Advances in Brief

Prostaglandin E₂ Stimulates the Growth of Colon Cancer Cells via Induction of Amphiregulin

Jinyi Shao, Sean B. Lee, Huiping Guo, B. Mark Evers, and Hongmiao Sheng

Department of Surgery and Sealy Center of Cancer Cell Biology, University of Texas Medical Branch, Galveston, Texas 77555 [J. S., H. G., M. B. E., H. S.] and National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892 [S. B. L.]

Abstract

Prostaglandin E₂ (PGE₂), a major product of cyclooxygenase enzymes, is implicated in colorectal carcinogenesis and has been shown to stimulate the growth of human colorectal carcinoma cells. Here, we show that PGE₂ activated the cyclic AMP/protein kinase A pathway, which induced the expression of amphiregulin (AR), an epidermal growth factor family member, through activation of a cyclic AMP-responsive element in the AR promoter. AR exerted a mitogenic effect on LS-174 cells and partially mediated the PGE₂-induced growth stimulation. In addition, PGE₂, in collaboration with transforming growth factor-α or K-Ras oncogene, synergistically induced AR expression and activated receptor tyrosine kinase-dependent signaling pathways. Our results provide novel mechanisms for cyclooxygenase-2 pro-oncogenic activity and suggest that PGE₂ may act with major oncogenic pathways in a synergistic fashion to activate the epidermal growth factor receptor signaling system through a ligand-dependent autocrine pathway.

Introduction

It is now clear that COX³-2 plays a role in the promotion of colorectal cancer (1). However, the effects of PGs generated by COX-2 have largely been unexplored. PGE₂, a major product of COX enzymes, acts via specific transmembrane GPCRs (2). Four PGE receptor (EP) subtypes have been identified. EP₁ receptor signals via generation of inositol 1,4,5-triphosphate and increased intracellular Ca²⁺. EP₂ and EP₃ receptors are coupled to Gs proteins and signal through increased cAMP, whereas the EP₄ receptor is coupled to inhibitory G proteins, which inhibit the generation of cAMP. It has been known for quite some time that short-term administration of PGE₂ causes significant stimulation of DNA synthesis; prolonged PGE₂ treatment markedly increases the weight, DNA, and RNA content of intestinal mucosa in rats (3). PGE₂ stimulates proliferation and growth of human colorectal cancer cells (4–6). These in vivo and in vitro findings suggest that COX-2-generated PGE₂ may provide a growth advantage to colorectal carcinomas.

The EGFR family and their cognate receptors (EGFRs), now referred to as the ErbB family, play critical roles in intestinal epithelial growth and transformation (7). Binding of the ligand to the EGFR leads to activation of RTKs that phosphorylate tyrosine residues of cellular signaling proteins and activate signaling pathways that are essential for intestinal epithelial proliferation (8). EGFR cross-communicates with GPCRs and can be transactivated by GPCR agonists (9). In a recent study, Pai et al. (4) reported that PGE₂ transactivates EGFR, triggers extracellular signaling-regulated kinase activation, and stimulates the proliferation of colorectal carcinoma cells. These studies demonstrate clearly that PGE₂ growth stimulation in colon cancer cells involves activation of the EGFR signaling system. We have reported previously that treatment with PGE₂ increases PI3K/Akt activity that is critical for the transformation of intestinal epithelial cells and that is required for the growth stimulation of PGE₂ in human colon cancer LS-174 cells (6). To better understand the mechanism(s) by which PGE₂ activates RTK-dependent signaling pathways and stimulates colon cancer cell growth, we investigated the regulation and functional role of a PGE₂ target gene, AR, in PGE₂ trophic activity. PGE₂ signaled through Gs-coupled receptors and activated PKA which, in turn, induced the expression of AR, a member of the EGF family, through transcriptional activation of a CRE in the AR promoter. AR exerted a mitogenic effect on LS-174 cells and mediated the trophic effect of PGE₂ in human colon cancer cells. Moreover, PGE₂, in cooperation with major oncogenic pathways, synergistically induced AR transcription. These results provide additional mechanisms mediating COX-2/PGE₂ pro-oncogenic actions in colorectal carcinogenesis.

