JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 115–123

Received June 14, 2002
Accepted September 27, 2002
DOI 10.1211/002235702504
ISSN 0022-3573

Involvement of nuclear factor-κB in lipoteichoic acid-induced cyclooxygenase-2 expression in RAW 264.7 macrophages

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Abstract

We have investigated the role of protein kinase C (PKC) and nuclear factor-κB (NF-κB) in cyclooxygenase-2 (COX-2) expression caused by *Staphylococcus aureus* lipoteichoic acid 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 lipoteichoic acid-induced COX-2 expression, while a phosphatidate phosphohydrolase inhibitor (propranolol) had no effect. Two PKC inhibitors (Go 6976 and Ro 31-8220) and the NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), also attenuated lipoteichoic acid-induced COX-2 expression. Lipoteichoic acid resulted in a decrease in PKC activity in the cytosol and an increase in PKC activity in membranes. The lipoteichoic acid-induced translocation of p65 NF-κB from the cytosol to the nucleus was inhibited by D-609, U-73122, Go 6976, Ro 31-8220, and PDTC, but not by propranolol. The results suggested that lipoteichoic acid might have activated PC-PLC and PI-PLC to induce PKC activation, which in turn initiated NF-κB activation, and finally induced COX-2 expression in RAW 264.7 macrophages.

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Funding: This work was supported by a research grant from the National Science Council of the Republic of China (NSC89-2314-B-038-048).

Introduction

Macrophages play important roles in the regulation of inflammation and immune responses. When activated, macrophages release growth factors, cytokines, and lipid mediators such as prostaglandins and leukotrienes. Secreted prostaglandins promote inflammation by increasing vascular permeability (Williams & Morley 1973) and vasodilation (Fletcher 1993) and by directing cellular migration into the site of inflammation through the production and release of proinflammatory cytokines (Williams & Shacter 1997). Induced prostaglandin synthesis is associated with the onset of symptoms resulting from acute immune system activation (Li et al 1999). Elevated prostaglandin levels are also associated with conditions of both chronic inflammation and cancer (Vane et al 1998). Due to the many potent effects of prostaglandins, control of prostaglandin synthesis is a critical element in the regulation of many physiological processes and the abatement of a number of pathophysiological conditions.

The synthesis of prostaglandins is dependent on the activity of the cyclooxygenase (COX) enzyme. COX converts arachidonic acid to prostaglandin H_2, which is then metabolized further to various prostaglandins, prostacyclin, or thromboxane A_2 (Vane et al 1998). Two COX isozymes, COX-1 and COX-2, have been identified in man and possess 60% homology (Xie et al 1991; Mitchell et al 1995). COX-1 is generally thought to produce prostaglandins, which serve to maintain cellular homeostasis, and to be expressed constitutively in many cell types including endothelial cells, platelets, and gastric mucosa (Vane 1994). COX-2, on the other hand, is induced by many pro-inflammatory stimuli, including cytokines and bacterial lipopolysaccharide (LPS) (Maier et al 1990; Mitchell et al 1993) in cells in-vitro and at the site of inflammation in-vivo (Vane et al 1994). Furthermore, COX-2 is believed to be responsible for the production of pro-inflammatory prostanoids in various models of inflammation (Chan et al 1995).

Although the incidence of Gram-positive infection has increased considerably over the last decade (Bone 1994), our knowledge regarding the mechanisms underlying the inflammatory responses caused by Gram-positive bacteria is still very limited. Cell walls of Gram-positive bacteria contain lipoteichoic acid and peptidoglycan, which themselves can activate leukocytes, stimulate the generation of pro-inflammatory cytokines, and hence cause a moderate systemic inflammatory response syndrome (Bhakdi et al 1991; Mattsson et al 1993). Lipoteichoic acid (a major component of Gram-positive bacterial cell walls) can also induce expression of inducible nitric oxide synthase (iNOS) in vascular smooth muscle cells (Auguet et al 1992) and macrophages (Kengatharan et al 1996). Recently, we have found that lipoteichoic acid inhibited platelet aggregation caused by collagen, thrombin, or ADP in human platelets (Sheu et al 2000). However, the expression of COX-2 induced by lipoteichoic acid has not been determined in RAW 264.7 macrophages, and the signal transduction events leading to the expression of COX-2 by lipoteichoic acid are unclear. The transcription factor nuclear factor-κB (NF-κB) plays a key role in transcriptional regulation of adhesion molecules, enzymes, and cytokines involved in chronic inflammatory diseases (Barnes & Karin 1997). Previous studies have shown a potential role of phosphatidylcholine-phospholipase C (PC-PLC) and NF-κB in lipoteichoic acid-induced iNOS expression in murine J744.2 macrophages (Kengatharan et al 1996). We have investigated the intracellular signalling pathway by which lipoteichoic acid induces COX-2 expression in RAW 264.7 macrophages.

