens was used to label the antibody, with diaminobenzidine (Zymed Laboratories, South San Francisco, CA) as the chromogen. Sections were counterstained briefly with Mayer's hematoxylin and dehydrated, and coverslips were applied.

Positive controls for iNOS were lung sections from rats that had been exposed to lipopolysaccharide (LPS, 10 mg/kg body wt) by intratracheal instillation 24 h before death; the AM in these animals were highly positive for iNOS using the above procedures. In control slides with 10% bovine serum albumin and no antibody, the mast cells were positive (in air- and silica-exposed animals), but there was no other staining. NT controls included an absorption control, in which the antibody was mixed with 10 mM NT before it was added to the sections, and a positive control, in which the sections were incubated in 1 mM sodium nitrite before application of

the antibody. In the absorption control, no staining for NT appeared in the lung sections.

Statistical analyses. The difference between air- and silica-exposed rats at each time point was tested using appropriate contrasts in the two-way analysis of variance model with interaction (30). A logarithmic transformation of the response variable was used to satisfy assumptions of normality and constant variance. To characterize toxicity changes over time, data were fit to a linear regression model that included any statistically significant polynomial terms up to a cubic. Predicted values and associated confidence intervals from the regression model (25) were used to identify significant differences between exposure times (i.e., time points with nonoverlapping confidence intervals) within the silica-exposed animals. This approach was preferable to pairwise tests of mean values, inasmuch as the regression model fits a single curve to the dose-response relationship over the entire range of data. Statistical significance was set at $P \leq 0.05$.

RESULTS

NO-dependent AM chemiluminescence. Except at 5 days of exposure, NO-dependent AM chemiluminescence was significantly higher for AM isolated from silica-exposed animals than from air-exposed controls (Fig. 1). Furthermore, NO-dependent AM chemiluminescence for AM isolated from silica-exposed rats from 10 to 116 days of exposure was significantly higher than that determined for AM isolated after 5 days of exposure (Fig. 1).

BAL fluid NO_x concentrations. Silica-exposed rats had significantly higher BAL fluid NO_x concentrations than air-exposed controls at every exposure time measured from 16 to 116 days of exposure (Fig. 2). For silica-exposed rats, BAL fluid NO_x concentrations at 79 days of exposure were significantly higher than those determined from 5 to 41 of days exposure. A further significant increase occurred at 116 days of exposure (Fig. 2).

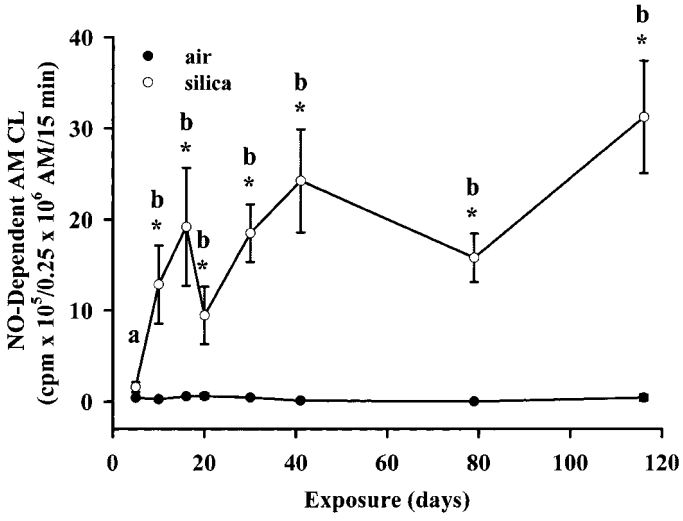

Fig. 1. Nitric oxide (NO)-dependent alveolar macrophage (AM) chemiluminescence (CL). Values are means ± SE (n = 5). *Significant difference ($P \leq 0.05$) between air-exposed controls and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters (a and b) are significantly different from each other ($P \leq 0.05$).

