Taylor & Francis
Taylor & Francis Group

Journal of Toxicology and Environmental Health Part A

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: https://www.tandfonline.com/loi/uteh20

IRON TETRAKIS (*N*-METHYL-4'-PYRIDYL) PORPHYRINATO (FeTMPyP) IS A POTENT SCAVENGING ANTIOXIDANT AND AN INHIBITOR OF STIMULANT-INDUCED NF-?B ACTIVATION OF RAW 264.7 MACROPHAGES

Jihee Lee Kang , Hui Su Lee , In Soon Pack , Stephen Leonard & Vincent Castranova

To cite this article: Jihee Lee Kang , Hui Su Lee , In Soon Pack , Stephen Leonard & Vincent Castranova (2001) IRON TETRAKIS (*N*-METHYL-4'-PYRIDYL) PORPHYRINATO (FeTMPyP) IS A POTENT SCAVENGING ANTIOXIDANT AND AN INHIBITOR OF STIMULANT-INDUCED NF-?B ACTIVATION OF RAW 264.7 MACROPHAGES, Journal of Toxicology and Environmental Health Part A, 64:4, 291-310, DOI: 10.1080/152873901316981295

To link to this article: https://doi.org/10.1080/152873901316981295

Journal of Toxicology and Environmental Health, Part A, 64:291–310, 2001

1528-7394/01 $12.00 + .00

IRON TETRAKIS (*N*-METHYL-4′-PYRIDYL) PORPHYRINATO (FeTMPyP) IS A POTENT SCAVENGING ANTIOXIDANT AND AN INHIBITOR OF STIMULANT-INDUCED NF-κB ACTIVATION OF RAW 264.7 MACROPHAGES

Jihee Lee Kang, Hui Su Lee, In Soon Pack

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

Stephen Leonard, Vincent Castranova

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

*Metalloporphyrins have been shown to be protective in oxidative stress models. However, the molecular basis for the antioxidative and antiinflammatory activities of iron tetrakis (*N*-methyl-4′-pyridyl) porphyrinato (FeTMPyP) is not known. The objective of this study was to determine whether FeTMPyP exhibited the ability to (1) scavenge reactive oxygen species (ROS), (2) inhibit the activation of nuclear factor kappa B (NF-κB), or (3) block the production of interleukin 1 (IL-1) in RAW 264.7 cultured macrophages. The results indicate that FeTMPyP is a potent scavenger of hydroxyl radicals and superoxide anion radicals, and an effective inhibitor of stimulant-induced NF-κB activation and IL-1 production by RAW 264.7 cells. Therefore, FeTMPyP may be a useful tool to investigate the molecular mechanisms involved in stimulant-induced signal pathways, and may possess therapeutic utility in diseases associated with overproduction of ROS.*

Nuclear factor kappa B (NF-κB) is an essential transcription factor that controls gene expression of cytokines, chemokines, growth factors, and cell adhesion molecules (Chen et al., 1999; Barnes & Karin, 1997). Recent evidence indicates that in vitro exposure of macrophages to silica or lipopolysaccharide (LPS) induces activation of NF-κB (Chen et al., 1998; Kang et al., 2000a). Activation of NF-κB in pulmonary phagocytes has also been demonstrated after in vivo exposure to silica (Sacks et al., 1998) or LPS (Blackwell et al., 1996). Therefore, activation of NF-κB binding to various gene promoter regions may be a key molecular event in the initiation of silica- or LPS-induced pulmonary diseases (Mayeux, 1997).

Received 17 January 2001; accepted 12 April 2001.

This work was supported by the National R&D Program (97-NS-01-01-A-13) for Women's Universities.

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

The predominantly characterized NF-κB complex is a p50–p65 heterodimer, which at rest is retained in the cytoplasm and is associated with an inhibitor molecule, IκB (Zabel & Baeuerle, 1990). In response to a variety of stimuli, IκB is phosphorylated and becomes dissociated from the NF-κB complex. Free NF-κB can then translocate to the nucleus, where it binds to the NF-κB motif and functions as a transcriptional regulator. A p65–p50 or p65–p52 heterodimer could be detected in silica- or LPS-stimulated RAW 264.7 macrophages (Chen et al., 1995; Goto et al., 1999). The DNA binding of NF-κB can be competitively inhibited by a nonlabeled NF-κB consensus DNA probe and supershifted by antibodies to NF-κB subunits (Chen et al., 1995).

Phosphorylation of serine residues of IκB-α is followed by the ubiquitination of this protein, leading to degradation of IκB-α by proteasomes (Brockman et al., 1995; Brown et al., 1995; DiDonato et al., 1996). 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 LPS (Mayeux, 1997; Imbert et al., 1996). Recent evidence indicates that exposure of T cells to hypoxia, reoxygenation, or pervanadate, or exposure of macrophages to silica resulted in phosphorylation of IκB-α on tyrosine 42 (Koong et al., 1994; Imbert et al., 1996; Kang et al., 2000b). These authors also reported an alternative mechanism of NF-κB activation in which tyrosine phosphorylation does not lead to degradation of the IκB-α through the proteasome pathway, unlike serine phosphorylation of IκB-α.

Exposure of lung phagocytes to silica results in the production of reactive oxygen species (ROS) (Castranova et al., 1996; DiMatteo et al., 1996), which are believed to be significantly involved in silica-induced cytotoxicity (Kim et al., 1999) and carcinogenicity (Saffiotti et al., 1985; Vallyathan et al., 1988; Fubini et al., 1990; Castranova, 1994; Shi et al., 1998). Our previous studies indicate that in vitro exposure RAW 264.7 cells to silica resulted in an increase in ROS production and that reactive oxidants play a role in silica-induced activation of NF-κB (Chen et al., 1998; Kang et al., 2000a). Indeed, catalase, superoxide dismutase, and formate have been shown to inhibit silica-induced NF-κB activation of macrophages in vitro (Chen et al., 1998; Kang et al., 2000a). Hydroxyl radical has been suggested as the key activation signal for silica-induced activation of NF-κB (Shi et al., 1999). Therefore, antioxidant agents that inhibit the activation of NF-κB may be potentially useful for therapeutic intervention.

Among the possibilities for such an agent is iron-tetrakis (*N*-methyl-4′-pyridyl) porphyrinato (FeTMPyP), a porphyrin analog that is a very stable redox active metal complex possessing an extensive conjugated ring system that undergoes reversible one-electron oxidation (Figure 1). Metalloporphyrins, including Fe- and Mn-porphyrins, have been shown to be protective in a wide variety of in vitro oxidative stress models involving

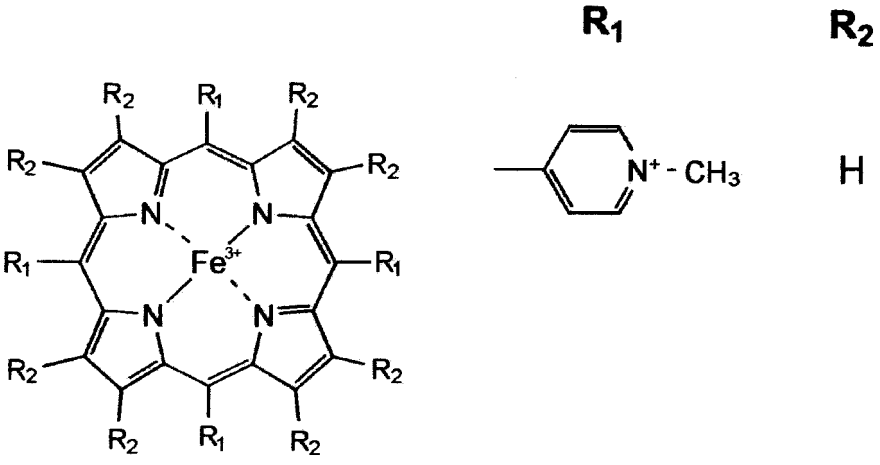

FIGURE 1. Chemical structure of FeTMPyP.

the generation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$) alone or in concert (Patel & Day, 1999). FeTMPyP has been shown to be an effective $ONOO^-$ decomposition catalyst in vitro and in vivo (Salvemini et al., 1998). It appears to be cytoprotective against endogenously generated $ONOO^-$ in LPS-stimulated RAW 264.7 cells, as well as in dissociated cultures of hippocampal neurons and glia exposed to cytokines (Misko et al., 1998). Metalloporphyrins inhibit heme oxygenase and other heme-dependent enzymes, such as guanylate cyclase and nitric oxide synthase (Bashir & Henley, 1993; Luo & Vincent, 1994). However, the molecular basis for the antioxidative and antiinflammatory activity assigned to FeTMPyP has not been fully identified in either in vitro or in vivo experimental models.

