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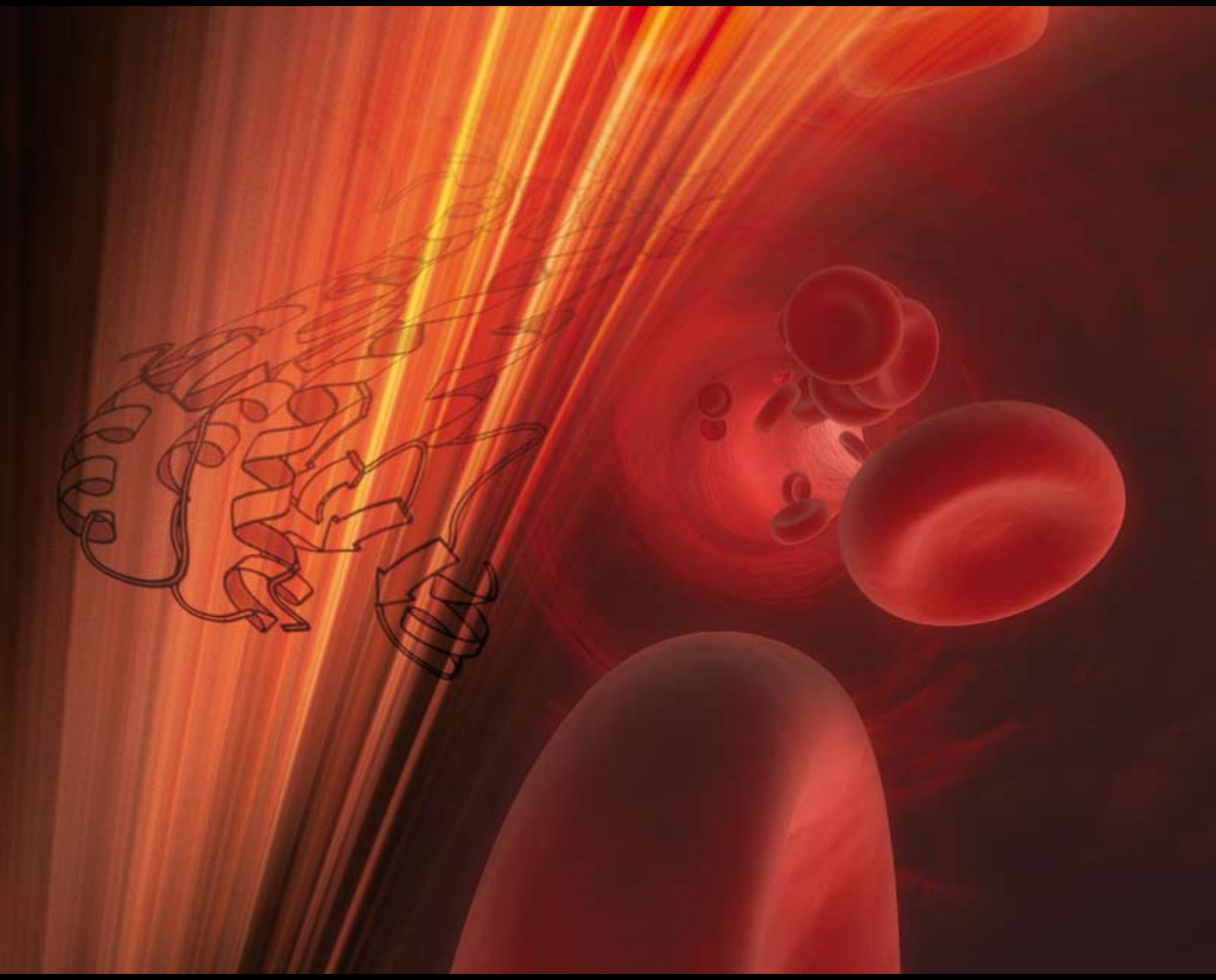
Development of Synthetic Modulators of PPARs: Current Challenges and Future Opportunities

Guest Editors: Francine M. Gregoire, Anne Reifel Miller, and Jane Pinaire



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Contents

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Editorial

Development of Synthetic Modulators of PPARs: Current Challenges and Future Opportunities

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The cloning of the mouse PPAR alpha gene in 1990 by Issemann and Green [1] stimulated intense interest in this family of nuclear receptors, and research efforts over the next decade established important roles for the PPAR isotypes in glucose and lipoprotein metabolism, inflammation, and atherosclerosis. Though the fibrates (PPAR-α agonists) had been used for the treatment of dyslipidemia for nearly 40 years, the discovery of the insulin-sensitizing effects of PPAR-γ agonists brought about the development and commercialization of the thiazolidinedione (TZD) class of oral anti-hyperglycemic medications: troglitazone, approved by the FDA in 1997, and pioglitazone and rosiglitazone, both approved by the FDA in 1999. The withdrawal of troglitazone from the market in 2000 was an early indicator of the potential safety issues of PPAR drugs. Even so, the clinical use of fibrates and TZDs has allowed for a better understanding of the safety profiles and safety issues of PPAR-α and PPAR-γ agonists [2, 3].

At the beginning of this decade, many pharmaceutical companies had development programs focused on delivering "new and improved" PPAR agonists to the market. For example, several programs were developing PPAR-α/γ dual agonists (i.e., glitazars) for the treatment of type 2 diabetes. Although preliminary data from various PPAR-α/γ dual agonist research programs was promising, nearly all of these research programs were discontinued due to safety issues identified during clinical testing and/or during preclinical testing [4–6]. More recently, the highly publicized (and controversial) meta-analysis of rosiglitazone reported by Nissen and Wolski [7] called into question the safety of the TZDs and prompted changes to the labels of both rosiglitazone and pioglitazone.

And yet, the promise of the therapeutic potential of PPAR drugs remains. A PubMed search using the term "peroxisome proliferator-activated receptor" yielded 1578 manuscripts (161 of which were review articles) between January 1 and December 1, 2008. In addition to the roles of the PPAR isotypes in lipid/lipoprotein/glucose metabolism, additional roles in diverse physiological processes and disease states are currently being investigated. As additional functions are identified; the PPARs will continue to be important molecular targets for identifying ligands (drugs) with potential applications to reproduction and fertility, normal development; function of the reproductive, gastrointestinal, respiratory, and central nervous systems; skin biology and wound healing; and cell cycle control and cancer.

Reports highlighting both challenges and opportunities in PPAR drug development are included in this special issue. For example, edema, weight gain, and a reduction in bone mass (particularly in women) are challenges limiting the clinical utility of the currently marketed TZDs. The renal and vascular mechanisms of TZD-induced fluid retention are reviewed by Yang and Soodvilai, and the recent clinical data describing the effects of TZDs on bone are reviewed by Schwartz. In contrast, several reports highlight opportunities in PPAR drug development. Deeg and Tan compare the effects of rosiglitazone and pioglitazone on lipids, lipoproteins, and apolipoproteins as reported in head-to-head, randomized clinical studies. Two papers describe the effects of PPARs/PPAR ligands on immune/inflammatory responses; Fernandez reviews the roles of the PPARs in modulating the immune/inflammatory response in atherosclerosis, while Yamashita reviews the receptor-independent effects of PPAR-α and PPAR-γ ligands on cysteinyl leukotriene production in mast cells as it relates to the development of potential anti-asthma medications. Technological and methodological approaches that may prove useful in the identification and assessment of new PPAR drugs are

also reported. Clarke et al. describe an approach used to determine the species differences in plasma protein binding to MBX-102, a novel PPAR-γ agonist currently in Phase 2 clinical development, and the corresponding differences in PPAR-γ activation across species. Cho et al. review the role of PPARs in metabolic disorders as well as various strategies and technologies used in the identification and assessment of PPAR drugs. Finally, Miyachi and Hashimoto describe the synthesis and SAR of subtype-specific PPAR agonists derived from a single 3,4-disubstituted phenylpropanoic acid "versatile template" scaffold, and Higgins and Mantzoros review the development and safety profile of INT-131, a potent non-TZD selective PPAR modulator (SPPARM) currently in Phase 2 clinical development.

In conclusion, though the full therapeutic potential of PPARs has yet to be realized, and serious safety issues are associated with the currently marketed PPAR drugs (PPAR-α and PPAR-γ), there remains intense interest in exploring new physiological roles of the PPARs and in the identification of new and improved PPAR agonists drugs.

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REFERENCES

[1] I. Issemann and S. Green, "Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators," *Nature*, vol. 347, no. 6294, pp. 645–650, 1990.

[2] A. Rubenstrunk, R. Hanf, D. W. Hum, J.-C. Fruchart, and B. Staels, "Safety issues and prospects for future generations of PPAR modulators," *Biochimica et Biophysica Acta*, vol. 1771, no. 8, pp. 1065–1081, 2007.

[3] B. G. Shearer and A. N. Billin, "The next generation of PPAR drugs: do we have the tools to find them?" *Biochimica et Biophysica Acta*, vol. 1771, no. 8, pp. 1082–1093, 2007.

[4] C. Fiévet, J.-C. Fruchart, and B. Staels, "PPARα and PPARγ dual agonists for the treatment of type 2 diabetes and the metabolic syndrome," *Current Opinion in Pharmacology*, vol. 6, no. 6, pp. 606–614, 2006.

[5] C. R. Waites, M. A. Dominick, T. P. Sanderson, and B. E. Schilling, "Nonclinical safety evaluation of muraglitazar, a novel PPARα/γ agonist," *Toxicological Sciences*, vol. 100, no. 1, pp. 248–258, 2007.

[6] P. Balakumar, M. Rose, S. S. Ganti, P. Krishan, and M. Singh, "PPAR dual agonists: are they opening Pandora's Box?" *Pharmacological Research*, vol. 56, no. 2, pp. 91–98, 2007.

[7] S. E. Nissen and K. Wolski, "Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes," *The New England Journal of Medicine*, vol. 356, no. 24, pp. 2457–2471, 2007.

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Review Article

Renal and Vascular Mechanisms of Thiazolidinedione-Induced Fluid Retention

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Recommended by Jane Pinaire

Thiazolidinediones (TZDs) are peroxisome proliferator-activated receptor subtype γ (PPARγ) activators that are clinically used as an insulin sensitizer for glycemic control in patients with type 2 diabetes. Additionally, TZDs exhibit novel anti-inflammatory, antioxidant, and antiproliferative properties, indicating therapeutic potential for a wide variety of diseases associated with diabetes and other conditions. The clinical applications of TZDs are limited by the common major side effect of fluid retention. A better understanding of the molecular mechanism of TZD-induced fluid retention is essential for the development of novel therapies with improved safety profiles. An important breakthrough in the field is the finding that the renal collecting duct is a major site for increased fluid reabsorption in response to rosiglitazone or pioglitazone. New evidence also indicates that increased vascular permeability in adipose tissues may contribute to edema formation and body weight gain. Future research should therefore be directed at achieving a better understanding of the detailed mechanisms of TZD-induced increases in renal sodium transport and in vascular permeability.

1. INTRODUCTION

Thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are highly effective for the treatment of type 2 diabetes and are widely prescribed. Unfortunately, fluid retention has emerged as the most common and serious side effect of TZDs and has become the most frequent cause of discontinuation of therapy. The incidence of TZD-induced fluid retention ranges from 7% in monotherapy and to as high as 15% when combined with insulin [1–3]. The fluid retention is often presented as peripheral edema, which can progress into pulmonary edema and congestive heart failure. TZD use leads to a 6-7% increase in blood volume in healthy volunteers [4, 5]. This blood volume expansion can dilute the red blood cell concentration, producing a reduced hematocrit. In fact, changes in hematocrit have been used as a surrogate marker for TZD-induced plasma volume expansion. The fluid retention is often resistant to loop diuretics but is reversed by withdrawing the drug. Many aspects of TZD-induced fluid retention have been covered by excellent review articles [6–12]. This review will emphasize renal sodium retention and vascular hyperpermeability as prominent mechanisms of TZD-induced fluid retention. We will also introduce several possible treatment strategies.

2. RENAL MECHANISM

The kidney is the key regulator of electrolyte balance and water conservation. Fluid retention at the renal level is suggested by evidence that TZD-induced edema is associated with reduced urinary sodium and water excretion. Song et al. reported that chronic three-day administration of rosiglitazone to Sprague Dawley rats significantly reduced urine volume (by 22%) and sodium excretion (by 44%) [13]. These findings lead us to speculate that renal mechanisms play a major role in TZD-induced fluid retention. TZDs may cause renal fluid reabsorption directly by affecting tubular transport, renal sodium retention, and vascular hyperpermeability or indirectly by affecting renal hemodynamics or processes. Yang et al. examined the effect of a PPARγ agonist, GI262570 (farglitazar), on the glomerular filtration rate, effective renal plasma flow, and renal filtration fraction in chronically catheter-implanted conscious rats [14]. In this

study, glomerular filtration rate was determined by using fluorescein isothiocyanate (FITC)-inulin and renal blood flow by using para-aminohippurate (PAH). A 10-day infusion of GI262570 decreased hematocrit, hemoglobin, and serum albumin (all $P < .05$), indicating volume expansion, but did not alter glomerular filtration rate, effective renal plasma flow, or renal filtration fraction. This indicates that PPARγ agonist-induced volume expansion is not related to changes in renal hemodynamics [14]. This observation is reinforced by a human study in which the six-week administration of pioglitazone to healthy volunteers led to sodium retention without a significant effect on glomerular filtration rate or renal blood flow [15]. This lack of change in renal hemodynamics is, however, not universally reported. The three-day administration of rosiglitazone in Sprague Dawley rates induced a 35% reduction in creatinine clearance, an indirect measure of the glomerular filtration rate [13]. It is unclear whether or not this discrepancy is related to differences in glomerular filtration rate measurement techniques or other experimental protocols.

The lack of solid evidence to support the alteration of renal hemodynamic parameters following treatment with PPARγ ligands suggests the possibility of a direct influence on tubular transport processes. The regulation of NaCl reabsorption in the kidney can occur at the level of sodium transport proteins lining the renal epithelia. These sodium transporters include basolateral Na-K-ATPase, and the following apical transporters that vary with individual nephron segments: the sodium hydrogenexchanger subtype III (NHE3) and the sodium phosphate cotransporter subtype II (NaPi-2) in the proximal convoluted tubule, the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2 or BSC1) in the thick ascending limb, the thiazide-sensitive Na-Cl cotransporter (NCC or TSC) in the distal convoluted tubule, and the amiloride-sensitive sodium channel (ENaC) in the collecting duct. The major water channel proteins (aquaporins, AQPs) in the kidney include AQP1-4, of which AQP1 and AQP2 function on the apical membrane, and AQP3 and AQP4 on the basolateral membrane [16]. The study of Song et al. is the first to provide a comprehensive examination of the effects of PPARγ agonists on various renal sodium and water transport proteins [13]. In that study, a three-day rosiglitazone treatment increasedthe whole kidney protein level of the α-1 subunit of Na-K-ATPase, NKCC2, NHE3, AQP2, and AQP3 [13]. These findings suggest that increases in sodium transport may occur in the proximal convoluted tubule and the thick ascending limb.

The collecting duct reabsorbs approximately 2-3% of the filtered sodium load primarily through ENaC, which is comprised of three subunits, α, β, and γ. These proteins are vital to day-to-day adjustment of sodium reabsorption and are regulated by the hormones aldosterone and insulin [17–19]. A key mediator of aldosterone activation of ENaC is serum and glucocorticoid regulated kinase 1 (SGK1) [20, 21]. Activated SGK1 prevents ENaC degradation by inactivating the ubiquitin ligase Nedd4-2 [22]. Nedd4-2 interacts with the PY motif of ENaC leading to endocytosis and degradation of the channel [22]. Prior to the conditional knockout (KO) studies, three major lines of evidence indicated that the activation of sodium transport processes in the distal nephron may underlie TZD-induced fluid retention. First, within the kidney, PPARγ is highly expressed in the renal medullary collecting duct, with lower expression levels in glomeruli, proximal tubules, and microvasculature. This was demonstrated by both RT-PCR and microdissection as well as by in situ hybridization techniques [23–25]. Second, in a cultured human cortical collecting duct (CCD) cell line, PPARγ agonists increased levels of cell surface α-ENaC. This is paralleled by an increase in SGK1 mRNA, which is abolished by pretreatment with a specific PPARγ antagonist, leading to increased levels of cell surface α-ENaC. Electrophoretic mobility shift assays further suggest that these effects are caused by the binding of PPARγ to a specific response element in the SGK1 promoter [20]. Third, in vivo evidence shows that GI262570 stimulates sodium and water reabsorption from the distal nephron in Sprague Dawley rats [26]. This evidence comes from increases in plasma sodium and chloride concentrations with concomitant decreases in plasma potassium concentration. Reciprocal changes in plasma NaCl and potassium levels are typically seen as a consequence of renal mineralocorticoid activation promoting NaCl reabsorption and potassium secretion in the distal nephron [26]. Additionally, mRNA levels for a group of genes involved in distal nephron sodium and water absorption in the kidney medulla are changed with GI262570 treatment [26].

The involvement of the distal nephron in TZD-induced fluid retention has been assessed in two independent studies using mice with a collecting duct-specific deletion of PPARγ (CD PPARγ KO) [27, 28]. In both studies, the expression of Cre recombinase was driven by an AQP2 promoter highly specific to the collecting duct. In these two studies, the experimental approaches for assessment of fluid retention were quite different: a combination of hematocrit, plasma aldosterone levels, and Evans blue (EB) dye-based measurement of plasma volume in one study (see Figure 1) [28] and determination of total water content in the other [27]. Remarkably, both studies reported a similar phenotype in that the conditional PPARγ knockout mice proved to be resistant to the rosiglitazone- or pioglitazone-induced body weight gain and plasma volume expansion found in mice expressing PPARγ in the collecting duct. As shown in Figure 1, a nine-day rosiglitazone treatment induced a gradual and significant increase in body weight in floxed mice when compared to untreated floxed controls (2.74 ± 0.25 versus 1.05 ± 0.16 gram, on day 9, $P < .05$). In contrast, body weight gains between rosiglitazone-treated and untreated CD PPARγ KO mice were not significantly different (0.90 ± 0.25 versus 0.81 ± 0.19 gram, on day 9, $P > .05$). Rosiglitazone treatment in the control mice induced plasma volume expansion, which was reflected by a significantly decreased hematocrit and plasma aldosterone levels as well as by a 32.2% increase in plasma volume as assessed by the EB dye technique. In contrast, rosiglitazone-treated CD PPARγ KO mice exhibited nonsignificant trends toward change in these parameters (see Figure 2). These two studies also provided evidence that exposure of primary collecting duct cells to PPARγ ligands leads to increased

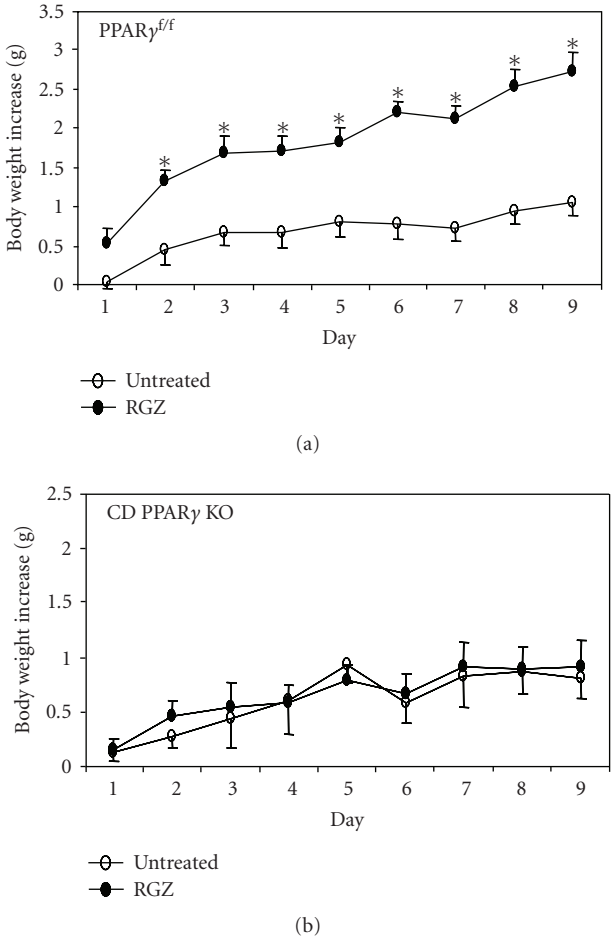

Figure 1: Body weight gains in untreated and rosiglitazone (RGZ)-treated PPAR$\gamma^{f/f}$ mice (a) and CD PPARγ knockout mice (b) (adapted from [28]). *, $P < .05$ versus vehicle at the corresponding time point.

sodium transport as assessed by measurements of $^{22}Na^{+}$ flux and transepithelial resistance.

