Research Article

Prostaglandin E_2 Enhances Intestinal Adenoma Growth via Activation of the Ras-Mitogen–Activated Protein Kinase Cascade

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Abstract

A large body of clinical, genetic, and biochemical evidence indicates that cyclooxygenase-2 (COX-2), a key enzyme for prostanoid biosynthesis, contributes to the promotion of colorectal cancer. COX-2–derived prostaglandin E_2 (PGE_2) is the most abundant prostaglandin found in several gastrointestinal malignancies. Although PGE_2 enhances intestinal adenoma growth in *Apc*^{min} mice, the mechanism(s) by which it accelerates tumor growth is not completely understood. Here we investigated how PGE_2 promotes intestinal tumor growth and the signaling pathways responsible for its effects. We observed that PGE_2 treatment leads to increased epithelial cell proliferation and induces COX-2 expression in intestinal adenomas. Furthermore, we show that PGE_2 regulation of COX-2 expression is mediated by activation of a Ras-mitogen–activated protein kinase signaling cascade. One intriguing finding is that COX-2–derived PGE_2 mimics the effects of constitutively active Ras through a self amplifying loop that allows for a distinct growth advantage. (Cancer Res 2005; 65(5): 1822-9)

Introduction

Colorectal cancer develops according to a complex and multistep process which involves genetic alterations and progressive changes in signaling pathways that regulate intestinal epithelial cell proliferation, differentiation, and apoptosis. A large body of data supports a role of the cyclooxygenase-2 (COX-2) enzyme in colorectal cancer progression (1, 2) and indicates that inhibition of COX-2 reduces tumor growth (3). Moreover, it is well established that cyclooxygenase enzymes play a key role in intestinal adenoma formation in *Apc*^{min} mice, a model frequently employed for studying colorectal cancer (4), and in *Apc*$^{\Delta 716}$ mutant mice (5). Tumor number decreases when *Apc* mutant mice are treated with COX-2-selective inhibitors (5). In addition, *in vitro* studies confirm that expression of COX-2 is correlated with intestinal epithelial cell proliferation and invasiveness, a reduction in the apoptotic rate, and induction of proangiogenic factors such as vascular endothelial growth factor (6–9).

COX-2 is an inducible gene which is regulated by a number of factors, including serum, growth factors, proinflammatory cytokines, hormones, oncogenes, or tumor promoters (10). Various extracellular stimuli regulate COX-2 expression through a mitogen-activated protein kinase (MAPK)–dependent pathway. For example, transforming growth factor α, INFγ, and platelet-derived growth factor induce COX-2 expression via activation of the extracellular signal–regulated kinase (ERK) signal transduction pathway in normal human epidermal keratinocytes, squamous carcinoma cells, and NIH 3T3 cells, respectively (11, 12).

COX-2 is the key enzyme for the conversion of arachidonic acid to prostaglandins, such as PGE_2, PGD_2, $PGF_2\alpha$, and PGI_2 (10, 13–15). PGE_2 is the most abundant prostaglandin found in human colorectal cancers, premalignant lesions, and cells derived from a number of solid malignancies (16–19). PGE_2 exerts its actions either in autocrine or in paracrine fashion via binding to G-protein coupled receptors (EP1-4), which belong to the family of rhodopsin-type receptors. Genetic studies using mice lacking the PGE_2 cell surface receptors EP1, EP2, or EP4 point to an important role for all three receptors in intestinal polyp formation (20–22). Moreover, EP1 or EP4 receptor antagonists decrease the incidence of intestinal adenomas in both the *Apc*^{min} and the carcinogen-treated mouse models (20, 21). These results provide strong evidence that PGE_2 plays a pivotal role in regulating intestinal adenoma formation. We recently reported that PGE_2 treatment increases adenoma burden in *Apc*^{min} mice (23). In addition, *in vitro* studies have shown that PGE_2 enhances clonogenicity and increases invasiveness of LS-174T carcinoma cells by activating the EP4-PI3k-Akt signaling cascade (24) and that PGE_2 promotes integrin $\alpha v\beta 3$–dependent endothelial cell adhesion and spreading (25). However, all of the mechanisms responsible for the effects of PGE_2 on intestinal adenoma growth are not known.

Ras mutations are found in a wide variety of human malignancies and in about 50% of colorectal carcinomas (26). In rodents, AOM-induced colonic carcinogenesis involves activation of the *K-Ras* gene (27). For example, K-Ras mutations were identified in 14 of 84 AOM-induced colonic tumors. Most importantly, a subset of tumors (18 of 70) had significantly higher activation of wild-type K-Ras compared with controls, suggesting that the activation of wild-type Ras is also involved in AOM-induced colonic carcinogenesis. Moreover, forced expression of constitutively active Ras (mutant Ras) up-regulates COX-2 expression and enhances cell proliferation in a variety of cell culture models (28–31). Therefore, we examined whether PGE_2 activates endogenous wild-type Ras that is known to regulate cell proliferation.

Note: R.N. DuBois is the Hortense B. Ingram Professor of Molecular Oncology. R.N. Dubois is a recipient of NIH MERIT award R37-DK47297.

