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Role of Tyrosine Kinase Activity in 2,2′,2″-Tripyridine-Induced Nitric Oxide Generation in Macrophages

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ABSTRACT. In this paper, we demonstrated that 2,2′,2″-tripyridine (TP, 1–20 μM) is a potent inducer of nitric oxide (NO) synthase in the cultured murine macrophage RAW 264.7 cell line. TP increased not only nitrite but also inducible NO synthase (iNOS) protein and mRNA production. Co-treatment with either NOS inhibitors (N^G-monomethyl-L-arginine and aminoguanidine) or cycloheximide and actinomycin D all inhibited TP-induced nitrite production, indicating the requirement of protein and mRNA synthesis. The signaling pathway of TP-induced iNOS expression was explored, and the results obtained suggested that increased tyrosine kinase activity followed by inhibitor of nuclear factor for immunoglobulin κ chain in B cells (IκB) degradation and then nuclear factor κB (NFκB) activation was involved in TP-induced iNOS expression. Tyrosine kinase inhibitors (e.g. genistein and tyrphostin AG126) inhibited both TP-induced nitrite and iNOS protein production. Whether the metalochelating property of TP was involved in these effects was explored by saturating TP with $FeCl_3$. Although the ferrated TP became inactive, the specific iron chelator desferrioxamine, at a very high concentration of 400 μM, induced only a weak enhancement of nitrite production in this RAW cell line. It was thereby concluded that TP induces NO production through an increase in iNOS expression, which is initiated by a signaling pathway via tyrosine kinases leading to an activation of NFκB. Since TP is much more potent than desferrioxamine in increasing nitrite production, it is suspected that the primary event induced by TP was possibly mediated by TP's interacting with certain macromolecules in addition to its metal-chelating property. BIOCHEM PHARMACOL **57**;12:1367–1373, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. 2,2′,2″-tripyridine; iNOS; tyrosine kinase; NFκB; Macrophage RAW 264.7

NO§ produced by the activated macrophages is believed to be an important mediator in the cytotoxic/cytostatic mechanism of non-specific immunity [1, 2]. NOS, the enzyme responsible for NO synthesis in various tissues, has been characterized into three types, neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). The calcium-independent iNOS in the macrophages could be activated by the bacterial endotoxin LPS, either alone or together with cytokines such as interleukin-1 or interferon-γ [3–5].

The *iNOS* gene from murine macrophages had been cloned and characterized [6]. The induction of iNOS by LPS and cytokines in macrophages is initiated by the activation of tyrosine kinases [7–9]. Thus, tyrosine kinase inhibitors blocked iNOS production at a point well downstream from the initial wave of LPS- and interferon-γ-mediated protein tyrosine phosphorylation [10]. As for the promoter of the murine gene encoding iNOS, a sequence for the NFκB/Rel binding site has been recognized [11–13]. Activation of NFκB enhances iNOS induction [14, 15]. In the cytoplasm, an inhibitory molecule of the IκB family bound to NFκB complexes and inhibited NFκB activation by masking the nuclear localization signals [16, 17]. Upon stimulation, phosphorylation of IκB-α on serine residues leads to a rapid degradation of IκB-α caused by protease [18, 19].

TP is a pyridine derivative known as a metal-chelating agent [20] and a synthetic by-product of the herbicide paraquat [21]. We previously demonstrated this metal chelator to be a strong mutagen. Since NO could be a DNA-damaging agent [22, 23] and the other metal chelators (pyrrolidine dithiocarbamate and desferrioxamine) have been shown to be capable of affecting the NOS system [14, 15, 24], it appeared worthwhile to examine the influences of TP on the NO/NOS system of the macrophages. In this paper, we demonstrate that TP is a potent inducer of NO synthase in the cultured murine macrophage RAW 264.7 cell line mediated by an initial activation of protein tyrosine kinase and NFκB.

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§ *Abbreviations:* iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NFκB, nuclear factor κB; IκB, inhibitor of nuclear factor for immunoglobulin κ chain in B cells; TP, 2,2′,2″-tripyridine; NO, nitric oxide; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; and NNMA, N^G-monomethyl-L-arginine.