Materials and Methods

Cell Culture. LS-174 cells were purchased from American Type Culture Collection (Manassas, VA) and were maintained in McCoy’s 5A medium containing 10% fetal bovine serum. The growth of cells in Matrigel (Collaborative Biomedical, Bedford, MA) was carried out as described previously (6). PGE₂ was purchased from Cayman Chemical (Ann Arbor, MI). H-89, LY-294002, and PD-153035 were purchased from Calbiochem (San Diego, CA). Dibutyryl cAMP was purchased from Sigma (St. Louis, MO). Amphiregulin, goat normal IgG, and anti-AR antibody were purchased from Research and Development Systems (Minneapolis, MN).

PKA Assay. cAMP-dependent PKA activity was measured by determining the transfer of the phosphate group of ATP to a synthetic peptide, which is a substrate for PKA (Calbiochem). The experiment was carried out according to the manufacturer’s instructions.

GeneChip Hybridization. This experiment was performed in the University of Iowa DNA Facility (10). Briefly, cRNA preparations from PGE₂ or vehicle-treated LS-174 cells were used to incorporate human GeneChip (U95A) expression arrays (Affymetrix, Inc.) based on a recommended protocol. Three replicate hybridizations were performed using PGE₂ or vehicle-treated RNA samples. Alterations in RNA transcript levels were analyzed using Affymetrix Analysis Suit 4.0 software. The fold change in expression between groups was calculated from the mean average difference scores.

RNA Extraction and Northern Blot Analysis. The extraction of total cellular RNA was carried out as described previously (11). RNA samples (20 μg/lane) were separated on formaldehyde-agarose gels and blotted onto nitrocellulose membranes. The blots were hybridized with cDNA probes labeled with [γ-³²P]dCTP by random primer extension (Stratagene, La Jolla, CA). After hybridization and washes, the blots were subjected to autoradiography.

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2 To whom requests for reprints should be addressed, at Department of Surgery, The University of Texas Medical Branch, Galveston, TX 77555. Phone: (409) 772-6661; E-mail: hosheng@utmb.edu.

3 The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; GPCR, G protein-coupled receptor; GS, stimulatory G; cAMP, cyclic AMP; EGFR, epidermal growth factor; EGF, EGF receptor; RTK, receptor tyrosine kinase; PKA, protein kinase A; CRE, cAMP-responsive element; CREB, CRE binding; RT-PCR, reverse transcription-PCR; TGF, transforming growth factor.
Transient Transfection and Luciferase Assay. The assays to determine the activity of the AR promoter were described previously (11). Reporter constructs pGL2-A, pGL2-B, pGL2-BΔCRE, pGL2-C, and pGL2-CΔCRE containing the 5′-flanking region of the human AR gene were described previously (12). For transient transfections, cells were cotransfected with 0.5 μg of one of the AR firefly luciferase plasmid constructs and 3 ng of the pRL-SV40 plasmid, containing the Renilla luciferase gene (Promega Corp., Madison, WI), using the FuGENE 6 procedure (Roche, Indianapolis, IN) as described in the manufacturer’s protocol. Transfected cells were lysed at the indicated times for luciferase assay. Firefly and Renilla luciferase activities were measured using a Dual-Luciferase Reporter assay system (Promega) and a luminometer. Firefly luciferase values were standardized to Renilla values.

Immunoblot Analysis. Immunoblot analysis was performed as described previously (11). The anti-phosphorylated Akt antibody and anti-pCREB antibody were purchased from Cell Signaling (Beverly, MA).

Real-Time RT-PCR. AR expression was quantified using real-time quantitative PCR or TaqMan technique (Applied Biosystems, Foster City, CA). The sequence of the primer/probe set was based on AR mRNA sequence (GenBank NM_001657) and includes: probe, AGTCCAGCTTAGAAGAC; forward primer, GCCTTTAGTCGCTTGATCTC; and reverse primer, CCTCACCTTCTCCTCATATTTCTC. 18S RNA TaqMan assay reagent was used for internal control. One-step RT-PCR was performed with 40 ng of RNA for both target gene and endogenous controls. Duplicate Ct values were analyzed in Microsoft Excel using the comparative Ct ($\Delta\Delta$Ct) method as described by the manufacturer (Applied Biosystems). The amount of target (2$^{-\Delta\Delta$Ct}) was obtained as normalized to 18S and relative to a calibrator.

Data Analysis. All statistical analyses were performed on a personal computer with the StatView 5.0.1 software (SAS Institute, Inc., Cary, NC). Analyses between multiple groups were determined by ANOVA. Analyses between two groups were determined using the unpaired Student t test. Differences of $P < 0.05$ were considered statistically significant.

