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Ligand activation of peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) and inhibition of cyclooxygenase 2 (COX2) attenuate colon carcinogenesis through independent signaling mechanisms

Holly E.Hollingshead[1,2], Michael G.Borland[1,2], Andrew N.Billin[3], Timothy M.Willson[3], Frank J.Gonzalez[4] and Jeffrey M.Peters[1,2,*]

[1]Department of Veterinary and Biomedical Sciences and The Center of Molecular Toxicology and Carcinogenesis, [2]Graduate Program in Biochemistry, Microbiology, and Molecular Biology, The Pennsylvania State University, University Park, PA 16802, USA, [3]Nuclear Receptor Discovery Research, GlaxoSmithKline, Research Triangle Park, NC 27709, USA and [4]Laboratory of Metabolism, National Cancer Institute, Bethesda, MD 20892, USA

[*]To whom correspondence should be addressed. Tel: +1 814 863 1387; Fax: +1 814 863 1696;
Email: jmp21@psu.edu

Cyclooxygenase (COX) 2-derived prostaglandin E_2 (PGE_2) promotes colorectal carcinoma growth and invasion, and inhibition of COX2 by non-steroidal anti-inflammatory drugs is known to inhibit these processes. There is controversy regarding the effect of ligand activation of peroxisome proliferator-activated receptor (PPAR)-β/δ on colon carcinogenesis, although collective evidence from independent laboratories suggest that ligand activation of PPARβ/δ leads to the induction of terminal differentiation coupled with inhibition of cell growth in a variety of models. The present study examined the hypothesis that ligand activation of PPARβ/δ and inhibition of COX2 attenuate colon cancer through independent mechanisms and that combining these two mechanisms will enhance this inhibition. Colon cancer was induced by administering azoxymethane to wild-type and PPARβ/δ-null mice. Cohorts of mice were treated with GW0742 (a PPARβ/δ ligand), nimesulide (a COX2 inhibitor) or a combination of GW0742 and nimesulide. Inhibition of COX2 by nimesulide attenuated colon cancer and ligand activation of PPARβ/δ by GW0742 had inhibitory effects. However, the combined treatment of GW0742 and nimesulide did not cause an enhancement in the attenuation of colon cancer. Mechanistically, the effects of these compounds occurred through independent mechanisms as increased levels of differentiation markers as a result of ligand activation of PPARβ/δ were not found with COX2 inhibition, and a reduction in PGE_2 levels resulting from COX2 inhibition was not observed in response to ligand activation of PPARβ/δ. Results from these studies effectively dissociate COX2 inhibition and PPARβ/δ activity during colon carcinogenesis.

Introduction

A growing body of evidence has linked cyclooxygenase (COX2) 2, an enzyme that catalyzes the conversion of arachidonic acid to prostaglandins, with colorectal cancer (CRC). Approximately 70–80% of human colorectal carcinomas exhibit increased levels of COX2 expression (1). Previous studies using genetic and pharmacologic approaches suggest that COX2 plays a causal role in the development of CRC (2,3). In addition, COX2-specific inhibitors have been shown to inhibit cell growth in a number of tumors including colon, skin epidermal, gall bladder, esophageal adenocarcinoma and pancreatic cancer cells (4–8). Selective inhibitors of COX2 have been shown to inhibit the growth of adenomatous polyps in patients with familial adenomatous polyposis, which helped to establish the basis for their use as drugs in the treatment and/or prevention of CRC (9). Though they have been associated with anti-neoplastic activity in the colon, the mechanisms by which COX2 inhibitors exert their activities remain unclear. COX2 derived prostaglandin E_2 (PGE_2) affects numerous tumorigenic processes, including cell proliferation, motility, immune function and apoptosis (10–15). In addition, PGE_2 has been shown to prevent chemically induced attenuation of epithelial cell proliferation in a mouse model of colonic injury (16). The primary mechanism by which PGE_2 exacerbates colon cancer is through binding to a family of G-protein coupled receptors known as prostaglandin type E receptors, which activate a signaling cascade that stimulates adenylate cyclase activity, leading to the accumulation of cyclic adenosine 3′,5′-monophosphate. Increased cyclic adenosine 3′,5′-monophosphate activates protein kinase A–cyclic AMP response element-binding-dependent gene expression in tumor cells, leading to increased cell proliferation and inhibition of apoptosis (17). Recent studies have suggested that PGE_2 may also activate tcf4/β-catenin-mediated transcription, leading to increased expression of genes such as *cyclin D*, *c-myc* and *VEGF*, thereby increasing cell proliferation and angiogenesis (18–20). Combined, these findings suggest that COX2 inhibitors exert their anti-CRC activity via inhibition of PGE_2, which leads to attenuation of colonic cell proliferation and increased apoptosis. While COX2 inhibition has generally been thought to underlie the anti-CRC activity of non-steroidal anti-inflammatory drugs (NSAIDs) (21,22), several COX-independent mechanisms of action have also been described in cultured CRC cells (23).

Some reports have suggested that peroxisome proliferator-activated receptor (PPAR)-β/δ may also participate in prostaglandin-mediated signaling (24–29). However, this hypothesis remains speculative as other findings are inconsistent with the hypothesis linking COX2 activity and PPARβ/δ activation. For example, sulindac-mediated apoptosis occurs in HCT116 colon cancer cells, in the absence of PPARβ/δ expression (30) and activation of PPARβ/δ does not occur in cells over-expressing prostacyclin synthase (31), suggesting that COX-derived $PG1_2$ is not an endogenous PPARβ/δ ligand. Further, there is no relationship between PPARβ/δ polymorphisms in humans and the ability of COX inhibition to attenuate colon cancer (32) and inhibition of chemically induced epithelial cancer by inhibition of COX activity is effective in the absence of PPARβ/δ expression (33). Collectively, these observations suggest that COX activity and PPARβ/δ functions are independent.

