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Jihee Lee Kang, Ewha Womans, Young Hyun Go, Kyu Chung Hur, Vincent Castranova

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SILICA-INDUCED NUCLEAR FACTOR-κB ACTIVATION: INVOLVEMENT OF REACTIVE OXYGEN SPECIES AND PROTEIN TYROSINE KINASE ACTIVATION

Jihee Lee Kang

Department of Physiology, College of Medicine, Division of Cell Biology, Ewha Medical Research Center and Center for Cell Signaling Research, Ewha Womans University, Seoul, Korea

Young Hyun Go

Department of Physiology, College of Medicine, Ewha Womans University, Seoul, Korea

Kyu Chung Hur

Department of Biological Science, College of Natural Science and Center for Cell Signaling Research, Ewha Womans University, Seoul, Korea

Vincent Castranova

Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA

Nuclear factor-κB (NF-κB) is a multiprotein complex that may regulate a variety of inflammatory cytokines involved in the initiation and progression of silicosis. The present study documents the ability of in vitro silica exposure to induce DNA-binding activity of NF-κB in a mouse peritoneal macrophage cell line (RAW264.7 cells) and investigates the role of reactive oxygen species (ROS) and/or protein tyrosine kinase in this activation. In vitro exposure of mouse macrophages to silica (100 μg/ml) resulted in a twofold increase in ROS production, measured as the generation of chemiluminescence (CL), and caused activation of NF-κB. Silica-induced CL was inhibited 100% by superoxide dismutase (SOD) and 75% by catalase, while NF-κB activation was inhibited by a variety of antioxidants (catalase, superoxide dismutase, α-tocopherol, pyrrolidine dithiocarbamate, or N-acetylcysteine). Further evidence for the involvement of ROS in NF-κB activation is that 1 mM H_2O_2 enhanced NF-κB/DNA binding and that this activation was inhibited by catalase. Specific inhibitors of protein tyrosine kinase, such as herbimycin A, genistein, and AG-494, prevented NF-κB activation in silica-treated cells. Genistein and AG-494 also reduced NF-κB activation in H_2O_2-treated cells. Results con-

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Address correspondence to Jihee Lee Kang, MD, PhD, Department of Physiology, College of Medicine, Ewha Womans University, 911-1 Mok-6-dong, Yangcheon-ku, Seoul 158-056, Korea. E-mail: jihee@mm.ewha.ac.kr

firm that tyrosine phosphorylation of several cellular proteins (approximate molecular mass of 39, 58–70, and 103 kD) was increased in silica-exposed macrophages and that genistein inhibited this silica-induced phosphorylation. In contrast, inhibitors of protein kinase A or C, such as H89, staurosporin, calphostin C, and H7, had no marked inhibitory effect on silica-induced NF-κB activation. The results suggest that ROS may play a role in silica-induced NF-κB activation in macrophages and that phosphorylation events mediated by tyrosine kinase may be involved in this activation.

Silicosis is an inflammatory and fibrotic lung disease caused by inhalation and deposition of silica dust (Lee, 1995). Interaction between silica and pulmonary alveolar macrophages is believed to play a crucial role in the development of silicosis (Lapp & Castranova, 1993). Upon contact with silica, alveolar macrophages produce a variety of inflammatory and fibrogenic factors, such as reactive oxygen species (ROS), lipid mediators, cytokines, chemotactic factors, and macrophage-derived growth factors, which have been proposed to be involved in the initiation and progression of silicosis (Lapp & Castranova, 1993; Lee, 1995; Driscoll & Guthrie, 1997).

Recently, it has been reported that silica can cause activation of nuclear transcription factor NF-κB (Chen et al., 1995a). NF-κB is a multiprotein complex that may regulate a variety of inflammatory cytokines, adhesion molecules, acute-phase proteins, and other transcription factors (Schmidt et al., 1995). In resting cells, NF-κB is predominantly a p50–p65 heterodimer, which is retained in the cytoplasm complexed to an inhibitory protein, IκB. Upon stimulation with diverse stimulants, such as lipopolysaccharide (LPS), tumor necrosis factor (TNF), interleukin-1 (IL-1), T-cell mitogens, ultraviolet (UV) light, oxidants, γ rays, asbestos, or nickel, IκB is phosphorylated and released from the NF-κB complex (Schmitz, 1995). The NF-κB then can translocate to the nucleus, where it binds to DNA at specific NF-κB binding regions.

A mode for NF-κB activation involving phosphorylation of IκB-α or p50 and p65 has been proposed by Mahon and O'Neil (1995) and Naumann and Scheidereit (1994). There are several candidate kinases that may be involved in the activation of NF-κB. It has been observed that IκB-α and -β could be phosphorylated in vitro by treatment with serine/threonine protein kinases A or C and heme-activated kinase (Ghosh & Baltimore, 1991; Link et al., 1992; Kerr et al., 1991). The involvement of tyrosine kinase in NF-κB activation is implicated by the observation that herbimycin A, a specific tyrosine kinase inhibitor, blocks NF-κB activation by modifying p50 subunits of the NF-κB complex (Chou-chi et al., 1994; Mahon & O'Neill, 1995).

