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Silica Induces Nuclear Factor-κB Activation through Tyrosine Phosphorylation of IκB-α in RAW264.7 Macrophages

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It was previously reported that protein tyrosine kinase (PTK) but not protein kinase C or A plays an important role in silica-induced activation of NF-κB in macrophages. The question is raised whether PTK stimulation and NF-κB activation in silica-stimulated macrophages are directly connected through tyrosine phosphorylation of IκB-α. Results indicate that stimulation of macrophages with silica led to NF-κB activation through tyrosine phosphorylation without serine phosphorylation. Specific inhibitors of protein tyrosine kinase, such as genistein and tyrophostin AG126, prevented tyrosine phosphorylation of IκB-α in response to silica. IκB-α protein levels remained relatively unchanged for up to 60 min after silica stimulation. Moreover, inhibition of proteasome proteolytic activity did not affect NF-κB activation by silica. Antioxidants, such as superoxide dismutase (SOD), *N*-acetylcysteine (NAC), and pyrrolidine dithiocarbamate (PDTC), blocked tyrosine phosphorylation of IκB-α induced by silica, suggesting reactive oxygen species (ROS) may be important regulatory molecules in NF-κB activation through tyrosine phosphorylation of IκB-α. The results suggest that tyrosine phosphorylation of IκB-α represents a proteasome proteolytic activity-independent mechanism for NF-κB activation that directly couples NF-κB to cellular tyrosine kinase in silica-stimulated macrophages. This proposed mechanism of NF-κB activation induced by silica could be used as a target for development of antiinflammatory and antifibrosis drugs. © 2000 Academic Press

Key Words: **silica; NF-κB; tyrosine phosphorylation; IκB-α; macrophages; reactive oxygen species.**

Pulmonary deposition of crystalline silica can result in a cycle of lung damage, fibroblast proliferation, and excess collagen production in the lung, causing lung fibrosis or silicosis (Craighead *et al.,* 1998). Upon contact with silica, alveolar macrophages produce a variety of inflammatory and fibrogenic factors, such as reactive oxygen species (ROS), lipid mediators, cytokines (IL-1, IL-6, TNFα), chemokines, and macrophage-derived growth factors (Lapp and Castranova, 1993; Shi *et al.,* 1998), which are critical to silica-induced pathogenesis.

Nuclear factor kappa B (NF-κB) is an essential transcription factor that regulates the gene expression of various cytokines, chemokines, growth factors, and cell-adhesion molecules (Chen *et al.,* 1999; Barnes and Karin, 1997). Therefore, activation of NF-κB binding to various gene promoter regions appears to be a key molecular event in the initiation of silica-induced pulmonary disease. Recent evidence indicates that *in vitro* exposure of macrophages to silica results in activation of NF-κB (Chen *et al.,* 1998; Kang *et al.,* 2000a). Silica-induced activation of NF-κB in pulmonary phagocytes has also been demonstrated after *in vivo* exposure to silica (Sacks *et al.,* 1998).

The most predominantly characterized NF-κB complex is a p50/p65 heterodimer, which is associated at rest with an inhibitor protein, IκB, and is retained in the cytoplasm (Zabel and Baeuerle, 1990). Phosphorylation is an important event of NF-κB activation at different levels. The active NF-κB can then translocate to the nucleus, where it binds to a NF-κB motif and functions as a transcriptional regulator.

Induced phosphorylation of IκB-α protein occurs at two conserved serine residues, serine 32 and 36, in the N-terminal domain of IκB-α (Brockman *et al.,* 1995; Brown *et al.,* 1995; DiDonato *et al.,* 1996). Phosphorylation of serine resides of IκB-α is followed by the ubiquitination of this protein, leading to degradation of IκB-α by proteasomes. Two cytokine-inducible kinases, called IκB kinases (IKK-α and IKK-β), were identified that phosphorylate IκB-α on serine 32 and 36 (DiDonato *et al.,* 1997; Mercurio *et al.,* 1997; Regnier *et al.,* 1997; Woronicz *et al.,* 1997; Zandi *et al.,* 1997). This regular pathway for NF-κB activation is triggered by tumor necrosis factor (TNF), interleukin (IL)-1β, phorbol 12-myristate 13-acetate (PMA), okadaic acid, or lipopolysaccharide (LPS).