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MATERIALS AND METHODS

Materials

LPS (*Escherichia coli* 0127:B8), TP, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, genistein, tyrphostin AG126, NMMA, aminoguanidine, actinomycin D, and cycloheximide were purchased from Sigma Chemical Co. [γ-^{32}P]ATP was obtained from Amersham Corp. Polynucleotide kinase and oligo(dT)18 were obtained from Pharmacia Biotech.

Cell Culture

RAW 264.7 cells, an Abelson virus-transformed murine macrophage cell line (American Type Culture Collection), was cultured in RPMI-1640 medium containing 10% [v/v] heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin, and were maintained in a humidified atmosphere of 5% CO_2/95% air at 37 $\pm$ 0.5°. For cell stimulation, RAW 264.7 cells were grown in 24-well plates (4×10^5 cells/well) in 0.5 mL of growth medium for 18 hr to allow the cell number to approximately double. The growth medium was replaced and the indicated stimulants were added. TP was dissolved in ethanol and the final concentration of ethanol maintained at less than 0.2% [v/v] to ensure that no toxic effect was exhibited. In the control experiment, an equivalent amount of ethanol vehicle was added.

Nitrite Assay

Nitrite levels in the cultured media, which reflect intracellular NOS activity, were determined by the Griess reagent [25]. Briefly, 100 μL of each culture medium was mixed with the same volume of Griess reagent (1% [w/v] sulfanilamide in 5% [v/v] phosphoric acid and 0.1% [w/v] *N*-(1-naphthyl)ethylenediamine dihydrochloride in water). Absorbance of the mixture at 550 nm was determined by using a microplate ELISA reader (Dynatech MR7000).

Western Blotting

Western blotting was performed according to a previously described method [26]. RAW 264.7 cells (1×10^6 cells/well) were grown in a six-well plate overnight. After stimulation, cells were washed with ice-cold PBS containing 1 mM Na_3VO_4, and then treated with 200 μL of lysis buffer (10 mM Tris–HCl, pH 7.5, 2 mM EDTA, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 3% [w/v] SDS, 0.1% [v/v] Nonidet P-40, 10 mM NaCl, and 0.15 mM $MgCl_2$). Cellular lysates were centrifuged at 20,000 *g* at 4 $\pm$ 0.5° for 30 min. The supernatants were collected and the protein contents determined by using the bicinchoninic acid protein assay reagents (Pierce) with BSA as standard. Each sample, which contained 50 μg of protein, was separated on SDS-polyacrylamide minigels (6% for iNOS and 10% for IκBα and tyrosine phosphorylation). After electrophoresis, gels were transferred to nitrocellulose paper. The loading and transfer of equal amounts of protein in each lane was verified by staining the protein bands with Amido Black. After extensive washing with distilled water to remove the protein stain, the membrane was incubated with 3% [w/v] BSA in PBS (containing 0.1% [v/v] Tween-20) to block non-specific immunoglobulins and then immunoblotted as described with mouse monoclonal anti-iNOS antibody (Transduction Laboratories) or PY20 monoclonal antiphosphotyrosine antibody (Transduction) or rabbit polyclonal anti-IκBα (Santa Cruz Biotechnology). The primary antibody was detected using anti-mouse immunoglobulin G-horseradish peroxidase (IgG-HRP) or anti-rabbit IgG-HRP (Santa Cruz Biotechnology) and the enhanced chemiluminescence kit (Amersham).

Reverse Transcription-Polymerase Chain Reaction

After the incubation of macrophages as described above, total RNA was isolated by using the acid guanidinium thiocyanate-phenol-chloroform method [13]. Total RNA (1 μg) was converted to cDNA with 1 mM oligo (dT) 18, 0.5 mM of each dNTP, 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, RNase inhibitor (1 U/mL), and Moloney's murine leukemia virus reverse transcriptase (10 U/mL) at 42° for 1 hr. The amplification of iNOS cDNA was performed by incubating 20 ng equivalents of cDNA in 100 mM Tris–HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin, 200 mM dNTPs, and 50 U/mL of Super Taq DNA polymerase with the following oligonucleotide primers: 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′. The cDNA sequence of glyceraldehyde-3-phosphate dehydrogenase was also amplified as control in a similar way using 5′-TGAAGGTCGGTGTGAACG-GATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ as primers. A thermal cycle of 45 sec at 95°, 45 sec at 65°, and 2 min at 72° was used for 30 cycles. Polymerase chain reaction products were analyzed on 1.8% agarose gels [14].

