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Regulation of Peroxisome Proliferator-Activated Receptor- β/δ by the APC/ β -CATENIN Pathway and Nonsteroidal Antiinflammatory Drugs

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Abstract

Studies indicate that peroxisome proliferator-activated receptor- β/δ (PPAR β/δ) can either attenuate or potentiate colon cancer. One hypothesis suggests that PPAR β/δ is upregulated by the adenomatous polyposis coli (APC)/ β -CATENIN pathway and a related hypothesis suggests that PPAR β/δ is downregulated by nonsteroidal anti-inflammatory drugs (NSAIDs). The present study examined these possibilities using in vivo and in vitro models. While APC/ β -CATENIN-dependent expression of CYCLIN D1 was observed in vivo and in vitro, expression of PPAR β/δ was not different in colon or intestinal polyps from wild-type or *Apc*^{min} heterozygous mice or in human colon cancer cell lines with mutations in *APC* and/or β -CATENIN. No difference in the level of PPAR β/δ was found in colon from wild-type or *Apc*^{min} heterozygous mice following treatment with NO-donating aspirin (NO-ASA). NSAIDs inhibited cell growth in RKO (wild-type *APC*) and DLD1 (mutant *APC*) human colon cancer cell lines but expression of PPAR β/δ was not downregulated in these cell lines in response to a broad concentration range of celecoxib, indomethacin, NS-398, or nimesulide. However, indomethacin caused an increase in PPAR β/δ mRNA and protein that was accompanied with increased expression of a known PPAR β/δ target gene. Interestingly, expression of PPAR α was also increased in the human colon cancer cell lines by several NSAIDs at the highest concentration examined. Results from these studies provide additional evidence indicating that PPAR β/δ is not upregulated by the APC/ β -CATENIN pathway. Further, these studies suggest that increased PPAR β/δ and/or PPAR α by NSAIDs in human colon cancer cell lines could contribute to the mechanisms underlying the chemopreventive effects of NSAIDs.

Keywords

colon cancer; PPAR β/δ ; gene expression; NSAIDs

INTRODUCTION

Peroxisome proliferator-activated receptor- β/δ (PPAR β/δ ; also referred to as PPAR β or PPAR δ) is a ligand-activated transcription factor with critical roles in regulating lipid and glucose homeostasis [1]. In response to ligand activation, PPAR β/δ modulates cellular function by directly modifying target gene expression. Since fatty acids likely act as endogenous ligands for PPARs, including PPAR β/δ , it is thought that PPAR β/δ acts as a cellular sensor. While the specific endogenous ligand(s) for PPAR β/δ remain to be identified, important physiological roles for PPAR β/δ have been established. For example, ligand activation of PPAR β/δ can decrease serum glucose concentration in diabetic mice [2], increase fatty acid oxidation in skeletal muscle [3], prevent high fat diet-induced obesity [3,4], increase serum high-density lipoprotein cholesterol [5], increase running endurance synergistically with exercise by increasing oxidative myofibers [6], prevent liver toxicity and fatty liver [7,8], and induce terminal differentiation in epithelial and neuronal cells [reviewed in 1]. For most of these effects, the essential requirement for PPAR β/δ has been established since these effects are not found in ligand-treated *Ppar β/δ -null* mice. In addition to direct regulation of target genes, PPAR β/δ also has potent anti-inflammatory activities that are likely mediated by PPAR β/δ interfering with other transcription factors [e.g., NF κ B, AP1, STAT3; reviewed in 9]. Thus, it is not surprising that there is considerable interest in targeting PPAR β/δ for the prevention and treatment of diseases including obesity, dyslipidemias, diabetes, and cancer.

