Chemoprevention of Chemically Induced Skin Tumorigenesis by Ligand Activation of Peroxisome Proliferator–Activated Receptor-β/δ and Inhibition of Cyclooxygenase 2

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Abstract

Ligand activation of peroxisome proliferator–activated receptor-β/δ (PPARβ/δ) and inhibition of cyclooxygenase-2 (COX2) activity by nonsteroidal anti-inflammatory drugs (NSAID) can both attenuate skin tumorigenesis. The present study examined the hypothesis that combining ligand activation of PPARβ/δ with inhibition of COX2 activity will increase the efficacy of chemoprevention of chemically induced skin tumorigenesis over that observed with either approach alone. To test this hypothesis, wild-type and Pparβ/δ-null mice were initiated with 7,12-dimethylbenz[a]anthracene (DMBA), topicaly treated with 12-O-tetradecanoylphorbol-13-acetate to promote tumorigenesis, and then immediately treated with topical application of the PPARβ/δ ligand GW0742, dietary administration of the COX2 inhibitor nimesulide, or both GW0742 and nimesulide. Ligand activation of PPARβ/δ with GW0742 caused a PPARβ/δ-dependent delay in the onset of tumor formation. Nimesulide also delayed the onset of tumor formation and caused inhibition of tumor multiplicity (46%) in wild-type mice but not in Pparβ/δ-null mice. Combining ligand activation of PPARβ/δ with dietary nimesulide resulted in a further decrease of tumor multiplicity (58%) in wild-type mice but not in Pparβ/δ-null mice. Biochemical and molecular analysis of skin and tumor samples show that these effects were due to the modulation of terminal differentiation, attenuation of inflammatory signaling, and induction of apoptosis through both PPARβ/δ-dependent and PPARβ/δ-independent mechanisms. Increased levels and activity of PPARβ/δ by nimesulide were also observed. These studies support the hypothesis that combining ligand activation of PPARβ/δ with inhibition of COX2 activity increases the efficacy of preventing chemically induced skin tumorigenesis as compared with either approach alone. Mol Cancer Ther; 9(12); 3267–77. ©2010 AACR.

Introduction

Cyclooxygenase (COX) signaling pathways have important roles in modulating skin carcinogenesis. COX is the central enzyme in prostanoid biosynthesis that catalyzes the conversion of arachidonic acid to prostaglandin H2, which is then converted to biologically active lipids such as thromboxane, prostaglandin E2 (PGE2), and prostacyclin by different enzymes (1). There are 2 isoforms of COX, COX1 and COX2. While COX1 is constitutively expressed, COX2 is induced by tumor promoters, growth factors, and cytokines (2). Results from experimental animal models have established a causal relationship between COX2 and skin carcinogenesis. For example, genetic disruption of both COX1 and COX2 can prevent skin tumorigenesis (3) and nonsteroidal anti-inflammatory drugs (NSAID) that inhibit COX activity as well as inhibit UV-induced and chemically induced skin carcinogenesis (4–7). The proliferative effects of COX2 are due primarily to increased synthesis of prostaglandins, which directly influence cell growth after binding to specific cell surface receptors, including the prostaglandin E, prostaglandin F, and prostaglandin I class of receptors (8, 9). For example, protumorigenic effect of PGE2 can be mediated by the EP2 receptor (10). Although prostaglandins can mediate their biological effects through specific prostaglandin receptors such as prostaglandin E, prostaglandin F, and prostaglandin I, they might also modulate the activities of peroxisome proliferator–activated receptors (PPAR).
Three distinct isoforms, PPARα, PPARβ (also referred to as PPARδ or PPARβ/δ), and PPARγ, exist, with essential roles in the regulation of adipogenesis, lipid metabolism, cell proliferation/apoptosis, cell differentiation, inflammatory responses, and carcinogenesis (11–16). PPARs regulate these pathways by modulation of gene expression through direct and indirect mechanisms. PPARβ/δ is found at very high levels in the nucleus of epithelium including intestine and in keratinocytes (17). In the absence of ligands, nuclear PPARβ/δ can also be coimmunoprecipitated with its heterodimerization partner RXRα, suggesting that PPARβ/δ has an important constitutive role in the epithelium (17). Thus, it is not surprising that important roles for PPARβ/δ have been observed in skin. For example, Pparβ/δ-null mice exhibit enhanced epidermal hyperplasia in response to phorbol ester treatment (18, 19) and exacerbated chemically induced skin tumorigenesis in a 2-stage carcinogenesis bioassay as compared with wild-type mice (20), suggesting that PPARβ/δ inhibits epidermal cell proliferation in response to stimuli. Consistent with this idea, PPARβ/δ-dependent inhibition of skin tumorigenesis is found after topical application of the PPARβ/δ ligand GW0742 (21). The chemopreventive effects of ligand activation of PPARβ/δ are mediated in part by the induction of unidentified target genes or nontranscriptional events that modulate terminal differentiation and inhibit cell proliferation and/or inhibition of proinflammatory signaling (reviewed in refs. 11, 14, 15).

