Peroxisome Proliferator-Activated Receptor β/δ Expression and Activation in Lung Cancer

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Peroxisome proliferator-activated receptor β/δ (PPAR β/δ) is a ligand-binding inducible transcriptional factor linked to carcinogenesis. Important functions of PPAR β/δ were demonstrated in series of human epithelial cancers; however, its role in lung cancer remains controversial. We investigated the differential expression level and localization of PPAR β/δ in tumors and adjacent normal lung tissue, and the effect of PPAR β/δ activation on lung cancer cell proliferation and apoptosis. PPAR β/δ was expressed in all studied human non-small cell lung cancers, and strong PPAR β/δ immunoreactivity was observed in epithelial cells of more than 75% of studied lung tumors. PPAR β/δ expression was consistently limited to the cancer cells in tumor tissue, while in adjacent normal lung tissue it was limited predominantly to the mononuclear cells. We found that ligand-binding activation of PPAR β/δ stimulates cell proliferation (an effect that was blocked by a dominant-negative construct of PPAR β/δ), stimulates anchorage-independent cell growth, and inhibits apoptosis in lung cancer cell lines. Importantly, the activation of PPAR β/δ induces Akt phosphorylation correlated with up-regulation of PDK1, down-regulation of PTEN, and increased expression of Bcl-xL and COX-2. These findings indicate that PPAR β/δ exerts proliferative and anti-apoptotic effects via PI3K/Akt1 and COX-2 pathways. In conclusion, PPAR β/δ is strongly expressed in the majority of lung cancers, and its activation induces proliferative and survival response in non-small cell lung cancer.

Keywords: non-small cell lung cancer; PPAR β/δ ; apoptosis; proliferation

Lung cancer is the third most common cancer in the United States, yet it causes more deaths than breast, colon, pancreatic, and prostate cancer combined. The objective of this study was to elucidate the role of peroxisome proliferator-activated receptor β/δ (PPAR β/δ) in lung cancer. Peroxisome proliferator-activated receptors α , β/δ , and γ are ligand-binding inducible transcriptional factors that belong to the nuclear hormone receptor family. PPARs are receptors for fatty acids, and their derivatives produced by lipoxygenase and cyclooxygenase pathways are known to regulate cell proliferation, differentiation, and survival, thereby controlling carcinogenesis. PPAR β/δ was the last identified, and remains least characterized among PPARs. PPAR β/δ was reported to play a pivotal role in neoplasia as a target of several pathways, including Apc- β -catenin tumor-suppressor pathway (1), oncogenic Ras (2), PI3K/Akt (3, 4), and COX-2 pathway (5, 6). The first link between PPAR β/δ and cancer was established in the colon, where it is expressed at high levels in tumor (1, 7, 8). The

CLINICAL RELEVANCE

This study provides a link between PPAR β/δ activity and the progression of lung cancer. Our findings contribute to a new and important area of research in lung cancer biology, and may have therapeutic implications.

consequence of modulation in PPAR β/δ expression and/or activity on colorectal cancer progression is considerably well studied; however, the role of PPAR β/δ activation in colorectal tumorigenesis *in vivo* is still controversial (9–12).

Little is known about the role of PPAR β/δ in the airway epithelium, and the effects of ligand activation of PPAR β/δ on transcriptional regulation and cellular functions are debated. PPAR β/δ protein was shown to be expressed in lung cancer cell lines and its ligand activation to induce epithelial cell proliferation through the up-regulation of EP4 gene expression (4). In contrast, others reported that PGI2 signaling contributes to the negative growth control of lung cancer cells, an effect mediated by activation of PPAR β/δ (13).

In this study, to elucidate the potential role of PPAR β/δ in lung cancer progression, we determined the expression pattern of PPAR β/δ in human lung primary tumors and in adjacent normal lung tissue. We also determined the effect of PPAR β/δ activation on cell proliferation and apoptosis in lung cancer.

