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Regulation of Cyclooxygenase-2 Expression by Phosphatidate Phosphohydrolase in Human Amnionic WISH Cells*

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Prostaglandins are known to play a key role in the initiation of labor in humans, but the mechanisms governing their synthesis in amnion are largely unknown. In this study, we have examined the regulatory pathways for prostaglandin E_2 (PGE_2) production during protein kinase C-dependent activation of human WISH cells. In these cells, PGE_2 synthesis appears to be limited not by free arachidonic acid availability but by the expression levels of cyclooxygenase-2 (COX-2). Concomitant with the cells being able to synthesize and secrete PGE_2, we detected significant elevations of both COX-2 protein and mRNA levels. Specific inhibition of COX-2 by NS-398 totally ablated PGE_2 synthesis. All of these responses were found to be strikingly dependent on an active phosphatidate phosphohydrolase 1 (PAP-1). Inhibition of PAP-1 activity by three different strategies (*i.e.* use of bromoenol lactone, propranolol, and ethanol) resulted in inhibition of COX-2 expression and hence of PGE_2 production. These data unveil a novel signaling mechanism for the regulation of PGE_2 production via regulation of COX-2 expression and implicate phosphatidate phosphohydrolase 1 as a key regulatory component of eicosanoid metabolic pathways in the amnion.

Prematurity is the leading cause of perinatal morbidity and mortality worldwide, affecting 5–10% of births (1). The prevention of premature birth is a goal not yet attained because of the lack of knowledge about basic mechanisms responsible for premature labor and delivery. Term and preterm labor are thought to share a common terminal pathway composed of uterine contractility, cervical dilation, and rupture of membranes. However the steps leading to these processes may be different. Whereas term labor results from physiological activation of the components of the common terminal pathway, preterm labor appears likely to be the result of a disease process that activates this common terminal pathway early (1).

It is well established that the prostaglandins (PG),[1] particularly PGE_2, are key mediators of the common terminal pathway (2–4). Arachidonic acid (AA) and prostaglandins accumulate in amniotic fluid in association with the onset of labor at both term (2–5) and preterm (6) labor. Consistent with this, cyclooxygenase (COX) activity increases in the amnion and chorion tissues both in term and preterm labor (7–9).

Both COX-1 and COX-2 are expressed in amnion and chorion, but only COX-2 increases near the onset of labor (10, 11), suggesting that both the increase in COX activity and subsequent PGE_2 production in the course of labor are attributable to COX-2 (10, 11). Also, recent evidence using COX-2 knockout mice show that the products of the cyclooxygenase pathway are required for every step of early pregnancy, including ovulation, fertilization, implantation and decidualization (12). The detailed ways that they act remain to be determined.

In this study, we have examined the expression and activity of the COX isoenzymes during activation of the human amnionic cell line WISH. Previously (13), we identified a novel pathway for AA mobilization in human amnionic WISH cells involving the participation of phosphatidate phosphohydrolase 1 (PAP-1) as a key regulatory element. We now demonstrate that PAP-1 is also implicated in the signaling cascade leading to induction of COX-2 in activated WISH cells. Therefore PAP-1 emerges as a novel key regulatory component of the eicosanoid response of amnion cells.

EXPERIMENTAL PROCEDURES

Materials—Human WISH cells (established amnion cell line) were obtained from the American Type Culture Collection (Manassas, VA). Iscove's modified Dulbecco's medium (endotoxin <0.05 ng/ml) was from BioWhittaker (Walkersville, MD). Fetal bovine serum was from Hyclone Labs (Logan, UT). Trypsin/EDTA solution was purchased from Irvine Scientific (Santa Ana, CA). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (specific activity 100 Ci/mmol) and [^{32}P]CTP (specific activity 6,000 Ci/mmol) were obtained from NEN Life Science Products. Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem. Arachidonic acid was from Biomol (Plymouth Meeting, PA). Bromoenol lactone (BEL) was from either Biomol or Calbiochem. The secretory PLA_2 inhibitor LY311727 was kindly provided by Dr. Edward Mihelich (Lilly Research Laboratories). Methyl arachidonyl fluorophosphonate (MAFP), aspirin, NS-398, indomethacin, COX-1 polyclonal antiserum, and COX-2 polyclonal antibody were from Cayman Chemical (Ann Arbor, MI). Casein solution (10×), streptavidin horseradish peroxidase, and biotinylated goat anti-rabbit $F(ab')_2$ fragments were from Vector Laboratories (Burlingame, CA). Propranolol and thimerosal were from Sigma. ExpressHyb hybridization solution was from CLONTECH (Palo Alto, CA). The 1.8-kilobase pair cDNA fragment encoding human COX-2 was a gift kindly provided by Dr. Timothy Hla (Dept. of Physiology, University of Connecticut). The β-actin probe used as a control in the Northern analysis was from Ambion (Austin, TX).

