ANNALS OF CLINICAL AND LABORATORY SCIENCE, Vol. 28, No. 1

Role of Hydroxyl Radical in Silica-induced NF-κB Activation in Macrophages*

FEI CHEN, Ph.D.,†
YONFJU LU, B.S.,†
LAURENCE M. DEMERS, Ph.D.,†
YON ROJANASAKUL, Ph.D.,‡
XIANGLIN SHI, Ph.D.,§
VAL VALLYATHAN, Ph.D.,§
and VINCE CASTRANOVA, Ph.D.§

†Department of Pathology,
The Pennsylvania State University College of Medicine,
The Milton S. Hershey Medical Center,
Hershey, PA 17033
and
‡Department of Basic Pharmaceutical Sciences,
West Virginia University, Morgantown, WV 26506
and
§Pathology and Physiology Research Branch,
Health Effects Laboratory Division,
National Institute for Occupational Safety and Health,
Morgantown, WV 26505

ABSTRACT

Nuclear transcription factor κB (NF-κB) is a multiprotein complex that regulates a variety of genes important for immunity and inflammation. The present study investigates the silica-induced activation of this transcription factor in mouse macrophage cell line RAW 264.7 cells, the role of free radical reactions in the mechanism of the activation, and its possible inhibition. Tetrandrine, a benzylisoquinoline alkaloid, which has been used as an antifibrotic drug to treat the lesions of silicosis and has been characterized as a hydroxyl radical (·OH) scavenger, inhibited the NF-κB activation induced by silica, lipopolysaccharide (LPS), and phorbol 12-myristate 13-acetate (PMA). Catalase, metal chelator, deferoxamine, and the silanol group (SiOH) blocker, poly(2-vinylpyridine-N-oxide) (PVPNO), also inhibited silica-induced NF-κB activation. Electron spin resonance (ESR) spin trapping measurements show that both deferoxamine and PVPNO decreased silica-mediated ·OH radical generation from H_2O_2. It is shown that Fe(II) and not Fe(III) is able to cause NF-κB activation. The antioxidant, ascorbate, attenuated the NF-κB activation induced by silica but not by LPS. The ·OH radical scavenger, sodium formate, inhibited NF-κB activation induced by silica but had only a minor effect on NF-κB

* Send reprint requests to: Laurence M. Demers, Ph.D., Department of Pathology, The Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Center, P.O. Box 850, Hershey, PA 17003 or Xianglin Shi, Ph.D., Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Mail Stop B167; Morgantown, WV 26505.

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activation induced by LPS. The results indicate that silica-mediated free radical generation via the Fenton or Fenton-like reaction ($M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^- + \cdot OH$) and silanol groups on the silica surface play an important role in silica-induced NF-κB activation.

Introduction

Epidemiologic and pathological studies have established that occupational exposure to crystalline silica leads to the development of pulmonary fibrosis.[1,2] Increasing evidence in recent years from epidemiologic and animal studies has also implicated crystalline silica as a potential carcinogen.[3,4] Although the exact biochemical mechanisms involved in silica-induced fibrosis and carcinogenesis are unclear, it is believed that silanol groups (SiOH), negative surface charge, silicon-based free radicals, and oxygen free radicals generated by silica-mediated reactions play a key role.[5,6,7,8,9,10,11,12]

Recently, it has been reported that silica can cause activation of nuclear transcription factor (NF)-κB.[13,14] The NF-κB protein is found in many different cell types and involved in the transmission of signals from the cytoplasm to the nucleus. It regulates a variety of genes involved in immune, inflammatory or acute phase responses, such as expression of various cytokines and surface receptors.[15,16,17,18] In cells which have inducible NF-κB activity, the active form of this factor is composed of two different subunits, p50 and p65. While NF-κB is found in many different cell types and tissues, it has been characterized best in cells of the immune system, such as pre-B cells, mature B and T lymphocytes, macrophages, and monocytes.[16] Although it is well documented that NF-κB is involved in the signal transduction pathways involved in inflammatory response, recently, strong evidence of the involvement of NF-κB in carcinogenesis of lymphoma has been collected.[19,20] It is known that the product of deregulated c-*myc* proto-oncogene plays a role in the pathogenesis of Burkitt's lymphoma, and NF-κB binding sites function to enhance elements in the c-*myc* gene.[18] Recent findings suggest that reactive oxygen species can induce NF-κB activation, and a variety of antioxidants inhibit its activation.[21,22] It is possible that signals from various stimuli are converged to a common signaling component which is regulated by reactive oxygen species. Although it has been reported that silica particles are able to generate free radicals, which are believed to be significantly involved in silica-induced toxicity and carcinogenicity,[4,7,8,9] whether or not silica can regulate NF-κB activation via free radical reactions is still an unanswered question.

At present there is no established method for pharmacologically mediated prevention and treatment of silicosis. However, tetrandrine, a benzylisoquinoline alkaloid, has been reported to retard and reverse the fibrotic lesions of silicosis in humans[23] and in rats.[24,25] Tetrandrine was also shown to be an effective anti-inflammatory drug in rats with established adjuvant-induced arthritis.[26] Recent studies have shown that tetrandrine is able to scavenge both hydroxyl ($\cdot OH$) and superoxide (O_2^-) radicals and inhibits silica-induced lipid peroxidation.[27] It is possible that tetrandrine may inhibit silica-induced NF-κB activation owing to its antioxidant property. Our hypothesis is that silica-mediated free radical reactions play a major role in the mechanism of silica-induced silicosis and carcinogenesis. Based on this hypothesis, our therapeutic strategy to prevent or attenuate silica-induced cellular injury is to develop proper antioxidants and chelators to inhibit silica-mediated free radical reactions. The current undertaking represents a part of our long term study toward this hypothesis and goal. In the present study, electron spin resonance (ESR) spin trapping technique was used to measure silica-mediated free radical generation and its inhibition. Electrophoretic mobility shift assay (EMSA) was used to measured NF-κB activation and its

attenuation. The major questions to be answered in the present study are as follows: (1) do hydroxyl radicals play a role in the mechanism of silica-induced NF-κB activation? and (2) do metal ion chelators, antioxidants, SiOH blockers, and tetrandrine attenuate the silica-induced NF-κB activation?