RESULTS

PGE2 Stimulated LS-174 Cell Growth through the cAMP/PKA Pathway. PGE2 may signal through four EP receptor subtypes, which act through different signaling pathways (2). By using single-cell [Ca$^{2+}$]$_i$ imaging, we found that PGE2 treatment did not alter intracellular Ca$^{2+}$ levels in LS-174 cells (data not shown). In contrast, PGE2 activated the cAMP/PKA signaling pathway (Fig. 1A). Stimulation with PGE2 rapidly increased PKA activity by ~7-fold in LS-174 cells. A selective PKA inhibitor, H-89, at 10 μM completely blocked the PGE2-induced PKA activity. Similar results were observed in LS-174 cells that were treated with dibutyryl cAMP (0.1 mM), suggesting that PGE2 signaled through Gs protein-coupled receptors and increased the levels of cAMP in LS-174 cells. In agreement with previous studies (13) that inhibition of PKA activity with antisense oligodeoxynucleotide impairs the growth of LS-174 cells, 10 μM H-89 significantly inhibited LS-174 cell growth in Matrigel (Fig. 1B). LS-174 cells formed colonies in Matrigel, and PGE2 treatment increased the volume of colonies ~4-fold. Addition of H-89 blocked the PGE2 stimulation of LS-174 cell growth in Matrigel. Thus, PKA activation appeared to be critical for PGE2 stimulation of LS-174 cell growth.

PGE2-induced AR Expression. To search for target genes that may mediate PGE2 growth-stimulatory activity in colon cancer cells, LS-174 cells were treated with PGE2 or vehicle for 2 h; cRNA preparations were hybridized to Affymetrix GeneChips. The expression of 14 genes, which included transcription factors, enzymes, immune-related genes, and growth factors, was increased 3–10.5-fold in response to PGE2 stimulation. Among these genes, AR, a member of the EGF family, was induced 9-fold after PGE2 treatment. Because AR stimulates the growth of colon cancer cells (14), we decided to further investigate roles of AR in PGE2 growth-stimulatory action. To confirm the findings generated from GeneChip assays, Northern analysis was conducted; levels of AR mRNA were increased 3–4-fold in LS-174 cells after treatment with PGE2 for 2 h and returned to basal levels by 8 h (Fig. 2A).

To determine the mechanism by which PGE2 induced AR expression, we investigated the role of PGE2 signaling in AR transcription. The nucleotide sequence of a 763-bp AR 5′-flanking region has been cloned and analyzed (12, 15). When LS-174 cells were transiently transfected with the reporter vector pGL2-A, which contains the AR promoter sequence from −850 to −87, PGE2 treatment rapidly increased the transcriptional activity of AR and resulted in an ~7-fold increase in luciferase activity (Fig. 2B). PKA activation was critical for the PGE2-stimulated AR transcription, which was completely blocked by the presence of H-89. These results suggest that PGE2-induced AR transcription was mediated by the PKA signaling pathway.

Roles of the CRE in PGE2 Activation of the AR Promoter. cAMP stimulates the expression of target genes through a conserved CRE. A CRE site has been identified previously in the AR promoter (15). The 155-bp sequence immediately upstream of the 5′ end of the mRNA start site includes a consensus TATA box (−238 to −233) and a CRE (−274 to −267). To elucidate the role of the CRE site in PGE2-induced AR transcription, LS-174 cells were transfected with pGL2-B, which contains 136 nucleotides (−328 to −192) including the Wilms’ tumor suppressor WT1 responsive element, the CRE, and the TATA box. Luciferase activity was increased (~5-fold) by PGE2 stimulation compared with vehicle-treated cells (Fig. 3A). Mutation of the CRE site from GAGCTCA to GAGCTAC (pGL2-BΔCRE) resulted in a significant reduction of AR promoter activity and almost completely attenuated PGE2-induced AR transcription. Using pGL2-C and pGL2-CΔCRE report vectors, which contain only 83 nucleotides

![Image](cancerres.aacrjournals.org)
To determine the role of AR in LS-174 cell growth. Treatment with AR and firefly luciferase values were standardized to Renilla values. Completely blocked by an EGFR tyrosine kinase inhibitor, PD-153035 increase in cell number. The growth-stimulatory effect of AR was assays performed in quadruplicate, based on three independent experiments; Renilla-adjusted luciferase values standardized to the controls. Plotted is the mean of /H11021 P

PGE2 treatment significantly increased the phosphorylation of CREB Ser-133 by the PKA catalytic subunit and interacts with the CRE. transcription factors of the CREB protein, which is phosphorylated at 3 protein at Ser-133 in LS-174 cells (Fig. 3

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A, middle panel). AR also increased the colony size of LS-174 cells that was partially blocked by an anti-AR neutralizing antibody before PGE2 treatment completely blocked PGE2-induced DNA synthesis (Fig. 4C, middle panel). PGE2 significantly increased the colony volume of LS-174 cells when grown in Matrigel; again, EGFR activity was critical for the growth-stimulatory effect of PGE2 (Fig. 4C, right panel). These results demonstrate clearly that PGE2 growth-stimulatory effects in LS-174 cells are mediated by the EGFR signaling system, which was likely activated by AR.

