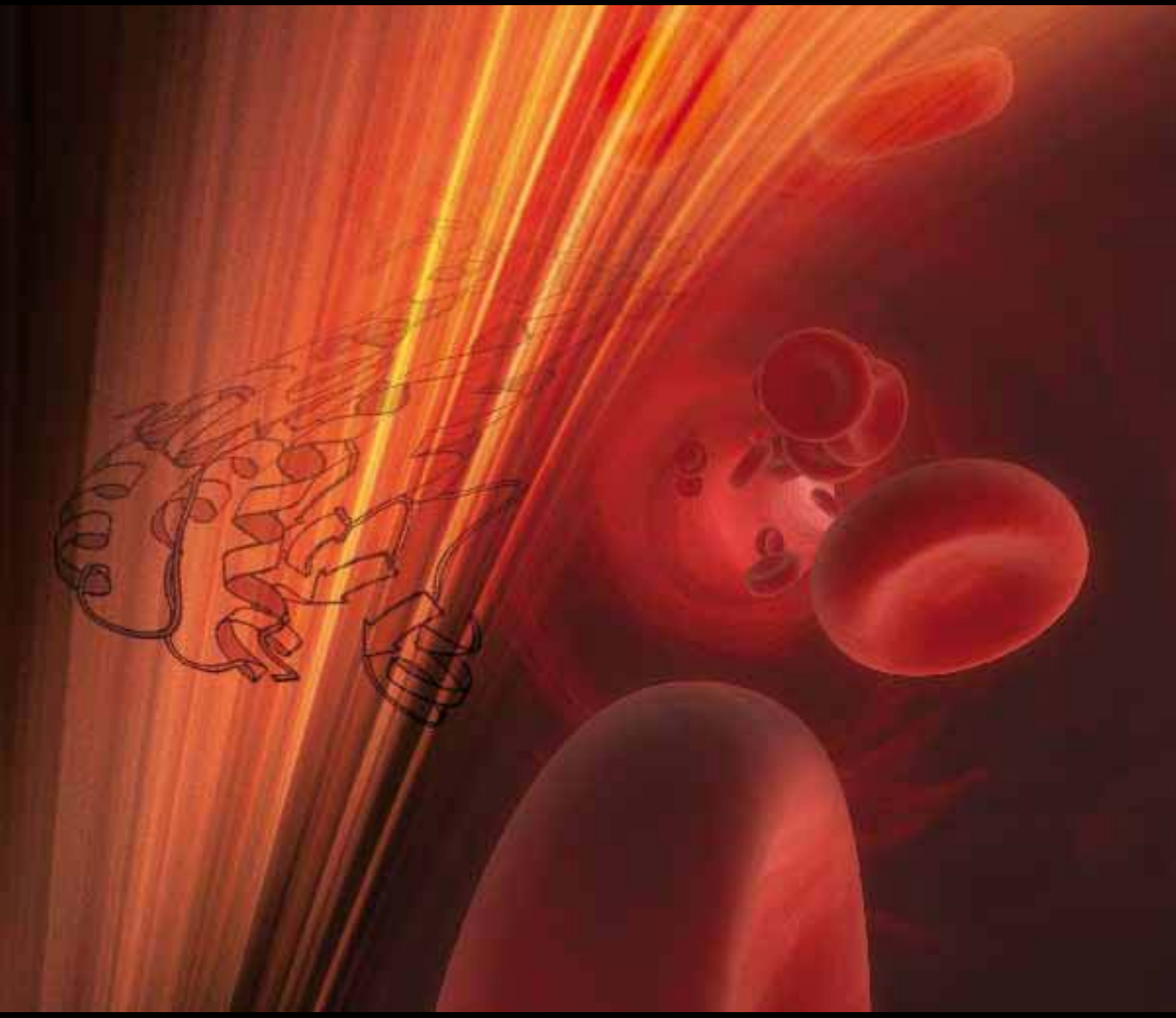
PPAR Research

Pharmacological and Toxicological Advances in PPAR-Related Medicines

Guest Editors: Yuji Kamijo, Christopher J. Nicol, and Stefan E. H. Alexson



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Editorial Board

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 940964, 2 pages
doi:10.1155/2012/940964

Editorial

Pharmacological and Toxicological Advances in PPAR-Related Medicines

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Received 13 August 2012; Accepted 13 August 2012

Peroxisome proliferator-activated receptors (PPARs) are involved in the pathophysiology of the various types of diseases. Many types of PPAR-related medicines developed and utilized clinically all over the world exert multiple effects, including regulation of hypolipidemic, antidiabetic, anti-inflammatory, antifibrotic, and antiproliferative pathways, with emerging potential benefits in other diseases. On the other hand, these medicines may also exert various toxicities, and some PPAR drugs are no longer in use clinically because of serious complications arising in some patients. Thus, the authors here have focused on the benefits and risks of these medicines, and aim to clarify their therapeutic potential for appropriate clinical utilization. This special issue in PPAR research includes 6 review articles and 6 research articles, as follows.

Review Articles. The paper "*The key to unlocking the chemotherapeutic potential of PPARγ ligands: Having the right combination*" by G. Skelhorne-Gross and C. J. B. Nicol is a review of the vast *in vitro, in vivo*, and human clinical trial studies, using chemotherapeutic combinations that include PPAR*γ* activating drugs. This review article reveals the novel chemotherapeutic potential of PPAR*γ* activating drugs, and provides a guide for further basic and clinical research. This information is certainly useful for optimization of chemotherapeutic interventions that will reduce the number of cancer related deaths.

The paper "*PPAR medicines and human disease: The ABCs of it all*" by A. J. Apostoli and C. J. B. Nicol is a review article that summarizes the advances of knowledge concerning effects of PPAR medicines on ATP-dependent binding cassette (ABC) transporters based on *in vitro, in vivo*, and human clinical trial studies. This review suggests the potential of PPAR-related medicines for controlling ABC transporter activity at the transcriptional level, and discusses their potential implications in human diseases with respect to cancer and atherosclerosis.

The paper "*The current knowledge of the role of PPAR in hepatic ischemia-reperfusion injury*" by M. Elias-Miró et al. is a review article concerning the roles of PPARs signaling pathways in hepatic ischemia reperfusion injury that is inherent to human liver transplantation and resection surgery. A shortage of available healthy livers for organ transplantation calls for the potential use of any available organ, including, for example, steatotic livers; however, steatotic livers are more susceptible to ischemia-reperfusion injury. This paper reviews PPAR-signaling pathways, summarizes some of the lesser known functions of PPARs in liver regeneration, and discusses potential therapies based on PPAR regulation that may minimize the observed side effects in liver surgery. This review emphasizes the need for further research into the roles of PPARs in various liver conditions and surgical procedures before being translated into treatment of human disease.

The paper "*Effects of PPARγ ligands in leukemia*" by Y. Tabe et al. is a review article that describes the antitumor advances of PPAR*γ* ligands, alone and in combination with retinoic acid receptor ligands in control of cell proliferation, differentiation, and apoptosis, and discusses their potential therapeutic applications in hematological malignancies.

Acute promyelocytic leukemia (APL, representing about 10% of AML patients) is unique among myeloid leukemias in that it is sensitive to all-trans-retinoic acid (ATRA). However, a number of APL patients relapse and develop ATRA resistance. This review article provides evidence on the consequences of the treatment with PPARγ ligands, in particular the triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), on the epigenetic/transcriptional events induced by retinoic acid in APL cells, and supports the clinical utility of ATRA/PPARγ-ligand combinations for treating hematological malignancies.

The paper "*Idealized PPARγ-based therapies: Lessons from bench and bedside*" by A. A. Amato and F. de A. R. Neves is a review about the knowledge acquired regarding efficacy and safety issues by PPARγ ligands. This body of work is attractive since the interest for PPARγ modulation as a strategy to treat metabolic diseases has increased recently, due to better understanding of PPARγ action.

The paper "*Nutraceuticals as ligands of PPARγ*" by M. Penumetcha and N. Santanam reviews the transcription factor PPARγ, which is the target for the thiazolidinediones, the first class of PPARγ agonist drugs used in the treatment of diabetes. Due to the increased adverse effects related to these drugs, newer safer drugs are being generated. This review paper describes some of the dietary components that have affinity for, and activate, PPARγ, as well as their pharmacology and potential toxicology.

Research Articles. The paper "*PPARα activation protects against anti-Thy1 nephritis by suppressing glomerular NF-κB signaling*" by K. Hashimoto et al. is the first to demonstratethe glomerular protective effects of treatment using a representative PPARα agonist, clofibrate, in rat mesangial proliferative glomerulonephritis model (MsPGN) anti-Thy1 nephritis. PPARα activation is known to exert anti-inflammatory effects in various cells and organs through suppression of NFκB signaling; however, its effect against glomerulonephritis has remained obscure. Because MsPGN is one of the significant factors leading to chronic kidney disease (CKD), the beneficial antinephritic effect of PPARα activation may provide a novel treatment strategy against CKD. Their findings may also be useful to create PPAR-based therapies to treat glomerular disease.

The paper "*Hepatic cerebroside sulfotransferase is induced by PPARα activation in mice*" by T. Kimura et al. is the first to examine sulfatide levels and the expression of enzymes related to sulfatide metabolism using wild-type (+/+), *Ppara*-heterozygous (+/−), and *Ppara*-null (−/−) mice given a control diet or one containing 0.1% fenofibrate, a typical PPARα activator. Recent studies have revealed a protective role of serum sulfatides against arteriosclerosis and hypercoagulation. Their results suggest that PPARα activation enhances hepatic sulfatide synthesis mainly through cerebroside sulfotransferase (CST) induction. Accordingly, CST may be a novel PPARα target gene product candidate with implications in disease prevention and treatment.

The paper "*Fatty acid accumulation and resulting PPARα activation in fibroblasts due to trifunctional protein deficiency*" by M. Wakabayashi et al. demonstrates free fatty acid accumulation, enhanced three acyl-CoA dehydrogenases, and PPARα activation in the fibroblasts from six patients with mitochondrial trifunctional protein deficiency, who had abnormalities in the second through fourth reactions in fatty acid β-oxidation system. These novel findings suggest that the fatty acid accumulation and resulting PPARα activation are major causes of the increase in the β-oxidation ability in the patients' fibroblasts, and that enhanced cell proliferation and increased oxidative stress relate to the development of specific clinical features. Additionally, significant suppression of the PPARα activation by means of MK886 treatment may provide a new method of treating this deficiency.

In the paper "*Global gene expression profiling in PPARγ agonist-treated kidneys in an orthologous rat model of human autosomal recessive polycystic kidney disease*" by D. Yoshihara et al., the authors explored the changes in gene expression by Pioglitazone (PIO), a PPARγ agonist, using polycystic kidney disease (PCK) rats. By analyzing globally, they successfully found that stearoyl-coenzyme A desaturase 1 (*Scd1*) was highly expressed in PCK kidneys, and PIO decreased its expression. Notably, they found that *Scd1* plays a role in the early cystogenesis, and this is the point where PIO may intervene in the process of cystogenesis.

The paper "*Plasticizers may activate human hepatic peroxisome proliferator-activated receptor α less than that of a mouse but may activate constitutive androstane receptor (CAR) in liver*" by Y. Ito et al. reported the species differences concerning activation of PPARα and CAR, which was induced by the oral exposure with industrial PPARα ligands, including dibutyl phthalate, di(2-ethylhexyl)phthalate, and di(2-ethylhexyl)adipate, between wild-type mice and humanized PPARα mice. These transcriptional species differences might cause different hepatic toxicities between murine model and human cases. This information would be valuable for the risk assessment of PPARα-related medicines.

The paper "*Peroxisome proliferator-activated receptor α agonists differentially regulate inhibitor of DNA binding expression in rodents and human cells*" by M. del C. González et al. reported rodent versus human species differences in the regulatory manner of inhibitor of DNA binding (Id2) via PPARα agonists. Since Id2 protein is involved in cell differentiation and proliferation, this finding may help to understand the species differences in toxicity of PPARα agonists.

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 504918, 16 pages
doi:10.1155/2012/504918

Review Article

PPAR Medicines and Human Disease: The ABCs of It All

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Received 24 February 2012; Revised 4 April 2012; Accepted 6 April 2012

Academic Editor: Yuji Kamijo

ATP-dependent binding cassette (ABC) transporters are a family of transmembrane proteins that pump a variety of hydrophobic compounds across cellular and subcellular barriers and are implicated in human diseases such as cancer and atherosclerosis. Inhibition of ABC transporter activity showed promise in early preclinical studies; however, the outcomes in clinical trials with these agents have not been as encouraging. Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate genes involved in fat and glucose metabolism, and inflammation. Activation of PPAR signaling is also reported to regulate ABC gene expression. This suggests the potential of PPAR medicines as a novel means of controlling ABC transporter activity at the transcriptional level. This paper summarizes the advances made in understanding how PPAR medicines affect ABC transporters, and the potential implications for impacting on human diseases, in particular with respect to cancer and atherosclerosis.

1. Introduction

Harnessing the energy released from adenosine triphosphate (ATP) hydrolysis, ATP-dependent binding cassette (ABC) transporters shuttle a wide range of substrates, including lipids, metabolites, and xenobiotics, across biological membranes in order to maintain normal cell metabolism. They represent the largest family of transmembrane proteins in humans, comprising 49 ABC genes, and are best reviewed elsewhere [1–3]. These genes are subdivided among seven subfamilies (A-G) based on sequence and structural homology and are highly conserved among eukaryotic species, suggesting that most appeared early in metazoan evolution [4]. The proteins encoded by ABC genes consist of two distinct domains: a transmembrane domain that recognizes specific compounds and transports them across cellular and subcellular barriers and a nucleotide-binding domain where ATP hydrolysis occurs to yield energy for substrate transport [5]. Typically, ABC proteins are unidirectional transporters expressed at the cell membrane, which move hydrophobic molecules internally for metabolic pathways, or externally for elimination from the cell and/or use by other tissues and organs. Thus, ABC transporters play important roles in a range of human physiologic, toxicologic, and pathologic functions. With respect to the latter, many preclinical reports that show promise in terms of regulating ABC transporters to overcome chemotherapeutic drug resistance in tumours, or modify lipid homeostasis in order to reduce atherosclerotic risk, have not achieved the same level of success in clinical trials.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate expression of a plethora of genes involved in sugar and fat metabolism, inflammation, and cancer [6–8]. Three PPAR homologs have been characterized—PPARα, PPARβ/δ, and PPARγ—each displaying a unique pattern of tissue-specific expression that reflect their distinctive functions [9–11]. Recently, there is mounting *in vitro* and *in vivo* evidence that activation of PPARs may alter ABC protein expression and/or function. Accordingly, this paper will summarize recent developments in an emerging field where PPAR medicines, capable of modulating ABC transporter genes at the transcriptional level, may prove useful when such modulation provides novel therapeutic options for treating cancer and atherosclerosis.

2. PPARs and Their Ligands

As members of the nuclear receptor superfamily, PPARs contain a ligand-binding domain that recognizes and binds specific PPAR agonists, and a DNA-binding domain that interacts with specific peroxisome proliferator-response elements (PPREs) within the genome [12]. PPARs are localized to the nucleus and dimerize with retinoid *X* receptor (RXR)α to form complexes that bind to PPREs in the promoter regions of a broad range of target genes [13]. In its resting state, the PPAR : RXRα complex associates with cell-specific corepressor molecules that aid in the silencing of target gene transcription. Ligand binding elicits a conformational change in PPAR that leads to the release of corepressors, and the recruitment of coactivator molecules that promote target gene transcriptional activity. Furthermore, ligand activation of PPARs may also repress signaling of some gene targets through direct interaction with other transcription factors or competition for available coregulators [14].

PPARα is highly expressed in the liver, heart, kidney, skeletal muscle, and large intestine [15]. It is activated by the "fibrate" class of drugs, such as bezafibrate, ciprofibrate, clofibrate, gemfibrozil, and fenofibrate, used to treat elevated triglycerides and low high-density lipoprotein (HDL) [16]. PPARβ/δ is more ubiquitously expressed with highest levels noted within the large intestine and placenta [15]. Similar to other PPAR subtypes, it may also be activated by various saturated and unsaturated fatty acids [12]. Because less is understood about PPARβ/δ, fewer synthetic activators have been developed; however, emerging evidence supports the potential therapeutic value of PPARβ/δ agonists, such as GW0742, GW501516, and MBX-8025, which remain to be clinically tested [17].

As a chief regulator of adipogenesis, PPARγ is abundantly expressed in adipose tissue [18], and like PPARα, is also detected in vascular and immune cells, as well as tissues such as the colon, breast, and prostate [19, 20]. Synthetic agents known as thiazolidinediones (TZDs) like troglitazone, ciglitazone, rosiglitazone, and pioglitazone are classic examples of PPARγ activators [21]. In North America, rosiglitazone and pioglitazone are still prescribed to treat type 2 diabetic patients. However, there are reports suggesting increased myocardial infarction risk with rosiglitazone use and bladder cancer risk with long-term use of pioglitazone [22, 23]. As a followup on the former, a safety review of rosiglitazone by a panel of international experts deemed the available data inconclusive and requiring further study. In the latter case, direct clinical evidence of this possible association is also required. Despite the need for more evidence, these drugs remain FDA approved, albeit with warning updates to package inserts clarifying the potential for risk [24, 25], and a Risk Evaluation and Mitigation Strategy (REMS) is in place to restrict access and distribution of rosiglitazone-containing medicines to those healthcare providers and their patients who confirm their awareness of the new warnings [26]. Nevertheless, the utility of these drugs remains valuable not only for their ability to provide mechanistic insight into the role of PPARγ-mediated target regulation, but also for their potential benefit in certain off-label uses.

Dual and pan PPAR ligands were also developed to enhance therapeutic potential via simultaneously activating two or more PPAR isoforms. Examples include PPARα/γ modulators like tesaglitazar, muraglitazar, and aleglitazar, and the pan PPAR$\alpha/(\beta/\delta)/\gamma$ agonist chiglitazar [27].

The reported links between the above listed PPAR medicines and their *in vitro* and *in vivo* effects on ABC transporters are summarized in Tables 1 and 2, respectively, and described in detail below in the context of several human diseases.

3. Cancer

The goal of chemotherapy is to target rapidly dividing cells or deregulated signaling pathways to suppress tumour growth, and ultimately, cure cancer patients; however, one primary roadblock to the success of chemotherapy is acquisition of multidrug resistance (MDR). A well-known cause of MDR is ABC transporter-driven drug efflux from cancer cells instilling resistance to multiple agents [28]. The well-known ABC transporters, P-glycoprotein (Pgp)/MDR1/ABCB1, multidrug resistance protein (MRP)1/ABCC1, and breast cancer-resistance protein (BCRP)/MXR/ABCG2, are overexpressed in a variety of different human cancers and transport a range of chemotherapeutic drugs [4]. Pgp, an important blood brain barrier component and regulator of intestinal drug absorption, was the first ABC transporter to be characterized in 1976 [29]. Its overexpression in tumours of the kidney, liver, colon, and breast correlates with chemoresistance [30–32]. Substrates of Pgp include anthracyclines, vinca alkaloids, taxanes, camptothecins, mitoxantrone, and methotrexate [33]. The second ABC gene discovered was the more ubiquitously expressed MRP1 [34], which transports anthracyclines, vinca alkaloids, and etoposide, in addition to organic anions and glutathione conjugates [28]. Its overexpression confers chemotherapy resistance in prostate, lung, breast, and neuroblastoma cancer [35, 36]. Finally, BCRP is normally expressed in placenta and small intestine, as well as various stem cell populations [37, 38]. Several drug-resistant cell lines also contain elevated levels of this ABC transporter, which contributes to the efflux of several antitumour agents such as doxorubicin, daunorubicin, mitoxantrone, and topotecan [39–41].

In addition to MDR, other functions of ABC transporters in cancer are beginning to emerge, further implicating these genes as important targets of chemotherapy. For example, Pgp expression, devoid of ATP-dependent drug transport, suppresses cell death in the presence of apoptotic signals in normal and cancer cells [42–44]. Furthermore, Pgp knockdown reduced the migration and invasion potential of MCF7 human breast cancer cells [45]. As a result of these studies, direct inhibition of ABC transporter activity has become an appealing undertaking for researchers in the development of improved cancer chemotherapeutics; however, several clinical trials using ABC inhibitors have proven unsuccessful [46].

Research has shown that PPAR activation induces expression of both mouse (Mdr1/Mdr1b/Abcb1b, Mdr2/Abcb4, and Mdr3/Mdr1a/Abcb1a) and human (MDR2/MDR3/ABCB4) homologs of Pgp, which efflux similar

Table 1: *In vitro* effects of PPAR ligands on ABC transporters.

ABC transporter	PPAR	PPAR Ligand	Cell line	Transporter effect	Reference
ABCA1	PPARα	Bezafibrate	Primary mouse fibroblasts THP1 human macrophages WI38 human fibroblasts	↑ ABCA1 and LXRα mRNA ↑ apoA1-mediated cholesterol efflux	[47]
			Immortalized human mesangial cells	↑ ABCA1 and LXRα mRNA ↑ apoA1-mediated cholesterol efflux	[48]
			Primary mouse hepatocytes HepG2 human hepatoma cells	↑ ABCA1 mRNA and protein ↑ HDL synthesis	[49]
		Clofibrate	Primary human foreskin keratinocytes	↑ ABCA1 mRNA	[50]
		Fenofibrate	Primary mouse fibroblasts THP1 human macrophages WI38 human fibroblasts	↑ ABCA1 and LXRα mRNA ↑ apoA1-mediated cholesterol efflux	[47]
			BALB/3T3 mouse fibroblasts RAW264.7 mouse leukemic macrophages THP1 human macrophages	↑ ABCA1 mRNA and protein ↑ apoA1-mediated cholesterol efflux	[51]
			Primary mouse hepatocytes HepG2 human hepatoma cells	↑ ABCA1 mRNA and protein ↑ HDL synthesis	[49]
		Gemfibrozil	Primary mouse fibroblasts THP1 human macrophages WI38 human fibroblasts	↑ ABCA1 and LXRα mRNA ↑ apoA1-mediated cholesterol efflux	[47]
			Primary mouse hepatocytes HepG2 human hepatoma cells	↑ ABCA1 mRNA and protein ↑ HDL synthesis	[49]
		LY518674	Primary mouse fibroblasts THP1 human macrophages WI38 human fibroblasts	↑ ABCA1 and LXRα mRNA ↑ apoA1-mediated cholesterol efflux	[47]
			Primary mouse hepatocytes HepG2 human hepatoma cells	↑ ABCA1 mRNA and protein ↑ HDL synthesis	[49]
		RPR-5	Primary human macrophages	↑ ABCA1 and LXRα mRNA	[52]
		WY14643	Immortalized human mesangial cells	↑ ABCA1 and LXRα mRNA ↑ apoA1-mediated cholesterol efflux	[48]
			Primary human macrophages	↑ ABCA1 and LXRα mRNA ↑ apoA1-mediated cholesterol efflux	[52]
			THP1 human macrophages	↑ ABCA1 mRNA	[52]
			BALB/3T3 mouse fibroblasts RAW264.7 mouse leukemic macrophages THP1 human macrophages	↑ ABCA1 mRNA and protein ↑ apoA1-mediated cholesterol efflux	[51]
			Primary canine gallbladder epithelial cells	↑ ABCA1 mRNA and protein	[53]

Table 1: Continued.

ABC transporter	PPAR	PPAR Ligand	Cell line	Transporter effect	Reference
	PPARα/γ	13-HODE	RAW264.7 mouse leukemic macrophages	↑ Abca1 and LXRα protein ↑ cholesterol efflux	[54]
		*c9t*11-CLA	RAW264.7 mouse leukemic macrophages	↑ Abca1 mRNA and protein ↑ LXRα mRNA ↑ HDL-mediated cholesterol efflux	[55]
		t10c12-CLA	RAW264.7 mouse leukemic macrophages	↑ Abca1 mRNA and protein ↑ LXRα mRNA ↑ HDL-mediated cholesterol efflux	[55]
		NO-pravastatin	Primary canine gallbladder epithelial cells	↑ ABCA1 mRNA and protein ↑ LXRα mRNA	[53]
		Pravastatin	Primary canine gallbladder epithelial cells	↑ ABCA1 mRNA and protein ↑ LXRα mRNA	[53]
		Simvastatin	Primary canine gallbladder epithelial cells	↑ ABCA1 mRNA and protein ↑ LXRα mRNA	[53]
	PPARγ	Pioglitazone	Primary mouse fibroblasts THP1 human macrophages WI38 human fibroblasts	↑ ABCA1 and LXRα mRNA ↑ apoA1-mediated cholesterol efflux	[47]
			RAW264.7 mouse leukemic macrophages THP1 human macrophages	↑ Abca1 mRNA and protein ↑ cholesterol efflux	[56]
		Rosiglitazone	Primary human macrophages	↑ ABCA1 and LXRα mRNA ↑ apoA1-mediated cholesterol efflux	[52]
			THP1 human macrophages	↑ ABCA1 mRNA	[52]
				↑ ABCA1 and LXRα mRNA ↑ cholesterol efflux	[57]
				↑ ABCA1 mRNA and protein	[58]
				↑ ABCA1 mRNA and protein ↓ intracellular cholesterol	[59]
		Troglitazone	Primary human macrophages	↑ ABCA1 and LXRα mRNA	[52]
			THP1 human macrophages	↑ ABCA1 mRNA	[52]
			Primary canine gallbladder epithelial cells	↑ ABCA1 mRNA and protein	[53]
		GW1929	HepG2 human hepatoma cells	↑ ABCA1, LXRα, and LXRβ mRNA ↓ ABCA1 and LXRβ protein	[60]

Table 1: Continued.

ABC transporter	PPAR	PPAR Ligand	Cell line	Transporter effect	Reference
		GW7845	THP1 human macrophages	↑ ABCA1 mRNA	[61]
		Mycophenolic acid	HepG2 human hepatoma cells	↑ ABCA1 mRNA and protein ↑ LXRα protein	[62]
		Prostaglandin J2	Immortalized human mesangial cells	↑ ABCA1 and LXRα mRNA ↑ apoA1-mediated cholesterol efflux	[48]
			Primary human macrophages	↑ ABCA1 and LXRα mRNA	[52]
		Telmisartan	RAW264.7 mouse leukemic macrophages	↑ Abca1 mRNA ↓ macrophage proliferation	[63]
	PPARβ/δ	GW501516	Primary mouse fibroblasts THP1 human macrophages WI38 human fibroblasts	↑ ABCA1 and LXRα mRNA ↑ apoA1-mediated cholesterol efflux	[47]
			THP1 human macrophages 1BR3N human fibroblasts	↑ ABCA1 mRNA ↑ apoA1-mediated cholesterol efflux	[61]
			FHS74 human intestinal cells	↑ ABCA1 mRNA	[61]
			Primary human skeletal muscle cells	↑ ABCA1 mRNA	[64]
			Primary human foreskin keratinocytes	↑ ABCA1 mRNA	[50]
ABCA12	PPARγ	Ciglitazone	Primary human foreskin keratinocytes	↑ ABCA12 mRNA and protein	[65]
		Troglitazone	Primary human foreskin keratinocytes	↑ ABCA12 mRNA	[65]
		GI251929X	Primary human foreskin keratinocytes	↑ ABCA12 mRNA	[65]
	PPARβ/δ	Ceramide	Primary human foreskin keratinocytes	↑ ABCA12 mRNA and protein	[66]
		GW610742	Primary human foreskin keratinocytes	↑ ABCA12 mRNA and protein	[65]
Pgp/MDR1/ABCB1	PPARα	Fenofibrate	Pgp-overexpressing L-MDR1 porcine kidney epithelial cells	↓ calcein efflux	[67]
	PPARα/γ	Simvastatin	Pgp-overexpressing L-MDR1 porcine kidney epithelial cells	↓ calcein efflux	[67]
	PPARγ	Rosiglitazone	Doxorubicin-resistant P388 mouse leukemia cells	↓ calcein efflux	[68]
		Troglitazone	Doxorubicin-resistant P388 mouse leukemia cells	↓ calcein efflux	[68]
			Doxorubicin-resistant K562 human leukemia cells Doxorubicin-resistant MCF7 human breast cancer cells	↓ Pgp protein ↑ sensitivity to doxorubicin	[69]

Table 1: Continued.

ABC transporter	PPAR	PPAR Ligand	Cell line	Transporter effect	Reference
			Vincristine-resistant SGC7901 human gastric cancer cells	↓ Pgp mRNA and protein ↓ Rh123 efflux ↑ sensitivity to vincristine	[70]
MDR2/MDR3/ABCB4	PPARα	Bezafibrate	HepG2 human hepatoma cells	↑ MDR2/MDR3 mRNA ↑ MDR2/MDR3 redistribution	[71]
				↑ MDR2/MDR3 mRNA ↑ MDR2/MDR3 redistribution ↑ phospholipid efflux	[72]
		Ciprofibrate	Primary mouse hepatocytes	↑ Mdr2 mRNA	[73]
		WY14643	Primary mouse hepatocytes	↑ Mdr2 mRNA	[73]
MRP2/ABCC2	PPARγ	Troglitazone	Primary rat hepatocytes	↓ Mrp2-associated bile efflux	[74]
ABCG1	PPARα/γ	13-HODE	RAW264.7 mouse leukemic macrophages	↑ Abcg1 and LXRα protein ↑ cholesterol efflux	[54]
	PPARγ	Pioglitazone	RAW264.7 mouse leukemic macrophages THP1 human macrophages	↑ ABCG1 mRNA and protein ↑ cholesterol efflux	[56]
		Rosiglitazone	THP1 human macrophages	↑ ABCG1 and LXRα mRNA ↑ cholesterol efflux	[57]
		Telmisartan	RAW264.7 mouse leukemic macrophages	↑ Abcg1 mRNA ↓ macrophage proliferation	[63]
BCRP/ABCG2	PPARα	Clofibrate	HCMEC/D3 human cerebral microvascular endothelial cells	↑ BCRP mRNA and protein ↑ mitoxantrone efflux	[75]
		GW7647	HCMEC/D3 human cerebral microvascular endothelial cells	↑ BCRP mRNA and protein	[75]
	PPARγ	Rosiglitazone	Primary human dendritic cells	↑ BCRP mRNA and protein ↑ Hoescht efflux ↑ mitoxantrone efflux ↑ sensitivity to mitoxantrone	[76]
			BCRP-overexpressing MDCKII canine kidney epithelial cells	↓ PhA efflux	[68]
			HuH7 human hepatoma cells	↑ BCRP mRNA	[68]
		Troglitazone	Primary human dendritic cells	↑ BCRP mRNA	[76]
			HuH7 human hepatoma cells	↑ BCRP mRNA	[68]
			Doxorubicin-resistant K562 human leukemia cells Doxorubicin-resistant MCF7 human breast cancer cells	↓ BCRP protein ↑ sensitivity to doxorubicin	[69]

Table 1: Continued.

ABC transporter	PPAR	PPAR Ligand	Cell line	Transporter effect	Reference
		GW7845	Primary human dendritic cells	↑ BCRP mRNA	[76]
		GW9662	Doxorubicin-resistant MCF7 human breast cancer cells	↓ BCRP protein	[69]

chemotherapy substrates as MDR1 [33]. Fasting-induced fatty acid release increased hepatic expression of Mdr2 mRNA and protein, as well as activity, in wild-type but not PPARα-knockout mice [77]. Similar results were observed in ciprofibrate-treated mice [73]. Interestingly, the latter trial demonstrated that elevated Mdr1 and Mdr3 mRNA expression accompanied Mdr2 induction in liver; however, in cultured mouse hepatocytes, only Mdr2 levels were elevated by PPARα agonists suggesting that *in vivo* induction of Mdr1 and Mdr3 may be influenced by PPARα activation in surrounding tissue. Furthermore, both ciprofibrate and clofibrate increased hepatic expression of Mdr2 mRNA in CF1 mice. This was associated with increased Mdr2 redistribution into bile canaliculi and enhanced biliary phospholipid secretion [78]. Similarly, in a chimeric mouse model with humanized liver, bezafibrate increased hepatic MDR2/MDR3 mRNA and protein, and promoted canalicular localization of the transporter [71]. Bezafibrate-treated HepG2 human hepatocellular liver carcinoma cells also showed elevated expression of MDR2/MDR3 mRNA. Although there was no subsequent change in protein levels, there was a redistribution of the transporter into pseudocanaliculi between cells, accompanied by enhanced apical localization of phospholipids, which could be attenuated by PPARα-specific knockdown [72].

Several MRP1 homologs may also be upregulated by PPARs, including MRP2/ABCC2, MRP3/ABCC3, and MRP4/ABCC4, which are known to transport substrates belonging to a variety of chemotherapy drug classes [33]. Although their normal physiological function remains elusive, it has been suggested that these transporters may play a role in MDR [79, 80]. Additionally, MRP4 expression may play a role in migration, as knockdown or pharmacological inhibition of this transporter appears to prevent human dendritic cell motility [81]. Moffit et al. examined the effect of clofibrate on hepatic transporters in mice. Following 10 days of dosing, clofibrate upregulated hepatic expression of Bcrp, Mrp3, and Mrp4 mRNA and protein in CD1 mice. Similar findings for Mrp3 and Mrp4 were detected in liver tissue isolated from clofibrate-treated wild-type SV129 mice, while no changes were seen in liver from similarly treated PPARα-knockout mice [82]. Liver expression of Mrp3 was also induced in C57BL mice treated with clofibrate, ciprofibrate, and diethylhexyl phthalate (DEHP) [83]. Maher et al. also reported the hepatic induction of Mrp3 and Mrp4 transcription in perfluorodecanoic-acid-(PFDA-) treated mice [84]. This was associated with elevated serum levels of serum-conjugated bilirubin and bile acids indicative of Mrp3- and Mrp4-specific hepatic efflux activity. These effects were attenuated in PPARα-knockout mice treated with PFDA. Several putative PPRE sequences were identified upstream of the Mrp3 and Mrp4 promoters, providing further evidence that PPARα may directly regulate transcription of these transporters in the liver.

Activation of PPARs may also induce expression of BCRP. PPARα agonists upregulate Bcrp transcription in mouse intestine [85]. Furthermore, PPARα-dependent activation induces BCRP expression and efflux activity in human cerebral endothelial cells [75]. Here, transporter induction is accompanied by binding of PPARα to a PPRE within the BCRP promoter. In human monocyte-derived dendritic cells, BCRP was directly induced by ligand-activated PPARγ through three functional PPRE sequences located within the gene's promoter [76]. This enhancement of BCRP activity elevated drug efflux and maintained intracellular low levels of mitoxantrone, which could be reversed by addition of a BCRP inhibitor. In doxorubicin-resistant MCF7 breast cancer and K562 human leukemia cell lines, troglitazone downregulated expression of BCRP, and restored sensitivity to doxorubicin treatment [69]. Although troglitazone may elicit effects that are PPARγ-dependent, it is also known to operate via pathways that are independent of this nuclear receptor [86]. Inhibition of PPARγ in untreated MCF7 cells reduced BCRP expression indicating that the observed effects of troglitazone were PPARγ-independent, and providing evidence that this TZD may suppress BCRP transcription in these cells by indirectly antagonizing PPARγ itself.

In contrast to the studies previously outlined, a number of reports indicate that PPAR activation may inhibit ABC transporter expression and activity. Chen et al. observed that troglitazone increased PPARγ activity and reversed Pgp-mediated chemoresistance in vincristine-resistant SGC7901 human gastric cancer cells [70]. Furthermore, Rajkumar and Yamuna performed genetic expression analysis on a doxorubicin-resistant 143B human osteosarcoma cell line and found increased expression of Pgp and Kruppel-like factor 2 [91]. Given that the latter is a known suppressor of PPARγ expression [92], these findings may implicate the PPARγ pathway as a negative regulator of Pgp transcription. Wang et al. also demonstrated that tumour necrosis factor (TNF)α could partially reverse MDR by inducing PPARα and suppressing Pgp in an adriamycin-resistant cell line derived from HepG2 cells [93]. In another study, PPARα agonists downregulated Mrp1 expression in mouse intestine [85]. Hepatic expression of Mrp2 protein was reduced in male Sprague-Dawley rats treated with the PPARα agonists, clofibrate, DEHP, and PFDA [89]. Furthermore, efflux of bile acids by Mrp2 may be suppressed by troglitazone in cultured rat hepatocytes [74]. Both rosiglitazone and troglitazone inhibited BCRP function in BCRP-overexpressing MDCKII

Table 2: *In vivo* effects of PPAR ligands on ABC transporters.

ABC transporter	Ligand	Receptor	Model	Transporter effect	Reference
ABCA1	PPARα	Fenofibrate	Hypertriglyceridemic patients	Differential HDL synthesis due to ABCA1 variants	[87]
		WY14643	SV129 mice	↑ Abca1 mRNA and protein in intestine ↓ intestinal absorption of cholesterol	[88]
	PPARγ	Telmisartan	ApoE−/− C57BL mice	↑ Abca1 mRNA in aorta ↓ atherosclerotic lesion size and number	[63]
Pgp/MDR1/ABCB1	PPARα	Ciprofibrate	SV129 mice	↑ hepatic Mdr1 & Mdr3 mRNA	[73]
MDR2/MDR3/ABCB4	PPARα	Bezafibrate	CF1 mice	↑ hepatic Mdr2 mRNA ↑ bile secretion of phospholipid	[78]
			Humanized liver-uPA/SCID chimeric mice	↑ hepatic MDR2/MDR3 mRNA and protein ↑ hepatic MDR2/MDR3 redistribution into bile canaliculi	[71]
		Ciprofibrate	SV129 mice	↑ hepatic Mdr2 mRNA and protein ↑ bile secretion of cholesterol and phospholipids	[73]
			CF1 mice	↑ hepatic Mdr2 mRNA ↑ Mdr2 redistribution into bile canaliculi ↑ bile secretion of phospholipid	[78]
		Clofibrate	CF1 mice	↑ hepatic Mdr2 mRNA ↑ Mdr2 redistribution into bile canaliculi ↑ bile secretion of phospholipid	[78]
		Fenofibrate	CF1 mice	↑ hepatic Mdr2 mRNA	[78]
		Gemfibrozil	CF1 mice	↑ hepatic Mdr2 mRNA	[78]
MRP1/ABCC1	PPARα	Ciprofibrate	C57BL mice	↓ hepatic Mrp1 mRNA	[83]
		Clofibrate	C57BL mice	↓ hepatic Mrp1 mRNA	[83]
		GW7647	C57BL mice	↓ Mrp1 mRNA in small intestine	[85]
		WY14643	C57BL mice	↓ Mrp1 mRNA in small intestine	[85]
MRP2/ABCC2	PPARα	Clofibrate	Sprague-Dawley rats	↓ hepatic Mrp2 protein	[89]
		DEHP	Sprague-Dawley rats	↓ hepatic Mrp2 protein	[89]
		PFDA	Sprague-Dawley rats	↓ hepatic Mrp2 protein	[89]
MRP3/ABCC3	PPARα	Ciprofibrate	C57BL mice	↑ hepatic Mrp3 mRNA	[83]
		Clofibrate	C57BL mice	↑ hepatic Mrp3 mRNA	[83]
			CD1 mice SV129 mice	↑ hepatic Mrp3 mRNA and protein	[82]

Table 2: Continued.

ABC transporter	Ligand	Receptor	Model	Transporter effect	Reference
		DEHP	C57BL mice	↑ hepatic Mrp3 mRNA	[83]
		PFDA	C57BL mice	↑ hepatic Mrp3 mRNA ↑ serum levels of bilirubin and bile acids	[84]
MRP4/ABCC4	PPARα	Clofibrate	CD1 mice SV129 mice	↑ hepatic Mrp4 mRNA and protein	[82]
		PFDA	C57BL mice	↑ hepatic Mrp3 mRNA ↑ serum levels of bilirubin and bile acids	[84]
ABCG1	PPARα	Fenofibrate	Zucker diabetic fatty rats	↑ Abcg1 mRNA ↑ HDL particle size	[90]
	PPARγ	Telmisartan	ApoE−/− C57BL mice	↑ Abcg1 mRNA in aorta ↓ atherosclerotic lesion size and number	[63]
BCRP/ABCG2	PPARα	Clofibrate	CD1 mice	↑ hepatic Bcrp mRNA and protein	[82]
			SV129 mice	↑ hepatic Bcrp mRNA	[82]
		GW7647	C57BL mice	↑ Bcrp mRNA in small intestine	[85]
		WY14643	C57BL mice	↑ Bcrp mRNA in small intestine	[85]

canine kidney epithelial cells, but induced its transcription in the HuH7 human hepatoma cell line [68]. These PPARγ activators also decreased Pgp-mediated drug efflux in doxorubicin-resistant P388 mouse leukemia cells. Moreover, fenofibrate suppressed Mdr1 transport activity in L-MDR1 porcine kidney epithelial cells [67]. Finally, in doxorubicin-resistant MCF7 and K562 cells, troglitazone downregulated expression of Pgp and reversed chemoresistance to doxorubicin [69]. However, among these studies it was not clarified if these activities were dependent on PPAR activation and signaling.

From the laboratory perspective, the involvement of ABC transporters in MDR and other cancer hallmarks necessitate these genes as vital targets of chemotherapy, whereas their precise role in the clinical manifestation of cancer remains elusive. This is likely why clinical trials with Pgp inhibitors failed to reduce drug efflux and subsequent chemoresistance [94]. Regulation of ABC gene transcription by PPARs may be another option, but primarily, a detailed understanding of the functional and clinical relevance of the entire ABC transporter family in tumour samples and cell lines is obligatory. Future studies may identify new roles for ABC transporters in cancer, which could be targeted by either pharmacological inhibition or regulation of PPARs. Most of the evidence implies that PPARs are positive regulators of cancer-related ABC genes, indicating that transporter expression can be suppressed by antagonizing PPARs. On the other hand, controversial findings have also been reported; therefore, improved understanding of the mechanism by which PPARs regulate ABC genes is required. In particular, delineating the effects of PPAR-dependent and -independent signaling on ABC gene transcription will determine the precise link between PPARs and ABC transporters in cancer and may predict the success of PPAR ligand therapy in reversing MDR. Additional studies exploring the effect of PPAR activation as an adjuvant to chemotherapy in a wide range of drug-resistant cancer cell lines may also prove insightful.

4. Atherosclerosis

The atherosclerotic condition is characterized by the thickening of arterial vessels as a result of an accumulation of oxidized low-density lipoproteins (LDL), and subsequently, cholesterol-laden macrophages as a consequence of a maladaptive immune response. The associated chronic inflammation and necrosis drives plaque formation and vessel hardening, which can invariably lead to coronary artery disease (CAD)—the leading cause of death worldwide [95]. Interestingly, recent evidence suggests that PPAR induction of ABC transporter expression may improve lipid profiles through enhanced cholesterol cycling and excretion, and thus represents a promising avenue to prevent cardiovascular disease progression.

As noted above, PPARα and PPARγ isoforms are also expressed in immune cells, such as mature macrophages, where they regulate genes involved in inflammation, differentiation, and TNF-α/IFN-γ-mediated apoptosis [96–98]. Expression of these two PPAR isoforms is also observed

in macrophage foam cells that constitute atherosclerotic lesions [20, 99–101]. Recent studies suggest activating PPARs exerts antiatherosclerotic properties via improved cholesterol homeostasis through the regulation of specific ABC transporters. ABCA1 is one such transporter that controls apolipoprotein-A1- (apoA1-) mediated cholesterol efflux in macrophages [102]. Another, ABCG1, also promotes the transport of cholesterol from macrophages to HDL, although the underlying mechanism remains unclear [103]. This efflux is a critical step in reverse cholesterol transport, a process that allows for cholesterol displacement and excretion by the liver, and represents a protective modality against atherosclerotic risk.

Activation of PPARγ stimulates apoA1-mediated cholesterol efflux from human and mouse macrophages and foam cells through a signaling cascade that culminates in ABCA1 induction [52, 57, 62]. This activity is mediated via PPARγ-dependent induction of liver X receptor (LXRα), an oxysterol-activated nuclear receptor, that triggers ABCA1 transcription via interaction with specific response elements in the ABCA1 promoter [104]. Although several putative PPRE sequences were initially identified in the LXRα promoter [105], only one was confirmed as a preferential PPARγ binding site in macrophages [57]. In addition, specific ligands for PPARα, PPARβ/δ, and PPARγ all increase LXRα and ABCA1 mRNA and protein and enhance apoA1-mediated lipid efflux and HDL synthesis in THP1 macrophages, suggesting that non-PPRE-dependent regulatory mechanisms may be responsible for some of these activities [47, 51]. In a similar study, THP1 macrophages treated with various PPAR ligands revealed that PPARβ/δ activation induced greater ABCA1 mRNA expression and apoA1-mediated cholesterol efflux compared to PPARα and PPARγ agonists [61]. Both rosiglitazone and pioglitazone treatment of THP1 macrophages also stimulated cholesterol efflux and induced ABCA1 mRNA and protein expression, implicating a regulatory role for PPARγ [56, 58, 59]. Correspondingly, treatment of mouse RAW264.7 macrophage-derived foam cells with conjugated linoleic acid (CLA) isomers (*c*9*t*11-CLA and *t*10*c*12-CLA) or the hydroxylated derivative of linoleic acid (13-HODE), known ligands of both PPARα and PPARγ, decreased cholesterol accumulation, enhanced cholesterol clearance, and induced expression of Abca1, and other genes involved in cholesterol homeostasis [54, 55]. Similarly, in other tissues, such as canine gallbladder epithelial cells, and human mesangial and skeletal muscle cells, PPAR activators upregulate LXRα-mediated ABCA1 transcription and prevent cholesterol accumulation [48, 53, 64].

Another PPARγ activator, telmisartan, induced Abca1 and Abcg1 expression in murine macrophages, and in the aorta of ApoE-deficient mice, where it suppressed macrophage proliferation and atherosclerotic progression [63]. It was also reported that the conditional deletion of PPARγ in macrophages led to decreased expression of LXRα, Abcg1, and ApoE in mice [106]. This was accompanied by a significant reduction in cholesterol efflux from macrophages to HDL. Furthermore, granulocyte macrophage colony-stimulating factor (GM-CSF) knockout mice showed reduced expression of PPARγ and Abcg1 in alveolar macrophages of the lung. Given that GM-CSF is a known positive regulator of PPARγ, reintroduction of PPARγ in alveolar macrophages increased Abcg1 expression and cholesterol efflux activity and decreased intracellular lipid content [107]. Consequently, PPARγ activation by pioglitazone induced cholesterol efflux activity and increased ABCG1 mRNA and protein in THP1 and RAW264.7 macrophages [56]. Fenofibrate also stimulated Abcg1 transcription, which was associated with increased HDL particle size, in Zucker diabetic fatty rats [90].

In the liver, ABCA1 is implicated in control of HDL synthesis, which represents another means of protecting against atherosclerosis. HDLs are specialized carrier molecules in the blood that transport cholesterol from peripheral tissues and cholesterol-laden macrophages to the liver for excretion [108]. This process is thought to be the main mechanism underlying HDL's antiatherosclerotic properties [109]. Indeed, plasma HDL levels correspond inversely with cardiovascular risk [110]. Consequently, impaired ABCA1 activity is associated with low plasma HDL, which is linked to Tangier disease, familial HDL deficiency, and accelerated atherosclerosis [111]. Furthermore, Abcg1-overexpressing transgenic mice have greater plasma HDL levels, improved cholesterol efflux from macrophages, and reduced atherosclerotic burden [112].

Several studies have demonstrated the ability of PPARs to regulate ABCA1 expression in the liver. In one study, PPAR activation with a variety of fibrates upregulated LXRα expression coupled with enhanced ABCA1 transcription and HDL biosynthesis in HepG2 cells [49]. Of the fibrates used, fenofibrate and LY518674 acted exclusively through PPARα, while bezafibrate and gemfibrozil preferred PPARγ and PPARβ/δ, respectively, in addition to PPARα activity. Accordingly, antagonism of PPARγ in HepG2 cells blocked upregulation of ABCA1 mRNA and protein; however, PPARγ activation also reduced ABCA1 protein levels in this cell line despite increased ABCA1 transcription [60]. In this model, activation of PPARγ caused the dissociation of LXRβ from ABCA1 at the cell membrane leading to increased ABCA1 protein degradation. Subsequently, translocation of LXRβ to the nucleus increased ABCA1 transcription via binding of this nuclear receptor to the promoter region of the ABCA1 gene. Whether this affected HDL biosynthesis or cholesterol efflux from HepG2 cells remains to be seen.

Fasting-associated fatty acid release induces hepatic expression of Abca1, Abcg5, and Abcg8 in wild-type but not PPARα-null mice [77]. Although these ABC transporters are involved in hepatobiliary cholesterol transport, maximal cholesterol excretion from the liver was decreased by ~50% after fasting. This raises the possibility of other PPARs and PPAR agonists playing a role in ABC transporter-mediated liver cholesterol efflux under normal conditions. More recently, a clinical trial examined the effect of fenofibrate treatment on HDL subclass particle concentrations on patients with triglycerides $\geq$150 mg/dL [87]. Following 3 weeks of therapy, stratification of participants by ABCA1 polymorphism genotypes revealed two variants (R1587K and R219K) that were associated with significant increases

in small HDL particles. This suggests a synergism between ABCA1 polymorphism and PPARα agonists.

One of the most intuitive ways to reduce the burden of atherosclerosis is to regulate the uptake of dietary cholesterol at the intestine. In mice, intestinal expression of Abca1 and Abcg8 is induced upon fasting [113]. Furthermore, normal mice maintained on a diet supplemented with a PPARα activator showed an increase in intestinal Abca1 gene transcription and protein compared to PPARα-deficient mice, which showed no effect to treatment [88]. This increased expression was associated with a reduction in cholesterol absorption, as well as decreased plasma and liver cholesterol concentrations.

Atherosclerotic heart disease is undoubtedly one of the most devastating diseases worldwide. While pharmacological and dietary interventions that lower LDL levels remain the current treatment paradigm for atherosclerosis, they may only decrease the incidence of cardiovascular events by ~30% [109]. The literature indicates that induction of ABCA1 and ABCG1 expression by PPAR activation may play a role in preventing atherosclerosis by improving cholesterol homeostasis and HDL synthesis. Moving forward, additional studies are required to address the clinical significance of these activities and to determine whether or not they are PPAR dependent. Clinical trials have begun to examine the effect of some PPAR activators in atherosclerosis, yielding a mixture of results. For example, fenofibrate treatment barely increased HDL levels and marginally lowered the incidence of CAD in high-risk patients with type 2 diabetes [114, 115]. In a similar study, gemfibrozil significantly reduced CAD, in part, by elevating HDL [116]. Studies have also demonstrated that TZDs promote the destabilization of atherosclerotic plaques in nondiabetic patients [117], while still others report that these PPAR activators may actually increase the risk of heart failure in type 2 diabetics [118]. Despite these findings, a better understanding of the pleiotropic effects of PPARs and their role in atherosclerosis is required in order to design and develop appropriate PPAR-based therapies devoid of detrimental effects.

5. Ichthyosis

Derived from the Greek *ichthys* for "fish," ichthyosis refers to a group of dermatological disorders generally described by severely dry, cracked, and flaky skin that is thought to bear resemblance to fish scales [119]. The main pathophysiological feature of this disease is a failure of skin barrier permeability, leading to a spectrum of conditions ranging from the most mild, such as the common ichthyosis vulgaris, to the most severe, such as Harlequin type ichthyosis, which is rare but fatal in newborns. Recently, mutations in ABCA12, a keratinocyte lipid transporter, were shown to underlie the latter phenotype [120, 121]. Under normal conditions, ABCA12 facilitates the uptake of lipids into specialized secretory granules, called lamellar bodies, within keratinocytes. These lipid-filled granules are then liberated from the cell where they release their cargo to the outermost layer of the epidermis, a requirement for normal formation of skin barrier permeability. On the other hand, ABCA12 deficiency prevents lipid loading into lamellar bodies, which leads to abnormal development of the skin and strikingly elevated rates of prenatal mortality [122].

While studies in this area are limited, they have demonstrated that ABCA12 may be regulated by PPARs, which may have important implications in Harlequin ichthyosis. Activation of PPARs promotes lamellar body secretion and improved epidermal barrier permeability in mice [123]. More recently, Jiang et al. demonstrated that ciglitazone, troglitazone, and the PPARβ/δ agonist, GW610742, induced expression of ABCA12 mRNA and protein in human keratinocytes [65]. Similarly, ceramide-induced transcription of ABCA12 was attenuated by siRNA knockdown of PPARβ/δ, indicating that this activity was dependent on PPARβ/δ [66]. In a separate experiment, Jiang et al. also demonstrated that clofibrate and the PPARβ/δ ligand, GW501516, increased expression of the ABCA1 cholesterol efflux pump in human keratinocytes [50]. Given that these cells require cholesterol for adequate formation of permeability barrier function [124], ABCA1 regulation by PPARs may also play an important role in understanding the pathophysiology of Harlequin ichthyosis. These findings implicate the potential utility of PPAR ligands for the treatment of this disease, which should be further validated *in vivo*.

