British Journal of Pharmacology (2001) 134, 543–552

www.nature.com/bjp

Induction of cyclooxygenase-2 protein by lipoteichoic acid from *Staphylococcus aureus* in human pulmonary epithelial cells: involvement of a nuclear factor-κB-dependent pathway

*,[1]Chien-Huang Lin, [1]I-Hui Kuan, [1]Horng-Mo Lee, [2]Wen-Sen Lee, [2]Joen-Rong Sheu, [1]Yuan-Soon Ho, [3]Chun-Hua Wang & [3]Han-Pin Kuo

[1]Graduate Institute of Biomedical Technology, Taipei Medical University, Taipei, Taiwan; [2]Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan and [3]Department of Thoracic Medicine II, Chang Gung Memorial Hospital, Taipei, Taiwan

1 This study investigated the role of protein kinase C (PKC) and transcription factor nuclear factor-κB (NF-κB) in cyclooxygenase-2 (COX-2) expression caused by lipoteichoic acid (LTA), a cell wall component of the gram-positive bacterium *Staphylococcus aureus*, in human pulmonary epithelial cell line (A549).

2 LTA caused dose- and time-dependent increases in COX-2 expression and COX activity, and a dose-dependent increase in PGE_2 release in A549 cells. The LTA-induced increases in COX-2 expression and COX activity were markedly inhibited by dexamethasone, actinomycin D or cyclohexamide, but not by polymyxin B, which binds and inactivates endotoxin.

3 The phosphatidylcholine-phospholipase C (PC-PLC) inhibitor (D-609) and the phosphatidate phosphohydrolase inhibitor (propranolol) reduced the LTA-induced increases in COX-2 expression and COX activity, while phosphatidylinositol-phospholipase C inhibitor (U-73122) had no effect. The PKC inhibitors (Go 6976, Ro 31-8220 and GF 109203X) and NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), also attenuated the LTA-induced increases in COX-2 expression and COX activity.

4 Treatment of A549 cells with LTA caused an increase in PKC activity in the plasma membrane; this stimulatory effect was inhibited by D-609, propranolol, or Go 6976, but not by U-73122.

5 Exposure of A549 cells to LTA caused a translocation of p65 NF-κB from the cytosol to the nucleus and a degradation of IκB-α in the cytosol. Treatment of A549 cells with LTA caused NF-κB activation by detecting the formation of NF-κB-specific DNA-protein complex in the nucleus; this effect was inhibited by dexamethasone, D-609, propranolol, Go 6976, Ro 31-8220, or PDTC.

6 These results suggest that LTA might activate PC-PLC and phosphatidylcholine-phospholipase D to induce PKC activation, which in turn initiates NF-κB activation, and finally induces COX-2 expression and PGE_2 release in human pulmonary epithelial cell line.
British Journal of Pharmacology (2001) **134,** 543–552

Keywords: Lipoteichoic acid; cyclooxygenase-2; prostaglandin E_2; protein kinase C; NF-κB; A549 cells

Abbreviations: COX-2, cyclooxygenase-2; DAG, diacylglycerol; EMSA, electrophoretic mobility shift assay; LTA, lipoteichoic acid; PC-PLC, phosphatidylcholine-phospholipase C; PC-PLD, phosphatidylcholine-phospholipase D; PDTC, pyrrolidine dithiocarbamate; PGE_2, prostaglandin E_2; PKC, protein kinase C; PI-PLC, phosphatidylinositol-phospholipase C

Introduction

Prostaglandins, lipid mediators involved in many processes including inflammation, are produced by many cell types (Mitchell *et al*., 1995). Cyclooxygenase (COX) converts arachidonic acid to prostaglandin H_2 which is then further metabolized to various prostaglandins, prostacyclin, or thromboxane A_2 (Vane *et al*., 1998). Two COX isozymes, COX-1 and COX-2, have been identified in humans bearing 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). 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 been 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. The cell wall of gram-positive bacteria contains lipoteichoic acid (LTA) and peptidoglycan, which themselves can activate leukocytes, stimulate the generation of pro-

*Author for correspondence at: Graduate Institute of Biomedical Technology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan; E-mail: chlin@tmu.edu.tw

inflammatory cytokines, and hence, cause moderate systemic inflammatory response syndrome (Bhakdi *et al*., 1991; Mattsson *et al*., 1993). LTA (a major component of the gram-positive bacterial cell wall) can also induce the expression of inducible nitric oxide synthase (iNOS) in vascular smooth muscle cells (Auguet *et al*., 1992) and macrophages (Kengatharan *et al*., 1996). Lately, we have found that LTA inhibited platelet aggregation caused by collagen, thrombin, or ADP in human platelets (Sheu *et al*., 2000). However, the expression of COX-2 induced by LTA has not been determined and the signal transduction events leading to the expression of COX-2 by LTA are unclear. The transcription factor NF-κB plays a key role in the transcriptional regulation of adhesion molecules, enzymes and cytokines involved in chronic inflammatory diseases (Barnes & Karin, 1997). In airway epithelial cells, pro-inflammatory cytokines, such as interleukin-1β (IL-1β), rapidly induce NF-κB DNA binding and cause up-regulation of NF-κB-dependent genes, including COX-2 (Newton *et al*., 1997) and iNOS (Nunokawa *et al*., 1996). Previous studies have shown a potential role of phosphatidylcholine-phospholipase C (PC-PLC) and NF-κB in LTA-induced iNOS expression in murine J744.2 macrophages (Kengatharan *et al*., 1996). In the present study, the intracellular signalling pathway by which LTA induces COX-2 expression in human airway epithelial cells was studied. The results show that LTA might activate PC-PLC and phosphatidylcholine-phospholipase D (PC-PLD) to induce PKC activation, which in turn initiates NF-κB activation, and finally induces COX-2 expression and PGE_2 release in the human pulmonary epithelial cell line (A549).

Methods

Materials

Lipoteichoic acid (LTA derived from *Staphylococcus aureus*), dexamethasone, actinomycin D, cyclohexamide, polymyxin B, propranolol, phorbol-12-myristate-13-acetate (PMA), pyrrolidine dithiocarbamate (PDTC), Trizma base, dithiothreitol (DTT), glycerol, phenylmethylsulphonyl fluoride (PMSF), pepstatin A, leupeptin, and sodium dodecyl sulphate (SDS) were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.). NS-398, Go 6976, Ro 31-8220, and GF 109203X were purchased from Calbiochem-Novabiochem (San Diego, CA, U.S.A.). D-609 and U-73122 were obtained from RBI (Natick, MA, U.S.A.). Penicillin/streptomycin, foetal calf serum (FCS) and Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 were purchased from Life Technologies, Inc. (Gaithersburg, MD, U.S.A.). PGE_2 enzyme immunoassay kit was obtained from Cayman Chem. Co. (Ann Arbor, MI, U.S.A.). A COX-2 monoclonal antibody, which cross react with human and mouse COX-2, was purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Antibodies specific for p65, IκB-α, and α-tubulin was purchased from Santa Cruz Biochemicals (Santz Cruz, CA, U.S.A.). Anti-mouse IgG conjugated alkaline phosphatase was purchased from Jackson Immuno Research Laboratories (West Grove, PA, U.S.A.). PKC [^{32}P] enzyme assay system was purchased from Amersham International plc (Buckinghamshire, U.K.). Digoxigenin (DIG) gel shift kit, 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, U.S.A.).

