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Nitric oxide up-regulates DNA-binding activity of nuclear factor-κB in macrophages stimulated with silica and inflammatory stimulants

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Abstract

Nitric oxide (NO), a reactive nitrogen species, plays an important role in inflammatory lung damage. In the present study, we investigated the role of NO in DNA-binding activity of NF-κB in macrophages stimulated with silica or other inflammatory stimulants. Treatment of mouse macrophages (RAW264.7 cells) with a selective inhibitor of inducible nitric oxide synthase (iNOS), L-N^6-(1-iminoethyl) lysine (L-NIL), or a nonselective iNOS inhibitor, Nω-nitro-L-arginine methylester (L-NAME), resulted in inhibition of silica-induced nitric oxide production as well as silica-induced NF-κB activation. L-NIL also effectively inhibited NF-κB activation induced by other inflammatory stimulants, such as lipopolysaccharide (LPS) or muramyl dipeptide (MDP). These inhibitory effects of L-NIL and L-NAME on silica- or LPS-induced NF-κB activation were also observed in primary rat alveolar macrophages. Furthermore, NO generating compounds, such as sodium nitroprusside (SNP) and 3-morpholinosydnonimine (SIN-1), caused a dose-dependent increase in NF-κB activation, which was positively correlated with the level of NO production. Specific inhibitors of protein tyrosine kinase, such as genistein and AG494, prevented NF-κB activation in SNP- or SIN-1 treated cells, suggesting involvement of tyrosine kinase in the NO signaling pathway leading to NF-κB activation. In contrast, inhibitors of protein kinase C or A, such as staurosporine or H89, had no inhibitory effect on SIN-1 induced NF-κB activation. Metalloporphyrins, such as tetrakis (N-methyl-4′-pyridyl) porphyrinato iron (III) (Fe-TMPyP) and Zn-TMPyP which are known to alter NO-dependent activity, markedly inhibited silica- and LPS-induced NF-κB activation. The results suggest that NF-κB activation in macrophages can be induced under certain conditions by nitric oxide and that nitric oxide produced by phagocytes exposed to inflammatory agents may up-regulate the activation of NF-κB. (Mol Cell Biochem **215**: 1–9, 2000)

Key words: nitric oxide, transcription factor, nuclear factor kappa B, macrophages, silica

Introduction

Inhalation of crystalline silica can occur in numerous occupational settings, such as, mining, sandblasting, surface drilling, tunneling, stone cutting, construction, pottery making and silica flour milling. Pulmonary deposition of silica dust can result in a cycle of lung damage and scarring known as silicosis, a fibrotic lung disease [1]. Furthermore, epidemiologic and animal studies suggest that silica exposure may also be associated with lung cancer [2, 3].

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 [4, 5]. Inflammatory cytokines and growth factors are thus critical to this pathogenesis. Nuclear factor kappa B (NF-κB) is an essential transcription factor which controls

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gene expression of a host of cytokines, chemokines, growth factors and cell adhesion molecules [6, 7]. 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.

NF-κB is a heterodimeric protein complex containing two members of the rel family of transcription factors, p50 and p65. At rest, the heterodimeric NF-κB complex is located in the cytoplasm bound to an inhibitory factor, IκB [8]. Upon stimulation, IκB is phosphorylated and proteolytically degraded or processed by proteasomes and other proteases. Free NF-κB then translocates into the nucleus where it binds to various gene promoter regions controlling the expression of various pro-inflammatory and proliferative agents [6].

Recent evidence indicates that in vitro exposure of macrophages to silica or LPS induces activation of NF-κB [9, 10]. Silica-induced activation of NF-κB in pulmonary phagocytes has also been demonstrated after *in vivo* exposure to silica [11]. Exposure of lung phagocytes to silica also results in the production of reactive oxygen species [12]. Evidence indicates that reactive oxidants play a role in silica-induced activation of NF-κB. Indeed, catalase and formate have been shown to inhibit silica-induced NF-κB activation of macrophages *in vitro* [9]. Hydroxyl radical has been suggested as the key activation signal for NF-κB [13].

Silica exposure has also been shown to up-regulate the production of another oxidant species, nitric oxide (NO), in pulmonary phagocytes harvested from exposed rats [14, 15]. Blackford *et al*. [16] have reported a correlation between the increase of mRNA levels for the inducible form of nitric oxide synthase (iNOS) and induction of NO production with the degree of inflammation in response to intratracheal instillation of occupational dusts with various pathogenicities. Currently, the role of NO in NF-κB activation is actively debated, with literature supporting both an inhibitory and a stimulatory effect of NO on NF-κB activation [17, 18].

