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p38 but Not p44/42 Mitogen-Activated Protein Kinase Is Required for Nitric Oxide Synthase Induction Mediated by Lipopolysaccharide in RAW 264.7 Macrophages

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ABSTRACT

Protein kinase C (PKC)-α, -βI, and -δ are known to be involved in the lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophages. The role of mitogen-activated protein kinases (MAPK) p44/42 and p38 in the LPS effect was studied further. LPS-mediated NO release and the inducible form of NO synthase expression were inhibited by the p38 inhibitor, SB 203580, but not by the MAPK kinase inhibitor, PD 98059. Ten-minute treatment of cells with LPS resulted in the activation of p44/42 MAPK, p38, and c-Jun NH_2-terminal kinase. Marked or slight activation, respectively, of p44/42 MAPK or p38 was also seen after 10-min treatment with 12-*O*-tetradecanoylphorbol-13-acetate, but c-Jun NH_2-terminal kinase activation did not occur. Tyrosine kinase inhibitor, genestein, attenuated the LPS-induced activation of both p44/42 MAPK and p38, whereas the PKC inhibitors, Ro 31-8220 and calphostin C, or long-term treatment with 12-*O*-tetradecanoylphorbol-13-acetate resulted in inhibition of p44/42 MAPK activation, but had only a slight effect on p38 activation, indicating that LPS-mediated PKC activation resulted in the activation of p44/42 MAPK. Nuclear factor-κB (NF-κB)-specific DNA-protein-binding activity in the nuclear extracts was enhanced by 10-min, 1-h, or 24-h treatment with LPS. Analysis of the proteins involved in NF-κB binding showed translocation of p65 from the cytosol to the nucleus after 10-min treatment with LPS. The onset of NF-κB activation correlated with the cytosolic degradation of both inhibitory proteins of NF-κB, IκB-α and IκB-β. IκB-α was resynthesized rapidly after loss (1-h LPS treatment), whereas IκB-β levels were not restored until after 24-h treatment. SB 203580 but not PD 98059 inhibited the LPS-induced stimulation of NF-κB DNA-protein binding. Thus, activation of p38 but not p44/42 MAPK by LPS resulted in the stimulation of NF-κB-specific DNA-protein binding and the subsequent expression of inducible form of NO synthase and NO release in RAW 264.7 macrophages.

Nitric oxide (NO) mediates a number of the host-defense functions of activated macrophages, including antimicrobial and tumoricidal activity (MacMicking et al., 1997). NO and its metabolites also have been implicated in the pathogenesis of the tissue damage associated with acute and chronic inflammation (Laskin and Pendino, 1995). Macrophages generate NO from the guanidino moiety of L-arginine through a reaction catalyzed by the inducible form of nitric oxide synthase (iNOS) (Leone et al., 1991). In contrast to the constitutive Ca^{2+}-dependent form of the enzyme found in the central nervous system and endothelial cells, iNOS can be induced by many immune stimuli. Changes in NO formation in iNOS-expressing cells usually correlate with similar changes in iNOS mRNA levels, indicating that a major part of iNOS regulation occurs at the level of transcription. The promoter region of the iNOS gene contains several binding sites for transcriptional factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), as well as for various members of the C/EBP, activating transcription factor/cAMP response element-binding protein (ATF/CREB), and Stat family of transcriptional factors (Lowenstein et al., 1993; Xie et al., 1993). Of these, proteins of the NF-κB family appear to be essential for the enhanced iNOS gene expression in macrophages exposed to the active components of endotoxin, lipopolysaccharide (LPS) (Sherman et al., 1993; Xie et al., 1994). In unstimulated cells, NF-κB is retained in the cytoplasm by binding to an inhibitory protein of NF-κB, IκB, but is released by signal induction and translocates to the nucleus and activates the responsive gene (Thanos and Maniatis, 1995). The macrophage iNOS is responsible for NO synthesis over a period of several hours after cell stimulation with LPS (Lyons et al., 1992).

The intracellular signaling pathways by which LPS causes iNOS expression are largely unresolved but involve a series

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ABBREVIATIONS: NO, nitric oxide; iNOS, inducible NO synthase; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; PDTC, pyrrolidine dithiocarbamate; EMSA, electrophoretic mobility-shift assay; MBP, myelin basic protein; PKC, protein kinase C; PI-PLC, phosphatidylinositol-phospholipase C; PC-PLC, phosphatidylcholine-PLC; MEK, MAPK kinase.

of events resulting in the transmission of the signal from the plasma membrane through the cytoplasm to the nucleus where iNOS gene expression is up-regulated. Previous studies have shown that LPS binds to LPS-binding protein and then binds to membrane CD14 and activates phosphatidylinositol-phospholipase C (PI-PLC) and phosphatidylcholine-PLC (PC-PLC) via tyrosine phosphorylation to induce protein kinase C (PKC) activation; this results in the stimulation of NF-κB-specific DNA-protein binding, initiating the expression of iNOS and, finally, the release of NO (Wright et al., 1990; Chen et al, 1998a). The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that participate in signaling pathways initiated by many extracellular stimuli, including growth factors and phorbol esters. These "classical" MAPKs are the p44 and p42 isoforms [also known as extracellular signal receptor-activated kinase (ERK)1 and ERK2] (Nishida and Gotoh, 1993; Davis, 1994). Recently, two novel MAPK-related enzymes have been identified (Davis, 1994): one is stress-activated protein kinase or c-Jun NH_2-terminal kinase (JNK) (Derijard et al., 1994), and the other is p38 (Han et al., 1994).

PKC can induce downstream activation of MAPK (Blumer and Johnson, 1994). In addition, LPS-induced activation of p44/42 MAPK, p38, and JNK has been reported in macrophages and other cell types (Han et al., 1993; Hambleton et al., 1996; Sanghera et al., 1996; Schumann et al., 1996), but their activation-signaling pathway and functional roles were not elucidated directly because of the lack of specific inhibitors at that time. In the present study, we therefore have studied the signaling pathway involved in LPS-induced p44/42 MAPK activation and its role in NO production using the MAPK kinase (MEK) inhibitor PD 98059; we have also studied the role of p38, using the p38-specific inhibitor, SB 203580.

