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Induction of Nitric Oxide Synthase in RAW 264.7 Macrophages by Lipoteichoic Acid from *Staphylococcus aureus:* Involvement of Protein Kinase C- and Nuclear Factor-κB-Dependent Mechanisms

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Key Words
Lipoteichoic acid · Inducible nitric oxide synthase · Nitric oxide · Protein kinase C · NF-κB · RAW 264.7 macrophages

Abstract
This study investigates the signaling pathway involved in inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) release caused by *Staphylococcus aureus* lipoteichoic acid (LTA) in RAW 264.7 macrophages. A phosphatidylcholine-phospholipase C (PC-PLC) inhibitor (D-609) and a phosphatidylinositol-phospholipase C (PI-PLC) inhibitor (U-73122) attenuated LTA-induced iNOS expression and NO release. Two PKC inhibitors (Go 6976 and Ro 31-8220), an NF-κB inhibitor (pyrrolidine dithiocarbamate; PDTC), and long-term (24 h) 12-phorbol-13-myristate acetate (PMA) treatment each also inhibited LTA-induced iNOS expression and NO release. Treatment of cells with LTA caused an increase in PKC activity; this stimulatory effect was inhibited by D-609, U-73122, or Ro 31-8220. Stimulation of cells with LTA caused IκB-α phosphorylation and IκB-α degradation in the cytosol, and translocation of p65 and p50 NF-κB from the cytosol to the nucleus. Treatment of cells with LTA caused NF-κB activation by detecting the formation of NF-κB-specific DNA-protein complexes in the nucleus; this effect was inhibited by Go 6976, Ro 31-8220, long-term PMA treatment, PDTC, *L*-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), and calpain inhibitor I. These results suggest that LTA might activate PC-PLC and PI-PLC to induce PKC activation, which in turn initiates NF-κB activation, and finally induces iNOS expression and NO release in RAW 264.7 macrophages.

Introduction

Nitric oxide (NO), a small radical gas, is involved in the regulation of vascular tone, platelet and leukocyte adhesion, neurotransmission, and mediation of excessive vasodilatation and cytotoxic actions of macrophages against microbes and tumor cells [20, 22]. NO is enzymat-

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ically formed from *L*-arginine by nitric oxide synthase (NOS). Two major classes of NOS have been described based on their expression and regulation. The constitutive form present in neurons or endothelial cells is a calcium-dependent enzyme. The inducible form (iNOS), on the other hand, present in macrophages and other cells, is regulated at the transcriptional level in response to lipopolysaccharide (LPS) or certain proinflammatory cytokines and does not require calcium for its activity [14, 21]. The production of large amounts of NO by iNOS has been implicated in the genesis of septic and cytokine-induced circulatory shock [30].

Septic shock can be defined as sepsis with hypotension resulting in impaired tissue perfusion despite adequate fluid resuscitation. Traditionally recognized as a consequence of gram-negative bacteremia, septic shock is also caused by gram-positive organisms, fungi, and probably viruses and parasites. Although relatively rare in the 1970s, the incidence of gram-positive septic shock has increased markedly over the past 15 years, and today between one third and one half of all cases of sepsis are caused by gram-positive organisms [4]. Endotoxin, a component of the outer membrane of gram-negative bacteria, has been identified as the prime initiator of gram-negative bacterial septic shock. In contrast to endotoxic shock, we know relatively little about the mechanisms of gram-positive bacterium-induced septic shock. However, lipoteichoic acid (LTA) from *Staphylococcus aureus* causes the induction of iNOS in murine macrophages [8] and in vascular smooth muscle cells [1]. LTA also produces circulatory failure (hypotension and vascular hyporeactivity to vasoconstrictor agents) in rats by induction of the iNOS protein and an increase in iNOS activity [9].

The promoter region for iNOS of the mouse gene has been characterized [32]. Several binding sites for transcription factors have been identified in the promoter region of the iNOS gene including nuclear factor-κB (NF-κB), activator protein-1, various members of the CCAAT/enhancer-binding protein, activating transcription factor (ATF)/cAMP response element binding protein, and the STAT family of transcription factors [5, 18, 34]. Of these transcription factors, only the activation of NF-κB has been shown to mediate enhanced expression of the iNOS gene in macrophages exposed to LPS [6]. Furthermore, previous studies have shown a potential role of tyrosine kinase and phosphatidylcholine-phospholipase C (PC-PLC) in the LTA-induced iNOS expression in murine J744.2 macrophages [15]. However, the signal pathways for LTA-induced iNOS expression are still unknown. In the present study, the intracellular signaling pathway by which LTA induces iNOS expression in RAW 264.7 macrophages was studied. The results show that LTA might activate PC-PLC and phosphatidylinositol phospholipase (PI-PLC) to induce protein kinase C (PKC) activation, which in turn initiates NF-κB activation, finally inducing iNOS expression and NO release in RAW 264.7 macrophages.

Materials and Methods

Materials

LTA (derived from *S. aureus*), LPS (derived from *Escherichia coli*), N^G-nitro-*L*-arginine methyl ester (*L*-NAME), 12-phorbol-13-myristate acetate (PMA), actinomycin D, cycloheximide, polymyxin B, pyrrolidine dithiocarbamate (PDTC), *L*-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), sulfanilamide, N-(1-naphthyl)-ethylenediamine, Trizma base, dithiothreitol (DTT), glycerol, phenylmethylsulphonyl fluoride (PMSF), pepstatin A, leupeptin, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chem. (St. Louis, Mo., USA). Go 6976, Ro 31-8220, and calpain inhibitor I were purchased from Calbiochem-Novabiochem (San Diego, Calif., USA). D-609 and U-73122 were obtained from RBI (Natick, Mass., USA). Penicillin/streptomycin, fetal calf serum (FCS), T4 polynucleotide kinase, and Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 were purchased from Life Technologies (Gaithersburg, Md., USA). Mouse monoclonal antibodies specific for the NF-κB subunit (p65 or p50) were purchased from Transduction Laboratories (Lexington, Ky., USA). Rabbit polyclonal antibodies specific for iNOS, IκB-α, phospho-IκB-α, and anti-rabbit-IgG-conjugated alkaline phosphatase were purchased from Santa Cruz Biochemicals (Santa Cruz, Calif., USA). Anti-mouse-IgG-conjugated alkaline phosphatase was purchased from Jackson Immuno Research Laboratories (West Grove, Pa., USA). The NF-κB probe was purchased from Promega (Madison, Wisc., USA). The PKC [^{32}P] enzyme assay system was purchased from Amersham International (Buckinghamshire, UK). 4-Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Boehringer Mannheim (Mannheim, Germany). Protein assay reagents were purchased from Bio-Rad (Hercules, Calif., USA).