The role of PPAR β/δ in cancer remains uncertain as some studies indicate that PPAR β/δ promotes tumorigenesis while others suggest that PPAR β/δ attenuates tumorigenesis [reviewed in 1,10,11]. It was originally hypothesized that PPAR β/δ was upregulated by the adenomatous polyposis coli (APC)/ β -CATENIN/transcription factor 4 (TCF4) pathway during colon cancer [12]. This was based in part on the observed decrease in PPAR β/δ expression in a human colon cancer cell line when APC expression was increased, coupled with the reported increase in PPAR β/δ expression in a small cohort of human colon tumors as compared to normal tissue [12]. This work led to the hypothesis that a mechanism by which nonsteroidal antiinflammatory drugs (NSAIDs) inhibit tumor growth is by suppressing cyclooxygenase-2 (COX2)-mediated formation of PPAR β/δ ligands, thereby limiting ligand activation of PPAR β/δ that putatively increased cell proliferation by upregulation of yet-to-be identified target genes [12]. However, while some reports support the initial finding that PPAR β/δ is upregulated by the APC/ β -CATENIN/TCF4 pathway, there are other studies that are not in concert with this view [reviewed in 1]. For example, disruption of *APC* in mouse colon does not result in an increase in PPAR β/δ expression [13] and increased expression of PPAR β/δ is not consistently found in human colon tumors [reviewed in 1]. A related hypothesis was also developed suggesting that NSAIDs inhibit growth of cancer cells by reducing expression of PPAR β/δ , however, not all reports support this hypothesis [reviewed in 1]. Thus, there is a pressing need to determine whether PPAR β/δ is regulated by an APC-dependent pathway and whether NSAIDs modulate PPAR β/δ expression. The present study examined the hypothesis that PPAR β/δ is regulated by the APC/ β -CATENIN/TCF4 pathway and/or NSAIDs using quantitative approaches with both in vivo and in vitro models.

MATERIALS AND METHODS

Cell Culture

RKO cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA). DLD1 and LS174T cells were cultured in RPMI 1640 medium (Invitrogen). HCT116 and HT29 cells were cultured in McCoy's 5A medium (Invitrogen). All cell lines were cultured with 10% fetal bovine serum at 37°C and 5% carbon dioxide. For quantitative Western blots, RKO or DLD1 cells were cultured for up to 48 h in culture medium containing one of the following NSAIDs: indomethacin (50, 200, or 600 μ M), nimesulide (10, 100, or 500

μM), NS-398 (0.1, 1.0, 10.0, or 100 μM) or celecoxib (1.0, 10, or 100 μM). Nuclear extracts were prepared using procedures as previously described [14] and stored at −80°C until further analysis.

Intestinal Tissue Samples

Colon tissue and small intestine polyps were dissected from female wild-type C57BL/6 or *Apc^{min}* heterozygous mice on a C57BL/6 background. Colon tissue from female wild-type C57BL/6 or *Apc^{min}* heterozygous mice on a C57BL/6 background treated with nitric oxide-donating aspirin (NO-ASA) were obtained from samples collected from a previously published study [15]. Nuclear extracts were prepared using procedures as previously described [14] and stored at −80°C until further analysis.

Cell Proliferation Analysis

For cell proliferation assays, RKO or DLD1 cells were plated on 12-well dishes at a density of ~20 000–40 000 cells per well and cultured in control medium for 24 h. After this 24 h culture period, cultured medium was changed to fresh medium containing DMSO (vehicle control), indo-methacin (50, 200, or 600 μM), nimesulide (10, 100, or 500 μM), NS-398 (1.0, 10, or 100 μM), or celecoxib (1.0, 10, or 100 μM). Cells were quantified every 24 h with a Z1 coulter particle counter® (Beckman Coulter, Fullerton, CA). Three independent samples for each treatment were used for each time point for every treatment, and each sample was counted in triplicate.

Western Blot Analysis

Thirty micrograms of nuclear extract per cell line sample or 50 μg of nuclear extract per tissue sample was resolved using SDS–polyacrylamide gels. The samples were transferred onto polyvinylidene fluoride membrane by electroblotting. The membranes were blocked with 5% dried milk in Tris-buffered saline/Tween-20 and incubated at 4°C overnight with primary antibodies. After incubation with biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), immunoreactive proteins on the membrane were detected with ¹²⁵I-labeled streptavidin using phosphorimaging analysis. Hybridization signals for the proteins of interest were normalized to the hybridization signal of the nuclear protein LAMIN. Minimum of three independent samples per treatment group were analyzed for all Western blots. For positive controls, lysate from COS1 cells transfected with expression vectors for mouse or human PPARα, PPARβ/δ, or PPARγ were used. The following antibodies were used: anti-LAMIN (SC-7293), anti-PPARβ/δ (H-74), anti-PPARγ (H-100, which detects both PPARγ1 and PPARγ2 isoforms), anti-cMYC (SC-42; Santa Cruz Biotechnologies, Santa Cruz, CA), or anti-CYCLIN D1 (Cell Signaling Technology, Danvers, MA). An anti-PPARβ/δ antibody [14] and an anti-PPARα antibody (Affinity Bioreagents, Golden, CO) were used for mouse samples.

