Prostaglandin E_2 promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor δ

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Summary

Cyclooxygenase-derived prostaglandin E_2 (PGE_2) is the predominant prostanoid found in most colorectal cancers (CRC) and is known to promote colon carcinoma growth and invasion. However, the key downstream signaling pathways necessary for PGE_2-induced intestinal carcinogenesis are unclear. Here we report that PGE_2 indirectly transactivates PPARδ through PI3K/Akt signaling, which promotes cell survival and intestinal adenoma formation. We also found that PGE_2 treatment of *Apc*^{min} mice dramatically increased intestinal adenoma burden, which was negated in *Apc*^{min} mice lacking PPARδ. We demonstrate that PPARδ is a focal point of crosstalk between the prostaglandin and Wnt signaling pathways which results in a shift from cell death to cell survival, leading to increased tumor growth.

Introduction

Colorectal cancer (CRC) leads to significant cancer-related morbidity and mortality in most industrialized countries. Chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs) and other cyclooxygenase (COX) inhibitors is known to reduce the risk for CRC in humans (Turini and DuBois, 2002). Identifying the molecular mechanisms underlying the protective effects of NSAIDs and selective COX inhibitors is important, since this may reveal other potential molecular targets for cancer prevention or treatment. There are two cyclooxygenase isoforms, COX-1 and COX-2, and both catalyze the conversion of arachidonic acid (AA) to endoperoxide intermediates that are ultimately converted to prostaglandins (PGE_2, PGD_2, $PGF_2\alpha$, PGI_2, and TxA_2) by specific PG synthases (DuBois et al., 1998). COX-1 is constitutively expressed in most cells and tissues under normal circumstances. Conversely, COX-2 is usually absent from most tissues or expressed at very low levels, but is highly induced in response to inflammatory mediators, growth factors, oncogene activation, and tumor promoters (DuBois et al., 1998).

There is both pharmacologic and genetic evidence supporting the hypothesis that NSAIDs reduce colorectal cancer risk via inhibition of COX-2 (Gupta and DuBois, 2001). For example, levels of the COX-2 enzyme are elevated in a large percentage of colorectal adenomas and adenocarcinomas (Eberhart et al., 1994), and COX-2 selective inhibitors have potent antitumor effects in several different animal models of colorectal cancer (Chulada et al., 2000; Jacoby et al., 2000; Oshima et al., 1996; Prescott and Fitzpatrick, 2000; Reddy et al., 2000; Sheng et al., 1997). Moreover, forced overexpression of COX-2 in intestinal epithelial cells results in resistance to apoptosis and increased cell adhesion to extracellular matrix (Tsujii and DuBois, 1995). Finally, deletion of the COX-2 gene results in a marked decrease of adenoma burden in both the small and large intestine in *Apc*$^{\Delta 716}$ mutant mice (Oshima et al., 1996) and in *Apc*^{min} mice (Chulada et al., 2000).

Although there is strong evidence to support a pro-oncogenic role for COX-2 in colorectal carcinogenesis, the molecular mechanism(s) by which the enzyme promotes tumor formation is less clear. Recent evidence points to an important role for

SIGNIFICANCE

Long-term use of cyclooxygenase (COX) inhibitors in humans leads to a 50% decrease in risk for colorectal cancer. It is not clear precisely how COX-2-derived prostaglandins induce intestinal adenoma formation or the key downstream signaling pathways required for these effects. This report demonstrates a novel link between the COX-2 derived PGE_2 and Wnt/β-catenin/Tcf signaling pathways that is crucial in regulating intestinal adenoma burden, and provides in vivo proof that PGE_2 regulates colorectal tumor growth. Furthermore, our study demonstrates that PPARδ is required for PGE_2 to stimulate colorectal adenoma growth. These results support the rationale for considering the development of PPARδ antagonists for use in cancer prevention and/or treatment.

the downstream metabolite PGE_2 in COX-2-mediated tumor promotion. PGE_2 levels are elevated in human colorectal cancers and adenomas in familial adenomatous polyposis (FAP) patients (Giardiello et al., 2004; Mahmoud et al., 1999; Pugh and Thomas, 1994; Rigas et al., 1993). Exposure of CRC cells to PGE_2 in vitro results in protection against programmed cell death (Sheng et al., 1998) and an increase in both cell proliferation and migration (Sheng et al., 2001b). Studies in humans revealed that adenoma regression was more effective when tissue PGE_2 levels were dramatically reduced following NSAID treatment (Giardiello et al., 2004). Finally, 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 (Mutoh et al., 2002; Sonoshita et al., 2001; Watanabe et al., 1999). However, the in vivo role of PGE_2 in promoting intestinal tumor growth remains unclear, but PGE_2 is known to regulate cell proliferation and apoptosis.

Peroxsiome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that are members of the nuclear hormone receptor superfamily (Willson et al., 2000). Putative endogenous ligands for PPARs include certain fatty acids and fatty acid metabolites. Three distinct PPAR isoforms (α, δ/β, and γ) have been isolated and characterized. The PPAR α and γ subtypes play essential roles in fatty acid catabolism and storage. Emerging evidence suggests that the PPARδ/β subtype is important for embryo implantation (Lim et al., 1999) and fatty acid oxidation (Wang et al., 2003), and as a regulator of cell survival in both the kidney following hypertonic stress (Hao et al., 2002) and in the skin following wound injury (Di-Poi et al., 2002, 2003).

There are also data linking PPARδ to CRC. For example, PPARδ is overexpressed in a high percentage of colorectal cancers (Gupta et al., 2000; He et al., 1999). Furthermore, Wnt/APC/β-catenin and K-Ras, two signaling pathways that are commonly affected during CRC development, directly regulate PPARδ expression. A majority of colorectal cancers contain inactivating mutations in the *APC* gene; a major consequence of this inactivation is increased expression of pro-oncogenic genes (e.g., c-MYC and cyclin D1) via the β-catenin/T cell factor-4 (TCF-4) transcriptional complex (He et al., 1998). PPARδ is also a target of the APC/β-catenin pathway (He et al., 1999). Restoration of wild-type APC in human colon cancer cells leads to a decrease in PPARδ levels, and mutant β-catenin activates the PPARδ promoter via TCF-4 response elements. Gain-of-function mutations in the K-Ras oncogene are also found in a majority of colorectal cancers. Recently, PPARδ expression and activity were found to be significantly increased in an intestinal epithelial cell line conditionally immortalized with mutant K-Ras (Shao et al., 2002).

Although the placement of PPARδ downstream of key mediators involved in the development of colorectal cancer is suggestive that the receptor is a tumor promoter in the intestine, there are conflicting data in support of a direct role for the receptor in oncogenesis. Somatic deletion of both PPARδ alleles from an established colorectal cancer cell line results in a decrease in tumorigenicity (He et al., 1999). In contrast, PPARδ was shown to be dispensable for small intestinal polyp formation in *Apc*^{min} mice (Barak et al., 2002) or to attenuate polyp formation in chemical and genetic models of colon cancer (Harman et al., 2004). However, one of these studies (Barak et al., 2002) was extremely limited by the small number (n = 3) of *PPAR*$\delta^{-/-}$/*Apc*^{min} mice available. We recently demonstrated that PPARδ activation by a high-affinity, subtype-selective agonist significantly increases the number and size of small intestinal polyps in *Apc*^{min} mice (Gupta et al., 2004). These findings suggest that while removing the *Ppard* gene has little effect, activation of PPARδ signaling plays a major role in accelerating intestinal polyp formation in vivo.

