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Lipoteichoic acid-induced cyclooxygenase-2 expression requires activations of p44/42 and p38 mitogen-activated protein kinase signal pathways

Chien-Huang Lin [a,b,*], I-Hui Kuan [a], Chun-Hua Wang [c], Horng-Mo Lee [a], Wen-Sen Lee [d], Joen-Rong Sheu [d], George Hsiao [d], Chih-Hsiung Wu [d], Han-Pin Kuo [c]

[a] *Graduate Institute of Biomedical Technology, Taipei Medical University, Taipei, Taiwan, ROC*
[b] *School of Respiratory Therapy, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan, ROC*
[c] *Department of Thoracic Medicine II, Chang Gung Memorial Hospital, Taipei, Taiwan, ROC*
[d] *Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan, ROC*

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Abstract

This study investigated the role of p44/42 and p38 mitogen-activated protein kinase (MAPK) in cyclooxygenase-2 expression caused by lipoteichoic acid in human pulmonary epithelial cell line (A549). Lipoteichoic acid-induced increases in cyclooxygenase activity and cyclooxygenase-2 expression were attenuated by tyrosine kinase inhibitors (genistein and tyrphostin AG126), a MAPK/extracellular signal-regulated protein kinase (MEK) inhibitor [2′-amino-3′-methoxyflavone] (PD 98059) and a p38 MAPK inhibitor [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole] (SB 203580). Lipoteichoic acid-induced p44/42 MAPK activation was inhibited by protein kinase C (PKC) inhibitors [12-(2-cyanoethyl)6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-a)pyrrolo(3,4-c)-carbazole] (Go 6976) and {3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide]} (Ro 31-8220), genistein and PD 98059. Lipoteichoic acid-induced increase in p38 MAPK activity was inhibited by Go 6976, Ro 31-8220, genistein and SB 203580. Lipoteichoic acid-mediated formation of nuclear factor-κB (NF-κB)-specific DNA–protein complex was inhibited by genistein, tyrphostin AG126, PD 98059 and SB 203580. These results suggest that the activations of both p44/42 and p38 MAPK by lipoteichoic acid result in stimulation of NF-κB-specific DNA–protein binding and subsequent cyclooxygenase-2 expression in A549 cells. Both events required activation of upstream tyrosine kinase and PKC.

Keywords: Lipoteichoic acid; Cyclooxygenase-2; p44/42 mitogen-activated protein kinase (MAPK); p38 MAPK; A549 cell

1. Introduction

Prostaglandins are ubiquitous compounds involved in various homeostatic and inflammatory processes throughout the body (Vane et al., 1998). They are formed by the combined action of phospholipase A_2, which liberates arachidonic acid from the *sn*-2 position of cellular membrane phospholipids, and cyclooxygenase, which converts arachidonic acid to the endoperoxide intermediate prostaglandin H_2. Prostaglandin H_2 is subsequently converted to various prostaglandins by the action of cell-specific synthases (Vane et al., 1998). Two cyclooxygenase isozymes, cyclooxygenase-1 and cyclooxygenase-2, have been identified in humans and bear 60% homology (Xie et al., 1991; Mitchell et al., 1995). Cyclooxygenase-1, constitutively expressed in most tissues, mediates physiological responses such as regulation of renal and vascular homeostasis and cytoprotection of the stomach. On the other hand, cyclooxygenase-2 is considered to be an inducible immediate-early gene product whose synthesis in cells can be up-regulated by mitogenic or inflammatory stimuli, including tumor promoters (Kujubu et al., 1991), platelet-derived growth factor (Habenicht et al., 1985), cytokines (Maier et al., 1990) and lipopolysaccharide (Mitchell et al., 1993) in vitro and at the site of inflammation in vivo (Vane et al.,

* Corresponding author. School of Respiratory Therapy, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan, ROC. Tel.: +886-2-27361661x3318; fax: +886-2-27324510.
E-mail address: chlin@tmu.edu.tw (C.-H. Lin).

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1994). Cyclooxygenase-2 is thought 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 risen considerably over the last decade (Bone, 1994), our knowledge regarding the mechanisms underlying 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; Mattson et al., 1993). Lipoteichoic acid 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 inhibits platelet aggregation caused by collagen, thrombin and ADP in human platelets (Sheu et al., 2000). Moreover, we demonstrated that lipoteichoic acid stimulates the expression of cyclooxygenase-2 in human pulmonary epithelial cells (Lin et al., 2001). However, the intracellular signaling pathways by which lipoteichoic acid causes cyclooxygenase-2 expression are largely undetermined. Recent reports have demonstrated that both p44/42 and p38 mitogen-activated protein kinase (MAPK) are activated by lipopolysaccharide or the pro-inflammatory cytokine, interleukin-1β (Chen and Wang, 1999; Netwon et al., 2000). These findings suggest that these two kinase pathways might be the important signaling mechanisms underlying the inflammatory process.

Previously, we showed that lipoteichoic acid activates phosphatidylcholine–phospholipase C (PC-PLC) and phosphatidylcholine–phospholipase D (PC-PLD) to induce protein kinase C (PKC) activation, which in turn initiates nuclear factor-κB (NF-κB) activation, and finally induces cyclooxygenase-2 expression and prostaglandin E_2 release in human pulmonary epithelial cells (Lin et al., 2001). However, the role of p44/42 and p38 MAPK in lipoteichoic acid-induced cyclooxygenase-2 expression has not yet been determined. In this study, we therefore studied the signaling transduction pathway of lipoteichoic acid-induced activations of p44/42 and p38 MAPK and their roles in lipoteichoic acid-mediated NF-κB activation and cyclooxygenase-2 expression in human pulmonary epithelial cells.