Recent evidence indicates exposure of T cells to hypoxia, reoxygenation, and pervanadate results in phosphorylation of IκB-α on tyrosine 42 (Koong *et al.,* 1994; Imbert *et al.,* 1996).

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These authors also reported an alternative mechanism of NF-κB activation by which tyrosine phosphorylation does not lead to degradation of the IκB-α through the proteasome pathway, unlike serine phosphorylation of IκB-α.

Recent evidence indicates that *in vitro* exposure of macrophages to silica induces tyrosine phosphorylation of proteins (Kang *et al.,* 2000a; Holian *et al.,* 1994). Silica-induced tyrosine phosphorylation of proteins has also been demonstrated after *in vivo* exposure to silica (Gossart *et al.,* 1996). Our previous study indicates that protein tyrosine kinase (PTK) but not protein kinase C or A plays an important role in silica-induced activation of NF-κB in macrophages, as shown by the use of various specific tyrosine or protein kinase inhibitors (Kang *et al.,* 2000a).

Recently, molecular approaches demonstrated that ROS can directly affect the cellular signaling apparatus and, consequently, the control of gene expression (Remacle *et al.,* 1995). Exposure of lung phagocytes to silica results in the production of reactive oxygen species (Castranova *et al.,* 1996). Evidence indicates that reactive oxidants play a role in silica-induced activation of NF-κB. Indeed, a variety of antioxidants (catalase, superoxide dismutase, α-tocopherol, pyrrolidine dithiocarbamate, or *N*-acetylcysteine) have been shown to inhibit silica-induced NF-κB activation of macrophages *in vitro* (Kang *et al.,* 2000a). Hydroxyl radical has been suggested as the key activation signal for NF-κB activation (Shi *et al.,* 1999). Furthermore, ROS have been reported to function as physiological regulators of tyrosine phosphorylation by their effects on oxidant-sensitive tyrosine kinase and/or tyrosine phosphatase (Fialkow *et al.,* 1993; Bauskin *et al.,* 1991). Indeed, NAC inhibited silica-induced tyrosine phosphorylation in both the absence and the presence of pervanadate, a protein tyrosine phosphatase inhibitor (Kang *et al.,* 2000b).

However, the mechanism involved in silica-induced activation of NF-κB is not completely understood. Whether PTK stimulation and NF-κB activation in silica-stimulated macrophages are directly connected through tyrosine phosphorylation of IκB-α remains a question.

Therefore, in the present study we investigated (1) whether silica induces tyrosine and/or serine phosphorylation of IκB-α in RAW264.7 macrophages; (2) whether the phosphorylation of IκB-α leads to degradation of IκB-α by proteasomes in response to silica; and (3) whether reactive oxygen species (ROS) are involved in induction of the phosphorylation of IκB-α in silica-stimulated macrophages.

METHODS

Reagents. Crystalline silica (Min-U-Sil, particle size <5 μm) was obtained from U.S. Silica Corp. (Berkeley Springs, WV). Prior to use, the silica samples were sterilized by heating at 160°C for 90 min in a dry oven. Silica particles were dispersed in DMEM (Life Technologies, Inc., Madison, WI) with supplements just before addition to culture plates. Antibodies used in this study were anti-IκB-α rabbit polyclonal (New England Biolabs, Inc., Beverly, MA), antiphospho-IκB-α (Serine 32) (New England Biolabs), and anti-phosphotyrosine 4G10 (Upstate Biotechnology, Lake Placid, NY). Genistein, AG126, superoxide dismutase (SOD), *N*-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC), and peptide Cbz-Ile-Glu (O-*t*-Bu)-Ala-leucinal (PSI) were purchased from Sigma Chemical Company (St. Louis, MO). MG115 and MG132 were purchased from Calbiochem (San Diego, CA). DNA polymerase and dNTP were purchased from Life Technologies (Gaithersburg, MD).