6. Conclusion

These studies describe compelling evidence for PPAR medicines in the regulation of ABC transporter expression and function. Beyond their respective individual roles in various human diseases, the overlap in tissue distribution and regulatory potential between PPARs and certain ABC transporters make this emerging story an attractive field for further research. They also provide an alternative approach when the targeting of ABC transporter genes in human cancer, atherosclerosis, or ichthyosis may suggest therapeutic advantages for patients. In addition, targeting ABC transporters at the transcriptional level may circumvent issues previously identified during focused inhibition of transporter activity. Furthermore, given the complex and multistage etiology of cancer and atherosclerosis, dual/pan PPAR modulators may prove especially useful in simultaneously regulating multiple PPAR isoforms and ABC transporters. For example, examining PPARα/γ agonists like aleglitazar, currently being assessed for cardiovascular safety in Phase 3 clinical trials, for synergistic effects on multiple ABC transporters may prove a fruitful area for future studies. Improving our understanding of the interactions between PPARs, their ligands, and ABC transporters will further aid in developing more targeted therapeutic strategies to mitigate the burden of human disease on patients and the healthcare system.

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 946943, 13 pages
doi:10.1155/2012/946943

Review Article

The Key to Unlocking the Chemotherapeutic Potential of PPARγ Ligands: Having the Right Combination

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Received 3 February 2012; Accepted 14 March 2012

Academic Editor: Yuji Kamijo

Despite extensive preclinical evidence that peroxisome proliferator-activated receptor (PPAR)γ activation protects against tumourigenesis, results from a few clinical trials using PPARγ ligands as monotherapy show modest success. In spite of this, several groups reported exciting results with therapeutic regimens that combine PPARγ ligands with other compounds: chemotherapeutic agents, retinoid x receptor (RXR)α agonists, statins, or cell-to-cell signaling molecules in preclinical cancer models and human trials. Here we have compiled an extensive review, consolidating the existing literature, which overwhelmingly supports a beneficial effect of treating with PPARγ ligands in combination with existing chemotherapies versus their monotherapy in cancer. There are many examples in which combination therapy resulted in synergistic/additive effects on apoptosis, differentiation, and the ability to reduce cell growth and tumour burden. There are also studies that indicate that PPARγ ligand pretreatment overcomes resistance and reduces toxicities. Several mechanisms are explored to explain these protective effects. This paper highlights each of these studies that, collectively, make a very strong case for the use of PPARγ ligands in combination with other agents in the treatment and management of several cancers.

1. Introduction

Cancer is the leading cause of death worldwide, with the projected number of associated deaths continuing to rise to an estimated 13.1 million people by 2030 [1]. For any given tumour, a concerted evaluation of type, stage, location, and size at the time of diagnosis influence the selection of one or more available treatment interventions, including surgery, radiotherapy, chemotherapy, or combinations as appropriate. Accordingly, improved understanding of how chemotherapeutic interventions can be optimized will assist with cancer prevention, as well as treatment and care of cancer patients.

Though many single agent treatments of solid or hematologic tumours are effective, they often select for resistant cells, and ultimately recurrent tumours, which no longer respond to the initial therapy [2]. To minimize the development of resistance, researchers and clinicians have expanded the use of combination drug therapies for some time. This approach favours combining individual classic chemotherapeutic agents aimed at forming new optimized regimens with additive/synergistic protective effects [3–5]. Of course, these combinations must also be chosen wisely to avoid similar synergism in toxicity. To achieve maximal chemotherapeutic potential and satisfy the previous conditions, many groups have explored combinations of traditional chemotherapies with the growing arsenal of targeted pathway-specific drugs [6], including those that activate an emerging target *peroxisome proliferator-activated receptor (PPAR)*γ. This paper is a review of the vast *in vitro, in vivo,* and human clinical trial studies, irrespective of cancer type, using chemotherapeutic combinations that include PPARγ-activating drugs. The aims are to evaluate the novel chemotherapeutic potential of PPARγ-activating drugs and provide

a guide for further basic and clinical research, in order to optimize chemotherapeutic interventions that will reduce the number of cancer-related deaths worldwide.

PPARγ is a candidate tumour suppressor gene and member of the nuclear receptor superfamily [7]. The gene encodes two isoforms, PPARγ1 and PPARγ2, derived from alternative splicing, which are preferentially expressed in nonadipogenic cell types and cells committed to the adipocyte lineage, respectively [8–10]. PPARγ normally associates with the retinoid X receptor (RXR)α and the resulting PPARγ; RXRα complex recognizes direct-repeat- (DR-) 1 motifs, referred to as peroxisome proliferator response elements (PPREs), in the promoters of target genes [11]. Complexed PPARγ is activated by ligands which include synthetic thiazolidinediones such as the gold standard activator rosiglitazone (ROSI) [12], used widely for >10 years to treat and prevent type II diabetes [13], as well as pioglitazone (PIO), troglitazone (TRO), ciglitazone (CIG), and many natural fatty acids and fatty acid metabolites, such as linoleic acid and signaling molecules like 15-deoxy-$D^{12,14}$-prostaglandin J_2(15d-PGJ_2) [14].

PPARγ ligands are reported to exert antitumourigenic properties *in vitro* and to induce tumour growth arrest or shrinkage in murine *in vivo* models [15–19]. Based on this, a few clinical trials have been performed to evaluate the effectiveness of PPARγ ligands in human cancer. In the most successful of these trials, three patients with advanced unresectable myxoid and pleiomorphic liposarcoma were treated with TRO. Serial biopsies revealed increased lipid accumulation, indicative of adipocyte differentiation, and a 2- to 4-fold decrease in the percentage of cells expressing the Ki-67 antigen, a marker of proliferation [20]. Unfortunately, further monotherapy trials using PPARγ ligands on more common epithelial-based cancers have not been as fortuitous. In separate phase II clinical trials, 22 women with refractory breast cancer and 25 patients with advanced colorectal cancer, respectively, treated with TRO experienced no objective tumour responses [21, 22]. Similarly, ROSI treatment did not prolong time to disease progression compared to placebo in 106 men with prostate carcinoma [23] or affect proliferation in breast tumours during a short pilot study [24].

Despite the limited success as a monotherapy, PPARγ agonists have shown tremendous potential for clinical utility when combined with traditional chemotherapeutics, RXRα ligands, statins, and cellular signaling molecules. Substantial evidence suggests that activating PPARγ synergistically enhances the protective effects of these agents, reduces their inherent toxicity, and even, in some cases, overcomes resistance. A summary of the preclinical and clinical work combining PPARγ ligands with various other compounds is provided in Tables 1 and 2, respectively. Extensive literature searches were performed using the US Library of Medicine and National Institute of Health's http://www.ncbi.nlm.nih.gov/pubmed/ for papers using treatment regimens that combined PPARγ agonists with other therapeutic agents. Any errors by omission are unintentional.

2. Chemotherapeutic Agents

2.1. Platinum Compounds. Platinum-based compounds have been widely used as chemotherapeutics since the 1970s to treat cancers of the breast, lung, ovary, testis, head, and neck [25]. These agents exert their cytotoxic effects by cross-linking DNA, which impairs DNA transcription and replication [26]. This damages cells which invoke DNA repair mechanisms and, when those fail, apoptosis [27]. Cisplatin, the first such compound available, is an extremely effective chemotherapeutic, although dosing is limited due to the associated risk of nephrotoxicity [28, 29]. Second and third generation drugs, carboplatin and oxaliplatin, are less damaging to kidneys but are associated with severe neuropathies [30]. PPARγ ligands in combination with platinum-based compounds have increased therapeutic efficacy, overcome resistance, and decreased toxicity in multiple cancer models.

Several cancer cell lines, including A549, Calu1, H23, H596, and H1650 non-small-cell lung cancer (NSCLC), Mosher colon cancer, and OVCA420, OVCA429, and ES2 ovarian cancer cells have demonstrated the synergy of combination treatment with platinum-based compounds and therapeutic doses of ROSI. These cells exhibited greater growth reduction, G2-M arrest, and increased apoptosis when treated with the combination than either agent, ROSI or chemotherapeutic, alone. *In vivo* xenograft mouse models using A549 lung cancer cells also suggest synergy, as low doses of ROSI and carboplatin reduced xenografted tumours to one-third the size of tumours from monotherapy controls [31]. In a separate study, ROSI pretreatment resulted in maximum reduction in mammary tumour volume when combined with cisplatin compared to treatment with cisplatin alone. The mammary tumours from cotreated mice also exhibited more glandular structures suggesting improved differentiation, an indication of less aggressive tumours which, clinically, would have a better prognosis [32]. Interestingly, another study, using TRO in combination with cisplatin in A549 and H522 non-small-cell lung cancer cells, found synergistic effects when TRO treatment followed cisplatin treatment but not vice versa, suggesting that the beneficial effects of PPARγ activation might depend on the sequence of drug administration [33]. The combinational regimen may also be effective to treat malignant pleural mesothelioma as TRO and cisplatin have an additive effect on EHMES-10 cells *in vitro* as well as tumour growth reduction and overall survival in xenograft mouse models, compared to either agent singularly in an animal model [34].

Many tumours, including ovarian and non-small-cell lung, that are initially responsive to platinum-based compounds eventually develop resistance [35]. The accruing resistant tumours grow unabated and are associated with poor prognosis [36]. Resistant tumours use multiple survival strategies including altered drug-uptake pathways, which prevent platinums from reaching DNA, or decreased DNA damage recognition and apoptosis network signaling [26].

Interestingly, combination treatment with PPARγ activators may be able to overcome this resistance. In one study, mice with EGFR- and K-Ras-driven lung adenocarcinomas, a model of platinum-resistant lung cancer, were treated with carboplatin, ROSI, or both. Neither monotherapy reduced tumour burden; however, combination therapy resulted in 80% reduction in tumour volume [57]. Microarray analysis from a separate study revealed that ROSI treatment reduces

Table 1: Synergistic effects between PPARγ ligands and other agents *in vitro*. Descriptions reflect most noteworthy finding of each study.

	Platinum-Based Compounds	Taxanes	Topoisomerase Inhibitors	Anti-metabolites	RXRα ligands	Cell Signalling Molecules	Statins
Rosiglitazone	(i) ↓ Cell growth in A549, Calu1, H23, H596 and H1650 NSCLC; Mosher colon; and OVCA420, OVCA429 and ES2 ovarian cancer [31]			(i) ↓ Cell viability in BEL-7402 and Huh-7 Hepatocellular carcinoma [37] (ii) ↑ Apoptosis in HT-29 colon cancer [40]	(i) ↑ Differentiating and growth-inhibitory effects in lymphoid Su-DHL, Sup-M2, Ramos, Raji, Hodgkin's cell lines, and primary chronic lymphocytic (lymphoid), and U937 and HL-60 (myeloid) leukemia [38] (ii) ↓ Cell viability in MCF-7, MCF-7TR1, SKBR-3, and T-47D breast cancer [41]	(i) ↑ Growth inhibition in MDA- MB-231 breast cancer [39] *pre-treatment	
Troglitazone	(i) ↑ Growth inhibition in A549 and H522 NSCLC [33] *post-treatment (ii) ↑ Growth inhibition in EHMES-10 mesothelioma [34]	(i) ↑ Growth inhibition in A549 and H522 NSCLC [33] *post-treatment			(i) ↑ Growth inhibition in MCF-7, T-47D, ZR-75-1 breast cancer [42]	(i) ↓ Cell numbers in HEY ovarian cancer [43] (ii)↑ Apoptosis in MCF-7, SKBR-3, and MDA-MB-453 breast cancer [44]	
Pioglitazone		(i) ↑ Growth inhibition in A549 and H522 NSCLC [33] *post-treatment			(i) ↑ Differentiation in harvested liposarcoma cells [45]		(i) Cytotoxic effects in U87, U138, LN 405 and rat RG II glioblastoma [46] (ii) ↑ Cytotoxic effects in IOMM Lee and KT21-MG1 meningioma [47]

Table 1: Continued.

	Platinum-Based Compounds	Taxanes	Topoisomerase Inhibitors	Anti-metabolites	RXRα ligands	Cell Signalling Molecules	Statins
Ciglitazone					(i) ↑ Growth inhibition in ZR-75-1 and T-47D breast; and Calu-6 lung cancer [48] (ii) Apoptosis in ANBL6 and 8226 multiple myeloma [50]; C6, U87MG andGL261 glioblastoma [51],and G361 melanoma [50, 52]	(i) ↓ Proliferation in HEY ovarian cancer [43]	(i) ↑ Cytotoxic and cytostatic effects in Panc 02 and MIA PaCa-2 pancreatic; C-26 colon; and EMT6 and MDA-MB-361 breast cancer [49]
RS5444		(i) ↑ Antiproliferative effects in DRO90-1 and ARO81 anaplastic thyroid carcinoma [53]					
15d-PGJ2		(i) ↑ Cytotoxicity in A549 and H460 NSCLC [54]	(i) ↑ Cytotoxicity in Cak-2 renal cell carcinoma [55]		(i) ↑ Differentiating and growth-inhibitory effects in Su-DHL, Sup-M2, Ramos, Raji, Hodgkin's cell lines, and primary chronic lymphocytic (lymphoid), and U937 and HL-60 (myeloid) leukemia [38] (ii) ↑ Apoptosis in ANBL6 and 8226 multiple myeloma [51]	(i) ↑ Growth inhibition in HEY ovarian cancer [43]	
LY 293111			(i) ↑ Cytotoxicity in MCF-7, MCF-7/adr and SKBR-3 breast; H460 lung; SW480 and RT4 colon; and HT1197 bladder cancer [6]				
Linoleic Acid					(i) ↑ Apoptosis and ↓ proliferation in MCF-7, MDA-MB-231, MDA-MB-468, T-47D and SKBR-3 breast cancer [56]		

Table 2: *In vivo* and clinical trials synergistic effects between PPARγ ligands and other agents. Descriptions reflect most noteworthy finding of each study.

	Platinum-based compounds	Taxanes	Topoisomerase inhibitors	Anti-metabolites
Rosiglitazone	(i) ↓ Volume of A549 NSCLC xenografted tumours [31] (ii) ↓ Volume in KRAS- and EGFRdriven lung tumours without disrupting immune system [57] (iii) ↓ Volume and ↑ differentiation of DMBA-induced breast tumours; treatment minimized nephrotoxicity [32] *pre-treatment			
Troglitazone	(i) ↓ Volume and ↑ overall survival of EHMES-10 malignant pleural mesothelioma xenografted tumours [34]			
Pioglitazone				*Phase 2 clinical trial:* (i) 30% of patients with high grade gliomas experienced disease stabilization; treatment well tolerated by all [58]
RS5444		(i) ↓ Volume of DRO90-1 and ARO81 anaplastic thyroid carcinoma xenografted tumours [53]		
15d-PGJ_2		(i) ↓ Volume of A549 and H460 NSCLC xenografted tumours [54]		
LY 293111			*Phase 1 clinical trial*: (i) ↓ GI toxicity in patients with advanced solid tumours [59]	(i) ↓ Volume of S2-013 pancreatic xenografted tumours; minimized side effects [60]

expression of five members of the metallothionein gene family [31]: metal-binding proteins that play a crucial role in platinum-drug resistance by sequestering platinum compounds outside the cell [61].

In addition to developed resistance, platinum-based compounds are associated with several morbidities, including nephrotoxicity, myelosuppression, and GI complications [26]. Given this, and the potential for an additional drug, in this case a PPARγ ligand, to exacerbate the inherent toxicity of platinums, the authors of the aforementioned lung adenocarcinoma study conducted extensive toxicological analysis on their treated mice. Fortunately, compared to monotherapy, combination therapy did not decrease markers of immune function, white blood cell counts, or hematocrit, and BUN and creatinine levels, indicative of kidney damage, were similarly unaffected [57].

Nephrotoxicity, experienced by 28–36% of patients after a single injection of cisplatin [62], may be, in part, exacerbated by TNF-α, a well-known mediator of inflammation [40]. Interestingly, PPARγ activators reduce inflammatory responses [63, 64]. Therefore, Tikoo et al. used a DMBA-induced murine breast cancer model to evaluate the ability of ROSI to decrease nephrotoxicity. They found that ROSI pretreatment significantly decreased circulating BUN, creatinine and TNF-α, and minimized tubular damage, suggesting that PPARγ activation ameliorated the nephrotoxicity associated with cisplatin treatment [32]. If this holds true in humans, ROSI treatment may allow physicians to use platinum-based compounds at higher, previously toxic, doses that may confer additional therapeutic benefit.

2.2. Taxanes. Taxanes, including paclitaxel and docetaxel, are commonly used chemotherapy agents for a large array of cancers which include ovarian, lung, head and neck, esophageal, breast, prostrate, and gastric cancers. Taxanes exert their effects by binding and immobilizing microtubules which prevents cell division [65]. There are multiple side effects associated with taxanes including reduced hematocrit, neuropathy, and myalgias/arthralgias [66].

A novel high-affinity PPARγ agonist, and thiazolidinedione derivative, RS5444, demonstrated additive antiproliferative activity on DRO90-1 and ARO81 anaplastic thyroid carcinoma cells, a particularly aggressive and dedifferentiated cancer [67]. RS5444 did not induce apoptosis by itself; however, when combined with paclitaxel, the apoptotic fraction of cells doubled. Using IC_{25} values experimentally derived from *in vitro* experiments, the group found that combination treatment with RS5444 and paclitaxel significantly reduced xenograft tumour volumes compared to either monotherapy alone [53].

Non-small-cell lung cancer is a leading cause of death from malignant disease in industrialized nations with a 5-year survival rate of approximately 15% [68, 69]. Novel therapeutic regimens involving PPARγ activators and traditional chemotherapeutics have shown some promise that they may someday improve this rate. An *in vitro* study indicated synergy between multiple PPARγ ligands (TRO and PIO) and paclitaxel in A549, H522 non-small-cell lung cancer cells that was dependent upon treatment order, with paclitaxel preceding TRO treatment [33]. Another group confirmed the synergistic effect of combining PPARγ activation with, this time, docetaxel. In this study, 15d-PJ_2 increased cytotoxicity in A549 and H460 cells *in vitro*. Extending this, they found that 15d-PJ_2 and docetaxel reduced A549 and H460 xenografted tumour volumes by 72%, nearly double the effect of docetaxel alone [54].

2.3. Topoisomerase Inhibitors. Both classes of topoisomerase inhibitors, type I (including irinotecan) and type II, work by binding and incapacitating topoisomerases: enzymes that are critical for DNA supercoiling and strain relief [70]. Ultimately, this binding prevents movement of the DNA replication fork which induces stress responses that can lead to apoptosis or the involvement of DNA damage repair mechanisms [71]. A topoisomerase I inhibitor, irinotecan, has demonstrated activity against a vast range of cancers [72] but is associated with significant GI toxicity and myelosuppression [73]. Budman and Calabro have shown synergistic cytotoxic increases in a variety of cell lines (MCF-7, MCF-7/adr, and SK-BR-3 breast cancer; H460 lung cancer; SW480 and RT4 colon cancer; HT1197 bladder cancer) between irinotecan and the PPARγ ligand LY293111 at clinically attainable doses [6], prompting human studies with this drug combination. To date, a phase I clinical trial has established a dosing schedule that minimized adverse GI events associated with LY293111 and irinotecan [59]. Another topoisomerase I inhibitor, camptothecin, enhanced the cytotoxicity of 15d-PGJ_2 in Cak-2 renal cell carcinoma cells. Interestingly, the authors did not find synergy when 15d-PGJ_2 was combined with other chemotherapeutics including doxorubicin, 5-FU, and cisplatin [55]. This synergism may allow clinicians to reduce the dose of topoisomerase inhibiting agents and thereby reduce associated toxicity, by combining treatment with PPARγ ligands.

2.4. Antimetabolites. Antimetabolites, including 5-Fluorouracil (5-FU), methotrexate, and others, are structurally similar compounds to vitamins, amino acids, or nucleic acid precursors which become incorporated into cellular macromolecules with disastrous consequences for cells such as inhibition of cell growth and division [74]. They have been used to treat several types of cancer including leukemia, breast, and ovarian but have been associated with myelosuppression, dermatitis, and diarrhea [75]. A phase II clinical trial was undertaken to evaluate the role of capecitabine, a precursor to 5-FU, in combination with PIO to treat recurrent high-grade gliomas. Only 29% of patients experienced disease stabilization after three months; however, the regimen was well tolerated by patients indicating potential for future therapeutic utility [58].

Hepatocellular carcinoma (HCC) and colorectal tumours are among the leading forms of cancer contributing to cancer-related deaths [69, 76]. HCC usually requires chemotherapy because tumours are often surgically unresectable due to advanced stage at diagnosis [77]. Treatment of both diseases often involves 5-FU; however, patients often respond

poorly as tumours develop multiple drug resistance [78–80] due to multiple mechanisms including increased drug efflux [81]. Interestingly, PPARγ may regulate ABC transporters, key proteins involved in drug efflux [82]. Accordingly, activation of PPARγ with ROSI, in combination with 5-FU treatment, has been evaluated in HCC and colon cancer. ROSI treatment decreased cell viability in two HCC cell lines (BEL-7402 and Huh-7) by 4- and 2-fold, respectively, compared to treatment with 5-FU alone. The authors also used siRNA to show that this effect was dependent on PPARγ [37]. Another group evaluated ROSI treatment with 5-FU in HT-29 colon cancer cells and found that ROSI treatment, at a low dose that did not affect proliferation or cell growth, enhanced 5-FU-induced apoptosis. Again, this effect was PPARγ dependent as it was ameliorated by the PPARγ antagonist GW9662 [83].

Another antimetabolite, gemcitabine, is a useful chemotherapeutic that arrests cell growth in multiple ways including incorporation into DNA and impeding cell division [84]. Gemcitabine is standard therapy for pancreatic cancer, a disease with a strikingly poor prognosis as most patients die within six months of diagnosis [85]. Gemcitabine only modestly prolongs survival but is useful as a palliative agent for several cancer-related morbidities. Hennig et al. evaluated the ability of the PPARγ activator LY293111 to enhance the activity of gemcitabine in an orthotopic pancreatic cancer model. Consistent with previous models, both gemcitabine and LY293111 significantly inhibited tumour growth and reduced the incidence of liver metastasis; however, the combination was more effective than either therapy alone. Furthermore, combination treatment maintained stable body weights, relieved tumour-induced cachexia, and decreased incidence of bowel obstruction [60]. This suggests that this combination may be effective, to not only treat aggressive pancreatic adenocarcinomas but also relieve side effects associated with monotherapy [86].

2.5. RXRα Ligands. The PPARγ binding partner, RXRα, is also a member of the nuclear receptor superfamily. RXR has three subtypes (α, β, and γ), which are activated by retinoids, a group of vitamin A analogues. After ligand binding, RXRα is able to modulate gene expression by binding retinoid X receptor responsive elements (RXREs), present in the promoter regions of target genes. Similar to PPARγ, RXRα activation profoundly affects multiple cellular activities that are pertinent to cancer including cellular growth, differentiation, apoptosis, and morphogenesis [87, 88].

Given this, multiple groups have investigated the combined use of PPARγ and RXRα ligands. The first report, from Tontonoz et al., indicated that simultaneous treatment of liposarcoma cells, selected from freshly harvested tumours, with both RXRα- and PPARγ-specific ligands, synergistically stimulated differentiation. Additionally, the authors showed that PPARγ is highly expressed in the major histological types of liposarcoma, suggesting that PPARγ-targeting agents, especially combined with RXRα ligands, may be useful therapy for human liposarcoma [45].

Since that time, beneficial effects have been reported for several types of malignancies, including hematological, breast, and lung cancer, for the combined treatment of PPARγ ligands and retinoids. Konopleva et al. reported that PPARγ is expressed in lymphoid (Su-DHL, Sup-M2, Ramos, Raji, Hodgkin's cell lines, and primary chronic lymphocytic leukemia) and myeloid (U937 and HL-60) cell lines, several of which undergo apoptosis when treated with PPARγ ligands including ROSI and 15d-PGJ_2. The apoptotic effects of PPARγ ligands were enhanced when combined with an RXRα agonist, LG100268, as reflected by mitochondrial depolarization and caspase activation [38]. Similarly, Ray et al. showed that PPARγ is expressed in ANBL6 and 8226 human multiple myeloma cell lines and that PPARγ ligands induce apoptosis, an effect which is enhanced by combination with 9-cis retinoic acid, a ligand of RXRα [51]. Elstner et al. found that PPARγ ligands were potentiated by RXRα ligands in multiple breast cancer cells (MCF-7, T-47D, ZR-75-) [42], work that was later confirmed by multiple groups [41, 56] including one study that also found protective effects in Calu-6 lung cancer cells [48]. One of these studies showed that combination treatment with ROSI and the RXRα ligand 9-cis retinoic acid inhibited cell viability in MCF-7, MCF-7TR1, SKBR-3, and T-47D breast cancer cells but did not affect MCF-10a normal immortalized breast epithelial cells. This exciting work suggests that the cytotoxic effect maybe specific to cancer cells. Mehta et al. took this approach into mouse models and found that the combination of LG10068, an RXRα-specific ligand, and TRO completely inhibited development of mammary tumours at micromolar concentrations in a DMBA-induced breast tumourigenesis model [17].

Many potential mechanisms are postulated to explain the synergistic protective effects of PPARγ and RXRα ligands. The protective effect at the whole body level may, in part, be mediated at the transcriptional level by the ability of PPARγ and RXRα ligands to inhibit aromatase secretion [89], enhance expression of glutathione S-transferases (GSTs) [90], or downregulate expression of matrix metalloproteinases (MMPs) [52]. Aromatase catalyzes the rate-limiting step in estrogen biosynthesis [91], which drives breast tumourigenesis by stimulating proliferation of breast tumour cells [92]. GSTs have multiple functions including the detoxification of several xenobiotics and carcinogens [93]. MMPs degrade extracellular matrix proteins carving pathways for migrating cancer cells and releasing sequestered growth factors [94]. Combined RXRα ligand and CIG treatment decreases cell growth and the invasive potential in G361 melanoma and U87MG glioblastoma cells by decreasing expression of matrix metalloproteinases [52].

Other groups have theorized that the synergistic effects of PPARγ and RXRα ligands may not be directly related to transcriptional effects. Ligands of PPARγ and RXRα recruit different subsets of transcriptional coactivators [95]; therefore, cotreatment may increase transcription as there is less competition [96]. Additionally, PPARγ activity may enhance proteosome inhibitors, allowing for RXRα accumulation and the enhancement of PPARγ : RXRα-mediated transcription [97]. Collectively, this work suggests that combining agents that activate both PPARγ and RXRα could synergistically enhance the protective effects of either agent alone.

3. Cell Signaling Molecules

Protective synergy with PPARγ ligands is not exclusive to traditional chemotherapeutic agents or RXRα ligands. There are a few reports of PPARγ activators combining with normal cell signaling molecules, including tumour necrosis factor (TNF)α, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), and Heregulin to confer an additive or synergistic protective effect. TNFα is a cytokine, chiefly produced by activated macrophages, that is involved in systemic inflammation, and leads to tumour regression [98, 99]. TRAIL, a member of the TNF family, induces apoptosis by binding receptors and recruiting the Fas-associated death domain and caspase-8, triggering apoptosis [100]. Heregulin is a soluble secreted growth factor that activates several classic tumourigenic signal transduction pathways including PI3K/Akt, Ras/MAPK, and JNK [101].

Based on evidence that ROSI upregulates p53 and p21, Mody et al. examined the ability of ROSI pretreatment to sensitize MDA-MB-231 breast cancer cells to therapies that act on these apoptosis/cell death pathways, such as TNFα. ROSI pretreatment dramatically increased TNFα-mediated growth inhibition by 9-fold versus control TNFα or ROSI alone. The authors also performed microarray analysis to evaluate genetic changes associated with ROSI treatment [39]. This may be a valuable tool to predict other agents which synergize with PPARγ ligand activity based on shared pathway utilization.

Partridge and Barnes evaluated the ability of multiple PPARγ ligands (CIG, TRO, and 15d-PGJ_2) to enhance the efficacy of TRAIL in a drug-resistant ovarian cancer cell line. Drug resistance is a serious problem in ovarian cancer, especially in advanced disease, where survival rates fall to 10–30% [102]. The combined treatment with CIG and TRAIL synergistically reduced proliferation in multiple cell lines, most notably the paclitaxel-resistant HEY ovarian cancer subclone. TRO treatment showed no effect on proliferation on its own; however, when combined with TRAIL, that reduced cell numbers in etoposide-, pemetrexed-, cisplatin-, docetaxel-, and gemcitabine-resistant cell lines. Similarly, 15d-PGJ_2 treatment inhibited growth in all cell lines, especially the HEY cell line which was developed by the authors [43].

Park et al. showed that Heregulin, which paradoxically drives tumourigenesis [103, 104], synergistically increases TRO-mediated breast cancer cell apoptosis and necrosis *in vitro* [44]. In light of previous reports that Heregulin plays a causal role in Tamoxifen- and Gefitinib-resistant breast cancer [105], Park's work provides evidence that combination therapy with Heregulin and PPARγ-activators may be a novel strategy for the treatment of resistant or refractory breast cancer [44].

4. Statins

Statins are another important class of drugs acting as inhibitors of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, a critical rate-limiting enzyme in cholesterol biosynthesis. Statins are commonly used to manage hypercholesterolemia and cardiovascular diseases and are some of the most frequently prescribed therapeutics for elderly patients. Recently, statins were evaluated for their protective effects in cancer and showed antiproliferative and pro-apoptotic effects *in vitro* [106–108]. Incubation with lovastatin and CIG for 48hrs exerted additive cytotoxic and cytostatic effects in multiple cancer cell lines (Panc 02 and MIA PaCa-2 pancreatic cancer, C-26 colon cancer, and EMT6 and MDA-MB-361 breast cancer) compared to either treatment alone [49, 50]. Further experiments on human U87, U138, LN 405, and rat RG II glioblastoma cells indicated cytotoxic synergy after 48- and 144-hour treatments with PIO and a variety of statins [46]. Additionally, treatment of two meningioma cell lines (IOMM-Lee and KT21-MG1) with PIO and statins showed significant synergistic cytotoxic effects [47]. It was also suggested that statins may signal through the transcription factor sterol response element-binding protein (SREBP) to encourage PPARγ-mediated upregulation of PTEN [109]. This evidence suggests yet another class of drugs that, combined with PPARγ-ligands, show synergistic protective effects in cancer.

5. Areas Needing More Work

The majority of literature in the field supports the view that combination cancer therapy with PPARγ ligands and chemotherapeutic agents produce beneficial effects. However, this trend is not universal. Multiple groups have evaluated combinations of chemotherapeutic cocktails that include PPARγ ligands and found no synergism. For example, Yamamoto et al. reported that the synergistic toxic effects of 15d-PGJ_2 in renal cell carcinoma were specific to its combination therapy with camptothecin, and not evident with two common chemotherapeutic agents: 5-fluorouracin and cisplatin [55]. Tapia-Perez's group also found that synergism in glioblastoma cells depends on the combination, this time the PPARγ ligand, as PIO + statin treatment produced a significant cytotoxic effect although the same was not true for ROSI + statin [46] Clearly, more work is needed to establish which combinations will be effective in which diseases, work that will be further complicated by factors such as dose and treatment timing (pre-, post-, cotreatment, etc.). Interestingly, the same combination regimen may not always be effective, even within the same disease, as Elstner et al. reported that only three (MCF-7, MDA-MB-231, and ZR-75-1) of the eight (MCF-7, BT20, BT474, MDA-MB-231, MDA-MB-436, SKBR3, T-47D, ZR-75-1) breast cancer cell lines they evaluated were sensitive to combinations of the PPARγ ligand TRO and RXRα ligand 9-cis retinoic acid. Interestingly, the sensitive cell lines all express high levels of the apoptosis protein bcl-2 [42]. This underscores the importance of work to evaluate the molecular mechanisms by which combination therapies exert their effects so that, someday, clinicians and researchers may predict treatment efficacy using molecular signatures. Most notably, extensive literature searches did not reveal reports of PPARγ ligands impeding the therapeutic efficacy of chemotherapeutic agents.

Synthetic PPARγ ligands are generally well-tolerated and nontoxic; however, multiple groups have reported adverse cardiovascular events associated with PPARγ ligands, including myocardial hypertrophy and congestive heart failure due to plasma volume expansion and edema, in humans and animal models [110–112]. To address this problem, the FDA convened leading experts in 2010 to carry out more research to definitively show whether PPARγ ligands are associated with increased cardiovascular risk. The committee observed no significant difference in acute myocardial infarction and acute heart failure between patients treated with ROSI or PIO versus matched control cases and recommended that further studies be performed to address this issue [113, 114]. While these studies are in progress, research should continue to evaluate PPARγ ligands for their efficacy and mechanisms of action given their well-documented protective effects in many diseases, including, but not limited to, cancer. Furthermore, a better understanding of the mechanisms by which activation of PPARγ-dependent signaling stops tumourigenesis may provide the basis for future development of more efficacious drugs to prevent and/or reduce cancer-related deaths.

6. Discussion

The studies summarized previously, and in Tables 1 and 2, suggest that the combination of PPARγ ligands plus standard chemotherapeutic agents, RXRα agonists, statins, and certain cellular signaling molecules holds promise as a novel therapy for several types of malignancy. In general, combined use of two or more therapeutic compounds is often advantageous because of the potential to use lower clinical doses of each, which decreases nonspecific toxicity. However, here we report several examples of synergistic/additive interactions between agents that activate PPARγ as well as reductions in toxicity and the ability to overcome resistance. The results here are largely preclinical, with a select few regimens being evaluated in human subjects and, even in those cases, early clinical trials which, naturally, focused largely on toxicity as opposed to efficacy. However, the volume of preclinical evidence suggests that a breakthrough in the clinical application of combination therapy with PPARγ agonists is very possible. Moving forward, studies should continue to evaluate mechanisms by which these regimens induce their therapeutic effects as this will ultimately lead to identification of patient populations with high probabilities of therapeutic efficacy. In summary, the types of combination therapy described here are promising strategies for the chemoprevention, management, and/or treatment of several types of cancer.

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 201284, 11 pages
doi:10.1155/2012/201284

Research Article

Plasticizers May Activate Human Hepatic Peroxisome Proliferator-Activated Receptor α Less Than That of a Mouse but May Activate Constitutive Androstane Receptor in Liver

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Received 31 January 2012; Revised 19 March 2012; Accepted 21 March 2012

Academic Editor: Yuji Kamijo

Dibutylphthalate (DBP), di(2-ethylhexyl)phthalate (DEHP), and di(2-ethylhexyl)adipate (DEHA) are used as plasticizers. Their metabolites activate peroxisome proliferator-activated receptor (PPAR) α, which may be related to their toxicities. However, species differences in the receptor functions between rodents and human make it difficult to precisely extrapolate their toxicity from animal studies to human. In this paper, we compared the species differences in the activation of mouse and human hepatic PPARα by these plasticizers using wild-type (*mPPARα*) and humanized *PPARα* (*hPPARα*) mice. At 12 weeks old, each genotyped male mouse was classified into three groups, and fed daily for 2 weeks per os with corn oil (vehicle control), 2.5 or 5.0 mmol/kg DBP (696, 1392 mg/kg), DEHP (977, 1953 mg/kg), and DEHA (926, 1853 mg/kg), respectively. Generally, hepatic PPARα of *mPPARα* mice was more strongly activated than that of *hPPARα* mice when several target genes involving β-oxidation of fatty acids were evaluated. Interestingly, all plasticizers also activated hepatic constitutive androstane receptor (CAR) more in *hPPARα* mice than in *mPPARα* mice. Taken together, these plasticizers activated mouse and human hepatic PPARα as well as CAR. The activation of PPARα was stronger in *mPPARα* mice than in *hPPARα* mice, while the opposite was true of CAR.

1. Introduction

Dibutylphthalate (DBP), di(2-ethylhexyl)phthalate (DEHP), and di(2-ethylhexyl)adipate (DEHA) are used as representative industrial plasticizers, though the use of the first two considerably decreased recently. These chemicals are involved in peroxisome proliferations, similar to endogenous fatty acids, exogenous fibrates, and thiazolidinediones [1–4]. Once most plasticizers are taken into the body, they are metabolized by lipase in several organs such as liver and small intestine, and their metabolites, especially monocarboxylic acids, activate peroxisome proliferator-activated receptor alpha (PPARα), and influence the receptor-related lipid metabolism, anti-inflammation, glucose metabolism, and ketogenesis [5].

Peroxisome proliferators (PPs) cause hepatocarcinogenesis in rodents, and PPARα is involved in the mode of action [6]. However, the lower expression of PPARα in human liver [7] and ligand affinity for the agonists [2, 3] has been discussed within the context of how the risk of these chemicals is extrapolated to human from the animal data [8]. Indeed, the International Agency for Research on Cancer downgraded the DEHP carcinogenicity potential from 2B to 3, which produced some conflicting views over the past decade [9–13], but then restored the potential to the 2B grade in 2011 [14]. In addition, recent results showed that not only mouse

but also human PPARα was eventually activated by several activators, such as trichloroacetic acid [15] or perfluorooctanoic acid [16], with species differences in PPARα-related gene activation [17]. These results further complicated the risk assessment of peroxisome proliferators.

PPARα-humanized (*hPPARα*) mice, so-called *hPPARα*$^{\text{Tet-OFF}}$, that express human PPARα only in the liver of *PPARα*-null mice were recently established [18]. This mouse line expresses human PPARα considerably higher than mouse PPARα in wild-type mice and is a useful tool to elucidate the former function: 0.1, 0.3 mg/kg b.w. of ammonium perfluorooctanoate-activated mouse PPARα, but not human PPARα, suggesting that the activation of the latter may be weaker than the former [16]. In contrast, when 0.1% Wy-14,643 (which is estimated at about 100 ∼ 130 mg/kg b.w.) was administered to wild-type and *hPPARα* mice, the functional activations of the target genes such as mitochondrial and peroxisomal β-oxidation enzymes were almost the same or slightly less in the latter than in the former [18–20]. Taken together, the activation of human PPARα may be weaker than that of mouse PPARα. However, it is doubtful whether the findings are always similar to the other peroxisome proliferators such as DEHP.

Constitutive androstane receptor (CAR) is a representative transcriptional regulator for drug-metabolizing enzymes such as cytochrome P450 (CYP), UDP-glucuronosyl transferase (UGT), or sulfotransferase and activated by xenobiotic ligand phenobarbital (PB) or 1,4-bis [2-(3,5-dichloropyridyloxyl)] benzene (TCPOBOP) [21–23]. Many peroxisome proliferators such as DEHP [24] or PFOA [25] are also xenobiotic ligands or activators. On the other hand, CAR plays an important role in lipid homeostasis because of the interactive action with PPARα and inhibition of PPARα-related oxidation of fatty acids [26]. Indeed, TCPOBOP treatment increased serum triglyceride (TG) [27] because of downregulation of β-oxidation and upregulation of fatty acid synthesis. However, there is no report whether other phthalates such as DBP and adipates activate CAR and influence lipid homeostasis. It is important to examine whether these phthalates act on CAR because CAR activation is related with liver toxicity, such as modulation of acetaminophen-induced hepatotoxicity [28] or PB-induced liver tumor development [29, 30].

In this study, we selected three plasticizers currently used worldwide, DBP, DEHP, and DEHA, to determine the differences among hepatic mouse and human PPARα and CAR activation in response to these plasticizers using two PPARα mouse lines, wild-type (*mPPARα*) and *hPPARα* mice. We also investigated how both receptor activations influence plasma and liver TG levels for detection of functional changes in hepatic PPARα and CAR by treatment of plasticizers.

2. Materials and Methods

2.1. Chemicals. Standard grades of DEHP (≥99.5%), DEHA (≥99.0%), and DBP (≥99.5%) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Experimental Animals. This study was conducted according to the Guidelines for Animal Experiments of The Nagoya University Animal Center. Two genotyped male mice with a Sv/129 genetic background, *hPPARα* [18] and wild-type *mPPARα*, were used to identify respective PPARα functions in the lipid metabolism. All mice were housed in a temperature- and light-controlled environment (25°C, 12 h light/dark cycle) and maintained on stock rodent chow and tap water *ad libitum*. At 12 weeks old, each genotyped mouse was classified into three groups: one group was treated with corn oil daily for two weeks by gavage (vehicle control group); the other two were treated with 2.5 or 5.0 mmol/kg DEHP (977, 1953 mg/kg), DEHA (926, 1853 mg/kg), or DBP (696, 1392 mg/kg), for two weeks. No significant differences were observed in the body weight at the start of the three plasticizer treatments (data not shown). On the next day after the last dose (18–20 hours later), all the mice were killed by decapitation, and the blood and livers were removed. The liver samples were stored at −80°C until use; as for the blood, after centrifuging at 3,500 g for 10 min, the plasma was stored at −80°C until use.

2.3. Nuclear Fraction. A nuclear fraction was extracted from a part of the frozen liver using a CelLytic NuCLEAR Extraction Kit (SIGMA, Tokyo, Japan).

2.4. Analysis of Protein Concentrations. Each tissue was homogenized with a three-fold volume of 10 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose. Protein concentrations of the homogenate samples were measured using a Protein Assay Kit (Bio-Rad, Tokyo, Japan).

2.5. Lipid Concentrations in Plasma and Liver. Lipid from liver was extracted using the method of Folch et al. [31]. TG in the liver and plasma measured using a TG-IE kit (Wako, Osaka, Japan).

2.6. Histopathological Analysis. The organs fixed in 10% neutral buffered formalin were embedded in paraffin and sliced into 2 μm sections. Tissue sections of the livers were stained with hematoxylin and eosin and examined under a light microscope using the BZ-8000 (Keyence Corporation, Osaka, Japan). Histopathological findings were scored according to the degree of lipid accumulation and necrosis with inflammatory cell infiltration.

2.7. Real-Time Quantitative PCR. Total RNA was isolated using RNeasy Mini Kit (QIAGEN, Tokyo, Japan). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using Oligo$(dT)_{20}$ primer. RNA quantity and quality were checked by a GeneQuant II RNA/DNA Calculator (Pharmacia Biotech, Framingham, MA). Primers were designed using Primer Express software (Applied Biosystems) based on the sequence of the respective GI number, as shown in the Supplemental Table available online at doi:10.1155/2012/201284. As for MTP and Cyp4a14, primers were used elsewhere [26, 32]. These mRNA levels were monitored by the ABI PRISM 7000 Sequence Detection

system (Applied Biosystems, Foster City, CA), as described previously [16, 33, 34].

2.8. Western Blotting. Western blotting was conducted by the method described previously [35]. Briefly, the samples for electrophoresis adjusted to 10 μg protein in liver homogenates of nuclear fraction were subjected to 10% SDS-PAGE and transferred to the nitrocellulose membranes. After blocking with 3% skim milk, each membrane was incubated with the primary antibody, followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immuno Research, West Grove, PA). The primary polyclonal antibody was prepared using purified medium-chain acyl-CoA dehydrogenase (MCAD) [36], keto-acyl-CoA thiolase (PT) [37], very long-chain acyl-CoA dehydrogenase (VLCAD) [38], and peroxisomal bifunctional protein (PH) [39]. These antibodies were already used elsewhere [15]. The primary polyclonal antibodies of PPARα were purchased from Santa Cruz Biotechnology, Inc. (CA). Each band was quantified using densitometry, the Lane & Spot Analyzer version 5.0 (ATTO Corporation, Tokyo, Japan) as described elsewhere [16, 33, 35]. Each band was normalized to the respective level of glyceraldehyde-3-phosphate dehydrogenase.

2.9. Electrophoretic Mobility Shift Assay (EMSA). The following oligonucleotides, synthesized by Sigma Aldrich Japan (Tokyo, Japan), were used as probes based on the sequence of DR-4 nuclear-receptor-(NR-) binding sites reported by Kim et al. [40]: NR-1 probe, 5′-biotin-TCTGTACTT-TCCTGACCTT-3′; NR-2 probe, 5′-biotin-TCAACTTGA-CTGACACC-3′. LightShift Chemiluminescent EMSA kit (Pierce Biotechnology, Rockford) was used with a slight modification. Sample mixture contained nuclear extract (4 μg), 0.2 mg/mL poly (dI-dC), 5% glycerol, 0.1% NP-40, 5 mM $MgCl_2$, 0.2 mM EDTA, 2% Ficol (400), 47 mg/mL transfer RNA, and 2 μM biotin-labeled double-stranded oligonucleotide. The reaction samples were resolved on non-denaturing electrophoresis (4% acrylamide) and transferred to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). Constitutive androstane receptor (CAR)-NR-1 and CAR-NR-2 complexes were detected with a Chemiluminescent Nucleic Acid Detection Module (Pierce Biotechnology) and visualized using a Lumi Vision PRO HS II (Aisin Seiki Co., Ltd., Japan).

2.10. Statistical Analysis. Comparisons were made using the two-way analysis of variance (ANOVA) and the Tukey-Kramer HSD post hoc test. A logarithmic transformation was applied to MTP-mRNA before statistical analysis. Values of $P < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Body and Liver Weights. No significant differences were observed in body weight after the treatments (Table 1). Exposure to 2.5 (low-dose) and 5.0 mmnol/kg (high-dose) DEHP and DEHA increased both liver weight and liver/body weight ratio only in *mPPARα* mice, but high-dose DBP increased only the absolute liver weights (Table 1). In contrast, treatment with any plasticizer failed to influence either the liver weight or the liver/body ratio in *hPPARα* mice.

3.2. TG in the Plasma and Liver. The plasma TG level in *mPPARα* control mice was similar to that in *hPPARα* controls (Table 1). High-dose DEHA increased plasma TG levels in *hPPARα* mice, but not in *mPPARα* mice. In contrast, the other plasticizers did not influence the levels. In each of the control mice, hepatic TG levels were significantly greater in *hPPARα* mice than in the *mPPARα* mice (Table 1). High-dose DEHP and DEHA decreased the levels in the liver of *mPPARα* mice. High-dose DEHP increased the levels in *hPPARα mice*, whereas DEHA did not. DBP did not influence the TG levels in both genotyped mice. Thus, the TG decrease due to the accelerated lipid metabolism was seen in *mPPARα* mice treated with DEHP or DEHA. In contrast, hepatic TG accumulation was seen in DEHP-treated *hPPARα* mice.

3.3. Histopathological Changes. In the control animals, no obvious differences in the scores of lipid accumulation, inflammatory and necrotic cell infiltrations were observed in the liver between both genotyped mice (Figure 1, scores not shown). As mentioned above, hepatic TG levels were greater in *hPPARα* controls than *mPPARα* controls; however no obvious histopathological differences in lipid accumulation were found between the two genotyped mice. The hepatocellular enlargements were prominently observed in *mPPARα* mice of the high-dose DEHP group and slightly in those of high-dose DEHA and DBP groups. Cytoplasmic vacuoles due to lipid accumulation were seen in *hPPARα* mice exposed to the three plasticizers, though the changes were not dose dependent. A focal necrosis with inflammatory cells was seen in two of five *hPPARα* mice exposed to high-dose DEHP, all animals exposed to high-dose DEHA and three of five animals exposed to low-dose DEHA. Moderate eosinophilic cytoplasm which may result from the increase in peroxisome or mitochondria was observed in all *mPPARα* mice treated with high-dose DEHP; however, the finding was minimal in those on the low dose. In contrast, only two of five animals on high-dose DBP and DEHA exhibited minimal or mild eosinophilic cytoplasm, respectively. Taken together, popular histopathological changes caused by peroxisome proliferators such as liver enlargement and eosinophilic cytoplasm were prominent in *mPPARα* mice treated with high-dose DEHP. On the other hand, focal necrosis was seen mainly in *hPPARα* mice exposed to high-dose DEHA.

3.4. PPARα and Target Genes. Low-dose DBP significantly increased PH- and PT-mRNA levels (2.7-fold and 2.0-fold, resp.) in *mPPARα* mice (Figure 2), whereas low-dose DEHP and DEHA did not. In high-dose groups, all plasticizers increased hepatic peroxisomal PH- and PT-mRNA in *mPPARα* mice, while DBP alone induced PT-mRNA in *hPPARα* mice. The increases were greatest in DEHP-treated *mPPARα* mice (7.1-fold and 4.1-fold, resp.), and those by

Table 1: Body, liver weights and TG levels after treatment with plasticizers for 2 weeks.

		B.W.	Liver weight	Liver weight/ B.W. (%)	Plasma TG	Liver TG
mPPARα	Control	23.9 ± 0.91	0.88 ± 0.11	3.68 ± 0.38	79.4 ± 16.3	14.8 ± 1.53
	DBP 2.5	25.9 ± 2.05	1.08 ± 0.13	4.14 ± 0.17	89.9 ± 24.8	12.5 ± 2.76
	DBP 5.0	26.7 ± 2.01	1.20 ± 0.10*	4.49 ± 0.40	113.9 ± 40.4	11.4 ± 1.68
	DEHP 2.5	22.1 ± 1.82	1.13 ± 0.11*	5.09 ± 0.24*	82.6 ± 13.8	11.6 ± 1.56
	DEHP 5.0	22.9 ± 0.92	1.26 ± 0.06*	5.54 ± 0.33*	84.0 ± 24.5	6.8 ± 0.90*
	DEHA 2.5	25.9 ± 0.85	1.20 ± 0.07*	4.63 ± 0.22*	136.9 ± 15.9	11.4 ± 0.90
	DEHA 5.0	24.2 ± 1.81	1.28 ± 0.18*	5.27 ± 0.35*	119.5 ± 36.3	7.5 ± 1.76*
hPPARα	Control	22.7 ± 2.20	1.04 ± 0.06	4.59 ± 0.25	97.0 ± 23.6	24.4 ± 5.51#
	DBP 2.5	25.0 ± 2.32	1.07 ± 0.08	4.29 ± 0.18	127.0 ± 35.0	22.6 ± 4.66
	DBP 5.0	23.1 ± 4.51	1.05 ± 0.28	4.76 ± 0.29	95.1 ± 26.0	31.9 ± 19.31
	DEHP 2.5	23.8 ± 2.58	1.12 ± 0.17	4.69 ± 0.25	111.5 ± 28.0	20.6 ± 4.66
	DEHP 5.0	21.6 ± 2.58	1.03 ± 0.17	4.52 ± 0.37	67.8 ± 35.0	30.9 ± 4.24*
	DEHA 2.5	24.9 ± 1.03	1.12 ± 0.08	4.48 ± 0.14	142.3 ± 59.9	23.1 ± 1.98
	DEHA 5.0	24.7 ± 2.94	1.23 ± 0.17	4.98 ± 0.25	176.0 ± 41.0*	28.4 ± 2.73

B.W: body weight.
Each value represents mean ± S.D. *Significantly different from respective controls ($P < 0.05$). #Significantly different from *mPPARα* controls ($P < 0.05$).

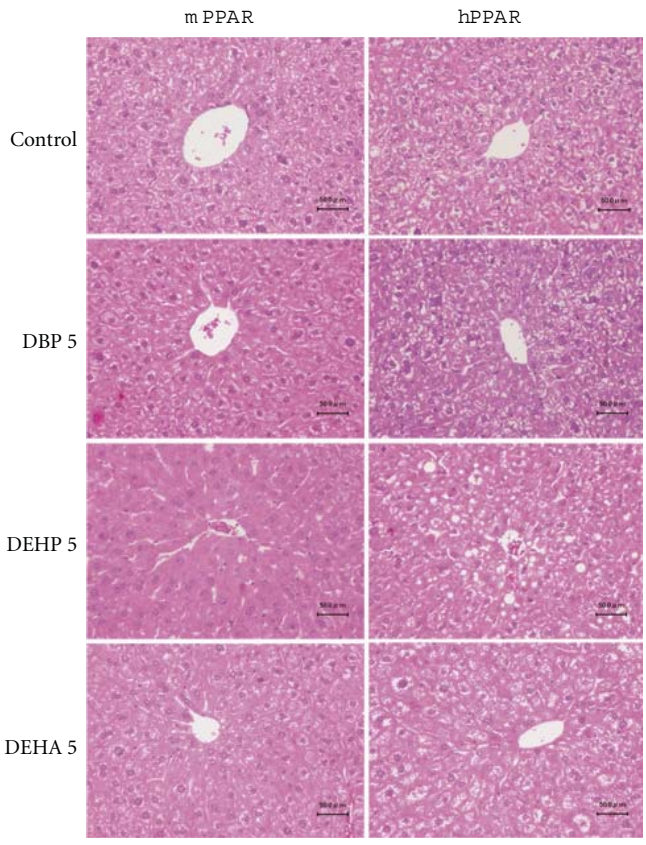


Figure 1: Histopathological changes in livers in *mPPARα* and *hPPARα* mice treated with control, high-dose DBP, DEHP, and DEHP for 2 weeks. Hepatocellular enlargements were prominently observed in *mPPARα* mice of DEHP group and slightly in those of DEHA and DBP. Moderate eosinophilic cytoplasm was observed in *mPPARα* mice treated with DEHP. Cytoplasmic vacuoles due to lipid accumulation were seen in *hPPARα* mice exposed to three plasticizers. Each scale bar indicates 50 μm.

DBP and DEHA treatments were almost the same (2.6-fold, 2.5-fold and 3.0-fold, 2.9-fold, resp.). All plasticizers at low dose did not influence hepatic mitochondrial MCAD- and VLCAD-mRNA levels. High-dose DEHP, however, increased both mRNA levels only in *mPPARα* mice, but only marginally (1.8-fold and 1.4-fold, resp.).