2. Materials and methods

2.1. Materials

Lipoteichoic acid (from *Staphylococcus aureus*), lipopolysaccharide (from *Escherichia coli*), phorbol-12-myristate-13-acetate (PMA), polymyxin B, Trizma base, dithiothreitol, glycerol, phenylmethylsulphonyl fluoride, pepstatin A, leupeptin and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). [2′-amino-3′-methoxyflavone] (PD 98059), [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole] (SB 203580), genistein, daidzein, tyrphostin AG126, tyrphostin A-1, [12-(2-cyanoethyl)6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-a)pyrrolo(3,4-c)-carbazole] (Go 6976) and {3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide]} (Ro 31-8220) were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Penicillin/streptomycin, Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 and fetal calf serum were purchased from Life Technologies (Gaithersburg, MD, USA). The prostaglandin E_2 enzyme immunoassay kit was obtained from Cayman Chem. (Ann Arbor, MI, USA). An antibody specific for cyclooxygenase-2 was purchased from Transduction Laboratories (Lexington, KY, USA). An antibody specific for phospho-p38 MAPK and the p38 MAPK activity assay kit were purchased from New England Biolabs (Beverly, MA, USA). Antibodies specific for phospho-p44/42 MAPK, p44/42 MAPK, p38 MAPK, CD14 and anti-rabbit immunoglobulin G (IgG)-conjugated alkaline phosphatase were purchased from Santa Cruz Biochemicals (Santa Cruz, CA, USA). An antibody specific for α-tubulin was purchased from Oncogene Science (Cambridge, UK). Anti-mouse IgG-conjugated alkaline phosphatase was purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). A digoxigenin (DIG) gel shift kit, 4-nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate were purchased from Boehringer Mannheim (Mannheim, Germany). Protein assay reagents were purchased from Bio-Rad (Hercules, CA, USA).

2.2. Cell culture

The A549 cell, a human pulmonary epithelial carcinoma cell line with type II alveolar epithelial cell differentiation, was obtained from American Type Culture Collection and grown in DMEM/Ham's F-12 nutrient mixture containing 10% fetal calf serum and penicillin/streptomycin (50 U/ml) in a humidified 37 °C incubator. After reaching confluence, cells were disaggregated in a trypsin solution, washed in DMEM/Ham's F-12 supplemented with 10% fetal calf serum, centrifuged at $125 \times g$ for 5 min, resuspended and then subcultured according to standard protocols.

2.3. Measurement of cyclooxygenase activity

The increase of cyclooxygenase activity was quantified by providing cells with exogenous arachidonic acid, the substrate for cyclooxygenase, and measuring its conversion into prostaglandin E_2. A549 cells were cultured in 12-well culture plates. After reaching confluence, cells were treated with vehicle, lipoteichoic acid (30 μg/ml), or pretreated with specific inhibitors as indicated followed by lipoteichoic acid, and then incubated in a humidified incubator at 37 °C for 24 h. Cells were then washed with phosphate-buffered

saline (PBS), and treated with fresh medium containing arachidonic acid (30 μg/ml) for 30 min at 37 °C. The medium was removed and stored at −80 °C until assay. Prostaglandin E_2 in the medium was assayed using the prostaglandin E_2 enzyme immunoassay kit according to the procedure described by the manufacturer.

2.4. Protein preparation and Western blotting

For determination of the expression of cyclooxygenase-2, α-tubulin, phosphorylated p44/42 MAPK, phosphorylated p38 MAPK, nonphosphorylated p44/42 MAPK and nonphosphorylated p38 MAPK in A549 cells, proteins were extracted and Western blotting analysis was performed as described previously (Lin et al., 2000). Briefly, A549 cells were cultured in 10-cm petri dishes. After reaching confluence, cells were treated with 30 μg/ml lipoteichoic acid or 10 nM PMA for indicated time intervals, and then incubated in a humidified incubator at 37 °C. In some experiments, cells were incubated with specific inhibitors as indicated before lipoteichoic acid treatment. After incubation, cells were washed with PBS (pH 7.4), incubated with extraction buffer (10 mM Tris [pH 7.0], 140 mM NaCl, 2 mM phenylmethylsulphonyl fluoride, 5 mM dithiothreitol, 0.5% NP-40, 0.05 mM pepstatin A and 0.2 mM leupeptin) with gentle shaking and then centrifuged at 12,500 × *g* for 30 min. The cell extract was then boiled in a ratio of 1:1 with sample buffer (100 mM Tris [pH 6.8], 20% glycerol, 4% SDS and 0.2% bromophenol blue). Electrophoresis was performed using 10% SDS–polyacrylamide gels (2 h, 110 V, 40 mA, 30 μg of protein per lane). Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (2 h, 40 V), nonspecific IgGs were blocked with 5% fat-free milk powder, and incubated for 2 h with specific antibodies for cyclooxygenase-2, α-tubulin, phospho-p44/42 MAPK, phospho-p38 MAPK, p44/42 MAPK, or p38 MAPK. The blot (for cyclooxygenase-2, α-tubulin, phospho-p44/42 MAPK and p44/42 MAPK) was then incubated with anti-mouse or anti-rabbit IgG linked to alkaline phosphatase for 2 h. Subsequently, the membrane was developed with 4-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate as a substrate. The blot (for phospho-p38 MAPK and p38 MAPK) was incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody for 2 h. After incubation, the immunoreactive band was detected with ECL-detecting reagents and developed with Hyperfilm-ECL. Quantitative data were obtained using a computing densitometer with Image-Pro plus software (Media Cybernetics, MD).