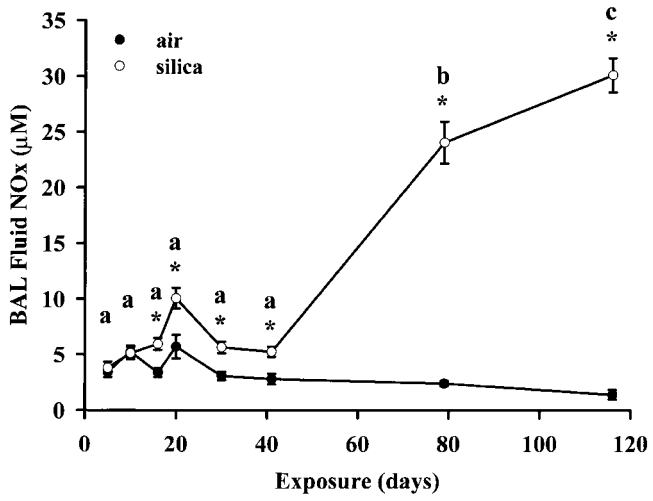

Fig. 2. Bronchoalveolar lavage (BAL) fluid NO_2^- + NO_3^- (NO_x) concentration. Values are means ± SE (n = 14–15). *Significant difference ($P \leq 0.05$) between air-exposed controls and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters (a–c) are significantly different from each other ($P \leq 0.05$).

Immunohistochemistry. Pathological changes, specifically histiocytic and suppurative alveolitis, alveolar epithelial cell hypertrophy and hyperplasia, and alveolar lipoproteinosis, were observed in all silica-exposed rat lungs after 79 and 116 days of exposure. Granulomatous inflammation was also seen in the bronchus-associated lymphoid tissue (BALT) at these time points. Cellular debris or possibly apoptotic bodies were observed in some of the alveoli, especially in regions of alveolitis and lipoproteinosis, after 41, 79, or 116 days of silica exposure. Similar pathological changes were previously described and quantified in air- and silica-exposed rat lungs from different animals in a previous report from this silica inhalation study (27).

The pattern of iNOS staining corresponded to the areas of inflammation within lung parenchyma (Fig. 3, *A* and *B*). There was very little staining with the iNOS antibody in animals exposed to silica or air for 10, 20, and 41 days. After 79 days of exposure, very little staining was present in the air-exposed controls, but in the silica-exposed animals, many regions of the lung stained positively, especially subpleural areas (Fig. 3*A*). The staining for iNOS was localized primarily in the periphery of the lung after 79 days, while after 116 days of exposure many of the central areas were positive as well.

AM and alveolar epithelial cells that appeared to be type II cells were positive for iNOS after 79 days of exposure to silica, while these cells were negative in air controls (Fig. 3, *C* and *D*). Neutrophils were unstained or only weakly positive. Intense iNOS staining was localized in silicotic granulomas in the parenchyma after 79 days of silica exposure (Fig. 3*E*). The areas of granulomatous inflammation in the BALT were positive for iNOS in three of five animals exposed to silica for 79 days (Fig. 3*F*) and in all the animals after 116