Materials and Methods

Reagents

Crystalline silica (particle diameter, 2 to 7 μm)* was a gift. Materials purchased included: deferoxamine,† lipopolysaccharide (LPS),† phorbol 12-myristate 13-acetate (PMA),† the spin trap, 5,5-dimethyl-1-pyrroline N-oxide (DMPO),‡ (purified by charcoal decolorization and vacuum distillation so that it did not contain any ESR detectable impurities), superoxide dismutase (SOD),§ and catalase.§ Inactivated SOD and catalase were obtained by heating these enzymes at 85°C overnight. Additional materials purchased were poly(2-vinylpyridine-N-oxide) (PVPNO),‖ all other molecular biology agents,¶ and [^{32}P]-dCTP.**

Free Radical Measurements

The ESR spin trapping technique[28,29] was used to detect short-lived free radical intermediates. This technique involves the addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product, the so-called spin adduct, which can be studied by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, and the hyperfine splittings of the spin adduct are generally characteristic of the original, short-lived, trapped radical. This method is specific and sensitive and is considered to be the best one for detection and identification of free radical generation. Hyperfine splittings were measured (to 0.1 G) directly from magnetic field separations using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as standards. The relative radical concentration was estimated by multiplying half of the peak height by $(\Delta H_{pp})^2$ (where ΔH_{pp} represents peak-to-peak width). All ESR measurements were conducted using a Varian E4 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured using potassium tetraperoxochromate (K_3CrO_8) and DPPH as reference standards. Reactants were mixed in test tubes to a total final volume of 450 μl.

Cell Line and Cell Culture

Mouse macrophage cell line RAW 264.7 cells were obtained from American Type Culture Collection (ATCC)†† The cells were maintained in Dulbecco's modified Eagle medium (DMEM)‡‡ supplemented with 10 percent fetal bovine serum, 2 mM glutamine, and 1000 units/ml penicillin-streptomycin. For stimulation assay, 5×10^6 RAW 264.7 cells were seeded into 6-well culture plates in 5 ml medium and stimulated with 100 μg/ml silica and indicated agents for 6 hours.

Isolation of Alveolar Macrophages

Alveolar macrophages were harvested from male pathogen-free Sprague-Dawley rats by bronchopulmonary lavage using a calcium- and magnesium-free Hank's balanced salt solution. Macrophages from ten 8-ml lavages were sedimented by centrifugation at 500 *g* for 5 min at 2°C and suspended in hydroxyethylpiperazine ethanesulphonic acid (HEPES)-buffered medium containing 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, and 5

* U.S. Silica Corp., Berkeley Springs, WV.
† Sigma Chemical Co., St. Louis, MO.
‡ Aldrich Chemical Co., Milwaukee, WI.
§ Boehringer Mannheim, Indianapolis, IN.
‖ Polysciences Inc., Warrington, PA.
¶ Promega, Madison, WI.
** DuPont NEN, Madison, WI.
†† Rockville, MD.
‡‡ Mediatech, Washington, DC.