Electrophoretic Mobility Shift Assay

Nuclear and cytoplasmic extracts were prepared by a modified method as described previously [27]. At the end of culture, the cells were suspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM PMSF) for 20 min on ice, vortexed for 10 sec every 5 min. Nuclei were pelleted by centrifugation at 12,000 *g* for 20 sec. The supernatants containing cytosolic proteins were collected. Pellet-containing nuclei were resuspended in buffer C (20 mM HEPES, pH 7.6, 20% [v/v] glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM PMSF) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 12,000 *g* for 2 min and

stored at −70°. For EMSA, each 5-μg nuclear extract was mixed with the labeled doubled-stranded NFκB oligonucleotide, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, and incubated at room temperature for 20 min. The incubation mixture included 1 μg of poly(dI-dC) in a binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% [v/v] Nonidet P-40, 5% [v/v] glycerol, and 50 mM NaCl). The DNA–protein complex was electrophoresed on 5% non-denaturing polyacrylamide gels in 0.5 × Tris/borate/EDTA buffer.

Statistics

The data in the text are given as means ± SE. The significance of difference was evaluated by the paired or unpaired Student's *t*-test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA). Probability values <0.05 were considered to be significant.

RESULTS

TP-induced NO Generation

TP induced a concentration-dependent increase in nitrite production in the culture medium of RAW 264.7 macrophages within 24 hr (Fig. 1A). The iron chelator desferrioxamine, at high concentrations of 100 and 400 μM, only moderately but not significantly enhanced TP in producing nitrite (Table 1). LPS by itself fully activated RAW maprophages and increased nitrite production by about 12-fold at 1 μg/mL, but did not further increase it at 10 μg/mL. The increase in nitrite accumulation was maximal at 24 hr. In order to test if LPS potentiated TP in producing nitrite, a submaximal concentration of LPS at 0.1 μg/mL was used. As shown in Fig. 1A, LPS shifted the curve to the right, showing a marked potentiation on TP-induced nitrite production.

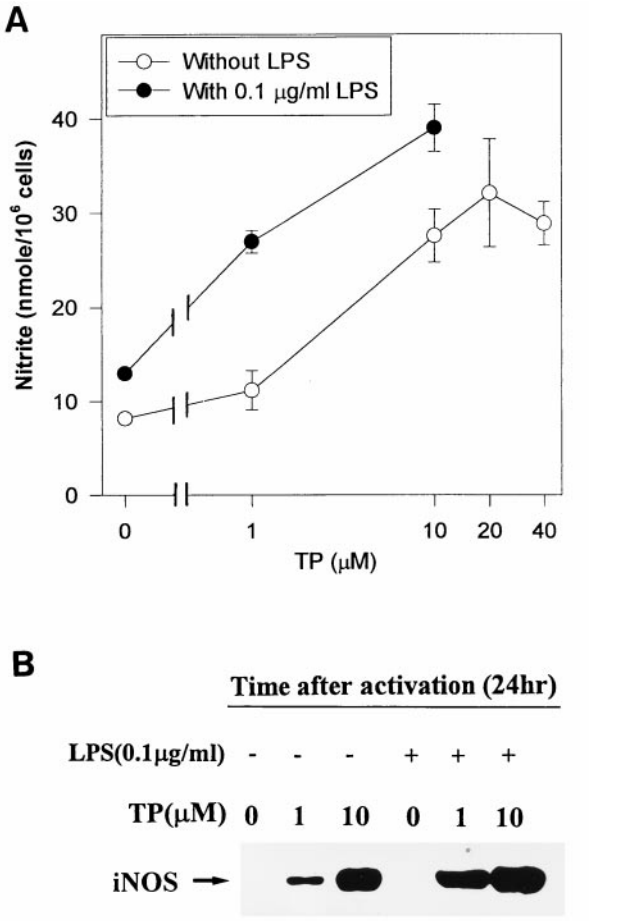

FIG. 1. Concentration-dependent increase in nitrite and iNOS protein production induced by TP in a cultured macrophage cell line. TP at various concentrations increased nitrite production after 24-hr incubation with RAW 264.7 cells. LPS (0.1 μg/mL) pretreatment for 5 min potentiates TP in both nitrite (A) and iNOS protein production (B) after 24-hr incubation. iNOS protein was determined by immunoblotting as described in Materials and Methods, and a representative result of three experiments is shown (B). Data (A) are presented as means ± SE (N = 6).

