Prostaglandins Enhance Epidermal Growth Factor-Induced DNA Synthesis in Hepatocytes by Stimulation of E Prostanoid 3 and F Prostanoid Receptors

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ABSTRACT
Prostaglandins stimulate hepatocyte proliferation in vivo and in vitro. We have examined the role of E prostanoid (EP) and F prostanoid receptors (FP) in enhancing the growth-stimulatory effect of epidermal growth factor (EGF) in cultured hepatocytes. The EP2 receptor agonist butaprost had no significant effect on DNA synthesis. EP1 receptor agonist butaprost had no significant effect on DNA synthesis. EP1 receptor-selective antagonists did not affect the enhancement by prostaglandin E2 of EGF-stimulated DNA synthesis. Sulprostone, misoprostol, and fluprostenol strongly enhanced DNA synthesis and inhibited glucagon-stimulated cAMP accumulation, indicating that they all activated EP3 receptors. Sulprostone and fluprostenol, and to a lesser extent misoprostol, stimulated accumulation of inositol phosphates. The effects of fluprostenol and sulprostone on phospholipase C (PLC) were inhibited by the FP receptor antagonist AL-8810, indicating that this effect was mediated by FP receptors. Inhibition of protein kinase C with GF109203X resulted in a partial reduction of the growth stimulation induced by fluprostenol, indicating a minor role of FP receptors. Combining fluprostenol with misoprostol, but not with sulprostone, resulted in partially additive effects on DNA synthesis, suggesting that both EP3 and FP receptors are involved. Combining sulprostone with misoprostol did not result in additive effects on DNA synthesis, suggesting that EP4 receptors were not involved. We conclude that, although a minor effect is exerted by EP receptors, the growth-stimulatory effects of prostaglandins in rat hepatocytes are mediated mainly by EP3 receptors. We have found no evidence of EP1 receptor involvement.

Prostaglandins stimulate proliferation in a number of different cell types (Watanabe et al., 1994; Sheng et al., 2001; Yau and Zahradka, 2003; Chang et al., 2005; Krysan et al., 2005), including neonatal and adult rat hepatocytes (Andreis et al., 1981; Skouteris et al., 1988; Refsnes et al., 1994; Hashimoto et al., 1997; Kimura et al., 2001). In primary cultures of rat hepatocytes, prostaglandins exert a small stimulatory effect on DNA synthesis on their own compared with the strong stimulation of DNA synthesis induced by mitogens, such as epidermal growth factor (EGF), and they act mainly by enhancing the growth stimulatory effect of mitogens (Christoffersen et al., 2000). Therefore, they belong to the group of substances that are termed comitogens (Michalopoulos, 1990). The serum concentration of prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α) following partial hepatectomy is transiently increased, and several studies indicate that prostaglandin signaling is required during liver regeneration (Little et al., 1988; Casado et al., 2001; Rudnick et al., 2001).

Prostaglandins exert their effect by binding to G protein-coupled receptors (Narumiya et al., 1999; Breyer et al., 2001). PGE2, PGF2α, prostaglandin D2, prostacyclin, and thromboxane A2 bind to EP, FP, D prostanoid receptor, prostacyclin

ABBREVIATIONS: EGF, epidermal growth factor; PGE2, prostaglandin E2; PGF2α, prostaglandin F2α; EP, E prostanoid receptor; FP, F prostanoid receptor; TP, thromboxane receptor; PLC, phospholipase C; GF109203X, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-maleimide; C25H24N10O2; IBMX, 3-isobutyl-1-methylxanthine; AH6809, 6-isopropoxy-9-oxoanthere-2-carboxylic acid, C16H12O4; SC-51089, 8-chloro-15-(2,3-dihydro-1H-inden-2-yl)-16,17,18,19,20-pentanor-prosta-5Z,13E-dien-1-oic acid; DAG, diacylglycerol; PKC, protein kinase C; ANOVA, analysis of variance.
Although our results did not provide evidence for a role of prostaglandins, but phospholipase C activation appeared to at least partly mediate the effect (Refsnes et al., 1995). We previously found that, in hepatocytes, adenylyl cyclase receptors in rat liver (Boie et al., 1997; Fennekohl et al., 2000). Several studies have examined the mRNA expression of prostanoid receptors that stimulate phospholipase C. EP1 receptors elicit the stimulatory effects of prostaglandins on EGF-induced DNA growth enhancement, using receptor-selective agonists and antagonists. The results show that, although FP receptors mediate most of the stimulatory effects of prostaglandins on EGF-induced DNA synthesis in rat hepatocytes.

