LUND UNIVERSITY

Conditional Expression of Human PPAR delta and a Dominant Negative Variant of hPPAR delta In Vivo

Higgins, Larry G.; Garbacz, Wojciech G.; Gustafsson, Mattias; Nainamalai, Sitheswaran; Ashby, Peter R.; Wolf, C. Roland; Palmer, Colin N. A.

Link to publication

Research Article

Conditional Expression of Human PPARδ and a Dominant Negative Variant of hPPARδ *In Vivo*

Larry G. Higgins,[1] Wojciech G. Garbacz,[1] Mattias C. U. Gustafsson,[2] Sitheswaran Nainamalai,[3] Peter R. Ashby,[1] C. Roland Wolf,[1] and Colin N. A. Palmer[1]

[1] *CRUK Molecular Pharmacology Unit, Medical Research Institute, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK*
[2] *Department of Laboratory Medicine, Division of Medical Microbiology, Lund University, Sölvegatan 23, SE-223 62 Lund, Sweden*
[3] *Division of Molecular Medicine, Medical Science Institute, College of Life Sciences, University of Dundee, Dundee DD1 4HN, UK*

Correspondence should be addressed to Colin N. A. Palmer, nuclear-receptor@dundee.ac.uk

The nuclear receptor, NR1C2 or peroxisome proliferator-activated receptor (PPAR)-δ, is ubiquitously expressed and important for placental development, fatty acid metabolism, wound healing, inflammation, and tumour development. PPARδ has been hypothesized to function as both a ligand activated transcription factor and a repressor of transcription in the absence of agonist. In this paper, treatment of mice conditionally expressing human PPARδ with GW501516 resulted in a marked loss in body weight that was not evident in nontransgenic animals or animals expressing a dominant negative derivative of PPARδ. Expression of either functional or dominant negative hPPARδ blocked bezafibrate-induced PPARα-dependent hepatomegaly and blocked the effect of bezafibrate on the transcription of PPARα target genes. These data demonstrate, for the first time, that PPARδ could inhibit the activation of PPARα *in vivo* and provide novel models for the investigation of the role of PPARδ in pathophysiology.

1. Introduction

There are three PPAR isoforms, PPARα, β/δ (herein referred to as δ), and γ. PPARα is found in tissues with a high rate of fatty acid catabolism where when agonist bound it activates the expression of genes involved in peroxisomal β-oxidation and mitochondrial β- and ω-oxidation and plays an important role in systemic fat catabolism, in the generation of ketone bodies and stimulating gluconeogenesis [1, 2]. Fibrate drugs cause PPARα-dependent liver peroxisome proliferation, hepatomegaly, and subsequently hepatocarcinoma when fed to rodents [2, 3]. Fibrate drugs are used successfully in the clinic to treat hyperlipidaemia and do not cause liver problems, most probably due to the lower level of PPARα in human liver [4, 5].

There are two forms of PPARγ, (γ1 and γ2, with differing amino termini). PPARγ is important for fatty acid and triglyceride anabolism and storage and essential for the differentiation of adipocytes [2].

PPARδ is the least known isoform in terms of biological function and the most abundant PPAR isoform in all tissues except liver and adipose tissue in rodents. PPARδ is downregulated in liver and kidney in response to fasting [6]. In all tissues examined, PPARδ protein was predominantly localised in the nucleus and could be immunoprecipitated with RXRα [7]. $ppar\delta^{-/-}$ mice have impaired placental function leading to viability problems [8–10]. Surviving knockouts were leaner and had reduced adipose stores, but this does not appear to be a direct effect of PPARδ, since adipose-specific $ppar\delta^{-/-}$ mice did not show the same phenotype [8]. PPARδ knockouts also showed increased keratinocyte proliferation in response to topical application of tetradecanoylphorbol acetate [9, 10].

PPARδ has been shown to play an important role in cellular differentiation [11–13] and in regulating energy homeostasis [8, 14]. Mice generated with PPARδ constitutively active in adipocytes were found to be lean, and leptin receptor mutant mice showed improved metabolic activity

and reduced fat deposits when fed GW501516 (a PPARδ-specific agonist) [15]. Muscle-specific PPARδ overexpression resulted in a net increase of muscle fibres with an oxidative metabolic phenotype with a concomitant decrease in body fat [16]. Comparably, mice with a cardio-restricted deletion of PPARδ showed decreased basal myocardial fatty acid oxidation followed by chronic lipid accumulation in the heart that led to cardiac hypertrophy and congestive heart failure [17].

The involvement of PPARδ in cancer promotion in various organs is clear; however, there is controversy about whether ligand activation of PPARδ is pro- or anti-tumourigenic. Following initiation, increased breast tumour development was evident in animals fed with GW501516 [18]. In animal models of colorectal cancer, PPARδ has been shown to both inhibit and promote the growth of intestinal polyps [8, 19, 20].

One study has shown that PPARδ was able to attract transcriptional corepressors and when bound to DNA in the absence of a PPARδ agonist more effectively than PPARα or PPARγ [21]. On the other hand, treatment of wild-type adipocytes with troglitazone, a potent PPARγ ligand, caused upregulation in PPARγ activity as measured by adipocyte differentiation and lipid accumulation assays. This effect was also almost entirely preserved in PPARδ-null adipocytes [22]; however, in another study, authors conclude that PPARδ suppressed PPARγ activity, but downregulation of PPARδ expression did not increase PPARγ expression levels [23]. A different study has shown that PPARδ is able to repress both PPARα and γ-dependent gene expression in $vitro$ [24]. Since PPARδ is the predominant PPAR isoform in many tissues [6], it was proposed that PPARδ could act as a PPRE gateway receptor [24]. However, deletion of the carboxyterminal exon of PPARδ did not result in increased expression of PPARα target genes in liver [25].

With confusing and opposing results being observed using knockout and drug models, we decided to take an alternative route to provide genetic models for both gain and loss of function of PPARδ. We show that we can control expression of hPPARδ and hPPAR$\delta\Delta$AF2 (a dominant negative derivative lacking the 11 carboxy-terminal aminoacid residues [26, 27]) in transgenic animals and that we can modulate PPAR-dependent gene expression in liver as well as the hepatomegaly associated with activation of PPARα. These new mouse models for studying PPARδ biology should thus be useful in resolving some of the uncertainties regarding this receptor.

2. Results

2.1. Conditional Expression of hPPARδ and hPPAR$\delta\Delta$AF2 in Transgenic Mice. Two mouse lines were generated that conditionally express human PPARδ and a dominant negative derivative thereof, hPPAR$\delta\Delta$AF2 (Figure 1). This approach allows for the manipulation of the levels of PPRE signaling through the conditional production of human PPARδ and a dominant negative derivative, hPPARδAF2, in transgenic mice. The $Cyp1a1$ gene is tightly regulated in $vivo$ and its expression is wholly dependent on the arylhydrocarbon

receptor (AhR), functioning as a heterodimer with the AhR nuclear translocator, Arnt, that binds the xenobiotic response element enhancer sequence in the regulatory 5′ UTR of the $Cyp1a1$ gene. The promoter of the rat $Cyp1a1$ gene has been used to conditionally express several genes in transgenic models [28–30]. The expression of genes under the control of the $Cyp1a1$ promoter is achieved by the administering of compounds that activate XRE-driven gene expression.

I3C, found in cruciferous vegetables, is converted to polyaromatic indolic compounds in the acid environment of the stomach and produces a potent and dose-dependent activation of XRE driven gene expression [31]. Feeding transgenic mice with a diet supplemented with I3C (0.5% (w/w)) or (0.25% (w/w)) resulted in a similar induction of both hPPARδ and hPPAR$\delta\Delta$AF2 transgenes in these animals (data not shown). Based on this result, we fed nontransgenic, hPPARδ and hPPAR$\delta\Delta$AF2 transgenic mice either on control diet or a diet supplemented with I3C (0.25% (w/w)) for 5 days and examined transgene expression in a range of organs (Figure 2). hPPARδ mRNA from livers of mice fed control diet was virtually undetectable, but hPPARδ message increased approximately 30,000-fold upon feeding I3C (0.25% (w/w)) for 5 days (Figure 2(a)). By contrast, hPPAR$\delta\Delta$AF2 animals on control diet had approximately 10-fold greater basal expression of the transgene than did the hPPARδ mice on the same diet. However, when these animals were fed on a diet containing I3C, the hPPAR$\delta\Delta$AF2 transgene expression in the liver increased (2900-fold) to levels similar to that of animals expressing the hPPARδ transgene (Figure 2(a)). Although I3C induced mRNA levels of both the hPPARδ and hPPAR$\delta\Delta$AF2 transgenes to similar levels in these mice, the corresponding protein expression in the livers of these mice differed. Using a monoclonal antibody raised against hPPARδ, animals expressing the hPPARδ transgene showed a robust increase in hPPARδ protein in their livers when fed on an I3C-supplemented diet compared to those on control diet (Figure 2(b)). When liver hPPAR$\delta\Delta$AF2 protein was examined in mice expressing this transgene, there was found to be a much less robust increase in protein expression (Figure 2(b), see arrow). It is possible that hPPAR$\delta\Delta$AF2-truncated protein may be less stable in the livers of these animals than the human full length form.

