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# Enhancement of nuclear factor-κB activation and protein tyrosine phosphorylation by a tyrosine phosphatase inhibitor, pervanadate, involves reactive oxygen species in silica-stimulated macrophages

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**Abstract**

Reactive oxygen species (ROS) and phosphorylation events mediated by tyrosine kinase are involved in silica-induced nuclear factor-kappa B (NF-κB) activation. Protein tyrosine phosphatase (PTPase) acts to limit protein tyrosine phosphorylation. In the present study, we investigated the role of PTPase in NF-κB activation and tyrosine phosphorylation in silica-stimulated macrophages, and the involvement of ROS in these responses. Treatment of mouse peritoneal macrophages (RAW264.7 cells) with a PTPase inhibitor, pervanadate, markedly enhanced the DNA-binding activity of NF-κB in the presence or absence of silica. The stimulatory effect of pervanadate on NF-κB activation was also demonstrated in LPS-stimulated macrophages. A specific inhibitor of protein tyrosine kinase (PTK), genistein, prevented the NF-κB activation induced by pervanadate in the presence of silica while inhibitors of protein kinase A or C, such as staurosporine or H7, had no inhibitory effect on NF-κB activation. A variety of antioxidants, such as catalase, superoxide dismutase, *N*-acetyl cysteine (NAC), and pyrrolidine dithiocarbamate, inhibited NF-κB activation induced by pervanadate in the presence of silica. Furthermore, pervanadate markedly enhanced silica- or LPS-induced protein tyrosine phosphorylation in cells. Treatment of macrophages with NAC abolished the increase in tyrosine phosphorylation in cells stimulated with the combination of pervanadate and either silica or LPS or with silica alone. The results suggest that PTPase may play a crucial role in the negative regulation of silica-signaling pathways leading to NF-κB activation in macrophages. Furthermore, ROS appear to be involved in downstream signaling between PTPase inhibition and NF-κB activation. 

*Keywords:* NF-κB; Pervanadate; Reactive oxygen species; Macrophages

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## 1. Introduction

Inhalation and deposition of silica dust result in a cycle of oxidant damage, pulmonary inflammation and fibrosis. Inflammatory cytokines and growth factors are considered critical to this pathogenesis. Nuclear factor-kappa B (NF-κB) is an essential transcription factor which controls gene expression of cytokines, chemokines, growth factors and cell adhesion molecules (Barnes and Karin, 1997; Chen et al., 1999). Recent evidence indicates that in vitro exposure of macrophages to silica or LPS induces activation of NF-κB (Chen et al., 1998; Kang et al., 2000). Silica-induced activation of NF-κB in pulmonary phagocytes has also been demonstrated after in vivo exposure to silica (Sacks et al., 1998). 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.

Various subunits of the NF-κB/Rel family of transcription factors have been identified including p50, p52, p65 (Rel A), C-Rel, Rel-B and the *Drosophila morphogen* Dorsal (Liou and Baltimore, 1993). The different family members can associate as various homo- or hetero-dimers through a highly conserved N-terminal sequence, called the NRD (NF-κB/rel/Dorsal, Grimm and Baeuerle, 1993), which is essential for DNA-binding and nuclear localization. The most predominantly characterized NF-κB complex is a p50–p65 heterodimer, which, in its resting state, is associated with an inhibitor molecule, IκB, and is retained in the cytoplasm (Zabel and Baeuerle, 1990). In response to a variety of stimuli, IκB is phosphorylated and dissociated from the NF-κB complex. The active NF-κB then can translocate to the nucleus where it binds to the NF-κB motif and functions as a transcriptional regulator. A p65–p50 or p65–p52 hetero-dimer could be detected in silica-stimulated RAW 264.7 macrophages. This NF-κB binding activity could be competitively inhibited by a non-labeled NF-κB consensus DNA probe (Chen et al., 1995).