ROS, such as superoxide anion, hydrogen peroxide, and hydroxyl radical, affect various molecular components of the cell, such as fatty acids, proteins, and DNA (Halliwell & Gutteridge, 1990), and excessive ROS have been associated with membrane damage and cell lysis (Castranova, 1994). Besides these well-known toxic effects, sublethal concentrations of ROS have been suggested to be responsible for the modulation of various cellu-

lar functions, including gene expression (Lo & Cruz, 1995; Devary et al., 1991), transcription factor activation (Nose et al., 1991), DNA synthesis (Schreck et al., 1991), and cellular proliferation (Murrell et al., 1990). Recently, molecular approaches demonstrated that ROS can directly affect the cellular signaling apparatus and, consequently, the control of gene expression (Remacle et al., 1995). Furthermore, a variety of antioxidants, such as pyrrolidine dithiocarbamate (PDTC), *N*-acetylcysteine (NAC), and vitamin E (Vit. E), have been reported to inhibit the expression of several genes under the regulation of NF-κB (Marui et al., 1993; Mendez et al., 1995) and activation of NF-κB (Remacle et al., 1995) in various cell systems and tissues, suggesting involvement of ROS in signal transduction associated with NF-κB activation.

In this study, the role of ROS and/or protein tyrosine kinase activation in DNA-binding activity of NF-κB in a mouse peritoneal macrophage cell line (RAW264.7 cells) was studied after silica stimulation.

METHODS

Reagents

Crystalline silica (Min-U-Sil, particle size <5 μm) was obtained from U.S. Silica Corporation (Berkeley Springs, WV). Prior to use, the silica samples were sterilized by heating at 160°C for 90 min in a dry oven and dispersed in Dulbecco's modified Eagle medium (DMEM, Mediatech, Washington, DC) with supplements just before addition to culture plates. Catalase, superoxide dismutase (SOD), vitamin E in the form of α-tocopherol succinate, pyrrolidine dithiocarbamate (PDTC), *N*-acetylcysteine (NAC), genistein, and H7 were purchased from Sigma Chemical Company (St. Louis, MO). Herbimycin A, AG494, H89, staurosporine, and calphostin C were purchased from Biomol Company (Plymouth Meeting, PA). DNA polymerase and dNTP were purchased from Life Technologies (Gaithersburg, MD). Anti-phosphotyrosine (PY-20) was obtained from Transduction Laboratories (Lexington, KY).

Cell Line and Cell Culture

RAW264.7 cells, from a mouse peritoneal macrophage cell line, were obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in DMEM (Mediatech, Washington, DC) supplemented with 5% fetal bovine serum (FBS, HyClone, Logan, UT), 2 m*M* glutamine, and 1000 Units/ml penicillin–streptomycin.

Measurement of Chemiluminescence Generation

The ability of silica to produce reactive oxygen species (ROS) by macrophages (RAW264.7 cells) was determined by measuring cellular chemiluminescence (CL) using a luminometer (Berthold, model LB9505AT, Wildbad,

West Germany). Briefly, cells were washed once with phosphate-buffered saline (145 m*M* NaCl, 5 m*M* KCl, 1.9 m*M* NaH_2PO_4, 9.35 m*M* Na_2HPO_4, 5.5 m*M* glucose, pH 7.4), centrifuged at 500 × g and 4°C for 5 min, and resuspended in HEPES-buffered medium (145 m*M* NaCl, 5 m*M* KCl, 10 m*M* NaHEPES, 5.5 m*M* glucose, 1 m*M* $CaCl_2$, pH 7.4). Cell counts were determined using an electronic cell counter equipped with a cell sizing attachment. Macrophages (2×10^6 cells/ml) were preincubated for 10 min at 37°C in a shaking water bath and then stimulated with silica (100–1000 μg/ml) in the presence or absence of SOD (615 U/ml) or catalase (3750 U/ml). Chemiluminescence was monitored continuously at 37°C for 10 min in the presence of 8 μg% luminol (Sigma Chemical Company, St. Louis, MO). The integral of counts per minute (cpm) versus time was used to compare the total CL between samples.

Nuclear Extracts

Nuclear extracts were prepared by a modified method of Sun et al. (1994). RAW 264.7 cells were cultured in 6-well plates at 5×10^6 cells/ml for 3 d; then the medium was replaced with fresh medium and cultured with 100 μg/ml of silica in the absence or presence of other agents as indicated for 4 h. At the end of culture, the cells were harvested and resuspended in hypotonic buffer A [100 m*M* HEPES, pH 7.9, 10 m*M* KCl, 0.1 *M* ethylenediamine tetraacetic acid (EDTA), 0.5 m*M* dithiothreitol, 1% nonidet P-40, and 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF)] for 10 min on ice, then vortexed for 10 s. Nuclei were pelleted by centrifugation at 12,000 × g for 30 s and were resuspended in buffer C (20 m*M* HEPES, pH 7.9, 20% glycerol, 0.42 *M* NaCl, 1 m*M* EDTA, and 0.5 m*M* PMSF) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 10,000 × g for 2 min and stored at –70°C.