The objective of this study was to determine if FeTMPyP exhibited the ability to scavenge reactive oxygen species, such as hydroxyl radical (•OH) and superoxide anion (O_2^-), and to inhibit the activation of NF-κB or the production of IL-1 in silica- or LPS-stimulated RAW264.7 macrophages.

METHODS

Reagents

Crystalline silica (Min-U-Sil, particle size <5 μm) was obtained from U.S. Silica Corporation (Berkeley Springs, WV). Prior to use, the silica samples were sterilized by heating at 160°C for 90 min in a dry oven. Silica particles were dispersed in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc., Madison, WI) with supplements just before addition to culture plates. LPS from *Escherichia coli* serotype 055B5 was purchased from Sigma Chemical Company (St. Louis, MO). FeTMPyP was

purchased from Mid-Century (Posen, IL). DNA polymerase and dNTP were purchased from Life Technologies (Gaithersburg, MD). Antibodies used in this study were anti-IκB-α rabbit polyclonal (New England Biolabs, Inc., Bevery, MA) and anti-phosphotyrosine 4G10 (Upstate Biotechnology, Lake Placid, NY).

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 supplemented with 5% fetal bovine serum (FBS, HyClone, Logan, UT), 2 m*M* glutamine, and 1000 U/ml penicillin–streptomycin.

Measurement of Cell Viability

Lactate dehydrogenase (LDH) is an abundant intracellular enzyme and its release into cell culture supernatants is a marker of lytic cell death (Lipton et al., 1993; Kim et al., 1999; DiMatteo et al., 1996). The activity of LDH was measured using a LDH determination kit (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, 100 μl of FeTMPyP (1–100 μ*M*) was added to 100 μl of adherent RAW 264.7 macrophages (10^4/ml) in given wells of a microplate. The cells were then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. After incubation, 100 μl of supernatant was added to 100 μl of reaction mixture and incubated for 30 min at room temperature. Absorbancy of the samples at 490 nm was measured using a microplate reader. Results were expressed as percent cell viability, referenced to the maximum LDH released when cells were lysed with detergent, using the formula:

$$\text{Percent viability} = 100 \times [1 - (\text{experimental} - \text{untreated}) / (\text{detergent} - \text{untreated})]$$

Free Radical Measurements

For detection and identification of short-lived radicals, a spin trapping method was used (Mottley & Mason, 1989; Shi et al., 1997). This technique involves the addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product, the so-called spin adduct, which can be studied by conventional electron spin resonance (ESR). The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, and the hyperfine splitting of the spin adduct is generally characteristic of the original, short-lived, trapped radical. This method is specific and sensitive and is considered to be the method of choice for the detection and identification of free radicals.

All ESR measurements were conducted using a Bruker ESP 300E ESR spectrometer and a flat cell assembly. Hyperfine couplings were mea-

sured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reference standards. The relative radical concentration was estimated by multiplying half of the peak height by $(\Delta H_{PP})^2$, where ΔH_{PP} represents peak-to-peak width. A SPEX 300 program (U.S. EPR, Inc., Clarksville, MD) was used for data acquisition and analysis. All experiments were performed at room temperature and under ambient air. Reactants were mixed in test tubes in a final volume of 1 ml. The reaction mixture was then transferred to a flat cell for ESR measurement. The concentrations given in the figure legends are final concentrations.

Nuclear Extracts

Nuclear extracts were prepared by a modified method of Sun et al. (1994). RAW 264.7 cells were cultured in 6-well plates at 5×10^6 cells/ml for 3 d; then the medium was replaced with fresh medium and cells were pretreated with FeTMPyP (1–100 μ*M*). After 2 h of pretreatment, cells were incubated with silica (100 μg/ml), LPS (10 μg/ml), or H_2O_2 (1 m*M*) in the absence or presence of FeTMPyP for a period of time as indicated. The concentrations of the stimulants 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 exposure, the cells were harvested and resuspended in hypotonic buffer A [100 m*M* HEPES, pH 7.9; 10 m*M* KCl; 0.1 *M* ethylenediamine tetraacetic acid (EDTA); 0.5 m*M* dithiothreitol; 1% nonidet P-40; and 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF)] for 10 min on ice and then vortexed for 10 s. Nuclei were pelleted by centrifugation at 12,000 × g for 30 s and were resuspended in buffer C (20 m*M* HEPES, pH 7.9; 20% glycerol; 0.42 *M* NaCl; 1 m*M* EDTA; and 0.5 m*M* PMSF) for 30 min on ice. The 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) nuclear extract protein, 2 μg poly(dI-dC)•poly(dI-dC) (Sigma Co., St. Louis, MO), and 40,000 cpm ^{32}P-labeled probe in binding buffer (4 m*M* HEPES, pH 7.9; 1 m*M* $MgCl_2$; 0.5 m*M* DTT; 2% glycerol; and 20 m*M* NaCl), were incubated for 30 min at room temperature. Protein–DNA complexes were separated on 5% non-denaturing polyacrylamide gels in 1 × Tris-borate/EDTA electrophoresis buffer (pH 7.5) and autoradiographed overnight. Autoradiographic signals for activated NF-κB were quantitated by densitometric scanning using an 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-stranded DNA containing NF-κB consensus sequence (5′-CCTGTGCTCCGGGAATTTCCCTGGCC-3′) labeled with [α-^{32}P]-dATP

(Amersham, Buckinghamshire, UK) using a DNA polymerase Klenow fragment.

Immunoprecipitation

The confluent cells grown on 100-mm plastic dishes were incubated in DMEM supplemented with 5% FBS, 2 m*M* glutamine, and 1000 U/ml penicillin–streptomycin for 3 d. Cells then were treated with silica (100 μg/ml) in the presence or absence of FeTMPyP (10 μ*M*) 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 m*M* Tris-HCl (pH 8), 150 m*M* NaCl, 1% nonidet P-40 (NP-40), 100 μg/ml PMSF, 1 μg/ml leupeptin, 1 m*M* Na_3 VO_4, 5 m*M* EDTA, and 1 m*M* benzamidine.

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

Western Blotting

The fractionated proteins for tyrosine phosphorylated IκB-α or cytoplasmic extracts (50 μg protein) from LPS (1 μg/ml)-treated cells for IκB-α were resolved on 10% SDS–polyacrylamide gels and electrophoretically transferred onto 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.

Measurement of IL-1 Activity in Cultured RAW 264.7 Macrophages

Cells were resuspended in RPMI-1640 media (Mediatech, Washington, DC) containing 2 m*M* glutamine, 100 U/ml mycostatin, and 10% FBS. Aliquots of 1 ml, containing 10^6 cells, were added to 24-well plates (Costar, Cambridge, MA) and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 2 h. The nonadherent cells were then removed by vigorously washing twice with 1 ml of RPMI media. The adherent cells were further incubated in 1 ml RPMI media containing 5 μg/ml LPS with or without FeTMPyP in the concentration range of 1–100 μ*M*. After incubating the cell culture for 24 h, the supernates were collected, filtered, and stored at –70°C until the thymocyte proliferation assay was performed.