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It is well known that activation of Ras triggers the downstream signaling pathways such as the Raf/MAPK kinase (MEK)/ERKs and PI3K/Akt pathways. In the Raf/MEK/ERKs pathway, activated Ras recruits Raf to the plasma membrane, which leads to phosphorylation of MEK, a dual specificity kinase that phosphorylates the Thr-*X*-Tyr motif in the activation loop of ERK. Upon activation, ERK translocates to the nucleus and regulates the activity of many transcription factors including Elk-1 (32, 33). Elk-1 is a member of the ternary complex factor family of Ets domain proteins that bind to serum response elements, a cis-element responsible for activation of immediate-early gene expression following mitogenic stimulation (34). It has been reported that serum, growth factors, and phorbol 12-myristate 13-acetate stimulate the phosphorylation of Elk-1 via the Raf-MEK-ERK pathway (35). Moreover, other seven transmembrane spanning G-protein-coupled receptor agonists have been shown to activate a Ras-MAPK signaling pathway (36). Because cytokines induce COX-2 expression by activating the ERK signal transduction pathway, we postulated that PGE$_2$ could up-regulate COX-2 through activation of Ras-MAPK signaling.

To investigate the mechanism responsible for the effect of PGE$_2$ to promote intestinal adenoma growth, we examined whether this lipid mediator stimulates intestinal epithelial cell proliferation and determined the downstream targets of PGE$_2$ using both *in vivo* and *in vitro* models. Here we show that PGE$_2$ enhances intestinal cell proliferation and induces COX-2 expression in Apc^{min} mouse adenomas. We further show that activation of Ras-MAPK pathway is required for PGE$_2$ to induce COX-2 expression and stimulate HCA-7 cell proliferation. Consistent with these findings, we also observed that PGE$_2$ enhances ERK and Elk-1 activity in intestinal adenomas and phospho-Elk-1 levels are dramatically elevated in human colorectal cancers compared with matched normal mucosa. To our knowledge, this is first report that COX-2 induction by PGE$_2$ is mediated by activation of the Ras-MAPK pathway.

Materials and Methods

Animals. C57BL/6J-Apc^{min} male mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age. After 1 week, Apc^{min} mice (n = 6) were randomly assigned to either a control or treatment group (vehicle and 150 μg PGE$_2$ per mice). Vehicle or PGE$_2$ in 100 μl sterile PBS was given twice daily via gavage feeding. After treatment for 7 weeks, mice were injected with 0.25 ml of BrdUrd labeling reagent (Zymed Laboratories, Inc., South San Francisco, CA) and sacrificed after 3 hours. The intestines were collected, opened longitudinally, fixed, and embedded in paraffin as reported previously (23). Sections were then used for cell proliferation assays and immunohistochemical staining.

***In vivo* Cell Proliferation Assays and Immunohistochemical Staining.** Proliferating cells in tissue sections were detected using an anti-BrdUrd antibody according to the instructions for ZYMED BrdUrd kit (Zymed Laboratories). In addition, tissue sections also were stained with mouse monoclonal antibodies against phospho-Elk-1 (Ser383) or phospho-ERK1/2 (Tyr204) at a dilution of 1:250 (Santa Cruz Biotechnology, Santa Cruz, CA). The immunohistochemical staining was completed by using a Zymed-Histostain-SP Kit (Zymed Laboratories).

Cell Culture. HCA-7 cells were maintained in McCoy's 5A medium containing 10% fetal bovine serum and penicillin-streptomycin. To arrest cell growth, cells were cultured in the absence of serum for 24 hours. The MEK1 (PD98059), the Ras (Ftase inhibitor III), and PI3K (LY294002) inhibitors (Calbiochem-Novabiochem Co., San Diego, CA) were prepared as a stock in DMSO (50 μmol/L).

Cell Proliferation ELISA and Cell Growth. Cell proliferation was measured using a Cell Proliferation ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's instructions. Briefly, HCA-7 cells (1×10^4 cells per well) grown on 96-well culture plates were pretreated with either DMSO or inhibitors for 1 hour and then treated with the indicated concentration of PGE$_2$ for 24 hours after serum starvation. The cells were then labeled with BrdUrd for an additional 6 hours. Incorporated BrdUrd was measured colorimetrically with an ELISA reader, Spectra MAX 340PC (Molecular Devices, Sunnyvale, CA). Cell vitality was measured using a trypan blue exclusion assay.

Whole Cell Extracts and Western Blot Analysis. Whole cell extracts were prepared from cells treated with either vehicle or PGE$_2$ for the indicated times and after serum starvation for 24 hours. Western blots were done following protocols provided by Santa Cruz Biotechnology. The cells were lysed in 0.6 ml of radioimmunoprecipitation assay buffer with protease inhibitor cocktail tablets (Boehringer Mannheim Co., Indianapolis, IN) and 0.2 μmol/L sodium orthovanadate. Fifty micrograms of soluble protein were fractionated in a 10% SDS-PAGE reducing gel, and then blotted onto a 0.2-μm nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% dry milk in TBS-T buffer for 1 hour and then incubated for 12 to 16 hours at 4°C in a 1:1,000 dilution of a pan-Ras antibody (AB-3; Oncogene Research Products, Cambridge, MA), an anti-phospho-ERK1/2, the anti-phospho-Elk-1, anti-Elk-1, anti-COX-2, or anti-ERK1/2 (Santa Cruz Biotechnology) in TBS-T buffer containing 5% dry milk. After three washings with TBS-T buffer, the membrane was incubated in a 1:3,000 dilution of the appropriate antimouse or antirabbit immunoglobulin conjugated with horseradish peroxidase (Boehringer Mannheim) in TBS-T buffer with 5% dry milk for 1 hour at room temperature. After three washings with TBS-T buffer, the protein bands were detected with the enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. The blots were stripped and then reprobed with anti-ERK2 or anti-Elk-1.