All plasticizers at low dose increased PH and PT protein in the liver of both genotyped mice except PH in DEHA-treated *hPPARα* mice and PT in DBP-treated *mPPARα* mice (Figures 3(a) and 3(b)). All plasticizers at high dose also increased PH and PT protein in the livers of both *mPPARα* and *hPPARα* mice. The inductions of PH were slightly stronger in *mPPARα* exposed to DBP and DEHP (DBP, 5.9-fold; DEHP, 6.0-fold; DEHA, 5.3-fold) than in *hPPARα* mice (3.9-fold, 1.9-fold, 5.1-fold, resp.). The increases of PT by DEHP or DEHA treatments were also stronger in *mPPARα* (2.8-fold and 1.8-fold, resp.) than in *hPPARα* mice (1.3-fold and 1.4-fold, resp.), although those by DBP were almost the same in both *mPPARα* and *hPPARα* mice.

In mitochondrial enzymes, three plasticizers at any doses increased hepatic VLCAD protein expressions in both *mPPARα* and *hPPARα* mice. The inductions appeared to be stronger in *mPPARα* mice exposed to DEHP and DEHA (DBP: 2.6-fold, DEHP: 5.4-fold, DEHA: 5.4-fold) than in corresponding *hPPARα* mice (2.3-fold, 1.4-fold, 1.5-fold, resp.), similar to peroxisomal enzyme PH. High-dose DEHP and DEHA increased hepatic MCAD levels in *mPPARα* and *hPPARα* mice, and in *hPPARα* mice, respectively, whereas DBP did not affect the levels in either *mPPARα* mice or *hPPARα* mice.

Low- and high-dose DEHA, DEHP, and DBP also increased hepatic Cyp4a14, a microsomal enzyme involved in ω-oxidation of many plasticizers, expressions only in *mPPARα* mice but not in *hPPARα* mice (Figure 2). Inductions in the former mice were 23-fold, 62-fold, and 21-fold at high-dose DBP, DEHP, and DEHA, respectively.

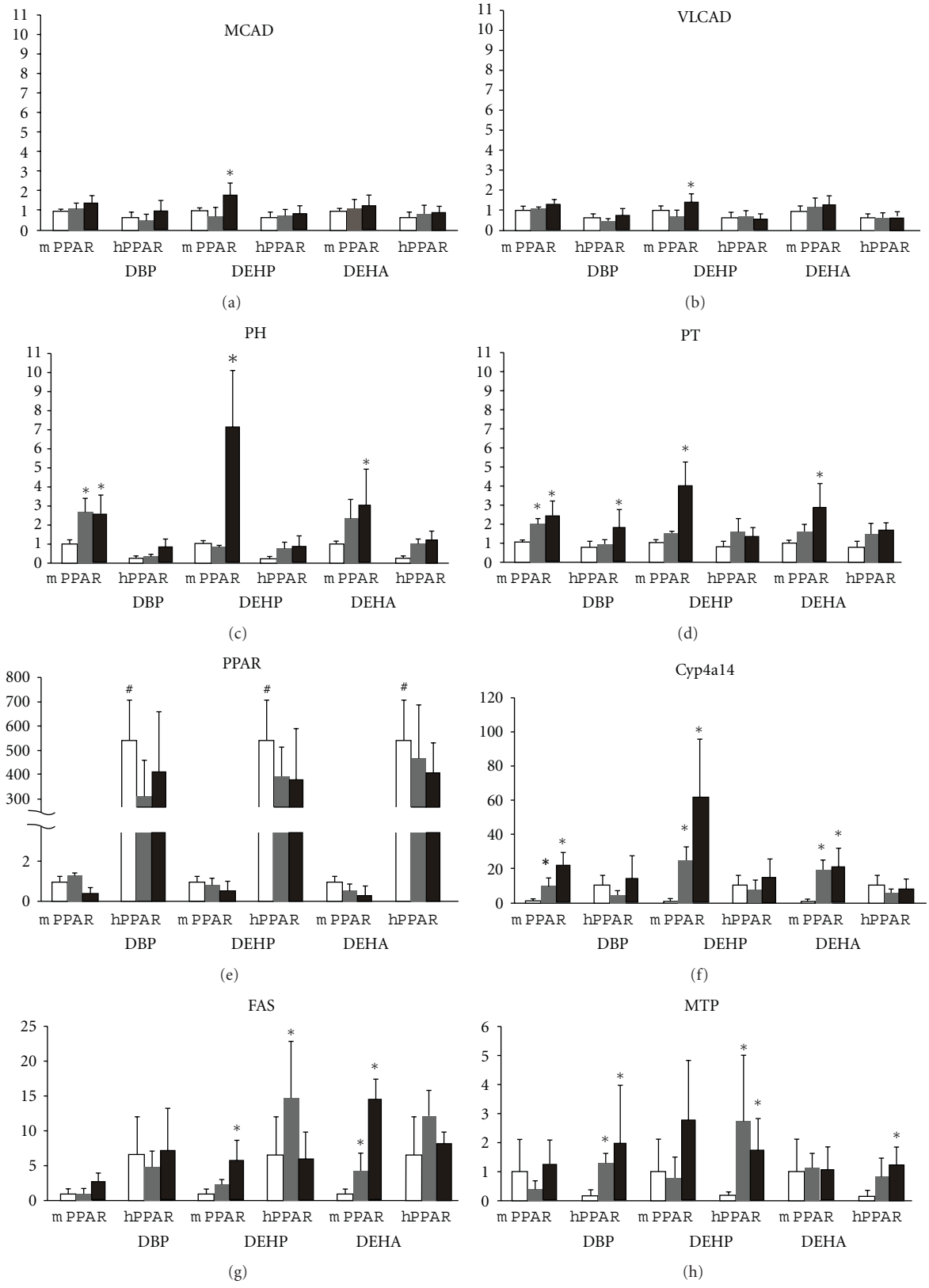
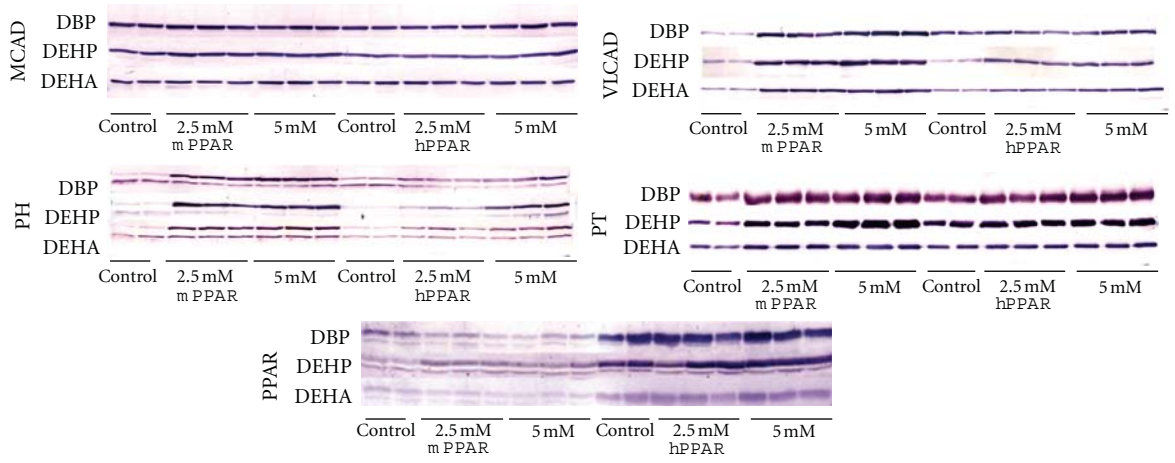
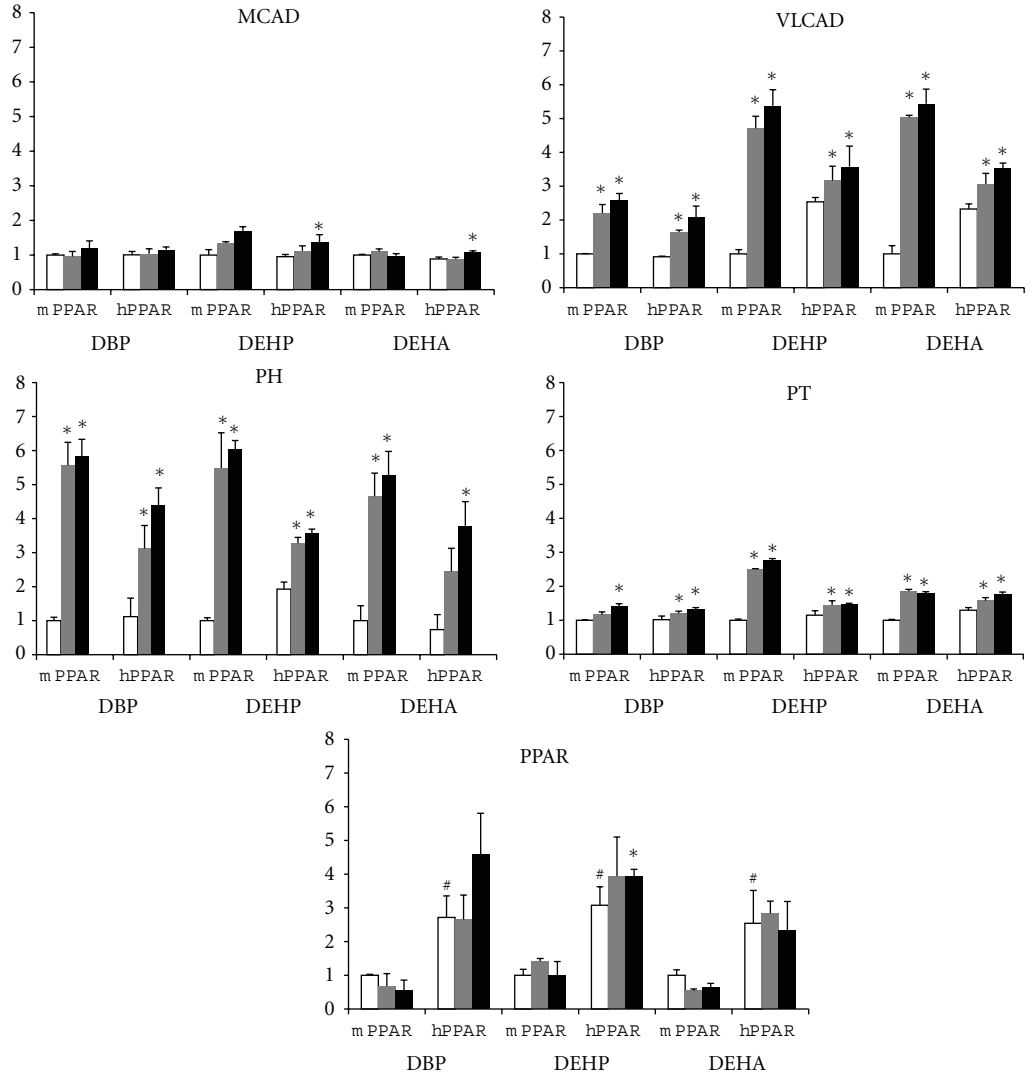


Figure 2: mRNA expressions of hepatic PPARα and its related genes in duplicate analyses. Expressions of mRNA were analyzed by quantitative real-time PCR. Each mRNA was normalized to the level of GAPDH-mRNA expression in the same preparation, and mean of control in *mPPARα* mice was assigned a value of 1.0. White, gray, and black columns represent control values, 2.5 mM- and 5.0 mM-treated group, respectively. Each column and bar represents mean ± S.D., respectively. A logarithmic transformation was applied to MTP-mRNA before statistical analysis. *Significantly different from respective controls ($P < 0.05$). #Significantly different among genotypes ($P < 0.05$).



(a)



(b)

Figure 3: (a) Western blotting analysis of hepatic PPARα and related genes. All mice from each treatment and genotype were examined across two gels, one of which is shown here. (b) Western blotting analysis of hepatic PPARα and related genes. Each band was quantified by densitometric analysis as described in Materials and Methods, and mean strength of control in *mPPARα* mice was assigned a value of 1.0. White, gray, and black columns represent control values, 2.5 mM- and 5.0 mM-treated group, respectively. Each column and bar represents mean ± S.D., respectively. *Significantly different from respective controls ($P < 0.05$). #Significantly different among genotypes ($P < 0.05$).

In the control group, the expression of PPARα was significantly greater in *hPPARα* mice than in *mPPARα* mice either in the mRNA (540-fold) or protein (about 3-fold) levels (Figures 2, 3(a), and 3(b)). No treatments elevated mouse and human PPARα-mRNAs. High-dose DEHP increased only PPARα protein expression in *hPPARα mice*, but other treatments did not.

Low- and high-dose DEHA and high-dose DEHP significantly increased FAS-mRNA to 4.4-fold and 14.7-fold, and 5.8-fold in *mPPARα* mice, respectively (Figure 2). Low-dose DEHP also increased it to 14.9-fold in *hPPARα* mice. However, DBP treatment did not influence FAS-mRNA in both genotype mice. We also measured MTP-mRNA levels in the liver: low- and high-dose DBP and DEHP increased the mRNA to 8.8-fold and 13.5-fold, and 18.8-fold and 11.8-fold, respectively, in *hPPARα* mice but not in *mPPARα* mice. Similarly, high-dose DEHA increased MTP-mRNAs (8.5-fold) only in *hPPARα* mice.

Collectively, inductions of peroxisomal, mitochondrial, and microsomal enzymes involved in β-oxidation were stronger in *mPPARα* mice than in *hPPARα* mice treated with plasticizers in terms of mRNA levels, whereas transporter enzyme was induced only in *hPPARα* mice exposed to plasticizers.

3.5. CAR and Target Gene. Low- and high-dose DEHA and high-dose DEHP and DBP decreased CAR-mRNA levels in *mPPARα* mice, but the levels in *hPPARα* mice were not affected at any dose (Figure 4(a)). In contrast, high-dose DEHP strongly induced typical CAR target gene, Cyp2b10-mRNA, in *hPPARα* mice (48.3-fold). Low- and high-dose DEHA induced Cyp2b10-mRNA levels in *hPPARα* mice (31.2-fold and 24.5-fold, resp.). The high-dose DEHA also elevated the mRNA levels in *mPPARα* mice (9.2-fold), but only marginally compared with those in *hPPARα* mice. In contrast, DBP did not influence the levels in both genotyped mice.

The treatments with all plasticizers dramatically induced NR-1 (Figure 4(b) A) and NR-2 (Figure 4(b) B) DNA-binding activity of hepatic CAR in *hPPARα* mice at high dose. The high-dose DEHP also induced NR-2-binding activity in *mPPARα* mice, but DBP or DEHA did not. The activities in *hPPARα* mice were strongest in the DEHP-treated group, followed by the DEHA- and DBP-treated group.

In summary, plasticizers, especially in DEHP or DEHA, bind to hepatic CAR and markedly induce CAR-target gene mainly in *hPPARα* mice.

4. Discussion

The present study clearly shows that three plasticizers (DEHP, DEHA, and DBP) significantly activated mouse hepatic PPARα in *mPPARα* mice, but the activation of human hepatic PPARα in *hPPARα* mice was weaker than that of the former mouse line even at the high-dose exposure, especially in peroxisomal β- or ω-oxidation. Among the three plasticizers, DEHP is the strongest from the standpoint of PPARα-mediated gene responses. These results are consistent with *in vitro* studies [3, 4] which demonstrated that mono (2-ethylhexyl) phthalate (MEHP) activated mouse PPARα at lower concentrations and exhibited a stronger response than those of human PPARα [4], and MEHP activated mouse and human PPARα at a lower concentration than the respective monoesters of DBP and DEHA [3, 4]. Interestingly, these species differences in PPARα activation were most prominent in microsomal PPARα-target gene, Cyp4a14, followed by mitochondrial (MCAD, VLCAD) or peroxisomal enzymes (PH, PT). Notably, all the plasticizers also activated CAR preferentially in *hPPARα* mice. The activation was also stronger in DEHP than DEHA judging from the target gene (Cyp2b10) as well as the DNA-binding (NR-1 and 2) activity analysis.

As mentioned above, DEHP and DEHA activated PPARα and CAR preferentially in *mPPARα* and *hPPARα* mice, respectively. Our finding is very similar to the fact that DEHP induced Cyp2b10 more strongly in the livers of *PPARα*-null mice than *mPPARα* ones [24, 41]. Although the reason why CAR induction was stronger in *hPPARα* mice than in *mPPARα* mice remains unclear, it is likely that CAR is more easily activated when the function of PPARα is weak, as with human PPARα in *hPPARa* mice [15] or lack of PPARα in *Pparα*-null mice [41]. CAR was reported to crosstalk with PPARα and suppress its related gene expressions such as Cyp4a14 and carnitine palmitoyltransferase 1α in the liver of mice [26, 27]. It is of interest that DEHP activated both receptors more than DEHA. However, the chemical form of the activator for each receptor may be different; since MEHP did not induce Cyp2b10 in JWZ-CAR cell line [42], the parent substance itself may be an activator of CAR. No report on DEHA indicated that either the parent substance itself or the metabolite(s) is a preferential activator for CAR. In the present study, DBP also induced binding activity of CAR in *hPPARα* mice but did not increase Cyp2b10-mRNA in that strain, though DBP has been reported to activate CAR in the liver of rats [43]. Interestingly, the CAR2 splice variant of human CAR is activated by DEHP [44], which suggests that human CAR may also play an important role in DEHP toxicity. Taken together, CAR-mediated effects by plasticizers should be noted as a novel aspect of their toxicities to provide a new rationale to evaluate toxicity correctly.

Species differences of mouse and human PPARα activation by Wy-14,643 have been investigated using *mPPARα* and *hPPARα* mice fed 0.1% Wy-14,643-containing feed for 2 weeks *ad libitum* [18], at a dose roughly estimated to be 0.3 ~ 0.4 mmol/kg/day. This dose significantly induced peroxisomal and mitochondrial fatty acid-metabolizing enzymes such as acyl-CoA oxidase, VLCAD, and MCAD, followed by a similar decrease in serum triglycerides in both mouse lines. Even a lower dose of Wy-14,643 than the plasticizers used in this study was presumed to activate mouse and human PPARα to a similar extent along with decreased plasma TG levels. This result suggests that there may not be a species difference in the activation by Wy-14,643. Since all plasticizers induced PPARα-related enzymes involved in β- or ω-oxidation in *mPPARα* mice but none of them influenced the plasma TG level, the PPARα activation by Wy-14,643 is not coincident with the present study from

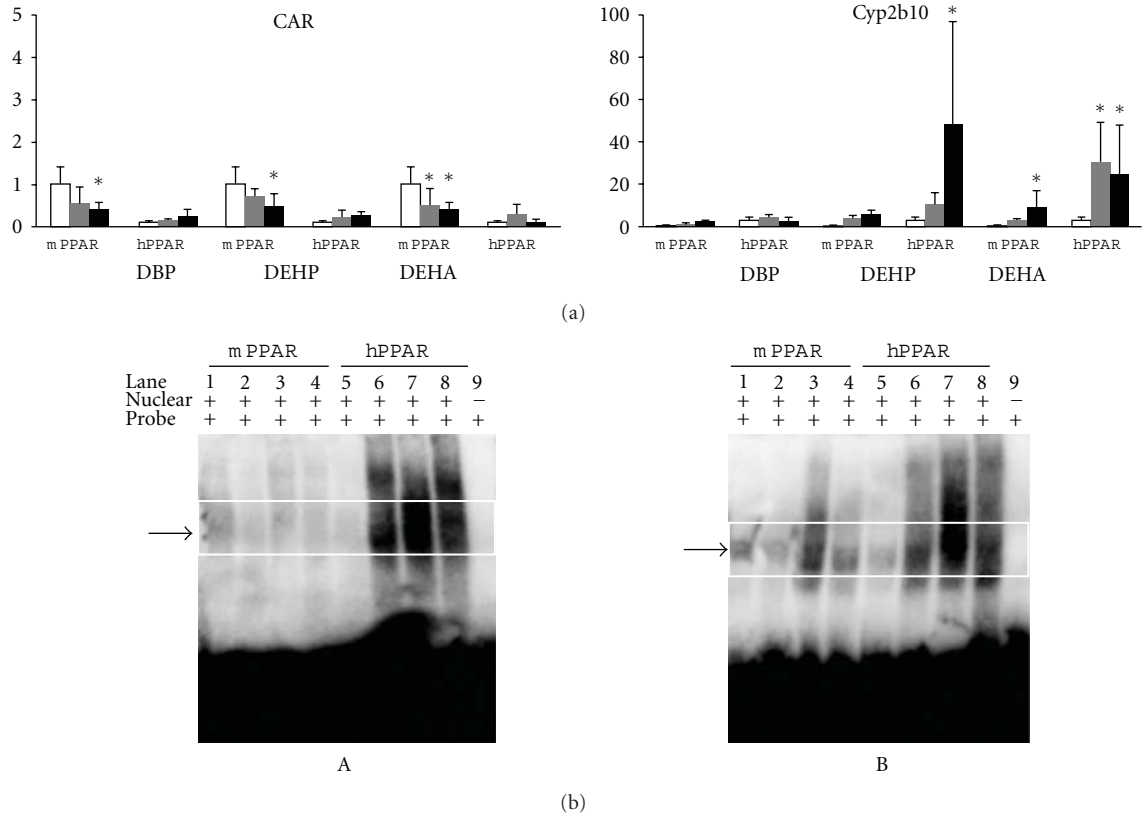


Figure 4: (a) Effects on hepatic expressions of CAR and Cyp2b10-mRNA levels. Each mRNA level was normalized to the level of GAPDH mRNA in the same preparation, and the mean of the control group in wild-type (*mPPARα*) mice was assigned a value of 1.0. White, gray and black columns represent control values, 2.5 mM- and 5.0 mM-treated group, respectively. Values are expressed as mean ± S.D. *Significantly different from respective control group ($P < 0.05$). (b) Electrophoresis mobility shift assays of CAR-NR-1 (A) and CAR-NR-2 (B) complexes in liver nuclear fraction from control or treated-*mPPARα* (wild-type) and *hPPARα* mice. Lanes 1 and 5, control of wild-type, respectively; lanes 2 and 6, wild-type and *hPPARα* mice treated with 5.0 mM DBP, respectively; lanes 3 and lane 7, wild-type and *hPPARα* mice treated with 5.0 mM DEHP, respectively; lanes 4 and lane 8, wild-type and *hPPARα* mice treated with 5.0 mM DEHA, respectively; lane 9, oligonucleotide for NR-1 or NR-2 only. Arrows indicate the shifted CAR-NR complex.

the standpoint of PPARα-target gene induction as well as plasma TG levels.

DEHP was the strongest inducer of PPARα-related β-oxidation enzymes in *mPPARα* mice among the three chemicals. It was also the strongest activator for CAR in both *mPPARα* and *hPPARα* mice in our study. However, Wy-14,643 did not activate CAR [41]. In this regard, the effect of Wy-14,643 on the nuclear receptors is different from that of DEHP. TCPOBOP, a CAR potent agonist, was suggested to cause an accumulation of serum TG [26, 27], whereas the PPARα agonist Wy-14,643 decreased it. These opposite actions by CAR and PPARα in TG homeostasis [45] may reflect the plasma TG unchanged by DEHP, because DEHP induced both PPARα and CAR. In contrast, the *hPPARα* mice exposed to high-dose DEHA had elevated plasma TG. In these mice, MTP-mRNA, which was involved in the transport of TG from liver to blood, was induced and may partly be the reason for the increased plasma TG, even though CAR was also induced by DEHA treatment.

As for TG levels in livers, the high dose of DEHP or DEHA decreased the levels in *mPPARα* mice, whereas DEHP increased the levels in *hPPARα* mice. The increase in *hPPARα* mice, as different from that in *mPPARα* mice, may be ascribed to the weaker inductions of enzymes involved in β- and ω-oxidation in *hPPARα* mice than in *mPPARα* mice. MEHP increased TG in hepatocyte culture of guinea pig because of the weak induction of β-oxidation and lauric acid hydroxylation, whereas it decreased TG in rat hepatocytes due to the significant induction of these enzymes [46]. The degree of β-oxidation-related enzyme inductions by DEHP was comparable between mice and rats [34]. Taken together, the difference in mouse and human PPPARα functions presumably produced the different effects of DEHP or DEHA on hepatic TG accumulation between *mPPARα* and *hPPARα* mice.

In the present study, we only investigated the effects of three kinds of plasticizers on the lipid metabolism and did not investigate DEHP- or DEHA-caused tumors in relation to PPARα. CAR is thought to mediate the hepatocarcinogenic effects of xenobiotics [29], suggesting that it may contribute to the PPARα-independent hepatocarcinogenesis observed in *PPARα*-null mice following chronic DEHP exposure [35]. DEHP at a 1150 mg/kg dose for 4 days induced CAR and Cyp2b10-mRNAs only in *PPARα*-null

mice, and 200 mg/kg DEHP induced them in both wild-type and *PPARα*-null mice [41]. The induced rate was greater in the latter than the former mice, suggesting that *PPARα*-null mice are more susceptible to DEHP-induced CAR signaling compared to that of *mPPARα* mice. DEHP activated not only PPARα but also CAR, though Wy-14,643 did not activate CAR [41]. This different signaling suggests that the molecular mechanism of carcinogenicity in phthalates may not always be the same as that of Wy-14,643.

Finally, hepatic mRNAs of cell cycle-related genes such as cyclin D1, protooncogene such as c-jun, and apoptosis-related gene Bax, were measured using *mPPARα* and *hPPARα* mice exposed to the plasticizers, but these mRNA levels did not increase in both genotyped mice; instead, decreases of cell cycle-related genes were observed in both genotyped mice (unpublished data), which is not consistent with the case of Wy-14,643 [19]. These results again suggest that DEHP-induced molecular signalings are not always the same as those by Wy-14,643. The reason for this is unclear, but the weaker affinity of DBP, DEHP, and DEHA for human and mouse PPARα than Wy-14,643 may be a possible explanation [4].

In conclusion, these plasticizers activated not only mouse and human hepatic PPARα but also CAR, and the activation of PPARα was stronger in *mPPARα* mice than in *hPPARα* mice, while that of CAR was the opposite. Thus, DEHP is not only a PPARα agonist but also a CAR activator, which may trigger each function.

Abbreviations

ANOVA: Analysis of variance
CAR: Constitutive androstane receptor
CV: Central vein
DBP: Dibutylphthalate
DEHP: Di(2-ethylhexyl)phthalate
DEHA: Di(2-ethylhexyl)adipate
DGAT: Diacylglycerol acyltransferase
EMSA: Electrophoretic mobility shift assay
hPPARα: Humanized PPARα mouse
MCAD: Medium-chain acyl-CoA dehydrogenase
MEHP: Mono(2-ethylhexyl)phthalate
mPPARα: Wild-type mouse
MTP: Microsomal triacylglycerol transfer protein
NR: DR-4 nuclear receptor binding site
PB: Phenobarbital
PH: Peroxisomal bifunctional protein
PPARα: Peroxisome proliferator-activated receptor α
PT: Keto-acyl-CoA thiolase
TG: Triglyceride
VLCAD: Very long-chain acyl-CoA dehydrogenase.

Acknowledgment

This study was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (B. 14370121, 17390169), Food Safety Commission, Japan (1002), and Health and Labour Sciences Research Grants from Research on Food Safety of the Ministry of Health, Labour and Welfare in Japan.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 858352, 7 pages
doi:10.1155/2012/858352

Review Article

Nutraceuticals as Ligands of PPARγ

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Received 27 February 2012; Revised 10 April 2012; Accepted 13 April 2012

Academic Editor: Christopher J. Nicol

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors that respond to several exogenous and endogenous ligands by modulating genes related to lipid, glucose, and insulin homeostasis. PPARγ, expressed in adipose tissue and liver, regulates lipid storage and glucose metabolism and is the target of type 2 diabetes drugs, thiazolidinediones (TZDs). Due to high levels of toxicity associated with the first generation TZDs, troglitazone (Rezulin), rosiglitazone (Avandia), and pioglitazone (Actos), there is a renewed search for newer PPAR drugs that exhibit better efficacy but lesser toxicity. In recent years, there has been a definite increase in the consumption of dietary supplements among diabetics, due to the possible health benefits associated with these nutraceutical components. With this impetus, investigations into alternative natural ligands of PPARs has also risen. This review highlights some of the dietary compounds (dietary lipids, isoflavones, and other flavanoids) that bind and transactivate PPARγ. A better understanding of the physiological effects of this PPAR activation by nutraceuticals and the availability of high-throughput technologies should lead to the discovery of less toxic alternatives to the PPAR drugs currently on the market.

1. Introduction

Peroxisome proliferator-activated receptor gamma (PPARγ), or NRIC3, is a ligand-activated transcription factor that belongs to the superfamily of nuclear receptors. PPARγ plays an important role in glucose and lipid homeostasis, inflammation, and adipocyte differentiation [1]. There are three known isoforms of PPARs: PPARα, PPARγ, and PPARβ/δ, each with different tissue specificity and physiological function [2]. All three isoforms share common molecular structure and functional domains similar to other nuclear receptor superfamilies consisting of the following: a distinct N-terminal ligand-independent transcriptional activation domain (AF-1), a DNA binding domain, the hinge region, and the ligand-binding domain which contains the ligand-dependent transcriptional activation domain (AF-2). Upon ligand binding, PPARγ forms a heterodimer with the retinoic acid receptor (RXR) and controls the expression of genes that have PPAR response elements (PPRE). This transcription factor is further regulated by commonly known coactivator proteins such as CBP/p300, the SRC family, TRAP 220, and corepressors such as SMART, NCoR, and RIP140 [1]. Two isoforms of PPARγ have been identified (PPARγ1 and PPARγ2), with a wide tissue distribution among various animal species [3].

Over the past two decades, there has been a flurry of research investigating the physiological significance of PPARγ activation. It is now generally accepted that both ligand dependent and independent activation of PPARγ mediate multiple metabolic pathways in the immune system [4], cardiovascular system [5], and the adipose tissue [6], thus modulating genes related to inflammation, lipid metabolism and adipogenesis. Most of these physiological functions of PPARγ were revealed because of the discovery of thiazolidinediones (TZD). These drugs are high affinity ligands of PPARγ with insulin sensitizing effects and used in the treatment of type 2 diabetes [7]. The identification of PPARγ as the molecular target of glitazones such as pioglitazone (TZD), came from seminal work by Kliewer et al. [8], Kletzien et al. [9], and Graves et al. [10]. Troglitazone

(Rezulin), rosiglitazone (Avandia), and pioglitazone (Actos) were the three originally approved TZD drugs for diabetes. Increased hepatic toxicity, edema, and cardiovascular risk associated with the use of the TZD drugs lead to the withdrawal of troglitazone (Rezulin) from the market and black box warnings on the other two available drugs [11]. Although these drugs are known PPARγ agonists, it is still not clear if the toxicities associated with these drugs are due to their interactions with the PPARγ receptor. A new generation of PPARγ drugs with equivalent insulin sensitizing activity like TZDs, but with lower toxicity, has been in development since the withdrawal of the earlier TZDs. These include (i) non-TZD like PPARγ agonists, (ii) PPAR α/γ dual agonists, (iii) PPAR pan agonists, (iv) PPARγ antagonists, and (iv) selective PPARγ modulating drugs (SPPARγM) [12, 13]. These newer agonists seem to have similar or better insulin sensitizing effects as compared to TZDs (rosiglitazone). Still, several of these new drugs exhibit some form of toxicity [14]. Yet, SPPARγM are purported to be less toxic because they are designed based on the ligand selective regulation of receptor function [13, 15–17]. Recent studies indicate that SPPARγM are mechanistically distinct from the TZDs in that these drugs interact at a site that is different than the AF-2 region, thus altering subsequent coregulator binding and resulting in favorable cellular responses [18]. The search will continue until better alternative drugs to the currently available TZDs with equal or greater beneficial effects, but fewer adverse effects are identified.

2. Natural Ligands of PPARγ

Although there is a renewed interest in identification of synthetic PPARγ modulators for the treatment of type 2 diabetes, developing known dietary components (nutraceuticals) that bind and activate PPARγ with more efficacy and safety, while promoting health benefits has become an absolute necessity [19]. The term nutraceutical is defined as any food (fruits, vegetables, nuts, tea, etc.) or part (extract) of a food, such as a dietary supplement that has a medical or health benefit including the prevention and treatment of disease [20]. However, there is no consensus on the definition or the regulation of nutraceuticals among scientists [21]. The majority of nutraceuticals are of plant origin. Thus, nutraceuticals are "pills" that contain concentrated forms of presumed bioactive phytochemicals extracted from the original food item (e.g., genistein from soy). Because of their plant origin, these compounds are considered safe and are popular among consumers. This review will elaborate on some of the currently well-known dietary constituents that act as PPARγ ligands, with a demonstrated ability to bind to and activate PPARγ. The subsequent biological responses that result from this activation is not the focus of this review. For the purposes of this review, any isolated dietary component used in cell based or animal studies is considered a nutraceutical. Dietary components that act as ligands of PPARγ include dietary lipids such as n-3 and n-6 fatty acids and their derivatives, isoflavones and flavonoids. Table 1 provides a partial list of dietary PPARγ ligands.

2.1. Exogenous and Endogenous Lipid Derivatives. The majority of available research has focused on understanding the physiological significance of the interactions between dietary lipids and their derivatives with PPARs [25, 32–38]. Dietary fats and oils are major sources of these ligands, which include both n-3 and n-6 lipids and their oxidized counterparts. Elegant structure-function studies have determined the binding efficiency of the dietary lipids with PPARs [25, 39–42] by comparing them to synthetic drugs (TZD). Though dietary lipids similar to synthetic ligands were able to bind to the ligand binding domain and cause conformational changes to activate the receptor, they are considered as weak PPARγ ligands because of their low physiological concentrations. One must keep in mind that most of the studies determining the binding efficiency of the nutraceuticals have been performed in either cell-free or cell-based systems. The specificity of the dietary compounds to act as ligands for PPARγ was determined by a lack of response when cells were either pretreated with a known antagonist of PPARγ or with constructs that lacked PPAR ligand binding domain. However, in cell based systems it is conceivable that a metabolite of the parent compound, not the parent compound itself, might be mediating the response through interactions with PPARγ. For example, 13-HODE (oxidized n-6 lipid), a known agonist of PPARγ, could be converted into 13-Ox-HODE prior to interacting with PPARγ.

2.1.1. Exogenous Lipids: Dietary Lipids. Many studies have demonstrated that nonesterified unsaturated fatty acids are better ligands of PPARγ as compared to saturated fatty acids [43]. Although unoxidized unsaturated fatty acids are present in abundance *in vivo*, evidence suggests that they are weak activators of PPARγ. However, there is compelling evidence that oxidized unsaturated fatty acids are potent ligands compared to their unoxidized counterparts. Using NMR spectroscopy, Itoh and colleagues [39] studied the crystal structure of PPARγ bound fatty acids. They determined that fatty acids that bound covalently to the receptor were strong activators of PPARγ and the binding was also dependent on the polar nature of the lipid. Furthermore, using a dual luciferase reporter system, they demonstrated that the oxidized forms of the docosahexaenoic acid (DHA), a dietary n-3 fatty acid, 4-hydroxy docosahexaenoic acid (4-HDHA), and 4-oxo docosahexaenoic acid (4-oxoDHA) were potent ligands (EC_{50} values of 3.7 μM and 0.4 μM) as compared to DHA (>10 μM). Fatty acids that are modified by oxidation or nitration can originate in the diet or can be generated *in vivo*. Research from our laboratory [44] and by others [45, 46], has shown that dietary oxidized lipids are absorbed by the intestine and incorporated into lipoproteins and tissues. A study by Ringseis et al. [47] showed increased PPARγ DNA binding in the intestinal cells of pigs fed oxidized (heated) sunflower oil compared to pigs fed unoxidized oil. Even though it was not possible to identify the specific ligands that bound to PPARγ, the findings from this study are important because they demonstrated that dietary oxidized fats were able to increase PPARγ interactions with the DNA, even though this activation of PPARγ was not

Table 1: Potential dietary PPARγ ligands.

Ligand	Binding affinity	Type of assay	Reference
Linoleic acid	$K_i > 1\,\mu$M	Competitive radio-labeled binding assay	[22]
Nitrolinoleic acid	$K_i = 133$ nM		
9-Hydroxyoctadecadienoic acid (9-HODE)			
13-Hydroxyoctadecadienoic acid (13-HODE)			
9/10-NO_2-linoleic acid	$IC_{50} = 0.6\,\mu$M	Scintillation proximity	[23]
12-NO_2-linoleic acid	$IC_{50} = 0.41\,\mu$M	Competitive binding assay	
13-NO_2-linoleic acid	$IC_{50} = 0.44\,\mu$M		
Azelaoyl phosphatidylcholine (in oxidized LDL)	40 nm	Radiolabeled binding assay	[24]
Docosahexaenoic acid (DHA)	$EC_{50} > 10\,\mu$m	Dual luciferase reporter system	[25]
4-Hydroxy docosahexaenoic acid (4-HDHA)	$EC_{50} = 3.7\,\mu$m		
4-Oxodocosahexaenoic acid (4-oxo-DHA)	$EC_{50} = 0.4\,\mu$M		
Conjugated linoleic acid isomers (CLA)	$IC_{50} = 3.2$–$7.4\,\mu$M	Competitive scintillation proximity assays	[26]
Isoflavones:			
Genistein	$K_i = 5.7\,\mu$M	Membrane-bound competitive PPARγ binding assay	[27]
Daidzein	20 μM	Luciferase reporter assay in 3T3-L1 cells	[28]
	$EC_{50} = 73\,\mu$M	Luciferase reporter assay in HeLa cells	[29]
Equol	20 μM	Luciferase reporter assay in 3T3-L1 cells	[28]
Biochanin A	$EC_{50} = 3.7\,\mu$m	Luciferase reporter assay in HeLa cells	[29]
	$EC_{50} < 1\,\mu$M	Luciferase reporter assay in HepG2 cells	[29]
Flavonoids:			
Psi-baptigenin	$EC_{50} = 2.9\,\mu$M	Transcriptional factor activity assay in ThP-1 cells	[30]
Hesperidin	$EC_{50} = 6.6\,\mu$M		
Quercetin (from dill, bay leaves, and oregano)	$EC_{50} = 2.8\,\mu$M	Ligand screening assay	[31]
2′-Hydroxy chalcone (cinnamon in polymeric form)	$EC_{50} = 3.8\,\mu$M	Ligand screening assay	[31]
Rosmarinic acid (marjoram)	$EC_{50} = 16\,\mu$M	Ligand screening assay	[31]

associated with concomitant NFκB mediated inflammation. Seminal work by Schopfer et al. [22] has shown that nitrolinoleic acid (LNO2), which acts as a PPARγ ligand, is present in the plasma of healthy humans and has a K_i of 133 nM as compared to a K_i of >1000 nM for linoleic acid. Additionally, it was capable of promoting adipogenesis and glucose uptake in the 3T3-L1 cell model. Another group of isomers of linoleic acid, conjugated linoleic acid (CLA), is present in dairy products and can also be produced *in vivo* by commensal bacteria. Based on competitive scintillation proximity assays, various CLA isomers had IC_{50} values of 3.2–7.4 μM for PPARγ, but had IC_{50} values in the nM range (140–260 nM) for PPARα [26, 48]. This suggests that CLA isomers are stronger activators of PPARα as compared to PPARγ. However, in the past few years there has been a flurry of research investigating the role of CLA isomers in experimental colitis [49] because PPARγ is abundantly expressed in this tissue, and it appears that the protective effects of CLA isomers are due to the activation of PPARγ. Future investigations should consider if these protective effects are being partially mediated by other PPAR isotypes.

2.1.2. Endogenous Lipids. The identification of an endogenous physiological ligand for PPARγ has been problematic, possibly due to its low abundance. Even though it has been well established that endogenous ligand-mediated activation of PPARγ leads to adipocyte differentiation, the identification of this ligand has not yet materialized. Is there any evidence that ligands of PPARγ are generated *in vivo*? Yes, since there are endogenous enzymes that generate lipid ligands that interact with PPARs. 12/15 lipoxygenase-derived oxidized fatty acids such as 13-HODE, 12-HETE, and 15-HETE have been shown to activate PPARγ in vascular smooth muscle cells [50, 51]. In addition, ligands such as 9-HODE, 13-HODE [52], and 1-O-hexadecyl-2-Azelaoyl-sn-glycero-3-phosphocholine (AZ-PC) [24], derived from

oxidized LDL, have also been shown to activate PPARγ in cell based studies. Similarly, ligands such as 15-deoxy-Δ,12,14-prostaglandin J2 (PGJ2) generated by the action of cyclooxygenase (COX) on arachidonic acid (n20 : 4) are excellent activators of PPARγ [53] but due to their low *in vivo* abundance are considered as weak ligands.

2.2. Dietary Isoflavones. The primary dietary sources of isoflavones that are used as supplements are extracted from legumes, especially soybeans. The isoflavones in soy are mainly daidzein, genistein, and glycitein. After hydrolysis in the gastrointestinal tract, isoflavones are further modified by intestinal microflora. Thus, the metabolites of isoflavones that end up in the circulation depend on the type of microflora that inhabits the intestine. Equol and O-desmethylangolensin (ODMA) are the most common metabolites of daidzein. Several studies have shown that genestein activates PPARγ at micromolar concentration [54, 55] but inhibits adipogenesis in 3T3-L1 adipocytes [56], primary human adipocytes [57], and in animal models [58, 59]. This antiadipogenic effect of genestein is attributed to mechanisms beyond PPARγ activation. For example, downregulation of adipocyte-specific genes such as C/EBPα and β, PPARγ, SREBP-1, and HSL has been reported [60]. A study by Dang et al. demonstrated that genistein has concentration-dependent effects on progenitor cells, that is, genistein can act as an agonist of the estrogen receptor at lower concentrations ($<1\,\mu$M) but become a PPARγ agonist at higher concentrations ($>1\,\mu$M) in mesenchymal progenitor cells, thus promoting either osteogenesis or adipogenesis, respectively [27]. Moreover, a role for the estrogen receptor cannot be overlooked because genistein down regulated ERα and ERβ in an animal study of ovariectomized mice [61]. Daidzein and its metabolite equol activated PPARγ [28] in luciferase reporter assays utilizing several cell types and promoted adipogenesis in 3T3-L1 cells at much lower concentrations (10–100 μm) than genistein [29].

2.3. Other Dietary Constituents. Fruits and vegetables are rich in flavonoids. By screening a natural product library, Salam and colleagues [30] identified two flavonoids, Ψ-baptigenin (EC_{50} = 2.9 μM) and hesperidin (EC_{50} = 6.6 μM) as strong agonists of PPARγ. Furthermore, these flavonoids promoted a strong induction of PPARγ in THP-1 cells which was abolished by treatment with the PPARγ antagonist GW9662. Interestingly, in a recent study [62], healthy humans who ingested a supplement of Red Clover had detectable levels of Ψ-baptigenin in their plasma, thus making this a plausible physiological ligand of PPARγ. The biological effects of these natural PPARγ agonists need further investigation. Other dietary components that have been studied are epigallocatechin gallate (EGCG, from green tea) and resveratrol (abundant in grapes, wine, and peanuts). Once again, there are very few studies that demonstrate the PPARγ binding ability of these compounds. Because of their ability to reduce lipid accumulation [63] by altering PPARγ expression [64], these agents are presumptive ligands of PPARγ. In an extensive review on culinary herbs and spices, Jungbauer and Medjakovic [31] identified components of herbs and spices such as cinnamon, oregano, and marjoram with PPARγ binding affinities between 2.8 and 23.7 μM. Interestingly, most of these components seem to be very weak transactivators of PPARγ.

In summary, it is obvious that dietary components can bind and activate PPAR gamma. What is lacking, however, is the delineation of the metabolic effects that are specific to this PPAR gamma activation. Thus, future efforts should focus on study methodologies and techniques that can demonstrate a cause and effect relationship between nutraceutical activation of PPAR gamma and its physiological function.

3. Toxicology of Nutraceuticals

Nutraceuticals are increasingly being used as nutritional supplements in treatment of diseases. Due to the plant origin of these supplements they are considered safe for human consumption. However, the levels of the active substance consumed vary when taken as a whole food, as compared to a nutritional supplement [65, 66]. Very few studies have reported on long-term effects of nutrition supplements in humans. High consumption of lipids is associated with high risk of cardiovascular disease, diabetes, obesity, and cancer [67, 68]. Higher consumption of flavonoid supplements can alter the physiological levels of iron, vitamins, and other nutrients [66]. Flavonoids also interact with cytochrome P450 enzymes thus altering pharmacodynamics and pharmacokinetics of various drugs [69–71]. Similar to reports on TZDs, some of the flavonoids such as genestein have been associated with increased cancer risk [72–75]. Therefore, unless safety profiles of these nutraceutical supplements in humans are available, caution should be used in their long-term use as PPAR modulators.

4. Conclusions

The study of nutraceuticals as PPAR ligands is in its infancy. Newer insights into the role of PPARs in physiology and pathophysiology will help design better therapeutics. Future studies utilizing both high throughput screening technology and tissue specific metabolic profiling should identify nutraceuticals that modulate PPARγ activity. Subsequent cell culture and animal studies followed by rigorous clinical trials should then be able to establish the pharmacological and toxicological profiles of these nutraceuticals and their potential in influencing human health.

Acknowledgments

The authors wish to acknowledge the financial support of NIH Grants RO1 HL074239 (NS) and P20RR016477-09S2 (NS). The authors thank Dr. Nadya Merchant and Ms. Lisa Daniels for proofreading our paper.

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 978687, 9 pages
doi:10.1155/2012/978687

Review Article

Idealized PPARγ-Based Therapies: Lessons from Bench and Bedside

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Received 6 February 2012; Accepted 23 April 2012

Academic Editor: Christopher J. Nicol

The incidence of type 2 (T2D) diabetes and other chronic conditions associated with insulin resistance is increasing at an alarming rate, underscoring the need for effective and safe therapeutic strategies. Peroxisome-proliferator-activated receptor gamma (PPARγ) has emerged as a critical regulator of glucose homeostasis, lipid homeostasis, and vascular inflammation. Currently marketed drugs targeting this receptor, the thiazolidinediones (TZDs), have proven benefits on insulin resistance and hyperglycemia associated with T2D. Unfortunately, they have been associated with long-term unfavorable effects on health, such as weight gain, plasma volume expansion, bone loss, cardiovascular toxicity, and possibly cancer, and these safety concerns have led to reduced interest for many PPARγ ligands. However, over the last years, data from human genetic studies, animal models, and studies with ligands have increased our understanding of PPARγ's actions and provided important insights into how ligand development strategies could be optimized to increase effectiveness and safety of PPARγ-based therapies.

1. Introduction

Peroxisome-proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily that heterodimerize with retinoid X receptors (RXRs) to modulate the transcription of target genes. They are activated by fatty acids [1] and are thus considered lipid sensors involved in the transcriptional regulation of energy metabolism [1]. Three isotypes of PPAR have been identified so far, namely, PPARα, PPARβ/δ, and PPARγ, each with a distinct pattern of tissue distribution and with unique physiological functions [2]. Briefly, PPARα is found in the liver, kidney, heart, and muscle and is implicated in the uptake and oxidation of fatty acids and lipoprotein metabolism. PPARβ/δ is expressed in most cell types and plays an important role in lipid metabolism and cell differentiation and growth. PPARγ actions are mediated by two isoforms, PPARγ1, which has a wide tissue expression, and PPARγ2, highly expressed in adipose tissue and considered the master regulator of adipocyte differentiation and function. It is noteworthy that PPARs are also expressed in macrophages, in which they are key modulators of the inflammatory response [3].

Consistent with their significance in metabolism physiology, this subfamily of nuclear receptors is an important target in metabolic disease. This is evidenced by the fact that PPARα is the molecular target for the lipid-lowering fibrate drugs and PPARγ is the target for the insulin-sensitizing TZDs. In fact, the identification of the lipid sensor PPARγ as a key regulator of glucose metabolism came from the discovery that TZDs are potent agonists for this receptor [4]. TZDs increase insulin action in diverse animal models of insulin resistance and also in patients with T2D. However, the molecular basis of improved insulin sensitivity by activation of this "pro-obesogenic" receptor is incompletely understood [5], especially considering that obesity and T2D do not represent states of PPARγ deficiency. Insights from tissue-specific animal knockout models of PPARγ and also from ligand studies suggest there are at least two plausible mechanisms [6]. Activation of PPARγ in adipose tissue improves its ability to store lipids, reducing lipotoxicity

in muscle and liver. Also, PPARγ agonists modulate the synthesis and release of a number of signaling molecules from the adipocytes and macrophages resident in the adipose tissue, with significant metabolic effects in other tissues [2]. There is also evidence that PPARγ activation outside the adipose tissue is important for the insulin-sensitizing actions of TZDs [7–9].

Despite their metabolic benefits, TZDs may have clinically significant adverse effects, such as increased body weight [6, 10], fluid retention [11], increased risk of heart failure [11], bone loss [12], increased risk of myocardial infarction [13], and a potential link with bladder cancer [14, 15]. Because of the concerns on cardiovascular toxicity, rosiglitazone has been withdrawn in many countries worldwide, and due to concerns over its possible association with bladder cancer, pioglitazone has been suspended in some European countries.

These safety issues regarding TZDs have raised a number of questions. Firstly, what are the mechanisms underlying these unfavorable effects? Is PPARγ still an attractive pharmacological target to treat metabolic disease? What are the tools to find safe and effective PPARγ ligands? Over the last years, basic research and clinical studies have provided many insights into how PPARγ-based therapies could be optimized.

2. What Are the Basis of TZDs' Adverse Events?

Three TZDs have been approved for the treatment of insulin resistance associated with T2D over the last 15 years: troglitazone (which was discontinued in 1998), rosiglitazone, and pioglitazone (which have been discontinued in some countries and restricted in others). Although they are effective agents for the treatment of T2D, their use is associated with a number of adverse events. Some of them are considered common to the TZD class of drugs, whereas others are unique to individual TZDs. The latter are best characterized by idiosyncratic hepatotoxicity associated specifically with troglitazone [16], which was the reason for its discontinuance. Well-established class adverse effects include fluid retention, increased risk of congestive heart failure, weight gain and bone loss. The mechanisms underlying some of these unfavorable effects have been defined, but those of many others remain to be defined, as is the case of increased risk of myocardial infarction seen with rosiglitazone treatment [13] or the possible association between bladder cancer and pioglitazone [14, 15].

2.1. Fluid Retention. TZD treatment is consistently associated with body fluid expansion, which is accompanied by hemodilution, peripheral edema, and the potential to increase the risk of congestive heart failure [11, 17]. The mechanisms underlying fluid retention are not completely defined, although PPARγ action in modulating sodium transport in the collecting duct (CD) in both animal models [18, 19] and humans [20] seems to be involved. PPARγ is mainly expressed in CD [21, 22] and CD-specific PPARγ knockout in mice reduces fluid retention induced by TZDs [18, 19]. Moreover, activation of PPARγ in CD cells results in increased expression of epithelium sodium channel (ENaC) [18, 19] and enhances apical localization of the β-subunit of the ENaC in cortical CD cells [23], which in turn increase sodium and fluid reabsorption. In addition, TZDs increase the activity of the ENaC and Na-K-ATPase system, independent of the increase in ENaC expression [24, 25]. There are also data to suggest that ENaC-independent mechanisms might be involved, since amiloride, an inhibitor of ENaC, fails to prevent TZD-induced fluid retention [24, 25]. Accordingly, aquaporin-2 has been also implicated in this phenomenon [26].

Plasma volume expansion secondary to renal fluid reabsorption results in increase luminal pressure in the microvasculature, which in turn leads to a rise in pressure gradient across the microvessel wall and hence in fluid flux to the interstitial compartment [3]. This is considered as the main mechanism of formation of peripheral edema, although a direct action of TZDs in endothelium cells to increase vascular permeability, mediated by PPARγ, has also been implicated [27–29].

In addition to peripheral edema, renal fluid retention by TZDs is associated with the potential to increase cardiac load and precipitate or exacerbate congestive heart failure [30–32]. This has been the rationale to contraindicate TZD treatment in patients with class III or IV heart failure according to the criteria of the New York Heart Association [17]. Despite the propensity to precipitate congestive heart failure, there has been an intense debate over the possibility of direct cardiotoxicity of TZDs, especially of rosiglitazone, as will be discussed later.

2.2. Weight Gain. Increases in body weight are seen with all TZDs in both animal studies including rodents and nonrodents [6] and clinical studies [10, 11]. This effect has been traditionally ascribed to increased adipogenesis and fluid retention resulting from PPARγ activation by TZDs in adipose tissue and collecting duct cells, respectively. Moreover, it has been recently suggested that TZDs might influence energy balance by activating PPARγ in the central nervous system (CNS) and inducing increased food intake [33, 34].

Increased body fat mass has been classically associated with insulin resistance and cardiovascular disease, and hence weight gain is considered unfavorable in the treatment of T2D patients, in whom overweight or obesity is already frequent. However, increased adipogenesis with TZD treatment is associated with fat redistribution characterized by an increase in subcutaneous adipose tissue and concomitant decrease in visceral adipose tissue [35, 36]. Because of the unfavorable effect of visceral fat on insulin sensitivity, this redistribution of fat by TZDs is generally considered as beneficial in spite of increased body adiposity [37].

