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Phosphoinositide 3-kinase activity leads to silica-induced NF-κB activation through interacting with tyrosine-phosphorylated IκB-α and contributing to tyrosine phosphorylation of p65 NF-κB

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Abstract

The role of the subunits of phosphoinositide (PI) 3-kinase in NF-κB activation in silica-stimulated RAW 264.7 cells was investigated. Results indicate that PI3-kinase activity was increased in response to silica. The p85α subunit of PI3-kinase interacted with tyrosine-phosphorylated IκB-α in silica-stimulated cells. PI3-kinase specific inhibitors, such as wortmannin and LY294003, substantially blocked both silica-induced PI3-kinase and NF-κB activation. The inhibition of NF-κB activation by PI3-kinase inhibitors was also observed in pervanadate-stimulated but not in LPS-stimulated cells. Furthermore, tyrosine phosphorylation of NF-κB p65 was enhanced in cells stimulated with silica, pervanadate or LPS, and wortmannin substantially inhibited the phosphorylation event induced by the first two stimulants but not LPS. Antioxidants, such as superoxide dismutase (SOD), N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC), blocked silica-induced PI3-kinase activation, suggesting that reactive oxygen species may be important regulatory molecules in NF-κB activation by mediating PI3-kinase activation. Our data suggest that p85 and p110 subunits of PI3-kinase play a role in NF-κB activation through interaction with tyrosine-phosphorylated IκB-α and contributing to tyrosine phosphorylation of p65 NF-κB. (Mol Cell Biochem **248**: 17–24, 2003)

Key words: PI3-kinase, NF-κB, reactive oxygen species, silica, RAW 264.7 cells

Introduction

Pulmonary deposition of silica dust can result in a cycle of lung damage and scarring known as silicosis, a fibrotic lung disease. It has been proposed that a cycle of oxidant damage, inflammation, and uncontrolled cell proliferation may be essential for the initiation and progression of silica-induced lung disease [1, 2]. Inflammatory cytokines and growth factors are thus critical to this pathogenesis. Nuclear factor kappa B (NF-κB) is an essential transcription factor which controls gene expression of a host of cytokines, chemokines, growth factors and cell adhesion molecules [3, 4]. 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.

Phosphorylation is an important event in NF-κB activation at different levels. Phosphorylation of serine resides of IκB-α is followed by the ubiquitination of this protein leading to

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degradation of IκB-α by proteasomes. This 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) [5]. Recent evidence indicates exposure of T cells to hypoxia, reoxygenation and pervanadate resulted in phosphorylation of IκB-α on tyrosine 42 [5, 6]. This different mechanism of NF-κB activation involving tyrosine phosphorylation is distinct from events involved in serine phosphorylation of IκB-α, which leads to degradation of the IκB-α through the proteasome pathway. Our results from the previous study also supported this mechanism of NF-κB activation in response to silica through tyrosine phosphorylation of IκB-α without serine phosphorylation [7]. Our results also indicate 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 [8].

Phosphoinositide (PI) 3-kinase is a heterodimer consisting of regulatory (p85) with Src homology 2 (SH2) domains and catalytic (p110) subunits [9]. Currently, the role of PI3-kinase subunits in the activation of NF-κB induced by different stimuli is actively debated. PI3-kinase has been reported to play a role in NF-κB activation by association through its SH2 domains with tyrosine-phosphorylated IκB-α after stimulation of T cells with pervanadate [10]. However, Reddy *et al*. [11] have demonstrated that the interaction between the SH2 domains of the p85 subunit and phosphotyrosine on IL-1 receptors activates NF-κB through the IL-1-receptor associated kinase in IL-1 stimulated cells. In addition, the role of p110 in NF-κB activation has not been well characterized, while activation of the p110 subunit in response to IL-1 led to phosphorylation of p65 NF-κB and transactivation of NF-κB, but not the nuclear translocation of NF-κB or the enhancement of DAN binding activity of NF-κB [9].

At present, it is suggested that PI3-kinase activation may be regulated by tyrosine phosphorylation, which is dependent on ROS-mediated cellular signaling [12, 13]. Exposure of RAW 264.7 cells to silica results in the production of ROS [8]. Evidence indicates that reactive oxidants play a role in activation of NF-κB [14] and in induction of tyrosine phosphorylation of IκB-α in silica-stimulated macrophages [7].