2.5. Measurement of p38 MAPK activity

The activity of p38 MAPK was measured using a p38 MAPK activity assay kit (New England Biolabs). Briefly, A549 cells were cultured in 10-cm petri dishes. After reaching confluence, cells were treated with 30 μg/ml lipoteichoic acid for indicated time intervals and incubated in a humidified incubator at 37 °C. In some experiments, cells were pretreated with specific inhibitors as indicated followed by lipoteichoic acid. After incubation, cells were washed with PBS (pH 7.4), incubated with lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4 and 1 μg/ml leupeptin) with gentle shaking, and then centrifuged at 12,500 × *g* for 30 min. Cell extracts were incubated with anti-phospho-p38 MAPK antibody which was immobilized to agarose hydrazide beads, and shaked at 4 °C for 24 h. The mixtures were then centrifuged at 12,500 × *g* for 30 s at 4 °C. Cell pellet was washed twice with lysis buffer, and then incubated with 50 μl of kinase buffer (25 mM Tris [pH 7.5], 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4 and 10 mM $MgCl_2$) supplemented with 200 μM ATP and 2 μg ATF-2 fusion protein for 60 min at 30 °C. The reaction was terminated by the addition of 3 × SDS sample buffer, after which it was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. Separated proteins were transferred to a nitrocellulose membrane, and incubated for 2 h with specific antibodies for phosphorylated ATF-2. The blot was then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000) and horseradish peroxidase-conjugated anti-biotin antibody (1:1000) for 1 h at 25 °C. The membrane was finally detected with LumiGLO chemiluminescent reagent and exposed to X-ray film. Quantitative data were obtained using a computing densitometer with Image-Pro plus software (Media Cybernetics).

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

A549 cells were cultured in 10-cm petri dishes. After reaching confluence, cells were pretreated with genistein (30 μM), tyrphostin AG126 (30 μM), PD 98059 (30 μM), or SB 203580 (1 μM) for 30 min before incubation of lipoteichoic acid (30 μg/ml) for 30 min. The cytosolic and nuclear protein fractions were then separated as described previously (Chen and Wang, 1999). Briefly, cells were washed with ice-cold PBS and pelleted. Cell pellet was resuspended in hypotonic buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.5 mM dithiothreitol, 10 mM aprotinin, 10 mM leupeptin and 20 mM PMSF) for 15 min on ice, and vortexed for 10 s. Nuclei were pelleted by centrifugation at 15,000 × *g* for 1 min. Supernatants containing cytosolic proteins were collected. A pellet containing nuclei was resuspended in hypertonic buffer (20 mM HEPES [pH 7.6], 25% glycerol, 1.5 mM $MgCl_2$, 4 mM EDTA, 0.05 mM dithiothreitol, 10 mM aprotinin, 10 mM leupeptin and 20 mM PMSF) for 30 min on ice. Supernatants containing nuclear proteins were collected by centrifugation at 15,000 × *g* for 2 min and then stored at −70 °C.

Electrophoretic mobility shift assay (EMSA) was performed using a DIG gel shift kit. Briefly, a double-stranded oligonucleotide probe containing NF-κB sequences (5′-AGTTGAGGGGACTTTCCCAGGC-3′ Promega) was purchased and end labeled with DIG using terminal transferase. The nuclear extract (5–10 μg) was incubated with 4 ng of DIG-labeled 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 dithiothreitol, 1% (w/v) 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. The gel was then transferred to a nylon membrane. The gel was incubated with 0.1% milk in Tris-base saline buffer containing 0.1% Tween 20 (TBST) at room temperature for 30 min, and then with anti-DIG linked to alkaline phosphatase for 30 min. The immunoreactive band was finally detected with disodium 3-[4-methoxyspiro{1,2-dioxetane-3,2′-[5-chloro]tricyclo[3.3.1.1^{3,7}]decan}-4-yl] phenyl phosphate (CSPD) detecting reagents and exposed to X-ray film. Quantitative data were obtained by using a computing densitometer with Image-Pro plus software (Media Cybernetics).

2.7. Statistical analysis

Results shown are the mean ± S.E.M. from three to four 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 to be statistically significant.

3. Results

3.1. Effect of polymyxin B on lipoteichoic acid-induced cyclooxygenase-2 expression

Exposure of A549 cells to lipoteichoic acid-stimulated prostaglandin E_2 release and the expression of 70-kDa cyclooxygenase-2 in concentration- and time-dependent manners (Lin et al., 2001), with the maximum being observed using 30 μg/ml lipoteichoic acid for 24 h; this condition was therefore used in the following cyclooxygenase-2 expression experiments. Pretreatment of cells with polymyxin B (0.5 μg/ml), which binds and inactivates endotoxin (Kengatharan et al., 1996), for 30 min attenuated lipopolysaccharide (1 μg/ml)-induced increases in cyclooxygenase-2 expression and cyclooxygenase activity as reflected by prostaglandin E_2 secretion (measured in the presence of 30 μM exogenous arachidonic acid for 30 min), while it had no effect on lipoteichoic acid-induced effects (Fig. 1A and B). These results indicate that the induction of cyclooxygenase-2 protein caused by lipoteichoic acid is not due to contamination with lipopolysaccharide. Pretreatment of A549 cells for 30 min with the CD14 antibody (2.5 μg/ml) almost completely inhibited the LTA-induced cyclooxygenase-2 expression. The CD14 antibody (2.5 μg/ml) alone did not affect the basal cyclooxygenase-2 expression (data not shown).