Materials and Methods

Materials. Affinity-purified rabbit polyclonal anti-iNOS antibody was obtained from Transduction Laboratories (Lexington, KY). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, penicillin, and streptomycin were purchased from GIBCO/BRL (Gaithersburg, MD). Rabbit polyclonal antibodies directed against p42 MAPK (ERK2), p38, JNK1, NF-κB (p65), IκB-α or IκB-β, and the NF-κB probe were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies directed against the phosphorylated form of Tyr-204 p44/42 MAPK, Tyr-182 p38, and Thr-183/Tyr-184 JNK and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was from L.C. Services Corp. (Woburn, MA). LPS (from *Escherichia coli* serotype 0127: B8), pyrrolidine dithiocarbamate (PDTC), sulfanilamide, *N*-(1-naphthyl)-ethylenediamine, protein A-Sepharose CL-4B, and myelin basic protein (MBP) were purchased from Sigma (St. Louis, MO), and genestein, calphostin C, and PD 98059 were purchased from Calbiochem (San Diego, CA). SB 203580 was a gift from SmithKline Beecham Pharmaceuticals (Dr. John C. Lee). Poly (dI/dc) was purchased from Pharmacia Biotech. Reagents for SDS-polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). Horseradish peroxidase-labeled donkey anti-rabbit second antibody and the enhanced chemiluminescence (ECL)-detecting reagent were purchased from Amersham International (Buckinghamshire, UK).

Cell Culture. RAW 264.7 cells, a murine macrophage cell line, were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For the nitrite assay, they were grown in 12-well plates, whereas for iNOS expression, p44/42 MAPK and p38 activation, and NF-κB gel-shift assay, they were grown in 10-cm dishes.

Determination of NO Concentration. NO production in the culture supernatant was evaluated by measuring nitrite, its stable degradation product, using Griess reagents. The DMEM first was changed to phenol red-free medium, and then the cells were stimulated with LPS (1 μg/ml) for 24 h. The isolated supernatant was centrifuged and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2% phosphoric acid) and incubated at room temperature for 10 min. The absorbance was measured at 550 nm in a microplate reader. Sodium nitrite was used as a standard. In pretreatment experiments, cells were incubated with genestein (tyrosine kinase inhibitor), Ro 31-8220, or calphostin C (PKC inhibitors), PD 98059, SB 203580, or PDTC (NF-κB inhibitor) for 30 min or with TPA for 24 h before addition of LPS. Calphostin C requires light for activation as indicated by the manufacturer. The presence of inhibitors did not affect cell viability.

Preparation of Cell Extracts and Western Blot Analysis of iNOS, Phosphorylated p44/42 MAPK, Phosphorylated p38, Phosphorylated JNK, ERK2, p38, and JNK1. After treatment with LPS or TPA or various inhibitors before challenge with LPS for 10 min, the cells were washed rapidly with PBS, then lysed with ice-cold lysis buffer [50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml of leupeptin, 20 μg/ml of aprotinin, 1 mM NaF, and 1 mM Na_3VO_4] as described previously (Chen and Chen, 1998), and the lysates were treated with 2× Laemmli buffer, then subjected to SDS-PAGE using a 7.5% (for iNOS) or 10% (for MAPKs) running gel. Proteins were transferred to nitrocellulose paper, and immunoblot analysis was performed as described previously (Chen et al., 1995). Briefly, the membrane was incubated successively with 0.1% milk in TTBS at room temperature for 1 h, with rabbit antibodies specific for iNOS or phosphorylated MAPKs or nonphosphorylated MAPKs for 1 h, and then with horseradish peroxidase-labeled anti-rabbit second antibody for 30 min. After each incubation, the membrane was washed extensively with TTBS and the immunoreactive band was detected with ECL-detecting reagents and developed with Hyperfilm-ECL. The quantitative data were obtained by using a computing densitometer with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation of p38 and p38 Activity Assay. The immunoprecipitation experiment was done as described previously (Chen and Chen, 1998). Briefly, 50 μg of total cell lysates was incubated with 1 μg of anti-p38 antibody for 1 h at 4°C and collected using protein A-Sepharose CL-4B beads. The beads were then washed three times with lysis buffer and incubated in 50 μl of kinase reaction mixture containing 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 μM Na_3VO_4, 0.3 mg/ml MBP, and 50 μM [γ-^{32}P]ATP (2000 cpm/pmol) for 30 min at 30°C. The reaction mixture was stopped by the addition of Laemmli buffer and subjected to 13% SDS-PAGE, and phosphorylated-MBP was visualized by autoradiography.

Preparation of Nuclear Extracts and the Electrophoretic Mobility-Shift Assay (EMSA). Control cells or cells pretreated with genestein, TPA, PD 98059, SB 203580, or PDTC were treated with 1 μg/ml of LPS for 1 h. The nuclear extracts then were isolated as described previously. Briefly, cells were washed with ice-cold PBS and pelleted. The cell pellet was resuspended in a hypotonic buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 1 mM NaF, and 1 mM Na_3VO_4] and incubated for 15 min on ice, then lysed by the addition of 0.5% Nonidet P-40 followed by vigorous vortexing for 10 s. The nuclei were pelleted and resuspended in extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM NaF, and 1 mM Na_3VO_4), and the tube was

shaken vigorously at 4°C for 15 min on a shaking platform. The nuclear extracts then were centrifuged, and the supernatants were aliquoted and stored at −80°C.

A double-stranded oligonucleotide probe containing NF-κB-binding sequences was purchased (5′-AGTTGAGGGGACTTTCCCAGGGC-3′, Santa Cruz Biotechnology) and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The nuclear extract (6–10 μg) was incubated at 30°C for 20 min with 1 ng of ^{32}P-labeled NF-κB probe (40,000–60,000 cpm) in 10 μl of binding buffer containing 1 μg poly(dI-dc), 15 mM HEPES, pH 7.6, 80 mM NaCl, 1 mM EGTA, 1 mM DTT, and 10% glycerol as described previously (Chen et al., 1998b). DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on a native 6% polyacrylamide gel, and then the gel was vacuum-dried and autoradiographed using an intensifying screen at −80°C.

In NF-κB (p65) translocation studies, both cytosolic and nuclear extracts were used; only cytosolic extracts were used in IκB-α or IκB-β degradation. The extracts were subjected to SDS-PAGE using a 10% running gel, and immunoblot analysis was performed as described above.