Low basal expression of both hPPARδ and hPPAR$\delta\Delta$AF2 mRNA was also observed in the adipose, brain, kidney, large intestine, muscle, ovary, and testis of animals on control diet with no significant induction of message by I3C observed in the brain, adipose, breast, heart, kidney, stomach, spleen, lung, muscle, ovary, or testis of mice harbouring either transgene. The large intestine showed the largest induction of both transgenes in response to I3C treatment. A greater basal expression of hPPARδ transcript was seen in breast, heart, small intestine, and stomach. These organs showed an increase in hPPARδ expression upon feeding animals with an I3C-supplemented diet, but this increase was significant only in small intestine, $P = 0.0007$ and $P = 0.005$ for hPPARδ and hPPAR$\delta\Delta$AF2, respectively. Lung and muscle showed a very different pattern of expression from the other tissues in that basal expression of hPPARδ was low and not inducible by I3C dietary supplementation. In lung, the
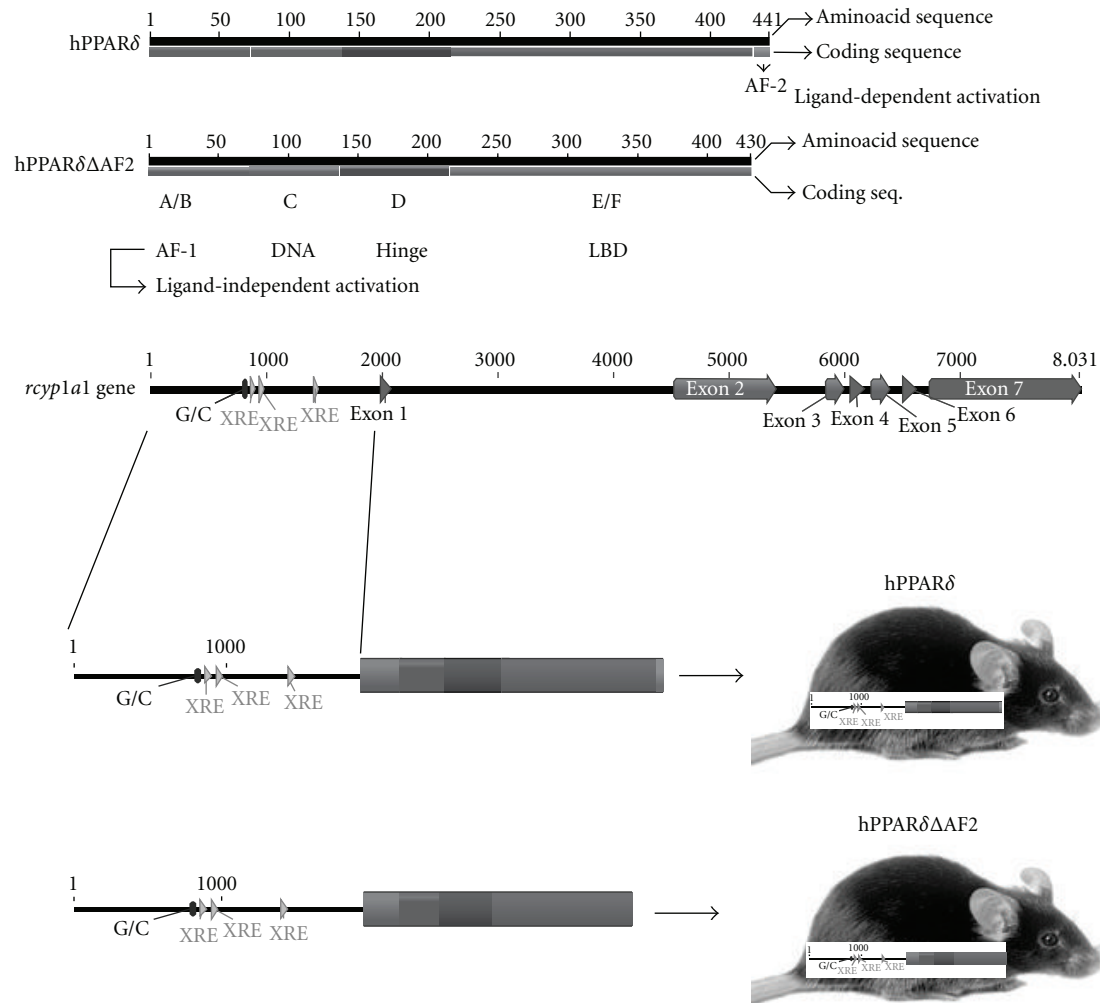
FIGURE 1: Graphic depicting the generation of mice conditionally expressing hPPARδ and hPPARδΔAF2 transgenes. Mice harbouring transgenes, encoding the coding sequence of hPPARδ or hPPARδΔAF2 fused to the rat *Cyp1a1* gene promoter and offering tight control of XRE enhancer driven transgene expression.

basal expression of hPPARδΔAF2 was high and induction by I3C observed but not significant. In muscle, however, basal expression of hPPARδΔAF2 was low but induced by I3C in the diet, $P = 0.033$. hPPARδ is known to be expressed in the sebaceous gland of the skin, and is induced upon feeding I3C in keratinocytes of hPPARδ transgenic mice [32].

2.2. Induction and Activation of hPPARδ Causes Body Weight Loss. PPARδ activation is known to produce favorable metabolic effects that include weight loss with increased metabolic rate in skeletal muscle, improved exercise endurance and insulin sensitivity [15, 33]. To investigate the role of activating hPPARδ and hPPARδΔAF2 on body weight, mice were fed either on normal diet supplemented with I3C (0.25% (w/w)) (I3C diet) or on an I3C diet supplemented with GW501516 (0.005% (w/w)) for 14 days. There was no difference in the weights of either nontransgenic, hPPARδ or hPPARδΔAF2 transgenic mice fed on I3C diet (Table 1 and Figure 3(a)). In nontransgenic mice, there was a small

although significant reduction in body weight when mice were given I3C diet containing GW501516 likely as a response of endogenous PPARδ activation by the ligand (Table 1 and Figure 3(a)). In animals carrying hPPARδ placed on the GW501516 diet, there was a marked reduction in body weight (direct weight difference ($P = 0.017$) and percentage of body weight ($P = 0.008$)) over the two-week period that was completely absent in nontransgenic animals or animals expressing hPPARδΔAF2 (Table 1 and Figure 3(a)).