Cell Culture

RAW 264.7 cells, a murine macrophage cell line, were obtained from American Type Culture Collection and grown in DMEM supplemented with 10% FCS and penicillin/streptomycin (50 U/ml) in a humidified 37°C incubator.

Measurement of NO Concentration

NO production was assayed by measuring nitrite (a stable degradation product of NO) in supernatant of cultured RAW 264.7 cells using the Griess reagent. Briefly, RAW 264.7 macrophages were cultured in 24-well plates. After reaching confluence, the culture medium was changed to phenol-red-free DMEM. Cells were then treated with LTA (1–30 μg/ml) for 24 h or LTA (10 μg/ml) for the indicated time intervals. The supernatant was collected, mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine, 2% phosphoric acid), and incubated at room temperature for 10 min. The absorbance measured at 550 nm

in a microplate reader was used as an indication of nitrite concentration. Sodium nitrite ($NaNO_2$) was used to produce a standard curve of nitrite concentration. In some experiments, cells were pretreated with specific inhibitors as indicated followed by 10 μg/ml LTA and then were incubated in a humidified incubator at 37°C for 24 h.

Protein Preparation and Western Blot Analysis

For determination of the expressions of iNOS and α-tubulin in RAW 264.7 cells, the preparation of total proteins and Western blot analysis were performed as described previously [17]. Briefly, RAW 264.7 cells were cultured in 10-cm petri dishes. After reaching confluence, cells were treated with LTA (1–30 μg/ml) for 24 h or LTA (10 μg/ml) for the indicated times and then were incubated in a humidified incubator at 37°C. In some experiments, cells were pretreated with specific inhibitors as indicated followed by LTA (10 μg/ml), and then incubated in a humidified incubator at 37°C. After incubation, cells were washed with phosphate-buffered saline (PBS, pH 7.4). Proteins were extracted with solution containing 10 m*M* Tris (pH 7.0), 140 m*M* NaCl, 0.5% NP-40, 2 m*M* PMSF, 5 m*M* DTT, 0.05 m*M* pepstatin A, and 0.2 m*M* leupeptin, centrifuged, mixed 1:1 with sample buffer [100 m*M* Tris (pH 6.8), 20% glycerol, 4% SDS, and 0.2% bromphenol blue], and boiled for 5 min. Electrophoresis was performed using 10% SDS-polyacrylamide gel (2 h, 110 V, 40 mA, 30 μg of protein per lane). Separated proteins were transferred to PVDF membranes (2 h, 40 V); nonspecific IgGs were blocked with 5% fat-free milk powder, and incubated for 2 h with specific antibodies against iNOS or α-tubulin. The blot was then incubated with anti-rabbit-IgG linked to alkaline phosphatase (1:1,000) for 2 h. Subsequently, the membrane was developed with NBT/BCIP as a substrate.

Analysis of PKC Activity

For the detection of PKC activity, cytosolic and membrane fractions were separated as described previously [16]. Briefly, RAW 264.7 cells were incubated with vehicle or LTA (10 μg/ml) the for indicated time intervals, or pretreated with specific inhibitors as indicated followed by LTA, and then incubated in a humidified incubator at 37°C. After incubation, cells were scraped, collected, homogenized in ice-cold homogenization buffer [20 m*M* Tris, 2 m*M* EDTA, 5 m*M* EGTA, 20% (v/v) glycerol, 2 m*M* PMSF, 1% (v/v) aprotinin, 5 m*M* DTT] for 20 min, sonicated for 10 s, and then centrifuged at 800 *g* for 10 min. The supernatant (cytosolic and membrane fraction) was removed and centrifuged at 25,000 *g* for 15 min. The supernatant (cytosolic fraction) was collected for further studies. The pellets (membrane fraction) were solubilized in homogenization buffer containing 0.1% NP-40. The PKC activity was assayed using the PKC activity assay kit (Amersham) according to the procedure described by the manufacturer.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay

RAW 264.7 macrophages were cultured in 10-cm culture Petri dishes. After reaching confluence, the cells were treated with vehicle or LTA (10 μg/ml) for the indicated time intervals, and then incubated in a humidified incubator at 37°C. In some experiments, the cells were pretreated with Go 6976 (1 μ*M*), Ro 31-8220 (1 μ*M*), PDTC (50 μ*M*), TPCK (30 μ*M*), or calpain inhibitor I (10 μ*M*) for 30 min, or with PMA (1 μ*M*) for 24 h followed by LTA (10 μg/ml), and then incubated in a humidified incubator at 37°C for 30 min. The cytosolic and nuclear protein fractions were then separated as described previously [6]. Briefly, the cells were washed with ice-cold PBS, and then centrifuged. The cell pellet was resuspended in hypotonic buffer [10 m*M* HEPES (pH 7.9), 10 m*M* KCl, 0.5 m*M* DTT, 10 m*M* aprotinin, 10 m*M* leupeptin, and 20 m*M* PMSF] for 15 min on ice, and vortexed for 10 s. The nuclei were pelleted by centrifugation at 15,000 *g* for 1 min. The supernatants containing cytosolic proteins were collected. A pellet containing nuclei was resuspended in hypertonic buffer [20 m*M* HEPES (pH 7.6), 25% glycerol, 1.5 m*M* $MgCl_2$, 4 m*M* EDTA, 0.05 m*M* DTT, 20 m*M* PMSF, 10 m*M* aprotinin, and 10 m*M* leupeptin] for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 15,000 *g* for 2 min and stored at −70°C. In studies of p65 or p50 translocation, both cytosolic and nuclear extracts were used; only cytosolic extracts were used for determination of the expressions of IκB-α and phospho-IκB-α. Extracts were subjected to SDS-PAGE using a 10% running gel, and Western blot analysis was performed as described above.