Guan et al. examined the effects of pioglitazone on the expression of α-, ß-, and γ-ENaC subunits in cultured inner medullary collecting duct (IMCD) cells [27]. Notably, within one hour following treatment of IMCDs with pioglitazone (1 μM), γ-ENaC mRNA expression increased roughly 10 folds before gradually diminishing. This stimulatory effect appeared to be specific for γ-ENaC mRNA, because α-ENaC and ß-ENaC mRNA levels did not show any change in response to treatment with pioglitazone. Interestingly, PPAR response elements (PPREs) are identified in intron 1 but not in the 5′ flanking region of the γ-ENaC gene. Chromatin immunoprecipitation (ChIP) of genomic DNAisolated from cultured mouse IMCDs revealed a physical interaction between PPARγ and γ-ENaC genomic DNA. Somewhat unexpectedly, the PPARγ binding site was shown to be located outside intron 1 of the γ-ENaC gene. Overall, these data support γ-ENaC as a direct target gene of PPARγ in the collecting duct cells, although the exact mechanism remains to be elucidated.

However, the role of ENaC as a direct target of PPARγ has not always been demonstrable. Nofziger et al. reported that, in collecting duct cell lines, PPARγ agonists failed to enhance basal or insulin-stimulated sodium transport as assessed by measurement of short-circuit current (Isc) [29]. This study also did not find that PPARγ-induced changes in the amount of SGK1 transcript or protein expression. Additionally, there is no solid evidence for major changes in renal expression of any of the ENaC subunits in response to PPARγ ligands in vivo [13, 26, 30]. More recently, Vallon et al. reported that collecting duct-specific gene inactivation of α-ENaC in the mouse does not attenuate the rosiglitazone-induced body weight gain [31]. In this study, the Hoxb-7 promoter was used to inactivate α-ENaC in the collecting duct, while leaving ENaC expression in the cortical connecting tubule (CNT) intact [32]. As expected, in the floxed control mice, rosiglitazone treatment (320 mg/kg diet) rapidly increased body weight (ΔBW day 11: 4.5 ± 0.8% versus 1.1 ± 0.6%, $P < .05$) and lowered hematocrit (44 ± 1.0% versus 47 ± 1%, $P < .0005$), while rosiglitazone treatment increased body weight (ΔBW: 7.3 ± 0.9% versus 0.9 ± 0.7%, $P < .0005$) and lowered hematocrit (42 ± 2% versus 47 ± 1%, $P < .05$) in α-ENaC collecting duct knockout mice. These data may argue against collecting duct ENaC playing a significant role in mediating the adverse effect of rosiglitazone. However, involvement of ENaC activity in the CNT cannot be ruled out. To resolve this issue, AQP2-Cre mice could be used to inactivate ENaC in the entire collecting duct system.

The negative results discussed above prompt consideration of alternative mechanisms for explaining PPARγ-mediated increases in distal tubular fluid reabsorption. There is a significant amiloride-insensitive component in the rosiglitazone-induced increases in sodium transport [28]. The possibility exists that increased reabsorption may occur by way of a paracellular route. For example, PPARγ may regulate the tight junction leading to altered permeability to sodium or other electrolytes. In an in vitromodel of differentiating normal human urothelial (NHU) cells, PPARγ activation in conjunction with epidermal growth factor receptor (EGFR) blockade led to the de novo expression of claudin 3 mRNA and protein and downregulation of claudin 2 transcription [33]. These results suggest a role for PPARγ and EGFR signaling pathways in regulating the tight junction formation in NHU cells. There is an intriguing possibility that a similar mechanism may operate in renal epithelial cells. Another possible mechanism is that PPARγ may regulate transport of ions other than sodium. Further studies are clearly needed to explore not only ENaC-dependent, but also ENaC-independent mechanisms, for TZD-activated fluid reabsorption in the distal nephron.

3. VASCULAR MECHANISM

PPARγ is expressed in the vascular system [34], including endothelial cells [35, 36], vascular smooth muscle cells (VSMC) [37] as well as monocyte/macrophages [38, 39]. Several lines of evidence suggest that PPARγ regulates various aspects of vascular function, including capillary permeability. Increased capillary permeability leads to

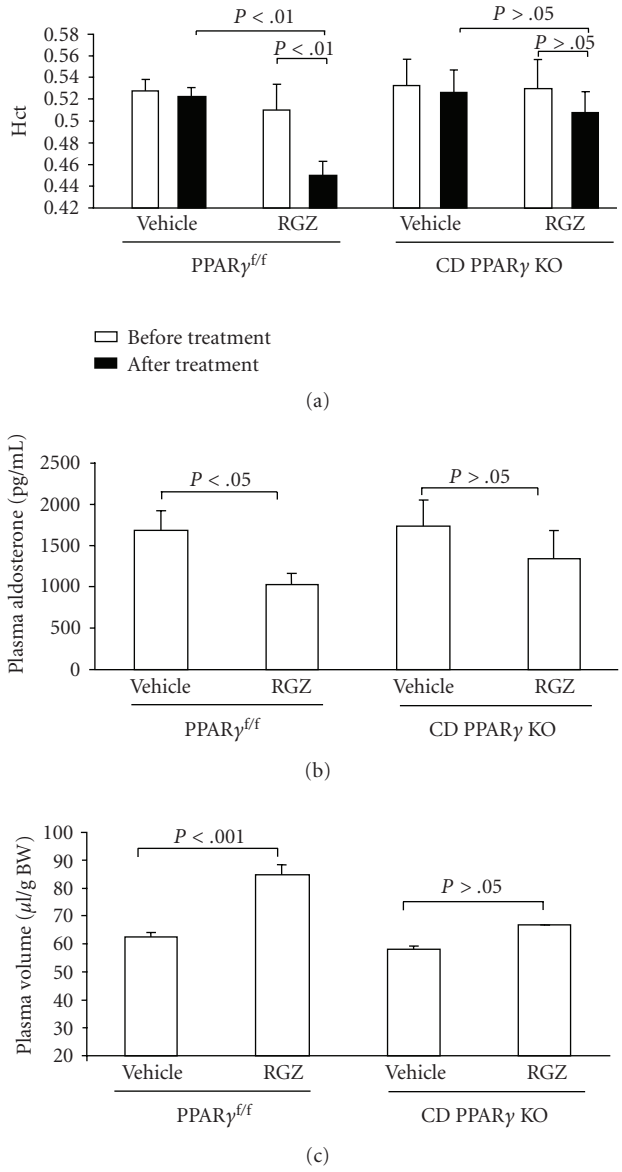

Figure 2: Changes in plasma volume in PPAR$\gamma^{f/f}$ and CD PPARγ knockout mice following rosiglitazone (RGZ) treatment (adapted from [28]). (a) Hematocrit (Hct) in PPAR$\gamma^{f/f}$ and CD PPARγ knockout mice before and after RGZ treatment. (b) Plasma aldosterone levels in PPAR$\gamma^{f/f}$ and CD PPARγ knockout mice following RGZ treatment. (c) Determination of plasma volume in PPAR$\gamma^{f/f}$ and CD PPARγ KO mice by the Evans blue (EB) dye technique.

extravasation of fluid and is thought to contribute to edema in patients treated with TZDs. Donnelly et al. were the first to examine the direct effect of rosiglitazone on endothelial barrier function using an in vitro system of pulmonary artery endothelial cell monolayers. Transendothelial albumin flux was measured using EB dye-labeled albumin. They found that exposure to high concentrations of rosiglitazone for four hours increased transendothelial albumin flux dose-dependently, with a noticeable effect at 10 μM and a maximal effect at 100 μM. This hyperpermeability response to high concentrations of rosiglitazone was fully reversible by washing rosiglitazone off the monolayer. After incubation for 24 to 48 hours, the effect of rosiglitazone began to subside. High concentrations of rosiglitazone (0.1–1 mM) are also needed to induce a vasodilator effect in isolated arteries [40]. Future studies, ideally employing gene knockout mice, may determine the extent of PPARγ mediation of the vascular response to high concentrations of TZDs. The mechanism of TZD-induced capillary permeability is not well characterized but may involve a number of factors, notably vascular endothelial growth factor (VEGF), nitric oxide, and protein kinase C, each of which is discussed below.

VEGF is a potent cytokine that augments vascular permeability in tumors, healing wounds, retinopathies, many important inflammatory conditions, and certain physiological processes, such as ovulation and corpus luteum formation [41]. VEGF is estimated to be 50 times more potent than histamine in enhancing vascular permeability [41]. The gene transfer of naked plasmid DNA encoding the 165-amino acid isoform of VEGF in patents with peripheral artery disease causes peripheral edema [42]. Evidence suggests an involvement of VEGF in TZD-induced edema. The study of Emorto et al. was the first to report that plasma levels of VEGF are significantly increased in troglitazone-treated subjects (120.1 ± 135.0 pg/mL) compared with those treated with diet alone (29.2 ± 36.1 pg/mL), sulfonylurea (25.8 ± 22.2 pg/mL), or insulin (24.6 ± 19.0 pg/mL). The effect of troglitazone on increased VEGF levels was further supported by plasma VEGF levels in five patients before treatment (20.2 ± 7.0 pg/mL), after three months of troglitazone treatment (83.6 ± 65.9 pg/mL), and three months after discontinuation (28.0 ± 11.6 pg/mL). These authors further demonstrated that troglitazone, as well as rosiglitazone, at the plasma concentrations observed in patients, increased VEGF mRNA levels in 3T3-L1 adipocytes. The finding suggests that PPARγ activation may directly stimulate expression of VEGF that leads to tissue edema. However, it is puzzling that several other studies show that PPARγ negatively regulates VEGF signaling. In transformed and primary endometrial cells rosiglitazone or 15-deoxy-delta 12,14-prostaglandin J_2 (15d-PGJ_2) decreased VEGF protein secretion [43]. In transiently transfected Ishikawa cells, rosiglitazone repressed VEGF gene promoter-luciferase activation with an IC [37] approximately 50 nM. By using truncated and mutated VEGF promoter constructs, this study further revealed that the PPARγ-regulated domain is a direct repeat (DR)-1 motif −443 bp upstream of the transcriptional start site [43]. Similarly, rosiglitazone attenuated VEGF-induced proliferation and migration of human pulmonary valve endothelial cells (HPVECs) [44]. Rosiglitazone also antagonized VEGF-induced nuclear factor translocation in activated T cells subtype c1 (NFATc1) [44]. Furthermore, rosiglitazone markedly decreased VEGF-induced tube formation and cell migration in human umbilical vein endothelial cells [45]. Taking these studies together, it seems likely that PPARγ exerts a dual effect on VEGF signaling, possibly depending on cell type.

Nitric oxide (NO) is a ubiquitous, naturally occurring molecule found in a variety of cell types and organ systems. Endothelial cells are rich in NO, which has been shown

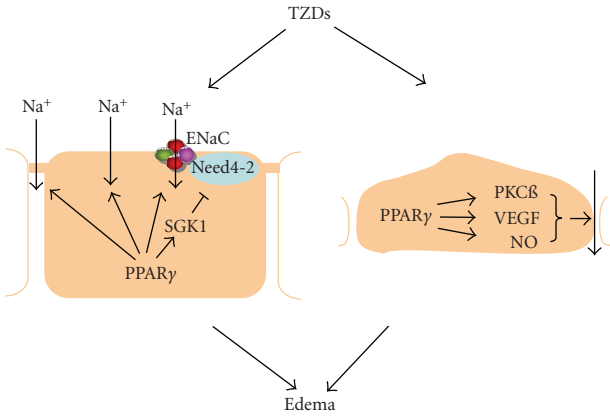

FIGURE 3: The mechanism for thiazolidinedione- (TZD-) induced edema. In the renal collecting duct, PPARγ activation increases sodium reabsorption through ENaC-dependent and independent mechanisms. In the blood vessels of adipose tissues, PPARγ ligands activate PKCß, VEGF, and NO, which together lead to increased endothelial permeability. The increased renal sodium retention at the level of the collecting duct in conjunction with increased vascular permeability may determine edema development.

to regulate many aspects of vascular function, including vascular permeability. Polikandriotis et al. report that 15d-PGJ_2 and ciglitazone increase cultured endothelial cell NO release without increasing the expression of endothelial nitric oxide synthase (eNOS) [46]. This study provided further evidence that PPARγ activation leads to eNOS ser1177 phosphorylation [46]. It seems plausible that the stimulation of eNOS-derived NO may contribute to TZD-induced edema. St-Pierre et al. examined the effect of rosiglitazone on muscle vasopermeability and NO system in the fructose-fed rat model [47]. In this study, extravasation of EB dye in vivo in specific muscle groups was used to assess vascular permeability. Fructose-fed rats treated with rosiglitazone had a 30–50% increase in extravasation of EB in the the Rectus femoris, soleus, gastrocnemius lateralis, vastus lateralis, and tibialis cranialis skeletal muscles [47]. In homogenates of skeletal muscles (vastus lateralis) from fructose-fed rats, rosiglitazone resulted in a significant increase in nitric oxide synthase (NOS) activity and eNOS immunoreactive content compared to the control animals [47]. Unexpectedly, the immunoreactive level of the most abundant muscle NOS isoforms, neuronal NOS (nNOS), remained unchanged.

Protein kinase C (PKC) plays a major role in determining vascular permeability through phosphorylation of the cytoskeleton proteins that form the tight intercellular junction [48–51]. In the study of Sotiropoulos et al., rosiglitazone treatment selectively activated PKC in fat and retinal tissues in parallel with the increased vascular permeability in these tissues [52]. The activation of PKC is evaluated by determining the enzyme activity together with tissue levels of diacylglycerol (DAG), a strong PKC activator [52]. These investigators tested the effect of PKCβ inhibition and gene knockout but did not determine specific PKC isoforms. They found that posttreatment with ruboxistaurin (RBX), a PKCβ inhibitor, effectively attenuated the increases in capillary permeability, water content, and weight of epididymal fat, as well as the increase in body weight associated with rosiglitazone treatment; this finding was also confirmed by using PKCβ KO mice [52].

4. POTENTIAL THERAPIES

4.1. *Inhibition of sodium transport in the collecting duct*

The use of diuretics for management of TZD-induced fluid retention has been evaluated by several case reports [2, 53] and, recently, by a controlled trial [54]. Most case reports show that the edema is refractory to a loop diuretic (furosemide) and that the symptoms resolve only after discontinuation of TZD. The recent controlled trial involved 381 patients with type 2 diabetes. It examined the effect of three diuretics that act with different mechanisms on rosiglitazone-induced body weight gain and plasma volume [54]. The diuretics included furosemide, which inhibits the Na-K-Cl cotransporter in the thick ascending limb of the loop of Henle, hydrochlorothiazide (HCTZ), which acts to inhibit the Na-Cl cotransporter in the distal convoluted tubule, and spironolactone (SPIRO), which is an ENaC inhibitor in the collecting duct. The degree of fluid retention in this study was evaluated by measuring changes in the hematocrit as an index of changes in plasma volume, body weight, total body water, and extracellular fluid changes determined by noninvasive bioelectrical impedance with an Akern soft tissue analyzer. SPIRO and HCTZ both effectively reduced fluid retention and body weight while furosemide had only a limited effect. The effectiveness of SPIRO may be attributable to the ability of this diuretic to interfere with the sodium retaining action of PPARγ in the collecting duct. It is unclear whether the same mechanism can explain the action of HCTZ. Thiazide diuretics act primarily in the proximal part of the distal convoluted tubules where they inhibit Na^+/Cl^- cotransport [55, 56], but they are also reported to inhibit salt and water reabsorption in the medullary collecting duct [57]. The reason for the lack of diuretic response of TZD-treated diabetics to furosemide is not entirely clear, but one possible explanation might be the lack of distal effect of this loop diuretic. Another possibility is that TZD-induced fluid retention may be associated with impaired transport machinery in the thick ascending limb. Possibly secondary to the volume expansion, the plasma level of atrial natriuretic factor (ANF) is elevated in TZD-treated diabetics [54]. ANF inhibits NaCl reabsorption in the loop of Henle as well as in other sites of nephron through the activation of guanylyl cyclase receptors that release cyclic GMP [58]. It also remains possible that PPARγ may negatively affect NaCl transport in the loop of Henle.

The experimental evidence favoring ENaC as a potential target of PPARγ in the distal nephron seems to provide a rationale for the use of amiloride as a specific ENaC inhibitor for treatment of TZD-induced fluid retention. Unfortunately, amiloride was not included in this clinical trial [54]. In the mouse, pretreatment with amiloride effectively prevents body weight gain and fluid retention produced

by pioglitazone. However, in the rat model, posttreatment with amiloride unexpectedly exacerbates the fluid retention induced by farglitazar. It is unclear whether this discrepancy between the studies is due to species differences, PPARγ ligand activity, or the different timing of amiloride treatment.

4.2. Combination of a PPARγ and a PPARα agonist

Boden et al. examined the effect of the combined use of rosiglitazone and fenofibrate in patients with type 2 diabetes [59]. Compared with rosiglitazone alone, rosiglitazone/fenofibrate proved significantly more effective in lowering fasting free fatty acid levels and tended to be more effective in achieving plasma glucose control. Interestingly, rosiglitazone/fenofibrate completely prevented the increase in body weight and body water content associated with rosiglitazone. This study is the first to show that the combined use of a PPARγ and a PPARα agonist can prevent rosiglitazone-induced fluid retention. The investigators did not propose a mechanism to explain this phenomenon. The two PPAR isoforms occur in different locations along the nephron. PPARα mRNA is found predominately in the cortex and is specifically localized in the proximal convoluted tubule (PCT). PPARγ is abundant in the renal inner medulla, specifically localized to the inner medullary collecting duct [23, 25]. The difference in nephron localization does not seem to favor the direct interaction between the two PPAR isoforms. However, it remains possible that low PPARα activity in the collecting duct may antagonize the sodium-retaining action of PPARγ. Future studies are needed to investigate whether an interaction occurs in the collecting duct or another location.

Dual PPARα/γ agonists have been developed by several pharmaceutical companies, and some have undergone or are currently undergoing clinical trials [60–62]. Unfortunately, muraglitazar, the first dual PPARα/γ agonist, has been associated with an excessive incidence of major adverse cardiovascular events, including myocardial infarction, stroke and transient ischemic attack, chronic heart failure and death [62]. This finding raises significant safety concerns about the dual agonists as well as the combination of a PPARγ and a PPARα agonist. In the study of Boden et al., rosiglitazone/fenofibrate appeared to be well tolerated [59]. The safety issues may be related to the ratio of PPARγ to PPARα. The ratios are fixed for the dual agonists, but can be varied by changing the proportion of PPARγ and PPARα agonists. It should be pointed out that Boden's study was limited to a small number of patients and a short period of treatment [59]. The safety issue regarding the combined use of a PPARγ and PPARα agonist needs to be carefully evaluated in larger-scale and longer-term clinical trials as well as animal studies.

4.3. Inhibition of protein kinase C

There is functional evidence suggesting the involvement of vascular permeability in TZD-induced body weight gain and fluid retention [52]. Therefore, targeting vascular permeability may provide a potential therapeutic strategy for this side effect of the TZDs. In an animal study, the use of a PKCβ inhibitor, RBX, to target vascular permeability effectively attenuated the increases in TZD-induced body weight gain [52]. Is there any safety issue related to RBX? In the animal models tested, including Zucker and lean fatty rats, and mice, RBX reduced rosiglitazone-induced capillary permeability, but had no significant effect on the baseline capillary permeability without rosiglitazone treatment. In this short-term animal study, the compound appears to be well tolerated. Another positive note is that RBX is being used in clinical trials for diabetic microvascular complications. In these trials, as well as in animal studies, RBX shows promise for treatment of diabetic retinopathy and nephropathy without noticeable side effects [63, 64].

5. CONCLUSIONS

The fluid retention and rapid body weight gain induced by TZD treatment are caused by increased fluid reabsorption in the distal nephron as well as increased vascular permeability in adipose tissues (see Figure 3). The molecular mechanisms of the effects of TZDs in renal collecting duct and in blood vessels remain unknown. Despite documentation of ENaC as a molecular target of TZDs in the collecting duct, increasing evidence indicates ENaC-independent mechanisms that may involve changes in paracellular transport. PKCß is shown to mediate TZD-induced vascular permeability in adipose tissues. More studies are required for determination of the signaling pathway responsible for PPARγ-dependent tissue-specific activation of PKCß. Currently, there are no effective therapies for the side effects of TZDs except drug withdrawal. A number of potential treatment strategies that target collecting duct sodium transport (amiloride) and vascular permeability (PKC inhibitors) have been developed from animal studies and should be evaluated by future clinical trials.

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REFERENCES

[1] M. Füchtenbusch, E. Standl, and H. Schatz, "Clinical efficacy of new thiazolidinediones and glinides in the treatment of type 2 diabetes mellitus," *Experimental and Clinical Endocrinology & Diabetes*, vol. 108, no. 3, pp. 151–163, 2000.

[2] I. B. Hirsch, J. Kelly, and S. Cooper, "Pulmonary edema associated with troglitazone therapy," *Archives of Internal Medicine*, vol. 159, no. 15, p. 1811, 1999.

[3] M. L. Thomas and S. J. Lloyd, "Pulmonary edema associated with rosiglitazone and troglitazone," *The Annals of Pharmacotherapy*, vol. 35, no. 1, pp. 123–124, 2001.

[4] (Pioglitazone) PIA. Takeda Pharmaceuticals, Inc.