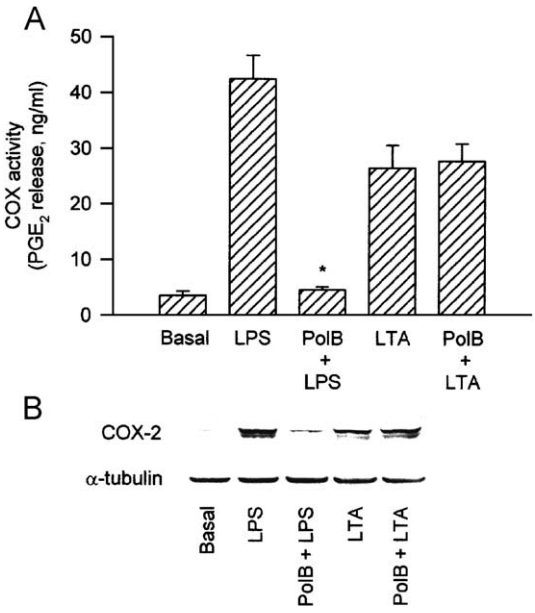

Fig. 1. Effects of polymyxin B on lipopolysaccharide- and lipoteichoic acid-induced increases in cyclooxygenase activity and cyclooxygenase-2 expression in A549 cells. In (A), cells were pretreated with polymyxin B (0.5 μg/ml) for 30 min before incubation with lipopolysaccharide (1 μg/ml) or lipoteichoic acid (30 μg/ml) for 24 h. The increase of cyclooxygenase activity was measured by examining prostaglandin E_2 formation in the presence of 30 μM exogeneous arachidonic acid for 30 min. Results are expressed as the mean ± S.E.M. ($n=3$). * $P<0.05$ as compared with the lipopolysaccharide- or lipoteichoic acid-treated group, respectively. In (B), cells were pretreated with polymyxin B (0.5 μg/ml) for 30 min before incubation with lipopolysaccharide (1 μg/ml) or lipoteichoic acid (30 μg/ml) for 24 h. Cells were then prepared for immunodetection using cyclooxygenase-2- or α-tubulin-specific antibody as described in Materials and methods. Equal loading in each lane was demonstrated by similar intensities of α-tubulin. LTA, lipoteichoic acid; LPS, lipopolysaccharide; PolB, polymyxin B, COX, cyclooxygenase; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2.

3.2. Roles of p44/42 and p38 MAPK in lipoteichoic acid-induced cyclooxygenase-2 expression

We have previously demonstrated that lipoteichoic acid might activate PC-PLC and PC-PLD to induce PKC activation, which in turn initiates NF-κB activation, and finally induces cyclooxygenase-2 expression and prostaglandin E_2 release in A549 cells (Lin et al., 2001). In order to examine whether p44/42 and p38 MAPK are also involved in the lipoteichoic acid-induced signal transduction pathway leading to cyclooxygenase-2 expression, cells were pretreated with a MAPK/extracellular signal-regulated protein kinase (MEK)-specific inhibitor (PD 98059) and a p38 MAPK inhibitor (SB 203580). Pretreatment of A549 cells for 30 min with PD 98059 (10 and 30 μM) or SB 203580 (1 and 10

μM) inhibited the lipoteichoic acid (30 μg/ml)-induced increase in cyclooxygenase activity (Fig. 2A). Treatment of A549 cells with these inhibitors at the indicated concentrations had no effect on basal cyclooxygenase activity (Fig. 2A). The lipoteichoic acid-induced increase of the cyclooxygenase-2 protein level was also inhibited by pretreatment of cells with 30 μM PD 98059 or 1 μM SB 203580 (Fig. 2C). Pretreatment of cells for 30 min with the tyrosine kinase inhibitor, genistein (10 and 30 μM), or tyrphostin AG126 (10 and 30 μM) markedly attenuated the lipoteichoic acid-induced increase in cyclooxygenase activity (Fig. 2B). None of these tyrosine kinase inhibitors affected basal cyclooxygenase activity (Fig. 2B). Lipoteichoic acid-induced cyclooxygenase-2 expression was also attenuated by 30 μM genistein or 30 μM tyrphostin AG126 treatment (Fig. 2D). However, neither daidzein (30 μM), an inactive analogue of genistein, nor tyrphostin A-1 (30 μM), an inactive analogue of tyrphostin AG126, affected the lipoteichoic acid-induced increase in cyclooxygenase activity and cyclooxygenase-2 expression (data not shown).