Despite the correlation between increased insulin sensitivity and adipogenesis and fat redistribution by TZD treatment, the need for increased adipogenesis to the antidiabetic effect of these drugs has been questioned. A substantial part of the insulin-sensitizing effect of TZDs has been ascribed to

their ability to induce adipocyte expression of adiponectin and reduce the expression other adipokines, which impair insulin action in peripheral tissues [2]. In addition, many PPARγ ligands with partial agonist activity have been shown to dissociate adipogenesis and weight gain from the insulin sensitizing effects [38], as will be discussed later.

Weight gain with TZD treatment has also been correlated with increased food intake for some years, at least in murine models [39]. Only recently, however, their effects on feeding have been dissociated from PPARγ activation on the adipose tissue. Complimentary reports by two independent research groups have suggested that PPARγ action in the CNS mediates its effects on food intake and energy balance [33, 34]. Ryan et al. showed that either acute or chronic activation of PPARγ by TZD treatment or hypothalamic overexpression of PPARγ, respectively, resulted in hyperphagia, positive energy balance, and weight gain. Conversely, inhibition of endogenous brain PPARγ action led to the opposite effects [33]. Lu et al. demonstrated that neuron-specific PPARγ knockout mice exhibited reduced food intake, increased energy expenditure during high-fat diet, resulting in reduced weight gain. Moreover, these animals were resistant to rosiglitazone-induced increase in feeding and weight gain [34].

2.3. Myocardial Infarction and Cardiovascular Mortality. Increased risk of congestive heart failure with TZD treatment has been traditionally associated with the propensity of these drugs to induce plasma volume expansion and increased cardiac load. However, the role of PPARγ in the heart has been controversial. Some animal studies have suggested that the direct action of PPARγ on the heart could be beneficial, since TZDs improve cardiac performance [40, 41], decrease cardiac hypertrophy [42–44], and may also have beneficial effects on left ventricular remodeling and function after ischemic injury [45, 46]. Other studies, in contrast, have suggested that TZDs induce cardiac hypertrophy in rodent models of diabetes [47, 48], although increased cardiac mass could not be attributed directly to PPARγ actions on the heart. Indeed, there are data to suggest that cardiac hypertrophy seen with TZDs may involve PPARγ-dependent and independent pathways, since cardiomyocyte-specific PPARγ-knockout mice were shown to develop cardiac hypertrophy and treatment of both wild-type and knockout mice with rosiglitazone also induced cardiac hypertrophy [49].

Clinical studies not primarily designed to address definite cardiovascular outcomes have also suggested no adverse effects of TZDs on cardiac performance or even a trend toward beneficial effects [40, 50]. Despite these potential favorable effects, in 2007 a meta-analysis indicated a significant increased risk for myocardial infarction and cardiovascular mortality in patients treated with rosiglitazone [13] and initiated concerns about the drug's cardiovascular safety. Since then, there has been no randomized controlled cardiovascular outcome trial sufficiently powered to confirm or refute these data [51–53]. Other meta-analyses conducted subsequently have either confirmed the initial findings or been inconclusive [54, 55], but none has refuted that rosiglitazone is associated with increased myocardial infarction risk. Moreover, the meta-analysis published in 2007 was updated in 2010 using alternative analysis to include trials with no cardiovascular events and confirmed the previous data that rosiglitazone increases risk for myocardial infarction [56].

The concerns regarding rosiglitazone's cardiovascular safety have raised the question of whether pioglitazone treatment is associated with a similar risk, since the mechanisms underlying increased risk for myocardial infarction with rosiglitazone have not been defined and it is therefore not known whether they are specific to this drug or represent a class effect. The Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROACTIVE trial) was a large randomized controlled trial designed to address cardiovascular outcomes that showed a benefit only in prespecified endpoints of death, myocardial infarction, and stroke [30]. It did not show statistically significant benefits in primary outcome, a broad composite of cardiovascular events. Smaller studies have similarly found that pioglitazone is not associated with increased cardiovascular risk other than the potential of exacerbation of congestive heart failure [57–59], whereas others have even suggested cardiovascular benefit [60].

Collectively, these data have raised two important questions. Firstly, what are the potential mechanisms underlying the cardiovascular adverse effects associated with rosiglitazone treatment? Further, what explains the differences between rosiglitazone and pioglitazone with respect to cardiovascular hazards? These questions remain unanswered, although conceivable mechanisms have been suggested. Clinical studies have shown that pioglitazone and rosiglitazone have different effects on lipid profiles. Rosiglitazone treatment increases low-density lipoprotein cholesterol levels and triglyceride levels [61], whereas pioglitazone reduces triglyceride levels and induces greater increases in high-density lipoprotein cholesterol levels [61]. In addition, the pattern of modulation of gene expression seems to be different when comparing both TZDs [62–64]. In a murine model of diabetes, rosiglitazone upregulated the expression of a matrix metalloproteinase gene in the heart, which encodes an enzyme implicated in plaque rupture [64].

2.4. Bone Loss and Increased Fracture Risk. Several clinical studies have linked both rosiglitazone and pioglitazone treatment to small but significant decreases in bone mineral density and increased fracture risk [12, 65–71], most frequently in women. Preclinical *in vivo* studies have greatly contributed to elucidate the mechanisms underlying this unfavorable effect. Treatment of mice with rosiglitazone suppresses osteoblast differentiation and increases marrow adipocytes [72], possibly by activating PPARγ in bone marrow stromal cells and diverting them from the osteoblast lineage into the adipocyte lineage [73]. Marrow insulin growth factor system may also be involved, since it is a key modulator of osteoblast differentiation and proliferation, and activation of PPARγ by rosiglitazone downregulates some components of this system [74]. Moreover, PPARγ

activation in hematopoietic precursors of the monocytic-macrophage lineage increases osteoclastogenesis and bone resorption [75].

2.5. Carcinogenesis. Concerns regarding the effect of TZDs on carcinogenesis are not recent; in 2005, pioglitazone and five of six dual PPARα/γ agonists were listed as having carcinogenic activity in rat bladder, and this has been [76] the rationale for FDA's official requirement, since 2006, that 2-year rodent carcinogenicity studies with PPAR ligands are conducted before clinical trials [77]. These concerns have been intensified recently, after the publication of observational clinical studies linking pioglitazone to bladder cancer risk [14, 15]. In contrast, there have been no preclinical and clinical data linking PPARα agonist to this type or cancer [78], neither there have been clinical data linking rosiglitazone to this type of cancer, although in a recent study rosiglitazone enhanced bladder tumors in rats pretreated with a bladder carcinogen [79].

Data from animal studies assessing the effects of PPAR ligands on tumorigenesis have been controversial. Some rodent studies have suggested that PPAR ligands may potentiate the development of diverse types of tumors, such as transitional cell carcinomas of the urothelium, hemangiosarcomas, liposarcomas, and sarcomatous tumors at various sites, whereas other animal studies have indicated a protective effect. These differences have been attributed to a number of factors, including ligand specificity (selective activation of PPARγ versus activation of other PPAR isotypes), the animal model (rodent versus non-rodent), and cancer type [3]. This issue is further complicated by data from *in vitro* studies suggesting the antiproliferation properties of PPARγ ligands [80]. Hence, the mechanisms underlying tumor formation are not established, and although the tumor types mentioned have been shown to express PPARγ it still discussed whether these effects are receptor dependent or -independent.

In particular, urothelium carcinomas have been associated with pioglitazone and some dual PPARα/δ agonists in different strains of rats (Sprague-Dawley, Fisher, Wistar). In these models, cellular hypertrophy has been an early finding in the bladder urothelium [81] although these effects have not been established as PPARγ-dependent. In addition, there are data to suggest that these compounds may result in the production of cytotoxic urinary solids that could induce regenerative proliferation in the urothelium in rats [82]. However, this effect is not seen in mice and is not likely to occur in primates [82]. The significance of these findings to humans is not clear, but recent observations have linked pioglitazone to bladder cancer. An interim analysis of an ongoing 10-year observational study with diabetic patients has not indicated a significant risk of bladder cancer with pioglitazone treatment for a median duration of 2 years. However, this risk was significantly increased in patients with longest duration of drug exposure or highest cumulative drug dose [14]. Further, data from the Adverse Event Reporting System of the FDA and the French Agency for the Safety of Health Products indicated a significantly increased risk of bladder cancer with pioglitazone treatment [15]. Pioglitazone was then withdrawn in France and Germany, and regulatory agencies in other countries have recommended that the drug should not be used in patients with active bladder cancer [83]. Notwithstanding, in a cohort study of 252,467 patients with a followup of less than 6 years, pioglitazone was not associated with increased risk of cancer at various sites, including prostate, female breast, lung/bronchitis, endometrium, colon, pancreas, kidney/renal pelvis, rectum, and also of non-Hodgkin lymphoma and melanoma [84].

3. A Historical Perspective on the Concept of Safety and Efficacy of PPARγ Ligands

The identification of PPARs as key regulators of diverse aspects of energy homeostasis has made them attractive pharmacological targets to treat metabolic diseases such as lipid disorders (drugs targeting PPARα or -δ), T2D (drugs targeting PPARγ), and obesity (drugs targeting PPARδ).

Initial strategies of ligand design aimed to develop potent full agonists or ligands acting on different PPAR isotypes to broaden their therapeutic effects. With respect to drugs targeting PPARγ, the clinical problems observed with the full agonists TZDs, as well as data from human genetic studies, animal knockout models, and preclinical and *in vitro* studies with ligands with different pharmacologic properties, have provided important insights into optimization of drug design strategies.

3.1. PPARγ Ligand Specificity. The possibility to target multiple risk factors associated with the metabolic syndrome by designing drugs with agonistic properties for more than one isotype of PPAR seemed very promising in the light of the diverse physiologic roles of this subfamily of nuclear receptors. Based on this rationale, some dual and pan-PPAR agonists were developed and some dual PPARα/γ agonists were evaluated in clinical trials, including muraglitazar, tesaglitazar, ragaglitazar, MK-767, and imiglitazar [3]. Failure with these ligands is probably best exemplified by the first PPARα/γ agonist, muraglitazar, which showed beneficial effects on glucose control and lipid levels of diabetic patients but was associated with a significantly increased risk of major cardiovascular events in a review of data from phase 2 and 3 clinical trials [85]. Other dual PPARα/γ agonists evaluated in clinical trials were also discontinued due to safety concerns [3]. It should be noted, however, that the reason for development discontinuation of these drugs was always compound specific, and therefore it is not clear if their adverse effects are a class effects or are unrelated to PPAR activation.

It is also noteworthy that the TZDs pioglitazone and rosiglitazone, although classically considered selective PPARγ ligands [4, 86], show weak agonist activity in both PPARα [87] and PPARδ [87, 88]. In fact, the favorable effects of pioglitazone on lipid profile accounted for its agonist properties on PPARα [89, 90]. As discussed before, although there are no data to attribute developmental failures with

dual PPARα/γ agonists to PPAR-dependent mechanisms, the properties of pioglitazone and rosiglitazone to activate both isotypes should be carefully considered.

3.2. Full versus Partial PPAR Agonists and Selective PPARγ Modulation. PPARγ agonists can be grouped into full agonists, classically represented by the TZDs, and partial agonists that, at saturating concentrations, result in lower levels of receptor activation than that of a full agonists. The interest for compounds with partial agonist activity comes from better understanding of PPARγ function with data from animal and human genetic studies and also from studies with ligands. The minor Ala allele of the human PPARγ2 polymorphism Pro12Ala [91] results in reduced binding affinity for responsive elements and reduced transcriptional activity [92, 93]. Clinically, this allele has been associated with improved insulin sensitivity and reduced risk of T2D [94–96] and seems to be associated with increased weight [97]. In addition, mice with germline heterozygous deletion of the gene encoding PPARγ resulting in reduced PPARγ activity exhibited increased insulin sensitivity as compared to wild-type mice [98] and were also resistant to high-fat diet-induced obesity and insulin resistance [99]. Collectively, these findings suggest that milder degrees of PPARγ activation, rather than its full activation, might be a better strategy to improve insulin sensitivity while preventing unfavorable effects of PPARγ action [100]. Based on this concept, partial PPARγ agonists are viewed as a strategy to maintain the benefits of PPARγ activation and at the same time reduce dose-dependent side effects observed with the full agonists, such as weight gain and plasma volume expansion. Indeed, in animal models and clinical studies many compounds with weak agonist activity minimize these unfavorable effects without loss of the insulin-sensitizing and antidiabetic activity [101]. Due to their ability to discriminate between the actions of PPARγ in different tissues, these compounds are also referred to as selective PPARγ modulators (SPPARγM) [101].

The molecular basis of the effects of SPPARγM is incompletely understood, but their effects probably stem from their distinct binding mode in the receptor's ligand binding pocket and differential recruitment of transcriptional cofactors [102], which can explain the different patterns of gene expression compared to that of full agonists [38]. However, the pattern of action of these ligands raise an important question: if the insulin-sensitizing and antidiabetic activity of PPARγ is closely correlated with their ability to activate PPARγ-induced transcription [86], why would ligands with weak agonist activity retain the favorable effects on glucose homeostasis, comparably to full agonists? Poor understanding of the mechanisms involved in the effects of partial PPARγ actions may have been one of the reasons for the reduced interest in these compounds in clinical trials in spite of their favorable effects in *in vitro* and preclinical studies.

A recent study by Choi et al. [103] greatly contributed to clarify important aspects of PPARγ action. This work showed that obesity-related inflammation activates cyclin-dependent kinase 5 (Cdk5) in the adipose tissue, which phosphorylates PPARγ at the serine residue at position 273 and results in dysregulation of a subset of PPARγ target genes, with reduced expression of genes with favorable metabolic effects, notably insulin sensitivity. They also showed that both full and weak agonists inhibit PPARγ phosphorylation by Cdk5 comparably. Moreover, this inhibition appears to be dissociated from classical receptor activation and is well correlated to the anti-diabetic effects of PPARγ ligands. These data suggest the rationale behind the action of these ligands and may not only renew interest for partial PPARγ ligands that have been already characterized *in vitro* and preclinically, but also be viewed as the basis for developing new PPARγ ligands. It is important to note that these data also raise important questions. Firstly, how does Cdk5-mediated phosphorylation of PPARγ lead to dysregulation of a subset PPARγ target genes? Further, how can the binding of a ligand to PPARγ inhibit S273 phosphorylation yet dissociate this effect from general transcriptional activity?

Based on the concept that the transcriptional effects of PPARγ ligands can be separated from the effects which result in insulin sensitization, in a subsequent work, Choi et al. [104] described a novel high-affinity synthetic PPARγ ligand (SR1664) completely devoid of classical transcriptional agonism but with full blocking activity of Cdk5-medidated phosphorylation. Treatment of wild-type mice with obesity and insulin resistance induced by high-fat and high-sugar diet with this ligand resulted in improvement of insulin sensitivity but in a nonstatistically significant reduction in glucose levels. As expected, in cell-based assays SR1664 antagonized transcriptional activity of PPARγ induced by rosiglitazone. Collectively, these data might indicate that a slight degree of partial agonism should be desirable for the benefits of PPARγ-based therapies.

4. Concluding Remarks

In the light of current knowledge regarding PPARγ action, optimized ligands would be those with mild agonistic activity, potent phosphorylation-inhibiting activity, and tissue-specific actions. With this profile, it might be possible to lower the risk of side effects while achieving maximal efficacy in treating insulin resistance. An important question is whether it would be cost-effective to search for new ligands with these features, since there are safe drugs currently available to treat T2D. The answer is probably yes, since metformin is the only marketed drug to treat insulin resistance, an important physiopathological component of the disease. Moreover, insulin resistance is associated with conditions other than T2D, such as obesity, cancer, and cardiovascular disease, and therefore new insulin-sensitizing agents could potentially have extensive clinical indications.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgment

This work was supported by Grants from Decanato de Pesquisa e Pós-Graduação, from University of Brasília,

Brazil (Edital 05/2012). Molecular Pharmacology Laboratory Group is supported by MCT/CNPq/CT-Infra/CT-Grant 620195/2008-8 and 485811/2011-1.

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 483536, 9 pages
doi:10.1155/2012/483536

Research Article

Peroxisome Proliferator-Activated Receptorα Agonists Differentially Regulate Inhibitor of DNA Binding Expression in Rodents and Human Cells

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Received 9 February 2012; Revised 2 April 2012; Accepted 4 April 2012

Academic Editor: Yuji Kamijo

Inhibitor of DNA binding (Id2) is a helix-loop-helix (HLH) transcription factor that participates in cell differentiation and proliferation. Id2 has been linked to the development of cardiovascular diseases since thiazolidinediones, antidiabetic agents and peroxisome proliferator-activated receptor (PPAR) gamma agonists, have been reported to diminish Id2 expression in human cells. We hypothesized that PPARα activators may also alter Id2 expression. Fenofibrate diminished hepatic Id2 expression in both late pregnant and unmated rats. In 24 hour fasted rats, Id2 expression was decreased under conditions known to activate PPARα. In order to determine whether the fibrate effects were mediated by PPARα, wild-type mice and PPARα-null mice were treated with Wy-14,643 (WY). WY reduced Id2 expression in wild-type mice without an effect in PPARα-null mice. In contrast, fenofibrate induced Id2 expression after 24 hours of treatment in human hepatocarcinoma cells (HepG2). MK-886, a PPARα antagonist, did not block fenofibrate-induced activation of Id2 expression, suggesting a PPARα-independent effect was involved. These findings confirm that Id2 is a gene responsive to PPARα agonists. Like other genes (apolipoprotein A-I, apolipoprotein A-V), the opposite directional transcriptional effect in rodents and a human cell line further emphasizes that PPARα agonists have different effects in rodents and humans.

1. Introduction

Fibrates have been effectively used to reduce plasma triglyceride levels under conditions of hypertriglyceridemia [1]. The molecular basis for the action of fibrates on lipid metabolism involves the activation of transcription factors, known as peroxisome proliferator-activated receptors (PPARs), principally the PPARα subtype expressed in liver ([2], for a review). Fibrates decrease the gene expression of apolipoprotein C-III, and increase the expression of fatty acid-catabolizing enzymes like acyl-coenzyme A oxidase [3] and 17β-hydroxysteroid dehydrogenase (17β-HSD) type IV [4] in rodent liver. Fibrates also display other effects, not directly related to the lowering of plasma lipids, including the modulation of immune and inflammatory responses. Thus, these drugs downregulate acute-phase protein expression, such as fibrinogen, C-reactive protein, and α2-macroglobulin [5–7].

PPARs also play an important role in glucose homeostasis. PPARα agonists, by upregulating fatty acid oxidation and ketone body production, are able to spare glucose. Several studies have indicated a beneficial effect of PPARα activation on insulin sensitivity [8, 9]. Thus, hyperinsulinemia and

hyperglycemia observed in mice subjected to a high-fat diet or in genetic insulin-resistant rodents [8] were sharply reduced by treatment with fibrates. The antidiabetic thiazolidinediones (TZD) drugs which are ligands of the PPARγ subtype, are prescribed for regulating glucose metabolism because they lower blood glucose by enhancing peripheral insulin sensitivity [10].

It has been shown that the levels of Id2, a member of the helix-loop-helix (HLH) transcriptional repressor protein family which includes Id1-4 [11, 12], are reduced in aortic smooth muscle cells by treatment with TZD suggesting that Id2 might play a role in their antidiabetic effects [10]. Furthermore, since glucose increases Id2 protein levels, Id2 could contribute to changes in cellular function that occur in insulin-resistant and diabetic states [13]. Interestingly, Id2 is upregulated in muscle, fat, and liver of obese ob/ob mice [14]. Park et al. [15] have demonstrated that Id2 is a transcriptional modifier of PPARγ expression and adipogenesis and found that Id2 expression is elevated in adipose tissues of diet-induced obese mice and humans leading to the hypothesis of a role for Id2 in obesity and insulin resistance. Furthermore, Id2 nullizygous mice show altered expression of genes involved in lipid metabolism which could be related to reduced lipid storage in liver and white adipose tissue [16]. These authors also found that genes involved in glucose homeostasis exhibited altered expression in Id2-null mice.

Id proteins participate in development, cell cycle control, differentiation, and tumorigenesis [17]. Id2 protein heterodimerizes with E proteins, a subset of basic HLH (bHLH) transcription factors [18] and sterol regulatory element-binding protein-1c (SREBP)-1c [19], but because Id2 lacks a DNA binding domain, Id2 acts as a dominant negative regulator of these transcription factors [11]. Additionally, Id2 is able to regulate the function of HLH transcription factors indirectly by sequestering E proteins [18].

Changes in lipid metabolism and insulin resistance during late pregnancy are comparable to that normally seen in type 2 diabetic patients, in which the use of fibrates is recommended [20]. For that reason, late pregnancy has been previously used by our group [2, 21–24] and other authors [25–28] to study the effect of PPAR agonists. We have used these experimental settings to discover new PPARα target genes in rodents [7]. Thus, we have used late-gestation rats to study the effect of fibrates in hepatic Id2 mRNA expression. In addition, since free fatty acids (FFA) are known to act as PPARα activators and fasting increases circulating FFA [29–31], the role of FFA on the Id2 mRNA expression was investigated in fasted rats. Furthermore, in order to determine whether the effect of fibrates on Id2 gene expression is mediated by PPARα, wild-type and PPARα-null mice were used. Finally, in order to study whether the effect of fibrates on Id2 gene expression is species-specific, the human hepatocarcinoma cell line (HepG2) was used as a model system.

2. Materials and Methods

2.1. Animals, Drug Administration, and Collection of the Samples

Study I. Female Sprague-Dawley rats weighing 180–210 g were fed *ad libitum* standard rat chow (B&K Universal, Barcelona, Spain) and housed under controlled light and temperature conditions (12 h light-dark cycle; $22 \pm 1^{\circ}$C). The experimental protocol was approved by the Animal Research Committee of the University San Pablo-CEU, Madrid, Spain. Half the animals were mated, and day 0 of pregnancy was determined by the appearance of spermatozoids in vaginal smears, whereas the remaining half were kept virgin. From day 16 of gestation, rats were given by oral gavage two daily doses of 0, 100 or 200 mg of fenofibrate (Sigma-Aldrich, St Louis, MO, USA)/kg of body weight, one at 8.00 h and the other at 18.00 h, suspended in 2% Tween-80 or Tween-80 alone. On the morning of the 20th day of pregnancy (after 4 days of treatment), corresponding to 14 h after receiving the last treatment, rats were decapitated and blood collected using tubes containing Na_2-EDTA. Liver was immediately removed, placed in liquid nitrogen and kept at -80°C until analysis. Virgin rats received the same treatment and were studied in parallel. There were 5-6 animals per group.

Study II. Female Sprague-Dawley rats weighing 180–210 g were mated, and half the animals were subjected to fasting for 24 h at day 19 of pregnancy. At day 20 of pregnancy, blood and liver were collected as before. Plasma aliquots were kept at -20°C until processing for the analysis of FFA by enzymatic commercial kit (Wako Chemicals GmbH, Neuss, Germany).

Study III. Male SV129 wild-type mice were purchased from Taconic (Germantown, NY, USA), and male SV129 PPARα-null mice [32] were a kind gift from Frank Gonzalez (National Cancer Institute, Bethesda, MD, USA). Control and treated mice ($n = 2$–5) were provided NIH-07 rodent chow (Ziegler Brothers, Gardner, PA, USA) and water *ad libitum*. This study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Chemical Industry Institute of Toxicology Institutional Animal Care and Use Committee (NC, USA). Lighting was on a 12 hr light/dark cycle. Mice were fed diet supplemented with either Wy-14,643 (WY) (ChemSyn Science Laboratories, Lenexa, KS, USA) (0.1%) or di-(2-ethylhexyl)phthalate (DEHP) (Aldrich Chemical, Milwaukee, WI, USA) (0.6%), or a control diet for 3 weeks. WY and DEHP were selected because of their different structural properties and uses. DEHP is considered a weak PPAR activator compared to WY. At the designated time after treatment, animals were anesthetized by pentobarbital injection and killed by exsanguination. Livers were removed, rinsed with isotonic saline, snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

Study IV. Human hepatocarcinoma cells (HepG2) were obtained from American Type Culture Collection (HB-8065) (Manassas, VA, USA) and cultured in EMEM media, supplemented with 1% glutamine, 1% nonessential amino acids, 3% antibiotics (100 U/mL penicillin and 100 μL/mL streptomycin), and 10% fetal bovine serum. All cells were grown in a 5% CO_2-humidified atmosphere at 37°C. After confluence, cells were cultured in serum-free medium (with 0.1% BSA) for 24 hours and different concentrations of fenofibrate (0, 10, 50, and 100 μM) in DMSO were added. After different times of incubation (2, 6, and 24 hours), media was collected and cells were washed with ice-cold PBS and removed with a cell scraper. After centrifugation, cell pellets were frozen and used for RNA extraction. In some cases, cells were preincubated for 30 minutes [33] with the PPARα antagonist MK-886 (Enzo Life Sciences Inc., Farmingdale, NY, USA) (10 μM) dissolved into DMSO. DMSO concentration in culture medium did not exceed 0.1%. An additional experiment was carried out in the same conditions as described above but the cells were instead cultured in serum-free medium for 36 hours, and then treated with the drugs.

2.2. Total RNA Preparation and Analysis

Studies I and II. Rat total hepatic RNA was isolated by a modification of the guanidium isothiocyanate method using Ultraspec RNA according to the manufacturer's instructions (Biotecx Labs, Houston, USA). Total RNA concentration was determined by absorbance measurement at 260 nm. The 260/280 absorption ratio of all samples was between 1.8 and 2.0. Total RNA-genomic DNA-free samples were used to analyse the expression of Id2 gene and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control, by semiquantitative RT-PCR, according to previously described protocols [7, 24]. Briefly, total RNA (2.5 μg) was digested with 5 U RNase free-DNaseI (Roche, USA) for 20 minutes at 37°C to remove traces of genomic DNA. The DNase was inactivated at 64°C for 10 minutes and cDNA was synthesized from total RNA by oligo(dT)-primed reverse transcription with Superscript II (Invitrogen, Life Technologies Ltd., Paisley, UK), according to the manufacturer's instructions. PCRs were performed in a 25 μL reaction mix containing 20 pmol of both forward and reverse primer, 10 mmol/L of each deoxyribonucleoside triphosphate, appropriate dilutions of the cDNA stock, 2.5 μL of PCR 10X buffer, and Accu Taq-polymerase (Sigma-Aldrich, St Louis, MO, USA). The sense and antisense primer sequences were 5′-GAAAAACAGCCTGTCGGACCA-3′ and 5′-CCAGGGCGATCTGCAGGT-3′ for Id2 (205 bp product); and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGT-3′ for GAPDH (450 bp product) [34, 35].

All reactions were performed in a PTC-100 Thermocycler (MJ Research, USA) in which samples underwent a 3 min initial denaturing step, followed by 35 cycles of 45 s to 1 min at 94°C, 45 s at the annealing temperature of 65°C for Id2 and 57°C for GAPDH, and a primer extension step at 72°C for 45 s to 1 min. The final extension step was 10 min at 72°C. The PCR products were analysed by agarose gel electrophoresis and DNA was visualized by ethidium bromide staining and using a UV-light box. Band intensity was determined by quantitative scanning densitometry (GS-700 Imaging Densitometer, BioRad, Hercules, CA, USA). To determine the linear range of the PCR, dilutions of the cDNA preparations were previously used for each gene and experimental group of rats. Results were normalized to the control gene (GAPDH).

Study III. Total RNA was isolated from mouse livers by a modification of the guanidinium isothiocyanate method using RNAzol according to manufacturer's instructions (Tel-Test, Friendswood, TX, USA). Twenty μg of denatured total RNA was separated on 1.2% agarose gels and transferred to nylon membranes in 20x SSC. The DNA probes for Northern blot analysis were labeled with [α-^{32}P]dCTP using the random primer DNA labelling kit provided by Amersham. Probes used were a rat L-bifunctional enzyme (Ehhadh) cDNA fragment, the complete cDNA of rat 17β-HSD type IV [4] and the PCR products generated as indicated above in the studies I and II, and using rat cDNA as a template. The probes were sequenced (ABI PRISM 377 Perkin Elmer DNA sequencer), and the sequences obtained were compared to Gene Bank sequences to confirm the accuracy of the probes used. Blots were prehybridized at 42°C for 2 h and hybridized overnight at the same temperature. Washing conditions were 0.1x SSC, 0.1% SDS at 53°C for 15 min three times, and membranes were exposed to appropriate screens (Imaging Screen K, BioRad) at 4°C from 1 h to three days and the images analyzed (Personal Molecular Imager FX, BioRad). Filters were stripped of label at 75–80°C for 1 h with 0.1x SSC, 0.5% SDS, 0.1% tetrasodium pyrophosphate and then rehybridized.

Study IV. Total RNA was isolated from HepG2 cell pellets by QIAcube automated protocol using spin-column kit (RNeasy Mini Kit, QIAGEN, Hilden, Germany). Total RNA concentration was determined by absorbance measurement at 260 nm. The 260/280 absorption ratio of all samples was between 1.8 and 2.0. Total RNA-genomic DNA-free samples were used to analyse the expression of Id2 and β-actin as a control, by reverse transcription and quantitative real time PCR (qPCR) assays, according to the following protocol: cDNA was synthesized from 1 μg total RNA by Transcriptor high fidelity cDNA synthesis kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. qPCRs were performed in a 20 μL reaction mix containing 20 pmol of both forward and reverse primer, SYBR Premix Ex Taq (Takara Bio Inc., Tokyo, Japan) and cDNA. Sense and antisense primer sequences were 5′-GAA AGCCTTCAGTCCCGTGAGGTCCGTT-3′ and 5′-CTG GTGATGCAGGCTGACAATAGTGGGATG-3′ for Id2 (271 bp) (Atlas RT-PCR Primer Sequences (Clontech, CA, USA); 5′-CCTGGCACCCAGCACAAT-3′ and 5′-GGGCCGGAC TCGTCATAC-3′ for β-actin (145 bp) [36]. Samples were analyzed in duplicate. All reactions were performed in a

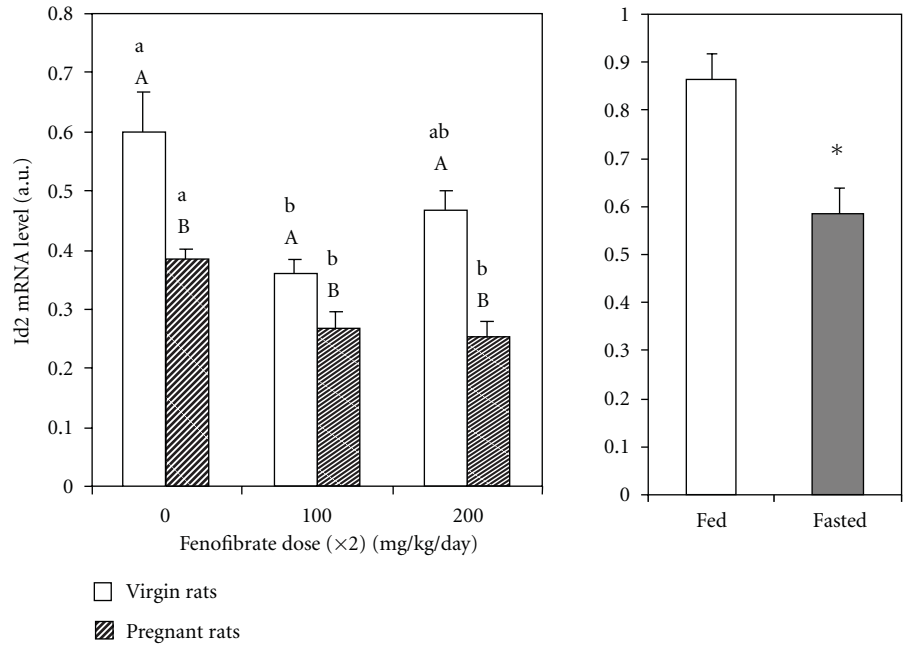

Figure 1: Fenofibrate downregulates Id2 gene expression in rats. Left panel: relative amount of mRNA of liver Id2, after 4-day treatment with or without fenofibrate in virgin and pregnant rats, measured by semiquantitative RT-PCR. Values were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and were represented using arbitrary units. Capital letters correspond to the statistical comparisons between pregnant and virgin rats receiving the same treatment. Small letters correspond to the statistical comparisons between rats receiving different drug doses. Values not sharing a common letter are significantly different at $P < 0.05$. Each value represents the mean ± standard error of five animals. Right panel: starvation downregulates Id2 gene expression. Relative amount of mRNA of liver Id2 from pregnant rats fed with standard pellet or fasted 24 h, measured by semiquantitative RT-PCR. Values were normalized against GAPDH expression and were represented using arbitrary units. Asterisk represents significantly different at $P < 0.05$.

LightCycler 5.0 (Roche). Optimal qPCR efficiency and linearity were previously confirmed for each target. Results for the expression of Id2 mRNA were expressed relative to the control gene (β-actin).

2.3. Statistical Analysis. Results were expressed as means ± S.E. Treatment effects were analyzed by one-way analysis of variance (ANOVA). When treatment effects were significantly different ($P < 0.05$), means were tested by Tukey multiple range test. For nonparametric data, the Mann-Whitney U test was used with differences between the two groups analyzed by Student t-test.

3. Results

3.1. Effect of Fenofibrate on Id2 Expression in Pregnant and Virgin Rats. As shown in Figure 1, hepatic Id2 mRNA levels were higher in virgin than in pregnant rats in the absence of treatment. In nonpregnant rats, hepatic Id2 mRNA levels were decreased by treatment with fenofibrate, although the effect at higher dose was not significant. PPARα agonist treatment for 4 days also decreased Id2 mRNA levels in pregnant rats (Figure 1) independently of the dose used, indicating that the lower dose was sufficient to reduce the expression of the Id2 gene. These results validate those previously found by our group when Id2 levels were evaluated using the same samples by macroarray technology (Atlas Nylon Arrays, Clontech, BD Biosciences, Palo Alto, CA, USA) (unpublished results).

3.2. Effect of Fasting on Id2 Expression. It is well known that several types of fatty acids are PPARα activators [29, 30]. The uptake of fatty acids into the liver as a result of their mobilization from adipose tissue after fasting would result in PPARα activation and changes in the expression of its target genes [31]. Plasma FFA levels in fed rats were 360.84 ± 22.15 μM, significantly different ($P < 0.05$) from those levels found in 24 h fasted rats: 1,503.90 ± 157.81 μM. As shown in Figure 1, 24 h fasting produced a significant decrease in hepatic expression of Id2 in comparison to the rats fed *ad libitum* correlating to the increase in circulating fatty acids.

3.3. Requirement for PPARα in Fibrate Regulation of Id2 Expression. Because PPARα has been shown to mediate several fibrate-inducible responses in the liver, we examined the dependence of fibrate-induced decreases in Id2 gene regulation on PPARα expression. Wild-type mice and PPARα-null mice [32] were fed a control diet or the same diet supplemented with either WY (0.1%) or DEHP (0.6%) for 3 weeks. As shown in Figure 2, when wild-type mice were fed WY there was a significant decrease in the liver expression of Id2 mRNA, whereas treatment with DEHP, a weaker PPARα activator, did not change the levels of Id2 mRNA. Treatment

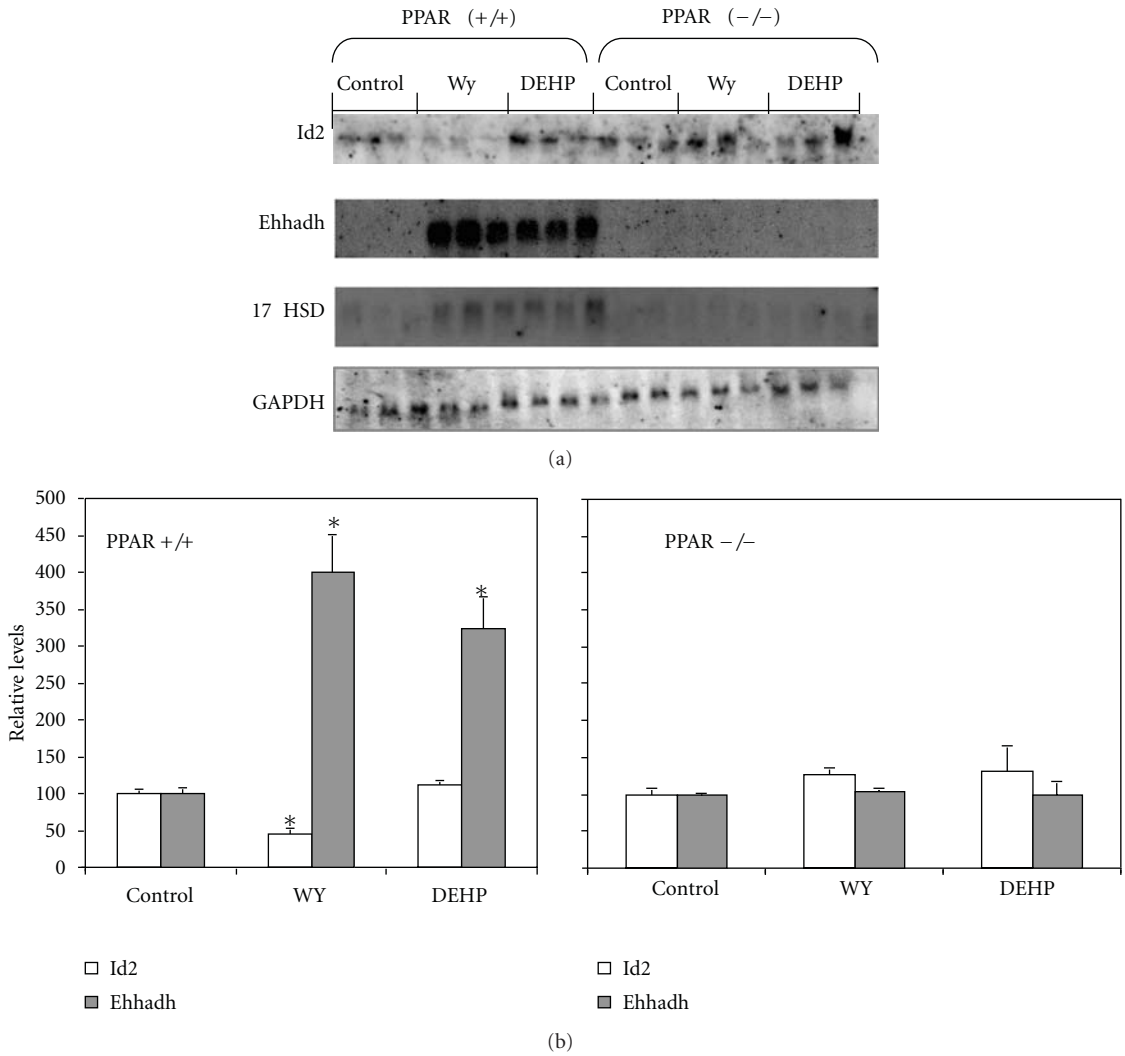

Figure 2: Downregulation of Id2 gene expression by WY is dependent on PPARα. Wild-type SV129 mice (+/+) or SV129 mice that lack PPARα (−/−) were fed a control diet (*Control*) or a diet containing WY (0.1%) or DEHP (0.6%) for 3 weeks. Total RNA isolated from liver was separated by 1.2% agarose, transferred to nylon, and analysed by northern blot using probes for Id2, Ehhadh, and 17βHSD, and GAPDH as a control. Northern autoradiograms (a) were densitometrically scanned and expression normalized to that of GAPDH (b). Each value represents the mean ± standard error of three animals. *significantly different from control ($P < 0.05$).

of PPARα-null mice with WY or DEHP resulted in no change in Id2 gene expression (Figure 2).

As a positive control of PPARα agonist regulation of gene expression, we also examined the levels of L-bifunctional enzyme (Ehhadh) mRNA. As expected [37, 38], Ehhadh mRNA expression was significantly enhanced by WY or DEHP treatments in wild-type mice but not in PPARα-null mice (Figure 2). A similar effect was found for 17β-HSD type IV gene expression (Figure 2), in accordance with our previously published results [4]. As a negative control, GAPDH mRNA levels remained constant under all conditions (Figure 2).

3.4. Effect of Fenofibrate on Id2 Expression in Human Cultured Cells. Since fibrates depressed Id2 hepatic expression in rodents in a PPARα mediated manner, we also determined if Id2 mRNA expression exhibits a similar behaviour in human cells. Unexpectedly, fenofibrate at 50 and 100 μM increased Id2 mRNA expression after 24 hours of treatment (Figure 3(a)). A previous report has shown that glucose could induce Id2 expression in cultured cells [13]. The EMEM media used here contained 5 mM glucose, therefore, we repeated the experiment in the presence of 20 mM glucose. The results observed in the presence of additional glucose were similar to those described in Figure 3(a) (data not shown).

To determine if the activation of Id2 expression is mediated by PPARα in HepG2 cells, the cells were preincubated with the PPARα antagonist MK-886 [39]. As shown in Figure 3(b), the effect of fenofibrate was not blocked by preincubation with MK-886, indicating that the induction

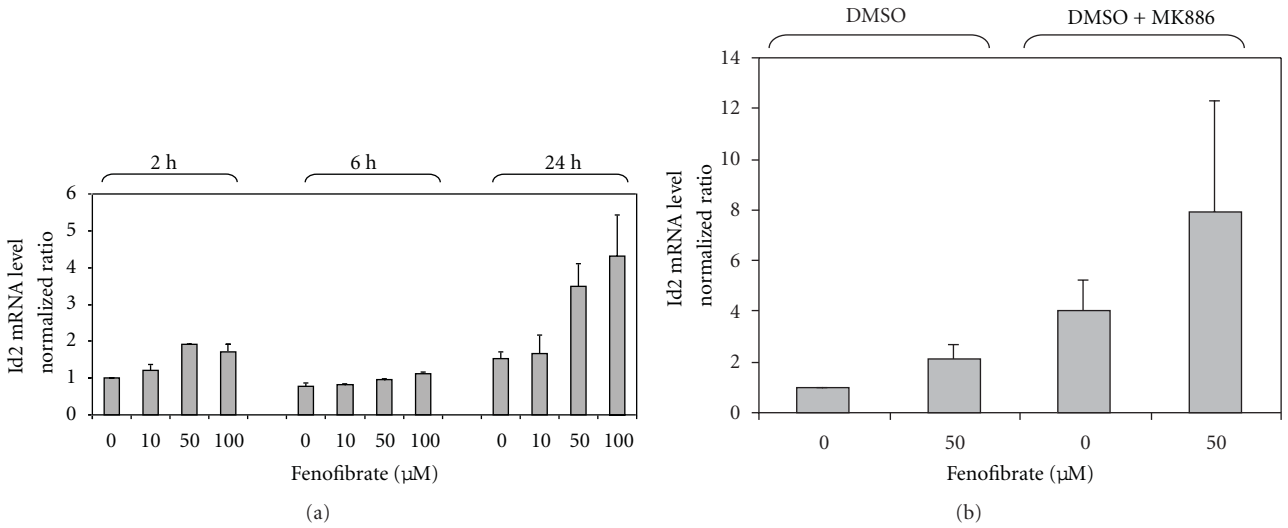

Figure 3: Fenofibrate upregulates Id2 gene expression in HepG2 cells. Panel (a) human hepatocarcinoma cells treated with different concentrations of fenofibrate (0, 10, 50, or 100 μM) for 2, 6, or 24 hours. Relative Id2 mRNA levels were measured by real-time PCR, normalized to β-actin levels and expressed in relative units to control. Values for Id2 mRNA are expressed as mean ± SD ($n = 3$). Panel (b) HepG2 cells were preincubated with the PPARα antagonist MK-886 (10 μM) where indicated and treated with different concentrations of fenofibrate (0 or 50 μM) for 24 hours.

was independent of PPARα. Instead, the PPARα antagonist showed an additive effect with fenofibrate on Id2 mRNA expression (Figure 3(b)). Glutathione S-transferase pi 1 (Gstp1), which expression has been recently shown to be modified by PPARα activators [40], was also measured. Fenofibrate increased Gstp1 expression (1.7-fold induction versus control without drug), whereas MK-886 abolished the effect of fenofibrate on HepG2 cells.

A recent report showed that Id2 expression could be influenced by circadian rhythm [16], and it has been previously established that serum is able to induce circadian gene expression [41, 42]. Therefore, an additional experiment was carried out with MK886 and fenofibrate, in which the cells were cultured in serum-free medium for 36 hours instead of 24 hours. Similar results to those observed in Figure 3(b) were obtained when the serum was substituted with BSA, 36 hours before the treatment with the drugs (data not shown).

4. Discussion

In this study, we found that fenofibrate treatment repressed liver Id2 mRNA expression both in pregnant and virgin rats. These findings agree with those of Yamazaki et al. [43] using cDNA microarrays from mice after 2 or 3 days oral administration of fenofibrate (100 mg/kg) or WY (30 mg/kg), and are consistent with those reported by Wong and Gill [44] after 1.0% DEHP in the diet for 13 weeks as studied by microarrays.

Glucocorticoids, whose circulating levels are augmented during pregnancy [45], have been described as repressors of Id2 expression in cells [46]. In agreement with this, basal levels of Id2 mRNA in pregnant rats were lower than in unmated rats, the difference being also observed after fenofibrate treatment. These findings emphasize the downregulatory effect of PPARα agonists, independent of whether Id2 mRNA levels are low, as in gestation, or elevated as in nonpregnant rats.

Fasting produces mobilization of fatty acids from adipose tissue. Fatty acids are natural activators of PPARα [29, 31, 47]. Therefore, the arrival of fatty acids in the liver as result of starvation, led to a significant decrease in hepatic expression of Id2. This finding reinforces the idea that PPARα activation produces a decrease in Id2 mRNA expression in liver.

Id2-null mice exhibit a decrease in adipose tissue and liver fat deposition compared to wild-type mice [16]. Consistent with that, Id2-overexpressing adipocytes show increased capacity for morphological differentiation and lipid accumulation [15]. In contrast, we found that PPARα activators decrease Id2 gene expression under the same conditions as we had previously reported an accumulation of lipids in the liver. Thus, hepatic triglyceride content in fasted rats was higher in comparison to fed condition [48], and it was also augmented in nonpregnant fenofibrate treated rats (in comparison to nontreated unmated rats) [24]. Nevertheless, although Id2 mRNA expression was also modified by fenofibrate in late gestation, hepatic triglyceride content was not affected by the drug in pregnant rats [24].

Since it has been reported that Id2 inhibits lipogenesis by interfering with the transcriptional activity of SREBP1c at the fatty acid synthase (FAS) promoter [19], our results might reflect an increased lipogenesis along with an Id2 repression. However, the hepatic expression of FAS was not significantly changed by fenofibrate in virgin or in pregnant rats [24], and 24 h fasting instead decreased FAS expression in pregnant rats (unpublished results).

We found a decrease in the expression of Id2 in mice treated with WY-14,643, yet this effect was not seen with

DEHP, indicating that the effect depends on PPARα activator potency. In the case of mice that lacked a functional form of PPARα, differences were not observed after the different treatments, suggesting that it was an effect mediated by PPARα. This finding is in agreement with that one recently described by el Azzouzi et al. [49] in murine cardiomyocyte cells using cDNA microarrays. One of gene listed by these authors to be specifically downregulated by WY was Id2. However, neither GW-5015160 (a PPARβ/δ agonist) nor rosiglitazone (a PPARγ agonist) produced any change. Thus, it can be assumed that fibrates affect Id2 gene expression through PPARα.

The decrease found in the expression of Id2 in mice by the potent PPARα activator WY agrees with the reduction observed in rats treated with fenofibrate. Since, it appears that both STAT3 and C/EBPβ are involved in regulation of Id2 [50, 51], and it is known that PPARα activation interferes with signalling pathways dependent on C/EBP and STAT [52], we hypothesize that PPARα may negatively regulate Id2 through inhibition of STAT3 or C/EBP.

In contrast to rodents, the negative effect of fibrates on Id2 gene expression was not observed in human cells. Fenofibrate enhanced Id2 mRNA levels in these cells after a 24 h incubation. Moreover, since Grønning et al. [13] have shown in murine macrophages that glucose induces increases in protein levels of this transcriptional repressor, we studied the effect of fibrates both at low glucose and high glucose and found a similar fenofibrate-inducing effect on Id2 mRNA expression. The effect of fenofibrate was observed after 24-h incubation, suggesting that regulation of Id2 expression by PPAR might occur by an indirect mechanism [53]. Nevertheless, several PPARα target genes [54] were also induced after 24-hour administration of fibrates but not earlier. Therefore, we studied the effect of MK-886, an antagonist of PPARα and found that fenofibrate-induced increases in Id2 mRNA expression were not abolished by preincubation with MK-886, confirming that the effect was not mediated by PPARα. In accordance with this finding, TZD, which also modulate Id2 mRNA levels in cultured human cells, use a PPARγ-independent mechanism [10]. Fenofibrate and MK-886 functioned synergistically to stimulate Id2 expression. The MK-886-induced increase in Id2 mRNA expression could be caused by two mechanisms: (i) specific inactivation of PPARα; (ii) other pathways, such as inhibition of leukotriene biosynthesis [39]. Therefore, it is assumed that fenofibrate-induced increases in Id2 expression occur by a PPARα-independent mechanism. How fenofibrate increases Id2 expression in HepG2 cells remains elusive.

Finally, Id proteins are HLH transcription factors that participate in development, cell cycle control, differentiation, and tumorigenesis [17]. However, the role of Id2 protein in the mechanism of action of fibrates has not been elucidated. Altogether, these findings confirm that Id2 gene expression is responsive to PPARα activators (fibrates and possibly fatty acids). However, as reported for other genes (apoA-I, apoA-V) ([2] and references therein), the effects are opposite in rodents versus humans. Since peroxisome proliferators function as hepatocarcinogenic agents in rodents, but not in humans [55], and considering the role of Id2 protein in cell proliferation and cancer [17], we speculate that the differential response to fibrate exposure might be related to the differences in liver tumorigenesis between species.

Abbreviations

PPAR: Peroxisome proliferator-activated receptor
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
Id2: Inhibitor of DNA binding 2
DEHP: Di-(2-ethylhexyl)phthalate
WY: WY-14,643
RT-PCR: Reverse transcriptase polymerase chain reaction
EMEM: Eagle's minimal essential medium
A.U.: Arbitrary units.

Acknowledgments

The authors would like to thank Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica (I+D+i), Instituto de Salud Carlos III-Subdirección General de Evaluación y Fomento de la Investigación (PI-09/02192), and European Community FEDER funds, as well as the Fundación Universitaria San Pablo-CEU (USP-PC 17/09) for financial support. M. Carmen González was a recipient of a predoctoral fellowship from the Universidad San Pablo-CEU, Madrid, Spain. The authors thank Milagros Morante for her excellent technical assistance and Drs. Mitch Rosen and Sheau-Fung Thai for a critical review of the paper. The information in this document has been supported by the U.S. Environmental Protection Agency. It has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 483656, 8 pages
doi:10.1155/2012/483656

Review Article

Effects of PPARγ Ligands on Leukemia

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Received 1 January 2012; Accepted 21 March 2012

Academic Editor: Stefan Alexson

Peroxisome proliferator-activated receptors (PPARs) and retinoic acid receptors (RARs), members of the nuclear receptor superfamily, are transcription factors that regulate a variety of important cellular functions. PPARs form heterodimers retinoid X receptor (RXR), an obligate heterodimeric partner for other nuclear receptors. Several novel links between retinoid metabolism and PPAR responses have been identified, and activation of PPAR/RXR expression has been shown to increase response to retinoids. PPARγ has emerged as a key regulator of cell growth and survival, whose activity is modulated by a number of synthetic and natural ligands. While clinical trials in cancer patients with thiazolidinediones (TZD) have been disappointing, novel structurally different PPARγ ligands, including triterpenoids, have entered clinical arena as therapeutic agents for epithelial and hematopoietic malignancies. Here we shall review the antitumor advances of PPARγ, alone and in combination with RARα ligands in control of cell proliferation, differentiation, and apoptosis and their potential therapeutic applications in hematological malignancies.

1. Introduction

Acute myelogenous leukemia (AML) remains incurable in most patients because of the likelihood of relapse and the development of resistant disease [1]. Many novel agents do not improve survival of patients once relapse occurs, which enforces the need for more effective treatment strategies for AML exploiting apoptosis and/or differentiation induction.

Ligands of nuclear hormone receptors (NHRs) have been shown to induce apoptosis and/or inhibiting proliferation in a variety of preclinical models. The most striking improvement in AML therapy was achieved by the treatment of acute promyelocytic leukemia (APL) using the retinoic acid (RA) receptor- (RAR-) specific ligand, all-trans RA (ATRA) [2, 3]. ATRA, combined with chemotherapy, results in complete remission (CR) rates ranging from 72% to 90% in APL patients with the oncogenic transcriptional repressor PML-RARα [4–8]. However, approximately 10% to 30% of patients relapse [8] and frequently develop resistance to ATRA [9, 10]. Acquisition of specific mutations in the ligand binding site, which leads to altered interactions with transcriptional coregulators, is a well-documented mechanism of acquired ATRA resistance [11, 12]. In addition, several alternative mechanisms such as DNA methylation [13] or impaired telomerase regulation [14] have been proposed to cause ATRA-resistant disease.

Considering the potential of using PPARγ ligands in APL "transcriptional" therapy, this paper summarizes the effects of endogenous and synthetic PPARγ ligands in AML and focuses on elucidating the mechanisms underlying the antitumor effects of novel synthetic PPARγ ligand 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) in APL.