RNA Analysis and Real-Time Quantitative Polymerase Chain Reaction

RKO and DLD1 cells were cultured as described above in medium containing 0.1% DMSO (control), GW0742 (0.1, 1.0, or 10 μM) and/or NSAIDs. Total mRNA was isolated using TRIzol and following manufacturer's recommended protocol (Invitrogen). The mRNA-encoding PPARβ/δ, adipose differentiation-related protein (*ADRP*), and angiopoietin-like 4 (*ANGPTL4*) were quantified using quantitative real-time polymerase chain reaction (qPCR). 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 number for the forward and reverse primers used to quantify mRNAs were: PPARα (L02932.1) forward, 5'-TGGACGAGTCTCCCAGTGG-3' and reverse, 5'-

CCCCGCAGATTCTACATTCG-3', PPAR β/δ (AY919140) forward, 5'-GACAGTGACCTGGCCCTATTCA-3' and reverse, 5'-AGGATGGTGTCTGGATAGCCT-3', *ADRP* (NM_000122) forward, 5'-CTGCTCTTCGCCTTTCGCT-3', and reverse, 5'-ACCACCCGAGTCACCACACT-3', and *ANGPTL4* (NM_020581) forward, 5'-TTCTCGCCTACCAGAGAAGTTGGG-3' and reverse, 5'-CATCCACAGCACCTACAACAGCAC-3'. The mRNA was normalized to the gene encoding *GAPDH* (BC083149) using the following primers: forward, 5'-GGTGGAGCCAAAAGGGTCAT-3' and reverse, 5'-GGTTCACACCCATCACAACAT-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 >85% efficiency. Relative expression levels of mRNA were normalized to *GAPDH* and analyzed for statistical significance using one-way analysis of variance (Prism 5.0).

RESULTS

Expression of PPAR β/δ in APC Mutant Mouse and Human Models

To critically examine the role of APC in modulating PPAR β/δ , expression of PPAR β/δ was quantitatively examined in mouse colon from wild-type and *Apc^{min}* heterozygous mice, and intestinal polyps from the latter genotype. No difference in the expression of PPAR β/δ was found in colon between either genotype, and no change in expression of PPAR β/δ was noted in the polyps as compared to normal colon tissue (Figure 1A). Expression of PPAR γ 1 and PPAR γ 2 was very low as compared to PPAR β/δ , and no difference in PPAR γ 1 or PPAR γ 2 levels was found between either genotype or in polyps (Figure 1A). PPAR α was not detected in any of the samples (Figure 1A). Interestingly, while expression of cMYC was not different between genotypes or between colon and polyp samples, increased expression of CYCLIN D1 was observed in the polyp samples as compared to colon from both wild-type and *Apc^{min}* heterozygous mice (Figure 1A). These data are consistent with past reports showing no difference in PPAR β/δ expression between wild-type and *Apc^{min}* heterozygous mice as determined by Western blotting [16–18].

To examine the role of APC in modulating PPAR β/δ expression in human colon cancer models, human colon cancer cell lines with different mutations in *APC* and β -*CATENIN* were utilized (Table 1). RKO human colon cancer cells were used as a control as they have wild-type *APC* and β -*CATENIN* alleles, while the other cell lines (DLD1, HT29, LS174T, HCT116) exhibit enhanced β -*CATENIN*/TCF4 signaling due to either mutant *APC* or gain-of-function mutations in β -*CATENIN*. Indeed, expression of CYCLIN D1 was significantly greater in DLD1, HT29, LS174T, and HCT116 cells as compared to RKO controls (Figure 1B), consistent with enhanced β -*CATENIN*/TCF4 signaling since CYCLIN D1 is a known target of this pathway. Expression of cMYC was markedly higher in LS174T cells as compared with all other cell lines (Figure 1B). Despite evidence of enhanced APC/ β -*CATENIN*/TCF4 signaling in DLD1, HT29, LS174T, and HCT116 cells, expression of PPAR β/δ was not different between any of the five human colon cancer cell lines (Figure 1B). Expression of PPAR α was not detected in any of the cell lines, and expression of PPAR γ 1 was similar between all of the cell lines (Figure 1B). Expression of PPAR γ 2 was higher in HT29 and LS174T cells as compared to the other cell lines (Figure 1B). Collectively, results from this analysis are consistent with results from the mouse models showing no change in PPAR β/δ expression due to APC-dependent upregulation.

