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Role of NF-κB Binding Sites in the Regulation of Inducible Nitric Oxide Synthase by Tyrosine Kinase

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In macrophages, lipopolysaccharide (LPS) alone or in combination with interferon-γ (IFN-γ) has been shown to release a nitric oxide (NO) through the increase of the transcription of the inducible nitric oxide synthase (iNOS) gene. To investigate the exact intracellular signaling pathway of the regulation of iNOS gene transcription by LPS plus IFN-γ, the effects of protein tyrosine kinase (PTK) inhibitor and protein kinase C (PKC) inhibitors on NO production, iNOS mRNA expression, nuclear factor-κB (NF-κB) binding activity and the promoter activity of iNOS gene containing two NF-κB sites have been examined in a mouse macrophage RAW 264.7 cells. LPS or IFN-γ stimulated NO production, and their effect was enhanced synergistically by mixture of LPS and IFN-γ. The PTK inhibitor such as tyrphostin reduced LPS plus IFN-γ-induced NO production, iNOS mRNA expression and NF-κB binding activity. In contrast, PKC inhibitors such as H-7, Ro-318220 and staurosporine did not show any effect on them. In addition, transfection of RAW 264.7 cells with iNOS promoter linked to a CAT reporter gene revealed that tyrphostin inhibited the iNOS promoter activity through the NF-κB binding site, whereas PKC inhibitors did not. Taken together, these suggest that PTK, but not PKC pathway, is involved in the regulation of the iNOS gene transcription through the NF-κB sites of iNOS promoter in RAW 264.7 macrophages by LPS plus IFN-γ.

Key Words: Nitric oxide synthase, NF-κB binding sites, Protein tyrosine kinase, Protein kinase C, Lipopolysaccharides, Macrophages

INTRODUCTION

NO is a short-lived molecule that mediates a wide range of biologic effects. It acts as an intracellular messenger (Lowenstein & Snyder, 1992; Bredt & Snyder, 1994; Schmidt & Walter, 1994), and plays a role in neurotransmission, antimicrobial defense and vascular homeostasis (Nathan, 1992; Knowles & Moncada, 1994; Nathan, 1997). NO is derived from L-arginine and can be produced by constitutively expressed NOS in cells such as endothelial cells, neurons and cardiac myocytes (Palmer et al, 1987; Garthwaite et al, 1988; Finkel et al, 1992), or iNOS in cells such as macrophages, hepatocytes and vascular smooth muscle cells (Stuehr & Marlelta, 1985; Busse & Mulsch, 1990; Hortelano et al, 1995). Although only recently uncovered as a physiological messenger, NO is increasingly appreciated as a major regulator in the nervous, immune and cardiovascular systems. iNOS is a high-output isoform compared with the two constitutive NOS isoforms; neuronal and endothelial NOS, and expressed from many cell types including macrophages after stimulation with endotoxin, cytokines including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IFN-γ as well as oxidative stress (Xie et al, 1992; Lorsbach et al, 1993; De Vera et al, 1996; Aona et al, 1997; Ogura et al,

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1997; Hur et al, 1999). Cellular NO release after iNOS induction is therefore thought to be important in many pathological conditions such as inflammation and ischemia.

Little is known about the intracellular signaling pathways of iNOS induction by LPS and IFN-γ in macrophages. A number of pathways have recently been implicated including the activation of PKC, particularly PKC-ε, tyrosine phosphorylation and activation of mitogen-activated protein kinase (MAPK) cascades (Paul et al, 1995; Diaz-Guerra et al, 1996; Da Silva et al, 1997; Chen et al, 1998; Cruz et al, 1999; Orlicek et al, 1999; Momose et al, 2000). The murine and human iNOS promoter have revealed the presence of 24 transcription factor binding sites, including NF-κB and AP-1 sites (Lowenstein et al, 1993; Chu et al, 1995). Also analysis of the transcriptional activity of iNOS promoter using deletional mutants has revealed that the two NF-κB binding motifs are essential in the control of iNOS expression (Lowenstein et al, 1993; Xie et al, 1993; Spink et al, 1995). However the functional role of NF-κB sites in the regulation of LPS and IFN-γ-induced transcription of the iNOS gene through PTK or PKC signal pathway has not been established.

