[CANCER RESEARCH 63, 5218–5223, September 1, 2003]

Advances in Brief

Prostaglandin E_2 Stimulates the Growth of Colon Cancer Cells via Induction of Amphiregulin[1]

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Abstract

Prostaglandin E_2 (PGE_2), 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 PGE_2 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 PGE_2-induced growth stimulation. In addition, PGE_2, 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 PGE_2 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 COX[3]-2 plays a role in the promotion of colorectal cancer (1). However, the effects of PGs generated by COX-2 have largely been unexplored. PGE_2, a major product of COX enzymes, acts via specific transmembrane GPCRs (2). Four PGE receptor (EP) subtypes have been identified. EP_1 receptor signals via generation of inositol 1,4,5-triphosphate and increased intracellular Ca^{2+}. EP_2 and EP_4 receptors are coupled to Gs proteins and signal through increased cAMP, whereas the EP_3 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 PGE_2 causes significant stimulation of DNA synthesis; prolonged PGE_2 treatment markedly increases the weight, DNA, and RNA content of intestinal mucosa in rats (3). PGE_2 stimulates proliferation and growth of human colorectal cancer cells (4–6). These *in vivo* and *in vitro* findings suggest that COX-2-generated PGE_2 may provide a growth advantage to colorectal carcinomas.

The EGF 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 PGE_2 transactivates EGFR, triggers extracellular signaling-regulated kinase activation, and stimulates the proliferation of colorectal carcinoma cells. These studies demonstrate clearly that PGE_2 growth stimulation in colon cancer cells involves activation of the EGFR signaling system. We have reported previously that treatment with PGE_2 increases PI3K/Akt activity that is critical for the transformation of intestinal epithelial cells and that is required for the growth stimulation of PGE_2 in human colon cancer LS-174 cells (6). To better understand the mechanism(s) by which PGE_2 activates RTK-dependent signaling pathways and stimulates colon cancer cell growth, we investigated the regulation and functional role of a PGE_2 target gene, *AR*, in PGE_2 trophic activity. PGE_2 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 PGE_2 in human colon cancer cells. Moreover, PGE_2, in cooperation with major oncogenic pathways, synergistically induced *AR* transcription. These results provide additional mechanisms mediating COX-2/PGE_2 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). PGE_2 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 PGE_2 or vehicle-treated LS-174 cells were used to inoculate human GeneChip (U95A) expression arrays (Affymetrix, Inc.) based on a recommended protocol. Three replicate hybridizations were performed using PGE_2 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 [α-^{32}P]dCTP by random primer extension (Stratagene, La Jolla, CA). After hybridization and washes, the blots were subjected to autoradiography.

Received 3/18/03; revised 6/26/03; accepted 7/11/03.

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[1] Supported by the John Sealy Memorial Endowment Fund for Biomedical Research and the Gastrointestinal Research Interdisciplinary Program (both to H. S.).

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[3] The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; GPCR, G protein-coupled receptor; Gs, stimulatory G; cAMP, cyclic AMP; EGF, epidermal growth factor; EGFR, EGF receptor; RTK, receptor tyrosine kinase; PI3K, phosphatidylinositol 3-kinase; AR, amphiregulin; 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, GCCTTTATGTCTGCTGTGATCCT; and reverse primer, CCTCAGCTTCTCCTTCATATTTCCT. 18S rRNA 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 C_T values were analyzed in Microsoft Excel using the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta CT}$) was obtained as normalized to 18 S 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

PGE_2 Stimulated LS-174 Cell Growth through the cAMP/PKA Pathway. PGE_2 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 PGE_2 treatment did not alter intracellular Ca^{2+} levels in LS-174 cells (data not shown). In contrast, PGE_2 activated the cAMP/PKA signaling pathway (Fig. 1*A*). Stimulation with PGE_2 rapidly increased PKA activity by ~7-fold in LS-174 cells. A selective PKA inhibitor, H-89, at 10 μM completely blocked the PGE_2-induced PKA activity. Similar results were observed in LS-174 cells that were treated with dibutyryl cAMP (0.1 mM), suggesting that PGE_2 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. 1*B*). LS-174 cells formed colonies in Matrigel, and PGE_2 treatment increased the volume of colonies ~4-fold. Addition of H-89 blocked the PGE_2 stimulation of LS-174 cell growth in Matrigel. Thus, PKA activation appeared to be critical for PGE_2 stimulation of LS-174 cell growth.