The objective of the present study was to elucidate the role of the subunits of PI3-kinase in NF-κB activation in response to silica in macrophages. In addition, whether ROS are involved in induction of PI3-kinase activation was also investigated. The data indicate that a p85α subunit interacts with tyrosine-phosphorylated IκB-α and a p110 subunit is specifically involved in tyrosine phosphorylation of p65 NF-κB, leading to NF-κB activation in response to silica. The PI3-kinase activity stimulated by silica appears to be regulated by ROS.

Materials and methods

Reagents

Crystalline silica (Min-U-Sil, particle size < 5 μm) was obtained from U.S. Silica Corporation (Berkeley Springs, WV, USA). 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, USA) with supplements just before addition to culture plates. Antibodies used in this study were: anti-IκB-α rabbit polyclonal (New England Biolabs, Inc., Beverley, MA, USA), anti-p85α polyclonal antiserum (New England Biolabs), polyclonal anti-p65, anti-PY20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-phosphotyrosine 4G10 (Upstate Biotechnology, Lake Placid, NY, USA). Wortmannin, LY294002, LPS (*Escherichia coli* lipopolysaccharide, 055:B5), superoxide dismutase (SOD), N-acetylcysteine (NAC), and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma Chemical Company (St. Louis, MO, USA). DNA polymerase and dNTP were purchased from Life Technologies (Gaithersburg, MD, USA).

Cell line and cell culture

RAW264.7 cells, a mouse peritoneal macrophage cell line, were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 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 1,000 units/ml penicillin-streptomycin for 3 days. Cells then were treated with silica, pervanadate or LPS in the presence or absence of a specific PI3-kinase inhibitor, wortmannin, 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-p85, anti-p65, or anti-IκB-α 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 at 13,000 × g for 30 sec. The pellet was then washed 3 times with ice-cold lysis buffer by centrifugation at 13,000 × g for 30 sec, dissolved in 20 μg of Laemmli's sample buffer, and separated on 10% SDS-polyacrylamide gels [15].

Western blotting

The fractionated proteins for p85α or tyrosine phosphorylated IκB-α and p65 NF-κB were resolved on 10% SDS-polyacrylamide gels and electrophoretically transferred onto a nitrocellulose paper. 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.* [16]. RAW 264.7 cells were cultured in 6-well plates at 5×10^6 cells/ml for 3 days, then the medium was replaced with fresh medium and cells pretreated with specific PI3-kinase inhibitors, such as wortmannin or LY294002. After a 2 h pretreatment, cells were cultured with silica, pervanadate or LPS in the absence or presence of an inhibitor as indicated for 4 h. The concentrations of silica, pervanadate 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 [8]. 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 sec. Nuclei were pelleted by centrifugation at 12,000 × g for 30 sec and were 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)·poly (dI-dC) (Sigma Co., St. Louis, MO, USA) 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% non-denaturing 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′-CTGTGCTCCGGGAATTTCCCTGGCC-3′) labeled with α-^{32}P] dATP (Amersham, Buckinghamshire, UK) using a DNA polymerase Klenow fragment.

PI3-kinase assay

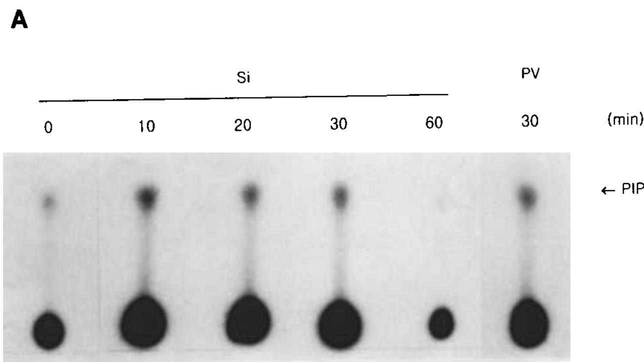
PI3-kinase activity was measured as described previously [17]. Briefly, immunoprecipitation was performed with anti-PY20. Equal amounts of protein were incubated with 1 μg of anti-PY20 for 4 h followed by a 1 h incubation with 50 μl of protein A-Sepharose (20% suspension). The bound beads were subjected to several washes with ice-cold buffers and were resuspended in kinase buffer containing 30 mM HEPES (pH 7.5), 200 μM adenosine, phosphatidylinositol (0.2 mg/ml), 10 μCi of [γ-^{32}P], 40 μM ATP and 30 mM $MgCl_2$ for 10 min. The reactions were terminated by adding 100 μl of 1 M HCl, and lipids were extracted, analyzed by thin-layer chromatography in chloroform-methanol-ammonium (45:35:10) and visualized by autoradiography.

