Brief Report

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GW501516 acts as an efficient PPARα activator in the mouse liver

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ABSTRACT: The peroxisome proliferator-activated receptor (PPAR) subtype specificity of GW501516, a well-known PPARδ-specific agonist, was studied by examining its effects on the expression of endogenous genes in primary hepatocytes and the liver of wild-type and PPARα-null mice. GW501516, like the PPARα-specific agonist Wy14,643, induced the expression of several PPAR target genes in a dose-dependent manner but this action was mostly absent in the cells and liver of PPARα-null mice. Results indicated that GW501516 acts as an efficient PPARα activator in the mouse liver.

Keywords: Peroxisome proliferator-activated receptor (PPAR), GW501516, subtype specificity, lipid metabolism

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated transcription factors that play important roles in lipid and glucose metabolism. The distinct PPAR subtypes PPARα, PPARδ, and PPARγ have been identified. All PPARs bind as heterodimers with the retinoid X receptor (RXR) to well-conserved response elements (PPRE) (*1*). Their functions are believed to overlap but diverge when acting on endogenous genes in various tissues although their expression patterns and ligand specificities also overlap (*2*). Knowledge of the distinct physiological functions of PPARs is based mostly on experimental results obtained using subtype-specific ligands (*3*) and gene-knockout mice (*4*) or gene-knockdown cells (*5*).

GW501516 has been widely used as a specific PPARδ agonist and experimental results have helped broaden understanding of the role of PPARδ (*6,7*). However, the effects of treatment with GW501516 are not always absent in gene-knockout animals, and thus the *in vivo* subtype specificity of the compound has not been confirmed. A previous study of PPAR agonists using primary cultured hepatocytes from wild-type and PPARα-null mice revealed that the effect of GW501516 on target gene expression could not be detected when hepatocytes derived from PPARα-null mice were used. The current study investigated this unexpected finding and concluded that GW501516 acts as an efficient PPARα activator in the mouse liver.

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2. Materials and Methods

2.1. *Compound*

GW501516 was purchased from Calbiochem (San Diego, CA, USA) or synthesized at Nippon Chemiphar (Misato, Japan) and the two compounds produced the same results. Troglitazone was synthesized at Nippon Chemiphar (*8*). (4-Chloro-6-(2,3-xylidino)-2-pyrimidinyl-thio)acetic acid (Wy14,643) and 2-(*p*-chlorophenoxy)isobutyric acid ethyl ester (clofibrate) were purchased from Tokyo-Kasei (Tokyo, Japan). Bezafibrate and gemfibrozil were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. *Animal experiments*

All protocols were approved by the Meiji Pharmaceutical University Committee for Ethics of Experimentation and Animal Care. Male C57BL or PPARα-null mice around 8 weeks in age were used as described (*9*). Mice were fed a control diet or a diet containing 0.01% GW501516 or 0.05% Wy14,643 for 2 days. The mice were sacrificed at the end of each treatment period and total RNA was isolated.

2.3. *Primary culture of mouse hepatocytes*

Mouse hepatocytes were isolated by a two-step *in situ* collagenase perfusion procedure (*10*). The livers of C57BL or PPARα-null mice were first perfused with Krebs-Ringer bicarbonate buffer at 7.2 mL/min and then with the buffer containing 0.3 mg/mL collagenase type IV at

4.6 mL/min. Cells were released from the liver into 20 mL of MEM, filtered through a cell strainer, and purified by sedimentation twice at 50 × g for 1 min. Cells were then plated on collagen-coated 6-well plates at a density of 0.35 × 10^6 cells/well. After 3 h, the medium was replaced to remove non-adherent hepatocytes and the treatment was started.

2.4. *Cell culture and DNA transfection*

CV-1 cells were maintained in DMEM (GIBCO®; Invitrogen, Carlsbad, CA, USA) with 10% (v/v) fetal bovine serum (FBS) as described (*11*). The cells were transiently transfected with plasmid DNA (0.4 μg/well) using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and a reporter assay was performed as described (*11*).

2.5. *Isolation of RNA and real-time PCR analysis*

Total RNA from mouse tissue and primary hepatocytes was prepared using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA) as described (*9*). Reverse transcription was done with the PrimeScript® RT reagent Kit (Takara Bio, Kyoto, Japan). Real-time PCR was done with a LightCycler 1.5 instrument and SYBR premix ExTaq (Takara Bio) as directed by the manufacturer. The primers for real-time PCR of PPARα-target gene transcripts were as described (*12*) and L32 was used as an internal control (*12*).