Materials and Methods

Materials

Lipoteichoic acid (derived from *Staphylococcus aureus*), lipopolysaccharide (LPS derived from *Escherichia coli*), actinomycin D, cycloheximide, polymyxin B, propranolol, phorbol-12-myristate-13-acetate (PMA), pyrrolidine dithiocarbamate (PDTC), Trizma base, dithiothreitol (DTT), glycerol, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, leupeptin, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chem. (St Louis, MO). Go 6976 and Ro 31-8220 were purchased from Calbiochem-Novabiochem (San Diego, CA). D-609 and U-73122 were obtained from RBI (Natick, MA). Penicillin/streptomycin, foetal calf serum and Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 were purchased from Life Technologies (Gaithersburg, MD). Antibodies specific for COX-2, p65, and α-tubulin were purchased from Transduction Laboratories (Lexington, KY). Anti-mouse IgG-conjugated alkaline phosphatase was purchased from Jackson Immuno Research Laboratories (West Grove, PA). Protein kinase C (PKC) ^{32}P enzyme assay system was purchased from Amersham (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, CA).

Cell culture

RAW 264.7 cells, a murine macrophage cell line, were obtained from the American Type Culture Collection and grown in DMEM supplemented with 10% foetal calf serum and penicillin/streptomycin (50 U mL^{-1}) in a humidified 37°C incubator.

Protein preparation and Western blotting

For determination of the expressions of COX-2 and α-tubulin in RAW 264.7 cells, the preparation of total proteins and Western blotting were performed as described by Lin et al (2000). Briefly, RAW 264.7 cells were cultured in 10-cm Petri dishes. After reaching confluence, cells were treated with lipoteichoic acid (1–30 μg mL^{-1}) for 24 h or lipoteichoic acid (10 μg mL^{-1}) for indicated times (0, 1, 2, 4, 6, 12 or 24 h) and incubated in a humidified incubator at 37°C. In some experiments, cells were incubated with specific inhibitors as indicated for 30 min before lipoteichoic acid (10 μg mL^{-1}) treatment. After incubation, cells were washed with phosphate-buffered saline (PBS, pH 7.4). Proteins were extracted with a solution containing (in mM) 10 Tris (pH 7.0), 140 NaCl, 0.5% NP-40, 2 PMSF, 5 DTT, 0.05 pepstatin A, and 0.2 leupeptin. They were then centrifuged, mixed 1:1 with sample buffer (100 mM Tris (pH 6.8), 20% glycerol, 4% SDS, and 0.2% Bromophenol blue) and boiled for 5 min. Electrophoresis was performed using a 10% SDS-polyacrylamide gel (2 h, 110 V, 40 mA, 30 μg 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 the mixture was incubated for 2 h with specific COX-2 or α-tubulin antibodies. The blot was then incubated with anti-mouse IgG linked to alkaline phosphatase (1:1000) for 2 h. Subsequently, the membrane was developed with NBT/BCIP as a substrate. Quantitative data were obtained using a computing densitometer with Image-Pro plus software (Media Cybernetics, Silver Spring, MD).

Analysis of PKC activity

For the detection of PKC activity, cytosolic and membrane fractions were separated as described by Li et al (1998). Briefly, RAW 264.7 cells were incubated with PMA (100 nM) or lipoteichoic acid (10 μg mL^{-1}) for 30 min, and incubated in a humidified incubator at 37°C. After incubation, cells were scraped and collected. Collected cells were homogenized in ice-cold homogenization buffer (in mM: 20 Tris, 2 EDTA, 5 EGTA, 20% (v/v) glycerol, 2 PMSF, 1% (v/v) aprotinin, 5 DTT) for 20 min, then sonicated for 10 s and centrifuged at 800 *g* for 10 min. The supernatant (cytosolic and membrane fraction) was re-

