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Involvement of Protein Kinase C-γ in IL-1β-Induced Cyclooxygenase-2 Expression in Human Pulmonary Epithelial Cells

CHIEN-HUANG LIN, SHENG-YUAN SHEU, HORNG-MO LEE, YUAN-SOON HO, WEN-SEN LEE, WUN-CHANG KO, and JOEN-RONG SHEU

Graduate Institutes of Biomedical Technology (C.-H.L., H.-M.L., Y.-S.H.) and Medical Sciences (S.-Y.S., W.-S.L., W.-C.K., J.-R.S.), Taipei Medical College, Taipei, Taiwan

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ABSTRACT

The signaling pathway of protein kinase C (PKC) is known to play a role in mediating the action of various cytokines. Here we examined the signal transduction pathway of PKC activation and the role of PKC isoforms in interleukin-1β (IL-1β)-mediated cyclooxygenase-2 (COX-2) expression in human pulmonary epithelial cell line (A549). The tyrosine kinase inhibitors (genistein and tyrphostin AG126) and phosphatidylcholine-phospholipase C inhibitor (D-609) prevented IL-1β-induced prostaglandin E_2 (PGE_2) release and COX-2 expression, whereas U-73122 (a phosphatidylinositol-phospholipase C inhibitor) and propranolol (a phosphatidate phosphohydrolase inhibitor) had no effect. The PKC inhibitors (Go 6976 and Ro 31–8220) and NF-κB inhibitor, pyrrolidine dithiocarbamate, also attenuated IL-1β-induced PGE_2 release and COX-2 expression. Western blot analysis using PKC isoenzyme-specific antibodies indicated that A549 cells expressed PKC-α, -γ, -ι, -λ, -ζ, and -μ. IL-1β caused the translocation of PKC-γ but not other isoforms from cytosol to the membrane fraction. Moreover, the translocation of PKC-γ was inhibited by genistein or D-609, but not by U-73122. IL-1β caused the translocation of p65 NF-κB from cytosol to the nucleus as well as the degradation of IκB-α in cytosol. Furthermore, the translocation of p65 NF-κB was inhibited by genistein, Go 6976, Ro 31–8220, or pyrrolidine dithiocarbamate. These results indicate that in human pulmonary epithelial cells, IL-1β might activate phosphatidylcholine-phospholipase C through an upstream tyrosine phosphorylation to elicit PKC activation, which in turn initiates NF-κB activation, and finally induces COX-2 expression and PGE_2 release. Of the PKC isoforms present in A549 cells, only activation of PKC-γ is involved in regulating IL-1β-induced responses.

Prostaglandins, a family of mediators, have numerous cardiovascular and inflammatory effects (Vane et al., 1998). Cyclooxygenase (COX) converts arachidonic acid to prostaglandin H_2, which is then further metabolized to various prostaglandins, prostacyclin, and thromboxane A_2 (Vane et al., 1998). It is now known that at least two distinct isoforms of COX have been identified (Xie et al., 1991; Mitchell et al., 1995). COX-1 is generally responsible for the production of prostaglandins under physiological conditions and is known to be expressed constitutively in many cell types including endothelial cells, platelets, and gastric mucosa (Vane, 1994). COX-2 is induced by proinflammatory stimuli, including cytokines (Maier et al., 1990) and bacterial lipopolysaccharide (Mitchell et al., 1993) in cells in vitro and in inflamed sites in vivo (Vane et al., 1994). Furthermore, COX-2 is thought to be the isoform responsible for the production of proinflammatory prostanoids in various models of inflammation (Chan et al., 1995).

Protein kinase C (PKC) represents a family of closely related serine/threonine kinases (Nishizuka, 1992; Hug and Sarre, 1993) that play a key role in different cellular signal transduction pathways (Nishizuka, 1988). Molecular cloning has shown that it consists of at least 12 isoforms with different tissue expressions, which have been shown to be related to specialized cell functions (Nishizuka, 1992; Hug and Sarre, 1993). They have been subdivided into conventional PKC isoforms (α, βI, βII, γ), novel PKC isoforms (δ, ϵ, η, θ), atypical PKC isoforms (ι, λ, ζ), and yet another subgroup (PKC μ) (Nishikawa et al., 1997). The conventional PKC members can be activated by calcium, phospholipids, diacylglycerol (DAG), and phorbol ester; the novel PKC members are activated by the same compounds but are calcium independent. The differential localization and activation properties of the PKC

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ABBREVIATIONS: COX, cyclooxygenase; IL-1β, interleukin-1β; PGE_2, prostaglandin E_2; PC-PLC, phosphatidylcholine-phospholipase C; PI-PLC, phosphatidylinositol-phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; PDTC, pyrrolidine dithiocarbamate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; NP-40, Nonident P-40.

isoforms have led us to determine the roles of individual PKC isoforms in the regulation of cellular functions.

The levels of cytokines are increased in inflammatory airway diseases, such as asthma (Barnes, 1994). It has been addressed that the concentration of interleukin-1β (IL-1β) increased in humans with an asthmatic attack and its increase is related to the disease (Mattoli et al., 1991). In rats, inhalation of IL-1β results in infiltration of neutrophils into the airways and increased airway responsiveness to inhaled bradykinin (Tsukagoshi et al., 1993). Lipopolysaccharide and certain cytokines, such as IL-1β, induced an increased expression of COX-2 in airway epithelial cells (Mitchell et al., 1994), airway macrophages (Lee et al., 1992), and monocytes (Hempel et al., 1994). However, the intracellular signal transduction mechanisms involved in the IL-1β-induced COX-2 expression are not fully understood. Previous reports have shown that the activations of tyrosine kinase (Akarasereenont and Thiemermann, 1996), PKC (Rzymkiewicz et al., 1996), and transcription factor NF-κB (Newton et al., 1997) are involved in the IL-1β-induced COX-2 expression. However, the relationships among these pathways are still unknown. Therefore, the purpose of the present study was to clarify the signaling transduction pathway of IL-1β-induced PKC activation and the role of PKC isoforms in IL-1β-mediated COX-2 expression in human airway epithelial cell line (A549).

