

Effect of Eicosapentaenoic Acid on E-type Prostaglandin Synthesis and EP4 Receptor Signaling in Human Colorectal Cancer Cells^{1,2}

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Abstract

The ω -3 polyunsaturated fatty acid eicosapentaenoic acid (EPA), in the free fatty acid (FFA) form, has been demonstrated to reduce adenoma number and size in patients with familial adenomatous polyposis. However, the mechanistic basis of the antineoplastic activity of EPA in the colorectum remains unclear. We tested the hypothesis that EPA-FFA negatively modulates synthesis of and signaling by prostaglandin (PG) E₂ in human colorectal cancer (CRC) cells. EPA-FFA induced apoptosis of cyclooxygenase (COX)-2–positive human HCA-7 CRC cells *in vitro*. EPA-FFA in cell culture medium was incorporated rapidly into phospholipid membranes of HCA-7 human CRC cells and acted as a substrate for COX-2, leading to reduced synthesis of PGE₂ and generation of PGE₃. Alone, PGE₃ bound and activated the PGE₂ EP4 receptor but with reduced affinity and efficacy compared with its “natural” ligand PGE₂. However, in the presence of PGE₂, PGE₃ acted as an antagonist of EP4 receptor–dependent 3',5' cyclic adenosine monophosphate induction in naturally EP4 receptor–positive LoVo human CRC cells and of resistance to apoptosis in HT-29-EP4 human CRC cells overexpressing the EP4 receptor. We conclude that EPA-FFA drives a COX-2–dependent “PGE₂-to-PGE₃ switch” in human CRC cells and that PGE₃ acts as a partial agonist at the PGE₂ EP4 receptor.

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Introduction

Cyclooxygenase (COX)–derived prostaglandin (PG) E₂ signaling is believed to play a critical role during colorectal carcinogenesis [1]. *In vitro* experiments have demonstrated that PGE₂ promotes colorectal cancer (CRC) cell proliferation and invasion, as well as resistance to apoptosis [2]. PGE₂ also has proangiogenic properties and may down-regulate the host antitumor immune response [2]. *In vivo*, PGE₂ has been demonstrated to drive intestinal tumorigenesis in the *Apc*^{Min/+} mouse model of familial adenomatous polyposis (FAP) [3] and in a rat model of carcinogen-induced CRC [4].

Epidemiological evidence and preclinical data suggest that omega (ω)-3 polyunsaturated fatty acids (PUFAs), which are found in large quantities in fish such as salmon and mackerel, have anti-CRC activity [5]. The mechanism(s) by which the main ω -3 PUFAs in dietary fish oil, namely 20:5 ω 3 eicosapentaenoic acid (EPA) and 22:6 ω 3 docosahexaenoic acid (DHA), have antineoplastic activity remains unclear [6]. One valid hypothesis is that the anti-CRC activity of EPA is explained by negative modulation of COX-PGE₂ signaling.

In “western” diets, the predominant substrate for both COX isoforms (“constitutive” COX-1 and “inducible” COX-2) is the ω -6 PUFA 20:4 ω 6 arachidonic acid (AA), from which two-series PGs such as PGE₂ are synthesized [7]. However, EPA can incorporate into the

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; CRC, colorectal cancer; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAP, familial adenomatous polyposis; FFA, free fatty acid; PG, prostaglandin; RCT, randomized controlled trial; TTBS, Tween–Tris-buffered saline

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²This article refers to supplementary materials, which are designated by Figures W1 and W2 and are available online at www.neoplasia.com.

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phospholipid bilayer, displace AA, and acts as an alternative substrate for the COX enzymes [7]. EPA turnover *in vitro* (measured as V_{max}) by COX-1 and COX-2 is 10% and 35%, respectively, of that for AA [7]. Importantly, COX converts EPA into PGH₃, rather than PGH₂, leading to production of equivalent three-series PGs such as PGE₃ [7]. It has been demonstrated that EPA administration to COX-2-positive A549 human lung cancer cells and BxPC-3 human pancreatic cancer cells leads to synthesis of PGE₃ at the expense of PGE₂ [8,9]. More recently, ω-3 fish oil administration to rats was shown to be associated with the appearance of PGE₃ and a reduction in PGE₂ levels in colorectal mucosa [10], confirming that a “PGE₂-to-PGE₃ switch” can occur *in vivo*.

Although a reduction in tissue levels of protumorigenic PGE₂ alone could explain the anticancer properties of EPA, it is possible that PGE₃ *per se* could contribute to the antitumorigenic activity of EPA. Consistent with this concept, Yang et al. [8] have demonstrated that exogenous PGE₃ increased apoptosis of A549 human lung cancer cells. However, the mechanistic basis of the antiproliferative activity of PGE₃ was not explored in that study.

PGE₂ signals through a family of four G protein-coupled receptors termed EP1 to EP4 (reviewed in Sugimoto and Narumiya [11]). At late stages of colorectal carcinogenesis (primary CRC growth and metastasis), preclinical evidence suggests a predominant role for the EP4 receptor in the protumorigenic activity of PGE₂ [12]. EP4 receptor expression is increased in mouse and human CRCs compared with normal colorectal mucosa [13,14]. Moreover, PGE₂-EP4 receptor signaling promotes tumorigenic behavior (proliferation, resistance to apoptosis, motility, and invasion) of human colorectal adenoma and CRC cells *in vitro* [13,14], whereas pharmacological antagonism of PGE₂-EP4 receptor signaling has been demonstrated to inhibit transplantable CRC cell tumor growth and liver metastasis in *Balb/c* mice [15]. Funahashi et al. [9] recently concluded that EPA had antiproliferative activity against BxPC-3 human pancreatic cancer cells through a mechanism involving the EP4 receptor on the basis that EPA activity was abrogated by the selective EP4 receptor antagonist ONO-AE3-208.

We have recently reported that EPA, in the free fatty acid (FFA) form (which is better absorbed from the human small intestine than EPA in the ethyl ester or triglyceride form [16]), 2 g daily for 6 months reduces rectal polyp number and size in a randomized controlled trial (RCT) of patients with FAP [17]. The aim of this study was to investigate the mechanistic basis of the antineoplastic activity of EPA-FFA in the colorectum by testing the hypotheses that EPA-FFA drives a switch from synthesis of PGE₂ to PGE₃ in human CRC cells and that PGE₃ acts through inhibition of EP4 receptor signaling, thereby contributing to the apoptotic activity of EPA against human CRC cells.

Materials and Methods

Reagents and Antibodies

EPA-FFA and Miglyol 810 (mixed capric and capryllic acid medium-chain triglycerides, which were used as the placebo in the RCT of EPA in FAP patients [17]) were kindly provided by SLA Pharma (Watford, UK). EPA-FFA was extracted from 500 mg of enteric-coated ALFA capsules using a sterile needle and diluted 1:100 in 95% (vol./vol.) ethanol immediately before use. A working solution of EPA was always freshly prepared from a new capsule to avoid auto-oxidation. AA (Sigma-Aldrich, Poole, UK) was dissolved in 95% (vol./vol.)

ethanol as a 200-mM stock solution and stored at -20°C. PGE₂ (20 mM stock solution in dimethyl sulfoxide [DMSO]) was also obtained from Sigma-Aldrich. PGE₃ (10 mM stock solution in DMSO) was obtained from Cayman Chemical Co (Ann Arbor, MI). Working solutions of PGE₃ were always freshly prepared from frozen stock that was then discarded to avoid freeze-thaw degradation. All other EP receptor agonists and antagonists were used as described previously [14]. SC-236 was a kind gift from Pfizer, Inc (Groton, CT). Methoxyamine HCl was obtained from Sigma-Aldrich, and all high-performance liquid chromatography-grade solvents were purchased from Fisher Scientific (Loughborough, UK).