Since PGE_2 and PPARδ are known to both promote cell survival and affect carcinoma growth in vitro, we tested the hypothesis that the ability of PGE_2 to accelerate adenomatous polyp growth was dependent on PPARδ. PGE_2 upregulated PPARδ transcriptional activity via activation of PI3K-Akt that led to increased cell survival. The antiapoptotic effect of PGE_2 was negated in cells expressing a dominant-negative PPARδ protein. Most importantly, PGE_2 was found to promote intestinal epithelial cell survival and colorectal adenoma growth in *Apc*^{min}, but not *PPAR*$\delta^{-/-}$/*Apc*^{min}, mice. Collectively, our results identify PPARδ as a critical downstream mediator in PGE_2-stimulated promotion of colorectal tumor growth.

Results

PGE_2 enhances PPARδ transcriptional activity via PI3K/Akt

As a first step in testing the hypothesis that PGE_2 promotes CRC growth via PPARδ, we determined the ability of PGE_2 to affect PPARδ transcriptional activity. We utilized LS-174T human colon carcinoma cells for these experiments, since these cells express EP4 receptors (Sheng et al., 2001b) and produce very little endogenous PGE_2 (0.13 ng/10^5 cells) compared to HCA-7 cells (4.2 ng/10^5 cells) (Shao et al., 2000). LS-174T cells were transfected with the PPRE3-tk-luciferase reporter vector in which luciferase gene expression is driven by three tandem repeats of the PPAR-response element (PPRE) from the acyl-CoA oxidase gene. A dose-dependent increase in luciferase activity (representing activation of endogenous PPAR receptor) was seen with either a high-affinity, subtype-selective synthetic ligand for PPARδ (GW1516) (Figure 1A) or PGE_2, but not with PGA_2 or $PGF_2\alpha$ (Figure 1B). Surprisingly, cotreatment of PGE_2 and GW1516 has synergistic effects resulting in a dramatic increase in PPAR transactivation (Figure 1C). Because all three PPAR subtypes can bind to and activate this PPRE reporter, PGE_2 could mediate its effects through any of the three PPAR subtypes. To determine the subtype selectivity of this response, cells were also transfected with expression vectors for PPARα, -δ/β, and -γ and treated with increasing doses of PGE_2. PGE_2 increased transactivation of the PPRE3-tk-luc reporter only in cells transfected with PPARδ/β, but not -α or -γ (Figure 1D), indicating that this response is specific for the PPARδ/β subtype. Similar results were obtained in the HCA-7 colorectal carcinoma cells (data not shown).

To further characterize the specificity and selectivity of this response, we tested the ability of dominant-negative PPARδ (dNPPARδ) to block the increase in PPARδ transcriptional activation seen in cells treated with PGE_2. The dNPPARδ construct we utilized contains an inactivating mutation in the AF-2 domain that encodes a receptor which retains its DNA binding domain but lacks the ability to recruit transcriptional coactivators. PPAR constructs containing such mutations in the AF-2 domain have been shown to be a valuable tool to specifically antagonize PPAR signaling (Gurnell et al., 2000). The ability of both PGE_2 and

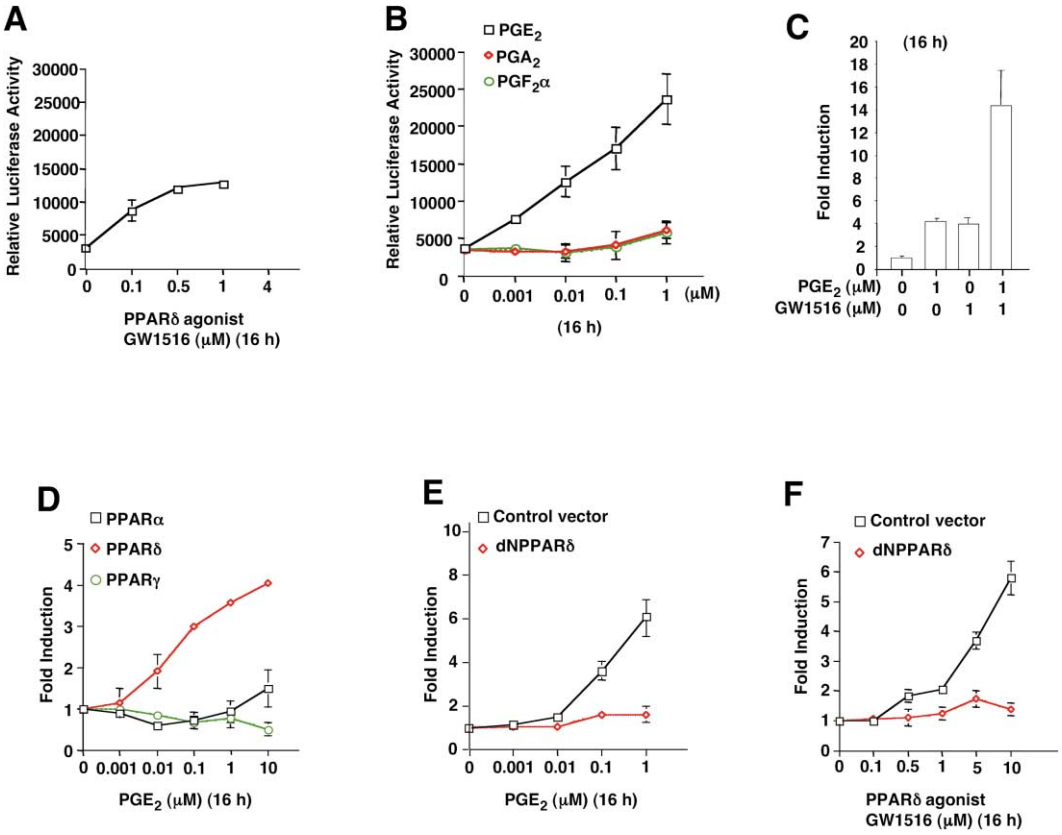

Figure 1. Exposure of LS174T cells to PGE_2 enhances PPARδ transcriptional activity

A–C: Cells were transiently transfected with $PPRE_3$-tk-luciferase and pRL-SV40 plasmids followed by treatment with a selective synthetic PPARδ agonist GW1516 (**A**), PGE_2, PGA_2, or $PGF_2\alpha$ (**B**), and cotreatment with PGE_2 and GW1516 (**C**). The dual luciferase assays were performed as described in the Experimental Procedures. Data are represented as the mean ± S.E.M. of relative luciferase activity from three independent experiments. The data were analyzed using the Student's paired t test ($p < 0.05$).

D: Cells were transiently cotransfected with a $PPRE_3$-tk-luciferase/pRL-SV40 and either PPARα, PPARδ, or PPARγ expression plasmids, followed by treatment with PGE_2. Data are represented as the mean ± S.E.M. of fold induction from three independent experiments. The relative luciferase activity of control cells was arbitrarily set to equal 1.0; all other comparisons were relative to this standard. The data were analyzed using the Student's paired t test ($p < 0.05$).

E and F: Cells were transiently cotransfected with $PPRE_3$-tk-luciferase/pRL-SV40 and either empty vector or dominant-negative PPARδ plasmids followed by treatment with a PGE_2 (**E**) or a PPARδ agonist (**F**). Data are represented as the means ± S.E.M. of fold induction from three independent experiments.

the PPARδ synthetic ligand GW1516 to activate endogenous PPARδ activity was blocked in LS174T cells cotransfected with dNPPARδ (Figures 1E and 1F).

