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

Dale W. Porter[1], Lyndell Millecchia[1], Victor A. Robinson[1], Ann Hubbs[1], Patsy Willard[1], Donna Pack[1], Dawn Ramsey[2], Jeff McLaurin[2], Amir Khan[2], Douglas Landsittel[1], Alexander Teass[2] and Vincent Castranova[1]

[1]National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Morgantown, WV 26505 and [2]National Institute for Occupational Safety and Health, Division of Applied Research and Technology, Cincinnati, OH 45226

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Address correspondence to Dale W. Porter, Ph.D., Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road, M/S 2015, Morgantown, WV, 26505, USA. Telephone: 304-285-6320 FAX: 304-285-5938 E-mail: DPorter@cdc.gov

ABSTRACT

In previous reports from this study, measurements of pulmonary inflammation, bronchoalveolar lavage (BAL) cell cytokine production and NF-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 hours/ day, 5 days/week) for 116 days. We report time-dependent changes in: (1) activation of alveolar macrophages (AM) and concomitant production of NO and ROS, (2) immunohistochemical localization of inducible nitric oxide synthase and the NO-induced damage product nitrotyrosine, (3) BAL fluid NOx and superoxide dismutase concentrations, and (4) lung lipid peroxidation levels. The major observations made in this study are: (1) NO and ROS production and resultant damage increased during silica exposure, and (2) the sites of iNOS activation and NO-mediated damage are associated anatomically with pathological lesions in the lungs.

Keywords: silicosis, fibrosis, oxidant injury, nitrotyrosine

INTRODUCTION

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), the hypothesis that oxidant-mediated lung damage may participate in the development of silica-induced pulmonary disease has been proposed (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 fluid of silica-exposed rats (16).

Other studies have suggested that nitric oxide (NO) may also participate in silica-induced pulmonary inflammation, damage and fibrosis. Messenger RNA levels for inducible nitric oxide synthase (iNOS), an inducible enzyme which produces NO, are increased in bronchoalveolar lavage (BAL) cells following intratracheal (IT) instillation of silica in rats (4, 17). Furthermore, pharmacological evidence suggests that NO production by AM is increased after IT 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 whose comprehensive goal was 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 NOx and superoxide dismutase (SOD) levels, and (3) immunohistochemical localization of iNOS and nitrotyrosine in silica-exposed rat lungs.

METHODS

Silica Chemical Analyses

The silica used in this study was MIN-U-SIL 5 (U.S. Silica, Berkeley Springs, WV). The bulk silica was analyzed for inorganic contaminants and desorbable organic carbon compounds, while aerosolized silica samples were analyzed for trace inorganic elements, and elemental and organic carbon. The results of these analyses have been reported (27).

Silica Aerosol Exposure of Rats

Pathogen-free male Fischer 344 rats (strain CDF, 75-100 g) were purchased from Charles River (Raleigh, NC) and housed in an AAALAC accredited animal facility using individual cages in two 5 m^3 Hinners-type inhalation chambers during this study. 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 hours per day, 5 days per week 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 on a 12 hour 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: a 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 ranged from 14.9 to 15.5 mg/m^3 silica during the study (27). Silica particle size averaged $\leq$2 μm as determined with an Anderson 8-stage cascade impactor, and the mass median aerodynamic diameter of the silica particles ranged from 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 exposed in this study ranged from 0.42 ± 0.05 mg SiO_2/lung after 1 day exposure to 6.27 ± 0.15 mg SiO_2/lung after 116 days exposure (27). Based on the volumetric model described by Oberdorster and colleagues (26) the percent of AM volume occupied by silica in this study was below overload levels (27). Furthermore, the silica burden versus exposure duration relationship exhibited equilibrium between 79 and 116 days of exposure, suggesting that the silica-exposed animals were not in pulmonary overload (27).

Bronchoalveolar Lavage

Rats were euthanized with an i.p. injection of ≥100 mg sodium pentobarbital/kg body weight after 5, 10, 16, 20, 30, 41, 79 and 116 days of exposure. Bronchoalveolar lavage (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 was conducted as described previously (27). BAL cells were resuspended in HEPES-buffered medium (10 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid], 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 (Coulter Multisizer II, Coulter Electronics, Hialeah, FL) as described previously (7).

BAL Fluid Superoxide Dismutase Activity

BAL fluid superoxide dismutase (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 NOx Concentrations

In this paper, we define NOx as the total of nitrite (NO_2^-) plus nitrate (NO_3^-) in a sample. To determine BAL NOx, it was necessary to reduce nitrate to nitrite in the BAL sample. To reduce the nitrate, a previously described nitrate reduction assay (14) was modified. The nitrate reduction reaction consisted of: 50 mM HEPES, 5 mM flavin adenine dinucleotide, 0.1 mM reduced nicotinamide adenine dinucleotide phosphate, 0.2 units 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 minutes. At the end of the incubation, the reaction was diluted with water and nitrite 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 x 10^6 AM/ml at 37°C for 20 minutes, followed by the addition of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) to a final concentration of 0.08 μg/ml followed by the measurement of chemiluminescence. To determine zymosan-stimulated chemiluminescence, 2 mg/ml of unopsonized zymosan was added immediately prior to 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 minutes. The integral of counts per minute versus time was calculated. Zymosan-stimulated chemiluminescence was calculated as the cpm in the zymosan-stimulated assay minus the cpm in the resting assay. NO-dependent chemiluminescence was determined by subtracting the zymosan-stimulated chemiluminescence from cells pre-incubated with 1 mM L-NAME from the zymosan-stimulated chemiluminescence without L-NAME. The use of unopsonized zymosan in the chemiluminescence assay allowed only AM chemiluminescence to be measured, because unopsonized zymosan stimulates AM chemiluminescence (5) but not PMN chemiluminescence (1, 15).

