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Triptolide inhibits murine-inducible nitric oxide synthase expression by down-regulating lipopolysaccharide-induced activity of nuclear factor-κB and c-Jun NH_2-terminal kinase

Young-Ho Kim[a,b], Sang-Han Lee[c], Jai-Youl Lee[b], Sang-Won Choi[d], Jong-Wook Park[a], Taeg Kyu Kwon[a,*]

[a] *Department of Immunology, School of Medicine, Keimyung University, 194 DongSan-Dong Jung-Gu, Taegu 700-712, South Korea*
[b] *Department of Microbiology, College of Natural Sciences, Kyungpook National University, Taegu, South Korea*
[c] *Korea Research Institute of Bioscience and Biotechnology, Yusong, Taejon 305-333, South Korea*
[d] *Department of Food Science and Nutrition, School of Home Economics, Catholic University of Daegu, South Korea*

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Abstract

Triptolide (PG490) is a natural, biologically active compound extracted from the Chinese herb *Tripterygium wilfordii*. It has been shown to possess potent anti-inflammatory and immunosuppressive properties. In Raw 264.7 cells stimulated with lipopolysaccharide (LPS) to mimic inflammation, triptolide inhibits nitric oxide (NO) production in a dose-dependent manner and abrogates inducible nitric oxide synthase (iNOS) gene expression. To investigate the mechanism by which triptolide inhibits murine iNOS gene expression, we examined activation of mitogen-activated protein kinases (MAP kinases) and nuclear factor-κB (NF-κB) in these cells. Addition of triptolide inhibited phosphorylation of c-Jun NH_2-terminal kinase (JNK) but not that of extracellular signal-regulated kinase (ERK) or p38 mitogen-activated protein kinase. In addition, triptolide significantly inhibited the DNA binding activity of NF-κB. Taken together, these results suggest that triptolide acts to inhibit inflammation through inhibition of NO production and iNOS expression through blockade of NF-κB and JNK activation.

Keywords: Triptolide; NO (Nitric oxide); iNOS; NF-κB; JNK

1. Introduction

The intercellular messenger nitric oxide (NO) is a short-lived free radical that participates in the physiology and pathophysiology of many systems (Moncada et al., 1991). NO is synthesized from L-arginine by nitric oxide synthase (NOS) with NADPH and oxygen as co-substrates (MacMicking et al., 1997). Three isoforms of NOS have been identified and are classified into two major categories: constitutive and inducible NOS. Constitutive NOS is found in the endothelia of blood vessels and brain neurons, and is Ca^{2+}/calmodulin-dependent. Inducible nitric oxide synthase (iNOS), which is normally not present in resting cells, is expressed in several pathophysiological conditions, and it produces large amounts of NO in response to inflammatory signals, such as cytokines and lipopolysaccharide (LPS) (Alderton et al., 2001; Liu and Hotchkiss, 1995; MacMicking et al., 1997; Moncada et al., 1991).

Expression of the iNOS gene in macrophages is regulated mainly at the transcriptional level, particularly by nuclear factor (NF-κB), activator protein-1 (AP-1), interferon regulatory factor 1 (IRF1) and signal transducer and activator of transcription 1 (STAT1) (Marks-Konczalik et al., 1998; Martin et al., 1994; Ohmori and Hamilton, 2001; Taylor et al., 1998; Xie et al., 1994). In particular, the NF-κB sites are essential for LPS-mediated (i.e. inflammatory) NO production (Xie et al., 1994). In the cytosol, NF-κB is constitutively present as homo- or heterodimers and is linked to inhibitory IκB proteins. Activation of NF-κB results in phosphorylation, ubiquitination, and proteasome-mediated degradation of the IκB proteins, followed by translocation of NF-κB to the nucleus and induction of gene transcription

* Corresponding author. Tel.: +82-53-250-7846; fax: +82-53-250-7074.
E-mail address: kwontk@dsmc.or.kr (T.K. Kwon).

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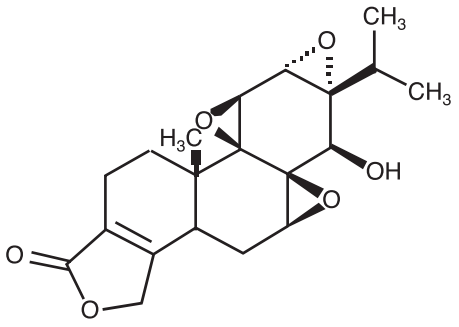

Fig. 1. Chemical structure of triptolide.

through its binding to the cis-acting NF-κB element (Baeuerle and Baltimore, 1996; Tak and Firestein, 2001).