PGE_2-induced AR Expression. To search for target genes that may mediate PGE_2 growth-stimulatory activity in colon cancer cells, LS-174 cells were treated with PGE_2 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 PGE_2 stimulation. Among these genes, *AR*, a member of the EGF family, was induced 9-fold after PGE_2 treatment. Because AR stimulates the growth of colon cancer cells (14), we decided to further investigate roles of *AR* in PGE_2 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 PGE_2 for 2 h and returned to basal levels by 8 h (Fig. 2*A*).

To determine the mechanism by which PGE_2 induced AR expression, we investigated the role of PGE_2 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, PGE_2 treatment rapidly increased the transcriptional activity of *AR* and resulted in an ~7-fold increase in luciferase activity (Fig. 2*B*). PKA activation was critical for the PGE_2-stimulated *AR* transcription, which was completely blocked by the presence of H-89. These results suggest that PGE_2-induced *AR* transcription was mediated by the PKA signaling pathway.

Roles of the CRE in PGE_2 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 PGE_2-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 PGE_2 stimulation compared with vehicle-treated cells (Fig. 3*A*). Mutation of the CRE site from GACGTCA to GACGTAC (pGL2-BΔCRE) resulted in a significant reduction of *AR* promoter activity and almost completely attenuated PGE_2-induced *AR* transcription. Using pGL2-C and pGL2-CΔCRE report vectors, which contain only 83 nucleotides

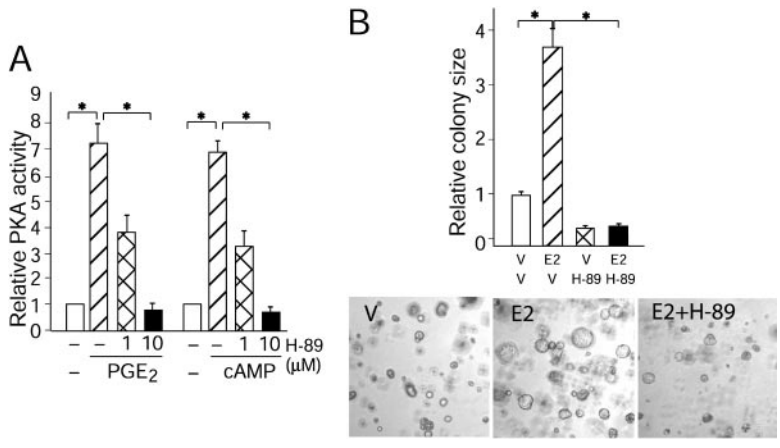

Fig. 1. PGE_2 activation of the cAMP/PKA pathway. *A,* PGE_2 induction of PKA. LS-174 cells were serum deprived for 48 h and then treated with PGE_2 (0.1 μM) or dibutyryl cAMP (0.1 mM) for 10 min in the presence or absence of H-89 at the indicated concentrations. PKA activity was measured by determining the transfer of the phosphate group of ATP to a synthetic peptide, which is a substrate for PKA. Results were normalized by the controls and expressed as relative PKA activity. Plotted is the means of assays performed in triplicate of two independent experiments; *bars,* SD. *, $P < 0.05$. *B,* PKA activity and LS-174 cell growth in Matrigel. LS-174 cells were grown in Matrigel with indicated treatment for 10 days (*V,* vehicle; *E2,* 0.1 μM PGE_2; *H-89,* 10 μM H-89). Colonies were photographed (×40), and the size of 60 colonies from each group was measured. Relative colony size to the control group is plotted. *, $P < 0.05$. This experiment was repeated three times.