Recent studies using immunohistochemical analysis suggest that PPAR β/δ expression was increased 10-fold in Apc^{min} heterozygous mouse colon as compared to wild type [19]. Further, it was also suggested that the chemopreventive effects of the NSAID, NO-ASA, was due in part to reduced expression of PPAR β/δ caused by NO-ASA [19]. Since it was recently shown that the background immunoreactivity of a highly specific anti-PPAR β/δ antibody is very high [14], samples of mouse colon from wild-type and Apc^{min} heterozygous mice treated with and without NO-ASA were obtained from a previously published study [15], and used to examine PPAR β/δ expression using quantitative Western blotting. In contrast to the previous report [19], no difference in the level of PPAR β/δ in colon samples was observed between wild-type and Apc^{min} heterozygous mice, and NO-ASA had no effect on PPAR β/δ expression (Figure 1C). No differences in the expression of PPAR α or PPAR γ 1 were detected between genotypes or treatment (Figure 1C).

Effect of NSAIDs on PPAR β/δ Expression in Human Colon Cancer Cell Lines

Some studies suggest that the cell growth inhibitory activity of NSAIDs may be due in part to inhibition of PPAR β/δ expression while others do not [reviewed in 1]. Thus, expression of PPAR β/δ protein was quantified in RKO and DLD1 human colon cancer cell lines treated with NSAIDs. A significant decrease in cell proliferation was observed in both RKO and DLD1 cells after 48 h of culture with 500 μ M nimesulide and by 72 h this effect was greater in DLD1 cells (Figure 2A). A significant decrease in cell proliferation was observed in both RKO and DLD1 cells after 48 h of culture by 600 μ M indomethacin (Figure 2B). Inhibition of cell growth was also observed after 72 h following culture in 200 μ M indomethacin in both cell lines (Figure 2B). In response to celecoxib, inhibition of cell proliferation was observed in both RKO and DLD1 after 48 h by a concentration of 100 μ M and after 72 h by concentrations greater than 1.0 μ M (Figure 2C). Significant inhibition of cell proliferation was found in RKO cells after 72 h of culture in either 10.0 or 100 μ M NS-398, and this effect was not observed in similarly treated DLD1 cells (Figure 2D). The decreases in cell proliferation by NSAIDs were likely due to increased cell death as determined by trypan blue exclusion (data not shown) and gross cell morphology (Supplemental Figure 1). These results show that RKO and DLD1 cells exhibit significant decreases in cell proliferation between 48 and 72 h following treatment with the indicated concentrations of NSAIDs and provide the rationale for the concentrations of NSAIDs that were used to evaluate the effect of NSAIDs on PPAR β/δ expression and function.

If NSAIDs-dependent inhibition of human colon cancer cell growth is mediated by downregulation of PPAR β/δ , then decreased expression of this receptor should precede the observed decreases in cell proliferation (Figure 2). Previous work by others also showed that decreased expression of PPAR β/δ by NSAIDs occurs between 24 and 48 h in human colon cancer cell lines [20,21]. Thus, expression of PPAR β/δ was examined in RKO and DLD1 cells treated with NSAIDs after 24 h. Expression of PPAR β/δ was either unchanged or increased by NSAIDs in both RKO and DLD1 cells (Figure 3). For example, PPAR β/δ was increased in RKO cells ~2-fold to 3-fold with 50–600 μ M indomethacin or 1.4-fold with 500 μ M nimesulide. In DLD1 cells, PPAR β/δ was increased ~2-fold treated with 500 μ M nimesulide or ~2-fold with 100 μ M celecoxib (Figure 3). The changes in PPAR β/δ protein were also reflected by similar changes in mRNA expression after 24 h of treatment (Figure 5A). Importantly, no decrease in PPAR β/δ protein or mRNA was observed in response to any of the NSAIDs after 24 h of treatment (Figure 3, Figure 5A). Interestingly, expression of PPAR α was increased by 2-fold to 6-fold in both RKO and DLD1 cells following exposure to 200 or 600 μ M indomethacin or 100 μ M celecoxib (Figure 3). Increased expression of PPAR α was also noted in DLD1 cells treated with 500 μ M nimesulide (Figure 3). The changes in PPAR α protein were also reflected by similar changes in mRNA expression after 24 h of treatment (Figure 5A). No changes in the level of PPAR γ were observed in either RKO or DLD1 cells treated with any of the NSAIDs (Figure 3). Expression of PPARs was also