Statistical Analyses. All data are expressed as mean ± S.E.M. Statistical analyses were done with Student's *t* test.

Results

LPS-Induced NO Production and iNOS Expression. Exposure of RAW 264.7 macrophages to LPS resulted in both nitrite production and the expression of the 130-kDa iNOS in a concentration- and time-dependent manner (Fig. 1). For an exposure period of 24 h, maximum nitrite release (47.6 ± 2.7 nmol/10^6 cells/24 h, n = 3) was obtained using 1 μg/ml of LPS (Fig. 1A). When cells were treated with 1 μg/ml of LPS for various times, nitrite release was significant at 6 h (9.8 ± 0.5 nmol, n = 3) and maximal at 24 h (Fig. 1B). In the following NO release experiments, the cells were treated with 1 μg/ml of LPS for 24 h. Under these conditions, as shown in Fig. 2, both transcriptional and translational inhibitors, actinomycin D and cycloheximide, dose-dependently inhibited the LPS-induced nitrite production, with 75.1% or 87.8% inhibition using 10 or 30 nM actinomycin D, respectively, and 69.8, 93.2, or 95.7% inhibition using 100, 300, or 1000 nM cycloheximide, respectively. LPS-induced iNOS expression also was inhibited using 30 nM actinomycin D or 300 nM cycloheximide. Because LPS could induce proinflammatory cytokine (IL-1β, TNF-α) release in macrophages, the effect of IL-1β and TNF-α on NO release was examined. IL-1β or TNF-α did not induce nitrite release (data not shown), indicating the direct effect of LPS on NO release in RAW cells.

Inhibitory Effect of p38 Inhibitor but Not MEK Inhibitor on LPS-Induced NO Production and iNOS Expression. Previous results have shown that, in RAW cells, LPS activated PI-PLC and PC-PLC via tyrosine phosphorylation, resulting in PKC activation, NF-κB activation, iNOS expression, and, finally, NO release (Chen et al., 1998a). The downstream signal for PKC involves activation of p44/42 MAPK. To determine whether activation of p44/42 MAPK was involved in the regulation of LPS-induced NO production, the MEK inhibitor, PD 98059, was used. Concomitantly, the p38 inhibitor, SB 203580, was also used to determine whether p38 was involved in the LPS response. As shown in Fig. 3, SB 203580 but not PD 98059 dose dependently inhibited LPS-induced nitrite production. SB 203580 (3 μM) had no effect on LPS-induced nitrite production, whereas 10 or 30 μM resulted in 58.4 or 62% inhibition, respectively. iNOS expression also was inhibited by 30 μM SB 203580 but not by 30 μM PD 98059 (Fig. 3).

LPS-Induced Activation of p38 and p44/42 MAPK. Because both LPS-induced NO production and iNOS expression was inhibited by SB 203580, indicating that activation of p38 was involved in LPS-induced NO production in RAW 264.7 macrophages, activation of p38 by LPS was examined. Activation of MAPKs requires phosphorylation at threonine and tyrosine residues. Immunoblot analysis using anti-phospho-specific p44/42 MAPK, p38, and JNK antibodies with phosphorylated tyrosine was performed. As shown in Fig. 4A, when cells were treated with 1 μg/ml of LPS for 10, 30, or 60 min, maximal activation of p44/42 MAPK and p38 was seen after 10- or 30-min treatment; less activation was seen after 60 min. On the other hand, activation of another MAPK, JNK, was also seen after 10-min treatment with LPS, reached a maximum at 30 min, and was lost after 60-min

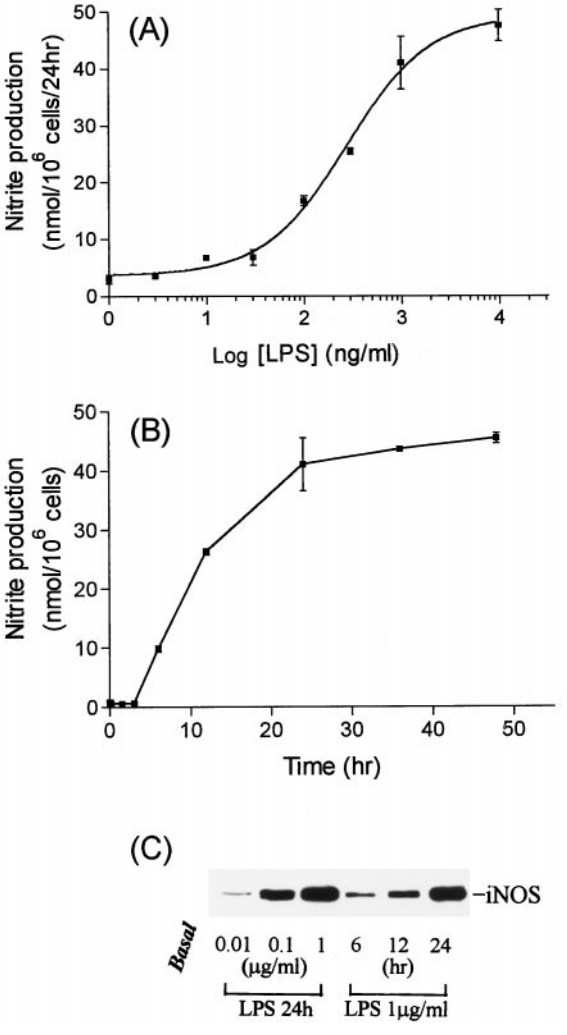

Fig. 1. Concentration- and time-dependent LPS-induced stimulation of nitrite release and iNOS expression in RAW 264.7 macrophages. Cells were incubated at 37°C with various concentrations of LPS for 24 h (A) or with 1 μg/ml of LPS for various time intervals (B), and then the medium was removed and analyzed for nitrite release. Results are expressed as mean ± S.E.M. of three independent experiments performed in triplicate. C, cells were incubated with indicated concentrations of LPS for 24 h or with 1 μg/ml of LPS for indicated time intervals, and then lysates were subjected to Western blotting using iNOS-specific antibody as described in *Materials and Methods*.

treatment. The quantitative data are shown in Fig. 4B. Ten-minute treatment with 1 μM TPA resulted in marked or slight activation of p44/42 MAPK or p38, respectively, whereas JNK was not activated; the expression of p42 MAPK, p38, and JNK1 was not affected by these treatments (Fig. 4).

