0026-895X/02/6103-614–619$3.00
MOLECULAR PHARMACOLOGY
Vol. 61, No. 3
1327/968357
Mol Pharmacol 61:614–619, 2002
Printed in U.S.A.
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Regulation of Cyclooxygenase-2 Expression by Phospholipase D in Human Amnion-Derived WISH Cells

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Received August 30, 2001; accepted December 13, 2001

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Prostaglandins (PGs) are known to play a key role in the initiation of labor, but the mechanisms regulating their synthesis in amnion are largely unknown. In this study, the regulatory mechanisms for PGE_2 production during phospholipase D (PLD) and p38-dependent activation of WISH cells were investigated. We found that the stimulation of WISH cells with interleukin (IL)-1β elicited dose-dependent synthesis of cyclooxygenase-2 (COX-2) mRNA, protein, and their products, PGE_2. Moreover, the treatment of [^{3}H]myristate-labeled cells in the presence of 1-butanol caused the dose-dependent formation of [^{3}H]phosphatidylbutanol (PBt), a product specific to PLD activity. Pretreating the cells with 1-butanol and Ro 31-8220 inhibited the IL-1β–induced COX-2 expression, but 3-butanol did not affect this response. In addition, evidence that PLD was involved in the stimulation of COX-2 expression was provided by the observations that COX-2 expression was stimulated by the dioctanoyl phosphatidic acid (PA) and that the prevention of PA dephosphorylation by 1-propranolol potentiated COX-2 expression by IL-1β. Moreover, IL-1β stimulation of the cells caused the phosphorylation of p38 and extracellular signal-regulated kinase (ERK), and IL-1β–induced COX-2 expression was inhibited by the pretreatment of WISH cells with a p38 inhibitor, in contrast ERK upstream inhibitor had no effect. Furthermore, Ro 31-8220 inhibited IL-1β–induced p38 phosphorylation but not ERK phosphorylation. The results of this study indicate that in human amnion cells, IL-1β might activate PLD through an upstream protein kinase C to elicit p38 and finally induce COX-2 expression.

It is well established that the PGs, particularly PGE_2, are key mediators of the common terminal pathway and thought to play a central role in the initiation of spontaneous labor in humans by mediating physiological effects, such as uterine contractions (Kniss et al., 1990; Maggi et al., 1994). The human amnion has the capacity to produce PGE_2, and it is known that changes in this capacity occur in association with parturition. Moreover, PGs accumulate in the amniotic fluid in association with the onset of labor (Gibb, 1998). Consistent with this, COX activity increases in the amnion during labor (Teixeira et al., 1994; Mijovic et al., 1997). Although COX-1 and COX-2 are expressed in the amnion, only COX-2 increases near the onset of labor (Slater et al., 1995; Fuentes et al., 1996; Zakar et al., 1998). Also, data from COX-2 knockout mice suggests that the products of COX enzyme are required for every step of early pregnancy, including ovulation, fertilization and implantation (Majerus, 1998). However, the detailed mechanisms that they regulate remain to be determined.

IL-1β is a pleiotropic cytokine that exerts a wide range of biological activities in labor processes, and increased levels of IL-1β are observed in the amniotic fluid of women with labor (Romero et al., 1990). IL-1β is also a known promoter of PGE_2 production in amnion cells (Albert et al., 1994). Previously, it was suggested that the secretory PLA_2 is the primary phospholipase involved in PG production (Munns et al., 1999). Other groups have documented that the cytosolic PLA_2 is the predominant phospholipase involved in PG production (Hansen et al., 1999; Wang et al., 2001). In addition, it has been also proposed that the PLD is one of the regulator in the agonist-stimulated signal transduction pathway (Mizunuma et al., 1993; Johnson et al., 1999). However, the downstream effectors linking IL-1β stimulation with COX-2 expression and PG production remains unidentified.

PLD catalyzes the hydrolysis of phospholipids, resulting in the generation of phosphatidic acid and the respective head groups. Moreover, PLD activation has been implicated in a

This work was supported by grant R02-2000-00138 from the basic Research Program of the Korea Science & Engineering Foundation.