3. Results and Discussion

To confirm that primary mouse hepatocytes were suitable for the PPAR agonist assay, the primary cultured hepatocytes were treated with several PPARα agonists and the levels of mRNA expressed by the endogenous PPARα-target genes were measured. Primary hepatocytes are known to maintain liver function and presumably these cells could be used to estimate the *in vivo* response of various agonists at various doses. Quantitative real-time PCR was used to measure the levels of hydratase-dehydrogenase bifunctional enzyme (HD) mRNA in control primary hepatocytes and primary hepatocytes treated with a PPARα agonist (Figure 1A). All of the PPAR-target genes examined, including the genes acyl-CoA oxidase (AOx) and liver-type fatty acid binding protein (L-FABP), responded to an extent to a wide variety of PPARα agonists in a dose-dependent manner (not shown). This response was much stronger in primary cultured mouse hepatocytes than in a standard transactivation assay system using CV-1 cells and the PPARα expression plasmid, in which no response to the weak agonist clofibrate was observed (Figure 1B).

In vitro studies suggest that GW501516 acts strictly as a PPARδ-specific agonist in a dose-dependent manner. To test this assumption, the response of wild-type primary hepatocytes to GW501516 was compared to the response in similar hepatocytes from PPARα-null mice. Using PPARα-null hepatocytes can exclude the possibility of the presence of significant residual activity of the receptor due to insufficient knockdown. Real-time PCR was used to measure changes in the mRNA levels of the most sensitive PPARα-target genes, HD (Figure 2A) and pyruvate dehydrogenase kinase 4 (PDK4) (Figure 2B) (*13*), in primary hepatocytes with various concentrations of PPAR agonists. No response to the PPARα agonist Wy14,643 was noted in PPARα-null hepatocytes and the response to the PPARγ agonist troglitazone was not significantly affected, as expected. In contrast, the dose-dependent response to GW501516 in hepatocytes was greatly reduced or eliminated by PPARα knock-out. The residual response to GW501516 in the PPARα-null hepatocytes was detectable only in the cells treated with the compound at concentrations much higher than those reported for specific activation of PPARδ. These results suggest that the increased levels of the mRNA of PPAR-target genes induced by treatment with GW501516 are achieved by activation of PPARα and that the remaining response was induced by activation of PPARδ or PPARγ or by some other unknown pathway.

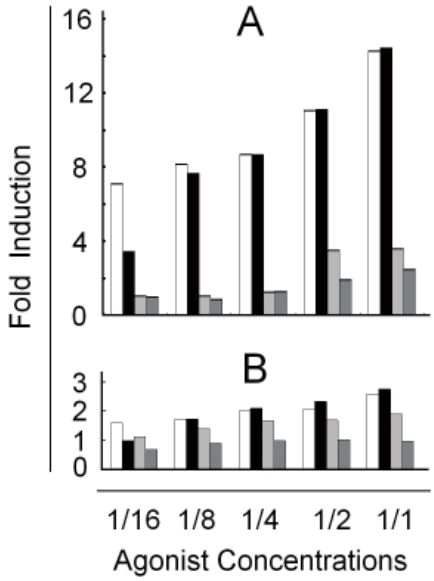

Figure 1. Cell-based PPAR ligand assay. PPARα agonist assays of various compounds were performed using the endogenous response in primary cultured hepatocytes from wild-type mice **(A)** or by reporter gene assay in cultured CV-1 cells **(B)**. **A)** Mouse primary hepatocytes were prepared and cultured with serial 2-fold dilutions of PPARα agonists for 18 h. The starting concentrations of the agonists were: Wy14,643 (open bar), 50 μM; bezafibrate (closed bar), 200 μM; gemfibrozil (lightly shaded bar), 200 μM; and clofibrate (darkly shaded bar), 200 μM. cDNA was synthesized from total RNA and the levels of mRNAs were quantified with real-time PCR. The levels of hydratase-dehydrogenase bifunctional enzyme (HD) mRNA are shown. Each agonist was assayed using independently isolated hepatocytes from at least three mice and a representative result is shown. **B)** A standard reporter gene assay was done with serial dilutions of the agonists in **A)**. Experiments were conducted at least three times and a representative result is shown. Experimental error was less than 30% in all cases.