Results

PI3-kinase activity is increased in silica-stimulated RAW 264.7 cells

The objective of the present study was to elucidate the role of the p85 and p110 subunits of PI3-kinase in NF-κB activation after stimulation of RAW 264.7 macrophages with silica. Therefore, it was essential to determine the induction of increased PI3-kinase activity in silica-stimulated cells. PI3-kinase activity was measured, using an *in vitro* kinase assay, with phosphatidylinositol as the substrate. RAW 264.7 cells were lysed after stimulation with silica, and the cellular extracts were collected for analysis for PI3-kinase activity. An increase in PI3-kinase activity was observed after 10 min of silica treatment, sustained through a 30 min exposure of cells to silica and declined after a 60 min exposure (Fig. 1A). The effect of pervanadate on PI3-kinase activity was also examined as a positive control. An increase in PI3-kinase was evident in RAW 264.7 cells stimulated with pervanadate for 30 min.

Figure 1B shows that LY294002 (50 μM) or wortmannin (100 nM) completely suppressed PI3-kinase activity induced by silica. The kinetics of silica or pervanadate-induced PI3-kinase activation preceded that for NF-κB activation in response to these stimuli (data not shown). Since the data presented thus far indicate that silica stimulates PI3-kinase activity, and that PI3-kinase is activated prior to NF-κB activation, it is possible the activation of PI3-kinase plays a role in silica-induced activation of NF-κB.



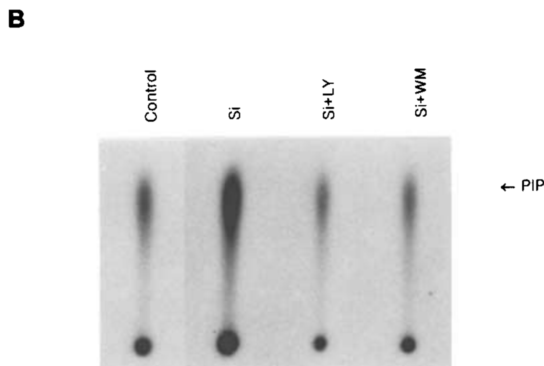


Fig. 1. Stimulation of PI3-kinase activity by silica or pervanadate (A). RAW264.7 cells were incubated with silica (100 μg/ml) or pervanadate (400 μM) for the indicated time periods (A). Effect of PI3-kinase inhibitors on silica-stimulated PI3-kinase activity (B). Cells were preincubated for 2 h with LY294002 (LY, 50 μM) or wortmannin (WM, 100 nM) before treatment with silica (100 μg/ml) for an additional 30 min. PI3-kinase activity was measured as the phosphorylation of phosphatidylinositol yielding phosphatidylinositol phosphate (PIP), indicated by an arrow.