2. PPARγ and PPARγ Ligands

PPARs belong to the NHR superfamily of ligand-dependent transcription factors, which includes RAR and RXR among others. Three PPAR isotypes have been identified: PPARγ,

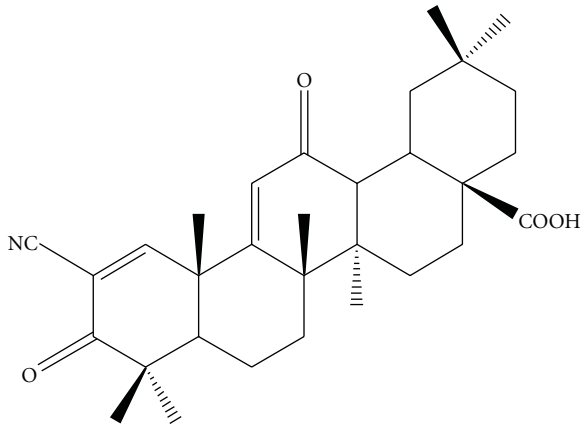

Figure 1: Molecular structure of CDDO 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO).

PPARα, and PPARβ/δ. PPARγ exists as a heterodimer with RXR, and upon activation by endogenous or synthetic ligands, PPARγ/RXR binds to the specific response elements PPRE in the promoter regions of target genes, respectively, which in turn functions as a transcription factor [15–17].

PPARγ modulates gene networks involved in controlling growth, cellular differentiation, and apoptosis [18]. PPARγ receptor can be activated by endogenous ligands (e.g., prostaglandin D2 (PGD2), 15-deoxy prostaglandin J2 (15dPGJ2), or 15-hydroxyeicosatetraenoic acid (15-HETE)) [19, 20], and synthetic ligands that include insulin sensitizing antidiabetic thiazolidinediones (TZD); troglitazone (TGZ), rosiglitazone (RGZ), ciglitazone (CGZ), or pioglitazone (PGZ) [21–23]; nonsteroidal anti-inflammatory compounds indomethacin, ibuprofen, flufenamic acid, or fenoprofen [24]; triterpenoids 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) [25] are a semisynthetic triterpenoid derived from oleanolic acid, whose structure contains two α, β-unsaturated carbonyl moieties. CDDO was shown to release nuclear receptor corepressor (NCoR) and recruit CCAAT/enhancer-binding protein (CBP/p300) to PPARγ [25] (Figure 1).

PPARγ ligands induce differentiation and inhibit proliferation in several tumor models [26–34]. The regulation of gene transcription by ligand-bound PPARγ involves cofactor proteins, which bridge transcription factors to the basal transcriptional machinery or modify chromatin structure. These include release of small accessory molecules known as corepressors (e.g., NCoR or silencing mediator for retinoid receptor and thyroid hormone receptors (SMRT)) and recruitment of coactivators (e.g., CBP/p300, cyclic adenosine monophosphate response-element binding protein (CREB), steroid receptor coactivator-1 (SRC-1), receptor interacting protein 140(RIP140), or PPARγ interacting protein (PRIP/RAP250) [35–40]. The multiprotein complex induces transcription by chromatin remodeling and interaction with the basal transcriptional machinery [41, 42], and the relative levels of cofactor expression (e.g., availability of cofactors CBP/p300 versus SRC-1) also control the specificity of the physiological response to target gene transcription [43].

3. Antitumor Effects of PPARγ in AML

High PPARγ expression was observed in normal bone marrow and peripheral blood $CD34^+$ progenitor cells [44]. Furthermore, significantly higher PPARγ mRNA expression was observed in primary AML cases compared to normal peripheral blood or bone marrow mononuclear cells [45, 46].

The mechanisms of cell differentiation and cell cycle arrest by activated PPARγ depend heavily on the specificity of PPARγ ligands. The induction of differentiation by activation of PPARγ may represent a promising novel therapeutic approach for cancer as already demonstrated for liposarcoma [27] and in xenograft models of prostate [47] and colon cancer [30]. Differentiation therapy may well play a role in acute myeloid leukemias, analogous to ATRA-induced differentiation in APL. PPARγ is known to be induced and/or expressed in cells of the myeloid/monocytic lineage [48, 49].

In PPARγ expressing AML cell lines, PPARγ ligand TGZ suppressed their clonal growth with G1 cell cycle phase arrest, induced differentiation into monocytes, and increased apoptosis at higher concentrations [50, 51]. Troglitazone-induced G0/G1 cell cycle arrest with upregulation of p21 mRNA in myeloid leukemia cell lines [52]. In concert with these findings, PPARγ ligand PGZ and 15dPGJ2 suppressed proliferation, and the combined treatment with ATRA synergistically induced myeloid differentiation in promyelocytic leukemia NB4 cells [53]. Furthermore, simultaneous treatment with TGZ and RXR or RAR ligands resulted in additive suppression of growth indicating that PPARγ ligand combined with a retinoid is a potent inhibitor of clonogenic growth of AML [50]. CDDO has been reported to induce monocytic differentiation of human myeloid leukemia cells and adipogenic differentiation of mouse fibroblasts [54].

CDDO-Me also induced granulo-monocytic differentiation in primary AML cells and cell lines. Combinations with ATRA or the RXR-specific ligand LG100268 enhanced the effects of CDDO-Me on cell viability and/or terminal differentiation of myeloid leukemic cell lines [54]. CDDO-Me-induced enhanced apoptosis when combined with ara-C and retinoids indicating potential activity in the future therapy for AML [55].

With respect to the mechanisms of PPARγ-ligand-induced differentiation, CCAAT enhancer-binding protein alpha (CEBPA) translational upregulation has been reported to be required for CDDO-induced granulocytic differentiation of AML patients samples and cell lines [56]. CDDO increases the ratio of transcriptionally active p42 and the inactive p30 CEBPA isoform, which in turn leads to transcriptional activation of CEBPA-regulated genes and associates with dephosphorylation of eIF2alpha and phosphorylation of eIF4E [56].

PPARγ ligands are additionally known to induce apoptosis. The mechanisms of apoptosis induction by activated PPARγ depend heavily on the specificity of PPARγ ligands. PPARγ activation by natural ligand 15dPGJ2 and synthetic ligand TGZ induce apoptosis accompanied by caspase-3 activation and downregulated c-myc gene expression in myeloid leukemic cells [57]. 15dPGJ2 and TGZ have been also

reported to induce upregulation of bax and downregulation of antiapoptotic proteins survivin and bcl-2 in AML and CML [58]. Furthermore, downregulation of cyclooxygenase-2 expression, disruption of mitochondrial membrane potential, activation of caspase-3, downregulation of Bcl-2, Bcl-Xl, and Mcl-1, and upregulation of Bax by these PPARγ agonists 15dPGJ2 and TGZ has been reported in human monocytic leukemia cells [59]. Semisynthetic oleanane triterpenoid CDDO has potent differentiating, antiproliferative, anti-inflammatory, and apoptosis-inducing properties [54]. CDDO has been reported to activate caspase-8 and -3 and to induce mitochondrial cytochrome *c* release in leukemic cells and in osteosarcoma cells [60–62]. CDDO has been further shown to activate the intrinsic pathway of apoptosis that involves the release of cytochrome *c* and AIF and initiates caspase-dependent and independent cell death in AML [63]. The C-28 methyl ester of CDDO, CDDO-Me [55], and C-28 imidazolide imide of CDDO (CDDO-Im) [64] has been shown to be more potent than CDDO in inducing apoptosis and differentiation of acute myeloid leukemia (AML) cells. CDDO-Me is 3- to 5-fold more active than CDDO in inhibiting the viability of AML cells in an MDR-1- and p53-independent manner, inducing apoptosis through a loss of mitochondrial membrane potential, and increasing caspase-3 cleavage and proapoptotic Bax protein. It has significantly less cytotoxicity against normal $CD34^+$ progenitor cells, assuring therapeutic window [55].

In addition, CDDO was shown to inhibit NF-κB-mediated gene expression in leukemic cells [62]. CDDO/tumor-necrosis-factor- (TNF-) induced apoptosis occurs through selective inhibition of NF-κB-dependent antiapoptotic proteins, bypassing potential mitochondrial resistance mechanisms [62]. CDDO-Me also inhibits both constitutive and inducible NF-κB through inhibition of IκB α kinase, leading to the suppression of expression of NF-κB-regulated gene products and enhancement of apoptosis induced by TNFα [65].

Notably, certain PPARγ ligands execute anti-tumor activities without requiring interaction with the PPAR ligand binding domain [66]. For example, CDDO, CDDO-Me, and CDDO-Im activate PPARγ-dependent and -independent pathways that inhibit cancer-cell growth [67]. They activate PPARγ in transactivation assays, and CDDO-induced apoptosis was diminished by dominant-negative PPARγ in myeloid HL-60 cells and by T007 in myeloid U937 cells [68], but CDDO-Im-induced differentiation in leukemia cells was not inhibited by the PPARγ antagonist GW9662 [61], and T007 did not affect inhibition of SKOV3 ovarian cancer cell growth by CDDO [69]. In these scenarios, interaction with the PPARγ receptor is irrelevant to the anti-cancer effects, which may depend on cell type, presence/activity of the receptor(s), and cellular abundance of coactivators/corepressors. PPAR-independent effects of PPARγ ligands are due in part to their electrophilic nature, proteasomal degradation of cell cycle-, and apoptosis-regulatory proteins, transcriptional repression, and other mechanisms [70–72]. Both, PPARγ-dependent and -independent pathways that contribute to inhibition of cancer cell growth may be beneficial for cancer chemotherapy [67].

4. Antitumor Effects of PPARγ-Active Triterpenoid CDDO on APL

RARs bind with high affinity to the RA-responsive element (RARE) as a heterodimer with RXR, which also heterodimerizes with other nuclear receptors, such as PPARγ.

In APL cells, the oncogenic transcription factor PML-RARα, a dominant negative transcriptional repressor, targets consist of two copies of an AGGTCA, a highly conserved consensus for RARα. PML-induced dimerization allows the two RARα moieties of PML-RARα to bind very distant monomeric DNA sites. The spectrum of response elements for PML-RARα and PML-RARα-RXR (DR1-DR16 response elements) is much broader than one for the wild-type RAR-RXR (DR1, DR2, and DR5), and PML-RARα-RXR oligomers silence a wide range of nuclear receptor target genes [73].

X-RARα fusion proteins in APL have been demonstrated to negatively affect transactivation of PPARγ [74], indicating that inhibition of PPARγ activity may contribute to the pathophysiology of the differentiation block in APL, and that PPARγ ligands could sensitize APL cells to the differentiating effects of ATRA including ATRA-resistant cells [45].

PML-RARα recruits the nuclear corepressors and histone deacetylase (HDAC), which leads to histone condensation and transcriptional repression [75–77]. ATRA acts by causing the PML-RARα/HDAC complex to dissociate, thereby converting PML-RARα into a transcriptional activator [76]. Reactivation of ATRA target genes by inducing an appropriate level of histone acetylation in their promoters is a potential strategy for restoring anticancer effects of ATRA in refractory APL [77]. Differentiating agents including ATRA, arsenic, cAMP, HDAC inhibitors, and rexinoids relieve this repression through various molecular mechanisms, allowing spontaneous differentiation of leukemic blasts [73].

In fact, it has been demonstrated that HDAC inhibitors (HDACI) such as trichostatin A (TSA), sodium phenylbutyrate (PB), and suberoylanilide hydroxamic acid (SAHA) can augment the cell growth inhibition induced by ATRA, and that ATRA combined with SAHA increased survival and induced remissions in APL transgenic mice harboring the PLZF-RARα translocation [78]. In addition, the PML-RARα fusion protein was observed to induce hypermethylation on *RAR* promoter, and the DNA methyltransferase inhibitor 5-asa-2′-deoxycytidine (5-Aza-dC) enhanced ATRA-induced *RAR* promoter transactivation in APL cells [13].

Induction of APL cell differentiation by ATRA is associated with modulation of several critical genes, including *RARβ2* [78], *C/EBPβ* [79], *p21* [80], *PU.1* [81], or a dominant repressor of RAR signaling *PRAME* [82]. Notably, PML-RARα has a significant affinity for DR1 [83], a binding site for RXR/PPARγ heterodimers, and negatively contributes to transactivation by ligand-activated PPRE.

The RA-target gene *RARβ* plays a crucial role in mediating the growth-inhibitory and tumor suppressive effects of retinoids in various cancer cells [84–87], and *RARβ* is silenced in many tumors [84, 87, 88] and myeloid leukemias [89, 90] including APL [13]. Its upregulation has been proposed as a general mechanism of retinoid-induced growth inhibition and differentiation induction

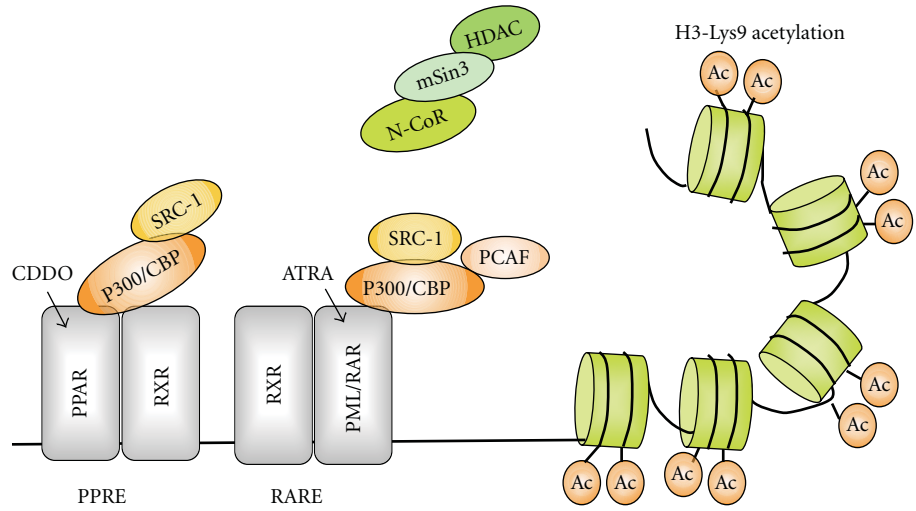

Figure 2: CDDO augments ATRA-induced reactivation of *RARβ2* in APL via histone acetylation. Combination of all-trans RA (ATRA) and 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) increases H3-Lys9 acetylation in *RARβ* P2 and RARβ2 transcription. CDDO-bound PPARγ may recruit coactivator proteins, including CBP-p300 and SRC-1 to PPARγ/RXR, which in turn induce histone acetylation and reactivation of ATRA target genes. Ac: acetylated histone H3-Lys9, HDAC: histone deacetylase, mSin3: mammalian homolog of the S. cerevisiae corepressor, Sin 3, NCoR: nuclear receptor corepressor, SRC-1: steroid receptor coactivator-1, CBP/p300: CCAAT/enhancer-binding protein, PCAF: P300/CBP-associated factor.

[72]. RARβ2 induction has been implicated in several tumor cell models in which retinoids inhibit growth and induce differentiation [91]. In HeLa cells, the transfected *RARβ2* transgene inhibits proliferation, while exogenous RA further increases the ability of the transgene to inhibit proliferation [92]. Disruption of RARβ2 expression in RARβ2 positive cancer cells abolishes RA effects of growth arrest [72], and the presence of *RARβ2* antisense predisposed the murine lung tissue to tumor formation [91].

Semisynthetic PPARγ ligand triterpenoid CDDO augmented the ATRA-induced reactivation of *RARβ2* in APL via histone acetylation [93]. In combination with ATRA, CDDO may activate the transcription of PPARγ target genes, which in turn increase the affinity of RARβ for βRARE. CDDO caused a prominent increase in RARβ2 binding to the response element in the gel shift assay, and ATRA/CDDO combination increased H3-Lys9 acetylation in *RARβ* P2 and RARβ2 transcription [93]. These findings support the concept that ligation of the PPARγ and RAR nuclear receptors is capable of inducing cell maturation and enhances proapoptotic effects of ATRA in APL cells. PPARγ and RXR form a complex with βRARE in the RARβ promoter, and the combination of ligands of PPARγ and RXR was reported to induce RARβ in ATRA-resistant breast cancer cells in the presence of histone deacetylase inhibitor [94]. Based on these findings, CDDO may induce recruitment of PPARγ/RXR to the RARE, which promotes affinity of RARβ for βRARE.

Ligand-bound RAR/RXR heterodimer has been shown to recruit the histone acetylase PCAF and the coactivator CBP/p300, which accumulates the HAT activity on the heterodimer/DNA complex and finally leads to enhanced retinoid-responsive transcription [95]. Likewise, the regulation of gene transcription by ligand-bound PPARγ involves the recruitment of coactivator proteins, including CBP/p300 and SRC-1 [17, 25, 39, 40]. CDDO has been shown to induce transactivation and PPARγ interaction with multiple coactivators including SRC-1, SRC-2, SRC-3, TRAP 220, CARM-1, and PGC-1 in colon cancer cells [67]. While CDDO alone did not recruit CBP to the *RARβ2* promoter, the CDDO/ATRA combination increased ATRA-induced CBP recruitment. Altogether, the ability of ATRA/CDDO to restore RAR signaling and to cause cell maturation might be in part dependent on the PPARγ-mediated induction of histone acetylation and reactivation of ATRA target genes (Figure 2).

ATRA is a nonselective retinoid capable of transactivating both, RARα and RXR receptors [96, 97]. Although PPARγ/RXR heterodimers promote transcriptional activity of PPARγ [16], RXR-selective ligand LG100268 and CDDO combination was not sufficient for *RARβ2* induction, suggesting that *RARβ2* gene induction is not due to ligand-induced RXR activation in APL cells [93].

Whereas CDDO alone failed to induce maturation of APL cells, the combination of CDDO with ATRA induced ATRA sensitive- and resistant-APL cells to differentiate into mature granulocytes with striking increase in Nitro Blue Tetrazolium (NBT) reduction positive and CD11b-positive cells above effects elicited by single agent ATRA [93]. Furthermore, the combined use of CDDO derivative CDDO-Me and ATRA in the murine model of APL resulted in the significant increase of mature granulocytic cells in peripheral blood and prolongation of survival compared to the single compound treatment of ATRA or CDDO. Ikeda et al. [64] also demonstrated that CDDO-Im selectively downregulated expression of PML-RARα fusion protein with an activation of caspase 8, which might contribute to enhanced ATRA-induced differentiation in APL cells, and arsenic-trioxide-

(ATO-) induced apoptosis in both ATRA-sensitive NB4 and resistant R2 cell lines and primary APL cells.

RA signaling is a common mechanism in AML other than APL, and HDAC inhibitors have been shown to restore RA-dependent transcriptional activation and trigger terminal differentiation of primary blasts from AML patients [89]. Recent reports of in vivo differentiation of the leukemic clone following HDAC inhibitor valproic acid/ATRA treatment in AML patients [98] further suggest the possibility that the ATRA/CDDO or its more potent derivatives combination may be useful transcriptional/differentiation therapy in non-APL AML. Randomized trial AML HD98B showed that administration of ATRA in addition to intensive chemotherapy improved the outcomes of the patients with genotype of "mutant (mt-) *NPM1* without *FLT3*-ITD" [99]. *NPM1* has been reported to be a possible transcriptional corepressor [100]. Inhibition of NPM1 oligomerization or knockdown of NPM1-induced apoptosis and sensitized to ATRA in mt-*NPM1*-bearing AML cells [101]. These findings suggest new avenues of exploration for ATRA and CDDO derivatives combination therapy targeting "mt-*NPM1* wt-*FLT3*" genotype AML.

Acknowledgment

The authors thank an anonymous for the paper review. There is no conflict of interests between the paper's authors and the companies involved in the paper.

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 802384, 14 pages
doi:10.1155/2012/802384

Review Article

The Current Knowledge of the Role of PPAR in Hepatic Ischemia-Reperfusion Injury

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Received 22 December 2011; Accepted 16 March 2012

Academic Editor: Stefan Alexson

Strategies to improve the viability of steatotic livers could reduce the risk of dysfunction after surgery and increase the number of organs suitable for transplantation. Peroxisome proliferator-activated receptors (PPARs) are major regulators of lipid metabolism and inflammation. In this paper, we review the PPAR signaling pathways and present some of their lesser-known functions in liver regeneration. Potential therapies based on PPAR regulation will be discussed. The data suggest that further investigations are required to elucidate whether PPAR could be a potential therapeutic target in liver surgery and to determine the most effective therapies that selectively regulate PPAR with minor side effects.

1. Introduction

Liver transplantation has evolved as the therapy of choice for patients with end-stage liver disease. However, the waiting list for liver transplantation is growing at a rapid pace, whereas the number of available organs is not increasing proportionately. The potential use of steatotic livers, one of the most common types of organs in marginal donors, for transplantation has become a major focus of investigation. However, steatotic livers are more susceptible to ischemia-reperfusion (I/R) injury, and the transplantation of steatotic levels results in a poorer outcome than that of nonsteatotic livers. Indeed, the use of steatotic livers for transplantation is associated with an increased risk of primary nonfunction or dysfunction after surgery [1, 2]. In hepatic resections, the operative mortality associated with steatosis exceeds 14%, compared with 2% for healthy livers, and the risks of dysfunction after surgery are similarly higher [2, 3]. Despite advances aimed at reducing the incidence of hepatic I/R injury (summarized in earlier reviews) [1, 2], the results to date are inconclusive. In this paper, we review the peroxisome proliferator-activated receptor alpha (PPARα) and PPARγ signaling pathways in steatosis, inflammation and regeneration, three key factors in steatotic liver surgery [1–5]. Our review of the different strategies pursued to regulate PPAR in liver diseases may motivate researchers to develop effective treatments for steatotic livers in patients undergoing I/R. The potential clinical application of strategies that regulate PPAR in the setting of steatotic liver surgery is also discussed.

2. Characteristics of PPAR

PPARs belong to the hormone nuclear receptor superfamily and consist of three isoforms: PPARα, PPARγ, and PPARβ/δ. Of these, our group and others have demonstrated that PPARα and PPARγ are important regulators of postischemic liver injury [1, 2, 6, 7] that exert their effects on steatosis and inflammation, which is inherent in steatotic liver surgery [8–12].

Previous results indicate that the presence of fatty infiltration by itself in the liver (without any surgical intervention) does not induce changes in PPARα or PPARγ levels, as no differences were observed in the levels of these transcription factors between steatotic and nonsteatotic livers of

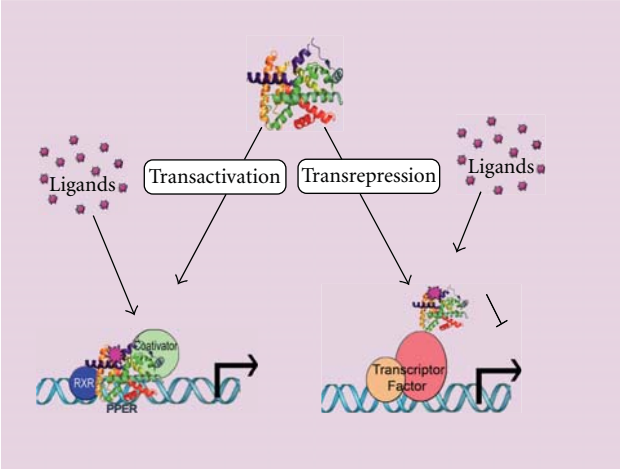

Figure 1: Basic mechanism of PPAR action. Receptor X retinoide, RXR; PPAR-response element, PPER.

a sham group of Zucker rats [13, 14]. These results contrast reports from the literature indicating high or low PPARγ levels in steatotic livers compared with those in nonsteatotic livers [15, 16]. These different results can be explained, at least in part, by differences in the level of PPARγ regulation between rats and mice [17], the different obesity experimental models evaluated, and the degree of steatosis. We reported that PPARγ expression levels in nonsteatotic livers during liver transplantation were similar to those observed in the sham group. However, increased PPARγ levels were observed in steatotic liver grafts [14, 18]. Thus, steatotic liver grafts are more predisposed to overexpress PPARγ. This is in line with clinical studies, in which PPARγ was upregulated in the livers of obese patients with nonalcoholic fatty liver disease (NALFD) [19]. Additionally, differences in PPARα expression were observed among different liver types. Indeed, steatotic livers are more predisposed to downregulate PPARα, when they are subjected to warm hepatic ischemia [13]. In line with these findings, PPARα is downregulated in the livers of obese patients with NALFD [20]. Findings such as these must be considered when applying the same pharmacological strategies indiscriminately to patients with steatotic and nonsteatotic livers because the effects may be very different.

PPARs can both activate and inhibit gene expression by two mechanisms: transactivation and transrepression. Transactivation is DNA- and ligand-dependent. PPARs activate transcription in a ligand-dependent manner by binding directly to specific PPAR response elements (PPREs) in target genes as heterodimers with retinoid X receptor (RXR). Agonist binding leads to the recruitment of coactivator complexes that modify the structure of chromatin and facilitate the assembly of the general transcriptional machinery at the promoter [21]. Transrepression is ligand-dependent and may explain the anti-inflammatory actions of PPARs [22]. PPARs repress transcription by antagonizing the actions of other transcription factors [21] (see Figure 1). Physiologically, PPAR-RXR heterodimers may bind to PPREs in the absence of a ligand. Although the transcriptional activation depends on the ligand-bound PPAR-RXR, the presence of unliganded PPAR-RXR at a PPRE has effects that vary depending on the promoter context and cell type [22]. Further investigations on the structures of PPARs and the mechanisms by which PPARs regulate gene transcription may be useful for designing certain strategies, such as the use of PPAR antagonists or agonists. As shown in the following sections, the currently used pharmacological strategies aimed at regulating PPAR could not be incorporated into liver surgery due to their potential side effects.

Given the antiobesity and anti-inflammatory properties of PPARα and PPARγ [8–12], pharmacological interventions targeting these transcription factors could be a promising strategy to treat hepatic steatosis in patients undergoing I/R. However, as shown in Figure 1, the effects of pharmacological strategies aimed at modulating PPARs depend on the type of ischemia (cold or warm ischemia), the length of ischemia, and the type of the liver (nonsteatotic or steatotic liver).

3. Effect of PPAR on Hepatic I/R

To the best of our knowledge, few studies have examined both the I/R-inducedexpression of hepatic PPARα and the potential benefits of PPARα agonists under these conditions. According to previous studies by our group, PPARα mRNA and protein levels in nonsteatotic livers during I/R were similar to those of the sham group, and PPARα did not play a crucial role in I/R injury in nonsteatotic livers [13]. This contrasts studies published by Okaya and Lentsch [23] and Xu et al. [24], who reported the benefits of PPARα agonists in postischemic liver injury. The protective effects were possibly associated with reductions in neutrophil accumulation, oxidative stress, and tumor necrosis factor (TNF) and interleukin-1 (IL-1) expression (Figure 2). Although the dose and pretreatment time of the PPARα agonist WY-14,643 were similar in both studies, Okaya and Lentsch [23] and Xu et al. [24], reported an ischemic period of 90 min [23, 24]; our ischemic period was 60 min, which is the ischemic period currently used in liver surgery [13]. Thus, 60 min of ischemia appears insufficient for inducing changes in PPARα levels in nonsteatotic livers. In nonalcoholic steatohepatitis (NASH) and simple steatosis, treatment of mice with the PPAR activator Wy-14,643 protects steatotic livers against I/R injury, and the benefits of this treatment potentially occur through the dampening of adhesion molecule and cytokine responses and activation of nuclear factor kappa B (NF-κB) and IL-6 production [25]. In steatotic livers undergoing warm ischemia, PPARα agonists can limit the damage induced by I/R. PPARα agonists as well as ischemic preconditioning (PC) through PPARα inhibited mitogen-activated protein kinases (MAPK) expression following I/R (Figure 2). This in turn inhibited adiponectin accumulation in steatotic livers and adiponectin worsening effects on oxidative stress and hepatic injury [13]. Given these data, PPARα regulation could be an alternative method for reducing the greater oxidative stress incurred by steatotic livers. Indeed, preventing I/R injury in steatotic livers via therapies aimed at inhibiting reactive oxygen species (ROS) production has proven difficult. Steatotic livers might produce SOD/catalase-insensitive ROS, which

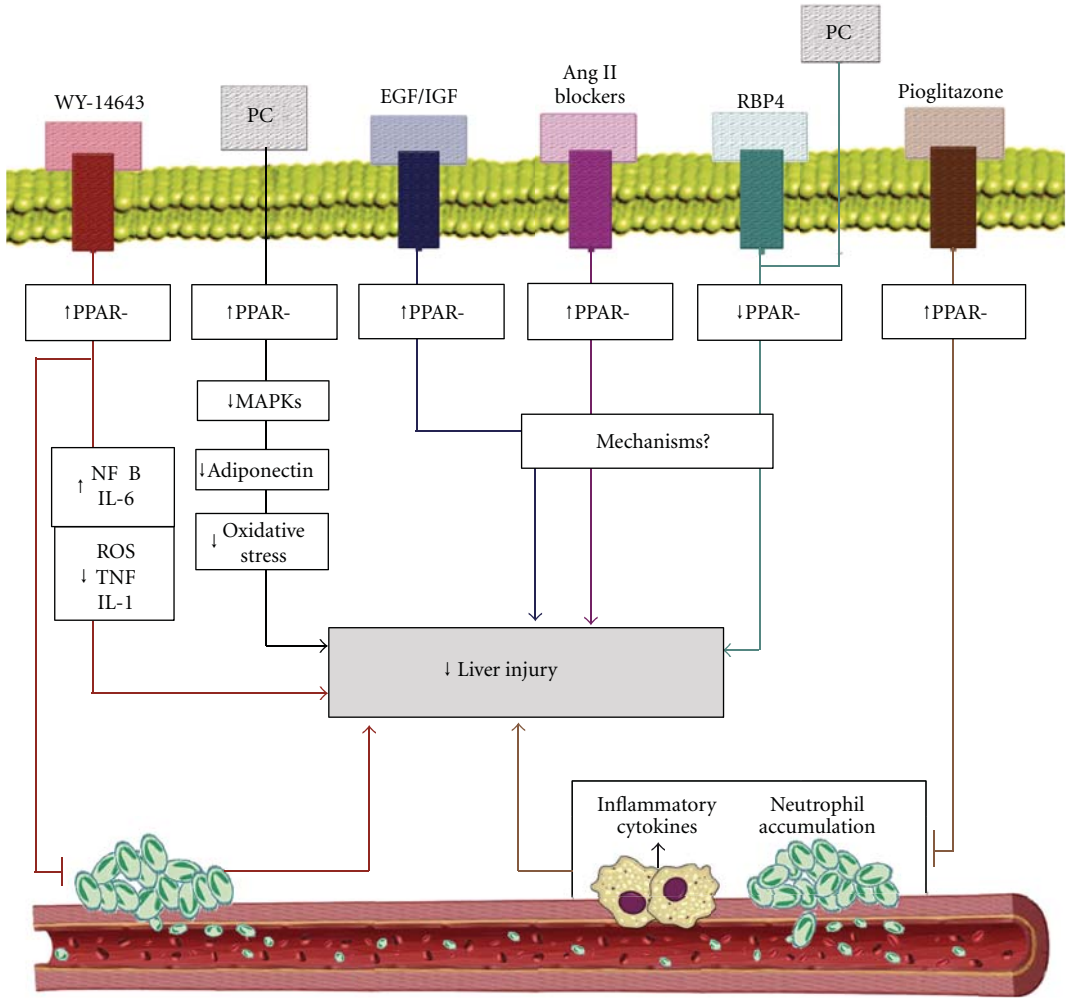

Figure 2: PPAR and hepatic I/R injury. Angiotensin II, Ang II; epidermal growth factor, EGF; insulin-like growth factor, IGF; interleukin-6, IL-6; mitogen-activated protein kinases, MAPKs; nuclear factor kappa B, NFκB; PPARα agonist; pioglitazone, peroxisome proliferator-activated receptors, PPAR; ischemic preconditioning, PC; retinol binding protein, RBP4, PPARα agonist; Wy-14,643.

may be involved in the mechanism of failure of steatotic livers after transplantation [26]. Moreover, gene therapy based on antioxidant overexpression is limited by the toxicity of the vectors [2, 27]. In a recent study of nonsteatotic livers undergoing warm hepatic ischemia, the dietary supplementation with n-3 polyunsaturated fatty acids (PUFAs) increased hepatic n-3 PUFA content and reduced hepatic n-6/n-3 PUFA content. This was associated with PPARα upregulation, which in turn reduced NF-κB signaling and oxidative stress, leading to a reduced inflammatory response [28].

The function of PPARγ in hepatic I/R injury is unclear. Previous results in liver transplantation studies indicated that I/R did not induce changes in PPARγ expression in nonsteatotic livers, and consequently, strategies based on PPARγ regulation had no effect on hepatic injury [14]. These results were different from those observed in nonsteatotic livers under warm ischemia conditions [6]. In that study, treatment with pioglitazone, a PPARγ agonist, significantly inhibited hepatic I/R injury (Figure 2). The protective effect was associated with the downregulation of several proinflammatory cytokines and chemokines and neutrophil accumulation [7]. This is in line with other results indicating that PPARγ-deficient mice displayed more severe injuries than untreated mice under warm ischemia conditions [6]. Furthermore, pioglitazone treatment inhibited apoptosis and significantly improved the survival of mice in a lethal model of hepatic I/R injury [7]. Previous studies indicated that PPARγ activation inhibits the release of TNFα, IL-1, and IL-6 by macrophages [29, 30], which could be of interest in steatotic livers. Indeed, under warm hepatic ischemia, higher IL-1 and lower IL-10 levels were detected in steatotic livers after reperfusion than in nonsteatotic livers [31]. This imbalance between pro- and anti-inflammatory ILs increased oxidative stress and decreased the tolerance of steatotic livers to I/R. In addition, different studies have reported proinflammatory and anti-inflammatory roles of TNF-α and IL-6, respectively, in the vulnerability of steatotic livers undergoing I/R [2, 32].

Previous results indicated that PPARγ activation in hepatocytes by rosiglitazone treatment increases autophagy and protects against hepatic I/R injury. Autophagy is

an evolutionarily conserved cellular process for recycling of old proteins and organelles via the lysosomal degradation [33]. Thus, these results suggest that PPARγ has anti-inflammatory properties and therefore may be relevant during hepatic I/R injury. In line with these data, PPARγ upregulation is a key mechanism of the benefits of different pharmacological or surgical strategies for steatotic livers undergoing I/R. Thus, some results based on isolated perfused livers indicated that the addition of growth factors (epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-I)) to University of Wisconsin (UW) preservation solution protected steatotic livers due to PPARγ overexpression [34]. Similarly, EGF pretreatment mediated by PPARγ overexpression protected steatotic livers undergoing warm ischemia [35] (Figure 2). Moreover, in warm hepatic ischemia, PPARγ upregulation was a key mechanism of the benefits of pharmacological blockers of angiotensin II (angiotensin-converting-enzyme (ACE) inhibitors and Ang II receptor antagonists) on steatotic livers [36]. However, the role of PPARγ in hepatic I/R injury could depend on the surgical conditions, as a recent study of liver transplantation indicated that treatment with a PPARγ antagonist was effective in steatotic livers, suggesting a detrimental role of PPARγ under these conditions [14]. In line with this finding, PPARγ inhibition was a key mechanism of the benefits of RBP4 treatment and PC on steatotic liver grafts [14]. Considering these results, drugs targeting PPARγ regulation can potentially increase the number of organs suitable for transplantation, as these drugs can improve the outcome for marginal grafts that would not otherwise have been transplanted. However, the data on PPARγ reported in steatotic liver transplantation models with standard liver graft sizes should not be extrapolated to small-size steatotic liver grafts. In the case of small liver transplants, the liver regeneration inherent in this surgical procedure and the mechanism of hepatic damage derived from the removal of hepatic mass should be considered [1, 31, 36]. In small liver grafts the periods of ischemia ranged 40–60 min, whereas the periods of ischemia ranged 6–8 hours for cadaveric donor liver transplantation.

4. Effect of PPAR on Hepatic Steatosis

Numerous studies suggest that the actions of PPARα can prevent steatosis. Mice deficient in PPARα develop hepatic steatosis when fasted or fed a high-fat diet [37, 46, 57]. Treatment with a PPARα agonist decreased hepatic steatosis in mice on a methionine- and choline-deficient (MCD) diet [37]. Activation of PPARα by the agonist Wy-14,643 ameliorated alcoholic fatty liver- and MCD-induced steatohepatitis [37, 38]. The critical role of PPARα in ameliorating steatosis is mediated through the regulation of a wide variety of genes involved in peroxisomal, mitochondrial, and microsomal FA β-oxidation systems in the liver [58]. When steatotic livers are submitted to certain stresses much as partial hepatectomy, the activation of PPARα by bezafibrate reduces the availability of FAs from circulation, reducing thus the hepatic sphingolipid synthesis [40] (see Table 1).

It is well known that n-3 PUFAs and their derivative FAs activate PPARα [59–61], which then heterodimerizes with RXR and liver X receptor, leading to the transcription of a large number of genes involved in lipid metabolism. It has been reported that n-3 PUFAs are more potent than the n-6 PUFAs as in vivo activators of PPARα [59]. In addition, PUFA metabolites such as eicosanoids or oxidized FAs have one to two orders of magnitude greater affinity for PPARα and are consequently far more potent transcriptional activators of PPARα-dependent genes [59].

The interaction of PPARα with its DNA recognition site is markedly enhanced by ligands such as hypotriglyceridemic fibrate drugs, conjugated linoleic acid, and PUFAs [59]. The discovery of PPARα led quickly to the idea that PPARα was a "master switch" transcription factor that was targeted by PUFA to coordinately suppress genes encoding lipid synthesis proteins and to induce genes encoding lipid oxidation proteins [59]. In line with this idea, recent studies suggested that n-3 FAs serve as important mediators of gene expression, working via the PPARs to control the expression of the genes involved in lipid and glucose metabolism and adipogenesis [61]. Neschen et al. [62] demostrated that the administration of dietary fish oil (n-3) to rats increases the FA capacity of their livers through its ability to function as a ligand activator of PPARα and thereby induces the transcription of several gene-encoding proteins affiliated with FA oxidation. Of interest, other studies examining the effects of fish oil feeding on the expression of several genes of PPAR knockout mice clearly indicated that hepatic gene regulation by fish oil feeding involves at least two different pathways: PPARα-dependent and PPARα-independent pathways. Enzymes for peroxisomal (CYP4A2) and microsomal (AOX) oxidation are PPARα-dependent and upregulated by fish oil feeding, whereas those for lipid synthesis (FAS; S14) are PPARα-independent and downregulated. This indicates that the FA regulation of de novo hepatic lipogenesis and FA oxidation are not mediated through a common factor (e.g., PPARα) [61].

Given all these data into in account, the regulation of PPARα by PUFA, particularly n-3 PUFA and possibly conjugated linoleic acid, may offer an explanation for the reported benefits of these FAs in different pathologies.

In obese NAFLD patients, the increased production of ROS leads to the depletion of n-3 PUFAs due to enhanced lipid peroxidation. As PPARα is activated through direct binding to n-3 PUFA, liver PPARα function is compromised in obesity. This prevented the upregulation of genes involved in lipid transport, FA β-oxidation and thermogenesis, favoring FA and triacylglycerol synthesis over FA β-oxidation and thus promoting hepatic steatosis [20]. Thus, PPARα activation by n-3 PUFA supplementation ameliorated hepatic steatosis in obese NAFLD patients [20]. In line with this, NASH patients have low levels of circulating n-3 PUFA, with a consequent increase of the n-6/n-3 FA ratio and impaired PPARα activity in the liver [42, 43]. NASH patients treated with eicosapentaenoic acid (EPA) or n-3 PUFAs, a mixture of EPA and docosahexaenoic acid, exhibited improvements in hepatic steatosis and necroinflammation in humans and rats with NASH, probably due to the reduction of hepatic TNFα expression and improvement of insulin sensitivity [41–43]. Moreover, PUFAs activate PPARα, leading to

Table 1: Effect of strategies that regulate PPAR on hepatic injury, steatosis, and regeneration in experimental models and patients. Angiotensin II: Ang II; choline deficient: CD; epidermal growth factor: EGF; high-fat diet: HFD; insulin-like growth factor 1: IGF-1; methionine choline deficient: MCD; nonalcoholic Steatohepatitis: NASH; peroxisome proliferator-activated receptors: PPARs; polyunsaturated fatty acids: PUFAs; ischemic preconditioning: PC; retinol binding protein-4: RBP4.

PPARα					
PPARα activators					
Strategies	Time	Effect	Experimental model and patients	Steatosis and hepatic injury	Regeneration
WY-14,643 (30 μmol/kg/d) [17]	3 weeks	↑ PPARα	Obese Zucker rats	↑ β-oxidation of fatty acids	Not evaluated
WY-14,643 (180 μmol/kg/d) [17]	1 week	↑ PPARα	Ob/ob mice	↑ β-oxidation of fatty acids; ↓ triglycerides	Not evaluated
WY-14,643 (10 mg/kg) [23, 24]	1 h before ischemia	↑ PPARα	Mice or Rats; warm ischemia (90 min)	↓ hepatic injury	Not evaluated
WY-14,643 (10 mg/kg) [13]	1 h before ischemia	↑ PPARα	Zucker obese rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
WY-14,643 (10 mg/kg) [25]	10 days before surgery	↑ PPARα	Foz/foz mice; steatotic livers; warm ischemia (90 min)	↓ hepatic injury	↑ cell cycle entry
Wy-14,643 (0.1%) [37]	5 weeks	↑ PPARα	Mice fed MCD diet	↓ steatohepatitis	Not evaluated
Wy-14,643 (0.1%) [38]	12 days	↑ PPARα	Mice fed MCD diet	↓ steatohepatitis; ↑ hepatic fatty acid oxidation	Not evaluated
Bezafibrate [39]	5 weeks	↑ PPARα	Mice fed MCD	↓ hepatic triglycerides; ↑ hepatic fatty acid oxidation	Not evaluated
Benzafibrate (75 mg/kg) [40]	7 days	↑ PPARα	Rats; partial hepatectomy	↓ availability of fatty acids; sphingolipid synthesis	↓ liver regeneration
PC (5 min/10 min) [13]	Immediately before ischemia	↑ PPARα	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
n-3 PUFA (EPA (270 mg/kg) and DHA (180 mg/kg)) [28]	7 days	↑ PPARα	Sprague-Dawley rats; warm ischemia	↓ hepatic injury, inflammation, and oxidative stress	Not evaluated
EPA (2700 mg/d) [41]	1 year	↑ PPARα	NAFLD patients	↓ steatosis, hepatic injury, necroinflammation, and oxidative stress	Not evaluated
n-3 PUFA (1 g/day) [42]	1 year	↑ PPARα	NAFLD patients	↓ steatosis, hepatic injury, and necroinflammation	Not evaluated
n-3 PUFA (2 g/day) [43]	6 months	↑ PPARα	NAFLD patients	↓ steatosis, hepatic injury, necroinflammation, and hepatic injury	Not evaluated
n-3 PUFA (2 g, 3 times daily) [44]	24 weeks	↑ PPARα	NAFLD patients with hyperlipidemia	↓ steatosis and hepatic injury	Not evaluated
Ω-3 FA (5 mL, thrice daily) [45]	24 weeks	↑ PPARα	NAFLD patients with dyslipidemia	↓ steatosis and hepatic injury	Not evaluated
Atorvastatin (20 mg/daily) [45]	24 weeks	↑ PPARα	NAFLD patients with dyslipidemia	↓ steatosis and hepatic injury	Not evaluated
Orlistat (120 mg, thrice daily) [45]	24 weeks	↑ PPARα	NAFLD patients with dyslipidemia	↓ steatosis and hepatic injury	Not evaluated

Table 1: Continued.

PPARα knockout					
Strategies	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
PPARα-knockout [23]	—	↓ PPARα	PPARα-null mice Warm ischemia (90 min)	↑ hepatic injury	Not evaluated
PPARα-knockout [46]	—	↓ PPARα	PPARα-null mice fed HF diet	↑ hepatic β-oxidation	Not evaluated
PPARα-knockout [47]	—	↓ PPARα	PPARα-null mice Partial hepatectomy	Not evaluated	↓ liver regeneration
PPARγ					
PPARγ activator					
Strategies	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
Rosiglitazone (10 mg/kg) [6]	30 min before ischemia	↑ PPARγ	PPAR$\gamma^{\pm}$ mice	↓ hepatic injury	Not evaluated
Rosiglitazone (2.5 μmol/kg/d) [17]	1 week	↑ PPARγ	Ob/ob mice	↓ triglycerides	Not evaluated
Rosiglitazone (3 mg/kg/day) [48]	5 weeks	↑ PPARγ	PPAR$\gamma^{fl/fl}$ mice fed HFD diet	↑ steatosis	Not evaluated
Rosiglitazone (1 mg/kg/day) [49]	12 weeks	↑ PPARγ	Obese C57BL/6J mice	↑ steatosis	Not evaluated
Rosiglitazone (10 mg/kg) [50]	2 days before surgery	↑ PPARγ	Mice partial hepatectomy	Not evaluated	↓ hepatic regeneration
Troglitazone (0.1%) + adPPARγ [51]	adPPARγ (5th day) troglitazone (5 days)	↑ PPARγ	PPARα-null mice fed CD diet	↑ steatosis	Not evaluated
Pioglitazone (500 μg/Kg) [52]	8 weeks	↑ PPARγ	Rat fed liquid diet + alcohol	↓ liver injury	Not evaluated
Pioglitazone (30 mg) [53]	96 weeks	↑ PPARγ	Patients with NASH	↓ steatosis	Not evaluated
Pioglitazone (25 mg/kg/day) [54]	5 days before surgery	↑ PPARγ	KK-A^{Y}, mice partial hepatectomy	Not evaluated	↑ hepatic regeneration
Pioglitazone (20 mg/kg) [7]	1.5 h before ischemia	↑ PPARγ	Mice Warm ischemia (60 min)	↓ hepatic injury	Not evaluated
Ang II blockers Captopril (100 mg/kg) or PD123319 (30 mg/kg) [36]	Immediately before ischemia	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
EGF and IGF-1 (10 μg/L) [34]	24 h in UW solution	↑ PPARγ	Obese Zucker rats; isolated liver perfused (24 h cold ischemia)	↓ hepatic injury	Not evaluated
EGF (100 μg/Kg) [35]	3 doses (every 8 h) starting before surgery	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
IGF-I (400 μg/Kg) [35]	2 doses (every 12 h) starting before surgery	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated
Adenovirus PPARγ + rosiglitazone (50 mg/kg/day) [55]	8 weeks	↑ PPARγ	C57BL/6J mice fed MCD diet	↓ steatohepatitis and fibrosis	Not evaluated
PC (5 min/10 min) [36]	Immediately before ischemia	↑ PPARγ	Obese Zucker rats; warm ischemia (60 min)	↓ hepatic injury	Not evaluated

Table 1: Continued.

PPARγ inhibitor					
Strategy	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
GW9662 (1 mg/kg) [14]	1 h before surgery	↓ PPARγ	Liver transplantation (6 h cold ischemia)	Does not change in hepatic injury	Not evaluated
GW9662 (1 mg/kg) [14]	1 h before surgery	↓ PPARγ	Steatotic liver transplantation (6 h cold ischemia)	↓ hepatic injury	Not evaluated
GW9662 (1 mg/kg, 3 times/week) [55]	8 weeks	↓ PPARγ	C57BL/6J mice fed MCD diet	↑ steatohepatitis, fibrosis and hepatic injury	Not evaluated
RBP4 (150 μg/kg) [14]	30 min before surgery	↓ PPARγ	Steatotic liver transplantation (6 h cold ischemia)	↓ hepatic injury	Not evaluated
PC (5 min/10 min) [14]	Immediately before ischemia	↓ PPARγ	Steatotic liver transplantation (6 h of cold ischemia)	↓ hepatic injury	Not evaluated
PPARγ inhibitor					
Strategies	Time	Effect	Experimental model	Steatosis and hepatic injury	Regeneration
PPARγ-knockout [56]	—	↓ PPARγ	Liver-specific PPARγ-null mice	↓ steatosis	Not evaluated

increased FA β-oxidation; hence, they can shift the energy balance from storage to consumption [41, 43]. n-3 PUFAs have also been proved as safe and efficacious for patients with NAFLD associated with hyperlipidemia, as indicated by reduced hepatic damage and serum lipid levels [44]. In another study, the efficacy and safety of three hypolipidemic, agents in patients with NAFLD with dyslipidemia were evaluated. In this context, predominantly hypertriglyceridemic, hypercholesterolemic, and overweight patients were treated with n-3 FAs, atorvastatin, and orlistat, respectively. The three different groups of patients exhibited reduced hepatic damage, normalized of hepatic steatosis, and reduced serum lipids [45].

Considering that steatosis is a risk factor in liver surgery, strategies aimed to reduce steatosis could increase the tolerance of steatotic livers to I/R. There is considerable evidence that liver regeneration is impaired in certain genetic models in which the liver contains excess fat. For example, steatotic livers from Ob mice exhibit defective liver regeneration and high mortality following partial hepatectomy [63]. Similarly, impaired liver regeneration was observed in steatotic livers undergoing partial hepatectomy under vascular occlusion compared with that in nonsteatotic livers [31]. On the contrary, drugs that reduce hepatic steatosis, such as PPARα regulators, should be considered with caution in clinical liver surgery, as other studies indicate that genetic or pharmacologic approaches that reduce lipid accumulation may also hinder liver regeneration [63–66]. Thus, a question is to what degree should we reduce steatosis in steatotic livers to protect this type of liver. Another question is whether we should reduce steatosis before the surgical procedure and therefore avoid the vulnerability of steatotic livers to I/R, or in contrast, should we use drugs aimed at reducing hepatic triglycerides during surgery and thus conserve the energy required for liver regeneration. Moreover, research evaluating whether the short-term administration of PPARα agonists might alleviate hepatic steatosis in steatotic livers before I/R would be of interest for clinical practice because there are obvious difficulties concerning the feasibility of long-term PPARα agonist administration in some I/R processes, in particular liver transplantation from cadaveric donors, because this is an emergency procedure in which there is very little time to pretreat the donor with PPARα agonists.

Several studies attribute a causal role to PPARγ in the development of steatosis by mechanisms involving the activation of lipogenic genes and de novo lipogenesis [48, 51]. In accordance, targeted deletion of PPARγ in hepatocytes protects mice against diet-induced hepatic steatosis [67], suggesting a prosteatotic role of PPARγ. Similarly, mice with liver-specific PPARγ silencing are protected against hepatic steatosis [56]. Additionally, treatment of ob/ob mice with rosiglitazone increased liver steatosis [49]. By contrast, different results have been reported regarding the effect of PPARγ on hepatic steatosis. Indeed, PPARγ-deficient mice develop more severe MCD-induced NAFLD, whereas adenovirus-mediated PPARγ overexpression attenuated the progression of NASH [55]. In line with this finding, rosiglitazone treatment prevented the development of NASH in a model of MCD-treated mice [55], and similar results were obtained using the PPARγ agonist pioglitazone [52, 53]. These different results can be partially explained by differences in the studies such as the species, type of PPAR agonist, method to induce hepatic steatosis, the type of genetic strategy used to induce PPARγ overexpression or deficiency in PPARγ expression as well as differences in the pretreatment times of the drugs used (see Table 1).

5. Effect of PPAR on Hepatic Regeneration

Recent studies demostrated that liver regeneration is impaired in a number of animal models of fatty liver disease [68–73]. PPARα-null mice subjected to partial hepatectomy (PH) have an impaired ability to regenerate hepatic mass. Emerging evidence suggests that PPARα is a critical modulator of the energy flux important for the repair of liver damage. For example, hepatocytes in the periportal regions, which divide and replicate after PH, require mitochondrial oxidation of FAs to generate energy [74]. PPARα controls the constitutive expression of genes involved in mitochondrial FA oxidation, including carnitine palmitoyltransferase-1 [46, 75]. In mice deficient in PPARα, the impaired hepatic regeneration is also associated with the altered expression of genes involved in cell cycle control and cytokine signaling. Studies with PPARα agonists indicate that PPARα upregulates genes involved in the cycle cell (Ccnd1 and cMyc) as well as IL1r1 and IL-6r [76] (Figure 3).

It is well known that PPARα affects the transcription of a number of genes involved in lipid turnover and peroxisomal and mitochondrial β-oxidation, resulting in the generation of ATP, which is required to "fuel" liver repair and regeneration [76]. By contrast, in conditions in which PPARα function and/or expression is altered such as hepatic steatosis, and small-size liver grafts, FA metabolism is deviated toward the accumulation of inadequately metabolized fat, favoring ROS generation. Consequently, ATP production is decreased, and the demise of hepatocytes via necrotic cell death is increased, halting liver repair [77] (Figure 3). Accordingly, mice with targeted PPARα disruption exhibit increased inflammation and necrosis and delayed liver regeneration following partial hepatectomy [47].

Previous results indicate that the impaired liver regeneration of steatotic rats was partially due to PPARα downregulation through the AdipoR2 axis. The inhibition of PPARα signaling, increased triglyceride (TG) accumulation in hepatocytes and inhibited the expression of hepatic enzymes that contribute to FA oxidation (Figure 3). This was associated with increased lipid peroxidation and decreased antioxidant levels [78].

