Differentiation of Human Neuroblastoma by Phenylacetate Is Mediated by Peroxisome Proliferator-activated Receptor γ

Shouwei Han, Randal K. Wada, and Neil Sidell

Department of Gynecology and Obstetrics, Emory University School of Medicine, Atlanta, Georgia 30322 [S. H., N. S.], and The Kapiolani Health Research Institute and the University of Hawaii, Honolulu, Hawaii 96813 [R. K. W.]

ABSTRACT

Phenylacetate (PA) is a member of a class of aromatic fatty acids that has demonstrated antitumor activity in experimental models and in humans. Previous reports have shown that PA and its analogues can act as ligands for the peroxisome proliferator-activated receptor (PPAR) and thereby regulate certain gene expression through peroxisome proliferator response elements. The role of this activity in the antitumor activity of PA has not been determined. To address this question, we have used the human neuroblastoma cell line LA-N-5, which expresses PPARγ and can be induced to differentiate with PA and with classical PPARγ ligands. Our results indicated that the PPARγ ligands 15-deoxy-Δ prostaglandin J2 and GW1929 as well as PA induced LA-N-5 cells to differentiate to a similar phenotype as evidenced by inhibition of cell proliferation, neurite outgrowth, increased acetylcholinesterase activity, and decreased N-myc gene expression. Furthermore, induction with all of the compounds was accompanied by up-regulation of mRNA levels of the nuclear retinoic acid receptor β (RARβ) and specific activation of a reporter gene construct (ΔSVPRE-CAT) that contains the canonical RA response element located in the RARβ promoter. All of the assessed functional and molecular effects of PA on LA-N-5 cells, as well as those of the classical PPARγ ligands, were inhibited by cotreatment with specific PPARγ antagonists (GW9662 and/or GW0072). Taken together, these studies have confirmed a role for PPARγ in neuroblastoma cell biology and indicated that the PPARγ signaling pathway plays a direct role in the PA-induced differentiation response of this cell type.

INTRODUCTION

Aromatic fatty acids, of which PA is a prototype, constitute a class of low toxicity drugs with demonstrated antitumor activity in experimental models and in humans. PA is a natural metabolite of phenylalanine, which was originally described as a plant growth hormone (1). Normally found in micromolar concentrations in human plasma, PA has a long clinical history as treatment for conditions associated with hyperammonemia such as urea cycle disorders in children (2, 3). This clinical experience has indicated that millimolar blood serum levels can be achieved without significant adverse effects.

As demonstrated in a variety of experimental in vitro and in vivo models, PA and its analogues can induce selective cytostasis and reduce the malignant potential of various hematological and solid neoplasms at nontoxic millimolar concentrations shown to be readily achievable in humans (4–7). Early clinical trials with PA have now documented activity in high-grade gliomas, hormone-independent prostatic carcinoma, and lymphoid malignancies. PB, which is metabolized to PA in humans, was also recently shown to benefit cancer patients who have failed conventional therapies (8, 9). For both compounds, the treatments were well tolerated, with a dose-limiting toxicity of somnolence. Despite this substantial experimental and clinical experience with PA compounds, their antitumor mechanism of action remains unknown.

In previous studies, we showed that PA can stimulate the differentiation of human nb cells by itself and can impact the differentiation program induced by RA by activating one of the retinoid nuclear receptors, RARβ (6, 10). It has also been shown that PA can activate other members of the steroid receptor superfamily, namely, the PPARs (11). Recently, Samid et al. (12) demonstrated that PA derivatives can directly act as ligands to PPARγ and that the cytostatic effects of these drugs on certain cell lines directly correlated with their PPAR-activating ability. In a previous study, we documented that the LA-N-5 human nb cell line and primary nb cells from patient tissue express abundant amounts of PPARγ and that PPARγ ligands can stimulate the differentiation of this cell type (13). These new results have now led us to address the question of whether the PPARγ signaling pathway is involved in the differentiation-inducing activity of PA on nb cells. Our present findings indicate that this is indeed the case.