When cells were pretreated with 30 μM genestein, LPS-induced activation of both p38 and p44/42 MAPK was attenuated (Fig. 5B). Overnight pretreatment with 1 μM TPA or 30-min pretreatment with 5 μM Ro 31-8220 or 300 nM calphostin C markedly inhibited the LPS-induced activation of p44/42 MAPK, whereas that of p38 was only slightly affected (Fig. 5, A and B). Thirty micromolar PD 98059 (MEK inhibitor) completely blocked the LPS-induced p44/42 MAPK activation without any effect on p38 (Fig. 5, A and C). In contrast, 30 μM SB 203580 (p38 inhibitor) also completely blocked the activation of p38 by LPS (Fig. 5 B and C), but had no effect on p44/42 MAPK (Fig. 5B). None of these treatments had any effect on the expression of p42 MAPK or p38 (Fig. 5, A and B).

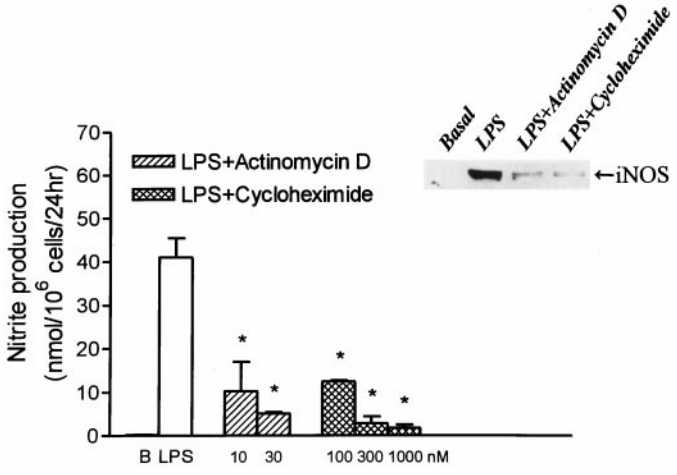

Fig. 2. Effect of actinomycin D or cycloheximide on LPS-induced nitrite release and iNOS expression in RAW 264.7 macrophages. Cells were pretreated with indicated concentrations of actinomycin D or cycloheximide for 30 min before incubation with 1 μg/ml of LPS for 24 h, and then medium was removed and analyzed for nitrite. Results are expressed as mean ± S.E.M. of three independent experiments performed in triplicate. $^*p < .05$ compared with LPS alone. For determination of iNOS expression, cells were pretreated with 30 nM actinomycin D or 300 nM cycloheximide for 30 min before incubation with 1 μg/ml of LPS for 24 h, followed by Western blotting of the lysates using iNOS-specific antibody, as described in *Materials and Methods*.

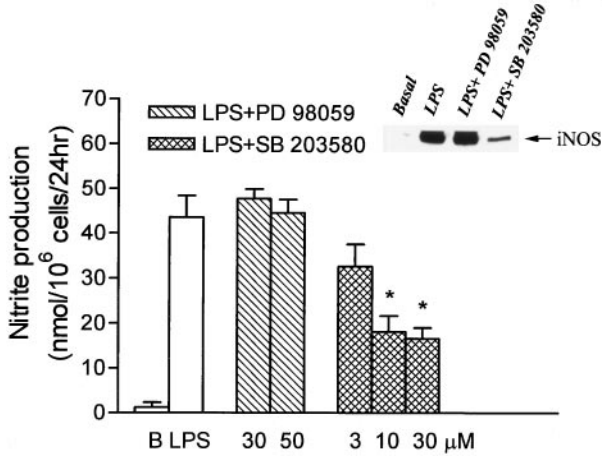

Fig. 3. Effect of PD 98059 or SB 203580 on LPS-induced nitrite release and iNOS expression in RAW 264.7 macrophages. Cells were pretreated with indicated concentrations of PD 98059 or SB 203580 for 30 min before incubation with 1 μg/ml of LPS for 24 h, and then medium was removed and analyzed for nitrite. Results are expressed as mean ± S.E.M. of three to five independent experiments performed in triplicate. $^*p < .05$ compared with LPS alone. For determination of iNOS expression, cells were pretreated with 30 μM PD 98059 or SB 203580 for 30 min before incubation with 1 μg/ml of LPS for 24 h, followed by Western blotting of lysates using iNOS-specific antibody as described in *Materials and Methods*.

