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Effect of inhaled crystalline silica in a rat model: Time course of pulmonary reactions

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Abstract

Numerous investigations have been conducted to elucidate mechanisms involved in the initiation and progression of silicosis. However, most of these studies involved bolus exposure of rats to silica, i.e. intratracheal instillation or a short duration inhalation exposure to a high dose of silica. Therefore, the question of pulmonary overload has been an issue in these studies. The objective of the current investigation was to monitor the time course of pulmonary reactions of rats exposed by inhalation to a non-overload level of crystalline silica. To accomplish this, rats were exposed to 15 mg/m^3 silica, 6 h/day, 5 days/week for up to 116 days of exposure. At various times (5–116 days exposure), animals were sacrificed and silica lung burden, lung damage, inflammation, NF-κB activation, reactive oxygen species and nitric oxide production, cytokine production, alveolar type II epithelial cell activity, and fibrosis were monitored. Activation of NF-κB/DNA binding in BAL cells was evident after 5 days of silica inhalation and increased linearly with continued exposure. Parameters of pulmonary damage, inflammation and alveolar type II epithelial cell activity rapidly increased to a significantly elevated but stable new level through the first 41 days of exposure and increased at a steep rate thereafter. Pulmonary fibrosis was measurable only after this explosive rise in lung damage and inflammation, as was the steep increase in TNF-α and IL-1 production from BAL cells and the dramatic rise in lavageable alveolar macrophages. Indicators of oxidant stress and pulmonary production of nitric oxide exhibited a time course which was similar to that for lung damage and inflammation with the steep rise correlating with initiation of pulmonary fibrosis. Staining for iNOS and nitrotyrosine was localized in granulomatous regions of the lung and bronchial associated lymphoid tissue. Therefore, these data demonstrate that the generation of oxidants and nitric oxide, in particular, is temporally and anatomically associated with the development of lung damage, inflammation, granulomas and fibrosis. This suggests an important role for nitric oxide in the initiation of silicosis. (Mol Cell Biochem **234/235**: 177–184, 2002)

Key words: silicosis, animal model, nitric oxide, reactive oxygen species, fibrosis

Introduction

Silicosis is a respiratory disease associated with the inhalation of crystalline silica [1]. Interstitial lung disease due to inhalation of crystalline silica is believed to be the consequence of pulmonary damage that results in inflammation, lung scarring and fibrosis. The data base from *in vitro* studies, animal exposures by intratracheal instillation or short-term inhalation, and studies of exposed workers has been used to develop a mechanistic framework for the initiation and progression of silicosis [2–4]. Proposed mechanisms have included: direct cytotoxicity of silica particles, stimulation of oxidant generation by exposed pulmonary phagocytes, activation of production of chemokines and inflammatory cytokines, and production of fibrogenic factors by lung cells.

Although previous studies provide substantial mechanistic information, most of these investigations have employed bolus exposure of rats to silica, i.e. intratracheal instillation or a short duration inhalation exposure to a high dose of silica, followed by evaluation of various pulmonary parameters at selected times post-exposure. Therefore, the question of pulmonary overload has been an issue in these studies. In addition, knowledge gaps exist concerning the detailed temporal relationships among silica lung burden, pulmonary inflamma-

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tion and damage, and development of pulmonary fibrosis [5]. This manuscript summarizes the results of a 6-month inhalation exposure of rats to crystalline silica (15 mg/m^3, 6 h/day, 5 days/week, for 116 exposure days). Parameters, such as lung burden, damage, inflammation, cytokine production, production of reactive oxygen and nitrogen species, and fibrosis, were monitored after 5, 10, 16, 20, 30, 41, 79, and 116 days of exposure.

Methods

The silica used in this study was Min-U-Sil 5 (US Silica, Berkeley Springs, WV, USA). X-ray analysis determined silica to be >98.5% crystalline silica. Aerosolized silica samples were examined for trace inorganic elements by proton-induced x-ray emission (PIXE) and for elemental and organic carbon using a thermal-optical analyzer. The silica dust contained small but detectable amounts of only iron, calcium, titanium and zinc (0.13% together), and 0.10% carbon.