A double-stranded oligonucleotide probe containing NF-κB sequences (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega) was purchased and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The nuclear extract (2.5–5 μg) was incubated with 1 ng of a ^{32}P-labeled NF-κB probe (50,000–75,000 cpm) in 10 μl of binding buffer containing 1 μg poly(dI-dc), 15 m*M* HEPES (pH 7.6), 80 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT, and 10% glycerol at 30°C for 25 min. DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on 6% polyacrylamide gels; then the gels were vacuum-dried and subjected to autoradiography with an intensifying screen at −80°C. Quantitative data were obtained using a computing densitometer with Image-Pro plus software (Media Cybernetics, Silver Spring, Md., USA).

Statistical Analysis

Results are expressed as the mean ± SEM from 3–4 independent experiments. One-way analysis of variance (ANOVA) followed by, when appropriate, Bonferroni's multiple-range test was used to determine the statistical significance of the difference between means. A p value of less than 0.05 was taken as statistical significance.

Results

Characterization of LTA-Induced NO Production and iNOS Expression in RAW 264.7 Cells

The basal level of nitrite released from RAW 264.7 cells was low (4.8 ± 0.8 μ*M*, n = 4). However, incubation of RAW 264.7 cells with bacterial LTA (1–30 μg/ml) for 24 h resulted in a dose-dependent increase in nitrite production and the expression of 130-kD iNOS (fig. 1a, b). LTA (10 μg/ml)-induced increases in nitrite production and iNOS expression were time dependent (fig. 1c, d). In the following experiments, cells were treated with 10 μg/ml LTA for 24 h. Pretreatment of cells with the transcriptional inhibitor, actinomycin D (0.3 μ*M*), or the translational inhibitor, cycloheximide (1 μ*M*), for 30 min markedly attenuated LTA-induced nitrite release and iNOS expression (data not shown) suggesting that iNOS expres-

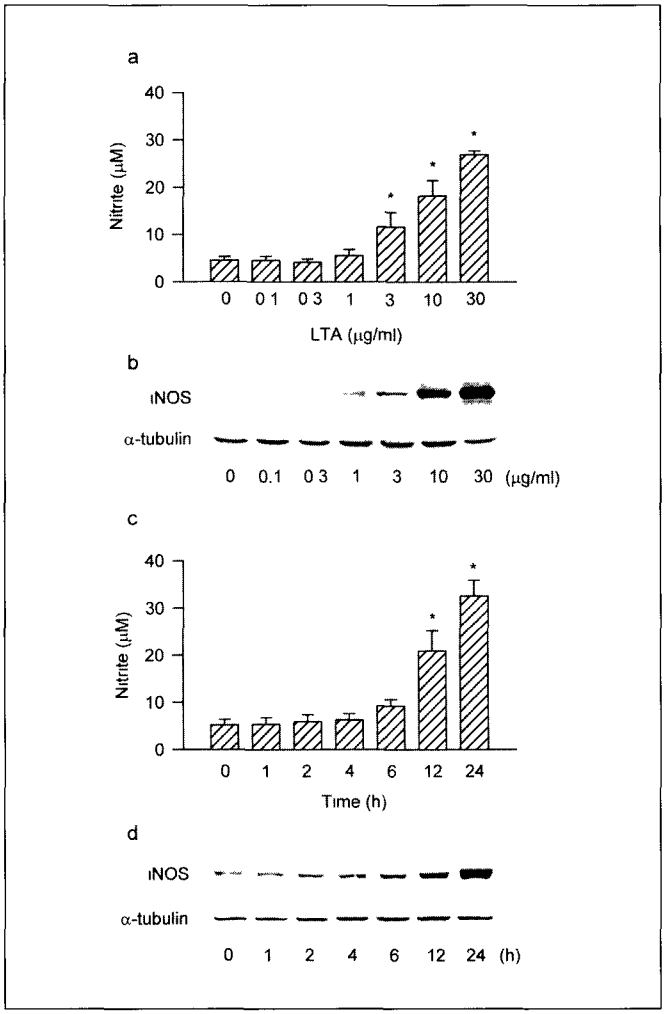

Fig. 1. Concentration- and time-dependent increases in nitrite release and iNOS expression caused by LTA in RAW 264.7 macrophages. Cells were incubated with various concentrations of LTA for 24 h (**a**) or with LTA (10 μg/ml) for various time intervals (**c**). Media were collected for nitrite determination. Results are expressed as the mean ± SEM of 4 independent experiments performed in triplicate. * $p < 0.05$ as compared with the basal level. Cells were incubated with various concentrations of LTA for 24 h (**b**) or with LTA (10 μg/ml) for the indicated time intervals (**d**), then immunodetected using specific antibodies against iNOS or α-tubulin as described in 'Methods'. Data are representative of 3 independent experiments which gave essentially identical results. Equal loading in each lane is demonstrated by similar intensities of α-tubulin.