The objective of the present study was to elucidate the role of NO in NF-κB activation in macrophages. To pursue this objective the effects of NOS inhibitors on NF-κB activation of macrophages in response to inflammatory stimulants were evaluated as well as the direct effects of NO generating systems on NF-κB activation. The results support the hypothesis that NO up-regulates DNA binding activity of NF-κB in macrophages.

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 then were dispersed in DMEM media with supplements just before addition to culture plates. Lipopolysaccharide (LPS) from *E. Coli* serotype 055B5, muramyl dipeptide (MDP), L-N^6-(1-iminoethyl)lysine (l-NIL), Nω-nitro-L-arginine methylester (L-NAME), and genistein were purchased from Sigma Chemical Company (St. Louis, MO, USA). AG494, H89, and staurosporine were purchased from Biomol Company (Plymouth Meeting, PA, USA). FeTMPyP and Zn-TMPyP were kindly provided by Dr. Won-Woo Nam (Ewha University, Seoul, Korea). 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 (Mediatech, Washington DC, USA) supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 2 mM glutamine, and 1,000 units/penicillin-streptomycin.

Isolation of rat alveolar macrophages

Alveolar macrophages were obtained from male Sprague-Dawley rats (250–280 g) by bronchoalveolar lavage [19]. Briefly, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt). The trachea was then cannulated and the lungs were lavaged 10 times with 8 ml aliquots of Ca^{2+}-, Mg^{2+}- free phosphate-buffered salt solution (145 mM NaCl, 5 mM KC1, 1.9 mM NaH_2PO_4, 9.35 mM Na_2 H PO_4, and 5.5 mM dextrose; pH = 7.4). Cells were washed with the same buffered solution and cell number, purity, and volume were measured using an electronic cell counter equipped with a cell sizing analyzer (Coulter Model ZBI with a channelizer 256; Coulter Electronics, Hialeah, FL, USA [20]. The cells were incubated in DMEM, supplemented with 5% FBS, 2 mM glutamine, and 1000 units/penicillin-streptomycin.

Nuclear extracts

Nuclear extracts were prepared by a modified method of Sun *et al*. [21]. RAW264.7 cells were cultured in 6-well plates at 5×10^6 cells/well for 3 days. The medium then was replaced with fresh medium and cells pretreated with NOS inhibitors, such as L-NIL (25–100 μM) and L-NAME (0.5–4 mM), metalloporphyrins, such as FeTMPyP and ZnTMPyP (10 μM), or protein kinase inhibitors, such as genistein (37 μM), AG494

(12 μM), staurosporine (0.01 μM) and H89 (0.05 μM). After a 2 h pretreatment, cells were cultured for 4 h with silica (100 μg/ml), LPS (10 μg/ml), MDP (10 μg/ml), SNP (1–100 μM), or SIN-1 (0.01–1 μM) in the absence or presence of inhibitor, as indicated. The concentrations of inflammatory stimulants and the duration of exposure used in this investigation were determined from previous concentration-response and time course studies for NF-κB activation [10]. At the end of the 4 h exposure, the cells were harvested, 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, and 0.5 mM PMSF) for 10 min on ice and then vortexted for 10 sec. Nuclei were pelleted by centrifugation at 12,000 × g for 30 sec 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., 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 × TBE buffer and autoradiographed overnight.

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

Nitrite assay in cultured cell

Cells were suspended in MEM (medium essential medium) with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at a final concentration of 1×10^6 macrophages/ml. Cells (1×10^6 macrophages) were added to 24 well plates (Costar, MA, USA) and incubated for 2 h at 37°C in a humidified atmosphere of 5% CO_2. The nonadherent cells then were removed by two 1 ml washes with the fresh MEM. Silica (100 μg/ml), SNP (1–100 μM) or SIN-1 (0.01–1 mM) was added to the cells. After incubating for 24 h, the cell cultures were centrifuged at 500 g for 15 min and the supernates stored at –70°C until they were assayed.

Nitric oxide production was determined using the Griess reaction to monitor nitrite levels in supernatant samples. Briefly, 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) was mixed with 50 μl samples of cell supernates. Optical density at 550 nm (OD_{550}) was measured using a microplate reader. Nitrite concentrations were calculated by comparison with OD_{550} values of standard solutions of sodium nitrite prepared in cell culture medium.