Some reports suggest that NSAIDs attenuates carcinogenesis by inhibiting PPARβ/δ expression and/or activities, although this view has yet to be experimentally confirmed, and there are many inconsistencies with this hypothesis in the literature (reviewed in refs. 14, 15). For example, the hypothesis that NSAIDs inhibit cancer by decreasing PPARβ/δ expression/function is inconsistent with the observation that PPARβ/δ expression following exposure to NSAIDs is either unchanged or increased in human cancer cell lines (22). Furthermore, inhibition of chemically induced skin tumorigenesis is found in both wild-type and Pparβ/δ-null mice following treatment with the COX1/COX2 inhibitor sulindac, suggesting that NSAIDs mediate chemoprevention of chemically induced skin tumorigenesis through PPARβ/δ-independent mechanisms (6). This is consistent with a recent report showing that combining COX2 inhibition with ligand activation of PPARβ/δ resulted in increased efficacy in the inhibition of preexisting skin tumor multiplicity (7). Collectively, these observations suggest that combining these 2 therapeutic approaches will increase the efficacy of chemoprevention as compared with either agent alone. Thus, the effect of combining COX2 inhibition and ligand activation of PPARβ/δ on chemoprevention of skin carcinogenesis was examined.

Materials and Methods

Two-stage chemical carcinogenesis bioassay

Female wild-type and Pparβ/δ-null mice on a C57BL/6 genetic background (19), 6 to 8 weeks of age, were initiated with 50 μg of 7,12-dimethylbenz[a]anthracene (DMBA; Sigma-Aldrich). One week after initiation, mice were treated topically with 5 μg of 12-O-tetradecanoyl-phorbol-13-acetate (TPA; NCI Chemical Carcinogen Reference Standard Repository), 3 days per week for 41 weeks. Mice from both genotypes were randomly divided into 1 of the following 4 groups: a) control diet and topical application of acetone; b) control diet and topical application of GW0742 (5 μmol/L); c) nimesulide diet (400 mg/kg) and topical application of acetone; or d) nimesulide diet (400 mg/kg) and topical application of GW0742 (5 μmol/L). Because C57BL/6 mice weighing 20 to 30 g typically consume approximately 4 g of food per day (23), the estimated dose of nimesulide ranged from 50 to 80 mg/kg of body weight per day. The concentrations of topical GW0742 and nimesulide in the diet were based on previous work showing inhibition of chemically induced skin tumorigenesis by GW0742 or nimesulide in related models (7, 21). After 42 weeks, mice were euthanized by overexposure to carbon dioxide. Tumor samples were either fixed or snap-frozen in liquid nitrogen for future analysis. Fixed tumor samples were embedded in paraffin, sectioned and stained with hematoxylin and eosin, and scored for benign or malignant pathology by 2 independent pathologists.

Short-term bioassay

Female wild-type and Pparβ/δ-null mice were acclimated to either a control or nimesulide diet (400 mg/kg) for 1 week and then treated topically with acetone or TPA dissolved in acetone (5 μg) followed 1 hour later by topical application of either acetone or GW0742 (5 μmol/L) every other day for a total of 3 applications. Mice were fed either the control or nimesulide diet during this period of topical GW0742 treatment. Mice were euthanized 6 hours after the last acetone or GW0742 treatment, and skin samples were obtained for RNA and protein isolation.

Keratinocyte culture

Primary mouse keratinocytes were isolated from 2-day postnatal wild-type and Pparβ/δ-null mice as described previously (24). Keratinocytes were cultured in low-calcium (0.05 mmol/L) Eagle’s minimal essential medium with 8% chelexed fetal bovine serum at 37°C and 5% carbon dioxide.