2.3. GW501516-Dependent Activation of Gene Expression Is Abolished, or Reversed, by hPPARδΔAF2. hPPARδΔAF2 has been shown to act in a dominant negative fashion *in vitro*. This effect was augmented by the addition of a PPARδ agonist [26]. To examine the effect of conditionally expressing hPPARδΔAF2 on PPARδ target gene expression, animals were fed on a diet containing either I3C (0.25% (w/w)) only or on a diet containing I3C and GW501516
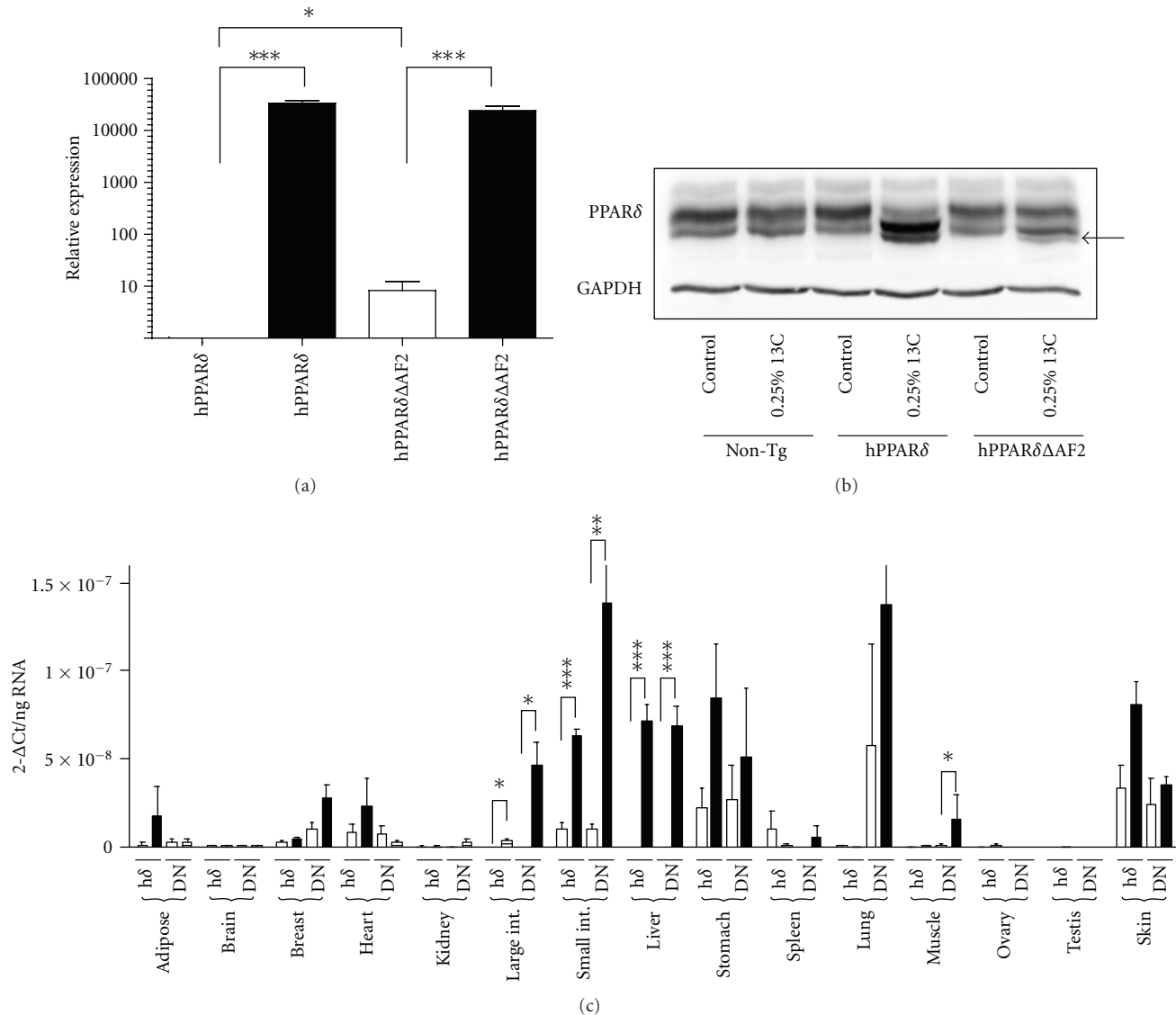
(a)

(b)

(c)

Figure 2: Mice conditionally express hPPARδ or hPPARδΔAF2 transgenes in response to feeding I3C. Animals ($n = 3$ per group, 10 weeks old, mixed male and female) were placed either on control diet or a diet containing I3C (0.25% (w/w)) for 5 days. (a) hPPARδ and hPPARδΔAF2 exhibit low basal mRNA expression (open bars) and highly inducible I3C-dependent expression (black bars) in mouse liver. (b) hPPARδ and hPPARδΔAF2 protein is expressed in livers in response to dietary supplementation with I3C. (c) hPPARδ (hδ) and hPPARδΔAF2 (DN) mRNA expression in various mouse organs in response to normal diet (open bars) and I3C-supplemented diet (black bars). Statistical significance where indicated was analysed using Mann-Whitney nonparametric test in GraphPad Prism version 5.0c, (GraphPad Software, San Diego, CA, USA). Significance is portrayed as ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$).

(0.25% (w/w) and 0.0025% (w/w), resp.) for 2 weeks prior to liver and muscle tissue being harvested and analyzed for relative mRNA expression levels of *Acox1*, *Adrp*, and mouse PPARδ (Figures 3(b)–3(f)).

Acox1, a known PPARδ target encoding the first enzyme of the fatty acid β-oxidation pathway, was upregulated in muscle ($P = 0.016$) but not in liver of nontransgenic animals fed a diet containing GW501516 (Figures 3(b) and 3(c)). This muscle upregulation of *Acox1* is most likely attributable to activation of endogenous mPPARδ. We found, however, that the hPPARδ transgene is required for up-regulation of *Acox1* mRNA in liver ($P = 0.036$), indicating the presence

of a functional hPPARδ protein. Up-regulation of muscle *Acox 1* in response to GW501516 was also observed in hPPARδ transgenic animals ($P = 0.015$) (Figure 3(c)). hPPARδ, however, is not likely to be involved in this response as expression of this transgene was not seen in muscle of hPPARδ-transgenic mice when administered I3C in the diet (Figure 2(c)), and a similar level of induction of *Acox1* was seen in nontransgenic mice (Figure 3(c)). In contrast, hPPARδΔAF2 was shown to be inducible in both liver and muscle of hPPARδΔAF2 transgenic mice by I3C (Figure 2(c)). The GW501516-dependent up-regulation of *Acox1* in both liver and muscle was completely inhibited in

(a)

(b)

(c)

(d)

(e)

(f)
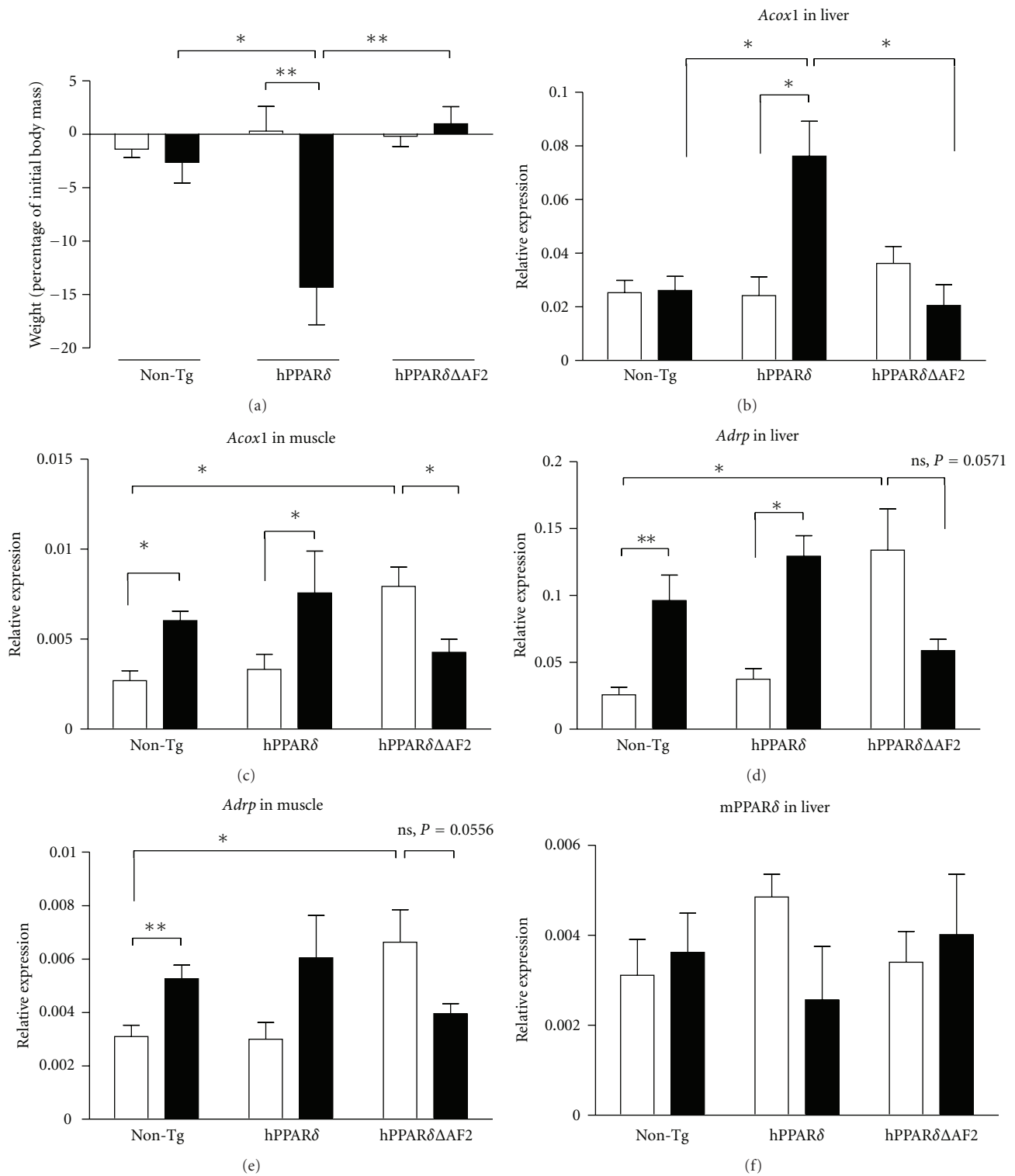
FIGURE 3: Mice expressing functional hPPARδ and hPPARδΔAF2 protein. Animals ($n = 5$ per group, 12 weeks old, mixed males and females) were placed on a diet containing I3C (0.25% (w/w)) (open bars) or a diet containing I3C (0.25% (w/w)) and GW501516 (0.0025% (w/w)) (black bars) for 14 days. (a) body weight of nontransgenic (Non-Tg), hPPARδ and hPPARδΔAF2 transgenic mice expressed as a percentage of initial body weight. (b–f) mRNA expression of *Acox1* in liver (b), muscle (c), mRNA expression of *Adrp* in liver (d), muscle (e), and mouse endogenous PPARδ (f) following treatments outlined above. Statistical significance ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$) where indicated was analysed using Mann-Whitney non-parametric test in GraphPad Prism version 5.0c, (GraphPad Software, San Diego, CA, USA).