Human embryonic kidney 293 cell EP4 and EP2 receptor membranes and [³H]-PGE₂ (185.6 Ci/mmol) were obtained from PerkinElmer LAS Ltd (Bucks, UK).

Polyclonal rabbit antibody against the human EP4 receptor (N-terminal) was obtained from Cayman Chemical Co. Polyclonal goat anti-COX-2 antibody (C-19) was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), and mouse monoclonal anti-chicken β-actin antibody was obtained from Sigma-Aldrich. IR Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies were obtained from Molecular Probes (Invitrogen, Paisley, UK). IRDye 700- and IRDye 800-conjugated secondary antibodies were obtained from Tebu-Bio Ltd (Peterborough, UK).

Cell Culture

Human CRC cell lines were all obtained from ECACC (Porton Down, UK) except mouse colon 26 (MC-26) cells, which were obtained from the National Cancer Institute (Frederick, MD). All cells were cultured in RPMI 1640 medium containing Glutamax supplemented with 10% (vol./vol.) heat-inactivated fetal bovine serum (all Invitrogen), except LoVo cells, which were cultured in Ham's F12 medium (Invitrogen) containing Glutamax supplemented with 10% (vol./vol.) heat-inactivated fetal bovine serum. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Cells were routinely subcultured using 0.25% (wt./vol.) trypsin (Invitrogen). Viable cells were counted using a hemocytometer in the presence of 0.04% (vol./vol.) trypan blue (Sigma-Aldrich).

Measurement of Apoptosis

Apoptosis was measured by counting 4% (wt./vol. in phosphate-buffered saline) paraformaldehyde-fixed, nonadherent HCA-7 and HT-29 human CRC cells as described and validated by us and other groups [14,18–25].

Immunoblot Analysis

Cells were lysed in ice-cold RIPA buffer (Sigma-Aldrich) and then passed through a QIAshredder homogenizer (Qiagen Ltd, Crawley, UK). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using 15 μg of total protein, and protein was transferred to polyvinylidene fluoride membranes. Membranes were blocked in Odyssey Blocking Buffer (LI-COR Biosciences Ltd, Cambridge, UK) for 1 hour before incubating with anti-COX-2 antibody diluted 1:1000 in 1:1 Odyssey Blocking Buffer-Tween-Tris-buffered saline (TTBS) for 1 hour at 25°C. Membranes were washed 3 × 3 minutes in TTBS followed by incubation with IRDye 700-labeled secondary antibody (1:2500) diluted in 1:1 Odyssey Blocking buffer-TTBS. After 3 × 3 minutes washes with TTBS, membranes were incubated with anti-β-actin antibody (1:20,000), and IRDye 800-labeled secondary antibody (1:2500) was diluted in 1:1 Odyssey Blocking buffer-TTBS

as above. Membranes were visualized using an Odyssey Infrared Imaging System and Odyssey v1.2 software (LI-COR).

Indirect Immunofluorescence

Immunofluorescence studies were performed with primary antibodies (anti-EP4, 1:25; anti-V5, 1:200) diluted in PBS containing 1% (wt./vol.) dried skimmed milk and 0.1% (vol./vol.) of Tween-20 as described [14], except that coverslips were mounted in Prolong Gold with 4',6 diamidino-2-phenylindole (Invitrogen).

Assay of Intracellular 3',5' Cyclic Adenosine Monophosphate Content

A total of 1×10^6 CRC cells were incubated overnight in triplicate 35-mm wells. Cells were pretreated with 50 μ M rolipram for 10 minutes before addition of 10 μ M ONO-AE3-208 (ONO Pharmaceutical Co Ltd, Osaka, Japan) for 45 minutes before addition of EP receptor agonists. Intracellular 3',5' cyclic adenosine monophosphate (cAMP) content was assayed using a Biotrak enzyme immunoassay (GE Healthcare Amersham, Amersham, UK) using the nonacetylation protocol.

Measurement of PGE₂ and PGE₃ Levels in CRC Cell-Conditioned Medium

PGE₂ and PGE₃ levels were measured in CRC cell-conditioned medium by high-sensitivity immunoassay (R&D Systems Ltd, Abingdon, UK) or liquid chromatography-tandem mass spectrometry (LC/MS/MS) based on the method of Murphey et al. [26]. PGE₂ and PGE₃ standards were prepared by converting PGE₂ and PGE₃ to their *O*-methyloxime derivatives. Methoximated samples were diluted to a total volume of 10 ml with water adjusted to pH 3.0. The aqueous sample was applied to a C-18 Isolute SPE cartridge (Kinesis, St Neots, Cambridgeshire, UK), preconditioned with 5 ml of methanol and 5 ml of water (pH 3.0). The SPE cartridge was washed with 10 ml of water (pH 3.0) and 10 ml of heptane. PGs were then eluted from the cartridge with 10 ml of ethyl acetate, and any residual aqueous material was removed from the eluate by aspiration. The eluate was evaporated using a centrifugal evaporator, and the dried residue was resuspended in 25 μ l of 95:5, 0.1 (vol./vol.) acetic acid-acetonitrile (mobile phase A).

LC was performed on an ACQUITY UPLC BEH C8 Column, 2.1 \times 100 mm, 1.7 μ m (Waters, Milford, MA) attached to an ACQUITY Ultra Performance LC (UPLC) System. Samples were separated by a gradient of 60% to 10% of mobile phase A in mobile phase B (50:50, 0.1 [vol./vol.] acetic acid-acetonitrile) for 4 minutes (total sample run time, 8 minutes) at a flow rate of 300 μ l/min before delivery to a bench-top tandem quadrupole mass spectrometer (Quattro Premier XE; Waters) operating in multiple reaction monitoring mode. Capillary and cone voltages were 3.5 kV and 20 V, respectively, with a collision voltage of 16 V, with source and desolvation temperatures of 150°C and 300°C, respectively. Multiple reaction monitoring channels were established for PGE₂ (m/z 380 \rightarrow 267.9 and 380.0) and PGE₃ (m/z 378 \rightarrow 265.9, 266.9, and 378.0).

Measurement of Fatty Acid Phospholipid Membrane Content

Fatty acid content in cell membranes was measured by gas chromatography-mass spectrometry as described [17].

[³H]-PGE₂ Binding Assay

EP4 receptor (20 μ g/well) and EP2 receptor (10 μ g/well) membrane preparations were incubated with [³H]-PGE₂, with or without

cold PGs, in 50 mM Tris pH 6.0 with 0.5% (wt./vol.) bovine serum albumin in 200- μ l reactions in a 96-well polystyrene microplate (PerkinElmer) at 27°C for 1 hour. The samples were transferred to a GF/B filter (PerkinElmer) presoaked in PBS with 0.5% (wt./vol.) bovine serum albumin using a Tomtec cell harvester, and unbound [³H]-PGE₂ was removed by washing eight times with ice-cold 50 mM Tris-HCl pH 7.4. The filter was left to dry overnight at room temperature before counting using a liquid scintillation counter (Wallac 1450 MicroBeta, PerkinElmer).

Statistical Analysis

The significance of differences associated with EPA and PG treatments was tested by one-way analysis of variance with least significant difference *post hoc* analysis. Significance was assumed if $P < .05$.

Results

EPA-FFA Induces Apoptosis of HCA-7 Human CRC Cells

We measured apoptosis of CRC cells by the established technique of nonadherent cell counting, which has been previously validated by fluorescence microscopy of 4',6 diamidino-2-phenylindole-stained cells [14,18–25] and is recognized to correlate strongly with other apoptosis assays based on measurement of caspase-3/7 activity [25] and appearance of cell surface annexin V [14].