Transient Transfection Assays. Cells (1.5×10^5 in 12-well plates) were transiently cotransfected with 0.2 μg of COX-2 (−327 to +59) luciferase reporter gene and 5 ng of pRL-SV40 by the LipofectAMINE Plus reagent following manufacturer's protocol (Life Technologies, Inc., Rockville, MA). For cotransfection assays, the cells were transiently cotransfected with 0.3 μg COX-2 (−327 to +59) luciferase reporter gene and 5 ng of pRL-SV40 and 0.4 μg of empty vector or dominant-negative PI3K plasmids. Three hours later, the cells were placed in fresh serum-free media and incubated for another 4 hours. The cells were then treated with PGE$_2$ after pretreatment with vehicle or inhibitors for 1 hour. After 16 hours, cells were harvested in 1× luciferase lysis buffer. Relative light units from firefly luciferase activity was determined using a luminometer, Monolight 3010, (BD Biosciences/PharMingen, San Diego, CA) and normalized to the relative light units from Renilla luciferase using the Dual Luciferase kit (Promega, Madison, WI).

Ras Activation Assays. Ras activity was measured using a Ras Activation Assay Kit (Upstate Biotechnology, Inc., Lake Placid, NY) following the manufacturer's instructions. Briefly, quiescent cells were stimulated with PGE$_2$ at indicated concentrations and for indicated times. Cells were washed twice with ice-cold HBS and lysed in 1× Mg^{2+} lysis/washing buffer containing protease inhibitor cocktail tablets (Roche Molecular Biochemicals) for 15 minutes at 4°C. Cell lysates were centrifuged at $1,000 \times g$ for 20 minutes. The supernatants were pretreated with glutathione-Sepharose-4B beads (Amersham Pharmacia Biotech) and the protein concentrations of the supernatants were then determined (Bio-Rad). Equal amounts of samples (400 μg) were immediately affinity-precipitated using 20 μg of recombinant glutathione *S*-transferase-c-Raf-1 ras binding domain (1149) fusion proteins conjugated glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 1 hour at 4°C. The precipitates were washed thrice with 1× Mg^{2+} lysis/washing buffer and eluted by boiling in 1× SDS-PAGE sample buffer. The proteins were separated on a 12% SDS-polyacrylamide gel and then immunoblotted with pan-Ras antibody (AB-3; Oncogene Research Products). To normalize the amount of GTP-bound Ras to total amount of Ras, equal volumes of cell lysate were also subjected to Western blot analysis using the pan-Ras antibody.

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Figure 1. PGE_2 induces intestinal epithelial cell proliferation and COX-2 expression in adenomas. Sections of small intestine were immunostained with anti-BrdUrd antibody (*A*) or COX-2 antibody (*B*). Representative section shows strong immunoreactive staining (*brown*) for incorporated BrdUrd in epithelial cells of adenomas taken from mice treated with PGE_2 and weak staining was found in the adenomas from mice treated with vehicle alone. *B,* levels of COX-2 expression in the adenomas from mice treated with PGE_2. *Top,* immunoreactive staining (*brown*) for COX-2. *Bar*, 100 μm. *Bottom*, three different Western blot experiments with similar results.

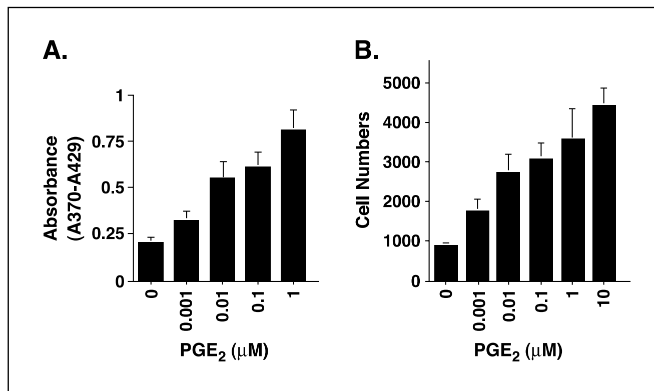

Figure 2. PGE_2 stimulates HCA-7 cell proliferation. HCA-7 cells (1×10^4) were seeded in a 96-well plate and incubated with 0.1 μmol/L PGE_2 for 24 hours after serum starvation for 24 hours. The cells were then labeled with BrdUrd for 6 hours. *A,* incorporated BrdUrd was measured colorimetrically by ELISA reader. *Columns*, means of absorbance ($A_{370\ nm}$-$A_{492\ nm}$) from three independent experiments done in triplicate; *bars,* ±SE. *B,* in addition, cells (1×10^4) were seeded in a 12-well plate and incubated with 0.1 μmol/L PGE_2 for 5 days after serum starvation for 24 hours. Cell numbers were counted from triplicate wells. *Columns,* means from three independent experiments; *bars,* ±SE.

Results

PGE_2 Induces Epithelial Cell Proliferation and COX-2 Expression in Intestinal Adenomas. Because epithelial cell proliferation may be one of the key mechanisms by which PGE_2 promotes intestinal adenoma growth, we investigated whether PGE_2 would affect the adenoma epithelial cell proliferation in the Apc^{min} mice. We treated 6-week-old Apc^{min} mice with vehicle (PBS) or PGE_2 for 7 weeks. Before sacrifice, mice were given an injection of BrdUrd to label the proliferating cell fraction. Significant BrdUrd uptake was seen in epithelial cells found in adenomas from PGE_2-treated mice as compared with controls (Fig. 1*A*). To further understand the molecular mechanism by which PGE_2 induces cell proliferation, we examined COX-2 expression in these adenomas because of our previous data indicating that elevated COX-2 expression was correlated with enhanced cell proliferation (37). Immunohistochemical staining and Western blot analyses were done to assess COX-2 expression levels. As shown in Fig. 1*B*, PGE_2 treatment resulted in a dramatic increase in COX-2 expression in intestinal adenomas. These results show that PGE_2 enhances COX-2 expression and stimulates epithelial cell proliferation in adenomas.