TABLE 1: Effect of GW501516 treatment on body weight (g) and hepatic triglycerides (mg/g of liver) in nontransgenic, hPPARδ and hPPARδΔAF2 mice.

Genotype	Treatment	Number of mice	Starting weight (g)[a]	Ultimate weight (g)[a]	P value[b]	Hepatic trigs (mg/g)	P value
Non-Tg	Control	5	24.35 ± 0.44	24.03 ± 0.53	0.1592	9.68 ± 1.18	
hPPARδ	Control	5	22.27 ± 0.98	22.15 ± 1.27	0.972	15.73 ± 3.62	ns 0.152[c]
hPARδΔAF2	Control	5	22.02 ± 1.03	21.96 ± 1.18	0.8675	52.98 ± 6.06	***0.0001[c]
Non-Tg	GW	5	21.36 ± 0.44	20.77 ± 0.53	0.1962	22.66 ± 2.31	**0.0011[d]
hPPARδ	GW	5	21.78 ± 0.98	18.4 ± 1.27	*0.0179	47.53 ± 6.24	**0.0024[d]
hPARδΔAF2	GW	5	21.97 ± 1.03	22.14 ± 1.18	0.5767	25.66 ± 2.16	**0.0023[d]

Values are the means ± S.E.M.
[a]Values adjusted for sex.
[b]P value of weight difference (ultimate body weight minus initial body weight; paired t-test).
[c]P value of difference between level of hepatic triglycerides: non-Tg control versus transgenics control (unpaired t-test).
[d]P value of difference between level of hepatic triglycerides: control versus treated groups (unpaired t-test).

the hPPARδΔAF2 transgenic mice (Figures 3(b) and 3(c)). Interestingly, *Acox1* expression was significantly increased in mice expressing hPPARδΔAF2 compared to nontransgenic mice in the absence of the PPARδ agonist. The increase in *Acox1* expression was lowered in the presence of PPARδ agonist (Figure 3(c)), indicating agonist-induced repression by hPPARδΔAF2 as described by Gustafsson et al. [26].

Adrp another PPAR target gene encodes a protein that coats lipid droplets and is expressed during lipid accumulation. Treatment of mice with GW501516 for 2 weeks leads to up-regulation of *Adrp* mRNA in both liver and muscle of nontransgenic mice ($P = 0.008$ for both organs). Again this is most likely due to GW501516 activation of endogenous mouse PPARδ (Figures 3(d) and 3(e)). In transgenic mice expressing hPPARδ, there was an up-regulation of *Adrp* by GW501516 in liver ($P = 0.036$) resulting in a 3.4-fold activation. Nontransgenic animals displayed an identical (3.4-fold) activation of *Adrp by GW501516* in their livers (Figure 3(d)). Levels of *Adrp* were also increased by GW501516 in the muscle of these animals but did not reach significance ($P = 0.151$) (Figure 3(e)). Thus, the extent of basal and GW501516-induced expression of *Adrp* in liver and muscle do not seem to be affected by hPPARδ transgene. Interestingly, basal levels of *Adrp* were significantly higher in mice expressing hPPARδΔAF2 compared to either nontransgenic or hPPARδ transgenic counterparts on I3C diet ($P = 0.016$ for both organs) (Figures 3(d) and 3(e)). The ability of hPPARδΔAF2 to repress expression of *Adrp* mRNA in the presence of GW501516 was evident in both liver and muscle of these animals with *Adrp* mRNA levels approaching the basal levels in nontransgenic and hPPARδ transgenic animals on I3C diet (Figure 3). This is most likely the result of ligand-augmented repression by hPPARδΔAF2 described by us previously [26]. Endogenous mouse PPARδ mRNA levels showed no change in expression pattern between treatment or transgene groups (Figure 3(f)).

Treatment of hPPARδ mice with GW501516 led to a greater than 200% increase in the accumulation of hepatic triglycerides when compared to untreated hPPARδ animal fed on control diet ($P = 0.0024$; Table 1).

Surprisingly, hPPARδΔAF2 animals fed on control diet had liver triglycerides levels comparable to the hPPARδ mice

fed the ligand-supplemented diet. However, hPPARδΔAF2 animals treated with GW501516 had a lower level of hepatic triglycerides (−108%), when compared to hPPARδΔAF2 fed on control diet ($P = 0.0023$; Table 1). Nontransgenic animals fed a diet supplemented with GW501516 displayed an increased hepatic triglyceride levels (122%) when compared to nontransgenic animals on control diet ($P = 0.0011$; Table 1).

2.4. Expression of PPARδ and PPARδΔAF2 Modulate Bezafibrate-Induced Hepatomegaly.

Bezafibrate has been shown to act as a dual PPARα/PPARδ agonist in mice and to cause hepatomegaly when administered in the diet at 0.5% (w/w). This response is completely absent in PPARα knockout mice [25]. To investigate whether or not PPARδ or PPARδΔAF2 expression could modulate PPARα-dependent hepatomegaly in mice fed with bezafibrate, mice were placed on a diet containing I3C (0.5% (w/w)) for 10 days to induce PPARδ transgene expression. Following this, GW501516 (0.005% (w/w)), a PPARδ-specific ligand, or the PPARα/δ dual ligand, bezafibrate (0.5% (w/w), was introduced to the diet containing I3C (0.5% (w/w)) for a further 10 days. Subsequently animals were sacrificed and liver weights recorded. Nontransgenic mice showed a decrease in liver weight ($P = 0.032$) when fed on GW501516-supplemented diet (Figure 4(a)). In contrast, nontransgenic animals fed bezafibrate supplemented diet displayed an increase in liver weight ($P = 0.003$) (Figure 4(a)). This finding is in agreement with previously published data [25]. Animals expressing hPPARδ or hPPARδΔAF2 showed no change in liver weight when fed with either bezafibrate or GW501516 containing diets (Figure 4(a)).