Synergy between PGE2 and Major Oncogenic Pathways. Malignant transformation of cells is an extremely complex process and involves a number of oncogenic signaling pathways, where cross-communication often occurs. TGF-α plays critical roles in colorectal carcinogenesis and often acts through an autocrine loop (16). Because both PGE2 and TGF-α may exert tumor-promoting effects to colon cancer cells, we sought to determine whether PGE2 and TGF-α act in a cooperative manner. As demonstrated in Fig. 5A, both PGE2 and TGF-α induced the levels of pAkt and pCREB; however, combined treatment with PGE2 and TGF-α resulted in a marked induction of PI3K activity and CREB activation. Next, we investigated whether PGE2 and TGF-α cooperatively induced AR transcription. LS-174 cells were transfected with pGL2-A and then treated with TGF-α or PGE2 plus TGF-α. TGF-α increased only luciferase activity ~2-fold; PGE2 increased AR promoter activity ~7-fold. However, the combination of PGE2 and TGF-α increased luciferase activity ~17-fold, suggesting that PGE2 and TGF-α synergistically activated the AR promoter. To demonstrate that the synergistic transcription of AR resulted from PGE2 and TGF-α treatment produced transcripts, we used real-time PCR to determine the expression of AR mRNA. PGE2 and TGF-α synergistically increased the levels of AR mRNA (Fig. 5C). Interestingly, a stronger synergy was observed at 24 h after PGE2

\((-275 \text{ to } -192\)) including the CRE and the TATA box, we confirmed the critical role of the CRE in PGE2-induced AR transcription (Fig. 3B). Transcriptional activation of the CRE requires activation transcription factors of the CREB protein, which is phosphorylated by the PKA catalytic subunit and interacts with the CRE. PGE2 treatment significantly increased the phosphorylation of CREB protein at Ser-133 in LS-174 cells (Fig. 3C).

AR Mediated the Trophic Action of PGE2. It was critical for us to determine the role of AR in LS-174 cell growth. Treatment with AR increased the growth rate of LS-174 cells and resulted in a ~2-fold increase in cell number. The growth-stimulatory effect of AR was completely blocked by an EGFR tyrosine kinase inhibitor, PD-153035 (1 μM; Fig. 4A, left panel). AR stimulated DNA synthesis in LS-174 cells that was partially blocked by an anti-AR neutralizing antibody (Fig. 4A, middle panel). AR also increased the colony size of LS-174 cells when grown in Matrigel that was completely attenuated by the addition of 1 μM PD-153035 (Fig. 4A, left panel). These results indicate that AR is a mitogenic growth factor for LS-174 cells.

Next, we investigated the functional involvement of AR in PGE2-induced growth stimulation. Both AR and PGE2 treatment increased PI3K activity, as noted by increased levels of pAkt that is critical for PGE2 stimulation of LS-174 cell growth (6). Inhibition of EGFR activity by PD-153035 (1 μM) blocked PGE2-induced pAkt (Fig. 4B), suggesting that PGE2 induction of PI3K activity required activation of EGFR. PGE2 treatment promoted the growth of LS-174 cells; this effect was blocked by inhibition of RTK activity (Fig. 4C, left panel). PGE2 modestly increased DNA synthesis in LS-174 cells; addition of an anti-AR neutralizing antibody before PGE2 treatment completely attenuated the PGE2-induced DNA synthesis (Fig. 4C, middle panel). PGE2 significantly increased the colony volume of LS-174 cells when grown in Matrigel; again, EGFR activity was critical for the growth-stimulatory effect of PGE2 (Fig. 4C, right panel). These results demonstrate clearly that PGE2 growth-stimulatory effects in LS-174 cells are mediated by the EGFR signaling system, which was likely activated by AR.

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and TGF-α treatment, although PGE2 alone had no significant effect on AR expression at this time point.