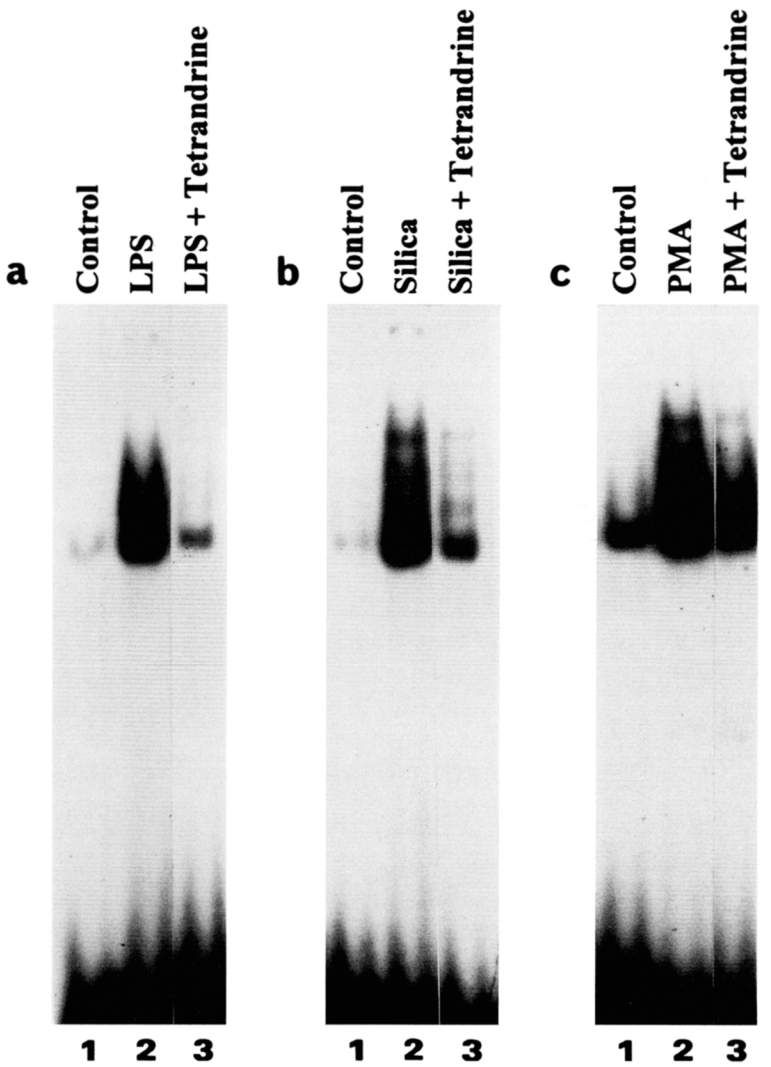

FIGURE 1. Induction of deoxyribonucleic acid (DNA) binding activity of nuclear transcription factor κB (NF-κB) protein by lipopolysaccharide (LPS) and silica and the effect of tetrandrine. The RAW 264.7 cells were adjusted to a density of 5×10^6/ml and treated for 6 h with different stimuli, then subjected to extraction of the nuclear proteins as described in the Materials and Methods. The DNA binding activity of the NF-κB protein was detected with a probe of ^{32}P-labeled double-stranded NF-κB binding oligonucleotide by electrophoretic mobility shift assay (EMSA). (a) Lane 1, untreated cells; lane 2, cells + 5 μg/ml LPS; lane 3, cells + 5 μg/ml LPS + 150 μM tetrandrine. (b) Lane 1, untreated cells; lanes 2, cells + 100 μg/ml silica; lanes 3, cells + 100 μg/ml silica + 150 μM tetrandrine. (c) Lane 1, untreated cells; lane 2, cells + 10 nM phorbol 12-myristate 13-acetate (PMA); lane 3, cells + 10 nM PMA + 150 μM tetrandrine.

mM glucose (pH 7.4). Cell viability counts were made using the trypan blue dye exclusion procedure.

NUCLEAR EXTRACTS

Nuclear extracts were prepared by a modified method of Sun et al.[30] The RAW 264.7 cells were cultured in 6-well plates at 2×10^6 cells/ml for 3 days. The medium then was replaced with fresh material and cultured with 100 μg/ml of silica combined with or without other agents, as indicated, for 6 h. At the end of culture, the cells were harvested and resuspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 M ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) for 10 min on ice, then vortexed for 10 sec. Nuclei were pelletted by centrifugation at 12,000 × *g* for 20 sec and were resuspended in buffer C (20 mM HEPES, pH 7.6, 25 percent glycerol, 0.4 NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 12,000 × *g* for 2 min and stored at −70°C.

Electrophoretic Mobility Shift Assay

The electrophoretic mobility shift assay (EMSa) is the method commonly used to measure NF-κB activation. The intensity of the band was determined by density photometer and used for relative quantitation. The preparation of ^{32}P-labeled double-stranded oligonucleotide containing NF-κB consensus sequence was performed as previously described.[14] Briefly, single-stranded deoxyribonucleic acid (DNA) was synthesized based on the κB-site in NF-κB2 (p100) gene promoter using a Millipore Cyclone Plus automated synthesizer. To prepare double-stranded DNA, the first strand DNA was annealed with a complementary decameric primer to its 3′-tail in 2 × anneal buffer. The second strand was extended with DNA polymerase Klenow fragment in a reaction mixture containing 250 μCi [^{32}P] deoxycytosine triphosphate (dCTP) and 5 mM adenosine triphosphate (dATP), quanosine triphosphate (dGTP), and thymidine triphosphate (dTTP). For EMSa, 4 μg of nuclear extract was mixed with the labeled double-stranded probe and incubated at room temperature for 30 min. The reaction solution was electrophoresed on native 6 percent polyacrylamide gel in 0.25 × TBE buffer for 2 to 3 h.

For all these described experiments, at least three parallel runs were performed for each sample.

Results

The mouse macrophage cell line RAW 264.7 cells were used to detect NF-κB activation by silica and other reagents. The cells were exposed for 6 h, and NF-κB was analyzed in the nuclear extracts. As shown in figure 1a, lane 1, the untreated cells did not exhibit any NF-κB activity. Upon treatment with LPS, the cells showed enhanced NF-κB binding activity (figure 1a, lane 2). As a negative particle control, TiO_2 did not exhibit any observable enhancement of NF-κB binding activity (data not shown). Tetrandrine inhibited LPS-induced NF-κB activation (figure 1a, lane 3). In figure 1b, lane 1 again are shown the untreated cells as a control. Silica induced significant NF-κB activation (figure 1b, lane 2). Tetrandrine inhibited silica-induced NF-κB activation (figure 1b, lane 3). Similar experiments were carried out using PMA as a stimulant. As shown in figure 1c, lanes 1–3, PMA also induced NF-κB activation and tetrandrine exhibited an inhibitory effect. In figure 2 is shown the concentration dependence of tetrandrine's inhibition on NF-κB activation induced by LPS, silica, and PMA. As shown in figure 2, tetrandrine caused a dose-dependent inhibition, and the degree of the effect was in the order of LPS > silica ≈ PMA.

The effect of catalase on silica-induced NF-κB activation was evaluated to study the role of H_2O_2. As shown in figure3a, catalase caused a dose-dependent inhibition of silica-induced NF-κB activation. In contrast, SOD enhanced silica-induced NF-κB activation (figure 3b). A stronger enhancement effect was observed at a higher SOD concentration. Inactivated catalase and SOD did not exhibit any observable effects (data not shown). The metal chelator, deferoxamine, also inhibited

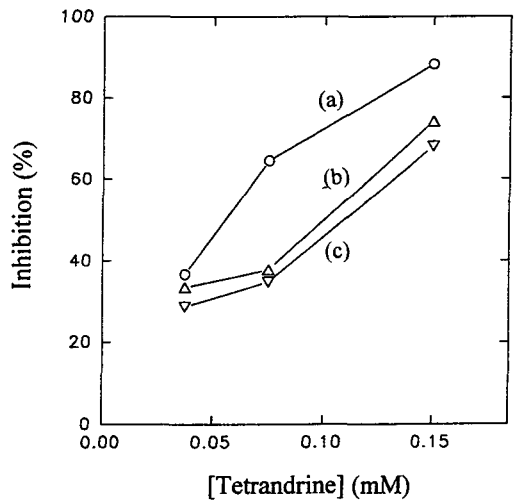

FIGURE 2. Induction of deoxyribonucleic acid (DNA) binding activity of nuclear transcription factor κB (NF-κB) protein by lipopolysaccharide (LPS), silica and phorbol 12-myristate 13-acetate (PMA) and effect of tetrandrine. (a) LPS, (b) silica and (c) PMA. Other experimental conditions are the same as those in Figure 1. The DNA binding activity of NF-κB was quantified by measuring the band intensity using density photometer.

silica-induced NF-κB activation (figure 3c, lanes 1, 2, and 3). Deferoxamine itself did not cause any NF-κB activation (figure 3c, lane 4). The involvement of iron-catalyzed reactions in the NF-κB activation is further implicated by the ability of Fe(II) and not Fe(III) to induce NF-κB activation in the order of Fe(II) > Fe(III) (figure 3d).