Lung Lipid Peroxidation

Rats, separate from those used for bronchoalveolar lavage, were euthanized with an i.p. injection of ≥100 mg sodium

pentobarbital/kg body weight after 10, 20, 41, 79 and 116 days exposure. Lungs were removed en bloc, washed with ice-cold 0.9% (w/v) NaCl, blotted dry and weighed. Lung tissue was processed and lipid peroxidation 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 bronchoalveolar lavage, were euthanized with an i.p. injection of ≥100 mg sodium pentobarbital/kg body weight after 10, 20, 41, 79, and 116 days exposure. The left lobe was inflated trans-pleurally with 2-3 ml of formalin, processed within 24 hours, and embedded in paraffin. Paraffin sections were cut at 5μm, deparaffinized in xylene and rehydrated. Slides were placed in citrate buffer (pH 6.0) and microwaved (32). After blocking endogenous peroxidase in a 1:1 mixture of 3% H_2O_2 and methanol, slides were placed in 10% bovine serum albumin (BSA) for 30 min at room temperature, then incubated overnight at 4°C in the primary antibody (monoclonal anti-iNOS, Transduction Laboratories, Lexington, KY, N32020, 1:50 dilution; polyclonal anti-nitrotyrosine, Upstate Biotechnology, Lake Placid, NY, #06-284, 1:100 dilution). The DAKO LSAB-2 kit (Carpenteria, CA) for rat specimens (K0609) was used to label the antibody, with

diaminobenzidine (Zymed Laboratories, South San Fransisco, CA) as the chromogen. Sections were counterstained briefly with Mayer's hematoxylin, dehydrated, and coverslipped.

Positive controls for iNOS were lung sections from rats that had been exposed to lipopolysaccharide (10 mg/kg body weight) by IT instillation 24 hours prior to sacrifice; the AM in these animals were highly positive for iNOS using the above procedures. In control slides with 10% BSA and no antibody, the mast cells were positive (both in air and silica-exposed animals), but there was no other staining. Nitrotyrosine (NT) controls included an absorption control, in which the antibody was mixed with 10 mM NT before adding 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-exposed 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 log 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 which 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 non-overlapping confidence intervals) within the silica-exposed animals. This approach was preferable to doing pair-wise tests of mean values, 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 exposure, NO-dependent AM chemiluminescence was significantly higher for AM isolated from silica-exposed rats in comparison to air-exposed controls (Figure 1). Furthermore, NO-dependent AM chemiluminescence for AM isolated from silica-exposed rats from 10-116 days exposure was significantly higher than that determined for AM isolated after 5 days exposure (Figure 1).

BAL Fluid NOx Concentrations

In comparison to air-exposed controls, silica-exposed rats had significantly higher BAL fluid NOx concentrations at every exposure time measured from 16-116 days exposure (Figure 2). For silica-exposed rats, BAL fluid NOx concentrations at 79 days exposure were significantly higher than that determined from 5-41 days exposure.

A further significant increase occurred at 116 days exposure (Figure 2).

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 (Figures 3A and 3B). 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 exposure, very little staining was present in the air controls, but the silica-exposed animals had many regions of the lung which stained positively, especially in subpleural areas (Figure 3A). The staining for iNOS was localized primarily in the periphery of the

lung after 79 days, while after 116 days 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 exposure to silica, while these cells were negative in air controls (Figures 3C and 3D). Neutrophils were unstained or only weakly positive. Intense iNOS staining was localized in silicotic granulomas in the parenchyma after 79 days silica exposure (Figure 3E). The areas of granulomatous inflammation in the BALT were positive for iNOS in 3 of 5 animals exposed to silica for 79 days (Figure 3F), and in all the animals after 116 days. Lipoprotein, present in peripheral areas after 79 days exposure, and present in almost all alveoli after 116 days 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 (Figures 4A and 4B). There were no major differences in NT staining between silica- and air-exposed animals after 10, 20, and 41 days exposure. After 79 and 116 days exposure, however, there was very pronounced staining in the silica-exposed animals (Figure 4A), with positive areas primarily in the periphery of the lung after 79 days silica exposure, and throughout the lung after 116 days exposure. AM and alveolar epithelial cells that appeared to be Type II cells were positive for NT after 79 days silica exposure, but were negative or weakly positive in the controls

(Figures 4C and 4D). Some of the AM in both silica-exposed and control animals were positive at all time points. The alveolar lipoprotein was positive for NT in 1 out of 5 animals after 79 days silica exposure, and in 4 out of 5 animals after 116 days (Figure 4C). Histiocytic aggregates in lipoprotein-rich regions were positive for NT (Figure 4E). The areas of granulomatous inflammation in the BALT were positive for NT in one animal after 79 days, and in four out of five animals after 116 days of silica exposure (Figure 4F).

Zymosan-Stimulated AM Chemiluminescence

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

BAL Fluid SOD Activity

In comparison to air-exposed controls, silica-exposed rats had significantly higher SOD activity in BAL fluid at every exposure time examined except 5 days (Figure 6). BAL fluid SOD activity in silica-exposed rats from 5-41 days exposure was significantly lower

than that determined at 79 and 116 days exposure (Figure 6).