A previous study from our laboratory suggested that phosphorylation events mediated by tyrosine kinase may be involved in NF-κB activation, and that silica-induced NF-κB activation in macrophages was blocked by various oxygen-radical scavengers (Kang et al., 2000). However, the molecular mechanisms by which ROS are involved in downstream signaling events between the tyrosine kinase and NF-κB activation remain unclear. One suggested mechanism is the inactivation of protein tyrosine phosphatase (PTPase) by oxidation of a conserved cystein residue within their catalytic domain, leading to increased protein tyrosine phosphorylation (Suzuki et al., 1997).

The level of tyrosine phosphorylation in cells is the result of a dynamic equilibrium between the opposing activities of tyrosine kinases and phosphotyrosine phosphatases, PTPase (Lee et al., 1998). The role of PTPase in the regulation of NF-κB activation is variable and appears to be cell type-specific. Imbert et al. (1994) have reported that a PTPase inhibitor, pervanadate, stimulated protein tyrosine phosphorylation and downstream events of the T-cell activation process, including induction of NF-κB binding to DNA. In contrast, Singh and Aggarwal (1995) have demonstrated that inhibitors of PTPase, including phenylarsine oxide (PAO), pervanadate, and diamide, blocked TNFα-dependent activation of NF-κB in human ML-1a cells. The role, if any, of PTPase in silica-induced activation of NF-κB in macrophages, however, is not yet understood.

The objectives of the present study, were to investigate the role of PTPase in DNA-binding activity of NF-κB and PTPase in a macrophage, RAW264.7, cell line after silica stimulation and to determine the involvement of ROS in this activation. To pursue these objectives, the effects of a PTPase inhibitor, pervanadate, in our model were studied, since pervanadate is a powerful phosphatase inhibitor that leads to the accumulation of phosphorylated protein (Reiland et al., 1996).

## 2. Methods

### 2.1. Reagents

Crystalline silica (Min-U-Sil, particle size $< 5$ μm) was obtained from US Silica Corporation (Berkeley Springs, WV). Prior to use, silica sam-

ples were sterilized by heating at 160°C for 90 min in a dry oven and dispersed in Dulbecco's modified eagle medium (DMEM) with supplements just before addition to culture plates. Lipopolysaccharide (LPS), catalase, superoxide dismutase (SOD), *N*-acetyl cysteine (NAC), pyrrolidine dithiocarbamate (PDTC), genistein, and H7 were purchased from Sigma Chemical Company (St. Louis, MO). Staurosporine was 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).

### 2.2. 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 (Mediatech, Washington, DC) supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT), 2 mM glutamine, and 1000 U/ml penicillin–streptomycin.

### 2.3. Preparation of pervanadate solutions

Pervanadate was prepared as described by Imbert et al. (1996a). Briefly, pervanadate was obtained by mixing vanadate (sodium orthovanadate) in incubation buffer (30 mM HEPES, pH 7.5; 150 mM NaCl; 4 mM KCl; 0.8 mM $MgSO_4$; 1.8 mM $CaCl_2$; 10 mM glucose) with 1 mM $H_2O_2$ for 15 min at 22°C. Catalase (200 μg/ml) was then added to remove the residual $H_2O_2$. The concentration of pervanadate generated is determined by the vanadate concentration added to the mixture.

### 2.4. 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 $5 \times 10^6$ cells per ml for 3 days. Then the medium was replaced with fresh medium and cells pretreated with inhibitors of protein kinases, such as genistein (74 μM), staurosporine (0.02 μM) and H7 (25 μM), or a variety of antioxidants, such as catalase (50 000 U/ml), SOD (1500 U/ml), NAC (1 μM) and PDTC (200 μM). After 2 h pretreatment, cells were cultured for 4 h with a combination of pervanadate (25–200 μM) plus silica (100 μg/ml) or LPS (1.0 μg/ml) in the absence or presence of a kinase inhibitor or antioxidant. At the end of this treatment period, 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 DTT; 1.0% NP40; 0.5 mM PMSF) for 10 min on ice, and then vortexed for 10 s. Nuclei were pelleted by centrifugation at $12\,000 \times g$ for 30 s and were resuspended in buffer C (20 mM HEPES, pH 7.9; 20% glycerol; 0.42 M NaCl; 1 mM EDTA; 0.5 mM PMSF) for 30 min on ice. The supernates containing nuclear proteins were collected by centrifugation at $10\,000 \times g$ for 2 min and stored at −70°C.