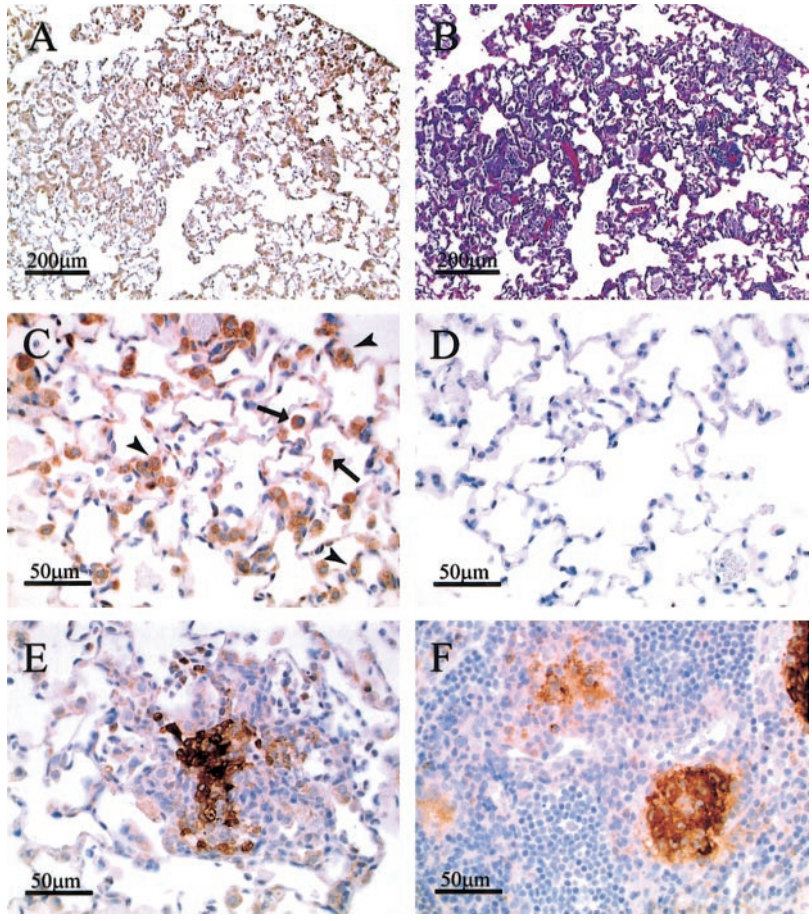

Fig. 3. Immunohistochemical localization of inducible NO synthase (iNOS) in rats exposed to air or silica. *A*: low-power view of alveoli in the periphery of a lung showing the pattern of iNOS staining in a rat lung exposed to silica for 79 days. *B*: low-power view of a section serial to *A*, stained with hematoxylin and eosin, showing the pattern of inflammation. *C*: alveolar region in an animal exposed to silica for 79 days, with positive macrophages (arrows) and epithelial cells (arrowheads). *D*: alveolar region in a control animal, with no significant staining for iNOS. *E*: intense staining for iNOS in a silicotic granuloma (79-day silica exposure). *F*: intense staining for iNOS in granulomatous regions within bronchial-associated lymphoid tissue (79-day silica exposure).

days. Lipoprotein, present in peripheral areas after 79 days of exposure and in almost all alveoli after 116 days of exposure, was not stained.

The pattern of NT staining was very similar to that of iNOS and corresponded to the areas of inflammation within the lung (Fig. 4, *A* and *B*). There were no major differences in NT staining between silica- and air-exposed animals after 10, 20, and 41 days of exposure. After 79 and 116 days of exposure, however, there was very pronounced staining in the silica-exposed animals (Fig. 4*A*), with positive areas primarily in the periphery of the lung after 79 days of silica exposure and throughout the lung after 116 days of exposure. AM and alveolar epithelial cells that appeared to be type II cells were positive for NT after 79 days of silica exposure but were negative or weakly positive in the controls (Fig. 4, *C* and *D*). Some of the AM in silica-exposed and control animals were positive at all time points. The alveolar lipoprotein was positive for NT in one of five animals after 79 days of silica exposure and in four of five animals after 116 days (Fig. 4*C*). Histiocytic aggregates in lipoprotein-rich regions were positive for NT (Fig. 4*E*). The areas of granulomatous inflammation in the BALT were positive for NT in one animal after 79 days and in four of five animals after 116 days of silica exposure (Fig. 4*F*).

Zymosan-stimulated AM chemiluminescence. Zymosan-stimulated AM chemiluminescence was significantly higher for AM isolated from silica-exposed rats than from air-exposed controls at every exposure time examined (Fig. 5). For silica-exposed rats, AM chemiluminescence from 10 to 79 days of exposure was significantly higher than that determined for AM isolated after 5 days of exposure, and a further significant increase occurred at 116 days of exposure (Fig. 5).