PGE_2-Up-Regulated COX-2 Expression Is Dependent on Ras-MAPK Activation. To further elucidate the signaling pathways

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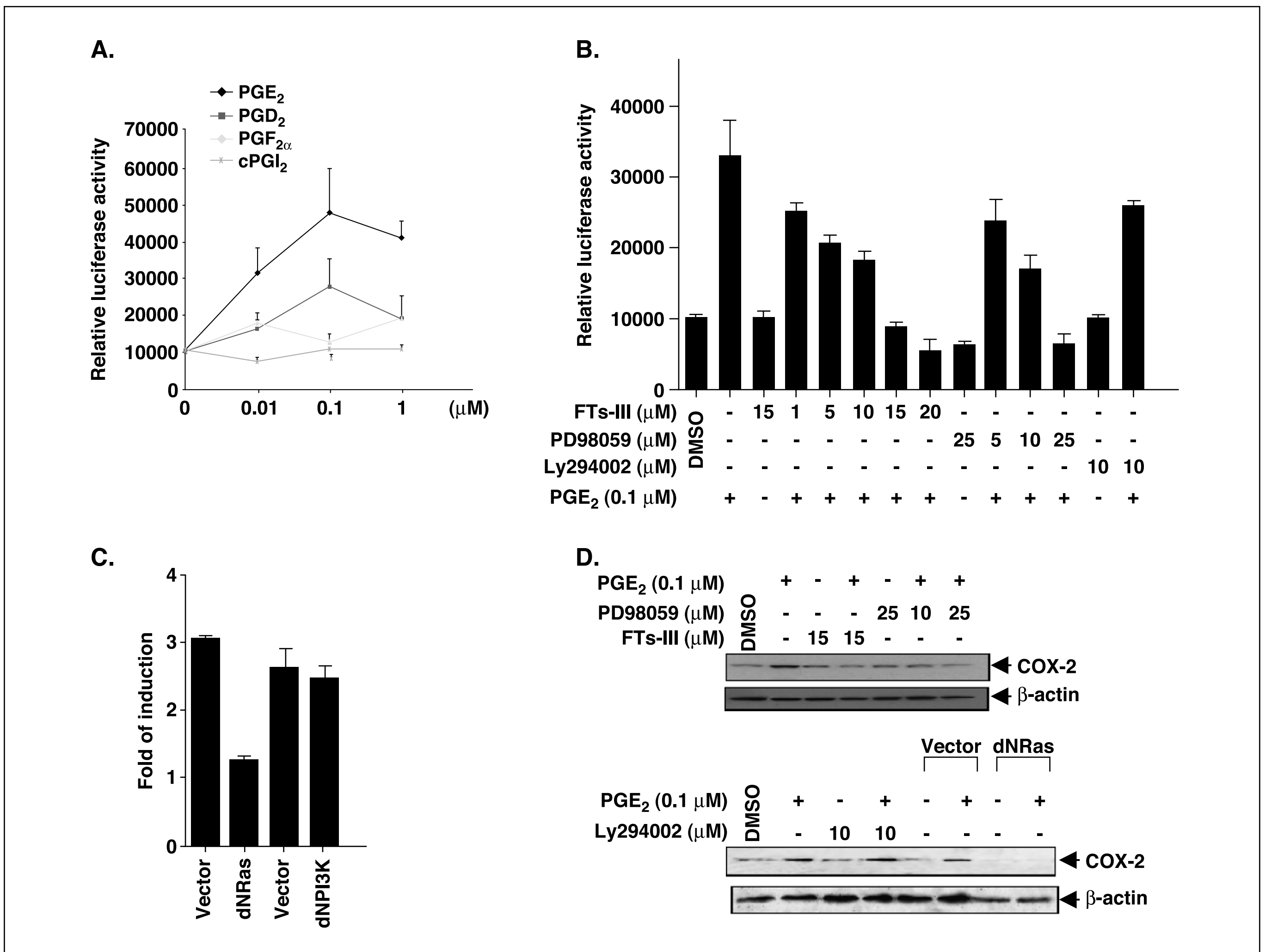

Figure 3. PGE_2 up-regulates COX-2 expression via activation of Ras-MAPK. *A,* HCA-7 cells were transiently transfected with COX-2 (−279 to +59) luciferase reporter gene and pRL-SV40 plasmids followed by treatment with PGE_2, PGD_2, $PGF_{2}\alpha$, $cPGI_2$. *B,* for inhibitor assays, the cells were pretreated with vehicle or increasing doses of inhibitors for 1 hour before the PGE_2 (0.1 μmol/L) treatment after transfection. The dual-luciferase assays were done as described in Materials and Methods after 16 hours of incubation. *Columns,* mean of relative luciferase activity from three independent experiments; *bars,* ±SE. *C,* effects of expression of a dominant negative Ras on PGE_2-up-regulated COX-2 promoter activity. HCA-7 cells were transiently cotransfected with COX-2 (−279 to +59) luciferase reporter gene and either empty vector, dominant-negative Ras, or dominant-negative PI3k plasmids together with pRL-SV40 plasmid followed by treatment with PGE_2 (0.1 μmol/L) treatment for 16 hours. *Columns,* fold induction relative to control (no PGE_2 treatment) from three independent experiments; *bars,* ±SE. *D,* effects of a Ras or MEK inhibitor on the induction of COX-2 protein expression by PGE_2. Cells were cultured and treated in the same conditions as noted in Fig. 2*A*. COX-2 protein levels were analyzed by Western blotting. The blot was subsequently reprobed with β-actin to evaluate protein loading. Representative of three different experiments with similar results.