It is noteworthy that the use of medicinal plants or their crude extracts in the prevention and/or treatment of several chronic diseases has been traditionally practiced in different ethnic societies worldwide. Triptolide (PG-490) is a diterpene triepoxide derived from the Chinese herb, *Tripterygium wilfordii* Hook. (Fig. 1) (Jiang, 1994; Kupchan et al., 1972; Qin et al., 1983; Tao et al., 1989). The herb *T. wilfordii* Hook. F (TWHF) has been used in traditional Chinese medicine for centuries. Extracts of *T. wilfordii* Hook. F have been shown to have positive effects on autoimmune diseases such as rheumatoid arthritis, nephritis, and systemic lupus erythematosus (Jiang, 1994; Qin et al., 1983; Tao et al., 1989). A primary component of most functional extracts of *T. wilfordii* Hook. F is triptolide (Kupchan et al., 1972). Recent studies have shown that triptolide inhibits mitogen-induced lymphocyte proliferation and shows immunosuppressive effects in skin allograft transplantation (Pu and Zhang, 1990; Yang et al., 1998). In addition, triptolide possesses anti-proliferative activities against L1210 and P388 mouse leukemia cells and suppresses colony formation in breast cancer cell lines (Kupchan et al., 1972; Wei and Adachi, 1991). However, the cellular and molecular mechanisms underlying triptolide-induced inhibition of NO production in macrophages are not clear.

In this study of triptolide anti-inflammatory properties, triptolide inhibited LPS-induced NO production and iNOS expression in Raw 264.7 cells, apparently through abrogating JNK activation and blockading NF-κB binding to the iNOS promoter, thereby inhibiting iNOS induction.

2. Materials and methods

2.1. Cells and materials

All reagents were purchased from Sigma-Aldrich unless otherwise stated. Triptolide was dissolved in dimethyl sulfoxide and freshly diluted in culture media for all in vitro experiments. The macrophage cell line Raw 264.7 was obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. The cells were subcultured twice weekly and grown on six-well plates at 1×10^6 cells/well, at 37 °C in fully humidified 5% CO_2 air. For experiments, cells were stimulated for 1–24 h in the presence of triptolide with or without inhibitors. Anti-iNOS was purchased from Santa Cruz Biotechnology. Anti-phospho-ERK, anti-phospho-JNK, and anti-phospho-p38 MAPK were purchased from New England Biolabs. The inhibitors RO31-8220 ({3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide; bisindolymaleimide IX, methanesulfonate]}, SB203580 ([4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidzole]), SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one), PD098059 (2′-amino-3′-methoxyflavone), PDTC (pyrrolidinedithiocarbamate) and LY294002 ([2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one]) were purchased from Biomol.

2.2. Western blotting

Cellular lysates were prepared by suspending 1.5×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS (4-morpholinepropanesulfonic acid), 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2), disrupted by sonication and extracted at 4 °C for 30 min. The proteins were electrotransferred to Immobilon-P membranes and detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

2.3. Nitrite quantification

NO_2^- accumulation in the medium was used as an indicator of NO production as previously described (Green et al., 1982). Raw 264.7 cells were plated at 1.5×10^6 cells/ml, and stimulated with LPS in the presence or absence of triptolide for 24 h. The isolated supernatants were mixed with an equal volume of Greiss reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring optical density at 550 nm.

2.4. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated according to Chomczynski and Sacchi (1987). Single-strand cDNA was synthesized from 2 μg of total RNA using M-MLV (Moloney–Murine leukemia virus) reverse transcriptase. The cDNA for iNOS and actin were PCR-amplified using the following specific primers: iNOS (sense) 5′-ATG GCT TGC CCC TGG AAG TTT CTC-3′ and (antisense) 5′-CCT CTG ATG

GTG CCA TCG GGC ATC TG-3′. PCR amplification was carried out as follows: 1 × (94 °C, 3 min); 30 × (94 °C, 45 s; 59 °C, 45 s; and 72 °C, 1 min); and 1 × (72 °C, 10 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

2.5. DNA transfection and luciferase assay

NF-κB reporter constructs were purchased from Clontech. The murine iNOS promoter-containing plasmid was a kind gift from Dr. C.C. Reddy's laboratory (Pennsylvania State University). NF-κB reporter plasmids or iNOS promoter plasmids were transfected into Raw 264.7 cells using the Lipofectamine reagent according to the manufacturer's instructions. To assess iNOS promoter driving of the luciferase gene, cells were collected and disrupted by sonication in lysis buffer (25 mM tris-phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were analyzed with the luciferase assay according to the manufacturer's instructions.

2.6. Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts from control or drug-treated cells was carried out as described previously (Baek et al., 2002). The sequences of the double-stranded oligonucleotides used to detect the DNA-binding activities of NF-κB, AP-1, IRF1 and STAT1 are as follows: NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; AP-1, 5′-CGC TTG ATG ACT CAG CCG GAA-3′; IRF1, 5′-GGA AGC GAA AAT GAA ATT GAC T-3′; and STAT1, 5′-CAT GTT ATG CAT ATT CCT GTA AGT G-3′. The reaction mixture for EMSA contained 20 mM Tris–HCl, pH 7.6, 1 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40 (NP-40), 1 μg poly (dI–dC) and 5 μg nuclear proteins. Unlabeled wild-type oligonucleotide was added into the reaction mixture and incubated for 10 min at room temperature. [^{32}P] labeled probe DNA (300,000 cpm) was added, and the binding reaction was allowed to proceed for another 20 min. Mixtures were resolved on 8% polyacrylamide gels at 150 V for 4 h. Gels were dried and subjected to autoradiography.