Electrophoretic Mobility Shift Assay

Binding reaction mixtures (10 μl) containing 5 μg (4 μl) nuclear extract protein, 2 μg poly (dI-dC) poly (dI-dC) (Sigma Co., St. Louis. MO), and 40,000 cpm of ^{32}P-labeled probe in binding buffer (4 m*M* HEPES pH 7.9, 1 m*M* $MgCl_2$, 0.5 m*M* DTT, 2% glycerol, and 20 m*M* NACl) were incubated for 30 min at room temperature. Protein–DNA complexes were separated on 5% nondenaturing polyacrylamide gels in 1× Tris-borate/EDTA electrophoresis buffer and autoradiographed overnight.

The oligonucleotide used as a probe for electrophoretic mobility shift assay (EMSA) was a double-stranded DNA containing NF-κB consensus sequence (5′-CCTGTGCTCCGGGAATTTCCCTGGCC-3′) labeled with [α-^{32}P]-dATP (Amersham, Buckinghamshire, UK) using DNA polymerase Klenow fragment.

Immunoprecipitation and Immunoblotting

The confluent cells grown on 100-mm plastic dishes were incubated in DMEM supplemented with 5% FBS, 2 m*M* glutamine, and 1000 U/ml

penicillin–streptomycin for 3 d. Cells then were treated with either silica (100 μg/ml) for 30 min or H_2O_2 (3 m*M*) as a positive control for 20 min in the presence or absence of genistein (74 μ*M*), and were washed with ice-cold phosphate-buffered saline (pH 7.4). The washed cells were lysed with 1 ml ice-cold lysis buffer containing 50 m*M* Tris-HCl (pH 8), 150 m*M* NaCl, 1% Nonidet P-40 (NP-40), 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 m*M* Na_3VO_4, 5 m*M* EDTA, and 1 m*M* benzamidine.

The cell lysate was centrifuged for 5 min at 13,000 × g. The resulting supernatant was incubated with 5 μg antiphosphotyrosine PY20 at 4°C for 1 h. After incubation at 4°C for 30 min with protein A- or G-conjugated sepharose (5 μg/ml) the antigen/antibody complexes were pelleted by centrifugation at 13,000 × g for 30 s. The pellet was then washed 3 times with ice-cold lysis buffer by centrifugation at 13,000 × g for 30 s, dissolved in 20 μl Laemmli's sample buffer (pH 7), and separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels (Laemmli, 1970).

The fractionated proteins on a 10% SDS–polyacrylamide gel were electrophoretically transferred onto a nitrocellulose paper as described by Towbin et al. (1979). Antibody labeling of protein bands was detected with enhanced chemiluminescence (ECL) reagents according to the supplier's protocol.

Statistics

Values were expressed as means standard errors of six or eight experiments. Data were compared with controls using one-way analysis of variance (ANOVA) and Student's *t*-test. Significance was set at $p \leq .05$.

RESULTS

The object of this investigation was to evaluate the role of ROS and/or tyrosine kinase in the silica-induced activation of NF-κB-DNA binding. In vitro exposure of macrophages to silica resulted in a concentration-dependent activation of the binding of NF-κB to DNA (Figure 1). Maximal NF-κB activation was observed after exposure to 100 μg/ml silica. Exposure of macrophages to 100 μg/ml silica also resulted in a twofold increase in ROS production, measured as the generation of chemiluminescence (CL) (Figure 2). Treatment of macrophages with either superoxide dismutase (SOD 615 U/ml) or catalase (3750 U/ml) inhibited silica-induced CL by approximately 100% and 75%, respectively. In contrast, SOD or catalase had no significant effect on basal generation of CL by unstimulated macrophages. The concentration dependence of the silica-induced increase in the generation of CL and the inhibitory effects of SOD or catalase at each concentration of silica are shown in Figure 3.

To investigate the role of ROS in silica-induced NF-κB activation, catalase or SOD was added to the cells 2 h before treatment with silica and the effects of these antioxidants on the silica-induced activation of

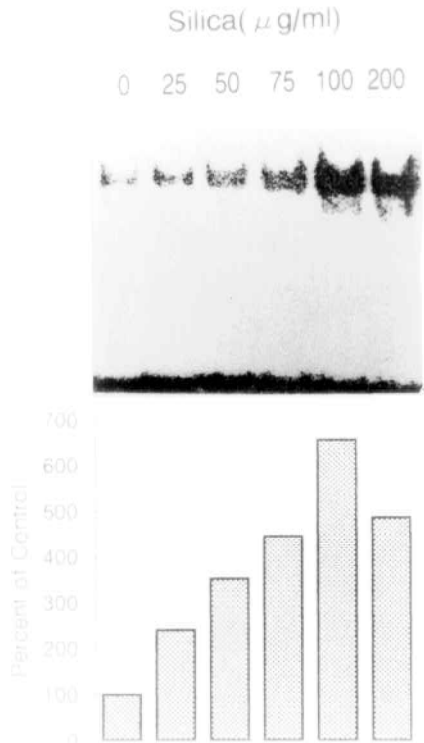

FIGURE 1. Activation of NF-κB by silica. Concentration dependence of NF-κB activation in RAW264.7 cells exposed in vitro to silica for 4 h. The results of an electrophoretic mobility shift assay are shown (upper panel). These data were quantified by densitometric analysis and are presented as a percentage of the response of unstimulated cells (lower panel).

