Ca$^{2+}$- and Protein Kinase C-dependent Signaling Pathway for Nuclear Factor-κB Activation, Inducible Nitric-oxide Synthase Expression, and Tumor Necrosis Factor-α Production in Lipopolysaccharide-stimulated Rat Peritoneal Macrophages*

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Xueyuan Zhou, Wenxiu Yang¹, and Junying Li
From the Department of Biophysics in the School of Physics, Key Laboratory of Bioactive Materials of Education Ministry, Nankai University, Tianjin 300071, Peoples Republic of China

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Lipopolysaccharide (LPS)-activated macrophages are pivotal in innate immunity. With LPS treatment, extracellular signals are transduced into macrophages via Toll-like receptor 4 and induce inflammatory mediator production via activating signal-pathways, including the nuclear factor-κB (NF-κB) pathway and the mitogen-activated protein kinase (MAPK) pathway. However, the mechanisms by which the intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]) increases and protein kinase C (PKC) is activated remain unclear. Therefore, we investigated the signaling pathways, including the nuclear factor-κB (NF-κB) pathway (7), for Ca$^{2+}$-activated macrophages. After LPS treatment, protein-tyrosine kinase inhibitors reduced phosphorylation of phospholipase C (PLC)γ2. Furthermore, a PLC inhibitor eliminated the transient [Ca$^{2+}$]i increase and decreased the amount of activated PKC. Therefore, these results revealed the following roles of Ca$^{2+}$ and PKC in the signaling pathway for NF-κB activation in LPS-stimulated macrophages. After LPS treatment, protein-tyrosine kinase mediates PLCγ1/2 phosphorylation, which is followed by a transient [Ca$^{2+}$]i increase. Several PKCs are activated, and PKC regulates phosphorylation of serine in MEKK1. Moreover, MEKKs regulate inhibitory κB kinase activation. Sequentially, NF-κB is activated, and inducible nitric-oxide synthase and tumor necrosis factor-α production is promoted.

Lipopolysaccharide (LPS),² one of the membrane components of Gram-negative bacteria, induces a variety of responses to severe infection, such as local inflammation, antibody production, and septic shock. Macrophages respond to LPS early in the infection and thus play a pivotal role in host defense. However, at high concentrations, LPS stimulates macrophages to release massive amounts of pro-inflammatory mediators such as interleukins (ILs), tumor necrosis factor α (TNF-α), superoxide, and nitric oxide (1, 2). These pro-inflammatory mediators can disturb normal cellular function, and this disruption can lead to multiple organ dysfunction syndromes or lethal septic shock (3, 4). Therefore, the investigation of LPS-induced signaling pathways in macrophages is important and necessary for discovering potential therapeutic targets and drugs.

In recent years, it has been found that LPS binds with LPS-binding protein and interacts with a complex that consists of CD14, MD2, and Toll-like receptor 4 (TLR4); the complex transduces the signal generated by its interaction with LPS into the interior of the cell (5, 6). The intracellular Toll/IL-1 receptor domain of TLR4 recruits myeloid differentiation factor 88, IL-1 receptor-associated kinase, and TNF receptor-associated factor 6 (TRAF6) to form a complex, which can transduce the signal further. Several signaling pathways are activated sequentially, including the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor-κB (NF-κB) pathway (7).

In LPS-stimulated macrophages, MAPKs are activated by a three-tiered cascade of kinases: 1) mitogen-activated/extracellular signal-regulated kinase kinase kinase (MEKK); 2) MAPK kinase (MEK); 3) MAPKs, including p38, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK). MAPKs can activate some transcription factors, such as activator protein 1 and activating transcription factor 2, and play important roles in the production of inflammatory mediators (8–11). However, how does LPS activate the MAPK path-

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1 To whom correspondence should be addressed. Tel: 86-22-23501005; Fax: 86-22-23501594; E-mail: yangwenx@nankai.edu.cn.

2 The abbreviations used are: LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor α; TLR4, Toll-like receptor 4; TRAF, TNF receptor-associated factor; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; MEKK, mitogen-activated/extracellular signal-regulated kinase kinase; MEK, mitogen-activated/extracellular signal-regulated kinase kinase; IKK, IκB kinase; PKC, protein kinase C; PTK, protein-tyrosine kinase; iκB, inhibitory κB; TAK, transforming growth factor β-activated kinase; TAK1, binding protein; Ca$^{2+}$, intracellular free Ca$^{2+}$ concentration; Ins(1,4,5)P$_3$, inositol 1,4,5-trisphosphate; INOS, inducible nitric-oxide synthase; PLC, phosphatidylinositol-specific phospholipase C; Fura-2/AM, Fura-2 acetoxymethyl ester; PMSF, phenylmethylsulfonfonyl fluoride; PBS, phosphate-buffered saline; HRP, horse-radish peroxidase; PtdIns(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; DAG, 1,2-diacylglycerol; BAPTA-AM, 1,2-bis(2-aminoxyethoxy)ethane-N,N,N’,N’-tetraacetic acid.
way in macrophages? Some reports have suggested that phosphoinositide 3-kinase, protein kinase C (PKC), and some protein-tyrosine kinases (PTKs) are responsible for MEKK activation (12, 13).