3. Results

3.1. Triptolide inhibition of LPS-induced NO, iNOS mRNA and protein expression in Raw 264.7 cells

Unstimulated Raw 264.7 cells do not contain iNOS, whereas addition of 5 ng/ml LPS induced iNOS synthesis in these cells (Fig. 2A). To elucidate the mechanisms by which LPS induced nitrite production, we examined the effects of LPS on iNOS mRNA and protein levels and found that LPS induced iNOS mRNA and protein accumulation in

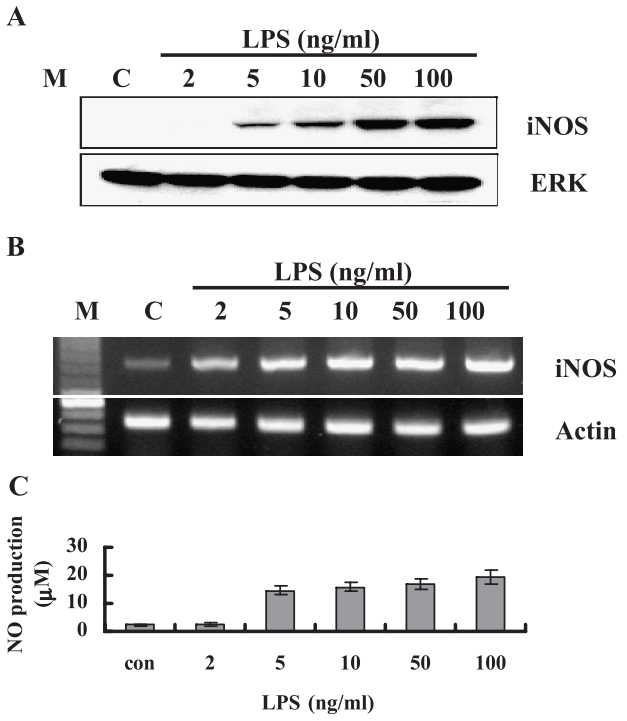

Fig. 2. LPS stimulates iNOS expression and nitrite production in Raw 264.7 cells. Raw 264.7 cells were treated with indicated concentrations of LPS for 24 h. The cells were lysed, and the lysates were analyzed by immunoblotting using anti-iNOS. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading (A). Cells were incubated for 20 h with the indicated concentrations of LPS, and RT-PCR analysis was performed (B). A representative result is shown; two additional experiments yielded similar results. Raw 264.7 cells were treated with the indicated concentrations of LPS for 24 h. Nitric oxide production was measured according to the Greiss method. iNOS protein and mRNA levels are representative of three independent experiments. The values for nitrite are averages ± S.D. from three independent experiments (C).

a dose-dependent manner (Fig. 2A and B). Unstimulated cells contained 2.3 ± 0.3 μM of NO, while cells stimulated with 50 ng/ml LPS contained 16.2 ± 1.1 μM of NO (from three independent experiments; Fig. 2C).

To investigate whether triptolide can inhibit LPS-induced NO and iNOS expression, Raw 264.7 cells were pretreated for 30 min with various concentrations of triptolide and subsequently treated with 50 ng/ml LPS. As shown in Fig. 3A, in cells untreated with triptolide, iNOS protein expression was barely detectable in unstimulated cells, but markedly increased 24 h after 50 ng/ml LPS treatment. Cells pretreated with triptolide showed a dose-dependent inhibition of iNOS protein expression following LPS stimulation. To assess the effect of triptolide on iNOS mRNA expression, we measured mRNA levels using RT-PCR. The expression of iNOS mRNA was hardly detectable in unstimulated cells. Raw 264.7 cells that were not treated with triptolide expressed high level of iNOS mRNA when stimulated with 50 ng/ml LPS for 18 h. Addition of triptolide inhibited this LPS-stimulated iNOS mRNA production in a dose-dependent manner (Fig. 3B). In untreated