Cell line and cell culture. RAW264.7 cells, a mouse peritoneal macrophage cell line, were obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in DMEM (Life Technologies) supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT), 2 mM glutamine, and 1,000 units/ml penicillin–streptomycin.

Immunoprecipitation. The confluent cells grown on 100-mm plastic dishes were incubated in DMEM supplemented with 5% FBS, 2 mM glutamine, and 1000 units/ml penicillin–streptomycin for 3 days. Cells then were treated with silica (100 μg/ml) in the presence or absence of specific tyrosine kinase inhibitors, such as genistein (74 μM) and AG126 (30 μM), or antioxidants, such as SOD (1500 units/ml), NAC (1 mM), and PDTC (250 μM), and washed with ice-cold phosphate-buffered saline (pH 7.4). The washed cells were lysed with 1 ml of ice-cold lysis buffer containing 50 mM Tris–HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 mM Na_3VO_4, 5 mM EDTA, and 1 mM benzamidine.

The cell lysate was centrifuged for 5 min at 13,000*g*. The resulting supernatant was incubated with anti-IκB-α rabbit polyclonal at 4°C for 1 h followed by 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 for 30 s. The pellet was then washed three times with ice-cold lysis buffer by centrifugation at 13,000*g* for 30 s, dissolved in 20 μl of Laemmli's sample buffer, and separated on 10% SDS–polyacrylamide gels (Laemmli, 1970).

Western blotting. The fractionated proteins for tyrosine-phosphorylated IκB-α or cytoplasmic extracts from silica (100 μg/ml) or LPS (1.0 μg/ml) -treated cells for IκB-α and phospho-IκB-α (Ser32) were resolved on 10% SDS–polyacrylamide gels and 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.

Nuclear extracts. Nuclear extracts were prepared by a modified method of Sun *et al.* (1994). RAW264.7 cells were cultured in six-well plates at 5×10^6 cells/ml for 3 days; then the medium was replaced with fresh medium and cells were pretreated with specific proteasome inhibitors, such as MG115 (25, 50 μM), MG132 (25, 50 μM), or a specific inhibitor of the chymotrypsin-like activity of the proteasome PSI (25, 50 μM). After a 2-h pretreatment, cells were cultured with silica (100 μg/ml) or LPS (1.0 μg/ml) in the absence or presence of inhibitor as indicated for 4 h. The concentrations of silica or LPS and the duration of exposure used in this investigation were determined from previous concentration–response and time-course studies for NF-κB activation (Kang *et al.,* 2000a). At the end of the 4-h exposure, the cells were harvested and resuspended in hypotonic buffer A (100 mM HEPES, pH 7.9; 10 mM KCl; 0.1 M EDTA; 0.5 mM dithiothreitol; 1% Nonidet P-40; and 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice and then vortexed for 10 s. Nuclei were pelleted by centrifugation at 12,000*g* for 30 s and resuspended in buffer C (20 mM Hepes, pH 7.9; 20% glycerol; 0.42 M NaCl; 1 mM EDTA; and 0.5 mM PMSF) for 30 min on ice. The supernates containing nuclear proteins were collected by centrifugation at 10,000*g* for 2 min and stored at −70°C.

Electrophoretic mobility shift assay (EMSA). Binding reaction mixtures (10 μl) containing 5 μg (4 μl) of nuclear extract protein, 2 μg of poly(dI-dC)· (Sigma Co.), and 40,000 cpm of ^{32}P-labeled probe in binding buffer (4 mM HEPES, pH 7.9; 1 mM $MgCl_2$; 0.5 mM DTT; 2% glycerol; and 20 mM 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 EMSA was a double-stranded DNA containing NF-κB consensus sequence (5′-CCTGTGCTCCGGGAATTTC-