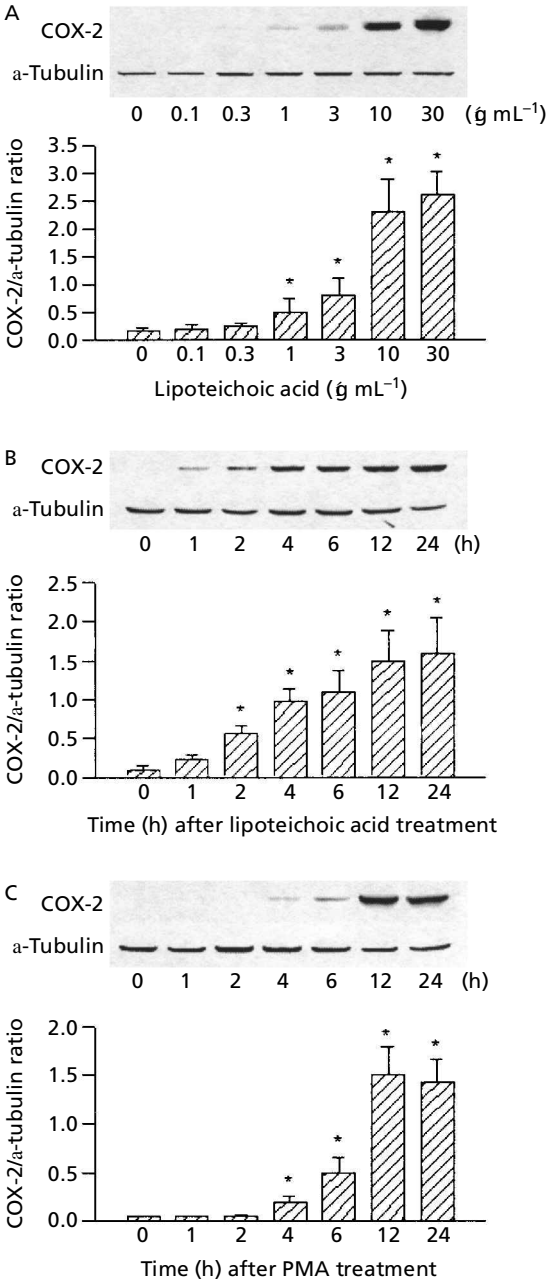

Figure 1 Dose- and time-dependent increases in COX-2 expression caused by lipoteichoic acid and PMA in RAW 264.7 macrophages. Cells were incubated with (A) the indicated doses of lipoteichoic acid for 24 h or with (B) lipoteichoic acid (10 μg mL^{-1}) or (C) PMA (100 nM) for the indicated time intervals, and the extracted proteins were then immunodetected with specific antibodies against COX-2 or α-tubulin. The extent of COX-2 and α-tubulin protein expression was quantitated using a densitometer with Image-Pro plus software. The relative level was calculated as the ratio of COX-2 to the α-tubulin protein level. Results are expressed as the mean$\pm$s.d. (n = 4). *$P < 0.05$ compared with the basal level.

moved and centrifuged at 25000 *g* for 15 min. The supernatant (cytosolic fraction) was obtained. The pellets (membrane fraction) were solubilized in homogenization buffer containing 0.1% NP-40. PKC activity was assayed using a PKC activity assay kit (Amersham) according to the procedure described by the manufacturer.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

RAW 264.7 macrophages were cultured in 10-cm culture Petri dishes. After reaching confluence, cells were treated with vehicle or lipoteichoic acid (10 μg mL^{-1}) for indicated time intervals (0, 5, 15, 30, 60 or 120 min), and then incubated in a humidified incubator at 37°C. The cytosolic and nuclear protein fractions were then separated as described by Chen et al 1998. Briefly, cells were washed with ice-cold PBS, and then centrifuged. The cell pellet was resuspended in hypotonic buffer (in mM: 10 HEPES (pH 7.9), 10 KCl, 0.5 DTT, 10 aprotinin, 10 leupeptin, and 20 PMSF) for 15 min on ice, and vortexed for 10 s. The nuclei were pelleted by centrifugation at 15000 *g* for 1 min. The supernatants containing cytosolic proteins were collected. A pellet containing nuclei was resuspended in hypertonic buffer (in mM: 20 HEPES (pH 7.6), 25% glycerol, 1.5 $MgCl_2$, 4 EDTA, 0.05 DTT, 20 PMSF, 10 aprotinin, and 10 leupeptin) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 15000 *g* for 2 min and stored at −70°C. Protein levels of p65 NF-κB in the cytosolic and nuclear fractions were determined by Western blotting analysis performed as described. In some experiments, cells were pretreated with D-609, U-73122, propranolol, Go 6976, Ro 31-8220, or PDTC for 30 min before lipoteichoic acid treatment. Protein levels of p65 NF-κB in the nuclear fraction were determined by Western blot analysis performed as described above.

A double-stranded oligonucleotide probe containing NF-κB sequences (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega) was purchased and end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The nuclear extract (2.5–5 μg) was incubated with 1 ng ^{32}P-labelled NF-κB probe (50000–75000 counts min^{-1}) in 10 μL binding buffer at 30°C for 25 min. The binding buffer contained 1 μg poly(dI-dc), 15 mM HEPES (pH 7.6), 80 mM NaCl, 1 mM EDTA, 1 mM DTT and 10% glycerol. 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).

Statistical analysis

Results are expressed as the mean$\pm$s.d. from three to four independent experiments. One-way analysis of variance 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 to be statistically significant.