[5] (Rosiglitazone) PIA. GlaxoSmithKline Pharmaceuticals.

[6] S. Z. Duan, M. G. Usher, and R. M. Mortensen, "Peroxisome proliferator-activated receptor-γ-mediated effects in the

vasculature," *Circulation Research*, vol. 102, no. 3, pp. 283–294, 2008.

[7] N. K. Hollenberg, "Considerations for management of fluid dynamic issues associated with thiazolidinediones," *The American Journal of Medicine*, vol. 115, no. 8, supplement 1, pp. 111–115, 2003.

[8] G. N. Kalambokis, A. A. Tsatsoulis, and E. V. Tsianos, "The edematogenic properties of insulin," *American Journal of Kidney Diseases*, vol. 44, no. 4, pp. 575–590, 2004.

[9] R. W. Nesto, D. Bell, R. O. Bonow, et al., "Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association," *Circulation*, vol. 108, no. 23, pp. 2941–2948, 2003.

[10] N. V. Niemeyer and L. M. Janney, "Thiazolidinedione-induced edema," *Pharmacotherapy*, vol. 22, no. 7, pp. 924–929, 2002.

[11] A. Rubenstrunk, R. Hanf, D. W. Hum, J.-C. Fruchart, and B. Staels, "Safety issues and prospects for future generations of PPAR modulators," *Biochimica et Biophysica Acta*, vol. 1771, no. 8, pp. 1065–1081, 2007.

[12] W. H. W. Tang and A. Maroo, "PPARγ agonists: safety issues in heart failure," *Diabetes, Obesity and Metabolism*, vol. 9, no. 4, pp. 447–454, 2007.

[13] J. Song, M. A. Knepper, X. Hu, J. G. Verbalis, and C. A. Ecelbarger, "Rosiglitazone activates renal sodium- and water-reabsorptive pathways and lowers blood pressure in normal rats," *Journal of Pharmacology and Experimental Therapeutics*, vol. 308, no. 2, pp. 426–433, 2004.

[14] B. Yang, L. G. Clifton, J. A. McNulty, L. Chen, K. K. Brown, and P. G. Baer, "Effects of a PPARγ agonist, GI262570, on renal filtration fraction and nitric oxide level in conscious rats," *Journal of Cardiovascular Pharmacology*, vol. 42, no. 3, pp. 436–441, 2003.

[15] A. Zanchi, A. Chiolero, M. Maillard, J. Nussberger, H.-R. Brunner, and M. Burnier, "Effects of the peroxisomal proliferator-activated receptor-γ agonist pioglitazone on renal and hormonal responses to salt in healthy men," *The Journal of Clinical Endocrinology & Metabolism*, vol. 89, no. 3, pp. 1140–1145, 2004.

[16] M. A. Knepper, J. B. Wade, J. Terris, et al., "Renal aquaporins," *Kidney International*, vol. 49, no. 6, pp. 1712–1717, 1996.

[17] J. Loffing, D. Loffing-Cueni, A. Macher, et al., "Localization of epithelial sodium channel and aquaporin-2 in rabbit kidney cortex," *American Journal of Physiology*, vol. 278, no. 4, pp. F530–F539, 2000.

[18] J. Loffing, L. Pietri, F. Aregger, et al., "Differential subcellular localization of ENaC subunits in mouse kidney in response to high- and low-Na diets," *American Journal of Physiology*, vol. 279, no. 2, pp. F252–F258, 2000.

[19] S. Masilamani, G.-H. Kim, C. Mitchell, J. B. Wade, and M. A. Knepper, "Aldosterone-mediated regulation of ENaC α, β, and γ subunit proteins in rat kidney," *The Journal of Clinical Investigation*, vol. 104, no. 7, pp. R19–R23, 1999.

[20] G. Hong, A. Lockhart, B. Davis, et al., "PPARγ activation enhances cell surface ENaCα via up-regulation of SGK1 in human collecting duct cells," *The FASEB Journal*, vol. 17, no. 3, pp. 1966–1968, 2003.

[21] J. A. Schafer, "Abnormal regulation of ENaC: syndromes of salt retention and salt wasting by the collecting duct," *American Journal of Physiology*, vol. 283, no. 2, pp. F221–F235, 2002.

[22] O. Staub and F. Verrey, "Impact of Nedd4 proteins and serum and glucocorticoid-induced kinases on epithelial Na^+ transport in the distal nephron," *Journal of the American Society of Nephrology*, vol. 16, no. 11, pp. 3167–3174, 2005.

[23] Y. Guan, Y. Zhang, L. Davis, and M. D. Breyer, "Expression of peroxisome proliferator-activated receptors in urinary tract of rabbits and humans," *American Journal of Physiology*, vol. 273, no. 6, pp. F1013–F1022, 1997.

[24] Y. Guan, Y. Zhang, A. Schneider, L. Davis, R. M. Breyer, and M. D. Breyer, "Peroxisome proliferator-activated receptor-γ activity is associated with renal microvasculature," *American Journal of Physiology*, vol. 281, no. 6, pp. F1036–F1046, 2001.

[25] T. Yang, D. E. Michele, J. Park, et al., "Expression of peroxisomal proliferator-activated receptors and retinoid X receptors in the kidney," *American Journal of Physiology*, vol. 277, no. 6, pp. F966–F973, 1999.

[26] L. Chen, B. Yang, J. A. McNulty, et al., "GI262570, a peroxisome proliferator-activated receptor γ agonist, changes electrolytes and water reabsorption from the distal nephron in rats," *Journal of Pharmacology and Experimental Therapeutics*, vol. 312, no. 2, pp. 718–725, 2005.

[27] Y. Guan, C. Hao, D. R. Cha, et al., "Thiazolidinediones expand body fluid volume through PPARγ stimulation of ENaC-mediated renal salt absorption," *Nature Medicine*, vol. 11, no. 8, pp. 861–866, 2005.

[28] H. Zhang, A. Zhang, D. E. Kohan, R. D. Nelson, F. J. Gonzalez, and T. Yang, "Collecting duct-specific deletion of peroxisome proliferator-activated receptor γ blocks thiazolidinedione-induced fluid retention," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 26, pp. 9406–9411, 2005.

[29] C. Nofziger, L. Chen, M. A. Shane, C. D. Smith, K. K. Brown, and B. L. Blazer-Yost, "PPARγ agonists do not directly enhance basal or insulin-stimulated Na^+ transport via the epithelial Na^+ channel," *Pflügers Archiv: European Journal of Physiology*, vol. 451, no. 3, pp. 445–453, 2005.

[30] S. Mittra, G. Sangle, R. Tandon, et al., "Increase in weight induced by muraglitazar, a dual PPARα/γ agonist, in *db/db* mice: adipogenesis/or oedema?" *British Journal of Pharmacology*, vol. 150, no. 4, pp. 480–487, 2007.

[31] V. Vallon, E. Hummler, T. Rieg, et al., "Collecting duct-specific inactivation of αENaC in the mouse kidney does not attenuate rosiglitazone-induced weight gain," *The FASEB Journal, Meeting Abstract*, vol. 22, 947.14, 2008.

[32] I. Rubera, J. Loffing, L. G. Palmer, et al., "Collecting duct-specific gene inactivation of αENaC in the mouse kidney does not impair sodium and potassium balance," *The Journal of Clinical Investigation*, vol. 112, no. 4, pp. 554–565, 2003.

[33] C. L. Varley, M. A. E. Garthwaite, W. Cross, J. Hinley, L. K. Trejdosiewicz, and J. Southgate, "PPARγ-regulated tight junction development during human urothelial cytodifferentiation," *Journal of Cellular Physiology*, vol. 208, no. 2, pp. 407–417, 2006.

[34] D. Bishop-Bailey, "Peroxisome proliferator-activated receptors in the cardiovascular system," *British Journal of Pharmacology*, vol. 129, no. 5, pp. 823–834, 2000.

[35] I. Inoue, K. Shino, S. Noji, T. Awata, and S. Katayama, "Expression of peroxisome proliferator-activated receptor α (PPARα) in primary cultures of human vascular endothelial cells," *Biochemical and Biophysical Research Communications*, vol. 246, no. 2, pp. 370–374, 1998.

[36] H. Satoh, K. Tsukamoto, Y. Hashimoto, et al., "Thiazolidinediones suppress endothelin-1 secretion from bovine vascular endothelial cells: a new possible role of PPARγ on vascular endothelial function," *Biochemical and Biophysical Research Communications*, vol. 254, no. 3, pp. 757–763, 1999.

[37] B. Staels, W. Koenig, A. Habib, et al., "Activation of human aortic smooth-muscle cells is inhibited by PPARα but not by

PPARγ activators," *Nature*, vol. 393, no. 6687, pp. 790–793, 1998.

[38] G. Chinetti, S. Griglio, M. Antonucci, et al., "Activation of proliferator-activated receptors α and γ induces apoptosis of human monocyte-derived macrophages," *Journal of Biological Chemistry*, vol. 273, no. 40, pp. 25573–25580, 1998.

[39] M. Ricote, A. C. Li, T. M. Willson, C. J. Kelly, and C. K. Glass, "The peroxisome proliferator-activated receptor-γ is a negative regulator of macrophage activation," *Nature*, vol. 391, no. 6662, pp. 79–82, 1998.

[40] M. J. Ryan, S. P. Didion, S. Mathur, F. M. Faraci, and C. D. Sigmund, "PPARγ agonist rosiglitazone improves vascular function and lowers blood pressure in hypertensive transgenic mice," *Hypertension*, vol. 43, no. 3, pp. 661–666, 2004.

[41] H. F. Dvorak, J. A. Nagy, D. Feng, L. F. Brown, and A. M. Dvorak, "Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis," *Current Topics in Microbiology and Immunology*, vol. 237, pp. 97–132, 1999.

[42] I. Baumgartner, G. Rauh, A. Pieczek, et al., "Lower-extremity edema associated with gene transfer of naked DNA encoding vascular endothelial growth factor," *Annals of Internal Medicine*, vol. 132, no. 11, pp. 880–884, 2000.

[43] L. L. H. Peeters, J.-L. Vigne, M. K. Tee, D. Zhao, L. L. Waite, and R. N. Taylor, "PPARγ represses VEGF expression in human endometrial cells: implications for uterine angiogenesis," *Angiogenesis*, vol. 8, no. 4, pp. 373–379, 2006.

[44] T. L. Sander, L. A. Noll, D. B. Klinkner, et al., "Rosiglitazone antagonizes vascular endothelial growth factor signaling and nuclear factor of activated T cells activation in cardiac valve endothelium," *Endothelium*, vol. 13, no. 3, pp. 181–190, 2006.

[45] W. H.-H. Sheu, H.-C. Ou, F.-P. Chou, T.-M. Lin, and C.-H. Yang, "Rosiglitazone inhibits endothelial proliferation and angiogenesis," *Life Sciences*, vol. 78, no. 13, pp. 1520–1528, 2006.

[46] J. A. Polikandriotis, L. J. Mazzella, H. L. Rupnow, and C. M. Hart, "Peroxisome proliferator-activated receptor γ ligands stimulate endothelial nitric oxide production through distinct peroxisome proliferator-activated receptor γ-dependent mechanisms," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 9, pp. 1810–1816, 2005.

[47] P. St-Pierre, L. Bouffard, and P. Maheux, "Rosiglitazone increases extravasation of macromolecules and endothelial nitric oxide synthase in skeletal muscles of the fructose-fed rat model," *Biochemical Pharmacology*, vol. 67, no. 10, pp. 1997–2004, 2004.

[48] H. Clarke, C. W. Marano, A. P. Soler, and J. M. Mullin, "Modification of tight junction function by protein kinase C isoforms," *Advanced Drug Delivery Reviews*, vol. 41, no. 3, pp. 283–301, 2000.

[49] H. Clarke, A. P. Soler, and J. M. Mullin, "Protein kinase C activation leads to dephosphorylation of occludin and tight junction permeability increase in LLC-PK1 epithelial cell sheets," *Journal of Cell Science*, vol. 113, no. 18, pp. 3187–3196, 2000.

[50] J. E. Stasek Jr. and J. G. N. Garcia, "The role of protein kinase C in α-thrombin-mediated endothelial cell activation," *Seminars in Thrombosis and Hemostasis*, vol. 18, no. 1, pp. 117–125, 1992.

[51] J. E. Stasek Jr., C. E. Patterson, and J. G. N. Garcia, "Protein kinase C phosphorylates caldesmon$_{77}$ and vimentin and enhances albumin permeability across cultured bovine pulmonary artery endothelial cell monolayers," *Journal of Cellular Physiology*, vol. 153, no. 1, pp. 62–75, 1992.

[52] K. B. Sotiropoulos, A. Clermont, Y. Yasuda, et al., "Adipose-specific effect of rosiglitazone on vascular permeability and protein kinase C activation: novel mechanism for PPARγ agonist's effects on edema and weight gain," *The FASEB Journal*, vol. 20, no. 8, pp. 1203–1205, 2006.

[53] F. Wang, L. M. Aleksunes, L. A. Reagan, and C. M. Vergara, "Management of rosiglitazone-induced edema: two case reports and a review of the literature," *Diabetes Technology & Therapeutics*, vol. 4, no. 4, pp. 505–514, 2002.

[54] J. Karalliedde, R. Buckingham, M. Starkie, D. Lorand, M. Stewart, and G. Viberti, "Effect of various diuretic treatments on rosiglitazone-induced fluid retention," *Journal of the American Society of Nephrology*, vol. 17, no. 12, pp. 3482–3490, 2006.

[55] D. H. Ellison, H. Velazquez, and F. S. Wright, "Thiazide-sensitive sodium chloride cotransport in early distal tubule," *American Journal of Physiology*, vol. 253, no. 3, pp. F546–F554, 1987.

[56] F. A. Gesek and P. A. Friedman, "Sodium entry mechanisms in distal convoluted tubule cells," *American Journal of Physiology*, vol. 268, no. 1, pp. F89–F98, 1995.

[57] D. R. Wilson, U. Honrath, and H. Sonnenberg, "Thiazide diuretic effect on medullary collecting duct function in the rat," *Kidney International*, vol. 23, no. 5, pp. 711–716, 1983.

[58] C. Bailly, "Transducing pathways involved in the control of NaCl reabsorption in the thick ascending limb of Henle's loop," *Kidney International. Supplement*, vol. 65, pp. S29–S35, 1998.

[59] G. Boden, C. Homko, M. Mozzoli, M. Zhang, K. Kresge, and P. Cheung, "Combined use of rosiglitazone and fenofibrate in patients with type 2 diabetes: prevention of fluid retention," *Diabetes*, vol. 56, no. 1, pp. 248–255, 2007.

[60] G. Boden and M. Zhang, "Recent findings concerning thiazolidinediones in the treatment of diabetes," *Expert Opinion on Investigational Drugs*, vol. 15, no. 3, pp. 243–250, 2006.

[61] G. J. Etgen, B. A. Oldham, W. T. Johnson, et al., "A tailored therapy for the metabolic syndrome: the dual peroxisome proliferator-activated receptor-α/γ agonist LY465608 ameliorates insulin resistance and diabetic hyperglycemia while improving cardiovascular risk factors in preclinical models," *Diabetes*, vol. 51, no. 4, pp. 1083–1087, 2002.

[62] S. E. Nissen, K. Wolski, and E. J. Topol, "Effect of muraglitazar on death and major adverse cardiovascular events in patients with type 2 diabetes mellitus," *Journal of the American Medical Association*, vol. 294, no. 20, pp. 2581–2586, 2005.

[63] P. W. Anderson, J. B. McGill, and K. R. Tuttle, "Protein kinase C β inhibition: the promise for treatment of diabetic nephropathy," *Current Opinion in Nephrology and Hypertension*, vol. 16, no. 5, pp. 397–402, 2007.

[64] M. Clarke and P. M. Dodson, "PKC inhibition and diabetic microvascular complications," *Best Practice & Research in Clinical Endocrinology & Metabolism*, vol. 21, no. 4, pp. 573–586, 2007.

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Review Article

TZDs and Bone: A Review of the Recent Clinical Evidence

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Over the past two years, evidence has emerged that the currently available thiazolidinediones (TZDs), rosiglitazone, and pioglitazone have negative skeletal consequences, at least in women, which are clinically important. Increased fracture risk in women, but not men, was reported for both TZDs, based on analyses of adverse event reports from clinical trials. In short-term clinical trials in women, both TZDs caused more rapid bone loss. In these trials, changes in bone turnover markers suggest a pattern of reduced bone formation without a change in resorption. Although limited, these results support the hypothesis based on rodent and in vitro models that reduced bone formation resulting from activation of peroxisome proliferator-activated receptor-γ (PPARγ) is a central mechanism for TZDs' effect on bone. Research is needed to better understand the mechanisms of bone loss with TZDs, to identify factors that influence susceptibility to TZD-induced osteoporosis, and to test treatments for its prevention.

1. INTRODUCTION

Recent reports have substantially advanced our knowledge of the clinical effects of TZDs on skeletal health. In early 2006, research into the skeletal effects in humans of rosiglitazone and pioglitazone, the currently prescribed TZDs, was limited to observational studies [1]. Although a body of evidence had developed from rodent and in vitro studies that these two TZDs cause bone loss, it was not known if these compounds had a similar effect in humans. Since then, rosiglitazone and piogltiazone were each linked to increased fracture risk among diabetic women, based on adverse event reports in clinical trials. And, in women, short-term clinical trials demonstrated substantial bone loss with both TZDs. Pioglitazone and rosiglitazone are widely used to treat diabetes, and better knowledge of their skeletal effects is crucial to guide clinical decisions. At the same time, because TZDs are ligands of PPARγ, a better understanding of their skeletal effects will help to clarify the role of PPARγ in bone metabolism and potentially shed light on the mechanisms of age-related bone loss. This review considers the recent clinical evidence regarding TZDs and skeletal health and discusses outstanding issues that warrant further research.

2. ROSIGLITAZONE AND FRACTURE RISK

Evidence that RSG increases fracture risk emerged with the results of the ADOPT trial published in 2006 [2]. A postproof note in the main report from the trial indicated increased fracture risk in women, but not men, enrolled in the trial. Since then, the fracture results have been published separately and in more detail [3]. ADOPT was designed to assess time to monotherapy failure for RSG compared to metformin and to a sulfonylurea, glyburide. The trial had three arms, corresponding to the three different treatments, and enrolled a total of 2511 men and 1840 women who were followed for a median of 4.0 years. The average age was 57 years. By self-report, 77% of women were postmenopausal. Participants were recently diagnosed with diabetes (<3 years), were drug naïve for hypoglycemic medications, and had an average A1C of about 7.4%.

Fractures, identified through adverse event reports, were specifically reviewed after the conclusion of the trial. Based on time to first fracture, the investigators found an increased risk among women in the RSG arm of 1.81 (95% CI: 1.17, 2.80) compared to metformin, and 2.13 (1.30, 3.51) compared to glyburide. The risk for men was

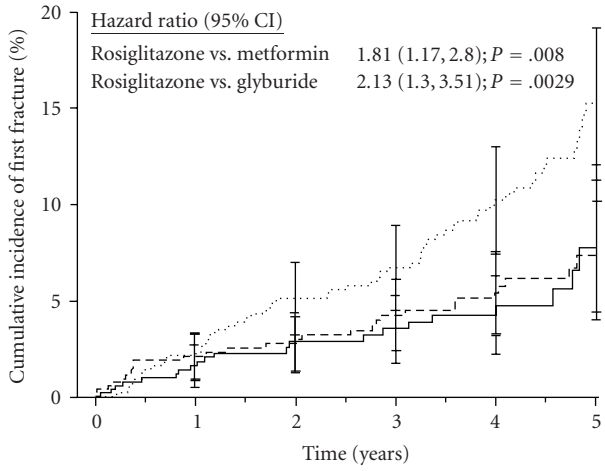

Figure 1: Kaplan-Meier estimates of the cumulative incidence of fractures at five years in women enrolled in ADOPT [3]. Bars represent 95% confidence intervals.

not increased compared with either metformin (RH 1.18; 95% CI: 0.72, 1.96) or glyburide (RH 1.08: 95% CI: 0.65, 1.79).

In women, risk was increased for both upper and lower limb fractures. Rate ratios calculated from fracture rates reported for ADOPT showed the largest increases in relative risk for foot (RR = 3.3), hand (RR = 2.6), and proximal humerus (RR > 8) fractures (see Table 1). There was no increased risk identified for clinical spine or hip fractures, but the numbers of these fractures, 3 clinical spine and 4 hip fractures among all women, were too small to draw firm conclusions. The small number of hip and spine fractures in the ADOPT population (average age 57 years) is not surprising since the rate of these fractures tends to be relatively low until after age 65.

For women, an examination of the survival curves from the ADOPT trial (see Figure 1) suggests that the increased risk of fracture with RSG is evident after about one year of treatment. In separate trials, discussed below, bone loss could be identified among women treated with RSG after only a few months of treatment. However, the ADOPT results suggest that bone loss with RSG does not make a noticeable difference in fracture risk until after about 12 months of treatment.