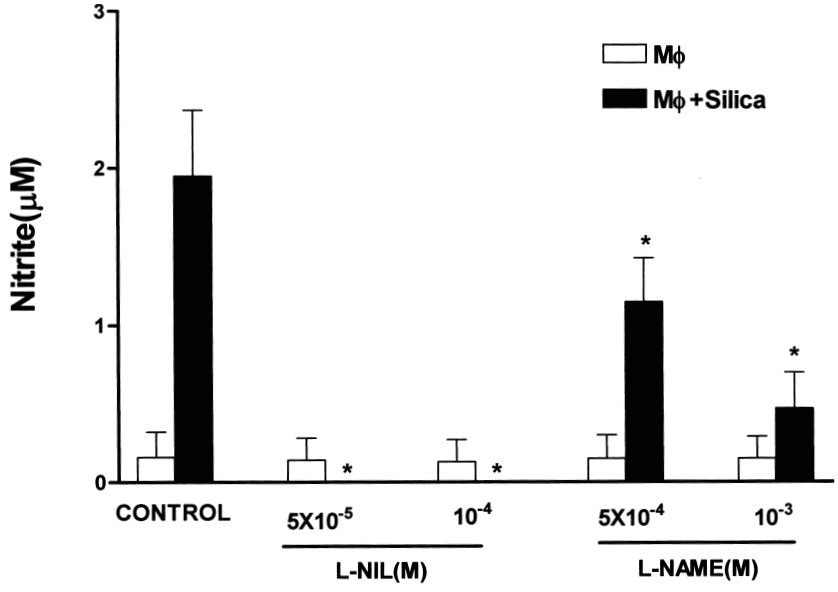

Fig. 1. Effects of inhibitors of NOS on the production of NO by silica-stimulated RAW264.7 cells. Macrophages (1×10^6/ml) were stimulated with silica (100 μg/ml) in the presence or absence of L-NIL (50 and 100 μM) or L-NAME (0.5 and 1 mM). After 24 h, supernates were assayed for nitrite using the Griess assay. Values are means ± S.E. of 5 separate experiments. *indicates a significant decrease ($p \leq 0.05$) compared to silica alone.

Statistics

Values were expressed as means ± S.E. of 6 or 8 experiments. Data were compared with controls using one way analysis of variance (ANOVA) and Student's *t*-test. Significant was set at $p \leq 0.05$.

Results

Exposure of RAW264.7 macrophages to crystalline silica resulted in an induction of nitric oxide production by these mouse phagocytes. As shown in Fig. 1, exposure of macrophages to 100 μg/ml silica for 24 h increased NO production by 12 fold. L-NIL, a specific, inhibitor of iNOS, completely blocked this silica-induced NO production, while L-NAME (1 μM), a non-specific inhibitor of iNOS, caused a 82% inhibition.

A previous report from our laboratory has shown that exposure of RAW264.7 macrophages to silica resulted in activation of NF-κB [10]. This activation occurred at silica concentrations which were not toxic to the cells, measured by trypan blue exclusion. Since silica caused both enhancement of NO production and NF-κB activation a question is raised as to whether NO-derived oxidants play a role in NF-κB activation. To test this hypothesis, the effect of inhibition of nitric oxide synthase on silica-induced NF-κB activation was investigated. As shown in Fig. 2, pretreatment of macrophages with NOS inhibitors decreased NF-κB activation in response to silica exposure. Maximal inhibition of 95 and 55% was detected at 100 μM L-NIL and 4 mM L-NAME, respectively.

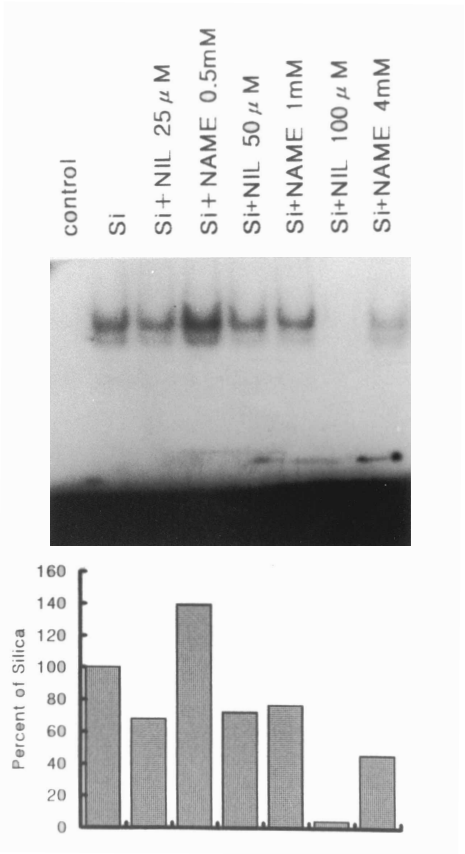

Fig. 2. EMSA illustrating the effect of inhibitors of NOS on silica-induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with L-NIL (25–100 μg/ml) or L-NAME (0.5–4 mM) and then stimulated with silica (100 μg/ml) for an additional 4 h. The results of EMSA are shown (upper panel) and quantitated by densitometric analysis as a percentage of the response of silica-stimulated cells (lower panel). Data are representative of at least 3 experiments.