NF-κB regulates the expression of many cytokines in LPS-stimulated macrophages and plays an important role in the immune system. Recently, considerable progress has been made in determining the function and regulation of NF-κB. In macrophages, NF-κB is a p65/p50 dimer and is inactivated by binding with inhibitory κB (IκB). The inhibitory κB kinase (IKK) β, a component of the IKK complex, can phosphorylate IκBα, and leads to the ubiquitination and degradation of IκB. Meanwhile, p65/p50 is translocated into the nucleus (14, 15). Therefore, the activation of the IKK complex plays a crucial role in NF-κB activation. Furthermore, two sets of adaptors are linked to IKK activation. The first involves transforming growth factor-β-activated kinase 1 (TAK1) and two associated adaptors, TAK1-binding protein 1 (TAB1) and TAB2. After stimulation, TRAF6 ubiquitylates itself or the component of the TAK1-TAB1-TAB2 complex and primes it for IKK activation. Another protein that has been reported to act as a bridge between TRAF6 and IKK is ECSIT. Moreover, MEKK1 and MEKK3 have been implicated in IKK activation in receptor/TLR4-mediated NF-κB activation pathways (7).

Although many studies have revealed that TRAF6 is related to MEKKS and IKK activation, the question about how TRAF6 activates them is unanswered. Intracellular free Ca2+ concentration ([Ca2+]i) is important for cellular function; however, few studies have been focused on the signaling pathway(s) involving [Ca2+]i, in LPS-stimulated macrophages. Several reports have indicated that LPS treatment can cause an increase in [Ca2+]i, which is related to TNF-α production in alveolar macrophages and Kuffer cells (16, 17). Our previous reports have also revealed that the transient increase in [Ca2+]i in LPS-stimulated rat peritoneal macrophages plays an important role in TNF-α production (18). Furthermore, LPS also induces a biphasic change in inositol 1,4,5-triphosphate (Ins(1,4,5)P3) in C3H/HeN mouse macrophages, and the change leads to the activation of a TNF-α gene. The later phase of the Ins(1,4,5)P3 change is mediated by a y2 type of phosphatidylinositol-specific phospholipase C (PLCγ2) (19). However, the mechanisms by which LPS induces a transient increase in [Ca2+]i, and the role of [Ca2+]i, in NF-κB and MAPK signaling pathways have not been elucidated. Additionally, LPS can activate PKC isoenzymes and regulate the expression of inflammatory mediators in macrophages (20). However, which kinds of PKC isoenzymes are responsible for NF-κB activation and how they work in the signaling pathway remain unknown.

In the present study, we investigated the [Ca2+]i- and PKC-dependent signaling pathway for NF-κB activation, inducible nitric-oxide synthase (iNOS) expression, and TNF-α production in LPS-stimulated rat peritoneal macrophages. On the basis of the experimental results, we propose a new mode of the signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Dimethyl sulfoxide, LPS (Escherichia coli serotype 0127:B8 prepared by phenol extraction), Fura-2 acetoxyethyl ester (Fura-2/AM), the PTK inhibitor (genistein), the PKC inhibitor (GF109203X), the PLC inhibitor (U73122), and EGTA were purchased from Sigma. BATPA-AM (intracellular Ca2+ chelator) was obtained from Molecular Probes. The MEKK inhibitor (PD98059), the PKCα-specific inhibitor (Go6976), and the PKCβ-specific inhibitor (Go6983) were purchased from Calbiochem. The PKCβ-specific inhibitor (LY379196) was acquired from Eli Lilly.

**Isolation and Culture of Macrophages**—Male Wistar rats (about 250 g), which were treated humanely in compliance with institutional guidelines, were killed. Hanks’ balanced salt solution was injected into the abdomen of each rat. Macrophages in Hanks’ balanced salt solution removed from the abdomen were centrifuged at 200 × g for 5 min. The cell pellet was collected, and the cells were cultured in RPMI 1640 and 10% fetal bovine serum for 6 h at 37 °C in 5% CO2. Adherent cells were harvested, re-suspended, and incubated for another 48 h before analysis. Nonspecific esterase staining showed that 95% of the adherent cells were macrophages.

**Immunoblot Assay to Detect PLC Phosphorylation**—Cells (10⁶) were scraped from dishes into cold lysis buffer (0.1 m Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 20 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml each of pepstatin and leupeptin) after they were washed twice in cold phosphate-buffered saline (PBS). After centrifugation (10,000 × g for 10 min at 4 °C), the soluble fraction, which contained PLC, was collected. The proteins in the soluble fraction were separated by SDS-PAGE (7%) and transferred to polyvinylidene fluoride membranes. Immunoblotting was carried out by treating the membrane with 4.5 μg of antibody specific for activated phosphotyrosines or phospho- serines of PLCγ1 (Tyr783), PLCγ2 (Tyr787), or PLCβ3 (Ser537) (Cell Signaling Technology; ratio of antibody dilution, 1:200) for 12 h. To normalize the amount of sample in each lane, immunoblotting was also carried out by using 4.5 μg of polyclonal antibody to PLCγ1, PLCγ2, or PLCβ3 (Cell Signaling Technology; ratio of antibody dilution, 1:200) for 12 h. Biotinylated antibody specific for rabbit immunoglobulin (Amersham Biosciences) was employed as the secondary antibody. The chemiluminescence of the streptavidin-horseradish peroxidase (HRP) conjugates was detected by film.