Self-reported menopausal status and baseline use of estrogen-containing hormones were available for women enrolled in ADOPT. As expected, premenopausal women had a lower rate of fracture than postmenopausal women, but both groups had an approximate doubling of fracture risk with RSG treatment. Menopausal status did not appear to substantially modify the effects of RSG on fracture. About 20% of women reported use of an estrogen-containing hormone at baseline. The effect of RSG on fracture risk did not appear to differ between those who did or did not report estrogen use.

It is possible, though not established, that poor glycemic control increases fracture risk [6]. However, this would not explain the ADOPT results as those in the RSG arm maintained glycemic control on monotherapy longer than those in the metformin or glyburide arms.

3. PIOGLITAZONE AND FRACTURE RISK

With the published report of increased fracture risk in the RSG arm of ADOPT, Takeda Pharmaceuticals, IL, USA the manufacturer of pioglitazone, reviewed their clinical trial databases and, in a letter to health care providers in 2007, reported an increased fracture risk with pioglitazone treatment in women, but not men [7]. The databases included 24 000 years of followup for over 8100 patients treated with pioglitazone and over 7400 patients in the comparison group. In these trials, the maximum duration of pioglitazone use was only 3.5 years. The magnitude of the increased risk reported for all clinical fractures was similar to the ADOPT results with a fracture rate of 1.9 per 100 person years in those using pioglitazone compared with a rate of 1.1 per 100 person years in those using placebo or an active comparator drug. The relative risk for men was not reported but was stated to be not statistically significant. Data on specific fracture sites was not provided although the letter stated that most of the fractures occurred in the distal upper limb or distal lower limb.

4. TZDs AND BONE LOSS

In 2007, Grey et al. reported the results of a 14-week randomized clinical trial comparing RSG (8 mg/day) with placebo in 50 postmenopausal women, average age 67 years, who did not have diabetes or osteoporosis [8]. The trial found modest reductions in two markers of bone formation. Procollagen type-I N-terminal propeptide was reduced by 13% ($P = .004$) and osteocalcin by 10% ($P = .04$) in the RSG arm compared with placebo. In contrast, the bone resorption marker, serum β-C-terminal telopeptide (S-CTX) of type I collagen, was stable in the RSG arm and did not differ significantly from placebo ($P = .9$). Substantial bone loss was reported at the total hip with RSG treatment. Women in the RSG group lost bone density (BMD) more rapidly at the total hip (−1.9% RSG versus −0.2% placebo, $P = .003$). For the total spine, bone loss was more rapid in the RSG arm but the difference was not statistically significant (−1.2% RSG versus −0.2% placebo, $P = .13$).

In a randomized, controlled, but unblinded trial, a lower dose of RSG (4 mg/day) for 12 weeks was compared with diet treatment alone in obese postmenopausal women with newly diagnosed diabetes [9]. Bone-specific alkaline phosphatase,

Table 1: Fracture rates comparing rosiglitazone with metformin or glyburide in ADOPT study. Table adapted from a Letter to Health Care Providers issued by GSK [4].

	Rosiglitazone		Metformin or glyburide		Relative rate (95% CI)	
Women						
Total followup (P-Y)	2187.20		3578.80			
Fracture site	*N*	Rate/100PY	*N*	Rate/100PY	RR	(95% CI)
Lower limb*	36	1.65	26	0.73	2.27	(1.33, 3.91)
Hip	2	0.09	2	0.06	1.64	(0.12, 22.57)
Foot	22	1.01	11	0.31	3.27	(1.52, 7.47)
Upper limb†	22	1.01	19	0.53	1.89	(0.98, 3.70)
Hand	8	0.37	5	0.14	2.62	(0.76, 10.17)
Humerus	5	0.23	0	0.00	‡	(1.50,‡)
Spine	1	0.05	2	0.06	0.82	(0.01, 15.72)
Other	5	0.23	8	0.22	1.02	(0.26, 3.55)
All fractures	64	2.93	55	1.54	1.90	(1.31, 2.78)
Men						
Total followup (P-Y)	2766.70		5570.40			
	N	Rate/100PY	*N*	Rate/100PY	RR	(95% CI)
Total participants with any fracture	32	1.16	57	1.02	1.13	(0.71, 1.77)

*Hip, foot, ankle, femur, fibula, lower limb (general), patella, tibia.
†Hand, humerus, clavicle, forearm, radius, upper limb (general), wrist.
‡Cannot estimate. No events in the comparison group.
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a bone formation marker, was decreased in the RSG arm (−21.5%) compared with diet only (−4.1%) ($P < .05$). Osteocalcin was decreased similarly in both arms (RSG −20%; diet only −17.6%) while urine deoxypyridinoline (DPD), a resorption marker, was not increased in the RSG arm (3%) compared with the diet only arm (17%).

The short-term effects of pioglitazone (30 mg/day) on bone density and markers have been tested in a 16-week randomized placebo-controlled trial among 30 premenopausal women with polycystic ovary syndrome (PCOS) [10]. BMD was reduced compared with placebo at the lumbar spine (−1.14% versus 0.00%), total hip (−0.18% versus 1.35%), and femoral neck (−1.45% versus 0.87%) (all $P < .05$). The magnitude of loss in the PIO group at the spine and femoral neck is similar to BMD losses reported with RSG use over 14 weeks in postmenopausal women [8]. Alkaline phosphatase, a marker of bone formation, was decreased in the PIO group compared to placebo but osteocalcin was not. Changes in the marker of bone resorption, S-CTX, were also not significantly different across treatment groups. The treated group experienced a significant decrease in fasting insulin compared to placebo. Since insulin may be anabolic for bone, this may have contributed to the bone loss observed with PIO although the authors reported that the changes in BMD and the changes in insulin were not significantly correlated. Estradiol and testosterone levels were not significantly altered in the PIO group.

Two observational studies have reported results for TZDs and changes in BMD or markers. The first clinical study to report increased bone loss with TZD use, combining troglitazone, rosiglitazone, and pioglitazone, was based on the Health, Aging, and Body Composition longitudinal observational study of older adults [11]. The cohort included 666 diabetic participants with an average age of 73 years. Of these, 69 participants reported any TZD use during four years of followup. Increased bone loss was found in diabetic women but not men. After controlling for potential confounders, the additional bone loss attributed to TZD use in women was −1.23% (95% CI: −2.06%, −0.40%) per year at the lumbar spine, −0.61% (−1.02%, −0.21%) per year for whole body, and −0.49% (−1.04%, 0.07%) for total hip. These estimates of increased bone loss are substantially lower than those reported by Grey et al. [8] for the trial of RSG use and by Glintborg et al. [10] for the trial of PIO use. The additional bone loss of 1.5–1.7% at the total hip over 14–16 weeks in these two trials, if sustained, would result in additional bone loss of 5-6% annually. While the observational study by Schwartz et al. may have underestimated the degree of bone loss associated with TZD use, it seems unlikely that bone loss of 6% per year is occurring with TZD use. Instead, there may be an initial period of more rapid bone loss, followed by continued loss at a lower rate, similar to the effect of glucocorticoids [12].

Although Schwartz et al. reported no increased bone loss with TZD use in diabetic men, Yaturu et al., in an observational study of 160 older diabetic men (average age 68 years), did report that RSG use ($N = 32$) was associated with increased bone loss of −1.05% per year at the total hip, −1.02% at the femoral neck, and −1.24% at the spine (all $P < .03$) [13]. However, the study did not have sufficient power to control for potential confounders such as A1C level, use of other medications, or diabetic complications.

4.1. Rodent and in vitro models

Results of rodent and in vitro models provided the first evidence that RSG and PIO cause bone loss. RSG has been more extensively studied in these models but both compounds are associated with bone loss in rodents [14, 15]. These findings have been reviewed previously [16, 17] and will not be discussed in depth here. However, a few points are worth noting as particularly relevant to future research in humans. In general, these models indicate a negative effect on osteoblast differentiation and activity with a decrease in bone formation. However, in a few reports, TZDs were associated with increased resorption. Notably, this occurred in ovariectomized rats [18] and in aged mice [19]. Sottile et al. reported that ovariectomized rats experienced bone loss with RSG, but intact female rats did not, and that the bone loss was characterized by increased resorption [18]. This suggests an interaction between RSG and estrogen levels that needs to be assessed in human studies. The results from Lazarenko et al. comparing the effects of RSG in young, adult, and aged mice suggest that the mechanism of action may be different in the aged mice [19]. In young and adult mice, bone loss with RSG treatment was driven by reduced formation while in older mice RSG treatment resulted in increased resorption. These results need to be explored in human studies as they would suggest different approaches to treatment for the prevention of TZD-induced osteoporosis.

5. FUTURE DIRECTIONS FOR CLINICAL RESEARCH

Substantial evidence has now emerged that RSG and PIO have clinically important negative skeletal effects. Increased fracture risk in women, but not men, has been reported for both RSG and PIO. Although this increased fracture risk was identified in the context of clinical trials, the fractures were identified through adverse event reports and were not a planned outcome of the trials. It is possible for adverse event results in a clinical trial to give a signal that is statistically significant due to chance rather than to an actual effect of the intervention. However, the fracture effect is consistent with two clinical trials demonstrating bone loss with RSG and PIO. And, the increased fracture risk and bone loss are consistent with the results of rodent and in vitro models. The combination of these studies provides a compelling argument that, in women, the two currently prescribed TZDs cause higher fracture risk due to bone loss.

Given this growing evidence of increased fracture risk and bone loss with TZD use, further exploration of the skeletal effects of TZDs is crucial to inform efforts to prevent TZD-induced osteoporosis and, more generally, to delineate the role of PPARγ in bone metabolism. Some of the key questions for clinical research are identified and discussed below.

5.1. What groups are at higher risk?

To inform clinical decisions and to better understand the mechanism of TZDs effects on the skeleton, it is important to ascertain if there are groups that are particularly vulnerable, or groups that are not susceptible, to increased fracture risk with TZD use. So far, the negative skeletal effects seem to be more important for women than for men, but results are not conclusive. Among women, menopausal status does not appear to modify the effect of RSG on the skeleton. The ADOPT results indicate that increased fracture risk extends to those who are premenopausal as well as postmenopausal. Both premenopausal [10] and postmenopausal [8] women have been shown to lose bone with TZD treatment.

A possible explanation for the lack of effect on the skeleton in men is the higher estrogen levels found in older men compared with older women. In a rat model, ovariectomized, but not intact, females had bone loss with RSG treatment, suggesting a protective effect from higher estrogen levels [18]. However, clinical results to date indicate that TZDs cause increased bone loss and fracture risk in pre- as well as postmenopausal women. Further research with measurements of endogenous estrogen levels could clarify whether there is an interaction between estrogen levels and TZD use.

5.2. What happens to bone density and turnover after 3-4 months of treatment?

The randomized trials with RSG and PIO have reported on treatment for 14–16 weeks. In both trials, the additional bone loss in the treated group was substantial, equivalent to a loss of 5-6% over a year, but it seems unlikely that this rate of loss is being sustained over longer treatment periods. Observational studies suggest increased loss of about 0.5–1% each year. Steroid treatment appears to cause initial rapid bone loss followed by continued loss but at a lower rate; the TZDs may present a similar pattern [12]. However, trials of longer duration are needed to assess the degree of loss over several years.

5.3. Effect on resorption as well as formation?

One of the key questions regarding the mechanism of action of the TZDs is whether bone resorption and formation, or only one, are affected. The clinical evidence to date, based on bone turnover markers, points to a reduction in bone formation without a change in bone resorption. However, these results are based on only three studies that included bone marker results [8–10]. Rodent models have generally shown reduced bone formation but, in aged mice and in ovariectomized rats, bone resorption is increased. Whether bone resorption is similarly increased with older age or with very low endogenous estrogen levels in human studies has not been fully explored.

5.4. Do effects on cortical and trabecular bone differ?

The increased fracture risk observed in the bones of the extremities, that have a relatively high proportion of cortical bone, suggests a negative impact on cortical bone. This pattern is distinct from glucocorticoids which have a particularly strong effect on trabecular bone and the risk of vertebral

fracture [12]. Studies using imaging techniques that can separate these two compartments, such as high resolution computed tomography, could clarify whether the effects of TZDs differ for cortical and trabecular bones.

5.5. Marrow adiposity

In most reports from rodent models, increased marrow adiposity accompanies bone loss with RSG treatment. Further investigation of this phenomenon has suggested that activation of PPARγ with RSG increases lineage allocation of stem cells towards adipocytes at the expense of osteoblasts in the marrow. To date, human studies have not measured bone marrow adiposity. Knowledge of the effect of TZDs on bone marrow fat would increase our understanding of the mechanisms underlying bone loss and fracture risk in humans with TZD use. In addition, an increase in bone marrow fat may cause an artificial decrease in BMD measured by DXA [20]. If marrow fat is increased, the degree of bone loss with TZD use may be overestimated by DXA measurements.

5.6. Effective treatment for TZD-induced osteoporosis

There are no studies to date on treatments that might prevent TZD-induced bone loss. Although the bisphosphonates mainly target bone resorption, the general reduction in bone turnover may be efficacious in preventing bone loss with TZD treatment. The bisphosphonates are successfully used for prevention of osteoporosis with corticosteroid treatment, also characterized by reduced bone formation [21]. However, TZDs have specific effects on bone, and bisphosphonate use should be explicitly tested to determine efficacy in this situation. Treatments that increase bone formation, currently limited to parathyroid hormone (PTH) and strontium ralenate, could theoretically prevent TZD-induced bone loss. PTH has been shown to prevent bone loss with glucocorticoid therapy [22], but neither treatment has been tested in relation to TZDs.

6. CONCLUSION

Research over the past two years has provided new clinical evidence that the currently prescribed TZDs increase fracture risk and bone loss, at least in women. Combined with the findings from rodent and in vitro models, these clinical results suggest that activation of PPARγ can play a role in bone loss. With the widespread use of TZDs as a diabetes treatment, further research is needed to delineate the groups that are most susceptible to TZD-induced osteoporosis, to determine the rate of bone loss with TZD treatment beyond 16 weeks, to assess the effects of TZDs on marrow adiposity, cortical and trabecular bones, and to identify treatments to prevent TZD-induced fracture risk. Addressing these questions will advance our ability to prevent TZD-induced osteoporosis and will provide a better understanding of the role of PPARγ activation in bone metabolism.

REFERENCES

[1] A. V. Schwartz, "Diabetes, TZDs, and bone: a review of the clinical evidence," *PPAR Research*, vol. 2006, Article ID 24502, 6 pages, 2006.

[2] S. E. Kahn, S. M. Haffner, M. A. Heise, et al., "Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy," *The New England Journal of Medicine*, vol. 355, no. 23, pp. 2427–2443, 2006.

[3] S. E. Kahn, B. Zinman, J. M. Lachin, et al., "Rosiglitazone-associated fractures in type 2 diabetes: an analysis from ADOPT," *Diabetes Care*, vol. 31, no. 5, pp. 845–851, 2008.

[4] GlaxoSmithKline. (GSK), Clinical trial observation of an increased incidence of fractures in female patients who received long-term treatment with Avandia® (rosiglitazone maleate) tablets for type 2 diabetes mellitus (Letter to Health Care Providers), February 2007, http://www.fda.gov/MedWatch/safety/2007/Avandia_GSK_Ltr.pdf.

[5] A. V. Schwartz and D. E. Sellmeyer, "Effect of thiazolidinediones on skeletal health in women with Type 2 diabetes," *Expert Opinion on Drug Safety*, vol. 7, no. 1, pp. 69–78, 2008.

[6] A. V. Schwartz, "Diabetes mellitus: does it affect bone?" *Calcified Tissue International*, vol. 73, no. 6, pp. 515–519, 2003.

[7] Takeda, Observation of an increased incidence of fractures in female patients who received long-term treatment with ACTOS® (pioglitazone HCl) tablets for type 2 diabetes mellitus. (Letter to Health Care Providers), March 2007, http://www.fda.gov/medwatch/safety/2007/Actosmar0807.pdf.

[8] A. Grey, M. Bolland, G. Gamble, et al., "The peroxisome proliferator-activated receptor-γ agonist rosiglitazone decreases bone formation and bone mineral density in healthy postmenopausal women: a randomized, controlled trial," *The Journal of Clinical Endocrinology & Metabolism*, vol. 92, no. 4, pp. 1305–1310, 2007.

[9] Z. Berberoglu, A. Gursoy, N. Bayraktar, A. C. Yazici, N. Bascil Tutuncu, and N. Guvener Demirag, "Rosiglitazone decreases serum bone-specific alkaline phosphatase activity in postmenopausal diabetic women," *The Journal of Clinical Endocrinology & Metabolism*, vol. 92, no. 9, pp. 3523–3530, 2007.

[10] D. Glintborg, M. Andersen, C. Hagen, L. Heickendorff, and A. P. Hermann, "Association of pioglitazone treatment with decreased bone mineral density in obese premenopausal patients with polycystic ovary syndrome: a randomized, placebo-controlled trial," *The Journal of Clinical Endocrinology & Metabolism*, vol. 93, no. 5, pp. 1696–1701, 2008.

[11] A. V. Schwartz, D. E. Sellmeyer, E. Vittinghoff, et al., "Thiazolidinedione use and bone loss in older diabetic adults," *The Journal of Clinical Endocrinology & Metabolism*, vol. 91, no. 9, pp. 3349–3354, 2006.

[12] T. P. van Staa, H. G. M. Leufkens, and C. Cooper, "The epidemiology of corticosteroid-induced osteoporosis: a meta-analysis," *Osteoporosis International*, vol. 13, no. 10, pp. 777–787, 2002.

[13] S. Yaturu, B. Bryant, and S. K. Jain, "Thiazolidinediones treatment decreases bone mineral density in type 2 diabetic men," *Diabetes Care*, vol. 30, no. 6, pp. 1574–1576, 2007.

[14] S. O. Rzonca, L. J. Suva, D. Gaddy, D. C. Montague, and B. Lecka-Czernik, "Bone is a target for the antidiabetic compound rosiglitazone," *Endocrinology*, vol. 145, no. 1, pp. 401–406, 2004.

[15] C. Jennermann, J. Triantafillou, D. Cowan, B. Pennink, K. Connolly, and D. Morris, "Effects of thiazolidinediones on bone turnover in the rat," *Journal of Bone and Mineral Research*, vol. 10, p. S241, 1995, (Abstract S361).

[16] B. Lecka-Czernik and L. J. Suva, "Resolving the two "bony" faces of PPAR-γ," *PPAR Research*, vol. 2006, Article ID 27489, 9 pages, 2006.

[17] A. Grey, "Skeletal consequences of thiazolidinedione therapy," *Osteoporosis International*, vol. 19, no. 2, pp. 129–137, 2008.

[18] V. Sottile, K. Seuwen, and M. Kneissel, "Enhanced marrow adipogenesis and bone resorption in estrogen-deprived rats treated with the PPARgamma agonist BRL49653 (rosiglitazone)," *Calcified Tissue International*, vol. 75, no. 4, pp. 329–337, 2004.

[19] O. P. Lazarenko, S. O. Rzonca, W. R. Hogue, F. L. Swain, L. J. Suva, and B. Lecka-Czernik, "Rosiglitazone induces decreases in bone mass and strength that are reminiscent of aged bone," *Endocrinology*, vol. 148, no. 6, pp. 2669–2680, 2007.

[20] T. N. Hangartner and C. C. Johnston, "Influence of fat on bone measurements with dual-energy absorptiometry," *Bone and Mineral*, vol. 9, no. 1, pp. 71–81, 1990.

[21] D. M. Reid, R. A. Hughes, R. F. J. M. Laan, et al., "Efficacy and safety of daily risedronate in the treatment of corticosteroid-induced osteoporosis in men and women: a randomized trial," *Journal of Bone and Mineral Research*, vol. 15, no. 6, pp. 1006–1013, 2000.

[22] N. E. Lane, S. Sanchez, G. W. Modin, H. K. Genant, E. Pierini, and C. D. Arnaud, "Parathyroid hormone treatment can reverse corticosteroid-induced osteoporosis. Results of a randomized controlled clinical trial," *The Journal of Clinical Investigation*, vol. 102, no. 8, pp. 1627–1633, 1998.

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Review Article

Pioglitazone versus Rosiglitazone: Effects on Lipids, Lipoproteins, and Apolipoproteins in Head-to-Head Randomized Clinical Studies

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Peroxisome proliferator-activated receptors (PPARs) play an important role in regulating both glucose and lipid metabolism. Agonists for both PPARγ and PPARγ have been used to treat dyslipidemia and hyperglycemia, respectively. In addition to affecting glucose metabolism, PPARγ agonists also regulate lipid metabolism. In this review, we will focus on the randomized clinical trials that directly compared the lipid effects of the thiazolidinedione class of PPARγ agonists, pioglitazone and rosiglitazone, head-to-head either as monotherapy or in combination with other lipid-altering or glucose-lowering agents

1. INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) play an important role in regulating both glucose and lipid metabolism. Agonists for both PPARα and PPARγ have been used to treat dyslipidemia and hyperglycemia, respectively. In addition to affecting glucose metabolism, PPARγ agonists also regulate lipid metabolism.