MATERIALS AND METHODS

Cell Culture

Lung cancer cell lines A549, H23 (adenocarcinoma), and H157 (squamous cell carcinoma) were obtained from the American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 50 IU/ml of penicillin/streptomycin. Before any experimental treatment, 10% FBS containing medium was replaced with 1% FBS-RPMI for overnight culturing.

Western Blotting

Total cell lysates were prepared with modified RIPA buffer containing protease complete mix (Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitors cocktail (Sigma-Aldrich, St. Louis, MO). Twenty to thirty micrograms of total protein per lane was resolved by either 10% or 4–20% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membrane. PPAR β/δ protein was detected with rabbit polyclonal antibody H-74 (Santa Cruz Biotechnology, Santa Cruz, CA). COX-2 antibody was from Oxford Biomedical Research (Oxford, MI); actin antibody was from Sigma; all other antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Secondary antibodies were horseradish peroxidase-conjugated anti-IgG (Promega, Madison, WI). Signal was detected using enhanced chemiluminescence kit (Pierce, Rockford, IL).

(Received in original form November 20, 2007 and in final form June 12, 2008)

This study was supported by the Damyon Runyon Cancer Research Foundation (Ci-# 19-03 to P.P.M.).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Cell Mol Biol Vol 39, pp 689–696, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-0426OC on June 19, 2008

Internet address: www.atsjournals.org

TMA Immunostaining and Evaluation

Two tissue microarrays (TMA), each consisting of 51 lung tumors with corresponding controls (adjacent nontumor tissue), were used for PPAR β/δ immunostaining. TMA preparation has been described previously (14). The major lung cancer subtypes were presented as follows: 47 adenocarcinomas, 42 squamous carcinomas, 6 large cell carcinomas, 3 small cell carcinomas, 2 carcinosarcoma tumors, and 2 large cell neuroendocrine carcinomas. Cases were selected randomly based on availability in tissue archive between years 1997 and 1999. Immunostaining was performed on paraffin-embedded tissue using PPAR β/δ antibody K-20 (Santa Cruz Biotechnology, Santa Cruz, CA). Tissue sections were processed as follows: antigen retrieval in sodium citrate buffer, quenching in 3% H₂O₂/methanol, blocking with 10% normal horse serum, incubation with primary antibody in 1% serum overnight at 4°C, biotinylated secondary antibody for 1 hour, and Vectastain mixture ABS Elite (Vector Laboratories), DAB staining followed by hematoxylin counterstaining. The intensity of immunoreactivity in TMA was evaluated using the semiquantitative scoring scale: no staining, 0; weak, 1; moderate, 2; and strong, 3. Scoring (0–3) was performed by two investigators (P.P.M. and A.L.G.) independently. Discrepancies in scoring were reviewed and a consensus score was determined. The average score for triplicate cases was used for statistical analysis.

WST-1 Proliferation Assay

Cells were grown for 1, 2, and 3 days in 1% FBS-RPMI medium containing various doses of compound GW501516—specific ligand for PPAR β/δ (15), purchased from Cayman Chemical Co. (Ann Arbor, MI). Cell proliferation/viability was estimated every day based on cleavage of the tetrazolium salt WST-1 (Roche, Indianapolis, IN) by cell mitochondrial dehydrogenases. Formazan dye produced from WST reagent by metabolically active cells was quantitated spectrophotometrically according to manufacturer's protocol.

Apoptosis Assay

Cells were seeded in 96-well plates at 10⁴ cells/well density in 200 μ l complete RPMI medium. Next day complete medium was replaced with 1% FBS-RPMI, containing 10 μ M of cisplatin to induce apoptosis. Increasing doses of GW501516 were introduced at the same time with cisplatin. After 2 days of cell culturing, cells were subjected to Cell Death ELISA (Roche Diagnostic, Indianapolis, IN) according to manufacturer's instructions. This quantitative photometric sandwich-enzyme-immunoassay detects histone-associated DNA fragments in the cytoplasm fraction of cell lysate. Increase in apoptosis enriches cytoplasmic fraction with nucleosomes, which leads to the higher absorbance reading in the well.