ABBREVIATIONS: PG, prostaglandin; COX, cyclooxygenase; IL-1β, interleukin-1β; PLD, phospholipase D; PA, phosphatidic acid; PBS, phosphate-buffered saline; PBt, phosphatidylbutanol; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; PAP, phosphatidic acid phosphohydrolase; PKC, protein kinase C; JNK, c-Jun NH_2-terminal kinase; PLA_2, phospholipase A_2.

wide range of cellular responses, including membrane trafficking (Liscovitch, 1996), mitogenesis (Boarder, 1994) and phagocytosis (Kusner et al., 1999). In mammals, at least two PLD isoforms are known to exist, PLD1 and PLD2 (Colley et al., 1997; Hammond et al., 1997; Lopez et al., 1998). Activation of PLD occurs through interaction with the small G-proteins of the ARF and Rac/Rho families as well as with PKC (Exton, 1999; Houle and Bourgoin, 1999). The relative contribution of these factors to PLD activation is highly dependent on the cell type and signaling model examined. Several lines of evidence have suggested a functional role for PLD in COX-2 regulation during cell activation (Sciorra and Daniel, 1996; Kaneki et al., 1998; Ueno et al., 2000). However, the contribution of PLD to COX-2 expression and PG production has not been as extensively studied. Therefore, we undertook to study the potential role of PLD and its signal transduction pathways in the COX-2 regulation using WISH amnion cells. In this study, the regulatory mechanisms for COX-2 expression and PGE_2 production during PLD and p38-dependent activation of WISH cells were investigated.

Experimental Procedures

Materials. The WISH human amnion cell line was obtained from the American Type Culture Collection (Manassas, VA). RPMI 1640, LipofectAMINE 2000, and the reverse transcription-polymerase chain reaction kit were purchased from Invitrogen (Carlsbad, CA), and fetal calf serum was purchased from Hyclone (Logan, UT). The human recombinant IL-1β was purchased from R&D Systems, Inc. (Minneapolis, MN). [^{3}H]Myristic acid and [γ-^{32}P]dCTP were from PerkinElmer Life Sciences (Buckinghamshire, UK), enhanced chemiluminescence reagents and the PGE_2 enzyme immunoassay kit were from Amersham Biosciences (Piscataway, NJ), rabbit polyclonal COX-1 and COX-2 antibodies were from Cayman Chemical (Ann Arbor, MI), phospho-ERK1/2, phospho-p38 and ERK2 antibodies were from New England Biolabs (Beverly, MA). 1-Propranolol, 1-butanol, and dioctanoyl PA were from Sigma (St. Louis, MO), and PD98059, SB203580, GF103290X, and Ro 31-8220 were from Biomol (Plymouth Meeting, PA) and were dissolved in dimethyl sulfoxide before addition to the cell culture. The final concentrations of dimethyl sulfoxide were 0.1% or less.

Cell Culture. The WISH cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. The cells were subcultured twice weekly by trypsinization and were seeded in either 12- (2×10^5 cells/well) or 6-well plates (5×10^5 cells/well). The cells were stimulated for various lengths of time ranging from a few minutes to 24 h in the presence of IL-1β with or without inhibitors

Assay of PLD Activity. PLD activity in WISH cells, the cellular phospholipids were labeled by incubating monolayers for 16 h with [^{3}H]myristic acid (2 μCi/ml) in growth medium. Thereafter, cells were washed three times in HBSS and resuspended in serum free medium. PLD activity was measured for 30 min at 37°C in a total volume of 1 ml, 1% 1-butanol and the indicated stimulatory agents. The reaction was then stopped and the specific PLD product [^{3}H]PBt was isolated and separated on silica gel 60 TLC plates. The formation of [^{3}H]PBt is expressed as a percentage of the total amount of labeled phospholipids.

PGE_2 Assay. PGE_2 levels were determined using an enzyme immunoassay kit according to the manufacturer's instructions. Briefly, 50 μl of standard or sample was pipetted into the appropriate wells. Aliquots of mouse polyclonal PGE_2 antibody and PGE_2 conjugated to alkaline phosphatase were then added to each well and allowed to incubate at room temperature for 1 h. After incubation, the wells were washed six times with 200 μl of PBS, including 0.05% Tween 20, followed by the addition of TMB substrate. Wells were read at 670 nm with an enzyme-linked immunosorbent assay reader 30 min after addition of substrate.