The dyslipidemia of type 2 diabetes mellitus is characterized by elevations in serum triglycerides and increased very low-density lipoprotein (VLDL) particle size, reduced high-density lipoprotein (HDL) cholesterol and HDL particle size, and the predominance of small, dense low-density lipoprotein (LDL) particles with generally normal LDL cholesterol. Many studies have examined the effect of improvements in glycemic control on serum lipids and lipoproteins utilizing a variety of glucose-lowering medications [1]. These include insulin, sulfonylureas, biguanides, thiazolidinediones, glucagon-like peptides, α-glucosidase inhibitors, and dipeptidyl peptidase-IV inhibitors. In general, improving glycemic control reduces serum triglycerides and increases HDL cholesterol. Numerous studies have compared the effect of thiazolidinediones with other oral glucose-lowering medications. In general, thiazolidinediones have better overall effects on lipids compared to sulfonylureas or insulin [2, 3]. In this review, we will focus on the randomized clinical trials that directly compared the lipid effects of the thiazolidinedione class of PPARγ agonists, pioglitazone and rosiglitazone, head to head either as monotherapy or in combination with other lipid-altering or glucose-lowering agents. The effects of troglitazone (Rezulin), which has been removed from the market, will not be discussed.

2. ROLE OF PPARγ IN REGULATING FATTY ACID/TRIGLYCERIDE METABOLISM

The whole-body response to activating PPARγ is storage of energy, as triglycerides, in adipocytes. This is accomplished by the coordinated regulation of tissue-specific gene expression in adipocytes, liver, and cells that utilize fatty acids for energy as well as various circulating factors that coordinate and regulate fatty acid synthesis and utilization. Although often only serum triglycerides are measured and monitored in patients, serum triglycerides represent just one compartment within which PPARγ medications affect whole-body triglyceride/fatty acid metabolism. Serum triglycerides within VLDL and chylomicrons may be considered the

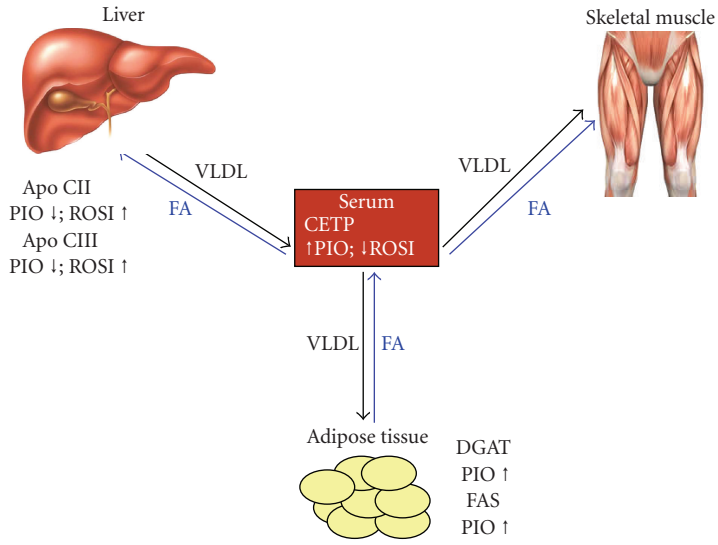

Figure 1

mechanism by which energy (as triglycerides) is transported from one tissue to another (Figure 1).

In the adipocyte, both pioglitazone and rosiglitazone increase the expression of genes associated with hydrolysis of triglyceride-rich lipoproteins and fatty acid uptake and storage [4, 5] (Figure 1). Thiazolidinediones also reduce fatty acid release from adipocytes. This in turn leads to less fatty acid delivery to the liver and a decrease in hepatic triglyceride synthesis. In addition, PPARγ medications influence secretion of adipokines that affect lipid and glucose metabolism. Pioglitazone and rosiglitazone therapies increase adiponectin [6, 7] and decrease retinol binding protein 4 [8] and resistin [9]. These adipokines influence lipid metabolism and insulin sensitivity.

In the liver, PPARγ therapy is associated with changes in expression of various genes involved in lipid metabolism including apolipoproteins CII and CIII. Apolipoproteins CII and CIII stimulate and inhibit lipoprotein lipase, respectively. Lipoprotein lipase is the major enzyme involved in hydrolyzing and removing triglyceride-rich lipoproteins from the serum.

3. COMPARISON OF LIPID EFFECTS OF PIOGLITAZONE AND ROSIGLITAZONE IN HEAD-TO-HEAD RANDOMIZED CLINICAL TRIALS

3.1. *Thiazolidinediones as monotherapy: effects on fasting lipids*

Goldberg et al. [10] and Deeg et al. [11] compared the effects of pioglitazone and rosiglitazone in patients with type 2 diabetes mellitus and dyslipidemia on non-lipid-altering medications. After discontinuing their glucose-lowering and lipid-altering medications, if they were on them, patients were randomized to pioglitazone or rosiglitazone. Patients were treated with 30 mg once a day (QD) of pioglitazone or 4 mg of rosiglitazone QD for 12 weeks with a forced titration to 45 mg QD and 4 mg twice a day (bid) for additional 12 weeks, respectively. Both medications reduced hemoglobin A1c (A1c), insulin resistance (as determined by HOMA-IR), and fasting free fatty acids to a similar extent. However, the effects on fasting triglycerides were divergent. Pioglitazone therapy was associated with a reduction in fasting triglycerides throughout the study, whereas rosiglitazone increased triglycerides within 4 weeks, which then declined with time. At the end of the study, triglycerides were decreased by 12% with pioglitazone, and elevated by 15% in patients on rosiglitazone.

The decrease in triglycerides with pioglitazone was associated with a decrease in large VLDL and intermediate density lipoproteins (IDLs), whereas the increase in triglycerides with rosiglitazone was associated with an increase in both large- and medium-sized VLDL and IDL concentrations. Pioglitazone decreased whereas rosiglitazone increased apolipoprotein CIII.

Both medications raised LDL cholesterol; however, the increase was significantly greater with rosiglitazone compared to pioglitazone (12.3% and 21.3%, resp.). Both therapies increased the average size of LDL particles, but the effect of pioglitazone was greater than that of rosiglitazone. Consistent with the changes in LDL cholesterol, pioglitazone did not significantly change apolipoprotein B levels but did reduce LDL particle concentration. Conversely, rosiglitazone increased both apolipoprotein B and LDL particle concentration. The clinical significance of the difference in particle concentration is unclear although decreased LDL particle concentration has been associated with a reduced risk for coronary heart disease [12, 13]. Both medications raised serum levels of lipoprotein (a).

As expected, both medications increased HDL cholesterol and the average size of HDL particles; however the

Table 1: Summary of clinical trials comparing lipid effects of pioglitazone and rosiglitazone.

	Concomitant glucose/lipid therapy	*N*	Duration	Pioglitazone effects	Rosiglitazone effects
Derosa et al. [16, 17]	Glimepiride	91	52 wk	↑HDL-C, apo AI ↓TC*, LDL-C*, ↓TG*, apo B*, Lp(a)*	↑TC*, LDL-C*, HDL-C, ↑TG*, apo AI, apo B*, lipoprotein (a)
Goldberg et al. [10], Deeg et al. [11]	None	802	24 wk	↑HDL-C*, LDL-C*, TC* ↓TG* ↑VLDL-P, HDL-P*, apo AI ↓LDL-P*, apo CIII*	↑TG*, HDL-C*, LDL-C*, ↑TC*, apo B* ↑VLDL-P, HDL-P*, LDL-P*, apo CIII* ↓apo AI*
Berhanu et al. [19]	Statins	305	17 wk	↓TG*, TC*, LDL-P, ↑LDL-C*, HDL-C (changes following switch from rosiglitazone to pioglitazone)	
Chappuis et al. [15]	None	17	12 wk	↓AUC-TG* ↑CETP*	↑AUC-TG* ↓CETP*
Derosa et al. [18]	Metformin	96	52 wk	↓TC*, LDL-C*, TG*, apo B* ↑HDL-C*, apo AI*	No significant changes in any lipid parameter
Berneis et al. [14]	None	9	12 wk	↑TC, HDL, LDL, LDL IIA* ↓TG*	↑TC, TG*, HDL, LDL, LDL-IIA

N = number of patients enrolled. Pioglitazone and rosiglitazone effects are summarized as % change from baseline and listed in parentheses. (*) indicates a statistically significant change from baseline. TC = total cholesterol, TG = triglycerides, LDL-C = LDL cholesterol, HDL-C = HDL cholesterol, LDL-P = LDL particle number, HDL-P = HDL particle number, apo = apolipoprotein, AUC-TG = area under the curve for TG.

increase in HDL cholesterol was significantly greater with pioglitazone therapy compared with rosiglitazone therapy (14.9% and 7.8%, resp.). Again, there was a difference in HDL particle subclasses between the medications. Pioglitazone increased total, large, and medium HDLs while decreasing small HDL concentration. Rosiglitazone, in contrast, decreased total, large, and small HDLs while increasing medium HDL particle concentration. These suggest that there are differences in HDL metabolism with these two agents. Pioglitazone had no effect on serum apolipoprotein AI levels, but rosiglitazone therapy was associated with a decrease in apolipoprotein AI levels.

3.2. Thiazolidinediones as monotherapy: effects on postprandial lipemia

Postprandial dyslipidemia is a feature of type 2 diabetes. Two small studies compared the effects of pioglitazone and rosiglitazone on postprandial lipemia using a prospective, randomized crossover design [14, 15]. After washing out both glucose-lowering (8 weeks) and lipid-altering medications (4 weeks), patients were randomized to either pioglitazone (30 mg QD for 4 weeks, then 45 mg QD for 8 weeks) or rosiglitazone (4 mg QD for 4 weeks followed by 4 mg bid for 8 weeks) with an 8-week washout during the crossover. Before and after each treatment, a standardized breakfast was served and postprandial glucose, lipids, and hormones were measured.

Both agents had similar effects on A1c and HOMA-IR. Pioglitazone reduced fasting and postprandial triglycerides that were associated with decreases in the smaller VLDL subfractions: VLDL-2 and VLDL-3. Rosiglitazone increased the postprandial triglycerides with increases in VLDL-2 and VLDL-3. There was no effect with either medication on fasting apolipoprotein B, AI, or CII/CIII ratio, and lipoprotein lipase or hepatic lipase activity did not differ between therapies. Cholesterol ester transfer protein activity decreased with rosiglitazone and increased after pioglitazone therapy. The second study demonstrated that pioglitazone was more effective than rosiglitazone in increasing larger LDL concentrations (fasting and postprandial) as well as in reducing levels of small, dense LDL particles [14].

3.3. Thiazolidinediones in combination with other oral antihyperglycemic medications

Derosa et al. [16] compared the effect of adding pioglitazone (15 mg QD) or rosiglitazone (4 mg QD) on patients with type 2 diabetes treated with glimepiride (4 mg QD). After 12 months, both groups had significant reductions in A1c (1.3%). The group treated with the pioglitazone combination had a reduction in total cholesterol, LDL cholesterol, lipoprotein (a), and apolipoprotein B with an increase in HDL cholesterol. The rosiglitazone group had increases in total cholesterol, LDL cholesterol, triglycerides, and apolipoprotein B but no effect on HDL cholesterol or

lipoprotein (a) [17]. Both groups showed a reduction in homocysteine.

In a similarly designed trial, patients with type 2 diabetes were treated with metformin and randomized to pioglitazone or rosiglitazone [18]. After 12 months, both groups had similar reductions in A1c and insulin resistance (as determined by HOMA-IR). Total cholesterol, LDL cholesterol, triglycerides, and apolipoprotein B decreased in the pioglitazone group with increases in HDL cholesterol and apolipoprotein AI. There were no changes observed in the rosiglitazone group.

3.4. Thiazolidinediones in combination with statins

Berhanu et al. [19] examined the changes in lipids when patients were switched from rosiglitazone and a statin to pioglitazone (30 mg) while maintaining a stable statin dose. At the end of the trial (17 weeks), although the A1c did not change, patients had a significant reduction in triglycerides, total cholesterol, and LDL particle concentration (189 nmol/L) and increases in LDL cholesterol, HDL cholesterol, and LDL particle diameter (0.23 nm). Apolipoprotein B did not change but apolipoprotein AI increased.

In summary, although the head-to-head and rosiglitazone-only [20] clinical trials demonstrate a benefit of rosiglitazone on HDL cholesterol, there isa relatively consistent and overall favorable impact of pioglitazone compared to rosiglitazone on serum lipids, lipoproteins, and apolipoproteins. It is also clear that the lipids' effects are unrelated to the changes in insulin sensitivity since [1] both agents have similar effects to improve insulin sensitivity and [2] the effect on insulin sensitivity can be clearly differentiated from lipid changes [21]. Thus, there must be other differences in the action of the thiazolidinediones that account for the divergent lipid effects.

3.5. Comparison of mechanisms of action on lipid metabolism

Whole-body fatty acid/triglyceride metabolism involves the interaction of numerous organs as described above. Since both pioglitazone and rosiglitazone have similar effects in the adipocyte on adipokines' expression and genes involved in fatty acid/triglyceride metabolism, the difference between these medications on serum triglycerides likely occurs within the liver and/or plasma compartment.

The most profound difference between the lipid effects of pioglitazone versus rosiglitazone is in fasting and postprandial triglycerides. As both medications have similar effects on glycemic control and insulin resistance, an additional mechanism must account for these differences. The differences in serum triglycerides occur in smaller VLDL particles which are produced in an insulin-independent fashion consistent with the observations that it is not the change in insulin resistance that accounts for the differences. One potential difference, which may account for the difference, is the effect on apolipoprotein CIII. Two studies have demonstrated that pioglitazone decreases and rosiglitazone increases apolipoprotein CIII [10, 22]. A decrease in apolipoprotein CIII would lead to an increase in lipoprotein lipase activity, and hence an increase in the hydrolysis of triglycerides and catabolic rate of triglyceride-rich lipoproteins including chylomicrons and VLDL [23]. This hypothesis is supported by the observation that pioglitazone increases the lipolysis of VLDL triglycerides without affecting the removal of VLDL particles [22]. Conversely, rosiglitazone increases the production and reduces the catabolism of triglyceride-rich lipoproteins including both VLDL and chylomicrons [21].

Another possibility is that genetic differences may contribute to the different lipid effects. Polymorphism of the PPARγ2 gene influences the glycemic response to rosiglitazone [24] but not to pioglitazone [25]. A lipoprotein lipase variant influences the glycemic effect of pioglitazone [26], while a polymorphism of the adiponectin [27] and perilipin [28] genes influences the glycemic and weight gain responses, respectively, to rosiglitazone. Since none of these studies directly compared both rosiglitazone and pioglitazone, it is unclear if polymorphism contributes to the differences. Most of these studies also did not show a linkage between lipid effects and polymorphisms, but a link between the adiponectin genotype at position 45 and the triglyceride effect of rosiglitazone did statistically approach significance [27]. Whether this occurs with pioglitazone has not been published to date.

It is possible that pharmacokinetic differences between pioglitazone and rosiglitazone may account for the differences in lipid effects; however, this is an unlikely contributor since the gene expression and pharmacodynamic effects of both agents exceed the presence of active drug in the serum.

Do the differences in lipid effects have clinical significance? Increased fasting and postprandial triglycerides [29, 30] as well as LDL particle concentration [12, 13] are risk factors for cardiovascular disease. Conversely, increases in large HDL and adiponectin are associated with reduction in risk. It is also likely that other effects influence the risk of coronary artery disease (CAD) events. It is likely that the integrated sum of these lipid effects, together with yet-defined factors, will determine the influence on atherosclerosis.

Clinical outcome trials with both pioglitazone and rosiglitazone have been published. Both pioglitazone and rosiglitazone improve endothelial function and reduce the progression of carotid intramedial thickness in patients [31–34]. These observations suggest a clinical benefit with both agents. In the PROACTIVE study, adding pioglitazone to the current treatment in patients with type 2 diabetes was associated with reductions in major atherosclerotic events as defined in the main secondary end-point [35], recurrent myocardial infarction [36], and recurrent stroke [37]. Meta-analysis of pioglitazone clinical trials showed a significantly lower risk of death, myocardial infarction, or stroke in patients with diabetes [20].

The effect of rosiglitazone on CAD events is more controversial. Some post hoc meta-analysis studies have suggested that rosiglitazone is associated with an increased risk of CAD events [38, 39]. However, in the RECORD trial, a prospective trial in patients with type 2 diabetes, no evidence for an increased event rate was found in an interim analysis

[40]. Completion of this along with other studies is needed to fully answer the effect of rosiglitazone on CAD events.

4. SUMMARY

Both pioglitazone and rosiglitazone reduce insulin resistance and improve glycemic control in patients with type 2 diabetes. However, the head-to-head clinical trials demonstrate a relatively consistent and favorable impact of pioglitazone compared to rosiglitazone on serum lipids, lipoproteins, and apolipoproteins. Whether these differences result in different outcomes that are clinically significant remains to be determined.

REFERENCES

[1] J. B. Buse, M. H. Tan, M. J. Prince, and P. P. Erickson, "The effects of oral anti-hyperglycaemic medications on serum lipid profiles in patients with type 2 diabetes," *Diabetes, Obesity and Metabolism*, vol. 6, no. 2, pp. 133–156, 2004.

[2] D. S. Bell, "Beneficial effects resulting from thiazolidinediones for treatment of type 2 diabetes mellitus," *Postgraduate Medicine*, pp. 35–44, 2003.

[3] H. Yki-Järvinen, "Thiazolidinediones," *The New England Journal of Medicine*, vol. 351, no. 11, pp. 1106–1118, 2004.

[4] G. Ranganathan, R. Unal, I. Pokrovskaya, et al., "The lipogenic enzymes DGAT1, FAS, and LPL in adipose tissue: effects of obesity, insulin resistance, and TZD treatment," *Journal of Lipid Research*, vol. 47, no. 11, pp. 2444–2450, 2006.

[5] G. D. Tan, G. Olivecrona, H. Vidal, K. N. Frayn, and F. Karpe, "Insulin sensitisation affects lipoprotein lipase transport in type 2 diabetes: role of adipose tissue and skeletal muscle in response to rosiglitazone," *Diabetologia*, vol. 49, no. 10, pp. 2412–2418, 2006.

[6] W.-S. Yang, C.-Y. Jeng, T.-J. Wu, et al., "Synthetic peroxisome proliferator-activated receptor-γ agonist, rosiglitazone, increases plasma levels of adiponectin in type 2 diabetic patients," *Diabetes Care*, vol. 25, no. 2, pp. 376–380, 2002.

[7] M. Bajaj, S. Suraamornkul, P. Piper, et al., "Decreased plasma adiponectin concentrations are closely related to hepatic fat content and hepatic insulin resistance in pioglitazone-treated type 2 diabetic patients," *The Journal of Clinical Endocrinology & Metabolism*, vol. 89, no. 1, pp. 200–206, 2004.

[8] A. Yao-Borengasser, V. Varma, A. M. Bodles, et al., "Retinol binding protein 4 expression in humans: relationship to insulin resistance, inflammation, and response to pioglitazone," *The Journal of Clinical Endocrinology & Metabolism*, vol. 92, no. 7, pp. 2590–2597, 2007.

[9] M. Bajaj, S. Suraamornkul, L. J. Hardies, T. Pratipanawatr, and R. A. DeFronzo, "Plasma resistin concentration, hepatic fat content, and hepatic and peripheral insulin resistance in pioglitazone-treated type II diabetic patients," *International Journal of Obesity*, vol. 28, no. 6, pp. 783–789, 2004.

[10] R. B. Goldberg, D. M. Kendall, M. A. Deeg, et al., "A comparison of lipid and glycemic effects of pioglitazone and rosiglitazone in patients with type 2 diabetes and dyslipidemia," *Diabetes Care*, vol. 28, no. 7, pp. 1547–1554, 2005.

[11] M. A. Deeg, J. B. Buse, R. B. Goldberg, et al., "Pioglitazone and rosiglitazone have different effects on serum lipoprotein particle concentrations and sizes in patients with type 2 diabetes and dyslipidemia," *Diabetes Care*, vol. 30, no. 10, pp. 2458–2464, 2007.

[12] L. Kuller, A. Arnold, R. Tracy, et al., "Nuclear magnetic resonance spectroscopy of lipoproteins and risk of coronary heart disease in the cardiovascular health study," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 22, no. 7, pp. 1175–1180, 2002.

[13] J. D. Otvos, D. Collins, D. S. Freedman, et al., "Low-density lipoprotein and high-density lipoprotein particle subclasses predict coronary events and are favorably changed by gemfibrozil therapy in the veterans affairs high-density lipoprotein intervention trial," *Circulation*, vol. 113, no. 12, pp. 1556–1563, 2006.

[14] K. Berneis, M. Rizzo, C. Stettler, et al., "Comparative effects of rosiglitazone and pioglitazone on fasting and postprandial low-density lipoprotein size and subclasses in patients with type 2 diabetes," *Expert Opinion on Pharmacotherapy*, vol. 9, no. 3, pp. 343–349, 2008.

[15] B. Chappuis, M. Braun, C. Stettler, et al., "Differential effect of pioglitazone (PGZ) and rosiglitazone (RGZ) on postprandial glucose and lipid metabolism in patients with type 2 diabetes mellitus: a prospective, randomized crossover study," *Diabetes/Metabolism Research and Reviews*, vol. 23, no. 5, pp. 392–399, 2007.