The present study was undertaken to determine the effects of inhibitors of PTK or PKC on iNOS gene expression and to characterize the molecular regulation of iNOS in RAW 264.7 macrophages. We observed that PTK, but not PKC, was involved in the signal transduction pathways of iNOS induction, and regulated the iNOS gene transcription through the binding of NF-κB sites of the iNOS promoter.

METHODS

Cells

RAW 264.7, a mouse macrophage cell line, were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) and 10 heat-inactivated FBS (Life Technologies/BRL, Gaithersburg, MD; complete medium), and were maintained at 37°C in a humidified incubator containing 5 CO_2.

Chemicals

Murine IFN-γ was purchased from Genzyme (Cambridge, MA). Poly(dI-dC) · poly(dI-dC) and dNTP were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). 1-Deoxydichloroacetyl-1-[^{14}C]chloramphenicol and [α-^{32}P]dCTP were purchased from Dupont-New England Nuclear Corp (Boston, MA). Restriction enzymes, Klenow fragment of DNA pol. I, Molony murine leukemia virus reverse transcriptase, Rnasin, bovine serum albumin (BSA) and acetyl-CoA were purchased from Boehringer Mannheim (Mannheim, Germany). Sequenase kit was obtained from USB (Cleveland, OH). Anti-murine p65, p50 and c-rel were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bacterial LPS (from *Escherichia coli*, serotype 0127: B8) was obtained from Sigma (St. Louis, MO). Other chemicals for electrophoresis were obtained from Bio-Rad (Richmond, CA).

Assay for nitrite concentration

Accumulated nitrite (NO_2^-) in culture medium was measured using an automated colorimetric assay based on the Griess reaction. Briefly, 100 μl of sample was reacted with the Griess reagent (1 sulfanilamide, 0.1 napthyl-ethylenediamine dihydrochloride/2.5 H_3PO_4) at room temperature for 10 minutes, and then NO_2^- concentration was determined by measuring the absorbance at 540 nm in a microplate reader (Molecular Device, USA). The standard curve was obtained using the known concentration of sodium nitrite. In all experiments, NO_2^- concentration in wells containing only medium was also measured as a blank control.

Reverse transcription and polymerase chain reaction (RT-PCR)

RAW 264.7 cells were lysed with RNAzol B reagent, and the total cellular RNA was purified according to the manufacturer's recommended procedure. Briefly, 5 μg of total RNA was reverse transcribed to the cDNA, and then amplified by PCR. Specific primers for PCR were designed according to the partial sequence of the mouse iNOS gene deposited in Gene Bank (accession no. M87039). The primer sequences for iNOS are as follows: sense, 5'-CAGAAGCAGAATGTGACCATC-3' and antisense. 5'-CTTCTGGTCGATGTCATGAGC-3'. The