The effects of the antioxidant, ascorbate, and the ·OH radical scavenger, formate, were also tested for their effect on NF-κB activation induced by both LPS and silica. As shown in figure 4a, ascorbate did not show any significant inhibitory effect on LPS-induced NF-κB activation. A weak inhibitory effect was observed for formate (figure 4b). In contrast, both ascorbate and formate significantly inhibited silica-induced NF-κB activation (figure 5).

A silanol (SiOH) blocker, PVPNO was used to test for a possible role of SiOH in the

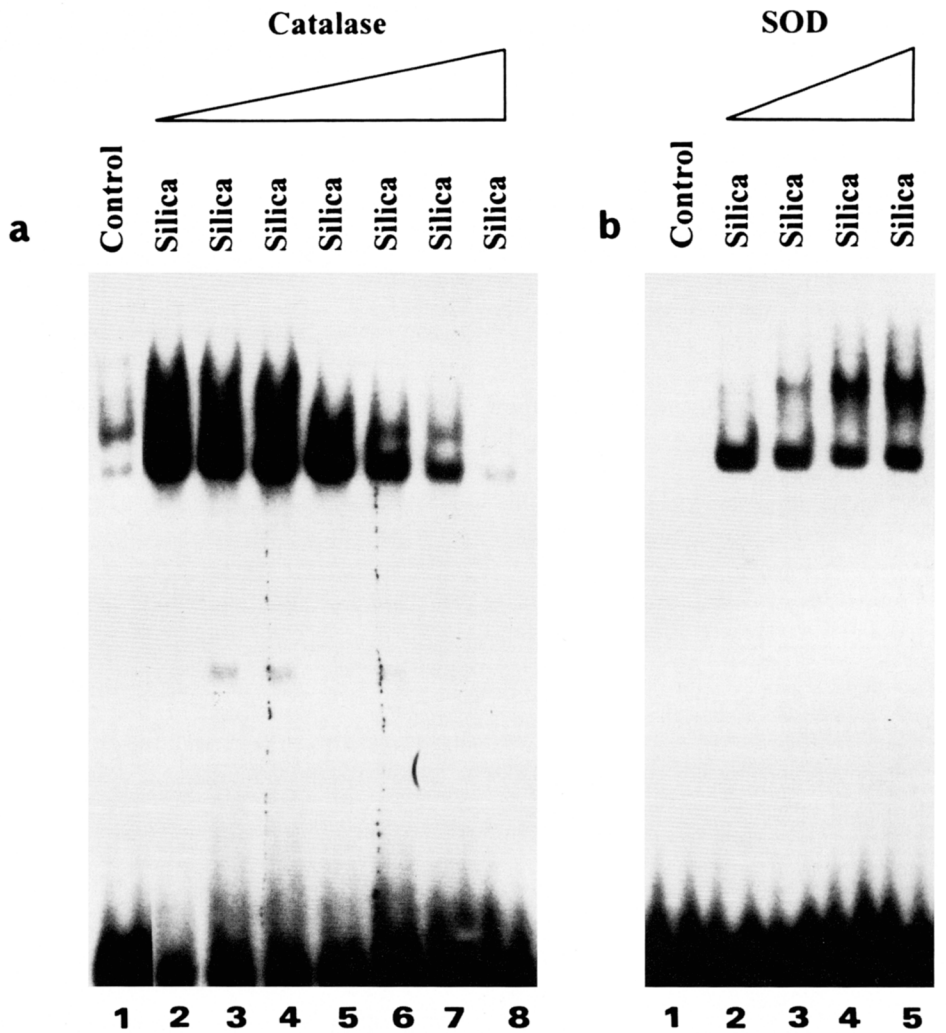

FIGURE 3. (a) Effect of catalase on silica-induced nuclear transcription factor κB (NF-κB) activation. Lane 1, untreated cells (5×10^6/ml); lane 2, cells + 100 μg/ml silica; lane 3, cells + 100 μg/ml silica + 1,250 units/ml catalase; lane 4, cells + 100 μg/ml silica + 2,500 units/ml catalase; lane 5, cells + 100 μg/ml silica + 5,000 units/ml catalase; lane 6, cells + 100 μg/ml silica + 10,000 units/ml catalase; lane 7, cells + 100 μg/ml silica + 20,000 units/ml catalase; lane 8, cells + 100 μg/ml silica + 40,000 units/ml catalase. (b) Effect of superoxide dismutase (SOD) on silica-induced NF-κB activation. Lane 1, untreated cells (5×10^6/ml); lane 2, cells + 100 μg/ml silica; lane 3, cells + 100 μg/ml silica + 160 units/ml SOD; lane 4, cells + 100 μg/ml silica + 640 units/ml SOD; lane 5, cells + 100 μg/ml silica + 1,280 units/ml SOD. →

mechanism of silica-induced NF-κB activation. As shown in figure 6, PVPNO caused a dramatic inhibition of NF-κB activity.

Similar to those outlined previously, silica is able to cause NF-κB activation in primary macrophage cells. Formate, deferoxamine and PVPNO inhibited the activation (data not shown).