Mechanism of TP-induced NO Production

The addition of the NOS inhibitors NMMA (1 mM) or aminoguanidine (100 μM) to the cells prevented the nitrite accumulation induced by TP (Table 1), suggesting an increase in NOS activity by TP. Treatment of cells with either 0.05 μM actinomycin D (an mRNA synthesis inhibitor) or 0.2 μM cycloheximide (a protein synthesis inhibitor) sufficiently prevented the increase in nitrite elicited by TP (Table 1). Thus, TP-increased NOS activity required synthesis of protein and mRNA. Saturating TP with 50 μM $FeCl_3$ significantly attenuated TP-induced nitrite production (Table 1). None of these NOS inhibitors used alone affected the cell viability.

The increase induced by TP and by LPS (as a positive control) of the expression of iNOS protein and mRNA is shown in Figs. 1B and 2. RAW 264.7 cells stimulated with TP expressed a protein of approximately 130 kDa, which was recognized by a specific antibody to macrophage iNOS (Fig. 1B; lanes 2 and 3). In contrast, unstimulated cells did not express detectable iNOS protein (Fig. 1B; lane 1). The TP-induced iNOS protein expression was enhanced by LPS (0.1 μg/mL) (Fig. 1B; lanes 5 and 6), while 0.1 μg/mL LPS by itself did not induce detectable iNOS protein expression (Fig. 1B; lane 4). A similar result was obtained in iNOS mRNA expression, which was increased by TP and LPS in a time-dependent manner (Fig. 2).

Signaling Pathway of TP-induced NO Production

To determine whether protein tyrosine kinase activity mediated NOS induction by TP, the tyrosine kinase inhibitor, either genistein (50 μM) or tyrphostin (100 μM), was simultaneously applied with 10 μM TP to cell cultures. After a 24-hr incubation, the cultured media were removed and cell extracts were made. As shown in Fig. 3, either

TABLE 1. The effects of various compounds on the nitrite generation induced by tripyridine in cultured RAW 264.7 cells

Treatment	Conc. (μM)	Nitrite (nmol/10^6 cells) Without TP	With TP
Control		9.3 ± 0.5	31.5 ± 3.5
NMMA	1000	7.5 ± 0.7	14.1 ± 2.3*
Aminoguanidine	100	7.8 ± 0.5	13.3 ± 1.1*
Actinomycin D	0.05	7.5 ± 0.5	9.8 ± 0.6*
Cycloheximide	0.2	7.6 ± 0.6	10.2 ± 0.5*
Desferrioxamine	100	7.8 ± 0.6	40.0 ± 2.1*
	400	8.0 ± 0.5	45.2 ± 2.5*
$FeCl_3$	50	8.2 ± 0.5	13.1 ± 1.5*

The cultured RAW 264.7 cells (2×10^5 cells/well in 24-well plates) were pretreated with different compounds for 5 min prior to the addition of 10 μM tripyridine (TP). The amount of nitrite released in the culture medium after 24-hr incubation was determined by Griess reagent. Data are presented as means ± SE from three separate experiments, each performed in triplicate.

*$P < 0.05$ as compared with that treated with 10 μM TP alone.

genistein or tyrphostin obviously inhibited not only TP-induced nitrite production (Fig. 3A) but also iNOS protein synthesis (Fig. 3B; lanes 3 and 5). These results confirmed the importance of tyrosine kinase activity for TP-induced NO production.

The cell protein extracts after vehicle or 10-μM TP treatments were separated by SDS-PAGE and then immunoblotted with an antibody specific for phosphotyrosine moieties. As shown in Fig. 4, TP treatment for 10–30 min prominently enhanced tyrosine phosphorylation of the proteins. Tyrosine phosphorylation of the proteins with molecular weights of 52 kDa and 60 kDa increased as early as 5 min of treatment and reached a maximum within 10 min, and then declined slowly remained at least one-third of maximum after 60 min of treatment. By contrast, the phosphorylation of the 120 kDa protein appeared later in the 10-min treatment and was no longer detected at 60 min.