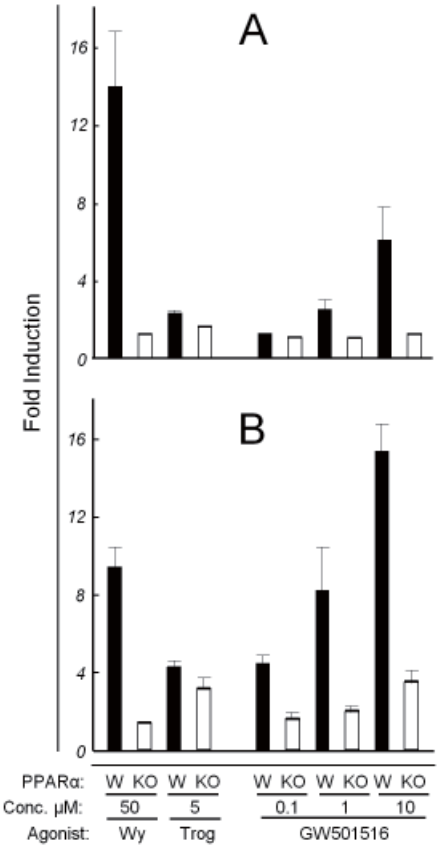

Figure 2. Effect of PPARα knockout. The effect of PPARα-knockout on the response of endogenous target genes in primary hepatocytes to the PPARδ agonist GW501516 was examined. Primary hepatocytes were prepared from wild-type and PPARα-null mice and cultured with various concentrations of GW501516 for 18 h. The PPARα agonist Wy14,643 (at 50 μM) and the PPARγ agonist troglitazone (5 μM) served as controls. cDNA was synthesized from total RNA and the levels of mRNAs of hydratase-dehydrogenase bifunctional enzyme (HD) **(A)** and pyruvate dehydrogenase kinase 4 (PDK4) **(B)** were measured with real-time PCR. Solid bars represent data from hepatocytes isolated from wild-type mice (W) and open bars represent the data from PPARα-null mice (KO). Data represent means (± S.D.) from 4 experiments.

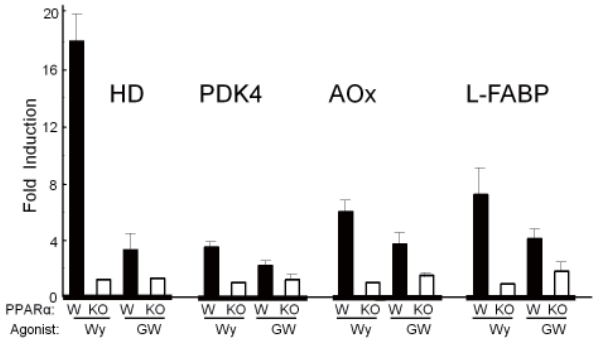

Figure 3. Response in the mouse liver. The effect of PPARα-knockout on the response of endogenous target genes to PPARδ agonist GW501516 in the mouse liver was examined. Wild-type (W) and PPARα-null mice (KO) were fed a control diet or a diet containing 0.01% GW501516 or 0.05% Wy14,643 (as the control) for 2 days. cDNA was synthesized from total RNA isolated from the liver and the levels of four PPAR-regulated mRNAs were measured with real-time PCR. Data represent means (± S.D.) from 3 experiments.

To examine whether the response of primary hepatocytes to PPAR agonists is a true reflection of *in vivo* response, wild-type and PPARα-null mice were fed a control diet or a diet containing the PPAR agonist GW501516 or Wy14,643. The levels of expression of mRNA of the target genes in the liver were then compared. As shown in Figure 3, the levels of HD, PDK4, AOx, and L-FABP mRNA increased with administration of GW501516 and Wy14,643 in the liver of wild-type mice but did not in the liver of PPARα-null mice, suggesting that the effect of GW501516 on the expression of PPAR-target genes in the liver was mostly due to activation of PPARα.