Thymocyte Proliferation Assay for IL-1 Activity

IL-1 activity in various macrophage-conditioned supernatants was determined by their capacity to stimulate thymocyte proliferation according to the method of Kang et al. (1992). Briefly, thymocytes were obtained

from male CD-1 mice (6 wk of age, 20–25 g) and suspended in RPMI-1640 media with 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 units/ml mycostatin, 10% FBS, and 2×10^{-5} *M* mercaptoethanol. Cells were counted using an electronic cell counter and adjusted to a concentration of 10×10^6 cells/ml. An aliquot of 100 μl of the RAW 264.7 macrophage-conditioned supernatants was added in quadruplicate to 96-well microculture plates, and 100 μl of thymocyte suspension was placed in each well. After 48 h of incubation at 37°C in 5% CO_2, the cultures were pulsed for 4–6 h with [^{3}H]thymidine (1 μCi/well, activity 2 Ci/mmol, Dupont NEN Products, Boston, MA), and harvested using a cell harvester (Brandle, Gaithersburg, MD). The radioactivity in the collecting glass filter disks was measured using a liquid scintillation counter (Beckman, Fullerton, CA). The levels of IL-1 activity in the tested RAW 264.7 macrophage supernates were expressed as counts per minute.

Statistics

Values were expressed as means ± standard errors. Data were analyzed using one-way analysis of variance (ANOVA) and Student's *t*-test. Significance was set at $p < .05$.

RESULTS

FeTMPyP Does Not Affect Cell Viability at Rest

The object of this investigation was to determine if FeTMPyP specifically altered the ability of macrophages to respond to stimulants. Therefore, it was essential to demonstrate that FeTMPyP was not cytotoxic under the conditions used for the functional assays employed in this study. Cellular viability was monitored as lactate dehydrogenase release. Viability of RAW 264.7 macrophages was not compromised after a 24-h in vitro exposure to FeTMPyP (1–100 *M*); that is, FeTMPyP did not increase the activity of LDH in the culture supernates.

FeTMPyP Is a Potent Antioxidant

To determine the role of FeTMPyP as a direct antioxidant, its scavenging ability was tested in an hydroxyl radical (•OH) generation system using ESR spectroscopy. Hydroxyl radicals were generated by the Fenton reaction of Fe^{2+} with H_2O_2. Figure 2a shows a typical ESR spectrum generated from a mixture containing $FeSO_4$ (5 m*M*) and H_2O_2 (1 m*M*) in the presence of DMPO (1 m*M*) as a spin trap. This spectrum consists of a 1:2:2:1 quartet with splittings of $a_H = a_N$, = 14.9 G. Based on these splittings constants, the 1:2:2:1 quartet was assigned to a DMPO/•OH adduct. Addition of FeTMPyP (1–10 μ*M*) reduced the ESR signal in a dose-dependent manner with almost complete scavenging of •OH at 10 μ*M* (Figure 2, b–d).

The reaction rate constant of FeTMPyP and •OH radical was calculated through spin trapping competition experiments as reported earlier (Shi et

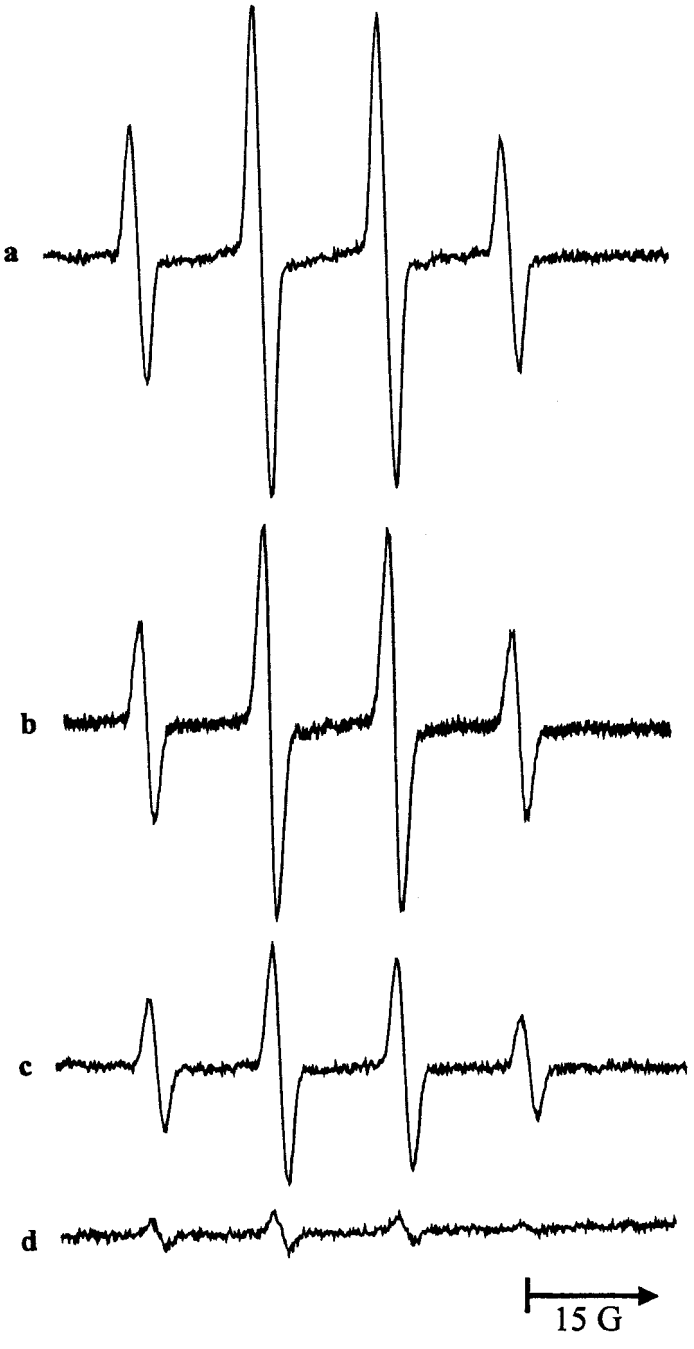

FIGURE 2. FeTMPyP as a hydroxyl radical scavenger: (a) ESR spectrum recorded 2 min after mixing 5 m*M* $FeSO_4$, 1 m*M* H_2O_2, and 1 m*M* DMPO in a pH 7.4 phosphate-buffered solution. (b, c, and d) Same as (a) but with 1, 5, or 10 μ*M* FeTMPyP added, respectively. The spectrum settings were: receiver gain, 2.51×10^4; modulation amplitude, 0.5 G; magnetic field, 3435 ± 50 G; scan time, 1 min. Data are representative of three separate measurements.

al., 1997). The rate constant for FeTMPyP was found to be 4.5×10^{10} M^{-1} s^{-1} (Figure 3). This is 3.6-fold higher than hypotaurine (1.24×10^{10} M^{-1} s^{-1}), a well-established antioxidant, and much (25-fold) greater than ZnTMPyP (1.8×10^{9} M^{-1} s^{-1}), a porphyrin analog that we have reported as having antioxidant activity (Kang et al., 2001).

To determine the potency of FeTMPyP as a superoxide anion (O_2^-) scavenger, a xanthine/xanthine oxidase system was used to generate O_2^- and the intensity of this radical was monitored by ESR using DMPO as a spin trap. A typical superoxide radical signal is shown in Figure 4a. Addition of FeTMPyP (1–25 μ*M*) reduced the O_2^- signal in a concentration-dependent manner with approximately 85% scavenging of O_2^- at 5 μ*M* (Figure 4, b–e). In addition, this signal reduction was greater than the inhibition observed in the presence of 5 μ*M* ascorbic acid (data not shown).

FeTMPyP Inhibits Stimulant-Induced NF-κB Activation

Previous reports have shown that exposure of RAW 264.7 macrophages to silica, LPS, or hydrogen peroxide resulted in activation of NF-κB (Meyer et al., 1993; Chen et al., 1998; Kang et al., 2000a). However, the initial signal transduction pathway leading to NF-κB activation induced by these stimulants has been shown to be different. Evidence indicates that ROS play a role in stimulant-induced activation of NF-κB. Since it was found FeTMPyP exhibited a potent ability to scavenge oxidants, a question was raised as to whether this drug would inhibit NF-κB activation induced by these stimulants. To examine this question, RAW 264.7 cells were preincubated for 2 h with different concentrations of FeTMPyP and then examined for NF-κB activation by treatment of cells for 4 h with silica (100 μg/ml) or LPS (10 μg/ml), or for 2 h with H_2O_2 (1 m*M*), at 37°C. The data shown in Figure 5, A, B, and C, indicate that FeTMPyP (1–100 μ*M*) inhibited the activation of NF-κB induced by silica, LPS, or H_2O_2 in a concentration-dependent manner with an inhibition of 100%, 78%, and 100% for silica, LPS, or H_2O_2, respectively, at 100 μ*M* FeTMPyP. These results suggest that FeTMPyP may act at a common step in the signal transduction pathways leading to NF-κB activation in response to each of these stimulants. DNA binding activity of NF-κB in unstimulated cells with FeTMPyP (1–100 μ*M*) alone was negligible (data not shown).