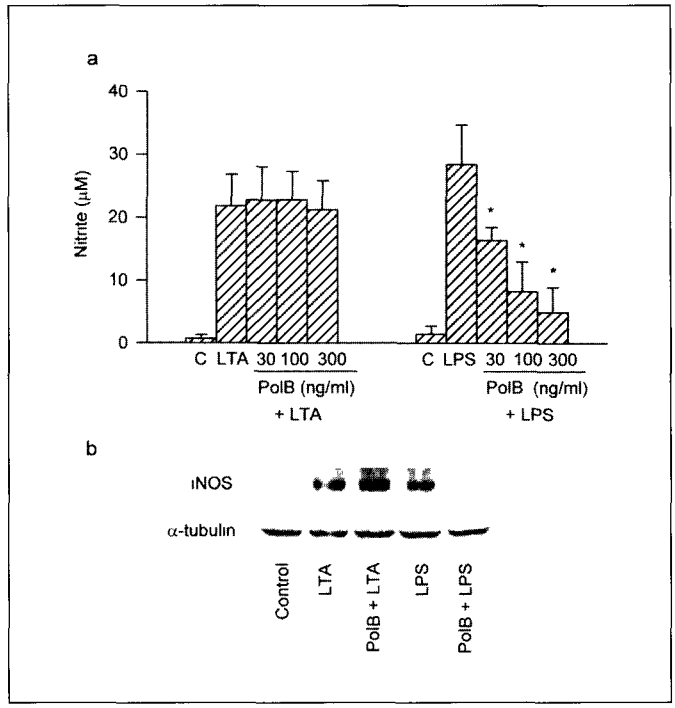

Fig. 2. Effects of polymycin B on LTA- or LPS-mediated nitrite release and iNOS expression in RAW 264.7 macrophages. **a** Cells were pretreated with various concentrations of polymycin B for 30 min followed by LTA (10 μg/ml) or LPS (1 μg/ml) treatment for 24 h. Media were collected for measurement of nitrite release. Results are expressed as the mean ± SEM of 3 independent experiments performed in triplicate. * $p < 0.05$ as compared with the LTA- or LPS-treated group, respectively. **b** Cells were pretreated with polymyxin B (300 ng/ml) for 30 min followed by LTA (10 μg/ml) or LPS (1 μg/ml) treatment for 24 h, and then immunodetected with specific antibodies against iNOS or α-tubulin as described in 'Methods'. Data are representative of 3 independent experiments which gave essentially identical results. Equal loading in each lane is demonstrated by similar intensities of α-tubulin. PolB = Polymycin B.

sion and NO production are dependent on de novo transcription and translation. Pretreatment of cells with polymyxin B (30–300 ng/ml), which binds and inactivates endotoxin, for 30 min dose-dependently attenuated LPS (1 μg/ml)-induced nitrite production, while it had no effect on LTA-induced effects (fig. 2a). Moreover, polymyxin B (300 ng/ml) markedly attenuated LPS- but not LTA-induced iNOS expression (fig. 2b). On the other hand, a nonselective NOS inhibitor, *L*-NAME (30, 100, and 300 μ*M*), markedly decreased LTA-induced nitrite release by 45.6, 75.8, and 97.9%, respectively.

Role of PKC in LTA-Induced NO Production and iNOS Expression

To determine whether PKC activation is involved in the signal transduction pathway leading to iNOS expression and NO production caused by LTA, the PKC inhibitors Go 6976 and Ro 31-8220 were used. Pretreatment of

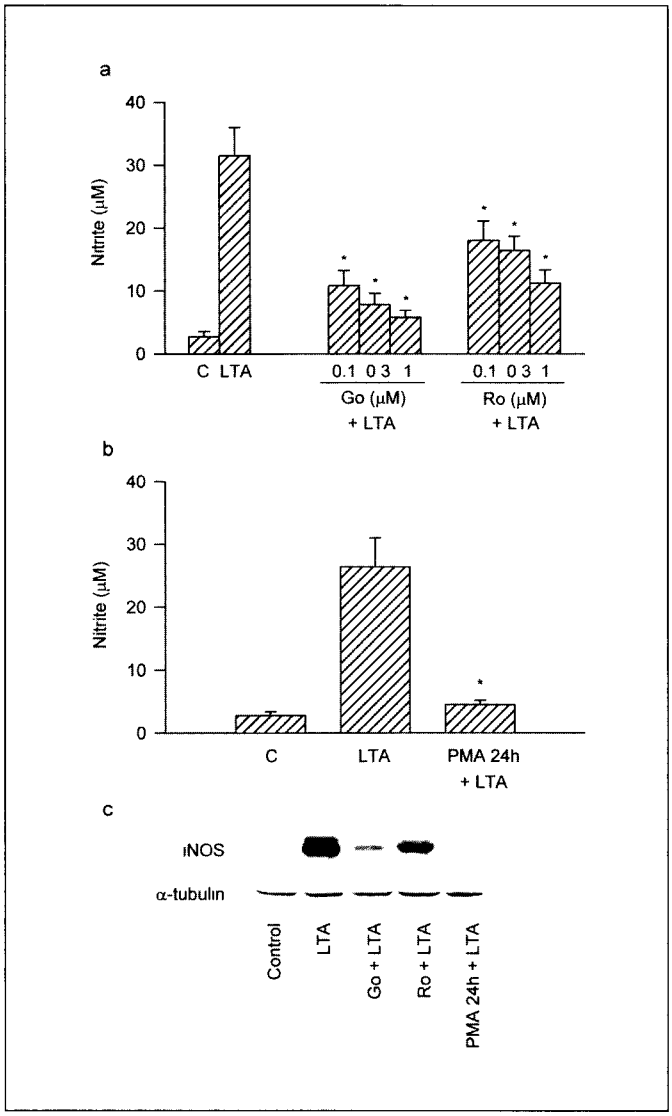

Fig. 3. Effects of Go 6976, Ro 31-8220, and long-term PMA treatment on LTA-mediated nitrite release and iNOS expression in RAW 264.7 macrophages. Cells were pretreated with various concentrations of Go 6976 or Ro 31-8220 for 30 min (**a**), or 1 μ*M* PMA for 24 h (**b**) followed by LTA (10 μg/ml) treatment for 24 h. The supernatant was collected for nitrite measurement. Results are expressed as the mean ± SEM of 3 independent experiments performed in triplicate. * $p < 0.05$ as compared with the LTA-treated group. **c** Cells were pretreated with Go 6976 (1 μ*M*) or Ro 31-8220 (1 μ*M*) for 30 min, or 1 μ*M* PMA for 24 h followed by LTA (10 μg/ml) treatment for 24 h, and then immunodetected using specific antibodies against iNOS or α-tubulin as described in 'Methods'. Data are representative of 3 independent experiments which gave essentially identical results. Equal loading in each lane is demonstrated by similar intensities of α-tubulin. Go = Go 6976; Ro = Ro 31-8220.