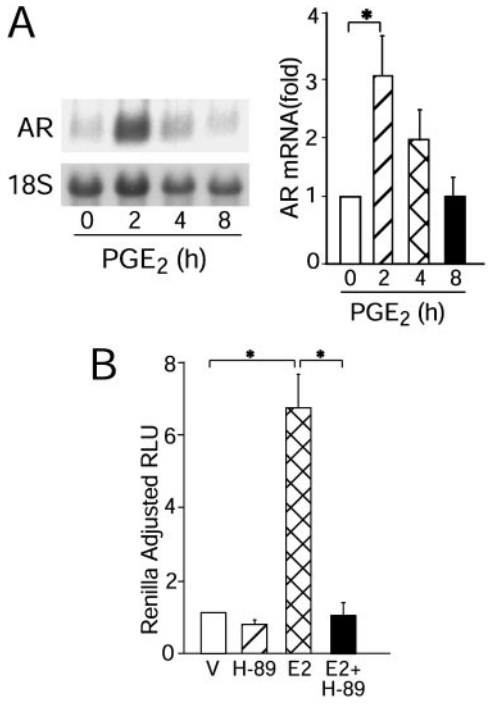

Fig. 2. PGE_2 induction of AR expression. *A,* Northern analysis of AR mRNA. LS-174 cells were serum deprived for 48 h and then treated with 0.1 μM PGE_2 for the indicated times. Total RNA was extracted, and levels of AR were detected using Northern blot analysis. The results were densitometrically analyzed using Image J software and standardized by the control values (0 h). Plotted is the mean of three independent experiments; *bars,* SD. *, $P < 0.05$. *B,* PGE_2 induction of *AR* promoter activity. LS-174 cells were transiently transfected with pGL2-A containing the nucleotide sequence of 763 bp (−850 to −87) of *AR* 5′-flanking region. Nucleotide numbers refer to promoter sequence (12, 15). Cells were serum deprived for 24 h before vehicle (*V*), H-89, PGE_2 (*E2*), or PGE_2 plus H-89 (*E2 + H-89*) treatment. Firefly and Renilla luciferase activities were measured, and firefly luciferase values were standardized to Renilla values. *Renilla Adjusted RLU,* Renilla-adjusted luciferase values standardized to the controls. Plotted is the mean of assays performed in quadruplicate, based on three independent experiments; *bars,* SD. *, $P < 0.05$.

(−275 to −192) including the CRE and the TATA box, we confirmed the critical role of the CRE in PGE_2-induced *AR* transcription (Fig. 3*B*). Transcriptional activation of the CRE requires activated transcription factors of the CREB protein, which is phosphorylated at Ser-133 by the PKA catalytic subunit and interacts with the CRE. PGE_2 treatment significantly increased the phosphorylation of CREB protein at Ser-133 in LS-174 cells (Fig. 3*C*).

AR Mediated the Trophic Action of PGE_2. 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. 4*A, left panel*). AR stimulated DNA synthesis in LS-174 cells that was partially blocked by an anti-AR neutralizing antibody (Fig. 4*A, 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. 4*A, 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 PGE_2-induced growth stimulation. Both AR and PGE_2 treatment increased PI3K activity, as noted by increased levels of pAkt that is critical for PGE_2 stimulation of LS-174 cell growth (6). Inhibition of EGFR activity by PD-153035 (1 μM) blocked PGE_2-induced pAkt (Fig. 4*B*), suggesting that PGE_2 induction of PI3K activity required activation of EGFR. PGE_2 treatment promoted the growth of LS-174 cells; this effect was blocked by inhibition of RTK activity (Fig. 4*C, left panel*). PGE_2 modestly increased DNA synthesis in LS-174 cells; addition of an anti-AR neutralizing antibody before PGE_2 treatment completely attenuated the PGE_2-induced DNA synthesis (Fig. 4*C, middle panel*). PGE_2 significantly increased the colony volume of LS-174 cells when grown in Matrigel; again, EGFR activity was critical for the growth-stimulatory effect of PGE_2 (Fig. 4*C, right panel*). These results demonstrate clearly that PGE_2 growth-stimulatory effects in LS-174 cells are mediated by the EGFR signaling system, which was likely activated by AR.

Synergy between PGE_2 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 PGE_2 and TGF-α may exert tumor-promoting effects to colon cancer cells, we sought to determine whether PGE_2 and TGF-α act in a cooperative manner. As demonstrated in Fig. 5*A,* both PGE_2 and TGF-α induced the levels of pAkt and pCREB; however, combined treatment with PGE_2 and TGF-α resulted in a marked induction of PI3K activity and CREB activation. Next, we investigated whether PGE_2 and TGF-α cooperatively induced *AR* transcription. LS-174 cells were transfected with pGL2-A and then treated with TGF-α or PGE_2 plus TGF-α. TGF-α increased only luciferase activity ~2-fold; PGE_2 increased *AR* promoter activity ~7-fold. However, the combination of PGE_2 and TGF-α increased luciferase activity ~17-fold, suggesting that PGE_2 and TGF-α synergistically activated the *AR* promoter. To demonstrate that the synergistic transcription of *AR* resulted from PGE_2 and TGF-α treatment produced transcripts, we used real-time PCR to determine the expression of AR mRNA. PGE_2 and TGF-α synergistically increased the levels of AR mRNA (Fig. 5*C*). Interestingly, a stronger synergy was observed at 24 h after PGE_2