Electron spin resonance spin trapping methodology was used to study the silica-mediated free radical generation from H_2O_2 and its possible inhibition by deferoxamine and PVPNO. In figure 7a is shown a typical ESR spectrum obtained from a mixture of silica particles and H_2O_2 in the presence of the spin trapping agent, DMPO. The spectrum consists of a 1:2:2:1 quartet with hyperfine splittings of $a_N = a_H = 14.9$ G. This spectrum was assigned to DMPO/·OH as reported earlier.[7,31] Addition of deferoxamine decreased the DMPO/·OH spin adduct signal (figure 7b). A similar result was obtained for PVPNO (figure 7c).

In table I are shown the relative ESR signal intensities obtained from a mixture of silica particles, H_2O_2, DMPO, and RAW cells in the presence of various inhibitors. As shown in this table, deferoxamine, formate, and PVPNO significantly reduce the free radical generation, demonstrating that the inhibitory effects on radi-

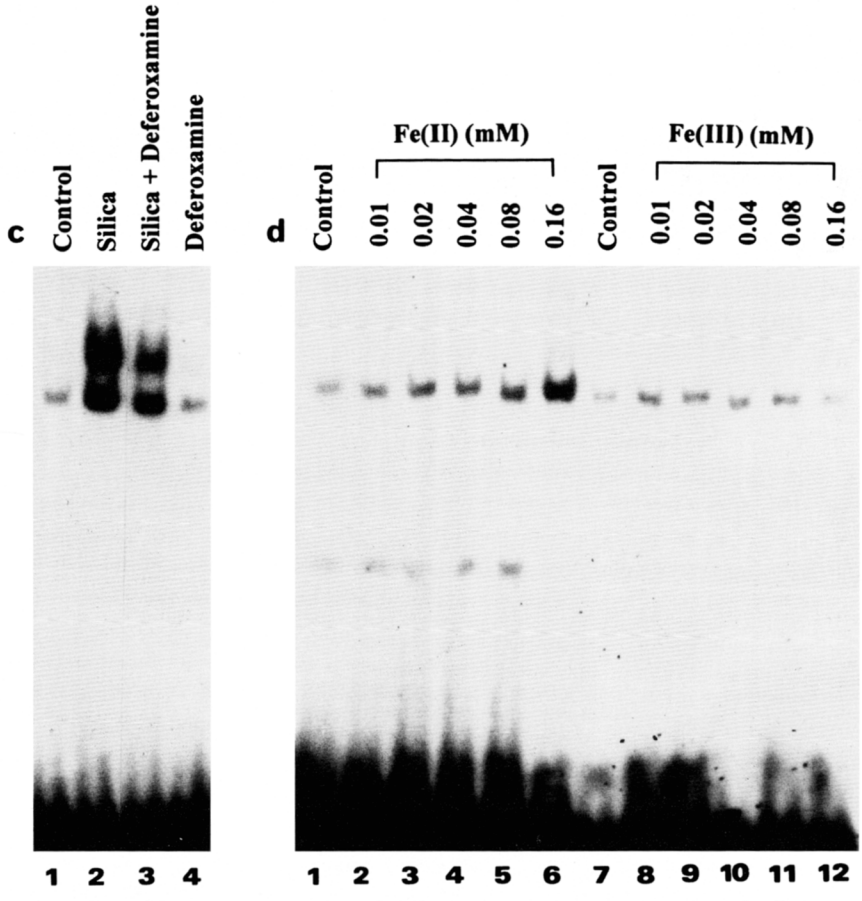

FIGURE 3. (c) Effect of deferoxamine on silica-induced NF-κB activation. Lane 1, untreated cells (5×10^6/ml); lane 2, cells + 100 μg/ml silica; lane 3, cells + 100 μg/ml silica + 1.6 mM deferoxamine; lane 4, cells + 1.6 mM deferoxamine. (d) NF-κB activation by Fe(II) and Fe(III). Lane 1, untreated cells (5×10^6/ml); lane 2, cells + 0.01 mM Fe(II); lane 3, cells + 0.02 mM Fe(II); lane 4, cells + 0.04 mM Fe(II); lane 5, cells + 0.08 mM Fe(II); lane 6, cells + 0.16 mM Fe(II), lane 7, untreated cells; lane 8, cells + 0.01 mM Fe(III); lane 9, cells + 0.02 mM Fe(III); lane 10, cells + 0.04 mM Fe(III); lane 11, cells + 0.08 mM Fe(III); lane 12, cells + 0.16 mM.