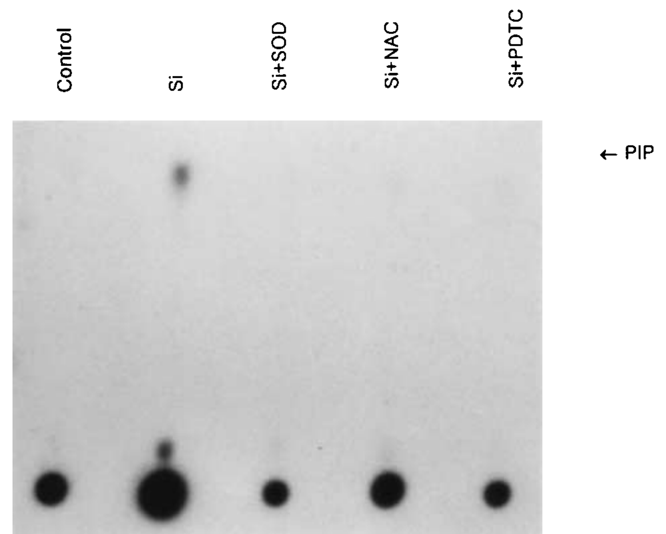


Fig. 2. Effects of anti-oxidants on PI3-kinase activity in silica-stimulated RAW 264.7 cells. Cells were preincubated for 2 h with superoxide dismutase (1,500 units/ml), N-acetylcysteine (1 mM) or pyrrolidine dithiocarbamate (200 μM) before treatment with silica (100 μg/ml) for an additional 30 min. PI3-kinase activity was measured as the phosphorylation of phosphatidylinositol yielding phosphatidylinositol phosphate (PIP), indicated by an arrow. Data are representative of at least 3 experiments.

Antioxidants block the PI3-kinase activity

Reactive oxidants have been suggested to be important regulatory molecules in NF-κB activation in silica-stimulated RAW 264.7 cells [8, 14]. Data in Fig. 2 indicate that antioxidants, such as SOD, NAC, or PDTC, completely blocked PI3-kinase activation induced by silica, suggesting that PI3-kinase activity, leading to NF-κB activation, may be regulated by ROS.

PI3-kinase p85α interacts with tyrosine-phosphorylated IκB-α

A previous report from our laboratory has shown that stimulation of RAW 264.7 cells with silica resulted in NF-κB activation through tyrosine phosphorylation of IκB-α [7]. PI3-kinase has been proposed as a candidate protein, which is known to specifically bind a phosphotyrosine-containing protein with its regulatory subunit p85 having SH2 domains [10, 11]. We, therefore, assessed the connection between PI3-kinase and NF-κB activation in silica-stimulated RAW 264.7 cells by evaluating the direct interaction of PI3-kinase p85α with IκB-α. RAW 264.7 cells were exposed to silica, and cell lysates from silica-treated or -untreated cells were then used for immunoprecipitation with an anti-IκB-α-specific antibody followed by Western blot analysis with the anti-p85α antibody (Fig. 3A). p85α was distinctly observed to associate with IκB-α at 20 min after silica stimulation, and this association increased further at 30 min. However, by 1 h after silica stimulation a decrease from maximal association was noted (data not shown). Figure 3B shows immunoprecipitation with anti-p85α antibody from silica-treated or untreated cells followed by Western blot analysis with the anti-p85α. Silica treatment did not change the amount or the mobility of the p85α band.

Silica-induced activation of NF-kB is blocked by PI3-kinase inhibitors

To assess the role of PI3-kinase in silica-induced NF-κB activation, RAW 264.7 cells were preincubated with PI3-kinase specific inhibitors, such as wortmannin and LY294003, and then examined for NF-κB activation in response to treatment

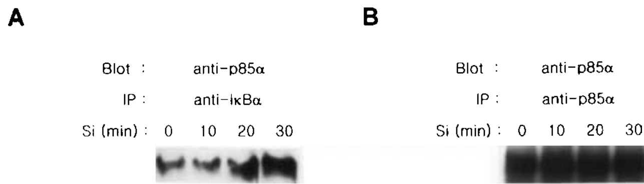


Fig. 3. Interaction of p85a with IκB-α in silica-stimulated RAW264.7 cells. RAW264.7 cells were incubated with silica (100 μg/ml) for 5–30 min. Lysates were immunoprecipitated with anti-IκB-α Ab (A) or anti-p85α (B). The presence of p85α in the complexes was detected by Western blotting.