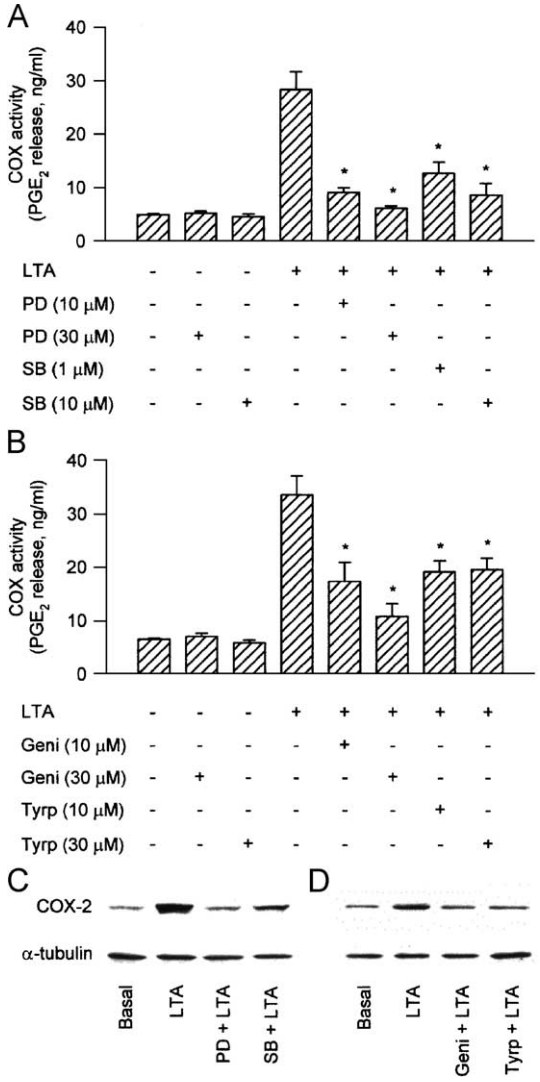

Fig. 2. Effects of various inhibitors on increases in cyclooxygenase activity and cyclooxygenase-2 expression caused by lipoteichoic acid in A549 cells. In (A) and (B), cells were pretreated with various concentrations of PD 98059, SB 203580 (A), genistein, or tyrphostin AG126 (B) for 30 min before incubation with 30 μg/ml lipoteichoic acid for 24 h. The increase of cyclooxygenase activity was measured by examining prostaglandin E_2 formation in the presence of 30 μM exogeneous arachidonic acid for 30 min. Results are expressed as the mean ± S.E.M. ($n = 4$). * $P < 0.05$ as compared with the lipoteichoic acid-treated group. In (C) and (D), cells were pretreated with 30 μM PD 98059, 1 μM SB 203580 (C), 30 μM genistein, or 30 μM tyrphostin AG126 (D) for 30 min before incubation with 30 μg/ml lipoteichoic acid for 24 h. Cells were then prepared for immunodetection using cyclooxygenase-2- or α-tubulin-specific antibody as described in Materials and methods. Equal loading in each lane was demonstrated by similar intensities of α-tubulin. LTA, lipoteichoic acid; PD, PD 98059; SB, SB 203580; Geni, genistein; Tyrp, tyrphostin AG126; COX, cyclooxygenase; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2.

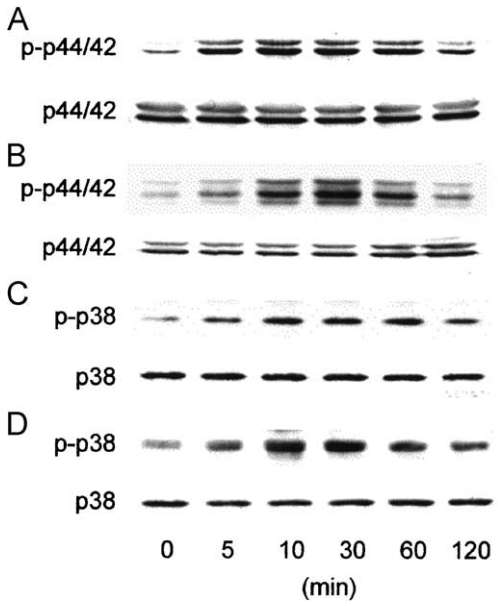

Fig. 3. Time-dependent activations of p44/42 and p38 MAPK caused by lipoteichoic acid and PMA in A549 cells. Cells were incubated with 30 μg/ml lipoteichoic acid (A, C) or 10 nM PMA (B, D) for indicated time intervals. Whole cell lysates were prepared and subjected to Western blot analysis using antibodies specific for phosphorylated p44/42 (p-p44/42), nonphosphorylated p44/42 MAPK (p44/42), phosphorylated p38 (p-p38), or nonphosphorylated p38 MAPK (p38) as described in Materials and methods.

3.3. Roles of PKC and tyrosine kinase in lipoteichoic acid-induced activations of p44/42 and p38 MAPK

Since activation of MAPKs requires phosphorylation at the threonine and tyrosine residues, immunoblot analysis was performed to examine MAPKs phosphorylation using anti-phospho-p44/42 MAPK-specific antibodies. Treatment of A549 cells with lipoteichoic acid (30 μg/ml) for various time intervals caused a marked activation of p44/42 MAPK with a maximal response at 10 min treatment (Fig. 3A). Furthermore, when cells were treated with 10 nM PMA, a PKC activator, for various time intervals resulted in an

activation of p44/42 MAPK. The activation of p44/42 MAPK peaked at 30 min after PMA treatment and then had declined by 60 min after treatment (Fig. 3B). The protein level of p44/42 MAPK was not affected by lipoteichoic acid and PMA treatment (Fig. 3A and B). Lipoteichoic acid-induced activation of p44/42 MAPK was markedly inhibited by pretreatment of cells for 30 min with the PKC inhibitors (Go 6976 [0.1 μM] or Ro 31-8220 [0.1 μM]), genistein (30 μM), or PD 98059 (30 μM), but not by SB 203580 (1 μM) (Fig. 4A,B and C). None of these treatments had any effect on p44/42 MAPK expression (Fig. 4A and B). Stimulation of cells with lipoteichoic acid (30 μg/ml) for 10–60 min resulted in marked activation of p38 MAPK. However, after 120 min treatment with lipoteichoic acid, the activation of p38 MAPK was decreased (Fig. 3C). Treatment of cells with 10 nM PMA