EPA-FFA promoted apoptosis of HCA-7 human CRC cells in a concentration-dependent manner (Figure 1A). EPA-FFA exposure equivalent to 0.8 μ mol/10⁶ cells, which represents addition of a 200- μ M absolute concentration of EPA-FFA into aqueous cell culture medium, was associated with a significant increase in apoptosis of HCA-7 human CRC cells above control cells (Figure 1A). By contrast, an equivalent level of exposure to the ω -6 PUFA AA did not induce apoptosis of HCA-7 human CRC cells (Figure 1B). Addition of the same vol./vol. amount of capric/caprylic acid medium-chain triglycerides (Miglyol 810), which were used as the placebo in the recent RCT of EPA [17], to cell culture medium was also not associated with increased apoptosis of HCA-7 human CRC cells (Figure 1C).

EPA-FFA in Aqueous Cell Culture Medium Is Rapidly Incorporated into CRC Cell Membranes

During the same 24-hour period, we demonstrated that EPA-FFA was incorporated into HCA-7 human CRC cell phospholipid membranes (Figure 2A). Exposure to EPA-FFA (0.8 μ mol/10⁶ cells) for 24 hours led to a large increase in EPA content so that EPA represented approximately 20% of the total PUFA content (Figure 2A). There was also a corresponding decrease in membrane AA content (Figure 2A). However, there was no evidence of EPA-DHA conversion in HCA-7 human CRC cells during the short-term exposure to EPA-FFA (Figure 2A).

EPA Incorporation Leads to Changes in E-type PG Synthesis by HCA-7 Human CRC Cells

HCA-7 human CRC cells constitutively express high levels of COX-2 protein and generate measurable levels of PGE₂ in conditioned medium [18]. Therefore, we investigated whether the proapoptotic activity linked to EPA incorporation was associated with the simultaneous changes in E-type PG synthesis in this human CRC cell line using LC/MS/MS (Figure W1).

Exposure to EPA-FFA for 24 hours was associated with a dose-dependent decrease in PGE₂ synthesis during a subsequent 4-hour

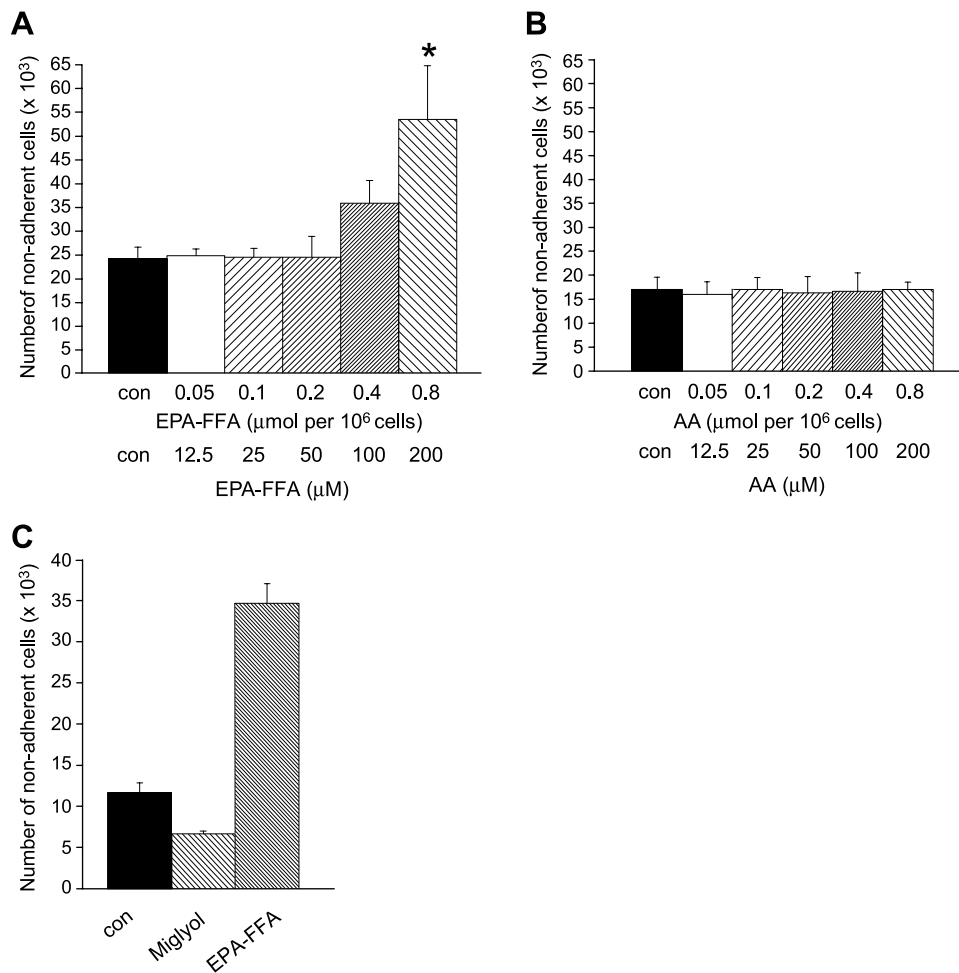


Figure 1. EPA-FFA induces apoptosis of HCA-7 human CRC cells. (A) Apoptosis of HCA-7 human CRC cells (measured by nonadherent cell counting; [14]) after treatment with increasing amounts of EPA-FFA (quoted as micromoles per 10^6 cells exposure and the equivalent working micromolar concentration below that) or the equivalent dilution (0.06% vol./vol.) of 95% (vol./vol.) ethanol carrier alone (con) to 0.8 μmol of EPA-FFA/ 10^6 cells (200 μM) for 24 hours after overnight incubation of 0.5×10^6 CRC in triplicate 35-mm wells. Columns and bars represent the mean and SEM of triplicate values, respectively. Data are representative of three independent experiments. * $P < .01$ for the comparison with con and EPA-FFA exposure less than 0.4 $\mu\text{mol}/10^6$ cells (100 μM). (B) Apoptotic HCA-7 human CRC cell counts after treatment with increasing amounts of AA (quoted as micromoles per 10^6 cells or the micromolar concentration) or the equivalent dilution (0.06% vol./vol.) of 95% (vol./vol.) ethanol carrier alone (con) to 0.8 μmol of AA/ 10^6 cells (200 μM) for 24 hours. Columns and bars represent the mean and SEM of triplicate values, respectively. (C) Comparison of the proapoptotic activity of EPA-FFA and Miglyol 810 (both 0.8 $\mu\text{mol}/10^6$ cells [200 μM] in 95% [vol./vol.] ethanol) on HCA-7 human CRC cells. Con represents the equivalent dilution (0.06% vol./vol.) of 95% (vol./vol.) ethanol alone. Columns and bars represent the mean and SEM of triplicate values, respectively.

period (Figure 2B), such that PGE₂ synthesis was inhibited by greater than 90% by amounts of EPA-FFA that promoted apoptosis (0.4–0.8 $\mu\text{mol}/10^6$ cells). In the presence of culture medium containing only the ethanol carrier, PGE₃ was not detected in HCA-7 human CRC cell-conditioned medium (Figure 2B). However, addition of EPA-FFA stimulated PGE₃ synthesis by HCA-7 human CRC cells (Figure 2B). Levels of PGE₃ in conditioned medium were an order of magnitude lower than the corresponding values for PGE₂. Moreover, there was no observable concentration-response relationship for PGE₃ synthesis. “Chronic” treatment of HCA-7 human CRC cells with EPA-FFA for 14 days was not associated with any further increase in PGE₃ levels in the conditioned medium (data not shown), suggesting that the PGE₃ levels measured in short-term experiments after a 24-hour EPA-FFA exposure represent maximal PGE₃ synthesis. EPA-FFA-dependent PGE₃ synthesis was also observed in MC-26 mouse CRC cells, which also constitutively express high levels of

COX-2 (data not shown). Pretreatment of HCA-7 human CRC cells with the selective COX-2 inhibitor SC-236 reduced PGE₃ synthesis by 87%, confirming that EPA-FFA-induced PGE₃ synthesis is predominantly a COX-2-dependent process (Figure 2C).