Experimental Procedures

Materials. IL-1β, actinomycin D, cyclohexamide, propranolol, pyrrolidine dithiocarbamate (PDTC), dithiothreitol (DTT), HEPES, EGTA, EDTA, glycerol, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, leupeptin, SDS, and Nonident P-40 (NP-40) were purchased from Sigma Chemical Co. (St. Louis, MO). Genistein, daidzein, tyrphostin AG126, tyrphostin A-1, NS-398, Go 6976, and Ro 31–8220 were purchased from Calbiochem-Novabiochem (San Diego, CA). D-609 and U-73122 were obtained from Research Biochemicals, Inc. (Natick, MA). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12, fetal calf serum (FCS), and penicillin/streptomycin were purchased from Life Technologies (Gaithersburg, MD). Prostaglandin E_2 (PGE_2) enzyme immunoassay kit was obtained from Cayman Chemical Co., Inc. (Ann Arbor, MI). Antibodies specific for COX-2, p65 NF-κB, and PKC isoforms (α, β, γ, δ, ϵ, θ, ι, λ, ζ, and μ) were purchased from Transduction Laboratories (Lexington, KY). An antibody specific for IκB-α was purchased from Santa Cruz Biochemicals (Santz Cruz, CA). 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). 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).

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 nutrient mixture containing 10% FCS and penicillin/streptomycin (50 U/ml) 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*g* for 5 min, then resuspended and subcultured according to standard protocols.

PGE_2 Enzyme Immunoassay. A549 cells were cultured in 12-well culture plates. After reaching confluence, the cells were treated with vehicle, IL-1β (1 ng/ml), or pretreatment inhibitors followed by IL-1β and incubated in a humidified incubator at 37°C. After incubation, the medium was removed and store at –80°C until assay. PGE_2 was assayed for the medium using the PGE_2 enzyme immunoassay kit according to the procedure described by the manufacturer. An antibody to PGE_2 had 18.7% cross-reactivity to PGE_1, 1% crossreactivity to 6-keto $PGF_{1\alpha}$, and less than 0.01% cross-reactivity to other prostaglandins.

Protein Preparation and Western Blotting. For the determination of the expressions of COX-2 and PKC isoforms 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 vehicle, IL-1β (1 ng/ml), or pretreatment inhibitors followed by IL-1β and incubated in a humidified incubator at 37°C. After incubation, cells were washed with PBS (pH 7.4) and incubated with extraction buffer (10 mM Tris pH 7.0, 140 mM NaCl, 2 mM PMSF, 5 mM DTT, 0.5% NP-40, 0.05 mM pepstatin A, and 0.2 mM leupeptin) with gentle shaking, and 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, glycerol 20%, SDS 4%, and bromophenol blue 0.2%). Electrophoresis was performed using 10% SDS-polyacrylamide gel (2 h, 110 V, 40 mA, 30 μg protein per lane). Separated proteins were transferred to polyvinylidene difluoride membranes (2 h, 40 V). Nonspecific IgGs were blocked with 5% fat-free milk powder, and the membranes were incubated for 2 h with specific antibodies for COX-2 or PKC isoforms (α, β, γ, δ, ϵ, θ, ι, λ, ζ, and μ). The membranes were then incubated with alkaline phosphatase (1:1000 v/v) conjugated with secondary antibody for 2 h. Subsequently, the Western blots were developed with 4-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate as a substrate.

Analysis of PKC Isoforms Translocation. For the detection of PKC translocation, cytosolic and membrane fractions were separated as described previously (Li et al., 1998). Briefly, A549 cells were incubated with vehicle or IL-1β (1 ng/ml) for 10, 30, and 60 min, then the cells were scraped and collected. The collected cells were homogenized in ice-cold homogenization buffer [20 mM Tris, 2 mM EDTA, 5 mM EGTA, 20% glycerol (v/v), 2 mM PMSF, 1% aprotinin (v/v), 5 mM DTT] for 20 min, then sonicated for 10 s and centrifuged at 800*g* for 10 min. The supernatant (cytosolic and membrane fraction) was removed and centrifuged at 25,000*g* for 15 min. The supernatant (cytosolic fraction) was obtained. The pellets (membrane fraction) were solubilized in homogenization buffer containing 0.1% NP-40. The protein levels of PKC isoforms (α, γ, ι, λ, ζ, and μ) in cytosolic and membrane fractions were determined by Western blotting analysis performed as described. In some experiments, cells were incubated with genistein, D-609, or U-73122 for 30 min before IL-1β treatment.

Analysis of p65 NF-κB Translocation and IκB-α Degradation. For the detection of p65 NF-κB translocation and IκB-α degradation, cytosolic and nuclear protein fractions were separated as described previously (Chen et al., 1998). A549 cells were incubated with vehicle or IL-1β for 10, 30, 60, and 120 min, then the cells were scraped and collected. The collected cells were suspended in ice-cold extraction buffer A [1 mM $NaVO_4$, 0.5 mM PMSF, 1% aprotinin (v/v), 1 mM DTT], and incubated for 20 min on ice, and then the cells were lysed by the addition of 0.5% NP-40, followed by vigorous vortexing for 10 s. The extracts were then centrifuged at 9000*g* for 5 min, and the supernatant (cytosolic fraction) was obtained. The pellets (nuclear fraction) were then resuspended in extraction buffer B [20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM $NaVO_4$, 1 mM PMSF, 1% aprotinin (v/v), 1 mM DTT], and sonicated for 5 s. The extracts were then centrifuged at 15,000*g* for 5 min, and the supernatants (nuclear fraction) were obtained. The protein levels of p65 NF-κB in the cytosolic and nuclear fractions and IκB-α in the cytosolic fraction were determined by Western blotting analysis performed as described. In some experiments, cells were preincubated with genistein, Go 6976, Ro 31–8220, or PDTC for 30 min before IL-1β treatment. The protein levels of p65 NF-κB in nuclear fractions were determined by Western blotting analysis performed as described.