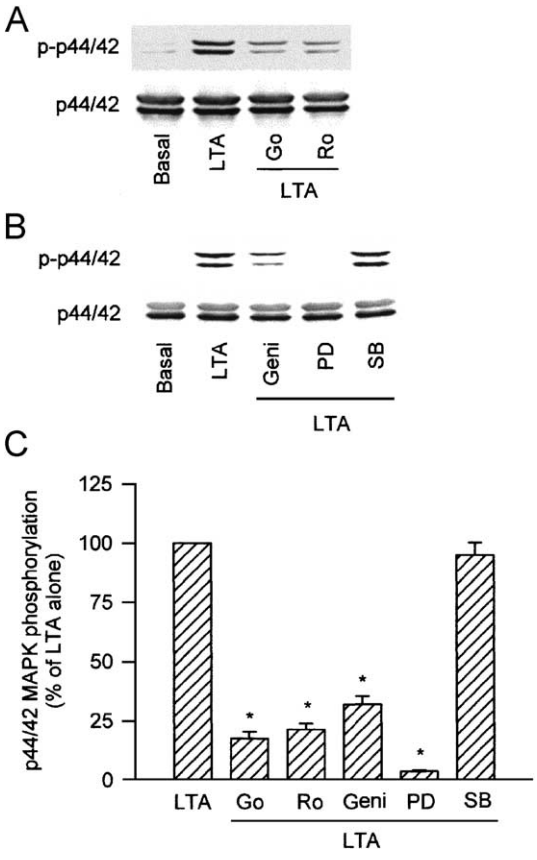

Fig. 4. Effects of various inhibitors on lipoteichoic acid-induced p44/42 MAPK activation in A549 cells. In (A) and (B), cells were pretreated with 0.1 μM Go 6976, 0.1 μM Ro 31-8220 (A), 30 μM genistein, 30 μM PD 98059, or 1 μM SB 203580 (B) for 30 min before incubation with lipoteichoic acid (30 μg/ml) for 10 min. Whole cell lysates were prepared and subjected to Western blot analysis using antibodies specific for phosphorylated p44/42 (p-p44/42) or nonphosphorylated p44/42 MAPK (p44/42) as described in Materials and methods. In (C), the extent of p44/42 MAPK activation was quantitated using a densitometer with Image-Pro plus software. Results are expressed as the mean ± S.E.M. ($n=3$). * $P<0.05$ as compared with the lipoteichoic acid-treated group. LTA, lipoteichoic acid; Go, Go 6976; Ro, Ro 31-8220; Geni, genistein; PD, PD 98059; SB, SB 203580.

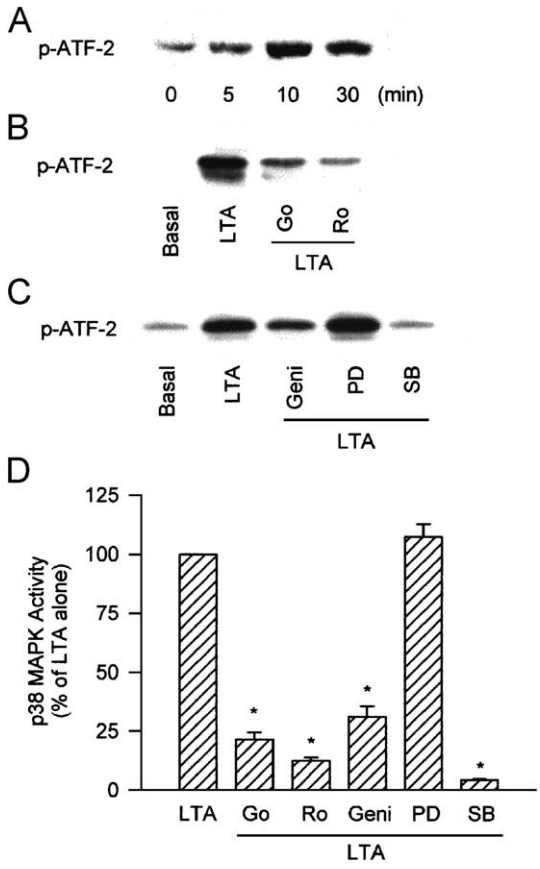

Fig. 5. Time-dependent increase in p38 MAPK activity caused by lipoteichoic acid and effects of various inhibitors on lipoteichoic acid-induced increase in p38 MAPK activity in A549 cells. In (A), cells were incubated with 30 μg/ml lipoteichoic acid for indicated time intervals. In (B) and (C), cells were pretreated with 0.1 μM Go 6976, 0.1 μM Ro 31-8220 (B), 30 μM genistein, 30 μM PD 98059, or 1 μM SB 203580 (C) for 30 min before incubation with lipoteichoic acid (30 μg/ml) for 10 min. Whole cell lysates were prepared and immunoprecipitated with anti-phospho-p38 MAPK antibody. The p38 MAPK activity was measured by phosphorylation of ATF-2 using antibody specific for phospho-ATF-2 (p-ATF-2) as described in Materials and methods. In (D), the extent of the increase in p38 MAPK activity was quantitated using a densitometer with Image-Pro plus software. Results are expressed as the mean ± S.E.M. ($n=3$). * $P<0.05$ as compared with the lipoteichoic acid-treated group. LTA, lipoteichoic acid; Go, Go 6976; Ro, Ro 31-8220; Geni, genistein; PD, PD 98059; SB, SB 203580.

for various time intervals also caused an activation of p38 MAPK and peaked at 30 min. The PMA-induced effects decreased gradually after 60 min treatment (Fig. 3D). The protein level of p38 MAPK was not affected by lipoteichoic acid and PMA treatment (Fig. 3C and D). When cells were treated with 30 μg/ml lipoteichoic acid, the p38 MAPK activity was increased and peaked at 10 min. After 30 min of treatment, the lipoteichoic acid-induced increase in p38 MAPK activity had declined (Fig. 5A). The lipoteichoic acid-induced increase in p38 MAPK activity was inhibited by pretreatment of cells for 30 min with Go 6976 (0.1 μM), Ro 31-8220 (0.1 μM), genistein (30 μM), or SB 203580 (1 μM), but not by PD 98059 (30 μM) (Fig. 5B,C and D).

3.4. Roles of tyrosine kinase, p44/42 and p38 MAPK in lipoteichoic acid-induced NF-κB activation

NF-κB is normally held in 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 is translocated to the nucleus where it activates the responsive gene (Baeuerle and Henkel, 1994; Barnes and Karin, 1997). Recently, we found that the NF-κB inhibitor, pyrrolidine dithiocarbamate, markedly inhibited lipoteichoic acid-induced increases in cyclooxygenase-2 expression and the binding activity of the NF-κB-specific DNA–protein complex in A549 cells, indicating that activation of NF-κB is critical in the induction of cyclooxygenase-2 protein caused by lipoteichoic acid in A549 cells (Lin et al., 2001). In nuclear extracts of unstimulated cells, a light staining of NF-κB-specific DNA–protein complexes formation was observed. Stimulation of cells with 30 μg/ml lipoteichoic acid for 30 min resulted in marked activation of NF-κB-specific DNA–protein complex formation (Fig. 6A). Pretreatment of cells for 30 min with genistein (30 μM), tyrphostin AG126 (30 μM), PD 98059 (30 μM), or SB 203580 (1 μM) markedly inhibited the lipoteichoic acid-induced activation of NF-κB-specific DNA–protein complex formation by 73.2%, 52.6%, 60.5%, or 56.8%, respectively (Fig. 6A and B).