Consistent with a role for changes in E-type PG levels in the proapoptotic activity of EPA, HT-29-EP4 human CRC cells, which do not generate detectable amounts of PGE₂ or PGE₃ in conditioned medium [14], in either the absence or presence of EPA-FFA (data not shown), were resistant to EPA-FFA-induced apoptosis (Figure 3).

Proapoptotic Activity of EPA-FFA Is Not Explained by Decreased COX-2 Protein Expression or Reduced PGE₂ Synthesis

It has previously been reported that EPA treatment decreases COX-2 gene expression in serum-starved HT-29 human CRC cells [27]. Therefore, reduced COX-2 protein levels could account for

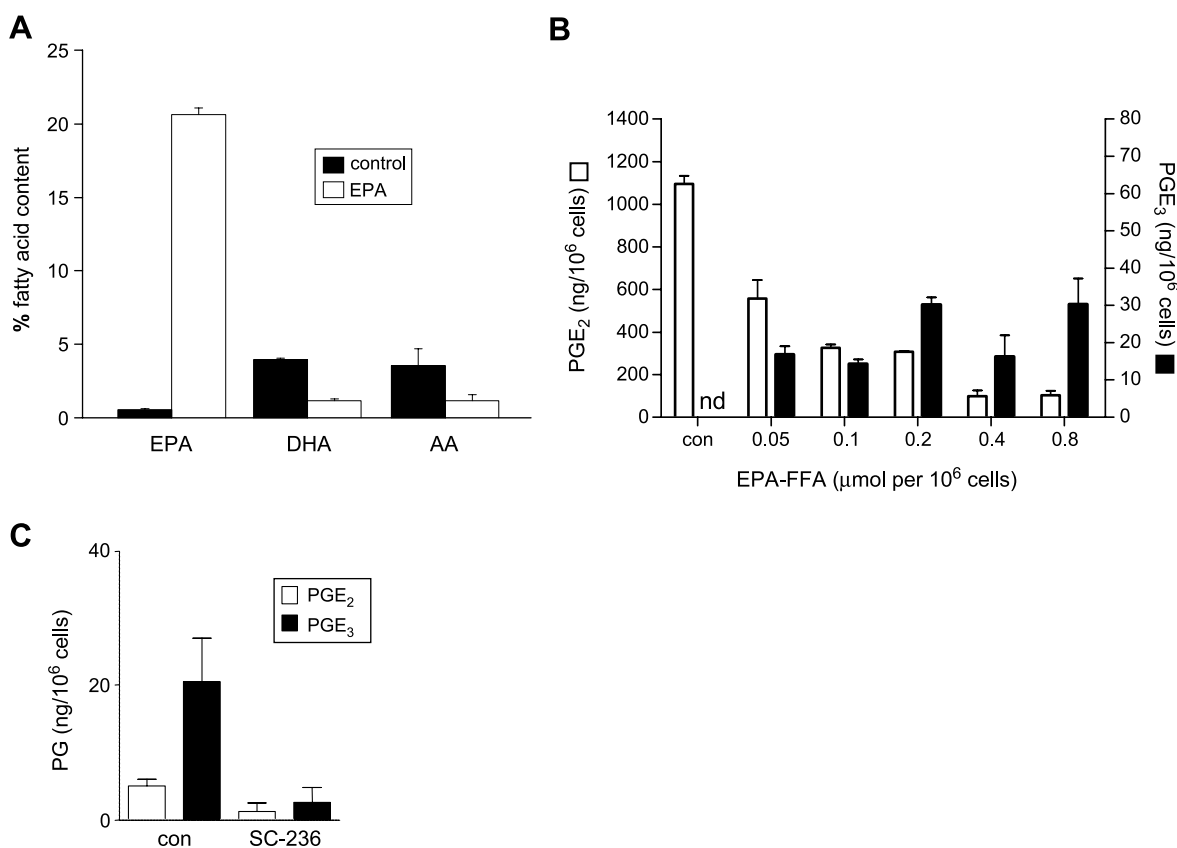


Figure 2. EPA-FFA incorporates into cell membranes and alters COX-2–dependent E-type PG synthesis in HCA-7 human CRC cells. (A) Changes in the fatty acid content of HCA-7 human CRC cell membranes after exposure to EPA-FFA (0.8 $\mu\text{mol}/10^6$ cells or 200 μM) or an equivalent dilution (0.06% [vol./vol.]) of 95% (vol./vol.) ethanol carrier alone (control) for 24 hours. Columns and bars represent the mean and SEM of triplicate values, respectively. (B) PGE₂ and PGE₃ levels in medium conditioned by HCA-7 human CRC cells in the absence or presence of EPA-FFA. PGE₂ and PGE₃ levels were measured by LC/MS/MS in medium conditioned by HCA-7 human CRC cells for 4 hours after 24 hours of prior exposure to EPA-FFA. Con represents the equivalent dilution (0.06% vol./vol.) of 95% (vol./vol.) ethanol alone. *nd* indicates not detected. Columns and bars represent the mean and SEM of triplicate values, respectively. Data are representative of three independent experiments. (C) The effect of SC-236 (1 μM) on EPA-FFA–dependent E-series PG synthesis by HCA-7 human CRC cells. SC-236 was added to the culture medium 90 minutes before EPA-FFA exposure (0.8 $\mu\text{mol}/10^6$ cells or 200 μM) for 24 hours. Fresh medium was conditioned for 4 hours as in panel B. The presence of SC-236 did not significantly change the viable cell number at the end of the experiment (data not shown). Columns and bars represent the mean and SEM of triplicate values, respectively.

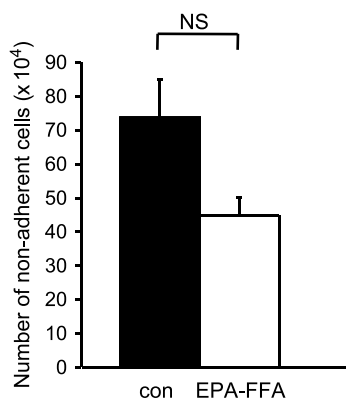


Figure 3. HT-29-EP4 human CRC cells are resistant to EPA-FFA–induced apoptosis. Apoptosis of HT-29-EP4 human CRC cells measured by nonadherent cell counting [14] after treatment with 0.8 μmol per 10^6 cells (200 μM) EPA-FFA or 95% (vol./vol.) ethanol carrier alone (0.06% [vol./vol.]) for 24 hours after overnight incubation of 1×10^6 CRC in triplicate 35-mm wells. Columns and bars represent the mean and SEM of triplicate values, respectively. *NS* indicates not statistically significant (unpaired *t* test).

reduced PGE₂ synthesis by HCA-7 human CRC cells treated with EPA-FFA. However, EPA exposure for 24 hours was not associated with changes in levels of COX-2 protein in HCA-7 human CRC cells (Figure 4A). Alternatively, reduced PGE₂ synthesis could explain the proapoptotic activity of EPA-FFA. Therefore, we tested whether exogenous PGE₂ “rescued” HCA-7 human CRC cells from EPA-FFA–induced apoptosis. In the absence of EPA-FFA, exogenously added PGE₂ significantly reduced spontaneous apoptosis of HCA-7 human CRC cells (Figure 4B). Addition of 1 μM PGE₂ partially “rescued” HCA-7 human CRC cells from the effect of EPA-FFA (a 15% decrease in apoptosis), but the presence of 10 μM PGE₂ did not alter EPA-FFA–induced apoptosis (Figure 4B).