A growing body of evidence indicates that PPARβ/δ attenuates CRC. For example, targeted deletion of the adenomatous polyposis coli alleles in mouse intestine results in reduced expression of PPARβ messenger RNA (mRNA) and protein accompanied with the expected increase in the level of mRNA encoding c-myc and accumulation of β-catenin (34). This is consistent with the findings that expression of PPARβ/δ in colon tumors from mouse and human models is lower as compared with normal colon epithelium (35,36). In the absence of PPARβ/δ expression, colon carcinogenesis is exacerbated in both genetic and a chemical mouse models of colon carcinogenesis (34,36,37), and ligand activation of PPARβ/δ in the colon results in inhibition of colon polyp formation in azoxymethane (AOM)-treated mice by increasing terminal differentiation in the colon (36). The latter finding is also supported by a number of reports from independent laboratories linking PPARβ/δ with inhibition of cell growth and/or terminal differentiation in a wide range of cell types (36,38–56). Additionally, there is good evidence that PPARβ/δ mediates

Abbreviations: APC, adenomatous polyposis coli; ADRP, adipocyte differentiation-related protein; ANGPTL4, angiogenin-related protein-like 4; AOM, azoxymethane; BrdU, bromodeoxyuridine; COX, cyclooxygenase; CRC, colorectal cancer; mRNA, messenger RNA; NSAID, non-steroidal anti-inflammatory drugs; PCR, polymerase chain reaction; PGE_2, prostaglandin E_2; PPAR, peroxisome proliferator-activated receptor.

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anti-inflammatory activity in a number of cell types (colon epithelium, macrophages, cardiomyocytes, immune cells, keratinocytes, myoblasts, endothelial cells and hepatocytes) (43,45,52,57–64). Collectively, these data suggest that PPARβ/δ attenuates CRC via activation of differentiation pathways with a subsequent reduction in cell proliferation and induction of apoptosis. Indeed, ligand activation of PPARβ/δ inhibits AOM-induced colon tumors in a PPARβ/δ-dependent mechanism that is due to the induction of terminal differentiation (36).

Combined, there is good evidence that inhibition of COX2 inhibits colon cancer by modulating prostaglandin-mediated signaling and that ligand activation of PPARβ/δ inhibits colon cancer by inducing terminal differentiation. This suggests that combining these two molecular pathways could effectively increase the efficacy of either targeted approach. This hypothesis is supported by a recent report showing that combining ligand activation of PPARβ/δ with the inhibition of COX enhances apoptosis and inhibits cell proliferation in human lung cancer cells (41). The present studies examined the hypothesis that combining the two treatments will increase the efficacy of inhibiting colon carcinogenesis.

Materials and methods

Cell proliferation analysis in HCT116 colon carcinoma cells

HCT116 colon carcinoma cells were obtained from American Type Culture Collection and maintained in McCoy's 5A medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum at 37°C and 5% carbon dioxide. For proliferation assays, cells were plated on 12-well dishes at a density of ∼500 000 cells per well 24 h prior to determining plating efficiency with a Z1 Coulter Particle Counter® at time 0 (Beckman Counter, Hialeah, FL). Cells were then serum starved for 24 h prior to ligand and nimesulide treatments. After this 24 h period, cells were maintained in respective culture medium with serum and treated with GW0742 for 24, 48 and 72 h at concentrations of 0 (dimethyl sulfoxide control) or 1 μM. This concentration of ligand was used because similar concentrations have been used by others, allowing for comparisons between these studies. In addition to ligand treatments, cells were treated with either the COX1/COX2 inhibitor indomethacin (Sigma-Aldrich, St Louis, MO) at a concentration of 600 μM or the COX2-specific inhibitor nimesulide (Sigma-Aldrich) at a concentration of 100 μM, either alone or in combination with GW0742. Cells were quantified every 24 h with a Z1 Coulter Particle Counter® (Beckman Counter). Triplicate samples for each treatment were used for each time point for every treatment, and each replicate was counted three times.

Colon cancer bioassay

Male wild-type and PPARβ/δ-null mice (50) (6–8 weeks of age, 10 mice per group) were injected intra-peritoneally with 10 mg AOM/kg body wt, once a week for 10 weeks as described previously (36). Four cohorts of mice from both genotypes were divided into one of the following groups: control, GW0742 alone (5 mg/kg body wt), nimesulide alone (400 mg/kg diet) or combined GW0742 and nimesulide. The mice being treated with GW0742 were administered 5 mg GW0742/kg body wt by oral gavage five times per week for 22 weeks. The mice being treated with nimesulide were fed a diet containing 400 mg nimesulide/kg diet (Bioserv, Piscataway, NJ) *ad libitum*. Twenty-two weeks after the initial injection with AOM, mice were euthanized by overexposure to carbon dioxide. The colons were flushed with phosphate-buffered saline, and lesions were counted and measured by inspection under a dissecting microscope.

Short-term nimesulide and ligand treatments

Male wild-type and PPARβ/δ-null mice were divided into one of three groups: (i) treated by oral gavage with GW0742 (5 mg/kg) once per day for 5 days, (ii) fed the nimesulide diet (400 mg/kg diet) for 5 days or (iii) fed the nimesulide diet (400 mg/kg diet) combined with GW0742 (5 mg/kg) via gavage once per day for 5 days. Two hours prior to euthanasia by overexposure to carbon dioxide, mice were injected intra-peritoneally with bromodeoxyuridine (BrdU) at a dose of 100 mg/kg. Mice were euthanized 8 h after the last dose of GW0742. The colons were carefully dissected and flushed with saline, cut into 3 mm serial sections and fixed in 10% buffered neutral formalin. After 24 h of fixation in formalin, colons were transferred to 70% ethanol and subsequently embedded in paraffin and cut into 3–4 μm sections for histological analyses. For PGE_2, protein and RNA analyses, sections of the colon were flash frozen in liquid nitrogen and stored at −80°C until ready for use.