At least three potential mechanisms that could explain the ability of PGE_2 to enhance PPARδ transactivation were considered: (1) PGE_2 directly binds to PPARδ and serves as an activating ligand; (2) PGE_2 upregulates PPARδ expression; and (3) PGE_2 modulates PPARδ transactivation through activation of EP receptor signaling. Certain prostaglandins have been shown to modify PPAR transcriptional activity through direct activation of the receptor. For example, the PGD_2 metabolite 15-deoxy PGJ_2 directly binds to and activates PPARγ (Forman et al., 1995; Kliewer et al., 1995). Additionally, the prostacyclin analog carbaprostacyclin has been shown in a modified ligand binding assay to directly bind to PPARδ (Forman et al., 1997), and endogenous prostacyclin production correlates with PPARδ activity (Gupta et al., 2000). It is unlikely, however, that PGE_2 is a direct ligand for PPARδ. Three independent groups have published data demonstrating that PGE_2 is incapable of directly binding to any of the three PPAR subtypes (Forman et al., 1997; Kliewer et al., 1995; Yu et al., 1995). Furthermore, we have also confirmed, using the PPAR-GAL4 ligand binding reporter assay, that PGE_2 is unable to bind directly to PPARδ (data not shown).

It is also possible that PGE_2 could increase the levels of PPARδ resulting in enhanced transactivation. However, treatment of LS174T cells with PGE_2 had no effect on the protein levels of PPARδ (Figure 2A). Finally, we hypothesized that PGE_2 may indirectly modify PPARδ transcriptional activity via a MAPK or/and the PI3K-Akt pathways, since PGE_2 can activate both pathways through the EP4 receptor in LS-174T cells (Sheng et al., 2001b). For example, PGE_2 induces phosphorylation of Akt in a dose-dependent manner, but does not affect Akt protein levels in LS-174T cells (Buchanan et al., 2003; Sheng et al., 2001b). PGE_2-induced activation of Akt was completely blocked by the PI3K inhibitor LY294002 (5–10 μM) (Buchanan et al., 2003; Sheng et al., 2001b). Cotreatment using PGE_2 and a MAPK inhibitor had no effect on the ability of PGE_2 to increase PPARδ activity (data not shown). However, pretreatment of LS174T

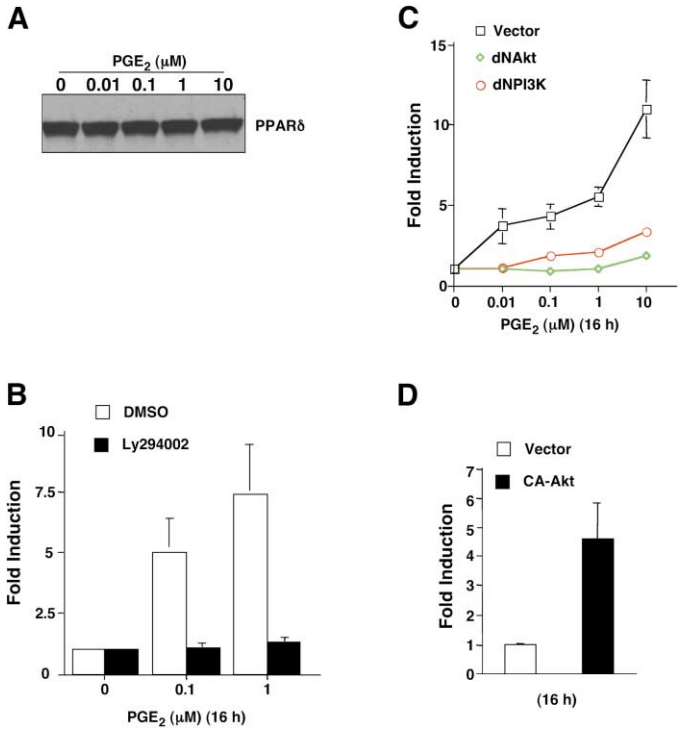

Figure 2. Mechanism by which PGE_2 regulates PPARδ activity

A: PGE_2 does not affect PPARδ protein levels. The LS-174T cells were treated with PGE_2 for 24 hr following overnight serum deprivation. PPARδ protein expression was determined by Western blot analysis.

B: The PI3K inhibitor LY294002 blocks PGE_2-upregulated PPARδ transactivation. Cells were transiently cotransfected with $PPRE_3$-tk-luciferase and pRL-SV40 plasmids. The transfected cells were pretreated with either DMSO as control or 5 μM PI3K inhibitor LY294002 for 1 hr before stimulation of PGE_2. Data are represented as the means ± S.E.M. of fold induction from three independent experiments.

C and D: The PI3K-Akt pathway is involved in PGE_2-mediated increased PPARδ transactivation. **C:** Cells were transiently cotransfected with $PPRE_3$-tk-luciferase/pRL-SV40 and either empty vector, dominant-negative Akt, or dominant-negative PI3K plasmids followed by treatment with PGE_2. **D:** Cells were transiently cotransfected with $PPRE_3$-tk-luciferase/pRL-SV40 and either empty vector or constitutively active Akt without PGE_2 treatment. Data are represented as the means ± S.E.M. of fold induction from three independent experiments.

cells with a specific PI3K inhibitor (LY294002) reduced PGE_2-induced PPARδ transcriptional activity (Figure 2B). In addition, PGE_2 was unable to induce PPARδ transactivation in cells transiently transfected with constructs expressing either dominant-negative PI3K or Akt (Figure 2C), while the expression of constitutively active Akt enhances PPARδ transactivation in the absence of exogenous PGE_2 (Figure 2D). These results suggest that PGE_2 indirectly enhances PPARδ transactivation via the PI3K/Akt cascade.

PGE_2 promotes cell survival via PPARδ in cultured CRC cells

The observation that exposure of cultured CRC cells to PGE_2 enhances PPARδ transactivation indicates that PPARδ is an important downstream mediator of PGE_2. PGE_2 has been shown to decrease the basal apoptotic rate in human CRC cells, and activation of PPARδ protects against apoptosis in renal medullary interstitial cells following hypertonic stress and in keratinocytes following wound injury. To determine whether PGE_2 promotes cell survival by increasing PPARδ activity, we tested the ability of a dNPPARδ to block the antiapoptotic effect of PGE_2 in the LS174T cells. Cultured LS-174T cells were treated with increasing concentrations of PGE_2 and a selective PPARδ agonist (GW1516) following serum deprivation. Serum starvation resulted in significant apoptosis (28%) compared to the low basal apoptotic rate (4%) of cells grown in 10% FBS (Figure 3A). However, treatment with PGE_2 or a PPARδ agonist attenuated apoptosis to that seen at basal levels (10% FBS conditions), suggesting that selective PPARδ activation mimics the effects seen following PGE_2 treatment (Figure 3A). To further evaluate whether PGE_2-induced cell survival requires activation of PPARδ, cells were transfected with either an empty vector or a dominant-negative PPARδ construct and then treated with PGE_2. Similar to parental cells, PGE_2 decreased apoptosis in the empty vector transfected cells, but failed to attenuate apoptosis in cells expressing the dominant-negative PPARδ protein (Figure 3B). Similarly, dominant-negative PPARδ also blocked the ability of GW1516 to inhibit apoptosis, suggesting that it was effective in blocking PPARδ activation (Figure 3C). These studies demonstrate that PGE_2-mediated cell survival requires activation of PPARδ.

Next we tested the effect of giving PGE_2 and GW1516 simultaneously on programmed cell death. We also determined whether the PI3K pathway mediates PGE_2-induced cell survival. As shown in Figure 3D, cotreatment with PGE_2 and GW1516 had synergistic effects on cell survival. In addition, the PI3K inhibitor LY294002 reversed the ability of PGE_2 to protect cells from undergoing apoptosis (Figure 3D). In contrast, LY294002 failed to inhibit GW1516-induced resistance to apoptosis (Figure 3D). These results further support the hypotheses that the PI3K/Akt pathway mediates the effects of PGE_2 on cell survival and that PPARδ acts downstream of the PI3k-Akt pathway.