Cell culture

A549 cells, a human pulmonary epithelial carcinoma cell line with type II alveolar epithelial cell differentiation, were obtained from American Type Culture Collection and grown in DMEM/Ham's F-12 neutrient mixture containing 10% FCS and penicillin/streptomycin (50 u ml^{-1}) in a humidified 37°C incubator. When confluent, cells were disaggregated in trypsin solution, washed in DMEM/Ham's F-12 supplemented with 10% FCS, centrifuged at $125 \times g$ for 5 min, then resuspended and subcultured according to standard protocols.

Measurements of PGE_2 release and COX activity

A549 cells were cultured in 12-well culture plates. For experiments designed to measure the release of PGE_2 due to endogeneous arachidonic acid, cells were treated with LTA (1 – 100 μg ml^{-1}) for 24 h, then the medium was removed and stored at −80°C until assay. PGE_2 was assayed by using the PGE_2 enzyme immunoassay kit according to the procedure described by the manufacturer. In separate experiments designed to measure the COX activity. COX activity was quantified by providing cells with exogeneous arachidonic acid, the substrate for COX, and measuring its conversion into PGE_2. The cells were treated with LTA (1 – 100 μg ml^{-1}) for 24 h or LTA (30 μg ml^{-1}) for indicated time intervals, after that cells were washed and fresh medium containing arachidonic acid (30 μM) were added for 30 min at 37°C, then the medium was removed for PGE_2 enzyme immunoassay. In some experiments, cells were treated with vehicle, LTA (30 μg ml^{-1}), or pretreatment with specific inhibitors as indicated followed by LTA and incubated in a humidified incubator at 37°C for 24 h. After incubation, the cells were washed, and fresh medium containing arachidonic acid (30 μM) was added for 30 min at 37°C; the medium was then removed for PGE_2 enzyme immunoassay.

Protein preparation and Western blotting

For determination of the expressions of COX-2 and α-tubulin in A549 cells, the preparation of total proteins and Western blotting were performed as described previously (Mitchell *et al*., 1994). Briefly, A549 cells were cultured in 10-cm petri dishes. After reaching confluence, cells were treated with LTA (1 – 100 μg ml^{-1}) for 24 h or LTA (30 μg ml^{-1}) for indicated times and incubated in a humidified incubator at 37°C. In some experiments, cells were treated with vehicle, LTA (30 μg ml^{-1}), or pretreatment with specific inhibitors as indicated followed by LTA and incubated in a humidified incubator at 37°C. After incubation, cells were washed with phosphate buffer saline (PBS, pH 7.4). Proteins were extracted with solution containing (mM): Tris 10; pH 7.0; NaCl 140; 0.5% NP-40; PMSF 2; DTT 5; pepstatin A 0.05 and leupeptin 0.2, centrifuged, mixed 1 : 1 with sample buffer (Tris 100 mM, pH 6.8; 20% glycerol; 4% SDS and 0.2% Bromophenol Blue), and boiled for 5 min. Electrophoresis

was performed using 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), blocked the nonspecific IgGs with 5% fat-free milk powder, and 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.

Analysis of PKC activity

For the detection of PKC activity, cytosolic and membrane fractions were separated as described previously (Li *et al*., 1998). Briefly, A549 cells were incubated with vehicle, LTA (30 μg ml^{-1}), or PMA (10 nM) 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, the cells were scraped, collected, homogenized in ice-cold homogenization buffer [Tris 20 mM, EDTA 2 mM, EGTA 5 mM, glycerol 20% (v v^{-1}), PMSF 2 mM, aprotinin 1% (v v^{-1}), DTT 5 mM] for 20 min, sonicated for 10 s, and then centrifuged at $800 \times g$ for 10 min. The supernatant (cytosolic and membrane fractions) was removed and centrifuged at $25{,}000 \times g$ for 15 min to obtain the cytosolic fraction (supernatant). The pellets (membrane fraction) were solubilized in homogenization buffer containing 0.1% NP-40. The PKC activity was assayed using the PKC enzyme assay system (Amersham International plc) according to the procedure described by the manufacturer.

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

A549 cells were cultured in 10-cm culture petri dishes. After reaching confluence, cells were treated with vehicle or LTA (30 μg ml^{-1}) for indicated times and incubated in a humidified incubator at 37°C. In some experiments, the cells were pretreated with dexamethasone (0.1 μM), D-609 (50 μM), propranolol (100 μM), Go 6976 (1 μM), Ro 31-8220 (1 μM), or PDTC (25 μM) for 30 min, treated with LTA (30 μg ml^{-1}), and then incubated in a humidified incubator at 37°C for 10 min. The cytosolic and nuclear protein fractions were then separated as described previously (Chen *et al*., 1998). Briefly, cells were washed with ice-cold PBS, and pelleted. The cell pellet was resuspended in hypotonic buffer (mM): HEPES 10, pH 7.9, KCl 10, DTT 0.5, aprotinin 10, leupeptin 10 and PMSF 20 for 15 min on ice, and vortexed for 10 s. Nuclei were pelleted by centrifugation at $15{,}000 \times g$ for 1 min. The supernatants containing cytosolic proteins were collected. A pellet containing nuclei was resuspended in hypertonic buffer (mM) HEPES 20, pH 7.6, 25% glycerol, $MgCl_2$ 1.5, EDTA 4, DTT 0.05, PMSF 20, aprotinin 10 and leupeptin 10, for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at $15{,}000 \times g$ for 2 min and stored at -70°C. In studies of p65 NF-κB translocation, both cytosolic and nuclear extracts were used; only cytosolic extracts were used in IκB-α degradation. The extracts were subjected to SDS–PAGE using a 10% running gel, and Western blotting analysis was performed as described above.

Electrophoretic mobility shift assay (EMSA) was performed using DIG gel shift kit. Briefly, a double-stranded oligonucleotide probe containing NF-κB sequences (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega) was purchased and end labelled with DIG using terminal transferase. The nuclear extract (10–15 μg) was incubated with 4 ng of DIG-labelled NF-κB probe in 10 μl binding buffer containing 10 μg poly(dI-dc), 1 μg poly L-lysine, 100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM DTT, 1% (w v^{-1}) Tween 20 and 150 mM KCl at 25°C for 15 min. DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on 6% polyacrylamide gel; then the gel was transferred to nylon membrane. The gel was incubated with 0.1% milk in TBST at room temperature for 30 min, and was incubated with anti-DIG linked to alkaline phosphatase for 30 min. The immunoreactive band was finally detected with CSPD detecting reagents and exposed to X-ray film. The quantitative data were obtained by using a computing densitometer with Image-Pro plus software (Media Cybernetics, Inc., MD, U.S.A.).