PGE₃ Antagonizes the Protumorigenic Activity of PGE₂ in HT-29-EP4 Human CRC Cells

An alternative hypothesis is that PGE₃, alone or in combination with reduced levels of protumorigenic PGE₂, underlies the proapoptotic activity of EPA-FFA. For initial experiments involving addition of exogenous PGE₂ and/or PGE₃, we used HT-29-EP4 human CRC

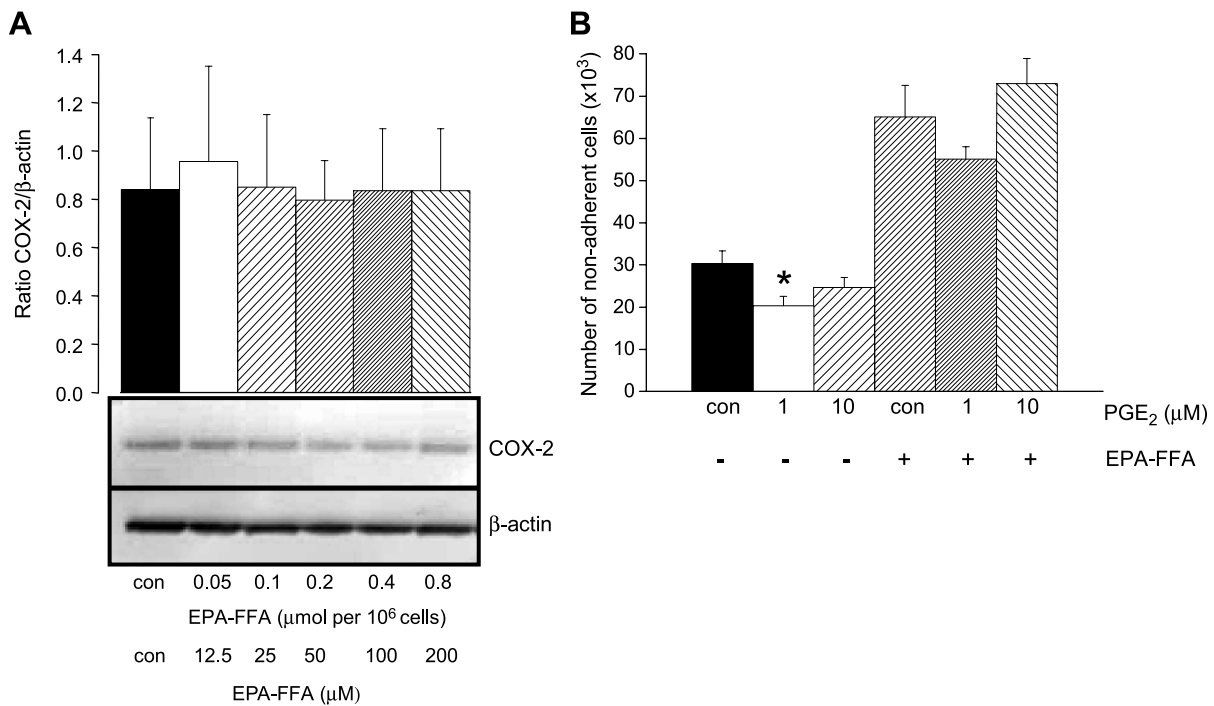


Figure 4. Decreased COX-2 protein expression or decreased PGE₂ synthesis alone does not explain the proapoptotic activity of EPA-FFA in human CRC cells. (A) Immunoblot analysis of COX-2 and β-actin protein levels in HCA-7 human CRC cells. Total protein cell lysates were obtained after treatment with 0.06% (vol./vol.) 95% (vol./vol.) ethanol alone (con) or differing amounts of EPA-FFA for 28 hours. Columns and bars represent the mean and SEM of fluorescence COX-2/β-actin ratios, respectively, in arbitrary units from two separate experiments. A representative immunoblot is included below. (B) Apoptosis of HCA-7 human CRC cells measured by nonadherent cell counting. Cells were cultured for 24 hours in the absence (0.06% [vol./vol.] dilution of 95% [vol./vol.] ethanol alone) or presence of 0.8 μmol per 10⁶ cells (200 μM) EPA-FFA with or without PGE₂. Columns and bars represent the mean and SEM of triplicate values, respectively. **P* < .05 for the comparison with control cells.

cells [14], thereby avoiding the confounding effect of endogenous PGE₂ production by CRC cells. As expected, PGE₂ decreased spontaneous apoptosis of HT-29-EP4 cells compared with cells treated with ethanol carrier alone (Figure 5A), as described previously [14]. How-

ever, equimolar amounts of PGE₃ alone did not significantly affect apoptosis of HT-29-EP4 cells (Figure 5A). Importantly, PGE₃ abrogated the antiapoptotic activity of PGE₂ when the two E-type PGs were present together at equimolar concentrations. This phenomenon

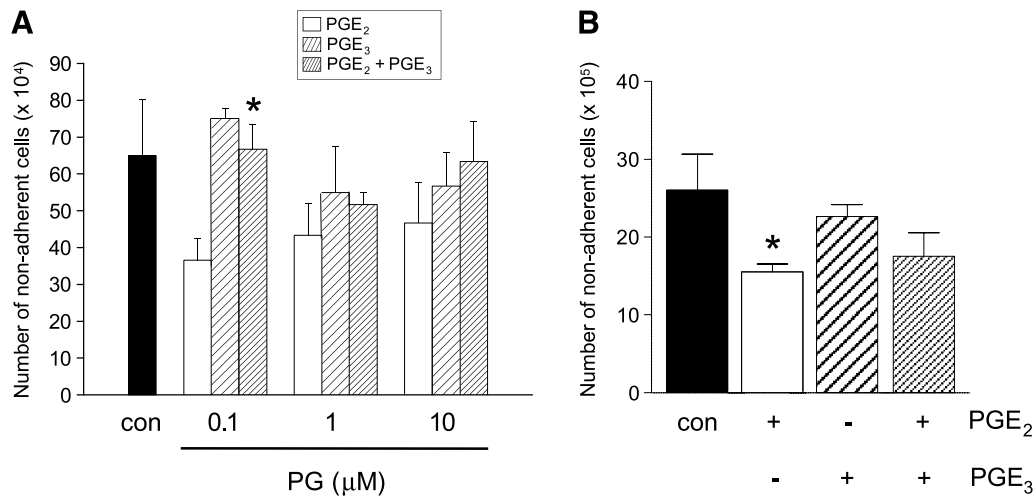


Figure 5. PGE₃ antagonizes the protumorigenic activity of PGE₂ in human CRC cells. (A) Apoptosis of HT-29-EP4 human CRC cells measured by nonadherent cell counting after treatment with PGE₃ and PGE₂, alone or in combination, for 24 hours. Columns and bars represent the mean and SEM of triplicate values for each condition, respectively. Con represents DMSO carrier (0.1% vol./vol.) alone. **P* < .05 for the difference from cells treated with 0.1 μM PGE₂ alone. (B) Apoptosis of HCA-7 human CRC cells measured by nonadherent cell counting after treatment with 1 μM PGE₃ and PGE₂, alone or in combination, for 24 hours. Columns and bars represent the mean and SEM of triplicate values for each condition, respectively. Con represents DMSO carrier (0.1% vol./vol.) alone. **P* < .05 for the comparison with control cells.

was most prominent at a PG concentration of 0.1 μM (Figure 5A). Similar observations were made with HCA-7 human CRC cells, in which 1 μM PGE₂, but not PGE₃, had significant antiapoptotic activity (Figure 5B). In a similar manner to HT-29-EP4 cells, the combination of PGE₂ and PGE₃ did not have an additive effect on the propensity of HCA-7 human CRC cells to apoptosis (Figure 5B). However, PGE₃ did not antagonize PGE₂ activity in HCA-7 human CRC cells to the degree

observed in HT-29-EP4 cells (Figure 5B), perhaps due to the high levels of PGE₂ already produced endogenously by these cells.