PGE_2 promotes intestinal adenoma formation and cell survival in *Apc^{min}* mice via PPARδ

A large percentage of human colorectal polyps are reported to contain inactivating mutations in the *APC* gene. Thus, *Apc^{min}* mice, in which the presence of a germline mutation in *APC* results in the development of multiple polyps in the small intestine, are a commonly used system to study intestinal polyposis. Deletion of the *COX-2* gene results in decreased tumor formation in the small intestine and colon of *Apc^{min}* and *Apc$^{\Delta 716}$* mutant mice (Chulada et al., 2000; Oshima et al., 1996), suggesting that COX-2 derived PGs promote adenoma growth in this model system.

Use of stable synthetic PGE_2 analogs in *Apc^{min}* mice has led to contradictory results (Hansen-Petrik et al., 2002; Wilson and Potten, 2000). We are not aware of any published reports describing the effects of exogenous administration of native PGE_2 on adenoma formation in *Apc^{min}* mice. *Apc^{min}* mice were treated with vehicle (PBS) or PGE_2 (150 or 300 μg/mouse twice daily). The body weights of mice treated with the vehicle (PBS) or PGE_2 were comparable throughout the study. However, after 5 weeks of treatment, 4 of 9 mice in the PGE_2-treated group (150 μg/mouse/twice daily) developed rectal bleeding (data not shown). Thus, all animals were sacrificed at 13 weeks of age to avoid undue pain and suffering. Plasma samples were analyzed for

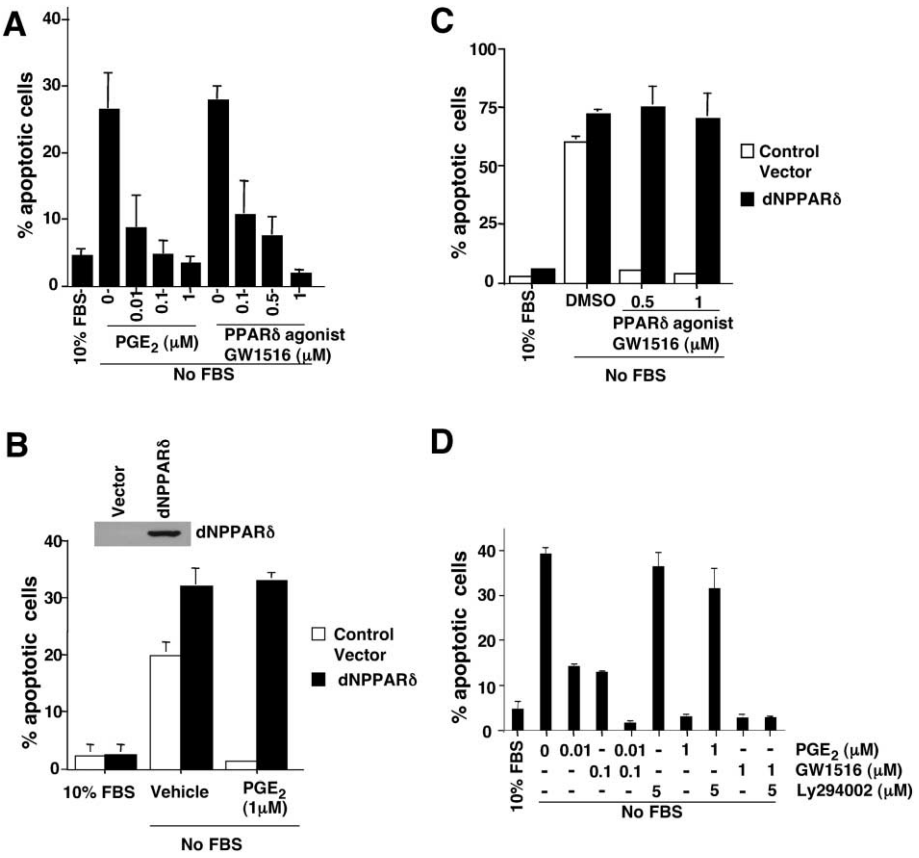

Figure 3. PPARδ is required for the antiapoptotic effects of PGE_2 on LS-174T cells

A: PGE_2 and a PPARδ selective agonist protect LS-174T cells from undergoing apoptosis following serum deprivation. The cells were treated with PGE_2 as indicated or GW1516 for 3 days after removal of serum. The number of apoptotic cells was determined by flow cytometry using an annexin V-FITC kit. Data are expressed as the mean ± S.E.M. of percent of apoptotic cells from three separate experiments.

B and C: Expression of a dominant-negative PPARδ protein inhibits the antiapoptotic effects of PGE_2. Cells transfected with empty vector or dominant-negative PPARδ were treated with PGE_2 (**B**) or the selective PPARδ agonist GW1516 (**C**) for 3 days following serum starvation. The apoptosis assays were performed as described above. The top panel in **B** represents dominant-negative PPARδ protein expression in LS-174T cells transfected with empty vector or dNPPARδ construct.

D: PGE_2 and a PPARδ agonist have synergistic effects on apoptosis, and the PI3K pathway is involved in the PGE_2-induced cell survival. The parental cells were either treated with each agent alone (PGE_2, GW1516, or LY294002) or co-treated with a combination of PGE_2 and GW1516, PGE_2 and LY294002, or GW1516 and LY294002 for 3 days after removal of serum. The apoptosis assays were performed as described above.

PGE_2 concentration, and the intestine (both small and large) was examined to determine polyp size and number.

Mice gavaged with PGE_2 (150 μg or 300 μg/mouse twice daily) had higher PGE_2 levels in plasma and intestinal tissue than the control group (vehicle) or the group treated with a lower dose of PGE_2 (75 μg/mouse/twice daily), demonstrating that exogenous administration of PGE_2 results in increased levels in intestinal target tissues (Figure 4A). All mice gavaged with PGE_2 at either 150 or 300 μg developed polyps in the large intestine, whereas only eleven percent of control mice (1/9) developed colorectal polyps (Figure 4B). More importantly, the average number of colorectal polyps per mouse at 150 and 300 μg PGE_2 significantly increased (3.3 ± 0.29 and 2.2 ± 0.58, respectively) compared to control mice (0.33 ± 0.33) (Figure 4B). As expected, *Apc^{min}* mice in all groups developed polyps in the small intestine. However, the average number of small intestinal polyps in the control group was 50.3 ± 5.5, and the number of polyps increased to 64.3 ± 4.2 and 100.8 ± 16.2, respectively, in mice treated with either 150 or 300 μg PGE_2 (Figure 4C). A similar trend was noted when measuring polyp size. The polyps in both the small and large intestine of mice treated with either dose of PGE_2 were significantly larger as compared to vehicle treated mice (Figures 4D and 4E). This was especially true in the colon (Figures 4D and 5A), where there was a dramatic increase in large polyps (>4 mm). However, the size distribution of the *Apc^{Min}* colon polyps following treatment with 150 μg PGE_2 showed a bimodal distribution (Figure 4D). These studies clearly establish that PGE_2 increases colorectal and small intestinal polyp burden in *Apc^{min}* mice.