Specific pathogen-free male Fischer 344 rats (strain CDF, 75–100g) were purchased from Charles River (Raleigh, NC, USA) and acclimated in the exposure chambers within an AAALAC-approved animal facility for 1 week prior to initiation of the study. Rats in one 5 m^3 Hinners-type inhalation chamber were exposed to filtered air (controls), while those in another chamber were exposed to silica. Characteristics of the exposure (dose, duration, particle size, etc.) are given in Table 1. Chamber atmospheres were maintained at 22.2–25.6°C, 40–70% humidity and <5 ppm ammonia throughout the study. Details of aerosol generation and characterization were given previously [6].

Rats were euthanized with an intraperitoneal injection of ≥100 mg sodium pentobarbital/kg body weight 40 h post-exposure. Whole blood was collected from anesthetized rats from the renal vein using a Vacutainer blood collection tube with Na_2 EDTA as the anticoagulant. Blood cell differentials were determined using a Cell-Dyn 3500R Hematology cell counter (Abbott Diagnostics, Abbott Park, IL, USA). Lungs were lavaged, tissue prepared or tissue fixed and stained to measure various indicators of pulmonary damage, inflammation, activation, oxidant production and fibrosis. These pulmonary responses are detailed in Table 2.

Table 1. Characteristics of exposure and sampling

Particle composition	>98.5% crystalline silica
Exposure concentration	15.3 mg/m^3*
Particle size	1.61 μm*
Exposure time and frequency	6 h/day; 5 days/week
Exposure duration	5–116 exposure days
Sacrifice	40 h post-exposure

*Average values of numerous determinations at 8 different time points during the 116 day exposure. Particle size is mass medium aerodynamic diameter.

Table 2. Pulmonary responses monitored

A. *Damage*
   1. Integrity of the lung air/blood barrier: lung weight (edema), acellular BALF protein and albumin levels
   2. Cellular membrane integrity: acellular BALF activity of LDH

B. *Inflammation*
   1. Pulmonary: BAL polymorphonuclear leukocyte (PMN) and alveolar macrophage counts, acellular BALF activity of β-NAG, histological scoring for alveolitis
   2. Systemic: peripheral blood PMN and monocyte counts

C. *Activation of transcription factors*
   NF-κB/DNA binding in BAL cells

D. *Cytokine production*
   TNF-α and IL-1 production by BAL cells

E. *Activation of alveolar type II epithelial cells*
   1. Morphology: histological evidence for hypertrophy and hyperplasia
   2. Lipid production: histological evidence of lipoproteinosis, levels of phospholipid in acellular BALF

F. *Fibrosis*
   1. Histological evidence: trichrome staining
   2. Chemical evidence: hydroxyproline levels in lung homogenates

G. *Production of oxidants*
   1. Alveolar macrophage: zymosan-stimulated chemiluminescence, L-NAME-inhibitable zymosan-stimulated chemiluminescence
   2. Lung: NO_x (NO_2^- and NO_3^-) in acellular BALF, immunohistochemical localization of lung iNOS and nitrotyrosine, lung lipid peroxidation, acellular BALF levels of SOD

Bronchoalveolar lavage (BAL) was conducted as described previously [6]. Briefly, a cannula was inserted into the trachea and the lungs lavaged with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) plus 5.5 mM D-glucose (pH = 7.4). The yield from the first 6 ml lavage was kept separate from that for the 9 subsequent lavages (8 ml each). BAL cells were isolated by centrifugation (650 × g, 10 min, 4°C). BAL cells were identified as alveolar macrophages (AM) or polymorphonuclear leukocytes (PMN) using an electronic cell counter/sizer (Coulter Multisizer II, Coulter Electronics, Hialeah, FL, USA) as described previously [7]. The acellular supernate from the first BAL (BAL fluid, i.e. BALF) was decanted and a number of parameters measured. BALF protein was determined colorimetrically at 540 nm using the Biuret reaction. BALF albumin was determined colorimetrically at 628 nm as albumin binding to bromcresol green. Lactate dehydrogenase (LDH) activity in the acellular BALF was measured by monitoring the LDH catalyzed oxidation of lactate coupled with the reduction of NAD at 340 nm. Phospholipid in the acellular BALF was measured as total phosphorus present in lipid extracts of BALF as described previously [6]. Inorganic phosphate was determined colorimetrically at 830 nm. Phospholipid content of BALF was calculated by multiplying the lipid phosphorous by 25 [8]. BALF superoxide