Following these early immediate tyrosine phosphoryla-

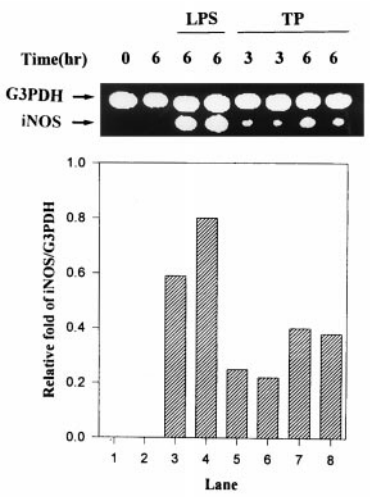

FIG. 2. Increase in iNOS mRNA expression by tripyridine or LPS in cultured RAW 264.7 macrophages. Analysis of mRNA of iNOS and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression from macrophages activated by either LPS (1 μg/mL) for 6 hr or TP (10 μM) for 3 and 6 hr, respectively. The iNOS/G3PDH ratio was determined by using an imaging desitometer. A representative curve of three experiments is shown.

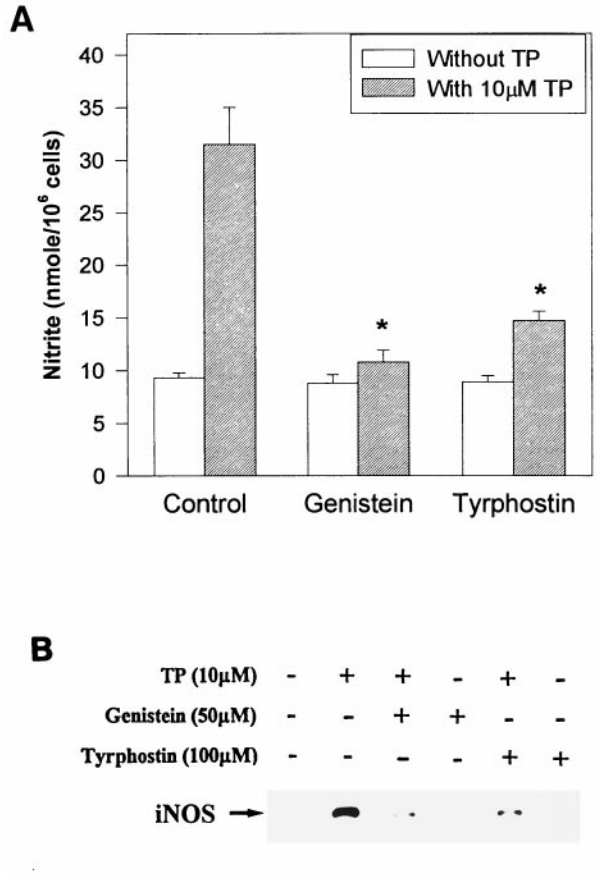

FIG. 3. Inhibition by genistein and tyrphostin of tripyridine-induced nitrite production and iNOS protein synthesis in macrophage cell line. RAW 264.7 cells were treated for 5 min with either genistein (50 μM) or tyrphostin (100 μM) prior to the addition of 10 μM tripyridine (TP) for 24 hr. Experimental details for assaying nitrite production and iNOS production are described under Materials and Methods. Data are presented as means ± SE. *$P < 0.05$ as compared with the respective control.

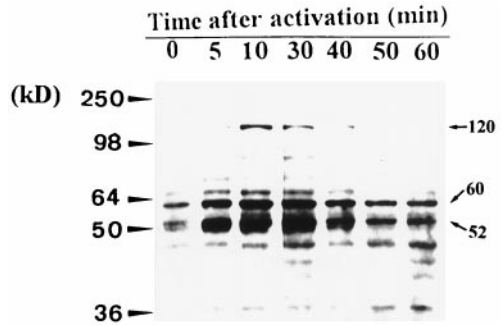

FIG. 4. Kinetics of TP-induced protein tyrosine phosphorylation. RAW 264.7 cells were incubated with 10 μM TP for various periods (0.5, 10, 30, 40, 50, and 60 min) and then lysed and resolved by SDS-PAGE. Protein tyrosine phosphorylation was immunoblotted with antiphosphotyrosine monoclonal antibody. The major protein bands (52 kDa, 60 kDa, and 120 kDa) increased by TP are indicated by arrows. One representative result of three experiments is shown.