MATERIALS AND METHODS

Cell Culture. The LA-N-5 human nb cell line was grown in RPMI 1640 (Cellgro, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum, HEPES buffer, 50 IU/ml penicillin/streptomycin, and 1 μg of amphotericin (complete medium) as described previously (14). 15d-PGJ2 was purchased from Cayman Chemical (Ann Arbor, MI) dissolved in methyl acetate. GW1929, GW9662, and GW0072 were synthesized by the Medicinal Chemistry Department at Glaxo Wellcome Research and Development and were a generous gift from Dr. T. M. Willson of the same institute (Glaxo Wellcome Co., Research Triangle Park, NC). Sodium PA was obtained from Elan Pharmaceutical Research Corp. (Gainesville, GA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

AChE Activity. Specific AChE activity was measured as a biochemical index of the relative state of differentiation of treated and untreated LA-N-5 cells. To measure AChE activity as described previously (14), cells were grown in 12-well plates for 6 days in the presence or absence of the indicated concentrations of PA, 15d-PGJ2, GW1929, GW0072, and/or GW9662. After washing twice with PBS, cells were collected, ice-cold 10 mm sodium phosphate buffer (pH 7) containing 0.5% Triton X-100 was added, and the suspension sonicated for 20 s. AChE activity was determined photometrically by following the hydrolysis of acetylthiocholine as described previously (14). Protein concentrations were determined with a Sigma Chemical Co. bicinchoninic acid protein assay kit using BSA as the standard. Results (in nmol/h/mg protein) are the means ± SE of triplicate wells in typical experiments and are expressed as a percentage of control. All experiments were repeated at least three times.

Cell Proliferation Assay. LA-N-5 cell proliferation was assessed based on the ability of the cells to stain with SRB (15). Cells were grown in 24-well culture plates for 6 days in the presence or absence of the indicated concentrations of PA, 15d-PGJ2, GW1929, GW0072, and/or GW9662. The untreated control cells were not less than 90% of confluence before harvest. The culture medium was removed, and the cells were washed thrice with PBS. Trichloroacetic acid (final concentration, 10%) was then added for fixation at 4°C. After
1 h of fixation, plates were washed five times with tap water. The plates were then air dried, and 0.4% SRB in 1% acetic acid was added for 30 min. Unbound SRB was removed by washing the plates four times with 1% acetic acid. After air drying, SRB dye within cells was dissolved for 5 min with 10 mm unbuffered Tris base (pH 10.5). The absorbance of the extracted SRB dye, which represented protein content, was measured with a spectrophotometer at 540 nm.

Northern Blot. LA-N-5 cells were cultured for the indicated number of days in the presence or absence of PA, 15d-PGJ2, GW1929, GW0072, and/or GW9662. Total RNA (30 μg) was extracted by using TRI reagent (Sigma Chemical Co.) as described previously (16), separated by electrophoresis in a denaturing formaldehyde agarose gel, and blotted onto a nylon transfer membrane (Micron Separations, Inc., Westborough, MA). The RNA was cross-linked to the membranes by irradiation for 1 min under UV light and baked for 5 min at 70°C. A random priming probe kit from Promega (Madison, WI) was used to label RARβ, N-myc, and GAPDH cDNA probes. The blots were hybridized overnight at 42°C with 32P-labeled RARβ, N-myc, or GAPDH probes. After washing twice with 2× SSC/0.1% SDS and twice with 0.1× SSC/0.1% SDS, membranes were exposed for 18–72 h at −70°C to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) with intensifying screens.

Cell Transfection and CAT Assay. All transfections were carried out with LA-N-5 cells by the non-liposome-mediated formulation FuGENE 6 transfection method (Roche Molecular Biochemicals, Indianapolis, IN). Cells were maintained in medium without phenol red that contained 10% fetal bovine serum for 24 h before transfection. Transfections (using 6-well plates of 70% confluent cells) contained 3 μl of FuGENE 6 reagent with 2 μg expression plasmid/well in serum-free medium. The ΔSV/BRE-CAT reporter construct was used to measure RARβ promoter activation (17, 18); ΔSV/CAT vector served as a control. RA, PA, GW1929, GW0072, and/or GW9662 were added to the cultures immediately after the transfection. Cells were harvested 24 h later and adjusted to 4×10^5 viable cells in 150 μl of PBS (pH 7.4). Cells were lysed by three freeze-thaw cycles. To assay CAT activity, we used the [1H]Jactetyl-CoA method as described previously (19). The lysate was thawed, and 50 μl of it placed into a glass scintillation vial containing 150 μl of PBS, 7.5 μl of 4× CAT buffer (32 mm chloramphenicol dissolved in ethanol), 22.5 μl of water, and 0.5 μl (0.1 μCi/ml) of [1H]Jactetyl-CoA per vial. Organic scintillation fluid was then added, and vials were loaded into the scintillation counter for counting at different time periods.