Statistical Analysis. Results shown are means ± S.E. from duplicate determinations (wells) from three to four separate experiments. One-way ANOVA followed by, when appropriate, Bonferroni's multiple range test was used to determine the statistical significance in the difference between means. A P value of less than .05 was taken as statistically significant.

Results

Characterization of COX-2 Expression Induced by IL-1β in A549 Cells. Treatment with IL-1β (0.001–10 ng/ml, for 24 h) caused a concentration-dependent increase in the release of PGE_2 (Fig. 1A) and the expression of a 70-kDa COX-2 protein (Fig. 1C) in A549 cells. Exposure of the cells to IL-1β resulted in a time-dependent release of PGE_2 (Fig. 1B) and expression of COX-2 protein (Fig. 1D). The earliest induction of COX-2 protein occurred at 1 h, and peaked at 6 h. The maximum production of PGE_2 was observed at 12 h. In the following experiments, the cells were treated with 1 ng/ml IL-1β for 24 h. Pretreatment of the cells with actinomycin D (1 μM) or cyclohexamide (10 μM) for 30 min markedly attenuated the IL-1β-induced release of PGE_2 by 93.1 and 80.2%, respectively. The IL-1β-induced expression of COX-2 was also attenuated (data not shown). When cells were pretreated for 30 min with the COX-1 inhibitor aspirin (3 μM) or the COX-2 inhibitor NS-398 (1 μM), the IL-lβ-induced PGE_2 release was markedly attenuated 96.3% by NS-398, whereas aspirin had no effect (data not shown).

Role of Tyrosine Kinase, Phospholipase C, and Phospholipase D on IL-1β-Induced PGE_2 Release and COX-2 Expression. To examine whether tyrosine kinase activation was involved in the signal transduction pathway leading to PGE_2 release and COX-2 expression caused by IL-1β, the tyrosine kinase inhibitors genistein and tyrphostin AG126 were used. Pretreatment of cells for 30 min with genistein (10 and 30 μM) or tyrphostin AG126 (10 and 30 μM) inhibited the IL-1β-induced PGE_2 release (Fig. 2A); the induction of COX-2 protein was also inhibited by genistein (30 μM) or tyrphostin AG126 (30 μM) (Fig. 2B). However, daidzein (30 μM), an inactive analog of genistein, or tyrphostin A-1 (30 μM), an inactive analog of tyrphostin AG126, did not affect the IL-1β-induced PGE_2 release and COX-2 expression (data not shown). When cells were pretreated for 30 min with the phosphatidylcholine-phospholipase C (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 IL-1β-induced PGE_2 release was inhibited 68.6% by D-609, whereas U-73122 or propranolol had no effect (Fig. 3A). Furthermore, D-609 (10–50 μM) caused a concentration-dependent inhibitory effect in the IL-1β-induced PGE_2 release (Fig. 3B). The IL-1β-induced COX-2 expression was also inhibited by D-609, but not by U-73122 or propranolol (Fig. 3C).

Role of PKC Isoforms on IL-1β-Induced PGE_2 Release and COX-2 Expression. To determine whether PKC activation was involved in the signal transduction pathway leading to PGE_2 release and COX-2 expression caused by IL-1β, the PKC inhibitors Go 6976 and Ro 31–8220 were used. Pretreatment of cells for 30 min with Go 6976 (3–20 μM) or Ro 31–8220 (3–20 μM) attenuated the IL-1β-induced PGE_2 release in a concentration-dependent manner (Fig. 4, A and B). The IL-1β-induced COX-2 expression was also inhibited by Go 6976 (20 μM) or Ro 31–8220 (20 μM) (Fig. 4C).

To examine which PKC isoform was involved in the IL-1β-mediated effects, the expression of each PKC isoform in A549 cells was examined by Western blotting analysis. Using anti-PKC isoform-specific antibodies demonstrated that PKC-α, -γ, -ι, -λ, -ζ, and -μ were detected in A549 cells, whereas PKC-β, -δ, -ϵ, and -θ were not detected (Fig. 5). Exposure of