Cell Culture—Human amnionic WISH cells (14) were maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 90% air and 10% CO_2. The cells were subcultured twice weekly by trypsinization and, when used for experiments, were seeded into 12-well plates (2×10^5 cells/well, Corning Inc.) or 6-well plates (3.7×10^5 cells/well, NUNC). After a 2-day growth, the cells, at 90% confluence, were rinsed with serum-free medium and incubated for 1 h before stimulation.

[^{3}H]AA Release—Cells were radiolabeled with [^{3}H]AA by including 0.5 μCi [^{3}H]AA/10^6 cells in the culture medium 20 h before stimulation. Unincorporated [^{3}H]AA was removed by washing the cells three times

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[1] The abbreviations used are: PG, prostaglandin; AA, arachidonic acid; COX, cyclooxygenase; BEL, bromoenol lactone; MAFP, methyl arachidonyl fluorophosphonate; PLA_2, phospholipase A_2; $iPLA_2$, Ca^{2+}-independent PLA_2; PAP, phosphatidate phosphohydrolase; PMA, phorbol myristate acetate.

with phosphate-buffered saline containing 1 mg/ml bovine serum albumin (fatty acid-free). Cells were stimulated with 25 ng/ml PMA for different time periods in the presence of 0.1 mg/ml bovine serum albumin. The supernatants were removed and cleared of detached cells by centrifugation, and radioactivity was counted by liquid scintillation. When inhibitors were used, except for ethanol, they were added to the cells 20 min before PMA was added to the medium. When ethanol was used as an inhibitor it was added to the cells just before the addition of PMA.

PGE_2 Assay—Cells were stimulated with 25 ng/ml PMA for different time periods in serum-free medium. The supernatants were removed and cleared of detached cells by centrifugation, and PGE_2 was quantitated using a specific radioimmunoassay (PerSeptive Biosystems, Framingham, MA). When inhibitors were used, except for ethanol, they were added to the cells 20 min before PMA was added to the medium. When ethanol was used as an inhibitor it was added to the cells just before the addition of PMA.

PAP Assay—PAP activity in homogenates from WISH cells were assayed exactly as described previously (16). The substrate [^{14}C]glycerol-labeled PA was presented as mixed micelles with Triton X-100 at a detergent/phospholipid mole ratio of 10:1. Assays were conducted at 37 °C. The incubation mixture contained in a final volume of 0.1 ml: 100 μM [^{14}C]PA substrate (0.025 μCi/assay), 1 mM Triton X-100, 50 mM Tris-HCl (pH 7.1), 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and the indicated amount of homogenate protein. After the indicated times the reaction was stopped and [^{14}C]PA and [^{14}C]diacylglycerol were separated by thin layer chromatography using the system *n*-hexane/ethyl ether/acetic acid (70:30:1). Total PAP activity in the homogenates arises from two separate enzymes: PAP-1 and PAP-2. To establish the relative contribution of each of these isoenzyme to total WISH cell PAP activity, experiments were carried out in the presence of 8 mM *N*-ethylmaleimide, an agent that completely blocks PAP-1 activity but has no effect on PAP-2 (16). By this procedure we determined that PAP-1 accounts for ~20% of total PAP activity in WISH cell homogenates. All assays were conducted under conditions of linearity with respect to time and protein concentration, and showed zero-order kinetics for the concentration of substrate used.