COX-2/PGE2 may exert pro-oncogenic effects on a variety of tumor types (17); therefore, it was critical to determine whether this synergistic effect also occurred in other cell types. Both PGE2 and TGF-α slightly increased the expression of AR mRNA in human kidney 293 cells; however, in combination, PGE2 and TGF-α synergistically induced the expression of AR mRNA (Fig. 5D).

The K-Ras oncogene plays a key role during the adenoma-to-carcinoma sequence of events involved in the neoplastic transformation of colonic epithelial cells. We found that PGE2 and K-Ras also induced AR transcription in a synergistic manner (Fig. 5E). Ectopic expression of K-RasVal12 increased AR promoter activity ~6-fold compared with empty vector-transfected LS-174 cells. PGE2 treatment increased luciferase activity ~7-fold in vector-transfected cells; however, a 27-fold increase was observed in K-RasVal12-transfected cells that were treated with PGE2.

Discussion

cAMP-dependent PKA consists of two isozymes, type I and type II, which are distinguished by their regulatory subunits (RI and RII). Increased expression of the type Iα regulatory subunit (Ria) has been correlated to the growth of an array of tumor cells including colon cancer cells (13). The ratio of RI to RII is significantly elevated in human colorectal carcinomas (18). The LS-174 cell line contains mainly type I PKA and is an excellent model for investigation of pro-oncogenic roles of the cAMP/PKA pathway (19). Treatment with Ria antisense oligonucleotide inhibits the growth of LS-174 cells (13). Results from this study demonstrated that PGE2 signaled through the Gs-coupled EP receptor and increased the activity of the cAMP/PKA pathway, which was essential for PGE2 growth-stimulatory activity. We show that AR, an EGFR ligand, was up-regulated by PGE2-induced cAMP/PKA activity and was a mediator for the PGE2 growth-stimulatory effect in LS-174 cells. Thus, our results establish a link between three crucial proneoplastic signaling systems, the COX-2/PGE2 pathway, the cAMP/PKA pathway, and the EGFR signaling system, where AR plays a central role. In this model, COX-2-generated PGE2 stimulates PKA activation through Ria; increased PKA activity induces the transcription of AR, which then activates RTK signaling pathways and stimulates proliferation and growth of colorectal carcinoma cells. Previous studies show that genetic disruption of either COX-2 or EP2 receptor decreases the number and size of intestinal polyps in ApcΔ716 mice (20). Tumor cell proliferation is significantly inhibited in adenomas of COX-2-deficient ApcΔ716 mice. These findings indicate the potential link between COX-2, PKA, and tumor cell proliferation in vivo. COX-2/PGE2-promoted tumor angiogenesis is thought to be one of the underlying mechanisms. Additional experiments are required to determine the roles of RTK in these animal models.

Our studies demonstrate that in response to PGE2 treatment, the
expression of AR is significantly increased in LS-174 cells. Cumulative evidence suggests that AR exerts tumor-promoting effects on colorectal carcinomas. AR mRNA is expressed in 60–70% of primary and metastatic human colorectal carcinomas but in only 2–7% of normal human colonic mucosa (21). AR plays critical roles in colon cancer cell proliferation and transformation that are required for the growth of human colon carcinoma xenografts (14). In agreement with these studies, we found that AR stimulated the proliferation and growth of LS-174 cells and mediated the PGE2-induced growth stimulation of colon cancer cells.

The CRE consists of an 8-bp palindrome (TGACGTCA) and is a 2–7% of normal human colonic mucosa (21). AR plays critical roles in colon cancer cell proliferation and transformation that are required for the growth of human colon carcinoma xenografts (14). In agreement with these studies, we found that AR stimulated the proliferation and growth of LS-174 cells and mediated the PGE2-induced growth stimulation of colon cancer cells.

In conclusion, our results link the COX-2/PGE2 pathway to the pro-oncogenic CAM/PKA pathway and the oncogenic EGFR signaling system, where an EGFR ligand, AR, serves as a central mediator. AR is up-regulated by the PGE2/cAMP/PKA pathway and, in turn, activates EGFR tyrosine kinases that transduce mitogenic signals and stimulate the growth of colorectal carcinoma cells. Future studies are aimed at the transcriptional regulation of gene expression through the PGE2/PKA/CRE pathway and the synergistic cooperation between PGE2 and major oncogenic pathways in a synergistic manner, thus providing additional insight into the mechanisms by which COX-2/PGE2 promotes colorectal carcinogenesis.

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References


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