sequences of the primers for β-actin are as follows: sense, 5'-GTGGGGCGCCCCAGGCACCA-3' and antisense, 5'-CTCCTTAATGTCACGCACGATTTC-3'. PCR was performed in a 30 μl reaction volume containing 1×Taq polymerase buffer (10 mM Tris, pH 8.3), 200 μM each deoxynucleotide mixture, 1.5 mM $MgCl_2$, 0.5 U Taq polymerase, 0.5 μM each oligonucleotide primer, and 2 μl of RT products. After preincubation for 5 min at 94°C, 30 cycles of amplification (94°C for 2 min, 65°C for 2 min, and 72°C for 2 min) were performed, and β-actin was used for an internal control. Each PCR reaction was analyzed by 1.2 agarose gel electrophoresis in Tris-acetate-EDTA buffer and stained with ethidium bromide.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts for EMSA were prepared from RAW 264.7 cells as previously described (Dignam et al, 1983) with minor modification. For binding reaction, 5 μg of nuclear extract was incubated at room temperature for 20 min with reaction buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, 200 μg/ml BSA, and 2 μg of poly(dI-dC) · poly(dI-dC). Then the ^{32}P-labeled double-stranded oligonucleotide (1 ng, ≥1×10^5 cpm) containing the NF-κB binding consensus sequence (5'-GGCAACTGGGGACTCTCCCTTT-3') was added to the reaction mixture for an additional 10 min at room temperature. The reaction products were fractionated on a nondenaturating 6% polyacrylamide gel, which was then dried and subjected to autoradiography. For competition assays, the excess oligonucleotide (50- to 100-fold molar excess) competitors were preincubated with nuclear extracts for 20 min at room temperature. A mutant NF-κB oligonucleotide used for the competition assay was as follows: 5'-GGCAACTGCTCACTCTCCCTTT-3'; the mutated sequences are underlined.

Generation of plasmid constructs

A 2,056 bp fragment, corresponding to the 5'-flanking region of mouse iNOS gene fused with a promoterless chloramphenicol acetyltransferase (CAT) reporter gene (piNOS 1974), was generously given by Dr. YM Kim & SG Paik (Department of Biology, Chungnam National University, Taejon, Korea). Plasmid piNOS 973 was constructed by deleting the upstream region of the iNOS promoter (−1974 to −974) from the plasmid piNOS 1974 after *Hind*III site was created at position −973 by PCR mutagenesis. By the same method used with piNOS 973, piNOS m973 (mNF-κBu, mNF-κBd) containing the mutated upstream and downstream NF-κB sites was constructed by PCR mutagenesis using oligonucleotide primers in which the GGG motif of the NF-κB sites was substituted by a CTC.

Transient transfection and CAT assay

RAW 264.7 cells were transfected by calcium phosphate-DNA co-precipitation method, as described previously (Gorman, 1986), with 20 μg of plasmid DNA containing the iNOS promoter construct. After 6 h, cells were washed twice with 1×PBS followed by a 2 min shock with 15% glycerol and stimulated with LPS plus IFN-γ. At least 15 h later, the cells were lysed by freezing and thawing. Cell lysate was heated at 65°C for 10 min to inactivate CAT inhibitors. Protein content was determined by Bradford assay (Bradford, 1976) and equal amount of proteins were assayed for CAT enzyme activity by TLC method. As an internal control for transfection efficiency, all cells were cotransfected with 5 μg of pCH110 plasmid (Pharmacia, Piscataway, NJ) for β-galactosidase assay.

RESULTS

Effects of signal blockers on the LPS plus IFN-γ-induced iNOS expression in RAW 264.7 macrophages

It has been reported that LPS alone or the combination of LPS and IFN-γ induced NO production from rat or mouse macrophage cells (Stuehr & Marletta, 1985; Lorsbach et al, 1993; Lowenstein et al, 1993). We also observed the synergic effects by LPS and IFN-γ on NO production (Fig. 1). Since NO production requires the enzymatic activity of NOS, its activity was measured by NO secretion using the method of Griess. NO (nitrite) production was induced by treatment of IFN-γ in a dose-dependent manner in the presence of 1 μg/ml of LPS (Fig. 1A). Alternatively, NO production dramatically increased in the presence of IFN-γ (10 U/ml) reaching maximal NO production by adding 10 μg/ml of LPS (Fig.