Lung Lipid Peroxidation

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

DISCUSSION

In two previous reports from this silica inhalation study, we presented data on the temporal relationships between silica-exposure, pulmonary inflammation, damage, and the development of fibrosis (27, 28). In the present report from this inhalation study, we examined 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 that AM isolated from rats exposed to silica for 10 days had NO-dependent chemiluminescence levels that were 46-fold higher versus air-

exposed controls, and by 116 days exposure this value had increased to 71-fold. Based on this observation, it might be expected that the pulmonary NO concentration may increase as the silica exposure progresses. Indeed, BAL NOx concentrations, which represents NO production not only from AM but also other cell types present in the lung, were significantly higher in silica-exposed rats versus air-exposed controls from 16-41 days exposure, followed by an explosive increase thereafter.

To examine the anatomical 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 all sites for either iNOS or NT positivity. The staining in regions of inflammation was very apparent in silica exposed animals after 79 and 116 days 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 either in AM, or in type II cells, when surfactant is being produced.

Despite the significant effect of silica exposure from 16-41 days on both BAL fluid NOx 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 exposure, BAL NOx 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 than that determined at earlier silica exposure times, as measured by NO-dependent AM chemiluminescence. However, the number of lavagable AM increased significantly versus control at these exposure times (28), and thus this increase in the number of AM may contribute to the increase in BAL NOx. 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 NOx concentrations. Interestingly, the time when the BAL fluid NOx 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 nitrite 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 from BAL cells isolated from the two coal miners demonstrated that both were higher than unexposed control, and that the miner with the abnormal chest x-ray had more iNOS mRNA than that from the miner with a normal chest x-ray (9). AM NO production was measured by NO-dependent chemiluminescence, and in comparison to unexposed control, the coal miners with normal and abnormal chest x-rays had 15- and 31-fold higher NO-dependent chemiluminescence, respectively

(9).

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 silica exposure, especially in regions of lipoproteinosis, corresponded to foci of apoptotic cells detected using the TUNEL assay (24). The apoptotic cells were limited to the airspaces, 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). Since 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 NOx, 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 by regulating 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-induced and LPS-induced NF-κB

activation (11). However, a later study which 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 NOx concentration, was significantly increased in silica-exposed rats but was relatively constant through the first 41 days of exposure, before steadily increasing thereafter. Thus, both the duration and NO concentration that the BAL cells were exposed to changed during this study. This makes it difficult to determine what 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 both the duration and concentration of NO in the lung is controlled.

Similar to rats, and equally controversial, one of the proposed mechanisms through which NO may regulate pulmonary disease in humans is by regulating 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 that an inverse relationship exists 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(γ)-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 hydrogen peroxide, which catalase subsequently converts to water and oxygen. It has been suggested that SOD is produced in proportion to the

oxidant stress present 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. Secondly, the data suggest that AM and epithelial cells of the lung contribute to NO production in the lung. Lastly, 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 at a time 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 is in

response to silica exposure.

Lastly, 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 non-linear, 61-fold increase over controls after 116 days exposure (28). However, this difference in the PMN response of the two species to silica may not be physiologically significant for three reasons. First, it has previously been demonstrated that pulmonary damage, as measured by BAL protein, LDH, and β-NAG, are not dependent on PMN infiltration into the lung (13). Secondly, 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. Lastly, histological examination of silica-exposed rat lungs in this study indicated that AM are the predominate 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 (6). 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 was 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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Fig. 1. NO-dependent AM chemiluminescence. Values represent means ± SE (N=5). An asterisk indicates significant difference ($p \leq 0.05$) between air-exposed control and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters are significantly different from each other ($p \leq 0.05$).

Fig. 2. BAL fluid NOx concentration. Values represent means ± SE (N=14-15). An asterisk indicates significant difference ($p \leq 0.05$) between air-exposed control and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters are significantly different from each other ($p \leq 0.05$).

Fig. 3. Immunohistochemical localization of inducible nitric oxide 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).

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).

Fig. 5. Zymosan-stimulated AM chemiluminescence. Values represent means ± SE (N=5). An asterisk indicates significant difference ($p \leq 0.05$) between air-exposed control and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters are significantly different from each other ($p \leq 0.05$).

Fig. 6. BAL fluid SOD activity. Values represent means ± SE (N=10-15). An asterisk indicates significant difference ($p \leq 0.05$) between air-exposed control and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters are significantly different from each other ($p \leq 0.05$).

Fig. 7. Lung lipid peroxidation. Values represent means ± SE (N=6). An asterisk indicates significant difference ($p \leq 0.05$) between air-exposed control and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters are significantly different from each other ($p \leq 0.05$).