NF-κB were then determined. A concentration-dependent inhibition of NF-κB activation was observed after SOD or catalase treatment (Figure 4). To verify that the inhibitory effects of these antioxidants are due to their ability to scavenge ROS, the effects of three other ROS scavengers, vitamin E (Vit. E), pyrrolidine dithiocarbamate (PDTC), and *N*-acetylcysteine (NAC), were examined on the silica-induced activation of NF-κB. Vitamin E (200 μg/ml), PDTC (0.2 m*M*), and NAC (1 and 10 m*M*) effectively inhibited silica-induced NF-κB activation by 100% (Figure 5).

To further study the ability of ROS to stimulate DNA binding activity of NF-κB, exogenous H_2O_2 was added to macrophages for 2 h, and the ability of H_2O_2 to stimulate DNA-binding activity of NF-κB was then analyzed. Figure 6 shows that exogenous H_2O_2 from 0.5 to 2 m*M* caused a concentration-dependent increase in NF-κB activation. The maximal response (increase by 100%) was detected at 1 m*M* H_2O_2, and catalase (12,000 U/ml) inhibited this activation of NF-κB induced by 1 m*M* H_2O_2 by 100%.

Phosphorylation events indicated by activation of protein kinases have been associated with the activation of NF-κB (Schmitz, 1995). To assess

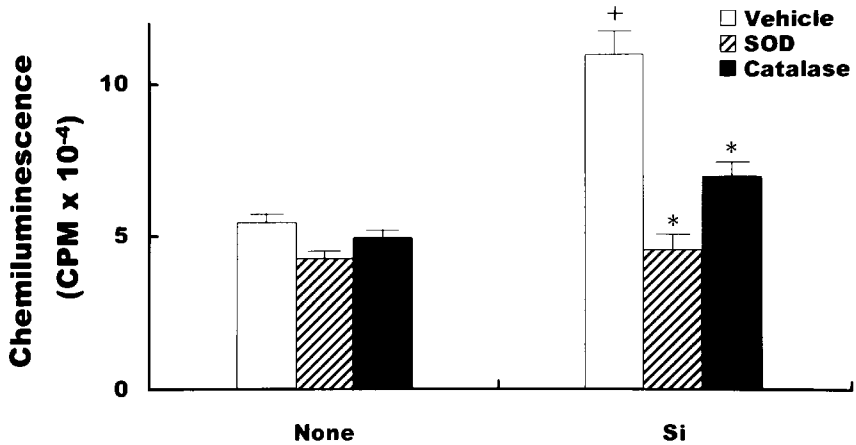

FIGURE 2. Generation of chemiluminescence from silica-stimulated RAW264.7 cells. The cells (2×10^6/ml) were preincubated at 37°C for 10 min and then stimulated with silica (100 μg/ml) in the presence or absence of SOD (615 U/ml) or catalase (3750 U/ml). Silica-stimulated chemiluminescence was expressed as the integral of cpm versus time. Values were means ± standard errors of six separate experiments. Plus sign indicates a significant increase ($p \leq .05$) from the vehicle control. Asterisk indicates a significant decrease ($p \leq .05$) compared to silica alone.

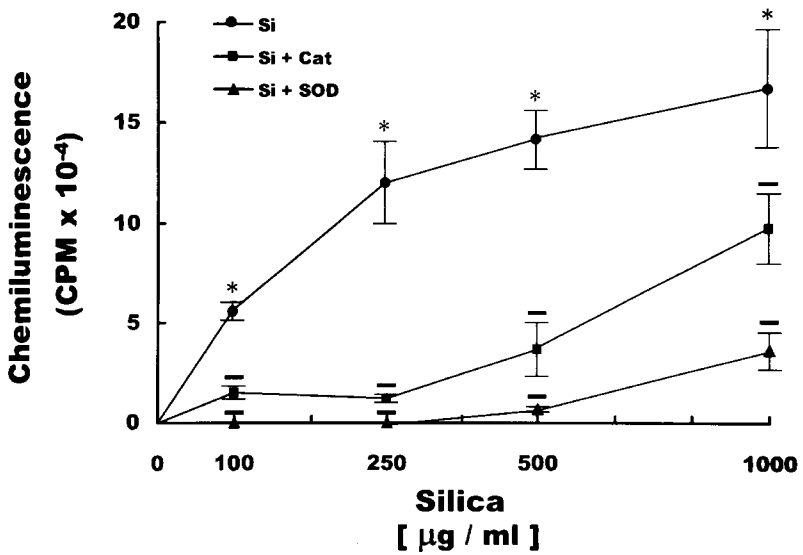

FIGURE 3. Concentration response of chemiluminescence generation from silica-stimulated RAW264.7 cells. The cells (2×10^6/ml) were preincubated at 37°C for 10 min and then stimulated with various concentrations of silica (100–1000 μg/ml) in the presence or absence of SOD (615 U/ml) or catalase (3750 U/ml). Silica-stimulated chemiluminescence was expressed as the integral of cpm versus time minus that generated from resting cells. Values were means ± standard errors of eight separate experiments. Asterisk indicates a significant increase from control, while minus sign indicates a significant decrease from silica alone ($p \leq .05$).