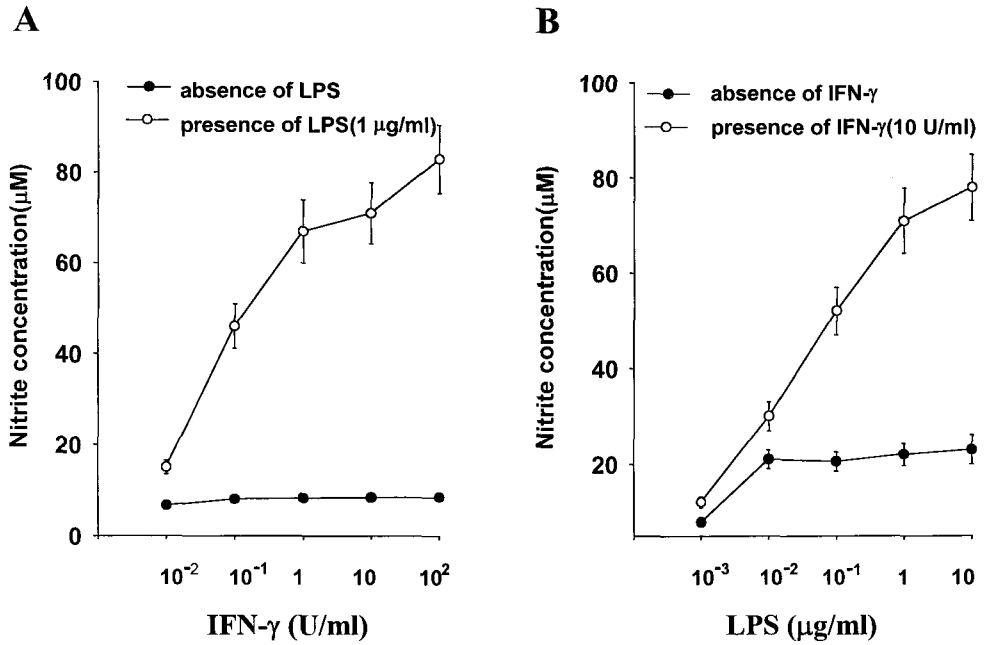

Fig. 1. Synergic effects of LPS and IFN-γ on NO production. (A) RAW 264.7 cells were cultured in the presence of different concentrations of IFN-γ with or without 1 μg/ml of LPS. After incubation for 24 h, the culture supernatants were collected and measured for nitrite concentration by Griess reaction. (B) RAW 264.7 cells were cultured in the presence of different concentrations of LPS with or without 10 U/ml of IFN-γ for 24 h, and the nitrite concentration in the culture supernatants was measured. The data represent the mean$\pm$SE of four different cultures.

1B). Based on these results, it seems that the signals of LPS and IFN-γ are sufficient to induce NO synthesis in RAW 264.7 cells.

Next, to examine the effects of PTK inhibitor (tyrphostin) and PKC inhibitors (H-7, Ro-318220 and staurosporine) on LPS plus IFN-γ-induced NO production, RAW 264.7 cells were treated with LPS plus IFN-γ in the presence or absence of various concentrations of them. The viability of these cells at used concentrations was proven to be more than 95% by the trypan blue dye exclusion method. As shown in Fig. 2, PTK activity was essential for NO production from RAW 264.7 cells. Tyrphostin dramatically inhibited NO production induced by LPS plus IFN-γ in a dose-dependent manner. Another PTK inhibitor, genistein also significantly decreased NO production (data not shown). However, PKC inhibitors (H-7, Ro-318220 and staurosporine) did not have any effects on NO production in the RAW 264.7 cells.

In addition, to verify whether the decrease in LPS plus IFN-γ-induced NO production by PTK inhibitor was due to transcriptional regulation, RT-PCR analysis for iNOS was performed. As expected, treatment of RAW 264.7 cells with LPS plus IFN-γ for 5 h dramatically increased iNOS mRNA expression (Fig. 3). The induced iNOS mRNA expression by LPS plus IFN-γ was almost completely inhibited by tyrphostin, whereas it was not inhibited by H-7, Ro-318220 or staurosporine. These data strongly suggest that PTK activity is essential for iNOS gene expression in RAW 264.7 cells.