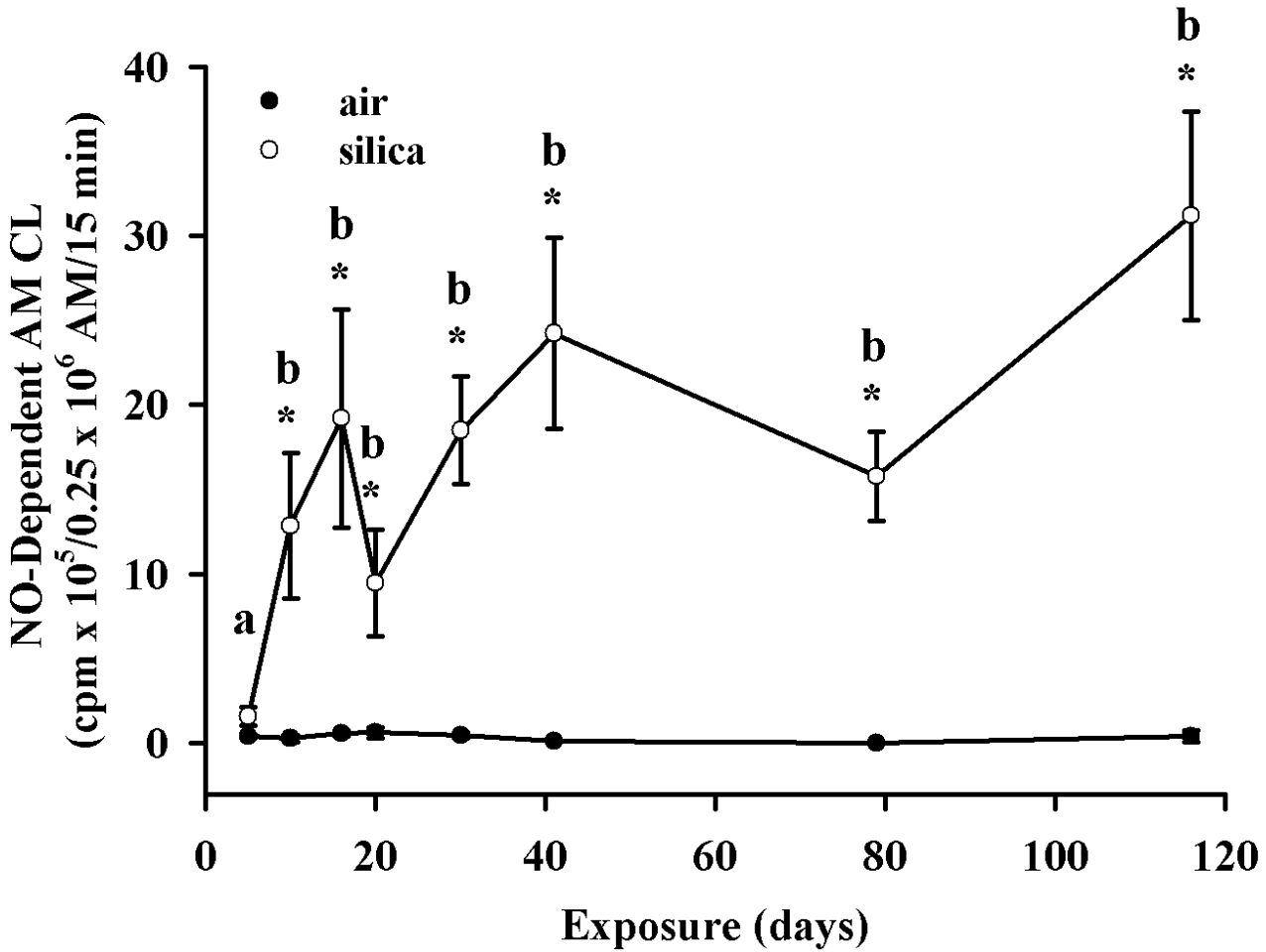
NO-Dependent AM CL
(cpm x 10^5/0.25 x 10^6 AM/15 min)
40
30
20
10
0
air
silica
a
b
*
0
20
40
60
80
100
120
Exposure (days)

Figure 1.

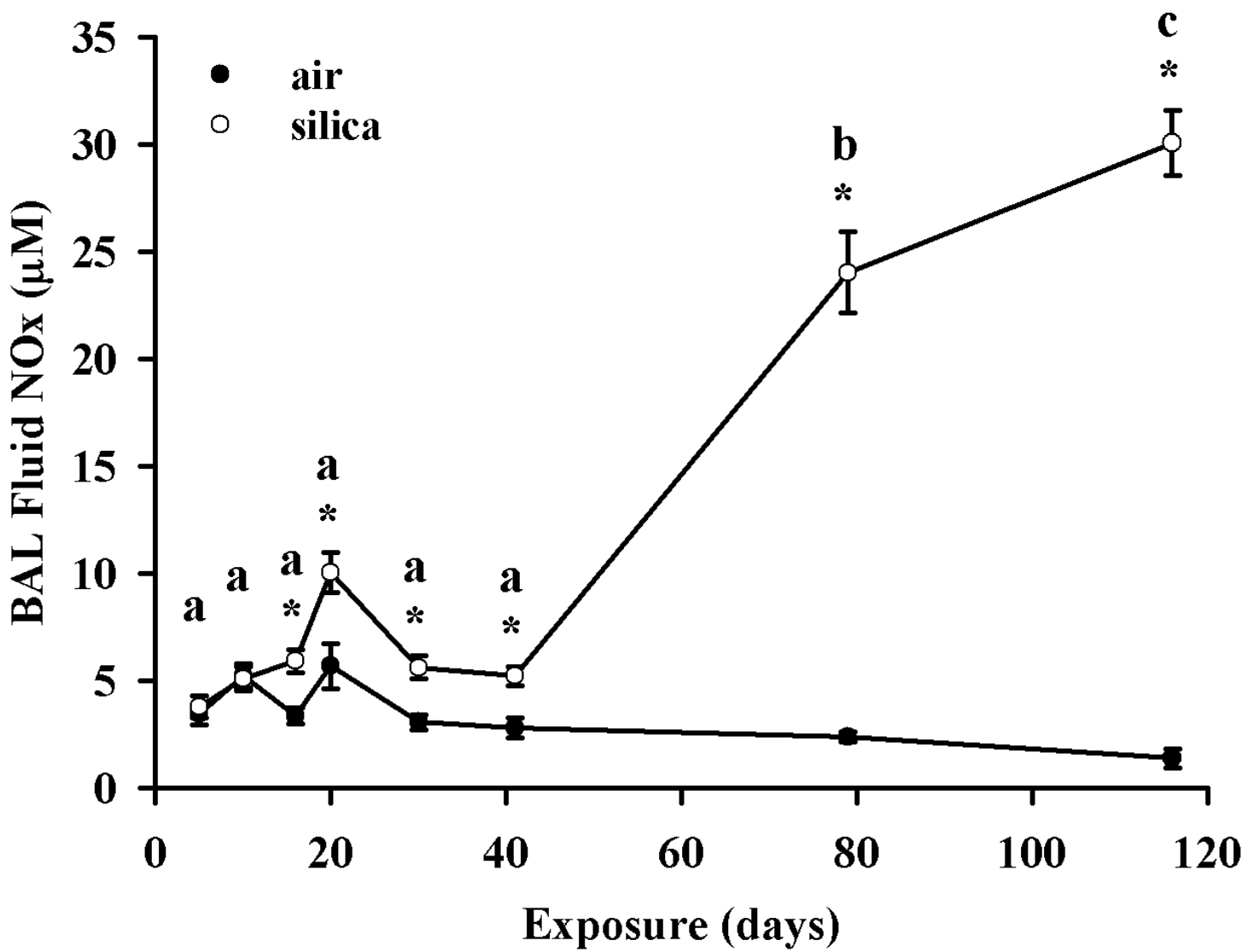
air
silica
BAL Fluid NOx (µM)
Exposure (days)
0
5
10
15
20
25
30
35
0
20
40
60
80
100
120
a
a
a
a
a
b
c
*