dismutase (SOD) activity was determined by monitoring the reduction of cytochrome c at 550 nm as described previously [9]. N-acetyl-β-D-glucosaminidase (β-NAG) activity in BALF was determined by monitoring the hydrolysis of 3-cresolsulfonphthaleiny-N-acetyl-β-D-glucosaminide, releasing 3-cresolsulfonphthalein, which was measured at 580 nm. The total nitrite (NO_2^-) and nitrate (NO_3^-), i.e. NO_x, in BALF was determined at 540 nm by a modified Griess reaction on samples treated to reduce nitrate to nitrite as follows: 50 mM HEPES, 5 mM favin adenine dinucleotide, 0.1 mM reduced nicotinamide adenine dinucleotide phosphate, 0.2 units of nitrate reductase and BALF to a total volume of 1 ml for 30 min at 37°C [10].

BAL cells were used to monitor transcription factor activation, oxidant production and cytokine production. Nuclear extracts of BAL cells were prepared as described previously [11]. The DNA binding activity of the transcription factor, nuclear factor kappa B (NF-κB), was determined using a [^{32}P]ATP-labeled oligonucleotide synthesized from the NF-κB binding sequence of the human interleukin-6 gene promoter as described by Isshiki *et al*. [12]. The DNA-protein binding reaction was conducted in a 24 µl reaction mixture containing 1 µg of poly (dI·dC), 3 µg of nuclear protein extract, 3 µg of bovine serum albumin, 4×10^4 cpm of [^{32}P]-labeled oligonucleotide probe, and 12 µl of reaction buffer (24% glycerol, 24 mM HEPES-pH 7.9, 8 mM Tris-HCl-pH 7.9, 2 mM EDTA and 2 mM 1,4-dithiothreitol). The mixture was incubated for 20 min at 22°C and then resolved on a 5% acrylamide gel with 0.5 × Tris-borate-EDTA at 200V for 90 min. After electrophoresis, the gel was dried and placed on Kodak X-OMAT film overnight at –70°C.

Oxidant production by AM was measured as zymosan-stimulated chemiluminescence (CL). Briefly, 1×10^6 AM/ml were preincubated in HEPES-buffered medium (10 mM HEPES, 145 mM NaCl, 5 mM KC1, 1 mM $CaCl_2$, 5.5 mM glucose; pH 7.4) for 20 min at 37°C. The reaction was initiated by the addition of 2 mg/ml of unopsonized zymosan and 0.08 µg/ml luminol, and CL measured as total cpm above background for 15 min with an automated luminometer (Berthold Autolumat LB 953, EG&G, Gaithersburg, MD, USA). NO-dependent CL was calculated as the decrease in zymosan-stimulated CL in cells pretreated for 20 min at 37°C with the nitric oxide synthase inhibitor, L-NAME (1 mM).

Tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) production from lavageable phagocytes was determined in the supernate of BAL cells cultured at 1×10^6 cells/ml in EMEM media supplemented with 10% heat-inactivated fetal bovine serum and 100 units/ml of penicillin and streptomycin at 37°C in 5% CO_2 for 18 h. TNF-α was determined by ELISA. IL-1 was determined using a thymocyte killing bioassay [13].

Other rats in the control and exposed groups were sacrificed but not lavaged. Lungs were excised and used for the following assays: lung weight, silica lung burden, lung hydroxyproline, histological scoring for alveolitis and fibrosis, and immunohistochemical localization of inducible nitric oxide synthase (iNOS) and nitrotyrosine. To measure silica lung burden, rats were euthanized with sodium pentobarbital and the lungs removed, weighed, frozen in liquid nitrogen, and pulverized with a mortar and pestle. A sample was weighed, lyophilized and transferred to a Griffin-style teflon beaker. Samples were treated with 10 ml of concentrated HNO_3 and 1 ml of 70% $HClO_4$, covered and refluxed overnight at 150°C. Covers were removed the next day, and samples were taken to perchloric fumes at 150°C. An additional 5 ml of HNO_3 was added, and samples taken to near dryness. The residues were dissolved in 0.5 ml hydrofluoric acid, quantitatively transferred to graduated centrifuge tubes, and diluted to 10 ml. Samples were analyzed for silica by inductively coupled plasma-atomic emission spectroscopy and compared to standards as described previously [6].