tions, we considered whether the degradation process of the inhibitory protein (IκB) of NFκB was enhanced by TP. As shown in Fig. 5A, IκB decreased in magnitude initially at 2 hr and maximally at 3 hr after treatment with 10 μM TP. Furthermore, the tyrosine kinase inhibitors genistein (50 μM; lane 3) and tyrphostin (100 μM; lane 5) effectively inhibited TP-induced IκB degradation (Fig. 5B). Moreover, the disappearance of IκB was found to be accompanied by the activation of NFκB, which translocated to nucleus as revealed by EMSA after 2-hr treatments with TP (Fig. 6). For a positive control, LPS (0.1 μg/mL) showed a similar enhancing effect on NFκB activation.

DISCUSSION

In this study, we have demonstrated that TP, a metal chelator, increased NO production in RAW 264.7 macrophages. The nitrate/nitrite level released into the culture

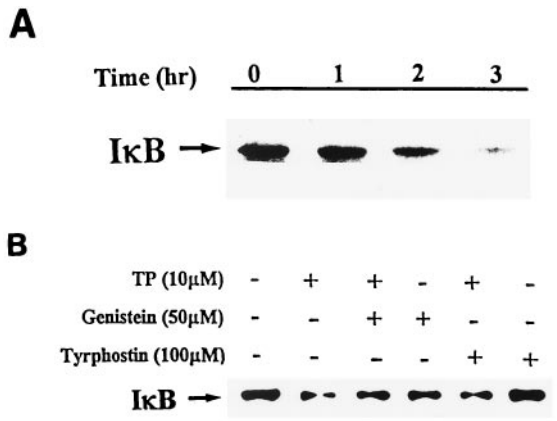

FIG. 5. Enhanced IκB degradation by TP in RAW 264.7 cells. Cytosolic extracts were prepared and analyzed by immunoblot with antibody directed toward IκB-α after incubating the cells with 10 μM TP for various periods (A). Pretreatment of cells for 5 min with either genistein (50 μM) or tyrphostin (100 μM) prior to the addition of TP for 3 hr inhibited IκB-α degradation (B). One representative result of three experiments is shown.

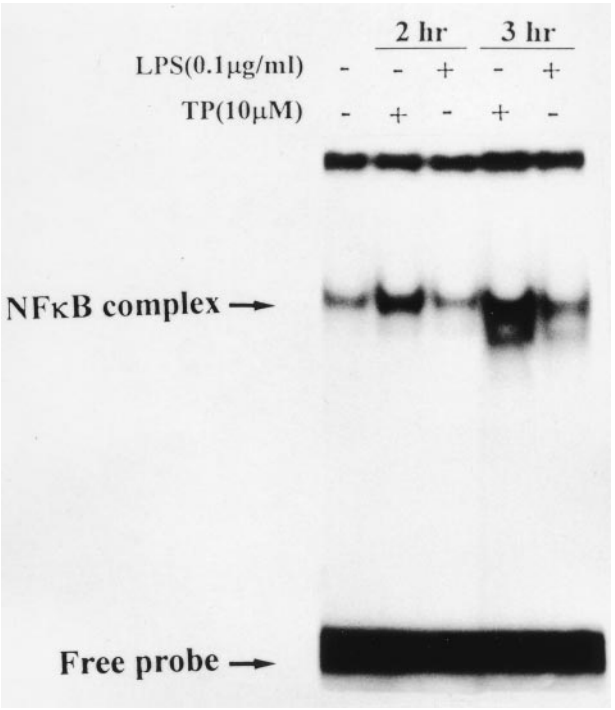

FIG. 6. Effect of tripyridine on NFκB activation in a macrophage cell line. RAW 264.7 cells were incubated with either 10 μM TP or 0.1 μg/mL LPS for 2 or 3 hr, and nuclear extracts were then prepared for EMSA. Note that TP and LPS markedly enhanced NFκB activation. Similar results were obtained from other three experiments.

medium was monitored for NOS activity because both nitrate and nitrite were known to be the spontaneously formed stable oxidation products of NO. This assay had been proven to be a good approximation of NOS activity [28]. Therefore, the increase in nitrite production by TP was attributed to an increase in NOS activity. Further evidence for this consideration is that the NOS inhibitors L-NMMA and aminoguanidine [29] attenuated TP-induced nitrite production, and TP definitely increased iNOS protein synthesis and iNOS mRNA expression as revealed by Western blotting the reverse transcription-polymerase chain reaction, respectively. Cyclohexemide (a protein synthesis inhibitor) and actinomycin D (an mRNA synthesis inhibitor) inhibited TP-induced nitrite production, confirming that TP effects were mediated by an increase in protein and mRNA synthesis.