Caspase 3/7 activity assay

Skin samples were ground to a fine powder in liquid nitrogen and then homogenized in buffer containing 10 mmol/L of Tris (pH = 7.5), 100 mmol/L of NaCl, 1 mmol/L of EDTA, and 0.01% Triton-X100. For in vitro analysis of caspase 3/7 activity, primary keratinocytes
were cultured as described earlier for 2 days before treatment with either DMSO, 1 μmol/L of GW0742, and 500 μmol/L of nimesulide or the combination of 1 μmol/L of GW0742 and 500 μmol/L of nimesulide for 24 hours. Cells were then trypsinized and lysed in the Tris buffer as described earlier for 30 minutes on ice. Homogenates were centrifuged at 16,000 × g, and the supernatant was used for analysis. Caspase 3/7 activity was measured using a luminescent assay (Promega).

Western blot analysis
Primary keratinocytes were cultured as described earlier for 2 days before treatment with either DMSO, 1 μmol/L of GW0742, and 500 μmol/L of nimesulide or the combination of 1 μmol/L of GW0742 and 500 μmol/L of nimesulide for 24 hours. Cells were then trypsinized and then lysed in buffer containing protease inhibitors. Samples were sonicated to facilitate cell lysis before centrifugation at 16,000 × g at 4°C for 30 minutes, and the supernatant was used for Western blot analysis. Protein from skin samples was isolated similarly with the same buffer. Separation of proteins by electrophoresis, transfer to membranes, and blocking was done as previously described (25). After overnight incubation at 4°C with the primary antibody, membranes were incubated with biotinylated secondary antibody (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature. The membranes were incubated with 125I-labeled streptavidin for 1 hour at room temperature. Hybridization signals for specific proteins were quantified with filmless autoradiographic analysis. The ratio of cleaved PARP to uncleaved PARP was calculated using Optiquant software.

RNA isolation and quantitative real-time PCR analysis
Total RNA was isolated from skin and tumor samples, using TRIZOL reagent (Invitrogen). Reverse transcription and quantitative real-time PCR (qPCR) were carried out as previously described (25). Primers for keratin 1 (K1), keratin 10 (K10), angiopoetin-like protein 4 (Angptl4), interleukin 6 (IL6; Il6), and tumor necrosis factor-α (TNFα; Tnfα) have been previously described (7, 21, 26, 27). The relative level of mRNA was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) or 18s RNA levels.

Statistical analysis
The significance of tumor incidence between each treatment and genotype was determined by chi-square test for trend analysis (Prism 5.0, GraphPad Software, Inc.). Fisher’s exact test was used to determine the significance of the incidence of mice with keratoacanthomas and/or squamous cell carcinomas (SCC). For all other analysis, a 1-tailed student t test was used.

Results
Ligand activation of PPARβ/δ and inhibition of COX2 enhances chemoprevention of chemically induced skin tumorigenesis
Combining ligand activation of PPARβ/δ with COX2 inhibition results in a modest decrease in multiplicity of preexisting tumors in a chemotherapeutic model (7). Because later stage tumors can be resistant to therapies designed to regress tumor growth, the effect of combining ligand activation of PPARβ/δ with COX2 inhibition was examined in a chemoprevention model. Marked changes were observed in both genotypes (Fig. 1; Supplementary Fig. 2). The onset of papilloma formation was sooner and the incidence of papilloma was greater in control Pparβ-null mice than in control wild-type mice prior to week 16 of the 2-stage bioassay (P ≤ 0.05; Fig. 1A), consistent with previous studies (7, 20, 21). Topical
application of the PPARβ/δ ligand GW0742, or dietary nimesulide, caused a delay in the onset of tumor formation \((P \leq 0.05\); Fig. 1A). These effects were not found in Pparβ/δ-null mice. Compared with control, combining ligand activation of PPARβ/δ with inhibition of COX2 activity caused a delay in the onset of tumor formation in wild-type mice, and this effect was not found in Pparβ/δ-null mice (Fig. 1A). In response to either GW0742 or nimesulide, the percentage of wild-type mice with skin tumors from week 11 to week 16 was lower but not statistically different than control wild-type mice (Fig. 1A). However, in response to both GW0742 and nimesulide, the percentage of wild-type mice with skin tumors from week 11 to week 16 was decreased as compared with control wild-type mice \((P \leq 0.05\); Fig. 1A). These effects of GW0742, nimesulide, or the combination of GW0742 and nimesulide were not found in Pparβ/δ-null mice (Fig. 1A). Skin tumor multiplicity was significantly greater (29%–30%) in control Pparβ/δ-null mice than in control wild-type mice from week 20 until week 42 of the 2-stage bioassay \((P \leq 0.05\); Fig. 1B). Ligand activation of PPARβ/δ with GW0742 resulted in decreased (20%–40%) tumor multiplicity in wild-type mice during week 37 to week 42 of the bioassay, and this effect was not found in Pparβ/δ-null mice \((P \leq 0.05\); Fig. 1B). Interestingly, skin tumor multiplicity was lower (24%–27%) in Pparβ/δ-null mice in response to topical GW0742 from week 20 to week 30 of the bioassay than in control Pparβ/δ-null mice \((P \leq 0.05\); Fig. 1B). Dietary nimesulide caused a decrease (30%–46%) in tumor multiplicity in wild-type mice during week 24 to week 42 of the bioassay, and this effect was not found in Pparβ/δ-null mice \((P \leq 0.05\); Fig. 1B). The combination of topical application of GW0742 and dietary nimesulide resulted in a marked decrease (57%–69%) of tumor multiplicity from week 22 onward in wild-type mice, and the effect was greater than either GW0742 or nimesulide treatment alone from week 21 to week 40 \((P \leq 0.05\); Fig. 1B). In Pparβ/δ-null mice, the combination of GW0742 with nimesulide caused a decrease (27%–42%) in tumor multiplicity from week 20 to week 30 \((P \leq 0.05\); Fig. 1B).