examined after 48 h (Figure 4) using concentrations of NSAIDs shown to cause inhibition of cell proliferation (Figure 3). Expression of PPAR β/δ was increased by 200 μ M indomethacin in RKO cells but not in DLD1 cells (Figure 4). No other changes in the expression of PPAR β/δ were noted in response to 500 μ M nimesulide, 10 μ M celecoxib, 100 μ M NS-398, or 150 μ M sulindac (Figure 4). While no change in PPAR γ 1 expression was observed in either cell type by NSAIDs, expression of PPAR α was significantly higher in DLD1 cells following treatment with 500 μ M nimesulide, 100 μ M NS-398, or 150 μ M sulindac in RKO cells following treatment with 100 μ M NS-398 (Figure 4).

To determine if any functional changes in PPAR β/δ activity are observed by NSAIDs, analysis of known PPAR β/δ target gene expression was performed. *ADRP* and *ANGPTL4* are well-characterized PPAR β/δ target genes [22–25]. Interestingly, there was a significant difference in the response to the PPAR β/δ ligand GW0742 between RKO and DLD1 cells. In RKO cells, no change in the expression of *ANGPTL4* mRNA was observed following treatment with GW0742 between 0.1 and 10 μ M while an increase in *ADRP* mRNA was observed in these cells with these concentrations of GW0742 (Figure 5B). In contrast, in DLD1 cells, no change in the expression of *ADRP* mRNA was observed following treatment with GW0742 between 0.1 and 10 μ M while an increase in *ANGPTL4* mRNA was observed in these cells with these concentrations of GW0742 (Figure 5B). Thus, *ADRP* mRNA was used as a marker for PPAR β/δ activity in RKO cells while *ANGPTL4* mRNA was used as a marker for PPAR β/δ activity in DLD1 cells. As compared to control, a significant increase in the level of mRNA encoding *ADRP* was found in RKO cells treated for 24 h with 600 μ M indomethacin, 500 μ M nimesulide, and 100 μ M celecoxib, but no decrease in expression of this PPAR β/δ target gene was observed with the other treatments (Figure 5B). A significant increase in the level of mRNA encoding *ANGPTL4* was found in DLD1 cells treated for 24 h with 600 μ M indomethacin and 500 μ M nimesulide as compared to control, but no decrease in expression of this PPAR target gene was observed with the other treatments as compared to control (Figure 5B). The increase in expression of the PPAR β/δ target genes *ADRP* and *ANGPTL4* correlate well with the observed increases in expression of PPAR β/δ observed in response to NSAIDs at both the mRNA (Figure 5A) and protein level (Figure 3 and Figure 4).

DISCUSSION

Results from these studies provide evidence indicating that PPAR β/δ is not upregulated by the APC/ β -CATENIN/TCF4 pathway. No difference in the level of PPAR β/δ protein was found between wild-type and Apc^{min} heterozygous mouse colon, or between colon and intestinal polyps in Apc^{min} heterozygous mice. Importantly, this is in contrast to the increase in CYCLIN D1 expression noted in intestinal polyps as compared to colon in both wild-type and Apc^{min} heterozygous mice. The lack of change in PPAR β/δ protein between wild-type and Apc^{min} heterozygous mouse colon and/or intestinal tumors is consistent with three previous reports [16–18]. Results from the present studies also show that the expression of PPAR β/δ is not different between five different human colon cancer cell lines with different mutations in *APC* and/or β -CATENIN (Table 1). Notably, as compared to RKO cells that have wild-type *APC* and β -CATENIN alleles, expression of PPAR β/δ was not different in DLD1, HT29, LS174T, or HCT116 cells. In contrast, expression of CYCLIN D1 was higher in DLD1, HT29, LS174T, or HCT116 cells as compared to RKO cells consistent with the known mutational spectrum in *APC* and β -CATENIN in these human colon cancer cell lines (Table 1). Thus, despite strong evidence that APC/ β -CATENIN/TCF4 signaling is enhanced in DLD1, HT29, LS174T, or HCT116 cells as compared to RKO cells, PPAR β/δ expression was not different. Collectively, data from an in vivo model of dysregulated APC (Apc^{min} heterozygous mice) and in vitro models of dysregulated APC indicate that PPAR β/δ is not upregulated by the APC/ β -CATENIN/TCF4 pathway. This is consistent with a large number of other studies including the lack of change in PPAR β/δ expression when *APC* is disrupted in the colon or when other