Anchorage-Independent Cell Growth Assay

For the anchorage-independent growth experiments, cells (5 $\times$ 10³ cells/well) were seeded in 0.35% agarose supplemented with complete culture medium. This suspension was layered over 1.5 ml of 0.7% agar-medium base layer in 6-well plates. One hundred nanomolars of GW501516 or 0.1% DMSO as a control was added in soft agar at the time of seeding and with culturing medium every 4 days. Plates were incubated for 14 days (A549 and H157 cells) or 21 days (H23 cells) at 37°C. The cell colonies were stained with 0.005% crystal violet for more than 1 hour. Colonies were counted by use of a dissecting microscope.

Cell Transfection with Dominant-Negative Human PPAR β/δ

A549 cells were seeded in 96-well plate (2.5 $\times$ 10³ cells/well). Cells were transfected with 0.06 μ g/well of pcDNA3.1(zeo) vector or pcDNA3.1(zeo)-dominant-negative human PPAR β/δ (DNhPPAR β/δ) (16) by the Lipofectamine 2000 reagent according to the manufacturer's protocol (Life Technologies, Inc., Rockville, MA). Efficiency of transient transfection was assessed by Western blot using PPAR β/δ antibody as described above and by fluorescence-activated cell sorter analysis using co-transfection with GFP-containing plasmid (pEGFP-C1, 0.006 μ g/well).

Statistical Analysis

For comparison between groups, unpaired *t*-tests and unpaired ANOVA tests were used. Statistical significance for this study was set at two-sided *P* < 0.05. Unless otherwise stated, results are given as the mean $\pm$ SD of three independently performed experiments. Kruskal-Wallis nonparametric test was used for immunostaining data evaluation.

RESULTS

Differential PPAR β/δ Protein Expression in Normal Lung and in Lung Cancer

We first assessed PPAR β/δ protein expression by Western blot in eight pairs of tumor and adjacent normal lung tissue. The PPAR β/δ immunoreactive band corresponding to 55 kD was present in all studied samples of lung adenocarcinoma, squamous carcinoma, and adjacent normal tissue (Figure 1A). Although six out of eight paired tumor/control samples showed elevated PPAR β/δ protein expression in tumors, the average difference was not statistically significant in either histologic group (Figure 1B).

To characterize the cellular localization of PPAR β/δ protein and its expression pattern in normal lung and lung cancers of different histologic subtypes, we used two tissue microarrays, each representing 51 lung tumors with corresponding adjacent nontumor tissue. The clinicopathologic characteristics of patients are described in Table 1. A total of 102 lung tumors were evaluated. Strong PPAR β/δ immunoreactivity was observed in all lung cancer subtypes studied: squamous carcinoma, adenocarcinoma including bronchioalveolar carcinoma (BAC), large cell carcinoma, large cell neuroendocrine carcinoma, carcinosarcoma tumors, and small cell carcinomas. The intensity of PPAR β/δ

Figure 1. Peroxisome proliferator-activated receptor (PPAR) β/δ expression in human lung tumor and normal tissue. (A) PPAR β/δ protein expression in lung cancer and normal lung by Western blot analysis. Actin was used as an internal control. (B) Densitometry data present relative expression of PPAR β/δ protein after normalization to actin expression. Data are shown as means (columns) with SD (bars) (*n* = 4). T, tumor; N, normal adjacent tissue; AdCa, adenocarcinoma; SqCa, squamous carcinoma.