Lipopolysaccaride (LPS) and muramyl dipeptide (MDP) are proinflammatory agents, which have been reported to stimulate iNOS expression and NO production in macrophages [22]. Therefore, we examined the ability of NOS inhibitors to decrease NF-κB activation induced by these agents. Figure 3 shows that L-NIL (100 μM) effectively inhibited LPS- or MDP- induced NF-κB activation by 46 or 63%, respectively. L-NAME was less effective, inhibiting LPS or MDP-induced NF-κB activation by 20–25%. The data from Figs 2 and 3 indicate that a potential role for NO in NF-κB activation is not limited to a specific inflammatory agent.

To determine if mouse peritoneal macrophage cell line results could be extended to primary alveolar macrophages, we examined the effects of NOS inhibitors on silica- or LPS-induced NF-κB activation in macrophages obtained by bronchoalveolar lavage of rats. Figure 4 shows that both silica and LPS are effective stimulants of NF-κB binding to DNA in primary alveolar macrophages. Similar to results in RAW264.7 cells, NOS inhibition caused significant declines in NF-κB activation with either silica- or LPS- exposed alveolar macrophages.

The data presented thus far suggest that stimulant-induced NO may play a role in NF-κB activation in macrophages. To test this hypothesis further, we investigated the effect of exogenous NO on macrophage NF-κB activity using NO generating systems, such as SNP or SIN-1. Addition of NO generating systems to RAW264.7 cells in culture resulted in a dose-dependent increase in NO levels detected in the supernate (Fig. 5). NO generation was substantially (10 fold) greater in response to SIN-1 (0.1 mM) than with 0.1 mM SNP. Exogenous NO resulted in a dose-dependent activation of NF-κB in mouse macrophages (Fig. 6). As with NO production, NF-κB activation was greater in the presence of 0.1 mM SIN-1 than 0.1 mM SNP.

A previous report from our laboratory indicated that silica exposure induced tyrosine phosphorylation in RAW264.7 cells and that silica-induced NF-κB activation was blocked by inhibition of tyrosine kinase but not protein kinase C or A [10]. Data in Fig. 7 indicate that inhibition of tyrosine kinase with genistein (37 μM) or AG494 (12 μM) also blocked NF-κB activation in response to exogenous NO by 49 or 99%,

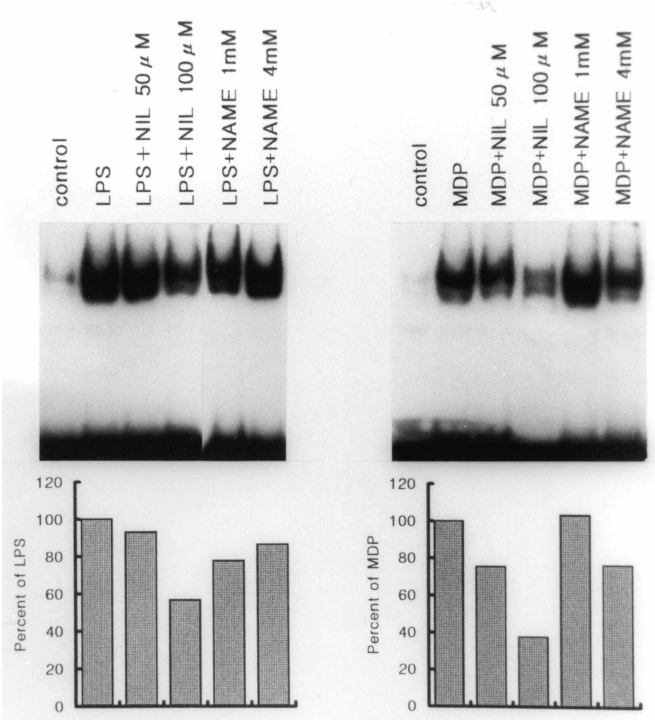

Fig. 3. EMSA illustrating the effect of inhibitors of NOS on LPS- (A) or MDP (B)-induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with L-NIL (50 or 100 μM) or L-NAME (1 or 4 mM) and then stimulated by LPS (10 μg/ml) or MDP (10 μg/ml) for an additional 4 h. The results of EMSA are shown (upper panels) and quantitated by densitometric analysis as a percentage of the response of LPS- or MDP- stimulated cells (lower panels). Date are representative of at least 3 experiments.