responsible for PGE_2-stimulated cell proliferation and COX-2 expression, we evaluated the effects of PGE_2 in HCA-7 cells that are known to exhibit COX-2-dependent proliferation (37, 38). HCA-7 cells have been carefully evaluated as an *in vitro* model to investigate COX-2-dependent cell growth (37, 38). These cells express PGE_2 receptors (EP1, EP3, and EP4) and contain wild type Ras. As a first step in identifying the signaling pathways involved, we examined the effect of PGE_2 on HCA-7 cell proliferation and COX-2 expression. A cell proliferation assay was used to directly measure the PGE_2-induced cell proliferation based on the measurement of BrdUrd incorporation during DNA synthesis. As shown in Fig. 2*A*, cell proliferation was stimulated following 24 hours of PGE_2 treatment in a dose-dependent manner. In addition, compared with untreated cells, PGE_2 treatment for 5 days increased cell number by 2-, 3.2-, 3.6-, 4.2-, and 5.2-fold at 0.001, 0.01, 0.1, 1, and 10 μmol/L, respectively (Fig. 2*B*). These results show that PGE_2 can stimulate HCA-7 cell proliferation in a dose-dependent manner.

Next, we determined the ability of PGE_2 to affect COX-2 promoter activity in HCA-7 cells. As shown in Fig. 3*A*, a dose-dependent increase in luciferase activity (representing COX-2 promoter activity) was seen with PGE_2, but not with PGD_2, $cPGI_2$, or $PGF_{2}\alpha$. Since our previous results showed that overexpression of constitutively active Ras up-regulates COX-2 expression (29), we determined whether the Ras-MAPK cascade mediates PGE_2 induction of COX-2 expression in colorectal cancer cells. Our results show that either a highly selective Ras inhibitor (Ftase inhibitor III) or a MEK inhibitor (PD98059) blocks the PGE_2-enhanced COX-2 promoter activity in a dose dependent manner, but not PI3k inhibitor (Ly294002; Fig. 3*B*). Overexpression of a dominant-negative Ras constuct also inhibited PGE_2-up-regulated COX-2 promoter activity, whereas expression of a dominant

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negative PI3k failed to inhibit PGE_2-mediated COX-2 promoter activity (Fig. 3*C*). Similarly, both inhibitors of Ras and MEK inhibit PGE_2-enhanced COX-2 protein expression, which is not affected by a PI3k inhibitor (Fig. 3*D*). Furthermore, overexpression of a dominant negative Ras also blocked both basal and PGE_2-induced COX-2 expression (COX-2 levels were barely detected; Fig. 3*D*, *bottom*). These results show that COX-2 is up-regulated by its own downstream product, PGE_2, and that this autoinduction is dependent on activation of the Ras-MAPK pathway.

PGE_2 Activates Ras-MAPK. To further show that PGE_2 can induce endogenous Ras (wild type) activity, HCA-7 cells were treated with PGE_2 for various times following 24 hours of serum starvation. Ras activation assays were done by selective affinity precipitation of GTP-bound Ras with immobilized glutathione *S*-transferase-c-Raf-1 ras binding domain and Ras-GTP was detected by Western blotting with a pan-Ras antibody. We found that PGE_2 increases activation of Ras in a biphasic manner (Fig. 4*A*). The early phase of Ras activation is dose dependent (Fig. 4*B*, *lanes 1*, *2*, *4*, and *6*), whereas the late phase of Ras activation is independent of PGE_2 (*lanes 1*, *3*, *5*, and *7*). Ras activation was evident as early as 1 minute, reached a maximum level at 5 minutes followed by return to the basal level by 20 minutes after 0.1 μmol/L PGE_2 treatment. However, Ras activation again increased at 180 minutes and persisted for a prolonged time (data not shown). To confirm that equivalent amounts of protein were loaded on the gel, the level of total Ras protein from all samples was examined by Western blotting. All the samples exhibited similar levels of total Ras protein (Fig. 4*A-B*, *bottom*). These results show that PGE_2 stimulates Ras activation in a time-dependent manner.

Because the MAPK cascade is one of the Ras downstream signaling pathways, we next examined whether PGE_2 can activate MAPK signaling. HCA-7 cells were treated with PGE_2 for 5 or 180 minutes. Western blotting was then done with an antibody which recognizes the phosphorylated (activated) form of ERK1/2 and its downstream target ELK-1. As shown in Fig. 4*C*, PGE_2 treatment enhanced phosphorylation of both ERK1/2 and ELK-1 without affecting the levels of total ERK1/2 and ELK-1 proteins. However, there is evidence that the activation of ERK by other G-protein coupled receptor agonists involves both Ras-Raf1-MEK dependent and independent effects. Thus, we examined whether Ras and MEK are required for PGE_2-enhanced ERK activation. HCA-7 cells were pretreated with the selective Ras and MEK inhibitors for 1 hour and then treated with PGE_2 for 5 minutes. Both of the inhibitors significantly blocked the PGE_2-stimulated ERK activation as well as its downstream target Elk-1 (Fig. 4*D*) without showing apparent signs of cytotoxicity at the concentrations and incubation times studied. Furthermore, the late phase of ERK and Elk-1 activation by PGE_2 (180 minutes) was also sensitive to both inhibitors (data not shown). These results show that a Ras-Raf-MEK cascade mediates the PGE_2-induced ERK and Elk-1 activation.