Materials and Methods

Materials. Dulbecco’s modified Eagle’s medium, HEPES, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA). William’s medium E was from Cambrex Bio Science Walkersville Inc. (Walkersville, MD). Prostaglandin E2, prostaglandin F2 alpha, butaprost, (+)-fluprostanol, sulprostone, iso-prostanoid carboxylic acid, free acid (free acid), AH6809 (6-isopropylox-9-oxoxygenide-2-carboxylic acid), and AL-8810 (9α,15β-dihydroxy-11β-fluoro-2-[2,3-dihy-dro-1H-inden-2-yl]-7,16,17,18,19,20-pentanor-prosta-5Z,13E-dien-1-ol acid) were obtained from Cayman Chemical (Ann Arbor, MI). Norepinephrine hydrochloride, timolol maleate salt, GF109203X [2-[(1-(3-dimethylaminopropyl)-1H-indol-3-yl)-male-imide]-collagenase (CO130), dexamethasone, EGF (from mouse), insulin, 3-isobutyl-1-methylxanthine (IBMX), and collagen (type I from rat tail) were obtained from Sigma Chemical Co. (St. Louis, MO). SC-51089 [8-chlorodibenzo[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-[1-oxo-3-(4-pyridinyl)propyl]hydrazide hydrochloride] and SC-51322 [8-chlorodibenzo[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-[3-fluoranyl(methyl)thio]-1-oxopropyl]hydrazide were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). [6-3H]Thymidine (20–30 Ci/mmol) and myo-[2-3H]inositol (15.0 Ci/mmol) were from GE Healthcare (Buckinghamshire, UK). Antibodies against EP1, EP2, EP3, and EP4 (C-terminal) receptors were obtained from Cayman Chemical; actin antibody (I-19) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals were of analytical quality.

Cell Isolation and Culture. Male Wistar rats (170–230 g) were fed ad libitum. Hepatocytes were isolated by collagenase perfusion as described previously (Refsnes et al., 1994). The hepatocytes were seeded onto Costar plastic culture wells (Corning Life Sciences (Acton, MA) at a density of 2 × 10^5/cm². The culture medium was a serum-free 1:1 combination of William’s medium E and Dulbecco’s modified Eagle’s medium (with final glucose concentration of 8.4 mM). The medium was supplemented with penicillin (67 μg/ml), streptomycin (100 μg/ml), collagen (3 μg/ml), insulin (100 nM), and dexamethasone (25 nM). The cultures were kept in 95% air/5% CO2 at 37°C.

Measurement of DNA Synthesis. Prostaglandins and prostaglandin analogs were added 3 h after plating unless otherwise indicated. GF109203X was added 2.5, 7, and 11 h after the cells were seeded in the indicated experiments. When cells were stimulated with norepinephrine, timolol (10 μM) was present for the last 15 min of the preincubation period to prevent binding to β-adrenergic receptors. EGF (5 nM) and [6-3H]thymidine were added at 24 h. The cells were harvested after 50 to 54 h, and DNA synthesis was measured as the amount of radioactivity incorporated into DNA.

Immunoblotting. Hepatocytes were harvested after 3 or 24 h of culturing. Aliquots with 12.5 μg of cell protein (total cell lysate prepared in Laemmli buffer) were electrophoresed on 12% (w/v) polyacrylamide gels (acrylamide/N,N’-bis-methylene acrylamide 30:0.08) followed by immunoblotting with a polyclonal antibody. Immunoreactive bands were visualized with ECL Western blotting detection reagents (GE Healthcare).

Inositol Phosphate Accumulation. Hepatocytes were cultured for 3 h as described above, with 5 μCi/ml myo-[2-3H]inositol added at the time of plating. Thirty minutes after agonist stimulation, medium was removed and replaced with Krebs-Ringer-HEPES buffer, pH 7.4, containing 10 mM glucose and 15 mM LiCl. Hepatocytes were stimulated with agonists as indicated, and the reaction was stopped by removing buffer (or medium) and adding 1 ml of ice-cold 0.4 M perchloric acid. Samples were harvested and neutralized with 4 M KOH, 1 M Tris, and 60 mM HEPES in the presence of a Universal indicator (Merck, Darmstadt, Germany). The neutralized supernatants were placed on columns containing 1 ml of Dowex AG 1-X8 resin (Sigma Chemical). The columns were washed with 20 ml of distilled water and 10 ml of 5 mM sodium tetraborate/60 mM ammonium formate, and inositol phosphates were eluted with 10 ml 1 M ammonium formate/0.1 M formic acid.