2.5. PPARδ and PPARδΔAF2 Modulate Bezafibrate Regulation of Gene Expression in the Liver.

To investigate whether or not the ability of hPPARδ and hPPARδΔAF2 to alleviate bezafibrate-induced hepatomegaly in these mice was associated with a repression of bezafibrate-induced gene expression by the hPPARδ and hPPARδΔAF2 transgenes, we examined mRNA expression from the livers of mice treated with bezafibrate or GW501516. The level of *Acox1* in nontransgenic mouse liver was strongly induced by bezafibrate but not by
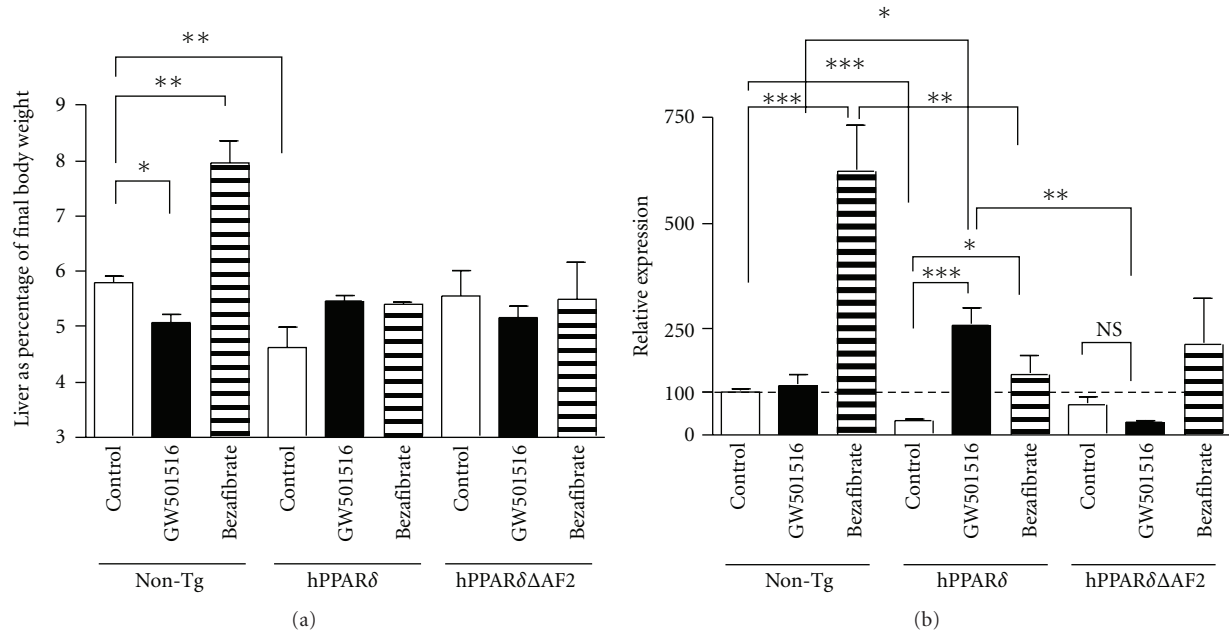
(a)

(b)

FIGURE 4: Expression of hPPARδ and hPPARδΔAF2 abolish bezafibrate-induced hepatomegaly in liver. Animals (n = 2–4 animals per group) were fed a diet containing I3C (0.5% (w/w)) for 10 days prior to being placed on diets containing I3C (0.5% (w/w)) (Control diet; open bars), or a diet containing I3C (0.5% (w/w)) and GW501516 (0.005% (w/w)) (GW501516 diet; black bars) or a diet containing I3C (0.5% (w/w)) and bezafibrate (0.5% (w/w)) (bezafibrate diet; striped bars) for a further 10 days. (a) Liver weights of animals are expressed as a percentage of body weight. (b) Liver *Acox1* mRNA expression. Graphs were analysed using the Mann-Whitney non-parametric test in GraphPad Prism version 5.0c, GraphPad Software, San Diego, California, USA. Significance is indicated as (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

GW501516 (Figure 4(b)). In contrast, hPPARδ transgenic mice displayed decreased constitutive levels of *Acox1* (P = 0.003), but retained inducibility with both GW501516 and bezafibrate (P = 0.001 and P = 0.011, resp.). Taken together, these observations suggest, that hPPARδ represses basal expression of *Acox1*, and that GW501516 activated-hPPARδ induces expression of *Acox1* in the livers of these animals (Figure 4(b)). The level of *Acox1* expression achieved by bezafibrate in the hPPARδ transgenic mice was much lower than that achieved in the nontransgenic animals (P = 0.0095). There were no significant changes in the liver expression levels of *Acox1* in the hPPARδΔAF2 animals in response to either GW501516 or bezafibrate (Figure 4(b)). Of note, even though the change was not statistically significant, we observed a trend to a ligand-dependent repression of PPARδ target gene expression in hPPARδΔAF2 animals similar to that observed in *in vitro* cellular assays [26] and in (Figures 3(b)–3(e) and Figure 4(b)). These observations provide further evidence for the dominant negative role of the hPPARδΔAF2 transgene in regulating both GW501516 (PPARδ specific agonist) and bezafibrate (PPARα/δ dual agonist) gene induction *in vivo* (Figure 4(b)).

3. Discussion

Attempts to resolve PPARδ biology have proven difficult with several groups reporting contradictory results obtained from both genetic and pharmaceutical modulation of PPARδ activity [8, 19, 20]. We believe that the interpretation of PPARδ experimentation results is confounded by the

ability of PPARδ to function as a repressor and activator of transcription depending on ligand binding status. We hypothesised that a more subtle modification of PPARδ *in vivo* would provide a useful tool to help delineate the biology of this nuclear receptor. To that end, we have in this study described two transgenic mouse models that conditionally expresses either human PPARδ or a dominant negative derivative of hPPARδ lacking the carboxy terminal 11 aminoacids comprising the activation function 2 domain of the protein. Both transgenes are functional, and human *PPARδ* mRNA is detectable in a range of tissues and protein expression is evident in the liver (Figure 2). In addition, these transgenic animals are refractory to bezafibrate-induced hepatomegaly (Figure 4) indicating that these mice are synthesising functional human PPARδ protein *in vivo*. In other model of humanized mice generated by Gross et al., the endogenous murine PPARδ was replaced with human PPARδ. Gene expression profiling in liver, soleus muscle, and macrophages showed similar gene patterns regulated by mouse and human PPARδ. In terms of regulation of lipid metabolism and inflammation, authors indicate that human PPARδ can compensate for mouse PPARδ [34].

The regulation of the rat *Cyp1a1* gene is well characterized and tightly controlled, and its promoter has been used for the conditional expression of genes in transgenic animals [28–30]. Our results demonstrate that expression of hPPARδ and hPPARδΔAF2 is highly inducible in the liver and intestine, but not in all tissues examined. For example, the stomach displayed a high basal expression of both PPARδ transgenes that was not significantly induced by feeding I3C.

In skin there was no significant induction of mRNA for either transgene. However, administration of dietary GW501516 to hPPARδ mice results in skin thickening with psoriasis-like lesions. In the skin of hPPARδ mice, basal hPPARδ protein is detected in the sebaceous gland. When exposed to I3C, these animals show inducible *PPARδ* mRNA expression in keratinocytes [32].

When the liver was examined for hPPARδ and hPPARδΔAF2 protein expression, mRNA and protein levels did not correlate well. In hPPARδ transgenic animals, there was a clear hPPARδ protein signal that was not substantially elevated above endogenous mouse PPARδ. In contrast, hPPARδΔAF2 protein expression was very weak despite a robust I3C-dependent increase in mRNA expression (Figure 2). Notwithstanding this, the hPPARδΔAF2 protein was clearly functional. In animals expressing hPPARδΔAF2, there was a complete inhibition of PPARδ-mediated induction of *Acox1* or *Adrp* in the liver and muscle in the presence of the PPARδ agonist GW501516 (Figures 3(c), 3(d), and 3(e)).

One noticeable observation is the higher basal levels of both *Acox1* in muscle and *Adrp* in liver and muscle of animals expressing hPPARδΔAF2 protein without an added PPARδ agonist in the diet. This may be as a result of the nonliganded dominant negative protein relieving endogenous mouse PPARδ-dependent repression of these genes as suggested by previous studies, particularly by the sequestration of RXR species from PPARδ-RXR heterodimers leading to PPRE being occupied by DR1 binding positive regulators that do not require RXR such as HNF4 or COUP-TFII [35–37]. Further work is required to confirm this at the genomic level.

In contrast to nontransgenic mice, the levels of *Acox1* and *Adrp* were actually reduced in the presence of PPARδ agonist in the hPPARδΔAF2 mice when compared to the levels in animals not receiving GW501516. This is in line with our previous observations where *in vitro* transfection experiments suggested that the hPPARδΔAF2-mediated repression is enhanced by a PPARδ agonist (most probably due to agonist enhanced hPPARδΔAF2/RXR heterodimerisation and thus increased PPRE affinity) [26]. This complex relationship between ligand-activated and dominant negative PPARδ in the control of gene expression reflects the findings of recent genomewide expression and chromatin immunoprecipitation studies by Adhikary et al., (2011), where differing modes of target gene regulation by PPARδ have been defined. In these studies, PPARδ was shown to elicit 3 differing transcriptional responses; (a) type 1 response: genes that were up-regulated by siRNA knock down of PPARδ, but were not induced by GW501516, (b) type II response: genes that were up-regulated by knock down of PPARδ and could be up-regulated by GW501516, and (c) type III response: genes that are downregulated by PPARδ siRNA that then showed either no response or a weak induction by GW501516 [38]. Ability to repress the gene expression by PPARδ was also shown in a study carried out by Kino et al.. In this work, overexpression of PPARδ enhanced the suppressive effect of GW501516 on transcriptional regulation of Interleukin-6 [39]. These observations support the complex interaction between activation and expression of PPARδ and the regulation of specific gene targets.