FeTMPyP Inhibits Silica-Dependent Tyrosine Phosphorylation of IκB-α

In a previous study, data suggested tyrosine phosphorylation of IκB-α represents a proteasome proteolytic activity-independent mechanism of NF-κB activation in silica-stimulated macrophages (Kang et al., 2000b). To determine whether the inhibitory action of FeTMPyP on NF-κB activation was due to an effect on silica-dependent tyrosine phosphorylation of IκB-α, cells were pretreated with FeTMPyP for 2 h before exposure to silica, and cell lysates from silica-treated cells in the presence or absence of the drug were then exposed to IκB-α-specific antibody followed by Western blot analysis with the anti-phosphotyrosine mAb. FeTMPyP (10 μ*M*) inhibited tyrosine phosphorylation of IκBα in cells exposed to silica by

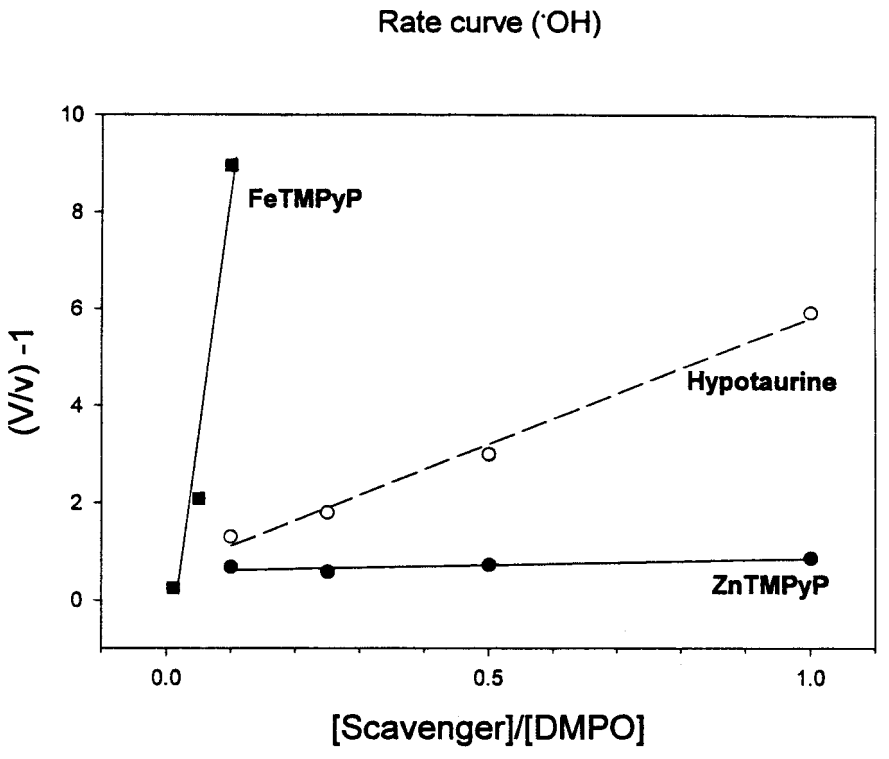

FIGURE 3. Scavenging of •OH by FeTMPyP, ZnTMPyP or hypotaurine. The •OH radicals were produced by the reaction of 5 m*M* $FeSO_4$ with 1 m*M* H_2O_2 in the presence of 1 m*M* DMPO. The data were plotted according to the equation $V/v - 1 = k_x[\text{scavenger}]/k_d[\text{DMPO}]$. K_{Fe} for FeTMPyP = 4.53×10^{10} M^{-1} s^{-1}. K_{Zn} for ZnTMPyP = 1.81×10^9 M^{-1} s^{-1}; K_h for hypotaurine = 1.24×10^{10} M^{-1} s^{-1}; V = peak height of control = 79.75 mm; v = peak height of scavengers. The rate constants of FeTMPyP, ZnTMPyP and hypotaurine are 4.53×10^{10} M^{-1} s^{-1} 1.8×10^9 M^{-1} s^{-1}, and 1.24×10^{10} M^{-1} s^{-1}, respectively. Data are representative of three determinations.

100% and 73% after 10 and 20 min of silica exposure, respectively (Figure 6).

FeTMPyP Inhibits LPS-Dependent Degradation of Iκ-α

Degradation of IκB-α by the proteasome preceded by phosphorylation of serine residues of IκB-α is essential for NF-κB activation by TNF, IL-1β, PMA, okadaic acid, or LPS. Therefore, the effect of FeTMPyP on degradation of IκB-α triggered by LPS was determined. Figure 7 shows that degradation of IκB-α occurred at 10 min after LPS stimulation with a relatively constant low level of IκB-α being maintained for up to 30 min. Thereafter, newly synthesized IκB-α protein was increased and IκB-α levels returned to the control level by 60 min. FeTMPyP (10 μ*M*) did not affect the cytoplasmic levels of IκB-α at rest. However, treatment of cells with

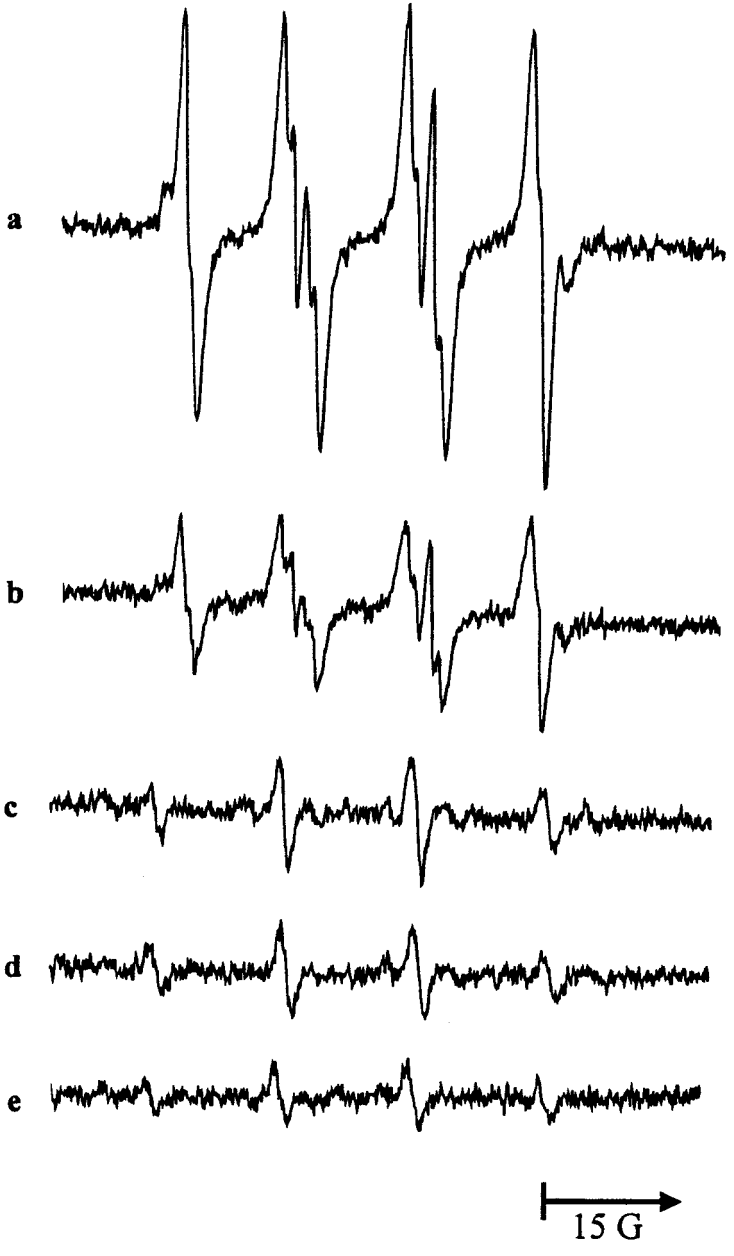

FIGURE 4. (a) Scavenging of O_2^- by FeTMPyP. The O_2^- radicals were produced by the reaction of 3.5 m*M* xanthine and 2 U xanthine oxidase in the presence of 100 m*M* DMPO in a pH 7.4 phosphate-buffered solution for 2 min. (b, c, d, and e) Same as (a) but with 1, 5, 10, and 25 μ*M* FeTMPyP added, respectively. The spectrum settings were: receiver gain, 2.51×10^4; modulation amplitude, 0.5 G; magnetic field, 3435 ± 50 G; scan time, 1 min. Data are representative of three separate measurements.