cAMP Accumulation. The cells were kept in culture for 3 or 20 h before stimulation. We found the results to be identical irrespective of time in culture, thus performing our experiments at 20 h for practical reasons. After 20 h of culturing, medium was replaced with Krebs-Ringer-HEPES buffer, pH 7.4, with glucose (10 mM), and cells were preincubated for 30 min. The cells were then incubated with 1 μM glucagon in the presence of 0.5 mM IBMX, in the absence or presence of increasing concentrations of prostaglandins for 3 min. The reaction was stopped by removing the incubation buffer and adding 5% trichloroacetic acid. The content was then scraped off with a rubber policeman, and the precipitate was spun down. After neutralization with CaCO3, cAMP in the supernatant was determined by radioimmunoadassay as described previously (Refsnes et al., 1995).

Other Methods. Protein was measured with the Coomassie Plus Protein Assay (Pierce, Rockford, IL) according to the manufacturer’s protocol.
Results

EP Receptor Expression in Hepatocytes. Prostanoid receptor expression in hepatocytes has previously been examined at the mRNA level (Boie et al., 1997; Fennekohl et al., 1999; Pérez et al., 2004). We examined EP receptor expression by Western blots and found that EP1, EP2, and EP3 receptors were expressed in our hepatocyte preparations. Furthermore, expression was similar in cells cultured for 3 or 24 h (Fig. 1). The molecular sizes of the different receptors (65, 52, and 53 kDa for the EP1, EP2, and EP3 receptors, respectively) were in agreement with published data (Southall and Vasko, 2001). We also detected a very faint band corresponding to the molecular mass of EP4 receptors (65 kDa; data not shown). Lysates from colorectal cancer cell lines were used as positive controls to verify the presence of EP2 and EP4 receptors (data not shown). An additional protein band at 51 kDa was detected with the anti-EP3 receptor antibody.

To characterize the prostanoid receptors involved in mediating the enhancement of DNA synthesis induced by EGF, we used receptor-selective agonists and antagonists and also examined agonist effects on signaling events immediately downstream of receptor activation to delineate their interaction with different prostanoid receptors.

Evidence against Involvement of EP1 and EP2 Receptors in Enhancement of EGF-Induced DNA Synthesis. As shown in Fig. 2A, the EP2-specific agonist butaprost (Boie et al., 1997; Kiriyama et al., 1997; Abramovitz et al., 2000) did not significantly affect DNA synthesis induced by EGF. The lack of effect of butaprost is in agreement with previous results published from our laboratory, suggesting that cAMP-mediated signaling is not involved in the enhancement of DNA synthesis (Boie et al., 1999; Peiréz et al., 2004). We examined EP receptor expression in hepatocytes, these results support the involvement of EP3 receptors in the growth enhancement (Fig. 3).

Role of EP3 Receptors in Enhancement of EGF-Induced DNA Synthesis. We and others have found that PGE2 depresses hormone-induced cAMP accumulation in hepatocytes through a pertussis toxin-sensitive step, presumably by a Gt-dependent inhibition of adenylyl cyclase (Bronstad and Christoffersen, 1981; Melien et al., 1988; Garatty et al., 1989). Figure 4A shows that sulprostone and misoprostol, which act as agonists on EP3 receptors (Boie et al., 1997; Kiriyama et al., 1997; Abramovitz et al., 2000), were equipotent (EC50, ~0.5 μM) and equally effective in inhibiting cAMP accumulation in response to glucagon. We also found that fluprostenol, like PGF2α, was able to inhibit glucagon-stimulated cAMP accumulation (Fig. 4B). Fluprostenol and PGF2α were approximately equipotent in inhibiting cAMP accumulation, with an IC50 of ~5 μM, but fluprostenol was less efficient, suggesting that it was acting as a partial agonist on EP3 receptors.

As shown in Fig. 2A, both sulprostone and misoprostol enhanced EGF-induced DNA synthesis approximately 3-fold. Fluprostenol stimulated DNA synthesis to a similar extent (Fig. 2B). Because all three agonists activated EP3 receptors in the hepatocytes, these results support the involvement of EP3 receptors in the enhancement of DNA synthesis. It is likely that misoprostol, which is an agonist on EP2 and EP4 receptors, as well as EP3 receptors (Boie et al., 1997; Kiriyama et al., 1997; Abramovitz et al., 2000), exerts its effect on DNA synthesis through EP3 receptors, since we have found no role of cAMP in the growth enhancement by prostanoids (Fig. 2A). However, we can not exclude a role of EP4 receptors, which may stimulate growth through cAMP-independent mechanisms (Kataoka et al., 2005; Fulton et al., 2006). Sulprostone, which is an agonist on EP1 and FP receptors, in addition to EP3 receptors (Kiriyama et al., 1997; Abramovitz et al., 2000), was more potent than misoprostol and also appeared to be more efficacious, although plateau effects were not reached, even when the agonists were present at the highest concentrations (100 μM). The higher potency of sulprostone compared with misoprostol may be due to differences in the rate of breakdown or stimulation of other prostanoid receptors, presumably FP receptors, since we have found no role for involvement of EP1 receptors in the growth enhancement (Fig. 3).