Effects of signal blockers on the LPS plus IFN-γ-induced iNOS promoter activity through the NF-κB binding sites

It has been known that NF-κB activation is an essential process for the induction of iNOS gene transcription, and that iNOS promoter has two functional NF-κB binding sites (Lowenstein et al, 1993; Xie et al, 1993; Spink et al, 1995). Thus, we examined the effects of signal blockers on LPS plus IFN-γ-induced NF-κB binding activity by EMSA. As

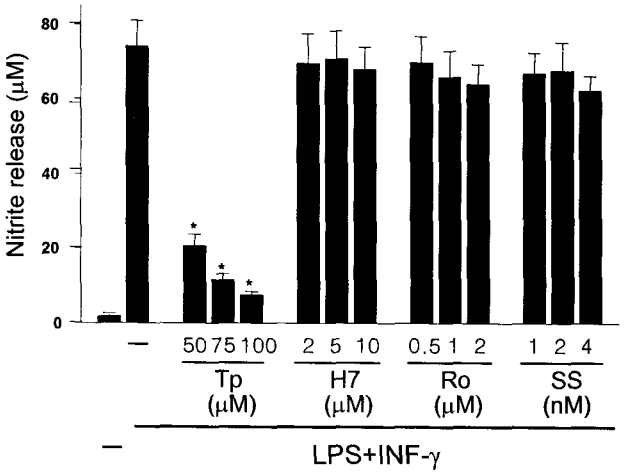

Fig. 2. Effects of signal blockers on LPS plus IFN-γ-induced NO production in RAW 264.7 cells. RAW 264.7 cells were preincubated with indicated concentrations of tyrphostin (TP), H-7 (H7), Ro-318220 (Ro) or staurosporine (SS), and then stimulated with 1 μg/ml of LPS and 10 U/ml of IFN-γ. After 24 h incubation, the concentration of nitrite released into media were measured by the Griess reaction. Each value is shown as mean ± SE of four different cultures. Asterisks indicate significant difference ($P < 0.05$), by Students' *t* test, compared with that of LPS plus IFN-γ stimulated cells.

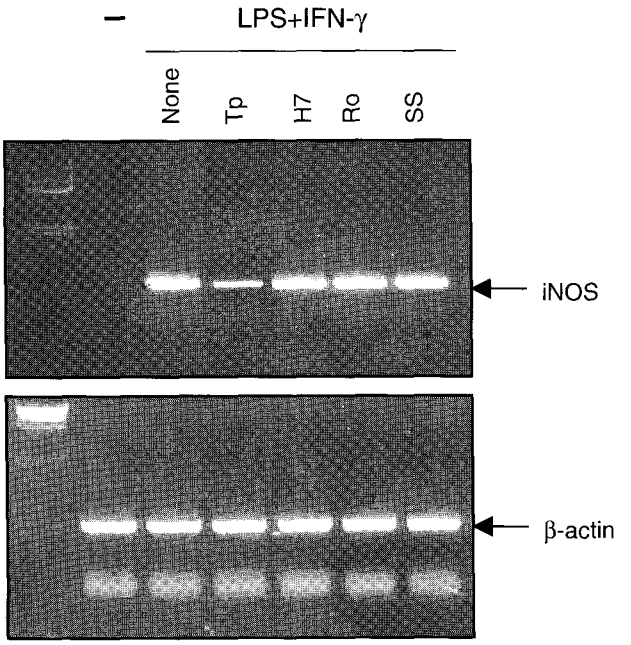

Fig. 3. Effects of signal blockers on LPS plus IFN-γ-induced iNOS mRNA expression. RAW 264.7 cells were pretreated with 100 μM of tyrphostin (TP), 10 μM of H-7 (H7), 2 μM of Ro-318220 (Ro) or 4 nM of staurosporine (SS) for 30 min, and then further cultured with LPS (1 μg/ml) plus IFN-γ (10 U/ml) for 6 h. RT-PCR was performed using iNOS or β-actin specific primers. The PCR products were separated on a 1.2 agarose gel and stained with ethidium bromide. β-actin was used as an internal control.