Results

Characterization of COX-2 expression induced by lipoteichoic acid in RAW 264.7 macrophages

Incubation of RAW 264.7 macrophages with bacterial lipoteichoic acid (1–30 μg mL^{-1}) for 24 h caused induction of COX-2 protein in a dose-dependent manner (Figure 1A). When cells were treated with 10 μg mL^{-1} lipoteichoic acid for various time intervals, the COX-2 protein levels were significantly increased by 2 h and peaked at 12–24 h (Figure 1B). In the following experiments, cells were treated with 10 μg mL^{-1} lipoteichoic acid for 24 h. Pretreatment of cells with actinomycin D (0.1 μM) or cycloheximide (3 μM) for 30 min markedly attenuated lipoteichoic acid-induced COX-2 expression (Figure 2A). Pretreatment of cells with polymyxin B (0.3 μg mL^{-1}), which binds and inactivates endotoxin (Kengatharan et al 1996), for 30 min completely attenuated LPS (1 μg mL^{-1})-induced COX-2 expression, while it had no effect on lipoteichoic acid-induced effects (Figure 2B).

Roles of phospholipase C and PKC in lipoteichoic acid-induced COX-2 expression

To determine whether PKC activation was involved in the signal transduction pathway leading to COX-2 expression caused by lipoteichoic acid, the PKC inhibitors Go 6976 and Ro 31-8220 were used. Pretreatment of cells for 30 min with Go 6976 (0.1–1 μM) or Ro 31-8220 (0.03–0.3 μM) attenuated lipoteichoic acid-induced COX-2 expression in a concentration-dependent manner (Figure 3A, B). On the other hand, stimulation of cells with PMA (100 nM), the PKC activator, caused an increase in COX-2 expression in a time-dependent manner. The induction of COX-2 protein was significantly increased at 4 h and peaked at 12 h after PMA treatment (Figure 1C). Treatment of RAW 264.7 cells with 100 nM PMA or 10 μg mL^{-1} lipoteichoic acid for 30 min resulted in a decrease in PKC activity in the cytosol and an increase in PKC activity in the membrane (Table 1). When cells were pretreated for 30 min with the PC-PLC inhibitor, D-609 (25 μM), or the phosphatidylinositol-phospholipase C (PI-PLC) inhibitor, U-73122 (5 μM), lipoteichoic acid-mediated COX-2 expression was markedly inhibited. Pretreatment for 30 min with the phosphatidate phosphohydrolase inhibitor, propranolol (100 μM), had no effect on lipoteichoic acid-mediated COX-2 expression (Figure 4).

The role of transcription factor NF-κB in LTA-induced COX-2 expression

To determine whether NF-κB activation was involved in the signal transduction pathway leading to COX-2 expression caused by lipoteichoic acid, cells were treated with the NF-κB inhibitor, PDTC, before lipoteichoic acid treatment. Pretreatment of cells for 30 min with PDTC (0.5–5 μM) attenuated lipoteichoic acid- and PMA-induced COX-2 expression in a concentration-dependent manner (Figure 5A, B). In nuclear extracts of the unstimulated cells,