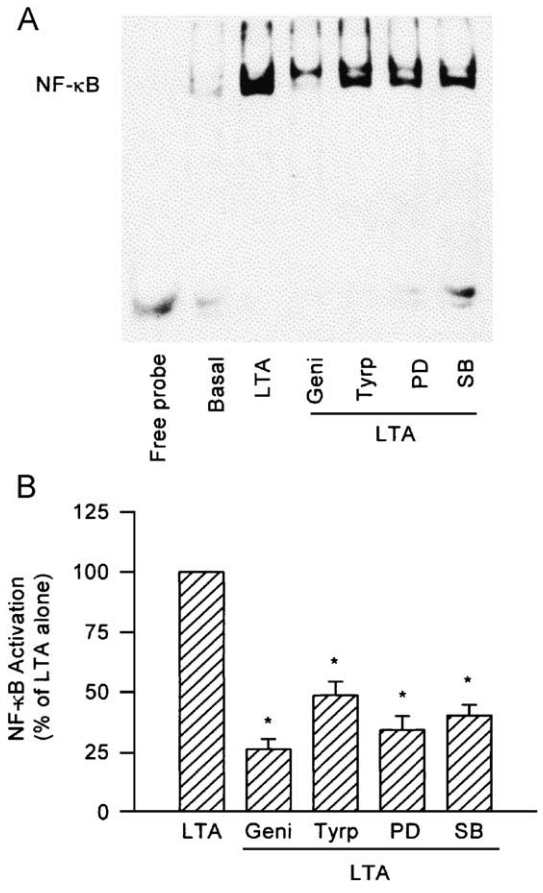

Fig. 6. Effects of genistein, tyrphostin AG126, PD 98059 and SB 203580 on lipoteichoic acid-induced NF-κB-specific DNA-protein complex formation in nuclear extracts of A549 cells. In (A), cells were pretreated with 30 μM genistein, 30 μM tyrphostin AG126, 30 μM PD 98059, or 1 μM SB 203580 for 30 min before incubation with lipoteichoic acid (30 μg/ml) for 30 min. Nuclear extracts were prepared for determination of NF-κB-specific DNA-protein binding activity by EMSA as described in Materials and methods. In (B), the extent of NF-κB activation was quantitated using a desitometer with Image-Pro plus software. Results are expressed as the mean ± S.E.M. ($n=3$). * $P<0.05$ as compared with the lipoteichoic acid-treated group. LTA, lipoteichoic acid; Geni, genistein; Tyrp, tyrphostin AG 126; PD, PD 98059; SB, SB 203580.

4. Discussion

Our previous studies indicated that PC-PLC, PC-PLD, PKC and transcription factor NF-κB might be involved in lipoteichoic acid-mediated signaling pathways leading to the expression of cyclooxygenase-2 protein in human pulmonary epithelial cells (Lin et al., 2001). In this study, we show that the CD14 antibody almost completely inhibited the lipoteichoic acid-induced cyclooxygenase-2 expression in A549 cells, suggesting CD14 is involved in the signal transduction leading to the expression of cyclooxygenase-2 protein caused by lipoteichoic acid. The MEK-specific inhibitor, PD 98059, inhibits lipoteichoic acid-induced increases in cyclooxygenase-2 expression and cyclooxygenase activity, suggesting that activation of MEK might also be involved in the induction of cyclooxygenase-2 caused by lipoteichoic acid. Treatment of A549 cells with lipoteichoic acid and the PKC activator, PMA, caused a marked activation of p44/42 MAPK. Furthermore, lipoteichoic acid-induced activation of p44/42 MAPK was inhibited by Go 6976, Ro 31-8220, genistein and PD 98059. These results suggest that activations of PKC, tyrosine kinase and MEK are upstream of lipoteichoic acid-induced p44/42 MAPK activation. Furthermore, activation of tyrosine kinase has been demonstrated to be involved in bradykinin-mediated p44/42 MAPK activation in mesangial cells (El-Dahr et al., 1998). Recently, activation of tyrosine kinase was suggested to be a key event in interleukin-1β-induced cyclooxygenase-2 expression in human pulmonary epithelial cells (Akarasereenont and Thiemermann, 1996; Lin et al., 2000). Previous studies have also shown a potential role of tyrosine kinase in lipoteichoic acid-induced iNOS expression in murine J744.2 macrophages (Kengatharan et al., 1996). In this study, we demonstrate that two structurally distinct tyrosine kinase inhibitors, genistein (competitive inhibitor at the ATP-binding site) and tyrphostin AG126 (competitive inhibitor at the substrate binding site), prevented lipoteichoic acid-induced increases in cyclooxygenase-2 expression and cyclooxygenase activity. In previous studies, we also demonstrated that PKC activation is involved in lipoteichoic acid-mediated cyclooxygenase-2 expression in A549 cells (Lin et al., 2001). Taken together, these results suggest that lipoteichoic acid might activate the pathways of PKC and tyrosine kinase to induce p44/42

MAPK activation, which in turn induces cyclooxygenase-2 expression in A549 cells.

Activation of p38 MAPK has been demonstrated to be involved in interleukin-1β-induced cyclooxygenase-2 expression and prostaglandin E_2 release in renal mesangial cells (Guan et al., 1998). We found herein that the p38 MAPK-specific inhibitor, SB 203580, inhibited lipoteichoic acid-induced increases in cyclooxygenase-2 expression and cyclooxygenase activity in A549 cells, suggesting that activation of p38 MAPK might also be involved in lipoteichoic acid-mediated cyclooxygenase-2 expression. In this study, we found that treatment of A549 cells with lipoteichoic acid and PMA resulted in a marked activation of p38 MAPK. Furthermore, lipoteichoic acid-mediated increase in p38 MAPK activity was inhibited by Go 6976, Ro 31-8220, genistein, or SB 203580. These findings suggest that lipoteichoic acid-induced p38 MAPK activation is downstream of the signal of PKC and tyrosine kinase.