Statistical analysis

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

Results

Characterization of COX-2 expression induced by LTA in A549 cells

The basal level of PGE_2 released from A549 cells was low (3.0 ± 0.3 ng ml^{-1}, $n = 4$). However, incubation of A549 cells with bacterial LTA (1–100 μg ml^{-1}) for 24 h resulted in a dose-dependent increase of PGE_2 release, and reached maximal level at 30 μg ml^{-1} LTA treatment (Figure 1A). In A549 cells, COX activity (measured in the presence of 30 μM exogenous arachidonic acid for 30 min) was dose-dependently elevated after 24 h LTA (1–100 μg ml^{-1}) treatment, with a maximal effect caused by 30 μg ml^{-1} LTA (Figure 1B). LTA (1–100 μg ml^{-1} for 24 h) also caused induction of COX-2 protein in a dose-dependent manner, with a maximal induction at 30 μg ml^{-1} LTA (Figure 1C). When cells were treated with 30 μg ml^{-1} LTA for various time intervals, the COX activity was increased significantly at 2 h and peaked at 24 h (Figure 2A). Treatment of A549 with 30 μg ml^{-1} LTA also increased the COX-2 protein levels in a time-dependent manner (Figure 2B). In the following experiments, the cells were treated with 30 μg ml^{-1} LTA for 24 h. Pretreatment of the cells with dexamethasone (0.1 μM), actinomycin D (0.1 μM), or cyclohexamide (3 μM) for 30 min markedly attenuated the LTA-induced increases in COX activity (Figure 3A). The LTA-induced expression of COX-2 was also attenuated (Figure 3B). Polymyxin B (0.5 μg ml^{-1}), which binds and inactivates endotoxin, did not affect the LTA-induced increases in COX activity and COX-2 expression (Figure 3A,B). Treatment of A549 cells with these

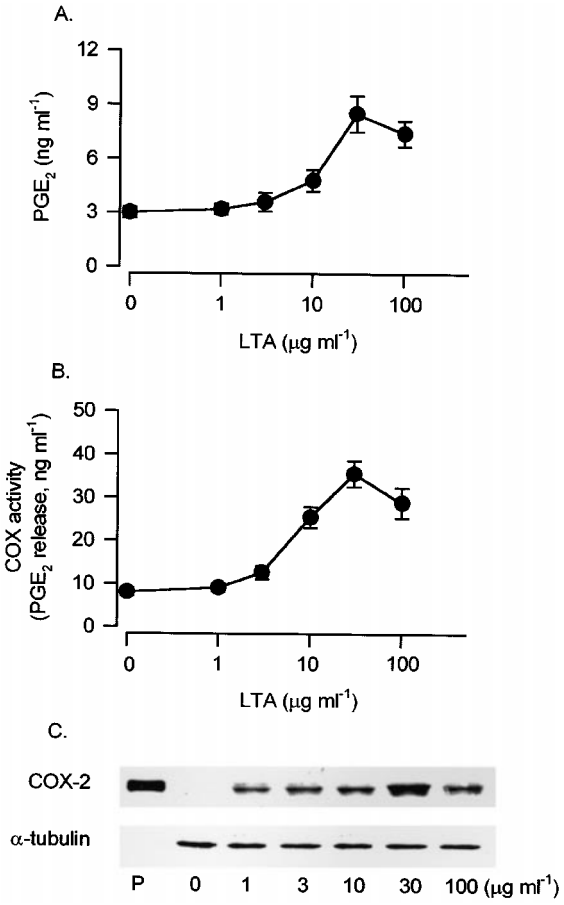

Figure 1 Concentration-dependent increases in the PGE_2 release, COX activity, and COX-2 expression caused by LTA in A549 cells. Cells were incubated with various concentrations of LTA for 24 h, and then the medium was removed for PGE_2 measurement (A). The LTA-mediated increase of COX activity was measured by examining the PGE_2 formation in the presence of 30 μM exogeneous arachidonic acid for 30 min (B). Results were expressed as mean$\pm$s.e.mean ($n=4$). In (C), cells were incubated with various concentrations of LTA for 24 h, and then immunodetected with COX-2 specific antibody as described in Methods. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin. Whole cell lysate of mouse macrophages (RAW 264.7) stimulated by LPS (1 μg ml^{-1}) and INFγ (10 ng ml^{-1}) for 12 h was used as a positive control (P).

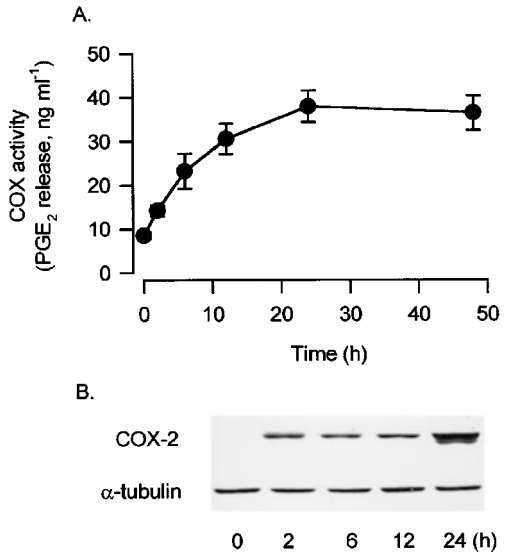

Figure 2 Time-dependent increases in COX activity and COX-2 expression caused by LTA in A549 cells. The increase of COX activity caused by LTA (30 μg ml^{-1}) for various time intervals was measured by examining the PGE_2 formation in the presence of 30 μM exogeneous arachidonic acid for 30 min (A). Results were expressed as mean$\pm$s.e.mean ($n=3$). In (B), cells were incubated with LTA (30 μg ml^{-1}) for indicated time intervals. The cells were then processed for immunodetection using COX-2 specific antibody as described in Methods. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

inhibitors at the indicated concentrations had no effect on the basal COX activity (Figure 3A). Moreover, NS-398 (1 μM), a selective COX-2 inhibitor, markedly decreased the LTA-induced PGE_2 release by 97.9% (data not shown).

The role of phospholipase C, phospholipase D, and protein kinase C on LTA-induced COX-2 expression

To determine whether protein kinase C (PKC) activation was involved in the signal transduction pathway leading to COX-2 expression caused by LTA, the PKC inhibitors, Go 6976, Ro 31-8220 or GF 109203X, were used. Pretreatment of cells for 30 min with Go 6976 (0.3 and 1 μM), Ro 31-8220 (0.3 and 1 μM), or GF 109203X (3 and 10 μM) markedly attenuated the LTA-induced increase in COX activity as reflected by PGE_2 secretion. None of these PKC inhibitors affected the basal COX activity (Figure 4A). The LTA-induced COX-2 expression was also inhibited by Go 6976 (1 μM), Ro 31-8220 (1 μM), or GF 109203X (10 μM) (Figure 4B). When cells were pretreated for 30 min with the PC-PLC inhibitor D-609 (50 μM), the phosphatidylinositol-phospholipase C (PI-PLC) inhibitor U-73122 (10 μM), or the phosphatidate phosphohydrolase inhibitor propranolol (100 μM), the LTA-mediated increase in COX activity was markedly inhibited by both D-609 and propranolol, while U-73122 had no effect. These inhibitors, at the concentration used, did not affect the basal COX activity (Figure 5A). Moreover, both D-609 and propranolol, but not U-73122, also inhibited the LTA-induced increase of the COX-2 expression (Figure 5B).

Treatment of the cells with LTA (30 μg ml^{-1}) for various time intervals resulted in a decrease in PKC activity in the cytosol fraction and an increase in the membrane fraction. Furthermore, stimulation of the cells for 30 min with 10 nM PMA, a PKC activator, caused a marked decrease in PKC activity in the cytosol fraction and an increase in the membrane fraction (Figure 6A). The LTA-mediated increase in PKC activity in the membrane fraction was inhibited by pretreatment of the cells 30 min with D-609 (50 μM), propranolol (100 μM), or Go 6976 (1 μM), but not with U-73122 (Figure 6B).

The role of transcription factor NF-κB on 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 LTA, the cells were treated with NF-κB specific inhibitor, PDTC, prior to LTA treatment. Pretreatment of the cells for 30 min with PDTC (10 and 25 μM) markedly attenuated the LTA-induced increase in COX activity.