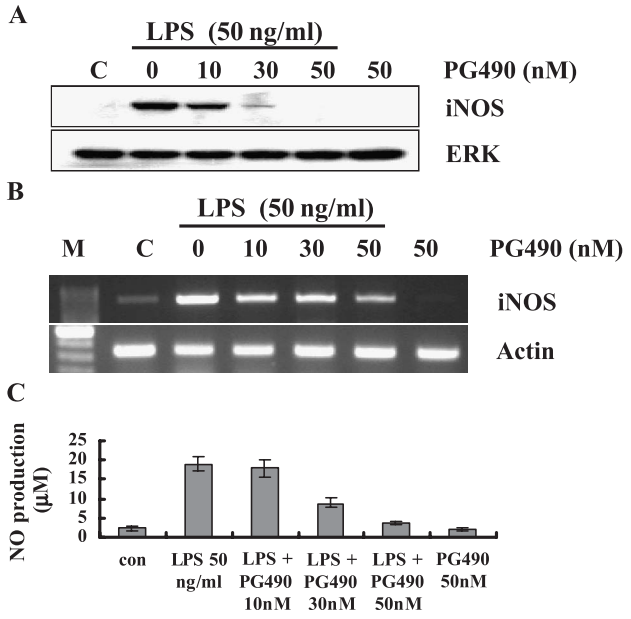

Fig. 3. Inhibition of nitrite production by triptolide in LPS-stimulated Raw 264.7 cells. Raw 264.7 cells were pretreated with the indicated concentrations of triptolide (PG490) for 1 h before incubation with LPS (50 ng/ml) for 24 h. The cells were lysed, and the lysates were analyzed by immunoblotting using anti-iNOS. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading (A). Total RNA was extracted and RT-PCR analysis was performed using probes specific for murine iNOS. A representative result is shown; two additional experiments yielded similar results (B). Raw 264.7 cells were pretreated with the indicated concentrations of triptolide (PG490) for 1 h before incubation with LPS (50 ng/ml) for 24 h. The culture supernatants were subsequently isolated and analyzed for nitrite levels. iNOS protein and mRNA levels are representative of three independent experiments. The values for nitrite are averages ± S.D. from three independent experiments (C).

Raw 264.7 cells, 50 ng/ml LPS evoked a 10-fold induction of nitrite production versus the unstimulated control; this induction was inhibited by triptolide treatment in a dose-dependent manner (Fig. 3C). The concentration and duration of triptolide treatment used in these studies had no significant effect on the viability of Raw 264.7 cells.

3.2. Triptolide is an inhibitor of LPS-induced phosphorylation of JNK in Raw 264.7 cells

To investigate whether the ERK, JNK, and p38 MAPK (mitogen-activated protein kinase) pathways are involved in LPS stimulation of Raw 264.7 cells, we examined the activation of the three MAPKs by detecting their dually phosphorylated forms in Western blots probed with specific anti-phosphokinase antibodies (Fig. 4A). LPS treatment induced a strong transient increase in phosphorylated JNK levels, which peaked at 30 min and declined thereafter. p38 MAPK was activated after 10 min and reached maximal activity at 30 min, and ERK was significantly activated (Fig. 4A). Overall, LPS treatment of macrophages stimulated these three MAPKs with similar kinetics. To investigate whether the inhibition of iNOS by triptolide is mediated through modulation of these pathways, we used Western blotting to examine JNK, ERK, and p38 MAPK phosphorylation following LPS stimulation of triptolide-pretreated Raw 264.7 cells. Treatment with triptolide significantly inhibited JNK phosphorylation in a dose-dependent manner (Fig. 4B), while ERK and p38 MAPK phosphorylation was unaffected (Fig. 4B).

To determine whether JAK/STAT pathway in LPS-mediated iNOS induction, Raw 264.7 cells were stimulated with LPS alone or LPS plus various concentrations of triptolide. The specific involvement of the JAK/STAT pathway in iNOS induction was tested using a specific anti-phospho-JAK2 antibodies and a specific inhibitor of the JAK2 tyrosine phosphorylation, the tyrphostin B42 (AG-490) (Meydan et al., 1996). As shown in Fig. 4C, treatment with LPS and triptolide in Raw 264.7 cells did not significantly alter the expression and phosphorylation levels of JAK2. We also tested the effects of AG490 on expression levels of iNOS in LPS-stimulated cells. However, we did not observe significant inhibition of iNOS in LPS and 100 μM AG490 treated Raw 264.7 cells (Fig. 4D). These results suggest that LPS signaling does not directly involve the JAK/STAT pathway in iNOS gene expression.

To more closely assess the relative roles played by MAPK and other signal regulatory proteins in LPS-mediated iNOS induction, we examined whether selective MAPK inhibitors could affect LPS-stimulated iNOS expression, iNOS mRNA and nitrite production. SP600125 (a JNK inhibitor) and PDTC (a NF-κB inhibitor) profoundly inhibited LPS-mediated iNOS induction and nitrite production. However, treatment with PD98059 (a mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor) only slightly decreased iNOS induction and nitrite production, while treatment with RO31-8220 (a protein kinase C inhibitor), SB203580 (a p38 MAP kinase inhibitor) and LY294002 (a phosphatidylinositol 3 kinase inhibitor) did not affect the LPS-stimulated iNOS induction and nitrite production (Fig. 5).

3.3. Role of JNK and NF-κB in LPS-mediated iNOS induction

To investigate whether JNK and NF-κB are involved in the LPS-stimulated induction of iNOS in macrophages, we investigated whether addition of their inhibitors could affect LPS-stimulated iNOS induction and nitrite production. Pretreatment of cells with SP600125 and PDTC inhibited LPS-stimulated iNOS induction, iNOS mRNA expression, and nitrite production in a dose-dependent manner (Fig. 6A and B).