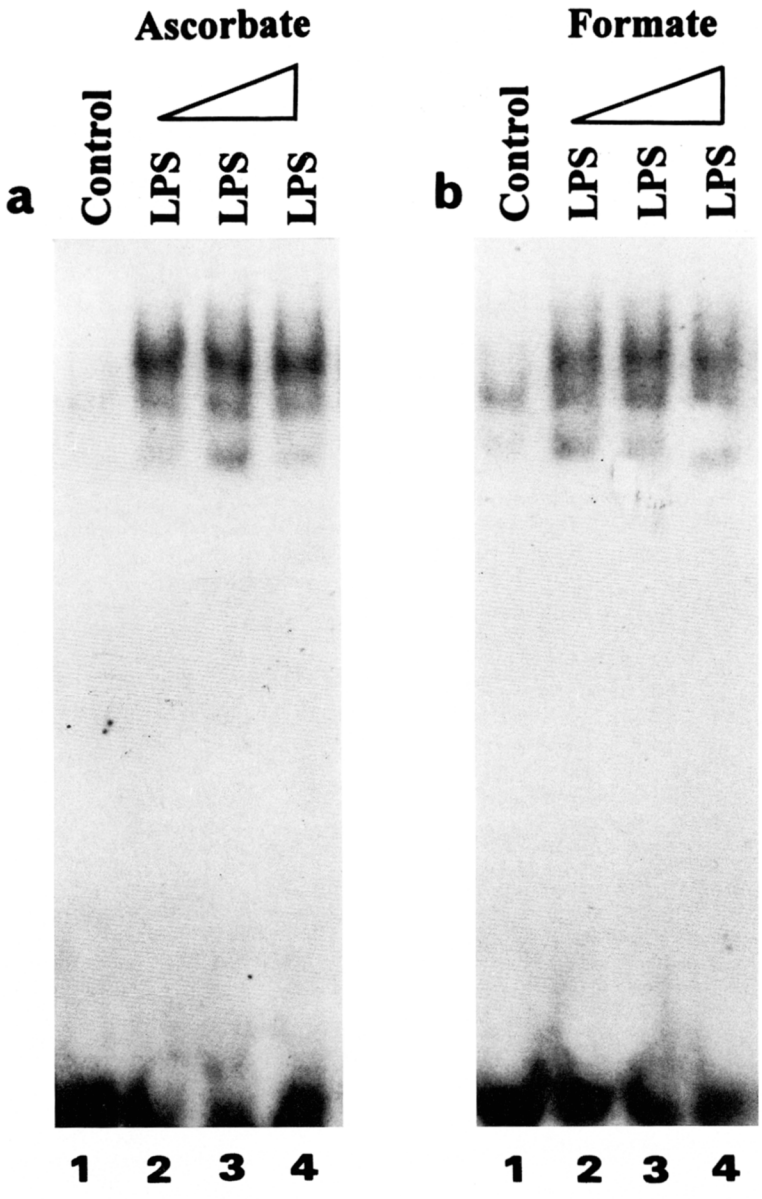

FIGURE 4. (a) Effect of ascorbate on lipopolysaccharide (LPS)-induced nuclear transcription factor κB (NF-κB) activation. Lanes 1, cells (5×10^6/ml) + 5 μg/ml LPS; lane 3, cells + 5 μg/ml LPS + 0.15 mM ascorbate; lane 4, cells + 5 μg/ml LPS + 0.8 mM ascorbate. (b) Effect of formate on LPS-induced NF-κB activation. Lanes 1, untreated cells (5×10^6/ml); lane 2, cells + 5 μg/ml LPS; lane 3, cells + 5 μg/ml LPS + 0.625 mM formate; lane 4, cells + 5 μg/ml LPS + 2.5 mM formate.

cal generation observed in non-cellular system also occur in the presence of RAW cells.

Discussion

This study has demonstrated that silica particles are able to activate NF-κB and ·OH radicals may play a key role in silica-induced NF-κB activation. The following experimental observations support this conclusion: (a) Silica is able to generate ·OH radical in the presence and absence of H_2O_2 as demonstrated by spin trapping measurements in earlier[7,31] and present studies: (b) Catalase, whose function is to remove H_2O_2 blocked the NF-κB activation. (c) Superoxide dismutase, whose function is to remove O_2^-, and generate H_2O_2, exhibited an opposite effect. It may be noted that earlier studies have shown that molecular oxygen was consumed in the generation of O_2^- in silica suspension.[11,32] Silica-induced DNA damage was inhibited in an argon atmosphere, indicating that the oxygen radicals responsible for DNA damage were generated from O_2 via O_2^- and H_2O_2 as intermediates (Equation [a]).[11,32]