[16] G. Derosa, A. F. G. Cicero, A. Gaddi, et al., "Metabolic effects of pioglitazone and rosiglitazone in patients with diabetes and metabolic syndrome treated with glimepiride: a twelve-month, multicenter, double-blind, randomized, controlled, parallel-group trial," *Clinical Therapeutics*, vol. 26, no. 5, pp. 744–754, 2004.

[17] G. Derosa, A. F. G. Cicero, A. D'Angelo, et al., "Effects of 1 year of treatment with pioglitazone or rosiglitazone added to glimepiride on lipoprotein (a) and homocysteine concentrations in patients with type 2 diabetes mellitus and metabolic syndrome: a multicenter, randomized, double-blind, controlled clinical trial," *Clinical Therapeutics*, vol. 28, no. 5, pp. 679–688, 2006.

[18] G. Derosa, A. D'Angelo, P. D. Ragonesi, et al., "Metabolic effects of pioglitazone and rosiglitazone in patients with diabetes and metabolic syndrome treated with metformin," *Internal Medicine Journal*, vol. 37, no. 2, pp. 79–86, 2007.

[19] P. Berhanu, M. S. Kipnes, M. A. Khan, et al., "Effects of pioglitazone on lipid and lipoprotein profiles in patients with type 2 diabetes and dyslipidaemia after treatment conversion from rosiglitazone while continuing stable statin therapy," *Diabetes and Vascular Disease Research*, vol. 3, no. 1, pp. 39–44, 2006.

[20] A. M. Lincoff, K. Wolski, S. J. Nicholls, and S. E. Nissen, "Pioglitazone and risk of cardiovascular events in patients with type 2 diabetes mellitus: a meta-analysis of randomized trials," *The Journal of the American Medical Association*, vol. 298, no. 10, pp. 1180–1188, 2007.

[21] H. Duez, B. Lamarche, K. D. Uffelman, et al., "Dissociation between the insulin-sensitizing effect of rosiglitazone and its effect on hepatic and intestinal lipoprotein production," *The Journal of Clinical Endocrinology & Metabolism*, vol. 93, no. 5, pp. 1722–1729, 2008.

[22] K. Nagashima, C. Lopez, D. Donovan, et al., "Effects of the PPARγ agonist pioglitazone on lipoprotein metabolism in patients with type 2 diabetes mellitus," *The Journal of Clinical Investigation*, vol. 115, no. 5, pp. 1323–1332, 2005.

[23] H. N. Ginsberg, N. A. Le, I. J. Goldberg, et al., "Apolipoprotein B metabolism in subjects with deficiency of apolipoproteins CIII and AI. Evidence that apolipoprotein CIII inhibits catabolism of triglyceride-rich lipoproteins by lipoprotein

lipase in vivo," *The Journal of Clinical Investigation*, vol. 78, no. 5, pp. 1287–1295, 1986.

[24] E. S. Kang, S. Y. Park, H. J. Kim, et al., "Effects of Pro12Ala polymorphism of peroxisome proliferator-activated receptor γ2 gene on rosiglitazone response in type 2 diabetes," *Clinical Pharmacology & Therapeutics*, vol. 78, no. 2, pp. 202–208, 2005.

[25] M. Blüher, G. Lübben, and R. Paschke, "Analysis of the relationship between the Pro12Ala variant in the PPAR-γ2 gene and the response rate to therapy with pioglitazone in patients with type 2 diabetes," *Diabetes Care*, vol. 26, no. 3, pp. 825–831, 2003.

[26] G. Wang, X. Wang, Q. Zhang, and Z. Ma, "Response to pioglitazone treatment is associated with the lipoprotein lipase S447X variant in subjects with type 2 diabetes mellitus," *International Journal of Clinical Practice*, vol. 61, no. 4, pp. 552–557, 2007.

[27] E. S. Kang, S. Y. Park, H. J. Kim, et al., "The influence of adiponectin gene polymorphism on the rosiglitazone response in patients with type 2 diabetes," *Diabetes Care*, vol. 28, no. 5, pp. 1139–1144, 2005.

[28] E. S. Kang, B. S. Cha, H. J. Kim, et al., "The 11482G > A polymorphism in the perilipin gene is associated with weight gain with rosiglitazone treatment in type 2 diabetes," *Diabetes Care*, vol. 29, no. 6, pp. 1320–1324, 2006.

[29] S. Bansal, J. E. Buring, N. Rifai, S. Mora, F. M. Sacks, and P. M. Ridker, "Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women," *The Journal of the American Medical Association*, vol. 298, no. 3, pp. 309–316, 2007.

[30] B. G. Nordestgaard, M. Benn, P. Schnohr, and A. Tybjærg-Hansen, "Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women," *The Journal of the American Medical Association*, vol. 298, no. 3, pp. 299–308, 2007.

[31] M. R. Langenfeld, T. Forst, C. Hohberg, et al., "Pioglitazone decreases carotid intima-media thickness independently of glycemic control in patients with type 2 diabetes mellitus: results from a controlled randomized study," *Circulation*, vol. 111, no. 19, pp. 2525–2531, 2005.

[32] T. Nakamura, T. Matsuda, Y. Kawagoe, et al., "Effect of pioglitazone on carotid intima-media thickness and arterial stiffness in type 2 diabetic nephropathy patients," *Metabolism*, vol. 53, no. 10, pp. 1382–1386, 2004.

[33] B. Hedblad, A. Zambanini, P. Nilsson, L. Janzon, and G. Berglund, "Rosiglitazone and carotid IMT progression rate in a mixed cohort of patients with type 2 diabetes and the insulin resistance syndrome: main results from the Rosiglitazone Atherosclerosis Study," *Journal of Internal Medicine*, vol. 261, no. 3, pp. 293–305, 2007.

[34] J. S. Sidhu, Z. Kaposzta, H. S. Markus, and J. C. Kaski, "Effect of rosiglitazone on common carotid intima-media thickness progression in coronary artery disease patients without diabetes mellitus," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 5, pp. 930–934, 2004.

[35] J. A. Dormandy, B. Charbonnel, D. J. Eckland, et al., "Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial in macroVascular Events): a randomised controlled trial," *The Lancet*, vol. 366, no. 9493, pp. 1279–1289, 2005.

[36] E. Erdmann, J. A. Dormandy, B. Charbonnel, M. Massi-Benedetti, I. K. Moules, and A. M. Skene, "The effect of pioglitazone on recurrent myocardial infarction in 2,445 patients with type 2 diabetes and previous myocardial infarction. Results from the PROactive (PROactive 05) study," *Journal of the American College of Cardiology*, vol. 49, no. 17, pp. 1772–1780, 2007.

[37] R. Wilcox, M.-G. Bousser, D. J. Betteridge, et al., "Effects of pioglitazone in patients with type 2 diabetes with or without previous stroke: results from PROactive (PROspective pioglitAzone Clinical Trial In macroVascular Events 04)," *Stroke*, vol. 38, no. 3, pp. 865–873, 2007.

[38] S. E. Nissen and K. Wolski, "Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes," *The New England Journal of Medicine*, vol. 356, no. 24, pp. 2457–2471, 2007.

[39] S. Singh, Y. K. Loke, and C. D. Furberg, "Long-term risk of cardiovascular events with rosiglitazone: a meta-analysis," *The Journal of the American Medical Association*, vol. 298, no. 10, pp. 1189–1195, 2007.

[40] P. D. Home, S. J. Pocock, H. Beck-Nielsen, et al., "Rosiglitazone evaluated for cardiovascular outcomes—an interim analysis," *The New England Journal of Medicine*, vol. 357, no. 1, pp. 28–38, 2007.

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Review Article

Peroxisome Proliferator-Activated Receptors in the Modulation of the Immune/Inflammatory Response in Atherosclerosis

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Inflammation has been recognized as an important hallmark of atherosclerosis. The pharmacological activation of PPAR-γ by the thiazolidinediones in diabetes, and of PPAR-α by the fibrates in hyperlipidemia has been shown to help to reduce inflammatory markers in preclinical and clinical studies. PPARs are known to modulate immune pathways through at least three different mechanisms: by direct binding to PPRE of anti-inflammatory cytokines genes; by transrepression of transcription factors like NF-κB and AP-1; or by corepression. The regulation of the inflammatory pathways by PPARs can be achieved on each one of the cells involved in the atherosclerotic process, that is, monocytes, macrophages, T cells, endothelial cells, and smooth muscle cells. Moreover, as each of these cellular components is interconnected with each other, PPAR activation in one cell type could affect the other ones. As activation of PPARs has clear ant-inflammatory benefits, PPARs ligands should be considered as a new therapeutical approach to ameliorate the exacerbated immune response in atherosclerotic diseases.

1. INTRODUCTION

Cardiovascular diseases represent the main cause of morbidity and mortality in western societies since the 20th century and mostly are a consequence of atherosclerosis, a prior established pathology. Atherosclerosis is defined as a progressive, chronic pathology characterized by the accumulation of lipids and fibrous elements in the large arteries. It is classified as an inflammatory disease, since in every phase of the atherosclerotic process, the immune response has a significant role [1]. Immune cells like monocytes, macrophages, and T-cells are crucial in the development of the atheroma and the stimulation and activation of endothelial cells (EC) and smooth muscle cells (SMC), cellular components of the vascular wall are extremely relevant for the recruitment of the cells responsible for the immune response.

Peroxisome proliferator-activated receptors (PPAR) and their agonists have been gaining more attention recently in regard to the study of the mechanisms involved in the etiology and pathogenesis of atherosclerosis. The expression of PPAR-α, -δ/β, and -γ in vascular wall cells and in immune cells, as well as in atherosclerotic lesions, has been described [2, 3]. The pharmacological modulations of both PPAR-α and -γ as therapeutic treatments for diabetes and hyperlipidemia have been linked to an improvement on the low-grade inflammation associated with these conditions [4, 5]. The inhibition of function and/or expression of certain genes critical for the initiation or maintenance of inflammatory cells recruitment, survival, proliferation, and activation has been shown to alter the progression of atherosclerotic lesions [6]. In this regard, the three PPAR isoforms certainly could play distinctive roles in modulating the inflammatory response in atherosclerosis.

2. PPAR: GENERAL CHARACTERISTICS

The PPARs belong to a subfamily of the nuclear receptors superfamily and are ligand-activated transcription factors which heterodimerize with the retinoic X receptor and

recognize PPAR response elements (PPRE) localized in the promoter region of target genes [2]. In addition to the direct involvement of PPAR in the gene-specific transcription, PPAR could also repress the transcription of certain genes brought about by the proinflammatory transcription factors, nuclear factor (NF)-κB, activation protein-1 (AP-1), and signal transducer and activator of transcription 1 (STAT-1), through the binding and sequestration of their corresponding cofactors [7].

Three PPAR forms have been described: PPAR-α (NR1C1), PPAR-δ/β (NR1C2), and PPAR-γ (NR1C3). These different receptors show a similar protein structure in spite of their different coding genes [3]. PPAR-α and PPAR-γ have been recognized to be key players in both cellular differentiation processes and anti-inflammatory regulation and, most recently, PPAR-δ/β has also been implicated in the immune response [8].

PPAR-α is mainly expressed in tissues characterized by a high rate of fatty acid catabolism (liver, kidney, heart, and muscle) and is the most abundantly expressed PPAR isoform in human endothelial cells (EC) [9]. The transcriptional activity of PPAR-α is stimulated by a variety of compounds (see Table 1) [2]. PPARα synthetic ligands, such as clofibrate, fenofibrate, and bezafibrate, were developed as hypolipidemic agents, through optimization of their lipid-lowering activity in rodents, even before the discovery of the PPARs [10]. PPAR-α is involved in the control of lipoproteins metabolism, fatty acid oxidation, and in the cellular uptake of fatty acids [10]. Studies in vitro suggest that PPAR-α also regulates the expression of genes that control inflammatory responses in EC, SMC, and macrophages, cells known to be implicated in the inflammatory response of vascular EC and in the pathology of atherosclerosis [9, 11].

PPAR-δ/β is ubiquitously expressed both in vascular SMC as well as in EC, besides liver, kidney, and abdominal adipose tissues. Several eicosanoids have been reported to activate PPAR-δ/β, including PGA_1 and PGD_2, and a synthetic prostaglandin carbaprostacyclin [10]. The important physiological roles of PPAR-δ/β are highlighted in genetically modified mouse models as deletion of PPAR-δ/β in mice leads to incomplete but very high penetration of a lethal phenotype and PPAR-δ/β heterozygous animals display abnormal wound healing [12]. Additionally, PPAR-δ/β has been implicated in the maintenance of lipid homeostasis [13], keratinocyte proliferation in response to injury [12, 14, 15], hyperplasic development of adipose tissue in animal under a high-fat diet [16], and was recently shown to have beneficial effects on muscle fat oxidation and lipid profiles in humans [17, 18].

The best characterized receptor in this group of nuclear factors is the PPAR-γ which plays a significant role in adipocyte differentiation and fat deposition [10, 19]. This receptor is expressed in adipose tissue, skeletal, and cardiac muscle and is also expressed in human peripheral blood monocytes and in monocytic cell lines. The large list of activators of PPAR-γ includes prostaglandin-derived 15-deoxi-delta 12,14PGJ_2 (15d-PGJ_2), the thiazolidinediones (TZD) troglitazone (Rezulin^R), pioglitazone (Actos^R), and rosiglitazone (Avandia^R), among others (see Table 1) [2, 10, 20]. The currently marketed TZDs are potent and selective PPAR-γ activators; they are antidiabetic agents that increase the insulin sensitivity of target tissues in animal models of non-insulin-dependent diabetes mellitus and in diabetic patients.

One of the features that characterize the PPAR is the large amounts of natural and synthetic molecules that can activate them. PPARs are differentially activated by naturally occurring eicosanoids and related molecules [21, 22]. Nitroalkene derivatives of fatty acids have also been characterized as endogenous PPAR ligands. Schopfer et al. [23], using CV-1 reporter cells cotransfected with plasmids containing the ligand-binding domain for PPAR-α, -δ/β, and -γ, found that nitrated linoleic acid (1 μM) (LNO_2) was capable to induce significant activation of PPAR-γ (620%), PPAR-α (325%), and PPAR-δ/β (221%), when compared to control cells. Concomitant works revealed the existence of LNO_2 and other fatty acid nitration products, generated by NO-dependent reactions, in human red cells, blood, and urine samples [24, 25].

The general approach used to study the effects of PPARs is through PPAR activation by natural or synthetic agonist. However, PPAR ligands have been shown to have both PPAR-dependent and -independent actions, which could be addressed by in vivo genetic manipulation, such as PPAR-knockout animals or in cell-based systems using small interfering RNA [26].

3. INFLAMMATION IS MODULATED BY Ox-LDL THROUGH PPAR

A primary initiating event in atherosclerosis is the accumulation of modified low-density lipoprotein (LDL) in the subendotelial matrix, such as oxidized-LDL (ox-LDL. These ox-LDL are taken up by macrophages, inducing the formation of foam cells, and stimulating the EC to produce a number of proinflammatory molecules, such as monocyte chemotactic protein (MCP)-1, whose effects are mediated by the G protein-coupled receptor CCR2, expressed mainly in monocytes, basophils, and certain subsets of T cells [27, 28].

One of the most studied factors involved in the atherosclerotic process is ox-LDL [29]. Ox-LDL provides ligands for PPAR-γ and PPAR-α [30, 31] and also seems to enhance the expression of PPAR-γ in differentiated macrophages [32]. Ox-LDL, oxidized linoleic acid, and metabolites derived from it, including 9-hydroxyoctadecaenoic acid (HODE) and 13-HODE, induce PPAR-γ activation in monocytes and monocytic cellular lines, stimulating the transcription of the ox-LDL receptor CD36/fatty acid translocase, through a PPRE in the promoter of CD36/fatty acid translocase gene, which leads to the formation of foam cells [19, 30, 33].

The first contact between ox-LDL and monocyte/macrophage cell elicits reactive oxygen species (ROS) formation, followed by a desensitization of macrophages via activation of PPAR-γ, which reduces ROS production, giving the ox-LDL a dual role in the activation/deactivation of macrophages [34]. Ox-LDL inhibited NF-κB-mediated IL-12

Table 1: Properties and agonists of PPARs.

Characteristic	PPAR-α	PPAR-γ	PPAR-δ/β
General distribution	Liver, heart, kidney, muscle. Endothelial cells	Heart, intestine, kidney, pancreas, spleen, muscle, adipose tissue	Liver, intestine, kidney, abdominal adipose tissue, skeletal muscle
Natural ligands	Saturated and unsaturated fatty acids; arachidonic acid-derived eicosanoids from the lipoxygenase pathway: 8-S-hydroxyeicosatetraenoic acid and leukotriene B4; insulin; oxidized LDL	Linoleic acid, linolenic acid, arachidonic acid, eicosapentenoic acid, 15-deoxy Δ12, 14-prostaglandin J2; 15-LOX metabolites (9-HODE and 13-HODE)	Saturated and unsaturated fatty acids; eicosanoids: PGA_1 and PGD_2
Synthetic agonists	Hypolipidemic fibrate drugs: fenofibrate, genfibrozil; plasticizers, ureidofibrates; WY14643, JTT-501, GW-2331 and PD72953	Antidiabetic thiazolidinediones: pioglitazone, troglitazone, rosiglitazone (BRL-49653), MCC-555; isoxazolidinedione JTT-501; tyrosine-based agonist: GI2-62570, GW-1929, and GW-7845 from GSK; α-alcoxy-β-phenylpropanoic acid; weak agonist: LTD4 receptor antagonist LY-171883; COX inhibitors: indomethacin, ibuprofen, fenoprofen, and flufenamic acid. Docosohexanoic derivatives	Leukotriene antagonist L-165041; phenylacetic derivatives L-796449 and L-783483; GW-2433, GW-501516, GW0742X; carbaprostacyclin
Gene/protein expression affected	Lipoprotein lipase, Apo CIII, Apo AI, Apo AII, fatty acid transporter protein, Acyl-CoA synthetase, mitochondrial HMG-CoA synthase, mitochondrial uncoupling protein 1	Acyl-CoA synthetase, fatty acid transporter protein, CD 36, lipoprotein lipase, TNF-α mitochondrial uncoupling protein 1–3; insulin-dependent glucose transporter 4	Fatty acid transporter protein, CD 36, fatty acid translocase, adipocyte lipid-binding protein, ABCA1, 14-3-3ε

production in LPS-stimulated mouse macrophages, involving both inhibition of the NF-κB-DNA interactions and physical interactions between NF-κB and PPAR-γ [35]. Activation of NF-κB is involved in the pathophysiology of many inflammatory chronic diseases, including atherosclerosis. Binding sites for NF-κB have been found in cellular adhesion molecules and chemokines [36, 37]. The NF-κB signaling pathway is activated by the proinflammatory cytokines TNF-α and IL-1α which are the major cytokine inducers of gene expression in EC. In resting macrophages, PPAR-γ ligands completely blocked the ox-LDL-mediated activation of NF-κB [38].

PPAR-α activation by ox-LDL in the vascular wall components seems to upregulate the inflammation. The activation of this receptor in human EC by oxidized components in LDL resulted in an increase in the production of chemotactic factors for monocytes (MCP-1 and IL-8), conferring it a proinflammatory effect [9].

4. MOLECULAR MECHANISMS FOR THE REGULATION OF INFLAMMATORY/IMMUNE RESPONSE IN ATHEROCLEROSIS BY PPAR AGONISTS

The adhesion of monocytes to the vascular wall is mediated by adhesion molecules expressed on the surface of the EC, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), P and E selectins [39], which is a phenotypic hallmark of EC activation and a critical step of many proinflammatory processes. The constitutive activation of PPAR-γ in ECs inhibited the expression of VCAM-1, ICAM-1, and E-selectin, by interference with NF-κB and AP-1 transactivation [40].

Many of the immune cells in the atheroma exhibit signs of activation and produce proinflammatory cytokines. In addition to monocytes and macrophages, T cells definitely play a significant role in the lesion, where CD4+ T cells dominate over CD8+ cells. CD4+ T cells differentiate into

Table 2: PPAR-mediated immune downregulation in monocytes/macrophages, T cells, EC, and SMC.

Monocyte/macrophage	T cell	EC	SMC
Reduced expression of TNF-α, CCR-2, synthesis of IL-6, IL-1 by PPAR-γ. Oxidative burst suppressed by PPAR-γ.	Reduced expression of TNF-α, IFN-γ, IL-2 by activation of PPAR-γ and -α.	Reduced expression of VCAM-1, ICAM-1, E-selectin and MHC-II by PPAR-γ, -α, and -δ/β; impaired TNF-α activation.	Reduced synthesis of IL6, COX-2, and prostaglandin by PPAR-α.

several subtypes, being T helper-1 cells (Th1) the predominant pattern in atherosclerosis. IFN-γ is a major proatherogenic Th1 cytokine, promoting macrophage and endothelial activation with production of adhesion molecules, cytokines, chemokines, radicals, proteases, and coagulation factors. In addition, IFN-γ inhibits cell proliferation, collagen production, and cholesterol efflux [41]. PPAR-α and PPAR-γ mRNA, and protein are expressed in isolated human CD4+ T cells, and the activation of each one of them by specific ligands reduces the secretion of IFN-γ, TNF-α, and IL-2 in these lymphocytes [42]. Furthermore, the effect of PPAR agonist on CD4+ T cells impaired their action on monocytes and EC, suggesting that PPAR modulation of inflammatory pathways in T cells may offer clinical benefits in atherosclerosis [43].