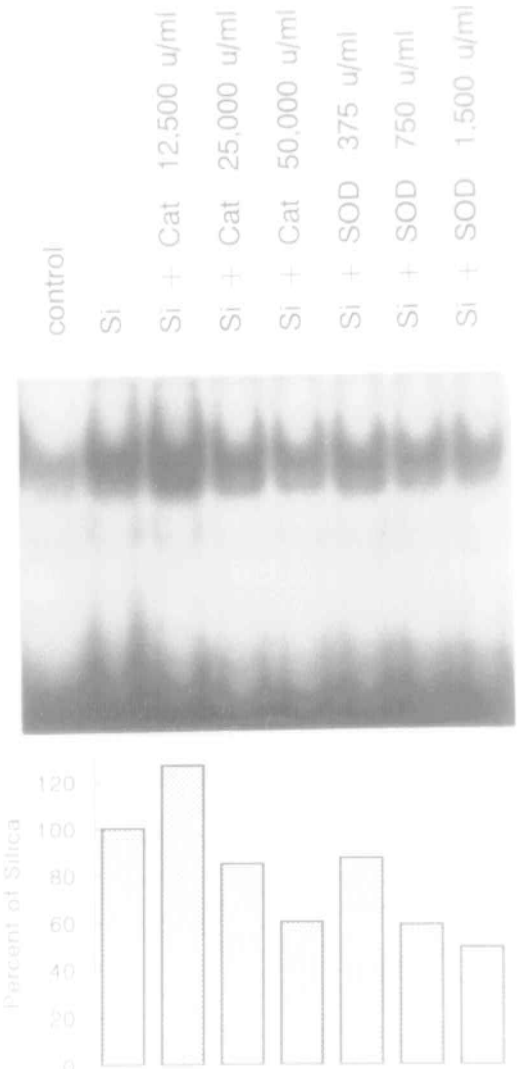

FIGURE 4. Electrophoretic mobility shift assay (EMSA) illustrating the effect of antioxidants on silica-induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with catalase or superoxide dismutase and then stimulated by silica (100 μg/ml) for an additional 4 h. The results of EMSA are shown (upper panel). These data were quantified by densitometric analysis and are presented as a percentage of the response of silica-stimulated cells (lower panel).

which protein kinase pathways may be involved in silica-induced NF-κB activation, various kinase inhibitors were added into the cells 2 h before exposure to silica. The nuclear extracts of the silica-stimulated cells were then examined for DNA-binding activity of NF-κB. Specific inhibitors of protein tyrosine kinase (PTK), such as herbimycin A (17 μ*M*), genistein (74 μ*M*), and AG494 (12 μ*M*), significantly prevented NF-κB activation in

silica-stimulated cells by 96, 85, and 90%, respectively (Figure 7). This inhibitory effect of genistein or AG494 also was demonstrated in the cells stimulated by exogenous H_2O_2 (Figure 8), suggesting that the ability of silica to turn on a PTK pathway leading to NF-κB activation may be related to ROS production in the stimulated cells.

Western blotting with anti-phosphotyrosine antibodies was employed in order to examine whether protein tyrosine phosphorylation in intact macrophages was affected by silica. Treatment of macrophages with 100 μg/ml

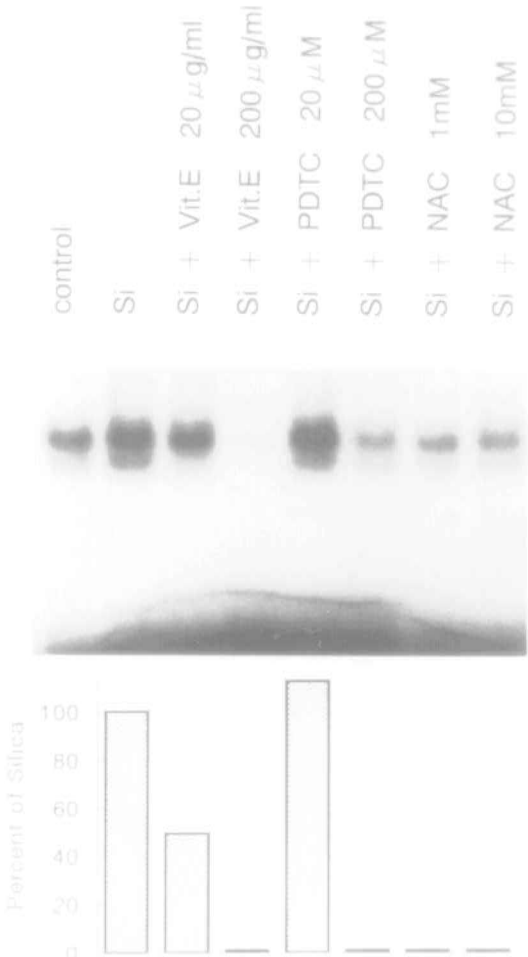

FIGURE 5. Electrophoretic mobility shift assay (EMSA) illustrating the effect of antioxidants on silica-induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with vitamin E (Vit. E), pyrrolidine dithiocarbamate (PDTC), or *N*-acetylcysteine (NAC) before stimulation by silica (100 μg/ml) for an additional 4 h. The results of EMSA are shown (upper panel). These data were quantified by densitometric analysis and are presented as a percentage of the response of silica-stimulated cells (lower panel).