of cells with silica. Wortmannin (100–500 nM) or LY294002 (100–500 μM) caused a measurable inhibition (i.e. 45 and 64% at 500 nM wortmannin and 500 μM LY294002, respectively) of silica-induced binding activity of NF-κB to DNA (Fig. 4A). However, wortmannin did not alter tyrosine-phosphorylation of IκB-α induced by silica, suggesting that PI3-kinase activity was not required for tyrosine phosphorylation of IκB-α (Fig. 5). In addition, as a positive control, the inhibitory effect of PI3-kinase inhibitors was also examined on NF-κB activation induced by pervanadate, since pervanadate-induced stimulation of NF-κB activation has been reported to depend on activation of PI3-kinase [10]. As expected, wortmannin inhibited pervanadate-induced NF-κB activation in a concentration-dependent (Fig. 4B). In contrast, wortmannin over the same range of concentrations had little effect on LPS-induced NF-κB activation (Fig. 4C). Wortmannin has been reported to irreversibly inactivate PI3-kinase by binding to its p110 catalytic subunit [18]. Therefore, these data suggest that the p110 subunit of PI3-kinase activity is required for silica- or pervanadate-, but not LPS-induced NF-κB activation through a pathway which does not affect the level of stimulant-induced tyrosine phosphorylation of IκB-α.

Wortmannin, a PI3-kinase inhibitor, blocks tyrosine phosphorylation of p65 induced by silica

Data from the present study suggest the involvement of not only p85 but also a p110 subunit of PI3-kinase in silica-induced NF-κB activation. We also previously reported that silica-induced NF-κB activation was blocked by inhibition of tyrosine kinase [8]. A question is, therefore, raised as to whether tyrosine phosphorylation of p65 was induced and, if so, whether a p110 catalytic subunit of PI3-kinase was involved in tyrosine phosphorylation of p65 in silica-treated cells. To test this hypothesis, the cell lysates from silica-treated or untreated cells were immunoprecipitated with control IgG (Fig. 6A , lanes 1 and 2) or with anti-p65 antibody (Fig. 6A, lanes 3–8) followed by Western blot analysis with the antiphosphotyrosine mAb. Substantial tyrosine phosphorylation of p65 was observed 10 min after silica stimulation