Role of FP Receptors in Enhancement of EGF-Induced DNA Synthesis. To examine activation of FP receptors, we determined phospholipase C activity in response to the different agonists. Sulprostone moderately stimulated

Statistics. Data were analyzed by t test or one-way ANOVA followed by Bonferroni’s post-test for comparison between groups using Prism version 4.00 for Windows (GraphPad Software, Inc., San Diego, CA). The criterion of significance was p < 0.05. All values in text and figures are means ± S.E.M.
the accumulation of inositol phosphates, with an EC$_{50}$ of 7.5 μM, whereas misoprostol only slightly elevated the level of inositol phosphates (Fig. 5A). Both PGF$_{2\alpha}$ and fluprostenol stimulated an increase in the accumulation of inositol phosphates, compatible with activation of FP receptors (Fig. 5B). The EC$_{50}$ of fluprostenol was 50 nM and that of PGF$_{2\alpha}$ was 1 μM. Thus, fluprostenol was approximately 100 times more potent in its stimulation of FP receptors than EP3 receptors, suggesting that its effects on DNA synthesis might be mediated mainly through activation of FP receptors. Pretreatment of the hepatocytes with the FP antagonist AL-8810 (Griffin et al., 1999) displaced the dose-response curve for fluprostenol to the right (Fig. 5C) and completely suppressed sulprostone-induced inositol phosphate accumulation (Fig. 5D), indicating that activation of phospholipase C was mediated through activation of FP receptors. Sulprostone was approximately 10-fold more potent on EP3 receptors than on FP receptors.

We previously found that a number of agonists acting on G$_q$-coupled receptors in hepatocytes exert their comitogenic effects, at least partly through sustained accumulation of diacylglycerol (DAG) and activation of protein kinase C (PKC) (Dajani et al., 1999). Having found that fluprostenol stimulated phospholipase C, we examined the role of PKC in the enhancement of EGF-induced DNA synthesis by fluprostenol. In these experiments, we used norepinephrine as a positive control since we have previously shown that its co-mitogenic effect is strongly dependent on PKC (Dajani et al., 1999). As shown in Fig. 6A, the effect of inhibiting PKC by
Additive Effects of EP3 and FP Receptor Stimulation on EGF-Induced DNA Synthesis. To further examine whether FP receptor activation contributed to the effect of fluprostanol on DNA synthesis, we combined fluprostanol in increasing concentrations with 100 μM misoprostol. As shown in Fig. 7A, combining the two agonists induced partially additive effects on DNA synthesis, particularly at the lower concentrations of fluprostanol, suggesting that misoprostol and fluprostanol are mediating their enhancement of DNA synthesis by activating partly different receptors, thus implying a role for both EP3 and FP receptors. In contrast, there was no additivity between fluprostanol and sulprostone (Fig. 7B), in agreement with the findings that sulprostone and fluprostanol are mediating their enhancement of DNA synthesis by activating partly different receptors, thus implying a role for both EP3 and FP receptors. Finally, fluprostanol-stimulated DNA synthesis was only slightly affected by PKC inhibition.

In agreement with a moderate prostaglandin-induced cAMP accumulation in cultured hepatocytes pretreated with pertussis toxin to inactivate G, (Melien et al., 1988; Fennekohl et al., 2000), we found that the hepatocytes expressed EP2 receptors, but we found no evidence of EP2 receptor involvement in the growth stimulatory effects of prostaglandins using the EP2 receptor-specific agonist butaprost. This is in agreement with our previous observation that cAMP does not mediate the proliferative effects of prostaglandins in the hepatocytes (Refsnes et al., 1995). Furthermore, although EP4 receptors have been found to stimulate cyclin D1 expression and phosphorylation of EGF receptor and extracellular signal-regulated kinase in murine hepatocytes (Kataoka et al., 2005), prostaglandins do not transactivate the EGF receptor in rat hepatocytes (Nilssen et al., 2004) but act to enhance subsequent stimulation of extracellular signal-regulated kinase and Akt by EGF (Dajani et al., 2007). In addition, we found no additive effects between sulprostone and misoprostol on DNA synthesis, suggesting lack of involvement of EP4 receptors.