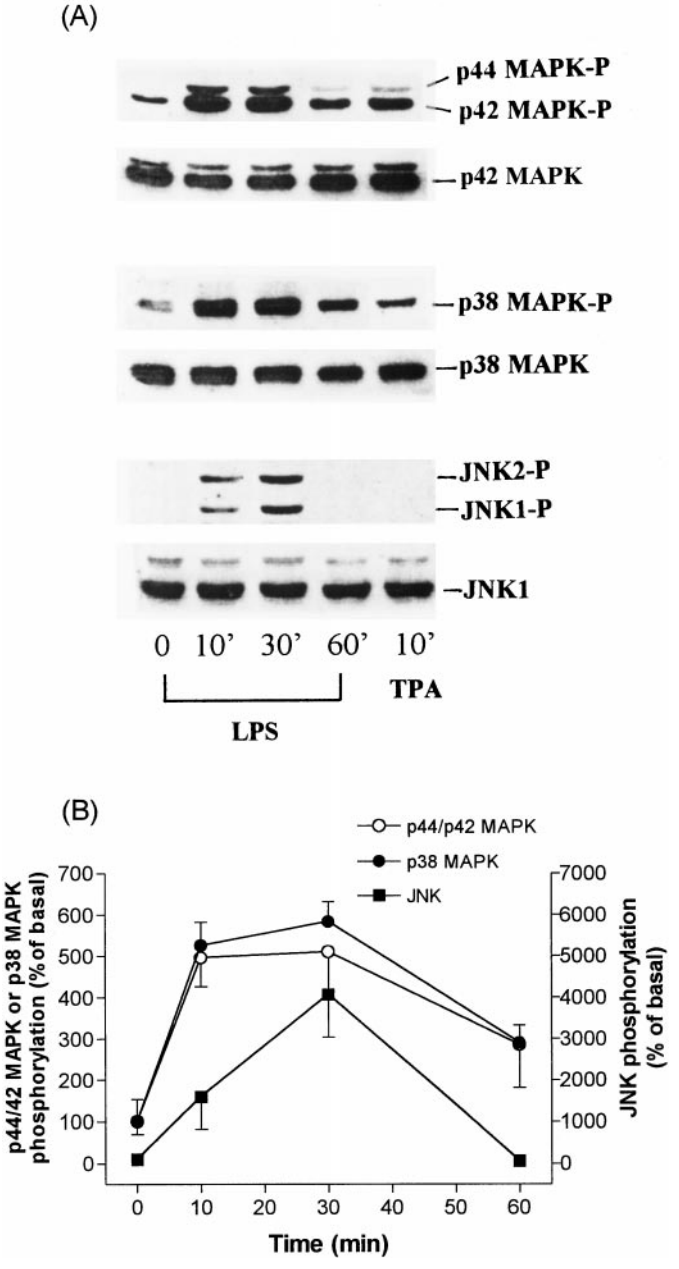

Fig. 4. Time-dependent activation of p44/42 MAPK, p38, and JNK by LPS or TPA in RAW 264.7 macrophages. A, cells were treated with 1 μg/ml of LPS for 10, 30, or 60 min or with 1 μM TPA for 10 min. Whole-cell lysates were prepared and subjected to Western blotting using antibodies specific for phosphorylated form of p44/42 MAPK, p38, or JNK or for p42 MAPK, p38, or JNK1 as described in *Materials and Methods*. B, extent of phosphorylation of these three MAPKs was quantitated using a densitometer with ImageQuant software. Data are presented as mean ± S.E.M. of three experiments.

Induction of NF-κB in the Nuclei of LPS-Stimulated RAW 264.7 Cells and the Inhibitory Effect of SB 203580 but Not PD 98059. In resting cells, the NF-κB p65/p50 heterodimer is retained in the cytosol by its association with IκB (Thanos and Maniatis, 1995). After stimulation of the cells with various agents, the cytosolic NF-κB/IκB complex dissociates and free NF-κB translocates to the nuclei. In macrophages, PDTC, an antioxidant that acts as a specific inhibitor of NF-κB activation (Schreck et al., 1992; Xie et al., 1994), blocks LPS-induced nitrite production and iNOS expression (Fig. 6) and the induction of nuclear-binding activity for NF-κB (Fig. 8). Thus, activation of NF-κB is indeed critical in the induction of iNOS by LPS. The time course of NF-κB activation after treatment with LPS was examined. Nuclear extracts prepared from RAW cells were assayed for activated NF-κB in an EMSA using radiolabeled oligonucleotides containing NF-κB recognition-site-like sequences found in the macrophage iNOS gene (Lowenstein et al., 1993). In nuclear extracts from unstimulated macrophages, two faint NF-κB-specific DNA-protein complexes were identified. LPS rapidly (10 min) activated NF-κB; similar activation was seen after 1 h (Fig. 7A), whereas after 24 h, less DNA-protein complex was seen, although it was still more abundant than in resting cells (Fig. 7A). These two banding patterns were identified as p65/p50 heterodimer and p50/p50 homodimer for the upper and lower complex, respectively (Xie et al., 1994; Chen et al., 1998a). To characterize the proteins involved in NF-κB activation, the amount of p65 in the cytosolic and nuclear extracts from activated cells was assessed by Western blots. As shown in Fig. 7B, p65 was translocated rapidly from the cytosol to the nuclear compartment in stimulated cells and remained constant for 1 h of LPS treatment.

Dissociation and degradation of the IκB moieties of the cytosolic NF-κB complexes is an important mechanism in controlling NF-κB translocation to the nucleus. Since the amount of NF-κB protein released to migrate to the nucleus is thought to be proportional to the degradation of IκB, both IκB-α and IκB-β protein levels in the cytosol were measured using Western blots. As shown in Fig. 7B, LPS rapidly induced complete degradation of IκB-α, but its level was fully restored after 1 h of LPS treatment. IκB-β also decreased in cells treated with LPS for 10 min and its levels was still low after 1 h of treatment, not being fully restored until 24 h.

After pretreatment of the cells with 30 μM genestein, 100 nM calphostin C, 30 μM SB 203580 or 25 μM PDTC but not with 30 μM PD 98059 for 30 min, the LPS-elicited activation of NF-κB specific DNA-protein complexes formation was inhibited (Fig. 8A and 8B).

Discussion

LPS induced activation of p38 in RAW 264.7 macrophages, and maximal activation occurred after 10 min treatment and was maintained for 30 min. LPS also induced activation of JNK, whereas maximal activation was not seen until 30-min