**Measurement of [Ca2+]i in Macrophages**—Intracellular free Ca2+ was detected by using the ratiometric fluorescent Ca2+ indicator dye Fura-2 and a microspectrofluorometer. Cells were incubated in 3 μM Fura-2/AM for 50 min at room temperature and washed twice with PBS. Changes in the fluorescence intensity of Fura-2 at excitation wavelengths of 340 and 380 nm and the emission wavelength of 510 nm were monitored in an individual peritoneal macrophage. [Ca2+]i was calculated by the following equation.

\[
[Ca^{2+}]_i = \frac{K_d \times [Ca-Fura-2]}{[Fura-2]} = \frac{K_d \times F_{340}}{F_{380}} \text{ (nmol/l)}
\]  

(Eq. 1)

Kd was the constant for Fura-2 chelating Ca2+, and its value was about 135 nmol/liter when the temperature was 22 °C. In the present experiment, F340/F380 was directly related to [Ca2+]i.
Measurement of Total PKC Activity—Total protein kinase C activity was determined as described by Lee and Wu (21) but with modifications. After treatment, cells were washed twice with PBS and scraped into cold lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 2.5 mM PMSF, 5 µg/ml leupeptin, and 5 µg/ml antipain). After solubilization, cell lysates were centrifuged at 10,000 × g for 30 min at 4 °C, and the supernatant was collected and aliquoted (200 µg/tube). PKC activity in the supernatant was measured immediately by using the Pierce Colorimetric PKC Assay Kit. The PKC-dependent phosphorylated peptide was quantified by spectroscopy at 570 nm.

Immunoblot Assay to Detect Phosphorylated PKC Isoenzymes—After treatment with LPS, macrophages were washed twice in ice-cold PBS. Cell pellets were resuspended in buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml antipain, 1% Nonidet P-40, 0.25% sodium deoxycholate) for 30 min at 4 °C. Lysates were centrifuged at 10,000 × g for 30 min at 4 °C, and the supernatants were collected. Proteins in the supernatant were separated by SDS-PAGE (12%). SDS-separated proteins were equilibrated in kinase buffer (20 mM Tris, pH 9.0–9.4, 40 mM glycerine, 0.375% SDS, 20% methanol) and electrotransferred to Immobilon-P membranes. Nonspecific binding sites were blocked by incubation for 1 h in Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 5% nonfat dry milk and 0.05% Tween 20. The membranes were then washed and incubated with 6 µg of anti-phospho-PKCα (Ser657) antibody (dilution ratio, 1:5000) (Santa Cruz Biotechnology), anti-phospho-PKCβ (Ser660) antibody (dilution ratio, 1:2500) (Santa Cruz Biotechnology), anti-phospho-PKCδ (Thr505) antibody (dilution ratio, 1:1000) (Cell Signaling Technology), or anti-phospho-PKCε (Ser729) antibody (dilution ratio, 1:2500) (Upstate) for 10 h at 4 °C. To normalize the amount of sample in each lane, immunoblotting was carried out by treating the membrane with 6 µg of polyclonal antibodies to PCKα (dilution ratio, 1:5000), PCKβ (dilution ratio, 1:2500), PCKδ (dilution ratio, 1:500), and PKCε (dilution ratio, 1:500) (Santa Cruz Biotechnology) for 10 h at 4 °C. An HRP-conjugated goat antimouse IgG antibody (dilution ratio, 1:20,000) was used as the second antibody, and the chemiluminescence was detected on film.

Immunoprecipitation and Immunoblot Analysis of Phosphorylated Serine in MEKK1—Cells were washed in ice-cold PBS and scraped into PBS. Cells were pelleted by centrifugation and lysed in whole cell extract lysis buffer (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1.5 mM MgCl2, 1% Triton X-100, 10% glycerol, 1.0 mM EDTA, 1 mM Na3VO4, 1 mM PMSF, 0.2 mM leupeptin, 0.2 mM pepstatin A) for 30 min on ice. The lysates were centrifuged at 10,000 × g for 10 min at 4 °C, and supernatants were isolated. After normalization (immunoblotting assay), cell lysates that contained equal amounts of total protein were incubated with 2 µg of polyclonal anti-MEK1 antibody (43-Y, Santa Cruz Biotechnology; dilution ratio, 1:200) for 2.5 h at 4 °C and then with 4.5 µg of protein G-conjugated agarose beads (Amersham Bioscieneces) for 1 h at 4 °C. After centrifugation, MEKK1 isolated from the beads was separated by SDS-PAGE (12%) and transferred onto a nitrocellulose membrane. Immunoblot analysis was carried out by using monoclonal antibody to phosphoserine (Santa Cruz Biotechnology; dilution ratio, 1:1000). Biotinylated antibody to rabbit Ig (Amersham Biosciences) was employed as the secondary antibody. The chemiluminescence of the streptavidin-HRP conjugates was detected by film.

Immunoprecipitation and IKK Protein Kinase Activity Assays—After LPS stimulation of macrophages, the cytosol was extracted by using 200 µl of IKK CE buffer (10 mM HEPES-KOH, pH 7.9, 250 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.2% Tween 20, 2 mM dithiothreitol, 1 mM PMSF, 20 mM β-glycerophosphate, 10 mM NaF, 0.1 mM Na3VO4), and the proteins in each extract were normalized using measurements from Bradford assays. Cytosolic extracts (100 µl) were incubated with 1 µg of monoclonal antibody specific for IKKα (BD Pharmingen) for 2 h at 4 °C, and then with protein G-conjugated agarose beads (Amersham Biosciences) for 1 h at 4 °C. After the beads were washed twice with IKK CE buffer and once with kinase buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 10 mM MgCl2, 2 mM dithiothreitol, 1 mM PMSF, 20 mM β-glycerophosphate, 10 mM NaF, 0.1 mM Na3VO4), they were incubated with 20 µl of kinase buffer containing 20 µM ATP, 10 µCi of [32P]ATP, and 0.5 µg of bacterially expressed GST-IκBα(1–54) substrate at 30 °C for 30 min. The reaction mixture underwent SDS-PAGE (10%), and the proteins were visualized. To normalize kinase activities, the proteins that appeared to be 50–175 kDa were transferred to polyvinylidene fluoride membranes (Amersham Biosciences), and the blots underwent standard immunoblotting techniques to detect IKK (22).