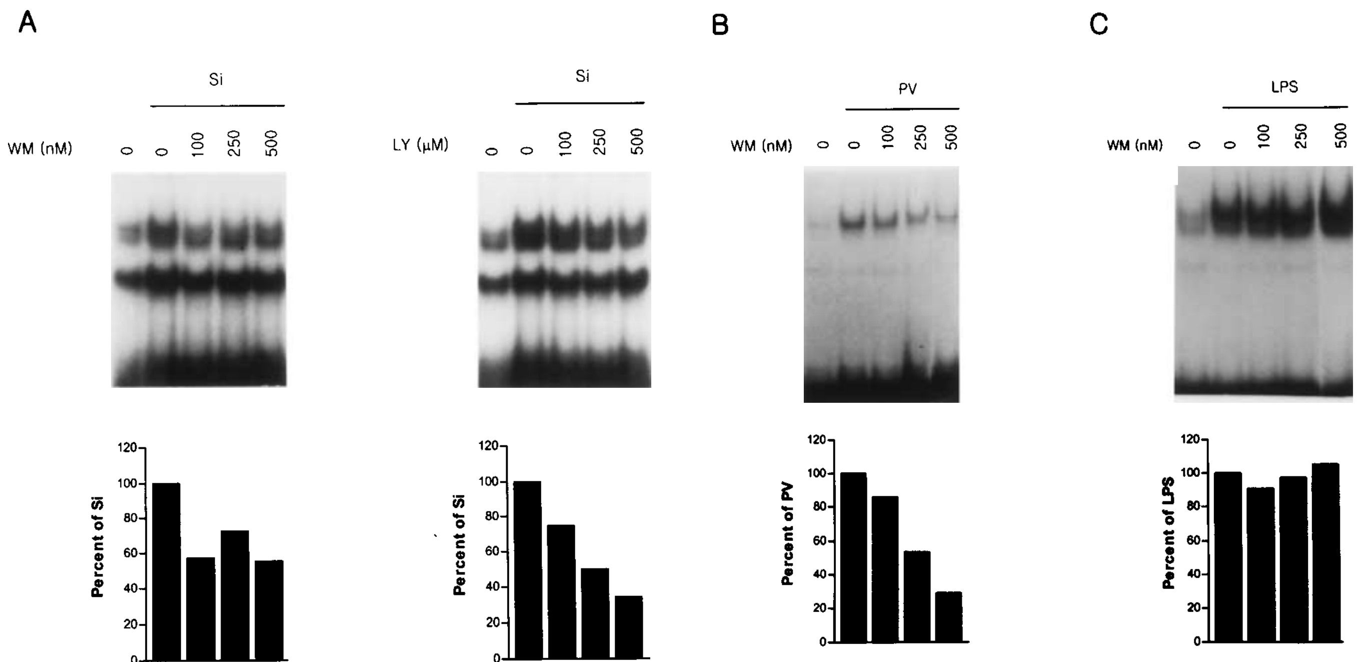


Fig. 4. Electrophoretic mobility shift assay (EMSA) illustrating the effects of PI3-kinase inhibitors on silica (A), pervanadate (B) or LPS (C) -induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with wortmannin (100–500 nM) or LY294002 (100–500 μM) and then stimulated by silica (100 μg/ml), pervanadate (100 μM) or LPS (10 μg/ml) for an additional 4 h. The results of EMSA are shown (upper panels) and quantitated by densitometric analysis as a percentage of the response to stimulant alone minus control (lower panels). Data are representative of at least 3 experiments.

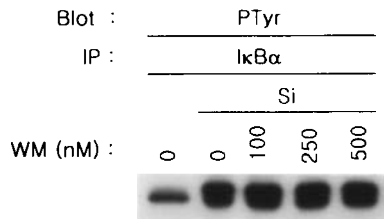


Fig. 5. Tyrosine phosphorylation of IκB-α in silica-stimulated RAW264.7 cells. Cells were preincubated for 2 h with wortmannin (100–500 nM) before treatment with silica (100 μg/ml) for an additional 20 min. The lysates were incubated with anti-IκB-α Ab before analysis of tyrosine phosphorylation by Western blotting with anti-phosphotyrosine mAb. Data are representative of at least 3 experiments.

and was sustained through a 30 min exposure of RAW 264.7 cells to silica. Tyrosine phosphorylation of p65 was not detected after 60 min of silica exposure. In contrast, treatment of RAW 264.7 cells with silica did not alter the tyrosine phosphorylation level of the IgG immunoprecipitate, indicating that the phosphorylated tyrosine residue was specifically presented on p65. No tyrosine-phosphorylated p65 was detected in unstimulated cells (Fig. 6A, lane 3). In contrast, tyrosine phosphorylation of p50 NF-κB was not observed in RAW 264.7 cells stimulated with silica for 10–60 min (data not shown). To examine that the role of PI3-kinase in tyrosine phosphorylation of P65 in silica-stimulated RAW 264.7 cells, wortmannin (100–500 nM) was added to cells 2 h before exposure to silica. As shown Fig. 6D, tyrosine phosphorylation of p65 in cells exposed to silica for 30 min was inhibited in a concentration-dependent manner by wortmannin. These data suggest that a PI3-kinase p110 subunit may result in an increase in tyrosine-phosphorylated p65, contributing to NF-κB activation in silica-treated cells. Tyrosine phosphorylation of p65 was also observed with a peak response at 20 or 10 min after pervanadate or LPS stimulation, respectively (Figs 6B and 6C). Wortmannin blocked this process in cells stimulated with pervanadate, but not with LPS (Figs 6E and 6F). These data suggest that tyrosine phosphorylation of p65 is a reaction common to several stimulants. However, PI3-kinase plays a specific role in silica- or pervanadate-stimulated cells but not those exposed to LPS.