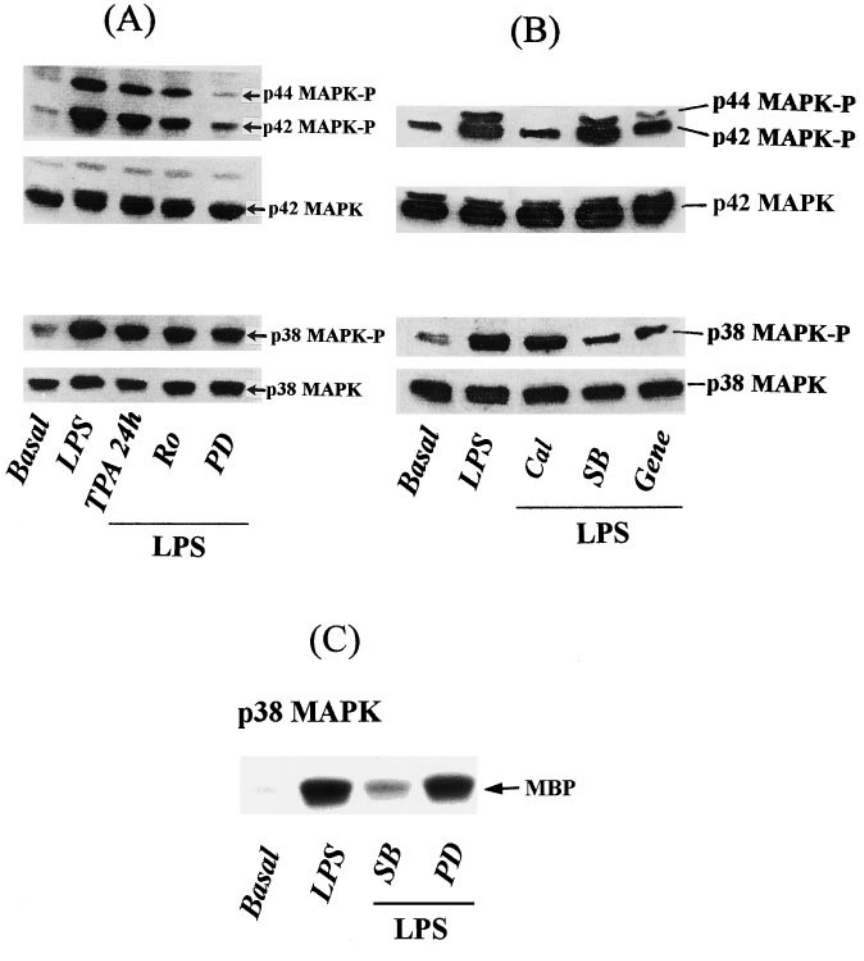

Fig. 5. Effect of genestein, long-term TPA treatment, Ro 31-8220, calphostin C, PD 98059, or SB 203580 on LPS induced p44/42 MAPK and p38 activation in RAW264.7 macrophages. Cells were pretreated with 1 μM TPA for 24 h or with 5 μM Ro 31-8220 (Ro) or 30 μM PD 98059 (PD) for 30 min (A) or with 300 nM calphostin C (Cal), 30 μM SB 203580 (SB), or 30 μM genestein (Gene) for 30 min (B) before incubation with 1 μg/ml of LPS for 10 min. Whole-cell lysates were prepared and subjected to Western blotting using antibodies specific for phosphorylated form of p44/42 MAPK or p38 or for p42 MAPK or p38 as described in *Materials and Methods*. Gels in A were run by SE-500 (Hoefer), and those in B were run by minigel (Bio-Rad). C, cell lysates were immunoprecipitated with anti-p38 antibody, and autoradiography of phosphorylated MBP was detected as described in *Materials and Methods*.

treatment (Fig. 4). Treatment with TPA alone resulted in a low level of activation of p38, but did not result in activation of JNK (Fig. 4). LPS-induced p38 activation was inhibited by genestein, but only slightly by Ro 31-8220, calphostin C, or overnight pretreatment with TPA (Fig. 5, A and B), indicating that p38 activation by LPS was the downstream signal of tyrosine kinase activation, whereas PKC was only slightly involved. We used the specific p38 inhibitor, SB 203580, to study the relationship between LPS-elicited activation of p38 and NO release in macrophages. This inhibitor completely blocked LPS-elicited p38 activation without any effect on p44/42 MAPK activation (Fig. 5, B and C) and also abrogated LPS-induced NO release and iNOS expression (Fig. 3), emphasizing the importance of p38 activation in mediating the LPS response in macrophages. This is the first study to directly explore the involvement of p38 in LPS-induced NO production and iNOS expression in macrophages. LPS-induced p38 activation has also been reported in human neutrophils, but, in this case, the intracellular-signaling pathway is PKC-independent and does not involve activation of p44/42 MAPK and JNK (Nick et al., 1996). In the present study, evidence is presented that the intracellular-signaling pathway of LPS acting via p38 activation requires the activation of an upstream protein tyrosine kinase, as suggested also by Sanghera et al. (1996). The involvement of PKC was negative or weak as reported previously in RAW cells, CD 14-expressing 70z/3 cells, and HeLa cells (Han et al., 1993; Raingeaud et al., 1995; Sanghera et al., 1996). However, significant p38 activation by TPA stimulation in human neutrophils has been reported (Nick et al., 1996). Recently, the kinase thought to be upstream of p38 has been identified as the MAP kinase kinase homolog, MKK3 or MKK6 (Cohen, 1997).

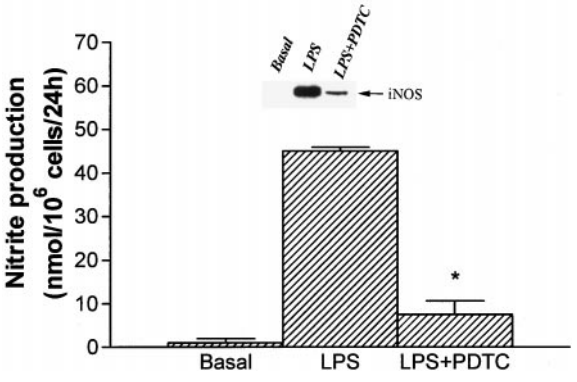

Fig. 6. Effect of PDTC on LPS-induced nitrite release and iNOS expression in RAW 264.7 macrophages. Cells were pretreated with 25 μM PDTC for 30 min before incubation with 1 μg/ml of LPS for 24 h, and then medium was removed and analyzed for nitrite. Results are expressed as mean ± S.E.M. of four independent experiments performed in triplicate. *$p < .05$ compared with LPS alone. For determination of iNOS expression, cells were pretreated with 25 μM PDTC for 30 min before incubation with 1 μg/ml of LPS for 24 h, followed by Western blotting of lysates using iNOS-specific antibody as described in *Materials and Methods*.

LPS is also a potent stimulator of the MAPK pathway in monocytes and macrophages, and many reports have demonstrated LPS-induced activation of the 44-kDa and 42-kDa isoforms of MAPK (Weinstein et al., 1992; Geppert et al., 1994; Reimann et al., 1994; Sanghera et al., 1996). This appears to be mediated by the dual-specificity kinase, MEK, which, in turn, is activated by Raf-1, a serine/threonine-specific kinase reported to be involved in the response to LPS (Geppert et al., 1994; Reimann et al., 1994). In this study, we used a specific MEK inhibitor, PD 98059, to study the relationship between the LPS-induced activation of p44/42 MAPK and NO release in macrophages. After 10 min of treatment with LPS, activation of p44/42 MAPK was seen, lasting for 30 min. PD 98059 completely blocked this activation, while not affecting LPS-induced NO release and iNOS expression (Figs. 3 and 5A), indicating that activation of p44/42 MAPK was not involved in this event. LPS-induced activation of p44/42 MAPK was also inhibited by genestein and the PKC inhibitors, Ro 31-8220 and calphostin C, and by down-regulation of PKC by overnight pretreatment with TPA (Fig. 5). Activation of PKC by short-term (10-min) treatment with TPA (Chen et al., 1998a) also resulted in marked activation of p44/42 MAPK. These results indicate that LPS-induced p44/42 MAPK activation is the downstream signal of PKC activation. However, p44/42 MAPK activation is not involved in NO production (present study) but might be related to the release of immunoregulatory molecules as suggested by Sanghera et al. (1996).