APC-related signaling molecules are repressed [reviewed in 1]. The present studies significantly extend the current understanding of potential roles of PPAR β/δ in colon cancer because to date, no comprehensive and quantitative analysis of PPAR β/δ protein expression in related models have been reported. Essentially all reports suggesting that PPAR β/δ is upregulated by the APC/ β -CATENIN/TCF4 pathway are based largely on mRNA measurement data [reviewed in 1]. Given the results from the present studies, it is clear that there remains a need for careful quantification of PPAR β/δ protein and function (e.g., PPAR β/δ target gene expression) from human samples comparing normal epithelium with tumors from patients with mutations in *APC* and/or β -CATENIN signaling genes since examination of mRNA may not reflect protein expression.

Expression of PPAR γ 1 and PPAR γ 2 was not different between wild-type and *Apc^{min}* heterozygous mice, but expression of PPAR γ 2 was markedly higher in HT29 cells and LS174T cells as compared to RKO, DLD1, and HCT116 cells. The lack of change in PPAR γ expression between wild-type and *Apc^{min}* heterozygous mice is in contrast to previous work by others showing increased expression of PPAR γ in *Apc^{min}* heterozygous mouse colon as compared to wild type [26]. However, the increased expression of PPAR γ 2 found in HT29 and LS174T cells is consistent with the findings that activation of β -CATENIN/TCF4 signaling can increase PPAR γ expression in SW480 cells [26]. The significance of these observations remains to be determined.

A related important finding from the present studies is the lack of change in PPAR β/δ protein in the colon from mice treated with NO-ASA, a relatively unique form of an NSAID. A previous report suggested that expression of PPAR β/δ was ~10-fold higher in the colonic nuclei of *Apc^{min}* heterozygous mice as compared to wild type and that treatment of NO-ASA caused downregulation of PPAR β/δ expression in the colon and polyps [19]. This putative downregulation was thought to contribute to the mechanisms underlying the chemopreventive effects of NO-ASA. However, results from the present studies quantitatively demonstrate that expression of PPAR β/δ protein is not higher in nuclear extracts from colon of *Apc^{min}* heterozygous mice as compared to wild type and that NO-ASA did not cause downregulation of PPAR β/δ in the colon. This discrepancy is likely explained by differences in the method of PPAR β/δ detection. The publication reporting these effects based their conclusion on immunohistochemical analysis of PPAR β/δ [19], whereas the present analysis used quantitative Western blotting using nuclear extracts from tissues from the same experiment. It was recently shown that there is considerable background immunoreactivity of even a highly specific anti-PPAR β/δ antibody [14] indicating that immunohistochemical analysis of PPAR β/δ is likely not suitable for quantifying PPAR β/δ . This is significant because there are a number of reports that used immunohistochemical analysis to examine PPAR β/δ expression in colon cancer models. For example, immunohistochemical analysis of PPAR β/δ suggests that expression of PPAR β/δ is increased in some human colon cancer patients but no quantified statistical analysis of Western blots was provided [27]. Additionally, another report suggested that expression of PPAR β/δ was higher in flat dysplastic adenomas from *Apc^{min}* heterozygous mice but Western blot analysis showed no change in the expression of PPAR β/δ in adenomas as compared to normal mucosa despite significant upregulation of CYCLIND1 [17]. Results from the present studies and the possible nonspecific immunoreactivity of anti-PPAR β/δ antibodies indicate that analysis of PPAR β/δ in colon cancer models should always include quantitative Western blotting. In addition to quantifying expression of PPAR β/δ , analysis of known PPAR β/δ target genes should also be performed in order to establish a functional role for potential differences in receptor expression since PPAR β/δ functions as a ligand-dependent transcription factor.