Most of the effects of GW501516 in the mouse liver that have been reported to activate lipid metabolism can be explained by its effects on PPARα instead of on PPARδ. If the subtype specificity of GW501516 is limited to PPARδ (*3*), the two nuclear receptors may play almost identical roles and there may be no physiological significance in having two overlapping receptors in the liver. This was the major impetus for the present study, which found that the effect of GW501516 on several PPAR target genes in primary hepatocytes and the liver depends on the expression of PPARα. The possibility that the effect of GW501516 on PPARα was overestimated because of the much lower level of expression of PPARδ compared to that of PPARα in the liver can be excluded given the results of a recent study by Girroir *et al.* (*14*). Using quantitative Western blotting, Girroir *et al.* found that liver tissue is one type of tissue that expresses the highest levels of PPARδ in mice. In addition, the doses of GW501516 used in the current study were lower than those suggested to be strictly specific to PPARδ. At the very least, the current doses were no higher than those used to specifically activate PPARδ in previously published studies (*5-7*). As the level of expression of PPARδ in the liver was not greatly affected by the absence of PPARα or by treatment with PPAR ligands (not shown), the present results strongly suggest that the effects of GW501516 in the liver are the result of activating PPARα. However, this does not necessarily exclude the possibility that activation of PPARα by GW501516 depends on PPARδ.

Activation of PPARα by GW501516 can be explained by two mechanisms: either by direct activation by binding of GW501516 to PPARα or by indirect activation through direct binding and activation of PPARδ. The first mechanism raises two important points; the *in vivo* subtype specificity of PPAR ligands might differ from that predicted by *in vitro* studies (*3*), and earlier experimental data using GW501516 as a PPARδ-specific ligand, and especially data from whole animal studies, may need to be re-interpreted. The subtype specificity of GW501516 for human PPARδ is reported to be > 500-fold higher than that for PPARα and PPARγ. This conclusion is based on the results

obtained using a cell-based transient transactivation assay and a binding assay using recombinant proteins. In such instances, only the ligand-binding domains (LBDs) of PPARs usually represent whole receptors. However, the LBD domains do not exist alone in the cell but interact with other domains of the receptors and with many other transcription factors, all of which affect the overall structures of the ligand-binding pockets (*15*). The results of GW501516 and PPAR-LBDs docking studies by I. Nakagome (Kitasato University, personal communication) suggest that the binding pocket of PPARα may be distorted or widened by such interactions for GW501516 to efficiently bind. Thus, the subtype specificity of a chemical compound for PPARs may differ somewhat between the subtype specificity according to an oversimplified assay system and that determined with an *in vivo* system. In a recent study of a physiologically relevant endogenous ligand for PPARα in the liver, Chakravarthy *et al*. reported a similar discrepancy in ligand specificity for *in vitro* estimations using LBD and the *in vivo* response of full-length receptors (*16*).

Indirect activation of PPARα by GW501516 could be achieved by increasing the level of expression of PPARα and its co-activators and/or by activating production of an endogenous ligand of PPARα. Since the level of PPARα expression in the mouse liver is high enough for a large response (*17*), increased production of an endogenous ligand may play a crucial role. Subsequent to the current study, Barroso *et al*. (*18*) suggested the possibility of a PPARδ-dependent effect of GW501516 on increased production of an endogenous PPARα ligand, 1-palmitoyl-2-oleoyl-phosphatidilcholine, although their suggestion is based solely on the assumption that GW501516 is a specific PPARδ ligand. They examined the long-term effect of GW501516 on high-fat diet-induced hyperglyceridemia and hepatic fatty acid oxidation in wild-type mice. Thus, increased production of an endogenous PPARα ligand was one of many changes observed, and evidence of a cause and effect relationship was not provided. PPARδ-null mice will need to be used to clarify the mechanism of PPARα activation by GW501516.

The results of the current study do not conflict with the position that PPARδ has an important physiological role in the liver. Shan *et al*. (*19*) showed that PPARδ protects against liver toxicity induced by environmental chemicals in PPARδ-null mice, so PPARδ could have a unique role in the liver. Sanderson *et al*. (*20*) suggested a unique function of PPARδ in the liver.

Preliminary results suggested that the response to GW501516 in skeletal muscle was only partially eliminated in PPARα-null mice. GW501516 appears to activate both PPARδ and PPARα in skeletal muscle. A primary cultured muscle cell system is needed for further analysis, but the effect of GW501516 is likely to differ somewhat between tissues.