shown in Fig. 4, incubation of RAW 264.7 cells with LPS plus IFN-γ generated prominent NF-κB complex binding. This NF-κB binding was significantly inhibited by tyrphostin treatment, but not by H-7, Ro-318220 or staurosporine treatment. Addition of a 100-fold excess of unlabeled wild-type probe specifically inhibited the NF-κB binding, while a probe bearing the mutated NF-κB binding sequence did not, indicating the binding specificity of NF-κB complex.

To examine the effects of signal blockers on iNOS promoter activity, RAW 264.7 cells were transiently transfected with a plasmid (piNOS 973) containing the mouse iNOS promoter region (position from −973 to +82) linked to CAT reporter gene by calcium phosphate co-precipitation method. In the unstimulated cells, a little CAT activity was found (Fig. 5), but significantly enhanced CAT expression was found in the stimulated cells by LPS plus IFN-γ. Addition of tyrphostin to LPS plus IFN-γ treated cells resulted in about 50 decrease of CAT activity compared to cells treated only with LPS plus IFN-γ. However, any addition of H-7, Ro-318220 or staurosporine did not affect on LPS plus IFN-γ-induced iNOS promoter activity. In addition, to determine the direct role of the NF-κB binding sites in the iNOS promoter, we constructed mutant plasmid, piNOS m973 that contained two site specific mutations within the upstream and downstream NF-κB sites of piNOS 973 (Fig. 5A). RAW 264.7 cells were transfected with piNOS m973 showed not only a marked reduction in CAT activities compared with cells transfected with the wild type piNOS 973 but also showed no difference in CAT activities by addition of PTK or PKC blockers. These data indicate that tyrosine kinase is critical in LPS plus IFN-γ-induced iNOS gene transcription, and NF-κB binding sites in the iNOS promoter are essential for this process.

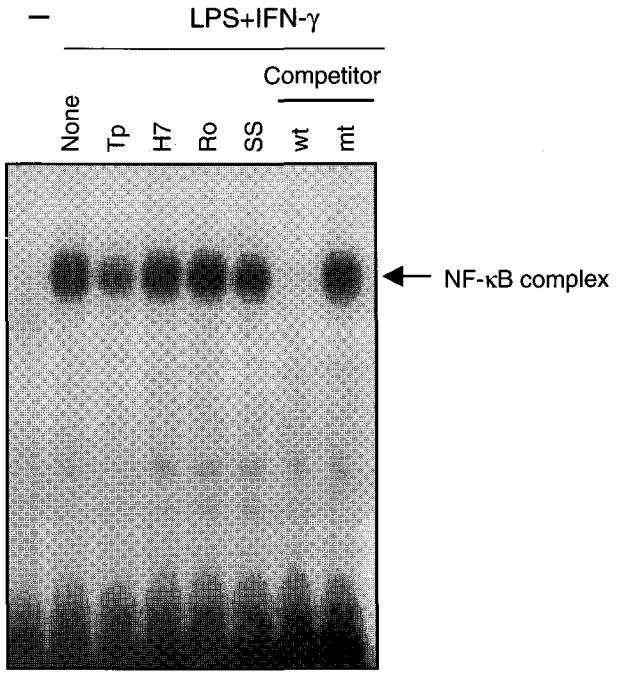

Fig. 4. Effects of signal blockers on LPS plus IFN-γ-induced NF-κB binding activity. RAW 264.7 cells were pretreated with 100 μM of tyrphostin (TP), 10 μM of H-7 (H7), 2 μM of Ro-318220 (Ro) or 4 nM of staurosporine (SS) for 30 min and then activated with LPS (1 μg/ml) plus IFN-γ (10 U/ml). After 5 h incubation, nuclear proteins were prepared and subjected to EMSA for NF-κB binding as described under Materials and Methods. The specificity of bands depict the binding of NF-κB in the nuclear extracts from cells treated with LPS plus IFN-γ for 5 h in the presence of 100-fold molar excess of unlabeled oligomers (wt) or oligomers containing the mutated NF-κB binding site (mt).