Histologic analysis showed that large, medium, or small size polyps in the colon represent advanced adenomas (Figures 5A and 5B) and microadenomas (data not shown). Overall, PGE_2-treated *Apc^{min}* mice had a greater number of larger adenomas than the control group along the entire length of the small and large intestine. In determining the true biological significance of a novel pathway, results obtained in vivo are generally considered to be much more meaningful than data obtained from cultured cells. Thus, we examined whether PGE_2 can activate PI3K-Akt signaling in *Apc^{min}* mouse adenomas in vivo. As demonstrated in Figure 5C, PGE_2 treatment resulted in a dramatic increase in phosphorylated Akt in adenomas from both the small and large intestine by immunohistochemistry (left panel) and by Western blot analysis (right panel). To further evaluate whether PGE_2 regulated apoptosis in vivo, TUNEL assays were performed to detect apoptotic cells within intestinal adenomas. The number of apoptotic cells was much lower in polyps from mice treated with PGE_2 (1 apoptotic cell/polyp) compared to that seen in control (vehicle-treated) mice (11 apoptotic cells/polyp) (Figure 5D).

To assess the physiologic relevance of our observation that PGE_2 regulates cell survival via PPARδ in cultured cells, we determined whether PGE_2 could induce polyp formation and promote cell survival in *PPARδ$^{-/-}$/Apc^{min}* mice. *PPARδ$^{-/-}$/Apc^{min}* male mice were generated by conventionally crossing the C57BL/6J-*Apc^{min}* mice with C57BL/6J- PPARδ null mice. We were able to obtain 12 viable male *PPARδ$^{-/-}$/Apc^{min}* mice through an extensive breeding effort that required several months. Six-week-old *PPARδ$^{-/-}$/Apc^{min}* mice were treated for

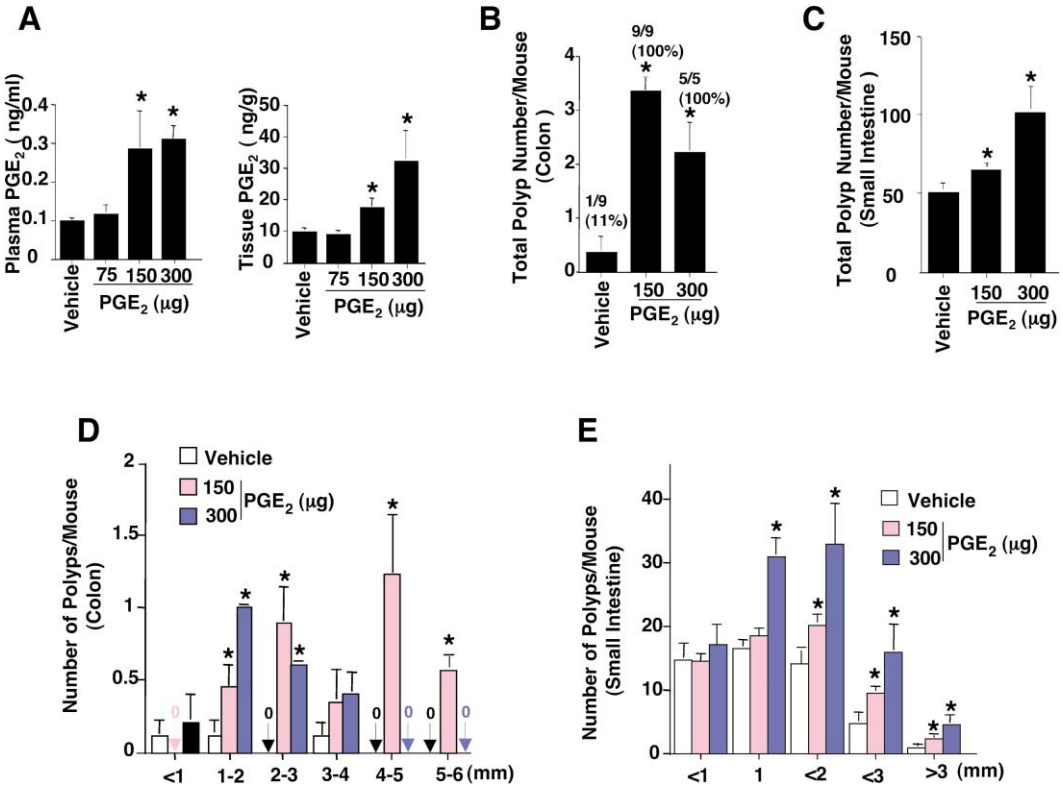

Figure 4. PGE_2 treatment increases adenoma number and size in *Apc*^{min} mice

A: Dose response of PGE_2 on prostaglandin levels in *Apc*^{min} mouse plasma (left panel) and intestinal tissues (right panel). Data are expressed as mean ± S.E.M. of vehicle (n = 9), 75 μg PGE_2 (n = 4), 150 μg PGE_2 (n = 9), and 300 μg PGE_2 group (n = 5). Asterisks represent statistical differences ($p < 0.05$; Student's t test).

B and C: The effect of PGE_2 on polyp number in the colon or small intestine. *Apc*^{min} mice were treated with vehicle or the indicated dose of PGE_2 as described in the text. After sacrifice, the large (**B**) and small (**C**) intestines were opened longitudinally and polyps were counted using a dissecting microscope (magnification 5×). Data are expressed as mean ± S.E.M. Asterisks represent statistical differences ($p < 0.05$; Student's t test).

D and E: The effect of PGE_2 on polyp size in the colon or small intestine. *Apc*^{min} mice were treated with vehicle or the indicated dose of PGE_2 as described in the text. After sacrifice, the large (**D**) and small (**E**) intestines were opened longitudinally and tumor diameter was measured with a digital caliper using a dissecting microscope (magnification 5×). Data are expressed as mean ± S.E.M. Asterisks represent statistical differences ($p < 0.05$; Student's t test).

seven weeks with PGE_2 (300 μg/each mouse/twice daily) or vehicle. The body weight of mice treated with vehicle or PGE_2 was comparable throughout the study. Mice in the PGE_2-treated group were normal and had no rectal bleeding. Unlike the results seen with wild-type *Apc*^{min} mice, administration of PGE_2 failed to affect colorectal polyp number (Figure 6A) or small intestinal polyp number (Figure 6B). In addition, PGE_2 treatment had no effect on the size of polyps in both colon and small intestine, as compared with mice treated with vehicle (data not shown). Furthermore, PGE_2 failed to affect apoptosis in intestinal adenomas taken from *PPAR*$\delta^{-/-}$/*Apc*^{min} mice (Figure 6C). However, PGE_2 treatment led to increased phosphorylation of Akt in small intestinal polyps of *PPAR*$\delta^{-/-}$/*Apc*^{min} mice (Figure 6D). These results demonstrate that the ability of PGE_2 to promote adenoma growth in *Apc*^{min} mice is dependent on the presence of PPARδ.

Discussion

Data obtained using cultured cells, animal models of intestinal cancer, and humans have implicated a direct role for COX-2 in the promotion of colorectal cancer. These studies have led to the evaluation of COX-2 selective inhibitors for the prevention and/or treatment of this disease. In fact, COX-2 inhibitors are currently approved for use in patients with the hereditary CRC syndrome familial adenomatous polyposis (FAP). For the larger population at-risk for CRC, the therapeutic potential of COX-2 selective inhibitor therapy for either primary or secondary prevention or as adjuvant therapy for advanced CRC is a question that has progressed beyond the laboratory and will now largely depend on the results of multiple clinical trials currently in progress.