Western Blot Analysis. The WISH cells were plated in a 6-well plate and treated with IL-1β for various times. They were then washed with cold-PBS, scraped off and pelleted at 700*g* and at 4°C. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). The preparation was then cleared by centrifugation and the supernatant saved as a whole-cell lysate. The proteins (20 μg) were separated by 8% reducing SDS-polyacrylamide gel electrophoresis and electroblotted in 20% methanol, 25 mM Tris, and 192 mM glycine onto a nitrocellulose membrane. The membrane was then blocked with 5% nonfat dry milk in Tris-buffered saline-Tween 20 (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween 20) and subsequently incubated with the antibodies for 4 h. Subsequently, the membrane was washed and incubated for 1 h with secondary antibodies conjugated to horseradish peroxidase. Finally, the membrane was washed and developed using an enhanced ECL system.

RNA Isolation and Northern Blot Analysis. The WISH cells were cultured for the indicated times at 37°C with various concentrations of IL-1β. The cells were subsequently washed three times with PBS containing 2% bovine serum albumin. The RNA was isolated using a Tri-Reagent kit (Molecular Research Center, Cincinnati, OH). Aliquots (2 μg) of the total RNA were denatured and fractionated by gel electrophoresis using a 1% agarose gel containing 2.2 M formaldehyde. The RNA was transferred by capillary action in 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) onto a nylon membrane. The blots were incubated with specific DNA probes for the human COX-2, which had been labeled with [α-^{32}P]dCTP by random priming using the Prime-α-Gene kit (Promega, Madison, WI). The glyceraldehyde-3-phosphate dehydrogenase probe was used as an internal control for the RNA loading.

Results

IL-1β Induces COX-2 mRNA and Protein Expression. Because COX-2 isoform seems to synthesize PGs in the setting of cell stimulation, we examined whether this isozyme was responsible for IL-1β–induced PGE_2 production in the culture medium. Cultures were incubated in serum-free medium in the presence or in the absence of various concentrations of human recombinant IL-1β (0.1–5 ng/ml) for 1 h and Northern blots analysis was performed using the human COX-2 probe. As shown in Fig. 1A, IL-1β induced the accumulation of the COX-2 transcripts at 0.5 and 1 ng/ml. When immunoblots of protein extracted from IL-1β–treated and control cells were probed with polyclonal anti–COX-2 antibodies, a migrating band was detected at approximately 72 kDa (Fig. 1B). The COX-2 proteins were not seen in control cells, but were markedly enhanced when cells were incubated with IL-1β. When protein blots were probed with polyclonal antibodies recognizing COX-1, no such regulation of expression was observed, and COX-1 was present at similar levels despite IL-1β stimulation (data not shown). In experiments designed to examine the time course of PGE_2 production in the medium of IL-1β–treated cells, we detected a rapid rise in the rate of synthesis. Therefore, 0.5 ng/ml of IL-1β was used for the experiments described below. To understand the molecular control of this rapid and transient synthesis of PGE_2 after IL-1β treatment, we probed Northern blots with a COX-2 cDNA after extraction of RNA species from cells exposed to 0.5 ng/ml IL-1β for 0 to 6 h. As shown in Fig. 2, COX-2 transcripts accumulated within 1 h of IL-1β treat-

ment, had rapidly declined by 2 h and persisted until 6 h after IL-1β treatment. COX-2 protein showed an increase within 2 h and continued to increase for 10 h, based on Western blot analysis.

Role of PLD on IL-1β–Induced COX-2 Expression. WISH cells produce large amounts of PGE_2 after prolonged exposure to IL-1β. To characterize the steps in the regulation of PGE_2 production that occur during the early stages of WISH cell activation, we measured PLD activity in the cells after incubation with 0.5 ng/ml IL-1β for different times. After a time lag, significant PBt formation was observed at 10 min, and reached a plateau at about 30 min (Fig. 3B). Typically, a 3- to 4-fold increase over basal unstimulated