BAL fluid SOD activity. SOD activity was significantly higher in BAL fluid from silica-exposed rats than from air-exposed controls at every exposure time except 5 days (Fig. 6). BAL fluid SOD activity in silica-exposed rats was significantly lower from 5 to 41 days of exposure than at 79 and 116 of days exposure (Fig. 6).

Lung lipid peroxidation. Lung lipid peroxidation levels were significantly higher in silica-exposed rats from 41 to 116 days of exposure than in air-exposed controls (Fig. 7). Lung lipid peroxidation levels in silica-exposed rats were significantly higher at 41 days of exposure than at 10 and 20 days of exposure; further significant increases occurred at 79 and 116 days of exposure (Fig. 7).

DISCUSSION

In two previous reports from this silica inhalation study, we presented data on the temporal relationships between silica exposure, pulmonary inflammation and damage, and the development of fibrosis (27, 28). In the present report from this inhalation study, we examined

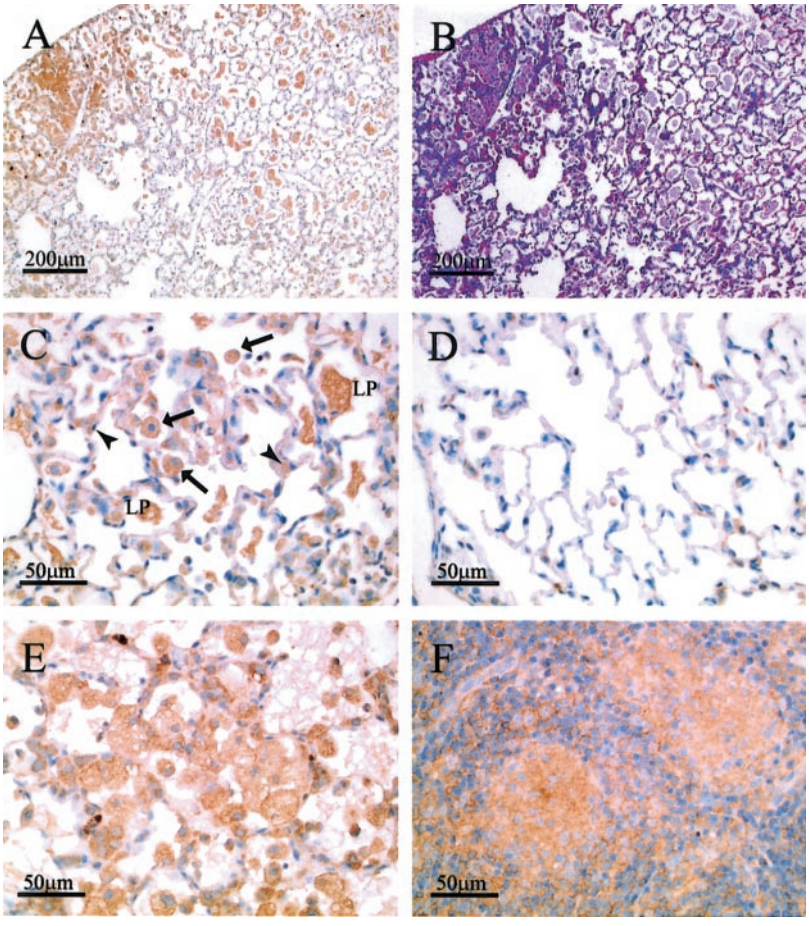

Fig. 4. Immunohistochemical localization of nitrotyrosine (NT) in rats exposed to air or silica. *A*: low-power view of alveoli in the periphery of a lung showing the pattern of NT staining in a rat lung exposed to silica for 116 days. *B*: low-power view of a section serial to *A*, stained with hematoxylin and eosin, showing the pattern of inflammation and lipoproteinosis. *C*: alveolar region in an animal exposed to silica for 79 days, with positive macrophages (arrows), epithelial cells (arrowheads), and lipoprotein (LP). *D*: alveolar region in a control animal, with no significant staining for NT. *E*: staining for NT in a lipoprotein- and macrophage-rich area (116-day silica exposure). *F*: staining for NT in granulomatous regions within bronchial-associated lymphoid tissue (116-day silica exposure).