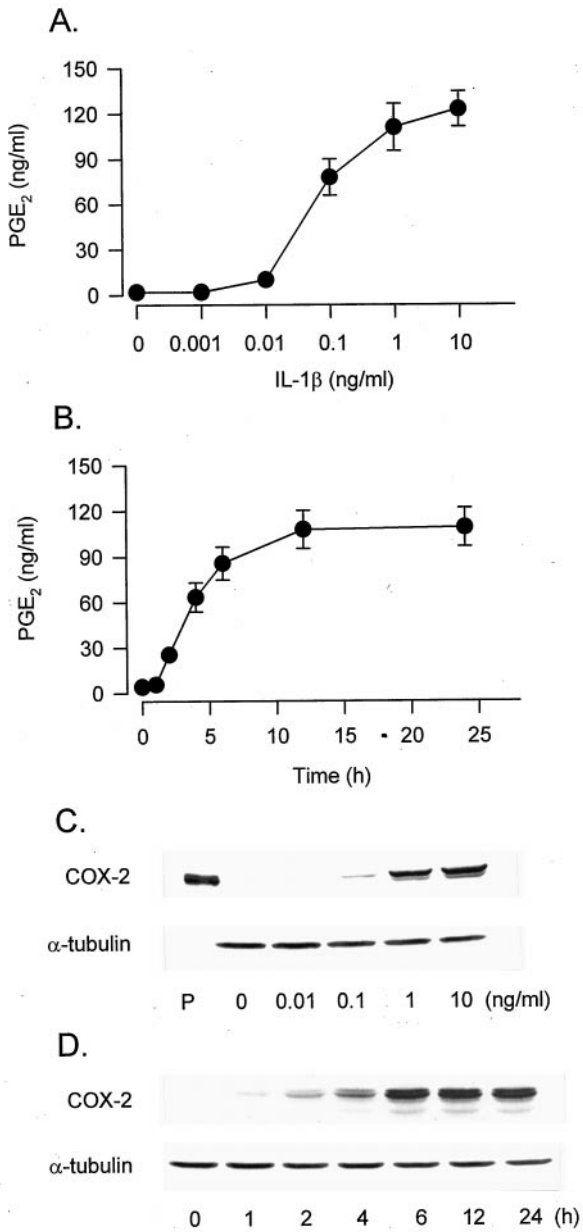

Fig. 1. Concentration- and time-dependent increase in the PGE_2 release and COX-2 expression caused by IL-1β in A549 cells. Cells were incubated with various concentrations of IL-1β for 24 h (A) or with IL-1β (1 ng/ml) for various time intervals (B), then the medium was removed, and the release of PGE_2 was measured. Results are expressed as means ± S.E. of three independent experiments performed in duplicate. Cells were incubated with the indicated concentrations of IL-1β for 24 h (C) or with IL-1β (1 ng/ml) for the indicated time intervals (D), and the extracted proteins were then immunodetected with COX-2 or α-tubulin specific antibody as described in *Experimental Procedures*. The equal loading in each lane was demonstrated by the similar intensities of α-tubulin. Whole cell lysates of mouse macrophages (RAW 264.7) stimulated by LPS (1 μg/ml) and INFγ (10 ng/ml) for 12 h were used as a positive control (P).

the cells to IL-1β (1 ng/ml) for 10, 30, and 60 min only caused translocation of PKC-γ (but not other) isoforms from cytosol to the membrane fraction (Fig. 6A). When cells were pretreated for 30 min with genistein (30 μM), D-609 (50 μM), or U-73122 (10 μM), the translocation of PKC-γ induced by IL-1β was inhibited by genistein or D-609, but not by U-73122 (Fig. 6B).

Role of Transcription Factor NF-κB on IL-1β-Induced PGE_2 Release and COX-2 Expression. To further study whether NF-κB was involved in the signal transduction pathway leading to PGE_2 release and COX-2 expression caused by IL-1β, the specific NF-κB inhibitor PDTC was used. Pretreatment of the cells for 30 min with PDTC (10–50 μM) attenuated dose dependently the IL-1β-induced PGE_2 release (Fig. 7A). The IL-1β-induced COX-2 expression was also attenuated by PDTC (50 μM) (Fig. 7B). After 10 to 30

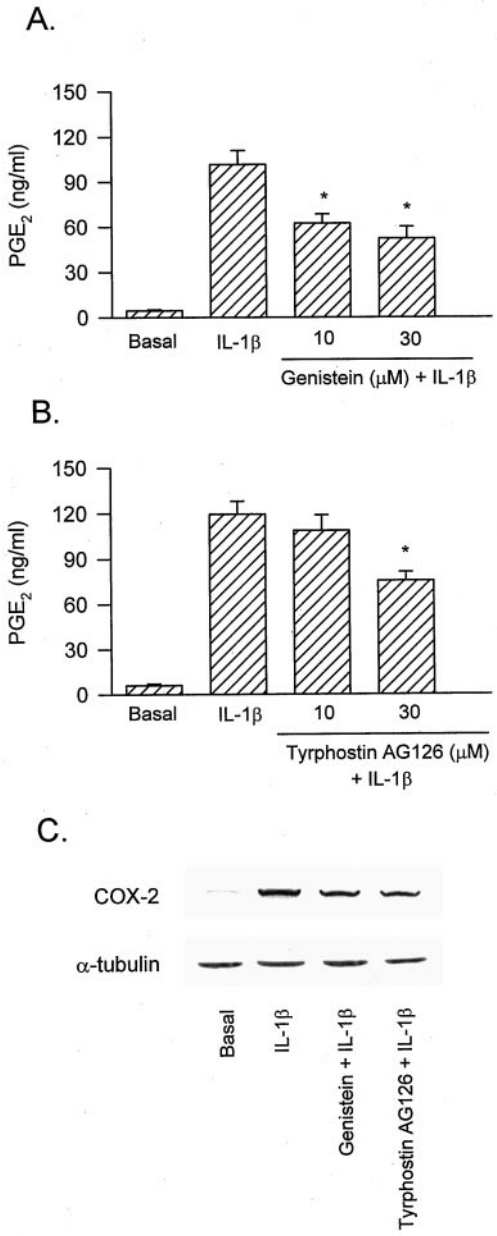

Fig. 2. Effects of genistein and tyrphostin AG126 on the PGE_2 release and COX-2 expression caused by IL-1β in A549 cells. Cells were pretreated with various concentrations of genistein (A) or tyrphostin AG126 (B) for 30 min before incubation with IL-1β (1 ng/ml) for 24 h. The medium was then removed, and the release of PGE_2 was measured. Results are expressed as means ± S.E. of four independent experiments performed in duplicate. *$P < .05$ as compared with treatment with IL-1β alone. C, cells were pretreated with genistein (30 μM) or tyrphostin AG126 (30 μM) for 30 min before incubation with IL-1β (1 ng/ml) for 24 h. Immunodetection with COX-2 or α-tubulin specific antibody was performed as described in *Experimental Procedures*. The equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