### 2.5. 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), 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; 20 mM NaCl) were incubated for 30 min at room temperature. Protein-DNA complexes were separated on 5% non-denaturing polyacrylamide gels in $1 \times$ TBE buffer and autoradiographed overnight. Autoradiographic signals for activated NF-κB were quantitated by densitometric scanning using UltroScan XL laser densitometer (LKB, Model 2222-020, Bromma, Sweden) to determine the intensity of each band.

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

### 2.6. Immunoprecipitation and immunoblotting

The confluent cells grown on 100 mm plastic dishes were incubated in DMEM supplemented with 5% FBS, 2 mM glutamine, and 1000 U/ml penicillin–streptomycin for 3 days. NAC (1 mM) was added to the cells 2 h prior to treatment. Cells then were treated with either silica (100 μg/ml) for 30 min or LPS (1.0 μg/ml) for 15 min in the presence or absence of pervanadate (50 μM). After treatment, cells were washed with ice-cold phosphate buffered-saline and lysed with 1 ml of ice-cold lysis buffer containing 50 mM Tris–HCl (pH 8.0), 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 supernate was incubated with 5 μg of PY-20 at 4°C for 1 h. After incubation for 30 min with protein A or G conjugated sepharose (5 μg/ml), the antigen/antibody complexes were pelleted by brief centrifugation. The pellet was resuspended in ice-cold lysis buffer and washed three times by alternate centrifugation at 13 000 × *g* for 30 s and resuspension in lysis buffer. The pellet was dissolved in 20 μl of Laemmli's sample buffer and analyzed on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE, Laemmli, 1970).

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

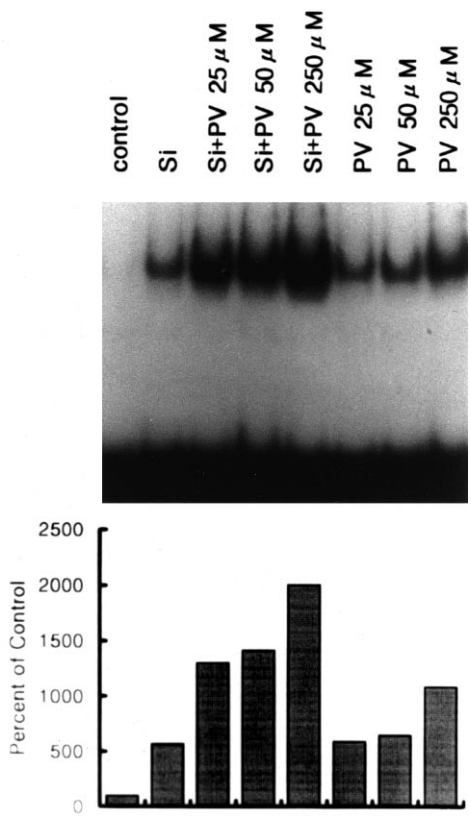


Fig. 1. EMSA illustrating the effect of pervanadate (PV) on silica-induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells treated with silica (100 μg/ml), PV (25–250 μM), or silica + PV for 4 h. The results of EMSA are shown (upper panel) or quantified by densitometric analysis and presented as a percentage of the control response (lower panel). These data are representative of results from three separate experiments.

## 3. Results

In vitro exposure of macrophages to pervanadate for 4 h resulted in a concentration-dependent activation of the binding of NF-κB to DNA (Fig. 1). Maximal NF-κB activation in the range of concentration of pervanadate from 25 to 250 μM was observed at 250 μM. Furthermore, the simultaneous treatment of pervanadate (25–200 μM) and silica (100 μg) showed an additive stimulation of binding activity of NF-κB to DNA. A greater than additive enhancement of DNA binding activity of NF-κB in macrophages was also observed when various concentrations of pervanadate were simultaneously added to cells treated with LPS (Fig. 2).