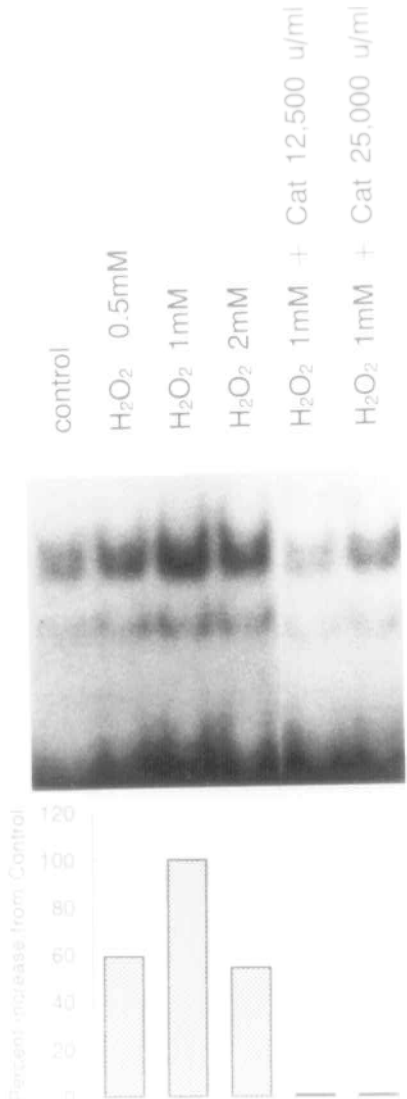

FIGURE 6. Electrophoretic mobility shift assay (EMSA) illustrating the effect of H_2O_2 on the activation of NF-κB in RAW264.7 cells. EMSA was conducted with nuclear extracts from cells treated with H_2O_2 for 2 h. The specificity of reaction was examined by addition of catalase 2 h prior to treatment with H_2O_2. The results of EMSA are shown (upper panel). These data were quantified by densitometric analysis and are presented as the percentage increase from the control response (lower panel).

silica for 30 min enhanced tyrosine phosphorylation of several proteins with molecular mass of approximately 39, 58–70, and 103 kD (Figure 9). Similar results were obtained after 20 min treatment of macrophages with 3 m*M* H_2O_2. Furthermore, pretreatment with a protein tyrosine kinase (PTK) specific inhibitor, genistein (74 μ*M*), for 2 h prevented this phos-

phorylation event in silica-treated cells. Genistein was somewhat less effective in inhibiting H_2O_2-induced protein tyrosine phosphorylation than silica-induced phosphorylation.

These data suggest involvement of protein tyrosine kinase (PTK) in silica-induced NF-κB activation. In contrast to the effects of inhibitors of PTK, pretreatment of macrophages with inhibitors of protein kinase A or C, such as H89 (0.005 or 0.05 μ*M*), staurosporine (0.02 or 0.2 μ*M*), calphostin C (0.05 or 0.5 μ*M*), or H7(2.5 or 25 μ*M*), did not result in marked inhibition of silica-induced NF-κB activation (Figures 10 and 11).

FIGURE 7. Electrophoretic mobility shift assay (ESMA) illustrating the effect of tyrosine kinase inhibitors on silica-induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with herbimycin A (1.7 or 17 μ*M*), genistein (37 or 74 μ*M*), or AG494 (6 or 12 μ*M*) and then stimulated by silica (100 μg/ml) for an additional 4 h. The results of EMSA are shown (upper panel). These data were quantified by densitometric analysis and are presented as a percentage of the response of silica-stimulated cells (lower panel).

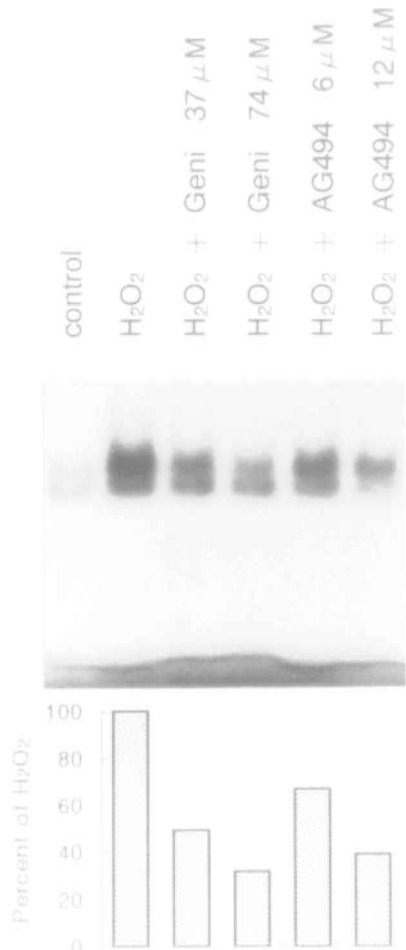

FIGURE 8. Electrophoretic mobility shift assay (EMSA) illustrating the effect of tyrosine kinase inhibitors on H_2O_2-induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with genistein (37 or 74 μ*M*) or AG494 (6 or 12 μ*M*) and then stimulated by H_2O_2 (1 m*M*) for an additional 2 h. The results of EMSA are shown (upper panel). These data were quantified by densitometric analysis and are presented as a percentage of the response of H_2O_2-stimulated cells (lower panel).