TABLE 1. THE CLINICOPATHOLOGIC DATA OF THE PATIENTS IN THE TISSUE MICROARRAY IMMUNOHISTOCHEMISTRY ANALYSIS

Characteristic	Number of Patients (%)
Sex	
Female	41 (40.2)
Male	61 (59.8)
Age, yr:	
Range	40–99
Mean ± SD	66 ± 11.7
Median	67
History of smoking*	
Current smoker	41 (40.2)
Ex-smoker	46 (45.5)
Never-smoker	10 (9.8)
Tumor stage†	
I	53 (52.0)
II	12 (11.8)
III	27 (26.5)
IV	8 (7.8)
Histology	
Adenocarcinoma	47 (46.1)
Squamous carcinoma	42 (41.2)
Large cell carcinoma	6 (5.9)
Carcinoid	2 (1.9)
Large cell neuroendocrine carcinoma	2 (1.9)
Small cell carcinoma	3 (2.9)

* Data available for 97 of 102 cases.
† Data available for 100 of 102 cases.

immunoreactivity in TMA was evaluated based on the following scoring scale: no staining, 0; weak staining, 1; moderate, 2; and strong, 3. Eighty out of 102 tumors (78%) demonstrated at least moderate immunoreactivity (Table 2). PPARβ/δ expression was consistently limited to the cancer cells in tumor tissue (Figures 2A and 2B). Evaluation of the normal lung at higher power demonstrates moderate to strong nuclear staining in alveolar macrophages, and occasional staining in type II pneumocytes (Figure 2C). Nuclear staining was predominant for

PPARβ/δ; however, cytosolic localization was also observed in few cases (Figure 2B). PPARβ/δ expression was increased as early as at stage I of lung cancer (Figures 2A and 2B). Next, we sought a correlation between immunostaining patterns and clinical variables. All tumors with neuroendocrine differentiation were excluded from this analysis because of the small samples size. There were no significant correlation between immunostaining intensity and history of smoking, tumor stage, or tumor histology from the tissue microarray analyses. Taken together, our results showed that PPARβ/δ is expressed in normal lung and in lung cancer. Importantly, PPARβ/δ protein is overexpressed in the majority of lung cancer and, therefore, may play the role in the pathogenesis and/or progression of lung cancer.

Ligand-Binding Activation of PPARβ/δ Stimulates Lung Cancer Cell Proliferation

To determine whether modulation of PPARβ/δ activity affects human lung cancer cell growth, we evaluated the proliferation of two adenocarcinoma cell lines (A549, H23) and one squamous carcinoma cell line (H157) after the treatment with PPARβ/δ highly specific ligand GW501516, since all three cell lines expressed PPARβ/δ transcript and protein (Figure 3A). Cells were treated with various doses of GW501516 for 3 days, and their proliferation/viability was determined by WST-1 assay. Proliferative effect of GW501516 treatment was observed in all three cell lines. All tested doses of GW501516 stimulated cell growth, and difference between treated and control cells was statistically significant at 10 nM of GW501516 (Figure 3B).

Ligand-Binding Activation of PPARβ/δ Inhibits Apoptosis in Lung Cancer Cells

Effect of ligand-binding activation of PPARβ/δ on apoptosis was evaluated by Cell Death ELISA-photometric enzyme-immunoassay that detects cytoplasmic histone-associated DNA frag-

Figure 2. PPARβ/δ expression in the human lung tumors. PPARβ/δ expression in (A) stage I adenocarcinoma, (B) stage I squamous carcinoma, and (C) nontumor tissue at ×10 and ×40 magnifications; the area presented at higher magnification is indicated by a square. PPARβ/δ immunostaining is limited to cancer cells in tumor tissue and predominantly to mononuclear cells in adjacent normal tissue.