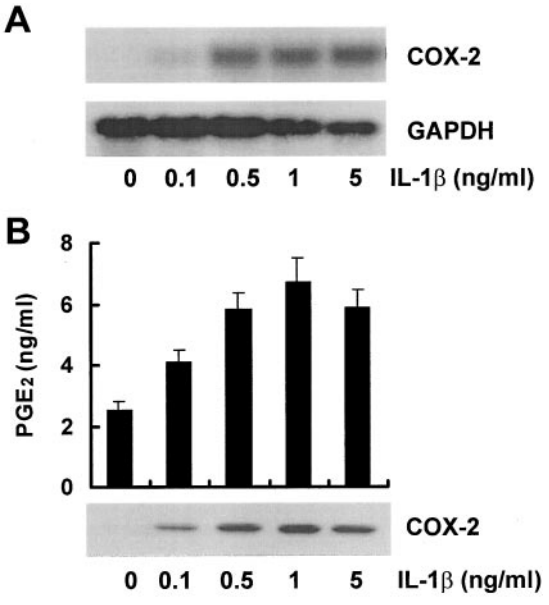

Fig. 1. Effect of IL-1β dosage on COX-2 mRNA and protein expression and PGE_2 production by WISH cells. The cells were treated with various concentrations of IL-1β for 1 h. Total RNA was extracted and Northern blot analysis was performed using probe specific for human COX-2. The COX-2 mRNA levels shown are representative of two independent experiments (A). Cells were treated with the indicated concentrations of IL-1β for 8 h, and then the release of PGE_2 was measured from supernatants and the extracted proteins was immunodetected with COX-2 specific antibody (B). The values for the PGE_2 production are represented as average ± S.E. and COX-2 protein levels shown are representative of three independent experiments.

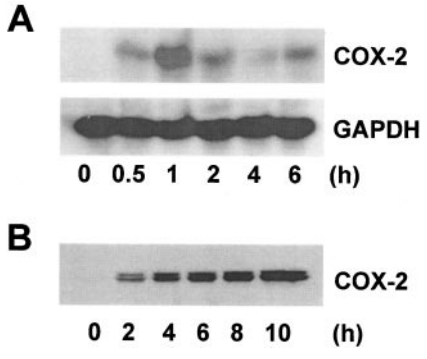

Fig. 2. Time-dependent increase in the COX-2 mRNA and protein expression by IL-1β. Cells were incubated with IL-1β (0.5 ng/ml) for the indicated times. Total RNA was isolated and Northern blot analysis was performed using a probe specific for human COX-2 (A). The cell lysates were extracted and Western blot analysis was performed using specific antibody for COX-2 (B). COX-2 mRNA and protein levels are representative of three independent experiments.

formation was detected at an optimal IL-1β concentration of 0.5 ng/ml. Many studies have demonstrated that 1-butanol exerts its anti-PLD action in part by suppressing PA formation. Therefore, we examined whether 1-butanol could inhibit IL-1β–induced COX-2 expression and PGE_2 production in WISH cells. The WISH cells were stimulated with IL-1β in the presence of 1% butanol for 8 h. As shown in Fig. 4A, 1-butanol inhibited IL-1β–induced COX-2 expression and PGE_2 production. In contrast, when the WISH cells were preincubated with 3-butanol and then challenged for 8 h with IL-1β no effect on IL-1β–induced COX-2 expression was observed. To further establish that the effect of 1-butanol on COX-2 expression and PGE_2 production is caused by the inhibition of PA formation by PLD, we used propranolol, which is a well-established PAP inhibitor. In experiments, the expression of COX-2 was determined in cells pretreated with propranolol. Figure 4B shows that propranolol conversely potentiated the COX-2 expression by IL-1β. Although the experiments showed that PLD was activated in IL-1β–treated WISH cells, they provided no direct evidence that PA was involved in the COX-2 expression. To test this, the cells were treated with various concentrations of a short-chain dioctanoyl PA. Figure 4C shows that dioctanoyl PA significantly induced COX-2 expression in a dose-dependent manner.