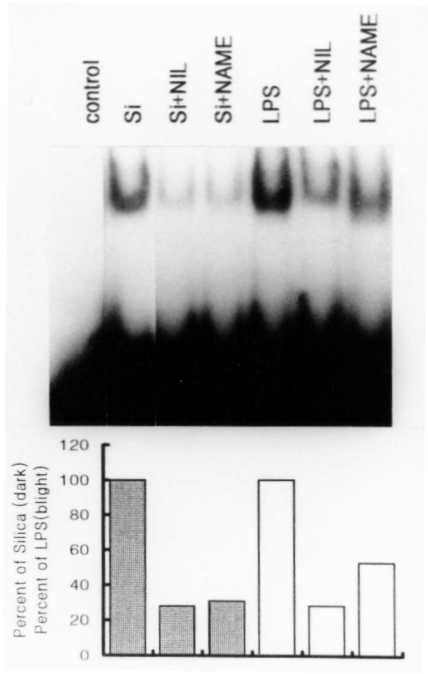

Fig. 4. EMSA illustrating the effect of inhibitors of NOS on activation of NF-κB in silica- or LPS-stimulated rat alveolar macrophages. Rat alveolar macrophages were obtained by bronchoalveolar lavage. Nuclear extracts were prepared from alveolar macrophages pretreated for 2 h with L-NIL (100 μM) or L-NAME (4 mM) and then stimulated with silica (100 μg/ml) or LPS (10 μg/ml) for an additional 4 h. The results of EMSA are shown (upper panel) and quantitated by densitometric analysis as a percentage of the response of silica- or LPS-stimulated cells (lower panel). Data are representative of at least 3 experiments.

respectively. In contrast, inhibitors of protein kinase C (staurosporine) or protein kinase A (H89) had no effect on NF-κB activation in the presence of a NO generator (Fig. 8).

Metalloporphyrins, such as FeTMPyP and ZnTMPyP, are known to inhibit NO-dependent cyclic GMP formation [23, 24]. In addition, metalloporphyrins have been reported to catalyze peroxinitrite decomposition [25]. Therefore, we investigated whether metalloporphyrins would affect silica- or LPS- stimulated NF-κB activation in RAW264.7 cells. As shown in Fig. 9, pretreatment of macrophages with metalloporphyrins for 2 h prior to silica or LPS exposure inhibited DNA binding activity of NF-κB by over 90%.

Discussion

The objective of the present investigation was to elucidate the effect of NO on stimulant-induced NF-κB activation in macrophages. Data indicate that in vitro stimulation of RAW-264.7 macrophages with silica induced both NO production and NF-κB activation. Inhibition of NOS activity with L-NIL or L-NAME resulted in inhibition of this silica-induced NF-κB activation, suggesting that endogenous NO may augment the activation of this transcription factor. Results indicate that this activating effect of NO on NF-κB occurred in response to various stimuli, i.e. silica, LPS, or muramyl dipeptide, and could be demonstrated in both a peritoneal macrophage cell line and in primary alveolar macrophages. In addition, direct activation of NF-κB by NO was demonstrated using exogenous NO generating systems (SIN-1 or SNP).

From the SIN-1 and SNP data presented in this study, it is difficult to construct a quantitative relationship between NO levels and the degree of activation of NF-kB. However, NO production by 10^{-4} M SNP or 10^{-5} M SIN-1 were similar and produced between a 3–3.5 fold increase in NF-κB-DNA binding. From the SIN-1 data, the relationship between NO and NF-κB activation also appears to be non-linear, exhibiting saturation between 0.1 and 1 mM SIN-1. SIN-1, in contrast to SNP, produces both superoxide and NO [36]. Therefore, part of the SIN-1 effect may be a response to peroxinitrite. Peroxinitrite has been shown to be an important mediator of several cell functions [37] as well as being a cytotoxic molecule [14]. However, the role of peroxynitrite formation on