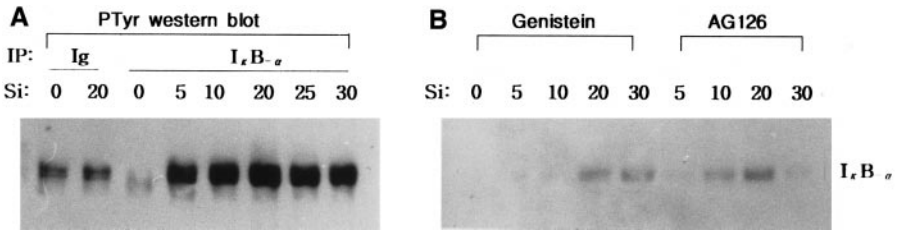

FIG. 1. Tyrosine phosphorylation of IκB-α in silica-stimulated RAW264.7 cells (A). RAW264.7 cells were incubated with silica (100 μg/ml) for 5–30 min. The lysates were incubated with control Ig (lanes 1 and 2) or anti-IκB-α mAb (lanes 3–8) before analysis of tyrosine phosphorylation by Western blotting with antiphosphotyrosine mAb. Effect of tyrosine kinase inhibitors on silica-induced tyrosine phosphorylation of IκB-α (B). Cells were preincubated for 2 h with genistein (74 μM) or AG126 (30 μM) before treatment with silica (100 μg/ml) for an additional indicated time (5–30 min). The lysates were incubated with anti-IκB-α mAb before analysis of tyrosine phosphorylation by Western blotting with anti-phosphotyrosine mAb. Data are representative of at least three experiments.

CCTGGCC-3′) labeled with [α-^{32}P]dATP (Amersham, Buckinghamshire, UK) using a DNA polymerase Klenow fragment.

RESULTS

A previous report from our laboratory has shown that exposure of RAW264.7 macrophages to silica (100 μg/ml) resulted in maximal NF-κB activation (Kang *et al.*, 2000a). In addition, we reported that silica-induced NF-κB activation was blocked by inhibition of tyrosine kinase but not protein kinase C or A (Kang *et al.*, 2000a).

To assess a direct connection between PTK stimulation and NF-κB activation in silica-stimulated macrophages through tyrosine phosphorylation of IκB-α, RAW264.7 macrophages were exposed to silica (100 μg/ml), and cell lysates from silica-treated or untreated cells were then used for immunoprecipitation with control IgG (Fig. 1A, lanes 1 and 2) or with IκB-α-specific antibody (lanes 3–8) followed by Western blot analysis with the antiphosphotyrosine mAb. Substantial tyrosine phosphorylation of IκB-α was observed after 5 min of silica stimulation and was sustained through a 30-min exposure of RAW264.7 cells to silica (Fig. 1A). In contrast, treatment of RAW264.7 cells with silica did not alter the tyrosine phosphorylation level of the IgG immunoprecipitate, indicating that the phosphorylated tyrosine residue was specifically presented on IκB-α. No tyrosine-phosphorylated IκB-α was detected in unstimulated cells (Fig. 1A, lane 3). To confirm that protein tyrosine kinase activation was directly connected to the NF-κB activation in silica-stimulated RAW264.7 macrophages, specific protein tyrosine kinase inhibitors, such as genistein (74 μM) and AG 126 (30 μM), were added to the cells 2 h before exposure to silica. As shown Fig. 1B, tyrosine phosphorylation of IκB-α in cells exposed to silica for 5–30 min was markedly blocked by either genistein or AG 126.

Although these data indicate that silica-induced NF-κB activation is mediated by tyrosine phosphorylation of IκB-α, it is possible that serine phosphorylation of IκB-α could also be a signal for NF-κB activation by silica treatment. To exam this possibility in our model, cells were exposed to silica or LPS, and cell lysates from stimulant-treated or untreated cells were then examined for serine phosphorylation of IκB-α by Western blot analysis with anti-phospho-IκB-α (serine 32) Ab. Serine phosphorylation of IκB-α was minimally induced after silica stimulation for 20 min. In contrast, LPS stimulation induced substantial serine phosphorylation within 5 min, which increased further at 10 min of LPS exposure (Fig. 2).