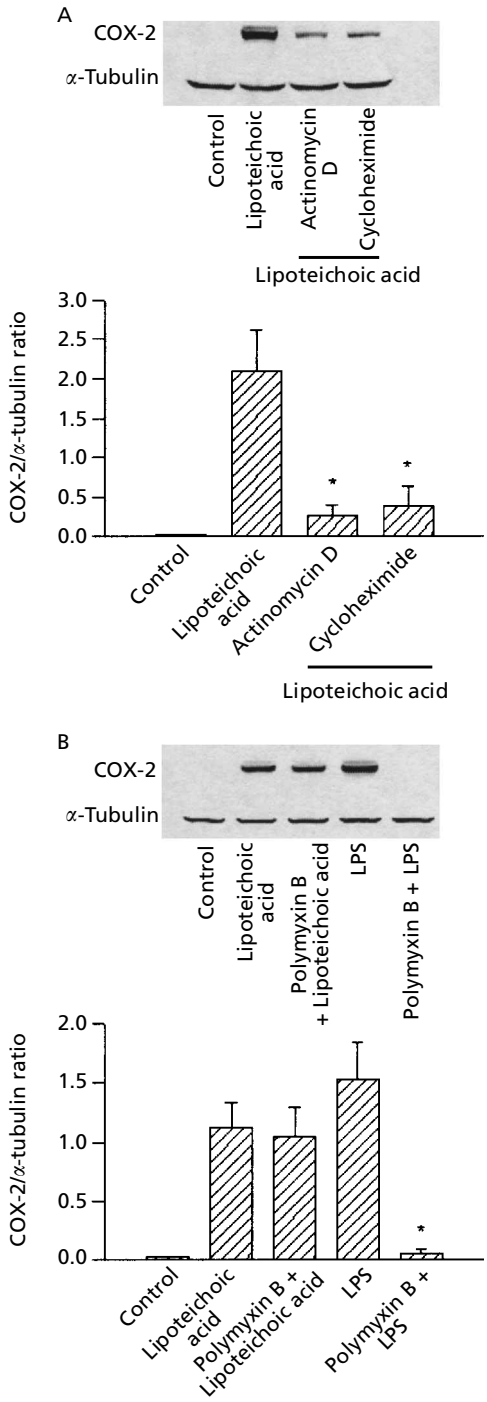

Figure 2 Effects of actinomycin D, cycloheximide, and polymyxin B on lipoteichoic acid-induced COX-2 expression in RAW 264.7 macrophages. (A) Cells were pretreated with actinomycin D (0.1 μM) or cycloheximide (3 μM) for 30 min followed by lipoteichoic acid (10 μg mL^{-1}) treatment for 24 h. (B) Cells were pretreated with polymyxin B (0.3 μg mL^{-1}) for 30 min before incubation with lipoteichoic acid (10 μg mL^{-1}) or LPS (1 μg mL^{-1}) for 24 h. Cell extracts were then prepared for immunodetection using specific antibodies against COX-2 or α-tubulin. The extent of COX-2 and α-tubulin protein expression was quantitated using a densitometer with Image-Pro plus software. The relative level was calculated as the ratio of COX-2 to the α-tubulin protein level. Results are expressed as the mean $\pm$ s.d. (n = 3). *$P < 0.05$ compared with the lipoteichoic acid- or LPS-treated group.

a slight intensity of formation of an NF-κB-specific DNA-protein complex was detected. Stimulation of cells with lipoteichoic acid (10 μg mL^{-1}) 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 (Figure 6A). Stimulation of cells with lipoteichoic acid (10 μg mL^{-1}) resulted in a marked translocation of p65 NF-κB from cytosol to the nucleus. The translocation of p65 NF-κB peaked at 30 min after lipoteichoic acid treatment and then declined by 60 min after treatment (Figure 6B). The lipoteichoic acid-induced translocation of p65 NF-κB was markedly inhibited by pretreatment of cells for 30 min with D-609 (25 μM), U-73122 (5 μM), Go 6976 (1 μM), Ro 31-8220 (0.3 μM), or PDTC (5 μM), but not by propranolol (100 μM) (Figure 6C, D).

Discussion

We have demonstrated that PC-PLC, PI-PLC, PKC, and transcription factor NF-κB may be involved in signal transduction leading to the expression of COX-2 protein in RAW 264.7 macrophages. Previous studies have shown that actinomycin D and cycloheximide are transcriptional and translational inhibitors, respectively (Chen & Wang 1999). In this study, we found that actinomycin D and cycloheximide markedly inhibited lipoteichoic acid-mediated COX-2 expression, suggesting that COX-2 expression was dependent on de novo transcription and translation. Pretreatment with polymyxin B, which binds and inactivates LPS (Kengatharan et al 1996), did not affect lipoteichoic acid-mediated COX-2 expression, which indicated that the induction of COX-2 protein caused by lipoteichoic acid was not due to contamination with LPS.

PKC is a family of serine/threonine kinases that appear to mediate various cellular functions (Nishizuka 1992; Hug & Sarre 1993). In renal mesangial cells, PKC-ζ was suggested to play an important role in IL-1β-mediated increases in COX-2 expression and PGE_2 production (Rzymkiewicz et al 1996). In our previous studies we demonstrated that PKC activation was involved in IL-1β-mediated signalling pathways leading to the expression of COX-2 protein in human pulmonary epithelial cells (Lin et al 2000). In this study, we have demonstrated that the PKC inhibitors Go 6976 and Ro 31-8220 prevented lipoteichoic acid-induced COX-2 expression. This suggested that PKC activation was an obligatory event in lipoteichoic acid-mediated COX-2 expression. PKC is activated by the physiological activator, diacylglycerol (Nishizuka 1992). The formation of diacylglycerol can be generated directly by the action of PI-PLC and PC-PLC (Nishizuka 1992; Exton 1994; Schutze et al 1994). An indirect pathway to generate diacylglycerol involves phosphatidylcholine cleavage by PC-PLD to generate phosphatidic acid, which can subsequently be converted to diacylglycerol by phosphatidate phosphohydrolase (Exton 1994). Schutze et al (1992) showed that D-609 selectively inhibited PC-PLC activity, without affecting the activities of PLA_2, PLD, or PI-PLC. Bleasdale et al (1990) demonstrated that U-73122