DISCUSSION

NF-κB has been known to be involved in signal transduction from cytoplasm to nucleus in cells exposed to a variety of stimuli (Schmitz, 1995). NF-κB plays an important role as a transcription factor that activates promoter genes regulating the expression of various cytokines involved in the initiation and progression of silicosis (Schmidt et al., 1995; Chen et al., 1998). Chen et al. (1995a) demonstrated silica stimulated DNA-binding activity of NF-κB and identified NF-κB components, such as p50, p65, and

p52, using a supershift assay. Recently, molecular and cellular approaches demonstrated that ROS may be responsible for the modulation of NF-κB activation (Remacle et al., 1995).

In the present study, the correlation between silica-induced production of ROS by macrophages (RAW264.7) and NF-κB activation was investigated. In vitro exposure of macrophages to 100 μg/ml silica resulted in a twofold increase in ROS production, measured as the generation of CL. This oxidant production was inhibited by SOD and catalase, suggesting a role for silica-induced production of superoxide anion and hydrogen peroxide in the chemiluminescent signal. This silica-induced oxidant generation was not the result of cytotoxicity, since cellular viability (measured as trypan blue exclusion) was unaffected by silica at the concentrations and exposure times employed in this study (data not shown).

It is believed that oxidants can act to cleave IκB from the p50–p65 heterodimer of NF-κB, allowing the migration of this transcription factor from the cytoplasm to the nucleus, where it will bind to promoter regions of DNA that regulate a variety of inflammatory cytokines and growth factors (Shi et al., 1998). The results of the present study suggest that activation of NF-κB by silica may depend on the redox state of the cells. This hypothesis is supported by recent studies in which catalase (an H_2O_2 scavenger) or deferoxamine (an iron chelator that reduces the ability of silica to generate ROS) inhibited NF-κB activation in silica-exposed macrophages (Chen et

FIGURE 9. Protein tyrosine phosphorylation induced by silica and H_2O_2. RAW264.7 cells were either unexposed or exposed for 2 h to genistein (74 μ*M*) and then 100 μg/ml silica for 30 min or 3 m*M* H_2O_2 for 20 min. Then Western blots with anti-phosphotyrosine antibodies were employed to monitor protein tyrosine phosphorylation. Sizes are indicated in kilodaltons, and arrows indicate protein bands whose phosphorylation is altered.

FIGURE 10. Electrophoretic mobility shift assay (EMSA) illustrating the effects of a protein kinase A inhibitor, H89, on silica-induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with H89 (0.005 or 0.05 μ*M*) and then stimulated by silica (100 μg/ml) for an additional 4 h. The results of EMSA are shown (upper panel). These data were quantified by densitometric analysis and are presented as a percentage of the response of silica-stimulated cells (lower panel).

al., 1997, 1998). These authors also reported that tetrandrine and poly-2-vinylpyridine *N*-oxide (PVPNO) inhibit silica-induced NF-κB binding to DNA. Evidence indicates that both treatments reduce silica-induced ROS production by macrophages. Similar inhibitory effects of antioxidants on the activation of NF-κB have been reported in a variety cells in response to stimuli, such as lipopolysaccharide (Chen et al., 1995a), asbestos (Simeonova & Luster, 1996), tumor necrosis factor α or interleukin-1 (Beg et al., 1993), and phorbol 12-myristate 13-acetate (Staal et al., 1990). Chen et al.

(1995b, 1997) have also reported that serine protease may play a role in silica-induced NF-κB activation, since this activation is inhibited by *N*-benzoyl-L-tyrosine ethyl ester (BTEE) or *N*-tosyl phenylalanine chloromethyl ketone (TPCK). In addition, they reported that overexpression of calpastatin, an inhibitor of the cysteine protease (calpain), blocked NF-κB activation by silica. In contrast to our observations, Suzuki et al. (1995) reported that transient overexpression of catalase in COS-1 cells did not block TNFα- or PMA-induced NF-κB activation. Brennan and O'Neill

FIGURE 11. Electrophoretic mobility shift assay (EMSA) illustrating the effects of protein kinase C or A inhibitors on silica-induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with staurosporine (0.02 or 0.2 μ*M*), calphostin C (0.05 or 0.5 μ*M*), and H7 (2.5 or 25 μ*M*) and then stimulated by silica (100 μg/ml) for an additional 4 h. The results of EMSA are shown (upper panel). These data were quantified by densitometric analysis and are presented as a percentage of the response of silica-stimulated cells (lower panel).

(1995) suggested that the role of ROS in NF-κB activation may be restricted to certain cell types, since micromolar H_2O_2 concentrations did not activate NF-κB in Jurkat T cells, EL4.NOB-1 T cells, or KB epidermal cells. However, Sundaresan et al. (1995) demonstrated that to achieve the intracellular concentration of H_2O_2 seen after platelet-derived growth factor (PDGF) stimulation, an extracellular concentration of H_2O_2 in the 0.1 to 1 m*M* range was required. This concentration of extracellular H_2O_2 appeared to induce the tyrosine phosphorylation of mitogen-activated protein (MAP) kinase. Our study also indicates that a similar range of extracellular H_2O_2 (0.5–2 m*M*) activated NF-κB, and this increase could be inhibited by the scavenging enzyme catalase. Reports indicate that H_2O_2 was also required for the lysophosphatidic acid-stimulated mitogen-activated protein kinase kinase activation pathway (Q. Chen et al., 1995) and PDGF-induced tyrosine phosphorylation of the p42 isoform of mitogen-activated protein kinase (Sundaresan et al., 1995). These results suggest H_2O_2 may act as a signal-transducing molecule after specific membrane perturbation by either soluble or particulate stimuli. However, the mechanism by which ROS are produced in response to the various NF-κB-activating stimuli has not yet been investigated fully.