We studied whether tyrosine phosphorylation of IκB-α leads the proteasome-dependent degradation of IκB-α similar to that reported for serine phosphorylation. The cell lysates were analyzed by Western blotting with anti-IκB-α Ab at various times after stimulation of RAW264.7 cells with silica or LPS. IκB-α protein levels were unchanged for up to 45 min of silica treatment and were increased slightly after 60 min, presumably due to newly synthesized IκB-α protein (Figs. 3A and 3C). In contrast, degradation of IκB-α occurred at 10 min after LPS stimulation and continued for up to 30 min. Newly synthesized IκB-α protein was increased for up to 240 min after LPS stimulation (Figs. 3B and 3D). Figure 4 shows the effect of specific proteasome inhibitors, such as MG 115 and MG 132, or a specific inhibitor of the chymotrypsin-like activity of the proteasome (PSI) on silica- or LPS-induced NF-κB activation in macrophages. Specific proteasome inhibitors did not prevent NF-κB activation in silica-treated cells (Fig. 4A). In contrast, MG 115 (50 μM), MG 132 (50 μM), or PSI (25 μM) inhibited NF-κB activation in LPS-stimulated cells by 90, 60, or 42%, respectively (Fig. 4B). The data presented thus far suggest that tyrosine phosphorylation of IκB-α in silica-stimulated cells does not lead to potent degradation of the IκB-α through the proteasome pathway.

Data in Fig. 5 indicate that antioxidants, such as SOD (1500 units/ml), NAC (1 mM), and PDTC (200 μM), blocked tyrosine phosphorylation of IκB-α induced by silica, suggesting

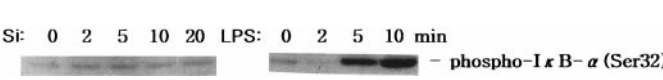

FIG. 2. Effects of silica or LPS on serine phosphorylation of IκB-α in RAW264.7 cells. RAW264.7 cells were incubated with silica (100 μg/ml) for 2–20 min or LPS (1.0 μg/ml) for 2–10 min. Serine phosphorylation was analyzed by anti-phospho-IκB-α (serine 32) Western blotting. Data are representative of at least three experiments.

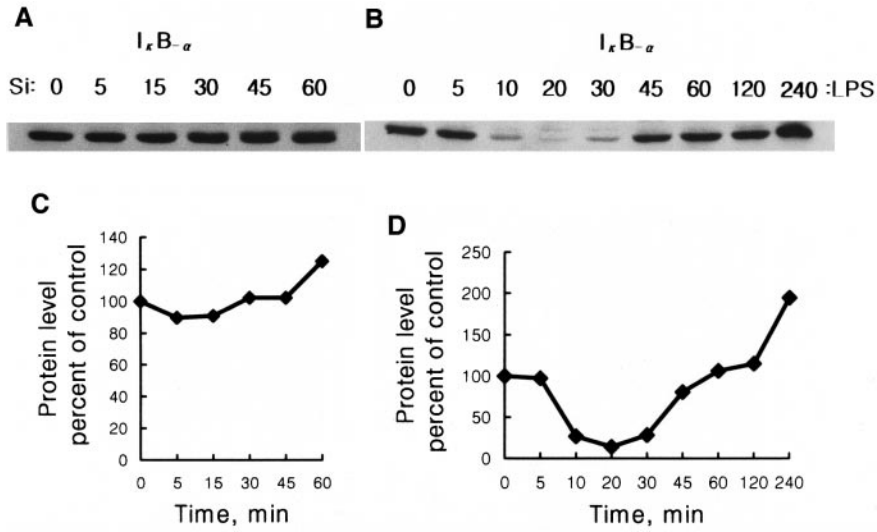

FIG. 3. Kinetics of degradation and resynthesis of IκB-α during exposure of RAW264.7 cells to silica or LPS. Cells were incubated with silica (100 μg/ml; A) or LPS (1.0 μg/ml; B) for the indicated times. Cell lysates were analyzed by IκB-α Western blotting. The levels of IκB-α, after stimulation with silica (C) or LPS (D) were evaluated by scanning the autoradiograms (A, B). Data are representative of at least three experiments.

that ROS may be important regulatory molecules in NF-κB activity through tyrosine phosphorylation of IκB-α.