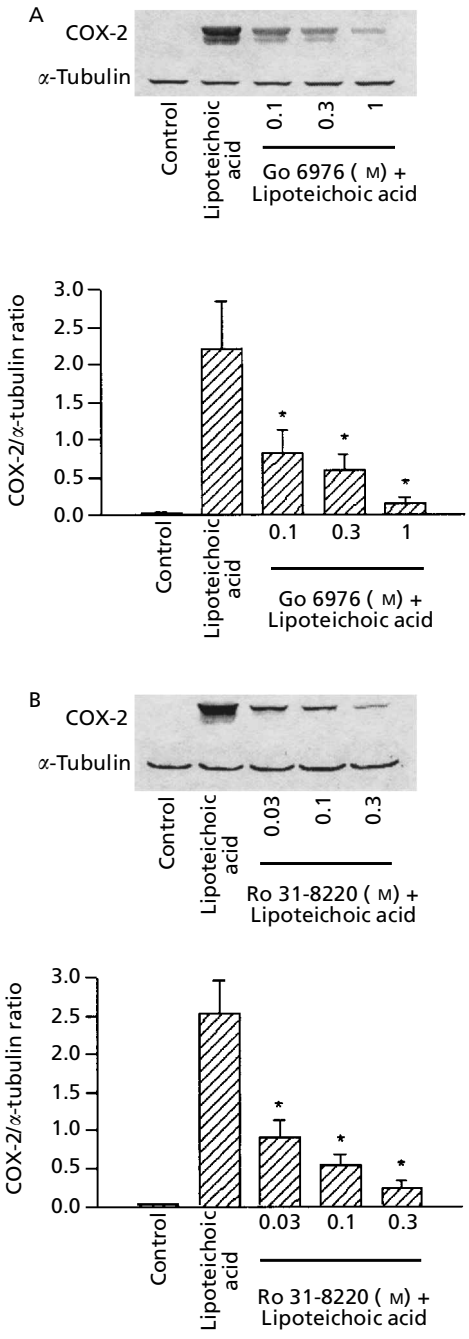

Figure 3 Effects of Go 6976 and Ro 31-8220 on lipoteichoic acid-induced COX-2 expression in RAW 264.7 macrophages. Cells were pretreated with (A) Go 6976 (0.1–1 μM) or (B) Ro 31-8220 (0.03–0.3 μM) for 30 min followed by lipoteichoic acid (10 μg mL^{-1}) treatment for 24 h. The extracted proteins were then immunodetected with specific antibodies against COX-2 or α-tubulin. The extent of COX-2 and α-tubulin protein expression was quantitated using a densitometer with Image-Pro plus software. The relative level was calculated as the ratio of COX-2 to the α-tubulin protein level. Results are expressed as the mean±s.d. (n = 3). *$P < 0.05$ compared with the lipoteichoic acid-treated group.

inhibited PI-PLC activation in human platelets and neutrophils. Recent reports have indicated that a higher concentration (> 10 μM) of propranolol inhibits phos-

Table 1 PKC activity caused by lipoteichoic acid and PMA in the cytosol and membrane fractions of RAW 264.7 macrophages.

	PKC activity (pmol phosphate min^{-1} (mg protein)$^{-1}$)	
	Cytosol	Membrane
Basal	0.39±0.06	0.21±0.06
PMA (100 nM)	0.09±0.05*	0.68±0.13*
Lipoteichoic acid (10 μg mL^{-1})	0.22±0.05*	0.43±0.07*

RAW 264.7 macrophages were treated with PMA (100 nM) or lipoteichoic acid (10 μg mL^{-1}) for 30 min. The subcellular (cytosol and membrane) fractions were then isolated. PKC activity in the cytosol and membrane was measured using a PKC activity assay kit (Amersham) according to the procedure described by the manufacturer. Results are expressed as the mean±s.d. (n = 3). *$P < 0.05$ compared with the basal level.

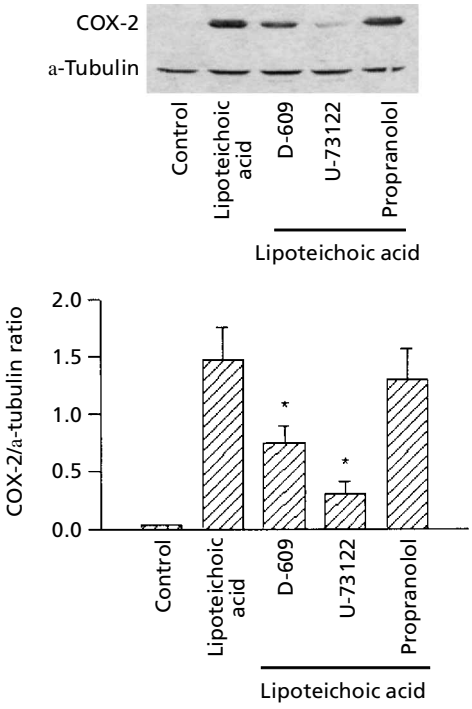

Figure 4 Effects of D-609, U-73122, and propranolol on lipoteichoic acid-induced COX-2 expression in RAW 264.7 macrophages. Cells were pretreated with D-609 (25 μM), U-73122 (5 μM), or propranolol (100 μM) for 30 min followed by lipoteichoic acid (10 μg mL^{-1}) treatment for 24 h. The extracted proteins were then immunodetected with specific antibodies against COX-2 or α-tubulin. The extent of COX-2 and α-tubulin protein expression was quantitated using a densitometer with Image-Pro plus software. The relative level was calculated as the ratio of COX-2 to the α-tubulin protein level. Results are expressed as the mean±s.d. (n = 3). *$P < 0.05$ compared with the lipoteichoic acid-treated group.