To verify that protein tyrosine kinase (PTK) may be critically involved in the NF-κB activation induced by pervanadate in the presence of silica, a specific inhibitor of PTK, genistein, was added to the cells 2 h before exposure to the combination of pervanadate (25–200 μM) and silica (100 μg/ml). Genistein (74 μM) dramatically prevented NF-κB activation in response to pervanadate plus

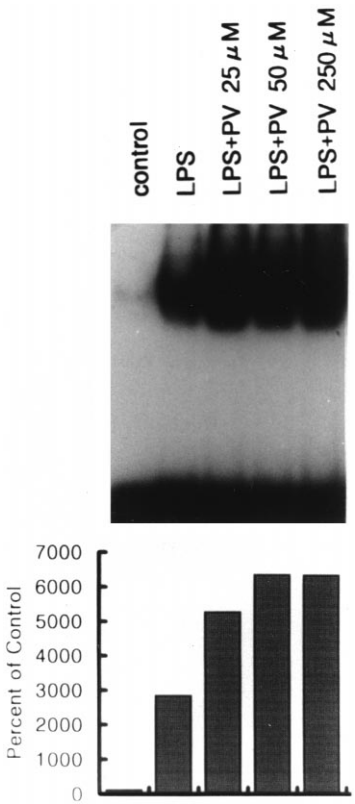


Fig. 2. EMSA illustrating the effect of PV on LPS-induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells stimulated with PV (25–250 μM) in the presence of LPS (1.0 μg/ml) for 4 h. The results of EMSA are shown (upper panel) or quantified by densitometric analysis and presented as a percentage of the control response (lower panel). This is a representative assay from three separate experiments.

silica (Fig. 3). In contrast to the effect of an inhibitor of PTK, pretreatment of macrophages with inhibitors of protein kinase A or C, such as staurosporine (0.02 μM) or H7 (25 μM), resulted in no inhibition of NF-κB activation induced by pervanadate (50 μM) in the presence of silica (Fig. 4).

Recently, molecular and cellular approaches demonstrated that ROS may play a role in the modulation of NF-κB activation (Remacle et al., 1995). We have previously reported that various antioxidants inhibited NF-κB activation induced by silica (Kang et al., 2000). The following experiments thus assessed the involvement of ROS in NF-κB activation induced by pervanadate in the presence of silica. Cells were pretreated with catalase, SOD, NAC, or PDTC for 2 h prior to treatment with pervanadate and silica. These antioxidants inhibited stimulant-induced NF-κB activation by approximately 35% (Fig. 5).

Tyrosine phosphorylation of cellular proteins is a crucial event in the transduction of activation signals leading to NF-κB activation in silica- or LPS-stimulated macrophages (Kang et al., 2000).