DISCUSSION

The objective of the present investigation was to elucidate the mechanistic relationship between PTK stimulation and NF-κB activation in silica-stimulated macrophages. Data indicate that *in vitro* stimulation of RAW264.7 macrophages with silica resulted in NF-κB activation through tyrosine phosphorylation of IκB-α without a noticeable decline in IκB-α levels. In addition, antioxidants such as SOD, NAC, and PDTC inhibited tyrosine phosphorylation of IκB-α after silica stimulation.

Data from the present study support an alternative pathway for NF-κB activation reported by Imbert *et al.* (1996), which involves tyrosine phosphorylation of IκB-α without appreciable degradation of IκB-α. This pathway has been identified in Jurkat-T cells treated with hypoxia, reoxygenation, and pervanadate (Koong *et al.,* 1994; Imbert *et al.,* 1996). Furthermore, Singh *et al.* (1996) have reported that in an *in vitro* reconstitution system, tyrosine-phosphorylated IκB-α was protected from degradation induced by pervanadate. In contrast to this alternative pathway, the regular pathway of NF-κB activation induced by TNFα, IL-1, okadaic acid, PMA, or LPS shows inducible phosphorylation of IκB-α at both serine 32 and 36 leading to its degradation through ubiguitination involving the 26S proteasome (DiDonato *et al.,* 1996; Scherer *et al.,* 1995; Chen *et al.,* 1995a; Alkalay *et al.,* 1995). Currently, two cytokine-inducible kinases (IKK-α and -β) have been identified that phosphorylate IκB-α on serine 32 and 36 (DiDonato *et al.,* 1997; Mercurio *et al.,* 1997; Regnier *et al.,* 1997; Woronicz *et al.,* 1997; Zandi *et al.,* 1997).

By site-specific mutation and deletion analysis, tyrosine 42 on IκB-α was identified as a phosphoregulation site (Imbert *et al.,* 1996; Singh *et al.,* 1996). Our data also indicate that rapid tyrosine phosphorylation of IκB-α can occur independent of serine phosphorylation. Therefore, the tyrosine phosphorylation of IκB-α may be an essential early event for the activation of NF-κB by silica.

Stimulants that induce NF-κB activation via the inducible phosphorylation of IκB-α also lead to the inducible phosphorylation of different subunits of NF-κB, such as p105 and p65. Diehl *et al.* (1995) reported the RelA (p65) subunit becomes rapidly phosphorylated in response to TNFα. Several of the NF-κB inducers lead to phosphorylation and carboxyl terminal degradation of the p105 precursor protein (Baeuerle and Henkle, 1994; Siebenlist *et al.,* 1994). A role for this inducible phosphorylation has been suggested to involve enhancement of DNA binding, but may also be correlated with release from IκB, nuclear translocation, and activation of transcription functions (Naumann and Scheidereit, 1994).

Our data indicate that IκB-α degradation was not apparent for 60 min after silica stimulation. Indeed, studies indicate no decline in IκB-α levels for up to 2 h after silica exposure (data not shown). Furthermore, specific proteasome inhibitors did not block NF-κB activation in silica-treated cells. In contrast, specific inhibitors of proteasome or the chymotrypsin-like proteasome, which were used in our model, have been shown to block LPS- or TNFα-induced IκB-α degradation and NF-κB activation (Chen *et al.,* 1997a; Traenckner *et al.,* 1995, 1994). Therefore, our data support the hypothesis that, unlike LPS