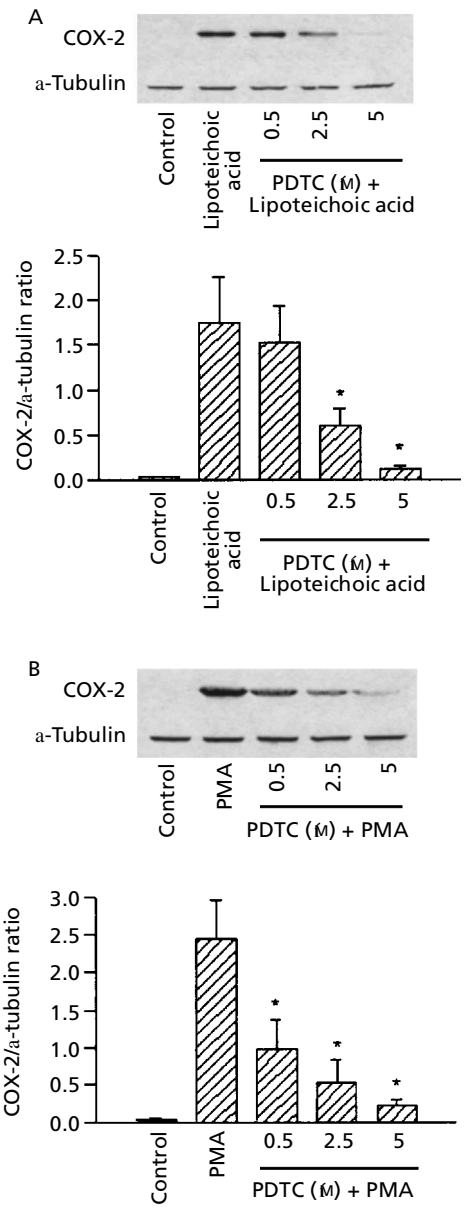

Figure 5 Effects of pyrrolidine dithiocarbamate (PDTC) on lipoteichoic acid- and PMA-mediated COX-2 expression in RAW 264.7 macrophages. Cells were pretreated with various concentrations of PDTC for 30 min followed by (A) lipoteichoic acid (10 μg mL^{-1}) or (B) PMA (100 nM) treatment for 24 h. The extracted proteins were then immunodetected with specific antibodies against COX-2 or α-tubulin. The extent of COX-2 and α-tubulin protein expression were quantitated using a densitometer with Image-Pro plus software. The relative level was calculated as the ratio of COX-2 to the α-tubulin protein level. Results are expressed as the mean±s.d. (n = 3). *$P < 0.05$ compared with the lipoteichoic acid- or PMA-treated groups, respectively.

phatidate phosphohydrolase activity (Billah et al 1989; Johnson et al 1999). In this study, we have demonstrated that D-609 and U-73122 inhibited lipoteichoic acid-induced COX-2 expression, whereas propranolol had no effect. Furthermore, we found that treatment of RAW 264.7 cells with lipoteichoic acid caused PKC activation. These results suggested that lipoteichoic acid might have activated PI-PLC and PC-PLC, but not the PC-PLD,