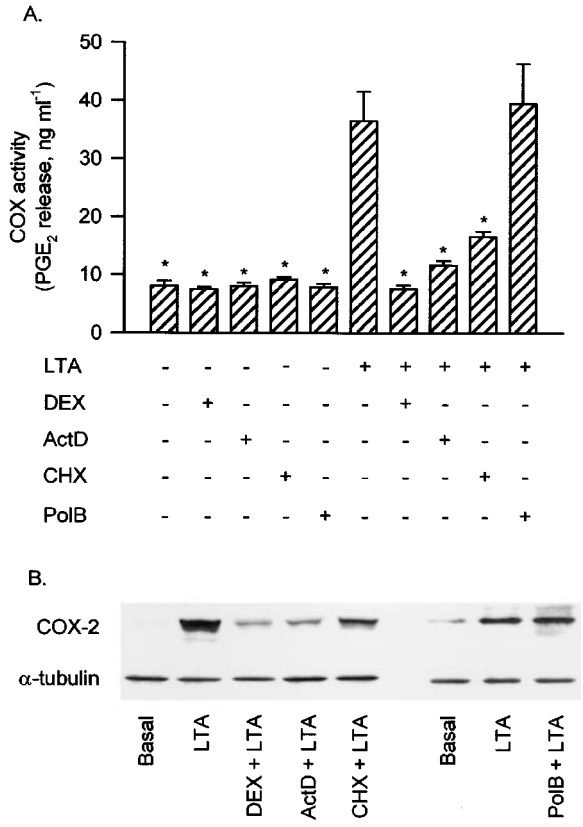

Figure 3 Effects of dexamethasone, actinomycin D, cyclohexamide and polymyxin B on the LTA-induced increase of COX activity and COX-2 expression in A549 cells. In (A), cells were pretreated with dexamethasone (DEX, 0.1 μM), actinomycin D (ActD; 0.1 μM), cyclohexamide (CHX; 3 μM) or polymyxin B (PolB, 0.5 μg ml^{-1}) for 30 min prior to a 24 h LTA (30 μg ml^{-1}) incubation. The increase of COX activity was measured by examining the PGE_2 formation in the presence of 30 μM exogeneous arachidonic acid for 30 min. Results were expressed as mean $\pm$ s.e.mean ($n=3$). *$P<0.05$ as compared with the LTA-treated group. In (B), cells were pretreated with DEX (0.1 μM), ActD (0.1 μM), CHX (3 μM) or PolB (0.5 μg ml^{-1}) for 30 min prior to a 24 h incubation with LTA (30 μg ml^{-1}). The cells were then prepared for immunodetection using COX-2 specific antibody as described in Methods. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

Treatment of A549 cells with PDTC (25 μM) alone had no effect on the basal COX activity (Figure 7A). The LTA-induced COX-2 expression was also attenuated by 25 μM PDTC (Figure 7B). Stimulation of the cells with LTA (30 μg ml^{-1}) for 10 min resulted in a marked translocation of p65 NF-κB from cytosol to the nucleus as well as a partial degradation of IκB-α in the cytosol. The LTA-induced effects disappeared gradually after 30 min treatment (Figure 8A). In nuclear extracts of the unstimulated cells, a slight intensity of NF-κB-specific DNA-protein complexes formation was observed. Stimulation of the cells with LTA (30 μg ml^{-1}) for 10–30 min resulted in marked activation of NF-κB-specific DNA-protein complexes formation. However, after 60–120 min treatment with LTA, the intensities of these DNA-protein complexes were decreased (Figure 8B). The formation of NF-κB complex was completely inhibited by the addition of excess 100$\times$ cold NF-κB consensus DNA sequence (data not shown), indicating that the DNA-protein interactions were sequence specific. When cells were pre-treated for 30 min with D-609 (50 μM), propranolol (100 μM), dexamethasone (0.1 μM), Go 6976 (1 μM), Ro 31-8220 (1 μM) or PDTC (25 μM), the LTA-induced activation of NF-κB-specific DNA-protein complexes formation was markedly inhibited by dexamethasone or PDTC, and partially inhibited by D-609, propranolol, Go 6976, or Ro 31-8220 (Figure 9A,B,C).

Discussion

The results of the present study demonstrated that the LTA-induced increase of PGE_2 release in human airway epithelial cells (A549) is a consequence of the induction of COX-2, and indicated that PC-PLC, PC-PLD, PKC, and transcription factor NF-κB might be involved in the signal transduction leading to the expression of COX-2 in these cells. In the absence of exogenous arachidonic acid, LTA caused the release of PGE_2 in A549 cells, suggesting that LTA might stimulate the expression of COX-2 as well as phospholipase A_2. Since actinomycin D and cyclohexamide markedly inhibited the LTA-mediated increases in COX-2 expression and COX activity, suggesting that the enhanced release of PGE_2 is dependent on *de novo* transcription and translation. Pretreatment with polymyxin B, which binds and inactivates LPS (Kengatharan *et al*., 1996), did not affect the LTA-mediated increases in COX-2 expression and COX activity, indicating that the induction of COX-2 protein caused by LTA is 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 increase in COX-2 expression and PGE_2 production (Rzymkiewicz *et al*., 1996). Our previous studies have also demonstrated that PKC activation is involved in the IL-1β-mediated signalling pathways leading to the expression of COX-2 protein in human pulmonary epithelial cells (Lin *et al*., 2000). In the present study, we demonstrated that the LTA-induced increases in COX-2 expression and COX activity were prevented by PKC inhibitors, Go 6976, Ro 31-8220, and GF 109203X, indicating that PKC activation is involved in the LTA-mediated signal transduction leading to the expression of COX-2 protein. Diacylglycerol (DAG) is a well-established activator of PKC (Nishizuka, 1992). Several mechanisms have been suggested to be responsible for the signal-mediated formation of DAG. The formation of DAG can be generated directly by the action of PI-PLC and PC-PLC (Exton, 1994; Nishizuka, 1992; Schutze *et al*., 1994). An indirect pathway to generate DAG involves phosphatidylcholine cleavage by PC-PLD to generate phosphatidic acid, which can be subsequently converted to DAG by phosphatidate phosphohydrolase (Exton, 1994). Previous reports have shown that D-609 selectively inhibited PC-PLC activity, without affecting the activities of PLA_2, PLD and PI-PLC (Schutze *et al*., 1992). It has been demonstrated that U-73122 inhibited PI-PLC activation in human platelets and neutrophils (Bleasdale *et al*., 1990). Propranolol at a lower concentration is a well-known β-adrenergic receptor antagonist. However, recent reports have indicated that higher concentration (>50 μM) of propranolol inhibits phosphatidate phosphohydrolase activity (Billah *et al*.,