cells for 30 min with Go 6976 (0.1–1 μ*M*) or Ro 31-8220 (0.1–1 μ*M*) concentration-dependently attenuated LTA-induced nitrite production (fig. 3a). Treatment of RAW 264.7 cells with Go 6976 (1 μ*M*) or Ro 31-8220 (1 μ*M*) alone had no effect on basal nitrite production (data not shown). LTA-induced iNOS expression was also inhibited by 1 μ*M* Go 6976 or 1 μ*M* Ro 31-8220 (fig. 3c). Furthermore, long-term (24 h) treatment of cells with PMA (1 μ*M*), which causes downregulation of PKC, also almost completely inhibited LTA-induced nitrite production and iNOS expression (fig. 3b, c). When cells were pretreated for 30 min with a PC-PLC inhibitor (D-609, 0.25–25 μ*M*) or a PI-PLC inhibitor (U-73122, 0.5–5 μ*M*), LTA-mediated nitrite release was inhibited in a concentration-dependent manner (fig. 4a). Treatment of RAW 264.7 cells with D-609 (25 μ*M*) or U-73122 (5 μ*M*) alone had no effect on basal nitrite production (data not shown). Furthermore, D-609 (25 μ*M*) and U-73122 (5 μ*M*) also individually inhibited LTA-induced iNOS expression (fig. 4a).

Treatment of RAW 264.7 cells with 10 μg/ml LTA for various time intervals resulted in a decrease in PKC activity in the cytosol and an increase in PKC activity in the membrane fraction, with a significant effect at 30 min, which was sustained to 120 min (fig. 5a). Exposure of cells to 1 μ*M* PMA for 30 min also resulted in a decrease in PKC activity in the cytosol and an increase in PKC activity in the membrane fraction (fig. 5a). The LTA-induced increase in PKC activity in the membrane was inhibited by a 30-min pretreatment with 25 μ*M* D-609, 5 μ*M* U-73122, or 1 μ*M* Ro 31-8220 (fig. 5b).

Role of Transcription Factor NF-κB on LTA-Induced NO Production and iNOS Expression

To determine whether NF-κB activation is involved in the signal transduction pathway leading to iNOS expression caused by LTA, cells were pretreated with an NF-κB inhibitor (PDTC) or IκB protease inhibitors (TPCK or calpain inhibitor I), followed by LTA treatment. Pretreatment of cells for 30 min with PDTC (10–50 μ*M*), TPCK (3 and 30 μ*M*), or calpain inhibitor I (3 and 10 μ*M*) attenuated LTA-induced nitrite production in a concentration-dependent manner (fig. 6a, b). Treatment of RAW 264.7 cells with PDTC (50 μ*M*), TPCK (30 μ*M*), or calpain inhibitor I (10 μ*M*) alone did not affect basal nitrite production (data not shown). LTA-induced iNOS expression was also attenuated by 50 μ*M* PDTC, 30 μ*M* TPCK, or 10 μ*M* calpain inhibitor I (fig. 6c). Stimulation of RAW 264.7 cells with LTA (10 μg/ml) for 10–60 min resulted in marked phosphorylation of IκB-α in the cytosol. How-

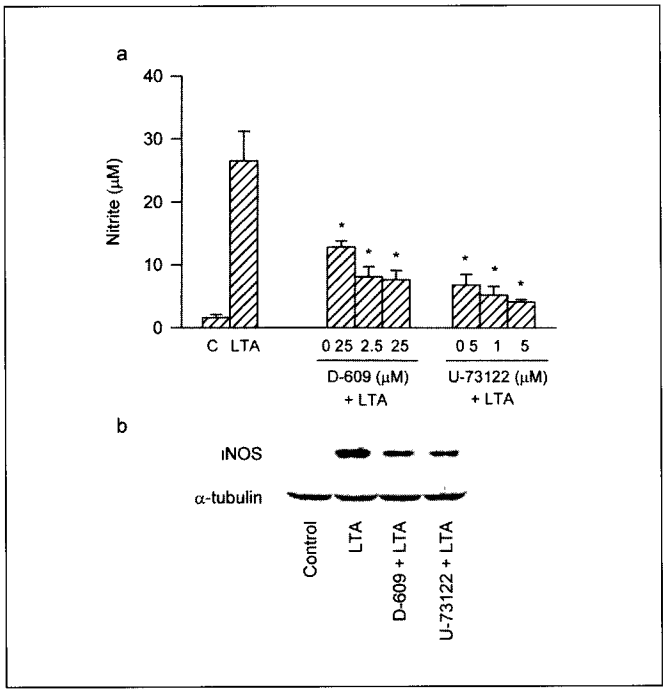

Fig. 4. Effects of D-609 and U-73122 on LTA-mediated nitrite release and iNOS expression in RAW 264.7 macrophages. **a** Cells were pretreated with various concentrations of D-609 or U-73122 for 30 min followed by LTA (10 μg/ml) treatment for 24 h. The supernatant was collected for nitrite measurement. Results are expressed as the mean ± SEM of 3 independent experiments performed in triplicate. * $p < 0.05$ as compared with the LTA-treated group. **b** Cells were pretreated with D-609 (25 μ*M*) or U-73122 (5 μ*M*) for 30 min followed by LTA (10 μg/ml) treatment for 24 h, and then immunodetected using specific antibodies against iNOS or α-tubulin as described in 'Methods'. Data are representative of 3 independent experiments which gave essentially identical results. Equal loading in each lane is demonstrated by similar intensities of α-tubulin.