For the determination of hydroxyproline, lungs were removed, washed in ice-cold 0.9% (w/v) NaCl, blotted dry and weighed. Lung hydroxyproline was determined after portions of lungs were chopped and hydrolyzed in 6N HCl for 48–72 h at 110°C according to the method of Kivirikko *et al*. [14]. Other portions of lung were processed to determine lipid peroxidation using a colorimetric assay at 586 nm following the protocol provided by the manufacturer (BIOXYTECH[7] LPO-586, Oxis International, Portland, OR, USA).

For histopathology, lungs were airway perfused by inflation with glutaraldehyde solution at 20 cm H_2O pressure. Lungs were then embedded in paraffin, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin and Masson's trichrome. Slides were examined by a board-certified veterinary pathologist, blinded to exposure status, and scored for severity and distribution of alveolitis and fibrosis as described previously [6].

For immunohistochemical localization of iNOS and nitrotyrosine, the left lobe of the lung was inflated transpleurally with 2–3 ml of formalin, processed within 24 h and embedded in paraffin. Sections were cut at 5 µm, deparaffinized in xylene and rehydrated. Slides were placed in citrate buffer (pH 6.0) and microwaved. After blocking endogenous peroxidase in a 1:1 mixture of 3% H_2O_2 and methanol, slides were placed in 10% bovine serum albumin for 30 min at 22°C, then incubated overnight at 4°C in primary antibody (monoclonal anti-iNOS, Transduction Laboratories, Lexington, KY, USA, N32020, 1:50 dilution; or polyclonal anti-nitrotyrosine, Upstate Biotechnology, Lake Placid, NY, USA, #06-284, 1:100 dilution). The DAKO LSAB-2 kit (Carpenteria, CA, USA) for rat specimens (K0609) was used to label the antibody with diaminobenzidine (Zymed Laboratories, South San Francisco, CA, USA) as the chromogen. Sections were counterstained briefly with Mayer's hematoxylin, dehydrated and coverslipped. Details of the procedure and controls for specificity of staining were given previously [10].

Results and discussion

The question of overload

Pulmonary overload is a condition in which dust burden is sufficiently high that clearance is non-specifically impaired and pulmonary inflammation and damage result [15]. These responses are due to the physical burden of a high dust load rather than to a characteristic toxicity of the dust. Most previous studies concerning animal models of silicosis have employed high bolus doses of silica, and the question of overload has been raised.

Three lines of evidence support the conclusion that dose and duration of the inhalation exposure to crystalline silica in the present study were not sufficient to place the rats into a condition of pulmonary overload. First, silica-exposed rats gained weight normally over the nearly 6 month duration of the inhalation study [6]. At no point throughout the study was body weight of the silica-exposed rats below that of the air-exposed controls. Indeed, at exposure times of 79 and 116 days, body weight of silica-exposed rats was slightly (4%) yet significantly above the controls. Therefore, there is no indication that the dust burden was sufficient to cause wasting of the rats. Secondly, the relationship between exposure duration and accumulation of silica in the lungs approached an equilibrium state between 79 and 116 days of exposure (Fig. 1). If rats were exposed to overload doses of dust, an equilibrium between continued deposition and elevated clearance would not be attained. Rather, clearance would decline and lung burden would increase exponentially [16]. A third indication that overload was not attained in the present study relates to the 'dust volume' hypothesis for overload. Oberdorster *et al*. [15] proposed that overload would begin, i.e. clearance would begin to decline, when dust burden equalled 6% of the total volume of the alveolar macrophages in the lung. Clearance would be completely inhibited when the total volume of the dust in the lung was 60% of the total alveolar macrophage volume. In the present study, dust volume was calculated as the silica burden after 116 days of exposure (6.2 mg/lung) divided by the density of silica (2.64×10^3 mg/ml). Total alveolar macrophage volume was estimated by the number of alveolar macrophages harvested by BAL after 116 days of exposure (2.72×10^7 cells/lung) times the mean cell volume (1200 μm^3/alveolar macrophage). Therefore, at 116 days of exposure, silica burden represented only 7% of the volume of the alveolar macrophages. It should be noted that BAL did not harvest all the alveolar macrophages in the lungs. Therefore, the silica burden is somewhat less than 7% of the macrophage volume. By this and the other two criteria described above, the dose and duration of exposure to silica was not sufficient to cause classical overload. Therefore, damage and inflammation in response to exposure reflects particle specific toxic characteristics of crystalline silica.