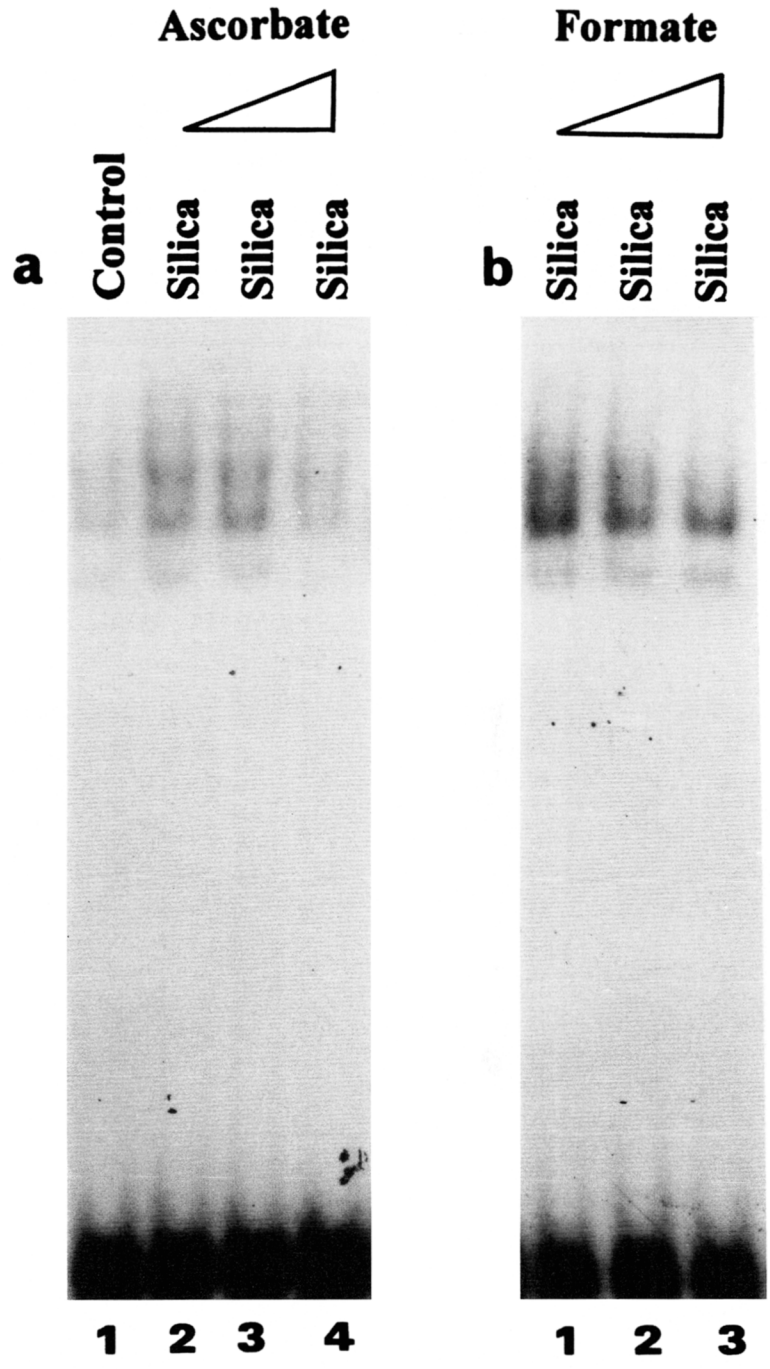

FIGURE 5. (a) Effect of ascorbate on silica-induced nuclear transcription factor κB (NF-κB) activation. Lane 1, untreated cells (5×10^6/ml); lane 2, cells + 100 μg/ml silica; lane 3, cells + 100 μg/ml silica + 0.15 mM ascorbate; lane 4, cells + 100 μg/ml silica + 0.8 mM ascorbate. (b) Effect of formate on silica-induced NF-κB activation. Lane 1, cells (5×10^6/ml) + 100 μg/ml silica; lane 2, cells + 100 μg/ml silica + 0.625 mM formate; lane 3, cells + 100 μg/ml silica + 2.5 mM formate.

$$O_2 \rightarrow O_2^- \rightarrow \cdot OH \qquad [a]$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH \qquad [b]$$

(d) Metal ions, Fe(II) and not Fe(III), enhanced the NF-κB activation. It is known that Fe(II) generates ·OH from H_2O_2 via the Fenton reaction (Equation [b]). Fe(III), on the other hand, is unable to generate ·OH radical without being first reduced to Fe(II). (e) Metal chelator, deferoxamine, also reduced the NF-κB activation. Deferox-

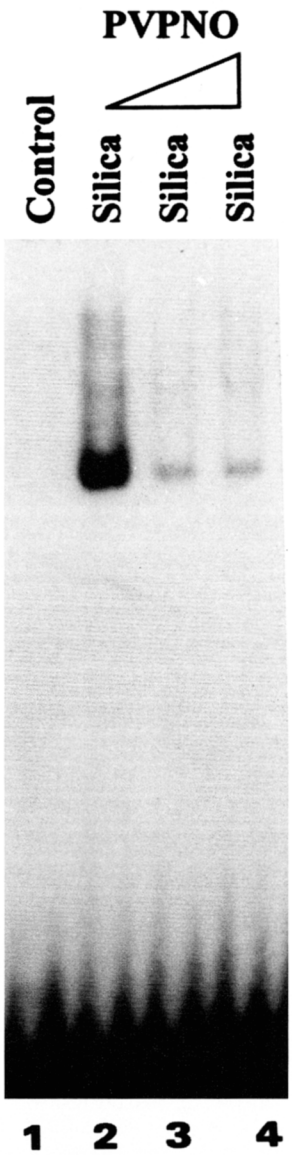

FIGURE 6. Effect of poly(2-vinylpyridine-N-oxide) [PVPNO] on silica-induced nuclear transcription factor κB (NF-κB) activation. Lanes 1, untreated cells (5×10^6/ml); lane 2, cells + 100 μg/ml silica; lane 3, cells + 100 μg/ml silica + 20 μg/ml PVPNO; lane 4, cells + 100 μg/ml silica + 50 μg/ml PVPNO.

amine chelates metal ions, such as Fe(II) or Fe(III), to make them less reactive toward H_2O_2 and thus attenuate the ·OH radical generation. (f) The antioxidant, ascorbate, and an ·OH radical scavenger, formate, inhibited the NF-κB activation. (g) Tetrandrine, which has been reported to inhibit H_2O_2 release from macrophage,[33] and also function as an ·OH radical scavenger,[27] inhibited the NF-κB activation.

As stated in the Introduction, silica is a fibrogenic agent owing to its ability to elicit resident macrophages to release inflammatory mediators and cytokines which can promote fibroblast proliferation and collagen deposition. It has been suggested that NF-κB activation is crucial in the cytoplasmic/nuclear signaling when cells are exposed to injury-producing conditions.[34] The NF-κB serves as a second messenger to induce a series of cellular genes in response to an environmental perturbation. Among cellular genes regulated by NF-κB are several proinflammatory and cytotoxic cytokines, including IL-2, IL-6 and TNF-α.[17] The NF-κB activates these genes by acting as a transcriptional factor and binding to the NF-κB consensus sequence in their promoters. Reactive oxygen intermediates have been suggested to be mediators of NF-κB activation from a variety of initiators such as Cr(VI) and phorbal esters.[21,22,35,36] Since silica

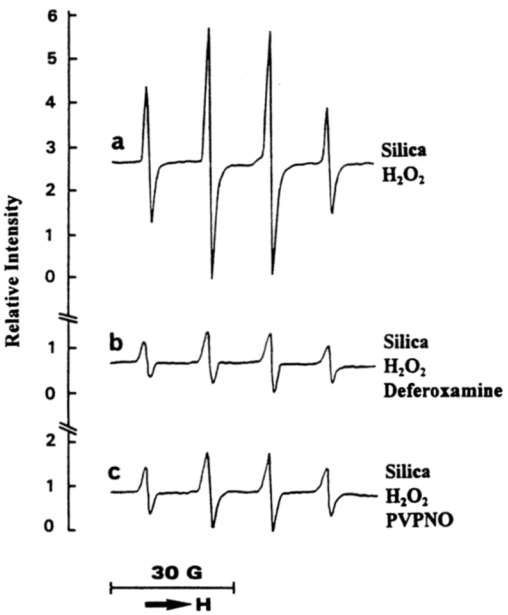

FIGURE 7. (a) electron spin resonance (ESR) spectrum recorded 2 min after mixing 100 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO), 100 mg/ml silica and 2 mM H_2O_2 in a pH 7.4 phosphate-buffered solution. (b) Same as (a) but with 1.5 mM deferoxamine added. (c) Same as (a) but with 100 μg/ml poly(2-vinylpyridine-N-oxide) [PVPNO] added.