Ligand-activated hPPARδ did not increase mRNA levels of *Acox1 Adrp* in muscle, or *Adrp* in liver over and above that seen by endogenous ligand-activated mPPARδ (Figure 3). In liver, hPPARδ mRNA and protein was up-regulated by feeding I3C in the diet. In the case of liver, this resulted in a ligand-dependent hPPARδ-specific activation of *Acox1*. *Acox1* was not induced by GW501516 alone in nontransgenic livers, suggesting that endogenous mPPARδ does not affect *Acox1* expression in the liver of these animals but does influence *Acox1* expression in the muscle (Figures 3(b) and 3(c)). *Adrp* expression, on the other hand, is activated by ligand-activated endogenous mouse PPARδ in both liver and muscle, but not modulated by ligand-activated conditionally-expressed human PPARδ in either of these organs. These results may highlight species differences in PPARδ and how it modulates gene expression differently depending on its environment. Taken in context of the work carried out by Adhikary et al. [38], PPARδ elicits a type II transcriptional response in regulating *Acox1* in mouse muscle and *Adrp* in liver and muscle, but mouse PPARδ does not regulate *Acox1* in mouse liver. Interestingly Adhikary et al. note that "the magnitude of induction by ligand approaches the effect of PPARδ depletion for individual genes showing a type II response." This is mirrored exactly in this model system. The induction (or relief of repression) of muscle *Acox1* and liver and muscle *Adrp* mRNA observed in the presence of hPPARδΔAF2 has a magnitude identical to the induction observed in the presence of GW501516 (Figures 3(c), 3(d), and 3(e)). Another striking observation in removing the AF2 domain of PPARδ (PPARδΔAF2) results in certain target genes being transcriptionally activated a similar manner to treatment of wild-type mice by the PPARδ ligand GW501516. The opposite is also true, hPPARδΔAF2 activated by GW501516 results in gene expression levels identical to those seen in hPPARδ transgenic animals in the absence of GW501516 (Figures 3(c), 3(d), and 3(e)). There is no evidence that hepatic levels of endogenous mouse PPARδ mRNA are being affected by transgene induction or GW501516 treatment (Figure 3(f)). PPARδ is known to be induced by exercise, fasting, and other factors rather than by self-activation [40].

PPARδ has a role in energy homeostasis as a key regulator of fatty acid oxidation, is expressed in skeletal muscle, with a higher expression in soleus muscle. Soleus muscle consists of fatigue resistant type 1 muscle fibres that have a high mitochondria content and use oxidative metabolism for energy production. Activation of PPARδ is known to promote weight loss [15, 33] that was particularly evident when hPPARδ animals were fed a diet containing GW501516 (Figure 3). Interestingly, the increased weight loss seen in the mice expressing hPPARδ cannot be a strictly muscle effect in these mice, as hPPARδ was not induced in muscle of these animals (Figure 2(c)).

So far, role of PPARδ in promoting or preventing hepatic steatosis is an open question [14, 41]. In nontransgenic mice, 2 weeks of PPARδ ligand treatment caused only moderate (2-fold) fluctuations in liver triglycerides (Table 1),

whereas in human PPARδ transgenic mice, two weeks of GW501516 treatment was sufficient to cause a significant accumulation of hepatic triglycerides (Table 1). A higher level of triglycerides was also found in livers of hPPAR$\delta\Delta$AF2 animals on control diet, but not in hPPAR$\delta\Delta$AF2 mice fed with a GW501516-supplemented diet. This role of the dominant negative enabling a "reverse agonism" action of GW501516 was first seen in our *in vitro* work, and it is intriguing that it is seen so clearly with the hepatic lipid accumulation as well as the target gene expression. This mechanism of this regulation of hepatic triglyceride accumulation is not clear and requires further investigation.

It is not unusual for PPARs to behave differently across species. PPARα is employed as a successful target for pharmaceutical intervention in humans with fibrate drugs being important in treatment of dyslipidemia and cardiovascular disease [42]. In rodents, however, fibrate drugs cause hepatomegaly and eventually hepatic carcinoma [2], which is not seen in humans, and is reflected in a much lower level of expression of PPARα and thus a different balance between PPARα and PPARδ in the human liver [4]. This interaction between PPARα and PPARδ levels in the regulation of hepatomegaly was borne out by our observation that bezafibrate-induced hepatomegaly was blocked by hPPAR$\delta\Delta$AF2. In addition, expression of hPPARδ blocked bezafibrate-dependent hepatomegaly and modulated bezafibrate-dependent gene expression. Taken together, this data demonstrates that unliganded PPARδ can, in certain circumstances, inhibit the action of PPARα.

In summary, there have been many conflicting and confusing studies attempting to understand PPARδ biology *in vivo*, which are complicated by PPARδ being able to both repress and activate gene expression. Genetic models of deleting *PPARδ* have been very insightful for elucidating the biology of this nuclear receptor. By conditionally expressing hPPARδ and hPPAR$\delta\Delta$AF2 in an otherwise normal endogenous mouse PPARδ background, we sought to generate animal models that would allow a more subtle examination of PPARδ biology without the complication of removing or inactivating a protein with a dual function. These animals will provide a useful tool to complement studies with knockout and drug models to help resolve the confusing results in the literature regarding PPARδ biology.

4. Materials and Methods

4.1. Reagents. All chemicals used were of the highest grade available and were, unless otherwise stated, purchased from Sigma/Aldrich, Gillingham, Dorset, UK. GW501516 was synthesised by AF ChemPharm Ltd., Sheffield, UK.

4.2. Transgenic Mouse Generation. Throughout this study all animals were treated in accordance with regulations contained in the Animals and Scientific Procedures Act (1996) of the United Kingdom, and with the approval of the University of Dundee ethical committee. Mice were housed in an environment with a temperature range of 19–23°C, under 12 hour light/dark cycles and given free access to food and water.

The animals were fed with RM1 laboratory animal feed (SDS Ltd., Wickam, UK).

The generation of mice expressing hPPARδ is described elsewhere [32]. To generate mice conditionally over-expressing a derivative of hPPARδ lacking the eleven carboxy-terminal aminoacids residues (hPPAR$\delta\Delta$AF2) [26, 27], the coding sequence of hPPARδ was amplified using primer PRMG15 (5′-CTAG<u>TCTAGA</u>**ATG**GAGCAGCCACAGGAG-GAAGC-3′) and PRMG16 (5′-CTAG<u>TCTAGA</u>TTAGTG-CAGCGAGGTCTCGGTTTC-3′), (*Xba*I-sites underlined, ATG start codon in bold). This PCR product was cleaved with *Xba*I and cloned into plasmid pUHD10-3 (M. Gossen, unpublished, Genbank accession number U89931) creating pMGD10. The integrity of the insert ion was confirmed by sequencing and cleaved out using *Bam*HI and ligated into pAHIR1-β-gal [28], digested with *Bgl*II resulting in plasmid pMGD18 (hPPAR$\delta\Delta$AF2). The correct orientation was confirmed by sequencing. Mice were generated as for hPPARδ as described previously [32]. Four founder lines on a C57BL/6 background were generated for each transgene and analysed for induction of transgene expression and suitable lines selected and brought forward for experimental analysis.

For experimental analysis, all animals were between 8 and 12 weeks of age. See figure legends of individual experiments for numbers and sex of the animals included.

Transgene expression was induced by supplementing the feed with indole 3-carbinol (I3C) at either 0.5% or 0.25% (w/w). Activation of hPPARδ andhPPARD$\delta\Delta$AF2 transgenes in these animals was achieved by supplementing the diet with bezafibrate (0.5% w/w) or GW501516 (0.005% or 0.0025 w/w). See figure legends of individual experiments for exact dietary supplementation and time frames.