TABLE 2. DISTRIBUTION OF THE PPAR β/δ IMMUNOREACTIVITY AMONG LUNG CANCER SUBTYPES

Histology of Lung Cancer	No Staining	Weak	Moderate	Strong
Adenocarcinoma (<i>n</i> = 47)	2 (4)	9 (19)	22 (47)	14 (30)
Squamous carcinoma (<i>n</i> = 42)	3 (7)	6 (14)	14 (33)	19 (45)
Large cell carcinoma (<i>n</i> = 6)	0	0	3 (50)	3 (50)
Carcinoid tumor (<i>n</i> = 2)	0	1 (50)	1 (50)	0
Large cell neuroendocrine carcinoma (<i>n</i> = 2)	1 (50)	0	0	1 (50)
Small cell carcinoma (<i>n</i> = 3)	0	0	0	3 (100)
Percent of total number of tumors (<i>n</i> = 102)	5.9	15.7	39.2	39.2
Percent of total number of adjacent lung tissue (<i>n</i> = 102)	57	41	2	0

Data represent number of tumors (% of subtype). PPAR β/δ immunoreactivity in different lung cancer subtypes was evaluated based on the staining intensity of 102 tissue samples (cases). Data are expressed as a number of samples (cases), and percentage of total number of cases for each group is shown in parentheses.

ments in cells. Cisplatin was used in this experiment to induce apoptosis (17). Cells were cultured for 2 days with 10 μ M of cisplatin added at the same time with GW501516. Cell Death ELISA results shown in Figure 4 demonstrated that PPAR β/δ -specific ligand GW501516 reduces apoptosis induced by cisplatin in all three NSCLC cell lines. The apoptosis rate was significantly lower in A549 and H23 cells treated with 10 nM or 100 nM of GW501516 compared with corresponding control cells treated with 0.1% DMSO. This effect was especially pronounced in the H23 adenocarcinoma cell line. Thus, PPAR β/δ ligand-binding activation increases resistance of lung cancer cells to apoptosis.

Ligand-Binding Activation of PPAR β/δ Induces Lung Cancer Cell Anchorage-Independent Growth

We tested effect of GW501516 on lung cancer cell anchorage-independent growth using cells colony formation in soft agar. As shown in Figure 4, PPAR β/δ activation significantly induced anchorage-independent growth in all three cell lines. Treatment with GW501516 increased number of colonies by 1.86-, 1.82-, and 1.92-fold for A549, H157, and H23 cell lines, respectively (Figure 4B). Colonies size was also significantly greater for cells treated with GW501516 compared with DMSO-treated cells (Figure 4A).

PPAR β/δ Exerts Tumorigenic Effect through PI3K/Akt and COX-2 Pathways

To ascertain the putative intracellular mechanism mediating the pro-tumor effects of PPAR β/δ , we tested several of its downstream targets. The data are presented for A549 cells; similar results were obtained for H157 and H23 cells (data provided in the online supplement). First, the expression of 3-phosphoinositide-dependent kinase-1 (PDK1) was evaluated. PDK1 is a PPAR β/δ direct target gene known to play a critical role in regulation of cell proliferation, differentiation, and survival (11). Cells were treated either with 0.1% DMSO (vehicle) or with 10 nM of GW501516 for 2 and 4 hours, and protein expression was detected by Western blot analysis. As shown in Figure 5, PPAR β/δ ligand-binding activation induced PDK1 expression in a time-dependent manner. Overexpression of PDK1 is one mechanism for activation of serine-threonine kinase Akt. This activation can be antagonized by phosphatase and tensin homolog deleted on chromosome 10 (PTEN). As shown in Figure 5, PPAR β/δ ligand-binding activation significantly decreased PTEN levels and was associated with various levels of Akt phosphorylation. GW501516 also induced the same pattern of antiapoptotic Bcl-xL protein expression (Figure 5). Moreover, our results showed that GW501516 treatment induced COX-2