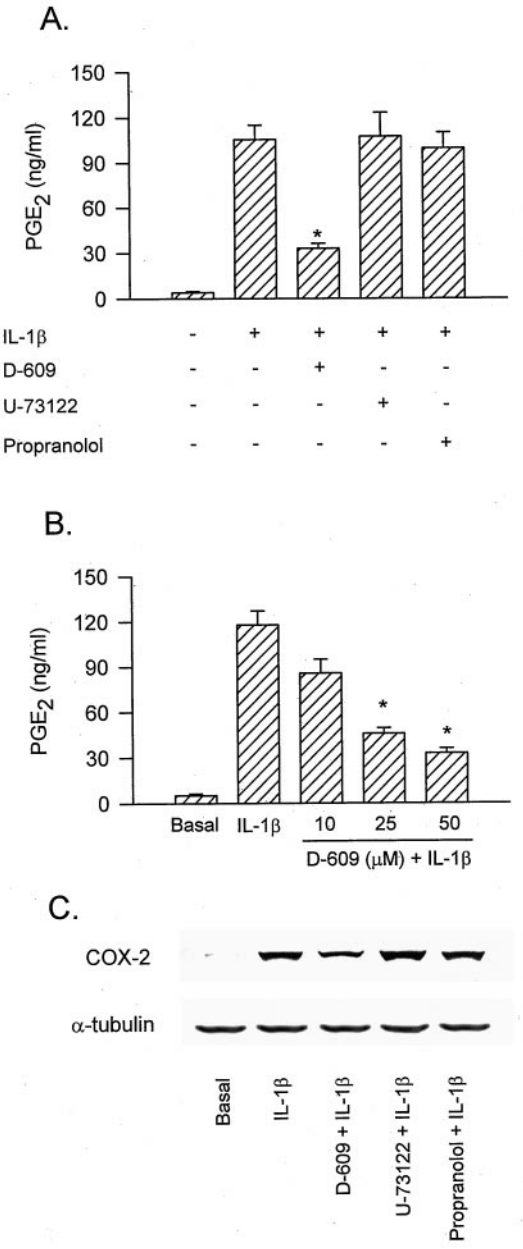

Fig. 3. Effects of D-609, U-73122, and propranolol on the PGE_2 release and COX-2 expression caused by IL-1β in A549 cells. Cells were pretreated with D-609 (50 μM), U-73122 (10 μM), or propranolol (100 μM) (A), or various concentrations of D-609 (B) for 30 min before incubation with IL-1β (1 ng/ml) for 24 h. The medium was then removed, and the release of PGE_2 was measured. Results are expressed as means ± S.E. of three independent experiments performed in duplicate. *$P < .05$ as compared with treatment with IL-1β alone. C, cells were pretreated with D-609 (50 μM), U-73122 (10 μM), or propranolol (100 μM) for 30 min before incubation with IL-1β (1 ng/ml) for 24 h. Immunodetection with COX-2 or α-tubulin specific antibody was performed as described in *Experimental Procedures*. The equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

min, stimulation of the cells with IL-1β (1 ng/ml) resulted in marked translocation of p65 NF-κB from cytosol to the nucleus (Fig. 8A) as well as the degradation of IκB-α in cytosol (Fig. 8B). The IL-1β-induced effects disappeared gradually after 30 min. After pretreatment of cells for 30 min with genistein (30 μM), Go 6976 (20 μM), Ro 31–8220 (20 μM), or PDTC (50 μM), the translocation of p65 NF-κB stimulated by IL-1β was inhibited (Fig. 9, A and B).