Average tumor size was greater in the Pparβ/δ-null mice than in wild-type mice, but this difference was not statistically significant (Fig. 1C). Topical GW0742 or the combined treatment of topical GW0742 and dietary nimesulide did not cause a significant decrease in average tumor size in either genotype (Fig. 1C). Dietary nimesulide caused a decrease in average tumor size in wild-type mice, and this effect was not observed in Pparβ/δ-null mice \((P \leq 0.05\); Fig. 1C). Closer examination of the distribution of the tumor size also revealed some striking differences (Fig. 2). The percentage of control Pparβ/δ-null mice with tumors in the 2- to 3-mm size range was greater than control wild-type mice (Fig. 2A). In addition, the average percentage of total tumors per mouse in the 1- to 2-mm range was greater in control wild-type mice than in control Pparβ/δ-null mice, and this difference was consistent with a greater percentage of total tumors per mouse in the 2- to 3-mm size range and greater than 5-mm size ranges in control Pparβ/δ-null mice as compared with control wild-type mice (Fig. 2B). In wild-type mice fed nimesulide, the percentage of mice with tumors in the 3- to 5-mm size range and the percentage of mice with tumors greater than 5-mm in size were significantly less as compared with control wild-type mice (Fig. 2A). Similarly, the average percentage of total tumors per mouse greater than 5 mm was lower in wild-type mice fed nimesulide as compared with control wild-type mice (Fig. 2B). The average percentage of total tumors per mouse in the 1- to 2-, 2- to 3, and 3- to 5-mm range was similar in wild-type mice fed nimesulide as compared with control wild-type mice (Fig. 2B). Dietary nimesulide had no effect on the distribution of tumors with different sizes in Pparβ/δ-null mice as compared with control Pparβ/δ-null mice (Figs. 2A and B). However, the average percentage of total tumors per mouse in the 1- to 2-mm size range was lower in Pparβ/δ-null mice fed nimesulide (Fig. 2B) than in wild-type mice fed nimesulide. This difference was due to the increase in the average percentage of total tumors per mouse in the 2- to 3-mm and greater than 5-mm size ranges in Pparβ/δ-null mice fed nimesulide as compared with similarly treated wild-type mice (Fig. 2B). In wild-type mice treated with GW0742, the percentage of mice with tumors in the 2- to 3-mm size range was greater, whereas the percentage of mice with tumors in the 3- to 5-mm and greater than 5-mm size ranges was less than that of control wild-type mice (Fig. 2A). This effect was not found in GW0742-treated Pparβ/δ-null mice (Fig. 2A). GW0742 had no effect on the average size distribution of total tumors per mouse in either genotype (Fig. 2B). The percentage of wild-type mice treated with both topical GW0742 and dietary nimesulide with tumors in all size ranges was markedly lower than that of control wild-type mice, and this effect was not found in similarly treated Pparβ/δ-null mice (Fig. 2A). The average percentage of total tumors per mouse in the 2- to 3-mm range and 3- to 5-mm ranges was lower in wild-type mice treated with both topical GW0742 and dietary nimesulide than in control wild-type mice; these effects were not found in similarly treated Pparβ/δ-null mice (Fig. 2B).