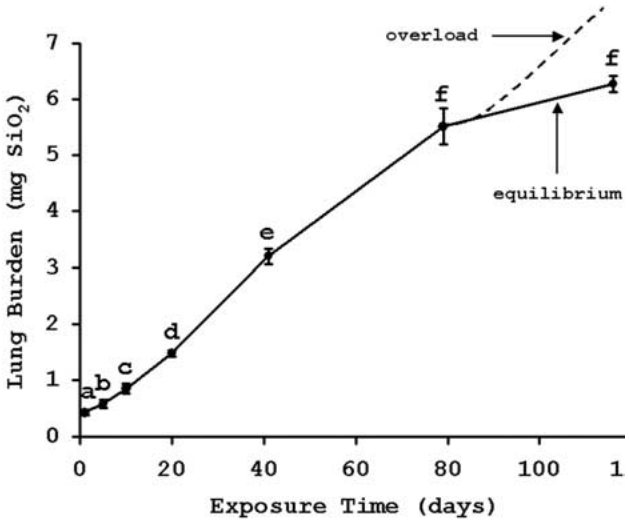

Fig. 1. Silica lung burden. Values represent means ± S.E. of 6 rats per exposure time. Deposited silica significantly increased with increasing exposure duration except at 116 days where burden was not significantly different from the preceding measurement time (79 days). This is taken as an indication that an equilibrium state is being attained.

A characteristic time course of pulmonary damage and inflammation in response to silica exposure

Pulmonary inflammation in response to silica exposure is characterized by an infiltration of PMN from the pulmonary capillaries to the airspaces as monitored by an increase in PMN harvested by bronchoalveolar lavage [11]. The time course of this inflammatory response is shown in Fig. 2. Recruitment of PMN into the airspaces is a rapid response to

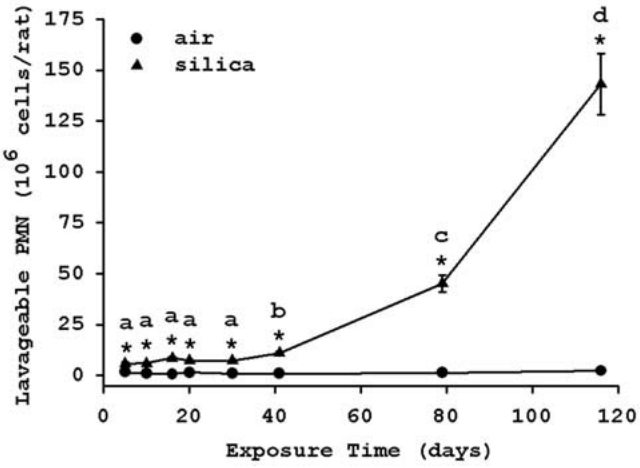

Fig. 2. PMN harvested by bronchoalveolar lavage. Values represent means ± S.E. of at least 14 rats for each exposure group at each time point. *Significant difference between silica-exposed rats and air-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters are significantly different from each other.

Table 3. Pulmonary parameters exhibiting an early rise to a significantly elevated but stable level followed by an explosive increase

Parameter	Time of initial increase	Time of explosive increase
Lung weight (edema)	10 days	42–79 days
BALF protein	16 days	42–79 days
BALF albumin	5 days	42–79 days
BALF LDH	10 days	42–79 days
BAL PMN	5 days	42–79 days
BALF β-NAG	16 days	42–79 days
Blood PMN	10 days	42–79 days
BALF phospholipid	5 days	42–79 days

inhalation of silica, with a significant increase in BAL PMN (250%) occurring after 5 days of exposure. This inflammation, although greater than air-exposed rats, was relatively stable for the first 41 days of exposure. After 41 days of exposure, there was an exponential increase in inflammation with BAL PMN increasing by 33- and 61-fold at 79 and 116 days of exposure, respectively.