The promoter region of the murine iNOS gene contains at least 24 elements homologous to consensus sequences for the binding of transcriptional factors involved in the inducibility of this gene by cytokines and LPS (Lowenstein et al., 1993). Of these, the NF-κB site is necessary for inducibility by LPS in mouse macrophages, because PDTC blocked both the pro-

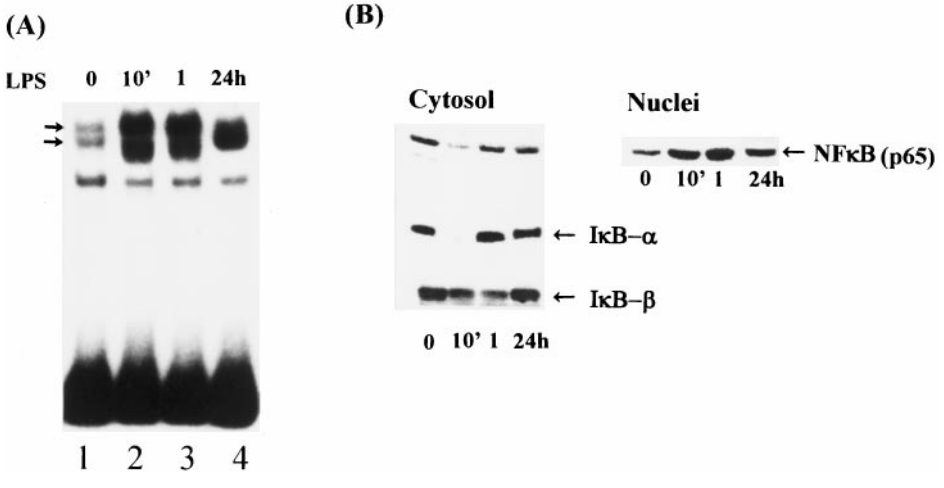

Fig. 7. Kinetics of LPS-induced NF-κB-specific DNA-protein complex formation, NF-κB translocation, and IκB degradation in RAW 264.7 macrophages. Cells were treated with 1 μg/ml of LPS for 10 min, 1 h, or 24 h (A), and then cytosolic and nuclear extracts were prepared. A, NF-κB-specific DNA-protein binding activity in nuclear extracts was determined by EMSA as described in *Materials and Methods*. B, Cytosolic and nuclear levels of NF-κB (p65) and cytosolic levels of IκB-α and IκB-β were immunodetected using NF-κB- (p65), IκB-α-, or IκB-β-specific antibodies, as described in *Materials and Methods*.

duction of nitrite and the nuclear NF-κB-binding activity in LPS-treated macrophages (Xie et al., 1994; Figs. 6 and 8). In unstimulated cells, NF-κB exists in an inactive form in the cytoplasm bound to the inhibitory protein IκBα or IκBβ (Baldwin, 1996). When these cells are stimulated, specific kinases phosphorylate IκB, causing its rapid degradation by proteasomes (Thanos and Maniatis, 1995; Chen et al., 1996; DiDonato et al., 1996). The release of NF-κB from IκB results in the translocation of NF-κB into the nucleus, where it binds to specific sequences in the promoter regions of target genes, then up-regulates gene expression (Henkel et al., 1993; Thompson et al., 1995). In the present study, EMSA studies showed rapid activation of NF-κB in response to LPS stimulation (10 min) (Fig. 7A), together with the paralleled translocation of p65 into the nucleus. Complete and partial degradation of cytosolic IκB-α and IκB-β, respectively, was also seen (Fig. 7B). It has been reported that all known NF-κB activators (e.g., IL-1, LPS, TNF, and TPA) induce degradation of IκB-α, but only persistent activators (e.g., IL-1 and LPS) have been shown to induce degradation of IκB-β (Thompson et al., 1995). Although LPS treatment resulted in the rapid loss of IκB-α protein as in several other cell types, it was resynthesized within 1 h (Fig. 7B) (Beg et al., 1993; Thompson et al., 1995; Diaz-Guerra et al., 1996; Han and Brasier, 1997). The renewed synthesis of IκB-α protein might be a result of activation of the IκB-α gene by activated nuclear NF-κB, because the IκB-α gene promoter contains kB-binding sites (Brown et al., 1993; Sun et al., 1993). The reappearance of IκB-α plays an autoregulatory role in the regulation of NF-κB function and could explain the transient activation of NF-κB by TNF-α (Thompson et al., 1995). However, sustained activation of NF-κB by 1-h treatment with LPS was seen despite the complete restoration of the levels of cytosolic IκB-α (Fig. 7A). This might be a result of the sustained degradation (1 h) of IκB-β (Fig. 7B), because a sustained reduction of IκB-β has been suggested to contribute to the persistent NF-κB activation (Thompson et al., 1995; Johnson et al., 1996). Levels of cytosolic IκB-β were restored after 24 h of LPS treatment; this result is similar to the effect of LPS seen in 70z/3 cells and hepatocytes and of IL-1 on human endothelial cells (Thompson et al., 1995; Diaz-Guerra et al., 1996; Johnson et al., 1996) but contrasts with the effect of TNF on endothelial cells and HepG2 cells in which cytosolic IκB-β levels do not recover (Johnson et al., 1996; Han and Brasier, 1997). After 24-h treatment with LPS, recovery of both IκB-α and IκB-β occurred; however, NF-κB activation still remained. The reason for this sustained activation is unknown, but might be due to IκB-β, which has been reported to act as a chaperone for NF-κB by protecting it from