The majority of representative skin lesions examined in all groups were squamous cell papillomas (data not shown). Skin lesions macroscopically suspected of being SCC were examined for histopathology. Skin lesions macroscopically suspected of being SCC were not observed in wild-type mice treated with nimesulide. For control, nimesulide-treated, GW0742-treated, and nimesulide + GW0742-treated wild-type mice, 2 of 8, 0 of 7, 3 of 10, and 2 of 10 mice, respectively, had lesions macroscopically suspected of being SCC. For control, nimesulide-treated, GW0742-treated, and nimesulide + GW0742-treated Pparβ/δ-null mice, 5 of 8, 3 of 10, 4 of 10, and 5 of 10 mice, respectively, had lesions macroscopically suspected of being SCC. Histopathologic analysis revealed that these lesions were typically either
keratoacanthomas or SCC. A higher incidence of keratoacanthomas was observed in control Ppara\(\beta\)-null mice (3/8) than in control wild-type mice (1/8; Supplementary Fig. 3A). No keratoacanthomas were found in wild-type mice fed dietary nimesulide, but GW0742, nimesulide, or their combined treatment did not cause any statistically significant changes in the incidence of keratoacanthoma in either genotype (Supplementary Fig. 3A). The average number of keratoacanthomas per mouse was comparable between both genotypes, although no keratoacanthomas were noted in wild-type mice fed nimesulide (Supplementary Fig. 3B). Although 25% of control wild-type mice (2/8) had SCC, no SCCs were found in wild-type mice treated with dietary nimesulide or topical GW0742 and only 10% of wild-type mice treated with both dietary nimesulide and topical GW0742 (1/10) had SCC (Supplementary Fig. 3C). SCCs were found in 25% of control Ppara\(\beta\)-null mice (2/8), 20% of nimesulide-treated Ppara\(\beta\)-null mice (2/10), none of GW0742-treated, and 40% of nimesulide + GW0742-treated Ppara\(\beta\)-null mice (4/10; Supplementary Fig. 3C). None of these differences achieved statistical significance. The average number of SCCs per mouse was comparable between both genotypes, although no SCCs were observed in nimesulide-treated or GW0742-treated wild-type mice or GW0742-treated Ppara\(\beta\)-null mice (Supplementary Fig. 3D). One hemangioma was observed in 1 GW0742-treated Ppara\(\beta\)-null mouse, and 1 malignant basal cell tumor was found in 1 nimesulide + GW0742-treated Ppara\(\beta\)-null mouse (data not shown). Interestingly, polymorphonuclear neutrophil infiltrates were more commonly observed in Ppara\(\beta\)-null mouse skin lesions.

Figure 2. Skin tumor size following ligand activation of PPAR\(\beta\)/\(\delta\) and inhibition of COX2. Wild-type (+/+ ) and Ppara\(\beta\)-null (−/− ) mice were treated with topical GW0742 (5 \(\mu\)mol/L), dietary nimesulide (400 mg/kg), or the combination of GW0742 and nimesulide during a 42-week, 2-stage bioassay (initiation with DMBA and promotion with TPA) as described in Materials and Methods. A, incidence of mice with different tumor sizes. These values represent the percentage of mice within a given group that exhibited skin tumors with the indicated size range. B, distribution of average tumor size for each treatment group. Mice within each treatment were used to calculate the percentage of tumors of that particular size range for each treatment group. *, significantly different from control wild-type, \(P \leq 0.05\).
Effect of GW0742 and nimesulide on terminal differentiation markers

Ligand activation of PPARβ/δ or inhibition of COX activity can both induce terminal differentiation in primary keratinocytes and skin (3, 30, 31). To determine whether the enhanced efficacy of inhibiting chemically induced skin tumorigenesis by combining GW0742 with nimesulide was due, in part, to modulation of terminal differentiation, expression of differentiation markers was examined. Dietary nimesulide, topical GW0742, and topical GW0742 in combination with dietary nimesulide increased expression of keratin 1 (K1) protein in wild-type mouse skin as compared with control, and this effect was not found in Pparβ/δ-null mouse skin (Fig. 3). Dietary nimesulide or topical GW0742 did not alter expression of keratin 10 (K10) protein in mouse skin from either genotype (Fig. 3). However, topical GW0742 in combination with dietary nimesulide increased expression of K10 protein in wild-type mouse skin as compared with control, and this effect was not found in Pparβ/δ-null mice (Fig. 3)