Immunoblot Assay to Detect Phosphorylated IκBα in the Cytoplasm—Cells (107) were washed twice with 5 ml of cold PBS and centrifuged at 350 × g for 5 min at 4 °C. Proteins were released by resuspension of the pellet in 250 µl of buffer (10 mM Tris-HCl, pH 7.8, 10 mM KCl, 2.5 mM NaH2PO4, 1.5 mM MgCl2, 1 mM Na2VO4, 0.5 mM dithiothreitol, 0.4 mM 4-(2-aminophenylazo)benzenesulfonyl fluoride/HCl, 2 µg/ml leupeptin, 2 µg/ml pepstatin A, and 2 µg/ml aprotinin). The suspension was incubated on ice for 10 min and then homogenized at a moderate speed. The homogenate underwent centrifugation at 4,500 × g for 5 min at 4 °C, and the supernatant was collected. After the proteins were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes and after the nonspecific binding sites on the membranes were blocked by incubation overnight at 4 °C with 1% bovine serum albumin, the membranes were then incubated with antibody specific for phospho-IκBα (Ser32) (dilution ratio, 1:2000; Cell Signaling) for 12 h at 4 °C. To normalize the amount of sample in each lane, immunoblotting analysis was also carried out by treating the membranes with 5 µg of polyclonal antibody to IκBα (dilution ratio, 1:1000; Cell Signaling) for 12 h at 4 °C. Biotinylated antibody to rabbit immunoglobulin (Amersham Biosciences) was employed as the secondary antibody. The chemiluminescence of the streptavidin-HRP conjugates was detected by film.

Western Blot Detection of p65 in Nuclei—Cells (107) were washed twice with cold PBS. Nuclei were released by resuspending the pellet in 250 µl of buffer A (10 mM Tris-HCl, pH 7.8, 10 mM KCl, 2.5 mM NaH2PO4, 1.5 mM MgCl2, 1 mM Na2VO4, 0.5 mM dithiothreitol, 0.4 mM 4-(2-aminophenylazo)benzenesulfonyl fluoride/HCl, 2 µg/ml leupeptin, 2 µg/ml pepsta-
tin A, and 2 μg/ml aprotinin). After incubation for 10 min on ice, the suspension was homogenized at a moderate speed. Nuclei were collected by centrifugation at 4,500 × g for 5 min at 4 °C and resuspended in 100 μl of buffer B (buffer A adjusted to 20 mM Tris-HCl, pH 7.8, 420 mM KCl, 20% (v/v) glycerol). Nuclei were then lysed at 4 °C for 30 min with gentle agitation, debris was cleared by centrifugation at 10,000 × g for 30 min at 4 °C, and the supernatant was collected. The proteins were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes. After nonspecific binding sites were blocked by incubation overnight at 4 °C with 1% bovine serum albumin, the membranes were then incubated with polyclonal antibody specific for p65 (Santa Cruz Biotechnology). To normalize the protein content of each sample, β-actin was detected with 15 μg of anti-β-actin antibody (Sigma, 1:1000). Biotinylated antibody specific for rabbit immunoglobulin (Amersham Biosciences) was employed as the secondary antibody. The chemiluminescence of the streptavidin-HRP conjugates was detected by film (23).

FIGURE 1. LPS induced phosphorylation of PLC, a transient increase in [Ca²⁺]i, PKC activation, phosphorylation of serine in MEKK1, IKK activation, IκBα phosphorylation, p65 translocation, NF-κB activation, iNOS expression, and TNF-α production in rat peritoneal macrophages. A, immunoblot analysis of phosphorylation of PLCγ1, PLCγ2, and PLCζ3 in cells that were treated with no LPS (Ⅰ) or with 10 μg/ml LPS. The antibodies were specific for phosphorylated PLCγ1, PLCγ2, or PLCζ3. The protein content of each sample was normalized by analysis using the appropriate antibodies against PLCγ1, PLCγ2, or PLCζ3. B, measurement of the transient [Ca²⁺]i increase in an individual macrophage. This experiment was repeated more than 9 times, and representative results of experiments in which extracellular calcium was present (Ⅰ) or absent (Ⅱ) are shown. The arrows indicate the times when reagents were added. C, enzyme-linked immunosorbent assay (Pierce Colorimetric PKC Assay) to measure total PKC activity. D, immunoblot analysis of phosphorylated PKC isoenzymes using antibodies to phosphorylated PKCa, -β, -δ, and -ε. The protein content of sample was normalized by immunoblot analysis with antibodies to PKCa, -β, -δ, and -ε. E, immunoprecipitation analysis of phosphorylated serine in MEKK1. After immunoprecipitation with antibody to MEKK1, the bound protein was analyzed by using an antibody to phosphorylated serine. The protein content of each sample was normalized by immunoblot analysis with an antibody to MEKK1. F, results of an immunoprecipitation and kinase assay to evaluate IKK activity. Glutathione S-transferase-IκBα served as a substrate in the assay, and its phosphorylation by IKK was quantified, normalized, and plotted. G, Western blot analysis of p65 translocation into nucleoli and immunoblot analysis of IκBα phosphorylation in the cytoplasm. The antibodies were specific for p65 and phosphorylated IκBα. The protein content of each sample was normalized by immunoblot or Western blot analysis with antibodies to IκBα or β-actin. H, results of a dual-luciferase activity assay to evaluate NF-κB activation. I, immunoblot analysis of iNOS expression in macrophages. The antibody was specific for iNOS. The protein content of each sample was normalized by immunoblot analysis with an antibody to β-actin. J, results of the TNF-α assay. The control was represented by ⬤, LPS by ☐.
Transient Transfection and Dual Luciferase Assays—Transfection was done by using a standard procedure for calcium phosphate precipitation. Macrophages were transfected with 3 µg of luciferase reporter plasmids that contained an NF-κB gene (pNF-κB-luc) and 3 µg of pRL-TK reporter plasmids (pRL-TK-Renilla). After 48 h, samples were harvested and prepared for luciferase assays according to the manufacturer's protocol (Promega Corp.). Firefly and Renilla luciferase activities were measured in each sample using the Dual Luciferase Assay (Promega Corp.).