Another major finding from the present studies is the lack of downregulation of PPAR β/δ by NSAIDs in human colon cancer cell lines. There are conflicting reports with some suggesting

that NSAIDs decrease expression of PPAR β/δ while others suggest that NSAIDs increase or have no effect on the expression of PPAR β/δ in cancer models [reviewed in 1]. The focus of the present studies was on RKO and DLD1 human colon cancer cell lines for several reasons including the fact that RKO cells express COX1/2 while DLD1 cells do not (this allows for distinguishing between COX1/2-dependent vs. COX1/2-independent effect), the fact that RKO cells have wildtype APC and β -CATENIN while DLD1 cells have a mutation in APC (this allows the ability to determine whether PPAR β/δ is upregulated by the APC/ β -CATENIN/TCF4 pathway) and a previous report suggesting that a variety of NSAIDs (indomethacin, sulindac, NS-398, and celecoxib) cause reduced expression of PPAR β/δ in the same cell lines [21]. Comprehensive and quantitative analysis of PPAR β/δ expression following treatment with NSAIDs at concentrations that effectively inhibited cell growth, and at time points preceding this inhibition, demonstrates that no decrease in expression or function of PPAR β/δ protein is observed in either RKO or DLD1 cells as compared to controls. This is indeed surprising since the same cell lines and time point (48 h) were examined using concentrations of indomethacin (200 μ M vs. 150 μ M), celecoxib (10 μ M vs. 12.5 μ M), and NS-398 (100 μ M vs. 120 μ M) that are comparable between the present study and former study [21], respectively. Others have recently suggested that NSAIDs (indomethacin and sulindac) induce apoptosis in colon cancer cell lines by PPAR β/δ -dependent suppression of 14-3-3 ϵ [20]. However, no decrease in PPAR β/δ protein expression was noted in DLD1 cells or HT29 cell (data not shown) by either NSAID. The reason for this and other discrepancies in the literature cannot be determined from the present studies. There are a number of problems with some of the reports suggesting NSAIDs cause a decrease in PPAR β/δ [reviewed in 1], including the lack of positive controls, no independent replicates, and the absence of quantification and statistical analysis. Examination of changes in PPAR β/δ -dependent gene expression in the present study strongly supports the Western blot analysis as no evidence of decreased PPAR β/δ activity was observed following treatment with NSAIDs. Future studies examining NSAID-induced changes in PPAR β/δ should include analysis of PPAR β/δ target gene expression to yield more convincing evidence rather than relying on receptor expression alone.

While no evidence of decreased PPAR β/δ following NSAIDs in human colon cancer cell lines was noted, increased expression of PPAR β/δ and PPAR α was observed for several NSAIDs. For example, increased expression of PPAR β/δ was observed in RKO cells by indomethacin after 24 and 48 h of treatment. This is consistent with mRNA expression as well. More importantly, the increase in expression of PPAR β/δ by indomethacin is also reflected by increased expression of known PPAR β/δ target genes. This is of interest because previous studies have also noted an increase in PPAR β/δ mRNA expression by indomethacin treatment in HCT116 cells [28] or SW-480 cells [29]. This suggests that increased expression of PPAR β/δ , not decreased expression might be mechanistically linked to the observed inhibition of cell proliferation induced by indomethacin. This finding is supported by another report showing enhanced cell proliferation by silencing PPAR β/δ expression in HCT116 cells [30]. Expression of PPAR α was also found to be significantly higher in RKO and DLD1 cells treated with the higher doses of NSAIDs. There is evidence that activation of PPAR α can inhibit colon tumorigenesis based on in vitro and in vivo analysis [31]. Thus, the significance of the observed increase in PPAR α expression and whether the increase in PPAR α function could mediate the cell growth modulatory effects of NSAIDs should also be examined based on these findings.

Collectively, results from these studies strongly suggest that the previously described mechanisms suggesting that the APC/ β -CATENIN/TCF4 pathway upregulates PPAR β/δ and that NSAIDs inhibit cell growth by decreasing PPAR β/δ expression should be evaluated more rigorously. Importantly, evidence of direct modulation of PPAR β/δ -dependent gene expression coupled with quantified statistical analysis of PPAR β/δ expression should be examined for this purpose. Indeed, results from the present studies strongly suggest that increased expression of PPAR β/δ by indomethacin could contribute to indomethacin-induced inhibition of cell

proliferation in human colon cancer cell lines. This idea is supported by a large body of literature demonstrating PPAR β/δ -dependent inhibition of cell proliferation [reviewed in 1].

Abbreviations

PPAR, peroxisome proliferator-activated receptor; APC, adenomatous polyposis coli; TCF4, transcription factor 4; NSAIDs, nonsteroidal antiinflammatory drugs; NO-ASA, nitric oxidizedonating aspirin; ADRP, adipose differentiation-related protein; ANGPTL4, angiopoietin-like 4; qPCR, quantitative real-time polymerase chain reaction; qPCR, quantitative real-time polymerase chain reaction.