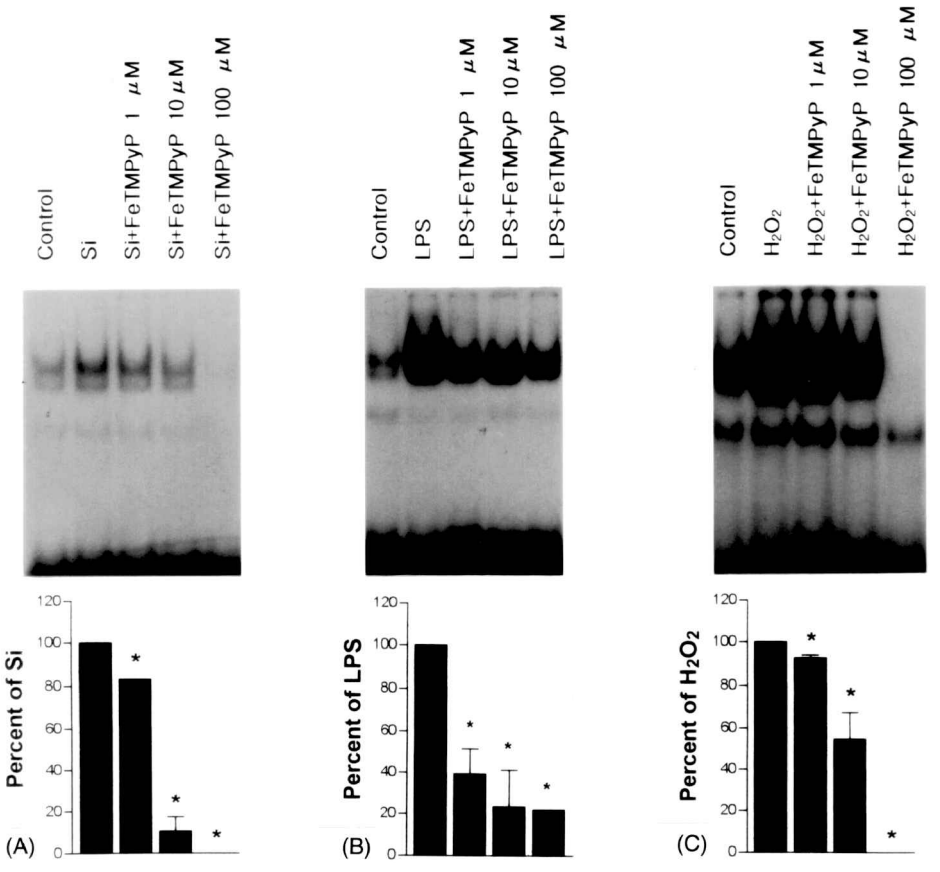

FIGURE 5. EMSA illustrating the effect of FeTMPyP on (A) silica, (B) LPS, or (C) H_2O_2 induced activation of NF-κB. Nuclear extracts were prepared from RAW264.7 cells pretreated for 2 h with FeTMPyP (1–100 μ*M*) and then stimulated with silica (100 μg/ml) or LPS (10 μg/ml) for an additional 4 h, or H_2O_2 (1 m*M*) for an additional 2 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). Values are means ± standard errors of three separate experiments. Asterisk indicates a significant inhibition by FeTMPyP compared to stimulant alone ($p < .05$).

FeTMPyP for 2 h before the addition of LPS inhibited the rate of degradation of IκB-α. The levels of IκB-α protein in cells exposed to LPS were increased in the presence of FeTMPyP (10 μ*M*) by 30, 238, and 71% after 5, 10, and 30 min of LPS exposure, respectively. However, FeTMPyP did not affect the minimum level of IκB-α protein in cells exposed to LPS for 20 min.

Dithiothreitol, a Reducing Agent, Reverses the Effect of FeTMPyP on NF-κB Activation

Intracellular thiols have been shown to play a key role in regulating NF-κB activation. FeTMPyP has been reported to be an effective $ONOO^-$

decomposition catalyst and can mediate the oxidation of thiol groups (Scorza & Minetti, 1998). Therefore, the ability dithiothreitol (DTT) to reverse the effect of FeTMPyP was examined in our study. Cells were preincubated with DTT (100 μ*M*) in the presence or absence of FeTMPyP (10 μ*M*) for 2 h and then the activation of NF-κB induced by silica was examined. Figure 8 shows that the DTT did not appear to have a significant effect on silica-induced NF-κB activation, but it substantially reversed the inhibitory effect of FeTMPyP by 64%. These results suggest that FeTMPyP inhibits NF-κB activation through alterations in intracellular thiols.

FeTMPyP Inhibits LPS-Induced IL-1 Production

IL-1 was chosen in our experiments as a representative proinflammatory cytokine because of its prominent role in pulmonary inflammatory and fibrotic responses (Kang et al., 1992) and because its gene expression is dependent on NF-κB (Baldwin, 1996). To examine the effect of FeTMPyP on the production of IL-1, thymocyte proliferation activity of RAW 264.7 macrophage supernatants was measured. Evidence indicates that over 90% of

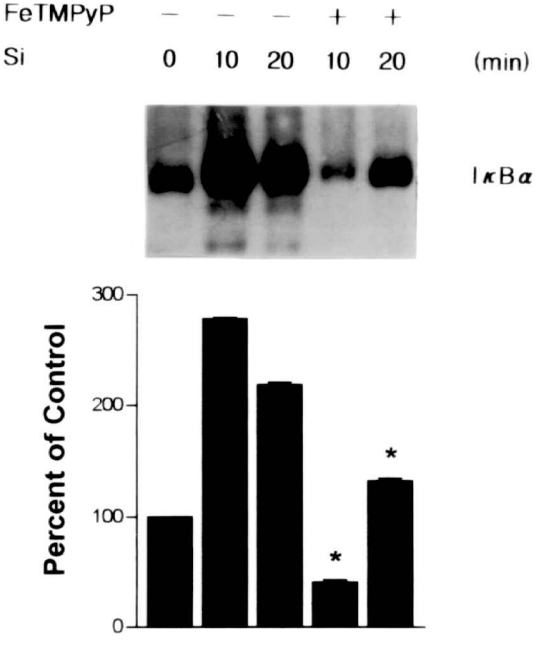

FIGURE 6. Effect of FeTMPyP on silica-dependent tyrosine phosphorylation of IκB-α. Cells were preincubated for 2 h with FeTMPyP (10 μ*M*) before treatment with silica (100 μg/ml) for an additional indicated time (10–20 min). The lysates were incubated with anti-IκB-α mAb before analysis of tyrosine phosphorylation by Western blotting with anti-phosphotyrosine mAb (upper panels). The levels of tyrosine phosphorylation of IκB-α are quantitated by densitometric analysis as a percentage of the control response (lower panels). Values are means ± standard errors of three separate experiments. Asterisk indicates a significant inhibition by FeTMPyP compared to silica alone at each time of incubation ($p < .05$).