Immunoblot Assays to Detect iNOS Expression—After LPS treatment, macrophages (10⁶) were lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue) and boiled for 5 min at 100 °C. Aliquots (20 µg per lane) underwent electrophoresis in a 10% polyacrylamide gel, and the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were treated with 10% nonfat milk for 1 hr to block nonspecific binding sites, rinsed, and incubated with rabbit polyclonal antibody to iNOS (Santa Cruz Biotechnology) overnight at 4 °C. To normalize the protein content of each sample, β-actin was detected with 15 µg of anti-β-actin antibody (Sigma, 1:1000). The membranes were then treated with HRP-conjugated antirabbit IgG (dilution ratio, 1:2000) for 1 h. Immune complexes were detected as described (24).

TNF-α Assays—Macrophages and L929 cells (mouse, C3H/An areolar and adipose cells) were cultured in 96-well plates. TNF-α was detected in each defined period. After collecting the macrophage supernatant, an equal volume of medium with or without reagents was added to the wells at different times. Supernatants of macrophage cell cultures (100 µl/well) were collected and added with actinomycin D (Sigma; final concentration, 1 µg/ml) to L929 cells. Twenty hours later, plates underwent centrifugation at 200 × g for 5 min. The supernatants were discarded, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (final concentration, 1 mg/ml; Sigma) without phenol red was added. After 4 h, the supernatants were discarded, and 100 µl of a solution of Me2SO and ethanol (ratio, 1:1) was added to each well. The A₅₇₀ values were measured by an enzyme-linked immunosorbent detector, and the quantity of TNF-α was calculated using a standard curve for recombinant human TNF-α (Pepro Tech EC LTD) (25).

Statistical Analysis—All assays were repeated a minimum of three times, and representative results from a single assay are shown. Some results are expressed as the mean ± S.E.
RESULTS

LPS-induced PLCγ Phosphorylation, a Transient Increase in \([Ca^{2+}]_{i}\), PKC Activation, Serine Phosphorylation in MEKK1, IKK, and NF-κB Activation, iNOS Expression, and TNF-α Production—Within 5 min of the start of LPS treatment (10 \(\mu\)g/ml), phosphorylated tyrosine in PLCγ1 and PLCγ2 was detected in rat peritoneal macrophages, and phosphorylation reached a maximum at 10 min. Nevertheless, phosphorylation of PLCβ3 was not detected (Fig. 1A). Moreover, a transient increase in \([Ca^{2+}]_{i}\), was evoked 110 s after the start of LPS treatment and continued for a total of 140 s. The peak of \([Ca^{2+}]_{i}\), was lower when 5 mM EGTA was added (the extracellular Ca\(^{2+}\) concentration was 1.25 mM), whereas the initial rates of Ca\(^{2+}\)-influx from the extracellular space. Therefore, the results indicate that the initial transient increase in \([Ca^{2+}]_{i}\), is due to intracellular Ca\(^{2+}\) release, which may be followed by Ca\(^{2+}\) influx from the extracellular space. Furthermore, total PKC activity increased between 0.25 and 1 h and then began to decline (Fig. 1C). The pattern of change seen in the quantities of phosphorylated isoenzymes PKCα, β, δ, and ε was similar to the pattern seen in total PKC activity over time (Fig. 1D).

Stimulation by LPS (10 \(\mu\)g/ml) resulted in the phosphorylation of serine in MEKK1, which was first detected at 0.25 h and increased for the next 0.75 h. From 1 to 3 h, MEKK1 was phosphorylated steadily (Fig. 1E). Moreover, IKK activity increased during the period of 1 to 3 h and then decreased (Fig. 1F). Accumulation of phosphorylated IkBα in the cytoplasm and translocation of p65 into the nucleus also increased markedly in the first 3 h of LPS treatment (Fig. 1G). Meanwhile, the pattern of NF-κB activation in response to LPS treatment resembled those of phosphorylated IkBα accumulation and p65 translocation (Fig. 1H). Finally, iNOS expression increased dramatically between 2 and 5 h of treatment (Fig. 1I). Different slightly from iNOS expression, TNF-α production markedly increased between 3 and 8 h (Fig. 1J).