BrdU analysis

Detection of BrdU-labeled cells was performed in the colon sections of short-term treated mouse colons using immunohistochemical methods. Sections were deparaffinized and rehydrated, and endogenous peroxidase was blocked with 3% H_2O_2 in methanol. Slides were incubated at 37°C for 30 min in a 0.08% trypsin solution for antigen retrieval, denatured by incubation in 2 N HCl at 37°C for 30 min and neutralized by incubation in 0.1 M borax for 10 min. Sections were blocked with 20% mouse serum for 30 min at room temperature, and subsequently blocked with Mouse-on-Mouse blocking reagent (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Primary mouse monoclonal BrdU antibody (Vector Laboratories) was applied to the sections at a 1:200 dilution and incubated at room temperature for 30 min. Slides were then incubated with secondary biotinylated anti-mouse IgG for (Vector Laboratories) 10 min at room temperature. These slides were incubated in avidin–biotin horseradish peroxidase (ABC kit, Vector Labarotories) for 5 min, followed by incubation in diaminobenzidine tetrahydrochloride for detection of positively labeled cells. The sections were counterstained with hematoxylin and visualized under a light microscope. BrdU-labeled colonocytes were quantified using light microscopy and labeling indices were quantified as a percentage of labeled cells per total cells in representative crypts counted.

Quantification of PGE_2 in colon and serum

PGE_2 levels were measured in the colons of short-term ligand-treated mice using an enzyme-linked immunosorbent assay-based assay (Assay Designs, Ann Arbor, MI). Extraction of PGE_2 from colon and serum was done as described previously (65). Tissues were homogenized briefly in prostaglandin extraction buffer [70% ethanol and 30% of 1 mM sodium phosphate (pH 4.0)] and incubated on ice for 30 min. All samples were then centrifuged at 3800 r.p.m. for 10 min, and the supernatant was collected. A fixed volume of each sample (250 μl) was dried under argon at 37°C and re-suspended in assay buffer. PGE_2 levels were determined following the manufacturer's recommended procedure and normalized to total protein content.

RNA analysis and real-time quantitative polymerase chain reaction

Total RNA was isolated from colon samples as described previously (36). The mRNAs encoding angiogenin-related protein-like 4 (ANGPTL4), COX2 and adipocyte differentiation-related protein (ADRP) were quantified using real-time polymerase chain reaction (PCR) analysis. The cDNA was generated using 2.5 μg total RNA with MultiScribe Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). Primers were designed for real-time PCR using the Primer Express software (Applied Biosystems). The sequence and GenBank accession numbers for the forward and reverse primers used to quantify mRNAs were *COX2* (NM_011198) forward, 5′-TTGCTGTACAAGCAGTGGCAAAGG-3′ and reverse, 5′-TGCAGCCATTTCCTTCTCTCCTGT-3′; *ANGPTL4* (NM_020581) forward, 5′-TTCTCGCCTACCAGAGAAGTTGGG-3′ and reverse, CATCCACAGCACCTACAACAGCAC-3′; and *ADRP* (NM_007408) forward, 5′-CACAAATTGCGGTTGCCAAT-3′ and reverse, 5′-ACTGGCAACAATCTCGGACGT-3′. All mRNAs examined were normalized to the gene encoding *GAPDH* (BC083149) using the following primers: forward, 5′-GGTGGAGCCAAAAGGGTCAT-3′ and reverse, 5′-GGTTCACACCCATCACAAACAT-3′. Real-time PCR reactions were carried out using SYBR green PCR master mix (Finnzymes, Espoo, Finland) in the iCycler and detected using the MyiQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The following conditions were used for PCR: 95°C for 15 s, 94°C for 10 s, 60°C for 30 s and 72°C for 30 s and repeated for 45 cycles. The PCR included a no template control reaction to control for contamination and/or genomic amplification. All reactions had >90% efficiency. Relative expression levels of mRNA were normalized to *GAPDH* and analyzed for statistical significance using one-way analysis of variance (Prism 4.0).

Quantitative western blot analysis

Protein samples were obtained from colon samples by homogenizing in 1× RIPA buffer. Samples were then centrifuged at 14 000 r.p.m. at 4°C for 45 min and supernatants were collected. Fifty micrograms of protein from each sample was resolved using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The samples were transferred onto a polyvinylidene difluoride membrane using an electroblotting method. After blocking in 5% milk in Tris buffered saline Tween-20, the membrane was incubated overnight at 4°C with primary antibody [COX2- (Santa Cruz Biotechnology, Santa Cruz, CA) or PPARγ (Affinity BioReagents, Golden, CO)], followed by incubation with a biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoreactive proteins were detected after incubation in [^{125}I]-labeled streptavidin (Amersham Biosciences, Piscataway, NJ) using phosphorimaging analysis. Hybridization signals for COX2 were normalized to the hybridization signals of the housekeeping protein, lactate dehydrogenase (Rockland, Gilbertsville, PA). Independent duplicate samples were used for analysis of each treatment group.

Chromatin immunoprecipitation assays

To confirm differential promoter occupancy on PPARβ/δ target genes after GW0742 treatment, chromatin immunoprecipitation assays were performed

using DNA from colonic epithelial cells from control and ligand-treated mice. Wild-type and PPARβ/δ-null mice were treated with vehicle (corn oil) or GW0742 (5 mg/kg) via oral gavage 4 h prior to euthanasia. After euthanasia, colons were removed, flushed with saline and epithelial cells scraped from the mucosa using a razor blade. Cells were cross-linked by adding formaldehyde to a final concentration of 1% in 7.8 M N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid. Chromatin immunoprecipitation assays were performed as described previously (66). Affinity-purified antibodies specific to cathepsin D (Santa Cruz Biotechnology) (negative control) or acetylated Histone H4 (Santa Cruz Biotechnology) were used for IP. No commercially available PPARβ/δ antibodies are suitable for immunoprecipitation (data not shown), precluding examination of PPARβ/δ promoter occupancy. Samples were PCR amplified with oligonucleotide primer pairs for the mouse 5′ *ANGPTL4* and *ADRP* proximal promoter elements using a standard PCR protocol. Primer sets for the *ANGPTL4* upstream promoter region were designed based on analysis of PPREs located in the 5′ upstream region. Primer pairs for the *ANGPTL4* proximal promoter region were forward, 5′-ACGTCGCTTATTAGGTCGCAAGGA-3′ and reverse, 5′-AGTGGGAGGGAGAGCATTGGA-3′. Primer sets for the *ADRP* upstream promoter region were designed based on previous evaluation of a peroxisome proliferator response element located in the 5′ upstream region spanning nucleotides −2004 to −1992 (67). Primer pairs for the ADRP proximal promoter region were forward, 5′-TCCTCCTTCCCTGGCAGACAAA-3′ and reverse, 5′-AGGAAGGTTGAGAACCACTGCTCT-3′.