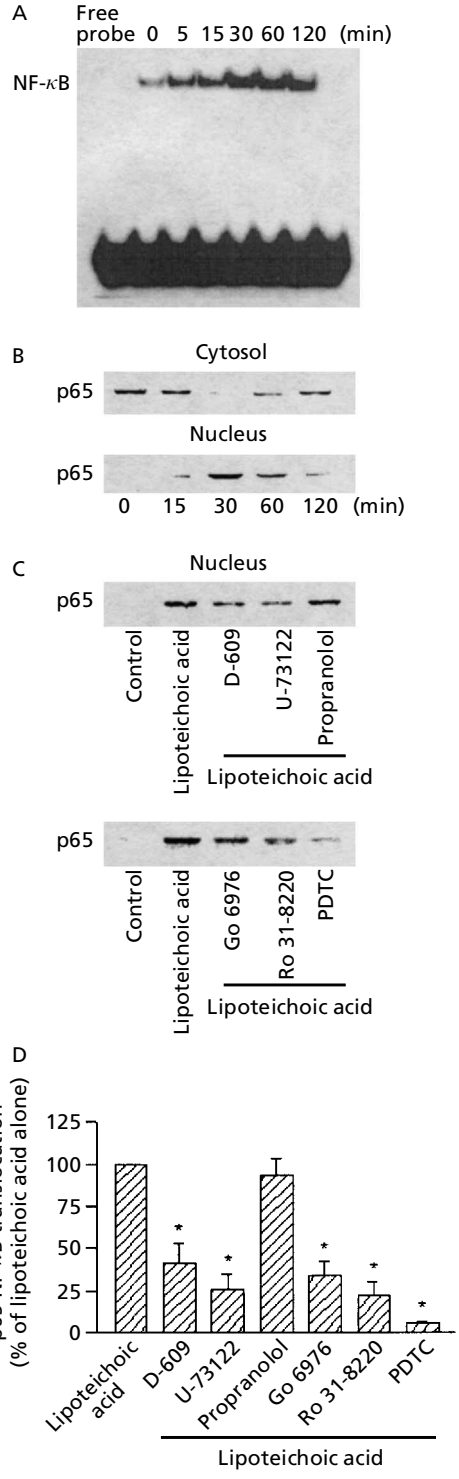

Figure 6 Kinetics of NF-κB-specific DNA–protein complex formation and p65 NF-κB translocation induced by lipoteichoic acid and effects of various inhibitors on lipoteichoic acid-mediated p65 NF-κB translocation in RAW 264.7 macrophages. Cells were treated with lipoteichoic acid (10 μg mL^{-1}) for various time intervals (A, B), or (C) pretreated with D-609 (25 μM), U-73122 (5 μM), propranolol (100 μM), Go 6976 (1 μM), Ro 31-8220 (0.3 μM), or PDTC (5 μM) for 30 min before incubation with lipoteichoic acid (10 μg mL^{-1}) for 30 min. Subcellular (cytosol and nucleus) fractions were then isolated. A. NF-κB–specific DNA–protein binding activity in nuclear extracts was determined using an electrophoretic mobility shift assay (EMSA). B. and C. The levels of cytosolic and nuclear p65 NF-κB were immunodetected with p65 NF-κB-specific antibody. D. The extent of p65 NF-κB translocation was quantitated using a densitometer with Image-Pro plus software. Results are expressed as the mean$\pm$s.d. (n = 3). *$P < 0.05$ compared with the lipoteichoic acid-treated group.

pathways to elicit PKC activation, which in turn initiated COX-2 expression. This is consistent with the finding that LPS-mediated induction of iNOS depended on the activation of PI-PLC and PC-PLC, but not PC-PLD, in RAW 264.7 macrophages (Chen et al 1998).

Newton et al (1997) demonstrated that transcription factor NF-κB was involved in the pro-inflammatory cytokine-induced expression of COX-2 protein. In this study, we have demonstrated that lipoteichoic acid-mediated COX-2 expression was inhibited by the NF-κB inhibitor PDTC, indicating that activation of NF-κB might be involved in the induction of COX-2 protein caused by lipoteichoic acid. The transcription factor NF-κB is constitutively present in cells as a heterodimer, consisting of a p50 DNA-binding subunit and a p65 trans-activating subunit. NF-κB is normally held in the cytoplasm in an inactivated state by the inhibitor protein, IκB-α. After activation, the cytosolic NF-κB/IκB-α complex dissociates, and free NF-κB translocates to the nucleus where it activates the responsive gene (Baeuerle & Henkel 1994; Barnes & Karin 1997). Recent studies have shown that lipoteichoic acid derived from *Enterococcus faecalis* activated the formation of NF-κB-specific DNA–protein complexes in basal uroepithelial cells (Elgavish 2000). We found that treatment of RAW 264.7 macrophages with lipoteichoic acid resulted in the translocation of p65 NF-κB from cytosol to the nucleus. Lipoteichoic acid-induced translocation of p65 NF-κB was inhibited by D-609, U-73122, Go 6976, and Ro 31-8220, but not by propranolol. These results suggested that lipoteichoic acid-induced translocation of p65 NF-κB might be via the pathways of PC-PLC, PI-PLC, and PKC, but not the PC-PLD pathway, in RAW 264.7 macrophages. Previous reports have shown that PC-PLC might be involved in TNF-α-induced activation of NF-κB (Schutze et al 1992). Chen et al (1998) demonstrated that activation of PC-PLC and PC-PLD was involved in LPS-induced NF-κB activation in RAW 264.7 macrophages. Furthermore, PKC has been implicated in IL-1β-mediated NF-κB activation, since the PKC inhibitors Go 6976 and Ro 31-8220 were able to inhibit the translocation of p65 NF-κB (Lin et al 2000). In this study, we have found that the PKC activator PMA stimulated COX-2 expression, and the effect was inhibited by PDTC, as was lipoteichoic acid-induced COX-2 expression. These results indicated that activation of NF-κB might occur downstream of PKC to induce COX-2 expression.

Conclusions

This study has demonstrated that lipoteichoic acid may have activated PI-PLC and PC-PLC to elicite PKC activation, which in turn initiated NF-κB activation, and

finally caused COX-2 expression in RAW 264.7 macrophages. Based on these results and a previous report (Lin et al 2001), we have found that the signal transduction pathway of lipoteichoic acid-induced COX-2 expression in RAW 264.7 macrophages is similar to that of the human pulmonary epithelial cell line (A549). The results indicated that COX-2 induction might be involved in inflammation elicited by Gram-positive organisms. By understanding these signal transduction pathways, we may be able to design therapeutic strategies to reduce airway inflammation caused by Gram-positive organisms.

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