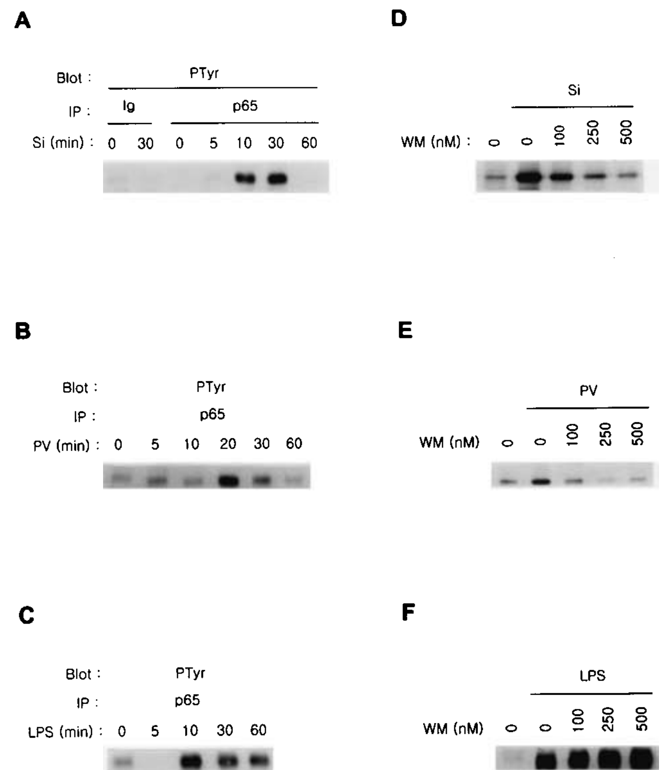


Fig. 6. Tyrosine phosphorylation of p65 NF-κB in silica-, pervanadate- or LPS-stimulated RAW264.7 cells. RAW264.7 cells were incubated with silica (100 μg/ml, A), pervanadate (100 μM, B) or LPS (10 μg/ml, C) for 5–60 min. The silica-treated lysates (A) were incubated with control Ig (lanes 1 and 2) or anti-p65 Ab (lanes 3–8) before analysis of tyrosine phosphorylation by Western blotting with anti-phosphotyrosine mAb. Cells were preincubated for 2 h with wortmannin (10–500 nM) before treatment with silica (D), pervanadate (E) or LPS (F) for an additional indicated time (30, 20, and 10 min, respectively). The lysates were incubated with anti-p65 Ab before analysis of tyrosine phosphorylation by Western blotting with anti-phosphotyrosine mAb. Data are representative of at least 3 experiments.

Discussion

The data from the present study support that (1) interaction between a p85 subunit of PI3-kinase and tyrosine-phosphorylated IκB-α may be involved in NF-κB activation independently of IκB-α degradation; (2) a p110 subunit of PI3 kinase activity is also required for silica- or pervanadate-, but not LPS-induced NF-κB activation through a pathway which does not affect the level of stimulant-induced tyrosine phosphorylation of IκB-α. Consistent with our data, Béraud *et al.* [10] have reported the interaction of the p85 subunit of PI3-kinase with tyrosine-phosphorylated IκB-α and involvement of p110 subunit in NF-κB activation in pervanadate-stimulated T cells. Unlike the role of the p85 subunit in NF-κB activation in response to silica or pervanadate, Reddy *et al.* [11] have demonstrated that a p85 subunit interacts with the IL-1 receptor in response to IL-1, leading to NF-κB activation. However, Sizemore *et al.* [9] have reported that specific PI3-kinase inhibitors have no effect on IκB-α degradation and DNA binding activity of NF-κB in response to IL-1. At this time, the differences in their results are not clearly explained, while the data from our present study support the specific role of PI3-kinase in NF-κB activation through tyrosine phosphorylation of IκB-α in response to silica or pervanadate, but not LPS. LPS, like IL-1, is a stimulant known to induce NF-κB activity through phosphorylation of serine in IκB-α and subsequent degradation of the inhibitor [5, 19].

Phosphorylation of IκB-α is a critical regulatory step in the activation of NF-κB. However, recent studies suggest that phosphorylation of NF-κB p65 may regulate DNA binding activity [20] or its transactivation potential [21]. Thus, phosphorylation of NF-κB was proposed to be an additional mechanism regulating the expression of NF-κB-dependent genes. Our results demonstrate, for the first time, that silica increases tyrosine-phosphorylation of p65. This process was also observed for stimulation with pervanadate or LPS. However, the p110 catalytic subunit of PI3-kinase appears essential for the process induced by silica or pervanadate but is not involved in LPS-induced tyrosine phosphorylation of p65. A role for p110 activity of PI3-kinase in induction of a pathway leading to the serine phosphorylation of p65 and transactivation of NF-κB, but not DNA binding activity of NF-κB in IL-1-stimulated cells has been reported [9]. In the present study, the effects of PI3-kinase on serine phosphorylation of p65 in silica-stimulated cells have not been examined. However, the p110 subunit plays a role in NF-κB activation in response to silica or pervanadate contributing to tyrosine phosphorylation of p65 NF-κB. Therefore, it is possible that different roles for p110 subunits may be determined by phosphorylation at different sites of p65 in response to the different signals.