Figure 2.

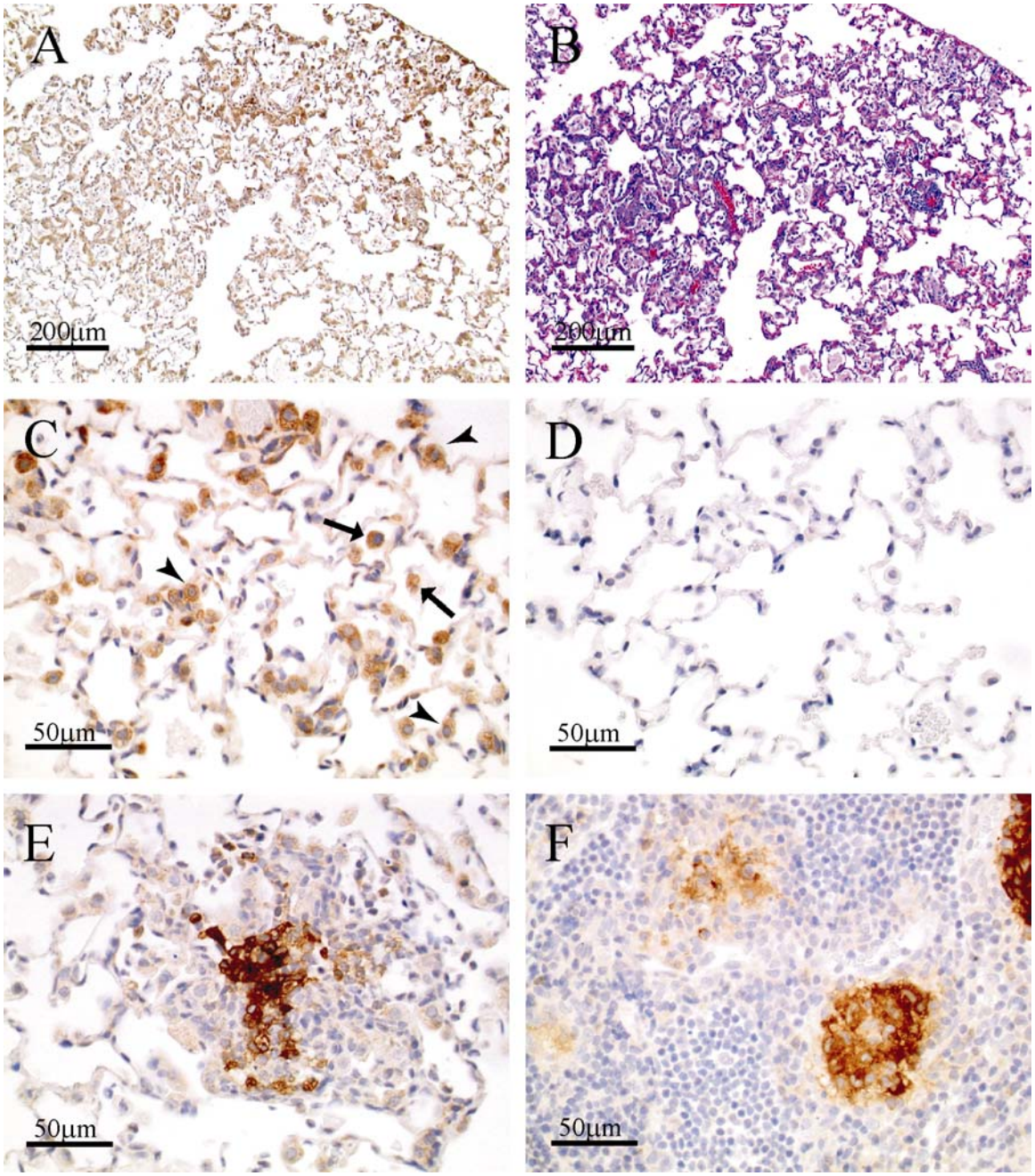

Figure 3.