3.4. Inhibition of NF-κB binding activity by triptolide in LPS-stimulated Raw 264.7 cells

NF-κB is known to be important in the induction of iNOS by LPS (Xie et al., 1994). To determine whether NF-

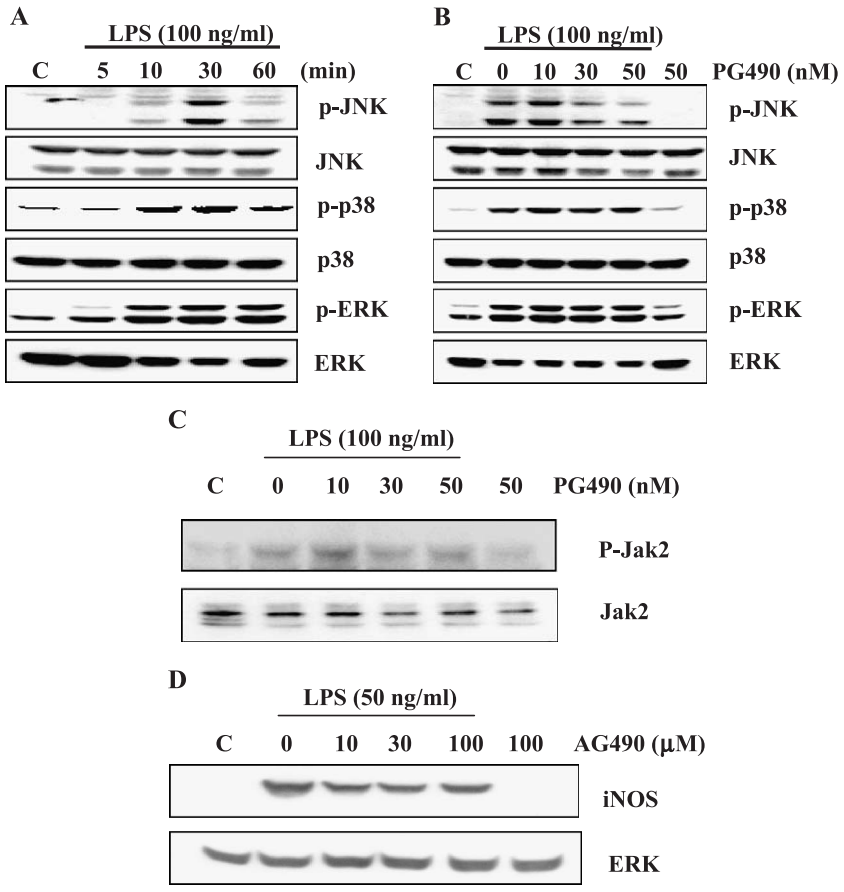

Fig. 4. Effect of triptolide on LPS-induced phosphorylation of MAPKs in Raw 264.7 cells. Raw 264.7 cells were stimulated with 100 ng/ml LPS and harvested at the indicated time points. A representative result is shown; two additional experiments yielded similar results (A). Raw 264.7 cells were treated with the indicated concentrations of triptolide (PG490) before incubation with 100 ng/ml LPS for 30 min. The whole-cell lysates were analyzed by immunoblot analysis using various antibodies against the activated MAPKs (dually phosphorylated on Tyr/Thr). To ascertain that the total level of each MAPK did not change, blots were stripped and reprobed with the antibodies raised against the corresponding phosphorylation-independent MAPK. The results presented are representative of three independent experiments (B). Raw 264.7 cells were treated with the indicated concentrations of triptolide (PG490) before incubation with 100 ng/ml LPS for 30 min. The whole-cell lysates were analyzed by immunoblot analysis using specific phospho-JAK2 antibodies against the activated JAK. To ascertain that the total level of JAK2 did not change, blots were stripped and reprobed with the antibodies raised against the corresponding phosphorylation-independent JAK2. The results presented are representative of three independent experiments (C). Raw 264.7 cells were pretreated with the indicated concentrations of AG490 for 1 h before incubation with LPS (50 ng/ml) for 24 h. The cells were lysed, and the lysates were analyzed by immunoblotting using anti-iNOS. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading (D).

κB is an important target for triptolide in Raw 264.7 cells, we performed an electrophoretic mobility shift assay. Treatment of Raw 264.7 cells with 100 ng/ml LPS caused a significant increase in the DNA binding activity of NF-κB within 60 min. To confirm that increasing bands were indeed NFκB-specific DNA–protein complexes, we tested binding of wild-type oligonucleotides against that of a mutant oligonucleotide lacking the NF-κB site. The wild-type competitor inhibited LPS-induced NF-κB binding activity, whereas the mutant type competitor did not (Fig. 7A), showing that the shift corresponded to NF-κB-specific DNA–protein complexes. In the presence of 30 nM triptolide, LPS-induced NF-κB binding was markedly suppressed (Fig. 7B). Interestingly, the expression of iNOS gene is regulated other transcriptional factors such as AP-1, IRF1 and STAT1 (Marks-Konczalik et al., 1998; Martin et al., 1994; Ohmori and Hamilton, 2001). To determine whether triptolide inhibits activation of AP-1, IRF1 and STAT1 through the inhibition of DNA binding of AP-1, IRF1 and STAT1, we examined the effect of triptolide on LPS-induced binding of AP-1, IRF1 and STAT1 by EMSA. Triptolide did not affect the intensity of the AP-1, IRF1 and STAT1 DNA complex induced by LPS or its migration in Raw 264.7 cells (negative data not shown).