Statistical Analysis. Data are expressed as the mean ± SE. ANOVA and Student’s t test (two-tailed) were used for statistical determinations (compared with control group). A level of P ≤ 0.05 was considered statistically significant. For analysis of PPARγ antagonist effects on PPARγ agonist-induced functional activity, differences between the combination treatment values (agonist + antagonist) minus untreated controls were compared with the differences between single treatment values (agonist alone) minus untreated controls. Because untreated control values were set to 100%, the ability of a compound to antagonize the functional activity of a PPARγ agonist is given by the following formula:

\[
\% \text{Antagonism} = \frac{\text{Activity}_{\text{agonist}} - \text{Activity}_{\text{agonist + antagonist}}}{\text{Activity}_{\text{agonist}}} \times 100
\]

where activity is expressed as a percentage of the control.

RESULTS

Effects of PA and PPARγ Ligands on the Growth of LA-N-5 Cells. Previous studies have demonstrated dose-dependent growth inhibition of LA-N-5 cells induced by both PA and PPARγ ligands (6, 13). In those studies, maximal growth-inhibitory effects were obtained at approximately 5 mM PA, 10 μM 15d-PGJ2, and 20 μM GW1929, respectively. Fig. 1 demonstrates that in the present studies, these concentrations of the compounds caused growth inhibition of 37% in the case of PA, 41% in the case of 15d-PGJ2, and 53% in the case of GW1929. As shown, the combination of PPARγ antagonist GW0072 with either PA, 15d-PGJ2, or GW1929 reduced the growth-inhibitory effects of treatments with the latter compounds alone. Thus, GW0072 antagonized the antiproliferative effects of PA, 15d-PGJ2, and GW1929 by 53%, 37%, and 63%, respectively. GW0072 alone had no significant effects on the growth of LA-N-5 cells. As a specificity control in these experiments, RA was used as a known differentiation/antiproliferative agent for these cells (17). Fig. 1 shows that RA inhibited the growth of the cells by 40%, but GW0072 did not significantly affect this growth-inhibitory activity.

Our previous studies have shown that PA and PPARγ ligands can induce neurite outgrowth from LA-N-5 cells at concentrations similar to those found to inhibit cell growth (6, 13). However, because neurite outgrowth is purely a visual qualitative phenomenon, experiments to determine whether GW0072 can antagonize this effect are difficult to interpret and therefore were not addressed at this time. GW0072 alone had no apparent morphological effects on LA-N-5 cells (data not shown).

AChE Activity. In LA-N-5 cells, AChE has been used extensively as a quantitative biochemical marker of differentiation after treatment with a variety of inducing agents including PA, 15d-PGJ2, and GW1929 (6, 13). As reported previously, Fig. 2 shows a 1.5–1.7-fold increase in AChE activity when LA-N-5 cells were treated with 5 mM PA, 10 μM 15d-PGJ2, or 20 μM GW1929 for 6 days. In contrast, the PPARγ antagonist GW0072 did not alter specific AChE activity. However, when added together, GW0072 antagonized the stimulatory effects of PA, 15d-PGJ2, and GW1929 on AChE by 67%, 54%, and 58%, respectively. GW0072 did not significantly alter RA-induced AChE activity.

N-myc Expression. N-myc expression is considered to be a molecular marker of differentiation of LA-N-5 cells and other nb cell lines (17, 20, 21). Previous work has shown that PA induces a moderate (15–35%) decrease of N-myc mRNA levels after long term-term treatment (>8 days; Ref. 6). In contrast, shorter treatments with PA (3–6 days) demonstrated no apparent effects on N-myc mRNA levels, although N-myc protein was found to be decreased (6). In our present studies, we have seen that the modest down-regulation of N-myc mRNA is largely reversed when the 8-day PA treatment is combined with PPARγ antagonists GW9662 or GW0072 (data not shown).

Nuclear RARβ Expression. We have demonstrated previously that PA can impact the RA differentiation program by up-regulating...
RARβ expression (6). Our studies showed that this effect is regulated at the level of transcription and mediated through the canonical RARE in the RARβ promoter (RAREβ; Ref. 10). To determine whether this activity of PA involves the PPARγ signaling pathway, we first assessed whether “classical” PPARγ agonists can also augment RARβ expression. Fig. 3a shows that this was indeed the case; densitometric scanning indicated a 5- to 25-fold increase (normalized to GAPDH) in RARβ mRNA levels induced by 15d-PGJ2 compared with controls and a 10–40-fold increase induced by GW1929 after 2 days of culturing. To confirm a role for PPARγ in this activity, we determined whether PPARγ antagonists could inhibit the PA- and 15d-PGJ2-induced increases in RARβ. Fig. 3b demonstrates that the addition of either GW9662 or GW0072 to the cultures completely blocked induction of RARβ expression in LA-N-5 cells. Culturing the cells in the PPARγ antagonists alone resulted in no differences in the expression of RARβ compared with that in control cells (data not shown).