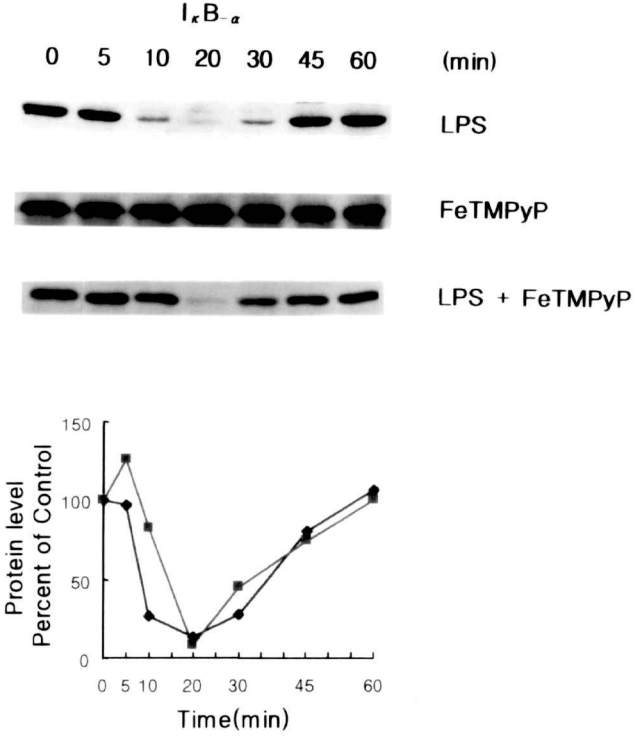

FIGURE 7. Effects of FeTMPyP on kinetics of degradation and resynthesis of IκB-α during exposure of RAW264.7 cells to LPS. Cells were preincubated for 2 h with FeTMPyP (10 μ*M*) before treatment with LPS (1 μg/ml) for an additional indicated time (upper). Cell lysates were analyzed for IκB-α by Western blotting. The levels of IκB-α, after stimulation with LPS (closed diamonds) or LPS + FeTMPyP (closed squares) were determined by scanning the autoradiograms (lower). Data are representative of at least three experiments.

this proliferation activity is due to IL-1 (Salem et al., 1990). Figure 9 shows the effect of FeTMPyP on RAW 264.7 macrophage production of IL-1 after stimulation by LPS. FeTMPyP appeared to exert little effect on the resting production of IL-1 from RAW 264.7 macrophages (data not shown). However, FeTMPyP inhibited LPS-stimulated IL-1 production by RAW 264.7 macrophages in a concentration-dependent manner, with the maximal inhibition of 100% at 50 μ*M* FeTMPyP. Direct treatment of thymocytes with FeTMPyP did not affect thymocyte proliferation at rest (data not shown).

DISCUSSION

Data presented in this study indicate that FeTMPyP at 10 μ*M* has a potent ability to scavenge hydroxyl radical (•OH) and superoxide anion (O_2^-). Indeed, its potency is higher than that of well-established antioxidants such as hypotaurine or ascorbic acid (Shi et al., 1997). Furthermore,

FeTMPyP substantially blocks stimulant-induced NF-κB activation and IL-1 production in RAW 264.7 macrophages. Metalloporphyrins, including Mn- and Fe-porphyrins, have the ability to alternate between reduced and oxidized states (Patel & Day, 1999). The ability of FeTMPyP to scavenge •OH and O_2^- is supported by the results of Imai et al. (1990), where Fe-porphyrin was reported to inhibit lipid peroxidation in rat liver homogenates. This antioxidant effect was not related to Fe^{3+}, even though it might be released from the parent porphyrin during incubation.

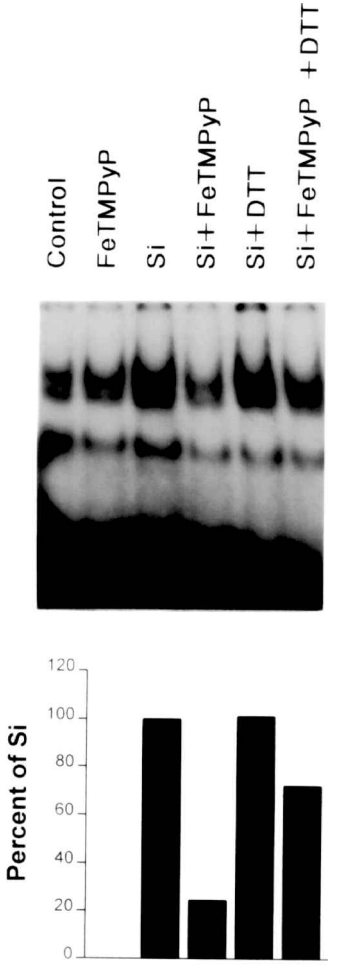

FIGURE 8. Effect of DTT on the FeTMPyP-dependent inhibition of NF-κB activation in silica-stimulated RAW 264.7 cells. Nuclear extracts were prepared from RAW 264.7 macrophages pretreated for 2 h with FeTMPyP (1–100 μ*M*) in the presence or absence of DTT (100 μ*M*) and then stimulated with silica (100 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 three experiments.

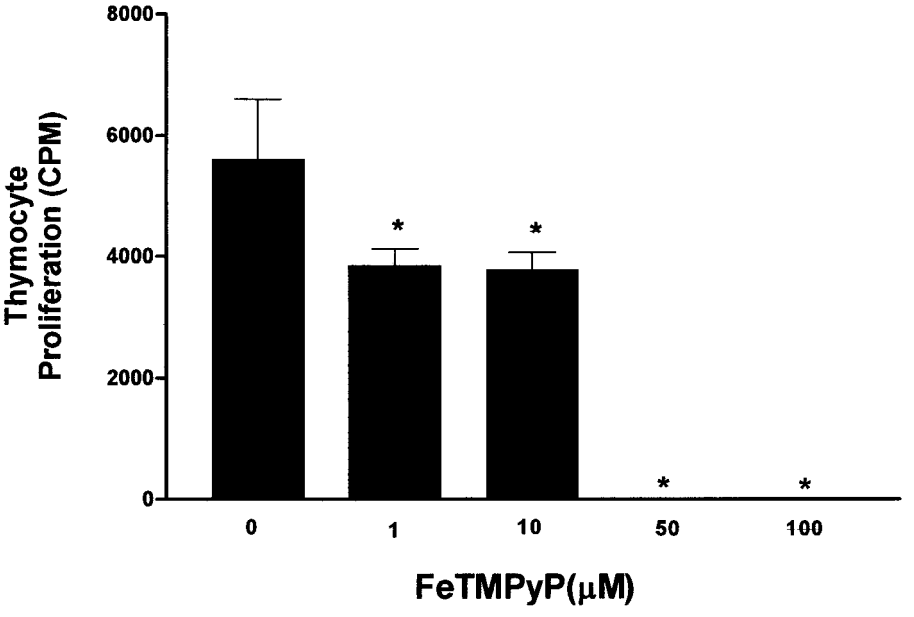

FIGURE 9. Effect of FeTMPyP on IL-1 production by LPS-stimulated RAW 264.7 macrophages in culture. RAW 264.7 macrophages (10^6/ml) were incubated in the absence of or after stimulation with LPS (5 μg/ml) in the presence or absence of FeTMPyP (1–100 μ*M*). After 24 h, IL-1 activity in the RAW 264.7 macrophage-conditioned supernates was measured by thymocyte incorporation of [^{3}H]thymidine. LPS-stimulated IL-1 activity was calculated as cpm minus that with resting cells. Values are means ± standard errors of three separate experiments. Asterisk indicates a significant difference compared to LPS alone ($p < .05$).

The mechanism by which FeTMPyP inhibits stimulant-induced NF-κB activation in response to various agents is not clear. ROS, protein tyrosine kinase, protein kinase C, protein tyrosine phosphatase, and proteases have been shown to play roles in the activation of NF-κB. As the stimulants employed in this study are known to induce ROS and/or PTK-dependent NF-κB activation in RAW264.7 cells (Kang et al., 2000a), it is possible that FeTMPyP exerts its effect by scavenging ROS and/or preventing PTK activation. Our findings that FeTMPyP can scavenge ROS and inhibit protein tyrosine phosphorylation induced by silica or LPS (data not shown) support this hypothesis.

Data from our previous study indicate that stimulation of RAW 264.7 macrophages with silica induces NF-κB activation through tyrosine phosphorylation of IκB-α without dependence on degradation of IκB-α (Kang et al., 2000b). The results from the present study show that FeTMPyP inhibited NF-κB activation by blocking tyrosine phosphorylation of IκB-α in silica-stimulated RAW 264.7. In contrast to silica-induced NF-κB activation, phosphorylation of serine residues of IκB-α leads to degradation of IκB-α by the proteasome, which is essential for NF-κB activation by LPS. Data show that FeTMPyP also inhibited degradation of IκB-α triggered by LPS.

