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Permalink
https://escholarship.org/uc/item/8k94t98b

Journal
The Journal of biological chemistry, 274(39)

ISSN
0021-9258

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Publication Date
1999-09-01

DOI
10.1074/jbc.274.39.27689

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

(Received for publication, March 11, 1999, and in revised form, June 16, 1999)

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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 E₂ (PGE₂) production during protein kinase C-dependent activation of human WISH cells. In these cells, PGE₂ 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 PGE₂, we detected significant elevations of both COX-2 protein and mRNA levels. Specific inhibition of COX-2 by NS-398 totally ablated PGE₂ 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 PGE₂ production. These data unveil a novel signaling mechanism for the regulation of COX-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.

Preamaturity 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), particularly PGE₂, 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 37 °C in a humidified atmosphere of 90% air and 10% CO₂. The cells were subcultured twice weekly by trypsinization and, when used for experiments, were seeded into 12-well plates (3.7 × 10⁵ cells/well, Corning Inc.) or 6-well plates (3.7 × 10⁶ 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.

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). 0.5 Ci/10⁶ cells in the culture medium 20 h before stimulation.

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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<sub>2</sub> 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<sub>2</sub> was quantitated in 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 [14C]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 [14C]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<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, and the indicated amount of homogenate protein. After the indicated time the reaction was stopped and [14C]PA and [14C]diacylglycerol were separated by thin layer chromatography using the system n-hexane/ethyl ether/acidic 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 isoenzymes 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 polyvinylidene fluoride (Millipore, Bedford, MA). The membranes were blocked for 1 h at room temperature with 2.5% casein in 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. The membranes were blocked for a total of 2 h at room temperature, increasing substantially afterward. The lack of a correlation between the time courses of AA release and PGE<sub>2</sub> production in PMA-stimulated WISH cells (e.g. Fig. 1 and Fig. 2) suggests that free AA availability is not rate-limiting for PGE<sub>2</sub> 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-stimulated PGE<sub>2</sub> 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<sub>2</sub> in response to PMA. Collectively these data suggest that the increased capacity of the WISH cells to produce PGE<sub>2</sub> 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. 5A. BEL is generally regarded as a Ca<sup>2+</sup>-independent phospholipase A<sub>2</sub> (iPLA<sub>2</sub>) 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. 5A are clearly not due to iPLA<sub>2</sub> inhibition, because MAFP, a dual cytosolic PLA<sub>2</sub>/iPLA<sub>2</sub> inhibitor (17) did not reproduce the effect. In agreement with the above data, BEL also inhibited the PMA-induced COX-2 mRNA levels (Fig. 5B). In perfect agreement with the data of Fig. 5, BEL strongly inhibited the PMA-stimulated PGE<sub>2</sub> production (Fig. 7).
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), PGE\(_2\) production (Fig. 7), and the induction of COX-2 protein (Fig. 8A) and mRNA (Fig. 8B).

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. 9A) and mRNA (Fig. 9B). 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...
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 PGE₂ production. Unexpectedly, our results reveal that PGE₂ is much more delayed in time. Appreciable PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂ by these cells. Moreover, PMA-stimulated PGE₂ 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 phosphohydrolyase, and finally cytosolic Group IV PLA₂ (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 PLA₂ 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.

Acknowledgments—We thank Dr. Timothy Hla of the Department of Physiology at the University of Connecticut for providing the cDNA fragment encoding human COX-2. We also thank Raymond Deems for helpful advice.

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J. Biol. Chem. 1999, 274:27689-27693.
doi: 10.1074/jbc.274.39.27689

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