The time course of PI3-kinase activity was consistent with that of tyrosine phosphorylation of p65 in silica-stimulated RAW 264.7 cells. These data suggest that tyrosine-phosphorylated IκB-α interacts with a p85α subunit of PI3-kinase together with NF-κB. The activation of a p110 subunit of PI3-kinase contributes to p65 phosphorylation, finally leading to dissociation of NF-κB from the NF-κB-IκB-α-PI3-kinase complex without requiring the inducible degradation of IκB-α. However, it is not clear whether these processes occur in simultaneously or in succession.

Compared with silica-induced NF-κB activation, silica-stimulated PI3-kinase activity occurred earlier (10 vs. 30 min after treatment with silica) and peaked earlier (30 min vs. 4–6 h after treatment with silica). These data suggest that PI3-kinase activation may act at a step following tyrosine phosphorylation of IκB-α or p65 possibly by direct interaction through the activities of its downstream effector molecules, such as the lipid kinase and the protein kinase, with the DNA binding sites in a way that would increase binding to DNA.

Recent evidence indicates that exposure of RAW 264.7 cells to silica results in the production of ROS, and that reactive oxidants play a role in silica-induced NF-κB activation [8]. Indeed, antioxidants inhibited tyrosine-phosphorylation of IκB-α in silica-stimulated RAW 264.7 cells [7]. Data from the present study suggest the involvement of ROS in the induction of PI3-kinase activation. This observation would add a novel activity for ROS in the activation of NF-κB induced by silica. Recent evidence indicates that small G proteins may couple hydrogen peroxide to extracellular signal-regulated kinase (ERK) activation through PI3-kinase [22]. From a mechanistic point of view, further study to investigate the involvement of small G proteins in linking silica-induced ROS production to PI3-kinase activity would be necessary.

Activation of the NF-κB pathway in macrophages appears to depend on the unique properties of the surface of crystalline silica. Coating crystalline silica with aluminum lactate to mask the negative surface charge [23] or with polyvinylpyridine *N*-oxide (PVPNO) to prevent the surface silanol groups from participating in H-bonding with cell membranes [24] significantly decreases the ability of quartz to activate NF-κB in macrophages. This indicates that the surface of the particle does play an important role in activation of transcription pathways. Indeed, Ding *et al.* [25] has reported that aging the surface of fractured quartz or coating it with PVPNO also significantly inhibits silica-induced MAP kinase pathways and AP-1 activation. To date, the effect of such coatings on the silica induction of the PI3-kinase pathway has not been reported.

In conclusion, this study shows that activated PI3-kinase in silica-stimulated RAW264.7 cells participates to a signaling pathway through directly interacting with tyrosine-phosphorylated IκB-α and contributing to tyrosine phosphorylation of p65, leading to NF-κB activation (Fig. 7). In addition, ROS appear to be important regulatory molecules in the activation of NF-κB by mediating PI3-kinase activation.

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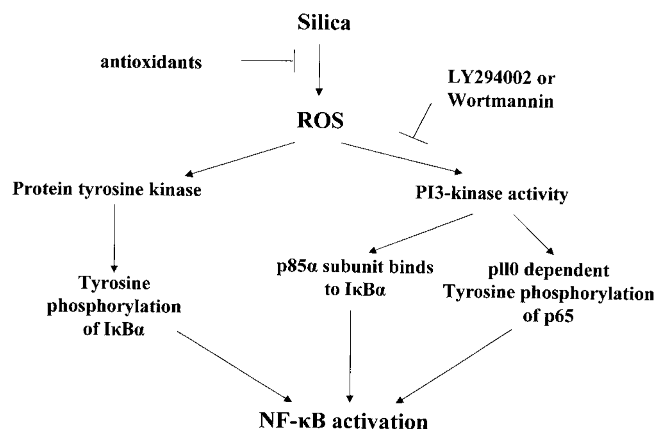

Fig. 7. A scheme depicting proposed PI3-kinase dependent and independent pathways for NF-κB activation.

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