the oxidative mechanisms underlying the development of silica-induced pulmonary disease, primarily focusing on NO and ROS.

The first step in our investigation of the relationship between NO and silica-induced pulmonary inflammation and damage was to characterize NO production in the lung. AM production of NO was measured by NO-dependent chemiluminescence and indicated NO-dependent chemiluminescence levels that were 46-fold higher in AM isolated from rats exposed to silica for 10

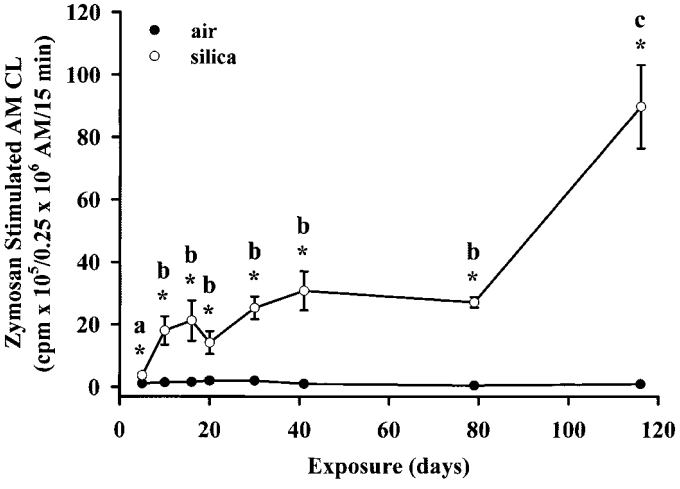

Fig. 5. Zymosan-stimulated AM chemiluminescence. Values are means ± SE (n = 5). *Significant difference ($P \leq 0.05$) between air-exposed controls and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters (a–c) are significantly different from each other ($P \leq 0.05$).

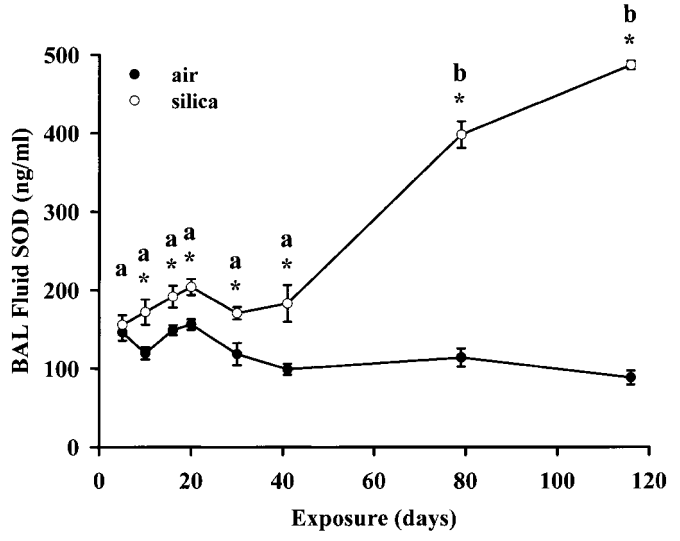

Fig. 6. BAL fluid superoxide dismutase (SOD) activity. Values are means ± SE (n = 10–15). *Significant difference ($P \leq 0.05$) between air-exposed controls and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters (a and b) are significantly different from each other ($P \leq 0.05$).