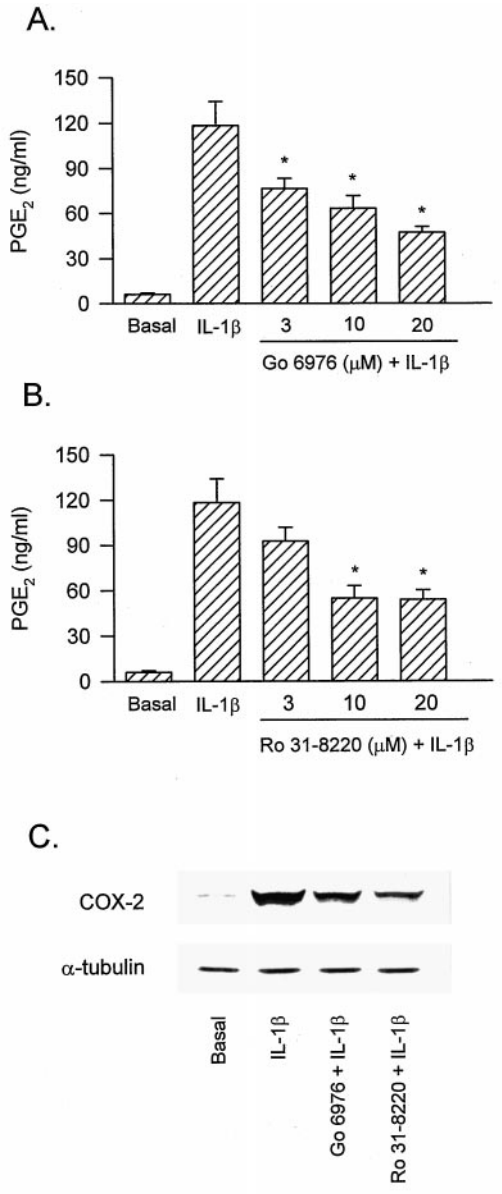

Fig. 4. Effects of Go 6976 and Ro 31–8220 on the PGE_2 release and COX-2 expression caused by IL-1β in A549 cells. Cells were pretreated with various concentrations of Go 6976 (A) or Ro 31–8220 (B) for 30 min before incubation with IL-1β (1 ng/ml) for 24 h. The medium was then removed, and the release of PGE_2 was measured. Results are expressed as means ± S.E. of four independent experiments performed in duplicate. *$P < .05$ as compared with treatment with IL-1β alone. C, cells were pretreated with Go 6976 (20 μM) or Ro 31–8220 (20 μM) for 30 min before incubation with IL-1β (1 ng/ml) for 24 h. Immunodetection with COX-2 or α-tubulin specific antibody was performed as described in *Experimental Procedures*. The equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

Discussion

This study demonstrated that the increase in the PGE_2 release by IL-1β in human pulmonary epithelial cells (A549) is a consequence of the induction of COX-2 and indicates that tyrosine kinase, PC-PLC, PKC, and transcription factor NF-κB may be involved in the signal transduction leading to the expression of COX-2 caused by IL-1β in these cells. Actinomycin D and cyclohexamide prevented the IL-1β-induced COX-2 expression and PGE_2 release, suggesting that the enhanced release of PGE_2 is dependent on de novo transcription and translation. In the absence of exogenous arachidonic acid, IL-1β caused the release of PGE_2 in A549 cells. This result is consistent with a previous study (Akarasereenont and Thiemermann, 1996) and suggests that IL-1β may stimulate the induction of COX-2 as well as phospholipase A_2.

PKC is a family of closely related serine/threonine kinases that appear to mediate various cellular functions (Nishizuka, 1992; Hug and Sarre, 1993). In the present study, we demonstrated that the IL-1β-induced COX-2 expression and PGE_2 release was prevented by PKC inhibitors Go 6976 and Ro 31–8220, indicating that PKC activation is involved in the signal transduction leading to the expression of COX-2 protein by IL-1β. DAG is a well established activator of PKC (Nishizuka, 1992). Several mechanisms may be responsible

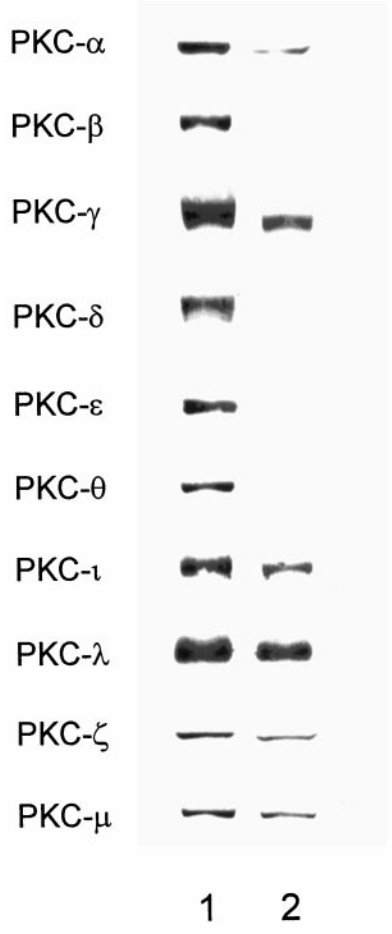

Fig. 5. Expression of PKC isoforms in A549 cells. Western blot analysis shows the levels of the ten PKC isoforms in rat brain, Jurkat cell (lane 1, positive control), and A549 cells (lane 2). Whole cell lysates of rat brain were used as a positive control of PKC-α, -β, -γ, -δ, -ϵ, -ι, -λ, or -ζ. Whole cell lysates of Jurkat cells were used as a positive control of PKC-θ or -μ.

for the signal-induced formation of DAG. The formation of DAG can be generated directly by the action of PI-PLC and PC-PLC (Nishizuka, 1992; Exton, 1994; Schütze et al., 1994). An indirect pathway to generate DAG involves phosphatidylcholine cleavage by phosphatidylcholine-phospholipase D, generating 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 (Schütze et al., 1992). It has been demonstrated that U-73122 inhibited PI-PLC activation in human platelets and neutrophils (Bleasdale et al., 1990); propranolol blocked PLD-derived DAG formation by inhibiting phosphatidate phosphohydrolase (Billah et al., 1989). We demonstrated that D609 inhibited IL-1β-induced PGE_2 release and COX-2 expression, whereas U-73122 and propranolol had no effect. These results indicated that IL-1β-induced PKC activation may be via the PC-PLC pathway, but not the PI-PLC or phosphatidylcholine-phospholipase D pathways. Indeed, the participation of PC-PLC in IL-1β-mediated signaling has been demonstrated by other laboratories (Rosoff et al., 1988; Kester et al., 1989). However, the mechanism involved in the activation of PC-PLC is still not well defined, but may involve tyrosine phosphorylation (Choudhury et al., 1991; Chen et al., 1998). Activation of tyrosine kinase has been suggested as a key event in the signal transduction leading to the expression of COX-2 by IL-1β (Akarasereenont and Thiemermann, 1996). We also demonstrated 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), inhibited the IL-1β-induced PGE_2 release and COX-2 expression. These results indicated that IL-1β may activate PC-PLC via an upstream tyrosine phosphorylation to induce PKC activation and which, in turn, induces COX-2 expression and PGE_2 release.