In contrast with the aforementioned data indicating the beneficial effects of PPARα on hepatic regeneration, a recent report indicated that PPARα activation by bezafibrate had negative effects on liver regeneration, which can be attributed to the inhibition of de novo sphingolipid synthesis [40]. Presumably, bezafibrate affects de novo sphingolipid synthesis by decreasing FA availability (Figure 3). The activation of PPARα by bezafibrate virtually obliterated the postoperative increase in plasma nonesterified FAs induced by PH. This can be explained by the inhibition of hormone-sensitive lipase activity in adipose tissue by PPARα ligands and their anti-inflammatory properties, which decrease the release of cytokines such as TNF and IL-6. Both events inhibited lipolysis in isolated white adipocytes, resulting in reduced FA release from extrahepatic sources after PH [40].

PPARγ activity is likely to be regulated during normal liver regeneration, and the disruption of this regulation could impair the regenerative response. Pioglitazone improved hepatic regeneration failure in obese mice. This effect was associated with reduced TNFα and IL-6 levels. Additionally, pioglitazone prevented the increased mRNA expression of signal transducer and activators of transcription-3 phosphorylation and suppressor of cytokine signaling-3 mRNA in the livers of obese mice [54]. However, inconsistent results have been obtained regarding the effect of PPARγ of liver regeneration. Indeed, rosiglitazone inhibited hepatocyte proliferation in mice undergoing partial hepatectomy by reducing p38 and cyclin expression [50] (see Figure 3).

On the basis of the inconsistent results reported to date on the role of PPAR in hepatic regeneration, it is difficult to discern whether we should attempt to inhibit PPAR or administer PPAR activators to promote liver regeneration in surgery.

6. Modulators of PPAR in Clinical Practice

Based on the data reported in experimental models (as reviewed above), different strategies (which have been summarized in Table 1) could exert effects on steatosis, inflammation, or regeneration by regulating PPAR. Whether these pharmacological approaches can be translated into treatments for clinical liver surgery remains unknown. For example, thiazolidinediones (TZDs) should not be applied in clinical liver surgery due to their potential side effects. TZDs (pioglitazone, troglitazone, and rosiglitazone) are synthetic PPARγ agonists that are widely used as antidiabetic agents [79–81]. However, prolonged treatment of obese and diabetic mice with TZDs resulted in the development of severe steatosis, which can lead to steatohepatitis and/or fibrosis. Troglitazone administration was associated with the development of idiosyncratic acute liver failure and was therefore withdrawn from clinical use [82, 83]. Hepatotoxicity has subsequently been reported in patients taking pioglitazone and rosiglitazone [83, 84]. These data provide support for current clinical practices in which these drugs are avoided or used judiciously in patients with known or suspected liver disease. Further experiments should be initiated to devise a pharmaceutical form appropriate for clinical use.

PPARα agonists are clinically and functionally relevant as fibrate therapeutics against hyperlipidemia and agents for reducing the complications of peripheral vascular disease in diabetic patients [85]. Despite their potentially beneficial roles, PPARα agonists should be used judiciously. Short-term administration in humans (1–10 days) would be unlikely to produce permanent genotoxic effects. However, long-term exposure to these drugs, which would be required to reduce hepatic steatosis, can result in oxidative DNA damage, among other effects [86–90] (Figure 4).

Further studies will also be required to elucidate whether growth factors, Ang II blockers, or RBP4 may be safer protective pharmacologic strategies for regulating PPAR in hepatic I/R injury in clinical practice (Figure 4). Nevertheless, none of the aforementioned strategies is specific for PPAR.

To avoid the potential side effects of PPAR agonists, strategies that regulate PPARα, such as the induction of PC could be of clinical interest. PC is an adaptive mechanism that consists of a brief period of I/R, resulting in marked

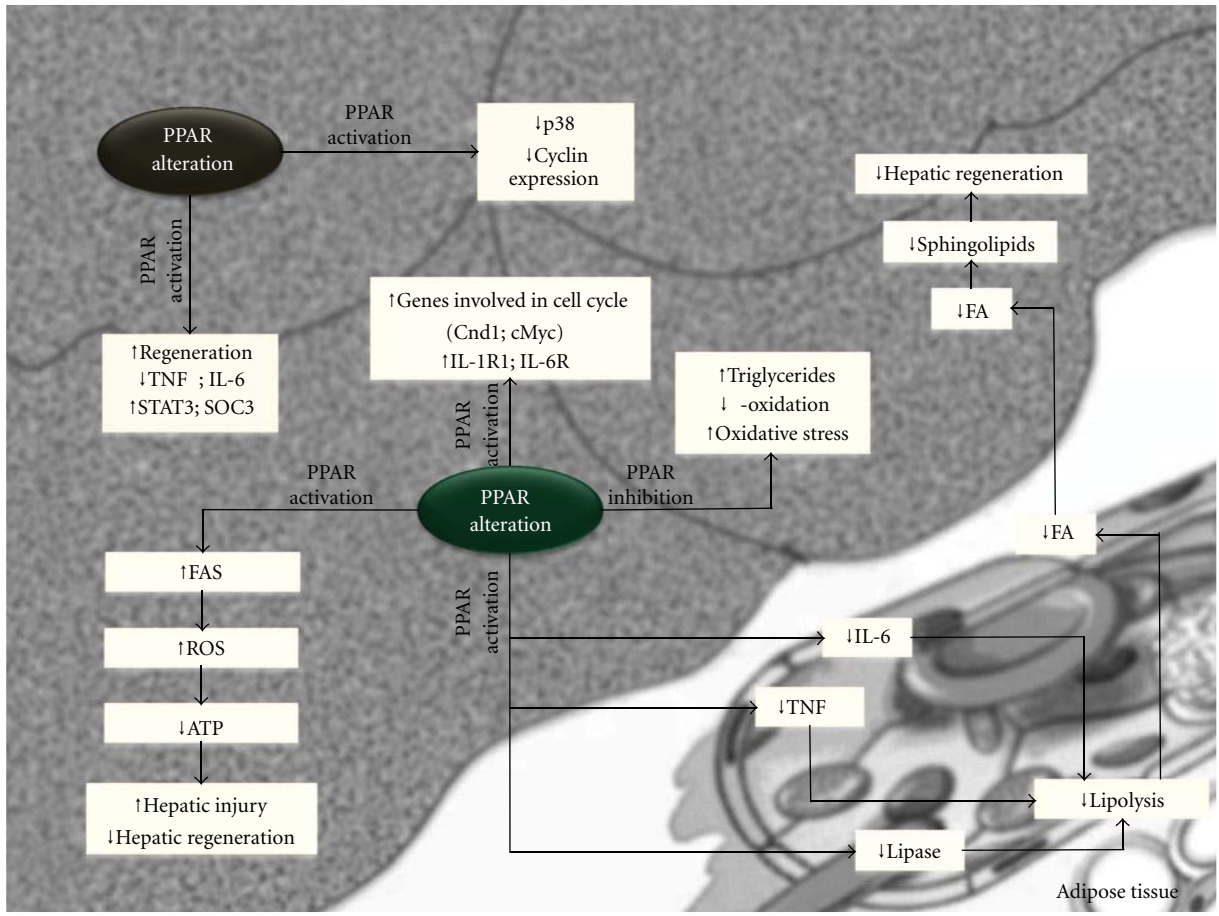

Figure 3: PPAR and hepatic regeneration. Adenosine triphosphate: ATP; fatty acid: FA; interleukin-1 receptor: IL-1R; interleukin-6: IL-6; interleukin-6 receptor: IL-6R; tumor necrosis factor-alpha: TNF-α; signal transducer and activator of transcription 3: STAT3; suppressor of cytokine signalling 3: SOC3; reactive oxygen species: ROS.

resistance in the liver, prior to a subsequent prolonged ischemic stress. Our successes regarding the efficacy of PC in nonsteatotic and steatotic livers undergoing warm ischemia (associated with PH) and liver transplantation [1, 2, 14, 91–93] have resulted in the clinical application of PC.

Several studies have demonstrated the effectiveness of PC in the resection of steatotic and nonsteatotic livers in clinical practice [94–96]. In such studies, the authors primarily performed liver resection via a continuous Pringle maneuver. However, other data indicate that PC does not improve postoperative liver function and does not affect morbidity or mortality after hepatectomy under vascular exclusion of the liver with the preservation of caval flow [97, 98]. The discrepancy between these differential effects of PC during hepatic resection might have arisen from the absence of back flow perfusion of the liver during vascular exclusion compared with that during the Pringle maneuver, which involves interruptions only to the inflow to the liver. In addition, the ischemic period used by Azoulay et al. [97] was longer (10 min on average) that that used by Clavien et al. [94]. All of these could explain, at least partially, the different effectiveness of PC in the clinical practice of liver surgery.

In the past decade, serious efforts have commenced to translate some of the robust benefits of PC against ischemia reperfusion to liver transplantation in clinical practice. It is fair to conclude that the overall clinical results have been less impressive than the observations in experimental animals. There are different data on the effectiveness of PC in I/R injury associated with liver transplantation [99–102]. However, these differential effects cannot be explained by the use of PC periods that have proved experimentally ineffective or by the clinical use of different cold ischemic times from those evaluated experimentally. However, the reduced proportion of subjects with steatosis enrolled in PC trials and the presence of brain death in clinical liver transplantation, which has thus far been evaluated in experimental studies of liver transplantation, should be considered.

As previously mentioned, the proportion of subjects with steatosis who have been enrolled in PC trials to date has been small (10%). Thus, in the future, clinical trials must make serious efforts to include a larger proportion of donor with steatotic livers to clarify the effectiveness of PC in liver transplantation in clinical practice. The benefits of PC are more likely to become clinically meaningful in patient

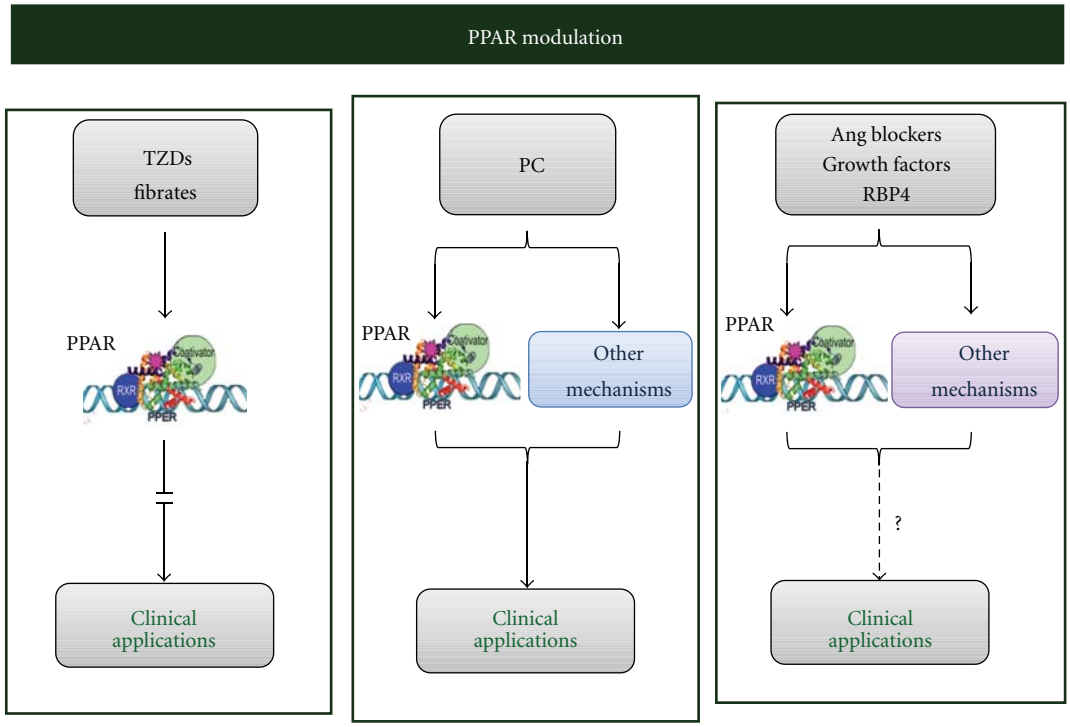

Figure 4: Clinical application of strategies that regulate PPARs. Angiotensin: Ang; peroxisome proliferator-activated receptors: PPARs; ischemic preconditioning: PC; retinol binding protein: RBP4; thiazolidinediones: TZDs.

groups with an increased risk of morbidity and mortality following PH, that is, in patients with hepatic steatosis and cirrhosis. In fact, in the largest prospective randomized study of PC in PH, Clavien et al. [94, 103] demostrated that PC was more effective in reducing reperfusion injury in patients with steatotic livers. Furthermore, Li et al. [104] reported that PC decreased the risk of hepatic insufficiency and shortened the hospital stay in patients with cirrhosis who underwent PH. There is the remote possibility that PC may not be effective in the context of brain death. Deceased organ donors have hemodynamic instability with decreased mean arterial pressure, portal venous, and hepatic tissue blood flow. Furthermore, brain death induces a multifaceted, intense systemic inflammatory response that is manifested in many organs, including the liver. It is very likely that such a framework of inflammatory response, well entrenched before the induction of PC, would interact with the various mechanistic aspects of PC and modulate the eventual PC response. To our knowledge, there are no studies of PC in the livers in brain-dead animals. Additional experimental studies of PC of the liver and other organs in brain-dead animals are needed to fill the knowledge gaps. The clinical observations suggest that PC alone may be insufficient to provide easily demonstrable clinical benefits in the presence of brain death. In that context, PC may be more effective when combined with physical, chemical, and pharmacological PC methods. Such experimental investigations could address an important clinical problem in liver transplantation, as more than 80% of livers used for transplantation are taken from cadaveric donors and approximately 20% of all brain-dead donors have a mild-to-moderate hepatic steatosis [105].

7. Conclusions and Perspectives

The use of experimental models has contributed to a better understanding of the multifaceted roles of PPARs. Strategies based on PPAR regulation have the potential to improve the postoperative outcomes of patients undergoing hepatic resections and to increase the number of organs suitable for transplantation, as these strategies may improve the outcomes of patients receiving marginal grafts that would not otherwise have been transplanted, leading to new possibilities for small steatotic liver transplants. Before a complete definition of a successful therapeutic strategy based on PPAR regulation is formed, several additional points need to be addressed. Comparative studies of the roles of different PPAR isoforms in hepatic I/R are required. We recently mapped the effects of PPAR on the pathways involved in the inflammatory process and lipid metabolism, and the effects of PPAR differ according the experimental model used. Therefore, therapeutic strategies targeting PPAR regulation also differ according to the surgical procedure. Moreover, the response of different types of liver to PPAR stimulation might differ and involve different signal transduction pathways that are at present marginally understood. Further research is required to select drugs that regulate PPAR with minimal side effects and optimize such potential treatments (e.g., dose and pharmacokinetics) before being translated into

treatments for human disease. Pharmacological strategies that specifically regulate PPAR including fibrates and TZDs might be inappropriate for clinical liver surgery due to their potential side effects. Conversely, surgical strategies such as PC have been applied in clinical surgery; however, these strategies do not exert their effects exclusively on PPAR, as they affect multiple aspects of I/R injury. Only a full appraisal of the role of PPAR in hepatic I/R and studies on the structure of this transcription factor will permit the design of new protective strategies for clinical liver surgery based on the specific regulation of PPAR without adverse effects.

Financial Support

This research was supported by the Ministerio de Ciencia e Innovación (Project Grant BFU2009-07410) Madrid, Spain; the ACC1Ó (Project Grant VALTEC08-2-0033) Barcelona, Spain.

Acknowledgments

The authors thank Bioscience Writers for revising the English text. M. Mendes-Braz is in receipt of a fellowship from CAPES Foundation, Ministry of Education of Brazilia, Brasília. M. B. Jiménez-Castro is in receipt of a fellowship from SETH Foundation (Sociedad Española de Transplante Hepático), Spain.

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 976089, 11 pages
doi:10.1155/2012/976089

Research Article

PPARα Activation Protects against Anti-Thy1 Nephritis by Suppressing Glomerular NF-κB Signaling

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Received 4 January 2012; Accepted 21 February 2012

Academic Editor: Christopher J. Nicol

The vast increase of chronic kidney disease (CKD) has attracted considerable attention worldwide, and the development of a novel therapeutic option against a representative kidney disease that leads to CKD, mesangial proliferative glomerulonephritis (MsPGN) would be significant. Peroxisome proliferator-activated receptor α (PPARα), a member of the steroid/nuclear receptor superfamily, is known to perform various physiological functions. Recently, we reported that PPARα in activated mesangial cells exerted anti-inflammatory effects and that the deficiency of PPARα resulted in high susceptibility to glomerulonephritis. To investigate whether PPARα activation improves the disease activity of MsPGN, we examined the protective effects of a PPARα agonist, clofibrate, in a well-established model of human MsPGN, anti-Thy1 nephritis, for the first time. This study demonstrated that pretreatment with clofibrate (via a 0.02% or 0.1% clofibrate-containing diet) continuously activated the glomerular PPARα, which outweighed the PPARα deterioration associated with the nephritic process. The PPARα activation appeared to suppress the NF-κB signaling pathway in glomeruli by the induction of IκBα, resulting in the reduction of proteinuria and the amelioration of the active inflammatory pathologic glomerular changes. These findings suggest the antinephritic potential of PPARα-related medicines against MsPGN. PPARα-related medicines might be useful as a treatment option for CKD.

1. Introduction

The vast increase in chronic kidney disease (CKD) has attracted considerable attention worldwide, since CKD is one of the most important risk factors for cardiovascular events, the induction of kidney replacement therapies, and death [1]. Among many types of primary kidney disease, mesangial proliferative glomerulonephritis (MsPGN) including IgA nephropathy is a representative proteinuric kidney disease that leads to CKD [2, 3]. Various medications such as angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, fish oil, statins, hydroxymethylglutaryl-CoA reductase inhibitors, immunosuppressive therapy, antiplatelets, and anticoagulants have been proposed; however, it remains difficult to control the nephritic activity associated with severe inflammatory pathologic glomerular changes [4]. It is known that the marked activation of nuclear factor kappa B (NF-κB) was detected in various kidney cells from MsPGN patients, including mesangial cells, glomerular endothelial and epithelial cells, tubular epithelial cells, and infiltrating cells and that the NF-κB transcriptional activation is significantly involved in the progression of kidney tissue injury [5]. Therefore, the development of a novel therapeutic option against NF-κB activation in active MsPGN would be significant.

Peroxisome proliferator-activated receptor α (PPARα), a member of the steroid/nuclear receptor superfamily of ligand-dependent transcription factors, is known to perform various physiological functions, including the maintenance of lipid and glucose homeostasis, the regulation of cell

proliferation, and anti-inflammatory effects via suppression of the NF-κB pathway [6–13]. Recently, we reported that the activated mesangial cells expressed a significant amount of PPARα and that the representative PPARα agonists, fibrates, exert anti-inflammatory effects in an *in vitro* study using murine mesangial cells stimulated by lipopolysaccharide [14]. Moreover, we also showed that a deficiency of PPARα resulted in high susceptibility to glomerulonephritis in an *in vivo* murine study [15]. These findings suggest that glomerular PPARα activation might contribute to the treatment of MsPGN.

To obtain basic evidence concerning the beneficial potential of PPARα ligand against MsPGN, we examined the glomerular protective effects of a PPARα agonist, clofibrate, in a well-established rat model of human MsPGN, anti-Thy1 nephritis. Anti-Thy1 nephritis, induced by anti-Thy1 antibody binding to the corresponding antigen on the membrane of mesangial cells, is marked by obvious transient inflammatory glomerular lesions, such as mesangial cell proliferation, mesangiolysis, glomerular capillary aneurysm formation, and extracapillary proliferation [16]. Several earlier studies demonstrated that upregulation of the NF-κB gene was greatly involved in the developmental process of anti-Thy1 nephritis [17, 18]. The current study reveals for the first time that PPARα activation via clofibrate treatment would attenuate the disease activity of anti-Thy1 nephritis by suppressing glomerular NF-κB signaling.

2. Materials and Methods

2.1. Animals and Experimental Design. Male Wistar rats were used in this study (age, 8 weeks; purchased from Nihon SLC, Hamamatsu, Japan). All rats were maintained in a facility free of specific pathogens, housed in a temperature- and light-controlled environment (25°C; 12-h light/dark cycle), and given tap water *ad libitum*. All procedures were performed in accordance with the guidelines of the Shinshu University, the National Institutes of Health, and the Association for Assessment and Accreditation of Laboratory Animal Care. The rats were divided into three groups: a regular diet group (Fib(−); $n = 24$), a low-dose clofibrate-containing-diet group (0.02% Fib; $n = 12$), and a high-dose clofibrate-containing-diet group (0.1% Fib; $n = 24$). The rats in the Fib(−) group were fed a regular diet throughout the experimental period. The clofibrate-treated rats were fed a 0.02 or 0.1% clofibrate-containing diet (drug weight/food weight) beginning 5 days before the injection of anti-Thy1 antibody, respectively. We measured the animals' body weight and daily food consumption every day. The mean body weight and food consumption values in each group did not change significantly throughout the experimental period, and did not differ among groups (Table 1). Using these data, the mean ± SD clofibrate dosage was calculated (Table 1). Clofibrate was obtained from Wako (Tokyo, Japan). Anti-Thy1 MsPGN was induced by a single intravenous injection of a mouse anti-Thy1 monoclonal antibody-containing solution. Concentrated anti-Thy1 antibody solution was obtained from Cedarlane Laboratories (Ontario, Canada, catalog no. CL005A). One vial of the commercial antibody solution was diluted with 300 μL of sterile saline, and it was injected into each rat at a dose of 25 μL/100 g body weight. No rat in any group died except those sacrificed according to the study protocol throughout the experimental period. Some rats were sacrificed for analysis according to the protocol at days 0, 4, 7, and 14. The numbers of rats subjected to analyses at days 0, 4, 7, and 14 were as follows: Fib(−) group, $n = 6$; 0.02% group, $n = 3$; 0.1% group, $n = 6$, at each day, respectively. The possibility of the induction failure of nephritis was checked by means of the measurement of urine protein excretion in the early phase, as described below. In the current study, all rats, which were injected to anti-Thy1 antibody solution, developed significant increases of proteinuria at day 2, indicating perfect induction of anti-Thy1 nephritis.

2.2. Pathological Analyses. Tissues from the kidneys of rats in each group were fixed in 4% paraformaldehyde. Deparaffinized sections were stained with hematoxylin & eosin, periodic acid Schiff, or periodic acid-methenamine-silver. Since anti-Thy1 nephritis markedly caused various acute inflammatory glomerular changes including mesangial cell proliferation, mesangiolysis, glomerular capillary aneurysm formation, and extracapillary proliferation, we evaluated these inflammatory glomerular changes using semiquantitative pathologic analyses. For the analyses, 50 randomly selected glomeruli from each kidney section were studied. The degree of mesangial cell proliferation was estimated using a scale that ranged from 0 to 3 (0, normal; 1, mild; 2, moderate; 3, severe). Indices were calculated using the following formula: Index $= (n_0 \times 0) + (n_1 \times 1) + (n_2 \times 2) + (n_3 \times 3)/50$ ($\sum_n = 50$). The levels of severity of the mesangiolysis, glomerular capillary aneurysm formation, and crescent formation were assessed by the appearance rate of each finding (% of the damaged glomeruli). These pathologic analyses were performed in a blinded manner by two observers who were unaware of the study protocol.

2.3. Intranuclear Transcription Factor Assay. The specific transcription factor DNA-binding activities of PPARα or NF-κB in nuclear extracts were analyzed using enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical, CA, USA). The specific double-stranded DNA sequence containing the PPAR or NF-κB response element was immobilized onto the bottoms of the wells of a 96-well plate. PPARα or NF-κB, contained in a nuclear extract, bound to each specific response element and was detected by the addition of a specific primary antibody. After secondary antibody binding, the DNA-binding activity was visualized calorimetrically. These ELISA assays are nonradioactive, sensitive established methods, and recently replaced the radioactive electrophoretic mobility shift assay. Nuclear protein was extracted from isolated glomeruli using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, MA, USA). Glomeruli were isolated from the kidney cortex of each rat by mechanical sieving techniques as described previously [19]. The nuclear protein samples, as well as

Table 1: Systemic change and estimated dose of clofibrate in anti-Thy1 nephritis rats.

Parameter	Fibrate (−) group				0.02% fibrate group				0.1% fibrate group			
	Day 0	Day 4	Day 7	Day 14	Day 0	Day 4	Day 7	Day 14	Day 0	Day 4	Day 7	Day 14
BW (g)	248 ± 26	254 ± 33	256 ± 38	267 ± 54	257±16	259 ± 48	264 ± 35	265 ± 47	254 ± 21	258 ± 39	263 ± 30	267 ± 40
sBP (mmHg)	152 ± 17	158 ± 20	158 ± 15	150±10	155 ± 10	149 ± 12	152 ± 18	153 ± 6	151 ± 16	157 ± 15	155 ± 20	158 ± 15
HR (beat/min)	400 ± 16	407 ± 30	410 ± 20	400 ± 20	393 ± 11	400 ± 10	406 ± 21	404±25	396 ± 26	405 ± 36	403 ± 30	402±15
FC (g/day)	20.1 ± 5	19 ± 3	18.5 ± 5	18.3 ± 5	18.2 ± 2	18.7 ± 2	19.6 ± 2	18.6 ± 5	18.9 ± 3	18.7 ± 1	19.5 ± 3	19 ± 6
Clo dose (mg/kg)	0	0	0	0	14.2	14.1	14.8	14.1	74.5	72.4	74.1	71.2

BW, body weight; sBP, systolic bood pressure; HR, heart rate; FC, food consumption; Clo, clofibrate.
These parameters were not affected by the induction of anti-Thy1 nephritis. There was no significant difference among groups.

Table 2: Primer sequences for quantitative real-time PCR assay.

Gene name	Primers	GenBank access no.
PPARα	Forward: 5′-GACAAGGCTCAGGATACCACTATG-3′ Reverse: 5′-TTGCAGCTTCGATCACACTTGTC-3′	NM_013196
ACOX	Forward: 5′-GGGCCTGACAGAAGCCTACAAG-3′ Reverse: 5′-AAGGTCGACAGAGGTTAGGTTCCA-3′	NM_017340
IκBα	Forward: 5′-TGACCATGGAAGTGATTGGTCAG-3′ Reverse: 5′-GATCACAGCCAAGTGGAGTGGA-3′	NM_001105720
COX2	Forward: 5′-GCGACTGTTCCAAACCAGCA-3′ Reverse: 5′-TGGGTCGAACTTGAGTTTGAAGTG-3′	NM_017232
ICAM1	Forward: 5′-ACAAGTGCCGTGCCTTTAGCTC-3′ Reverse: 5′-GATCACGAAGCCCGCAATG-3′	NM_012967
TNFα	Forward: 5′-AACTCGAGTGACAAGCCCGTAG-3′ Reverse: 5′-GTACCACCAGTTGGTTGTCTTTGA-3′	NM_012675

PPARα, peroxisome proliferator-activated receptor α; ACOX, acyl-CoA oxidase; IκBα, inhibitory factor κBα; COX2, cyclooxygenase-2; ICAM1, intercellular adhesion molecule-1; TNFα, tumor necrosis factor-α.

commercial positive control protein reagents and blank samples, were subjected to ELISA in triplicate. The mean optical density (OD) of the blank sample was subtracted from the OD of each sample, and the value was normalized to each nuclear protein amount and subsequently expressed as the change relative to the value of the control rats (Fib(−) group at day 0).

2.4. Analyses of mRNA. Analyses of mRNA were performed using quantitative real-time PCR as described previously [20–22]. One microgram of total RNA, extracted from isolated glomeruli obtained from each rat, was reverse-transcribed using oligo(dT) primers and Superscript reverse transcriptase (Invitrogen, CA). The cDNAs were quantified with an ABI PRISM 7700 sequence detection system (Applied Biosystems, CA) using specific primers and SYBR Green double-stranded DNA binding dye I. The specific primers were designed as shown in Table 2. For relative quantification of mRNA, glyceraldehyde-3-phosphate dehydrogenase was used as an internal control, and the relative expression of RNA was calculated by the comparative threshold cycle (Ct) method. The expression was expressed in terms of the change relative to the expression of the control [Fib(−) group of rats at day 0]. PCR reactions were carried out in triplicate and averaged for analysis.

2.5. Miscellaneous Methods. Throughout the experimental period, urine collections were carried out daily. Urine protein concentrations were measured as described previously [7]. Serum urea nitrogen and serum creatinine were measured by enzymatic methods using a clinical analyzer (JCA-BM2250; JEOL, Tokyo, Japan).

2.6. Statistical Analysis. Analysis of significant differences with respect to the interactive effects of the two factors (fibrate treatment and anti-Thy1 antibody injection) was performed using one-way ANOVA. Throughout the paper, significant differences from the respective day 0 group are indicated with number signs ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$), while significant differences between regular-diet and clofibrate-diet groups are indicated with asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

3. Results

3.1. The Antiproteinuric Effect by Clofibrate Treatment in Anti-Thy1 Nephritis. Pretreatment with clofibrate for 5 days and the inductive procedure of anti-Thy1 nephritis did not cause any systemic changes to body weight, food consumption, urine volume, blood pressure, or heart rate (Table 1). Pretreatment with clofibrate did not affect the urinalysis in any group of rats; however, the anti-Thy1 antibody injection immediately and dramatically increased daily urine protein excretion in all groups (Figure 1). Especially in the Fib(−) group, massive proteinuria appeared within 2 days and then gradually decreased. The clofibrate treatment attenuated the marked elevation of proteinuria throughout the experimental period in a dose-dependent manner. Serum levels of urea

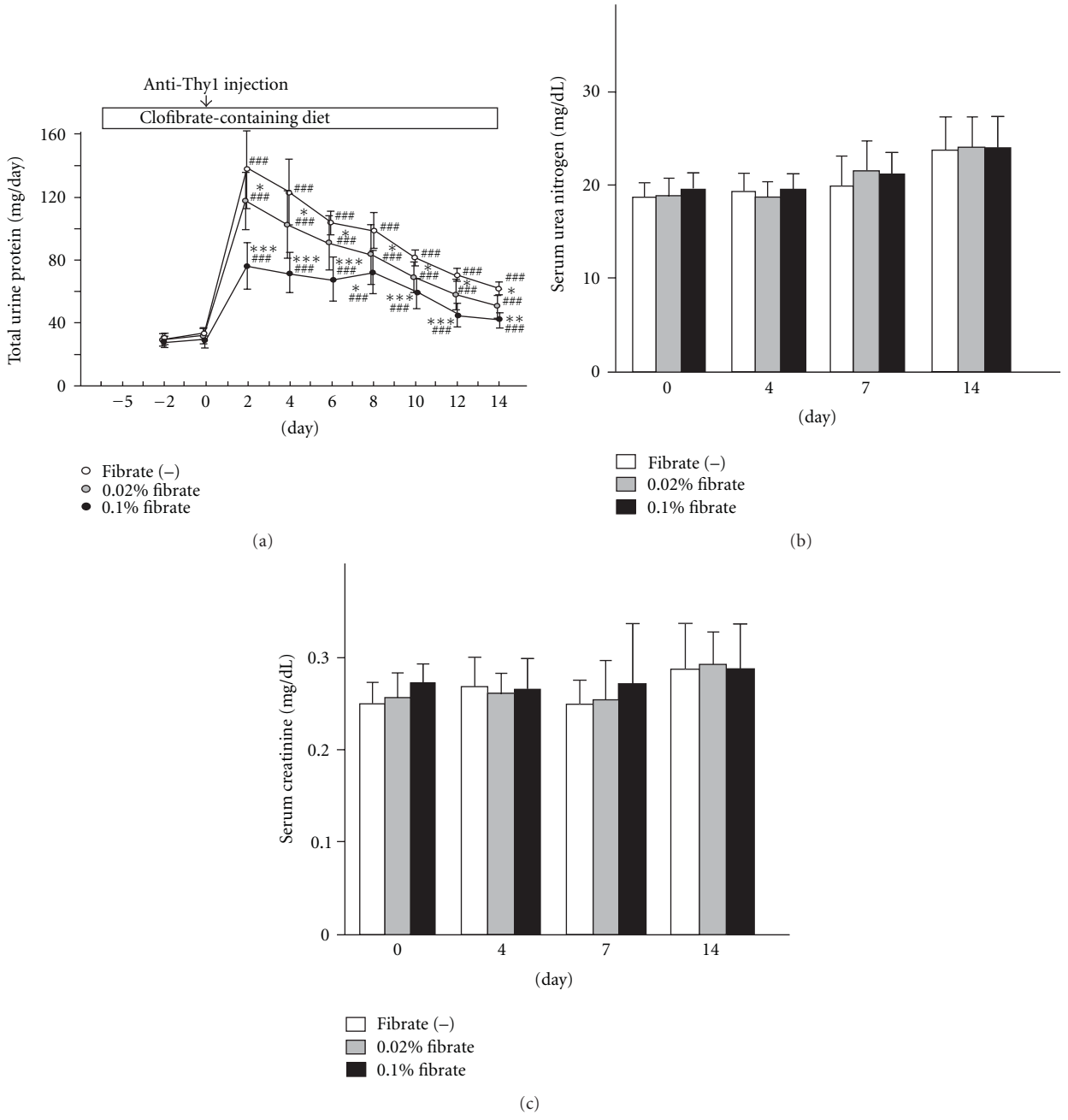

Figure 1: Alteration of kidney function by clofibrate pretreatment in anti-Thy1 nephritis rat. (a) Time course of the daily urinary protein excretion in anti-Thy1 nephritis rats. ((b) and (c)) Serum concentrations of urea nitrogen and creatinine, respectively. The clofibrate-pretreatment group was fed a 0.02% or 0.1% clofibrate-containing diet from 5 days before anti-Thy1 antibody injection. The start time of the anti-Thy1 antibody injection was designated as day 0. Values represent means ± SD ($n = 24$, 12, and 24 for the Fib(−), 0.02% fibrate, and 0.1% fibrate groups, resp.). Significant differences from the respective day 0 groups are indicated with number signs ($^{\#\#\#}P < 0.001$), while significant differences between regular-diet and clofibrate-diet groups are indicated with asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

nitrogen and creatinine were prone to increase in all groups; however, there were no significant differences among the three groups. These findings suggest an antiproteinuric effect of clofibrate treatment against anti-Thy1 nephritis.

3.2. The Amelioration of Glomerular Active Lesions by Clofibrate Treatment. To evaluate kidney damage, we carried out pathological analyses. In the Fib(−) group, an acute finding of mesangial damage, mesangiolysis, induced by anti-Thy1 antibody appeared within 4 days, followed by various severe glomerular inflammatory changes, such as glomerular capillary aneurysm formation, crescent formation, and mesangial cell proliferation (Figure 2). The semiquantitative pathological analyses demonstrated that the levels of severity of these acute glomerular lesions reached peak levels on day 7

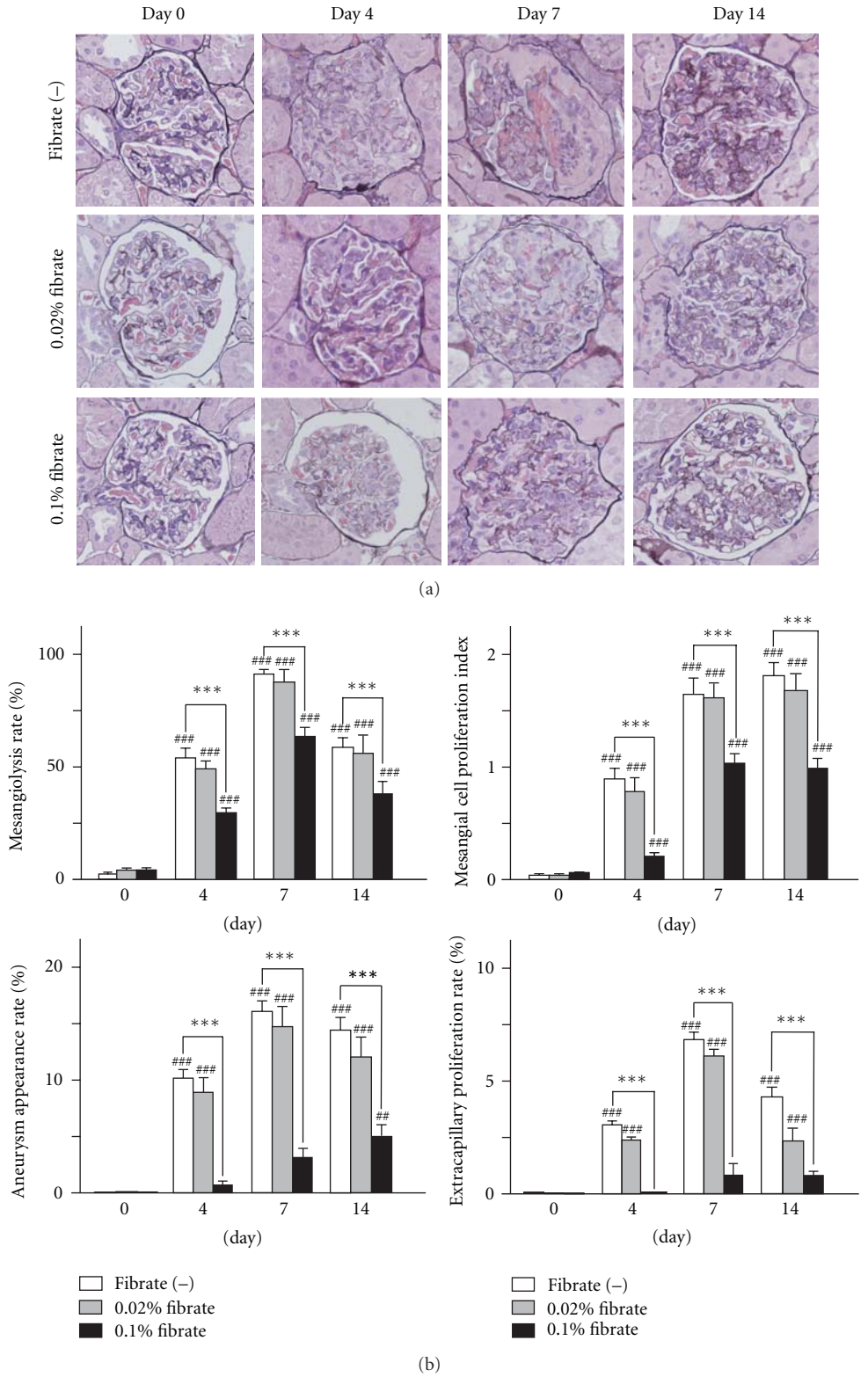

Figure 2: Light microscopic analyses of kidney injuries. (a) Representative micrographs of the kidney in anti-Thy1 nephritis rats. Kidney sections were stained with periodic acid methenamine silver (PAM). (b) Semiquantification of pathologic changes including mesangial cell proliferation, mesangiolysis, glomerular capillary aneurysm formation, and crescent formation. Values are means ± SD. Significant differences from the respective day 0 groups are indicated with number signs ($^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$), while significant differences between regular-diet and clofibrate-diet groups are indicated with asterisks ($^{***}P < 0.001$).

in the Fib(−) group. The findings of mesangiolysis, capillary aneurysm, and crescent improved spontaneously on day 14, while the high level of mesangial cell proliferation continued in this group. The pretreatment with clofibrate caused no glomerular change at day 0. This treatment markedly moderated the acute findings induced by anti-Thy1 antibody in a dose-dependent manner throughout the experimental period. These findings suggest that pretreatment with clofibrate ameliorated the glomerular active lesions of anti-Thy1 nephritis.

3.3. The Activation of Glomerular PPARα by Clofibrate Treatment. To investigate the degree of PPARα activation via clofibrate treatment, we examined the binding activities of intranuclear PPARα with PPAR response element (PPRE), using nuclear protein samples from isolated glomeruli of each group. In the Fib(−) group, the induction of anti-Thy1 nephritis obviously decreased the PPRE binding activity of PPARα at days 7 and 14 in a time-dependent manner (Figure 3). The pretreatment with clofibrate increased the glomerular PPARα activity at day 0 (before the anti-Thy1 antibody injection), in a dose-dependent manner. In spite of the induction of anti-Thy1 nephritis, the high-dose clofibrate treatment further enhanced the increase of PPARα activity, and the low-dose treatment maintained the activated level as of day 7. Then, the PPARα activities of both clofibrate groups decreased at day 14, but the level of activity was still high as compared to that of the control rats. These findings suggest that the PPRE binding activity of PPARα deteriorated due to the development of anti-Thy1 nephritis in the control group; however, the pretreatment with clofibrate outweighed this deterioration and continuously activated glomerular PPARα. To verify the enhancement of the transcriptional activity of glomerular PPARα, we next examined the mRNA expression levels of PPARα and of its representative target molecule, acyl-CoA oxidase (ACOX). In the Fib(−) group, the induction of anti-Thy1 nephritis decreased the mRNA expressions of PPARα and ACOX, a result that was identical to the results of the PPRE binding assay and suggesting the deterioration of glomerular PPARα (Figure 4). The pretreatment with clofibrate increased the mRNA expression of PPARα and ACOX at day 0 in a dose-dependent manner. The induction of anti-Thy1 nephritis decreased these expressions in each group; however, the PPARα and ACOX expressions of both clofibrate treatment groups remained over the baseline level of the control rats. These findings support the finding of continuous activation of PPARα via the clofibrate treatment. This activation was resistant to the PPARα deterioration associated with the nephritic process.

3.4. The Suppression of the NF-κB Pathway by Clofibrate Treatment. Since many earlier studies have demonstrated that the activated PPARα exerts anti-inflammatory effects through suppression of the NF-κB pathway [23, 24], we next examined the binding activities of nuclear NF-κB (p65) with a NF-κB response element. The response element binding activities of NF-κB did not differ among the groups at day 0. However, anti-Thy1 antibody injection increased the NF-κB binding activities in the Fib(−) and low-dose clofibrate groups (Figure 3). The peak phase of NF-κB activation in both groups appeared to be around day 7. On the other hand, the high-dose clofibrate treatment dramatically suppressed the NF-κB activation throughout the experimental period. The time course of NF-κB activation appeared to be consistent with that of the pathological activities of anti-Thy1 nephritis in each group. It is known that activated PPARα suppresses the NF-κB pathway via the induction of the inhibitory factor κBα (IκBα) [23]. The mRNA analyses demonstrated that the high-dose clofibrate treatment continuously induced IκBα expression and decreased the high mRNA levels of proinflammatory mediators including cyclooxygenase-2 (COX2), tumor necrosis factor-α (TNFα), and intercellular adhesion molecule-I (ICAM1), which were NF-κB target molecules (Figure 4). These findings suggest that the anti-Thy1 antibody injection induced the continuous activation of the NF-κB signaling pathway in glomeruli followed by an increase of proinflammatory mediators and that this proinflammatory pathway was suppressed considerably by the induction of IκBα, which might be mediated by PPARα activation.

4. Discussion

The current study demonstrated that pretreatment with clofibrate exerted antiproteinuric effects and ameliorated the active glomerular pathologic inflammatory changes in rat anti-Thy1 nephritis. The pretreatment with clofibrate continuously activated the glomerular PPARα, which outweighed the PPARα deterioration associated with the nephritic process. This glomerular PPARα activation would suppress the NF-κB signaling pathway via the induction of IκBα and result in beneficial antinephritic effects. These findings indicate the anti-nephritic potentiality of PPARα-related medicines.

Several metabolic experimental studies, including murine studies employing a high-fat-diet-induced glomerular injury model or a diabetic nephropathy model, have also demonstrated the beneficial properties of the PPARα agonist fibrates in reducing glomerular lesions [25–28]. These studies suggested various beneficial glomerular protective effects of fibrates as follows. First, PPARα activation improves the lipid metabolic abnormality in glomeruli. Second, PPARα activation attenuates the glomerular oxidative stress. Third, PPARα activation ameliorates systemic insulin resistance, lipid abnormality, energy homeostasis, hypertension, and vascular injuries. These pathogenic abnormalities are known to induce secondary activation of the NF-κB signaling pathway and accumulation of the extracellular matrix in glomeruli, resulting in glomerular failure [25]. Using these metabolic experimental models, it might be difficult to detect whether PPARα agonists have direct anti-inflammatory effects that protect glomeruli. In contrast to these metabolic models, the mechanism of glomerular injury of anti-Thy1 nephritis is due to the direct inflammatory response by complement (C5b-9)-induced activation of the NF-κB signaling pathway [16, 17].

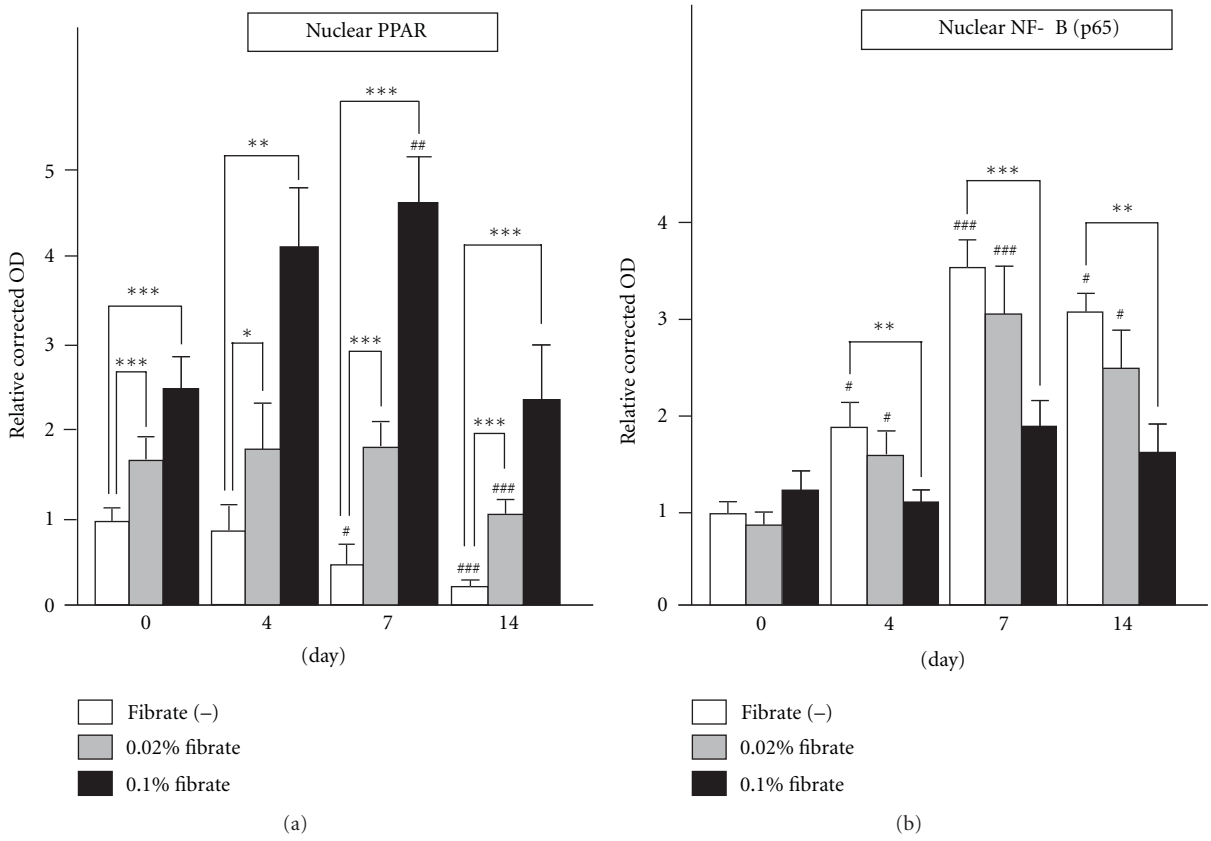

Figure 3: Alteration of intranuclear transcription factor activities. (a) The PPAR response element-binding activities of intranuclear PPARα from glomeruli in each group of anti-Thy1 nephritis rats. (b) The NF-κB response element-binding activities of intranuclear NF-κB in each group. For these assays, the nuclear protein samples were subjected to ELISA in triplicate. The optical density (OD) for each sample was corrected by that of a blank sample and by protein amount in each sample. The data were expressed as changes relative to the value for the control rats (Fib(−) group of rats at day 0). Values are means ± SD. Significant differences from the respective day 0 groups are indicated with number signs ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$), while significant differences between regular-diet and clofibrate-diet groups are indicated with asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Therefore, this animal model appeared to be suitable to demonstrate the direct anti-inflammatory effects of PPARα in glomerulonephritis. Another earlier experimental study using a rat antiglomerular basement membrane crescentic glomerulonephritis model also indicated the direct anti-inflammatory effects of PPARα, thus supporting our results [29]. The NF-κB-suppressing effects of glomerular PPARα might be useful for the treatment of the various types of glomerulonephritis, including MsPGN, immune complex kidney disease, crescentic glomerulonephritis, and lupus nephritis, as well as metabolic abnormality-based glomerulonephropathy.

In the current study, the NF-κB-suppressing effects of clofibrate might be obscure in the low-dose fibrate treatment group; however, the antiproteinuric effect in this group was rather obvious, suggesting the existence of another mechanism of the anti-proteinuric effect of PPARα agonist. A recent study reported that a PPARα agonist, fenofibrate, effectively reduced proteinuria and attenuated the reduction level of glomerular nephrin, an important molecule regulating glomerular permeability, following doxorubicin-induced podocyte injuries [30]. This study also demonstrated that PPARα-null mice exhibited susceptibility to doxorubicin-induced proteinuria, which was associated with lower expression of nephrin compared with wild-type mice. This paper suggests the existence of an anti-proteinuric effect of PPARα agonist via the maintenance effect of nephrin. Several previous studies reported that the nephrin protein expression in the glomeruli of anti-Thy1 nephritis was weak and exhibited a discontinuous pattern as determined by immunostaining [31]. Therefore, the anti-proteinuric effects of PPARα agonists might be derived from this protective effect of podocytes, as well as from NF-κB-suppressing effects.

It is known that PPARα is expressed more highly in proximal tubular epithelial cells (PTECs) than in glomeruli, and tubular PPARα exerts a protective effect in PTECs via the amelioration of fatty acid catabolism, the decreasing of oxidative stress and apoptosis, and the suppression of NF-κB singling [13, 32]. These tubular protective effects of PPARα were detected in various types of tubulointerstitial injury models, such as protein-overload nephropathy (the toxicity of excess fatty acids), unilateral ureteral obstruction, 5/6 nephrectomy, ischemia/reperfusion injury, and cisplatin

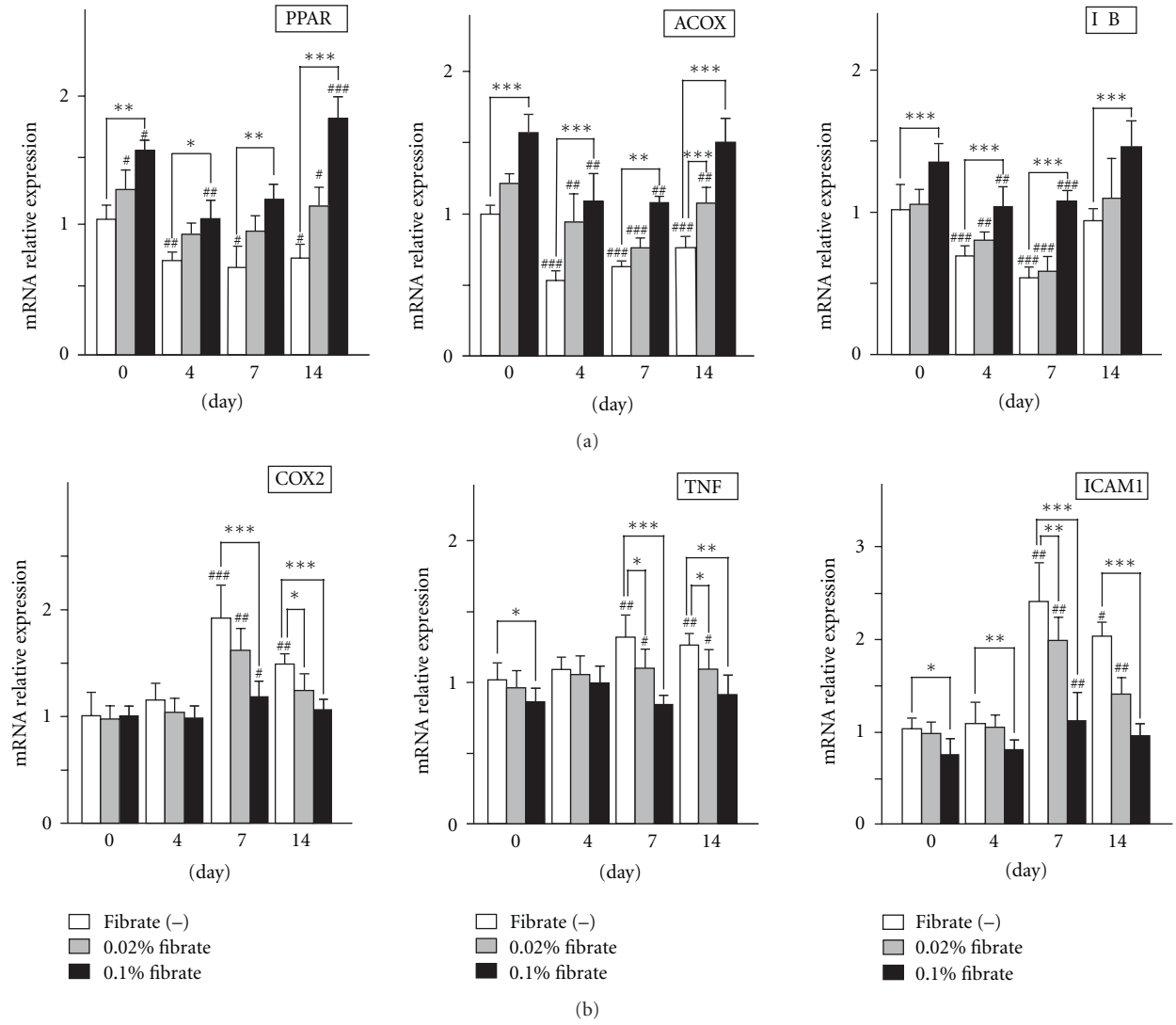

Figure 4: Alteration of mRNA expression of target molecules of transcription factors. mRNAs were obtained from glomeruli of each group of rats. The expression of mRNAs for the target molecules of PPARα (a) and NF-κB (b), including PPARα, ACOX, IκBα, COX2, TNFα, and ICAM1, was measured with real-time PCR. For relative quantification of mRNA, glyceraldehyde-3-phosphate dehydrogenase was used as an internal control, and the relative expression of RNA was calculated by the comparative threshold cycle (Ct) method. The expression was expressed as the change relative to that of the control rats (Fib(−) group of rats at day 0). PCR reactions were carried out in triplicate and averaged for analysis. Values represent means ± SD. Significant differences from the respective day 0 groups are indicated with number signs ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$), while significant differences between regular-diet and clofibrate-diet groups are indicated with asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

injury [13, 33–35]. In the current study, anti-Thy1 nephritis induced a high level of proteinuria, a representative tubulotoxic factor; however, the proteinuria was transient, so tubulointerstitial changes were scarcely detected throughout the experimental period. Therefore, we could not detect the protective effects of PPARα against tubulointerstitial injuries derived from a high level of proteinuria in this model. In order to detect such effects, we would have to perform another experiment using models exhibiting continuous excretion of proteinuria in the future.