Effect of GW0742 and nimesulide on the inflammatory response

Inflammation can influence different stages of tumorigenesis. Secretion of proinflammatory signaling molecules by immune and somatic cells such as TNFα and IL6 can act on cancer cells and promote tumor growth and malignant conversion (reviewed in ref. 32). The NSAID nimesulide is known to attenuate inflammation by inhibiting COX2 activity and the subsequent production of arachidonic acid metabolites. In addition, ligand activation of PPARβ/δ is also known to have anti-inflammatory activities in rodent and human models (reviewed in refs. 14, 15, 33). To determine whether attenuation of inflammation could, in part, underlie the observed inhibition of chemically induced skin tumorigenesis, expression of the mRNA encoding 2 important proinflammatory cytokines, TNFα and IL6, was examined in both the tumor samples and mouse skin. Tumors from Pparβ/δ-null mice from all treatment groups had a higher level of Il6 mRNA (2- to 20-fold) and Tnfα mRNA (2- to 3-fold) than that of similarly treated wild-type mice (Fig. 4A and B). Dietary nimesulide caused a significant decrease of both Il6 mRNA (53% lower) and Tnfα mRNA (79% lower) in tumors from wild-type mice but not in tumors from Pparβ/δ-null mice. Tumors from wild-type mice treated only with topical GW0742 or the combination of topical GW0742 and dietary nimesulide exhibited a decrease in mRNA encoding Il6 and Tnfα, but this change was only statistically significant for Tnfα mRNA (73%–77% lower; Fig. 4B). No change in expression of Il6 or Tnfα mRNA was found in tumors from Pparβ/δ-null mice treated with topical GW0742 or the combination of topical GW0742 and dietary nimesulide (Fig. 4A and B). These data are consistent with the presence of polymorphonuclear neutrophil infiltrates found more commonly in Pparβ/δ-null mice than in wild-type mice (Supplementary Fig. 4).

A short-term bioassay was also carried out using wild-type and Pparβ/δ-null mice that were acclimated to either a control or nimesulide diet for 1 week and then treated with or without TPA followed 1 hour later with either acetone (vehicle control) or GW0742. The rationale for this approach is that TPA is known to increase inflammatory signaling that could influence tumor promotion. Expression of Il6 mRNA was similar in both control wild-type and control Pparβ/δ-null mouse skin (Fig. 4C). Expression of Il6 mRNA was increased in both wild-type and Pparβ/δ-null mouse skin in response to

![Figure 3](image)
TPA treatment but was markedly higher (8-fold vs. 106-fold, respectively) in \( Ppar_b\)-null mouse skin than in wild-type mouse skin (Fig. 4C). Expression of \( Il6 \) mRNA was not influenced by GW0742, nimesulide, or GW0742 + nimesulide treatment in either control or TPA-treated wild-type mouse skin or control \( Ppar_b\)-null mouse skin (Fig. 4C). Dietary nimesulide in \( Ppar_b\)-null mice resulted in lower \( Il6 \) mRNA (63% lower) following topical TPA, and a similar effect was also found in \( Ppar_b\)-null mice that were treated with GW0742 and nimesulide (84% lower) following topical TPA treatment (Fig. 4C). Expression of \( Tnf_a \) mRNA was similar in both control wild-type and control \( Ppar_b\)-null mouse skin (Fig. 4D). Expression of \( Tnf_a \) mRNA was increased in both wild-type and \( Ppar_b\)-null mouse skin following TPA treatment, and this effect was greater in \( Ppar_b\)-null mouse skin than in wild-type mouse skin (Fig. 4D). Expression of \( Tnf_a \) mRNA was not influenced by GW0742, nimesulide, or combined GW0742 and nimesulide treatment in either control or TPA-treated mouse skin from either genotype (Fig. 4D). Whereas the relative change in expression of \( Tnf_a \) mRNA in response to TPA was approximately 2-fold in all groups of both genotypes, the relative fold change in expression of \( Tnf_a \) mRNA was less (1.1-fold) in TPA-treated wild-type mouse skin treated with dietary nimesulide and topical GW0742 than in similarly treated control wild-type mouse skin, and this effect was not observed in \( Ppar_b\)-null mice (Fig. 4D).