Role of the \([Ca^{2+}]_{i}\), Increase in LPS-induced PKC Activation, Phosphorylation of Serine in MEKK1, and IKK Activation—To identify the role of the \([Ca^{2+}]_{i}\), increase in the signaling pathway for NF-κB activation, extracellular or intracellular Ca\(^{2+}\) was eliminated, and the effect was assessed. Pretreatment of macrophages with 50 \(\mu\)M BAPTA-AM (an intracellular Ca\(^{2+}\)-chelator) for 1 h or with 5 mM EGTA for 5 min had no effect on LPS-induced phosphorylation of PLCγ1 and PLCγ2 (data not shown). However, total PKC activity and phosphorylation of PKCs, especially Ca\(^{2+}\)-dependent phosphorylation of PKCα and PKCβ, were reduced (Fig. 2A, B, E, and F). Nevertheless, the quantity of phosphorylated PKCβ and PKCe, whose phosphorylation is Ca\(^{2+}\)-independent, was not affected obviously. Moreover, LPS-induced phosphorylation of serine in MEKK1 and activation of IKK were inhibited (Fig. 2, C, D, G, and H). Therefore, activation of PKCα and PKCβ depends on the transient increase in \([Ca^{2+}]_{i}\), in LPS-stimulated rat peritoneal macrophages.

Role of PKC in LPS-induced Phosphorylation of Serine in MEKK1 and Activation of IKK—To elucidate the relationship between PKC and other molecules in the signaling pathway for

![Figure 3](http://www.jbc.org/)

**FIGURE 3.** The PKC inhibitor (GF109203X) and PKCβ inhibitor (LY379196) reduced LPS-induced PKC activation, phosphorylation of serine in MEKK1, and IKK activation in rat peritoneal macrophages. Macrophages were not pretreated (−) or were pretreated (+) with GF109203X (1 \(\mu\)M) or LY379196 (30 \(\mu\)M) for 30 min, and then LPS (10 \(\mu\)g/ml) was added. A and D, enzyme-linked immunosorbent assay (Pierce Colorimetric PKC Assay) to measure total PKC activity. B and F, immunoprecipitation and immunoblot analysis to evaluate phosphorylated serine in MEKK1. The antibodies were specific for MEKK1 and phospho-

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NF-κB activation in LPS-stimulated rat peritoneal macrophages, the cells were treated with PKC inhibitors and their effects were examined. After pretreatment of macrophages with 1 μM GF109203X (a PKC inhibitor) for 30 min, LPS-induced total PKC activation was inhibited sufficiently (Fig. 3A). Furthermore, phosphorylation of serine in MEKK1 and activation of IKK were decreased (Fig. 3B and C). However, phosphorylation of PLCγ was unaffected (data not shown). Pretreatment of macrophages with Go6976 (10 μM; a PKCα inhibitor) or Go6983 (10 μM; a PKCβ inhibitor) for 30 min had no effect on LPS-induced phosphorylation of serine in MEKK1 and on activation of IKK and NF-κB (data not shown). Nevertheless, after pretreatment with LY379196 (10 μM; a PKCβ inhibitor) for 30 min, LPS-induced phosphorylation of serine in MEKK1 and activation of IKK were inhibited markedly (Fig. 3, F and G). Additionally, LPS-induced total PKC activation and PKCβ phosphorylation were also inhibited greatly (Fig. 3, D and E). Therefore, the results suggest that PKC, especially PKCβ, participates in the phosphorylation of serine in MEKK1 in LPS-stimulated rat peritoneal macrophages. Therefore, the results suggest that PKC, especially PKCβ, participates in the phosphorylation of serine in MEKK1 in LPS-stimulated rat peritoneal macrophages. (Fig. 4C). The remainder of PKC activation may be due to calcium- and DAG-independent PKCs. Therefore, it can be concluded that PLCγ1 and PLCγ2 regulate intracellular Ca²⁺ release in LPS-stimulated rat peritoneal macrophages. Moreover, it also can be concluded that PLC activation plays an important role in regulating PKC activation.

A PKT Inhibitor Reduced LPS-induced Phosphorylation of PLCγ, [Ca²⁺]i Increase, PKC Activation, Phosphorylation of Serine in MEKK1, and IKK Activation—Only after tyrosine phosphorylation can PLCγ be activated. To investigate the signal upstream of PLC, macrophages were pretreated with the PKT inhibitor genistein (30 μM) for 30 min and then LPS was added (10 μg/ml). LPS-induced tyrosine phosphorylation of PLCγ1 and PLCγ2 was inhibited almost completely (Fig. 5A). Meanwhile, the transient increase in [Ca²⁺]i was inhibited completely (Fig. 5B). Total PKC activation, phosphorylation of serine in MEKK1, and IKK activation were also inhibited (Fig. 4, C–E). However, the inhibition of total PKC activation was not complete (Fig. 4C). The results indicate that tyrosine phosphorylation of PLCγ1 and PLCγ2 is PKT-dependent. They also suggest that...
PTK is involved in regulating other detected effectors in LPS-stimulated rat peritoneal macrophages.

A MEKK Inhibitor Reduced IKK Activation, NF-κB Activation, iNOS Expression, and TNF-α Production—The results of the present study demonstrated that the phosphorylation of serine in MEKK1 is PKCβ-dependent in LPS-stimulated rat peritoneal macrophages (Fig. 3). However, the question about how MEKK1 transduces the signal downstream is unanswered. To investigate the role of MEKK in the signaling pathway of NF-κB activation, macrophages were pretreated with the MEKK inhibitor PD98059 (20 μM; a MEKK1-specific inhibitor is not available) for 30 min. After pretreatment, LPS-induced IKK activation was decreased (Fig. 6A). In addition, NF-κB activation, iNOS expression, and TNF-α production were also inhibited (Fig. 6B–D). These results indicate that MEKKs participate in the regulation of IKK activation in LPS-stimulated rat peritoneal macrophages.