To gain further insight into the mechanism of triptolide-mediated regulation of NF-κB, we examined the effects of inhibitions of triptolide on IκB phosphorylation and degradation. As shown in Fig. 7C, LPS treatment of Raw 264.7 cells results in a stimulation of phosphorylation of IκB and degradation of IκB. Exposure of cells to various concentrations of triptolide for 1 h prior to LPS stimulation did not significantly alter IκB phosphorylation and degradation. To determine the effect of triptolide on LPS-stimulated NF-κB-dependent reporter gene expression, we used a pNF-κB-Luc

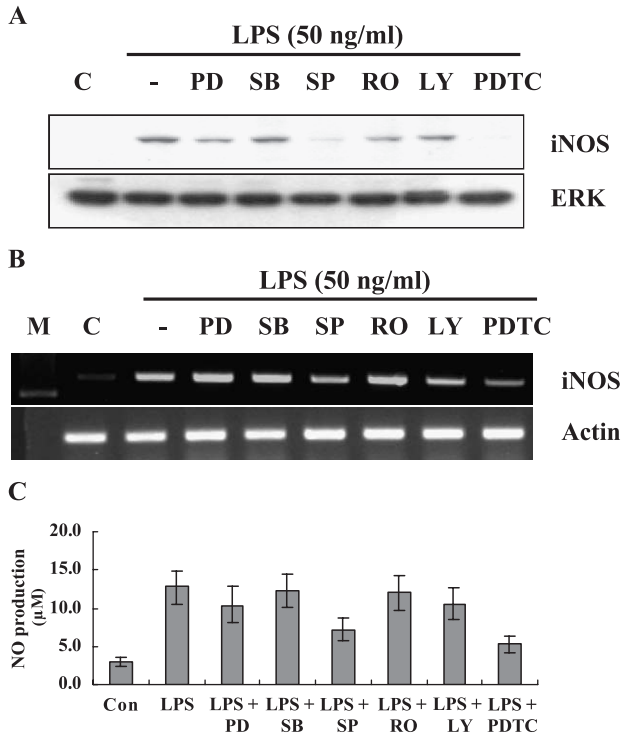

Fig. 5. Effect of various signal pathway inhibitors on LPS-induced nitrite production and iNOS mRNA and protein levels. Raw 264.7 cells were pretreated with PD98059 (PD; 50 μM), SB203580 (SB; 10 μM), SP600125 (SP; 20 μM), RO31-8220 (RO; 2 μM), LY294002 (LY; 25 μM), for PDTC (100 μM) for 30 min followed by stimulation with 50 ng/ml LPS for 24 h. iNOS expression was determined by Western blot analysis. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading (A). Total RNA was isolated and mRNA accumulation was determined by RT-PCR. A representative result is shown; two additional experiments yielded similar results (B). Nitrite production was determined in the culture supernatant. The iNOS protein and mRNA data are representative of two independent experiments. The values for nitrite are averages ± S.D. from three independent experiments (C).

plasmid, which was generated by inserting four spaced NF-κB binding sites into the pLuc-Promoter vector. Raw 264.7 cells were transiently transfected with the pNF-κB-Luc plasmid and then stimulated with 50 ng/ml LPS either in the presence or absence of triptolide. Triptolide treatment significantly reduced the LPS-induced increase in NF-κB-dependent luciferase enzyme expression (Fig. 7D). To further investigate the effect of triptolide on iNOS promoter transcriptional activity, Raw 264.7 cells were transiently transfected with a plasmid containing the iNOS promoter, which contains several potential transcriptional factor binding sites, including one for NF-κB, within −1589 bases upstream of the transcription start site (Prabhu et al., 2002). Triptolide treatment significantly decreased the activity of the iNOS promoter in LPS-stimulated cells (Fig. 7E).

4. Discussion

Triptolide is known to have anti-inflammatory and immunosuppressive effects. Here, we demonstrate that triptolide inhibits NO production and iNOS gene expression in LPS-stimulated cultured macrophages, and that these effects are mediated through the inhibition of JNK and the transcription factor NF-κB. Macrophage-derived NO is an important host defense and microbial and tumor cell killing agent, as well as a regulator of proinflammatory genes in vivo (MacMicking et al., 1997). Indeed, it is possible that modulation of iNOS expression could potentially control chronic and acute inflammatory diseases (Guo et al., 2000; Nathan, 1997; Vodovotz et al., 1996), so it is important that we understand the inhibitory effect of triptolide on iNOS gene expression.