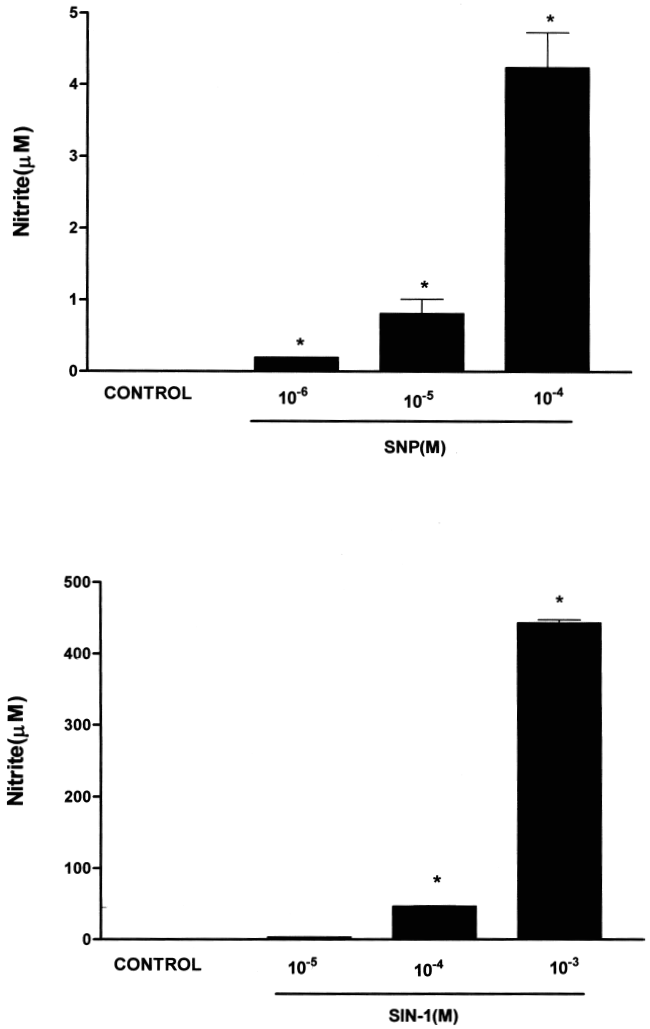

Fig. 5. Dose response of NO production by NO generating compounds. RAW264.7 cells (1×10^6/ml) were treated with SNP (1–100 μM; A) or SIN-1 (0.01–1 mM; B). After 24 h, the supernates were assayed for nitrite. Values were means ± S.E. of 5 separate experiments. *indicates a significant increase ($p \leq 0.05$) compared to controls.

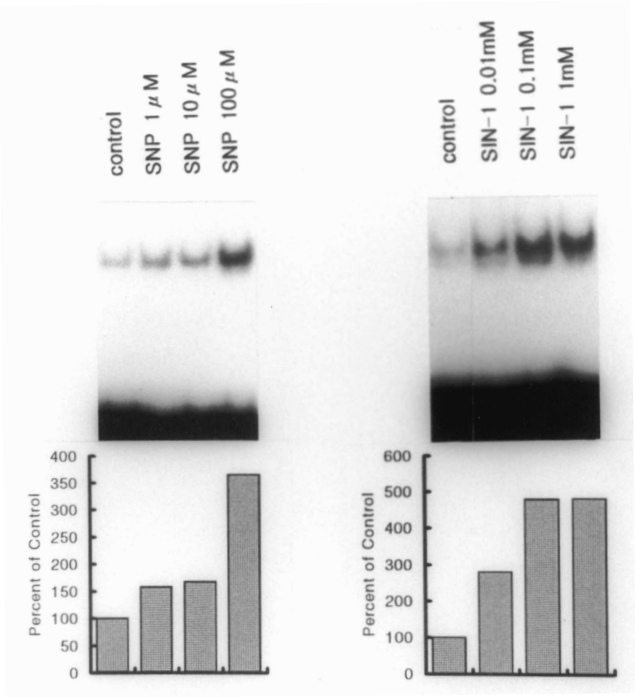

Fig. 6. EMSA illustrating the effect of NO generating compounds on the activation of NF-κB in RAW264.7 cells. EMSA was conducted with nuclear extracts from cells treated with various concentrations of SNP (A) or SIN-1 (B) for 4 h. The results of EMSA are shown (upper panels) and quantitated by densitometric analysis as the percentage increase from the control response (lower panels). Data are representative of at least 3 experiments.

NF-κB activation in the cells treated with SIN-1 remains to be determined.

Data from the present study support the hypothesis that NO augments the activation of NF-κB in macrophages and, therefore, may play a role in producing a positive cycle of inflammation. A stimulatory effect of NO on NF-κB activation has also been reported in human lymphocytes exposed to exogenous NO generating systems [18]. NO has also been reported to enhance TNFα or PMA-induced NF-κB activation in endothelial cells [26]. Schreck and Baeuerle [27] reported that NO enhanced the dissociation of the IκB-NF-κB complex. This is supported by the demonstration that NO increased the activity of IKK (IκB kinase) in TNFα or PMA-stimulated endothelial cells [26]. Data suggest that this stimulatory effect of NO on NF-κB activation may be mediated by a G-protein pathway, since a G-protein inhibitor decreased NO activation of NF-κB in lymphocytes [28]. NO has been shown to stimulate guanylate cyclase [24] and activate GTPase activity [28]. Evidence indicates that NO increased the activity of $p21^{ras}$ (a G protein family member) and increased $p21^{ras}$-GTP binding [29]. Since stimulants failed to activate NF-κB in $p21^{ras}$ defective cells, it was proposed that NO-induced activation of NF-κB involved $p21^{ras}$.

Our data indicate that metalloporphyrins inhibited silica- or LPS-induced NF-κB activation in macrophages. Since metalloporphyrins have been shown to enhance the decomposition of peroxynitrite [25], inhibition via a NO mechanism is proposed. Indeed, metalloporphyrins have been shown to inhibit NO-dependent activation of guanylyl cyclase [24] and decrease NO induction of cGMP [23]. These data support the hypothesis that NO activates NF-κB through a G-protein mediated pathway.