Statistical analysis

Data were analyzed for statistical significance using analysis of variance and the Bonferroni post test (Prism 4.0).

Results

Ligand activation of PPARβ/δ and inhibition of COX enhance attenuation of cell proliferation in HCT116 colon cancer cells

Cell proliferation was significantly decreased as early as 24 h after treatment in response to either indomethacin or nimesulide, and continued to decrease over the 72 h treatment period (Figure 1A and B). No significant change in cell proliferation was observed following GW0742 treatment in the first 24 h of exposure. However, a significant decrease in cell proliferation was observed in HCT116 cells after 48 h of GW0742 treatment (Figure 1A and B), and this inhibition in cell proliferation continued for the duration of the experimental period. Consistent with a previous report (41), co-treatment of indomethacin or nimesulide with GW0742 resulted in an enhancement in the inhibition of cell proliferation as compared with either of the treatments alone (Figure 1A and B). Increased expression of mRNA encoding PPARβ/δ was found in response to both indomethacin and nimesulide

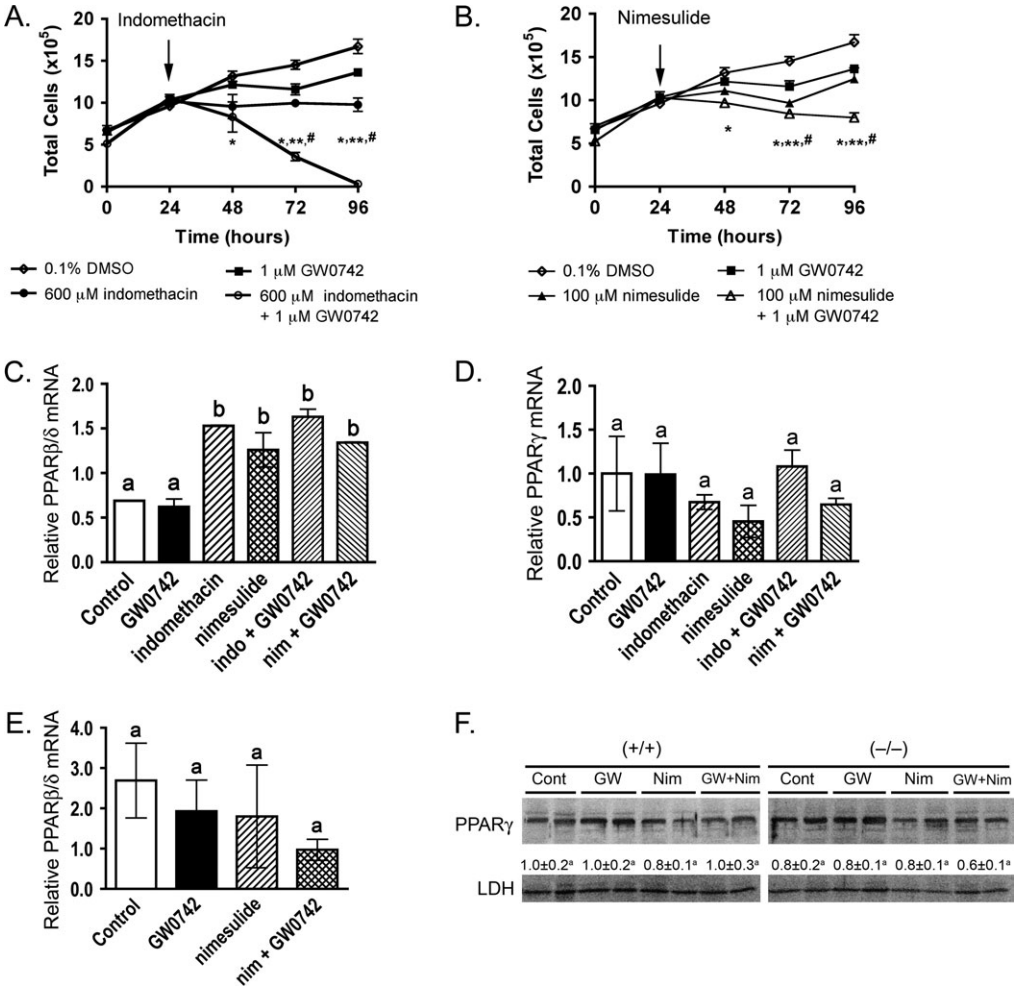

Fig. 1. Effect of GW0742 and COX inhibitors on cell proliferation in HCT116 colon cancer cells and PPARβ/δ and PPARγ mRNA expression *in vitro* and *in vivo*. HCT116 cells were treated with 1 μM GW0742, 600 μM indomethacin (**A**) or 100 μM nimesulide (**B**) or a combination of both indicated by the arrow, and cell number was quantified daily using Coulter counting as described in Materials and Methods. Values represent the mean ± SEM. *Indomethacin, nimesulide and combined treatment significantly different than dimethyl sulfoxide control, $P \leq 0.05$; **GW0742 treatment significantly different than dimethyl sulfoxide control, $P \leq 0.05$; #indomethacin or nimesulide treatment significantly different than GW0742 treatment, $P \leq 0.05$. Real-time PCR quantification of mRNA encoding (**C**) PPARβ/δ, (**D**) PPARγ from HCT116 cells treated as described above or (**E**) PPARβ/δ in colon of mice treated with either GW0742, dietary nimesulide or GW0742 and nimesulide. (**F**) Quantitative western blot analysis of PPARγ expression in colon of mice treated with either GW0742 and dietary nimesulide or GW0742 and nimesulide. Values with different letter are significantly different at $P \leq 0.05$.