In the current study, we used clofibrate to investigate the antinephritic potential of PPARα-related medicines, since this molecule was established as a representative beneficial medicine activating PPARα. However, we recommend the careful use of fibrates when clinical physicians treat kidney disease patients because the renal toxicity by excess serum accumulation of the fibrates was often detected in the animal models of kidney dysfunction [36]. The mechanism of the renal toxicity of fibrates was not fully understood; however, our earlier studies reported that excess-dose clofibrate treatment induced considerable oxidative stress due to a PPARα-dependent mechanism, such as the induction of PPARα-regulated ROS-generating enzymes (acyl-CoA oxidase, cytochrome P450 4A, and NADPH oxidase) and

the enhancement of mitochondrial fatty acid β-oxidation [13, 37]. Furthermore, our recent study reported that fibrates could also enhance oxidative stress in a PPARα-independent manner, as well as by a PPARα-dependent mechanism [36]. The unfavorable oxidative effect of fibrates treatment appeared to surpass the antioxidative effects of PPARα activation in the situation of excess drug accumulation in the serum; therefore adequate dose management would be essential for patients with kidney dysfunction. The earlier study demonstrated that the marked elevation of oxidative stress, induced by excess serum accumulation of fibrates, exerted proximal tubular epithelial cell toxicities such as tubular dilatation, tubular atrophy, and tubular cast formation [36]. Interestingly, the glomerular toxicity of fibrates was not detected, suggesting that excess fibrates exert only tubular toxicity without glomerular toxicity [36]. In the current study, the anti-Thy1 nephritic process resulted in transient glomerular damage without tubular damage; therefore, this limited situation might contribute to the good results of fibrate effects, obvious glomerular protection, and less tubular toxicity. On the other hand, in the human case of many types of chronic progressing glomerulonephritis, a considerable level of gradual secondary tubular damage generally appears; therefore, the tubular toxic effects of fibrates might become obvious, especially after CKD has progressed. With regard to the safe use of fibrates, we must be clear that the results of this study would not provide long-term safety verification for CKD patients. Furthermore, in order to prevent excess drug accumulation and the associated toxicities, we employed a pretreatment protocol established via past animal study [36] in which an adequate dose of fibrate was started before the appearance of apparent kidney dysfunction. In these specific situations, we succeeded in detecting beneficial anti-nephritic effects of fibrates without adverse renal effects in the current study. We believe that the results are important when considering the beneficial potential of PPARα-related medicine in treating glomerulonephritis. In humans, two clinical trials have reported that fibrates suppressed microalbuminuria in patients with early diabetic nephropathy; however, kidney dysfunction was not obviously improved [38, 39]. The results of these clinical trials might be derived from the delicate balance between the beneficial effects of PPARα activation and the renal toxicity of fibrates. In the future, the development of a novel PPARα agonist exhibiting stable pharmacokinetics under kidney dysfunction is needed.

5. Conclusions

Taken together, the current results suggest that pretreatment with a representative PPARα agonist, clofibrate, exerts a protective function against anti-Thy1 nephritis via the suppression of glomerular NF-κB signaling for the first time. The developmental process of anti-Thy1 nephritis decreased glomerular PPARα expression and weakened its function, while the pretreatment with an appropriate dose of clofibrate appeared to outweigh this deterioration. However, there are several limitations to our study. First, the use of pretreatment before nephritis might not fit the actual clinical situation of the treatment for human kidney disease. The investigation of the beneficial effects of a treatment administered after the initiation of anti-Thy1 nephritis using a novel medicine, a high serum concentration of which is not caused by kidney dysfunction or scarcely exerts toxicity, is needed in the future. Second, there are known to be species differences in PPARα activation via fibrate treatment between rodents and humans [40]. Therefore, we could not directly apply the results of the current study to human patients. In order to evaluate the anti-nephritic effect of human PPARα function, an investigation using PPARα-humanized mice might be useful [41]. Third, anti-Thy1 nephritis is a very famous rat model resembling human MsPGN; however this nephritis could be produced only in rats. Therefore, we have to verify the anti-nephritic effects of PPARα agonists using various nephritic models in the future. Nevertheless, the potential anti-nephritic effects of PPARα activation suggested in the current study will be valuable for the development of a useful therapeutic strategy to treat glomerulonephritis.

Conflict of Interests

The authors declare that there are no conflicts of interest.

Authors' Contribution

Koji Hashimoto and Yuji Kamijo contributed equally to this work.

Acknowledgments

This work was supported by a grant (Grant number 22590238) from the Japan Society for the Promotion of Science (JSPS).

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 695898, 10 pages
doi:10.1155/2012/695898

Research Article

Global Gene Expression Profiling in PPAR-γ Agonist-Treated Kidneys in an Orthologous Rat Model of Human Autosomal Recessive Polycystic Kidney Disease

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Received 18 January 2012; Accepted 17 February 2012

Academic Editor: Yuji Kamijo

Kidneys are enlarged by aberrant proliferation of tubule epithelial cells leading to the formation of numerous cysts, nephron loss, and interstitial fibrosis in polycystic kidney disease (PKD). Pioglitazone (PIO), a PPAR-γ agonist, decreased cell proliferation, interstitial fibrosis, and inflammation, and ameliorated PKD progression in PCK rats (*Am. J. Physiol.-Renal*, 2011). To explore genetic mechanisms involved, changes in global gene expression were analyzed. By Gene Set Enrichment Analysis of 30655 genes, 13 of the top 20 downregulated gene ontology biological process gene sets and six of the top 20 curated gene set canonical pathways identified to be downregulated by PIOtreatment were related to cell cycle and proliferation, including EGF, PDGF and JNK pathways. Their relevant pathways were identified using the Kyoto Encyclopedia of Gene and Genomes database. Stearoyl-coenzyme A desaturase 1 is a key enzyme in fatty acid metabolism found in the top 5 genes downregulated by PIO treatment. Immunohistochemical analysis revealed that the gene product of this enzyme was highly expressed in PCK kidneys and decreased by PIO. These data show that PIO alters the expression of genes involved in cell cycle progression, cell proliferation, and fatty acid metabolism.

1. Introduction

Polycystic kidney diseases (PKD) are characterized by progressive enlargement of numerous fluid-filled cysts in both kidneys, often leading to chronic kidney disease (CKD). Autosomal dominant PKD (ADPKD) is one of the most common hereditary disorders in humans with an incidence of 1 : 500–1,000, caused by mutations in the *PKD1* or *PKD2* gene. Progressive kidney enlargement is due to aberrant proliferation of the cystic epithelia, together with an accumulation of fluid into the cyst cavities due to transepithelial chloride (Cl^-) and fluid secretion [1–3]. Autosomal recessive PKD (ARPKD) is known as a juvenile-type cystic disease with an incidence of 1 : 20,000 [3]. Kidneys in ARPKD patients are characterized by cystic fusiform dilations of the collecting ducts accompanied by increased cell proliferation and fluid secretion, leading to massive kidney enlargement and renal failure occurring in the first few years after birth [4]. Increased cell proliferation, stimulated fluid secretion, and interstitial fibrosis are often observed in cystic liver disease in ARPKD as well [5].

Peroxisome proliferator-activated receptors (PPARs) belong to a nuclear receptor superfamily of ligand-activated transcription factors with subtypes α, β/δ, and γ. PPAR-γ is widely expressed in several organs including kidneys and known to be activated by fatty acids [6, 7]. Antidiabetic agents, pioglitazone (PIO), troglitazone, ciglitazone, and rosiglitazone, are used to control blood sugar levels in patients with diabetes mellitus. These PPAR-γ agonists also have important roles in regulation of cell cycle, inhibition of fibrosis, infiltration and metastasis of cancer cells, and modulation of inflammatory cytokines.

Treatment with PIO improved survival and ameliorated cardiac defects and the degree of renal cystogenesis in embryos of *Pkd1*$^{-/-}$ mice in a previous study [8]. In addition, long-term treatment of this agonist improved endothelial function by increasing production of nitric oxide in adult heterozygous *Pkd1*$^{+/-}$ mice [8]. Another PPAR-γ agonist, rosiglitazone attenuated PKD progression and prolonged survival of Han: SPRD Cy rats [9]. In our recent study, daily treatment of PIO ameliorated polycystic kidney disease through inhibiting Raf/MEK/ERK and AKT/mTOR/S6 signaling cascades in the PCK rat, an orthologous model of human ARPKD [10]. These findings suggest that PPAR-γ agonists may have therapeutic value in ARPKD via altering several cellular signaling pathways. In the current study, we applied global gene expression profiling to explore novel cellular signaling pathways potentially related to the ameliorating effects of PIO in PCK rat kidneys.

2. Methods

2.1. PCK Rat and Study Design. PCK rats were originally derived from a strain of Sprague-Dawley rats in Japan and descendants of this colony have been maintained at the Education and Research Center of Animal Models for Human Diseases, Fujita Health University. PCK rats and normal Sprague Dawley rats (+/+; Charles River Japan Inc., Kanagawa, Japan) were allowed free access to water and food throughout the study. Female PCK and +/+ rats, aged 4–20 weeks ($n = 10$ per gender) were randomly assigned to one of two groups: treatment with 10 mg/kg PIO (Takeda Pharmaceutical Company Limited, Osaka, Japan) or vehicle control (0.5% DMSO) by gavage every day as previously reported [10]. The protocol for the ethics and use of these animals was approved by the Animal Care and Use Committee at Fujita Health University.

At 20 weeks of age, rats were anesthetized with sodium pentobarbital (Schering-Plough Corp., Kenilworth, NJ), and the kidneys were removed rapidly, causing lethal exsanguination. Half of the left kidney was frozen in liquid nitrogen for RNA extraction. Half of the right kidney was immersed in 4% paraformaldehyde, embedded in paraffin, and sectioned for immunohistochemistry.

2.2. RNA Extraction. RNA was extracted from kidneys of rats with or without PIO treatment using a monophasic solution of phenol/guanidine isothiocyanate and TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) in accordance with their manual, and the samples were incubated with RNase-free DNase I (Ambion, TX, USA). The quality and concentration of each sample was confirmed by spectrophotometry (NanoDrop ND-1000; Asahi glass Co. Ltd., Tokyo, Japan). Total RNA obtained from three females was pooled in each PIO-treated or control vehicle-treated (CONT) group in accordance with our previous report [11].

2.3. Microarrays. DNA microarray experiments were performed essentially as described previously [11]. Briefly, 500 ng aliquots of total RNA obtained from kidneys of five rats were labeled using a Quick Amp Labeling Kit, one-color (Agilent Technologies, Inc., Santa Clara, CA, USA), according to the manufacturer's instructions. The pooled renal RNA of PIO- or vehicle-treated PCK rats were labeled with the Cy3-fluorescence dye. After determination of labeling efficiency, 1.65 μg aliquots of Cy3-labeled RNA were hybridized using the Gene Expression hybridization kit (Agilent Technologies) onto Rat Oligo Microarrays (Agilent Technologies, product no. G4130A) according to the manufacturer's hybridization protocol. The microarray slides were examined with an Agilent microarray scanner and software. Data analysis was performed with Agilent Feature Extraction software (version A.7.1.1).

Data from microarray experiments of PIO- or vehicle-treated rats were analyzed independently. Primary microarray data are available from the Gene Expression Omnibus (GEO) (accession number GSE00000). Evaluation of signal intensity was divided into three classes, {0}: nondetected, {1}: weakly detected, and {2}: strongly detected transcription product. Gene ontology analysis of biological process (C5BP) and curated gene sets of canonical pathways (C2CP) were analyzed by importing the data into Gene Set Enrichment Analysis (GSEA version 2, the Broad Institute/Massachusetts Institute of technology, USA) [12].

Using the GeneSpring software, the changed probes were listed as "Log 2 ratio was over 1 (over 2-fold) or less than -1 (less than 1/2-fold) between PIO group and CONT group" and "the signal evaluation was {2} (strongly detected) in both groups". In the changed genes, Kyoto Encyclopedia of Gene and Genomes (KEGG) analysis was used [13].

2.4. Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). cDNA was produced from total RNA by reverse transcriptase using random hexamer primers (SuperScript II First Strand Synthesis System; Invitrogen Co., Carlsbad, CA, USA). To compare gene expression patterns of PCK kidneys with PIO or vehicle treatment, we selected a key enzyme in fatty acid metabolism, stearoyl-coenzyme A desaturase 1 (*Scd1*), and uncoupling protein 1 (*Ucp1*). Gene expression was detected by real-time RT-PCR (ABI 7300 real-time PCR system; Applied Biosystems, Foster City, CA, USA) using the TaqMan reagent-based chemistry protocol. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene was used for data normalization. The probes of *Scd1*, *UCP-1*, and *GAPDH* were CCCACATGCTCCAAGAGATCTCCAG, CTCTTCAGGGAGAGAAACGCCTGCC, and AACCCATCACCATCTTCCAGGAGCG, respectively (TaqMan Gene

Table 1: (a) Top 20 downregulated gene ontology biological process gene sets in PIO-treated compared with vehicle-treated kidneys. The name of biological process is listed in order of nominal *P* value. (b) Upregulated gene ontology biological process gene sets in PIO-treated compared with vehicle-treated kidneys. The name of biological process is listed in order of nominal *P* value.

(a)

Name of biological process gene sets	Number of genes in the gene set	Nominal *P* value
Defense response	98	0.000
Regulation of cell proliferation	136	0.000
Cell cycle phase	53	0.000
Positive regulation of cell proliferation	64	0.000
Cell cycle process	61	0.000
Positive regulation of cellular process	258	0.000
Cellular morphogenesis during differentiation	22	0.001
Positive regulation of developmental process	91	0.001
Immune system process	128	0.001
Cellular defense response	19	0.003
Neuron differentiation	35	0.004
Negative regulation of cell proliferation	73	0.004
Neurite development	27	0.005
G Protein signaling coupled to ip3 second messenger phospholipase C activating	22	0.005
Inflammatory response	56	0.005
Regulation of response to stimulus	15	0.006
Neuron development	30	0.007
M phase	27	0.007
Interphase	29	0.008
Axonogenesis	21	0.009

(b)

Name of biological process gene sets	Number of genes in the gene set	Nominal *P* value
Nitrogen compound catabolic process	17	0.000
Amine catabolic process	15	0.000
Amino acid metabolic process	46	0.000
Amino acid and derivative metabolic process	58	0.000
Organic acid metabolic process	106	0.000
Carboxylic acid metabolic process	104	0.022

Expression Assays; Applied Biosystems). Relative quantification of gene expression was compared to one in SD control vehicle-treated (CONT) kidneys (set to 1.0).

2.5. Immunohistochemistry. Kidney sections were fixed, embedded, and sectioned for immunoreaction as described previously [10, 11]. Sections were incubated with *Scd1* antibody (1 : 250 ab19862 Abcam, Cambridge, UK) in PBS containing 1% BSA plus 0.05% NaN_3 overnight at 4°C. To test for a specific *Scd1* immunoreaction in the kidney, mouse IgG2b, κ isotype control antibody (1 : 200 400323 BioLegend, San Diego, CA), was used. Sections were incubated with secondary antibody Histofine MAX-PO (MULTI: for anti-mouse/rabbit IgG, IgA, and IgM) obtained from Nichirei Biosciences (Tokyo, Japan). Immune reaction products were developed using 3,3′-diaminobenzidine (ENVISION kit HRP Dako Cytomation K3466, Dako Japan Inc., Tokyo, Japan).

2.6. Statistical Analysis. Results are expressed as the arithmetic mean ± standard error. Statistical comparisons between groups were performed by Student's *t*-test and two-way analysis of variance, and differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Identification of Differentially Expressed Genes by Expression Profiling. Previous report indicates that PPAR-γ agonistic action decreases expression of endothelin receptor type A (EDNRA) [14], suggesting that *EDNRA* is one of the down-stream target gene of PPAR-γ agonists. In our current study, expression of *Ednra* was also downregulated in PIO-treated kidneys (Log2 ratio = −1.30). *EDNRA* expression is increased in human ADPKD, and overexpression of *Ednra* causes cyst formation in transgenic mouse kidneys [15]. Because not only *EDNRA* but also various genes

Table 2: (a) Downregulated gene ontology biological process gene sets from genes with greater than 1.25-fold changes in PIO-treated compared with vehicle-treated kidneys. The name of biological process is listed in order of nominal P value. (b) Common genes found in cell cycle or cell proliferation gene sets downregulated by PIO treatment. ✓: listed gene in the gene set, —: non-listed gene in the gene set. (c) Upregulated gene ontology biological process gene sets with greater than 1.25-fold changes in PIO-treatmed compared with vehicle-treated kidneys.

(a)

Name of biological process gene sets	Number of genes in the gene set	Nominal P value
Carbohydrate METABOLIC PROCESS	16	0.019
Cell proliferation GO 0008283	70	0.024
Organelle organization and biogenesis	34	0.025
Cell cycle GO 0007049	44	0.027
Negative regulation of cell proliferation	28	0.032
Cell cycle process	31	0.037

(b)

Gene symbol	Description	Name of biological process gene sets			
		Cell cycle GO 0007049	Negative regulation of cell proliferation	Cell cycle process	Cell proliferation GO 0008283
Brca2	Breast cancer 2	✓	✓	✓	✓
Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	✓	✓	✓	✓
Chek1	CHK1 checkpoint homolog	✓	✓	✓	—
BUB1B	Cell cycle checkpoint protein kinase Bub1 Fragment	✓	✓	—	✓
PLK1	Pololike kinase 1	✓	✓	—	✓
Cdkn1c	Cyclin-dependent kinase inhibitor 1C	✓	✓	✓	—
Cul5	Cullin 5	✓	✓	✓	✓
Tgfb2	Transforming growth factor, beta 2	✓	—	✓	✓
Bcat1	Branched chain aminotransferase 1	✓	✓	—	✓
PTPRC	Protein tyrosine phosphatase, receptor type, C	✓	✓	—	✓
POLA1	Polymerase (DNA directed), alpha 1	✓	✓	—	✓

(c)

Name of biological process gene sets	Number of genes in the gene set	Nominal P value
Neurological System Process	40	0.032

may be influenced by PPAR-γ agonistic actions, it became intriguing to determine the expression of other potential gene targets of PIO in PCK rat kidneys. 30,655 of 43,379 probes yielded detectable signals in both PIO- and vehicle-treated kidneys of PCK rats. The 11,809 genes represented by these 30,655 probes were analyzed by GSEA. In gene ontology analysis of biological process (C5BP) gene sets, 334 were formed from these 11,809 genes. 293 of those 334 gene sets were downregulated in PIO-treated kidneys compared with vehicle-treated kidneys, of which 77 were significantly different ($P < 0.05$, Table 1(a)). In the top 20 downregulated C5BP gene sets with the greatest significant differences, 13 were related to cell proliferation, cell cycle, morphogenesis, differentiation, and development, and 4 gene sets were related to cellular defense and inflammation. On the other hand, 41 of the 334 gene sets were upregulated in PIO-treated

kidneys compared with vehicle-treated kidneys, of which 6 were significantly different ($P < 0.05$, Table 1(b)). These gene sets were related to catabolic and metabolic processes.

To examine the gene sets with the greatest changes, only 2,611 genes, which changed more than 1.25-fold in PIO-treated kidneys compared to vehicle-treated kidneys, were analyzed. 141 gene sets were formed from these 2,611 genes. 112 of those 141 gene sets were downregulated in PIO-treated kidneys compared with vehicle-treated kidneys of PCK rats. Of these, 6 gene sets were significantly different ($P < 0.05$, Table 2(a)). 4 of these 6 gene sets are related to cell cycle and cell proliferation (Table 2(a)). Common genes in these gene sets include G1/S or G2/M checkpoint related genes, breast cancer 2 (*Brca2*), cyclin-dependent kinase inhibitor 2B (*Cdkn2b*), CHK1 checkpoint homolog (*Chek1*), cell cycle checkpoint protein kinase Bub1 fragment (*BUB1B*), pololike kinase 1 (*PLK1*), and cyclin-dependent kinase inhibitor 1C (*Cdkn1c*) (Table 2(b)). Of the remaining 29 of the 141 gene sets that were upregulated in PIO-treated kidneys compared with vehicle-treated kidneys, only one, related to neurological system processes, was significantly elevated ($P < 0.05$) (Table 2(c)).

In curated gene sets of canonical pathways (C2CP), 257 were formed from the 11,809 genes detected. 201 of these 257 gene sets were downregulated in PIO-treated kidneys compared with vehicle-treated kidneys, of which 33 were significantly lower ($P < 0.05$). From the 20 downregulated C2CP gene sets with the highest significant differences (lowest P values), 6 gene sets were related to cell cycle and cell proliferation including c-Jun N-terminal kinase (*JNK*), epidermal growth factor (*EGF*), and platelet-derived growth factor (*PDGF*) pathways, and 3 gene sets were related to inflammatory signals including interleukin-1 receptor (*IL1R*) and interleukin-6 (*IL6*) pathways (Table 3(a)). One gene set, extracellular matrix (ECM) receptor interaction, also was in the top 20 downregulated in C2CP. On the other hand, 56 of 257 gene sets were upregulated in PIO-treated kidneys compared with vehicle-treated kidneys, of which 5 gene sets were significantly higher ($P < 0.05$, Table 3(b)). 3 of these 5 gene sets are related to glutamate, alanine, and aspartate metabolism.

GSEA is a computational method that determines whether an a priori defined set of genes shows statistically significant and concordant differences between two biological states and can detect important biological processes or canonical pathways by using the list rank information without using a threshold [12]. Among the 43,379 probes spotted on the microarray slide, 189 probes were significantly changed. From these 189 probes, 31 genes were identified by KEGG analysis. 23 of those 31 genes were downregulated in PIO-treated compared with vehicle-treated kidneys (Table 4(a)). Two key enzymes in fatty acid metabolism, stearoyl-coenzyme A desaturase 1 (*Scd1*) and uncoupling protein 1 (*Ucp1*), which are involved in PPAR signaling were in the top 15 genes downregulated by PIO treatment. On the other hand, 8 of the 31 genes were upregulated in PIO-treated kidneys compared with vehicle-treated kidneys (Table 4(b)).

Table 3: (a) Top 20 downregulated curated gene sets of canonical pathways in PIO-treated compared with vehicle-treated kidneys. The name of biological process is listed in order of nominal P value. (b) Upregulated curated gene sets of canonical pathways in PIO-treated compared with vehicle-treated kidneys. The name of biological process is listed in order of nominal P value.

(a)

Name of biological process gene sets	Number of genes in the gene set	Nominal P value
HSA04640 hematopoietic cell lineage	32	0.000
HSA04610 complement and coagulation cascades	37	0.000
HSA04510 focal adhesion	110	0.001
Breast cancer estrogen signaling	60	0.001
HSA04060 cytokine cytokine receptor interaction	99	0.002
HSA04912 GNRH Signaling Pathway	64	0.002
HSA04110 cell cycle	44	0.003
HSA01430 cell communication	39	0.004
IL1R pathway	15	0.007
Eicosanoid synthesis	15	0.009
HSA04512 ECM receptor interaction	41	0.009
Cell cycle KEGG	34	0.012
ST JNK MAPK pathway	17	0.017
EGF pathway	23	0.023
PDGF pathway	23	0.028
FCER1 pathway	26	0.029
GSK3 pathway	18	0.029
Prostaglandin and leukotriene metabolism	19	0.032
IL6 pathway	17	0.032
HSA02010 ABC transporters general	21	0.033

(b)

Name of biological process gene sets	Number of genes in the gene set	Nominal P value
HSA00190 oxidative phosphorylation	37	0.000
Glutamate metabolism	15	0.000
HSA00252 alanine and aspartate metabolism	17	0.010
HSA00710 carbon fixation	15	0.019
HSA00251 glutamate metabolism	17	0.019

3.2. Cellular Expression and Distribution of Scd1 in Rodent Polycystic Kidneys. For *Scd1* and *Ucp1*, in order to confirm the mRNA expression by DNA microarray screening above, real-time RT-PCR analysis was performed. The mRNA level of *Scd1* in the kidney was increased in PCK rats compared

Table 4: (a) Downregulated genes in PIO-treated compared with vehicle-treated (CONT) kidneys of PCK rats. The name of gene symbol was listed in order of PIO/CONT Log2 ratio. (b) Upregulated genes in PIO-treated compared with vehicle-treated (CONT) kidneys of PCK rats. The name of gene symbol was listed in order of PIO/CONT Log2 ratio.

(a)

Gene symbol	Description	KEGG pathway	PIO/CONT Log 2 ratio
Olr1436	Olfactory receptor 1436	Olfactory transduction	−3.27
Xylt1	Xylosyltransferase 1	Glycosaminoglycan biosynthesis-chondroitin sulfate/glycosaminoglycan biosynthesis-heparan sulfate/metabolic pathways	−3.03
Map3k10	Mixed-lineage kinase 2	MAPK signaling pathway	−2.24
Icoslg	—	Cell adhesion molecules (CAMs)/intestinal immune network for IgA production	−2.15
Scd1	Stearoyl-coenzyme A desaturase 1	Biosynthesis of unsaturated fatty acids/PPAR signaling pathway	−2.01
Ucp1	Uncoupling protein 1	PPAR signaling pathway/Huntington's disease	−1.91
Oxt	Oxytocin, prepropeptide	Neuroactive ligand-receptor interaction	−1.81
Chrm1	Cholinergic receptor, muscarinic 1	Calcium signaling pathway/Neuroactive ligand-receptor interaction/regulation of actin cytoskeleton	−1.75
Avp	Arginine vasopressin	Neuroactive ligand-receptor interaction/vascular smooth muscle contraction/vasopressin-regulated water reabsorption	−1.58
Lpcat2	Lysophosphatidylcholine acyltransferase 2	Glycerophospholipid metabolism/ether lipid metabolism/metabolic pathways	−1.37
Il12rb1	Interleukin 12 receptor, beta 1	Cytokine-cytokine receptor interaction/jak-STAT signaling pathway	−1.34
EDNRA	Endothelin receptor type A	Calcium signaling pathway/neuroactive ligand-receptor interaction/vascular smooth muscle contraction	−1.30
Cfd	Complement factor D (adipsin)	Complement and coagulation cascades	−1.20
Serpinb5	Serine (or cysteine) peptidase inhibitor, clade B, member 5	p53 signaling pathway	−1.19
Htr2b	5-Hydroxytryptamine (serotonin) receptor 2B	Calcium signaling pathway/neuroactive ligand-receptor interaction/gap junction	−1.19
Cox8b	Cytochrome c oxidase, subunit VIIIb	Oxidative phosphorylation/metabolic pathways/cardiac muscle contraction/Alzheimer's disease/Parkinson's disease/Huntington's disease	−1.17
Peg12	Paternally expressed 12	Wnt signaling pathway	−1.11
Sema3d	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	Axon guidance	−1.07
Atp1a2	ATPase, Na+/K+ transporting, alpha 2 polypeptide	Cardiac muscle contraction/aldosterone-regulated sodium reabsorption/proximal tubule bicarbonate reclamation/salivary secretion/gastric acid secretion	−1.05
Dll3	Delta-like 3	Notch signaling pathway	−1.05
Brca2	Breast cancer 2	Homologous recombination/pathways in cancer/pancreatic cancer	−1.04
Aqp4	Aquaporin 4 (Aqp4), transcript variant 2	Vasopressin-regulated water reabsorption	−1.02
Gys2	Glycogen synthase 2	Starch and sucrose metabolism/insulin signaling pathway	−1.01

KEGG pathway: Koto Encyclopedia of Gene and Genomes pathway.

(b)

Gene symbol	Description	KEGG pathway	PIO/CONT Log 2 ratio
Gucy2d	Guanylate cyclase 2d (Gucy2d)	Purine metabolism/olfactory transduction/phototransduction	1.59
Cyp2b1	Cytochrome P450, family 2, subfamily b, polypeptide 1 (Cyp2b1), mRNA	Arachidonic acid metabolism/retinol metabolism/metabolism of xenobiotics by cytochrome P450/drug metabolism-cytochrome P450/metabolic pathways	1.45
Cyp2d3	Cytochrome P450, family 2, subfamily d, polypeptide 3 (Cyp2d3)	Drug metabolism-cytochrome P450	1.20
Tarsl2	Threonyl-tRNA synthetase-like 2 (Tarsl2), mRNA	Aminoacyl-tRNA biosynthesis	1.17
Prl	Prolactin (Prl), mRNA	Cytokine-cytokine receptor interaction/neuroactive ligand-receptor interaction/jak-STAT signaling pathway	1.17
Olr1331	Olfactory receptor 1331 (Olr1331), mRNA	Olfactory transduction	1.17
Dync1h1	Dynein cytoplasmic 1 heavy chain 1 (Dync1h1), mRNA	Phagosome/vasopressin-regulated water reabsorption	1.11
Olr297	Olfactory receptor 297 (Olr297)	Olfactory transduction	1.06

KEGG pathway: Koto Encyclopedia of Gene and Genomes pathway.

to SD rats and was decreased by PIO treatment in PCK rats (Figure 1(a)). On the other hand, the mRNA level of *Ucp1* was not significantly different between PCK and SD rats (data not shown).

Scd1 is involved in cell proliferation via growth factors in some type of cancer cells [16–18]. To determine the cellular distribution of *Scd1* in PCK and SD kidneys, immunohistochemistry was used. In normal SD kidneys, *Scd1* was hardly detected. On the other hand, in untreated PCK kidneys, *Scd1* was present in the cytoplasm of normal-shaped tubule epithelia diffusely but not in growing cysts. With PIO treatment, the distribution of *Scd1* decreased in those normal-shaped cells (Figures 1(b) and 1(c)). These findings suggest that *Scd1* may relate to the onset of renal cyst formation originated from normal-shaped tubules.

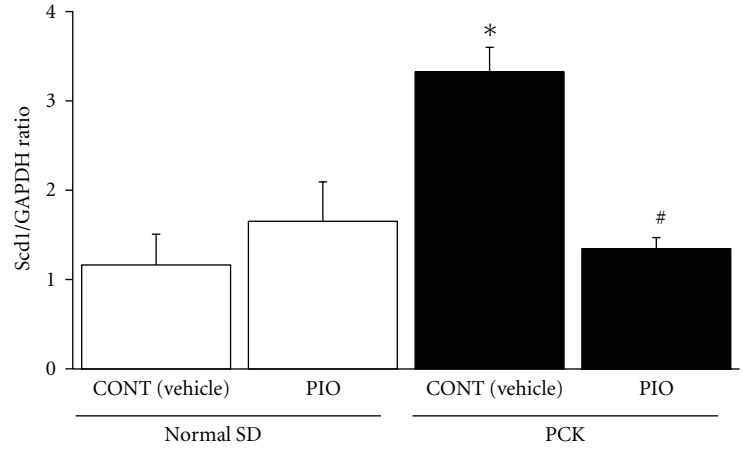
4. Discussion

In our previous report, we demonstrated that PIO treatment in PCK rats inhibited renal Raf/MEK/ERK and AKT/mTOR/S6 activity and reduced proliferation of diseased renal cells [10]. In the current study, we analyzed DNA microarray using GSEA and KEGG pathway analysis in order to detect gene-based effects of PIO treatment [12, 13]. The results of GSEA analysis of C5BP and C2CP are consistent with our previous findings, as a number of gene sets related to cell cycle and cell proliferation are downregulated in kidneys of PIO-treated PCK rats.

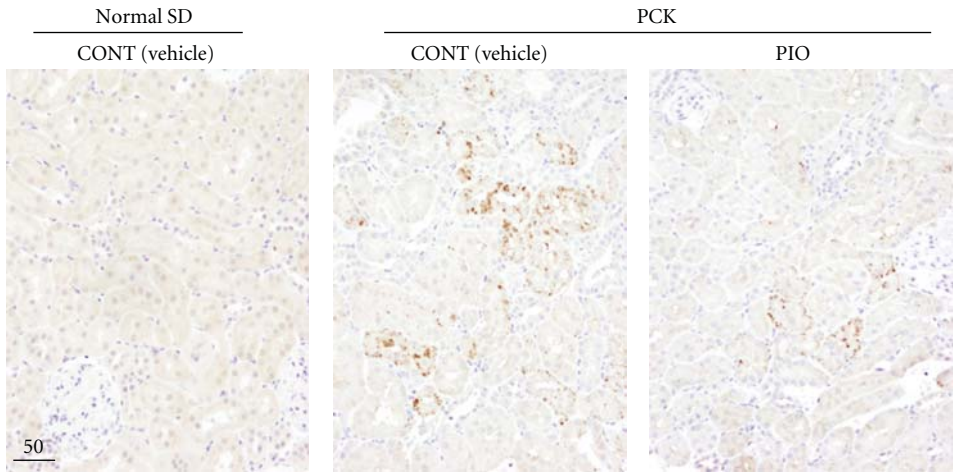
Both EGF and PDGF pathways were downregulated by PIO treatment (Table 3(a)). In PKD cystic epithelial cells, growth factors such as EGF and PDGF activate the Raf/MEK/ERK pathway via receptor binding and tyrosine kinase activation [19–21]. Therefore, PIO may ameliorate PKD in PCK rats by inhibiting cell proliferation through suppression of the activity of EGF and PDGF pathways. Further, in PKD patients, several reports show that cystic kidneys have significant levels of apoptosis [22, 23]. The JNK pathway is known to have critical roles in cell apoptosis, and JNK is overexpressed in cystic epithelial cells in Pkd1 conditional knockout mice [23, 24]. In the current study, the JNK MAPK pathway also was downregulated by PIO treatment. Therefore, PIO may have antiapoptotic effects via inactivation of the JNK pathway.

PIO, as well as other PPAR-γ agonists rosiglitazone and troglitazone, is known to induce cell cycle arrest and cell apoptosis in human cancer cells [25–27]. Although it has recently been reported that rosiglitazone inhibits cell proliferation by inducing G1 cell cycle arrest in ADPKD cyst-lining epithelial cells [28], the inhibitory mechanism of PIO is under studied in PKD. In the current analysis, *Brca2, BUB1B, Cdkn1c, Cdkn2b, Chek1,* and *PLK1* were downregulated. These genes are involved in cell cycle regulation, G0/G1, G1/S and/or G2/M checkpoints [29–35], suggesting that the antiproliferative effect of PIO may be related to cell cycle arrest.

After searching each gene expression with significant change by PIO treatment, we then focused on *Scd1* because it is known to stimulate cell proliferation in cancer cells through phosphorylation of AKT [16–18], one of the responsible kinases in cystic cell proliferation in PKD [10, 36]. Immunohistochemical analysis demonstrated that *Scd1* expression was increased in noncystic tubules in PCK kidneys, and PIOtreatment reduced its overexpression, suggesting that *Scd1* may relate to the onset of cell proliferation in initial cyst formation through phosphorylation of AKT. In addition, activation of the cell cycle increases syntheses of phospholipids and cholesterol [37–39], and *Scd1* controls the balance of saturated and monounsaturated fatty acids,



(a)



(b)

Rat strain	Treatment	Cysts			Noncystic tubules			
Normal SD	CONT (vehicle)	–			0.3	±	0.1	
	PIO	–			0.3	±	0.1	NS
PCK	CONT (vehicle)	0	±	0	10.9	±	0.3	
	PIO	0	±	0	6.2	±	0.7	**

(c)

Figure 1: Cellular expression and distribution of *Scd1* in rodent polycystic kidneys. (a) Relative gene expression levels for *Scd1*. mRNA expression levels are shown for vehicle-treted (CONT) or PIO-treated SD and PCK kidneys as compared to vehicle-treated (CONT) SD kidneys (set to 1.0) ($^{*}P < 0.05$ SD (CONT) versus PCK (CONT), $^{\#}P < 0.05$ PCK (CONT) versus PCK (PIO)). Expression levels were normalized to GAPDH. (b) Renal *Scd1* distribution in vehicle-treated (CONT) or PIO-treated SD and PCK rats. Representative kidney sections from vehicle-treated (CONT) or PIO-treated SD and PCK rats were stained with an antibody to *Scd1*. Mouse IgG2b, κ isotype control antibody, did not show any reaction in the kidney. (c) Ratio of *Scd1*-positive cysts or noncystic tubules in kidney sections. Positive-stained cysts or non-cystic tubules were counted in five random fields of kidney sections obtained from five rats in each group by a naive observer using a 20x objective. ($^{**}P < 0.01$ PCK (CONT) versus PCK (PIO) in noncystic tubules in the kidney section).

regulating the composition of cholesterol esters and phospholipids in cell membrane structure [16]. Therefore, PIO may reduce cell proliferation by the downregulation of *Scd1* gene expression not only through reducing AKT signaling activity but also through altering fatty acid synthesis. In abnormal cell proliferation in cancer, *Scd1* expression is increased, and the cell proliferation is suppressed by treatment with PPAR-γ agonists, although the changes in *Scd1* expression are not always consistent [16, 40, 41]. On the other hand, in diabetes mellitus with insulin resistance,

adipose tissue or skeletal muscle *Scd1* expression is decreased and increased by PPAR-γ agonists [42–44]. Therefore, the expression level of *Scd1* and the effect of PPAR-γ agonists may depend on the disease and/or the state of cell proliferation.

Clinically, increased body weight, oedema, and urinary bladder tumors are concerned as possible side effects of PPAR-γ agonists. Although those phenomena were not observed in both genders of PCK rats in the current PIO treatment, the effect of longer term treatment with different doses will need to be studied carefully. Since ameliorative effects are reported in several animal models of PKD [8–10, 45], PPAR-γ agonists are thought to be a potential candidate for therapeutic interventions in both ARPKD and ADPKD patients.

5. Conclusions

In the current study, PIO reduced PKD progression and altered the expression of renal genes involved in cell proliferation, cell cycle progression, and fatty acid metabolism in an orthologous rat model of human ARPKD. In addition to the previously demonstrated inhibition of Raf/MEK/ERK and AKT/mTOR/S6 signaling pathways by treatment of PCK rats with 10 mg/kg PIO for 16 weeks [10], suppression of cell proliferation may also be related to reductions in EGF, PDGF, and JNK pathways, cell cycle arrest related to *Brca2, BUB1B*, *Cdkn1c*, *Cdkn2b*, *Chek1,* and *PLK1* genes, and alteration of fatty acid metabolism related to *Scd1*.

Acknowledgments

This work was supported by Grant-in-Aid for scientific research (no. 22590351 to S. Nagao, and no. 23390033 to T. Abe) and 21st Century COE Program (to Fujita Health University) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Canadian Institutes of Health Research (MOP230564 to H. Aukema).

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 371691, 7 pages
doi:10.1155/2012/371691

Research Article

Fatty Acid Accumulation and Resulting PPARα Activation in Fibroblasts due to Trifunctional Protein Deficiency

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Received 31 January 2012; Accepted 24 February 2012

Academic Editor: Christopher J. Nicol

To examine fatty acid accumulation and its toxic effects in cells, we analyzed skin fibroblasts from six patients with mitochondrial trifunctional protein deficiency, who had abnormalities in the second through fourth reactions in fatty acid β-oxidation system. We found free fatty acid accumulation, enhanced three acyl-CoA dehydrogenases, catalyzing the first reaction in the β-oxidation system and being assumed to have normal activities in these patients, and PPARα activation that was confirmed in the experiments using MK886, a PPARα specific antagonist and fenofibrate, a PPARα specific agonist. These novel findings suggest that the fatty acid accumulation and the resulting PPARα activation are major causes of the increase in the β-oxidation ability as probable compensation for fatty acid metabolism in the patients' fibroblasts, and that enhanced cell proliferation and increased oxidative stress due to the PPARα activation relate to the development of specific clinical features such as hypertrophic cardiomyopathy, slight hepatomegaly, and skeletal myopathy. Additionally, significant suppression of the PPARα activation by means of MK886 treatment is assumed to provide a new method of treating this deficiency.

1. Introduction

The presence of an excessive level of fatty acids may cause significant toxicity in many organs and tissues. We recently examined the close relation between fatty acid toxicity and peroxisome proliferator-activated receptor (PPAR) functions. In some of our experiments, acute kidney injury was induced by albumin-overload nephropathy, in which PPARα protected proximal tubular cells from acute toxicity induced by fatty acids bound to albumin [1]; furthermore, pretreatment with low-dose fibrates protected against the fatty-acid-induced renal tubule toxicity by counteracting PPARα deterioration [2]. In our other experiments, hepatic steatosis and hepatocellular carcinoma in hepatitis C virus core protein transgenic mice were caused through fatty-acid-induced PPARα activation [3, 4]. These *in vivo* experiments provided important results concerning fatty acid toxicity at the organ and tissue levels; however, the degree of the toxicity differed greatly, even among the same types of cells. We, therefore, undertook several experiments using cultured cells to elucidate the detailed mechanisms in the cell toxicity. We adopted fibroblasts from patients having a certain abnormality in the mitochondrial fatty acid β-oxidation system.

Trifunctional protein (TP), catalyzing fatty acid β-oxidation in mitochondria, is a multienzyme complex composed of four molecules of the α-subunit containing the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase domains and four molecules of the β-subunit containing the 3-ketoacyl-CoA thiolase domain. An inborn error of this enzyme complex can cause sudden infant death

syndrome, hepatomegaly accompanying fat accumulation, hepatic encephalopathy, skeletal myopathy, or hypertrophic cardiomyopathy with rather high frequency. This deficiency is classified into two different phenotypes: one represents the existence of both subunits and the lack of only the 3-hydroxyacyl-CoA dehydrogenase activity and the other represents the absence of both subunits and the lack of the three activities, although the clinical features of these two phenotypes are similar [5–7].

2. Materials and Methods

2.1. Chemicals. MK886, a PPARα-specific antagonist [8] and fenofibrate were obtained from Wako Pure Chemical (Osaka, Japan) and Sigma Chemical Company (St. Louis, MO, USA), respectively.

2.2. Source of the Cells and Culture Method. The case histories of the TP patients were reported elsewhere [5–7]. Skin fibroblasts were collected from the patients [5, 9, 10] and cultured in the medium containing Dulbecco's modified Eagle's medium, 10% (v/v) fetal calf serum, 0.1 mM nonessential amino acids, 1 X antibiotic-antimycotic solution (Invitrogen Life Technologies Corp., Carlsbad, CA, USA) and 4.5 mg D-glucose/mL.

2.3. Acyl-CoA Dehydrogenase Activity. About 1-2 mg of fibroblasts was suspended in 150 μL of solution containing 67 mM potassium phosphate (pH 7.5), 200 mM sodium chloride, and 0.6% (w/v) Triton X-100. The suspension was gently sonicated, and the solution was centrifuged at 3,000 $\times$g for 5 min. Fifty μL of the supernatant fraction was mixed with a solution containing 67 mM potassium phosphate (pH 7.5), 40–50 μM palmitoyl-CoA (or octanoyl-CoA), and 0.4 μM electron transfer flavoprotein, in a final volume of 1.5 mL. The mixture without the electron transfer flavoprotein was preincubated for 2 min at 37°C with gentle bubbling of nitrogen gas to exclude oxygen. The reaction was started by addition of electron transfer flavoprotein, and run under nitrogen gas. Electron transfer flavoprotein reduction was measured by using a fluorometer (Hitachi F-2000) with excitation at 342 nm and emission at 496 nm [11]. The activities in fibroblasts were also measured by the method coupling with ferricenium ion [12] in order to confirm them.

2.4. Immunoblot Analysis. Protein concentrations were measured colorimetrically with a BCA Protein Assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). Whole-cell lysates (60 μg protein) were subjected to 10% SDS-polyacrylamide gel electrophoresis [13]. After electrophoresis, the proteins were transferred to nitrocellulose membranes, which were incubated with the primary antibody and then with alkaline phosphatase-conjugated goat anti-rabbit IgG. Antibodies against VLCAD, LCAD, and MCAD were described previously [14, 15]. The band of actin was used as the loading control. Band intensities were measured densitometrically, normalized to those of actin, and then expressed as fold changes relative to the averaged value of the three control fibroblasts.

2.5. Analysis of mRNA. Total RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and samples of 2 μg of RNA were reverse transcribed using oligo-dT primers and SuperScript II reverse transcriptase (Invitrogen Life Technologies Corp.). Levels of mRNA were quantified by real-time polymerase chain reaction using a SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) on a Thermal Cycler Dice TP800 system (Takara Bio) [3, 16]. Specific primers were designed by Primer Express software (Applied Biosystems, Foster City, CA, USA): 5′-GAGCCACGGACTTCCAGATA-3′ and 5′-GCATTCATCTGTCACCTTCCA-3′ for the VLCAD gene; 5′-TCACTCAGAATGGGAGAAAGC-3′ and 5′-CTCCAATTCCACCAAGATGCT-3′ for the LCAD gene; 5′-TAACCAACGGAGGAAAAGCT-3′ and 5′-CTGCTTCCACAATGAATCCA-3′ for the MCAD gene; 5′-GTGAAATCGGGACCCATAAG-3′ and 5′-CGATGGTTGTCCATTTTGAG-3′ for the peroxisomal acyl-CoA oxidase gene; 5′-CCATTCGATCTCACCAAGGT-3′ and 5′-GGATTCCGGTTTAAGACCAGTT-3′ for the catalase gene; 5′-GGAGGGAGCTGACTGATACACT-3′ and 5′-TCAGCAGGTTGGCAATCTC-3′ for the c-Fos gene; 5′-GGACTATCCTGCTGCCAAGA-3′ and 5′-CTGGTGCATTTTCGGTTGTT-3′ for the c-Myc gene; 5′-CACTGGTGGTCCATGAAAAAG-3′ and 5′-ACTTCCAGCGTTTCCTGTCT-3′ for the Cu, Zn-superoxide dismutase gene; 5′-CCGAGAAGCTGTGCATCTACA-3′ and 5′-GGTTCCACTTGAGCTTGTTCA-3′ for the cyclin D1 gene; 5′-TGTATGGAAGAGCCCAGATTC-3′ and 5′-GCACAGTACAGGCACAAAGGT-3′ for the NADPH oxidase 4 gene; 5′-GGCGTGAACCTCACCAGTAT-3′ and 5′-GCGTTATCTTCGGCCCTTAG-3′ for the proliferating cell nuclear antigen gene; 5′-CCTCAAGATCATCAGCAATGC-3′ and 5′-GGTCATGAGTCCTTCCACGAT-3′ for the GAPDH gene. The mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal control. Measurements of mRNA levels were normalized to those of GAPDH and then expressed as fold changes relative to the averaged value of the three control fibroblasts.

2.6. Assays for DNA Binding Activity of PPARs. The DNA-binding activity of PPARα, PPARβ, and PPARγ was determined using the PPARα, PPARβ, and PPARγ Transcription Factor Assay kits (Cayman Chemical, Ann Arbor, MI, USA) [17–19], respectively. These assays are based on an enzyme-linked immunosorbent assay using PPAR response element- (PPRE-) immobilized microplates and specific PPAR antibodies, thus offering similar results to those from the conventional radioactive electrophoretic mobility shift assay. DNA-binding assays were carried out according to the manufacturer's instructions using whole-cell lysates (100 μg protein). Results are expressed as fold changes relative to the averaged value of the three control fibroblasts.

2.7. Analyses of TG and FFA. To determine the content of triglycerides (TGs) and free fatty acids (FFAs), lipids were extracted according to a method reported by Hara and Radin [20]. TG and FFA were measured with Triglyceride *E*-test kit

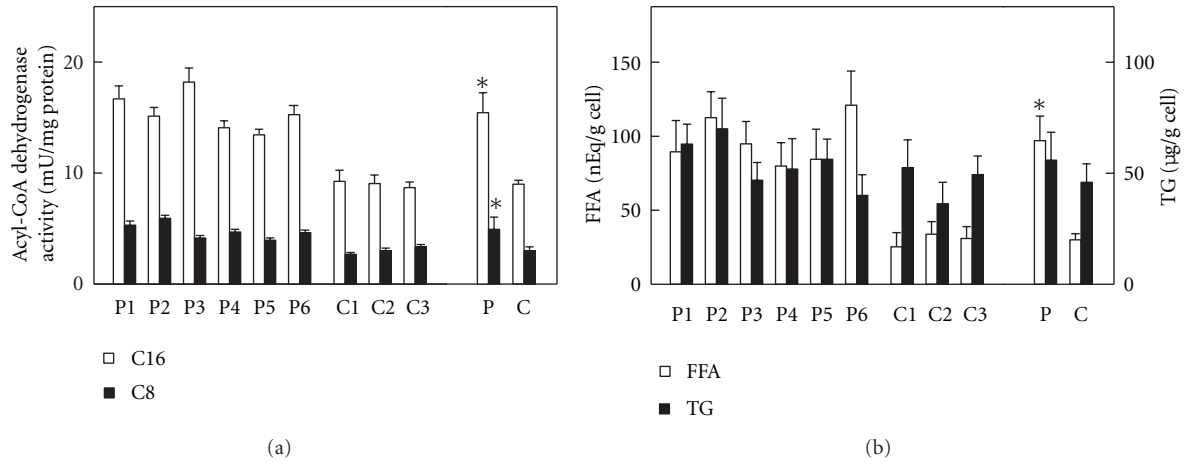

Figure 1: Acyl-CoA dehydrogenase activity and FFA/TG contents in fibroblasts. Assay methods were, respectively, described in Section 2. (a) Indicates palmitoyl-CoA (open bar, C16) and octanoyl-CoA (closed bar, C8) dehydrogenase activities, respectively. (b) Indicates FFA (open bar) and TG (closed bar) contents, respectively. P1–P6, individual patient's fibroblast; C1–C3, individual control fibroblast; P, means ± SD in six patients' fibroblasts; C, means ± SD in three control fibroblasts. $^*P < 0.05$ versus controls.

and an NEFA *C*-test kit (Wako Pure Chemical, Osaka, Japan), respectively.

2.8. Statistical Analysis. All data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA with Bonferroni correction (SPSS Statistics 17.0; SPSS Inc, Chicago, IL, USA). Correlation coefficients were calculated using Spearman's rank correlation analysis. A probability value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Acyl-CoA Dehydrogenase Activity and the Content of TG/FFA in Fibroblasts. Six strains of skin fibroblasts were prepared from the individual TP patients, as well as the three strains from the healthy adult men as described in Section 2. The reproductive rate of these fibroblasts was similar in all strains. TP deficiency is based on abnormalities in the second through fourth reactions in the mitochondrial fatty acid β-oxidation system; therefore, acyl-CoA dehydrogenase, catalyzing the first reaction in the β-oxidation system, was assumed to be normal in the patients' fibroblasts. Additionally, increased levels of FFA/TG due to the impaired β-oxidation ability in these fibroblasts were expected. Thus, these parameters were analyzed first. Palmitoyl-CoA and octanoyl-CoA dehydrogenase activities in the patients' fibroblasts were 1.72- and 1.64-fold higher than those in the control fibroblasts, respectively, (Figure 1(a)). FFA content in the patients' fibroblasts was 3.2-fold higher than that in the control fibroblasts, while TG levels were similar in both fibroblasts. These FFA and TG levels were much lower than those in human serum (Figure 1(b)).