Figure 3. PPAR β/δ stimulates lung cancer cell proliferation and apoptosis. (A) PPAR β/δ mRNA and protein is expressed in lung cancer epithelial cell lines as shown by RT-PCR and Western blot, respectively. (B) Cells were treated with DMSO (vehicle) or various doses of GW501516 and grown for 3 days. Cell proliferation was estimated by WST-1 assay. Mean values with SD are shown (*n* = 5). **P* < 0.05 compared with vehicle (DMSO-treated cells). (C) Cells were treated with 10 μ M cisplatin along with various doses of GW501516. After 40 hours, an apoptosis rate was estimated by Cell Death ELISA. Results are presented as percentages of inhibition of apoptosis compared with vehicle (0.1% DMSO). Two experiments were performed in four replicates each. Data are shown as means of two experiments (columns) with SD (bars). **P* < 0.05 compared with vehicle.

expression in all three cell lines (Figure 5). COX-2 has been shown to have an antiapoptotic effect in several cancers, including lung cancer, and there is compelling evidence linking PPAR β/δ activation with COX-2 activity (5, 7, 11, 18). Collectively, these results show that PPAR β/δ modulates critical regulators of cellular

Figure 4. PPAR β/δ ligand-binding activation increases cell anchorage-independent growth. Anchorage-independent growth was measured by counting colonies formed in soft agar after exposure to GW501516 or DMSO (vehicle). (A) Representative images of colonies after each treatment are shown, with (B) average colony number. Two independent experiments with triplicates were performed. Data are shown as means of two experiments (columns) with SD (bars). * $P < 0.05$ compared with DMSO control.

proliferation and survival, supporting its role in lung tumor progression.

Functional Inhibition of PPAR β/δ Prevents Downstream Signaling and Decreases Cell Growth

To prove that demonstrated effects of PPAR β/δ ligand-binding activation on lung cancer cells are specific for PPAR β/δ , we

used a dominant-negative PPAR β/δ (DNhPPAR β/δ) construct. A549 cells were transiently transfected with either empty vector (EV) or vector containing DNhPPAR β/δ construct (DN). This DNhPPAR δ construct contains an inactivating mutation in the AF-2 domain, and, as a result, PPAR β/δ receptor lacks the ability to recruit transcriptional co-activators (16). Efficiency of transfection was 52.8% and 53.5% for cells transfected with EV

Figure 5. Effect of PPAR β/δ ligand-binding activation on cell survival-associated proteins. (A) Representative Western blot showing time-dependent changes in PDK1, PTEN, pAkt, Bcl-xL, and COX-2 protein expression in A549 cells treated with 10 nM of GW501516. Actin or total Akt were used as an internal control. (B) Densitometry data present relative expression of proteins after normalization to actin or total Akt (for pAkt) expression. Data are shown as means of three experiments (columns) with SD (bars). * $P < 0.05$ compared with DMSO control.

or DN vectors, respectively. Overexpression of dominant-negative PPAR β/δ was demonstrated by Western blotting (Figure 6A) performed on cells collected at Days 1, 2, and 3 after transfection (2-d time-point is presented). These transfected cells were used to prepare cell lysate for Western blotting and to perform WST-1 proliferation assay. As expected, the functional inhibition of PPAR β/δ prevented downstream signaling (Figure 6B). Changes in PTEN, COX-2, and Bcl-xL expression under GW501516 treatment observed in A549 cells transfected with empty vector were abrogated in cells transfected with DNhPPAR β/δ , as shown by Western blotting (Figure 6B). Introducing of dominant-negative PPAR β/δ into A549 cells also inhibited cell growth, as shown in Figure 6C. Thus, the effect of GW501516 treatment on lung cancer cells is mediated by PPAR β/δ .

DISCUSSION

This study revealed several novel observations. We demonstrate for the first time that PPAR β/δ protein is expressed in the majority of primary lung cancers. The activation of PPAR β/δ induces cell proliferation, anchorage-independent cell growth, and inhibition of apoptosis in NSCLC cell lines. PI3/Akt and COX-2 pathways mediate the effects of PPAR β/δ . Taken together, our findings provide evidence for a role of PPAR β/δ in lung tumor progression.