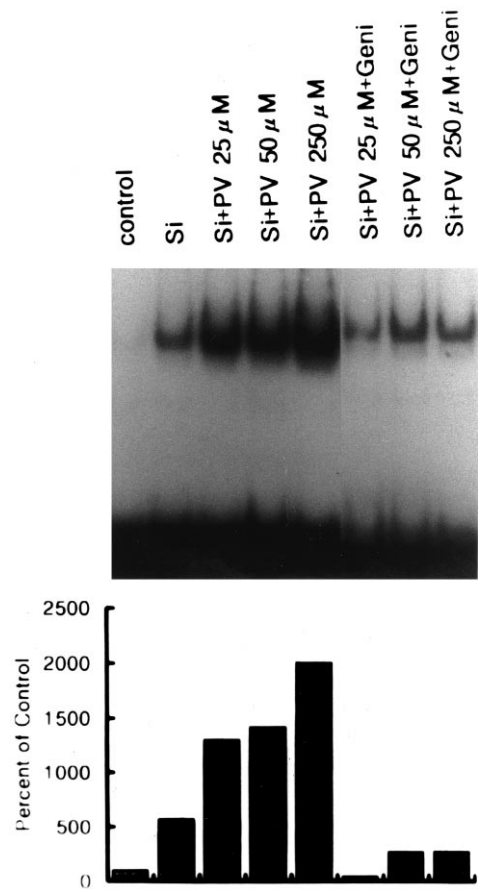


Fig. 3. EMSA illustrating the effect of a tyrosine kinase inhibitor, genistein, on NF-κB activation induced by silica + PV. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with genistein (74 μM) and then stimulated by silica (100 μg/ml) + PV (25–250 μM). The results of EMSA are shown (upper panel) or quantified by densitometric analysis and presented as a percentage of the control response (lower panel). This is a representative assay from three separate experiments.

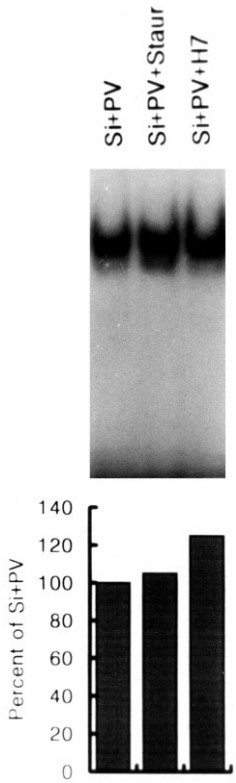


Fig. 4. EMSA illustrating the effects of a protein kinase C or A inhibitor on NF-κB activation induced by silica + PV. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with staurosporine (0.02 μM) or H7 (25 μM) and then stimulated by silica (100 μg/ml) + PV (25–250 μM). The results of EMSA are shown (upper panel) or quantified by densitometric analysis and presented as a percentage of the silica + PV response (lower panel). This is a representative assay from three separate experiments.

Pervanadate has been reported to induce activation of the tyrosine kinases lck and fyn and to dramatically increase tyrosine phosphorylation of cellular proteins in Jurkat human leukemic T-cells (Imbert et al., 1996b). Therefore, we examined the effect of pervanadate on silica- or LPS-induced tyrosine phosphorylation in RAW 264.7 macrophages. Treatment of macrophages with the combination of pervanadate plus silica for 30 min or pervanadate plus LPS for 15 min dramatically elevated tyrosine phosphorylation of many proteins compared with tyrosine phosphorylation in the cells stimulated with silica or LPS alone (Fig. 6). The involvement of ROS in the tyrosine phosphorylation induced by these stimulants alone, or in combination was further evaluated by using a potent reducing agent, NAC. Treatment with NAC effectively inhibited protein tyrosine phosphorylation in the cells stimulated with the combination of pervanadate and either silica or LPS as well as silica alone. Thus, these data suggest that enhancement of protein tyrosine phosphorylation during cell stimulation in the presence of pervanadate may be modified by ROS.

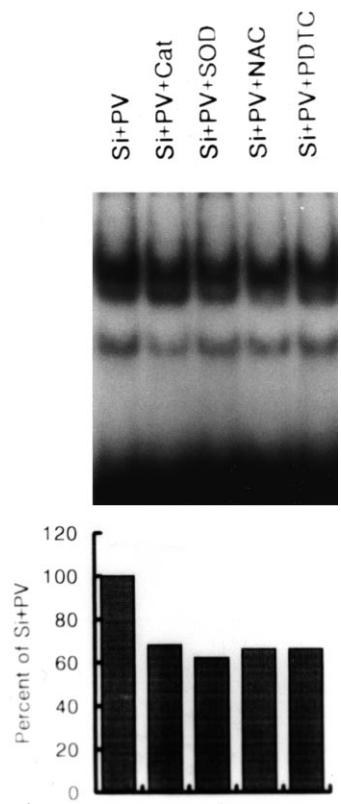


Fig. 5. EMSA illustrating the effect of antioxidants on NF-κB activation induced by PV in the presence of silica. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with catalase (50 000 U/ml), SOD (1500 U/ml), NAC (1 mM), or PDTC (200 μM), and then simultaneously stimulated by the combination of PV and silica. The results of EMSA are shown (upper panel) or quantified by densitometric analysis and presented as a percentage of the response of silica + PV (lower panel). This is a representative assay from three separate experiments.