**PPARγ-mediated Activation through RAREβ.** We have previously demonstrated that PA activation of RARβ occurs through the canonical RARE designated RAREβ located in the gene’s promoter (10). We now tested whether PA activation of this response element is mediated by PPARγ by first evaluating the effects of the PPARγ agonists on the RAREγ-containing CAT reporter construct ΔSV/βRE-CAT in transient transfection experiments with LA-N-5 cells. This construct contains a single copy of RAREβ cloned at the unique HinIII site present in a basal promoter CAT construct, ΔSV-CAT. This vector, which served as a control for ΔSV/βRE-CAT, was originally constructed by replacing the TK promoter in TK-CAT (22) with the Spl-HindIII fragment of the SV40 early promoter (18). As can be seen in Fig. 4, in the presence of PA and GW1929, CAT activity from ΔSV/βRE-CAT was 1.8- and 1.6-fold above untreated levels, respectively. As an additional positive control for this system, RA caused a 1.75-fold increase in CAT activity.

Next we determined whether the PPARγ antagonist GW9662 would specifically retard the PA-mediated activation of ΔSV/βRE-CAT. Fig. 4 shows that this was indeed the case; whereas GW9662 had no significant activity on the reporter itself, it reduced both PA- and GW1929-mediated induction by 54% and 60%, respectively. In contrast, GW9662 had no significant effect on RA induction of CAT activity, confirming that the specificity of the inhibitory activity of GW9662 was mediated by PPARγ. Under the same treatment conditions, activity of the basal construct ΔSV-CAT in the presence of any of the compounds never differed significantly from that of control cultures.

**DISCUSSION**

Although there have been scores of published laboratory studies and a few clinical trials, the antitumor effects of PA and its analogues remain unclear. Recently, PA combined with RA was shown to induce remission in a patient with acute promyelocytic leukemia whose disease was resistant to RA as a single agent (23). However, this effect of PA was speculated to be due to the ability of the butyrate moiety of this compound to function as a histone deacetylase inhibitor rather than to the metabolic conversion of PB to PA. Early studies suggested that the ability of PA to deplete serum of glutamine due to the formation of phenylacetylglutamine might serve to selectively inhibit the growth of certain types of tumor cells (24). However, because many tumor cells known to be affected by PA are not particularly...
sensitive to glutamine depletion, this mechanism alone could not
explain the antitumor activity of this compound. Other investigations
demonstrated that PA, due to its ability to block β-hydroxy-β-meth-
ylglutaryl CoA reductase, inhibits cholesterol synthesis and protein
prenylation in glioma, melanoma, prostatic carcinoma, and nb cells (5,
6). These cells, like most cancer cells, are dependent on intracellular
synthesis of isoprenoids for growth and survival. The possibility that
inhibition of protein prenylation may play a role in the antitumor
effects of PA has been supported by studies demonstrating that the
prenylation-inhibitory activity of different PA derivatives correlated
with their cytostatic activity (5). In particular, PA inhibition of p21
isoprenylation has been linked to its ability to induce phenotypic
reversion of ras-transformed cells (25, 26). Farnesyl transferase
inhibitors, which also block ras isoprenylation, have demonstrated sim-
ilar antitumor effects (27, 28). What this posttranslational mechanism
cannot explain, however, is the ability of the PA derivatives to alter
transcription of certain genes such as RARβ, which is known to be
involved in cell growth and differentiation (6, 10), in a protein
synthesis-independent fashion.

In this study, we have addressed the possibility that at least part of
the differentiation-inducing activity of PA on human nb cells is
mediated by its ability to act as a ligand for PPARγ. In the studies by
Samid et al. (12), it was demonstrated that PA and its analogues can
bind directly to PPARγ with Kᵦ values in the range of 0.2–6.0 μM, in
contrast to the reported Kᵦ values of 15d-PGJ2 and GW1929 of about
20 μM (29) and <40 nM (30), respectively. Furthermore, the low
affinity of PA-PPARγ binding corresponded to the potency of the PA
derivatives to act as inhibitors of the proliferation of breast and glioma
cancer cells (12). Thus, PA compounds can be considered ligands of
PPARγ, but they bind with low affinity. Our results have shown that in
human nb cells, which express abundant levels of PPARγ (13),
PPARγ antagonists GW9662 and GW0072 can suppress the differ-
etiation-inducing activity of PA. GW9662, an irreversible antagonist
of PPARγ, was reported to inhibit the induction of CD36 by interleu-
kin 4 and to block the action of BRL49653, a PPARγ agonist (31).