Despite these advances, there is compelling evidence to suggest that research on the COX-2 signaling pathway in CRC will continue to have direct relevance to the treatment of gastrointestinal malignancies (and perhaps other types of solid cancers as well). For example, inhibitors that target PG receptor subtypes downstream of COX-2 could be more effective with fewer side effects than COX-2-selective inhibitors. In addition, use of some selective COX-2 inhibitors has been associated with an increase in cardiovascular events, such as myocardial infarctions, presumably due to an imbalance between the COX-2-derived production of PGI_2 and the COX-1-derived production of TXA_2. Hence, attacking important targets downstream of COX-2 may improve the safety profile considerably by not af-

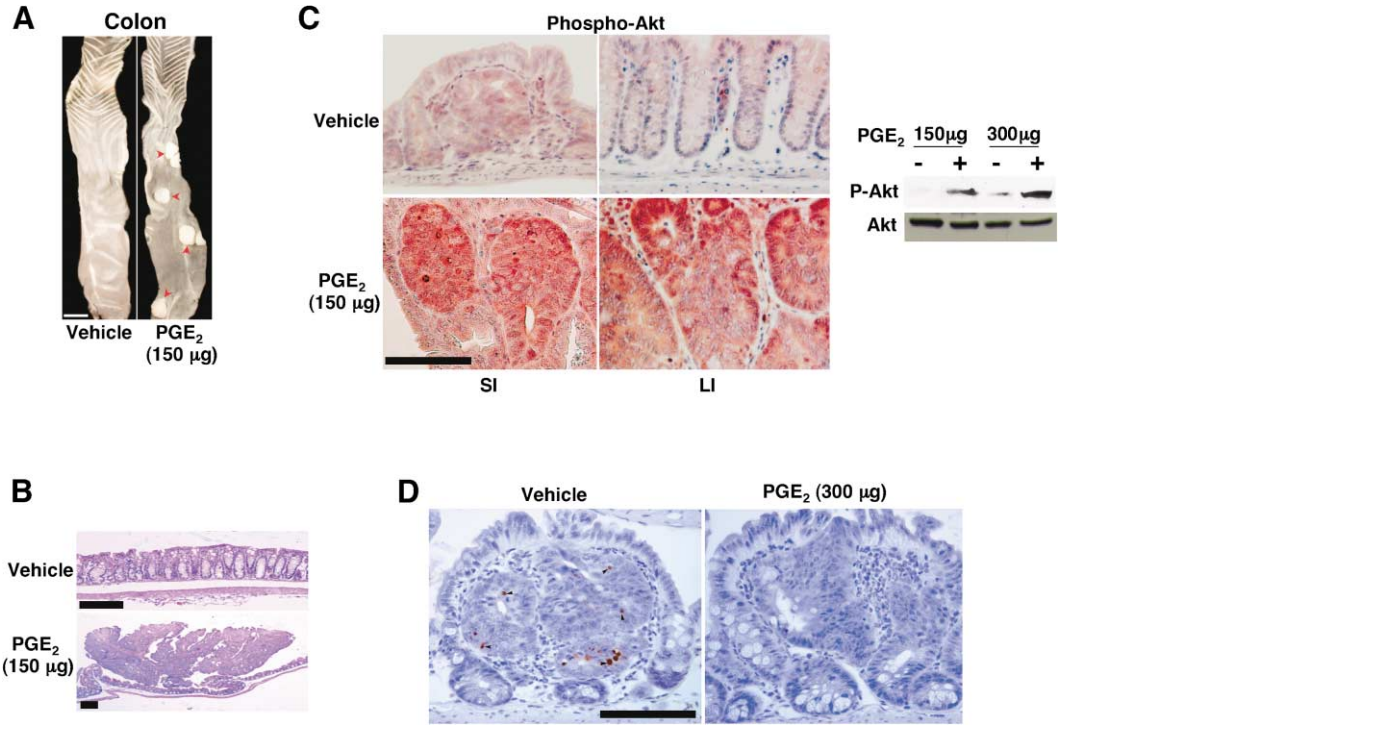

Figure 5. Morphologic and histologic analysis of colorectal adenomas in *Apc*^{min} mice following treatment with PGE_2

A: Gross view of colonic polyps in *Apc*^{min} mice treated with vehicle or PGE_2. Scale bar, 0.5 cm.

B: Histological sections of colonic polyps from *Apc*^{min} mice treated with vehicle or PGE_2. Sections with 5 μm in thickness were deparaffined with xylene and rehydrated by ethanol treatment and then stained with hematoxylin and eosin to examine intestinal adenomas. The top panel is a representative section from vehicle treated animals, while the lower panel is a typical adenoma from PGE_2-treated mice (the scale bar = 50 μm).

C: PGE_2 stimulates Akt activation in *Apc*^{min} mice. Sections of small (SI) and large (LI) intestine were immunostained with phospho-Akt (Ser473) antibody (left panel). A representative section shows strong immunoreactive staining (red/brown) for phospho-Akt in the epithelial cells of polyps throughout the intestine after treatment with PGE_2 (150 μg/mouse, twice daily) for 7 weeks. Faint staining was observed in the small intestinal adenomas, and very weak staining was found in normal colonic tissue of vehicle-treated mice. Scale bar, 100 μm. The right panel of **C** represents the levels of phospho-Akt and Akt in the small intestinal adenomas taken from mice treated with either vehicle or PGE_2 (150 or 300 μg/mouse). Each sample included 40 polyps collected from two animals.

D: TUNEL staining of an intestinal adenoma taken from mice treated with vehicle (left panel) or PGE_2 (right panel). Apoptotic nuclei are stained dark brown by the DeadEnd colorimetric TUNEL system as described in the Experimental Procedures. The arrows denote a group of TUNEL-positive stained epithelial cells undergoing apoptosis. Scale bar, 100 μm.

fecting the balance of prostaglandins important for regulating vascular tone and platelet aggregation.

PGE_2 is the major prostaglandin product of the cyclooxygenase pathway found in many solid tumors, including CRC. However, the role of PGE_2 in promoting intestinal tumor growth remains controversial. Therefore, we have focused on determining the key downstream signaling molecules responsible for regulating adenoma growth.

Regulation of PPARδ transcriptional activity by PGE_2

Exposure of CRC cells to PGE_2 results in a dose-dependent increase in PPARδ transcriptional activity. The effect is specific for the PPARδ/β subtype, based on the finding that it was only seen when cells were transfected with PPARδ but not PPARα or PPARγ. Moreover, expression of a dominant-negative PPARδ protein blocked the ability of PGE_2 to modulate PPARδ activity. However, PPARs form obligate heterodimers with the RXR family of nuclear receptors. Further, the PPRE3-tk-luciferase reporter gene can be activated by changes in RXR activity. Thus, there is a possibility that PGE_2, in some type of unknown complex interaction, activates RXR signaling only in the presence of PPARδ but not in the presence of -α or -γ subtypes. We completed experiments which demonstrated that PGE_2 treatment had no effect on RXR activity to rule out this possibility (data not shown).

Nuclear hormone receptor activity is regulated by a number of different variables in a complex set of interactions. Most simplistically, a receptor can be activated by the addition of an activating ligand. Multiple reports have shown in ligand binding assays that PGE_2 has no binding affinity for any of the three PPAR subtypes. We have confirmed these results using the PPAR-GAL4 ligand binding reporter assays. We did not observe any increase in PPARδ binding activity in the PPARδ-LBD-GAL4 binding reporter assays following prolonged treatment of cells with PGE_2 (data not shown), indicating the lack of PGE_2 binding or induction of an endogenous ligand. We did show that the ability of PGE_2 to regulate PPARδ is dependent on the PI3K/Akt kinase pathway. How the PI3K/Akt pathway regulates PPARδ activity will be addressed in the future.