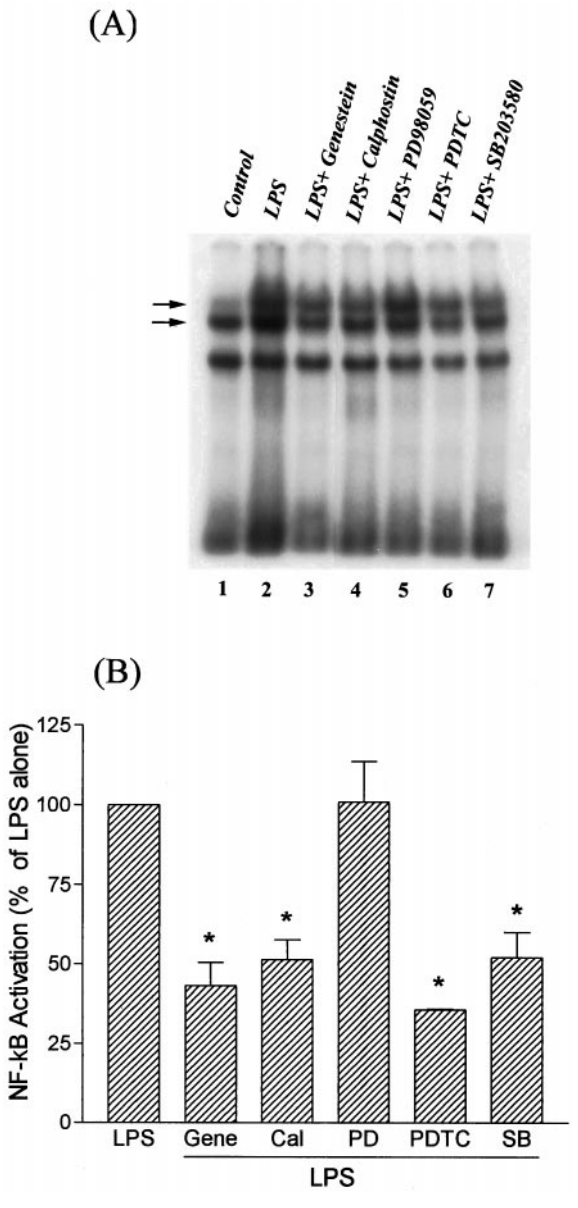

Fig. 8. Effect of various inhibitors on LPS-induced NF-κB-specific DNA-protein complex formation in nuclear extracts of RAW 264.7 macrophages. A, cells were pretreated with 30 μM genestein, 100 nM calphostin C, 30 μM PD 98059, 30 μM SB 203580, or 25 μM PDTC for 30 min before challenge with 1 μg/ml of LPS for 1 h, and then nuclear extracts were prepared and their NF-κB DNA-protein-binding activity was determined by EMSA as described in *Materials and Methods*. B, extent of NF-κB activation was quantitated using a densitometer with ImageQuant software. Data are presented as mean ± S.E.M. of three experiments. $*p < .05$ compared with LPS alone.

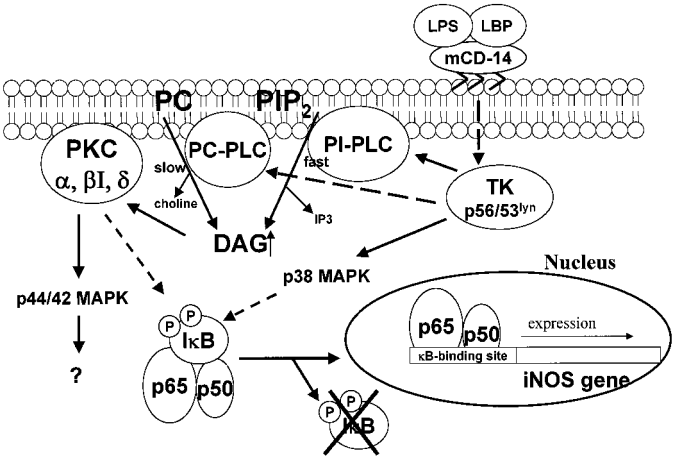

Fig. 9. Schematic representation of the signaling pathway of LPS-induced NO release in RAW 264.7 macrophages. LPS binds to LPS-binding protein (LBP), then binds to membrane CD14 (mCD14), and activates PI-PLC and PC-PLC via tyrosine phosphorylation to induce PKC activation. Tyrosine phosphorylation also induces p38 activation. These result in stimulation of NF-κB-specific DNA-protein binding, initiating iNOS expression and, finally, NO release. LPS-elicited PKC activation induces downstream p44/42 MAPK stimulation, which is, however, not involved in NO release.

IκB-α and allowing it to be transported to the nucleus (Suyang et al., 1996).

In this study, the LPS-induced activation of NF-κB-specific DNA-protein complex formation was inhibited by a tyrosine kinase inhibitor, a PKC inhibitor, or a p38 inhibitor but not by a MEK inhibitor. Phosphorylation of Ser-32 and Ser-36 in IκB-α and Ser-19 and Ser-23 in IκB-β triggers polyubiquitination of the IκBs and targets them for rapid degradation by the 26S proteasome (Chen et al., 1996; DiDonato et al., 1996). Recently, IκB-α kinase has been cloned and identified and has been shown to be activated by TNF-α or IL-1 and trigger the phosphorylation of IκB-α, thus removing its inhibitory effect on NF-κB (DiDonato et al., 1997; Regnier et al., 1997). Whether IκB-α kinase exists in macrophages and LPS stimulates this kinase is still unknown. However, LPS, by acting on PKC and p38 activation, might contribute to the degradation of IκB-α and IκB-β in macrophages, resulting in NF-κB activation (Fig. 8).

In summary, LPS induced activation of both p38 and p44/42 MAPK; however, only p38 is involved in the stimulation of NF-κB DNA-protein binding and the subsequent expression of iNOS and NO release in macrophages. Both events required the activation of an upstream protein tyrosine kinase. The stimulation of p38 was mainly PKC-independent, whereas that of p44/42 MAPK was downstream of LPS-elicited PKC activation. A schematic representation of the signaling pathway of LPS-induced NO release in RAW 264.7 macrophages is shown in Fig. 9.

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