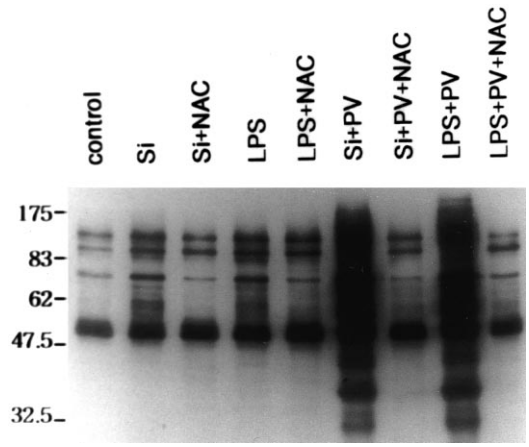


Fig. 6. Protein tyrosine phosphorylation induced by PV in the presence or absence of silica or LPS. RAW264.7 cells were incubated in the absence or presence of NAC (1 mM) for 2 h. After pretreatment, cells were incubated with silica (100 μg/ml) + PV (50 μM) for 30 min or LPS (1.0 μg/ml) + PV (50 μM) for 15 min. Then Western blots with anti-phosphotyrosine antibodies were employed to monitor protein tyrosine phosphorylation. Sizes are indicated in kilodaltons. This is a representative assay from three separate experiments.

## 4. Discussion

Data presented in this study indicate that pervanadate, an inhibitor of PTPase, can directly stimulate NF-κB activation in mouse macrophages. These data suggest that PTPase plays a role in attenuating NF-κB activation. Direct stimulation of NF-κB activation by pervanadate has also been found in Jurkat leukemic T cells (Imbert et al., 1994). This pervanadate-induced NF-κB activation was not due to residual $H_2O_2$ remaining in pervanadate preparation, since 200 μM $H_2O_2$ alone or $H_2O_2$ in the presence of catalase had no stimulatory effect (data not shown). Data presented in the current study also demonstrate an additive effect of pervanadate and silica on NF-κB activation. Such additive stimulation of NF-κB has also been reported by Imbert et al. (1996a) in Jurkat T cells treated with pervanadate plus PMA. As with NF-κB activation, additive effects of pervanadate in combination with either anti-CD3 antibody or PMA have been reported for cell surface expression of two activation markers, CD69 and interleukin-2 receptor α-chain (CD25) and IL-2, by Jurkat T cells (Imbert et al., 1996b). Furthermore, Diaz-Guerra et al. (1999) reported that treatment of peritoneal macrophages with pervanadate plus interferon (IFN)-γ or LPS augmented activation of NF-κB and expression of inducible nitric oxide synthase (iNOS). In contrast, inhibitors of PTPase, such as PAO, pervanadate or diamide inhibited NF-κB activation in tumor necrosis factor (TNF)-stimulated human myeloid ML-1a cells (Singh and Aggarwal, 1995) and human dermal microvessel endothelial cells (Dhawan et al., 1997). These authors point out, that such results seem paradoxical in view of the reports that PAO, pervanadate, and diamide are all inhibitors of dephosphorylation and thus should increase the levels of NF-κB activation, since phosphorylation of IkB has been shown to be essential for activation of NF-κB. In the present study, PAO (25 μM) or diamide (0.5 mM) had an additive effect on silica- or LPS-induced NF-κB activation in RAW 264.7 cells (data not shown). This disagreement may be due to different sensitivity of various cell types to PTPase and/or cellular oxidative potential in the presence of an inhibitor of PTPase. Indeed, Menon et al. (1993) suggested that such differences in induction of NF-κB activation may reflect intrinsic differences in the intracellular oxidative state between different cell types. Schieven et al. (1993) have suggested that the stimulatory effects of ROS and vanadate, a prototype of pervanadate, on tyrosine phosphorylation were due to the kinase activation in addition to phosphatase inhibition. In addition, Zor et al. (1993) have shown that ROS formation induced by PMA in association with vanadate was essential for protein tyrosine phosphorylation and phospholipase $A_2$ activation. In the present study, we demonstrated that antioxidants, such as catalase, SOD, NAC and PDTC, inhibited NF-κB activation in macrophages stimulated by pervanadate plus silica. These data thus support the interpretation that the effect of pervanadate on NF-κB activation during cell stimulation is dependent on cellular redox state. In addition, there are various IκB species in different cell types. Therefore, specific subpopulations of NF-κB-IκB complex may be responsive to a particular stimulus (Beraud et al., 1999). This allows for cell type specific regulation of NF-B.