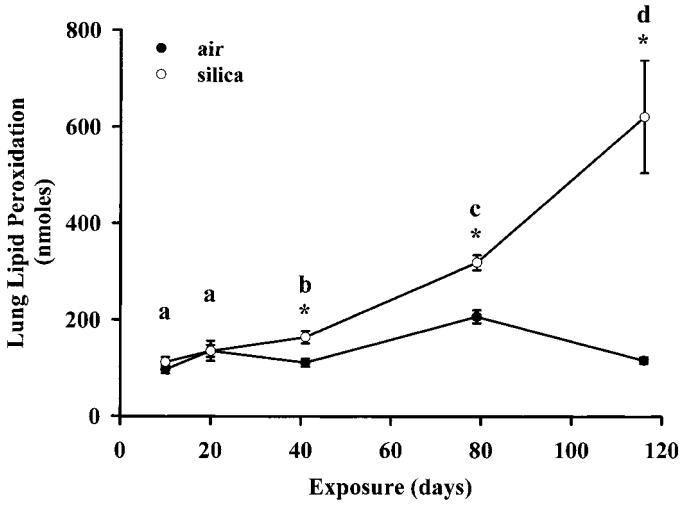

Fig. 7. Lung lipid peroxidation. Values are means ± SE (n = 6). *Significant difference ($P \leq 0.05$) between air-exposed controls and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters (a–d) are significantly different from each other ($P \leq 0.05$).

days than in AM from air-exposed controls, and by 116 days of exposure this value had increased to 71-fold. On the basis of this observation, it might be expected that the pulmonary NO concentration may increase as the silica exposure progresses. Indeed, BAL NO_x concentration, which represents NO production not only from AM but also other cell types present in the lung, was significantly higher in silica-exposed rats than in air-exposed controls from 16 to 41 of days exposure and explosively increased thereafter.

To examine the anatomic association between NO production and pulmonary inflammation, damage, and fibrosis, immunohistochemical studies were conducted. The pattern of iNOS and NT staining corresponded very well to the localization of pathological changes observed in silica-exposed animals. Specifically, areas of granulomatous inflammation of BALT, lipoproteinosis, type II cell hyperplasia, and alveolitis were sites for iNOS or NT positivity. The staining in regions of inflammation was very apparent in silica-exposed animals after 79 and 116 days of exposure and was absent in air-exposed animals.

Peroxynitrite, produced by a reaction between superoxide anion and NO (2), reacts with tyrosine residues on proteins to form NT. In this study, NT localization is highly congruent with iNOS localization. Localization of NT indicates that peroxynitrite is being produced in the immediate vicinity. This would also account for positive staining for NT in the lipoprotein, probably from increased iNOS activity in AM, or in type II cells, when surfactant is being produced.

Despite the significant effect of silica exposure from 16 to 41 days on BAL fluid NO_x and AM NO production (as measured by NO-dependent AM chemiluminescence), immunohistochemistry detected no significant iNOS or NT staining in the AM or epithelial cells at these early times. This apparent inconsistency is probably due to the difference in the sensitivity of the NO-dependent AM chemiluminescence and immunohistochemistry assays, the chemiluminescence assay being more sensitive. At 79 and 116 days of exposure, BAL NO_x concentrations from silica-exposed rats further increased. During this period, AM NO production remained significantly higher than control for silica-exposed rats but was not different from that determined at earlier silica exposure times, as measured by NO-dependent AM chemiluminescence. However, the number of lavagable AM increased significantly vs. control at these exposure times (28), and thus this increase in the number of AM may contribute to the increase in BAL NO_x. At these same exposure times, 79 and 116 days, immunohistochemistry detected significant iNOS and NT staining in the AM and pulmonary epithelial cells. These data suggest that AM, as well as pulmonary epithelial cells, contribute to BAL fluid NO_x concentrations. Interestingly, the time when the BAL fluid NO_x increases dramatically coincides with the rapid increase in pulmonary inflammation, cytotoxic damage, and fibrosis previously described (27, 28).