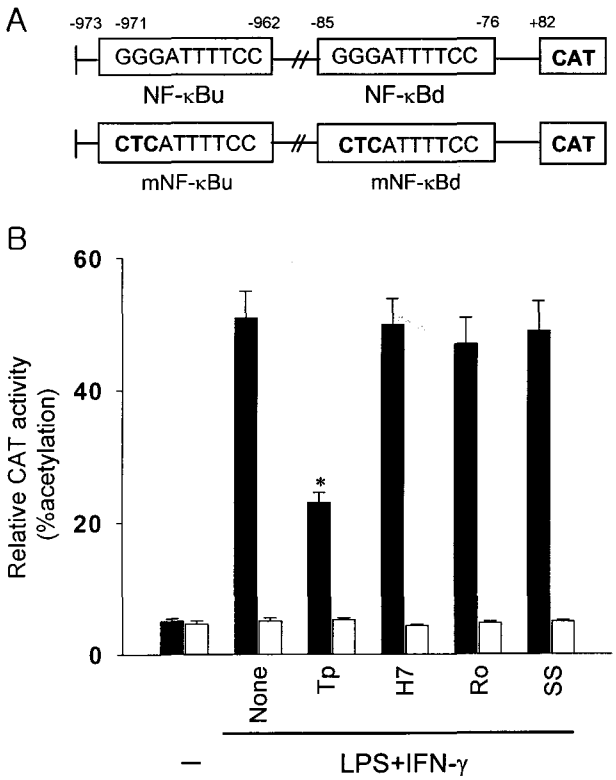

Fig. 5. Effects of signal blockers on the LPS plus IFN-γ-induced iNOS promoter activity via NF-κB site. (A) CAT construct (piNOS m973) containing the mutated upstream and downstream NF-κB sites in the region from -973 to $+82$ of wild type piNOS 973 plasmid. Mutated nucleotide of the upstream and downstream NF-κB sites in CAT constructs are shown as bold characters. (B) The RAW 264.7 cells were transfected with plasmid DNA (piNOS 973, solid bars) containing the 973 bp fragment of iNOS promoter region, or plasmid with two NF-κB sites mutated (piNOS m973, open bars). Thereafter the cells were incubated with 100 μM of tyrphostin (TP), 10 μM of H-7 (H7), 2 μM of Ro-318220 (Ro) or 4 nM of staurosporine (SS) in the presence or absence of LPS (1 μg/ml) plus IFN-γ (10 U/ml) for 15 h, and CAT activity was assayed by TLC. Results were indicated as percent acetylation and the mean $\pm$ SE of four independent experiments. Asterisk indicates significant difference ($P<0.05$), by Students' t test, compared with that of LPS plus IFN-γ stimulated cells.

DISCUSSION

NO, the smallest known biologic mediator produced by mammalian cells, is involved in a diverse array of activities, including vasodilatation, neurotransmission and antimicrobial functions (Nathan, 1992; Knowles & Moncada, 1994; Nathan, 1997). Because iNOS can produce a large amount of NO, its induction plays an influential role in cell death, tissue damage, and inflammation (Nathan Xie, 1994; Kuo et al, 1996). Maximal induction of iNOS depends on synergistically combined stimuli; the most effective stimuli vary with cell type. These stimulatory compounds include TNF-α, IL-1β, IFN-γ and LPS (Xie et al, 1992; Lorsbach et al, 1993; De Vera et al, 1996; Aona et al, 1997; Ogura et al, 1997). We also showed that LPS induced NO production and iNOS mRNA expression in a concentration dependent manner in RAW 264.7 macrophages (Fig. 1, Fig. 2). This response was potentiated by the presence of IFN-γ.