3.2. Expression of Three Acyl-CoA Dehydrogenases. Palmitoyl-CoA and octanoyl-CoA dehydrogenation are catalyzed by three forms of acyl-CoA dehydrogenase; therefore, their expression levels were examined. The protein levels of VLCAD, LCAD, and MCAD in the patients' fibroblasts were 1.55-, 2.15-, and 1.97-fold higher than those in the control fibroblasts, respectively, (Figure 2(a)). The mRNA contents of VLCAD, LCAD, and MCAD in the patients' fibroblasts were 2.00-, 2.92-, and 2.63-fold higher than those in the control fibroblasts, respectively, (Figure 2(b)). These data were consistent with the observations shown in Figure 1(a). The simultaneous increases in the expression levels of the three forms of acyl-CoA dehydrogenase strongly suggested the presence of PPARα activation in the patients' fibroblasts, since the three forms are known as PPARα target gene products [15]. The presence of PPARα activation was thereby examined in detail.

3.3. Assays for DNA-Binding Activity of PPARs. Immunoblot analysis using whole-cell lysates from the fibroblasts and specific antibodies was performed and provided very faint bands for PPARβ and no bands for PPARα and PPARγ. mRNA analysis was also done as described in Section 2, and indicated that the PPARα, β, and γ mRNAs were $10^{-6} \sim 10^{-4}$ levels for GAPDH mRNA in the fibroblasts, meaning that the data from the immunoblot and mRNA analyses were unreliable for detecting PPAR activation. The PPRE-binding assay was then done, which demonstrated an increase of PPRE-binding activity only for PPARα in the whole-cell lysates from the patients' fibroblasts (Figure 3). These data supported the presence of PPARα activation in the patients' fibroblasts.

3.4. Treatments with MK886 and Fenofibrate. To confirm the appearance of PPARα activation in the patients' fibroblasts, the fibroblasts were treated with MK886, a PPARα-specific antagonist and fenofibrate, a PPARα specific agonist, respectively. The expression level of MCAD, a representative PPARα

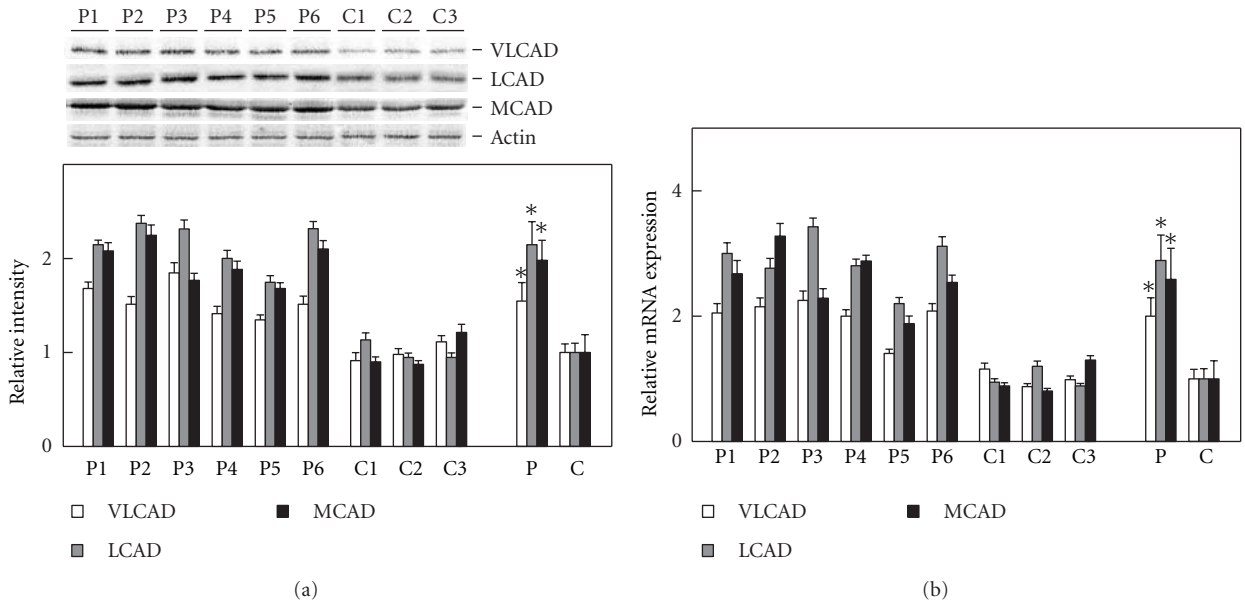

Figure 2: Expression levels of three Species of acyl-CoA dehydrogenase. Assay methods were, respectively, described in Section 2. (a) Shows relative quantification of expression levels of three acyl-CoA dehydrogenases. Upper panel indicates protein bands in immunoblot analysis. The band of actin was used as the loading control. Lower panel indicates relative protein amounts obtained by immunoblot and densitometric analyses. (b) Shows relative mRNA expression. Open bar, VLCAD; gray bar, LCAD; closed bar, MCAD. P1–P6, individual patient's fibroblast; C1–C3, individual control fibroblast; P, means ± SD in six patients' fibroblasts; C, means ± SD in three control fibroblasts. $^*P < 0.05$, versus controls.

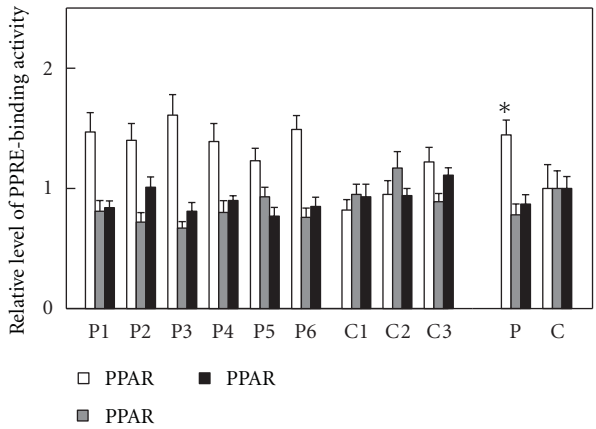

Figure 3: PPRE-binding activity. Assay methods were described in Section 2. Open bar, PPARα; gray bar, PPARβ; closed bar, PPARγ. P1–P6, individual patient's fibroblast; C1–C3, individual control fibroblast; P, means ± SD in six patients' fibroblasts; C, means ± SD in three control fibroblasts. $^*P < 0.05$, versus controls.

target gene product, was investigated. In the patients' fibroblasts, the MK886 treatment evidently reduced MCAD expression both in the protein and mRNA levels, and the fenofibrate treatment left this expression unchanged. In the control fibroblasts, the MK886 treatment did not affect this expression, and the fenofibrate treatment increased it both in the protein and mRNA levels (Figure 4). These data demonstrated that a considerable level of PPARα activation constitutively functioned in the patients' fibroblasts.

4. Discussion

This study demonstrated the occurrence of FFA accumulation, increased palmitoyl-CoA and octanoyl-CoA dehydrogenase activities, coordinated enhancement in the expression of three acyl-CoA dehydrogenases, a significant increase of PPRE-binding activity only for PPARα, and reduced MCAD expression as a result of PPARα-specific antagonist treatment in all of the fibroblasts from six patients with TP deficiency who had abnormalities in the second through fourth reactions in the mitochondrial fatty acid β-oxidation system. These results demonstrated that a considerable level of PPARα activation constitutively functioned in the patients' fibroblasts, in which FFA seemed to act as endogenous ligands toward PPARα as reported elsewhere [21–23]. FFA seems to work not toxically but protectively in the patients' fibroblasts, since the FFA accumulation and the resulting PPARα activation probably compensated for the impaired fatty acid metabolism in the patients' fibroblasts. It would be interesting to investigate whether this compensation appears in the patients' liver and heart, where considerable increases of TG/FFA and much higher levels of PPARα expression are expected [3, 24, 25]. From this viewpoint, the results obtained by using the patients' fibroblasts in the current study are useful for understanding the PPARα function.

This PPARα activation might induce cell proliferation in the patients' fibroblasts. To examine it, the mRNA levels with several oncogene products and cell cycle regulators were analyzed. The mRNA levels in the patients' fibroblasts were 2.5 ± 0.5-fold for c-Fos, 3.0 ± 0.6-fold for c-Myc, 2.4 ± 0.7-fold for cyclin D1, and 2.1 ± 0.3-fold for proliferating cell nuclear

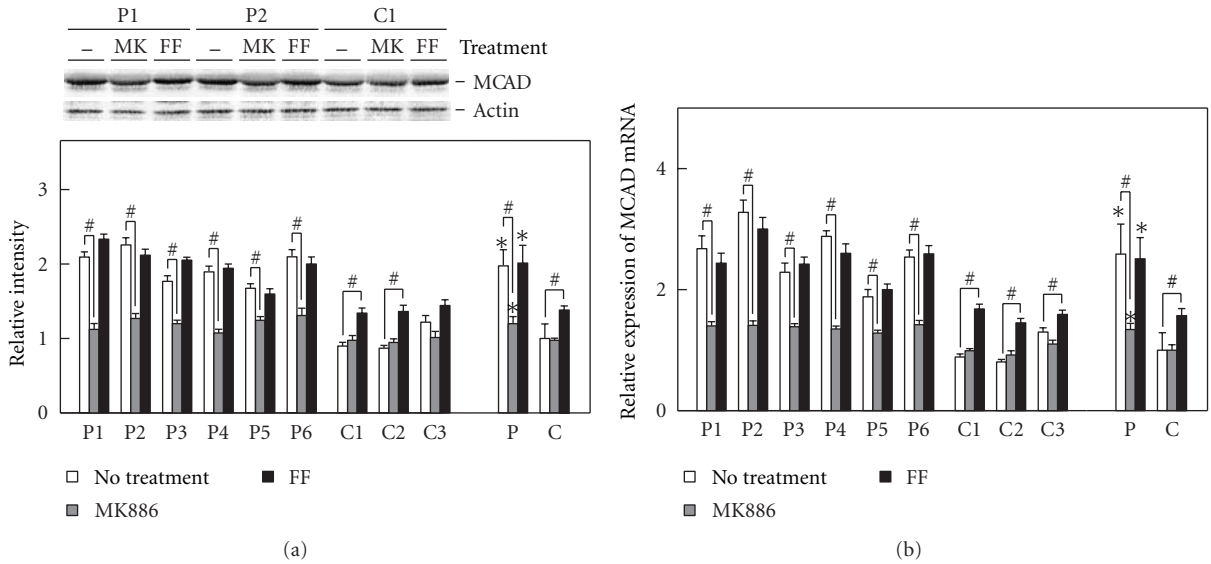

Figure 4: Effects of the MK886 or fenofibrate treatment on MCAD expression. The fibroblasts were plated in dishes and allowed to grow to 80% confluence. MK886 (30 μM final concentration) and fenofibrate (200 μM final concentration) were added to cell culture media, respectively. Both chemicals were dissolved in DMSO, and the final concentration of DMSO in media was maintained at 0.05% (v/v) in all cases. After 6 h, fibroblasts were harvested and used to analyze MCAD expression. (a) Shows relative quantification of expression level of MCAD protein. Upper panel indicates protein bands in immunoblot analysis. The band of actin was used as the loading control. Protein bands of two patients (P1 and P2) and a control (C1) are shown due to space limitation. Lower panel indicates relative protein amounts obtained by immunoblot and densitometric analyses. (b) Shows relative mRNA expression. Open bar, no treatment; gray bar, MK886 treatment; closed bar, fenofibrate (FF) treatment. P1–P6, individual patient's fibroblast; C1–C3, individual control fibroblast; P, means ± SD in six patients' fibroblasts; C, means ± SD in three control fibroblasts. $^{*}P < 0.05$, versus controls; $^{\#}P < 0.05$, no treatment versus MK886 or fenofibrate treatment.

antigen, which are all known as possible PPARα target gene products [3], when compared with those in the control fibroblasts. These results suggest the presence of promoted cell proliferation in the patients' fibroblasts, which appears to be helpful for elucidating the mechanisms of hypertrophic cardiomyopathy and hepatomegaly that occur in TP-deficient patients. Additionally, this work described increased oxidative stress in the patients' fibroblasts. Biochemical analysis measuring 4-hydroxyalkenals and malondialdehyde with the use of an LPO-586 kit demonstrated 2.2 ± 0.2 times greater lipid peroxides contents in the patients' fibroblasts than in the control fibroblasts, implying enhanced levels of oxidative stresses in the former fibroblasts. This finding was consistent with the results of mRNA analysis: the mRNA levels in the patients' fibroblasts were 2.7 ± 0.3-fold for peroxisomal acyl-CoA oxidase, which is known as a representative PPARα target gene product [3, 15], 1.3 ± 0.4-fold for catalase, 0.9 ± 0.3-fold for Cu, Zn-superoxide dismutase, and 1.1 ± 0.4-fold for NADPH oxidase 4, when compared with those in the control fibroblasts. This increased oxidative stress might help to elucidate the mechanisms of skeletal muscle weakness and hepatic encephalopathy, which occur in many TP-deficient patients. The FFA accumulation and the resulting PPARα activation seem to exert not protective but toxic effects on the patients' fibroblasts, since the activation aggravates intracellular circumstances by increasing oxidative stresses and promoting cell proliferation, which counteracts the protective role mentioned above of compensating for the impaired fatty acid metabolism in the patients' fibroblasts.

Additionally, this research pointed out the significant suppression of the PPARα activation by the MK886 treatment, which might be useful to eliminate the toxic effects of the activation. Thus, the MK886 treatment together with the administration of glucose or sucrose to supply energy might offer a new method for treating this deficiency.

By the way, short-chain (SCAD), medium-chain, long-chain, and very-long-chain acyl-CoA dehydrogenases are known to catalyze the first reaction in the mitochondrial β-oxidation system. Among the four isozymes, the presence of SCAD, MCAD, and VLCAD deficiencies has been reported. Patients with SCAD deficiency occasionally represented skeletal muscle weakness and developmental delay [26, 27], and those with MCAD deficiency frequently exhibited fasting intolerance and hypoketotic hypoglycemia [28, 29], which is rather dissimilar to the clinical features of patients with TP deficiency. On the other hand, patients with VLCAD deficiency frequently presented with fasting coma, skeletal muscle weakness, skeletal myopathy, hypertrophic cardiomyopathy, cardiomegaly, and slight hepatomegaly with fat accumulation [30–32], conditions which are similar to the clinical features of patients with TP deficiency. This similarity may depend on the PPARα activation induced by accumulated long-chain fatty acids and their derivatives. A future study using fibroblasts from patients with VLCAD deficiency is expected to confirm the mechanisms mentioned in this paper.

Abbreviations

FFA: Free fatty acids
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
LCAD: Long-chain acyl-CoA dehydrogenase
MCAD: Medium-chain acyl-CoA dehydrogenase
PPAR: Peroxisome proliferator-activated receptor
PPRE: PPAR response element
TG: Triglycerides
TP: Trifunctional protein
VLCAD: Very-long-chain acyl-CoA dehydrogenase.

Conflict of Interests

The authors have declared that no conflict of interests exists.

Acknowledgments

The authors thank Trevor Ralph for his English editorial assistance. The authors have declared that no financial support was provided.

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Hindawi Publishing Corporation
PPAR Research
Volume 2012, Article ID 174932, 10 pages
doi:10.1155/2012/174932

Research Article

Hepatic Cerebroside Sulfotransferase Is Induced by PPARα Activation in Mice

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Received 4 January 2012; Accepted 16 February 2012

Academic Editor: Christopher J. Nicol

Sulfatides are one of the major sphingoglycolipids in mammalian serum and are synthesized and secreted mainly from the liver as a component of lipoproteins. Recent studies revealed a protective role for serum sulfatides against arteriosclerosis and hypercoagulation. Although peroxisome proliferator-activated receptor (PPAR) α has important functions in hepatic lipoprotein metabolism, its association with sulfatides has not been investigated. In this study, sulfatide levels and the expression of enzymes related to sulfatide metabolism were examined using wild-type (+/+), *Ppara*-heterozygous (+/−), and *Ppara*-null (−/−) mice given a control diet or one containing 0.1% fenofibrate, a clinically used hypolipidemic drug and PPARα activator. Fenofibrate treatment increased serum and hepatic sulfatides in *Ppara* (+/+) and (+/−) mice through a marked induction of hepatic cerebroside sulfotransferase (CST), a key enzyme in sulfatide synthesis, in a PPARα-dependent manner. Furthermore, increases in CST mRNA levels were correlated with mRNA elevations of several known PPARα target genes, and such changes were not observed for other sulfatide-metabolism enzymes in the liver. These results suggest that PPARα activation enhances hepatic sulfatide synthesis via CST induction and implicate CST as a novel PPARα target gene.

1. Introduction

Sulfatides are sphingoglycolipids composed of sphingoid, fatty acid, galactose, and sulfate [1] that are distributed in various tissues such as the central nervous system, kidney, liver, and gastrointestinal tract [1–4]. Glycolipids are also present in the serum as one of the major components of lipoproteins [1]. Several studies have revealed a protective role for serum sulfatides against arteriosclerosis and hypercoagulation [5]. Serum levels of sulfatides are markedly decreased in humans with end-stage renal failure [6] but normalize after renal transplantation [7]. However, the precise mechanism regulating serum sulfatide concentrations in humans remains unclear. Previously studies demonstrated that serum sulfatide levels were strongly correlated with hepatic, but not renal, sulfatide levels in mice with protein overload nephropathy, and that decreased serum sulfatide levels were also associated with the downregulation of hepatic expression of cerebroside sulfotransferase (CST), a key enzyme in sulfatide synthesis [8]. These and previous findings suggest the possible participation of hepatic peroxisome proliferator-activated receptor (PPAR) in the regulation of serum and liver sulfatide metabolisms. To examine this possibility, serum and liver sulfatide concentrations and hepatic expression of a series of sulfatide-metabolizing enzymes were analyzed using *Ppara*-homozygous (+/+), *Ppara*-heterozygous (+/−), and *Ppara*-null (−/−) mice fed a control diet or one containing fenofibrate, a typical PPARα activator.

2. Materials and Methods

2.1. Mice and Treatment. All animal experiments were conducted in accordance with animal study protocols approved by the Shinshu University School of Medicine. Wild-type (+/+), *Ppara* (+/−), and *Ppara* (−/−) mice on a 129/Sv genetic background were generated as described previously [9–11]. These mice were maintained in a pathogen-free environment under controlled conditions (25°C; 12 h light/dark cycle) with tap water *ad libitum* and a standard rodent diet. Twelve-week-old male wild-type (+/+), *Ppara* (+/−), and *Ppara* (−/−) mice weighing 25–30 g were used for the following experiments. Mice of each genotype were randomly divided into two groups ($n = 6$ in each group of the same genotype). One mouse group was treated with a regular diet containing 0.1% fenofibrate (Wako Pure Chemical Industries, Osaka, Japan), and the other group was continued on a regular diet as a control. In an additional experiment, *Ppara* (+/+), *Ppara* (+/−), and *Ppara* (−/−) mice were randomly divided into two groups ($n = 6$ in each group of the same genotype) and were treated with a regular diet with or without 0.5% clofibrate (Wako Pure Chemical Industries). Seven days after commencing treatment, the mice were sacrificed under anesthesia for collection of blood and tissues.

2.2. Extraction and Measurement of Lipids. Total lipids in the serum and liver were extracted using the hexane/isopropanol method [12], and serum/liver sulfatides were determined as forms of lysosulfatides (LS; sulfatides without fatty acids) by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) as previously described [13]. Sulfatides levels were calculated as the sum of the levels of seven LS molecular species: LS-sphingadienine (LS-d18 : 2), LS-(4*E*)-sphingenine (LS-d18 : 1), LS-sphinganine (LS-d18 : 0), LS-4D-hydroxysphinganine (LS-t18 : 0), LS-(4*E*)-icosasphingenine (LS-d20 : 1), LS-icosasphinganine (LS-d20 : 0), and LS-4D-hydroxyicosasphinganine (LS-t20 : 0). Triglyceride (TG) levels in the serum and liver were measured using a Triglyceride *E*-test kit (Wako Pure Chemical Industries).

2.3. Immunoblot Analysis. Liver nuclear and cytosolic fractions were prepared from each mouse using NE-PER Nuclear and Cytoplasmic Extraction Regents (Thermo Fisher Scientific, Rockford, IL, USA) [14], and their protein concentrations were determined with a BCA protein assay kit (Thermo Fisher Scientific) [15]. Nuclear fractions (10 μg protein) were used for immunoblot analysis of PPARs and TATA box-binding protein (TBP). For detection of other proteins, cytosolic fractions (5 μg protein) were employed. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary antibodies followed by alkaline phosphatase-conjugated secondary antibodies [16–18]. Primary antibodies against long-chain acyl-CoA synthase (LACS), liver fatty acid-binding protein (L-FABP), and medium-chain acyl-CoA dehydrogenase (MCAD) were prepared as described previously [19–21]. Antibodies against other proteins were purchased commercially: cerebroside sulfotransferase (CST) from Abnova (Taipei, Taiwan), arylsulfatase A (ARSA) from Everest Biotech (Oxfordshire, UK), TBP from Abcam (Cambridge, UK), and ceramide galactosyltransferase (CGT), galactosylceramidase (GALC), microsomal transfer protein (MTP), PPARα, PPARβ/δ, PPARγ, and actin from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TBP and actin were used as loading controls for nuclear and cytosolic protein extracts, respectively. Band intensities were measured densitometrically, normalized to those of TBP or actin, and then expressed as fold changes relative to those of *Ppara* (+/+) mice treated with a control diet.

2.4. Analysis of mRNA. Total liver RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and samples of 2 μg of RNA were reverse-transcribed using oligo-dT primers and SuperScript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). Levels of mRNA were quantified by real-time polymerase chain reaction (PCR) using an SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) on a Thermal Cycler Dice TP800 system (Takara Bio) [10, 16]. Specific primers were designed by Primer Express software (Applied Biosystems, Foster City, CA, USA) as shown in Table 1. The mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal control. Measurements of mRNA levels were normalized to those of GAPDH and then expressed as fold changes relative to those of *Ppara* (+/+) mice treated with a control diet.

2.5. Assays for DNA-Binding Activity of PPARs. The DNA-binding activity of nuclear PPARαPPARβ/δ, and PPARγ was determined using PPARα, PPARβ/δ, and PPARγ Transcription Factor Assay kits (Cayman Chemical, Ann Arbor, MI, USA) [22–24], respectively. These assays are based on an enzyme-linked immunosorbent assay using PPAR response element (PPRE) immobilized microplates and specific PPAR antibodies, thus offering similar results to those from the conventional radioactive electrophoretic mobility shift assay. DNA-binding assays were carried out according to the manufacturer's instructions using nuclear extracts (50 μg protein) prepared as described previously. Results are expressed as fold changes relative to those of *Ppara* (+/+) mice treated with a control diet.

2.6. Statistical Analysis. All data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA with Bonferroni correction (SPSS Statistics 17.0; SPSS Inc, Chicago, IL, USA). Correlation coefficients were calculated using Spearman's rank correlation analysis. A probability value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Fenofibrate Increased Serum/Liver Sulfatides in a PPARα-Dependent Manner. Fenofibrate treatment increased serum, and more notably liver, sulfatide concentrations in *Ppara*

Table 1: Primer pairs used for the RT-PCR.

Gene	GeneBank accession number		Primer sequence
ARSA	NM_009713	F	5′-ACCACCCCTAACCTGGATCAGT-3′
		R	5′-ATGGCGTGCACAGAGACACA-3′
CGT	NM_011674	F	5′-TGGGTCCAGCCTATGGATGT-3′
		R	5′-GCAGCGTTGGTCTTGGAAAC-3′
CST	NM_016922	F	5′-ATGGCCTTCACGACCTCAGA-3′
		R	5′-CGGTCTTGTGCGTCTTCATG-3′
GALC	NM_008079	F	5′-GAGTGAGAATCATAGCGAGCGATA-3′
		R	5′-AGTTCCTGGTCCAGCAGCAA-3′
GAPDH	M32599	F	5′-TGCACCACCAACTGCTTAG-3′
		R	5′-GGATGCAGGGATGATGTTCTG-3′
LACS	NM_007981	F	5′-TCCTACGGCAGTGATCTGGTG-3′
		R	5′-GGTTGCCTGTAGTTCCACTTGTG-3′
L-FABP	NM_017399	F	5′-GCAGAGCCAGGAGAACTTTGAG-3′
		R	5′-TTTGATTTTCTTCCCTTCATGCA-3′
MCAD	NM_007382	F	5′-TGCTTTTGATAGAACCAGACCTACAGT-3′
		R	5′-CTTGGTGCTCCACTAGCAGCTT-3′
MTP	NM_008642	F	5′-GAGCGGTCTGGATTTACAACG-3′
		R	5′-GTAGGTAGTGACAGATGTGGCTTTTG-3′
PPARα	NM_011144	F	5′-CCTCAGGGTACCACTACGGAGT-3′
		R	5′-GCCGAATAGTTCGCCGAA-3′
PPARβ/δ	XM_128500	F	5′-TCAACATGGAATGTCGGGTT-3′
		R	5′-ATACTCGAGCTTCATGCGGATT-3′
PPARγ	NM_011146	F	5-TTCCACTATGGAGTTCATGCTTGT-3′
		R	5′-TCCGGCAGTTAAGATCACACCTA-3′

F: forward sequence; R: reverse sequence.

(+/+) and (+/−) mice only (Figure 1(a)). However, the increases in serum/liver sulfatides were not detected in *Ppara* (−/−) mice with fenofibrate treatment. These results demonstrate that fenofibrate increases serum/liver sulfatide levels in a PPARα-dependent manner. The treatment did not affect the composition of sulfatides (Table 2). Fenofibrate also decreased serum/liver TG levels in a PPARα-dependent manner (Figure 1(b)), which was in agreement with previous reports [25, 26].

3.2. Fenofibrate Upregulated Hepatic CST in a PPARα-Dependent Manner. We assessed several major hepatic sulfatide-metabolizing enzymes to determine the mechanistic basis of the changes observed in sulfatide concentrations. CST and ARSA, respectively, catalyze the forward and reverse reactions from galactosylceramides to sulfatides, and a similar relationship exists for CGT and GALC in the synthesis of galactosylceramides from ceramides [8]. Fenofibrate treatment significantly increased levels of mRNA encoding CST in *Ppara* (+/+) and (+/−) mice (Figure 2(a)), with the extent of induction higher in the *Ppara* (+/+) group. Upregulation of CST expression by fenofibrate was not observed in *Ppara* (−/−) mice. PPARα-dependent increases in CST mRNA corresponded to increases in CST protein levels (Figure 2(b)). Fenofibrate treatment did not affect expression of the other sulfatide-metabolizing enzymes, ARSA, CGT, and GALC, at either the mRNA or the protein level (Figure 2). Since hepatic CST mRNA levels were strongly correlated with sulfatide levels in the serum ($r = 0.886$, $P = 0.019$) and liver ($r = 0.943$, $P = 0.005$), the increased serum/liver sulfatide levels found after treatment were viewed as mainly due to the significant induction of hepatic CST.

3.3. Hepatic CST Was Induced by PPARα Activation. As expected, fenofibrate treatment significantly enhanced hepatic expression of PPARα and several representative PPARα target genes, including LACS, MCAD, L-FABP, and MTP (Figures 3 and 4) [27–29]. The DNA binding activity levels of PPARα were also elevated by fenofibrate (Figure 3(b)). The treatment did not influence the expression and activity of PPARβ/δ or PPARγ (Figure 3), nor did it affect levels of CST mRNA or protein in the livers of *Ppara* (−/−) mice (Figure 2). The mRNA levels of CST were strongly correlated with those of PPARα target gene products ($r = 0.886$, $P = 0.019$ for LACS; $r = 0.928$, $P = 0.008$ for MCAD; $r = 0.943$, $P = 0.005$ for L-FABP; and $r = 0.943$, $P = 0.005$ for MTP). PPARα-dependent induction of CST mRNA levels was also observed in mice treated with clofibrate, another typical PPARα activator (Figure 5). These results indicated that

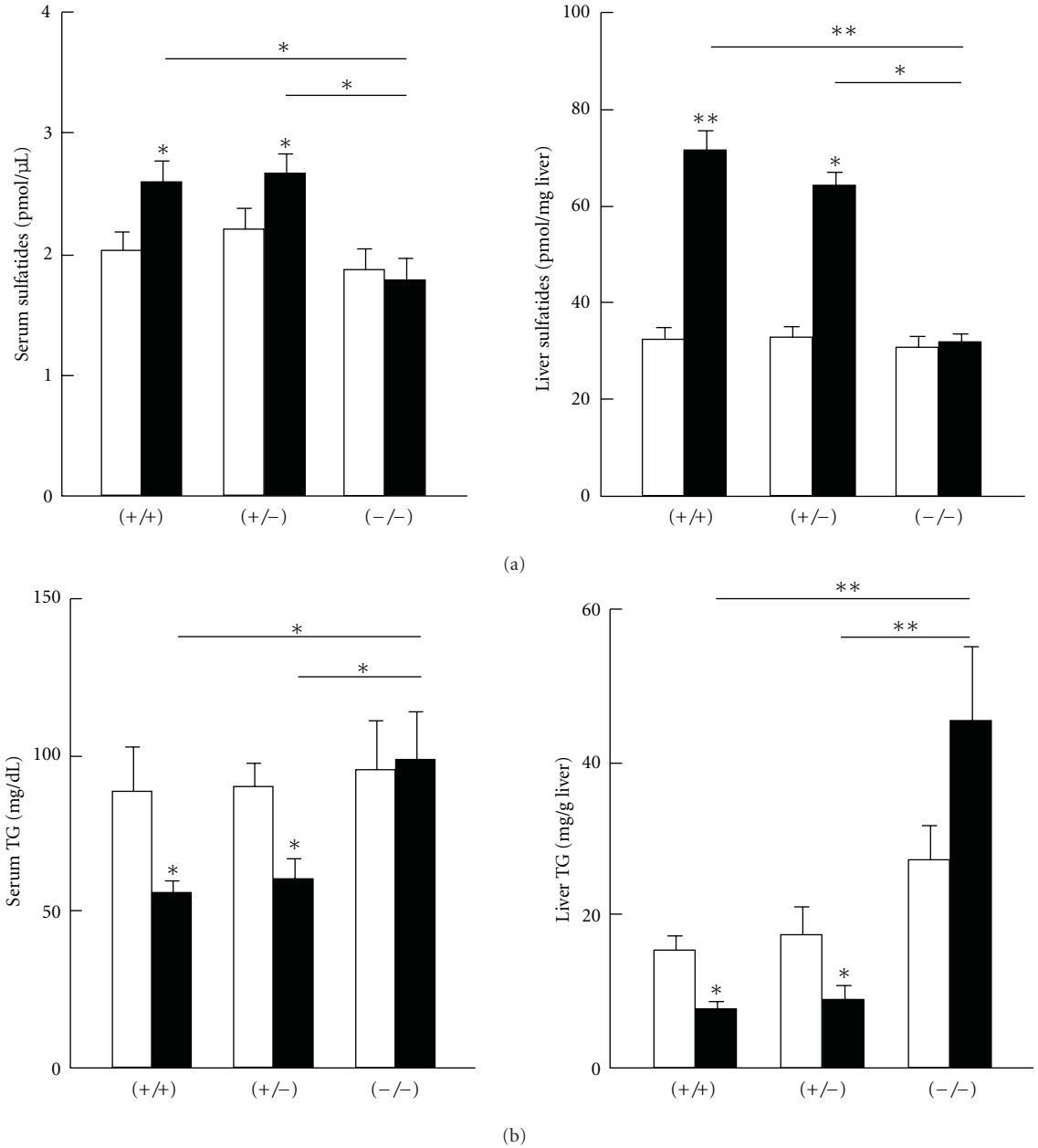

Figure 1: Changes in serum and hepatic levels of sulfatides (a) and TG (b). *Ppara* (+/+), (+/−), and (−/−) mice were treated without (open bars) or with (closed bars) 0.1% fenofibrate for 7 days. Results are expressed as mean ± SD ($n = 6$/group). $^{*}P < 0.05$; $^{**}P < 0.01$.

the induction of hepatic CST was closely associated with PPARα activation in mice.

4. Discussion

The present study revealed that fenofibrate treatment increased serum/liver sulfatide levels and the expression of hepatic CST mRNA and protein through PPARα activation. As CST mRNA levels were closely correlated with those of four known PPARα target genes, these findings suggest that CST may be a novel PPARα target gene candidate.

While CST is a key enzyme in sulfatide metabolism, little is known about its transcriptional regulation. We recently reported that an increase in hepatic oxidative stress downregulated CST expression in mice [8], although the mechanism remains unclear. A search for putative PPRE regions in the mouse CST gene [30, 31] revealed several candidates: −1,434/−1,422 (AGGTCTAAGGGCA), −1,202/−1,190 (TGGACTTTGCCCT), and −896/−884 (AGGACAAAGAGCA) from exon 1a; −1,499/−1,487 (AGGCTACAGTTCA) from exon 1e; and −1,569/−1,557 (AGGTCAGAGCACA) and −302/−290 (AGGACAGAGCCCA) from exon 1f. These regions may be useful for analysis in future in vitro experiments.

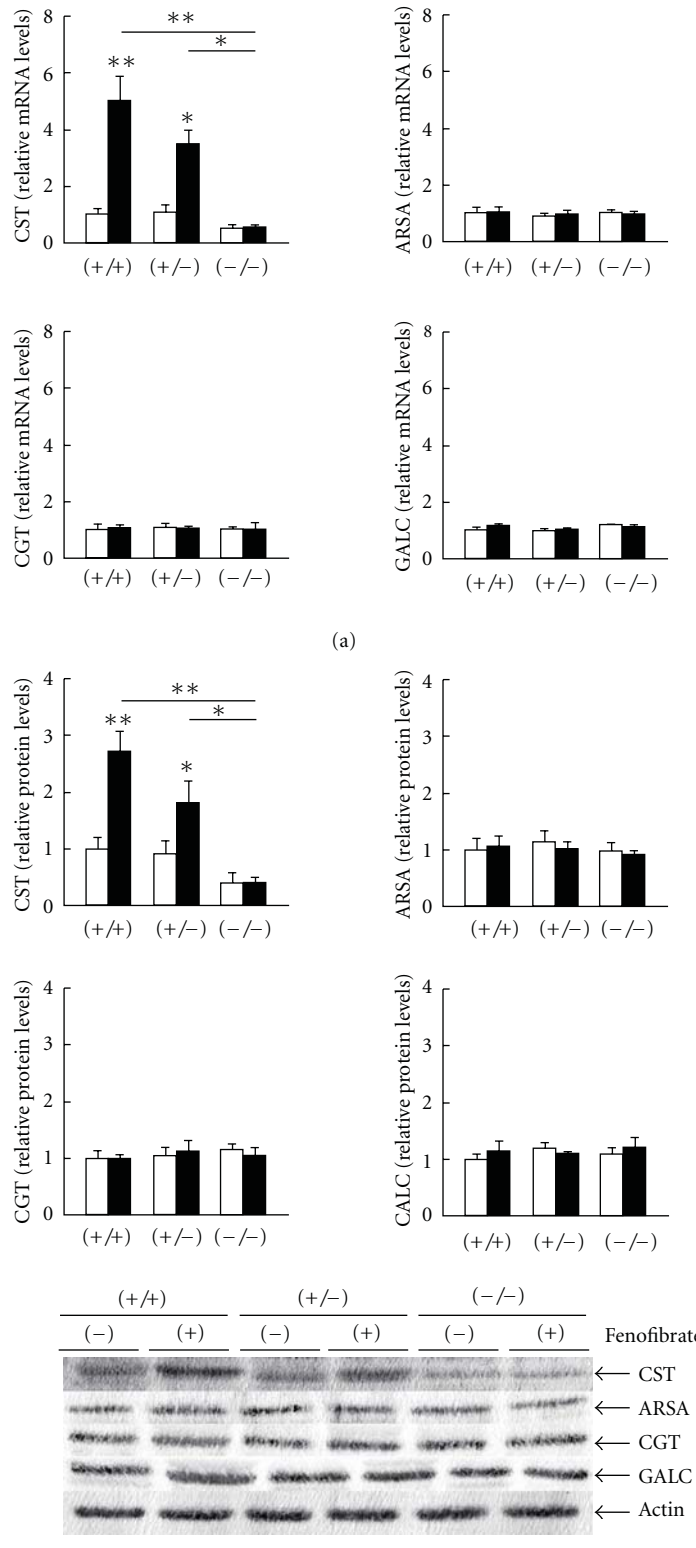

Figure 2: Changes in hepatic expression of sulfatide-metabolizing enzymes by fenofibrate treatment. Open and closed bars indicate mice treated without or with 0.1% fenofibrate, respectively. Data are expressed as mean ± SD ($n = 6$/group). $^{*}P < 0.05$; $^{**}P < 0.01$. (a) The mRNA levels of CST, ARSA, CGT, and GALC. Hepatic mRNA levels were normalized to those of GAPDH and then expressed as fold changes relative to those of *Ppara* (+/+) mice treated with a control diet. (b) Immunoblot analysis. Actin was used as the loading control. Band intensities were measured densitometrically, normalized to those of actin, and then expressed as fold changes relative to those of *Ppara* (+/+) mice treated with a control diet.

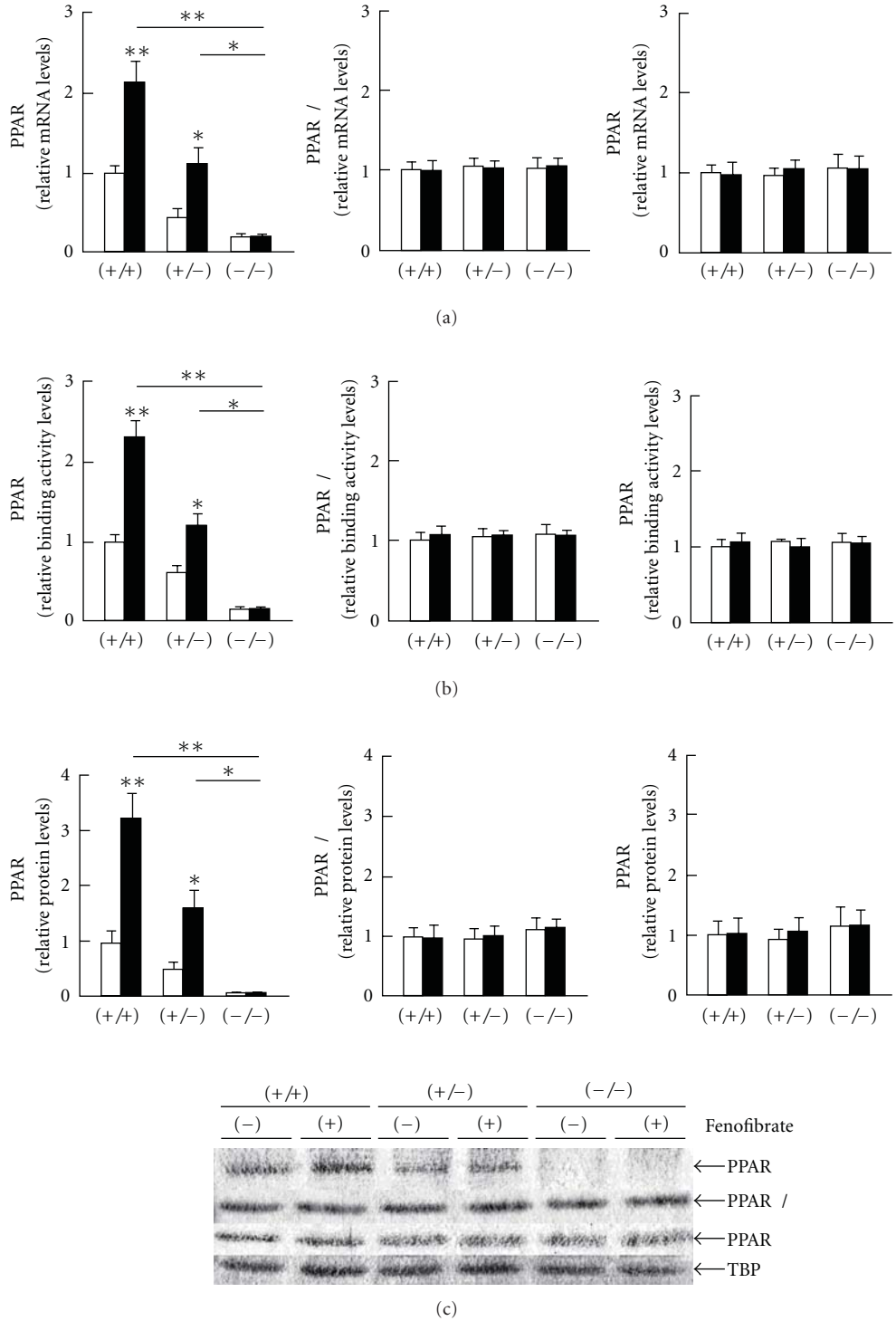

Figure 3: Changes in hepatic expression of PPARs by fenofibrate treatment. Open and closed bars indicate mice treated without or with 0.1% fenofibrate, respectively. Data are expressed as mean ± SD ($n = 6$/group). $^{*}P < 0.05$; $^{**}P < 0.01$. (a) The mRNA levels of PPARs. Hepatic mRNA levels were normalized to those of GAPDH and then expressed as fold changes relative to those of *Ppara* (+/+) mice treated with a control diet. (b) PPAR-binding activity based on an enzyme-linked immunosorbent assay. Detailed protocols are described in Section 2. Results are expressed as fold changes relative to those of *Ppara* (+/+) mice treated with a control diet. (c) Immunoblot analysis. TBP was used as the loading control. Band intensities were measured densitometrically, normalized to those of TBP, and then expressed as fold changes relative to those of *Ppara* (+/+) mice treated with a control diet.

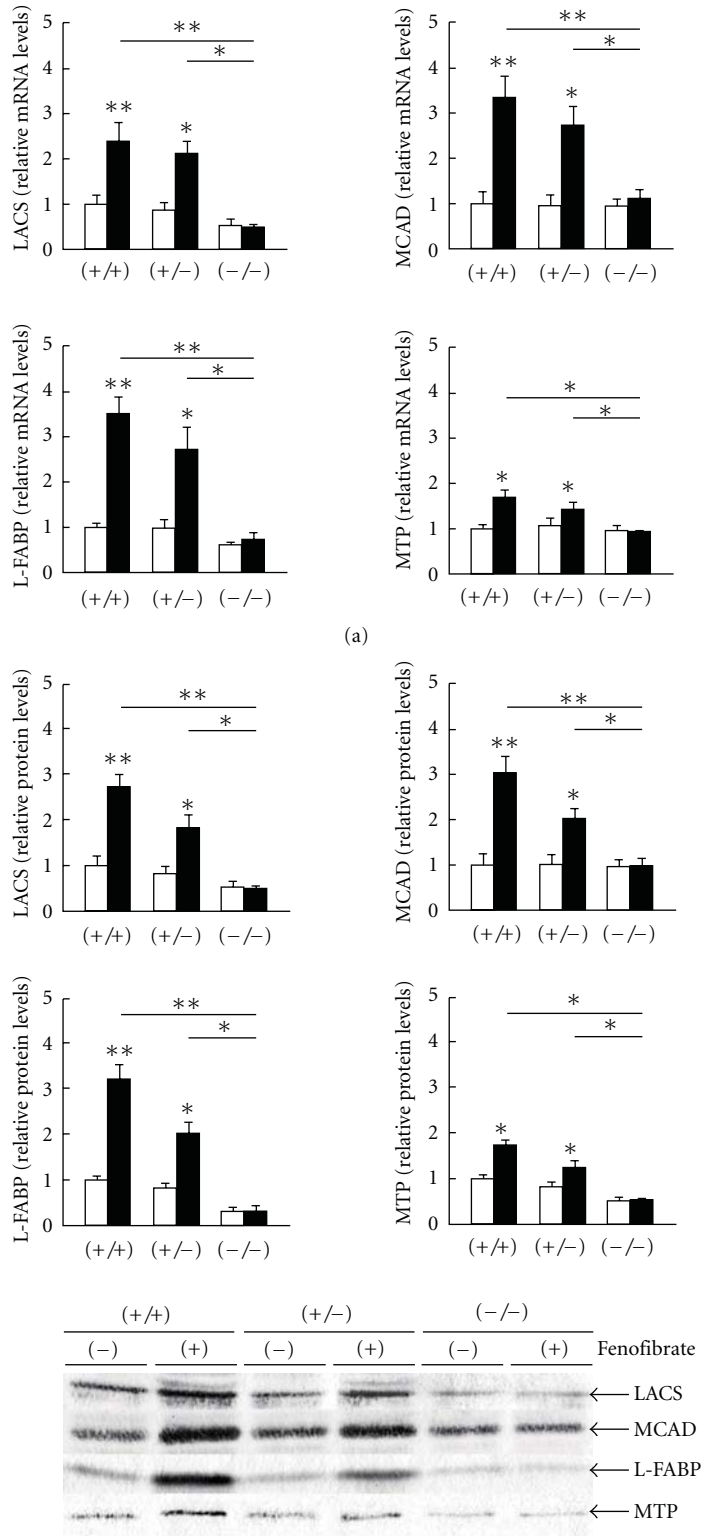

Figure 4: Changes in hepatic expression of conventional PPARα target genes by fenofibrate treatment. Open and closed bars indicate mice treated without or with fenofibrate, respectively. Data are expressed as mean ± SD (n = 6/group). $^{*}P < 0.05$; $^{**}P < 0.01$. (a) Analysis of mRNA. Hepatic mRNA levels were normalized to those of GAPDH and then expressed as fold changes relative to levels of *Ppara* (+/+) mice treated with a control diet. (b) Immunoblot analysis. Band intensities were measured densitometrically, normalized to those of actin, and then expressed as fold changes relative to those of *Ppara* (+/+) mice treated with a control diet.

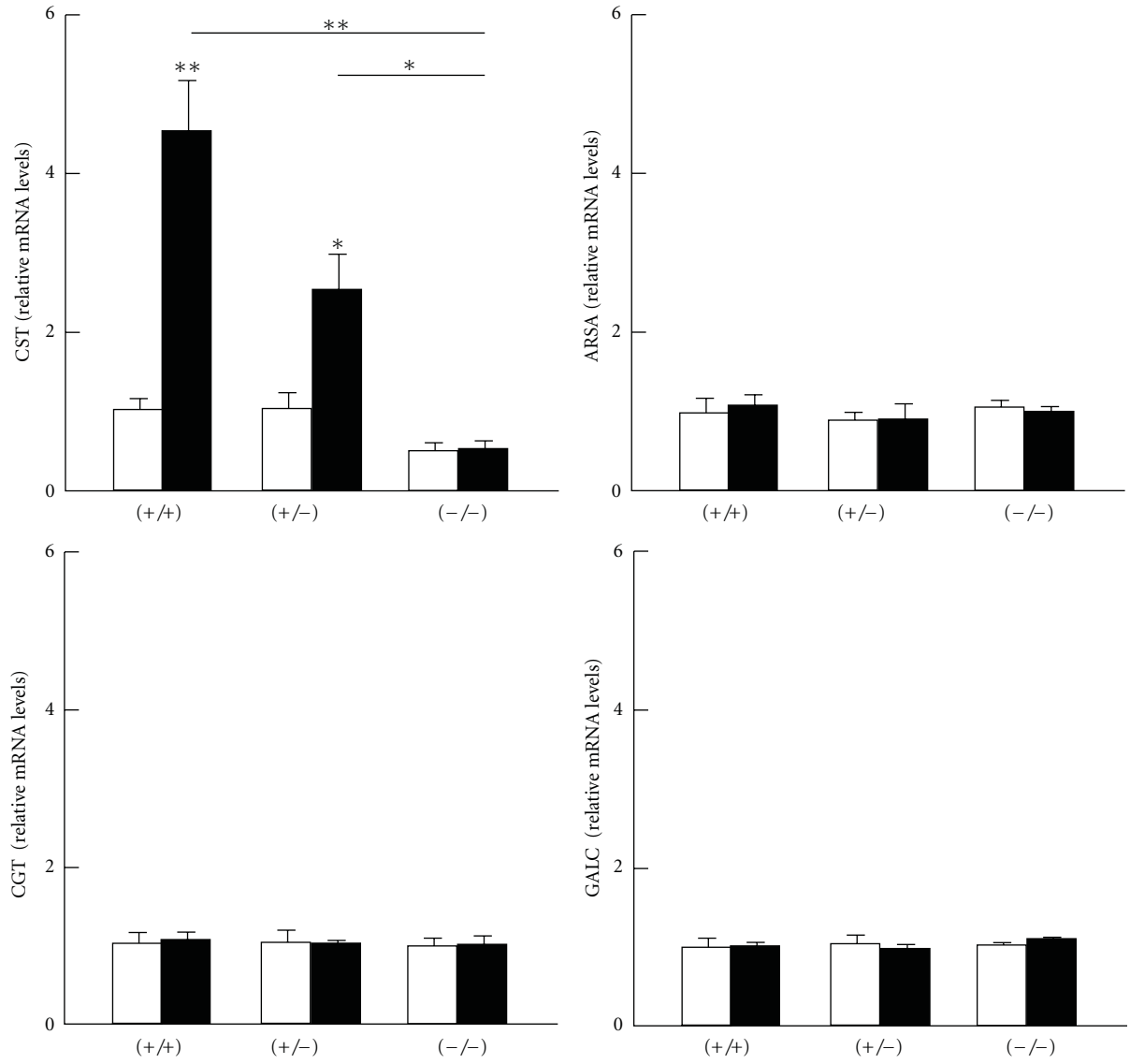

Figure 5: PPARα-dependent induction of CST mRNA levels by clofibrate treatment. Open and closed bars indicate mice treated without or with 0.5% clofibrate, respectively. Data are expressed as mean ± SD ($n = 6$/group). $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 2: Composition of serum and liver sulfatides.

	Serum						Liver					
	(+/+)		(+/−)		(−/−)		(+/+)		(+/−)		(−/−)	
	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)
d18 : 2	7	9	8	7	8	7	12	11	12	13	11	12
d18 : 1	34	31	33	36	33	35	29	30	30	28	30	31
d18 : 0	11	11	12	10	11	10	11	10	12	11	10	12
t18 : 0	7	9	8	7	8	7	6	6	6	5	7	6
d20 : 1	8	11	9	8	9	8	12	11	10	12	10	10
d20 : 0	5	7	6	6	6	6	10	9	9	10	9	8
t20 : 0	28	22	24	26	25	27	20	23	21	21	23	21

(−): mice treated with a control diet; (+): mice treated with fenofibrate; d18 : 2: sphingadienine; d18 : 1: (4*E*)-sphingenine; d18 : 0: sphinganine; t18 : 0: 4D-hydroxysphinganine; d20 : 1: (4*E*)-icosasphingenine; d20 : 0: icosasphinganine; t20 : 0: 4D-hydroxyicosasphinganine.
Data are expressed as percentages.

The degree of increases in serum sulfatides was lower than that in hepatic sulfatides by fenofibrate treatment (1.27-fold in the serum versus 2.20-fold in liver in *Ppara* (+/+) mice and 1.22-fold in the serum versus 1.95-fold in the liver of *Ppara* (+/−) mice). Sulfatides synthesized in the liver are secreted into the blood together with TG as a component of very-low-density lipoprotein (VLDL) [32]. Thus, hepatic TG content was reduced by fenofibrate treatment probably due to the enhanced of mitochondrial β-oxidation ability resulting in a reduction of hepatic VLDL synthesis as seen in other experiments using cultured hepatocytes [33]. Further studies are required to determine sulfatide metabolism in the serum and liver since they are significantly influenced by numerous pathophysiological events and treatments, including acute kidney injury [8, 34], clofibrate pretreatment [35], chronic kidney disease [6], and kidney transplantation [7].

The role of PPARα has been clarified in several liver diseases. For instance, PPARα is downregulated in alcoholic liver disease [11, 36] as well as after liver transplantation[37]. Persistent activation of PPARα ameliorates hepatic steatosis and inflammation in mice but may also induce hepatocarcinogenesis [10]. The association between liver disease and sulfatide metabolism may be of interest for further research.

Lastly, several animal studies have uncovered a protective role for serum sulfatides against arteriosclerosis and hypercoagulation [5]. We also reported a close relationship between lower serum sulfatide concentrations and higher incidences of cardiovascular disease in patients with end-stage renal failure [6], in whom sulfatide levels returned to normal following kidney transplantation [7]. Accordingly, increasing or maintaining serum sulfatide levels using fibrates may be useful in reducing the risk of cardiovascular events, which is consistent with the known beneficial effect of fibrates seen in randomized controlled studies [38]. Furthermore, these findings show a need to examine sulfatide metabolism in cardiomyocytes, endothelial cells, and vascular smooth cells to disclose any novel protective roles of PPARα in cardiovascular inflammation and atherosclerosis, particularly in relation to CST upregulation.

Abbreviations

ARSA: Arylsulfatase A
CGT: Ceramide galactosyltransferase
CST: Cerebroside sulfotransferase
GALC: Galactosylceramidase
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
LACS: Long-chain acyl-CoA synthase
L-FABP: Liver fatty acid-binding protein
LS: Lysosulfatides
MCAD: Medium-chain acyl-CoA dehydrogenase
MTP: Microsomal triglyceride transfer protein
PCR: Polymerase chain reaction
PPAR: Peroxisome proliferator-activated receptor
PPRE: PPAR response element
TG: Triglycerides.

Conflicts of Interests

The authors have declared that no conflict of interests exists.

Acknowledgment

The authors thank Trevor Ralph for his English editorial assistance.

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