The transcription factor, NF-κB, is critical to the induction of iNOS by lipoteichoic acid in macrophages (Kengatharan et al., 1996). In A549 cells, lipoteichoic acid increased the levels of the NF-κB-specific DNA–protein complex in nuclear extracts, and resulted in the translocation of p65 NF-κB from cytosol to the nucleus, as well as degradation of IκB-α in the cytosol (Lin et al., 2001). The NF-κB inhibitor, pyrrolidine dithiocarbamate, inhibited lipoteichoic acid-induced increases in cyclooxygenase-2 expression and the binding activity of NF-κB-specific DNA–protein complex, indicating that NF-κB is also critical in the induction of cyclooxygenase-2 protein caused by lipoteichoic acid in A549 cells (Lin et al., 2001). In the present study, we found that activation of NF-κB-specific DNA–protein complex formation caused by lipoteichoic acid was inhibited by genistein, tyrphostin AG126, PD 98059, or SB 203580. Previous studies have also demonstrated that the PKC inhibitors, Ro 31-8220 and Go 6976, inhibit lipoteichoic acid-mediated activation of NF-κB-specific DNA–protein complex formation (Lin et al., 2001). These results indicate that, in addition to PKC, lipoteichoic acid might also act through the pathways of tyrosine kinase, p44/42 MAPK and p38 MAPK to induce NF-κB activation, which in turn induces cyclooxygenase-2 expression in A549 cells. In fact, p38 MAPK activation has been demonstrated to be upstream of activation of NF-κB caused by lipopolysaccharide in RAW 264.7 macrophages (Chen and Wang, 1999).

In this study, the L-alanine content of the LTA (Sigma) preparation using a phenolic extraction procedure is 30%. However, Hartung et al. prepared highly purified LTA with a 70% L-alanine content by the method of butanol extraction (Morath et al., 2001). The discrepancy in the potency of LTA might be due to its L-alanine content with different preparation; the butanol-extracted LTA increased tumor necrosis factor formation in monocytes, with a maximal effect at 0.1 μg/ml LTA and the phenol-extracted LTA (Sigma) possessed less potent activity in the formation of tumor necrosis factor (Morath et al., 2001). In fact, we also found that the phenol-extracted LTA used in this study (Sigma) concentration-dependently induced cyclooxygenase-2 expression and prostaglandin E_2 release in A549 cells, and reached maximal level at 30 μg/ml LTA treatment (Lin et al., 2001).

In conclusion, lipoteichoic acid might induce activation of both p44/42 and p38 MAPK, which in turn, initiates NF-κB activation, and finally causes cyclooxygenase-2 expression in A549 cells. Both events require the activation of upstream protein tyrosine kinase and PKC. The present study, together with our previous report (Lin et al., 2001), delineate, in part, the signal transduction pathways of lipoteichoic acid-induced cyclooxygenase-2 expression. A schematic representation of the signaling pathway of lipoteichoic acid-induced cyclooxygenase-2 expression in human pulmonary epithelial cell line is shown in Fig. 7. By understanding these signal transduction pathways, we may be able to design therapeutic strategies to reduce airway inflammation caused by Gram-positive organisms.

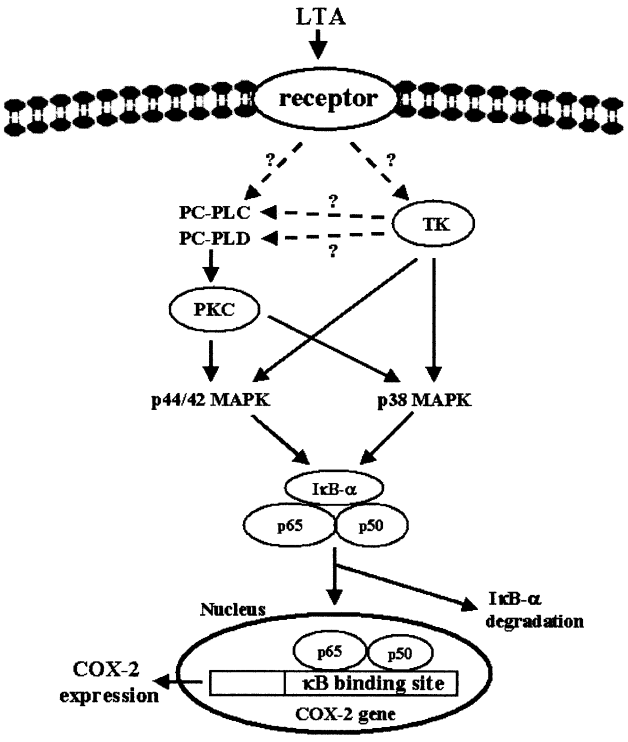

Fig. 7. Schematic representation of the signaling pathway of lipoteichoic acid-induced cyclooxygenase-2 expression in a human airway epithelial cell line (A549). Lipoteichoic acid binds to membrane receptors, and then activates phosphatidylcholine–phospholipase C and phosphatidylcholine–phospholipase D to induce PKC activation. Lipoteichoic acid might induce activations of both p44/42 and p38 MAPK. Activations of p44/42 and p38 MAPK require the activation of upstream protein tyrosine kinase (TK) and PKC. These result in stimulation of NF-κB-specific DNA-protein binding and subsequent cyclooxygenase-2 expression. LTA, lipoteichoic acid; COX-2, cyclooxygenase-2; PC-PLC, phosphatidylcholine–phospholipase C; PC-PLD, phosphatidylcholine–phospholipase D; TK, tyrosine kinase.

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