**Effect of GW0742 and nimesulide on apoptosis**

Because combining ligand activation of \( PPAR_b/\delta \) with GW0742 with inhibition of COX2 activity by dietary nimesulide caused the most marked effect on tumor multiplicity, the effect of these treatments on apoptosis was examined. There is compelling evidence that 1 mechanism by which nimesulide inhibits tumorigenesis is through the induction of apoptosis (reviewed in ref. 34). In contrast, the effect of ligand activation of \( PPAR_b/\delta \) on apoptotic signaling remains uncertain. This is due to conflicting studies suggesting that ligand activation of \( PPAR_b/\delta \) causes proapoptotic signaling, anti-apoptotic signaling, or has no effect on apoptosis (reviewed in refs. 14, 15). Caspase 3/7 activity and poly(ADP-ribose) polymerase (PARP) cleavage were measured to determine whether ligand activation of \( PPAR_b/\delta \) on apoptotic signaling remains uncertain. This is due to conflicting studies suggesting that ligand activation of \( PPAR_b/\delta \) causes proapoptotic signaling, anti-apoptotic signaling, or has no effect on apoptosis (reviewed in refs. 14, 15). Caspase 3/7 activity and poly(ADP-ribose) polymerase (PARP) cleavage were measured to determine whether ligand activation of \( PPAR_b/\delta \) with GW0742 and/or inhibition of COX2 by nimesulide modulate apoptosis in mouse skin and keratinocytes. In control wild-type and \( Ppar_b\)-null mouse skin, dietary nimesulide or topical GW0742 did not modulate caspase 3/7 activity compared with control in either genotype (Fig. 5A). However, the combined treatment of GW0742 with nimesulide caused an increase in caspase 3/7 activity in wild-type mouse skin, and this effect was
not seen in Pparb/d-null mouse skin. Because different cell types can influence apoptosis, the effect of GW0742 and nimesulide was examined in primary keratinocytes from wild-type and Pparb/d-null mice. Consistent with results obtained from the analysis of whole skin, GW0742 did not alter caspase 3/7 activity or PARP cleavage in either wild-type or Pparb/d-null primary keratinocyte (Fig. 5B–D). In contrast, culturing primary keratinocytes with nimesulide increased apoptotic signaling in primary keratinocytes in both genotypes, as evidenced by an increase in caspase 3/7 activity and PARP cleavage (Fig. 5B–D). Cotreatment of wild-type primary keratinocytes with nimesulide and GW0742 led to enhanced caspase 3/7 activity and PARP cleavage as compared with that observed with either compound alone, but this increase was not found in Pparb/d-null keratinocytes (Fig. 5B–D).

**Effect of GW0742 and nimesulide on expression and function of PPARb/d**

Nimesulide is not known to activate PPARb/d by acting as an agonist. One mechanism that may explain some of the modest PPARb/d-dependent changes resulting from nimesulide treatment is increased expression and function of PPARb/d. Indeed, increased expression of PPARb/d has been observed following exposure to NSAIDs including nimesulide (22). Thus, expression and function of PPARb/d were examined. Interestingly, dietary nimesulide, topical GW0742, and the combined treatment of GW0742 and nimesulide all increased expression of Pparb/d mRNA in wild-type mouse skin (Fig. 6A). This increase in expression was also found at the protein level, but the changes were not statistically significant (Fig. 6B). However, examination of expression of the PPARb/d target gene Angptl4 showed that dietary nimesulide, topical GW0742, and the combined treatment of GW0742 and nimesulide all increased expression of Angptl4 mRNA in wild-type mouse skin, and this effect was not found in similarly treated Pparb/d-null mouse skin (Fig. 6C).

**Discussion**

Consistent with past studies (20, 21), chemically induced skin tumorigenesis was exacerbated in Pparb/d-null mice as compared with wild-type mice, as assessed by differences in the onset tumor formation, the incidence of keratoacanthomas, and tumor multiplicity. Furthermore, ligand activation of PPARb/d inhibited chemically induced skin tumorigenesis through PPARb/d-dependent mechanisms similar to results from past studies (7, 21). Dietary nimesulide was also effective for chemoprevention as shown by decreased tumor multiplicity and a decrease in tumor size distribution. As compared with dietary nimesulide or topical GW0742, the combination of dietary nimesulide and topical GW0742 enhanced the chemopreventive activity of either agent alone, most notably by the prolonged marked decrease in tumor multiplicity. Interestingly, the effect of GW0742, nimesulide, and the combined treatment of nimesulide and GW0742 seems to be due, in part, to the
modulation of PPARβ/δ-dependent and PPARβ/δ-independent mechanisms that influence differentiation, inflammation, and apoptosis.