The MAP kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular

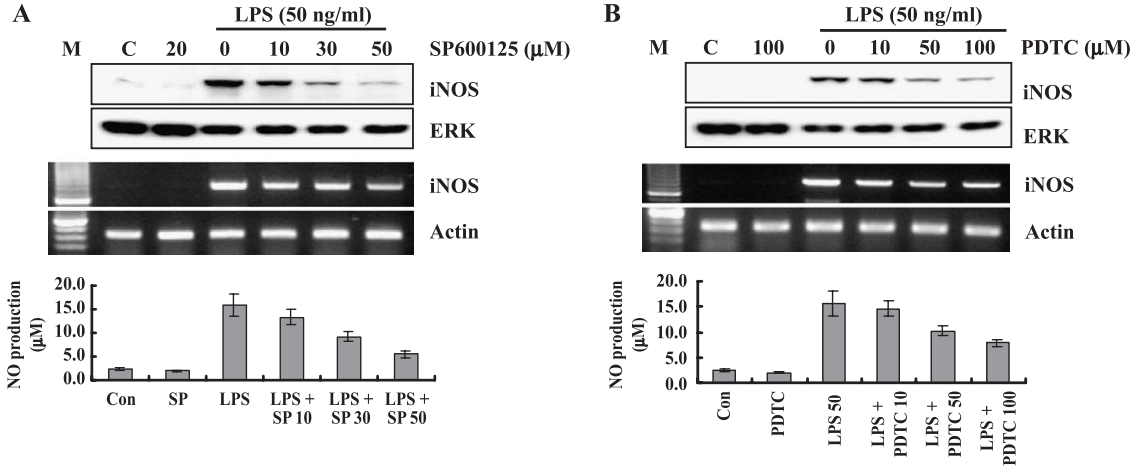

Fig. 6. Effect of SP600125 and PDTC on LPS-induced nitrite production, iNOS mRNA and iNOS protein. Raw 264.7 cells were pretreated with indicated concentrations of SP600125 (A) and PDTC (B) for 30 min followed by stimulation with 50 ng/ml LPS for 24 h. iNOS expression was determined by Western blot analysis. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading. Total RNA was isolated and mRNA accumulation was determined by RT-PCR. Nitrite production was determined in the culture supernatant. The values for nitrite are averages ± S.D. from three independent experiments. The iNOS protein and mRNA data presented are representative of three independent experiments.