4.3. Determination of Transgene Detection. All animals were analysed for the presence of the transgene by PCR. DNA was extracted from ear notches and the hPPARδ or hPPAR$\delta\Delta$AF2 transgene was amplified using primer set PRMG159 (5′-CCAACCACCCTGTCCCAGCTTG-3′) and PRMG160 (5′-ACAAACTCTGCCCTGCTCTATG-3′) using HotStarTaq DNA polymerase and Q-solution (Qiagen).

4.4. RNA Isolation and Semiquantitative Real-Time PCR. Total RNA from mouse tissue was isolated using the RNeasy Mini Kit (Qiagen) from kidney, liver, spleen, lung, ovary, and testis. The RNeasy Lipid kit (Qiagen) was used to isolate total RNA from brain, adipose, and breast. Total RNA isolation from heart, muscle, large and small intestines, stomach, and skin was isolated using RNeasy Mini Fibrous kit (Qiagen). All tissue samples were snap frozen in liquid N_2 upon harvest and kept frozen until homogenization in the relevant RNA extraction kit buffers using a rotor-stator homogenizer. RNA isolation kits were used as per manufacturer's instructions, and in all cases an on-column DNAse digestion step was included prior to total RNA elution to ensure complete removal of any genomic DNA from the preparation. Purified total RNA was stored at −80°C. First strand cDNA synthesis form 200 ng of RNA was performed using Omniscript Reverse Transcription kit (Qiagen). Upon completion, the

reaction was diluted in water, and a volume representing 5 ng of starting material was used for PCR reactions. The measurement of mRNA levels was achieved using quantitative real-time PCR using TaqMan chemistry on an Applied Biosystems 7900 sequence detector instrument. The primer and probe sets used to amplify hPPARδ were published previously [43]. The primer and probe set used to amplify mouse *Acox1*mRNA was primer: 5′-TGACCGTTAAGG-TCTTTGCAGA-3′ and 5′-AGGTTCCTCAGCACGGCTT-3′ with probe 5′-[Fam]-AACTCCCCAAGATTCAAGACA-GAGCCGT-[Tamra]-3′. Mouse *Adrp* mRNA was amplified using primer 5′-CAGCCAACGTCCGAGATTG-3′ and 5-CACATCCTTCGCCCCAGT-3′; probe 5′[FAM]-TGCCAG-TGCCAGAGGTGCCGT-[Tamra]-3′.

4.5. Lipid Measurements. Total hepatic lipids were extracted according to Folch method [44]. Lipid analysis of liver extracts was performed using RX Daytona clinical analyser (Randox, UK) following manufacturer's instructions.

4.6. Western Blot. Soluble lysates were prepared from frozen liver as described previously [45]. Protein concentrations were determined by the Bradford dye-binding assay (Bio-Rad). For immunoblotting, 30 μg aliquots of liver lysate were resolved by SDS/polyacrylamide-gel electophoresis, transferred to immobilon-P membranes and blocked with reconstituted dried milk (10% w/v) in TBS-tween. Membranes were probed with antihuman PPARδ/NR1C2 monoclonal antibody (R&D systems) at a concentration of 1 μg/mL in TBS-tween supplemented with dried milk powder (10% (w/v)). Following washing the membrane was incubated with secondary antimouse IgG coupled to horseradish peroxidase (1 : 10,000 dilution) (Dako). Bands were visualized using enhanced chemiluminescence (Millipore), and images were captured using the Fujifilm LAS3000 mini imager. To confirm equal loading of samples, blots were reprobed with antibodies against GAPDH (Sigma).

4.7. Statistical Analysis. Data was analysed using GraphPad Prism Software (Graphpad Software Inc., CA, USA). Statistical significance was calculated using the non-parametric Mann-Whitney U test. Statistical significance is described in the text and is indicated in the figures as ($^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$).

Authors' Contribution

Participated in research design: L. G. Higgins, W. G. Garbacz, M. C. U. Gustafsson, P. R. Ashby, C. R. Wolf, and C. N. A. Palmer. Conducted experiments: L. G. Higgins, W. G. Garbacz, S. Nainamalai, P. R. Ashby, and M. C. U. Gustafsson. Performed data analysis: L. G. Higgins and C. N. A. Palmer. Wrote or contributed to writing of the paper: L. G. Higgins, C. N. A. Palmer, and M. C. U. Gustafsson. Other: C. N. A. Palmer acquired funding for the research.

References

[1] S. Kersten, J. Seydoux, J. M. Peters, F. J. Gonzalez, B. Desvergne, and W. Wahli, "Peroxisome proliferator-activated receptor α mediates the adaptive response to fasting," *Journal of Clinical Investigation*, vol. 103, no. 11, pp. 1489–1498, 1999.

[2] B. Desvergne and W. Wahli, "Peroxisome proliferator-activated receptors: nuclear control of metabolism," *Endocrine Reviews*, vol. 20, no. 5, pp. 649–688, 1999.

[3] S. S. T. Lee, T. Pineau, J. Drago et al., "Targeted disruption of the α isoform of the peroxisome proliferator- activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators," *Molecular and Cellular Biology*, vol. 15, no. 6, pp. 3012–3022, 1995.

[4] C. N. A. Palmer, M. H. Hsu, K. J. Griffin, J. L. Raucy, and E. F. Johnson, "Peroxisome proliferator activated receptor-α expression in human liver," *Molecular Pharmacology*, vol. 53, no. 1, pp. 14–22, 1998.

[5] S. Yu, W. Q. Cao, P. Kashireddy et al., "Human peroxisome proliferator-activated receptor α (PPARα) supports the induction of peroxisome proliferation in PPARα-deficient mouse liver," *Journal of Biological Chemistry*, vol. 276, no. 45, pp. 42485–42491, 2001.

[6] P. Escher, O. Braissant, S. Basu-Modak, L. Michalik, W. Wahli, and B. Desvergne, "Rat PPARs: quantitative analysis in adult rat tissues and regulation in fasting and refeeding," *Endocrinology*, vol. 142, no. 10, pp. 4195–4202, 2001.

[7] E. E. Girroir, H. E. Hollingshead, P. He, B. Zhu, G. H. Perdew, and J. M. Peters, "Quantitative expression patterns of peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) protein in mice," *Biochemical and Biophysical Research Communications*, vol. 371, no. 3, pp. 456–461, 2008.

[8] Y. Barak, D. Liao, W. He et al., "Effects of peroxisome proliferator-activated receptor δ on placentation, adiposity, and colorectal cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 1, pp. 303–308, 2002.

[9] L. Michalik, B. Desvergne, N. S. Tan et al., "Impaired skin wound healing in peroxisome proliferator-activated receptor (PPAR)α and PPARβ mutant mice," *Journal of Cell Biology*, vol. 154, no. 4, pp. 799–814, 2001.

[10] J. M. Peters, S. S. T. Lee, W. Li et al., "Growths, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor β(δ)," *Molecular and Cellular Biology*, vol. 20, no. 14, pp. 5119–5128, 2000.

[11] F. Varnat, B. B. Heggeler, P. Grisel et al., "PPARβ/δ regulates paneth cell differentiation via controlling the hedgehog signaling pathway," *Gastroenterology*, vol. 131, no. 2, pp. 538–553, 2006.

[12] K. Nadra, S. I. Anghel, E. Joye et al., "Differentiation of trophoblast giant cells and their metabolic functions are dependent on peroxisome proliferator-activated receptor β/δ," *Molecular and Cellular Biology*, vol. 26, no. 8, pp. 3266–3281, 2006.

[13] N. Di-Po, N. S. Tan, L. Michalik, W. Wahli, and B. Desvergne, "Antiapoptotic role of PPARβ in keratinocytes via transcriptional control of the Akt1 signaling pathway," *Molecular Cell*, vol. 10, no. 4, pp. 721–733, 2002.

[14] C. H. Lee, P. Olson, A. Hevener et al., "PPARδ regulates glucose metabolism and insulin sensitivity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 9, pp. 3444–3449, 2006.

[15] Y. X. Wang, C. H. Lee, S. Tiep et al., "Peroxisome-proliferator-activated receptor δ activates fat metabolism to prevent obesity," *Cell*, vol. 113, no. 2, pp. 159–170, 2003.

[16] S. Luquet, J. Lopez-Soriano, D. Holst et al., "Peroxisome pro-liferator-activated receptor δ controls muscle development and oxidative capability," *FASEB Journal*, vol. 17, no. 15, pp. 2299–2301, 2003.