Kang *et al*. [10] have shown that protein tryosine kinase (PTK) plays an important role in silica-induced activation of NF-κB in macrophages. Data from the present study, indicate that inhibitors of PTK decreased NF-κB activation in response to exogenous NO generators. A link between NO,

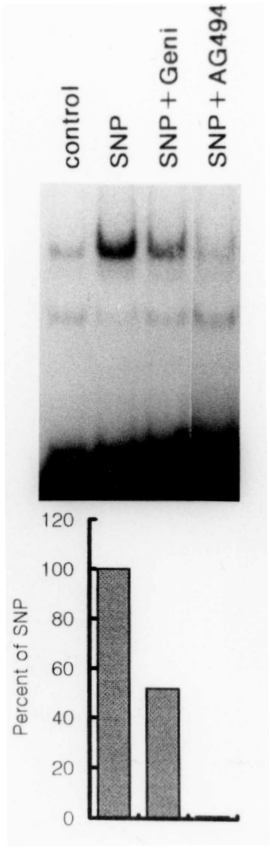

Fig. 7. EMSA illustrating the effect of tyrosine kinase inhibitors on SNP-induced activation of NF-κB. Nuclear extracts were prepared from RAW-264.7 cells pretreated for 2 h with genistein (37 μM) or AG494 (12 μM) and then stimulated by SNP (100 μM) for an additional 4 h. The results of EMSA are shown (upper panels) and quantitated by densitometric analysis as a percentage of the response of SNP-treated cells (lower panels). Data are representative of at least 3 experiments.

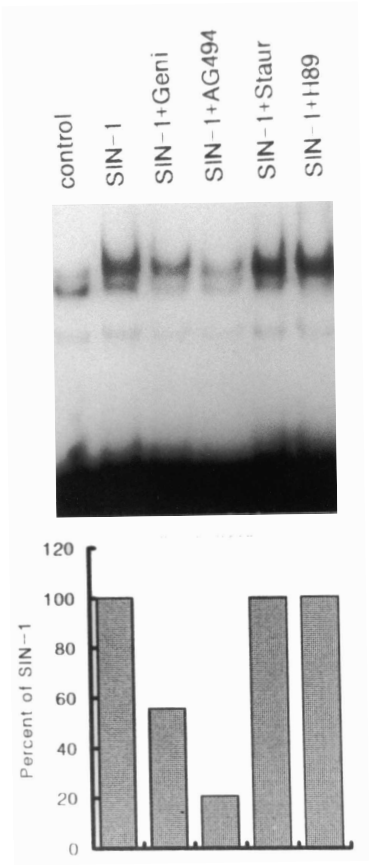

Fig. 8. EMSA illustrating the effects of inhibitors of tyrosine kinase, protein kinase C or protein kinase A on SIN-1-induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with genistein (74 μM), AG494 (12 μM), staurosporine (0.01 μM) or H89 (0.05 μM) and then stimulated by SIN-1 (100 μM) for an additional 4 h. The results of EMSA are shown (upper panel) and quantitated by densitometric analysis as a percentage of the response of SIN-1-treated cells (lower panel). Data are representative of at least 3 experiments.

PTK, and NF-κB activation is supported by Lander *et al*. [18] who demonstrated that treatment of lymphocytes with NO generating systems increased the activity of PTK, specifically $p56^{lck}$ kinase activity.

In contrast to data presented in the present study, Chen *et al*. [17, 30] reported that NOS inhibitors enhanced silica- or LPS-induced NF-κB activation in RAW264.7 macrophages. A negative effect of NO on the activity of NF-κB was also reported in alveolar macrophages [31]. In addition, Raychaudhuri *et al*. [31] reported that NO generating systems blocked the LPS-induced decrease in IκB levels in these pulmonary phagocytes. This result is supported by data suggesting that relatively high concentrations of a NO generator (0.5 mM S-itrosoglutathione) stabilized IκB, preventing activation of NF-κB [32]. Evidence has also been reported indicating that NO caused direct nitrosation of cysteine 62 of the p50 subunit of NF-κB [33]. Such S-nitrosation would decrease NF-κB-DNA binding and, thus, block signaling pathways for several pro-inflammatory cytokines [31]. It has also been proposed that NO can scavenge reactive oxygen species, thus inhibiting stimulant-induced activation of NF-κB [6, 9].