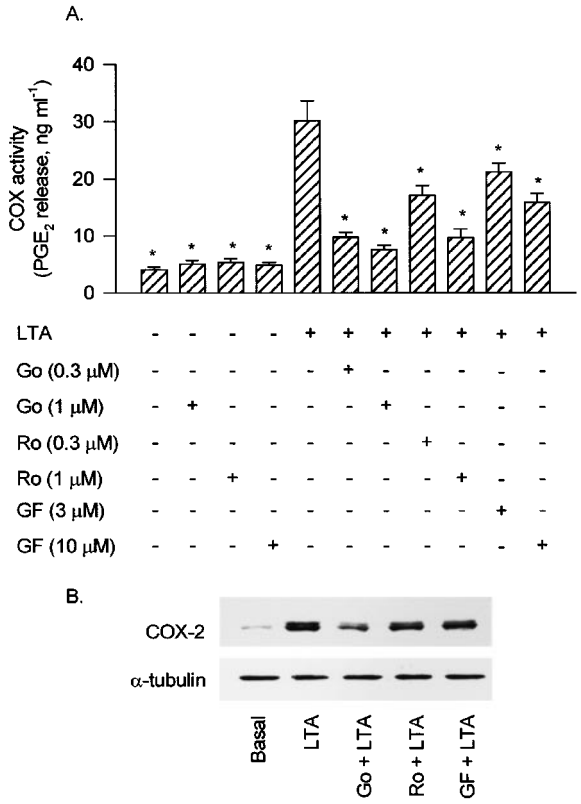

Figure 4 Effects of Go 6976, Ro 31-8220 and GF 109203X on the increases of COX activity and COX-2 expression caused by LTA in A549 cells. In (A), cells were pretreated with various concentrations of Go 6976 (Go), Ro 31-8220 (Ro) or GF 109203X (GF) for 30 min prior to a 24 h incubation with LTA (30 μg ml^{-1}). The increase of COX activity was measured by examining the PGE_2 formation in the presence of 30 μM exogeneous arachidonic acid for 30 min. Results were expressed as mean$\pm$s.e.mean ($n=4$). *$P<0.05$ as compared with the LTA-treated group. In (B), cells were pretreated with Go (1 μM), Ro (1 μM) or GF (10 μM) for 30 min prior to a 24 h incubation with LTA (30 μg ml^{-1}). The cells were then prepared for immunodetection using COX-2 specific antibody as described in Methods. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

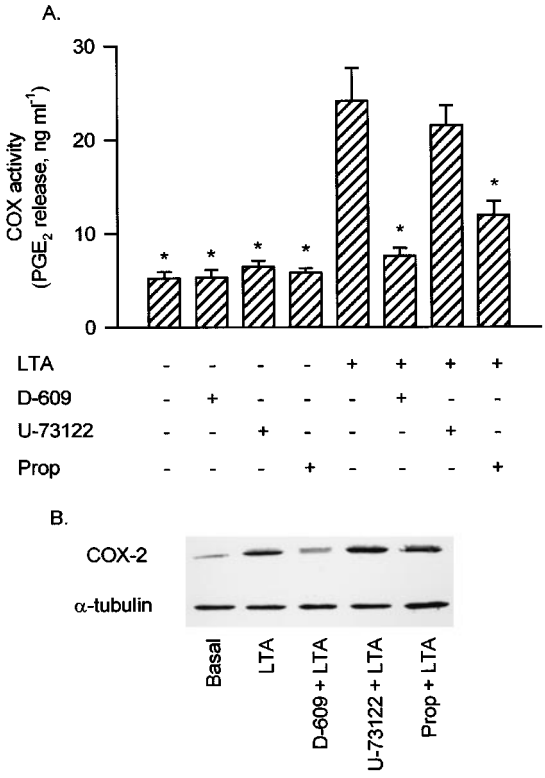

Figure 5 Effects of D-609, U-73122 and propranolol on the increases of COX activity and COX-2 expression caused by LTA in A549 cells. In (A), cells were pretreated with D-609 (50 μM), U-73122 (10 μM) or propranolol (Prop, 100 μM) for 30 min prior to a 24 h incubation with LTA (30 μg ml^{-1}). The increase of COX activity was measured by examining the PGE_2 formation in the presence of 30 μM exogeneous arachidonic acid for 30 min. Results were expressed as mean$\pm$s.e.mean ($n=3$). *$P<0.05$ as compared with the LTA-treated group. In (B), cells were pretreated with D-609 (50 μM), U-73122 (10 μM) or Prop (100 μM) for 30 min prior to a 24 h incubation with LTA (30 μg ml^{-1}). The cells were then prepared for immunodetection using COX-2 specific antibody as described in Methods. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

1989; Johnson *et al*., 1999). In the present study, we demonstrated that both D609 and propranolol inhibited LTA-induced increases in COX-2 expression and COX activity, whereas U-73122 had no effect. These results indicated that LTA-induced COX-2 expression might be *via* the PC-PLC and PC-PLD pathways, but not the PI-PLC pathway. This is consistent with the finding that the LPS-mediated induction of iNOS also depends on the activation of PC-PLC and PC-PLD in astrocytes (Chen *et al*., 1998). In resting cells, PKCs are located predominately in the cytosol (Hecker *et al*., 1993). After activated, the PKCs translocate from the cytosol to the membrane fraction (Mochly-Rosen, 1995). We found that treatment of A549 cells with LTA caused an increase in PKC activity in the membrane fraction. Moreover, the LTA-mediated increase in PKC activity in the membrane fraction was inhibited by D-609, propranolol, or Go 6976. The results of the present study indicated that LTA might activate PC-PLC and PC-PLD to induce PKC activation, which in turn induces COX-2 expression and PGE_2 release. However, the mechanism involved in the activation of PC-PLC and PC-PLD is still not well defined, but may involve tyrosine phosphorylation (Choudhury *et al*., 1991).

It has been demonstrated that transcription factor NF-κB is involved in the pro-inflammatory cytokines-induced expression of COX-2 protein (Newton *et al*., 1997; Lin *et al*., 2000). In the present study, we showed that PDTC, which inhibits NF-κB activation, markedly inhibited the LTA-induced increases in COX-2 expression and COX activity. This finding suggests that activation of NF-κB is also critical in the induction of COX-2 caused by LTA. NF-κB is constitutively presented in cells as a heterodimer, consisting of a p50 DNA-binding subunit and a p65 trans-activating subunit. In resting cells, the inhibitor subunit IκB-α is bound to the p50/p65 heterodimer of NF-κB in the cytoplasm (Thanos & Maniatis, 1995). Treatment of cells with IL-1β or TNF-α results in the specific phosphorylation of two serine residues on IκB-α (Scherer *et al*., 1995) followed by the ubiquitination (Roff *et al*., 1996) and degradation of this subunit (Thanos & Maniatis, 1995). The release of IκB-α causes an activation of NF-κB, which then translocates to the nucleus and activates transcription (Thanos & Maniatis,

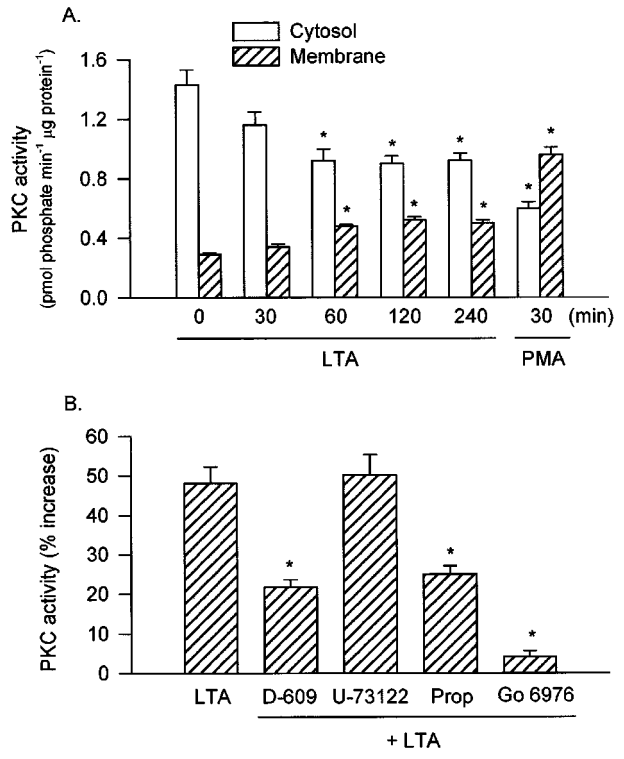

Figure 6 The PKC activity caused by LTA in the cytosol and membrane and effects of D609, U-73122, propranolol, and Go 6976 on the LTA-induced increase in PKC activity in membrane fraction of A549 cells. The cells were treated with LTA (30 μg ml^{-1}) for various time intervals, or PMA (10 nM) for 30 min (A), or pretreated with D609 (50 μM), U-73122 (10 μM), propranolol (Prop, 100 μM), and Go 6976 (1 μM) for 30 min before incubation with LTA (30 μg ml^{-1}) for 60 min (B). The subcellular (cytosol and membrane) fractions were then isolated. The PKC activities in the cytosol and membrane were measured as described in Methods. Results are expressed as mean$\pm$s.e.mean of three independent experiments performed in duplicate. *$P<0.05$ as compared with basal level (A) or LTA alone (B).