**DISCUSSION**

The signaling pathway for LPS-induced production of proinflammatory mediators in macrophages has been studied for many years. TLR4 associates with several kinases and adaptors to transduce signals downstream via TRAF6. Sequentially, MAPKs are activated by a three-tiered cascade of kinases, and NF-κB is activated after IKK phosphorylates IκBα. However, the question about what kinds of signaling molecules connect TRAF6 to IKK and MAPKs has remained unanswered (6, 7, 27–29). In the present study, we investigated the characteristics, mechanisms, and roles of the transient increase in [Ca2+], and PKC activation in the signaling pathway for LPS-induced NF-κB activation, iNOS expression, and TNF-α production in rat peritoneal macrophages. We systematically monitored LPS-induced dynamic changes, such as those in tyrosine phosphorylation of PLC, the transient increase in [Ca2+], activation of PKC, phosphorylation of serine in MEKK1, activation of IKK, accumulation of phosphorylated IκBα in the cytoplasm, translocation of p50/p65 in the nucleolus, activation of NF-κB, expression of iNOS, and production of TNF-α (Fig. 1).

As a second messenger, intracellular Ca2+ is essential for many cellular responses. In the present study, LPS aroused a rapid transient increase in [Ca2+]i in rat peritoneal macrophages. When extracellular Ca2+ was removed, the initial rate of [Ca2+]i increase did not differ from that seen when extracellular Ca2+ was present, but the peak in [Ca2+]i was lower when extracellular Ca2+ was absent (Fig. 1). Thus, it indicates that the LPS-induced transient increase in [Ca2+]i begins with the release of Ca2+ from intracellular Ca2+ pools in rat peritoneal macrophages, and this release may be followed by Ca2+ influx from the extracellular space. In non-excitable cells such as rat peritoneal macrophages, Ca2+ is released mainly through the opening of the Ins(1,4,5)P3 receptor in the endoplasmic reticulum when intracellular Ins(1,4,5)P3 increases (30). Ins(1,4,5)P3 is produced in the cytoplasm when active PLC catalyzes the hydrolysis of PtdIns(4,5)P2 into Ins(1,4,5)P3 and DAG (19). The PLC family is composed of several PLC isoenzymes. One subfamily includes PLCβ and PLCε, both of which are activated by the receptor-coupled Gq protein in the membrane (31, 32). Another subfamily is PLCγ, whose tyrosine phosphorylation depends on the activation of PTK (33, 34), but it is unknown which type(s) of PLC isoenzymes that mediate the transient increase in [Ca2+]i, are activated in LPS-stimulated rat peritoneal macrophages. Results from the present study provide insight into this issue.
FIGURE 6. A MEKK inhibitor (PD98059) decreased LPS-induced IKK and NF-κB activation, iNOS expression, and TNF-α production in rat peritoneal macrophages. Macrophages were not pretreated (−) or were pretreated (+) with PD98059 (20 μM) for 30 min, and then LPS (10 μg/ml) was added. A, results of an IKK immunoprecipitation kinase assay. B, results of a dual luciferase assay to evaluate the extent of NF-κB activation. C, results of immunoblot analysis to evaluate iNOS expression. The antibody was specific for iNOS. D, results of TNF-α assays. The filled circle indicates the values for the control cells; the filled triangle, the values for cells treated with LPS alone; and the filled square, the values for cells treated with LPS plus PD98059.

First, in the present study, the phosphorylation of a member of the PLCβ subfamily, PLCβ3, was not detected, whereas tyrosine residues in PLCγ1 and PLCγ2 were phosphorylated substantially (Fig. 1). Second, when rat peritoneal macrophages were pretreated with the PTK inhibitor genistein, LPS-induced phosphorylation of PLCγ1 and PLCγ2 was inhibited completely, and the LPS-induced transient increase in [Ca^{2+}]_{i} was also absent (Fig. 5). Third, the PLC inhibitor U73122 blunted the transient increase in [Ca^{2+}]_{i} (Fig. 4). Fourth, the LPS-activated receptor TLR4 is not coupled to the G_{i} protein in macrophages. Moreover, no report has revealed that a G_{i} protein-coupled receptor is activated in LPS-stimulated macrophages. Fifth, there is no evidence demonstrating that PLCα, -β, -δ, and -ε are activated in LPS-stimulated macrophages. Taken together, the present information suggests that PTK-mediated phosphorylation of PLCγ1 and PLCγ2 regulates Ca^{2+} release.

The present results indicate that PTK mediates activation of PLCγ1 and PLCγ2. When a PTK inhibitor (genistein) was used, there was complete inhibition of a series of biological events induced by LPS, including phosphorylation of PLCγ1 and PLCγ2, a transient increase in [Ca^{2+}]_{i}, total PKC activation, phosphorylation of serine in MEKK1, and activation of IKK (Fig. 5). PTK also affects a series of biological effectors in rat peritoneal macrophages. However, the specific PTK that mediates phosphorylation of PLCγ1 and PLCγ2 in rat peritoneal macrophages has not been identified. Recently, the activation of some non-receptor PTKs was detected in the TLR4 signaling pathway. The tyrosine kinase Btk is involved in TLR4-mediated activation of NF-κB. In LPS-stimulated macrophages, Btk regulates NF-κB activation and participates in the production of several pro-inflammatory mediators (35–37).