PPARβ/δ-dependent chemoprevention of chemically induced skin tumorigenesis by GW0742 is likely due, in part, to enhanced terminal differentiation, as observed in the present study and previous reports (7, 21, 28, 29, 31). However, reduced expression of Tnfα mRNA was also observed in skin tumors from GW0742-treated wild-type mice but not in similarly treated Pparβ/δ-null mice. Because activating PPARβ/δ is known to inhibit inflammatory signaling (reviewed in ref. 33), it is possible that inhibition of inflammatory signaling by PPARβ/δ also contributes to the mechanisms underlying the chemopreventive effects of GW0742 in this model. This is consistent with the reduced accumulation of infiltrating polymorphonuclear neutrophils in GW0742-treated skin tumors. The mechanism underlying GW0742-dependent inhibition of skin tumor multiplicity in both wild-type and Pparβ/δ-null mice is uncertain, but this has been found previously (21). One possible mechanism is that GW0742 inhibits myeloperoxidase activity through direct enzyme inhibition (28). Because myeloperoxidase is found in neutrophils that accumulate during tumor promotion with TPA, it is possible that GW0742 inhibits the activity of infiltrating neutrophils. Additional studies are needed to determine how GW0742 inhibits chemically induced skin tumorigenesis through PPARβ/δ-independent mechanisms.

It is of interest to note that the chemopreventive effect of nimesulide was also dependent on PPARβ/δ. Indeed, delayed onset of skin tumorigenesis, reduced tumor multiplicity, and larger proportion of smaller versus larger tumors were all observed in wild-type mice fed the nimesulide diet, and these effects were diminished in similarly treated Pparβ/δ/-null mice. One possible mechanism that may underlie this effect is the observed increase in PPARβ/δ function resulting from nimesulide treatment.

Similar increases in PPARβ/δ expression and function have also been observed in colon cancer cell lines, and these changes were also associated with the inhibition of cell growth by nimesulide (22). The increase in PPARβ/δ expression by nimesulide could lead to enhanced terminal differentiation or anti-inflammatory activities. This is consistent with the observed increase in K1 expression and the inhibition of Il6 and Tnfα mRNA in skin tumors found in wild-type mice treated with dietary nimesulide but not in similarly treated Pparβ/δ/-null mice. Although dietary nimesulide at the concentration used in the present study is known to inhibit COX2 activity in mouse skin (7), expression of COX2 is also known to be higher in phorbol ester–treated Pparβ/δ/-null mouse skin than in control (35). Furthermore, inhibition of COX2 activity is found in wild-type mouse skin and this effect is diminished in Pparβ/δ/-null mouse skin (7). Thus, the observed PPARβ/δ-dependent chemoprevention by nimesulide could be due to differences in stoichiometry between nimesulide and COX2.

Further studies are needed to examine this possibility.

The efficacy of chemoprevention of chemically induced skin tumorigenesis was greatest when nimesulide was combined with GW0742. This was most evident by the prolonged inhibition of tumor multiplicity. Interestingly, this effect was due to both PPARβ/δ-dependent and PPARβ/δ-independent mechanisms. Inhibition of tumor multiplicity was observed in both wild-type and Pparβ/δ/-null mice from week 20 to week 32, after which this was found in wild-type but not in Pparβ/δ/-null mice. This is of interest because dietary nimesulide was only effective for inhibiting tumor multiplicity in wild-type mice but not in Pparβ/δ/-null mice, whereas GW0742 was effective in both genotypes during this time frame. Combining inhibition of COX2 activity with inhibition of myeloperoxidase activity could result in synergistic or additive effects that contribute to the observed enhanced chemoprevention by both nimesulide and GW0742. However, results from the present study also show that nimesulide effectively increases apoptotic signaling in...
mouse keratinocytes in both genotypes. This suggests that the observed PPARα/δ-independent inhibition of tumor multiplicity resulting from the combination of nimesulide and GW0742 could be influenced, in part, by increased apoptotic signaling. Why the observed chemoprevention becomes dependent on PPARα/δ during the later stages of the bioassay is uncertain but could be due to the combined effects on differentiation and anti-inflammatory activities that become more dominant during this period. Because of the striking enhanced chemoprevention of chemically induced skin tumorigenesis by combining ligand activation of PPARα/δ with the inhibition of COX2 activity, as compared with either agent alone, it will be of great interest to determine whether this approach can be used for UV-induced skin tumorigenesis, a more predominant etiologic risk factor for skin cancer in humans. Alternatively, whether inhibiting EP receptor activity and activating PPARα/δ will provide a safer approach, due to known issues associated with COX2 inhibitors, should be of interest based on these original studies. Combining inhibition of COX2 signaling with ligand activation of PPARα/δ could provide a new approach for chemoprevention of skin tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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