(Figure 1C). No change in the expression of PPARγ mRNA was found in response to any of the treatments (Figure 1D). These results suggest that ligand activation of PPARβ/δ with COX inhibition can increase the efficacy of colon carcinogenesis inhibition.

Ligand activation of PPARβ/δ and inhibition of COX2 attenuate colon polyp multiplicity and/or cell proliferation independently

Administration of GW0742 (5 mg/kg, five times per week, 22 weeks) did not result in a significant decrease in colon polyp number, consistent with previous findings (36). In contrast, dietary nimesulide caused a significant decrease in colon polyp number, consistent with previous work by others (68) (Figure 2A). Co-treatment with GW0742 and nimesulide did not result in an enhancement in chemoprevention. Similar to previous reports, more colon polyps were found in PPARβ/δ-null mice as compared with similarly treated wild-type animals (36,37) (Figure 2A). Interestingly, dietary nimesulide treatment resulted in a significant decrease in colon polyp formation in PPARβ/δ-null mice as compared with control and GW0742-treated PPARβ-null mice (Figure 2A). No difference in the expression of mRNA encoding PPARβ/δ in colon was found between any of the treatment groups (Figure 1E). Additionally, no difference in the expression of PPARγ was observed between any of the treatment groups (Figure 1F).

A short-term experiment revealed that ligand activation of PPARβ/δ with GW0742 in wild-type mice caused a significant decrease in cell proliferation, and this effect was not observed in similarly treated PPARβ-null mice (Figure 2B). Nimesulide treatment also resulted in a significant decrease in cell proliferation in wild-type mice but not in PPARβ/δ-null mice (Figure 2B, supplementary Figure 1 is available at *Carcinogenesis* Online). Co-treatment with GW0742 and nimesulide did not enhance the reduction of cell proliferation (Figure 2B, supplementary Figure 1 is available at *Carcinogenesis* Online).

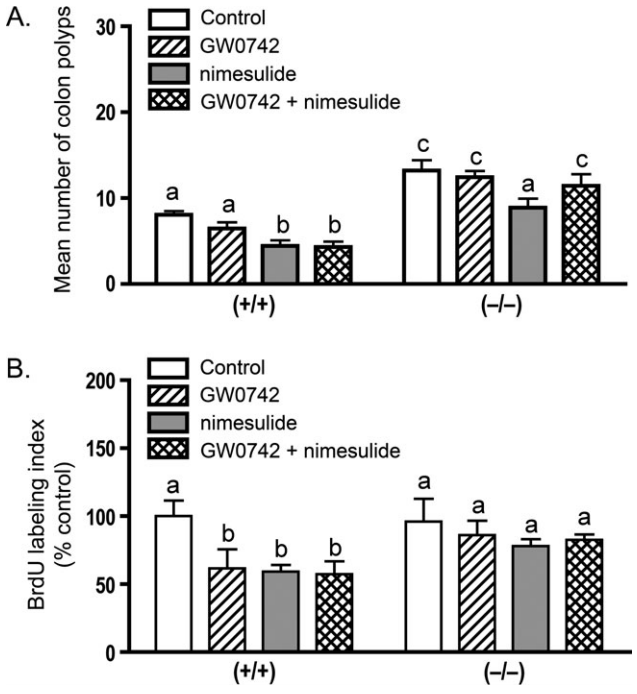

Fig. 2. Effects of GW0742 and nimesulide treatment on colon polyp multiplicity and cell proliferation in a colon cancer mouse model. (**A**) Wild-type mice (+/+) and PPARβ/δ-null mice (−/−) were treated with GW0742 (5 mg/kg) or dietary nimesulide (400 mg/kg diet) or a combination of both for 22 weeks as described in Materials and Methods. Colon polyp multiplicity was determined at the end of the experiment. (**B**) Wild-type mice (+/+) and PPARβ/δ-null mice (−/−) were treated with GW0742 (5 mg/kg) or nimesulide (400 mg/kg diet) or a combination of both for 5 days as described in Materials and Methods. Colons were fixed and immunohistochemical analysis was performed to examine BrdU incorporation in colonocytes. Relative BrdU incorporation was quantified as a percent of control. For all figures, values with different letters are significantly different at $P \leq 0.05$ as determined by analysis of variance.

Nimesulide inhibits COX2 activity in the colon and GW0742 does not

Nimesulide treatment caused a significant decrease in the level of PGE_2, and this decrease occurred in both wild-type and PPARβ/δ-null mice (Figure 3A), demonstrating that nimesulide treatment inhibited COX2. In contrast, GW0742 treatment had no effect on the level of colonic PGE_2 in either genotype (Figure 3A). In addition, co-treatment with GW0742 and nimesulide did not cause an enhancement in the reduction of PGE_2. The level of colonic COX2 mRNA and protein were unchanged in either nimesulide or GW0742-treated wild-type or PPARβ/δ-null mice (Figure 3B and C).