Involvement of PKC in IL-1β–induced COX-2 Expression. Activation of PKC, particularly the α isoform, has previously been shown to constitute a major route to PLD activation in a wide variety of cell types (Houle and Bourgoin,

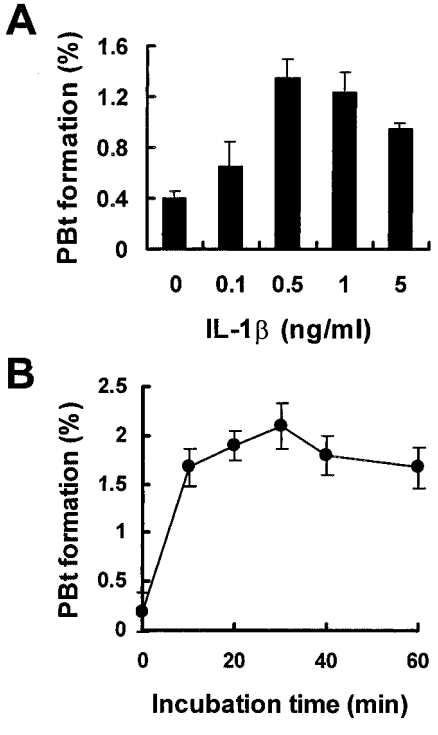

Fig. 3. Dose and time course of IL-1β–stimulated PLD activity. Cells were labeled with [^{3}H]myristic acid for 16 h and stimulated with the indicated concentrations of IL-1β for 30 min (A) or stimulated for the indicated times with 0.5 ng/ml of IL-1β (B). PLD activity was measured as described under *Experimental Procedures*. Data are expressed as the percentage of [^{3}H]PBt to total ^{3}H-labeled lipids. Values shown are the means ± S.E. of three independent experiments performed in duplicate in different batches of cells.

1999). In addition, it was reported that the PKC regulates COX-2 expression and PGE_2 production by various agonists (Lin et al., 2000; Molina-Holgado et al., 2000). To assess whether or not COX-2 expression was mediated by PKC activation, experiments were conducted in the presence of PKC inhibitor Ro 31-8220. Ro 31-8220 suppressed COX-2 expression, as determined by Western blot analysis (Fig. 5). Involvement of PKC in IL-1β–induced COX-2 expression was confirmed by using of other inhibitor GF109203X, a potent and selective PKC inhibitor. Like Ro 31-8220, GF109203X also suppressed IL-1β–induced COX-2 expression (data not shown).

Characterization of p38 and ERK Activation by IL-1β in WISH Cells. We also studied the IL-1β–induced activation of p38 and ERK in WISH cells using phosphospecific antibodies that bind only activated forms of p38 and ERK. Western blot analysis demonstrated that IL-1β activated the p38 and ERK in a dose-dependent manner (Fig. 6A). The maximal level of stimulation was reached at 1 ng/ml for both MAPKs. As shown in Fig. 6B, IL-1β induced a time-dependent phosphorylation of p38. The phosphorylation of p38 was apparent at the earliest analysis time of 3 min, and attained a maximal level at 20 min. We also determined the ERK activity by immunoblot. As for p38 activation, IL-1β–induced ERK phosphorylation occurred very early and reached a maximal level at 20 min.

p38 Activation Mediates IL-1β–Induced COX-2 Expression. We used MAPK inhibitors to examine whether MAPK activation was involved in the signal transduction pathway leading to COX-2 expression caused by IL-1β. Moreover, the MAPK-specific inhibitors PD98059 and SB203580 were used to examine the kind of MAPK isoform involved in the IL-1β–mediated effect. It is evident from Fig. 7 that p38 inhibitor SB203580 suppressed IL-1β–induced COX-2 expression and p38 phosphorylation in WISH cells. However, the ERK upstream inhibitor PD98059 did not alter IL-1β–induced COX-2 expression, although this compound strongly inhibited IL-1β–induced ERK phosphorylation. To determine whether the p38-mediated COX-2 expression is exerted via PKC, we stimulated the cells with the IL-1β in the presence of PKC inhibitors and determined the change of MAPK phosphorylation. As shown in Fig. 8, pretreatment with the PKC inhibitor Ro 31-8220 suppressed IL-1β–induced p38 phosphorylation. In contrast, this inhibitor did not affect IL-1β–induced ERK phosphorylation.