Some investigators have reported that human AM fail to express iNOS and induced production of NO in response to various stimulants and, therefore, question the relevance of silica-induced NO in rats to human disease. However, recent evidence indicates that, after in vivo stimulation, human AM can produce NO and that the level of NO production correlates with the degree of human pulmonary pathology.

Similar to rats, human pulmonary NO, measured as the concentration of NO_2^- in epithelial lung lining fluid and exhaled NO concentration, is highly correlated with iNOS expression in AM isolated from subjects with primary lung cancer (22). Increased NO production has also been reported in humans with silica-induced lung disease. Specifically, iNOS mRNA levels and NO production from BAL cells were determined from a silica-exposed coal miner with an abnormal chest X-ray, a silica-exposed coal miner with a normal chest X-ray, and an unexposed control. iNOS mRNA was higher from BAL cells isolated from the two coal miners than from BAL cells from the unexposed control, and iNOS mRNA was higher in the miner with the abnormal chest X-ray than in the miner with the normal chest X-ray (5). AM NO production was measured by NO-dependent chemiluminescence, and the coal miners with normal and abnormal chest X-rays had 15- and 31-fold higher NO-dependent chemiluminescence, respectively, than the unexposed control (5).

Further examination of the data suggests some possible mechanisms through which NO may contribute to silica-induced pulmonary disease. In this study, the cellular debris observed in the alveoli at 41, 79, and 116 days of silica exposure, especially in regions of lipoproteinosis, corresponded to foci of apoptotic cells detected using the TdT-mediated dUTP nick end labeling assay (24). The apoptotic cells were limited to the air spaces, were not in the interstitium, and increased significantly in number after 41, 79, and 116 days of silica exposure (23). It was also noted that the apoptotic cells were not highly loaded with silica particles

(23). Because we localized iNOS and NT in the same regions, it is possible that NO and/or peroxynitrite is having a direct effect on AM, perhaps stimulating apoptosis. The observation that the time course for elevation of AM apoptosis (23, 31) correlates well with pulmonary NO concentrations as measured by BAL fluid NO_x further supports this hypothesis. Thus it is proposed that AM apoptosis may be regulated by pulmonary NO concentrations and not by AM phagocytosis of silica particles.

Another mechanism through which NO may influence silica-induced disease is regulation of nuclear factor-κB (NF-κB) activation. The effect of NO on NF-κB activation is controversial. Studies using the mouse monocyte-macrophage cell line RAW 264.7 have indicated that exogenous NO inhibits silica- and LPS-induced NF-κB activation (11). However, a later study that also used RAW 264.7 cells demonstrated that exogenous NO can stimulate NF-κB activation (19). Furthermore, NF-κB activation is stimulated in rat primary AM exposed to silica and LPS in vitro, and iNOS inhibitors reduced this NF-κB activation (19). The contradictory results of these studies may reflect differences in NO concentrations, the duration of NO exposure, and/or the basal activity of macrophages (19), since these have been shown to alter the effect of NO on NF-κB activation in other studies (12, 33).

We previously reported that NF-κB is activated in BAL cells isolated from silica-exposed rats in this inhalation study and that the level of activation progressively increased throughout the silica exposure (28). Data presented in this report demonstrate that the NO concentration in the lung, evidenced by the BAL fluid NO_x concentration, was significantly increased in silica-exposed rats but was relatively constant through the first 41 days of exposure before steadily increasing thereafter. Thus the duration and NO concentration to which the BAL cells were exposed changed during this study. This makes it difficult to determine the effect NO may have had on the progressive increase in NF-κB activation previously reported for the BAL cells (28). To further investigate the relationship between NO and NF-κB activation in BAL cells in vivo, it will be necessary to conduct additional experiments in which the duration and concentration of NO in the lung are controlled.