In determining the biological significance of a signaling pathway, results obtained *in vivo* are helpful to verify the data obtained from cultured cells. Thus, we examined whether PGE_2 can activate the MAPK cascade in Apc^{min} mouse adenomas *in vivo*. As shown in Fig. 5*A*, PGE_2 treatment resulted in dramatic increases in both phosphorylated ERK1/2 and Elk-1 in intestinal adenomas as determined by immunostaining. Moreover, because ERK and c-jun-NH_2-kinase activities increase modestly in a subset of human colorectal carcinomas (39), we examined whether the Elk-1 activation is also increased in human colorectal cancers. Western blotting was done to examine Elk-1 activation in human colon cancers and matched normal tissues. Among the 15 pairs of human colon cancer and the matched normal mucosal samples, eight pairs showed high levels of Elk-1 activation (phosphorylation) in the cancer specimens (Fig. 5*B*). To our knowledge, this is the first report that Elk-1 activity is increased in colorectal cancer.

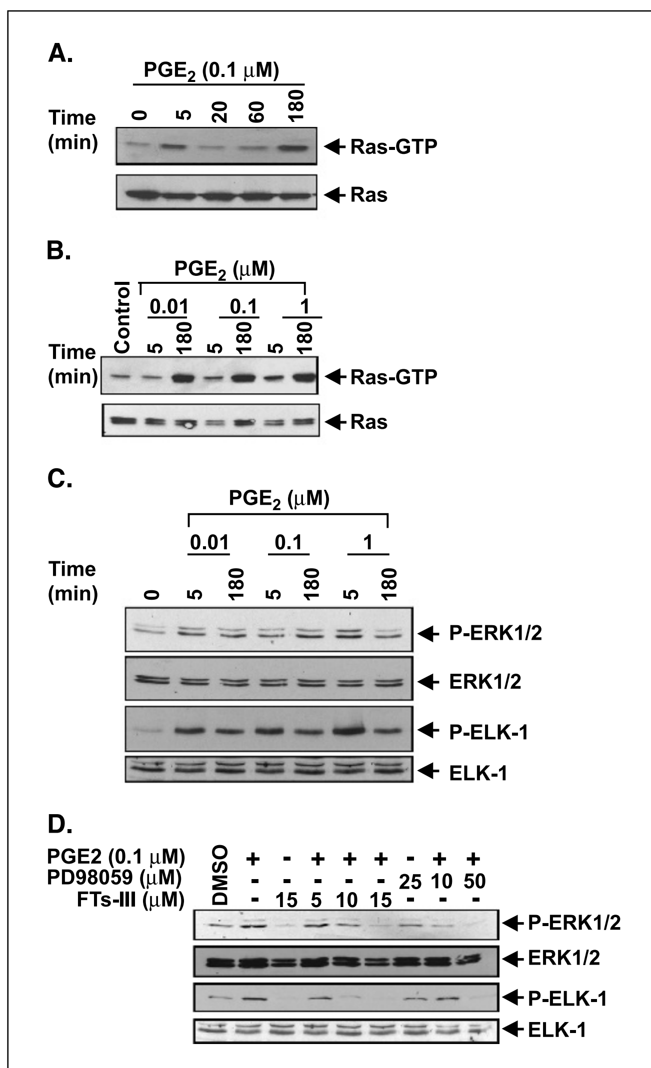

Figure 4. PGE_2 induces activation of the Ras-MAPK cascade. *A,* HCA-7 cells were treated with PGE_2 (0.1 μmol/L) for the indicated times after serum-starved for 24 hours. GTP-bound Ras was affinity-precipitated from 400 μg of whole cell extract using a GST-c-Raf-1/Ras binding domain fusion protein. GTP-bound Ras proteins were detected by Western blotting using a pan-Ras antibody. *Bottom,* amount of total Ras protein showing equal protein in each sample. Representative of three different experiments showing similar results. *B,* HCA-7 cells were serum-starved for 24 hours before PGE_2 treatment at indicated concentrations of PGE_2 for 5 and 180 minutes. Ras activation assays were done as mentioned above. Representative of three different experiments with similar results. *C,* PGE_2 enhances activation of ERK and Elk-1. HCA-7 cells were treated with PGE_2 at indicated concentrations for 5 and 180 minutes after serum-starved for 24 hours. ERK1/2 and Elk-1 activation was detected by measuring the levels of phosphorylated ERK1/2 and Elk-1 by Western blotting using anti-phospho-ERK1/2 (Tyr204) or anti-phospho-Elk-1 (Ser383) antibody, respectively. The blots were reprobed with ERK1/2 or Elk-1 antibody to monitor equal loading of samples. This figure is representative of three different experiments with similar results. *D,* effect of a Ras inhibitor (Fts-III) or MEK inhibitor (PD98059) on PGE_2-induced ERK1/2 and Elk-1 activation. The cells were pretreated with the indicated inhibitors for 1 hour after serum starvation for 24 hours and then incubated with 0.1 μM PGE_2 for 5 minutes. ERK1/2 and Elk-1 activation was measured following the same procedure as mentioned above. Representative of three different experiments with similar results.