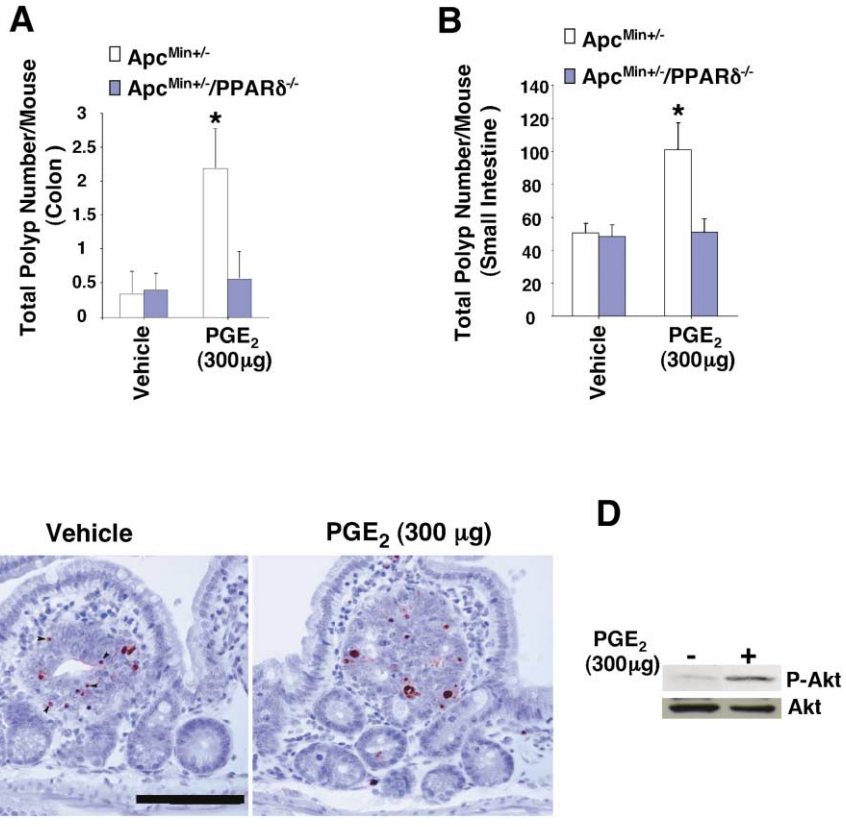

Figure 6. PGE_2 has no effect on adenoma number *in* Apc^{min} mice lacking PPARδ

A and B: Six-week-old $PPAR\delta^{-/-}/Apc^{min}$ mice were treated with vehicle (n = 6) or the PGE_2 (n = 6) as described in the text. After sacrifice, the large (**A**) and small (**B**) intestines were opened longitudinally and polyps were counted using a dissecting microscope (magnification 5×). Data are expressed as mean ± S.E.M. Asterisks represent statistical differences ($p < 0.05$; Student's t test).

C: TUNEL staining of intestinal adenomas from $PPAR\delta^{-/-}/Apc^{min}$ mice treated with vehicle (left panel) or PGE_2 (right panel). Scale bar, 100 μm.

D: Western blot analysis of phospho-Akt and Akt in the small intestinal polyps taken from $PPAR\delta^{-/-}/Apc^{min}$ mice treated with either vehicle or PGE_2 (300 μg/mouse). Each sample includes 40 polyps collected from two animals.

PGE_2 regulation of apoptosis and intestinal polyp growth is dependent on PPARδ

We have shown both in vitro and in vivo that the ability of PGE_2 to promote resistance to apoptosis and to accelerate intestinal adenoma growth is dependent on PPARδ. The addition of either PGE_2 or GW1516 (a selective PPARδ agonist) attenuates growth factor withdrawal-mediated apoptosis in cultured CRC cells. Further, expression of a dominant-negative PPARδ protein blocks the ability of PGE_2 to promote cell survival. Using a dominant-negative approach has a number of caveats. For example, the introduction of dominant-negative PPARδ could have multiple biological effects independent of its ability to inactive endogenous PPARδ activity. To overcome these limitations, we tested the importance of our in vitro findings in a physiologically relevant in vivo model system.

Apc^{min} mice spontaneously develop intestinal adenomas; however, these occur mainly in the small intestine, with very few in the colon. A recent report showed that Apc^{min} mice have higher levels of PGE_2 than the wild-type mice at 15 weeks of age (Kettunen et al., 2003). After 15 weeks, the concentration of PGE_2 correlates well with the number of polyps (Kettunen et al., 2003). Treatment of Apc^{min} mice with PGE_2 leads to a 2- to 3-fold increase in PGE_2 concentrations in both the serum and intestine compared to vehicle alone (Figure 1). This increase is comparable to that seen when comparing 15-week-old wild-type and Apc^{min} mice. Furthermore, PGE_2 treatment promotes a significant increase in polyp size in both the small and large intestine. Prior studies examining the effects of exogenous PGE_2 administration on tumor growth have relied on synthetic analogs that have differential activity for members of the EP receptor family. To our knowledge, no one has generated data testing whether native PGE_2 has an effect on intestinal adenoma growth. Our results establish that treatment with the endogenous form of PGE_2 has pro-oncogenic activity in an in vivo model system. Most importantly, the ability of PGE_2 to both inhibit apoptosis and increase intestinal polyp growth is essentially negated in Apc^{min} mice that are genetically null for PPARδ, although PGE_2 still induces activation of Akt in small intestinal adenomas of these animals.

The role of PPARδ in CRC is not well understood. Although activation of the receptor using a synthetic agonist promotes intestinal adenoma growth, genetic loss-of-function experiments in different model systems continue to produce variable results. Deletion of PPARδ from an established CRC cell line causes a reduction in tumorigenic potential (Park et al., 2001). However, experiments that examine polyp formation in $PPAR\delta^{-/-}/Apc^{min}$ animals have found either no effect (Barak et al., 2002) or, paradoxically, an increase in polyp number and size (Harman et al., 2004). The explanation for these disparate results may relate to inherent differences in model systems (an established cancer cell line versus developing adenomas) or to the particular background strain of PPARδ null mice being evaluated. We have noted that the number of adenomas which develop in Apc^{min} mice is quite variable, depending on the background strain and fat content of the diet. Our results using Apc^{min} mice null for PPARδ support the view that PPARδ is pro-oncogenic in this model system. Why we see results contrary to prior studies may be due to differences in the genetic background of Apc^{min} mice or possibly to differences in the type of targeting strategy employed to delete PPARδ.

The mechanism by which PPARδ regulates cell survival in CRC cells is not known. Recent studies have convincingly established that PPARδ promotes cell survival in keratinocytes (Di-Poi et al., 2002; Michalik et al., 2001). In these experiments, ablation of PPARδ in vivo resulted in increased apoptosis in the mouse epidermis. The receptor was found to regulate a number of important apoptotic-related genes, including several members of the caspase family. The same group subsequently demonstrated that PPARδ promoted cell survival via activation of Akt1. Our data indicating that the PI3K/Akt pathway also upregulates PPARδ activity suggest that a positive feedback loop might exist between PPARδ and Akt to regulate apoptosis under certain physiological conditions. Whether the apoptotic-related target genes of PPARδ in keratinocytes are the same as those regulated by the receptor in intestinal epithelial cells is not known.

In summary, our results provide evidence that PGE_2 promotes intestinal adenoma formation via PPARδ. Previous studies have documented that PPARδ is also downstream of the Wnt/β-catenin and K-Ras pathways in CRC cells. Our data linking the COX pathway to PPARδ suggest that the receptor is an integral component of the crosstalk that occurs between multiple pro-oncogenic signaling pathways during the shift in balance between cell survival and cell death that promotes colorectal carcinogenesis. Activators of PPARδ have hypolipidemic effects, and compounds that activate the receptor are currently in large-scale clinical trials to examine their potential use for the treatment of patients with hyperlipidemic states. Our results would suggest that PPARδ is an important pro-oncogenic factor in CRC, and that agonists of the receptor may have proneoplastic side effects in certain populations at risk for CRC (particularly those cancers initiated by loss of function mutations in *APC*). As a corollary, antagonists of the receptor might prove to be useful in the chemoprevention and/or treatment of CRC.