Similar to rats, and equally controversial, one of the proposed mechanisms through which NO may regulate pulmonary disease in humans is regulation of NF-κB activation. In vitro, LPS-induced NF-κB activation in human primary AM is decreased in a dose-dependent manner by NO (29). Furthermore, in vivo studies of humans with asthma or primary pulmonary hypertension indicate an inverse relationship between NF-κB activation and airway NO concentrations (29). In contrast, human AM isolated from patients with pulmonary tuberculosis have enhanced NO production and NF-κB activation, and inhibition of NO by N^G-monomethyl-L-arginine decreased NF-κB activation, indicating a positive correlation between NO production and NF-κB activation (36).

Oxidative damage, not mediated by NO but by other forms of ROS, was monitored in this study by measuring lung lipid peroxidation levels. We determined that lung lipid peroxidation increased steadily in the lungs of silica-exposed rats. This indicated that the lungs were in a state of oxidative stress, which is consistent with previous studies of silica-exposed rat lungs (35). SOD catalyzes the conversion of superoxide to H_2O_2, which is subsequently converted to water and oxygen by catalase. It has been suggested that SOD is produced in proportion to the oxidant stress in the lung as a defensive response (34). However, the increase in BAL SOD activity does not appear to be sufficient to protect the lung from ROS-mediated damage, since lung tissue lipid peroxidation levels continued to increase throughout the silica exposure.

Several significant observations were made in this study. First, immunohistochemistry studies determined that the sites of iNOS activation and NT damage are associated anatomically with pathological lesions in the lungs of silica-exposed rats. Second, the data suggest that AM and epithelial cells of the lung contribute to NO production in the lung. Finally, there is an apparent positive association between the pulmonary NO concentration reported in this study and increased AM apoptosis (23, 31) and BAL cell NF-κB activation (28) previously described in other reports from this study. The regulatory interactions between NO, NF-κB, and apoptosis are complex and remain to be completely eludicated, but the data from this study suggest that AM NO production and NF-κB activation in BAL cells occur when pulmonary NO concentration and silica lung burden are low, whereas AM apoptosis peaks at 79 days (23, 31), when the pulmonary NO concentration and silica lung burden are higher. Although this does not assist in determining the regulatory interactions between NO, NF-κB, and apoptosis, it does indicate that NF-κB activation and NO production by AM are much earlier events than AM apoptosis in response to silica exposure.

Finally, one should consider the human relevance of this rat model. Previously reported data from this silica inhalation study demonstrated that silica-exposed rats developed lipidosis and diffuse pulmonary fibrosis (27), characteristics similar to those observed in humans with accelerated silicosis (10). Unlike accelerated silicosis in humans, BAL PMN yield from rats exposed to silica exhibited a nonlinear, 61-fold increase over controls after 116 days of exposure (28). However, this difference in the PMN response of the two species to silica may not be physiologically significant for three reasons: *1*) It has previously been demonstrated that pulmonary damage, as measured by BAL protein, lactate dehydrogenase, and *N*-acetyl-β-glucosaminidase, is not dependent on PMN infiltration into the lung (13). *2*) Immunohistochemical data in this study indicate that rat PMN have little to no iNOS protein and, thus, would make a negligible contribution to pulmonary NO production. *3*) Histological examination of silica-exposed rat lungs in this study indicated that AM are the predominant inflammatory cell (27), but this was not

reflected in BAL AM yields, because activated AM are difficult to lavage, and thus their numbers are underestimated in exposed rats (9). Thus similarities in silica-induced AM NO production and NO-dependent regulatory mechanisms in rats and humans suggest that the proposed role of NO in the initiation and progression of silica-induced pulmonary disease in this rat model may also be operating in humans with accelerated silicosis. Furthermore, data from our silica inhalation study were used to model the relationship between lung burden and pulmonary response in the rat and have also been used to model the human response using coal miner data (20). Results of this modeling indicate that the rat exposed to silica is a relevant model for human pneumoconiosis.

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