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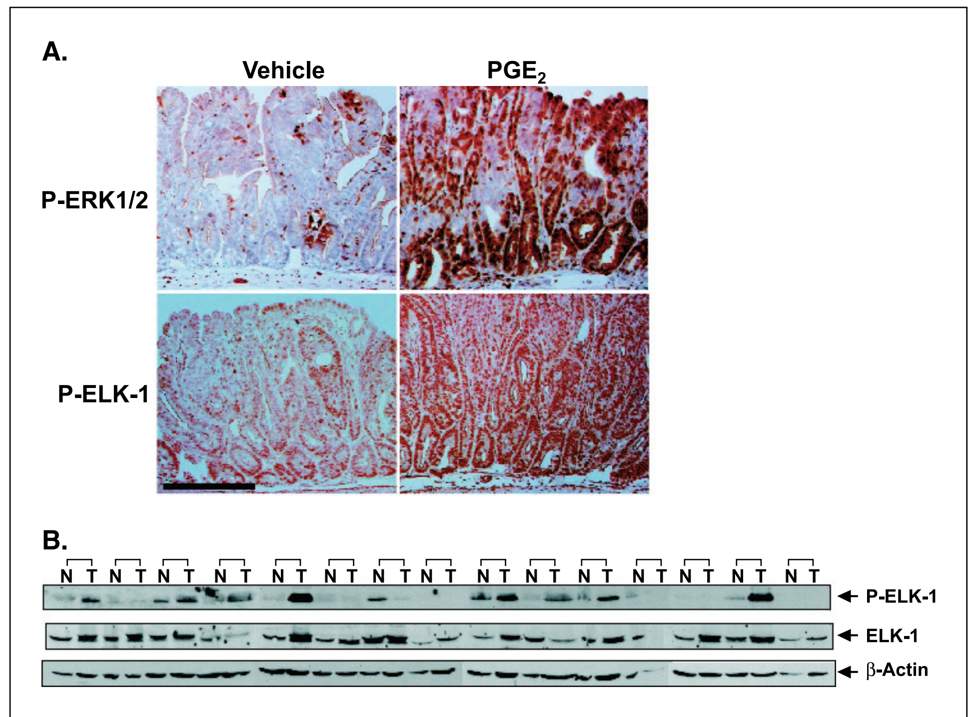

Figure 5. PGE_2 induces activation of ERK and Elk-1 *in vivo*. *A,* PGE_2 induces activation of ERK and Elk-1 in the intestinal adenomas of Apc^{min} mice. Sections of small intestine were immunostained with phospho-ERK1/2 antibody (*top*) or phospho-Elk-1 antibody (*bottom*). Representative section of strong immunoreactive staining (*red*) for phospho-ERK1/2 or phospho-Elk1 in the epithelial cells of the adenomas taken from mice treated with PGE_2 and weak staining was found in the adenomas taken from mice treated with vehicle. *Bar*, 100 μm. *B,* Elk-1 activation in human colon tumor and matched normal tissue. Whole cell protein lysates from 15 different matched pairs of normal (*N*) and cancer tissue (*T*) were analyzed for Elk-1 phosphorylation and expression by using immunoblot analysis. Each blot was subsequently reprobed for β-actin to evaluate protein loading.

Taken together, these results show that PGE_2 up-regulates COX-2 expression through activation of the Ras-MAPK pathway.

A Ras-MAPK Cascade Is Required for PGE_2-Stimulated Cell Proliferation. Finally, we examined whether the Ras-MAPK pathway is required for PGE_2-induced cell proliferation. HCA-7 cells were pretreated with either the Ras or MEK inhibitor for 1 hour, and then treated with indicated concentration of PGE_2. Both cell proliferation by ELISA and cell counting were used to measure cell proliferation and cell growth. We observed that PGE_2-induced cell proliferation was totally blocked by the Ras inhibitor at 20 μmol/L or MEK inhibitor at 25 μmol/L, respectively (Fig. 6). These data show that the Ras-MAPK cascade is required for PGE_2-stimulated cell proliferation and growth.

Discussion

There is considerable preclinical evidence indicating that COX-2 plays a role in the development of various types of cancers, including colorectal, breast, bladder, skin and lung cancer. COX-2 derived PGE_2 was found to promote colorectal adenoma growth in Apc^{min} mice. The aim of our current study was to understand the molecular mechanism(s) by which PGE_2 promotes intestinal tumor growth.

Our laboratory was the first to report significant elevation of COX-2 expression in 85% of human colorectal carcinomas and in ~50% of colorectal adenomas (1). However, the mechanism(s) by which COX-2 is highly expressed in a number of solid malignancies is not yet completely understood. COX-2 mRNA and protein are normally very low or undetectable in normal intestinal tissues, but are rapidly induced in response to inflammation, cytokines, growth factors, oncogenes, endotoxins, and other chemicals. Here we show that PGE_2 can amplify the expression of COX-2, a key regulatory enzyme in the PG biosynthetic pathway, in colorectal carcinoma cells through a positive feedback loop. This self-amplifying loop may help explain why COX-2 is constitutively overexpressed in majority of colorectal cancers.