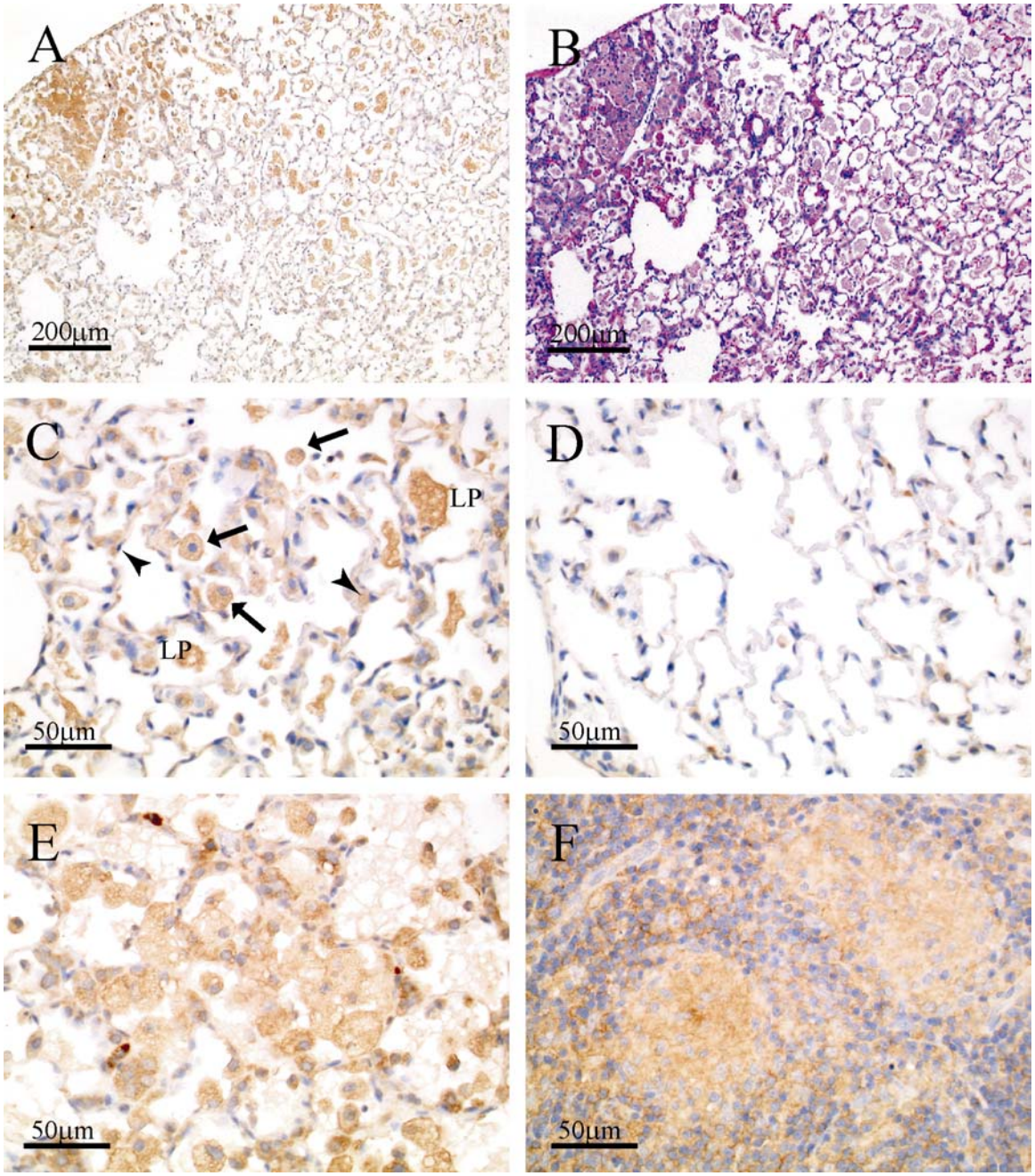

Figure 4.