As with silica-induced NF-κB activation (Chen et al., 1998; Kang et al., 2000), the antioxidant, NAC, inhibited protein tyrosine phosphorylation induced by pervanadate in the presence of silica or LPS. These data suggest that endogenous ROS may play a regulatory role in the protein tyrosine phosphorylation as well as NF-κB activation. The exact mechanism in which ROS activate NF-κB is not fully understood. However, hydroxyl radicals ($^{\bullet}OH$) seem to play a key role in silica-induced NF-κB activation. Indeed, Chen et al. (1998) have demonstrated the production of $^{\bullet}OH$ from silica-exposed macrophages by electron spin resonance spectroscpoy. They proposed that $H_2O_2$ produced during the respiratory burst by silica-exposed macrophages could participate in a Fenton reaction with trace ferrous iron on the silica particles to generate $^{\bullet}OH$. Blocking this $^{\bullet}OH$ generation with catalase (a $H_2O_2$ scavenger) or metal chelators resulted in inhibition of silica-induced NF-κB activation (Chen et al., 1998). Our data further the understanding of the role of ROS by indicating that ROS are not only involved in NF-κB activation, but act through the stimulation of tyrosine phosphorylation.

The role of PTPase in the silica- or LPS-induced activation of NF-κB in macrophages is not completely understood. However, findings suggest that pervanadate-sensitive tyrosine PTPase may be important as a negative modulator. Potent activation of NF-κB by pervanadate-induced inhibition of PTPase appears to be mediated by phosphorylation of IκB-α on tyrosine (Imbert et al., 1996a). Tyrosine-phosphorylated peptide can be specifically recognized by Src homology 2 (SH2) domains (Pawson, 1995). The regulatory subunit of phosphoinositide 3-kinase (p85α) has been shown to associate through its Src homology 2 domains with tyrosine-phosphorylated IκB-α in vitro and in vivo after stimulation of T cells with pervanadate (Beraud et al., 1999). This association could provide a mechanism by which tyrosine-phosphorylated IκB is released from NF-κB. Whether the stimulatory effect of pervanadate is due to inactivation of PTPase or activation of protein-tyrosine kinase or both is not certain. However, Schieven et al. (1993) have suggested that the combination of kinase activation and phosphatase inhibition provides a mechanism for the hyperinduction of tyrosine phosphorylation. This signal would be expected to amplify downstream signaling events such as NF-κB activation. In addition, depending on the abundance of IκB species in the cells, the stimulatory effect of pervanadate on NF-κB activation in response to other stimulants can be understood.

In summary, the stimulatory effect of pervanadate on silica-induced NF-κB activation in macrophages implies that pervanadate may increase the gene expression and inflammatory responses to silica that are dependent on NF-κB. In addition, ROS may be involved, directly or indirectly, in the signaling pathways between PTPase inhibition and NF-κB activation.

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