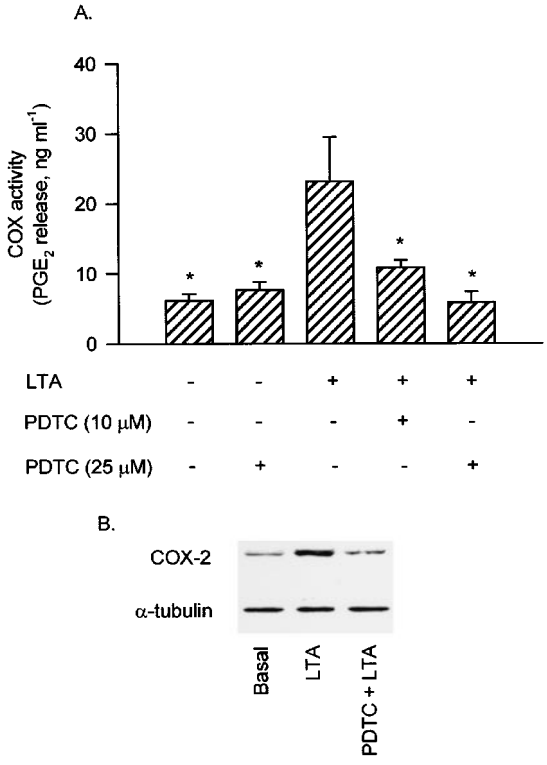

Figure 7 Effects of pyrrolidine dithiocarbamate (PDTC) on the increases of COX activity and COX-2 expression caused by LTA in A549 cells. In (A), cells were pretreated with various concentrations of PDTC for 30 min prior to a 24 h incubation with LTA (30 μg ml^{-1}). The increase of COX activity was measured by examining the PGE_2 formation in the presence of 30 μM exogeneous arachidonic acid for 30 min. Results were expressed as mean$\pm$ s.e.mean ($n=4$). *$P<0.05$ as compared with the LTA-treated group. In (B), cells were pretreated with PDTC (25 μM) for 30 min prior to a 24 h incubation with LTA (30 μg ml^{-1}). The cells were then processed for immunodetection using COX-2 specific antibody as described in Methods. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

1995). We found that treatment of A549 cells with LTA resulted in the translocation of p65 NF-κB from cytosol to the nucleus as well as the degradation of IκB-α in the cytosol. LTA also increased the level of the NF-κB-specific DNA-protein complex in nuclear extracts of A549 cells. Recent studies have shown that LTA derived from *Enterococcus faecalis* can activate NF-κB-specific DNA-protein complexes formation in basal uroepithelial cells (Elgavish, 2000). The activation of NF-κB-specific DNA-protein complexes formation caused by LTA was inhibited by D-609, propranolol, Go 6976, Ro 31-8220, and PDTC (Figure 9). These results indicated that LTA might act through the pathways of PC-PLC, PC-PLD, and PKC to induce NF-κB activation in A549 cells. Previous reports have shown that the PC-PLC might be involved in the TNF-α-induced activation of NF-κB (Schutze *et al*., 1992). It has been demonstrated that the activations of PC-PLC and PC-PLD were involved in LPS-induced NF-κB activation in astrocytes (Chen *et al*., 1998). Furthermore, PKC has also 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). However, the PKC inhibitors, Go 6976, Ro 31-8220, or GF 109203X were not able to completely block the LTA-mediated increases in COX-2 expression and COX activity, suggesting that other signal pathways might also be involved in the LTA-mediated COX-2 expression. Indeed, we have recently found that activations of p44/42 mitogen-activated protein kinase and protein kinase A are also involved in the LTA-mediated COX-2 expression, and these pathways are not dependent on PKC activation (unpublished observations).

Cytokines are also indicated in the regulation of COX-2 expression. Previous reports have shown that LTA can induce the release of cytokines, including IL-1β, TNF-α, and IL-6 in cultured human monocytes (Bhakdi *et al*., 1991). Moreover, LPS and certain pro-inflammatory cytokines, such as IL-1β, have been demonstrated to induce an increased expression of COX-2 protein in airway epithelial cells (Mitchell *et al*., 1994), airway macrophages (Lee *et al*., 1992) and monocytes (Hempel *et al*., 1994). Therefore, the LTA-induced COX-2 expression might occur through cytokines release. Whether LTA can directly induce COX-2 expression or not remains to be determined.

In this study we have demonstrated that the LTA-induced increases in COX-2 expression and COX activity are inhibited by the anti-inflammatory steroid, dexamethasone.

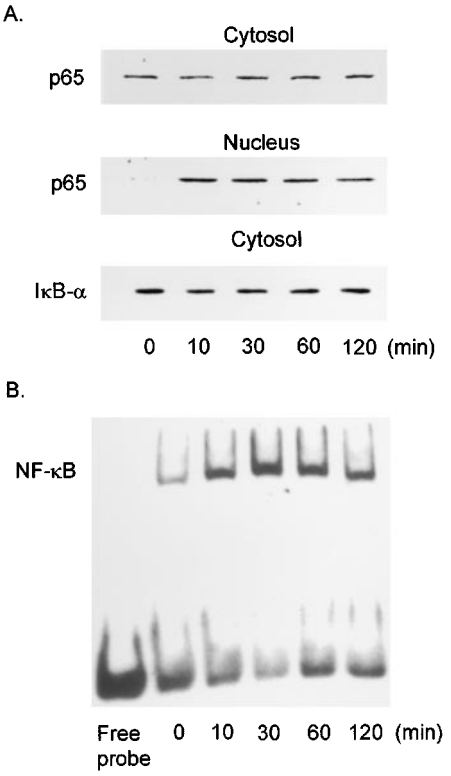

Figure 8 Kinetics of p65 NF-κB translocation, IκB-α degradation and NF-κB-specific DNA-protein complex formation induced by LTA in A549 cells. Cells were pretreated with vehicle or LTA (30 μg ml^{-1}) for various time intervals, and then prepared for subcellular (cytosol and nucleus) fractions as described in Methods. In (A), the levels of cytosolic and nuclear p65 NF-κB and cytosolic IκB-α were immunodetected with p65 NF-κB and IκB-α specific antibodies, respectively, as described in Methods. In (B), NF-κB-specific DNA-protein binding activity in nuclear extracts was determined by electrophoretic mobility shift assay (EMSA) as described in Methods.