PGE₃ Directly Binds and Activates the EP4 Receptor on Human CRC Cells

Given the close structural similarity between PGE₂ and PGE₃, we hypothesized that PGE₃ antagonizes PGE₂ activity by binding to

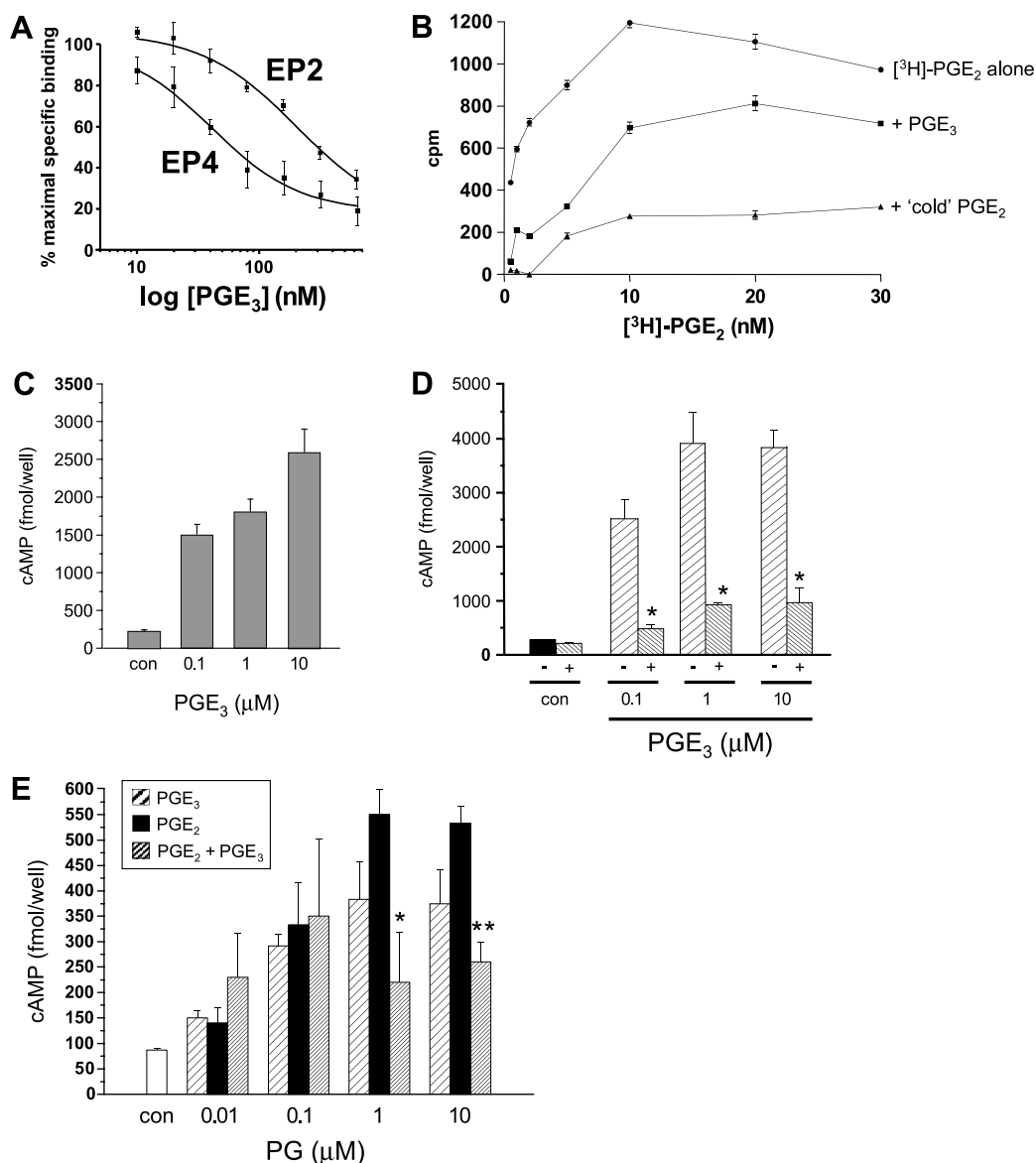


Figure 6. PGE₃ binds and acts as a partial agonist at the EP4 receptor in human CRC cells. (A) The effect of "cold" PGE₃ on [³H]-PGE₂ (20 nM) binding to purified EP4 and EP2 receptor membranes. Data represent the mean and SEM percentage specific binding ($n = 3$) compared with a "no PGE₃" control. (B) Competition of "cold" PGE₃ or PGE₂ (20 nM) with [³H]-PGE₂ binding to purified EP4 receptor membranes. Data represent the mean and SEM of triplicate measurements in counts per minute (cpm). (C) The effect of PGE₃ on cAMP levels in HT-29-EP4 human CRC cells at 5 minutes. Cells were preincubated with the phosphodiesterase inhibitor rolipram. Columns and bars represent the mean and SEM of triplicate values for each condition, respectively. Con represents 0.1% (vol./vol.) of DMSO carrier alone. Levels of cAMP after stimulation with all three concentrations of PGE₃ were significantly different from control cAMP levels ($P < .001$). (D) The effect of PGE₃ alone on cAMP levels in LoVo human CRC cells at 5 minutes, in the absence (-) or presence (+) of an EP4 receptor antagonist (10 μM ONO-AE3-208 for 60 minutes). Columns and bars represent the mean and SEM of triplicate values for each condition, respectively. Con represents 0.1% (vol./vol.) of DMSO carrier alone. * $P < .01$ for the differences in cAMP levels in the absence and presence of ONO-AE3-208. (E) The effect of PGE₃ and PGE₂, alone or in combination, on cAMP levels in LoVo human CRC cells at 5 minutes. Columns and bars represent the mean and SEM of triplicate values for each condition. Con represents 0.1% (vol./vol.) of DMSO carrier alone. * $P < .05$ for the difference in cAMP levels between cells treated with PGE₂ alone versus equimolar amounts of PGE₂ and PGE₃. ** $P < .01$ for the difference in cAMP levels between cells treated with PGE₂ alone versus equimolar amounts of PGE₂ and PGE₃.

PGE₂ EP receptors. Evidence is strongest for a role for EP4 and EP2 receptors in PGE₂ signaling during colorectal carcinogenesis [12–14,28,29]. Consistent with the concept that PGE₃ abrogates PGE₂ signaling through EP receptors, PGE₃ competed with PGE₂ (20 nM) for binding to purified human EP4 receptor (half maximal inhibitory concentration [IC₅₀] = 48 nM) and, to a lesser extent, EP2 receptor (IC₅₀ = 310 nM) membrane preparations in radioligand binding assays (Figure 6A). However, direct comparison of PGE₃ and PGE₂ in a radioligand competition assay using a purified human EP4 receptor preparation demonstrated that PGE₃ bound the EP4 receptor with less affinity than the “natural” ligand PGE₂ across a range of PGE₂ concentrations (Figure 6B).

Moreover, PGE₃ treatment led to a concentration-dependent rise in cAMP levels in our HT-29-EP4 human CRC cell model of EP4 receptor overexpression [14], confirming that PGE₃ can act as an EP4 receptor agonist (Figure 6C). We also tested the effect of PGE₃ on LoVo human CRC cells, which do not synthesize PGE₂ (detection limit 2 pg/ml per 10⁵ cells; data not shown) and which naturally exhibit predominant PGE₂-EP4 receptor signaling (Figure W2). PGE₃ also induced a concentration-dependent rise in cAMP levels in EP4 receptor-positive LoVo human CRC cells (Figure 6D). Cyclic AMP induction by PGE₃ was inhibited by preincubation with the selective EP4 receptor antagonist ONO-AE3-208 [14], thus confirming EP4 receptor agonist properties of PGE₃ in a second “natural” human CRC cell line (Figure 6D).