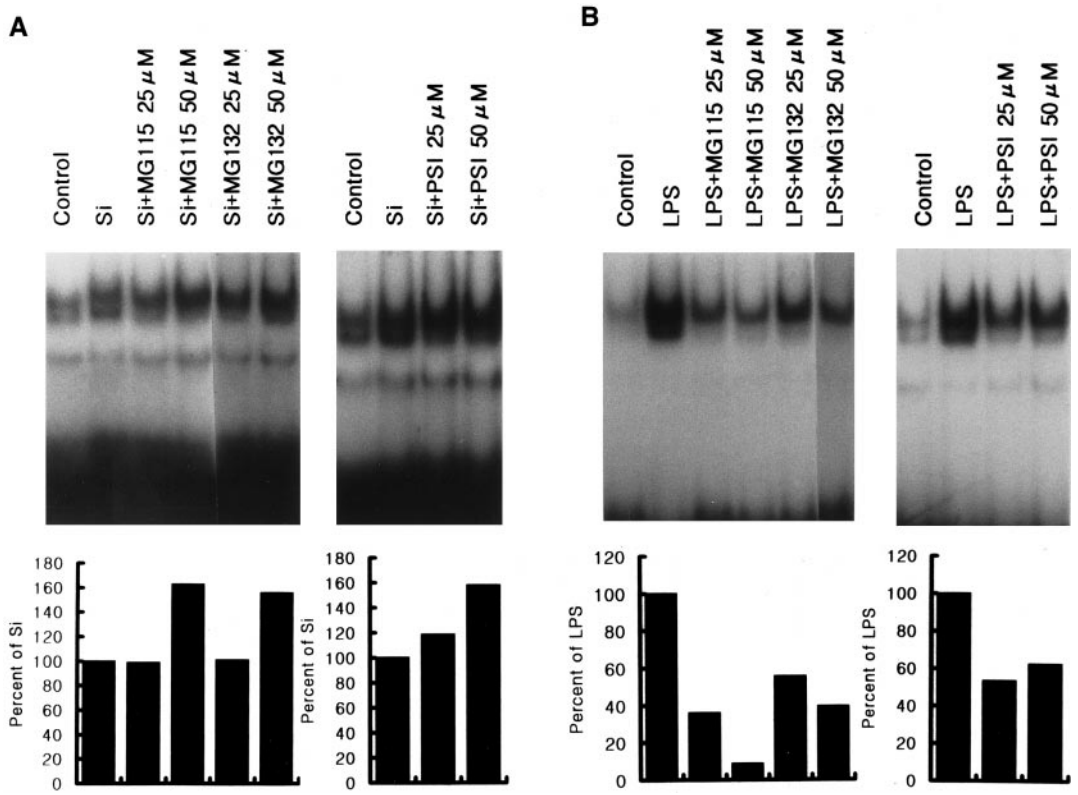

FIG. 4. Electrophoretic mobility shift assay (EMSA) illustrating the effects of proteasome inhibitors on silica- (A) or LPS- (B) induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with proteasome inhibitors (25 or 50 μM), such as MG115, MG 132, or an inhibitor of chymotrypsin-like activity of the proteasome (PSI, 25 or 50 μM), and then stimulated by silica (100 μg/ml) or LPS (5.0 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- or LPS-stimulated cells (lower panel). Data are representative of at least three experiments.

stimulation, NF-κB activation through phosphorylation of IκB-α in silica-stimulated RAW264.7 cells is not dependent on a proteasomal pathway. Consistent with our data, Chen *et al.* (1997b) have also reported the lack of an effect of a proteasome inhibitor, MG132, on silica-induced NF-κB activation. They concluded that it was improbable that the absence of inhibition was due to nonspecific absorption or binding of MG132 by silica particles. It has been also shown that inhibition of proteasome proteolytic activity did not affect NF-κB induction in pervanadate-stimulated T cells (Imbert *et al.*, 1996). Chen *et al.* (1995b, 1997b) also have 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 cholorom-ethyl ketone (TPCK). In addition, overexpression of calpastatin, an inhibitor of the cystein protease (calpain), blocked NF-κB activation by silica.

In vitro phosphorylation/dephosphorylation experiments strongly suggested that tyrosine phosphorylation directly interfered with the interaction between IκB-α and NF-κB (Imbert *et al.*, 1996). However, it has not been known how tyrosine phosphorylation released IκB-α. Beraud *et al.* (1999) have found that the regulatory subunit of phosphoinositide 3-kinase (PI3-kinase) associates through its Src homology 2 domains with tyrosine-phosphorylated IκB-α *in vitro* as well as *in vivo.* This could explain how tyrosine phosphorylation of IκB-α can lead to NF-κB activation without degradation of IκB-α. However, how PI3-kinase activity contributes to NF-κB activation or how phosphorylation of IκB-α by PI3-kinase causes the