According to the present results, LPS-induced transient increase in [Ca^{2+}]_{i} is due to intracellular Ca^{2+} release and extracellular Ca^{2+} influx. When extracellular or intracellular Ca^{2+} was chelated, phosphorylation of PKCα and PKCβ was eliminated almost completely, whereas phosphorylation of PKCδ and PKCε was not affected obviously. Furthermore, total PKC activity was inhibited greatly (Fig. 2). Because activated PLCγ can catalyze PtdIns(4,5)P_{2} hydrolysis to produce DAG and Ins(1,4,5)P_{3}, we believe that these results suggest that phosphorylation of PLCγ and the transient increase in [Ca^{2+}]_{i}, lead to Ca^{2+}- and DAG-dependent activation of PKCα and PKCβ. The activation of other PKCs (PKCδ and PKCε) is DAG-dependent in LPS-stimulated rat peritoneal macrophages.

When phosphorylation of PLCγ1 and PLCγ2 was inhibited completely by U73122, total PKC activation was not eliminated completely (Fig. 4). Nevertheless, when the PTK inhibitor genistein inhibited phosphorylation of PLCγ1 and PLCγ2 completely, it also eliminated total PKC activation (Fig. 5). These results suggest that total PKC activation may depend on factors other than PLC. PTK may contribute to some Ca^{2+}- and DAG-dependent activation of PKC in rat peritoneal macrophages. Recent reports have revealed that LPS can activate atypical PKCs in macrophages (38).

In other types of cells, MEKK1 is regulated by particular kinases such as the germinal center kinase and NF-κB-inducing kinase (39, 40). Until now, no report has suggested that PKCβ regulates phosphorylation of serine in MEKK1 in rat peritoneal macrophages. In our study, the PKC inhibitor GF109203X decreased LPS-induced phosphorylation of serine in MEKK1 and activation of IKK and NF-κB. Moreover, the PKCβ inhibitor (LY379196) reduced this serine phosphorylation dramatically (Fig. 3), but other PKC isoenzyme inhibitors (Go6976, which inhibits PKCα, and Go6983, which inhibits PKCe) had no obvious effect (data not shown). Therefore, these results suggest that PKCβ participates in phosphorylation of serine in MEKK1 in LPS-stimulated rat peritoneal macrophages.

The MEKK family includes kinases such as MEKK1, MEKK2, and MEKK3 (41–43). In some cells, MEKK1 and MEKK3 are involved in the activation of IKK in the signaling pathway for IL-1 receptor/TLR4-mediated activation of NF-κB (44, 45). However, similar studies of peritoneal macrophages have been few. In our study, we were unable to detect a relationship between MEKK1 alone and IKK because no MEKK1-specific inhibitor was available. When the MEKK inhibitor PD98059 was used, LPS-induced activation of IKK and NF-κB were inhibited partially (Fig. 4). A possible reason for this result may be that kinases other than MEKKs regulate IKK activation. For example, TAK1, phosphoinositide 3-kinase, and NF-κB-inducing kinase regulate IKK activation (26, 46–50). Thus, these
[Ca\(^{2+}\)]_i and PKC Are Related to LPS-induced NF-κB Activation

results suggest that MEKK participates in, but is not the sole one responsible for IKK activation in LPS-stimulated rat peritoneal macrophages.

An important transcription factor for the expression of iNOS, ILs, and TNF-α, NF-κB is a p50/p65 dimer in macrophages. After binding with IκBα, NF-κB is inhibited. When IκBα is phosphorylated by IKK, the p50/p65 dimer dissociates from the complex and relocates to the nucleus; this process leads to NF-κB activation (14, 15). In the present study, after the activation of IKK, there was an increase in phosphorylated IκBα in the cytoplasm, p65 in the nucleolus, and NF-κB activation (Fig. 1).

In general, LPS evokes a series of events in rat peritoneal macrophages. The above analysis of these cells after treatment with kinase or enzyme inhibitors revealed a series of biological events upon which we propose the components and their sequence in the Ca\(^{2+}\) - and PKC-dependent signaling pathway for NF-κB activation (Fig. 7). First, LPS acts with TLR4 and mediates PTK activation in macrophages. Second, PTK may induce PLC\(\gamma\)1 and PLC\(\gamma\)2 phosphorylation, which leads to PtdIns(4,5)\(P_2\) hydrolysis and thus production of DAG and Ins(1,4,5)\(P_3\). With the increase of Ins(1,4,5)\(P_3\) in the cytoplasm, intracellular pools release Ca\(^{2+}\), and this release may be followed by Ca\(^{2+}\) influx from extracellular spaces. Activated PLC\(\gamma\) and the transient increase in [Ca\(^{2+}\)]\(_i\), activate PKC isoenzymes. Next, PKCB regulates the phosphorylation of serine in MEKK1. Activated MEKK participates in IKK activation, and activated IKK in turn activates NF-κB by phosphorylating IκBα and inducing the translocation of the p65/p50 dimer into the nucleus. Finally, the expression of iNOS and the production of TNF-α are elevated.

The PLC inhibitor, the PKC inhibitor, and the MEKK inhibitor did not inhibit LPS-induced IKK activation completely, but the PTK inhibitor eliminated all of these events. Therefore, it is reasonable to regard this LPS-activated pathway as one involved in NF-κB activation. In fact, LPS activates IKK and NF-κB by a complex signaling network. Meanwhile, LPS also activates MAPKs. Then, activator protein-1, ATF, and other transcription factors are activated. Therefore, we hypothesize that the cooperation of these signaling pathways may be responsible for the activation of transcription factors that promote the expression and release of inflammatory mediators.

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Ca$^{2+}$- and Protein Kinase C-dependent Signaling Pathway for Nuclear Factor-κB Activation, Inducible Nitric-oxide Synthase Expression, and Tumor Necrosis Factor-α Production in Lipopolysaccharide-stimulated Rat Peritoneal Macrophages

Xueyuan Zhou, Wenxiu Yang and Junying Li

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