Discussion

Previous studies with the WISH human amnion cell line have demonstrated that the stimulation of these cells with IL-1β leads to increased COX-2 expression and PGE_2 release (Albert et al., 1994). We have now extended these previous

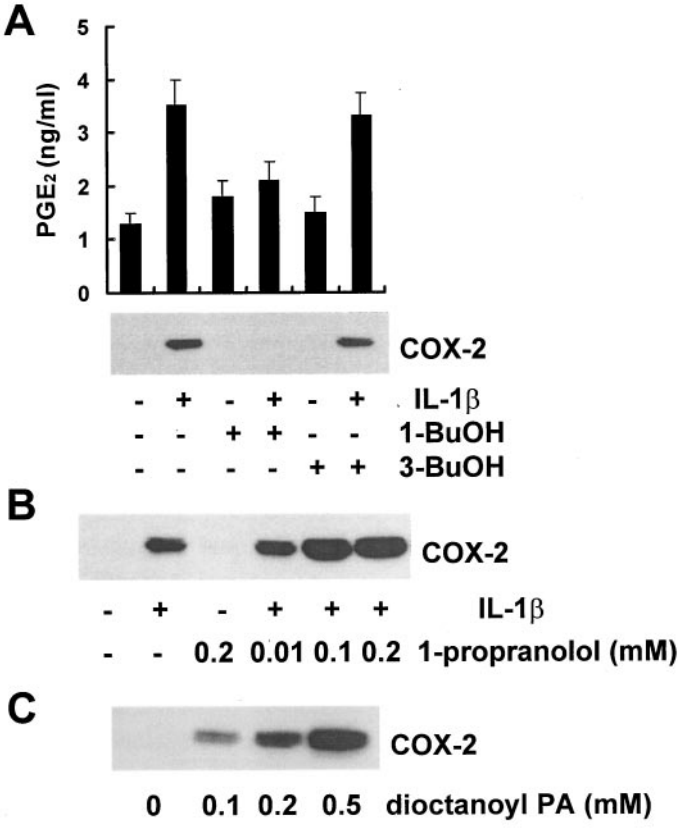

Fig. 4. Effect of PLD inhibitors and dioctanoyl PA on COX-2 expression. Cells were stimulated with IL-1β in the presence of 1% 1-butanol or 3-butanol for 8 h (A). Cells were treated with IL-1β in the presence of varying concentrations of 1-propranolol for 8 h (B). Cells were stimulated by the indicated concentrations of dioctanoyl PA for 8 h (C). The release of PGE_2 was measured from supernatants and the extracted proteins were immunodetected with COX-2 specific antibody. The values for the PGE_2 production are representative as average ± S.E. and the COX-2 protein levels are represented from three independent experiments.

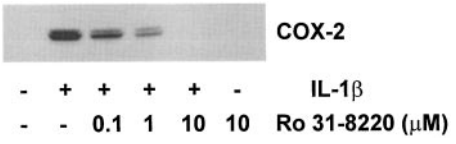

Fig. 5. Effect of the PKC inhibitor Ro 31-8220 on IL-1β–induced COX-2 expression. Cells were preincubated with the different concentrations of Ro 31-8220 for 30 min then stimulated with IL-1β for 8 h. The cell lysates were analyzed by Western blot with COX-2 antibody. The COX-2 protein levels are represented from three independent experiments.

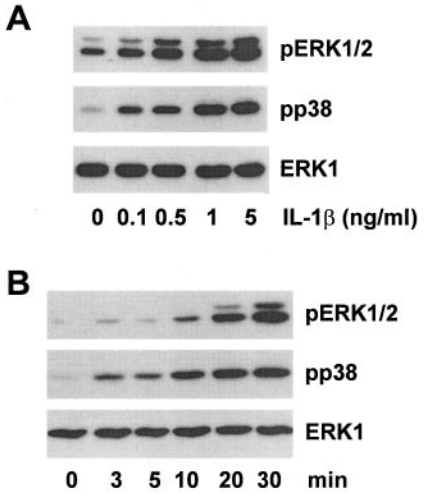

Fig. 6. Concentration and time response of p38 and ERK phosphorylation by IL-1β. Cells were stimulated with the indicated concentrations of IL-1β for 20 min (A) and the indicated times with IL-1β (B). Cells were lysed and samples were analyzed by Western blot analysis. Equal amounts of proteins were loaded and p38 and ERK phosphorylation were detected by immunoblot with phosphospecific antibodies. MAPK phosphorylation levels are represented from three independent experiments.