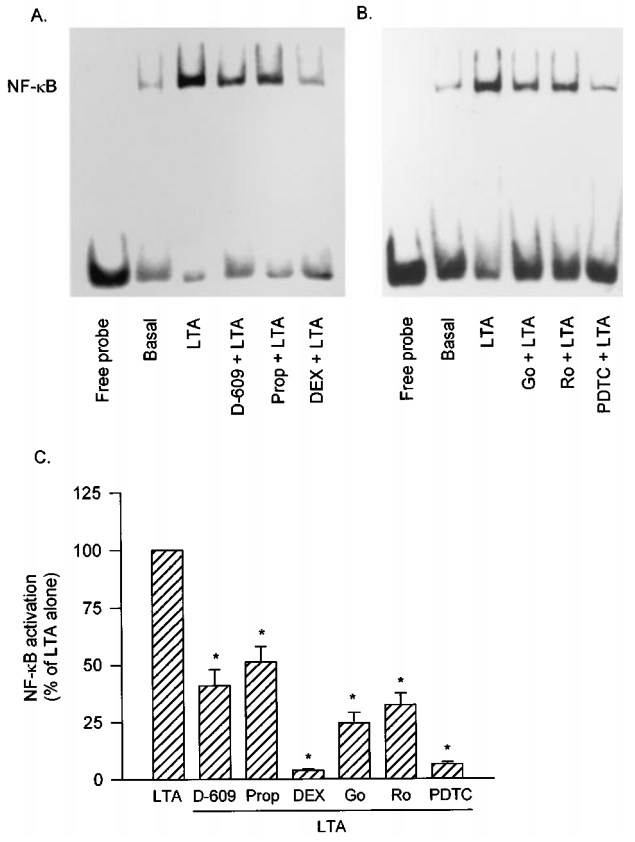

Figure 9 Effects of D-609, propranolol, dexamethasone, Go 6976, Ro 31-8220, or PDTC on the LTA-induced NF-κB-specific DNA-protein complex formation in nuclear extracts of A549 cells. Cells were pretreated with D-609 (50 μM), propranolol (Prop, 100 μM), dexamethasone (DEX, 0.1 μM) (A), or Go 6976 (Go, 1 μM), Ro 31-8220 (Ro, 1 μM), or PDTC (25 μM) (B) for 30 min prior to a 10 min incubation with LTA (30 μg ml^{-1}). The nuclear extracts were prepared for determination of NF-κB-specific DNA-protein binding activity by EMSA as described in Methods. In (C), extent of NF-κB activation was quantitated using a densitometer with Image-Pro plus software. Results were expressed as mean$\pm$s.e.mean ($n=3$). *$P<0.05$ as compared with the LTA-treated group.

Transcriptional suppression of inflammatory genes, such as IL-8 and iNOS, by glucocorticoids has been demonstrated to be mediated, at least in part, by suppression of NF-κB activity (Kleinert *et al*., 1996; Mukaida *et al*., 1994). Since the human COX-2 gene has two putative NF-κB binding sites in the promoter region (Appleby *et al*., 1994), it is possible that the suppression of LTA-mediated COX-2 expression by dexamethasone might occur by a similar mechanism. Indeed, our present study has demonstrated that the LTA-mediated activation of NF-κB-specific DNA-protein complexes formation was inhibited by dexamethasone, indicating the inhibitory action of dexamethasone on LTA-induced COX-2 expression are intimately linked to the suppression of the activity of this transcription factor. Based on the findings of the present study, we attempt to speculate that the anti-inflammatory action of glucocorticoids in inflammatory airway diseases caused by gram-positive bacteria might be associated with the inhibitory action of these compounds on induction of immediate early genes such as COX-2, which subsequently suppresses the prostanoids release at the site of inflammation.

In conclusion, LTA might activate PC-PLC and PC-PLD to elicit PKC activation, which in turn initiates NF-κB activation, and finally causes COX-2 expression and PGE_2 release. This is the first study showing LTA can induce COX-2 expression and PGE_2 release in human pulmonary epithelial cells. The results indicate that COX-2 induction and the subsequent enhanced release of PGE_2 may be involved in airway inflammation elicited by gram-positive organisms. An understanding of the molecular mechanisms involved in the regulation of COX-2 expression by LTA promotes new insights into the pathophysiology of inflammation and may lead to new therapeutical strategies capable of interrupting the inflammatory cascade at key points.

This work was supported by a research grant from the National Science Council of the Republic of China (NSC89-2320-B-038-031).

References

APPLEBY, S.B., RISTIMAKI, A., NEILSON, K., NARKO, K. & HLA, T. (1994). Structure of the human cyclo-oxygenase-2 gene. *Biochem. J.,* **302,** 723 – 727.

AUGUET, M., LONCHAMPT, M.O., DELAFLOTTE, S., GOULIN-SCHULZ, J., CHABRIER, P.E. & BRAQUET, P. (1992). Induction of nitric oxide synthase by lipoteichoic acid from *Staphylococcus aureus* in vascular smooth muscle cells. *FEBS Lett.,* **297,** 183 – 185.

BARNES, P.J. & KARIN, M. (1997). Nuclear factor-κB – a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.,* **336,** 1066 – 1071.

BHAKDI, S., KLONISCH, T., NUBER, P. & FISCHER, W. (1991). Stimulation of monokine production by lipoteichoic acids. *Infect. Immun.,* **59,** 4614 – 4620.

BILLAH, M.M., ECKEL, S., MULLMANN, T.J., EGAN, R.W. & SIEGEL, M.I. (1989). Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils. Involvement of phosphatidate phosphohydrolase in signal transduction. *J. Biol. Chem.,* **264,** 17069 – 17077.

BLEASDALE, J.E., THAKUR, N.R., GREMBAN, R.S., BUNDY, G.L., FITZPATRICK, F.A., SMITH, R.J. & BUNTING, S. (1990). Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J. Pharmacol. Exp. Ther.,* **255,** 756 – 768.

BONE, R.C. (1994). Gram-positive organisms and sepsis. *Arch. Intern. Med.,* **154,** 26 – 34.

CHAN, C.C., BOYCE, S., BRIDEAU, C., FORD-HUTCHINSON, A.W., GORDON, R., GUAY, D., HILL, R.G., LI, C.S., MANCINI, J., PENNETON, M., PRASIT, P., RASORI, R., RIENDEAU, D., ROY, P., TAGARI, P., VICKERS, P., WONG, E. & RODGER, I.W. (1995). Pharmacology of a selective cyclooxygenase-2 inhibitor, L-745,337: a novel nonsteroid antiinflammatory agent with an ulcerogenic sparing effect in rat and non-human primate stomach. *J. Pharmacol. Exp. Ther.,* **274,** 1531 – 1537.

CHEN, C.C., WANG, J.K., CHEN, W.C. & LIN, S.B. (1998). Protein kinase C-η mediates lipopolysaccharide-induced nitric oxide synthase expression in primary astrocytes. *J. Biol. Chem.,* **273,** 19424 – 19430.

CHOUDHURY, G.G., SYLVIA, V.L., WANG, L.M., PIERCE, J. & SAKAGUCHI A.Y. (1991). The kinase insert domain of colony stimulating factor-1 receptor is dispensable for CSF-1 induced phosphatidylcholine hydrolysis. *FEBS Lett.,* **282,** 351 – 354.

ELGAVISH, A. (2000). NF-kappa B activation mediates the response of a subpopulation of basal uroepithelial cells to a cell wall component of *Enterococcus faecalis*. *J. Cell Physiol.,* **182,** 232 – 238.

EXTON, J.H. (1994). Phosphatidylcholine breakdown and signal transduction. *Biochim. Biophys. Acta.,* **1212,** 26 – 42.

HECKER, M., LUCKHOFF, A. & BUSSE, R. (1993). Modulation of endothelial autacoid release by protein kinase C: feedback inhibition or non-specific attenuation of receptor-dependent cell activation? *J. Cell Physiol.,* **156,** 571 – 578.