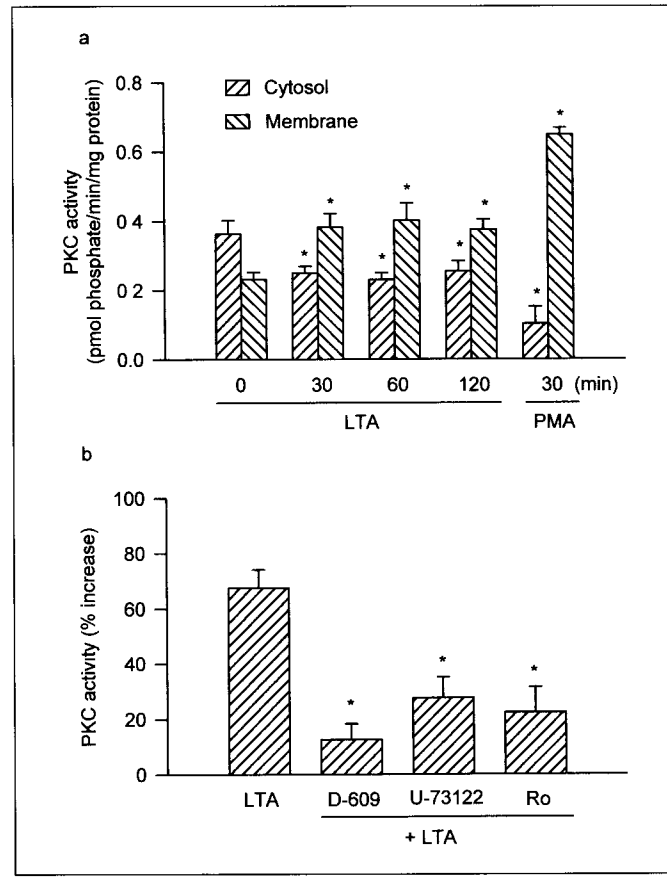

Fig. 5. PKC activity caused by LTA and PMA in the cytosol and membrane fractions of RAW 264.7 macrophages and effects of various inhibitors on LTA-mediated increases in PKC activity in the membrane fraction of RAW 264.7 macrophages. Cells were treated with LTA (10 μg/ml) for various time intervals, or PMA (1 μ*M*) for 30 min (**a**), or pretreated with D-609 (25 μ*M*), U-73122 (5 μ*M*), or Ro 31-8220 (1 μ*M*) for 30 min followed by LTA (10 μg/ml) treatment for 30 min (**b**). The subcellular (cytosol and membrane) fractions were then isolated for the measurement of PKC activities in the cytosolic and membrane fractions as described in 'Methods'. Results are expressed as the mean ± SEM of 3 independent experiments performed in duplicate. * $p < 0.05$ as compared with the basal level (**a**) or LTA alone (**b**). Ro = Ro 31-8220.

ever, it only caused a partial degradation of IκB-α in the cytosol after 10–30 min of treatment. Stimulation of cells with LTA for various time intervals also caused translocation of p65 and p50 NF-κB from the cytosol to the nucleus, with a maximal effect at 30 min. LTA-induced translocations of p65 and p50 NF-κB gradually decreased after 60–120 min of treatment (fig. 7a). In nuclear extracts of unstimulated cells, a slight intensification of the formation of an NF-κB-specific DNA-protein complex was detected. Stimulation of cells with LTA (10 μg/ml) for 10–30 min resulted in marked activation of the formation of the NF-κB-specific DNA-protein complex. However, after 60–120 min of treatment, intensities of these DNA-protein complexes had decreased (fig. 7b). The formation of the NF-κB complex was completely inhibited by addition of the 50× cold NF-κB consensus DNA sequence (fig. 7b), indicating that the DNA-protein interactions were sequence specific. When cells were pretreated for 30 min with 1 μ*M* Go 6976, 1 μ*M* Ro 31-8220, 50 μ*M* PDTC, 30 μ*M* TPCK, or 10 μ*M* calpain inhibitor I, or for 24 h with 1 μ*M* PMA, LTA-induced activation of the formation of the NF-κB-specific DNA-protein complex was partially inhibited by Go 6976, Ro 31-8220, or long-term PMA treatment, and markedly inhibited by PDTC, TPCK, or calpain inhibitor I (fig. 8a, b).