Elucidation of the contribution from different prostaglandin receptors is hampered by the lack of commercially avail-
able receptor-specific ligands. Misoprostol is an agonist on EP2, EP3, and EP4 receptors but has been found to have a greater affinity for EP3 receptors in all of the species examined (Boie et al., 1997; Kiriyama et al., 1997; Abramovitz et al., 2000). Because we found no evidence for a role of EP2 or EP4 receptors, misoprostol is likely to exert its effects on DNA synthesis through activation of EP3 receptors. This was supported by the demonstration that misoprostol inhibited glucagon-stimulated cAMP accumulation. The EP3 receptor exists in several isoforms of which both the EP3α and EP3β have been identified in rat hepatocytes and both appear to couple primarily to G1 (Neuschafer-Rube et al., 1994; Fennekohl et al., 1999). We also observed that misoprostol slightly stimulated PLC activity in the hepatocytes. The cloned EP3 receptors from several species have been found to activate PLC; this may occur through either G1- or Gq-coupling (Narumiya et al., 1999, 2003). Sulprostone stimulated inositol phosphate accumulation in the hepatocytes, but this effect was mediated by FP receptors because it was completely blocked by the FP receptor antagonist AL-8810. Although sulprostone was reported to be a partial agonist on cloned rat EP1 receptors, it exerted 72% of the maximal response induced by PGE2 (Boie et al., 1997). Thus, the lack of effect of sulprostone through EP1 receptors on PLC in the hepatocytes may either be due to poor coupling of EP1 receptors to PLC (Katoh et al., 1995) or low expression of EP1 receptors in the hepatocytes. The lack of effect of three different EP1 receptor-selective antagonists on PGE2-stimulated DNA synthesis further supports the lack of involvement of EP1 receptors in stimulation of hepatocyte growth. AH6809 and SC-51089 were used at concentrations approximately 10 times their Kd values determined in binding studies (Boie et al., 1997; Abramovitz et al., 2000), and SC-51322 was used at a concentration approximately 70 times its Kd (Abramovitz et al., 2000), without causing any shift in the dose-response curve of PGE2-induced growth stimulation. SC-51332 is also an antagonist at EP3 receptors (Abramovitz et al., 2000), and at the concentration used here (5 μM), it slightly right-shifted the dose-response curve of misoprostol in the inhibition of glucagon-stimulated cAMP accumulation (data not shown).

Of the agonists used in the present study, fluprostenol is generally believed to be the most selective receptor agonist, acting preferentially on FP receptors (Kiriyama et al., 1997; Sharif et al., 2003). However, in agreement with previous binding studies (Abramovitz et al., 2000; Sharif et al., 2003), we found that fluprostenol also activated EP3 receptors shown by inhibition of glucagon-stimulated cAMP accumulation, but with a 100-fold lower affinity than its affinity toward FP receptors. In addition, the results suggested that fluprostenol is a partial agonist on EP3 receptors in the hepatocytes, as demonstrated by a lower efficacy compared with PGF2α. This may account for the partial additivity between fluprostenol and misoprostol on DNA synthesis, particularly at lower concentrations of fluprostenol, where it would interact mainly with FP receptors.

FP receptors are Gq-coupled receptors that activate PLC. We previously found that the comitogenic effects of vasopressin, angiotensin II, and norepinephrine, acting on Gq-coupled receptors, are mediated, at least partly, through sustained accumulation of DAG and activation of PKC (Dajani et al., 1999). Furthermore, we have shown that PGF2α, like vasopressin, angiotensin II, and norepinephrine, induces sustained DAG accumulation, but to a lesser extent than the other agonists (Nilssen et al., 2005). However, repeated addition of the PKC inhibitor GF109203X affected fluprostenol-stimulated DNA synthesis to a minor extent, compared with norepinephrine-stimulated DNA synthesis, suggesting that the Gq-mediated pathway was more important. It is possible that the increase in DAG induced by activation of FP receptors is insufficient to result in significant PKC activation. Taken together, our results suggest that, although a minor effect is exerted by FP receptors, EP3 receptors mediate most of the growth stimulatory effects of prostaglandins in hepatocytes. Thus, we have confirmed the involvement of EP3 receptors as described by Hashimoto et al. (1997); however, we could not detect a significant contribution from EP1 receptors (Kimura et al., 2001).

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References


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