In resting cells, PKCs are located predominately in the cytosol (Hecker et al., 1993). After activation, the PKCs translocate from cytosol to the membrane fraction (Mochly-Rosen, 1995). Western blot analysis showed that PKC-α, -γ, -ι, -λ, -ζ, and -μ were detected in A549 cells (Fig. 5). Among these isoforms, IL-1β caused only PKC-γ translocation from cytosol to the membrane fraction, indicating the activation of the PKC-γ isoform. Moreover, the activation of PKC-γ was inhibited by genistein and D-609, but not by U-73122, sug-

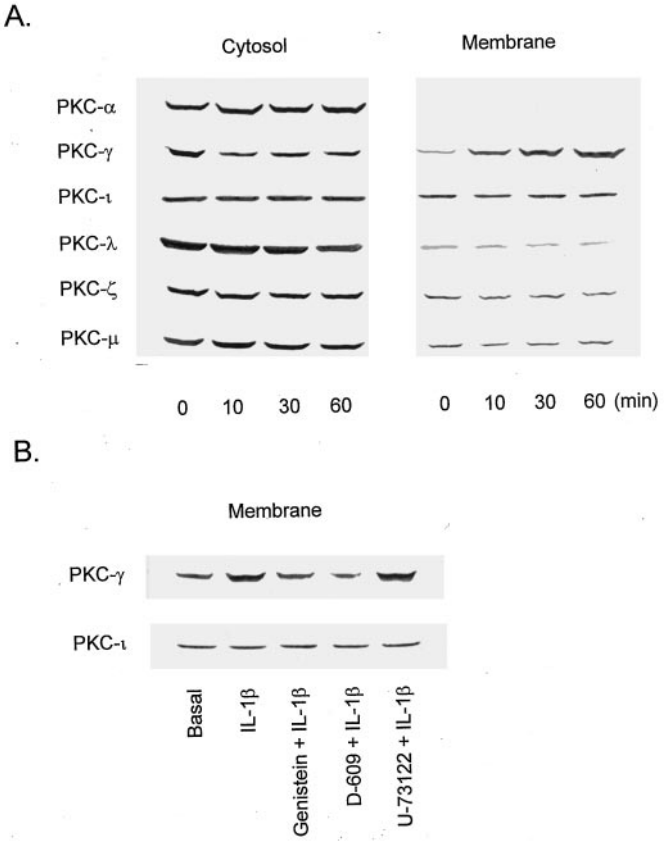

Fig. 6. The translocation of PKC isoforms induced by IL-1β and effects of genistein, D-609, and U-73122 on the translocation of PKC-γ caused by IL-1β in A549 cells. A, cells were pretreated with vehicle or IL-1β (1 ng/ml) for 10, 30, and 60 min. The subcellular (cytosol and membrane) fractions were isolated and then immunodetected with antibodies specific for PKC-α, -γ, -ι, -λ, -ζ, or -μ as described in *Experimental Procedures*. B, cells were pretreated with genistein (30 μM), D-609 (50 μM), or U-73122 (10 μM) for 30 min before incubation with IL-1β (1 ng/ml) for 30 min. The levels of PKC-γ in membrane fraction were immunodetected with PKC-γ or -ι specific antibody as described in *Experimental Procedures*. The equal loading in each lane was demonstrated by the similar intensities of PKC-ι.

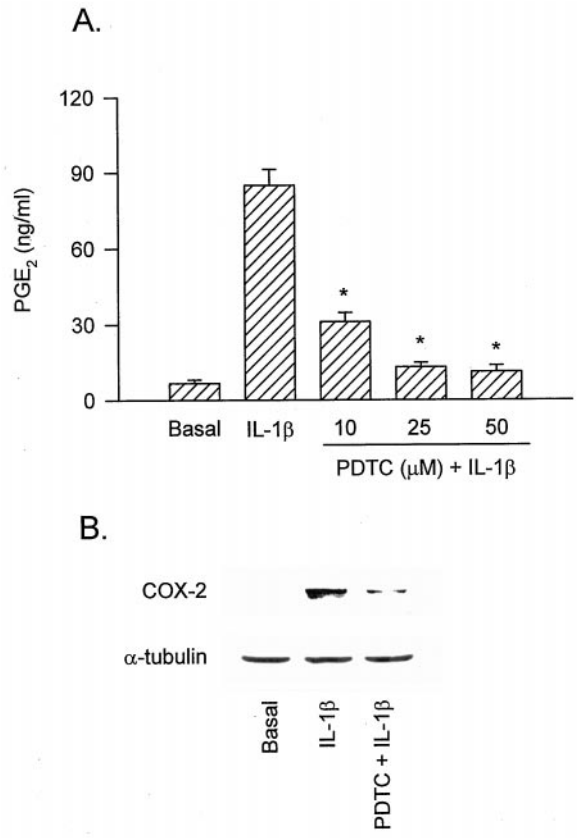

Fig. 7. Effects of PDTC on the PGE_2 release and COX-2 expression caused by IL-1β in A549 cells. Cells were pretreated with various concentrations of PDTC (A) for 30 min before incubation with IL-1β (1 ng/ml) for 24 h. The medium was then removed and the release of PGE_2 was measured. Results are expressed as means ± S.E. of three independent experiments performed in duplicate. $^*P < .05$ as compared with treatment with IL-1β alone. B, cells were pretreated with PDTC (50 μM) for 30 min before incubation with IL-1β (1 ng/ml) for 24 h. Immunodetection with COX-2 or α-tubulin specific antibody was performed as described in *Experimental Procedures*. The equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

gesting that IL-1β may act through the activation of tyrosine kinase and PC-PLC to induce PKC-γ activation. In renal mesangial cells, PKC-ζ was suggested to play an important role in the increase of PGE_2 production caused by IL-1β (Rzymkiewicz et al., 1996). On the other hand, PKC-η has been shown to be involved in lipopolysaccharide-induced nitric oxide synthase expression in primary astrocytes (Chen et al., 1998). This is additional evidence that different members of the PKC family within single cells elicit specific physiological responses.