Western Blot Analyses—The cells were lysed using phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 0.5% Triton X-100 in phosphate-buffered saline. Samples (50 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide gel) and transferred to Immobilon-P (Millipore, Bedford, MA). The membranes were blocked for 1 h at room temperature with 2.5% casein in 50 mM Tris-HCl (pH 7.5), 154 mM NaCl, and 0.2 mM thimerosal. The blocking solution was then removed, and the membranes were incubated with anti-COX antibody (1:1,000 dilution) in blot buffer (50 mM Tris-HCl, 200 mM NaCl, 0.05% Tween 20, 1% casein, pH 7.5) overnight at 4 °C. The membranes were then washed in Tris-buffered saline/Tween 20 (50 mM Tris-HCl, 200 mM NaCl, pH 7.5, containing 0.05% Tween 20) three times for 5 min. The membranes were then incubated with biotinylated goat anti-rabbit $F(ab')_2$ fragments (1:2,000 dilution) in blot buffer for 30 min at room temperature and then washed (3 washes, 5 min each) in Tris-buffered saline/Tween 20. The membranes were then incubated with streptavidin horseradish peroxidase (1:5,000 dilution) in blot buffer for 30 min at room temperature and washed (3 washes, 5 min each) in Tris-buffered saline/Tween 20. The bands were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Preparation of RNA and Northern Analyses—Total cellular RNA was isolated from unstimulated or PMA-stimulated cells by the TRIZOL reagent method (Life Technologies, Inc.), exactly as indicated by the manufacturer. Fifteen μg of total RNA per lane was electrophoresed in a 1% formaldehyde/agarose gel and transferred to nylon-supported membranes (Hybond, Amersham Pharmacia Biotech) in 10× SSC buffer. After UV cross-linking, the membranes were hybridized in ExpressHyb hybridization solution according to the manufacturer's protocol. cDNA probes were labeled using Ready-to-Go DNA labeling beads from Amersham Pharmacia Biotech according to the manufacturer's protocol. ^{32}P-labeled probes for COX-2 and β-actin were incubated with the filters for 1 h at 68 °C. When incubated with the COX-2 probe, the membranes were washed once for 30 min in 0.2× SSC containing 0.5% SDS at 68 °C, followed by two washes with 0.1× SSC containing 0.1% SDS for 15 min at 68 °C. When incubated with the β-actin probe, the membranes were washed twice with 0.1× SSC containing 0.1% SDS for 15 min at 68 °C. Bands were visualized by autoradiography.

Data Presentation—Assays were carried out in duplicate or triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data presented are from representative experiments.

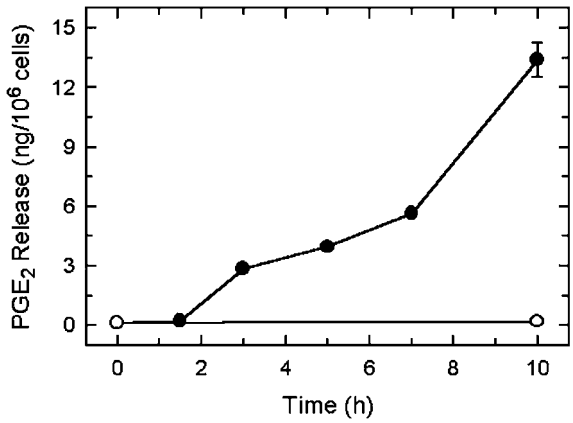

FIG. 1. **PMA-stimulated long term PGE_2 production.** Time-course of PGE_2 release in WISH cells in the absence (○) or presence (●) of 25 ng/ml PMA.

RESULTS

PGE_2 Production in PMA-activated WISH Cells—Previous experiments in our laboratory established that PMA induces AA release in WISH cells in a time-dependent manner with the maximal response being reached almost 90 min after cell stimulation (13). As shown in Fig. 1, PMA-stimulated WISH cells also produced measurable amounts of PGE_2, albeit with a distinct time dependence. Stimulated PGE_2 was barely detectable within the first 2 h of stimulation, increasing substantially afterward. The lack of a correlation between the time courses of AA release and PGE_2 production in PMA-stimulated WISH cells (*e.g.* Fig. 1 and Fig. 1A in Ref. 13) suggests that free AA availability is not rate-limiting for PGE_2 biosynthesis in this cell line.

The above notion directed us to the study of the expression and activity of the AA-metabolizing enzymes COX-1 and COX-2. Immunoblot analysis of the COX isoforms expressed by the WISH cells revealed that COX-2, but not COX-1, increased after PMA treatment in a time- and concentration-dependent manner (Fig. 2). Maximal COX-2 expression was observed at 4–6 h, which corresponds well with the kinetics of PMA-induced PGE_2 production shown above. The increases in COX-2 protein were confirmed by analyzing mRNA levels for both COX-1 and COX-2. As shown in Fig. 3, COX-2 mRNA levels substantially increased after PMA treatment; whereas, COX-1 mRNA levels did not change (data not shown). Fig. 4 shows that the COX-2 specific inhibitor NS-398 abolished the production of PGE_2 in response to PMA. Collectively these data suggest that the increased capacity of the WISH cells to produce PGE_2 in response to PMA is due to the increased expression of COX-2 protein.