HEMPEL, S., MONICK, M.M. & HUNNINGHAKE, G.W. (1994). Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J. Clin. Invest.,* **93,** 391 – 396.

HUG, H. & SARRE, T.F. (1993). Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.,* 291, 329 – 343.

JOHNSON, C.A., BALBOA, M.A., BALSINDE, J. & DENNIS, E.A. (1999). Regulation of cyclooxygenase-2 expression by phosphatidate phosphohydrolase in human amnionic WISH cells. *J. Biol. Chem.,* **274,** 27689 – 27693.

KENGATHARAN, M., DE KIMPE, S.J. & THIEMERMANN, C. (1996). Analysis of the signal transduction in the induction of nitric oxide synthase by lipoteichoic acid in macrophages. *Br. J. Pharmacol.,* **117,** 1163 – 1170.

KLEINERT, H., EUCHENHOFER, C., IHRIG-BIEDERT, I. & FORSTERMANN, U. (1996). Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-κB. *Mol. Pharmacol.,* **49,** 15 – 21.

LEE, S.H., SOYOOLA, E., CHANMUGAM, P., HART, S., SUN, W., ZHONG, H., LIOU, S., SIMMONS, D. & HWANG, D. (1992). Selective expression of mitogen-inducible cyclo-oxygenase in macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.,* **267,** 25934 – 25938.

LI, H., OEHRLEIN, S.A., WALLERATH, T., IHRIG-BIEDERT, I., WOHLFART, P., ULSHOFER, T., JESSEN, T., HERGET, T., FORSTERMANN, U. & KLEINERT, H. (1998). Activation of protein kinase C α and/or ε enhances transcription of the human endothelial nitric oxide synthase gene. *Mol. Pharmacol.,* **53,** 630 – 637.

LIN, C.H., SHEU, S.Y., LEE, H.M., HO, Y.S., LEE, W.S., KO, W.C. & SHEU, J.R. (2000). Involvement of protein kinase C-γ in IL-1β-induced cyclooxygenase-2 expression in human pulmonary epithelial cells. *Mol. Pharmacol.,* **57,** 36 – 43.

MAIER, J.A., HLA, T. & MACIAG, T. (1990). Cyclo-oxygenase is an immediate early gene induced by interleukin-1 in human endothelial cells. *J. Biol. Chem.,* **265,** 10805 – 10808.

MATTSSON, E., VERHAGE, L., ROLLOF, J., FLEER, A., VERHOEF, J. & VANDIJK, H. (1993). Peptidoglycan and teichoic acid from *Staphylococcus epidermidis* stimulate human monocytes to release tumor necrosis factor-α, interleukin-1β and interleukin-6. *FEMS. Immunol. Med. Microbiol.,* **7,** 281 – 287.

MITCHELL, J.A., AKARASEREENONT, P., THIEMERMANN, C., FLOWER, R.J. & VANE, J.R. (1993). Selectivity of non-steroid anti-inflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc. Nal. Acad. Sci. U.S.A.,* **90,** 11693 – 11697.

MITCHELL, J.A., BELVISI, M.G., AKARASEREENONT, P., ROBBINS, R.A., KWON, O.-J., CROXTALL, J., BARNES, P.J. & VANE, J.R. (1994). Induction of cyclo-oxygenase-2 by cytokines in human pulmonary epithelial cells: regulation of dexamethasone. *Br. J. Pharmacol.,* **113,** 1008 – 1014.

MITCHELL, J.A., LARKIN, S. & WILLIAMS, T.J. (1995). Cyclooxygenase-2: regulation and relevance in inflammation. *Biochem. Pharmacol.,* **50,** 1535 – 1542.

MOCHLY-ROSEN, D. (1995). Location of protein kinases by anchoring proteins: a theme in signal transduction. *Science (Washington DC),* **268,** 247 – 251.

MUKAIDA, N., MORITA, M., ISHIKAWA, Y., RICE, N., OKAMOTO, S., KASAHARA, T. & MATSUSHIMA, K. (1994). Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-κB is target for glucocorticoid-mediated interleukin 8 gene repression. *J. Biol. Chem.,* **269,** 13289 – 13295.

NEWTON, R., KUITERT, L.M., BERGMANN, M., ADCOCK, I.M. & BARNES, P.J. (1997). Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta. *Biochem. Biophys. Res. Commun.,* **237,** 28 – 32.

NISHIZUKA, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science (Washington DC),* **258,** 607 – 614.

NUNOKAWA, Y., OIKAWA, S. & TANAKA, S. (1996). Human inducible nitric oxide synthase gene is transcriptionally regulated by nuclear factor-κB dependent mechanism. *Biochem. Biophys. Res. Commun.,* **223,** 347 – 352.

ROFF, M., THOMPSON, J., RODRIGUEZ, M.S., JACQUE, J.M., BALEUX, F., ARENZANA SEISDEOS, F. & HAY, R.T. (1996). Role of IκB-α ubiquitination in signal-induced activation of NF-κB *in vivo*. *J. Biol. Chem.,* **271,** 7844 – 7850.

RZYMKIEWICZ, D.M., TETSUKA, T., DAPHNA-IKEN, D., SRIVASTAVA, S. & MORRISON, A.R. (1996). Interleukin-1β activates protein kinase Cζ in renal mesangial cells. Potential role in prostaglandin E_2 up-regulation. *J. Biol. Chem.,* **271,** 17241 – 17246.

SCHERER, D.C., BROKMAN, J.A., CHEN, Z., MANIATIS, T. & BALLARD, D.W. (1995). Signal-induced IκB-α degradation of requires site-specific ubiquitination. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 11259 – 11263.

SCHUTZE, S., MACHLEIDT, T. & KRONKE, M. (1994). The role of diacylglycerol and ceramide in tumor necrosis factor and interleukin-1 signal transduction. *J. Leukoc. Biol.,* **56,** 533 – 541.

SCHUTZE, S., POTTHOFF, K., MACHLEIDT, T., BERKOVIC, D., WEIGMANN, K. & KRONKE, M. (1992). TNF activate NF-κB by phosphatidylcholine-specific phospholipase C-induced 'acidic' sphingomyelin breakdown. *Cell,* **71,** 765–776.

SHEU, J.R., LEE, C.R., LIN, C.H., HSIAO, G., KO, W.C., CHEN, Y.C. & YEN, M.H. (2000). Mechanisms involved in the antiplatelet activity of *Staphylococcus aureus* lipoteichoic acid in human platelets. *Thromb. Haemost.,* **83,** 777–784.

THANOS, D. & MANIATIS, T. (1995). NF-κB: a lesson in family values. *Cell,* **80,** 529–532.

VANE, J.R. (1994). Pharmacology: towards a better aspirin. *Nature (Lond),* **367,** 215.

VANE, J.R., BAKHLE, Y.S. & BOTTING, R.M. (1998). Cyclooxygenases 1 and 2. *Annu. Rev. Pharmacol. Toxicol.,* **38,** 97–120.

VANE, J.R., MITCHELL, J.A., APPLETON, I., TOMLINSON, A., BISHOP-BAILEY, D.A., CROXTALL, J. & WILLOUGHBY, D. (1994). Inducible isoforms of cyclooxygenase and nitric oxide synthase in inflammation. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 2046–2050.

XIE, W.L., CHIPMAN, J.G., ROBERSTON, D.L., ERIKSON, R.L. & SIMMONS, D.L. (1991). Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 2692–2696.

(Received May 21, 2001
Accepted July 17, 2001)