References

1. Schoonjans K, Staels B, Auwerx J. The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. Biochim Biophy Acta. 1996; 1302:93-109.
2. Forman BM, Ruan B, Chen J, Schroepfer GJ Jr, Evans RM. The orphan nuclear receptor LXRalpha is positively and negatively regulated by distinct products of mevalonate metabolism. Proc Natl Acad Sci U S A. 1997; 94:10588-10593.
3. Oliver WR Jr, Shenk JL, Snaith MR, Russell CS, Plunket KD, Bodkin NL, Lewis MC, Winegar DA, Sznaidman ML, Lambert MH, Xu HE, Sternbach DD, Kliewer SA, Hansen BC, Willson TM. A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. Proc Natl Acad Sci U S A. 2001; 98:5306-5311.
4. Peters JM, Lee SS, Li W, Ward JM, Gavrilova O, Everett C, Reitman ML, Hudson LD, Gonzalez FJ. Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor beta(delta). Mol Cell Biol. 2000; 20:5119-5128.
5. Krämer DK, Al-Khalili L, Guigas B, Leng Y, Garcia-Roves PM, Krook A. Role of AMP kinase and PPARdelta in the regulation of lipid and glucose metabolism in human skeletal muscle. J Biol Chem. 2007; 282:19313-19320.
6. Wang YX, Lee CH, Tiep S, Yu RT, Ham J, Kang H, Evans RM. Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. Cell. 2003; 113:159-170.
7. Tanaka T, Yamamoto J, Iwasaki S, *et al*. Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle and attenuates metabolic syndrome. Proc Natl Acad Sc U S A. 2003; 100:15924-15929.
8. Fukui Y, Masui S, Osada S, Umesono K, Motojima K. A new thiazolidinedione, NC-2100, which is a weak PPAR-gamma activator, exhibits potent antidiabetic effects and induces uncoupling protein 1 in white adipose tissue of KKAy obese mice. Diabetes. 2000; 49:759-767.
9. Motojima K, Passilly P, Peters JM, Gonzalez FJ, Latruffe N. Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor alpha and gamma activators in a tissue- and inducer-specific manner. J Biol Chem. 1998; 273:16710-16714.
10. Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol. 1976; 13:29-83.
11. Sato O, Kuriki C, Fukui Y, Motojima K. Dual promoter structure of mouse and human fatty acid translocase/CD36 genes and unique transcriptional activation by peroxisome proliferator-activated receptor alpha and gamma ligands. J Biol Chem. 2002; 277:15703-15711.
12. Hirai T, Fukui Y, Motojima K. PPARalpha agonists positively and negatively regulate the expression of several nutrient/drug transporters in mouse small intestine. Biol Pharm Bull. 2007; 30:2185-2190.
13. Motojima K. A metabolic switching hypothesis for the first step in the hypolipidemic effects of fibrates. Biol Pharm Bull. 2002; 25:1509-1511.
14. Girroir EE, Hollingshead HE, He P, Zhu B, Perdew GH, Peters JM. Quantitative expression patterns of peroxisome proliferator-activated receptor-beta/delta

(PPARbeta/delta) protein in mice. Biochem Biophys Res Commun. 2008; 371:456-461.
15. Stanley TB, Leesnitzer LM, Montana VG, Galardi CM, Lambert MH, Holt JA, Xu HE, Moore LB, Blanchard SG, Stimmel JB. Subtype specific effects of peroxisome proliferator-activated receptor ligands on corepressor affinity. Biochemistry. 2003; 42:9278-9287.
16. Chakravarthy MV, Lodhi IJ, Yin L, Malapaka RR, Xu HE, Turk J, Semenkovich CF. Identification of a physiologically relevant endogenous ligand for PPARalpha in liver. Cell. 2009; 138:476-488.
17. Palmer CN, Hsu MH, Griffin KJ, Raucy JL, Johnson EF. Peroxisome proliferator activated receptor-alpha expression in human liver. Mol Phamacol. 1998; 53:14-22.
18. Barroso E, Rodriguez-Calvo R, Serrano-Marco L, Astudillo AM, Balsinde J, Palomer X, Vázquez-Carrera M. The PPARβ/δ activator GW501516 prevents the down-regulation of AMPK caused by a high-fat diet in liver and amplifies the PGC-1α-Lipin 1-PPARα pathway leading to increased fatty acid oxidation. Endocrinoligy. 2011; 152:1848-1859.
19. Shan W, Nicol CJ, Ito S, Bility MT, Kennett MJ, Ward JM, Gonzalez FJ, Peters JM. Peroxisome proliferator-activated receptor-beta/delta protects against chemically induced liver toxicity in mice. Hepatology. 2008; 47:225-235.
20. Sanderson LM, Degenhardt T, Koppen A, Kalkhoven E, Desvergne B, Müller M, Kersten S. Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) but not PPARalpha serves as a plasma free fatty acid sensor in liver. Mol Cell Biol. 2009; 29:6257-6267.

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