Inhibition of COX-2 Protein Expression by BEL—The effect of BEL on PMA-induced COX-2 protein is shown in Fig. 5*A*. BEL is generally regarded as a Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) inhibitor (15), but recent studies have demonstrated that it affects another cellular phospholipase, namely PAP-1 (16). We show in Fig. 6 that BEL directly inhibits WISH cell PAP-1 activity. Importantly, the effects of BEL on COX-2 expression shown in Fig. 5*A* are clearly not due to $iPLA_2$ inhibition, because MAFP, a dual cytosolic PLA_2/$iPLA_2$ inhibitor (17) did not reproduce the effect. In agreement with the above data, BEL also inhibited the PMA-induced COX-2 mRNA levels (Fig. 5*B*). In perfect agreement with the data of Fig. 5, BEL strongly inhibited the PMA-stimulated PGE_2 production (Fig. 7).

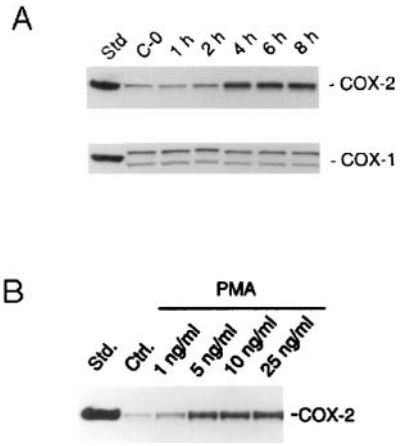

FIG. 2. **PMA-stimulated COX expression.** *A*, time course of COX-1 and COX-2 expression in WISH cells stimulated by 25 ng/ml PMA for the time indicated (h), as assessed by immunoblot. C-0 denotes a control incubation (no PMA) at 0 h. *B*, dose response of the effect of PMA on COX-2 protein levels at 6 h.

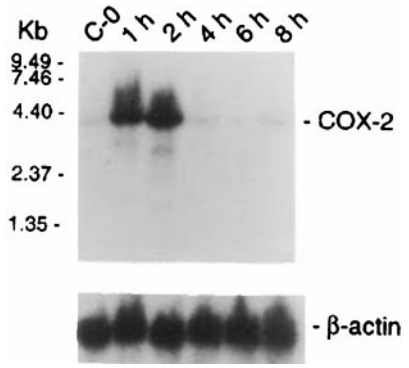

FIG. 3. **Time-course of COX-2 mRNA production in cells stimulated with 25 ng/ml PMA for the time indicated (h) as assessed by Northern blot.** C-0 denotes a control incubation (no PMA) at 0 h.

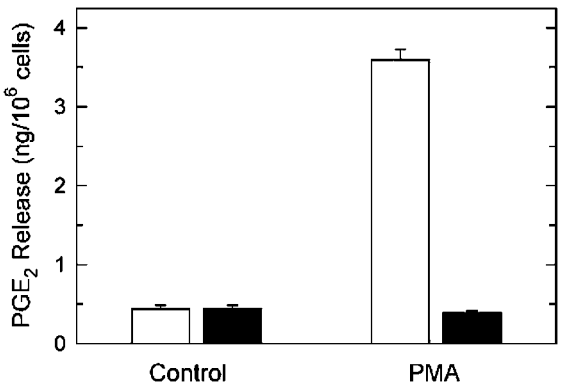

FIG. 4. **Effect of NS-398 on PGE_2 release.** PGE_2 release was measured in unstimulated cells (*Control*) or cells stimulated with 25 ng/ml PMA, in the absence (*open bars*) or presence (*closed bars*) of NS-398 (5 μM). NS-398 was added 20 min before the addition of PMA. The cells were then incubated for 10 h. PGE_2 in the supernatants was measured using a specific radioimmunoassay.