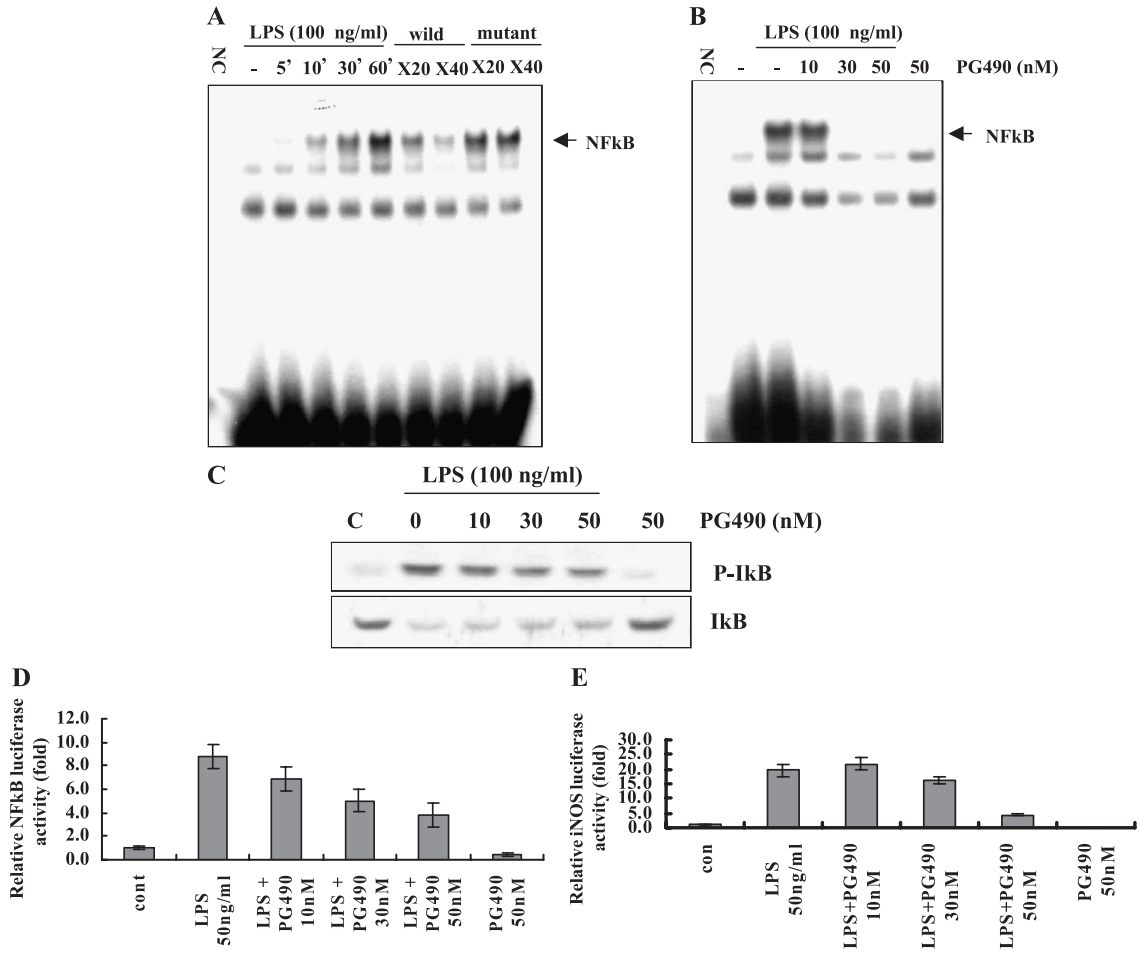

Fig. 7. Effect of triptolide on NF-κB DNA binding activity and iNOS promoter activity in LPS-stimulated cells. Raw 264.7 cells were stimulated with 100 ng/ml LPS and harvested at the indicated time points, and nuclear proteins were extracted. EMSA analysis of the nuclear extracts was conducted using a [^{32}P]-labeled NF-κB oligonucleotide probe. Binding specificity was determined using the unlabeled wild-type probe or mutant-type containing the NF-κB binding sequence (20- or 40-fold in excess) to compete with the labeled oligonucleotide (A). Raw 264.7 cells were pretreated with the indicated concentrations of triptolide (PG490) for 30 min before incubation with 100 ng/ml LPS for 30 min. NF-κB binding was determined as described (B). Raw 264.7 cells were pretreated with indicated concentrations of triptolide (PG490) for 1 h followed by stimulated with 100 ng/ml LPS. IκB and IκB Ser-32 phosphorylation were determined by Western blot analysis (C). Inhibition of NF-κB-dependent reporter gene expression by triptolide (PG490) in LPS-stimulated Raw 264.7 cells. Cells were transiently transfected with a pNF-κB-Luc plasmid containing four copies of the NF-κB binding site, treated with the indicated concentrations of triptolide (PG490) and 50 ng/ml LPS for 24 h. The cells were lysed and luciferase activity was measured. Data represent the mean ± S.D. of at least three independent experiments (D). To elucidate the effects of triptolide (PG490) on iNOS promoter activities, a murine iNOS promoter plasmid was transfected. Raw 264.7 cells were treated with or without varying concentrations of triptolide (PG490) in presence of 50 ng/ml LPS. Luciferase activity was measured. Data represent the mean ± S.D. of at least three independent experiments (E).

responses to cytokines and stresses (Johnson and Lapadat, 2002). Moreover, MAP kinases are involved in the signaling pathway for LPS-induced iNOS expression (Caivano, 1998). In agreement with the report of Ajizian et al. (1999), we found that reduction of LPS-induced iNOS expression following addition of a MEK inhibitor indicated that the ERK signal pathway is involved in LPS-induced iNOS expression in Raw 264.7 cells. Also, our study clearly demonstrated the participation of JNK in LPS-induced NO production in these cells. Recently, it was found in the yeast two-hybrid system that JNK may associate with the c-rel subunit of NF-κB and directly enhance NF-κB activation in an overexpression system (Meyer et al., 1996). We also investigated the effects of triptolide on the LPS-induced phosphorylation of MAP kinases in Raw 264.7 cells. Treatment with triptolide caused a significant inhibition of JNK phosphorylation, but not that of ERK or p38 MAPK, suggesting that JNK is involved in the inhibitory effect of triptolide on LPS-stimulated NF-κB binding in Raw 264.7 cells.

The promoter region of the murine iNOS gene contains two transcriptional regulatory regions, an enhancer and a basal promoter (Lowenstein et al., 1993; Xie et al., 1993). A proximal region at −48 to −209 functions as the basal promoter. It contains an octamer element and an NF-κB binding site, which mediates responsiveness to LPS. The distal region (−913 to −1029) functions as an enhancer element and responds to LPS and interferon-γ through NF-

κB and interferon regulatory factor-1 (Lowenstein et al., 1993; Xie et al., 1993). Kleinert et al. (1996) reported that three different signal transduction pathways can induce iNOS expression in 3T3 cells: the receptor tyrosine kinase pathway (by interferon-γ and LPS), the protein kinase A pathway (by forskolin and 8-bromo-cAMP), and the protein kinase C pathway (by phorbolmyristate acetate (PMA)). All these pathways seem to converge in the activation of the NF-κB transcription factor. Therefore, the NF-κB sites are essential for LPS-mediated NO production. The nuclear translocation and DNA binding of the NF-κB transcription factor is preceded by the phosphorylation and degradation of IκB. To determine the specific signal transduction pathway that is involved in the inhibition of NF-κB by triptolide, we examined the effects of triptolide on the activation of NF-κB induced by LPS, finding that pretreatment of Raw 264.7 cells with triptolide did not inhibit the LPS-induced IκB phosphorylation and degradation. However, LPS-induced NF-κB binding activity and NF-κB-dependent luciferase activity are significantly inhibited by triptolide.

In summary, triptolide inhibits LPS-induced NO production and expression of iNOS mRNA and protein in macrophages. These effects are mediated, at least in part, by inhibition of JNK and NF-κB activation. The fact that NF-κB is negatively regulated by triptolide is important because this transcription factor plays a critical role in the regulation of a variety of genes involved in inflammatory responses. In view of the facts that NO plays an important role in mediating inflammatory responses and that triptolide may be an important determinant of clinical response in inflammatory diseases, further efforts to explore this therapeutic strategy appear warranted.

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