Experimental procedures

Animals

C57BL/6J-*Apc*^{min} male mice were obtained from Jackson Laboratory (Bar Harbor, ME) at five weeks of age. C57BL/6J-*Apc*^{min} and C57BL/6J-*Apc*^{min}/*PPAR*δ$^{-/-}$ were housed and fed with standard mouse diet in the Animal Care Facility according to National Institutes of Health and institutional guidelines for laboratory animals. At the age of 6 weeks, *Apc*^{min} mice (n = 26) were randomly grouped into four groups according to the PGE_2 treatments (vehicle, 75 μg PGE_2/each mouse, 150 μg PGE_2/each mouse, and 300 μg PGE_2/each mouse), and *PPAR*δ$^{-/-}$/*Apc*^{min} mice (n = 12) were randomly grouped into two groups treated with vehicle or 300 μg PGE_2/each. Vehicle or differing amounts of PGE_2 in 100 μl sterile PBS was administered twice daily via gavage feeding. After treatment for 7 weeks, mice were sacrificed. Blood and entire intestinal tissues were collected from mice in each group for analysis of levels of PGE_2. The entire intestines were then immediately flushed with ice-cold PBS (pH 7.4) and filled with 10% neutral buffered formalin for 24 hr. The intestines were transferred to 70% ethanol for 24 hr, opened longitudinally, and examined under a magnification dissecting microscope (magnification 5×) to count polyps. The tumor diameter was measured with a digital caliper. After tumors were counted, intestinal tissues were fixed flat on the sheets of filter paper and embedded in paraffin. For histology, sections with 5 μm in thickness were deparaffined with xylene and rehydrated by ethanol treatment and then stained with hematoxylin and eosin to examine intestinal adenomas. The unstained sections were subjected to TUNEL assays and immunohistochemical staining.

PGE_2 levels

Intestinal tissues were homogenized and PGE_2 was acidified and extracted from the homogenate and plasma as previously described (DuBois et al., 1994). The levels of PGE_2 in the plasma and tissue were quantified using gas chromatography negative ion chemical ionization mass spectrometry as described (DuBois et al., 1994). The concentration of PGE_2 in the samples was calculated by comparing the ratios of its peak areas to the internal standard.

Cell culture and reagents

LS-174T cells were purchased from ATCC (Manassas, VA) and maintained in McCoy's 5A medium with 10% fetal bovine serum. Ly 294002 was obtained from Calbiochem (La Jolla, CA). PGE_2, PGA_2, and $PGF_{2\alpha}$ were purchased from Cayman Chemical (Ann Arbor, MI). GW1516 was obtained from CareX (Strausburg, France). The constructs of constitutively active Akt (myristylated form of Akt1), dominant-negative Akt (kinase-deficient mutants of Akt1-K179M), and dominant-negative PI3K (pSG5-Δp85) were described previously (Sheng et al., 2001a).

TUNEL assays

The fragmented DNA of apoptotic cells in tissue sections was end-labeled using the Dead-End colorimetric TUNEL system according to the manufacturer's instructions (Promega, Madison, WI). Sections were deparaffinized, rehydrated, incubated with 20 μg/ml proteinase K, washed with PBS, and then immersed in a TDT reaction mixture for 60 min at 37°C. The slides were then washed in 2× SSC for 15 min to stop the reaction. Following another wash and blocking cycle, the slides were incubated with Streptavidin peroxidase for 30 min at room temperature and then stained with DAB.

Immunohistochemical staining

Tissue sections (5 μm thick; n = 5 per animal) were stained with a rabbit polyclonal antibody against phospho-Akt (Ser473) at a dilution of 1:250 (Cell Signaling, Beverly, MA). The immunohistochemical staining was completed by using a Zymed-Histostain-SP Kit (Zymed, South San Francisco, CA) as described previously (Gupta et al., 2004).

Stable transfection and Western blot

The LS-174T cells were transfected with 0.4 μg empty vector, dominant-negative PPARδ by the LipofectAMINE Plus reagent according to the manufacturer's protocol (Life Technologies, Inc., Rockville, MA). Stable transfectants were selected by growth in zeocin (Invitrogen, Carlsbad, CA). Whole cell extracts were prepared from parental LS-174T cells expressing a dominant-negative PPARδ protein or empty vector-transfected cells treated with vehicle or PGE_2 for 24 hr. For Western blot analysis, the cells or polyps taken from the intestine were washed at 4°C with 1× PBS and lysed in 0.6 ml of RIPA buffer containing protease inhibitor tablets (Boehringer Mannheim Corp., Indianapolis, IN). Fifty micrograms of soluble protein was boiled, subjected to electrophoresis on a 10% SDS-PAGE reducing gel, and then electrophoretically transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% dry milk in TBS-T buffer for 1 hr and then incubated for 12–16 hr at 4°C in a 1:500 dilution of the PPARδ antibody (Perseus Peoteomics Inc., Japan), Akt, or phospho-Akt antibody (Cell Signaling, Beverly, MA) in TBS-T buffer which contained 5% dry milk. After three washes with TBS-T buffer, the membrane was incubated in a 1:3000 dilution of the anti-mouse or anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Boehringer Mannheim Corp.) for 1 hr at room temperature. After three washes with TBS-T buffer, the protein bands were detected with the ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions.

Apoptosis assays

Parental LS-174T cells, dominant-negative PPARδ cells, or cells transfected with empty vector (2.5×10^5/each well) were plated in 6-well plates. After culture overnight, the cells were washed twice with PBS and then incubated in serum-free media containing either 10% FBS, vehicle, LY294002, PGE_2, and/or GW1516 for 3 days. For the PGE_2 experiments, the culture media was replaced with fresh serum-free media containing PGE_2 every 24 hr. The assays for apoptotic cells were performed using a TACS Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions (R&D System, Inc., Minneapolis, MN). For this assay, the cells were harvested, washed, and then incubated with Annexin V-FITC and propidium iodide. The cells were again washed and then analyzed by flow cytometry. The

combination of Annexin V-FITC and propidium iodide allowed for the differentiation between early apoptotic cells (Annexin V-FITC positive), late apoptotic cells (Annexin V-FITC and propidium iodide positive), necrotic cells (propidium iodide positive), and viable cells (both negative).

PPAR transcriptional assays

The LS-174T cells (2.0×10^5/well in 12-well plates) were transiently cotransfected with 0.3 μg PPRE3-tk-luciferase/5 ng of pRL-SV40 and 0.4 μg of empty vector, PPARα, PPARδ, PPARγ, dominant-negative PPARδ, dominant-negative Akt, constitutively active Akt, or dominant-negative PI3K plasmids using LipofectAMINE Plus reagent according to the manufacturer's instructions (Life Technologies, Inc., Rockville, MA). After a 3 hr incubation, fresh media without serum was added, and the cells were incubated for an additional 4 hr. These cells were then treated with either vehicle, GW1516, and/or PGE_2 for 16 hr. For experiments evaluating a PI3K inhibitor, cells were pretreated with Ly294002 for 1 hr before treatment with vehicle or PGE_2 16 hr. Following the experiment, the cells were harvested and lysed. Luciferase activity was measured using a Dual Luciferase kit (Promega) and a Monolight 3010 luminometer (BD Biosciences/Pharmingen, San Diego, CA). The relative luciferase activity was determined and normalized to *Renilla* luciferase.

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