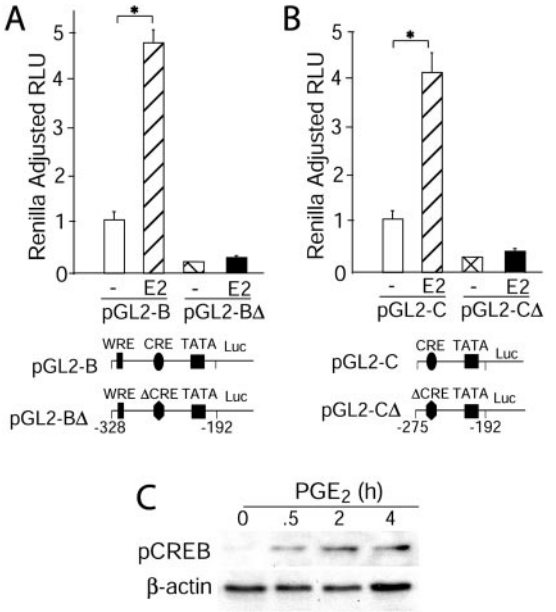

Fig. 3. PGE_2 activation of the CRE in the *AR* promoter. *A,* the role of the CRE in PGE_2 activation of *AR* transcription. LS-174 cells were transiently transfected with pGL2-B or pGL2-BΔCRE (*pGL2-B*Δ). Cells were then treated with vehicle (*V*) or PGE_2 (0.1 μM; *E2*) for 6 h. Firefly and Renilla luciferase activities were measured and standardized. Plotted is the mean of assays performed in quadruplicate; *bars,* SD. *, $P < 0.05$. Results shown are representative of five separate experiments. *B,* LS-174 cells were transiently transfected with pGL2-C or pGL2-CΔCRE (*pGL2-C*Δ). Cells were then treated with vehicle (*V*) or PGE_2 (0.1 μM; *E2*) for 6 h. Firefly and Renilla luciferase activities were measured and standardized. Plotted is the mean of assays performed in quadruplicate; *bars,* SD. *, $P < 0.05$. Results shown are representative of five separate experiments. *C,* PGE_2 induction of pCREB. LS-174 cells were serum deprived for 48 h before PGE_2 treatment. Cellular protein was collected at the indicated time points for detection of phosphorylated CREB (Ser-133). This experiment was repeated three times.