The expression of PPAR β/δ assessed by Western blot showed elevated level of PPAR β/δ protein in lung tumor compared with adjacent normal tissue in six pairs of tissue. The similar expression profile of PPAR β/δ was recently reported for human gastric and colorectum cancers (5, 19). We found a differential expression pattern for PPAR β/δ in lung tumor and adjacent normal tissue. PPAR β/δ immunoreactivity was specific for epithelial tumor cells, and was limited predominantly to mononuclear cells in adjacent normal tissue. We observed predominantly nuclear localization of PPAR β/δ staining. Seventy-eight percent of evaluated NSCLCs exhibited intensive PPAR β/δ immunoreac-

tivity in tissue microarray. Importantly, PPAR β/δ expression was significantly up-regulated at early stages of lung cancer, suggesting a significant role of this receptor in tumor progression.

The role of sustained activation of PPAR β/δ in lung cancer has been a subject of controversy. Published reports document that activation of PPAR β/δ promotes cell growth in some models (4, 9, 20), and inhibits it in others (13, 21). Our data clearly demonstrated that the expression and activation of PPAR β/δ promotes lung cancer cells survival, proliferation, and anchorage-independent cell growth. Fukumoto and colleagues (13) reported PPAR β/δ as a key molecule in PGI₂ signaling with negative growth control properties in A549 cells. These results were obtained with carbarprostacyclin (an agonist for both PGI₂ receptor IP and PPAR β/δ) and L-16504 (a specific agonist for PPAR β/δ). The discrepancy between findings may be attributed to different types and concentrations of PPAR β/δ agonists used in our and Fukumoto's studies. High concentration (20 μ M) of PPAR β/δ ligand L-165041 could overcome PPAR β/δ specificity, as even 1 μ M of L-165041 was shown to activate PPAR γ luciferase reporter gene expression (22). In our study, the role of PPAR β/δ activation in cell proliferation was evaluated by using the synthetic pharmacologic ligand GW501516. It is a potent and selective PPAR β/δ agonist, with EC50 of 1.1 nM and with 1,000-fold greater selectivity over other human subtypes PPAR α and γ (15, 22, 23). GW501516 was shown to increase proliferation of the lung squamous carcinoma cells H157 starting from 1 μ M, and this effect was abolished by introducing PPAR β/δ siRNA (4). We observed proliferative effect of GW501516 treatment on H157 cells and also on two adenocarcinoma cell lines (A549 and H23) at as low and PPAR β/δ -selective a dose as 10 nM. We demonstrated that introduction of dominant-negative PPAR β/δ into A549 cells inhibits cell growth and prevents downstream signaling, confirming PPAR β/δ specificity of the observed effect.

Similarly, the role of PPAR β/δ activation in apoptosis has been debated in literature. There are reports indicating an

Figure 6. Effect of PPAR β/δ functional inhibition in lung cancer cells. (A) Western blot analysis of DNhPPAR β/δ expression in A549 cells after 48 hours of transient transfection. Actin was used as an internal control. EV, cells transfected with empty vector; DN, cells transfected with DNhPPAR β/δ -containing vector. (B) Western blot showing expression of cell survival-associated proteins in A549 cells, transfected either with DNhPPAR β/δ -containing vector (DN) or empty vector (EV). Numbers indicate relative protein expression normalized to actin as detected by densitometry. (C) Functional inhibition of PPAR β/δ attenuates A549 cell growth. Cells were transiently transfected either with empty vector (EV) or with DNhPPAR β/δ -containing vector (DN), and were growing for 3 days. Cell viability/proliferation was estimated by WST-1 assay every day. Two experiments were run in five replicates. Data are shown as means of two experiments (columns) with SD (bars).

antiapoptotic role of PPAR β/δ (24–26) as well as its proapoptotic role (10, 21, 27). We demonstrated that GW501516 treatment inhibits cisplatin-induced apoptosis in NSCLC cells in a dose-dependent manner. Inhibition of apoptosis upon PPAR β/δ activation was demonstrated for all three cell lines; however, this effect was not significant in H157 cells. Lower susceptibility of H157 cells to apoptosis has been reported in the literature (28, 29).