[17] L. Cheng, G. Ding, Q. Qin et al., "Cardiomyocyte-restricted peroxisome proliferator-activated receptor-δ deletion per-turbs myocardial fatty acid oxidation and leads to cardiomy-opathy," *Nature Medicine*, vol. 10, no. 11, pp. 1245–1250, 2004.

[18] Y. Yin, R. G. Russell, L. E. Dettin et al., "Peroxisome pro-liferator-activated receptor δ and γ agonists differentially alter tumor differentiation and progression during mammary carcinogenesis," *Cancer Research*, vol. 65, no. 9, pp. 3950–3957, 2005.

[19] R. A. Gupta, D. Wang, S. Katkuri, H. Wang, S. K. Dey, and R. N. DuBois, "Activation of nuclear hormone receptor perox-isome proliferator-activated receptor-δ accelerates intestinal adenoma growth," *Nature Medicine*, vol. 10, no. 3, pp. 245–247, 2004.

[20] F. S. Harman, C. J. Nicol, H. E. Marin, J. M. Ward, F. J. Gonzalez, and J. M. Peters, "Peroxisome proliferator-activated receptor-δ attenuates colon carcinogenesis," *Nature Medicine*, vol. 10, no. 5, pp. 481–483, 2004.

[21] A. M. Krogsdam, C. A. F. Nielsen, S. Neve et al., "Nuclear receptor corepressor-dependent repression of peroxisome-proliferator-activated receptor δ-mediated transactivation," *Biochemical Journal*, vol. 363, no. 1, pp. 157–165, 2002.

[22] K. Matsusue, J. M. Peters, and F. J. Gonzalez, "PPARβ/δ poten-tiates PPARγ-stimulated adipocyte differentiation," *FASEB Journal*, vol. 18, no. 12, pp. 1477–1479, 2004.

[23] X. Zuo, Y. Wu, J. S. Morris, J. B. Stimmel, and L. M. Leesnitzer, "Oxidative metabolism of linoleic acid modulates PPAR-beta/delta suppression of PPAR-gamma activity," *Oncogene*, vol. 25, no. 8, pp. 1225–1241, 2006.

[24] Y. Shi, M. Hon, and R. M. Evans, "The peroxisome pro-liferator-activated receptor δ, an integrator of transcriptional repression and nuclear receptor signaling," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 5, pp. 2613–2618, 2002.

[25] J. M. Peters, T. Aoyama, A. M. Burns, and F. J. Gonzalez, "Bezafibrate is a dual ligand for PPARalpha and PPARbeta: studies using null mice," *Biochimica et Biophysica Acta*, vol. 1632, pp. 80–89, 2003.

[26] M. C. Gustafsson, D. Knight, and C. N. Palmer, "Ligand mod-ulated antagonism of PPARgamma by genomic and non-genomic actions of PPARdelta," *PLoS ONE*, vol. 4, article e7046, 2009.

[27] P. Targett-Adams, M. J. McElwee, E. Ehrenborg, M. C. Gustafs-son, C. N. Palmer, and J. McLauchlan, "A PPAR response element regulates transcription of the gene for human adipose differentiation-related protein," *Biochimica et Biophysica Acta*, vol. 1728, no. 1-2, pp. 95–104, 2005.

[28] S. J. Campbell, F. Carlotti, P. A. Hall, A. J. Clark, and C. R. Wolf, "Regulation of the CYP1A1 promoter in transgenic mice: an exquisitely sensitive on-off system for cell specific gene regulation," *Journal of Cell Science*, vol. 109, no. 11, pp. 2619–2625, 1996.

[29] S. Kantachuvesiri, S. Fleming, J. Peters et al., "Controlled hypertension, a transgenic toggle switch reveals differential mechanisms underlying vascular disease," *Journal of Biological Chemistry*, vol. 276, no. 39, pp. 36727–36733, 2001.

[30] R. D. Finn, A. W. McLaren, D. Carrie, C. J. Henderson, and C. R. Wolf, "Conditional deletion of cytochrome P450 oxidore-ductase in the liver and gastrointestinal tract: a new model

for studying the functions of the P450 system," *Journal of Pharmacology and Experimental Therapeutics*, vol. 322, no. 1, pp. 40–47, 2007.

[31] M. I. Kleman, L. Poellinger, and J. A. Gustafsson, "Regulation of human dioxin receptor function by indolocarbazoles, receptor ligands of dietary origin," *Journal of Biological Chem-istry*, vol. 269, no. 7, pp. 5137–5144, 1994.

[32] M. Romanowska, L. Reilly, C. N. Palmer, M. C. Gustafs-son, and J. Foerster, "Activation of PPARbeta/delta causes a psoriasis-like skin disease in vivo," *PLoS ONE*, vol. 5, article e9701, 2010.

[33] Y. X. Wang, C. L. Zhang, R. T. Yu et al., "Regulation of muscle fiber type and running endurance by PPARδ," *PLoS Biology*, vol. 2, no. 10, article e294, 2004.

[34] B. Gross, N. Hennuyer, E. Bouchaert, C. Rommens, and D. Grillot, "Generation and characterization of a humanized PPARdelta mouse model," *British Journal of Pharmacology*, vol. 164, pp. 192–208, 2011.

[35] C. N. A. Palmer, M. H. Hsu, K. J. Griffin, and E. F. Johnson, "Novel sequence determinants in peroxisome proliferator sig-naling," *Journal of Biological Chemistry*, vol. 270, no. 27, pp. 16114–16121, 1995.

[36] C. N. A. Palmer, M. H. Hsu, A. S. Muerhoff, K. J. Griffin, and E. F. Johnson, "Interaction of the peroxisome proliferator-activated receptor α with the retinoid X receptor α unmasks a cryptic peroxisome proliferator response element that overlaps an ARP-1-binding site in the CYP4A6 promoter," *Journal of Biological Chemistry*, vol. 269, no. 27, pp. 18083–18089, 1994.

[37] I. P. Torra, Y. Jamshidi, D. M. Flavell, J. C. Fruchart, and B. Staels, "Characterization of the human PPARα promoter: identification of a functional nuclear receptor response ele-ment," *Molecular Endocrinology*, vol. 16, no. 5, pp. 1013–1028, 2002.

[38] T. Adhikary, K. Kaddatz, F. Finkernagel, A. Schonbauer, and W. Meissner, "Genomewide analyses define different modes of transcriptional regulation by peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta)," *PLoS ONE*, vol. 6, article e16344, 2011.

[39] T. Kino, K. C. Rice, and G. P. Chrousos, "The PPARδ ago-nist GW501516 suppresses interleukin-6-mediated hepatocyte acute phase reaction via STAT3 inhibition," *European Journal of Clinical Investigation*, vol. 37, no. 5, pp. 425–433, 2007.

[40] T. Fritz, D. K. Kramer, H. K. Karlsson, D. Galuska, and P. Engfeldt, "Low-intensity exercise increases skeletal muscle protein expression of PPARdelta and UCP3 in type 2 diabetic patients," *Diabetes/Metabolism Research and Reviews*, vol. 22, pp. 492–498, 2006.

[41] X. Qin, X. Xie, Y. Fan et al., "Peroxisome proliferator-activated receptor-δ induces insulin-induced gene-1 and suppresses hepatic lipogenesis in obese diabetic mice," *Hepatology*, vol. 48, no. 2, pp. 432–441, 2008.

[42] S. Kersten, B. Desvergne, and W. Wahli, "Roles of PPARS in health and disease," *Nature*, vol. 405, no. 6785, pp. 421–424, 2000.

[43] V. Young, M. Ho, H. Vosper, J. J. F. Belch, and C. N. A. Palmer, "Elevated expression of the genes encoding TNF-α and throm-boxane synthase in leucocytes from patients with systemic sclerosis," *Rheumatology*, vol. 41, no. 8, pp. 869–875, 2002.

[44] J. Folch, M. Lees, and G. H. Sloane Stanley, "A simple method for the isolation and purification of total lipides from animal tissues," *The Journal of biological chemistry*, vol. 226, no. 1, pp. 497–509, 1957.

[45] V. P. Kelly, E. M. Ellis, M. M. Manson et al., "Chemoprevention of aflatoxin B1 hepatocarcinogenesis by coumarin, a natural benzopyrone that is a potent inducer of aflatoxin B1-aldehyde reductase, the glutathione S-transferase A5 and P1 subunits, and NAD(P)H:Quinone oxidoreductase in rat liver," *Cancer Research*, vol. 60, no. 4, pp. 957–969, 2000.