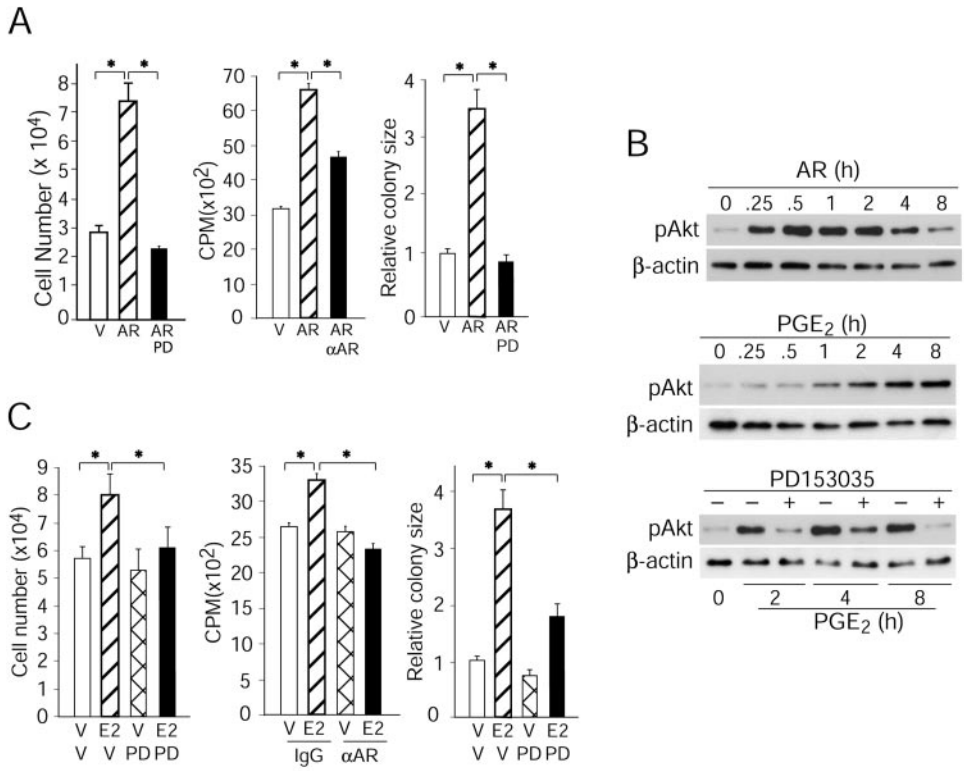

Fig. 4. AR in PGE_2 trophic action. *A,* AR stimulation of LS-174 cell growth. *Left panel,* 10^4 LS-174 cells were seeded in 12-well plates and treated with vehicle (*V*), AR (100 ng/ml), or AR plus PD-153035 (1 μM) for 4 days. *, $P < 0.05$. *Middle panel,* LS-174 cells were seeded in 24-well plates and treated with vehicle (*V*), AR (100 ng/ml), or AR plus anti-AR antibody (20 μg/ml) for 24 h. [^{3}H]]Thymidine incorporation was measured. *, $P < 0.05$. *Right panel,* LS-174 cells were grown in Matrigel and treated with vehicle (*V*), AR (100 ng/ml), or AR plus PD-153035 (1 μM) for 10 days. Relative colony size was determined. *, $P < 0.05$. All growth studies in *A* were repeated three times. *B,* AR and PGE_2 induction of PI3K activity. LS-174 cells were serum deprived for 48 h before the indicated treatments (*AR,* 100 ng/ml; *PGE_2,* 0.1 μM; *PD153035,* 1 μM). Protein lysates were extracted at the indicated time points and subjected to Western analysis. Results were similar in two independent experiments. *C,* inhibition of RTK and PGE_2 growth stimulation. *Left panel,* 2.5×10^4 LS-174 cells were seeded in 12-well plates and grown in serum-deprived medium with the indicated treatments for 4 days. *V,* vehicle; *E2,* PGE_2 (0.1 μM); *PD,* PD-153035 (1 μM). Cell numbers were counted, and values are means from triplicate wells; *bars,* SD. *, $P < 0.05$. Results were similar in three independent experiments. *Middle panel,* serum-deprived LS-174 cells were treated with vehicle (*V*) or PGE_2 (0.1 μM) in the presence of normal IgG or anti-AR antibody (αAR) for 24 h. [^{3}H]]Thymidine incorporation was measured. *, $P < 0.05$. Results were similar in two independent experiments. *Right panel,* LS-174 cells were grown in Matrigel with the indicated treatment for 10 days. *V,* vehicle; *E2,* PGE_2 (0.1 μM); *PD,* PD-153035 (1 μM). Colonies were photographed, and the size of 60 colonies from each group was measured. Relative colony size to the control group is shown. *, $P < 0.05$. Results were similar in two independent experiments.

and TGF-α treatment, although PGE_2 alone had no significant effect on AR expression at this time point.

COX-2/PGE_2 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 PGE_2 and TGF-α slightly increased the expression of AR mRNA in human kidney 293 cells; however, in combination, PGE_2 and TGF-α synergistically induced the expression of AR mRNA (Fig. 5*D*).

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 PGE_2 and K-*Ras* also induced *AR* transcription in a synergistic manner (Fig. 5*E*). Ectopic expression of K-Ras^{Val12} increased *AR* promoter activity ~6-fold compared with empty vector-transfected LS-174 cells. PGE_2 treatment increased luciferase activity ~7-fold in vector-transfected cells; however, a 27-fold increase was observed in K-Ras^{Val12}-transfected cells that were treated with PGE_2.