A number of other parameters of pulmonary damage and inflammation exhibited a time course of response to silica exposure which was similar to that for BAL PMN [6, 10, 11]. That is, there was a significant increase relatively early in the exposure, which remained relatively stable through 41 days of exposure, after which there was an explosive increase in the pulmonary response. Pulmonary parameters which exhibit this characteristic time course are listed in Table 3. They include measures of damage to the lung air/blood barrier (increases in lung weight, BALF protein and BALF albumin), an indicator of cytotoxicity (increases in BALF LDH), indicators of inflammation (increases in BAL PMN, BALF β-NAG, and peripheral blood PMN), and an indicator of hyperactivity of alveolar type II cells (increases in BALF phospholipid).

Time course of pulmonary fibrosis

Data from Fig. 2 and Table 3 suggest that disease progression during silica exposure is not linear. Rather, for a significant period of time, silica-induced lung damage and inflammation, although elevated from control, are maintained at a relatively stable new set point, suggesting that the lung is successfully coping with this stressor. At some point (between 42 and 79 days of exposure under the present exposure conditions) compensatory responses are exceeded and there is a rapid progression of pathology. This hypothesis is supported by data for the time course of pulmonary fibrosis (Table 4). There is no indication, histologically or chemically, of a fibrotic response when parameters of lung injury and inflammation were elevated but stable. The first indication of fibrosis is noted at 79 days of exposure, i.e. a time of the steep rise of damage and inflammation.

Table 4. Pulmonary fibrosis[a]

Exposure days	Lung hydroxyproline[b]	Fibrotic score[c]
10	0	0
20	0	0
41	0	0
79	0	3.8 ± 1.0
116	1.72 ± 0.17	5.2 ± 0.2

[a]Values are given as increases from the air controls (means ± S.E. of at least 5 determinations); [b]Values in mg hydroxyproline/lung; [c]Sum of the scores for trichrome staining intensity and distribution.

Time course of other pulmonary responses

Compared to the rapid elevation in BAL PMN, the BAL AM response was relatively slow [11]. BAL AM were not significantly increased above control until 41 days of exposure. The steep increase in BAL AM was not noted until the 116 day exposure time point. Likewise, blood monocyte levels were not significantly elevated from control until 79 days of exposure [11]. Therefore, an increase in BAL AM does not appear to correlate with the early increases in pulmonary damage and inflammation. In addition, the steep rise in AM harvested by BAL appears to lag behind the initiation of the fibrotic response. However, BAL may not harvest all of the AM in the lung. Indeed, histopathological scores for alveolitis are significantly elevated after 20 days of exposure and increase progressively through 116 days of exposure. Therefore, the role of AM in the fibrotic response cannot be discounted.

Increases in the activation of the transcription factor, NF-κB, have been linked to the production of a number of inflammatory cytokines and growth factors [17]. Therefore, information concerning silica induction of NF-κB activity is vital to understanding mechanisms involved in the initiation and progression of silicosis. Activation of NF-κB has been reported after *in vitro* exposure of a mouse monocyte-macrophage cell line (RAW 264.7) to silica [18, 19]. Recently, activation of NF-κB has been reported in BAL cells of rats after intratracheal instillation of silica [20]. However, these responses were monitored only for relatively short periods after exposure to high doses of silica. Therefore, an objective of the present study was to determine whether NF-κB is activated in BAL cells harvested from rats exposed by inhalation to a non-overload dose of silica. NF-κB activity, measured as NF-κB binding to DNA, was significantly increased (by 15%) after 5 days of silica exposure and increased linearly thereafter to 2.5-fold the control level at 116 days of exposure [11]. It is of note that NF-κB activity continued to increase when most measures of inflammation and damage were relatively stable, i.e. for the first 41 days of exposure.