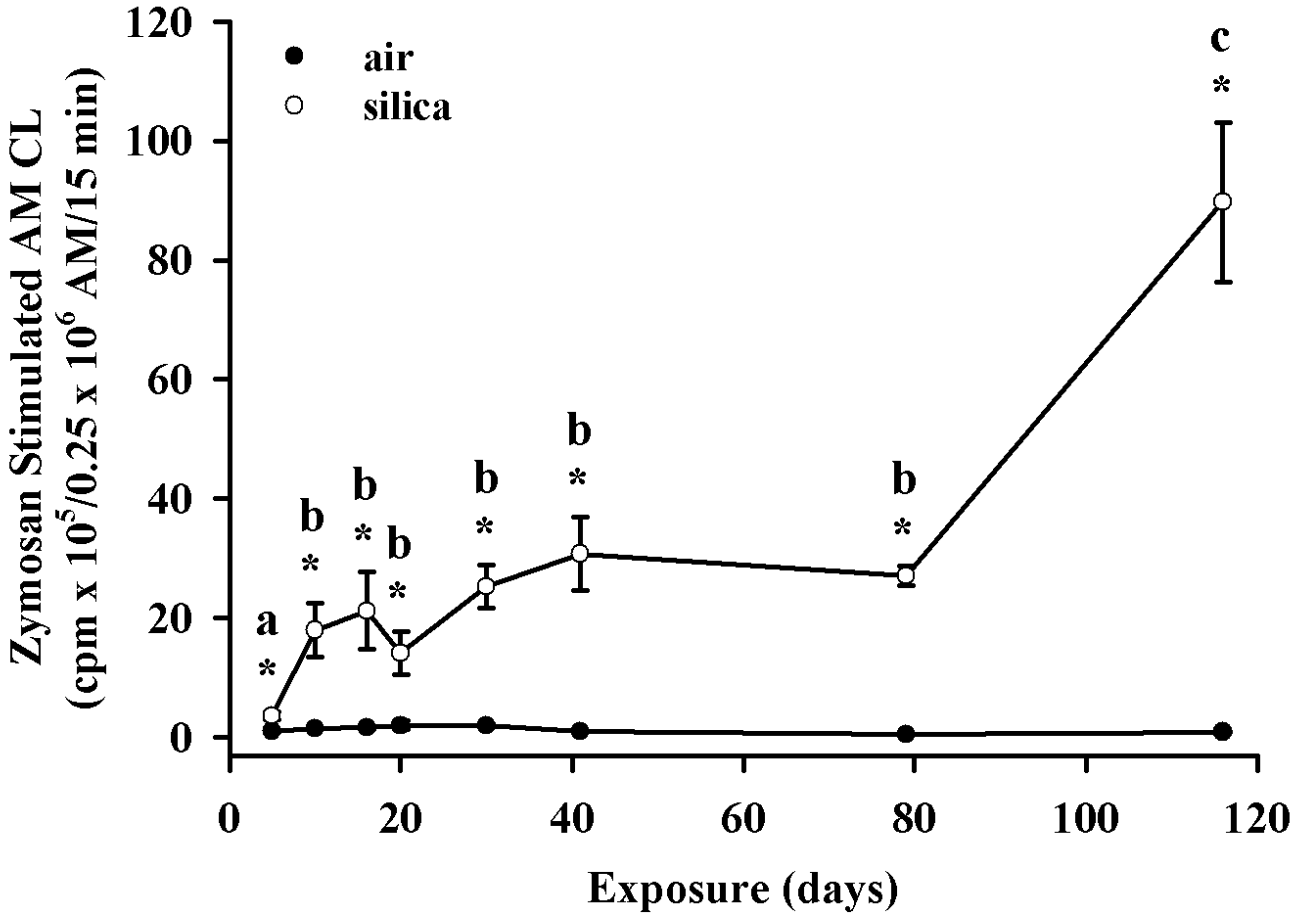
Zymosan Stimulated AM CL
(cpm x 10^5/0.25 x 10^6 AM/15 min)
air
silica
Exposure (days)
0
20
40
60
80
100
120
a
b
b
b
b
b
c
*

Figure 5.

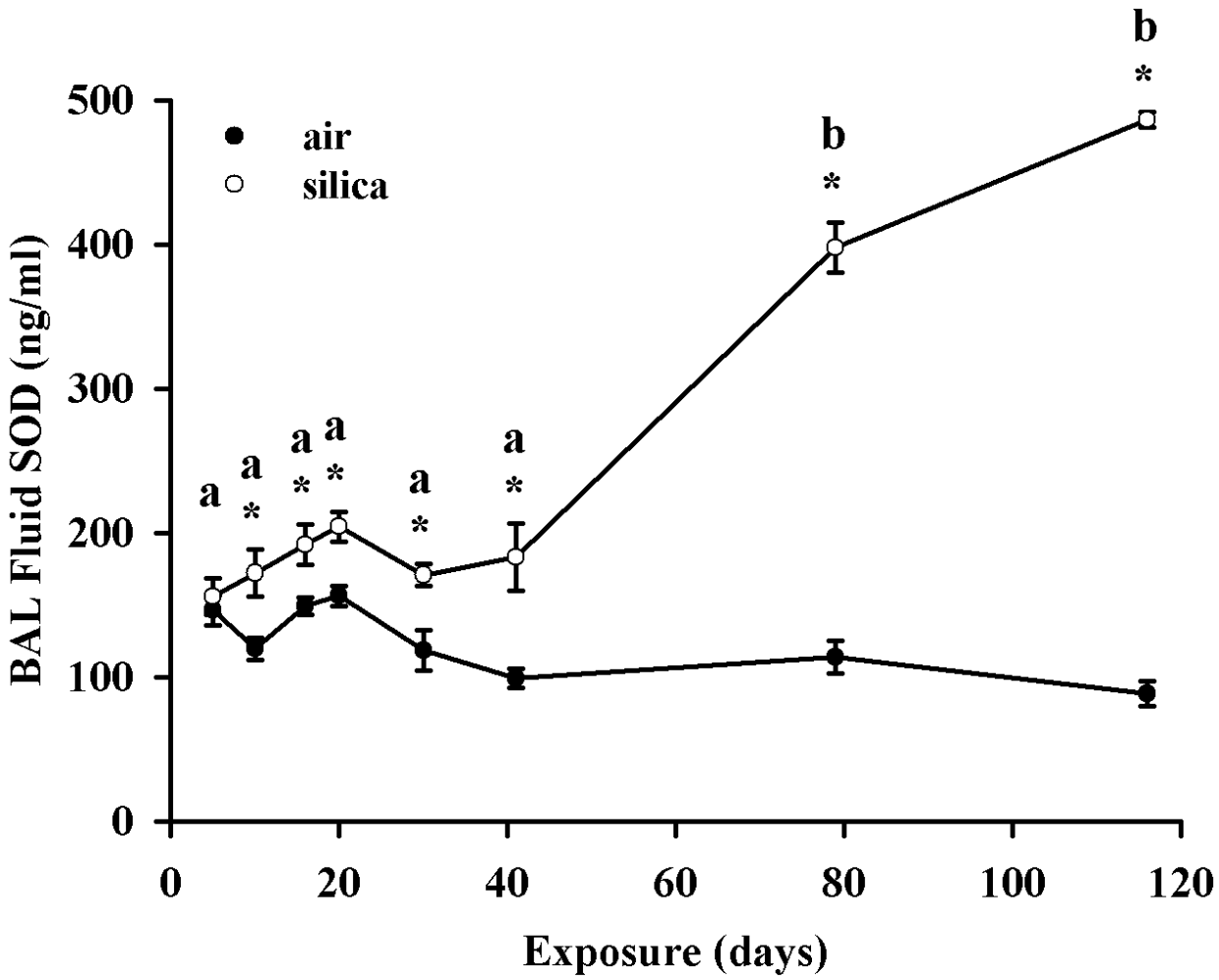
BAL Fluid SOD (ng/ml)
500
400
300
200
100
0
air
silica
a
a
a
a
a
b
b
*
0
20
40
60
80
100
120
Exposure (days)