TNFα is a catabolic proinflammatory cytokine, produced by Th1 cells and macrophages, that exerts a wide range of effects on cells and tissues, through the activation of the transcription factor NF-κB. Gene targeting of TNFα leads to reduced atherosclerosis [42]. It has been shown that human aortic EC activation by TNFα could be prevented by incubation with MCC-555, a novel TZD, while pioglitazone and rosiglitazone did not [44].

PPAR-α has been shown to inhibit transcriptional responses to inflammatory stimuli by interfering with the activation of NF-κB, leading to the reduction of VCAM-1 in EC [11]. In vascular SMC, PPAR-α agonists inhibited IL-1-induced IL-6 expression, cyclooxygenase-2 (COX-2) and prostaglandin production [45]. The upregulation of antioxidant enzymes activity by the PPAR-α activators reduced the oxidative stress and, as the result, it might inhibit the NF-κB activation and subsequent inflammatory response [46].

The regulation of chemokine-receptor expression may be a crucial mechanism to control monocyte responses to chemokines. Monocytic-line THP-1 cells incubated with rosiglitazone reduced CCR2 surface expression by about 50–60% ($P < .001$) compared with untreated control cells [29]. PPAR-γ agonists suppress monocyte elaboration of inflammatory cytokines TNFα, IL1, and IL6 [47]. Table 2 summarizes the general effects of PPAR activation on each cell type.

In murine hypercholesterolemic models, the administration of PPAR-γ ligands inhibited the development of atherosclerosis, in spite of the high expression of CD36 in the vascular wall [48]. Downstream PPARγ-dependent anti-inflammatory effects of 15d-PGJ_2 include the inhibition of transcriptional activation by NF-κB via IκB, which affect gene expression of inducible nitric oxide synthase (iNOS), TNFα, COX-2, IFN-α, IL-1, IL-6, and LPS-induced transcription of AP-1 and STAT-1 [49]. Oxidative burst in macrophages is also attenuated by PPAR-γ activation [50].

Other regulatory mechanism that could be attributed to PPAR is the selective activation of anti-inflammatory cytokines, like IL-10. IL-10 has potent deactivating properties in macrophages and T cells and modulates many cellular processes that may interfere with the development and stability of the atherosclerotic plaque [51]. Using nanomolar concentrations of rosiglitazone, Thompson et al. have demonstrated the upregulation of IL-10, likely through a functional PPRE found in the promoter region of IL-10 gene [52].

Major histocompatibility complex class II molecules (MHC-II) play a critical role in the induction of immune responses by presenting peptides of foreign antigens to CD4+ T lymphocytes, which result in their activation and proliferation. Human ECs are capable of expressing MHC-II under treatment with IFN-γ and this induction is repressed by PPAR-γ ligands [53].

PPAR-δ/β seems to have dual effects in regard to inflammation in atherosclerotic models [8, 54–57]. Although results from both PPAR-δ/β–/– and PPAR-δ/β overexpressing macrophages suggested a proinflammatory role for PPAR-δ/β, treatment of cells with PPAR-δ/β agonist GW501516 suppressed the expression of MCP-1 and IL-1β in a receptor-dependent manner, indicating that activation of PPAR-δ/β had an anti-inflammatory effect [54]. The pharmacological modulation of PPAR-δ/β in atherosclerotic LDLR–/– mice showed decreased expression of MCP-1, TNFα, and ICAM-1 [55] and similarly, proinflammatory modulators were suppressed in apoE–/– mice treated with GW501516 [56]. Fan et al. also found an anti-inflammatory effect of PPAR-δ/β agonists in TNFα-activated EC [8]. In addition, Takata et al. have found that PPAR-δ/β agonist GW0742 substantially inhibited vascular proinflammatory gene expression, macrophage content, and atherosclerosis in an angiotensin II-induced high fat-fed male LDLR–/– mouse model of accelerated atherosclerosis [57]. Furthermore, promising results were obtained in a clinical evaluation of the PPAR-δ/β agonist GW501516 in six obese males [17]. Although inflammatory markers were not considered in this study, the wide range of beneficial effects by the pharmacological activation of PPAR-δ/β could suggest an improvement on the inflammatory grade of proatherogenic conditions and an attractive therapeutic target for drug development to treat atherosclerosis [56].

Post-translational modifications have been found to modulate transcriptional activity of PPAR-γ [57]. One of these modifications is sumoylation, the covalent attachment

of a small ubiquitin-like proteins (Ubl) called SUMO-1. Pascual et al. [58] proposed a novel pathway mediating ligand-dependent transrepression of inflammatory response genes by PPAR-γ in macrophages which involves ligand-dependent sumoylation of the PPAR-γ ligand-binding domain. This targets PPAR-γ to nuclear receptor corepressor (NCoR)/histone deacetylase-3 (HDAC3) complexes on inflammatory gene promoters, which in turn prevents recruitment of the ubiquitylation/19S proteosome machinery that normally mediates the signal-dependent removal of corepressor complexes required for gene activation. As a result, NCoR complexes are not cleared from the promoter and target genes are maintained in a repressed state. This mechanism provides an explanation for how an agonist-bound nuclear receptor can be converted from an activator of transcription to a promoter-specific repressor of NF-κB target genes that regulate immunity and homeostasis [59].

5. CONCLUSION

Basic and clinical research points out towards an intrinsic interplay between immune/inflammatory mediators and PPAR activation in the pathogenesis and development of atherosclerosis. Each one of PPARs seems to have and share different output in order to reach the cellular homeostasis. It could be seen that Inflammation, as a disruption of homeostasis, has not only internal control by its own but an external regulation through the activation of PPARs. However, it is still not clear what are the "real functional in vivo" natural ligands of PPAR. Meanwhile, the search for the ideal synthetic ligand that would combine the beneficial effects of PPAR activation is ongoing. Such "magical" drug would be prescribed either as hypoglycemic, hypolipemic and anti-inflammatory agent, but would need to be chronically administered. Overall, pharmacological activation of PPARs might be a better approach to cover all the underlying inflammatory features of the atherosclerosis.

ABBREVIATIONS

ABC: ATP-binding cassette
ALBP: Adipocyte lipid binding protein
AP-1: Activation protein-1
COX: Cyclooxygenase
EC: Endothelial cells
FAT: Fatty acid translocase
HDAC3: Histone deacetylase-3
HDL: High-density lipoprotein
HMG-CoA: Hydroxy methyl glutaryl coenzyme A
HODE: Hydroxy octadecaenoic acid
ICAM-1: Intercellular adhesion molecule 1
IFN: Interferon
IL: Interleukin
LDL: Low-density lipoprotein
LNO_2: Nitrated linoleic acid
LPS: Lipopolysaccharide
MCP: Monocyte chemotactic protein
MHC-II: Major histocompatibility complex class II molecules (MHC-II)
NCoR: Nuclear receptor corepressor
NF-κB: Nuclear factor κB
NOS: Nitric oxide synthase
PG: Prostaglandin
PPAR: Peroxisome proliferator-activated receptors
PPRE: PPAR response elements
ROS: Reactive oxygen species
SMC: Smooth muscle cells
STAT-1: Signal transducer and activator of transcription 1
Th: T helper cells
TNF: Tumor necrosis factor
TZD: Thiazolidinediones
Ubl: Ubiquitin-like proteins
VCAM-1: Vascular cell adhesion molecule-1.

REFERENCES

[1] R. Ross, "Atherosclerosis—an inflammatory disease," *The New England Journal of Medicine*, vol. 340, no. 2, pp. 115–126, 1999.
[2] G. Chinetti, J.-C. Fruchart, and B. Staels, "Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation," *Inflammation Research*, vol. 49, no. 10, pp. 497–505, 2000.
[3] B. P. Neve, J.-C. Fruchart, and B. Staels, "Role of the peroxisome proliferator-activated receptors (PPAR) in atherosclerosis," *Biochemical Pharmacology*, vol. 60, no. 8, pp. 1245–1250, 2000.
[4] S. Cuzzocrea, B. Pisano, L. Dugo, et al., "Rosiglitazone, a ligand of the peroxisome proliferator-activated receptor-γ, reduces acute inflammation," *European Journal of Pharmacology*, vol. 483, no. 1, pp. 79–93, 2004.
[5] R. Adamiec, M. Gacka, T. Dobosz, S. Szymaniec, D. Bednarska-Chabowska, and A. Sadakierska-Chudy, "Stimulation of the peroxisome proliferator-activated receptor γ (PPARγ) and the expression of selected blood monocyte cytokine genes in diabetic macroangiopathy," *Atherosclerosis*, vol. 194, no. 2, pp. e108–e115, 2007.
[6] P. K. Shah, "Circulating markers of inflammation for vascular risk prediction: are they ready for prime time," *Circulation*, vol. 101, no. 15, pp. 1758–1759, 2000.
[7] G. Chinetti, S. Lestavel, V. Bocher, et al., "PPAR-α and PPAR-γ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway," *Nature Medicine*, vol. 7, no. 1, pp. 53–58, 2001.
[8] Y. Fan, Y. Wang, Z. Tang, et al., "Suppression of pro-inflammatory adhesion molecules by PPAR-δ in human vascular endothelial cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 2, pp. 315–321, 2008.
[9] H. Lee, W. Shi, P. Tontonoz, et al., "Role for peroxisome proliferator-activated receptor α in oxidized phospholipid-induced synthesis of monocyte chemotactic protein-1 interleukin-8 by endothelial cells," *Circulation Research*, vol. 87, no. 6, pp. 516–521, 2000.
[10] T. M. Willson, P. J. Brown, D. D. Sternbach, and B. R. Henke, "The PPARs: from orphan receptors to drug discovery," *Journal of Medicinal Chemistry*, vol. 43, no. 4, pp. 527–550, 2000.
[11] N. Marx, G. K. Sukhova, T. Collins, P. Libby, and J. Plutzky, "PPARα activators inhibit cytokine-induced vascular cell adhesion molecule-1 expression in human endothelial cells," *Circulation*, vol. 99, no. 24, pp. 3125–3131, 1999.

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

[13] T. E. Akiyama, G. Lambert, C. J. Nicol, et al., "Peroxisome proliferator-activated receptor β/δ regulates very low density lipoprotein production and catabolism in mice on a Western diet," *The Journal of Biological Chemistry*, vol. 279, no. 20, pp. 20874–20881, 2004.

[14] D. W. Gilroy and P. R. Colville-Nash, "New insights into the role of COX 2 in inflammation," *Journal of Molecular Medicine*, vol. 78, no. 3, pp. 121–129, 2000.

[15] N. S. Tan, L. Michalik, N. Noy, et al., "Critical roles of PPARβ/δ in keratinocyte response to inflammation," *Genes & Development*, vol. 15, no. 24, pp. 3263–3277, 2001.

[16] C. Jehl-Pietri, C. Bastie, I. Gillot, S. Luquet, and P. A. Grimaldi, "Peroxisome-proliferator-activated receptor δ mediates the effects of long-chain fatty acids on post-confluent cell proliferation," *Biochemical Journal*, vol. 350, no. 1, pp. 93–98, 2000.

[17] U. Risérus, D. Sprecher, T. Johnson, et al., "Activation of peroxisome proliferator-activated receptor (PPAR)δ promotes reversal of multiple metabolic abnormalities, reduces oxidative stress, and increases fatty acid oxidation in moderately obese men," *Diabetes*, vol. 57, no. 2, pp. 332–339, 2008.

[18] W. R. Oliver Jr., J. L. Shenk, M. R. Snaith, et al., "A selective peroxisome proliferator-activated receptor δ agonist promotes reverse cholesterol transport," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 9, pp. 5306–5311, 2001.

[19] M.-B. Debril, J.-P. Renaud, L. Fajas, and J. Auwerx, "The pleiotropic functions of peroxisome proliferator-activated receptor γ," *Journal of Molecular Medicine*, vol. 79, no. 1, pp. 30–47, 2001.

[20] J. M. Lehmann, J. M. Lenhard, B. B. Oliver, G. M. Ringold, and S. A. Kliewer, "Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs," *The Journal of Biological Chemistry*, vol. 272, no. 6, pp. 3406–3410, 1997.

[21] K. Yu, W. Bayona, C. B. Kallen, et al., "Differential activation of peroxisome proliferator-activated receptors by eicosanoids," *The Journal of Biological Chemistry*, vol. 270, no. 41, pp. 23975–23983, 1995.

[22] J. U. Scher and M. H. Pillinger, "15d-PGJ2: the anti-inflammatory prostaglandin?" *Clinical Immunology*, vol. 114, no. 2, pp. 100–109, 2005.

[23] F. J. Schopfer, Y. Lin, P. R. S. Baker, et al., "Nitrolinoleic acid: an endogenous peroxisome proliferator-activated receptor γ ligand," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 7, pp. 2340–2345, 2005.

[24] P. R. S. Baker, Y. Lin, F. J. Schopfer, et al., "Fatty acid transduction of nitric oxide signaling: multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands," *The Journal of Biological Chemistry*, vol. 280, no. 51, pp. 42464–42475, 2005.

[25] P. R. S. Baker, F. J. Schopfer, S. Sweeney, and B. A. Freeman, "Red cell membrane and plasma linoleic acid nitration products: synthesis, clinical identification, and quantitation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 32, pp. 11577–11582, 2004.

[26] R. Cunard, M. Ricote, D. DiCampli, et al., "Regulation of cytokine expression by ligands of peroxisome proliferator activated receptors," *The Journal of Immunology*, vol. 168, no. 6, pp. 2795–2802, 2002.

[27] A. J. Lusis, "Atherosclerosis," *Nature*, vol. 407, no. 6801, pp. 233–241, 2000.

[28] K. H. Han, M. K. Chang, A. Boullier, et al., "Oxidized LDL reduces monocyte CCR2 expression through pathways involving peroxisome proliferator-activated receptor γ," *The Journal of Clinical Investigation*, vol. 106, no. 6, pp. 793–802, 2000.

[29] J. Galle, T. Hansen-Hagge, C. Wanner, and S. Seibold, "Impact of oxidized low density lipoprotein on vascular cells," *Atherosclerosis*, vol. 185, no. 2, pp. 219–226, 2006.

[30] L. Nagy, P. Tontonoz, J. G. A. Alvarez, H. Chen, and R. M. Evans, "Oxidized LDL regulates macrophage gene expression through ligand activation of PPARγ," *Cell*, vol. 93, no. 2, pp. 229–240, 1998.

[31] G. Leonarduzzi, M. C. Arkan, H. Başağa, E. Chiarpotto, A. Sevanian, and G. Poli, "Lipid oxidation products in cell signaling," *Free Radical Biology and Medicine*, vol. 28, no. 9, pp. 1370–1378, 2000.

[32] M. Ricote, J. Huang, L. Fajas, et al., "Expression of the peroxisome proliferator-activated receptor γ (PPARγ) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 13, pp. 7614–7619, 1998.

[33] J. Feng, J. Han, S. F. A. Pearce, et al., "Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-γ," *Journal of Lipid Research*, vol. 41, no. 5, pp. 688–696, 2000.

[34] B. Fischer, A. von Knethen, and B. Br"une, "Dualism of oxidized lipoproteins in provoking and attenuating the oxidative burst in macrophages: role of peroxisome proliferator-activated receptor-γ," *The Journal of Immunology*, vol. 168, no. 6, pp. 2828–2834, 2002.

[35] S. W. Chung, B. Y. Kang, S. H. Kim, et al., "Oxidized low density lipoprotein inhibits interleukin-12 production in lipopolysaccharide-activated mouse macrophages via direct interactions between peroxisome proliferator-activated receptor-γ and nuclear factor-κB," *The Journal of Biological Chemistry*, vol. 275, no. 42, pp. 32681–32687, 2000.

[36] R. Schreck, K. Albermann, and P. A. Baeuerle, "Nuclear factor κB: an oxidative stress-responsive transcription factor of eukaryotic cells," *Free Radical Research Communications*, vol. 17, no. 4, pp. 221–237, 1992.

[37] S. Schoonbroodt and J. Piette, "Oxidative stress interference with the nuclear factor-κB activation pathways," *Biochemical Pharmacology*, vol. 60, no. 8, pp. 1075–1083, 2000.

[38] C.-Y. Han, S.-Y. Park, and Y. K. Pak, "Role of endocytosis in the transactivation of nuclear factor-κB by oxidized low-density lipoprotein," *Biochemical Journal*, vol. 350, no. 3, pp. 829–837, 2000.

[39] D. T. Price and J. Loscalzo, "Cellular adhesion molecules and atherogenesis," *The American Journal of Medicine*, vol. 107, no. 1, pp. 85–97, 1999.

[40] N. Wang, L. Verna, N.-G. Chen, et al., "Constitutive activation of peroxisome proliferator-activated receptor-γ suppresses pro-inflammatory adhesion molecules in human vascular endothelial cells," *The Journal of Biological Chemistry*, vol. 277, no. 37, pp. 34176–34181, 2002.

[41] G. K. Hansson, A.-K. L. Robertson, and C. S"oderberg-Nauclér, "Inflammation and atherosclerosis," *Annual Review of Pathology*, vol. 1, no. 1, pp. 297–329, 2006.

[42] L. Brånén, L. Hovgaard, M. Nitulescu, E. Bengtsson, J. Nilsson, and S. Jovinge, "Inhibition of tumor necrosis factor-α reduces atherosclerosis in apolipoprotein E knockout mice,"

Arteriosclerosis, Thrombosis, and Vascular Biology, vol. 24, no. 11, pp. 2137–2142, 2004.

[43] N. Marx, B. Kehrle, K. Kohlhammer, et al., "PPAR activators as anti-inflammatory mediators in human T lymphocytes: implications for atherosclerosis and transplantation-associated arteriosclerosis," *Circulation Research*, vol. 90, no. 6, pp. 703–710, 2002.

[44] S. Kurebayashi, X. Xu, S. Ishii, M. Shiraishi, H. Kouhara, and S. Kasayama, "A novel thiazolidinedione MCC-555 down-regulates tumor necrosis factor-α-induced expression of vascular cell adhesion molecule-1 in vascular endothelial cells," *Atherosclerosis*, vol. 182, no. 1, pp. 71–77, 2005.

[45] P. Delerive, K. De Bosscher, S. Besnard, et al., "Peroxisome proliferator-activated receptor α negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-κB and AP-1," *The Journal of Biological Chemistry*, vol. 274, no. 45, pp. 32048–32054, 1999.

[46] A. Tedgui and Z. Mallat, "Anti-inflammatory mechanisms in the vascular wall," *Circulation Research*, vol. 88, no. 9, pp. 877–887, 2001.

[47] C. Jiang, A. T. Ting, and B. Seed, "PPAR-γ agonists inhibit production of monocyte inflammatory cytokines," *Nature*, vol. 391, no. 6662, pp. 82–86, 1998.

[48] A. C. Li, K. K. Brown, M. J. Silvestre, T. M. Willson, W. Palinski, and C. K. Glass, "Peroxisome proliferator-activated receptor γ ligands inhibit development of atherosclerosis in LDL receptor-deficient mice," *The Journal of Clinical Investigation*, vol. 106, no. 4, pp. 523–531, 2000.

[49] Y. Ye, S. P. Nishi, S. Manickavasagam, et al., "Activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) by atorvastatin is mediated by 15-deoxy-$\Delta^{12,14}$-PGJ_2," *Prostaglandins & Other Lipid Mediators*, vol. 84, no. 1-2, pp. 43–53, 2007.

[50] A. von Knethen and B. Br"une, "Delayed activation of PPARγ by LPS and IFN-γ attenuates the oxidative burst in macrophages," *The FASEB Journal*, vol. 15, no. 2, pp. 535–544, 2001.

[51] Z. Mallat, S. Besnard, M. Duriez, et al., "Protective role of interleukin-10 in atherosclerosis," *Circulation Research*, vol. 85, no. 8, pp. e17–e24, 1999.

[52] P. W. Thompson, A. I. Bayliffe, A. P. Warren, and J. R. Lamb, "Interleukin-10 is upregulated by nanomolar rosiglitazone treatment of mature dendritic cells and human CD4+ T cells," *Cytokine*, vol. 39, no. 3, pp. 184–191, 2007.

[53] B. R. Kwak, S. Myit, F. Mulhaupt, et al., "PPARγ but not PPARα ligands are potent repressors of major histocompatibility complex class II induction in atheroma-associated cells," *Circulation Research*, vol. 90, no. 3, pp. 356–362, 2002.

[54] C.-H. Lee, A. Chawla, N. Urbiztondo, D. Liao, W. A. Boisvert, and R. M. Evans, "Transcriptional repression of atherogenic inflammation: modulation by PPARδ," *Science*, vol. 302, no. 5644, pp. 453–457, 2003.