To further establish that the effects of BEL on COX-2 expression and attendant PGE_2 production are actually due to inhibition of PAP-1, we used two other independent strategies to achieve inhibition of the enzyme. In the first place we used propranolol, a well established PAP-1 inhibitor (18) that is both structurally and mechanistically unrelated to BEL. Propranolol also completely inhibited WISH cell PAP-1 activity (Fig. 6),

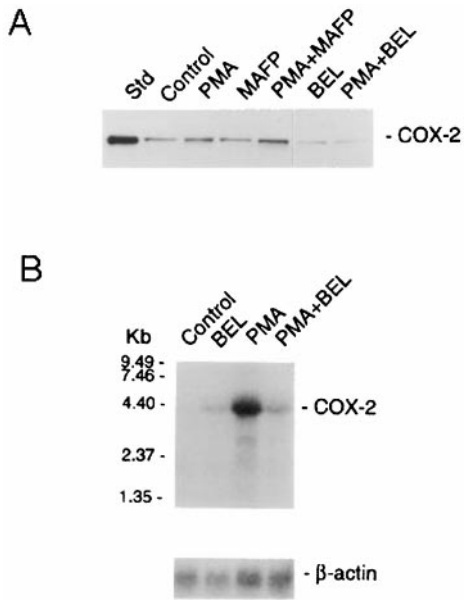

FIG. 5. **COX-2 expression in the presence of BEL.** *A*, effect of BEL and MAFP on COX-2 protein. The cells were pretreated with the inhibitors 20 min before the addition of 25 ng/ml PMA or vehicle (*Control*) and incubated for 6 h. COX-2 protein content was analyzed by immunoblot as described under "Experimental Procedures." *B*, effect of BEL on COX-2 mRNA levels. The cells were pretreated with 25 μM BEL for 20 min and then stimulated with 25 ng/ml PMA and incubated for 2 h. Total RNA was extracted and subjected to Northern blot analysis as described under "Experimental Procedures."

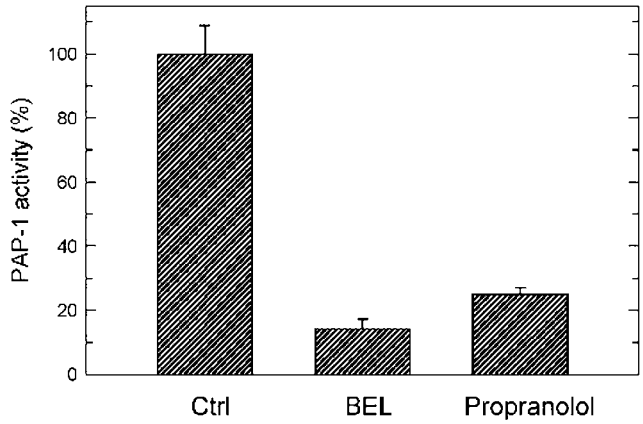

FIG. 6. **PAP activity in WISH cell homogenates.** WISH cell homogenates were incubated with 25 μM BEL, 100 μM propranolol or neither (*Ctrl*) for 10 min before assaying PAP activity as described under "Experimental Procedures."

PGE_2 production (Fig. 7), and the induction of COX-2 protein (Fig. 8*A*) and mRNA (Fig. 8*B*).

As a third approach, we used ethanol. By forming phosphatidylethanol instead of phosphatidic acid by phospholipase D, this alcohol depletes the substrate for PAP-1, therefore blunting the action of the enzyme. The overall effect is thus the same as if PAP-1 was being directly inhibited. Ethanol totally blocked PGE_2 release (Fig. 7) and dramatically decreased the levels of both COX-2 protein (Fig. 9*A*) and mRNA (Fig. 9*B*). Collectively, these data do suggest the involvement of PAP-1 as an upstream component of the signaling cascade triggered by PMA that leads to enhanced COX-2 expression and attendant PGE_2 release.

DISCUSSION

Our previous studies with the human amnionic cell line WISH have demonstrated that acute stimulation of these cells

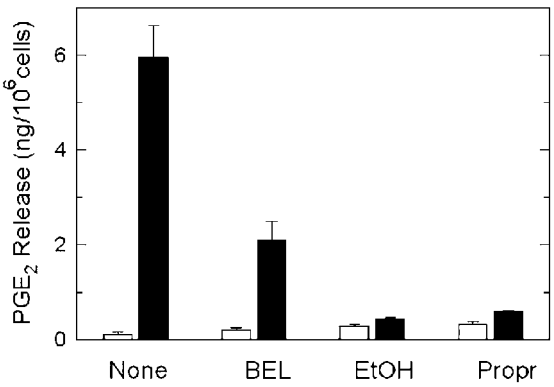

FIG. 7. **PGE_2 release in the presence of PAP inhibitors.** PGE_2 release was measured in cells incubated in the absence (*open bars*) or presence (*closed bars*) of 25 ng/ml PMA, and, where indicated, in the presence of BEL (25 μM), ethanol (EtOH, 2%), propranolol (*Propr*, 100 μM). The cells were then incubated for 6 h. Afterward, PGE_2 in the supernatants was determined with a specific radioimmunoassay.