Similar effects on tyrosine phosphorylation as well as degradation of IκBα have been reported with antioxidants and specific inhibitors of PTK (Kang et al., 2000b; Natarajan et al., 1998; Imbert et al., 1996). These findings suggest that FeTMPyP may act on the step that links reactive oxidants, PTK, and NF-κB activation. However, the inhibitory effect FeTMPyP on the DNA-binding activity of NF-κB in RAW 264.7 cells stimulated with either silica or LPS for 4 h appears much more dramatic than that on tyrosine phosphorylation or degradation of IκBα for the short-term exposure. These data suggest that FeTMPyP acts more effectively at a step following phosphorylation or degradation of IκBα, possibly by direct interaction with the NF-κB proteins or the DNA binding site, in a way that would prevent binding to DNA.

Intracellular thiols have been demonstrated to play a key role in regulating NF-κB activation in that low thiol levels are required for activation and high thiol levels inhibit activation (Staal et al., 1990). L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK), a protease inhibitor (Finco et al., 1994), herbimycin, a tyrosine kinase inhibitor (Mahon & O'Neill, 1995), and diamide and phenylarsine oxide (PAO) (Singh & Aggarwal, 1995), protein tyrosine phosphatase inhibitors, have been shown to suppress NF-κB activation (Chen et al., 1995). The effects of these inhibitors on NF-κB activation were completely reversed by DTT, a reducing agent. These results suggest that the redox state of the cell through the shifts in intracellular thiols is crucial to the control of NF-κB activity. FeTMPyP has been shown to be an effective $ONOO^-$ decomposition catalyst and mediates the oxidation of the thiol group of both cysteine and glutathione through competing one- and two-electron pathways (Scorza & Minetti, 1998). The ability of DTT to reverse the FeTMPyP-dependent inhibition of NF-κB activation in silica-stimulated cells supports this hypothesis.

It has been postulated that ROS can directly affect the cellular signaling apparatus and consequently the control of gene expression. Indeed, ROS have been suggested to play a regulatory role in tyrosine phosphorylation as well as NF-κB activation (Suzuki et al., 1997; Remacle et al., 1995). ROS have also been implicated as important mediators in silica-induced lung injury (Kang et al., 1992; Shi et al., 1998), and ROS may serve as mediators of production of cytokines, such as IL-1 and TNFα, in stimulated cells (Simeonova & Luster, 1996; Kawashima et al., 1998). In the present study, increases in NF-κB binding to DNA as well as production of a NF-κB dependent cytokine, IL-1, by LPS-stimulated RAW 264.7 macrophages were inhibited by FeTMPyP. However, the effect of this drug on NF-κB-dependent gene expression of IL-1 and promoter activation specific to IL-1 as well as other cytokines remains to be understood.

In conclusion, our findings indicate that FeTMPyP is a potent antioxidant that can scavenge ROS. FeTMPyP is effective in preventing stimulant-induced NF-κB activation and IL-1 activity in RAW 264.7 macrophages. Therefore, FeTMPyP may be a useful tool to investigate the molecular mechanisms involved in stimulant-induced signal pathways leading to inflammation and disease development. FeTMPyP may also have therapeutic

utility in diseases associated with the overproduction of hydroxyl radicals and superoxide radicals.

REFERENCES

Baldwin, A. S., Jr. 1996. The NF-κB and IκB proteins: New discoveries and insights. *Annu. Rev. Immunol.* 14:649–683.

Barnes, P. J., and Karin, M. 1997. Nuclear factor-kappa B: A pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* 366:1066–1071.

Bashir, Z. I., and Henley, J. M. 1993. The French connection: a magnum of excitatory amino acids in Marseilles. *Trends Pharmacol. Sci.* 14:387–390.

Blackwell, T. S., Blackwell T. R., Holden, E. P., Christman, B. W., and Christman, J. W. 1996. In vivo antioxidant treatment suppresses nuclear factor-kappa B activation and neutrophilic lung inflammation. *J. Immunol.* 157:1630–1637.

Brockman, J. A., Scherer, D. C., McKinsey, T. A., Hall, S. M., Qi, X., Lee, W. Y., and Ballard, D. V. 1995. Coupling of a signal response domain in IκB-α to multiple pathway for NF-κB activation. *Mol. Cell. Biol.* 15:2809–2818.

Brown, K., Gerstburger, S., Carlson, L., Franzoso, G., and Siebenlist, U. 1995. Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science* 267:1485–1488.

Castranova, V. 1994. Generation of oxygen radicals and mechanisms of injury prevention. *Environ. Health Perspect.* 102(suppl. 10):65–68.

Castranova, V., Antonini, J. M., Reasor, M. J., Wu, L., and Van Dyke, K. 1996. Oxidant release from pulmonary phagocytes. In *Silica and silica-induced lung diseases,* eds. V. Castranova, V. Vallyathan, and W. E. Wallace, pp. 185–195. Boca Raton, FL: CRC Press.

Chen, F., Sun, S. C., Kuh, D. C., Gaydos, L. J., and Demers, L. M. 1995. Dependence and reversal of nitric oxide production on NF-κB in silica and lipopolysaccharide-induced macrophages. *Biochem. Biophys. Res. Commun.* 214:839–846.

Chen, F., Lu, Y., Demers, L. M., Rojanasakul, Y., Shi, X., Vallyathan, V., and Castranova, V. 1998. Role of hydroxyl radical in silica-induced NF-κB activation in macrophages. *Ann. Clin. Lab. Sci.* 28:1–13.

Chen, F., Castranova, V., Shi, X., and Demers, L. M. 1999. New insights into the role of nuclear factor-κB, a ubiquitous transcription factor in the initiation of diseases. *Clin. Chem.* 45:7–17.

DiDonato, J. A., Mercurio, F., Rosette, C., Wu-Li, J., Sutyang, H., Ghosh, S., and Karin, M. 1996. Mapping of the inducible I kappa B phosphorylation sites that signal its ubiquitination and degradation. *Mol. Cell. Biol.* 16:1295–1304.

DiMatteo M., Antonini, J. M., Dyke, K. V., and Reasor, M. J. 1996. Characteristics of the acute-phase pulmonary response to silica in rats. *J. Toxicol. Environ. Health* 47:93–108.

Finco, T. S., Beg, A. A., and Baldwin, A. S., Jr. 1994. Inducible phosphorylation of I kappa B alpha is not sufficient for its dissociation from NF-kappa B and is inhibited by protease inhibitors. *Proc. Natl. Acad. Sci. USA* 91:11884–11888.

Fubini, B., Giamello, E., Volante, M., and Bolis, V. 1990. Chemical functionalities at the silica surface determining its reactivity when inhaled. Formation and reactivity of surface radicals. *Toxicol. Ind. Health* 6:571–598.

Goto, M., Katayama, K. I., Shirakawa, F., and Tanaka, I. 1999. Involvement of NF-kappa B p50/p65 heterodimer in activation of the human pro-interleukin-1 beta gene at two subregions of the upstream enhancer element. *Cytokine* 11:16–28.

Imai, K., Aimoto, T., Sato, M., and Kimura, R. 1990. Antioxidative effect of several porphyrins on lipid peroxidation in rat liver homogenates. *Chem. Pharm. Bull.* 38:258–260.

Imbert, V., Rupec, R. A., Livolsi, A., Pahl, H. L., Traenckner, E. B., Mueller-Dieckmann, C., Farahifar, D., Rossi, B., Auberger, P., Baeuerle, P. A., and Peyron, J. F. 1996. Tyrosine phosphorylation of IκB-α activates NF-κB without proteolytic degradation of IκB-α. *Cell* 86:787–798.

Kang, J. H., Lewis, D. M., Castranova, V., Rojanasakul, Y., Banks, D. E., Ma, J. Y., and Ma, J. K. 1992. Inhibitory action of tetrandrine on macrophage production of interleukin-1 (IL-1)-like activity and thymocyte proliferation. *Exp. Lung Res.* 18:715–729.