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 (RIα) 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 RIα antisense oligonucleotide inhibits the growth of LS-174 cells (13). Results from this study demonstrated that PGE_2 signaled through the Gs-coupled EP receptor and increased the activity of the cAMP/PKA pathway, which was essential for PGE_2 growth-stimulatory activity. We show that AR, an EGFR ligand, was up-regulated by PGE_2-induced cAMP/PKA activity and was a mediator for the PGE_2 growth-stimulatory effect in LS-174 cells. Thus, our results establish a link between three crucial proneoplastic signaling systems, the COX-2/PGE_2 pathway, the cAMP/PKA pathway, and the EGFR signaling system, where AR plays a central role. In this model, COX-2-generated PGE_2 stimulates PKA activation through RIα; 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 EP_2 receptor decreases the number and size of intestinal polyps in *Apc*Δ716 mice (20). Tumor cell proliferation is significantly inhibited in adenomas of COX-2-deficient *Apc*Δ716 mice. These findings indicate the potential link between COX-2, PKA, and tumor cell proliferation *in vivo*. COX-2/PGE_2-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 PGE_2 treatment, the

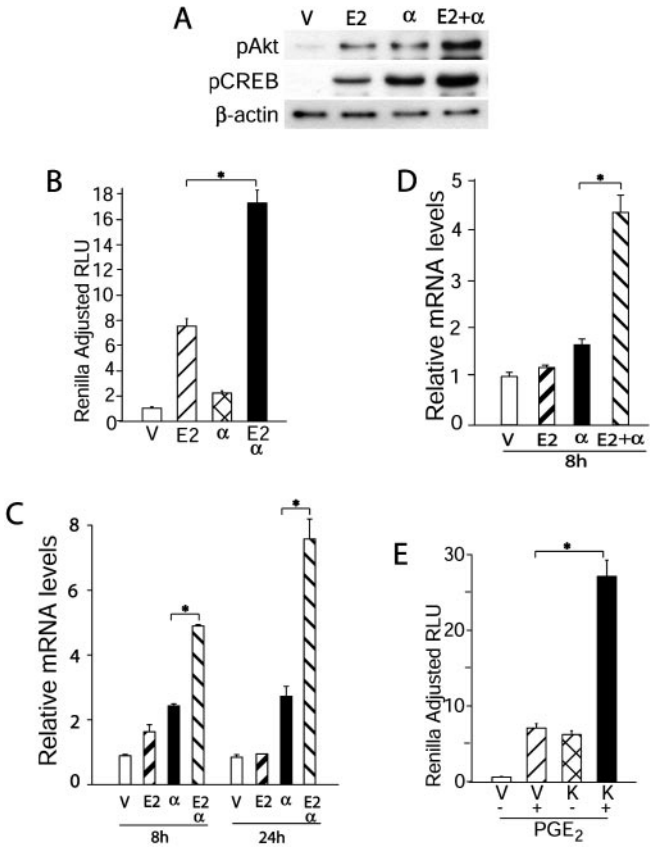

Fig. 5. Synergy between PGE_2 and oncogenic pathways. *A,* PGE_2 and TGF-α induction of pCREB and pAkt. LS-174 cells were serum deprived for 48 h before the indicated treatment. *E2,* PGE_2 (0.1 μM); α, TGF-α (100 ng/ml). Protein lysates were extracted after a 2-h incubation and subjected to Western analysis for pAkt and pCREB. Results shown are representative of three separate experiments. *B,* synergistic induction of *AR* transcription by PGE_2 and TGF-α. LS-174 cells were transiently transfected with pGL2-A and treated with vehicle (*V*), PGE_2 (*E2*), TGF-α (α), or both PGE_2 and TGF-α. Firefly and Renilla luciferase activities were measured, and firefly luciferase values were standardized to Renilla values. Plotted is the mean of assays performed in quadruplicate; *bars,* SD. *, $P < 0.05$. Results were similar in five independent experiments. *C,* synergistic induction of AR mRNA by PGE_2 and TGF-α in LS-174 cells. LS-174 cells were subjected to the indicated treatment (*V,* vehicle; *E2,* 0.1 μM PGE_2; α, 100 ng/ml TGF-α). mRNA was used as a template for a real-time RT-PCR assay to measure relative levels of AR expression. *, $P < 0.05$. Results were similar in two independent experiments. *D,* synergistic induction of AR mRNA by PGE_2 and TGF-α in 293 cells. Human 293 cells were subjected to the indicated treatment (*V,* vehicle; *E2,* 0.1 μM PGE_2; α, 100 ng/ml TGF-α). mRNA was used as a template for a real-time RT-PCR assay to measure relative levels of AR expression. *, $P < 0.05$. Results were similar in two independent experiments. *E,* synergistic induction of AR transcription by PGE_2 and K-*Ras*. LS-174 cells were cotransfected with pGL2-A and pcDNA-3/K-Ras^{Val12} (*K*) or pGL2-A and empty pcDNA-3 vector (*V*). Cells were serum deprived for 24 h before a 6-h PGE_2 treatment. Firefly and Renilla luciferase activities were measured, and firefly luciferase values were standardized to Renilla values. Plotted is the mean of assays performed in quadruplicate; *bars,* SD. *, $P < 0.05$. Results were similar in four independent experiments.