Ligand activation of PPARβ/δ directly modulates target gene expression associated with terminal differentiation and inhibition of metastasis

The mRNA encoding ANGPTL4 and ADRP were increased by GW0742 treatment in wild-type mice and this increase was not observed in similarly treated PPARβ/δ-null mice (Figure 4A). This demonstrates that GW0742 causes increased expression of these PPARβ/δ target genes. Increased expression of these PPARβ/δ target genes was not found after nimesulide treatment (Figure 4A). Similarly, ligand activation of PPARβ/δ in the presence of the COX2 inhibitor nimesulide did not significantly alter the increase in ADRP expression, although a marginal enhancement of ANGPTL4 mRNA was found with co-treatment (Figure 4A). Neither of these changes observed with co-treatment were found in PPARβ/δ-null mice. Since mRNA encoding ADRP (a marker of differentiation in the colon) was up-regulated by ligand activation of PPARβ/δ in the colon (Figure 4A), this suggests that PPARβ/δ activation induces differentiation in the colon as shown previously (36). In contrast, nimesulide treatment did not influence mRNA encoding ADRP (Figure 4A), suggesting that COX2 inhibition does not have a role in modulating terminal differentiation in the colon.

To demonstrate that induction of ANGPTL4 and ADRP is mediated by direct transcriptional modulation, chromatin immunoprecipitation analysis using an acetylated histone antibody was performed as no commercially available PPARβ/δ antibodies are suitable for IP (data not shown). Immunoprecipitation of chromatin with the affinity-purified anti-acetylated histone H4 antibody showed increased promoter occupancy on both the *ANGPTL4* and *ADRP* promoter in wild-type mouse colon after treatment with GW0742, and this effect was not found in either control or ligand-treated PPARβ/δ-null samples (Figure 4B). This is consistent with PPARβ/δ-dependent increased activity of histone acetyl transferase, which occurs to facilitate histone remodeling, allowing for ligand activated PPARβ/δ to up-regulate target genes.

Discussion

The effect of ligand activation of PPARβ/δ on colon carcinogenesis remains controversial due to disparities in the literature. Thus, it is essential to clarify and delineate specific mechanisms resulting from ligand administration. A number of recent reports in the literature suggest that PPARβ/δ is a target of the COX2 pathway and promotes CRC growth and invasion. However, these reports are inconsistent with data showing that PPARβ/δ represses inflammation, induces differentiation and suppresses cell proliferation, effects one would predict would lead to the attenuation of cancer. Findings from the present studies address three general areas of interest: (i) the role of PPARβ/δ in modulating COX-dependent activities, (ii) the effect of ligand activation of PPARβ/δ in a colon-specific carcinogenic model and (iii) whether combining ligand activation of PPARβ/δ with inhibition of COX2 will increase the efficacy of chemoprevention.

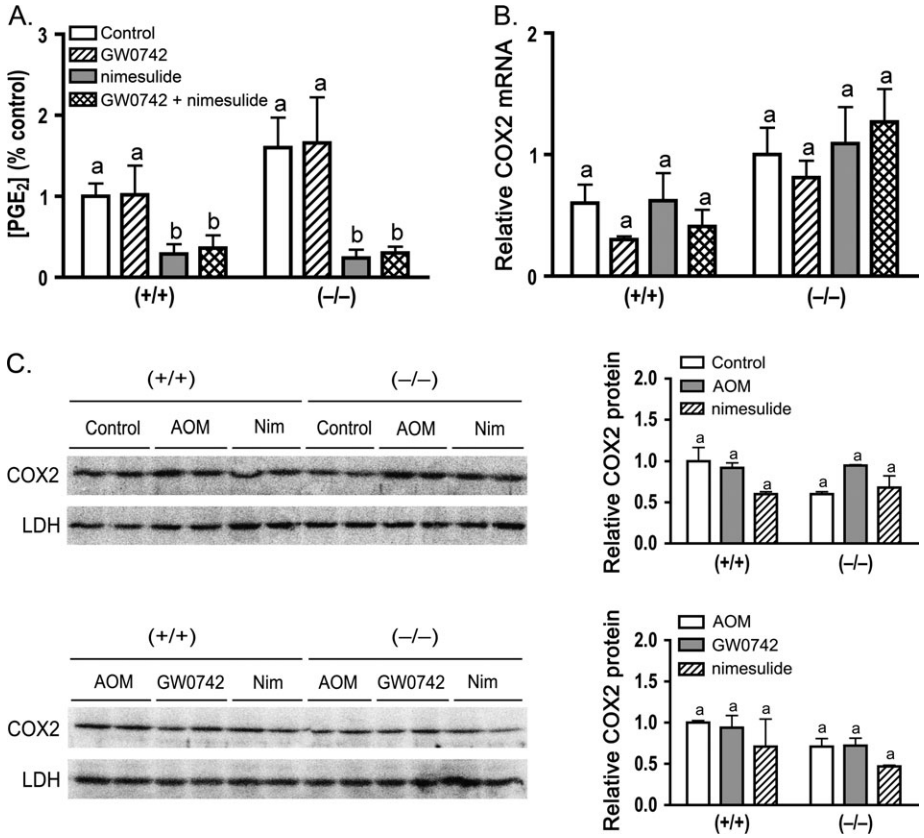

Fig. 3. Effect of GW0742 and nimesulide administration on PGE_2 concentration and COX2 expression in the colon. (**A**) Effect of GW0742 treatment (5 mg/kg/one times daily/5 days) or nimesulide treatment (400 mg/kg diet/5 days) or a combination of both on PGE_2 concentration in the colons of wild-type (+/+) and PPARβ/δ-null (−/−) mice. PGE_2 concentration is presented as pg PGE_2/mg protein and expressed as a percent of control. (**B**) Quantitative real-time PCR analysis on mRNA encoding COX2 in the colon of wild-type (+/+) and PPARβ/δ-null (−/−) mice treated with GW0742 (5 mg/kg) or nimesulide (400 mg/kg diet) or a combination of both. Values represent the mean fold change as compared with respective control ± SEM. (**C**) Quantitative western blot analysis of COX2 expression in the skin from control, AOM, GW0742 and nimesulide-treated wild-type (+/+) and PPARβ/δ-null mice (−/−). Values are presented as the mean ± SEM from independent samples. Values with different letters are significantly different at $P \leq 0.05$ as determined by analysis of variance.