PGE₃ Is a Partial Agonist of the EP4 Receptor in Human CRC Cells

A direct comparison of EP4 receptor activation by PGE₂ and PGE₃ in LoVo human CRC cells demonstrated that, although PGE₃ is an agonist at the EP4 receptor, PGE₃ is less potent at activating the EP4 receptor than PGE₂ (Figure 6E) with a 50% effective dose (ED₅₀) of less than 100 nM, consistent with the lower binding affinity of PGE₃ noted earlier (Figure 6B). In keeping with the effect of PGE₃ on human CRC cell apoptosis (Figure 5), exposure of equimolar amounts of PGE₂ and PGE₃ to LoVo human CRC cells was not associated with an additive effect on cAMP elevation (Figure 6E). Instead, the combination of PGE₃ and PGE₂ was associated with a lower cAMP response than either ligand alone, suggesting a negative interaction between the three- and two-series PGs (Figure 6E). Similar observations were made in HT-29-EP4 human CRC cells (data not shown). Therefore, PGE₃ is a partial agonist at the EP4 receptor such that PGE₃ alone acts as an EP4 agonist, but in the presence of the natural ligand PGE₂, it behaves as an EP4 receptor antagonist. The partial agonist activity of PGE₃ on EP4 receptor-dependent cAMP elevation is consistent with a decrease in the antiapoptotic activity of PGE₂ in the presence of PGE₃ that was observed in EP4 receptor-positive HT-29-EP4 (Figure 5A).

Discussion

We report, for the first time, that the ω -3 PUFA EPA, in the FFA form, reduces COX-2-dependent PGE₂ synthesis and simultaneously drives a “PGE₂-to-PGE₃ switch” in human CRC cells *in vitro*. We have also elucidated that PGE₃ antagonizes protumorigenic signaling at the EP4 receptor by its natural ligand PGE₂ in human CRC cells. The relative contributions of reduced PGE₂ synthesis and PGE₃ activity to the anti-CRC effects of EPA *in vivo* remain to be determined. We did not address potential COX-independent mechanisms of the antineoplastic activity of EPA [5,6] in our experiments, including modulation of lipid raft behavior and lipid peroxidation.

Although EPA has previously been demonstrated to induce apoptosis of human CRC cells [5,6], ours is the first report concerning the proapoptotic activity of EPA-FFA formulation that has been shown to have antineoplastic effects in humans with FAP [17]. Our data are consistent with the increase in apoptotic cell frequency in normal rectal mucosa, which was observed in a previous phase 2 trial of EPA-FFA in patients with a history of “sporadic” colorectal adenomas [30].

Although EPA can act as a substrate for COX-2 with a K_M value similar to AA, enzymatic turnover values are approximately 35% those of AA [31]. Therefore, in the absence of any short-term change in COX-2 protein levels, reduced EPA substrate handling may explain the combination of a marked dose-dependent decrease in PGE₂ synthesis with appearance of PGE₃ at significantly lower levels than those observed for PGE₂. However, other factors are also likely to contribute to “inefficient” E-type PG synthesis when EPA acts as a COX-2 substrate, which may include decreased PGE synthase activity on PGH₃ compared with PGH₂ [31]. Studies are currently underway in our laboratory that will address whether a similar magnitude “PGE₂-to-PGE₃ switch” occurs in EPA-FFA-treated mouse and human CRC tissue *in vivo*. It is interesting to note that in the one published study that has examined whether a tissue “PGE₂-to-PGE₃ switch” occurs *in vivo*, colonic mucosal PGE₂ levels were reduced by approximately 75%, along with appearance of PGE₃ (albeit at much lower concentrations), in rats administered a fish oil- and pectin-containing diet [10].

Another important observation is that EPA, in the FFA form, is incorporated efficiently into membrane phospholipids of human CRC cells when delivered into the aqueous cell culture medium. Our observations concur with those in murine epidermal cells [32], human breast cancer cells [33], and human endothelial cells [34], in which significant EPA incorporation occurred within 24 hours of addition to the cell culture medium in an ethanol carrier. These *in vitro* findings mirror the increase in the EPA content of rectal mucosa of FAP patients, who received EPA-FFA 2 g daily for 6 months [17]. Not unexpectedly, there was no evidence of ω -3 PUFA interconversion from EPA to the other main ω -3 PUFA DHA in short-term cell culture, which is likely explained by low elongase and Δ^6 -desaturase activities in intestinal epithelial cells necessary for EPA-DHA conversion [35].

An important component of our study was a functional screen of a panel of human CRC cells for dominant PGE₂-EP4 receptor signaling based on the identification of PGE₂-dependent cAMP induction that could be inhibited by a selective EP4 receptor antagonist, in combination with immunoreactive protein localization. Identification of dominant PGE₂-EP4 receptor activity has significant advantages over previous semiquantitative reverse transcription-polymerase chain reaction measurements of EP receptor messenger RNA expression, which have uncertain functional significance [29,36]. Our functional screen identified LoVo and HCA-7 human CRC cells as exhibiting predominant EP4 receptor-dependent induction of cAMP by PGE₂, for use in future *in vitro* studies of EP4 receptor function during colorectal carcinogenesis, alongside stably transfected HT-29-EP4 cells [14].

Previous studies using stably transfected human embryonic kidney 293 cells expressing human EP receptors have demonstrated that PGE₃ acts as a partial agonist at EP1 to EP3 receptors, with the binding affinity and receptor activation potency of PGE₃ being significantly less than PGE₂ [31]. However, the 3.5-fold decrease in affinity and 6-fold decrease in potency (measured by cAMP induction) of PGE₃ (ED₅₀ = 3.5 nM) compared with PGE₂ (ED₅₀ = 0.58 nM) at

the EP4 receptor in 293 cells failed to reach statistical significance in this study [30]. Our data confirm that PGE₃ does indeed act as a partial agonist at the EP4 receptor in human CRC cells, which naturally express the EP4 receptor. PGE₃ has been previously reported to act in an EP4 receptor-dependent manner in BxPC-3 human pancreatic cancer cells, although direct PGE₃-EP4 receptor binding was not confirmed in this study [9].

Exogenous PGE₃ has been reported to decrease proliferation of COX-2-positive human A549 lung cancer cells and BxPC-3 human pancreatic cancer cells [8,9] and decrease invasiveness of COX-2-positive 70W human melanoma cells [37]. However, treatment with PGE₃ did not have any significant effect on the proliferation of NIH 3T3 fibroblasts [38] or nontransformed mouse mammary epithelial cells [39] and actually stimulated interleukin 6 production by unactivated mouse RAW264.7 macrophages, in a similar manner to PGE₂ [38]. Partial agonist activity of PGE₃ at EP receptors may explain these discrepant findings such that exogenously added PGE₃ has antagonist properties on a background of ongoing PGE₂-dependent signaling in COX-2-positive cells but has stimulatory activity when present alone with cells that do not synthesize PGE₂.

Our novel insights into EPA-dependent alterations in E-type PG synthesis and EP receptor signaling in human CRC cells have important implications for future translational human studies of the anti-CRC activity of the ω-3 PUFA EPA. The role of COX-2 in EPA-dependent alterations in PG synthesis suggests that combination therapy of EPA with a COX-2 inhibitor could abrogate the antineoplastic activity of both agents. Consistent with this concept, the selective COX-2 inhibitor celecoxib and the nonselective COX inhibitor indomethacin have both been demonstrated to decrease the proapoptotic and antiproliferative effects of EPA, respectively [8,40]. Therefore, ω-3 PUFA and selective COX-2 inhibitor treatment should only be considered for evaluation as a combination anticancer therapy with caution. Measurement of erythrocyte and/or tissue ω-3 PUFA levels is established as a biomarker of ω-3 PUFA intake and bioavailability [41,42]. However, the degree of membrane ω-3 PUFA incorporation might not necessarily mirror bioactivity. We suggest that measurement of PGE₃ (or its stable metabolite) levels in tissue, plasma, or urine should be investigated as a potential biomarker of COX-2-dependent EPA activity and therapeutic response in clinical studies.