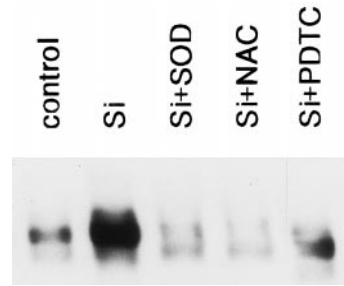

FIG. 5. Effects of antioxidants on tyrosine phosphorylation of IκB-α in silica-stimulated RAW264.7 cells. Cells were preincubated for 2 h with superoxide dismutase (1500 units/ml), *N*-acetylcysteine (1 mM), or pyrrolidine dithiocarbamate (200 μM) before treatment with silica (100 μg/ml) for an additional 20 min. The lysates were incubated with anti-Iκ-αB mAb before analysis of tyrosine phosphorylation by Western blotting with antiphosphotyrosine mAb. Data are representative of at least three experiments.

release of IκB-α from the NF-κB complex remains unclear. The exact role of PI3-kinase activity in NF-κB activation in silica-stimulated macrophages and development of silicosis is being investigated in our laboratory.

Tyrosine phosphorylation of IκB-α and NF-κB activation were impaired in Lck-deficient Jurkat varients, suggesting that IκB-α could be phosphorylated by Lck or by a Lck-activated PTK (Imbert *et al.,* 1996). Koong *et al.,* (1994) have reported that both dominant negative alleles of *Ha-Ras* and *Raf-1* inhibited NF-κB induction by hypoxia, suggesting that the hypoxia-induced pathway for NF-κB activation is dependent on *Ras* and *Raf-1* kinase activity. In macrophages, both IκB kinase (IKK) and stress-activated protein kinase/ERK kinase (SEK1), an intermediate kinase within the mitogen-activated protein kinase (MEKK1) to c-Jun N-terminal kinase (JNK) cascade, are involved in vanadate-induced NF-κB activation. However, which PTK is involved in downstream signaling events leading to NF-κB activation or which PTK directly catalyzes tyrosine phosphorylation of IκB-α in silica-stimulated macrophages has not been identified. Consistent with data presented in the present study, Rupec and Baeuerle (1995) reported that IκB-α tyrosine phosphorylation was induced after reoxygenation of hypoxic Jurkat cells, suggesting that ROS could stimulate tyrosine phosphorylation of IκB-α. ROS have been reported to play a regulatory role in the protein tyrosine phosphorylation as well as NF-κB activation (Suzuki *et al.,* 1997; Remacle *et al.,* 1995). Which tyrosine kinase is targeted by ROS in macrophages and the exact role in which ROS induce phosphorylation of IκB-α in models of silica exposure remain to be understood.

Chen *et al.* (1998) reported that SOD enhanced silica-induced NF-κB activation in macrophages. They concluded that SOD increased H_2O_2 levels and thus induced hydroxyl radical production via a Fenton reaction with Fe^{2+} on the silica. Since silica-induced NF-κB was inhibited by sodium formate, they proposed that hydroxyl radical was a key modulator of NF-κB activation. In contrast, results from the present study indicate that SOD inhibited rather than increased silica-induced tyrosine phosphorylation of IκB-α. In addition, our previous data indicate that SOD decreased silica-induced NF-κB activation in a dose-dependent manner (Kang *et al.,* 2000a). A possible reason for this difference is that trace iron levels may have been lower in the radical production. In such a case, SOD would inhibit rather than increase hydroxyl radical generation and the resultant activation of NF-κB. Such a possibility is summarized by the reaction scheme

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-.$$

In conclusion, results of the present study indicate that stimulation of RAW264.7 macrophages with silica induces NF-κB activation through tyrosine phosphorylation of IκB-α. ROS appear to be important regulatory molecules in this IκB-α tyrosine phosphorylation. However, this silica-enhanced tyrosine phosphorylation of IκB-α and activation of NF-κB do not appear to be dependent on proteasome-induced degradation of IκB-α.

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