reports to PGE_2 production and investigated the mechanism of signal transduction for the COX-2 regulation by IL-1β. Our study shows that the activation by IL-1β of the human amnion cell line WISH induces COX-2 expression and PGE_2 production as well as PLD activity. Furthermore, this study demonstrates that p38 mediates in IL-1β–stimulated COX-2 induction and suggests that PLD is implicated in the signaling cascade leading to the induction of COX-2 in activated WISH cells.

The production of PGs in amnion is important in the physiology of human parturition, and numerous studies have reported their key role (Slater et al., 1995; Zakar et al., 1998). The proposed mechanism for the initiation of labor, which involves the release of arachidonic acid and the expression of COX-2 for PG biosynthesis, has been thoroughly documented with biological evidence. However, information is still scarce as to the signaling mechanisms involved in COX-2 regulation during labor. Previous reports have demonstrated that COX-2 induction by IL-1β is mediated by PKC (Lin et al., 2000; Molina-Holgado et al., 2000), PLA_2 (Hansen et al., 1999; Munns et al., 1999) and tyrosine kinase (Akarasereenont and Thiemermann, 1996; Yucel-Lindberg et al., 1999). In addition, it has been suggested that PLD is one of the regulators of COX-2 expression. Angel et al. (1994) has reported that IL-1β amplifies bradykinin-induced PGE_2 production via a PLD-linked mechanism. Kaneki et al. (1998) showed that PMA-induced COX-2 expression in osteoblast like UMR-106 cells was dependent upon PLD activity. Johnson et al. (1999) suggested that the PAP, PLD downstream effector, was involved in PMA-induced COX-2 expression. However, none of those studies found a direct link between PLD and COX-2 in IL-1β–induced WISH cells. To elucidate the role of PLD in the COX-2 regulation, we made use of the property that PLD must catalyze a transphosphatidylation reaction. The most common alcohol used for this purpose is 1-butanol or ethanol, which leads to the formation of PBt or phospatidylethanol. We used 3-butanol to rule out the nonspecific effect of 1-butanol, and found that 1-butanol inhibited IL-1β–induced COX-2 expression and PGE_2 production, whereas 3-butanol, as expected, did not. Interestingly, the IL-1β–induced COX-2 protein was strongly potentiated by 1-propranolol. This compound has been shown to inhibit PAP. This result provides evidence that IL-1β–induced COX-2 expression is mediated by the PLD. A role for PLD in the pathway leading to COX-2 expression received further support when dioctanoyl PA was used to induce COX-2 expression. These results demonstrate that PLD activity and the intracellular accumulation of PA are importantly involved in IL-1β–dependent COX-2 expression.

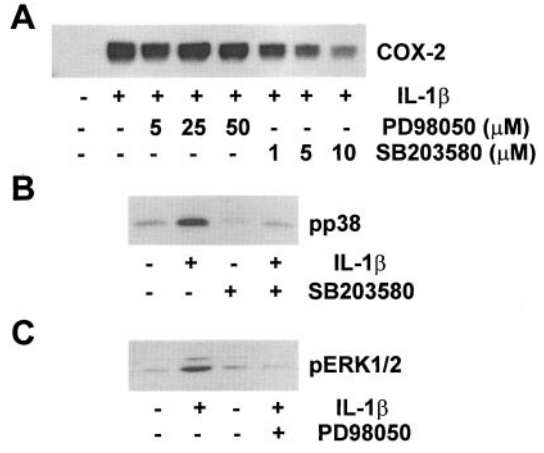

Fig. 7. Effect of MEK and p38 inhibitor on IL-1β–induced COX-2 expression. WISH cells were pretreated with the indicated concentrations of PD98059 or SB203580 for 30 min followed by stimulation with IL-1β for 8 h (A). The COX-2 expression was determined by Western blot analysis using COX-2 antibody. Cells were pretreated with PD98059 (B) or SB203580 (C) for 10 min followed by stimulation with IL-1β for 20 min. MAPK phosphorylation were determined by Western blot analysis using phosphospecific antibodies. The COX-2 protein and MAPK phosphorylation levels are represented from three independent experiments.