Kang, J. L., Go, Y. H., Hur, K. C., and Castranova, V. 2000a. Silica induced nuclear factor-κB activation: Involvement of reactive oxygen species and protein tyrosine kinase activation. *J. Toxicol. Environ. Health A* 60:27–46.

Kang, J. L., Pack, I. S., Hong, S. M., Lee, H. S., and Castranova, V. 2000b. Silica induced nuclear factor-κB activation through tyrosine phosphorylation of IκB-α in RAW264.7 macrophages. *Toxicol. Appl. Pharmacol.* 169:59–65.

Kang, J. L., Pack, I. S., Hong, S. M., Lee, H. S., Hah, J. H., Nam, W., Leonard, S., and Castranova, V. 2001. Zinc tetrachis (*N*-methyl-4′-pyridyl) porphyrinato (ZnTMPyP) is an effective inhibitor of stimulant-induced activation of RAW 264.7 cells. *Toxicol. Appl. Pharmacol.* 172:140–149.

Kawashima, S., Hayashi, M., Takii, T., Kimura, H., Zhang, H. L., Nagatsu, A., Sakakibara, J., Murata, K., Oomoto, Y., and Onozaki, K. 1998. Serotonin derivative, *N*-(*p*-coumaroyl) serotonin, inhibits the production of TNF-alpha, IL-1 alpha, IL-1 beta, and IL-6 by endotoxin-stimulated human blood monocytes. *J. Interferon Cytokine Res.* 18:423–428.

Kim, J. K., Lee, W. K., Lee, E. J., Cho, Y. J., Kim, H. S., Chung, Y., Kim, K. A., and Lim, Y. 1999. Mechanism of silica- and titanium dioxide-induced cytotoxicity in alveolar macrophages. *J. Toxicol. Environ. Health A* 58:437–450.

Koong, A. C., Chen, E. Y., Mivelchi, N. F., Denko, N. C., Stambrook, P., and Giaccia, A. J. 1994. Hypoxic activation of nuclear factor κ-B is mediated by a *Ras* and *Raf* signaling pathway and does not involve MAP kinase (ERK1 or ERK2). *Cancer Res.* 54:5273–5279.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (*Lond.*) 227:680–685.

Lipton, S. A., Choi, Y. B., Pan, Z. H., Lei, S. Z., Chen, H. S., Sucher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. S. 1993. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364:626–632.

Luo, D., and Vincent, S. R. 1994. Metalloporphyrins inhibit nitric oxide-dependent cGMP formation in vivo. *Eur. J. Pharmacol.* 267:263–267.

Mahon, T. M., and O'Neill, L. A. 1995. Studies into the effect of the tyrosine kinase inhibitor herbimycin A on NF-kappa B activation in T lymphocytes. Evidence for covalent modification of the p50 subunit. *J. Biol. Chem.* 270:28557–28564.

Mayeux, P. R. 1997. Pathobiology of lipopolysaccharide. *J. Toxicol. Environ. Health* 51:415–435.

Meyer, M., Schreck, R., and Baeuerle, P. A. 1993. H_2O_2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J.* 12:2005–2015.

Misko, T. P., Highkin, M. K., Veenhuizen, A. W., Manning, P. T., Stem, M. K., Currie, M. G., and Salvemini, D. 1998. Characterization of the cytoprotective action of peroxynitrite decomposition catalysts. *J. Biol Chem.* 273:15646–15653.

Mottley, C., and Mason, R. P. 1989. Nitroxide radical adducts in biology: Chemistry, applications, and pitfalls. *Biol. Magn. Reson.* 8:489–546.

Natarajan, K., Manna, S. K., Chaturvedi, M. M., and Aggarwal, B. B. 1998. Protein tyrosine kinase inhibitors block tumor necrosis factor-induced activation of nuclear factor-κB, degradation of IκBα, nuclear translocation of p65, and subsequent gene expression. *Arch. Biochem. Biophys.* 352:59–70.

Patel, M., and Day, B. J. 1999. Metalloporphyrin class of therapeutic catalytic antioxidants. *Trends Pharmacol. Sci.* 20:359–364.

Remacle, J., Raes, M., Toussaint, O., Renard, P., and Rao, G. 1995. Low levels of reactive oxygen species as modulators of cell functions. *Mutat. Res.* 316:103–122.

Sacks, M., Gordon, J., Bylander, J., Porter, D., Shi, X. L., Castranova, V., Kaczmarczyk, W., Van Dyke, K., and Reasor, A. J. 1998. Silica-induced pulmonary inflammation in rats: Activation of NF-kappa B and its suppression by dexamethasone. *Biochem. Biophys. Res. Commun.* 253:181–184.

Saffiotti, U., Williams, A. O., Daniel, L. N., Kaighn, M. E., Mao, Y., and Shi, X. 1985. Carcinogenesis by crystalline silica: animal, cellular, and molecular studies. In *Silica and silica-induced lung diseases,* eds. V. Castranova, V. Vallyathan, and W. E. Wallace, pp. 345–381. Boca Raton, FL: CRC Press.

Salem, P., Deryckx, S., Dulioust, A., Vivier, E., Denizot, Y., Damais, C., Dinnrello, C. A., and

Thomas, Y. 1990. Immunoregulatory functions of paf-acether IV. Enhancement of IL-1 production by muramyl dipeptide-stimulated monocytes. *J. Immunol.* 144:1338–1344.

Salvemini, D., Wang, Z. Q., Stem, M. K., Currie, M. G., and Misko, T. P. 1998. Peroxynitrite decomposition catalysts: Therapeutics for peroxynitrite-mediated pathology. *Proc. Natl. Acad. Sci. USA* 95:2659–2663.

Scorza, G., and Minetti, M. 1998. One-electron oxidation pathway of thiols by peroxynitrite in biological fluids: Bicarbonate and ascorbate promote the formation of albumin disulphide dimers in human blood plasma. *Biochem. J.* 329:405–413.

Shi, X., Flynn, D. C., Porter, D. W., Leonard, S. S., Vallyathan, V., and Castranova, V. 1997. Efficacy of taurine based compounds as hydroxyl radical scavengers in silica-induced peroxidation. *Ann. Clin. Lab. Sci.* 27:365–374.

Shi, X., Castranova, V., Halliwell, B., and Vallyathan, V. 1998. Reactive oxygen species and silica-induced carcinogenesis. *J. Toxicol. Environ. Health B* 1:181–197.

Shi, X., Ding, M., Dong, Z., Chen, F., Ye, J., Wang, S., Leonard, S. S., Castranova, V., and Vallyathan, V. 1999. Antioxidant properties of aspirin: characterization of the ability of aspirin to inhibit silica-induced lipid peroxidation, DNA damage, NF-κB activation and TNFα production. *Mol. Cell. Biochem.* 199:93–102.

Simeonova, P. P., and Luster, M. L. 1996. Asbestos induction of nuclear transcription factors and interleukin 8 gene regulation. *Am. J. Respir. Cell Mol. Biol.* 15:787–795.

Singh, S., and Aggarwal, B. 1995. Protein-tyrosine phosphatase inhibitors block tumor necrosis factor-dependent activation of the nuclear transcription factor NF-κB. *J. Biol. Chem.* 270:10631–10639.

Staal, F. J., Roederer, M., Herzenberg, L. A., and Herzenberg, L. A. 1990. Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 87:9943–9947.

Sun, S. C., Elwood, J., Beraud, C., and Greene, W. C. 1994. Human T-cell leukemia virus type I Tax activation of NF-kappa B/Rel involves phosphorylation and degradation of I kappa B alpha and RelA (p65)-mediated induction of the c-rel gene. *Mol. Cell. Biol.* 14:7377–7384.

Suzuki, Y. J., Forman, H. J., and Sevanian, A. 1997. Oxidants as stimulators of signal transduction. *Free Radical Biol. Med.* 22:269–285.

Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350–4354.

Vallyathan, V., Shi, X., Dalal, N. S., Irr, W., and Castranova, V. 1988. Generation of free radical from freshly fractured silica dust. Potential role in acute silica-induced lung injury. *Am. Rev. Respir. Dis.* 138:1213–1219.

Zabel, U., and Baeuerle, P. A. 1990. Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. *Cell* 61:255–265.