Figure 6.

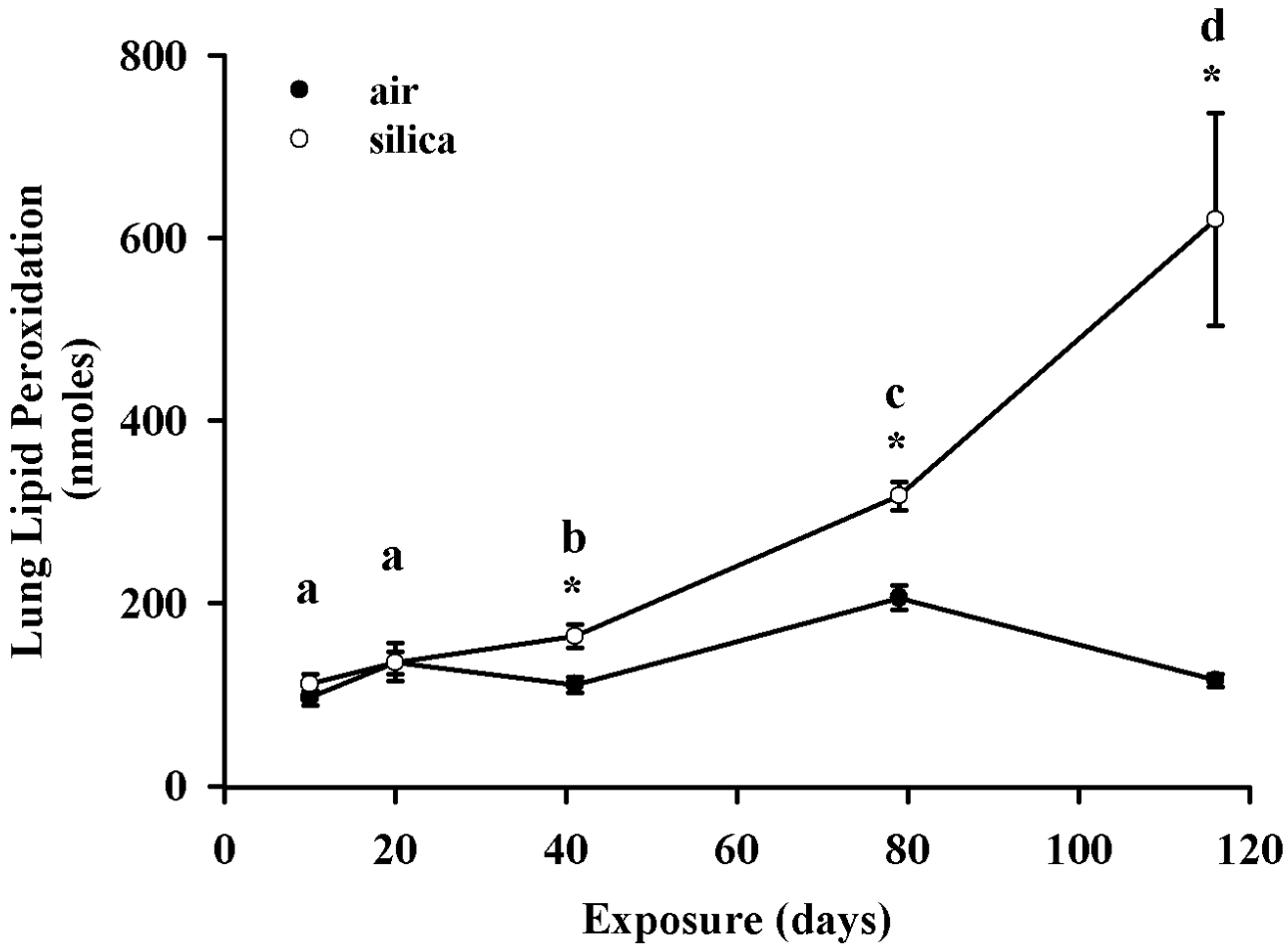
800
600
400
200
0
Lung Lipid Peroxidation
(nmoles)
air
silica
a
a
b
*
c
*
d
*
0
20
40
60
80
100
120
Exposure (days)

Figure 7.