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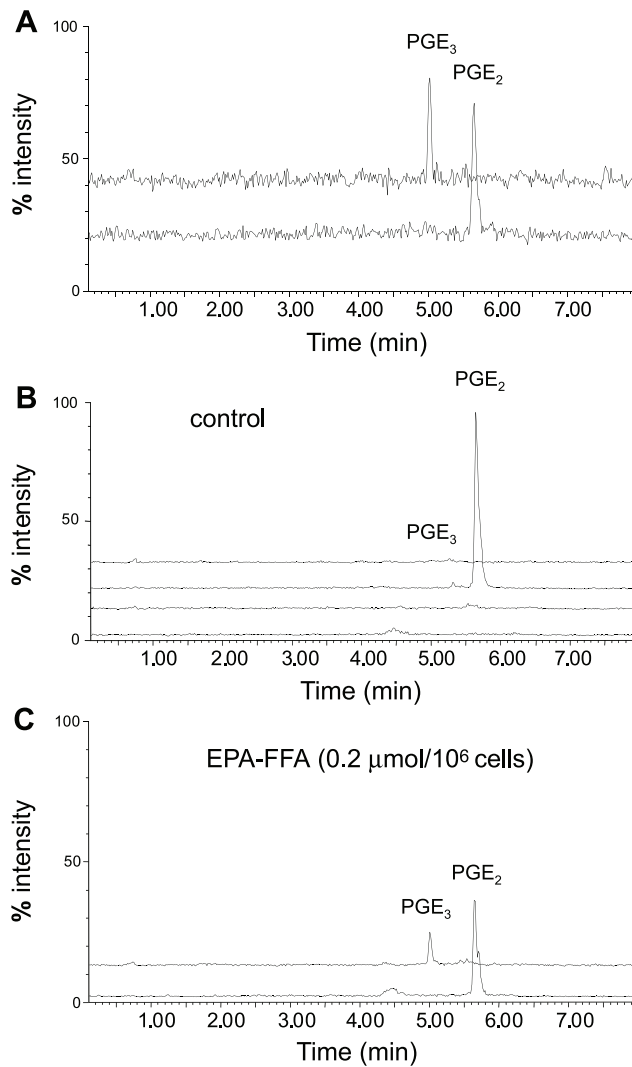


Figure W1. LC/MS/MS measurement of PGE₂ and PGE₃ in HCA-7 human CRC cell-conditioned medium. (A) Chromatogram of authentic PGE₂ (100 ng/ml) and PGE₃ (50 ng/ml) running at 5.7 and 5.0 minutes, respectively. (B) Chromatograms of extracted cell culture medium. The lower traces are PGE₃ and PGE₂ channels for unconditioned medium. The top two traces are PGE₃ and PGE₂ channels for medium conditioned by cells cultured in the presence of 95% (vol./vol.) ethanol carrier alone for 24 hours. Note the absence of a PGE₃ peak and a large PGE₂ peak. (C) Chromatograms of extracted conditioned medium from cells cultured in the presence of EPA-FFA (0.2 μmol/10⁶ cells [equivalent to a 50-μM concentration]) for 24 hours. PGE₃ is now present in conditioned medium along with lesser amounts of PGE₂ compared with medium conditioned in the absence of EPA-FFA (compare with B).

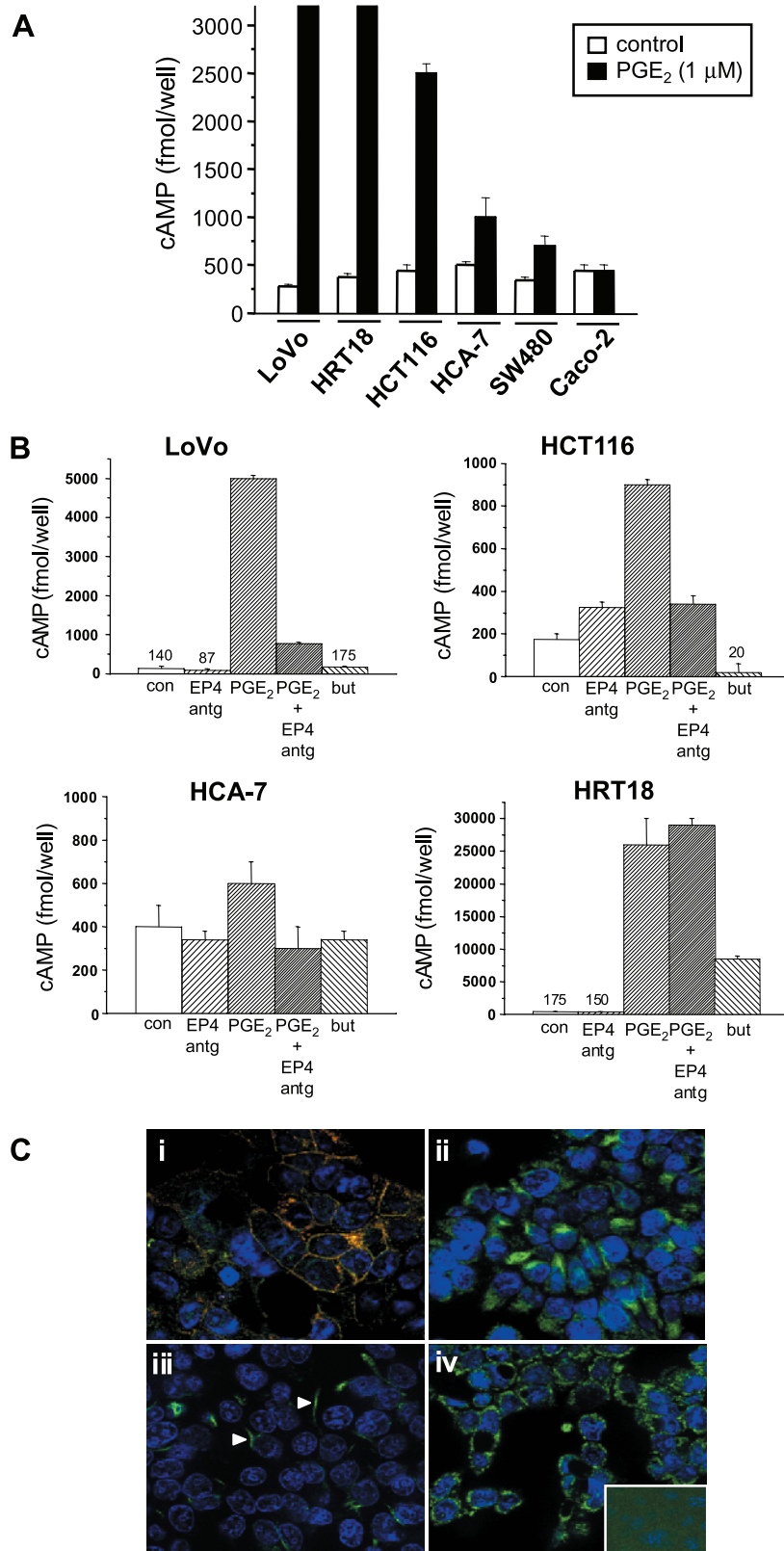


Figure W2. EP4 receptor signaling in human CRC cells. (A) Human CRC cell lines that generated a PGE₂-induced increase in intracellular cAMP at 5 minutes were identified. (B) cAMP induction by PGE₂ (1 μM) was inhibited by the EP4 receptor antagonist (EP4 antg) ONO-AE3-208 (10 μM) but not mimicked by the EP2 receptor agonist butaprost (but; 1 μM) in LoVo, HCT116, and HCA-7 cells, confirming dominant PGE₂-EP4 receptor signaling in these human CRC cell lines (but not HRT18). (C) Immunofluorescence detection of EP4 receptor protein in i) HT-29-EP4 cells (membranous colocalization [yellow] of anti-V5 tag [red] and EP4 receptor [green]), ii) and iii) LoVo cells demonstrating cytoplasmic and membranous localization (arrowheads), and iv) PGE₂ (1 μM)-treated LoVo cells demonstrating ligand-induced receptor internalization (inset—no primary antibody control).