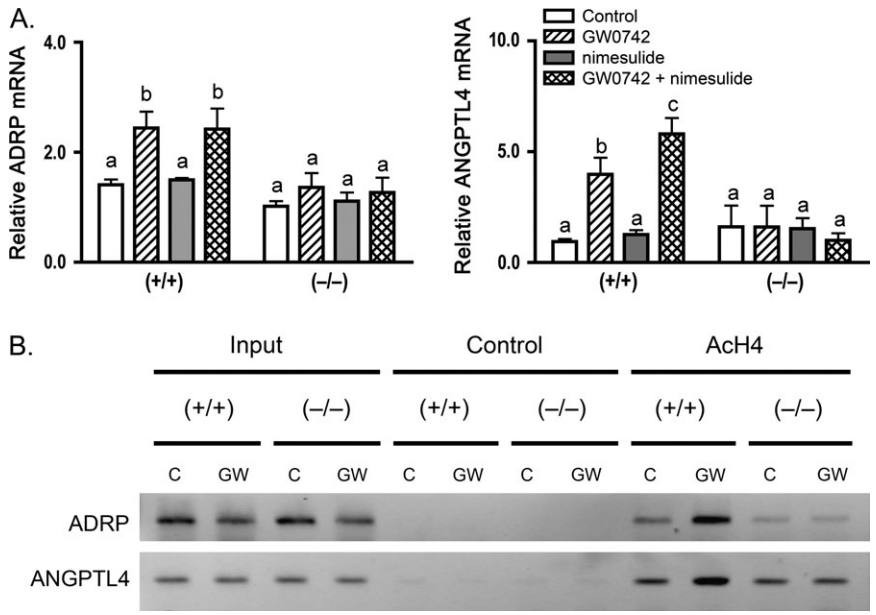

Fig. 4. Effect of GW0742 and nimesulide administration on PPARβ/δ target genes. (**A**) Effect of administration of GW0742 (5 mg/kg), dietary nimesulide (400 mg/kg diet) or a combination of both on mRNA encoding ANGPTL4 and ADRP in the colon of wild-type (+/+) or PPARβ/δ-null (−/−) mice. Values represent the mean fold change as compared with respective control ± SEM. (**B**) Effect of administration of GW0742 (5 mg/kg) on differential promoter occupancy in (+/+) and (−/−) mouse colonocytes in the 5′ regulatory region of *ADRP* or *ANGPTL4* using chromatin immunoprecipitation analysis with an anti-acetylated histone H4 antibody.

The current findings confirm a direct role of ligand activation of PPARβ/δ in differentiation in the colon, and more importantly distinguish COX2 and PPARβ/δ signaling as distinct molecular pathways, independent of each other.

In 1999, it was suggested that PPARβ/δ was a downstream target of the APC/β-catenin/tcf4 pathway (26). This and other observations led to the hypothesis that one mechanism by which inhibition of COX2-inhibited colon cancer was by reducing COX-derived PPARβ/δ ligands, which putatively led to increased PPARβ/δ transcription that mediated increased cell growth of colon tumors. This idea was supported by a number of related observations including that inhibition of COX activity by sulindac can inhibit PPARβ/δ activity and over-expression of PPARβ/δ in HCT116 cells prevents sulindac-mediated apoptosis (26). Inhibition of COX by sulindac is also reported to down-regulate PPARβ/δ in HT29 cells and lead to an overall increase in the level of apoptosis (69). However, results from the present studies and many others as well are inconsistent with the hypothesis that inhibition of colon cancer by COX2 inhibition is mediated in part by reducing COX-derived PPARβ/δ ligands, which limits PPARβ/δ transcription that facilitates increased cell growth of colon tumors. The combined observations from the present studies showing that inhibition of colon-specific carcinogenesis by inhibition of COX2 activity by nimesulide occurs in the absence of PPARβ/δ expression coupled with the observation that inhibition of chemically induced skin cancer occurs in mice that do not express a functional PPARβ/δ (33) dissociates the effect of COX inhibition and ligand activation of PPARβ/δ in tumorigenesis. Further, there is strong evidence that PPARβ/δ is not a downstream target of the APC/β-catenin/tcf pathway (34–36,70). Recent findings also show that PGI_2 does not activate PPARβ/δ transcription, as over-expression of prostacyclin synthase does not increase PPARβ/δ activity (31). HCT116 cells lacking PPARβ/δ are also not resistant to sulindac-mediated apoptosis (30), which is of interest because this finding comes from the same lab that originally suggested that PPARβ/δ is linked to sulindac-induced apoptosis (26) and is inconsistent with a more recent report (69). It is also important to note that there is no correlation between PPARβ/δ polymorphisms and the ability of NSAIDs to inhibit colon cancer in humans (32). Thus, these findings strongly suggest that the mechanisms underlying chemoprevention of colon cancer by inhibition of COX2 activity is more probably due to decreased PGE_2 signaling and related mechanisms, but are not mediated by PPARβ/δ. However, it also remains possible that inhibition of cell growth and tumorigenesis observed in these studies by inhibition of COX2 are mediated in part through mechanisms that are independent of PGE_2 as anti-tumorigenic effects of COX2 inhibitors are found in COX2-null mice (reviewed in ref. 71).

The recent hypothesis that NSAID-induced apoptosis is mediated by suppression of PPARβ/δ expression (69) is also not supported by the present findings. No decrease in PPARβ/δ was found in HCT116 cells by either indomethacin or nimesulide. Similarly, no changes in PPARβ/δ expression were found in colonocytes after exposure to the COX2 inhibitor nimesulide *in vivo*. This demonstrates that an NSAID-induced change in PPARβ/δ expression that putatively modulates apoptosis is not consistently observed. Indeed, increased expression of PPARβ/δ was found in response to NSAIDs, consistent with results obtained from treating renal cell carcinomas with NSAIDs (49). The reason for these differences could be due to differences in model systems (HT29 cells versus HCT116 cells), but the lack of change in an *in vivo* cancer model indicates that PPARβ/δ expression is not modulated by NSAIDs.