To confirm the tumorigenic effect of PPAR β/δ activation, we tested the effect of GW501516 on lung cancer cell anchorage-independent growth. Colony formation in soft agar is a property closely associated with cancer cell malignancy. Treatment with PPAR β/δ ligand significantly augmented colony formation by all three cell lines, increasing both size and number of colonies.

The functional role of PPAR β/δ activation in lung tumor progression was supported by its effect on several molecular targets important for cell proliferation and survival pathways. In this study, GW501516 activation of cancer cells induced up-regulation of PDK1, down-regulation of PTEN, and increased phosphorylation of Akt. PDK1 was identified previously as a direct target for PPAR β/δ (11, 25). Increased expression of PDK1 in response to GW501516 treatment provides further evidence for PPAR β/δ activation in three studied cell lines. PDK1 is involved in the PI3K/Akt pathway, which is pivotal for cell differentiation, survival, and apoptosis. Activation of Akt-1 occurs through two critical phosphorylation events. The first one is phosphorylation at threonine (T308) in the catalytic domain by PDK1 (30). Subsequent phosphorylation at serine (Ser473) in hydrophobic motif is required for full activation, and it can be mediated also by PDK1 (31). The up-regulation of PDK-1 was shown to increase Akt1 activity in keratinocytes, and it was sufficient to suppress apoptosis in cell culture (25). Tumor suppressor PTEN may prevent translocation and activation of PDK1 and Akt. The loss of PTEN expression results in increased Akt activity and continued cell survival and proliferation (32, 33). We observed a similar effect in NSCLC cells upon PPAR β/δ activation: up-regulation of PDK1 and pAkt with PTEN suppression, followed by increased cell proliferation/viability and inhibition of cisplatin-induced apoptosis. In addition, GW501516 treatment induced the expression of Bcl-xL in all three cell lines, an important regulator of mitochondrial-mediated apoptosis, which further supports a role of PPAR β/δ in lung cancer progression.

We report an increase in COX-2 expression after PPAR β/δ ligand-binding activation in NSCLC cells. Increased level of COX-2 have been associated with resistance to apoptosis and increased proliferation rate in many cancer cell types. The link between COX-2 expression and PPAR β/δ was established in colorectal and gastric cancers, cholangiocarcinoma, and endometrial adenocarcinoma (5, 7, 34, 35). PPAR β/δ activation by GW501516 in hepatocellular carcinoma cells resulted in increased COX-2 promoter activity, induction of COX-2 expression, and increased cellular proliferation (6). Although no peroxisome-proliferator response element has been identified in COX-2 promoter, PPAR β/δ is known to mediate its transcriptional activity through interaction with other transcriptional factors for COX-2, such as NF- κ B, C/EBP, and CRE (4, 11, 25, 36). Further studies addressing the exact mechanism of COX-2 regulation by PPAR β/δ are required.

In summary, we found that PPAR β/δ is overexpressed in a majority of lung cancer subtypes. We showed that PPAR β/δ ligand-binding activation promotes proliferation and inhibits apoptosis in lung tumor epithelial cells, increasing their tumorigenicity. We demonstrated that the tumorigenic effect of PPAR β/δ ligand-binding activation in NSCLC cells involves PI3K/Akt and COX-2 pathways. *In vivo* animal studies are required to

ascertain the biological role of PPAR β/δ in lung cancer. If the tumorigenic role of PPAR β/δ is confirmed, PPAR β/δ -specific antagonist may serve as an effective agent in cancer therapy.

Conflict of Interest Statement: R.N.D. is on the scientific advisory board for Tragara and received \$10,000 per year. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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