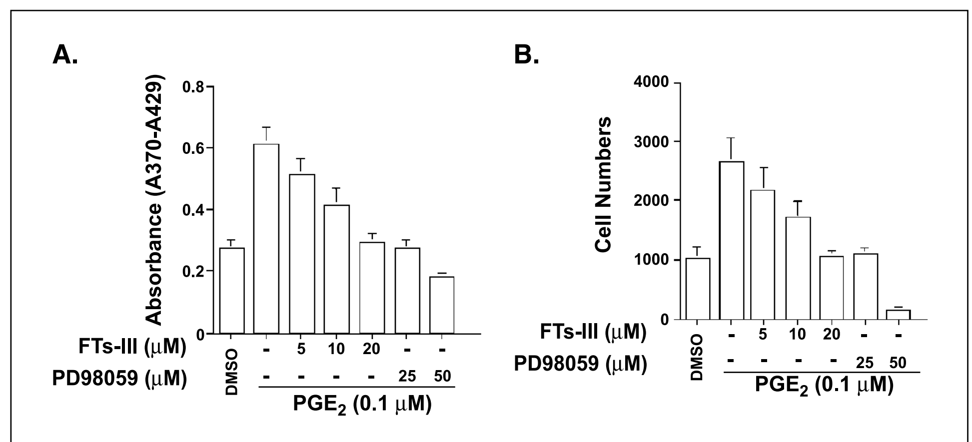

Figure 6. A Ras-MAPK cascade is involved in PGE_2-stimulated cell proliferation. *A,* HCA-7 cells (1×10^4) were seeded in a 96-well plate and pretreated with indicated inhibitors for 1 hour after serum starvation for 24 hours. The cells were incubated with 0.1 μmol/L PGE_2 for 24 hours. Means ± SE of absorbance ($A_{370\ nm}$-$A_{492\ nm}$) from three independent experiments in triplicate. *B,* in addition, cells (1×10^4) were seeded in a 12-well plate and pretreated with indicated inhibitors for 1 hour after serum starvation for 24 hours. The cells were incubated with 0.1 μmol/L PGE_2 for 5 days. Cell numbers were counted from triplicate wells. Means ± SE from three independent experiments.

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Our results show that PGE_2 activates Ras at early (5 minutes) and late times (3-6 hours). A likely explanation for this observation is that there is a negative feedback loop that inactivates EP receptor function following ligand binding, which in turn inhibits Ras activation. In general, ligand binding to G-protein coupled receptors results in receptor phosphorylation, desensitization, and sequestration. For example, chemokine receptor CXCR2 desensitization occurs within 1 minute, whereas receptor sequestration is a much later event (30-60 minutes) (40). We postulate that PGE_2 binding to its cognate receptors also leads to the desensitization and sequestration of EP receptors, which provides negative feedback after 5 minutes of PGE_2 stimulation (Fig. 4*A*). Another possible explanation is that PGE_2 is metabolized to an inactive cyclopentenone PGA_2 in cultures similar to that has been observed in other biological fluids (41). Therefore, Ras activation in the late activation phase may depend primarily upon newly synthesized PGE_2 through an autocrine loop.

Our results also show that Ras activation during the early phase results in increased COX-2 expression via a MAPK-dependent pathway. We have previously shown that COX-2 expression is elevated within 30 minutes after transforming growth factor α or 12-*O*-tetradecanoylphorbol-13-acetate treatment in rat intestinal epithelial (RIE-1) cells (42). In addition, PGE_2 increases COX-2 mRNA expression in human prostatic carcinoma PC-3 cells with the highest levels of stimulation seen at 3 hours (43). These and our present results suggest that the constitutive (late phase) Ras activation is dependent on the newly synthesized PGE_2 by COX-2. Alternatively, increased levels of PGE synthase in addition to COX-2 may drive new synthesis of PGE_2. This is consistent with the observation that PGE synthase is up-regulated after activation of the MAPK pathway (44). Our present findings suggest a novel mechanism by which COX-2-derived PGE_2 constitutively activates Ras in a positive autocrine feedback loop. Thus, PGE_2 may mimic the effects of an aberrant Ras function even in the absence of an actual *Ras* gene mutation. This implies that the influence of wild-type Ras in human colorectal cancer is even greater than what is expected based on the known frequency of Ras mutations.

Activated ERK can directly phosphorylate transcription factors, such as Elk-1, which binds to the serum-responsive element of the c-*fos* promoter (45, 46). In this respect, PGE_2 can induce the expression of early response genes, such as *c-fos*, *c-jun*, *jun B*, and *egr-1* (47) and formation of heterodimers or homodimers of the Jun and Fos family members form the activator protein−1 transcription factor complex. A putative activator protein−1 cis-activating consensus element is present in the COX-2 promoter and activator protein−1 activation mediates the induction of COX-2 in intestinal epithelial cells in response to the G-protein-coupled receptor ligand, Bombesin (48). Thus, it is likely that PGE_2 signals through a Ras-MAPK-dependent pathway to activate activator protein−1, which then regulates *COX-2* gene expression, leading to increased cell proliferation. However, further investigation is needed to confirm this speculation. Nonetheless, analysis of signaling pathways affected by PGE_2 reveals that a Ras-ERK-Elk-1 pathway mediates PGE_2 downstream signaling leading to increased cell proliferation and up-regulation of COX-2 expression. In conclusion, we show for the first time that a key oncogenic signaling pathway mediate the regulation of COX-2 by PGE_2 in colorectal carcinoma cells. Our data support a novel mechanism by which COX-2-derived PGE_2 promotes human cancer cell growth by autoregulation of COX-2 expression, which depends primarily on PGE_2-induced Ras-MAPK pathway. An understanding of PGE_2 downstream signaling transduction pathways critical for tumor growth control may lead to the development of novel therapeutic and chemoprotective options for colorectal cancer patients.

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