TABLE I

Inhibition of ·OH Radical by Inhibitors[a]

Inhibitor	*Relative ESR Signal Intensity of DMPO/·OH*
0	1.0
Deferoxamine (1 mM)	0.24
Sodium formate (10 mM)	0.15
PVPNO (100 μg/ml)	0.35

[a]The reaction mixture contained 100 mM DMPO, 0.5 mM H_2O_2, 100 mg/ml silica, 1 million/cells, and various inhibitors in pH 7.4 phosphate buffered solution.

particles are able to cause NF-κB activation possibly via free radical reactions, it is feasible that signals for a variety of silica-induced responses are due to a common signaling component, which is regulated by reactive oxygen species. It may be noted that a recent study has shown that N-acetyl-cysteine did not block silica-induced NF-κB activation.[13] Although N-acetyl-cysteine is considered an antioxidant, it is not an efficient ·OH scavenger and thus may not inhibit silica-induced NF-κB activation via OH initiated reactions.

The results obtained from the present study show that tetrandrine is able to inhibit silica-induced NF-κB activation. While tetrandrine has been reported to retard and reverse the fibrotic lesions of silicosis in human and in rats, its mechanism of action is unclear. As mentioned earlier, tetrandrine is an effective inhibitor of silica induced H_2O_2 production by macrophages.[33] In addition, recent studies have shown that tetrandrine is capable of scavenging OH radicals and inhibiting silica-induced lipid peroxidation.[27] Tetrandrine has been shown to be an effective inhibitor of IL-1 secretion from alveolar macrophages activated by silica or LPS.[37]

As demonstrated in the present study, tetrandrine inhibited LPS-induced NF-κB activation. It is possible that silica or LPS causes increased secretion of IL-1 or other cytokines via activation of NF-κB. It appears that the mechanism of NF-κB activation induced by LPS, although it can be inhibited by tetrandrine, is different from that by silica. For silica, both ascorbate and formate exhibited inhibitory effect. For LPS, the effect is weak. While it is likely that one of the steps involves antioxidant activity of tetrandrine toward ·OH radical, other mechanism may also exist. For example, tetrandrine increases intracellular calcium concentration by its channel blocker effect and inhibiting the production of NO in activated macrophages.[38] The latter is specially important since the production of NO has a direct effect on silica-induced NF-κB activation.[14] It may be noted that although other transcription factors, such as AP-1, are also important, NF-κB may be particularly relevant in inflammatory, immune and acute phase response after silica exposure.

In the present study, deferoxamine was shown to reduce silica-mediated ·OH radical generation and attenuate silica-induced NF-κB activation. Deferoxamine is widely used for the prevention and treatment of iron overload.[39,40] It inhibits OH radical generation from H_2O_2 by transition metals, including Fe, Cr and V.[41,42] A large dose of deferoxamine (50 mg/kg/day) can be safely injected into humans.[40] Thus, further investigation on the use of deferoxamine or other metal chelators may offer a possible preventative strategy against silica-induced fibrosis and carcinogenesis.

Another important result obtained in the present study is that PVPNO inhibited silica-induced NF-κB activation. SiOH groups on the silica surface have been considered to be involved in silica-induced cellular damage.[4,5,9,43] Chemical modifications of the silica surface can be used to reduce toxicity *in vitro* and fibrosis *in vivo*. It is known that when silica particles are exposed to water, surface silicon-oxygen bonds (Si-O) are hydrated, resulting in the formation of SiOH groups; PVPNO is able to bind to SiOH groups. It has been reported to inhibit silica-induced toxicity,[44] to decrease and delay the development of silicosis in experimental animals and in

humans,[45,46] and to block the interaction of the silica surface with the phosphate groups of DNA *in vitro*.[16] It has also been reported that PVPNO inhibited silica-induced production of oxygen radicals in cells.[47,48] The ESR spin trapping measurements in the present study have demonstrated the inhibitory effect of PVPNO on ·OH radical generation by silica plus H_2O_2, implying the involvement of SiOH group in the generation of ·OH radical from H_2O_2 by silica.

It may be noted that the *in vitro* model of transcription factor activation may be related to *in vivo* effects in the lung. However, it is difficult to infer from *in vitro* studies whether or not *in vivo* effects are similar in the presence of biological defense mechanism. The major goal of the present study is to examine whether or not silica can induce NF-κB activation via free radical reactions. This study is a part of our long term goal of understanding the role of silica-mediated free radical reactions in the mechanisms of silica-induced silicosis and carcinogenesis and the possible prevention. Based on the findings obtained in this *in vitro* study, plans have been formed to carry out *in vivo* exposure experiments to examine whether or not silica is able to induce similar NF-κB activation and whether or not certain antioxidants and chelators can block or attenuate the NF-κB activation.

On the basis of the previous results, the following conclusions were derived: (a) silica induces NF-κB activation in macrophages and silica-mediated free radical reactions may play an important role; (b) silica-induced NF-κB activation can be attenuated by the antioxidant (ascorbate), ·OH radical scavenger (formate), metal chelator (deferoxamine), and SiOH blocker (PVPNO); (c) tetrandrine reduced NF-κB activation by silica, LPS and PMA; and (d) both deferoxamine and PVPNO inhibited the ·OH radical generation by silica from H_2O_2. The results may not only help improve the understanding of silica-induced fibrosis and carcinogenesis but also help develop a better therapeutic agent to prevent or attenuate silica-induced fibrogenic and carcinogenic effects.

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