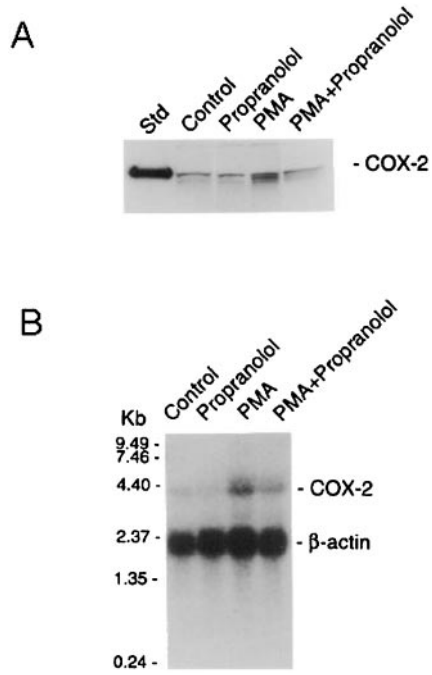

FIG. 8. **Effect of propranolol on COX-2 expression.** *A*, effect on COX-2 protein. The cells were treated with 100 μM propranolol for 20 min before treatment without or with 25 ng/ml PMA. The incubations proceeded for 6 h. Afterward COX-2 protein was analyzed by immunoblot. *B*, effect on COX-2 mRNA. The cells were treated with 100 μM propranolol for 20 min before treatment without or with 25 ng/ml PMA. The incubations proceeded for 2 h. Afterward COX-2 mRNA was analyzed by Northern blot.

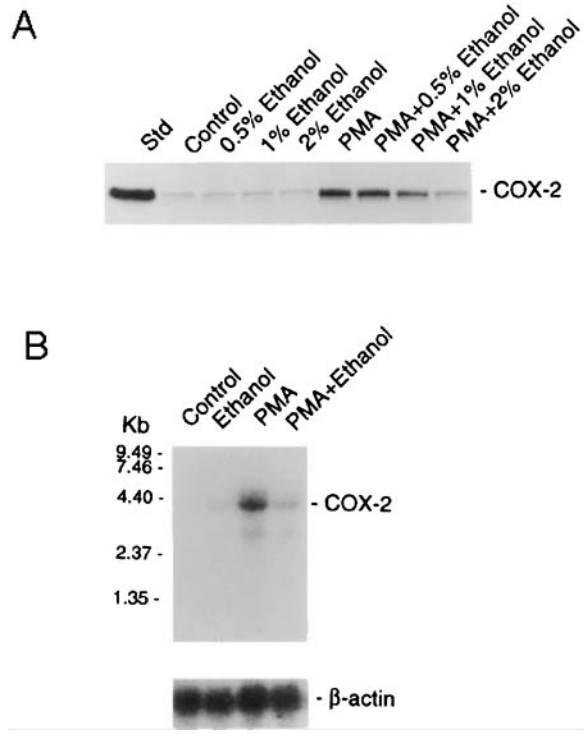

FIG. 9. **Effect of ethanol on COX-2 expression.** *A*, effect on COX-2 protein. The cells were treated with different concentrations of ethanol in the absence or presence of 25 ng/ml PMA. The incubations proceeded for 6 h. Afterward COX-2 protein content was analyzed by immunoblot. *B*, effect on COX-2 mRNA levels. The cells were treated with 2% ethanol in the absence or presence of 25 ng/ml PMA. The incubations proceeded for 2 h. Afterward COX-2 mRNA levels were analyzed by immunoblot.