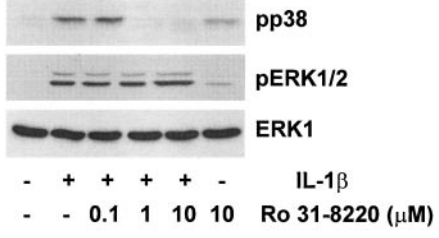

Fig. 8. Effect of PKC on IL-1β–induced p38 and ERK phosphorylation. Cells were pretreated with the different concentrations Ro 31-8220 for 10 min followed by stimulation with IL-1β for 20 min. Cells were lysed and samples were analyzed by Western blot analysis. Equal amounts of proteins were loaded and p38 and ERK phosphorylation were detected by immunoblot with phosphospecific antibodies. MAPK phosphorylation levels are represented from three independent experiments.

PKC is a family of closely related serine/threonine kinases that seem to mediate various cellular functions. The signaling pathway of PKC is known to play a role in mediating the action of various cytokines, including IL-1β. Activation of PKC has been suggested to be key event in the signal transduction leading to COX-2 expression by IL-1β (Lin et al., 2000; Molina-Holgado et al., 2000). In the present study, we also demonstrated that IL-1β–induced COX-2 expression was prevented by the PKC inhibitors Ro 31-8220 and GF103290X, indicating that PKC activation is involved in the signal transduction leading to COX-2 expression by IL-1β. PKC is well established as a major physiological regulator of PLD. Studies have identified the modes of regulation of PLD by PKC in detail (Gustavsson et al., 1994; Lopez et al., 1995; Exton, 1999). Therefore, our results suggested that IL-1β–induced COX-2 expression may be via the PKC/PLD pathway.

IL-1β–induced signaling events have been shown to include activation of MAPKs downstream of PKC (Fiebich et al., 2000; Molina-Holgado et al., 2000). In addition, one major route for the production of the PGE_2 by COX-2 is MAPK-mediated signal transduction (Bartlett et al., 1999). In the WISH cell, IL-1β treatment resulted in the phosphorylation of the ERK and p38 cascades, with maximal stimulation of both activity at 1 ng/ml. Furthermore, we used MAPK inhibitors to examine whether MAPK activation was involved in the signal transduction pathway leading to COX-2 expression caused by IL-1β. Moreover, the MAPK-specific inhibitors PD98059 and SB203580 were used to examine the kind of MAPK isoform involved in the IL-1β–mediated effect. SB203580, a selective inhibitor of p38, strongly suppressed IL-1β–induced COX-2 expression. Our results do not rule out the possibility that IL-1β–induced COX-2 expression is mediated by JNK. However, we do not believe this to be the case. Because treatment of cells with PKC inhibitors failed to inhibit IL-1β–induced JNK phosphorylation, we concluded

that JNK activation does not play a significant role in the mechanism by which IL-1β modulates COX-2 expression.

To elucidate the role of PKC in the p38 activation, we treated Ro 31-8220, and found that this compound inhibited IL-1β–induced p38 phosphorylation. Moreover, inhibition of p38 phosphorylation by Ro 31-8220 produced concentration dependent effects on IL-1β–stimulated COX-2 expression, indicating a clear causal relationship between p38 activation and PGE_2 synthesis. Therefore, our results provide evidence that one of the signal transduction pathways initiated by IL-1β leading to COX-2 expression involves PKC and p38 activation. Several reports have showed that PLD is downstream of MAPK molecules. Indeed, previous reports have found that PLD and its product, PA mediate insulin-dependent MAPK activation in Rat-1 fibroblasts (Rizzo et al., 1999). Very recently, Bechoua and Daniel (2001) reported that the PLD is required in the signaling pathway leading to p38 activation by fMLP in neutrophil-like HL-60 cells. However, the biological significance of the each MAPK activation is not yet fully understood, but it may be related to the novel function that is PLD-dependent MAPK activation. Further studies are necessary to determine overall signal transduction pathways that are associated with IL-1β–induced COX-2 regulation.

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