GW0072 was identified as a high affinity PPARγ ligand that was a
weak partial agonist of PPARγ transactivation. However, this com-
 pound has been shown to be a potent antagonist of adipocyte differ-
etiation (32). When added at micromolar concentrations, GW9662
and GW0072 inhibited the antiproliferative activity, neurite out-
growth, increased ACHe activity, and reduction of N-myc expression
in LA-N-5 cells caused by millimolar concentrations of PA. On the
other hand, differentiation effects induced by RA were not affected by
cotreatment with the PPARγ antagonists, confirming that the speci-
ficity of their activity is mediated through PPARγ. These findings are
consistent with the contention that low affinity binding of PA to
PPARγ is at least partially responsible for the differentiation-inducing
activity of PA in nb cells. The observation that high affinity PPARγ
agonists at lower (micromolar) concentrations produce effects similar
to those of PA provides more evidence to support this contention and
indicates that functional PPARγ binding is sufficient for inducing
certain aspects of nb cell differentiation.

Up-regulation of RARβ has been shown to be an important early
event in the RA-induced differentiation response of many types of
cancer cells, including nb (33, 34). We have shown previously that PA
can also induce RARβ mRNA expression in human nb cells and that
this effect is regulated at the level of transcription and mediated
through RAREβ (10). Our data have now demonstrated that the
PPARγ ligands 15d-PGJ2 and GW1929, which is a high affinity
synthetic PPARγ ligand (35), also up-regulate RARβ expression.
Furthermore, we have shown that RARβ induction by PA, as well as
that by 15d-PGJ2, was blocked by the PPARγ antagonists.

To interpret our findings of increased ΔSV/re-CAT activation by
PA and GW1929, it should be remembered that this construct does not
contain a significant portion of the RARB gene promoter (36) but only
contains the small regulatory element RAREβ (a tandem repeat of the
AGGTCA half-site) that has been shown to directly bind and be
activated by all of the RA nuclear receptors (α, β, and γ), either in the
form of homodimers or complexed with RXR as a heterodimer (37).
Thus, specific activation of ΔSV/re-CAT by PPARγ ligands must be
mediated directly through RAREβ. PPAR/ RXR heterodimers have been
shown to bind weakly to the RAREβ in DNA gel shift assays (38), and,
along with the present data, this observation suggests the occurrence of
functionally significant cross-binding of this response element with “ac-
tivated” PPARγ/RXR heterodimers. This type of cross-talk has now been
confirmed between other members of the steroid hormone receptor su-
perfamily. For example, glucocorticoid and progesterone receptors share
common binding sequences in the promotors of the uteroglobin and
metallothionein genes (39, 40). RARs can bind and activate genes
through certain thyroid hormone response elements (41), and a novel
class of common cis-acting response elements for retinoid, vitamin D,
and estrogen receptors has been described previously (42). Future studies
will test the hypothesis that liganded PPARγ complexed with other
Cellular cofactors (e.g., RXR) can directly bind and activate RAREβ.

In conclusion, we have demonstrated that high affinity PPARγ
ligands can mimic the differentiation-inducing activity of PA on
human nb cells using a variety of functional and molecular criteria.
The contention that signaling through PPARγ plays a direct role in
PA-induced differentiation was supported by the ability of specific
PPARγ antagonists to inhibit this activity. Recently, RA has been
shown to have an effective compound for prolonging the remission time,
increasing the survival, and reducing the recurrence of nb when used in
the setting of minimal residual disease (43). Our demonstration of an
apparent cross-talk between the PPARγ and RA signaling pathways
that results in up-regulation of RARβ suggests a novel approach using
PPARγ ligands to enhance the retinoid sensitivity of nb cells or to
overcome the retinoid resistance that is sometimes seen in the clinic.

Fig. 4. Effect of PA and PPARγ ligands on ΔSV/RE-CAT expression. LA-N-5 cells
were transfected with the ΔSV-CAT (Ⅲ) or ΔSV/re-CAT (Ⅳ) constructs by the Fu-
GENE 6 noliposomal formulation method and immediately treated as indicated with 5
μM RA, 5 μM PA, 10 μM GW1929, or solvent control (Con) in either the absence (−)
or presence (+) of 20 μM GW9662. Cell extracts from normalized cell number were assayed
for CAT activity and expressed as cpm/μl lystate. Solvent controls without GW9662 were
set at 100%. Bars represent the mean ± SE of three independent experiments. When using
the ΔSV-CAT construct, no significant differences in CAT activity were noted between
treatment conditions. When using the ΔSV/re-CAT construct, * indicates a signifi-
cant difference between treated cells in the absence of GW9662 and solvent control
(P ≤ 0.05); ** indicates significance between treated cells in the presence of GW9662 and
the corresponding single treatment values (P ≤ 0.05).
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