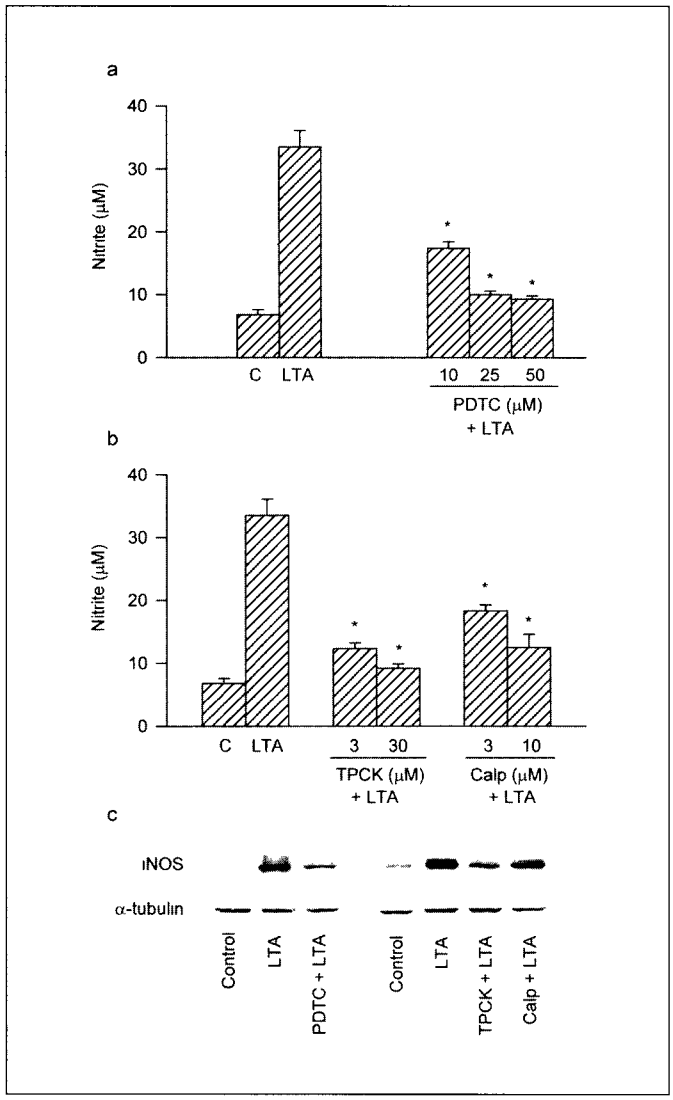

Fig. 6. Effects of PDTC, TPCK, and calpain inhibitor I on LTA-mediated nitrite release and iNOS expression in RAW 264.7 macrophages. Cells were pretreated with various concentrations of PDTC (**a**), TPCK, or calpain inhibitor I (**b**) for 30 min followed by 10 μg/ml LTA treatment for 24 h, and then the media were collected for nitrite measurement. Results are expressed as the mean ± SEM of 3 independent experiments performed in triplicate. * $p < 0.05$ as compared with the LTA-treated group. **c** Cells were pretreated with 50 μ*M* PDTC, 30 μ*M* TPCK, or 10 μ*M* calpain inhibitor I for 30 min followed by 10 μg/ml LTA treatment for 24 h, and then immunodetected using specific antibodies against iNOS or α-tubulin as described in 'Methods'. Data are representative of 3 independent experiments which gave essentially identical results. Equal loading in each lane is demonstrated by similar intensities of α-tubulin. Calp = calpain inhibitor I.

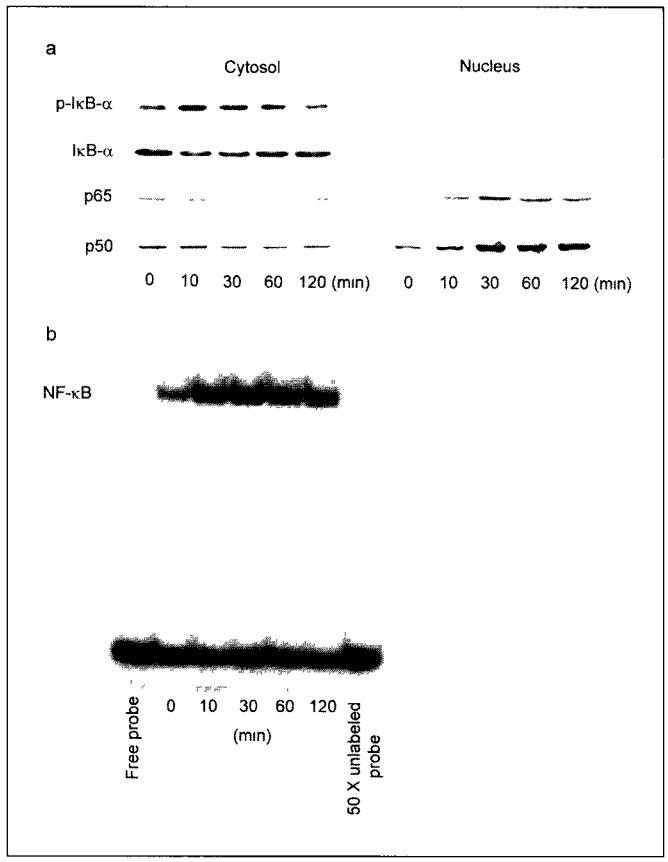

Fig. 7. Kinetics of LTA-induced IκB-α phosphorylation, IκB-α degradation, NF-κB translocation, and NF-κB-specific DNA-protein complex formation in RAW 264.7 macrophages. Cells were pretreated with LTA (10 μg/ml) for various time intervals, and then subcellular (cytosolic and nuclear) fractions were prepared as described in 'Methods'. **a** Levels of phosphorylated IκB-α (p-IκB-α) and IκB-α protein in the cytosol, and p65 and p50 in the cytosolic and nuclear fractions were immunodetected with specific antibodies against phospho-IκB-α, IκB-α, p65, or p50, respectively, as described in 'Methods'. **b** NF-κB-specific DNA-protein-binding activity in nuclear extracts was determined using an electrophoretic mobility shift assay as described in 'Methods'. Data are representative of 3 independent experiments which gave essentially identical results.

Discussion

The results of the present study demonstrate that the LTA-induced increase in NO release in RAW 264.7 macrophages is a consequence of the induction of iNOS, and indicate that PC-PLC, PI-PLC, PKC, and transcription factor NF-κB might be involved in signal transduction leading to the expression of iNOS in these cells. Pretreatment of cells with polymyxin B, which binds and inacti-