Evidence from *in vitro* studies reports that activation of NF-κB induces the production of pro-inflammatory cytokines by macrophages [17, 21, 22]. Therefore, the time course of silica-

induced production of TNF-α and IL-1 from BAL cells was investigated [11]. A significant increase in TNF-α production (150%) was observed after 30 days of silica inhalation. IL-1 was significantly increased by 75% at day 10. In both cases, the steep explosive increase in cytokine production (TNF-α 300-fold and IL-1 270-fold compared to control, respectively) occurred relatively late, i.e. after 116 days of exposure. Therefore, the explosive rise in parameters of damage and inflammation, as well as initiation of pulmonary fibrosis, preceded the steep rise in these cytokines. Therefore, the time course of cytokine production from BAL cells did not correlate with other pulmonary responses to silica exposure. These results are in contrast with results from short term high dose exposures where it has been proposed that TNF-α plays an active role in driving pulmonary damage, inflammation, and fibrosis [1, 23–25]. A possible explanation for the lack of correlation in the present study may be that bronchoalveolar lavage failed to harvest the most adherent and thus the most activated alveolar phagocytes. In addition, cytokine production by alveolar type II cells, which exhibit hyperplasia and hypertrophy at 79 days of exposure, was not evaluated [6]. Also of note is that the time course for silica-induced activation of NF-κB is strikingly different from that for TNF-α and IL-1 production from BAL cells.

Production of oxidants in response to silica inhalation

Chemiluminescence in the absence or presence of L-NAME (an inhibitor of nitric oxide synthase) was determined as an indicator of the production of oxidant species (in general) and nitric oxide (specifically) by AM [10]. Zymosan-stimulated chemiluminescence increased significantly to 4-fold and 12-fold the control value at 10 and 41 days of silica inhalation, respectively. An explosive increase (80-fold) in chemiluminescence from AM occurred at 116 days. Similarly, L-NAME-inhibitable chemiluminescence was 5-fold and 10-fold the control level at days 10 and 41 of exposure, respectively, before exhibiting a steep increase (53-fold) at day 116. The time course of total and L-NAME-inhibitable chemiluminescence suggests that oxidant production from lavageable AM alone can not explain the steep rise in damage, inflammation and fibrosis seen at 79 days of silica inhalation. It is possible that the non-lavageable AM contribute substantially to the production of reactive oxygen species and NO in the lung, since these strongly adherent cells should be more activated than lavageable phagocytes.

In contrast to L-NAME-inhibitable chemiluminescence for lavageable AM, the levels of the nitric oxide products (NO_2 and $NO_3 = NO_x$) measured in acellular BALF exhibit a time course similar to that for most of the parameters of damage and inflammation [10]. As shown in Fig. 3, BALF levels of NO_x increased significantly at 16 days and remained at a stable elevated level through 41 days of exposure, before rising

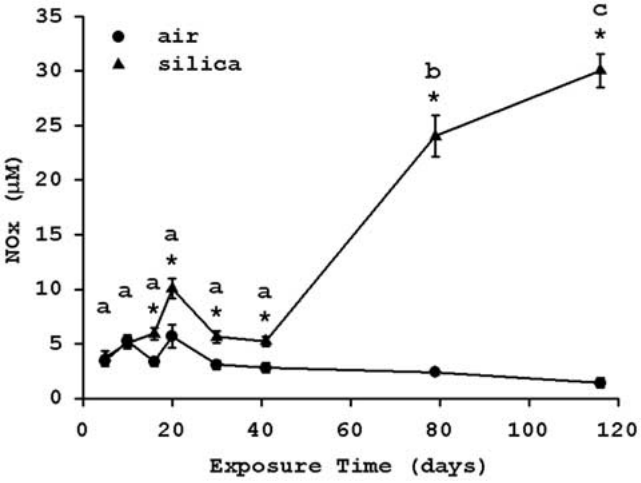

Fig. 3. BALF levels of NO_x. Values represent means ± S.E. of 14 rats for each exposure group at each time point. *Significant difference between silica-exposed and air-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters are significantly different from each other.

sharply between 42 and 79 days. Two other markers of oxidant stress, i.e. BALF levels of SOD and lung lipid peroxidation, also exhibit this characteristic time course [10]. As shown in Table 5, SOD levels in acellular BALF increased significantly (by 41%) at 10 days of exposure and explosively (by 255%) at the 79 day exposure time. Elevated levels of SOD in BALF have been taken as a pulmonary response to oxidant stress [26]. Furthermore, when excess oxidative species react with lung tissue, lipid peroxidation can occur. As shown in Table 5, lung lipid peroxidation was significantly increased (by 58%) at 41 days of exposure. The level of lung lipid peroxidation rose further at 79 days and still more at 116 days to a final level which was 5.3-fold higher than control.