with PMA leads to increased AA release in a short phase that plateaus at about 2 h. We have now extended these previous experiments to PGE_2 production. Unexpectedly, our results reveal that PGE_2 is much more delayed in time. Appreciable PGE_2 is observed only after 2 h of incubation with the phorbol ester and proceeds continuously for at least 10 h. Given that free AA is the precursor of prostaglandins, this discrepancy between the time courses of both AA and PGE_2 generation suggests that the production of the latter is not limited by availability of the former. Thus, expression and activity of COX-2 protein would appear to be the most plausible candidate for limiting PGE_2 biosynthesis in WISH cells once AA release is activated. In accord with this, PMA was found to induce a dose- and time-dependent increase in the expression of COX-2 mRNA and protein, which corresponds very well with the production of PGE_2 by these cells. Moreover, PMA-stimulated PGE_2 production by WISH cells is abolished by the specific COX-2 inhibitor NS-398. On the other hand, immunoblot analysis demonstrates that COX-1 is constitutively expressed and remains unchanged in WISH cells activated with PMA. Thus, this study supports a key role for COX-2 in PG biosynthesis during parturition, which is in accord with data by others in amnion cells under different experimental settings (4, 19–22). However, information is still scarce as to the signaling mechanisms involved in COX-2 protein up-regulation under stimulation conditions.

Recently, we unveiled a novel signaling mechanism operating in activated WISH cells (13). According to this mechanism, acute protein kinase Cα activation results in the sequential activation of phospholipase D, phosphatidate phosphohydrolase, and finally cytosolic Group IV PLA_2 (13). The message delivered by this pathway is the early (2 h) mobilization of free AA. As indicated above, free AA is not immediately used for PG synthesis, which suggests the possibility that at short times it may serve a signaling role. Thus we initially considered the possibility of whether this early burst of free AA was somehow involved in the delayed up-regulation of COX-2. Several lines of evidence suggest however that this may not be the case. Treatment of the cells with MAFP, which inhibits the cytosolic PLA_2 and hence AA mobilization (13), does not alter COX-2 message or protein levels. In addition, we have noted that incubating the cells with micromolar quantities of free AA does not increase COX-2 mRNA or protein levels (data not shown). Thus, if the early burst of free AA release produced in WISH cells actually serves a signaling role, it is unrelated to COX-2 up-regulation. In our AA release experiments, we use bovine serum albumin in the incubation medium to prevent cellular re-utilization of the fatty acid. Thus the bulk of the AA released

accumulates in the extracellular medium (13). However, in the absence of albumin, conditions under which prostaglandin production is measured, most of the liberated fatty acid is reacylated back into phospholipids, meaning that it would not be used for prostaglandin production. This suggests that delayed PG production is primarily determined by the expression levels of COX-2 and not by the increased availability of fatty acid precursor, which is the prevailing limiting step in the short term (minutes) PG production of most cell types (23).

Interestingly, the induction of both COX-2 protein and mRNA was strongly blunted by BEL. This compound has recently been shown to inhibit both the Group VI $iPLA_2$ and Mg^{2+}-dependent PAP-1 with similar potencies (16). MAFP, another compound that inhibits $iPLA_2$ (17), did not have any effect on COX, thus suggesting that PAP-1 is the enzyme whose blockage leads to inhibition of COX-2 expression. In accordance with this view, inhibition of PAP-1 by two other unrelated strategies, namely direct inhibition of the enzyme by propranolol and PAP substrate depletion by ethanol, gave the same inhibitory effect on COX-2 expression. Collectively, these results strongly implicate PAP-1 as an upstream component of the PMA-triggered sequence of events that culminate in COX-2 protein expression and hence, increased PGE_2 production. This is a very interesting concept because our previous studies (13) have unveiled the crucial role that PAP-1 plays in the short term PMA signaling that leads to cytosolic PLA_2 activation and attendant AA release. Thus the results of our studies suggest that PAP-1 is a central enzyme in protein kinase C-dependent AA metabolism in amnionic cells by regulating the two major enzymes involved in the response. On one hand, PAP-1 channels the short term signals originating from protein kinase C stimulation to cytosolic PLA_2 activation and AA mobilization (13). On the other hand, PAP-1 appears to be a key component of the sequence of mechanisms that trigger long term COX-2 induction and PGE_2 production (this study).

Unlike its Mg^{2+}-independent counterpart, PAP-2, PAP-1 had traditionally been thought to be primarily involved in the regulation of glycerolipid synthesis (24). In addition to that role, this study adds to the increasingly attractive notion (25) that PAP-1 does indeed serve a signaling role in cells. Ongoing studies in our laboratory are attempting to elucidate other components of the PAP-1-regulated pathway leading to activation of the COX-2 gene.

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