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 PGE_2-induced growth stimulation of colon cancer cells.

The CRE consists of an 8-bp palindrome (TGACGTCA) and is typically found within 100 nucleotides of the TATA box. Although the CRE is conserved in the *AR* promoter, thus far, the role of the CRE in the regulation of *AR* transcription is not completely understood (15). Wilms tumor suppressor transcription factor (WT1) activates the CRE in the *AR* promoter through an undefined mechanism in human osteosarcoma cells (12). Here, we show that the CRE is critical for the PGE_2-induced transcriptional activation of *AR* in LS-174 cells. CRE sites are frequently located adjacent to other regulatory elements, such as Sp1. Several Sp1 sequences are found adjacent to the CRE of the *AR* promoter, and their functional roles are under investigation. These results suggest that genes that contain an active CRE site in their promoter may be potentially regulated by PGE_2. Further identifying PGE_2/PKA target genes and their functional roles will contribute to the understanding of the molecular pathway of colorectal carcinogenesis.

The mechanisms by which GPCR transactivates RTK signaling pathways are complex. It was shown that PGE_2-mediated transactivation of EGFR and downstream signaling involves TGF-α, an EGFR ligand, which is likely released by the c-Src-activated matrix metalloproteinase pathway (4). In the present study, we demonstrate that PGE_2 activated EGFR through induction of AR expression. Apparently, PGE_2 may target multiple EGF family members through different mechanisms in colon cancer cells. The PI3K/Akt pathway acts as a transducer of input initiated by growth factor receptors and plays critical roles in the growth and transformation of colorectal carcinoma cells (22). We have reported that the PGE_2 growth-stimulatory effect on LS-174 cells is dependent on PI3K activity (6). Results from this study demonstrate that PGE_2-induced PI3K activation was dependent on EGFR tyrosine kinase activity, which was likely activated by PGE_2-induced autocrine AR.

Although it is now clear that COX-2 plays an important role in the promotion of colorectal cancer (1), COX-2 is not defined as an oncogene. In previous studies (5, 6), we have demonstrated that PGE_2 activates oncogenic signaling pathways and enhances transformed phenotypes including cell proliferation, motility, and morphogenesis. In the present study, we show that PGE_2, in conjunction with TGF-α and the K-*Ras* oncogene, synergistically induced *AR* transcription and stimulated the activation of PKA and PI3K. These results suggest that PGE_2 alone may exert temporary effects on tumor cells; however, in combination with other oncogenic signaling pathways, PGE_2 may play a key role in neoplastic transformation. To our knowledge, this study is the first to demonstrate that PGE_2 acts with major oncogenic signaling pathways in a synergistic manner, thus providing additional insight into the mechanisms by which COX-2/PGE_2 promotes colorectal carcinogenesis.

In conclusion, our results link the COX-2/PGE_2 pathway to the pro-oncogenic cAMP/PKA pathway and the oncogenic EGFR signaling system, where an EGFR ligand, AR, serves as a central mediator. AR is up-regulated by the PGE_2/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 PGE_2/PKA/CRE pathway and the synergistic cooperation between PGE_2 and major oncogenic pathways, which will be critical for the understanding of COX-2 proneoplastic activity in colorectal carcinogenesis.

Acknowledgments

We thank Dr. Raymond N. DuBois for valuable comments on the manuscript and Dr. Kevin Knudtson (University of Iowa) for performing Affymetrix GeneChip Assays.

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