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Figure 1.

Quantitative expression of PPARs in vivo and in vitro. (A) Representative Western blot analysis of PPARs, cMYC, and CYCLIN D1 in wild-type (APC^{+/+}), *Apc*^{min} heterozygous (APC^{+/-}), and intestinal polyps from *Apc*^{min} heterozygous mice (APC polyps (+/-)). Normalized values are presented as the mean $\pm$ SEM and were calculated from examining independent samples from nine mice. Values within a row with different superscripts are significantly different at $P \leq 0.05$. +, positive control; see Materials and Methods Section. A positive control was not used for cMYC or CYCLIN D1. (B) Representative Western blot analysis of PPARs, cMYC, and CYCLIN D1 in human colon cancer cell lines. PPAR γ 1 (53 000 M_r) and PPAR γ 2 (56 000 M_r) were detected in the cancer cell lines. Normalized values are

presented as the mean $\pm$ SEM and were calculated from examining a minimum of three independent samples per cell line. Values within a row with different superscripts are significantly different at $P\leq 0.05$. Positive control is designated by “+”; see Materials and Methods Section. A positive control was not used for cMYC or CYCLIN D1. (C) Representative Western blot analysis of PPARs in wild-type (APC^{+/+}) or Apc^{min} heterozygous (APC^{+/-}) mice treated with either vehicle or NO-donating aspirin (NO-ASA). Normalized values are presented as the mean $\pm$ SEM and were calculated from examining independent samples from six mice. Values within a row with different superscripts are significantly different at $P\leq 0.05$. Positive control is designated by “+”; see Materials and Methods Section.

Figure 2.

Effect of NSAIDs on cell proliferation in human colon cancer cell lines. RKO or DLD1 cells were cultured for 72 h in the presence or absence of (A) nimesulide, (B) indomethacin, (C) celecoxib, or (D) NS-398 at the indicated concentrations. Values represent the mean $\pm$ SEM and were calculated from a minimum of three independent samples per treatment. *Significantly less than control at $P\leq 0.05$.

Figure 3.

Expression of PPARs in RKO or DLD1 cells after 24 h of culture with NSAIDs. RKO or DLD1 cells were cultured as described in Materials and Methods Section for 24 h with the indicated NSAID. Quantitative Western blot analysis of nuclear extracts was performed. Normalized values were calculated from a minimum of three independent samples per treatment and represent the mean ± SEM. Average values with a different superscript are significantly different at $P \leq 0.05$.

Figure 4.

Expression of PPARs in RKO or DLD1 cells after 48 h of culture with NSAIDs. RKO or DLD1 cells were cultured as described in Materials and Methods Section for 48 h with either control (DMSO), 200 μ M indomethacin, 500 μ M nimesulide, 10 μ M celecoxib, 100 μ M NS-398, or 150 μ M sulindac. Quantitative Western blot analysis of nuclear extracts was performed. Normalized values were calculated from a minimum of three independent samples per treatment and represent the mean $\pm$ SEM. Average values with a different superscript are significantly different at $P \leq 0.05$.

Figure 5.

Effect of NSAIDs on PPAR target gene expression in RKO or DLD1 cells. (A) RKO or DLD1 cells were cultured in the presence of the indicated concentration of indomethacin, nimesulide, NS-398, or celecoxib for 24 h. RNA was isolated and used for qPCR analysis of *PPAR β/δ* or *PPAR α* mRNA as described in Materials and Methods Section. Values represent the mean $\pm$ SEM and were calculated from three independent samples per treatment group. Values with different letters are significantly different at $P \leq 0.05$. (B) RKO or DLD1 cells were cultured as described in Materials and Methods Section for 24 h with the *PPAR β/δ* ligand GW0742 or the indicated concentration of NSAID. Quantitative real-time PCR was performed to examine expression of mRNA encoding either *ADRP* or *ANGPTL4*. Normalized values were calculated

from a minimum of three independent samples per treatment and represent the mean $\pm$ SEM. SEM. Average values with different letters are significantly different at $P\leq 0.05$.

Table 1

Genotype of Human Colon Cancer Cell Lines Used for These Studies

Cell line	APC	β -CATENIN
RKO	Wild type	Wild type
DLD1	Mutant	Wild type
HT29	Mutant	Wild type
LS174T	Wild type	Mutant
HCT116	Wild type	Codon 45 deletion