The second important finding from the present studies is the demonstration that ligand activation of PPARβ/δ directly increased expression of a differentiation-related target gene (ADRP) and inhibited replicative DNA synthesis in colonocytes, but did not potentiate tumorigenesis as reported by others (72). Treatment with GW0742 in wild-type mice with a dose of 5 mg/kg did not result in significant reduction of colon polyp formation, consistent with a previous report (36). However, short-term treatment with this dose of ligand caused a PPARβ/δ-dependent inhibition of cell proliferation in the colon as determined by relative BrdU incorporation. Deletion of PPARβ/δ expression also resulted in an increase in the average polyp number, as shown previously (36,37). The protective nature of PPARβ/δ is probably due to the direct modulation of terminal differentiation as shown in previous studies (36), which did not occur in response to inhibition of COX2 activity. Alternatively, ligand activation of PPARβ/δ could inhibit tumorigenesis via induction of ANGPTL4, which can inhibit tumor cell motility and invasiveness (73). These observations are consistent with work showing that treatment with two PPARβ/δ-specific ligands (GW0742 and GW501516) in three different human colon cancer cell lines (HCT116, HT29 and LS-174T) and one human liver cell line (HepG2) does not result in an increase of cell proliferation (74) and two independent studies showing that GW501516 does not cause increased cell proliferation in HT29 cells (75,76). The observed inhibition of cell growth found in HCT116 cells in the present studies and inhibition of colon tumorigenesis reported in a previous study (36) after ligand activation of PPARβ/δ is consistent with a number of other studies showing PPARβ/δ-mediated inhibition of cell growth in a number of different model systems (36,38–56). The present studies cannot determine why previous work by others suggests that ligand activation of PPARβ/δ potentiates colon carcinogenesis (72). However, it is worth noting that these studies (72) did not examine colon cancer using the colon-specific carcinogen AOM, and more importantly used mice on a mixed genetic background. Thus, it is possible that these results are confounded by the presence of modifiers of min alleles (77–79), which can significantly modulate the incidence of intestinal tumorigenesis in the APC^{min} mouse model. It is also worth noting that the variation observed in the average number of intestinal polyps simply within control APC^{min} mice from one laboratory ranges from 30 to >100 (27,72,80), while treatment-related effects exhibit similar large variation. This raises serious concern regarding the suitability of the APC^{min} mouse model for studying the role of PPARβ/δ in colon cancer. Thus, it remains possible that the contention that PPARβ/δ potentiates colon carcinogenesis is incorrect due to inherent limitations of the model. Combined, there is good evidence to suggest that both inhibition of COX2 activity and ligand activation of PPARβ/δ can both inhibit colon carcinogenesis, which supports the hypothesis that combining these two approaches will increase the efficacy of chemoprevention, which was also examined in these studies.

Interestingly, combining ligand activation of PPARβ/δ with inhibition of COX2 either additively or synergistically inhibited cell growth of HCT116 cells, consistent with the proposed hypothesis and similar to findings made in a lung tumor cell line (41). The mechanisms underlying this effect are uncertain but could include a synergistic or additive effect resulting from the combined effect of PPARβ/δ-dependent induction of terminal differentiation and inhibition of cell growth (81) coupled with NSAID-mediated suppression of PGE_2 signaling or NSAID modulation of COX-independent activities. Additionally, since PPARs and NSAIDs such as nimesulide can both inhibit nuclear factor kappa beta activity (82,83), it is possible that combining ligand activation of PPARβ/δ with COX2 inhibitors more effectively interferes with nuclear factor kappa beta signaling that leads to enhanced inhibition of cell growth. The effect of ligand activation of PPARβ/δ and COX2 inhibition was also examined using a mouse colon cancer model. Dietary nimesulide treatment resulted in a significant decrease in COX2 activity, colon polyp formation and cell proliferation in both genotypes. This is consistent with past work showing that inhibition of chemically induced skin cancer by sulindac is independent of PPARβ/δ (33). Co-treatment of GW0742 and nimesulide did not improve the inhibition of colon polyp formation or cell proliferation and no changes in PGE_2 levels were observed as a result of co-treatment with nimesulide and GW0742. Thus, despite promising data obtained from *in vitro* analysis, combining ligand activation of PPARβ/δ with inhibition of COX2 did not improve colon cancer chemoprevention at the doses examined in this work. It is unclear why the *in vitro* findings, suggesting that COX2 inhibitors work additively or synergistically with PPARβ/δ ligands to inhibit cell proliferation, are inconsistent with the *in vivo* findings showing no combinatorial effects between the two molecular targets. It remains possible that this

is due in part to species-specific differences between mouse and human, which could include differences in the ability to induce terminal differentiation, modulate COX2 activities and/or differences in nuclear factor kappa beta signaling. It is also possible that an additive or synergistic effect could be observed in the mouse model by varying the amounts of compounds, in particular by increasing the PPARβ/δ ligand. Further research is necessary to elucidate the mechanisms for these possible species-specific disparities or the possible additive or synergistic effects of these two compounds.

Collectively, the results of this study clearly distinguish COX2 and PPARβ/δ signaling as functionally distinct pathways. There are many reports in the literature that show that ligand activation of PPARβ/δ mediates cellular terminal differentiation, leading to inhibition of cell proliferation. This supports the hypothesis that PPARβ/δ attenuates a number of cancers. The present findings are highly inconsistent with the idea that PPARβ/δ potentiates the development of colon cancer via the COX2 pathway, and it gives strong evidence that PPARβ/δ attenuates colon cancer in a mechanism independent from that of the COX2 pathway. Further studies should be undertaken to examine the idea that combining ligand activation of PPARβ/δ with COX2 inhibitors and/or other molecular targets could lead to establishing chemotherapeutic/chemopreventive approaches with greater efficacy than currently available for the treatment of colon cancer.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: A.N.B. and T.M.W. are employed by Glaxo Smith Kline that has an interest in developing PPAR agonists as therapeutic agents.

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