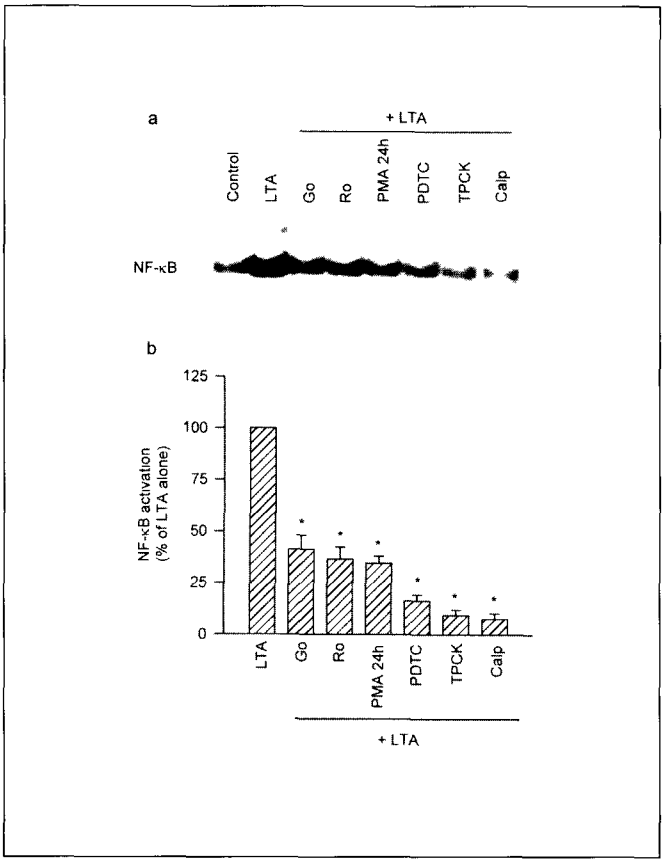

Fig. 8. Effects of various inhibitors or long-term PMA treatment on LTA-induced formation of the NF-κB DNA-protein complex in nuclear extracts of RAW 264.7 macrophages. **a** Cells were pretreated for 30 min with 1 μ*M* Go 6976, 1 μ*M* Ro 31-8220, 50 μ*M* PDTC, 30 μ*M* TPCK, or 10 μ*M* calpain inhibitor I for 30 min, or 1 μ*M* PMA for 24 h followed by 10 μg/ml LTA for 30 min. The nuclear extracts were prepared for assaying the NF-κB DNA-protein binding activity by electrophoretic mobility shift assay as described in 'Methods'. **b** The extent of NF-κB activation was quantitated using a densitometer with Image-Pro plus software. Results are expressed as the mean ± SEM of 3 independent experiments. * $p < 0.05$ as compared with the LTA-treated group. Go = Go 6976; Ro = Ro 31-8220; Calp = calpain inhibitor I.

vates LPS [15], did not affect LTA-mediated iNOS expression or NO release, indicating that the inductions of iNOS protein and NO release caused by LTA were not due to LPS contamination.

PKC is a family of serine/threonine kinases that appear to mediate various cellular functions [13, 23]. In the present study, we demonstrate that LTA-mediated iNOS expression and NO release were prevented by 2 PKC inhibitors (Go 6976 and Ro 31-8220) and by long-term (24 h) PMA treatment. Furthermore, we found that treatment of RAW 264.7 cells with LTA caused an increase in PKC activity. These results indicate that PKC activation is involved in LTA-mediated signal transduction leading to expression of the iNOS protein. Diacylglycerol (DAG) is a physiological activator of PKC [23]. Several mechanisms have been suggested to be responsible for the signal-mediated formation of DAG. The formation of DAG can be generated by the action of PI-PLC and PC-PLC [11, 23]. Previous reports have shown that D-609 selectively inhibits PC-PLC activity without affecting the activities of phospholipase A_2, phospholipase D, or PI-PLC [26]. It has been demonstrated that U-73122 inhibits PI-PLC activation in human platelets and neutrophils [3]. In the present study, we demonstrate that D-609 and U-73122 inhibit LTA-mediated increases in PKC activity, iNOS expression, and NO release, indicating that LTA might activate PC-PLC and PI-PLC to induce PKC activation, which in turn induces iNOS expression and NO release.

It has been demonstrated that transcription factor NF-κB is involved in LPS-induced expression of the iNOS protein [6]. In the present study, we also demonstrate that LTA-mediated iNOS expression and nitrite release are inhibited by the NF-κB inhibitor, PDTC, and the IκB protease inhibitors, TPCK and calpain inhibitor I. These results indicate that NF-κB activation is involved in LTA-mediated signal transduction leading to expression of the iNOS protein. NF-κB is constitutively present in cells as a heterodimer, consisting of a p50 DNA-binding subunit and a p65 trans-activating subunit. In cells in a resting condition, the inhibitor subunit IκB-α is bound to the p50/p65 heterodimer of NF-κB in the cytoplasm [29]. Treatment of cells with IL-1β or TNF-α results in the specific phosphorylation of 2 serine residues on IκB-α followed by the ubiquitination and degradation of this subunit [24, 25, 29]. The release of IκB-α causes activation of NF-κB, which in turn is translocated to the nucleus and activates transcription [29]. We found that treatment of RAW 264.7 cells with LTA resulted in an increase in IκB-α phosphorylation, a partial degradation of IκB-α in the cytosol, and translocations of p65 and p50 NF-κB from cytosol to the nucleus. Recent studies have shown that LTA derived from *Enterococcus faecalis* can activate the formation of an NF-κB-specific DNA-protein complex in basal urothelial cells [10]. We also demonstrate that LTA increases the level of the NF-κB-specific DNA-protein complex in nuclear extracts of RAW 264.7 macrophages. The formation of the NF-κB-specific DNA-protein complex activated by LTA was inhibited by Go 6976, Ro 31-8220, and long-term PMA treatment (fig. 8). These

results indicate that PKC activation might be involved in LTA-induced NF-κB activation in RAW 264.7 macrophages.

Previous reports have shown that LTA can induce the release of cytokines, including IL-1β, TNF-α, and IL-6 in cultured human monocytes [2, 19]. Moreover, proinflammatory cytokines have been demonstrated to induce increased expression of iNOS protein in RAW 264.7 macrophages [21]. Therefore, the LTA-induced iNOS expression might occur through the release of cytokines. Whether LTA can directly induce iNOS expression or not remains to be determined.

In conclusion, LTA might activate PC-PLC and PI-PLC to elicit PKC activation, which in turn initiates NF-κB activation, finally causing iNOS expression and NO release in RAW 264.7 macrophages. The results suggest that iNOS induction and the subsequent enhanced release of NO may be involved in inflammatory responses and septic shock elicited by gram-positive organisms. Based on the results of the present study and a previous report from Chen et al. [7], we found that the signal transduction pathway of LTA-induced iNOS expression is similar to that of LPS-induced iNOS expression in RAW 264.7 cells. One possible explanation for this similarity is that both LTA and LPS bind to the same receptor, CD 14 [12, 33], which is a glycosylphosphatidylinositiol-linked membrane receptor lacking an intracellular signaling domain. Moreover, a transmembrane co-receptor (signal-transducing receptor) for CD14 has been postulated [31]. Recently, toll-like receptor 2 (TLR2) and TLR4 have been identified as the signal-transducing receptors for LTA and LPS, respectively [27, 28]. It seems very likely that TLR2 and TLR4 possess a similar signal transduction pathway. By understanding these signal transduction pathways, we may be able to design therapeutic strategies to reduce inflammatory responses and septic shock caused by gram-positive organisms.

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