The possible signaling pathways of TP-induced nitrite production were explored in this study. It has been demonstrated that the expression of *iNOS* gene induced by either cytokines or LPS is mediated by transcription factors such as NFκB [11, 30]. NFκB, which is constitutively present in cells as a heterodimer consisting of a p50 DNA-binding subunit and a p65-transactivating subunit, is normally held in the cytoplasm in an inactive state by the inhibitor protein IκB [30]. Inflammatory cytokines activate cell surface receptors, leading to the dissociation of IκB from the NFκB complex following phosphorylation of IκB, and then proteolytic degradation of the phosphorylated IκB

by protease [30]. Eventually, the activated NFκB is translocated into the nucleus and bound to the relevant DNA-binding sites on the promoter region of the *iNOS* gene. Therefore, degradation of IκB is essential for the activation of NFκB, and inhibiting the phosphorylation of IκB by various agents may lead to the activation of NFκB [30].

Since the effect of TP could be enhanced by LPS, we investigated whether the tyrosine kinase pathway coupled with the activated NFκB was involved in TP effects. The results obtained showed that TP rapidly and prominently increased tyrosine phosphorylation of several proteins including 52 kDa, 60 kDa, and 120 kDa within 10 min. Subsequent reduction of cytoplasmic IκB accompanied by the activation of NFκB were induced by TP. These findings were similar to those induced by LPS and interleukin-1β. Furthermore, all of these events apparently occurred prior to the initiation of iNOS mRNA and protein synthesis. Read *et al.* have suggested that inhibition of tyrosine kinase with genistein inhibits the activation of NFκB, implying that tyrosine kinase activation precedes NFκB activation [31]. It has also been suggested that there is a direct connection between protein tyrosine kinase stimulation and NFκB activation through tyrosine phosphorylation of IκB-α [32]. Based on these contentions together with the results obtained in this study, we propose that TP increases NOS activity through a signaling pathway of increasing tyrosine kinase → IκB degradation → NFκB activation → *NOS* gene expression.

There is substantial evidence suggesting that LPS binds to a receptor protein (CD14) to initiate the induction of iNOS. It remains to be clarified whether TP also binds to a similar CD14-like protein prior to the induction of iNOS. The chemical nature of TP belongs to a pyridine derivative with three tertiary nitrogen atoms, which was originally adopted as a metal chelator. In our laboratory, we found that TP behaves as a curare-like antagonist on the nicotinic acetylcholine receptor, perhaps by a direct binding to the receptor protein [33]. These findings suggest that the TP molecule may possess a flexible configuration capable of binding to macromolecules in addition to its originally known metal chelation. Although the saturated, ferrated TP became inactive in promoting nitrite production, perhaps only the free form of TP is active in binding to the macromolecules. This proposal is further supported by the finding that a specific potent iron chelator, desferrioxamine, exerted only a weak enhancement of nitrite production in cultured RAW 264.7 macrophages. However, it must be pointed out that the regulatory machinery of nitrite production by iron and metal chelators in the different cell lines, e.g. RAW 264 macrophages in this study and J744 macrophages in the study of desferrioxamine enhancement by Weiss *et al.* [24], may be quite different, because we have found the amount of nitrite production induced by LPS alone in J744 cells to be only about one-one hundredth that of RAW cells*; LPS alone can fully activate nitrite production in RAW cells, but interferon γ is required in combination with LPS to obtain full activation in J744 cells.

In conclusion, we have demonstrated in this study that TP could facilitate iNOS expression in the RAW 264.7 macrophages. The signaling pathway for TP-induced nitrite production was identified as increased tyrosine kinase activity followed by the degradation of IκB and then activation of the transcription factor NFκB. Whether NO production induced by TP is responsible for its mutagenic effect is under investigation.

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