There are several possible explanations for this conflicting evidence regarding the role of NO in NF-κB activation. Umansky *et al*. [26] have shown that results vary greatly with NO level. That is, activation of NF-κB was demonstrated with low levels of NO, while inhibition occurred at high NO levels. Since various cell types function at different oxidant/antioxidant states, this could explain the variation in responses among cell types. Timing of the exposure to NO may also be important. Indeed, Diaz-Cazorla *et al*. [34] reported that NO activated NF-κB after short exposures and inhibited NF-κB following longer exposures. The response of cells to NO may also be affected by the activation state of the cells. Indeed, NO was found to stimulate NF-κB activity of endothelial cells treated with TNF-α or PMA, but had no effect

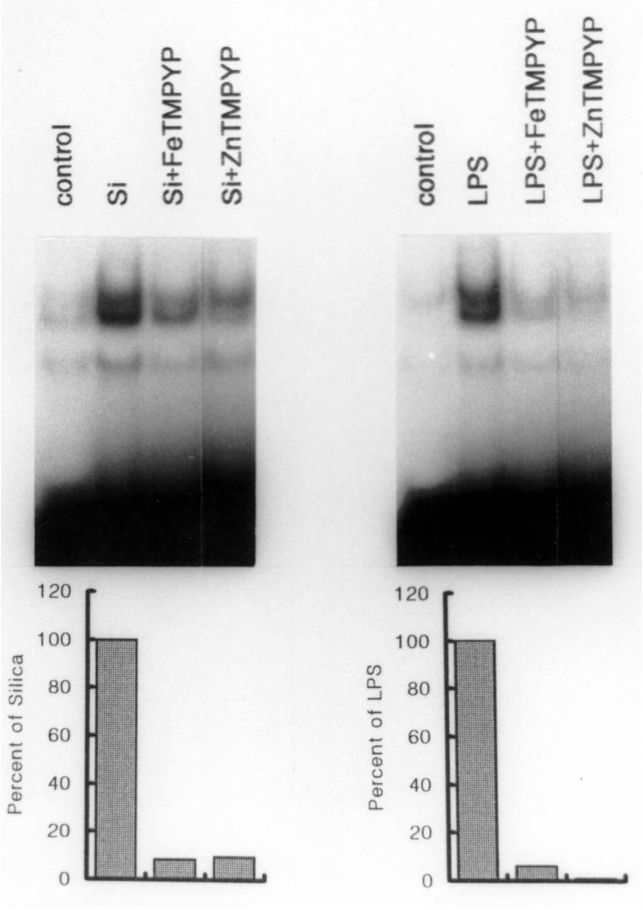

Fig. 9. EMSA illustrating the effect of metalloporphyrins on silica (A)- or LPS (B)-induced activation of NF-kB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with FeTMPyP (10 μM) or ZnTMPyP (10 μM) and then stimulated by silica (100 μg/ml) or LPS (10 μg/ml) for an additional 4 h. The results of EMSA are shown (upper panels) and quantitated by densitometric analysis as a percentage of the response of silica-stimulated cells (lower panels). Data are representative of a least 3 experiments.

in unstimulated cells [26]. This may explain the discrepancy between the current study and those of Chen *et al*. [17, 30]. In our study, RAW264.7 macrophages were stimulated by silica or LPS while being incubated in media containing serum. We found that NOS inhibitors decreased NF-κB activation. However, in the studies of Chen *et al*. [17, 30], RAW macrophages were serum starved before stimulation with silica or LPS. Therefore, the basal activity of NF-κB was lower in their study than in ours. Another possible reason for the different responses to NOS inhibitors may be that we preincubated the macrophages with NOS inhibitors for 2 h prior to stimulation with silica, while Chen and colleagues added silica and NOS inhibitors simultaneously. Therefore, it is likely that greater cytoplasmic levels of NOS inhibitor were achieved in our study.

Exposure of rats to silica or LPS has been shown to increase mRNA levels for iNOS and stimulate NO production by pulmonary phagocytes [14, 15]. Blackford *et al*. [16] investigated the pulmonary response of rats exposed to particles of varying pathogenicity, i.e. silica, coal dust, titanium dioxide, and carbonyl iron. They found that the magnitude of inflammation and damage seen in the animal model correlated with human pathogenicity and that there was a direct relationship between the level of inflammation and damage caused by a dust and the induction of mRNA for iNOS and NO production. A similar correlation between NO induction and disease severity was reported in silica-exposed miners with various degrees of pneumoconiosis [35]. Data from the present study suggest that NO may act to amplify inflammation by activating NF-κB. Indeed, NF-κB is involved in the up regulation of numerous pro-inflammatory cytokines [6], which have been linked to disease initiation and progression in both animal models and in dust-exposed workers [4]. Therefore, NO may play an important role in the development of silicosis. It is clear that further investigation is required to resolve the conflicting literature concerning the role of NO in NF-κB activation and in development of pneumoconiosis as well as other diseases.

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