Since phosphorylation events have been implicated in signal transduction for NF-κB action, the effects of various protein kinase inhibitors on silica-induced NF-κB activation in RAW 264.7 macrophage cells were examined. Herbimycin A, genistein, and AG494, specific inhibitors of protein tyrosine kinase (PTK) that are reported to act by different mechanisms (Mahon & O'Neill, 1995), markedly prevented NF-κB activation. It was further demonstrated that protein tyrosine phosphorylation was increased after treatment with silica as well as H_2O_2. These phosphorylation events were also potently inhibited by genistein. This is the first demonstration of a role for PTK in the activation of NF-κB in silica-stimulated macrophages. A role of PTK in the activation of this transcription factor has been reported in other cell systems, such as lipopolysaccharide stimulation of blood monocytes (Geng et al., 1993) and interleukin-1 stimulation of T cells (Iwasaki et al., 1992). In contrast, inhibitors of protein kinase A or C, such as H89, staurosporin, calphostin C, and H7, did not markedly affect the activation of NF-κB when used in concentrations that would not affect PTK. However, Diaz-Meco et al. (1993) reported that overexpression of a PKC isotype, ξPKC, results in stimulation of the translocation of NF-κB into the nucleus of NIH 3T3 fibroblasts. Li and Sedivy (1993) found the Raf-1 kinase can mobilize NF-κB by phosphorylating IκB in the cytoplasmic IκB–NF-κB complex to release active NF-κB.

The molecular mechanisms by which ROS are involved in downstream signaling events between the tyrosine activation and NF-κB activation remain unclear. One suggested mechanism is the inactivation of protein tyrosine phosphatase by oxidation of a conserved cysteine residue

within its catalytic domain, leading to increased protein tyrosine phosphorylation (Suzuki et al., 1997). Indeed, Grabowski et al. (1995) linked intracellular oxidant generation to the inhibition of tyrosine phosphatase activity in pulmonary macrophages treated with vanadium. Therefore, ROS may bridge the gap between the tyrosine kinase activation and NF-κB downstream signaling by this molecular mechanism.

In the present study, higher concentrations of silica were required to stimulate ROS production (chemiluminescence) than to activate NF-κB. The chemiluminescence assay required a higher concentration of silica because exposure duration was much lower, that is, 10 min for stimulation of chemiluminescence versus 4 h for activation of NF-κB. Although stimulatory levels of silica were not comparable for the two assays, the association of ROS production with NF-κB activation is strengthened by the inhibitory effects of SOD, catalase, and antioxidants on silica-induced NF-κB activation.

When employing in vitro exposures, the relationship between cellular exposure levels and human exposures is often problematic. In the present study of NF-κB activation, the macrophage to silica ratio was 5×10^4 cells/μg silica. Although this is a relatively high exposure, it is not beyond what can be seen in workers in high-exposure jobs, such as rock drilling or sandblasting. Indeed, whole-lung lavage of rock drillers with acute silicosis yielded as much as 500 μg of silica from the lung (Wilt et al., 1996). Stone et al. (1992) reported that a normal human lung would contain 6×10^9 alveolar macrophages. Therefore, the macrophage to silica ratio in this acute silicotic patient would be 1×10^4 cells/μg silica, which is in the same range as the present in vitro exposure.

In the present study, RAW 246.7 cells were used as a model system for macrophages. RAW 246.7 cells are mouse peritoneal macrophages that possess excellent viability and growth characteristics in culture (Raschke et al., 1978). Therefore, they are well suited for in vitro exposure studies. Although not identical to alveolar macrophages, RAW 246.7 cells demonstrate many properties that model the response of pulmonary macrophages to occupational agents. Similar to alveolar macrophages, RAW 246.7 cells exhibit the following: (1) activation of the cycloxygenase pathway, secretion of tumor necrosis factor α (TNFα) and release of interleukin 1 after in vitro exposure to silica (Chen et al., 1995b); (2) secretion of tumor necrosis factor α and upregulation of mRNA for TNFα after phagocytosis of silica (Claudio et al., 1995); (3) secretion of TNFα after in vitro exposure to fiberglass (Ye et al., 1999); (4) phagocytosis of extracts of yeast cell walls (Raschke et al., 1978); and (5) sensitivity to lipopolysaccharide (Raschke et al., 1978). Indeed, Rojansakul et al. (1999) have demonstrated that silica-induced activation of NF-κB and the concentration dependence of stimulation of TNFα production in response to silica are nearly identical in alveolar macrophages and RAW 246.7 cells. Claudio et al. (1995) demonstrated that, in contrast to RAW 246.7 cells, other monocyte cell lines were

poor models for macrophage response to silica. For these reasons, the use of RAW 246.7 cells has become commonplace.

In conclusion, this study has shown that ROS play a role in silica-induced NF-κB activation in macrophages and that phosphorylation events mediated by PTK may be involved in this activation.

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