Characterization of signal transduction pathways involved in iNOS expression is important to under-

stand the regulation of NO production. It has been reported that PKC, phospholipase A_2, MAPK and PTKs are involved in iNOS expression (Paul et al, 1995; Diaz-Guerra et al, 1996; Da Silva et al, 1997; Chen et al, 1998; Cruz et al, 1999; Orlicek et al, 1999; Momose et al, 2000). In these experiments, we demonstrated that PTK inhibitors such as tyrphostin and genistein significantly inhibit NO production and iNOS mRNA expression (Fig. 2, Fig. 3). Our results appear to be consistent with those of most studies in which the PTK pathway was involved in the regulation of iNOS expression for example: studies involving macrophages (Orlicek et al, 1999; Knapp English, 2000), dendritic cells (Cruz et al, 1999) and microglial cells (Lockhart et al, 1998). However, PKC inhibitors such as H-7, Ro-318220 and staurosporine did not show any effects on them. In many cells, stimulation of the PKC pathway has little or no effect on iNOS induction by itself, but potentiates cytokine induction (Simmons and Murphy, 1994; Kleinert et al, 1996). The apparent discrepancies between the several results including ours suggest that the pathway of iNOS induction may be differentially modulated by PKC in a cell- or tissue-specific manner. Paul & co-workers (1995) showed that PKC inhibitors suppressed cytokine induced NO production as well as iNOS enzyme activity in RAW 264.7 cells. Although the concentrations of PKC blockers (5 to 10 μM of Ro-318220) used in their experiment showed strong inhibition of NO production and iNOS enzyme activity, they caused severe cytotoxic effect in our study as evaluated by trypan blue dye exclusion or MTT assay.

Functional analysis of the 5'-flanking region of murine iNOS promoter revealed that the region between −48 and −209 bp containing NF-κB and NF/IL-6 site is critical for inducibility by LPS, whereas the region between −1029 and −913 bp containing NF-κB, IRF-1 and ISRE/GAS sites mediate IFN-γ-potentiation of LPS induction in mouse macrophages (Lowenstein et al, 1993; Xie et al, 1993). Two essential *cis*-acting elements, a promoter proximal region (position −85 to −76, downstream NF-κB site) and a more distal region (position −971 to −962, upstream NF-κB site), are known to mediate transcriptional induction of iNOS. Thus, the analysis of NF-κB activation is important to understand the mechanisms that control iNOS expression by signal blockers. In this experiment, tyrphostin significantly suppressed LPS plus IFN-γ-induced NF-κB activation, but PKC inhibitors did not (Fig. 4). Although the results of current study including ours demonstrate that the PTK inhibitors suppressed NF-κB activation induced by LPS plus IFN-γ in macrophage cells, the effects of PTK or PKC blockers on iNOS promoter activity have not been shown. By measuring iNOS promoter activity transfected in RAW 264.7 cells with piNOS 973 containing two NF-κB sites, we found that PTK blocker dramatically inhibited iNOS promoter activity induced by LPS plus IFN-γ, whereas no significant differences were observed in cells treated with PKC blockers (Fig. 5). The specific role of both NF-κB sites on the regulation of iNOS expression by PTK blocker was in an agreement with the absence of the effect of PTK blocker upon the iNOS promoter with mutated NF-κB sites (piNOS m973) after transient transfection.

These results support the conclusion that iNOS expression by LPS plus IFN-γ in RAW 264.7 macrophages is dependent on PTK but not PKC, and the PTK blocker inhibited iNOS gene transcription through the NF-κB sites in iNOS promoter.

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