Data from Table 5 indicate that a sharp rise in BALF NO_x (79 days) occurred before the steep increase in L-NAME-inhibitable chemiluminescence from lavageable AM (116 days). This suggests that lavageable AM were not the only source of silica-stimulated nitric oxide production in exposed lungs. Immunohistochemical staining of rat lungs for iNOS indicates induction of this enzyme in silica-exposed lungs after 79 and 116 days of dust inhalation [10]. As shown in Fig. 4, iNOS staining was seen in alveolar type II epithelial

Table 5. Parameters of oxidant generation

Parameter	Time of initial increase	Time of explosive increase
AM CL	10 days	80–116 days
AM L-NAME-inhibitable CL	10 days	80–116 days
BALF NO_x	16 days	42–79 days
BALF SOD	10 days	42–79 days
Lung lipid peroxidation	41 days	42–79 days

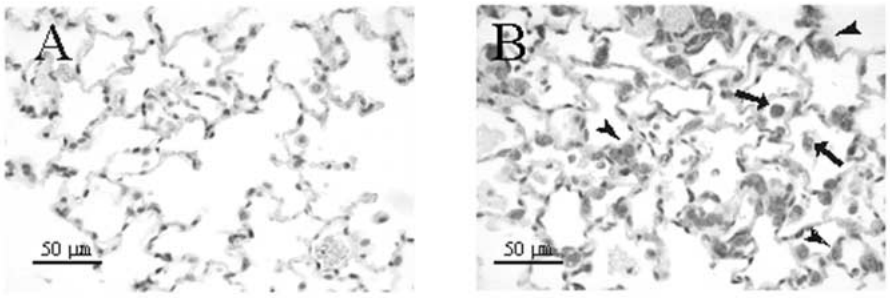

Fig. 4. Immunohistochemical localization of inducible nitric oxide synthase (iNOS) in rats exposed to air (A) or silica (B) for 79 days. Arrows indicate positively staining alveolar macrophages. Arrowheads indicate positively staining alveolar type II cells.

cells as well as alveolar macrophages, suggesting that type II cells may also be a source of NO_x in BALF. Porter *et al*. [10] have also shown that iNOS staining was associated with regions of lung inflammation and was particularly pronounced in areas of silica-induced granulomas in lung tissue and bronchial associated lymphoid tissue. Likewise, immunohistochemical staining for nitrotyrosine, i.e. the reaction product of peroxynitrite with protein tyrosine residues, became prominent after 79 days of silica inhalation [10]. As expected from the cellular localization of iNOS, nitrotyrosine was localized in AM and type II cells. Nitrotyrosine staining was prominent in highly inflamed regions of the lung and in granulomatous regions of bronchial associated lymphoid tissue [10].

Conclusions

In summary, the time course of pulmonary injury and inflammation in response to inhalation of non-overload doses of crystalline silica is unique. There is a rapid and significant increase in numerous markers of damage and inflammation, which remain at a stable new set point for the first 41 days of exposure. This suggests that over this range of lung burdens the lung is handling the silica insult and controlling lung damage. Somewhere between 41–79 days of exposure, the lungs compensatory defenses appear to fail and markers of damage and inflammation rise sharply. It is only during this phase of exposure that fibrosis begins. Kuempel *et al*. [27] have developed a toxicokinetic model using these data and found that the kinetics of response in the rat are relevant to the burden-dependent development of pneumoconiosis in silica-exposed workers. In addition, data from the present investigation indicate that (1) the time course of BALF NO_x correlates with the time course of parameters of damage and inflammation; (2) the steep rise in NO_x levels correlates with the induction of pulmonary fibrosis in response to silica; and (3) localization of iNOS and nitrotyrosine indicates heavy staining in areas of granulomas. These data suggest an important role of NO in silica-induced lung injury, inflammation and disease progression.

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