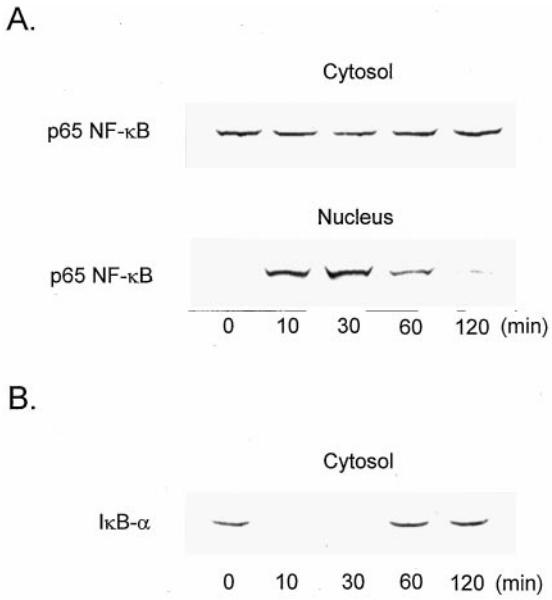

Fig. 8. The translocation of p65 NF-κB and IκB-α degradation induced by IL-1β in A549 cells. Cells were pretreated with vehicle or IL-1β (1 ng/ml) for 10, 30, 60, and 120 min. The subcellular (cytosol and nucleus) fractions were prepared for immunodetection. The cytosolic and nuclear levels of p65 NF-κB (A) and cytosolic levels of IκB-α (B) were immunodetected with p65 NF-κB and IκB-α specific antibodies as described in *Experimental Procedures*.

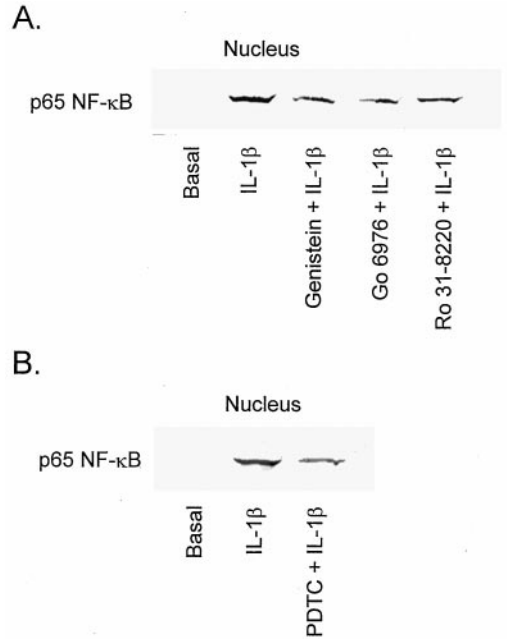

Fig. 9. Effects of genistein, Go 6976, Ro 31–8220, and PDTC on the translocation of p65 NF-κB caused by IL-1β in A549 cells. Cells were pretreated with genistein (30 μM), Go 6976 (20 μM), Ro 31–8220 (20 μM) (A), or PDTC (3 μM) (B) for 30 min before incubation with IL-1β (1 ng/ml) for 30 min. The subcellular (cytosol and nucleus) fractions were prepared for immunodetection. The nuclear levels of p65 NF-κB were immunodetected with p65 NF-κB specific antibody as described in *Experimental Procedures*.

In the present study, we showed that PDTC, which inhibits NF-κB activation, inhibited the IL-1β-induced PGE_2 release and COX-2 expression, suggesting that activation of NF-κB may be also involved in the induction of COX-2 caused by IL-1β. Similar findings have been reported by other laboratories (Newton et al., 1997). 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 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 (Grimm and Baeuerle, 1993; Baeuerle and Henkel, 1994). We found that IL-1β resulted in the translocation of p65 NF-κB from cytosol to the nucleus as well as the degradation of IκB-α in cytosol in A549 cells (Fig. 8). Furthermore, the translocation of p65 NF-κB stimulated by IL-1β was inhibited by genistein, Go 6976, Ro 31–8220, and PDTC. Previous reports have shown that the tyrosine kinase inhibitors, such as herbimycin and genistein, inhibit the activation of NF-κB caused by IL-1 (Iwasata et al., 1992) or lipopolysaccharide (Read et al., 1993). These results indicated that IL-1β may act through tyrosine kinase and PKC pathways to induce NF-κB activation in A549 cells. However, genistein, tyrphostin AG126, D-609, Go 6976, or Ro 31–8220 was not able to completely block the IL-1β-induced COX-2 expression and PGE_2 release, suggesting that other signal pathways may also be involved in the IL-1β-mediated COX-2 expression. Indeed, we have recently demonstrated that activation of p44/42 mitogen-activated protein kinase is also involved in the IL-1β-mediated COX-2 expression, and the pathway is not dependent on PKC activation (unpublished observations).

In conclusion, IL-1β might activate PC-PLC through an upstream tyrosine phosphorylation to elicit PKC activation, which in turn initiates NF-κB activation, and finally causes COX-2 expression and PGE_2 release. Of the PKC isoforms present in A549 cells, only PKC-γ activation is involved in regulating the IL-1β-induced responses. The molecular mechanisms involved in the regulation of COX-2 expression by IL-1β promote new insights into the pathophysiology of inflammation and may lead to new therapeutic strategies capable of interrupting the inflammatory cascade at key points.

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Send reprint requests to: Chien-Huang Lin, Ph.D., Institute of Biomedical Technology, Taipei Medical College, 250 Wu-Hsing St., Taipei 110, Taiwan. E-mail: chlin@tmc.edu.tw