[55] T. L. Graham, C. Mookherjee, K. E. Suckling, C. N. A. Palmer, and L. Patel, "The PPARδ agonist GW0742X reduces atherosclerosis in $LDLR^{-/-}$ mice," *Atherosclerosis*, vol. 181, no. 1, pp. 29–37, 2005.

[56] G. D. Barish, A. R. Atkins, M. Downes, et al., "PPARδ regulates multiple proinflammatory pathways to suppress atherosclerosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 11, pp. 4271–4276, 2008.

[57] Y. Takata, J. Liu, F. Yin, et al., "PPARδ-mediated anti-inflammatory mechanisms inhibit angiotensin II-accelerated atherosclerosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 11, pp. 4277–4282, 2008.

[58] G. Pascual, A. L. Fong, S. Ogawa, et al., "A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-γ," *Nature*, vol. 437, no. 7059, pp. 759–763, 2005.

[59] T. Ohshima, H. Koga, and K. Shimotahno, "Transcriptional activity of peroxisome proliferator-activated receptor γ is modulated by SUMO-1 modification," *The Journal of Biological Chemistry*, vol. 279, no. 28, pp. 29551–29557, 2004.

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Review Article

PPARα/γ-Independent Effects of PPARα/γ Ligands on Cysteinyl Leukotriene Production in Mast Cells

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Peroxisome proliferator-activated receptor (PPAR) α ligands (Wy-14,643, and fenofibrate) and PPARγ ligands (troglitazone and ciglitazone) inhibit antigen-induced cysteinyl leukotriene production in immunoglobulin E-treated mast cells. The inhibitory effect of these ligands on cysteinyl leukotriene production is quite strong and is almost equivalent to that of the anti-asthma compound zileuton. To develop new aspects for anti-asthma drugs the pharmacological target of these compounds should be clarified. Experiments with bone-marrow-derived mast cells from PPARα knockout mice and pharmacological inhibitors of PPARγ suggest that the inhibitory effects of these ligands are independent of PPARs α and γ. The mechanisms of the PPAR-independent inhibition by these agents on cysteinyl leukotriene production are discussed in this review.

1. INTRODUCTION

Asthma is defined as "a common chronic disorder of the airways that is complex and characterized by variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammation" [1]. Many types of inflammatory cells, neutrophils, eosinophils, lymphocytes, and mast cells contribute to the development of asthma.

Mast cells are differentiated from bone marrow stem cells and release various mediators of inflammation, such as histamine, through degranulation and arachidonic acid metabolites through *de novo* synthesis in response to pathological stimuli in asthma, atopic dermatitis, and other conditions. Immunoglobulin (Ig) E, a protein from B lymphocytes, increases in the serum of patients with type I allergic diseases [2].

Arachidonic acid is metabolized into many biologically active lipids, such as prostaglandins via cyclooxygenase, and leukotrienes (LTs) via 5-lipoxygenase (5-LOX). Arachidonic acid liberated from membrane phospholipids by phospholipase A_2 is then metabolized into LTA_4 by the 5-LOX/5-LOX activating protein (FLAP) complex (Figure 1). LTA_4 is metabolized into LTC_4 by conjugating cysteine, glycine, and glutamic acid via LTC synthase [3]. LTC_4 is subsequently metabolized into LTD_4 and LTE_4 via the contribution of dipeptidases [4] or cytochrome P450 [5] by glutamic acid and glycine degradation (Figure 2). The LTs C_4, D_4, and E_4 are called cysteinyl LTs (cysLTs) because they contain cysteine in their molecules. The cysLTs are regarded as main mediators of asthma because of their potent constricting effects on bronchiolar smooth muscle [6]. Specific receptors of cysLT are known [7, 8], and the inhibitors of the receptor [9] and the inhibitors of 5-LOX/FLAP activity [10–12] have been used to treat asthma.

Peroxisome proliferator-activated receptors (PPARs) are a family of transcription factors that are part of the nuclear receptor superfamily. The PPARs have 3 subtypes from the independent genes α, β (also called δ), and γ. A group of hypolipidemic agents, such as clofibrate and fenofibrate, are known to be ligands for PPARα, and some agents used to treat type 2 diabetes mellitus, such as rosiglitazone, pioglitazone, and ciglitazone, are known to be ligands for PPARγ. Some physiological fatty acids, such as leukotriene B_4 and 15-deoxy-$\Delta^{12\text{-}14}$ prostaglandin J_2, are reported to be ligands for PPARα and PPARγ, respectively [15, 16].

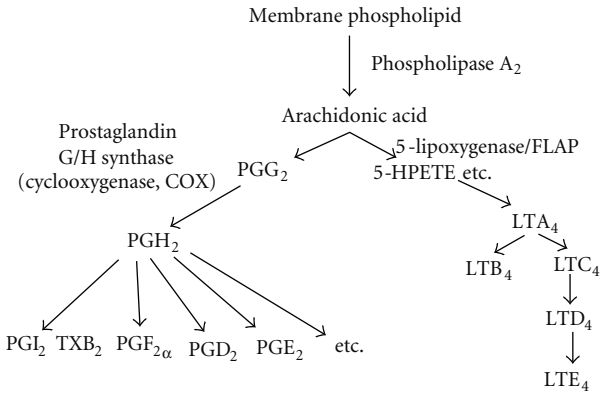

Figure 1: Diagram of arachidonic acid metabolism.

2. LIGANDS FOR PPARγ INHIBIT cysLT PRODUCTION IN MAST CELLS

Troglitazone (1 μM), a PPARγ ligand formerly used to treat type 2 diabetes mellitus, inhibits LTB_4, LTC_4, and LTE_4 production induced by the type I allergy mechanism in a mast cell line, RBL-2H3 [17]. The inhibitory effects of troglitazone on these LTs are strong and similar to those of the clinically-used 5-LOX inhibitor zileuton (1 μM) [17]. Another PPARγ ligand, ciglitazone (30 μM), also inhibits LTC_4 production [18]. Neither troglitazone nor ciglitazone affects hexosaminidase release, the index for mast cell degranulation, or prostaglandin D_2 production via cyclooxygenase [17, 18]. The observations that 0.1 μM of the PPARγ antagonist GW9662, which inhibits the PPARγ activation of $(AOx)_3$-TK-Luc promoter induced by the PPARγ ligand rosiglitazone [19], did not affect LTC_4 production [18] and that 30 μM of GW9662 inhibits LTC_4 production (our unpublished data) in the IgE-sensitized, and Ag-treated RBL-2H3 mast cell line obscures the contribution of PPARγ on LT production in mast cells.

3. LIGANDS FOR PPARα ALSO INHIBIT cysLT PRODUCTION IN MAST CELLS

Whether PPARα ligands affect LT production in mast cells has been examined, and the PPARα ligands fenofibrate (100 μM) and Wy-14,643(30 μM) have been reported to inhibit calcium ionophore A23187-induced cysLT production by the RBL-2H3 mast cell line [13]. However, Wy-14,643 does not significantly inhibit cysLT production by the IgE-sensitized and Ag-treated RBL-2H3 mast cell line. Neither fenofibrate (100 μM) nor Wy-14,643 (30 μM) affects radioactivity released from the IgE sensitized [^{3}H]-arachidonic acid prelabeled RBL-2H3 mast cell line following treatment with Ag, which is an index of arachidonic acid release from mast cells. Neither fenofibrate (100 μM) nor WY-14,643 (30 μM) affects lipid peroxidation, which is an index of 5-LOX activation, whereas troglitazone (1 μM) and zileuton (1 μM) strongly inhibit lipid peroxidation [13].

4. ARE THE INHIBITORY EFFECTS OF THESE PPARs LIGANDS VIA PPARs?

Subsequently, the mRNA levels of PPARs α and γ were examined in mast cells. There were no significant PPARα [13] and PPARγ (our unpublished data) bands on Northern blot analysis of the RBL-2H3 mast cell line or of mouse bone marrow-derived mast cells (BMMCs). Then, PPARα [13] and γ [14] mRNA levels in RBL-2H3 mast cell line were measured with the real-time semiquantitative polymerase chain reaction (PCR) and compared with levels in other organs. The PPARα mRNA level is less than the level in 1000-times diluted liver, and the PPARγ mRNA level is almost the same as the level in 100-times diluted white adipose tissue (Figure 3).

These observations that mast cells have very low levels of PPARα/γ mRNA lead to another question: are these PPARs in mast cells effective?

Studies have examined whether fenofibrate (100 μM) raises acyl-CoA oxidase mRNA levels, which are known to be induced by PPARα activation [20, 21], and have shown that fenofibrate does not increase acyl-CoA oxidase mRNA levels in the RBL-2H3 mast cell line [13]. The effects of these PPARα ligands on BMMCs from PPARα-null mice were thoroughly examined, and both fenofibrate (100 μM) and Wy-14,643 (30 μM) were found to inihbit cysLT production [13]. It has been concluded that these compounds inhibit cysLT production independently of PPARα.

We have observed that the immunoreactivity of anti-PPARγ IgG in the RBL-2H3 mast cell line though ciglitazone (30 μM) does not induce the mRNA level of acyl-CoA binding protein [18], which is a target gene of PPARγ [22]. Diaz et al. [23] have examined PPARγ protein in mouse BMMCs by SDS-PAGE immunoblot analysis and reported that the amount of PPARγ in BMMCs is equivalent to that in the Jurkat T-cell line, which is known to have effective PPARγ [24]. Maeyama et al. [25] have demonstrated that rosiglitazone (1–30 μM) increases the proliferation of BMMCs, but that the proliferation is not observed in BMMCs from PPARγ heterozygous deficient mice. Ward and Tan [26] have reviewed the contents of PPARs in various types of cells and have concluded that the PPARγ in mast cells might play a role, and Paruchuri et al. [27] have recently reported that LTE_4-induced COX-2 induction, prostaglandin D_2 production, and ERK phosphorylation are sensitive for the interference of PPARγ in the human mast cell sarcoma line LAD2 and may indicate a role of PPARγ in mast cells. Further studies of the role of PPARγ in mast cells are necessary.

5. WHAT IS THE TARGET?

The experimental findings that PPARs α and γ in mast cells seem not to be effective at very low mRNA levels lead to another question: what is the target of these compounds?

Fenofibrate (25 mg/kg p.o. for 10 days) induces proliferation of peroxisomes even in PPARα-null mice [28]. Wy-14,643 (75 μM) induces plasminogen activator inhibitor I with the induction of p38 and p42 mitogen-activated protein

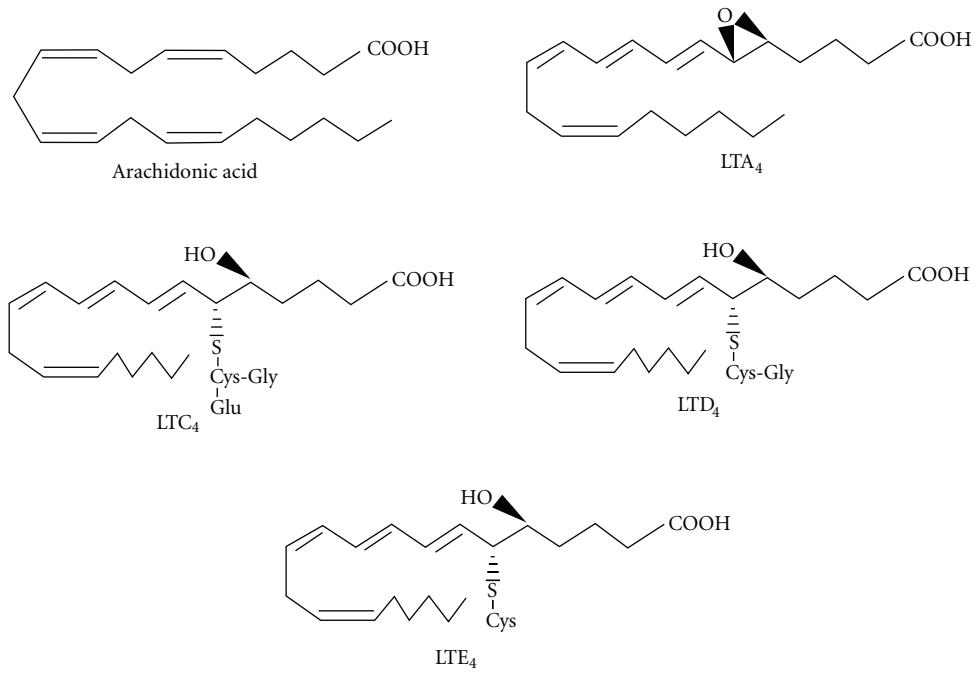

Figure 2: Chemical structures of arachidonic acid and cysteinyl leukotrienes.

kinase (MAPK) phosphorylation 5 minutes after treatment, which would be too early for the induction to occur via transcription [29]. The ligand Wy-14,643 (1 μM) leads to the phosphorylation of extracellular signal-regulated kinase (ERK) after 5 minutes of treatment but does not increase acyl-CoA oxidase mRNA levels [30].

The PPARγ ligands ciglitazone (20 μM) and 15-deoxy-$\Delta^{12\text{-}14}$ prostaglandin J_2(15 μM) induce ERK, c-Jun N-terminal kinase, and p38 MAPK after 15 minutes of treatment, which might be earlier than transcription occurs [31]. The inducible effects of PPARγ ligands on MAPK have been reported elsewhere [32, 33], and most authors have concluded that these effects are independent of PPARγ.

MAPK is reported to induce 5-LOX activity in human polymorphonuclear cells and the Mono Mac 6 human monocytic leukemia cell line [34], and these findings may support the presence of PPAR-independent effects of PPAR α and γ ligands. However, MAPK phosphorylation has not been observed in mast cells treated with these PPAR ligands. The stimulating effect of these compounds on MAPK seems not to be the main mechanism of the PPAR-independent inhibition of cysLT production because it might increase the production of cysLTs.

The cysLT concentration is determined by subtracting degradation from production, and the PPAR-independent activation of MAPK increases cysLT production in mast cells. The degradation of cysLTs could be another mechanism of these drugs. The responsible enzymes of cysLT metabolism remain unclear. Recent findings that LTC_4 is metabolized into LTD_4 by γ-glutamyltransferase and γ-glutamylleukotrienase and that of double knockout mice of these enzymes do not metabolize LTC_4 into LTD_4 may indicate that these enzymes are the enzymes responsible for LTC_4 degradation [35]. The degradation of LTD_4 into LTE_4 is reported to occur partly because of dipeptidase [36], but the responsible enzyme is still unclear. Induction of cytochrome P450 (CYP) 2B1/2 by phenobarbital in rats and the decrease in LTC_4 concentrations in liver extract suggest the involvement of CYP2B1/2 in LTC_4 degradation [37]. The CYP family comprises a large number of enzymes, and we do not yet have sufficient information on the contribution of CYP to cysLT metabolism.

Fujimura et al. [38] have reported that incubation with prostaglandin A_1 (as PPARβ/δ ligand) and 15-deoxy-$\Delta^{12\text{-}14}$ prostaglandin J_2(as PPARγ ligand) for more than 6 hours decreases the surface IgE receptor Fc ε RI in the KU812 human basophilic cell line, whereas LTB_4 (as PPARα ligand) does not. The PPARα and γ ligands were preincubated for 2 hours before antigen treatment in mast cells [13, 17, 18], and the decrease of Fc ε RI on the surface of mast cells is not the main mechanism of the PPAR-independent inhibition of cysLT production. Regulation of the sensitivity to antigens is of pathological interest in allergic diseases, including asthma, and the interaction of mast cells with other inflammatory cells in pathological conditions should be examined.

6. CONCLUSION

These findings show that some effects of ligands of PPARs α and γ occur through a mechanism independent of PPARs α and γ. The involvement of PPARs α and γ should be examined in pharmacological experiments of PPAR ligands and of ligands of other nuclear receptors.

The involvement of PPARα in the effects of PPAR ligands can be investigated in PPARα-null mice [39] and at lower cost in mast cells, as described above.

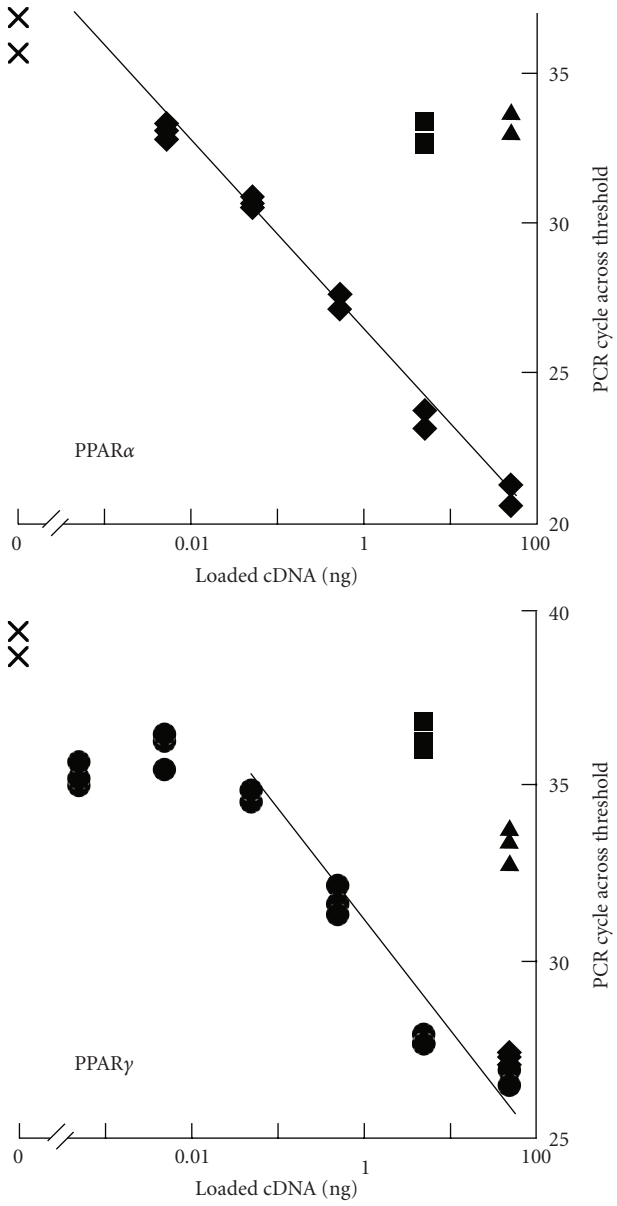

Figure 3: Measurement of mRNA levels of PPARα (upper panel) and PPARγ (lower panel) with real-time semiquantitative PCR. Total RNA (1 μg) extracted from white adipose tissue (●), liver (▲), BMMC (■), and RBL-2H3 mast cells (♦) was supplemented with 50 pg of chloramphenicol acetyltransferase RNA and then reverse-transcribed. The indicated amounts of cDNA were applied to real-time PCR. PCR performed without cDNA was used as a negative control (×) of the reaction. Data are presented as the number of PCR cycles to cross the threshold. Messenger RNA levels in these tissues were extrapolated from the PCR cycle of the liver for PPARα or white adipose tissue for PPARγ and then corrected by the chloramphenicol acetyltransferase cDNA content in each sample and presented in the manuscripts [13, 14].

PPARγ-null mice die at 10.5 to 11.5 days post coitum because of placental dysfunction [40], and the contribution of PPARγ cannot be examined in PPARγ-homozygous knockout mice. One of the mutants of the PPARγ2 subtype, $^{\text{Pro}}12^{\text{Ala}}$, reduces transcription of wildtype tk-Luc-linked PPARγ-related acyl-CoA oxidase, the peroxisome proliferator-responsible element, and lipoprotein lipase promoter by 40%, and persons homogenous for Ala-mutated PPARγ have lower body mass indexes and higher serum levels of high-density lipoprotein cholesterol [41]. A 50% reduction in PPARγ activity seems to have some biological effects, and PPARγ heterozygous knockout mice, which are expected to have 50% lower levels of PPARγ activity, and conditional knockout mice could be useful experimental models. Some RNA interference probes are available to inhibit PPARγ transcription and would be useful tools for investigating PPARγ involvement in cells, although the nonspecific interference by off-target effects should be noted.

Further investigations of the involvement of PPARs and other nuclear receptors in arachidonic acid metabolism are necessary to develop more effective and specific compounds as anti-asthma drugs.

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REFERENCES

[1] Natioal Heart Lung and Blood Institute, "Guidelines for the diagnosis and management of asthma (EPR-3)," Tech. Rep. NIH 08-4051, Natioal Heart Lung and Blood Institute, Bethesda, Md, USA, 2007.

[2] J. M. Brown, T. M. Wilson, and D. D. Metcalfe, "The mast cell and allergic diseases: role in pathogenesis and implications for therapy," *Clinical & Experimental Allergy*, vol. 38, no. 1, pp. 4–18, 2008.

[3] P. Christmas, B. M. Weber, M. McKee, D. Brown, and R. J. Soberman, "Membrane localization and topology of leukotriene C_4 synthase," *The Journal of Biological Chemistry*, vol. 277, no. 32, pp. 28902–28908, 2002.

[4] M. Raulf, M. Stuning, and W. Konig, "Metabolism of leukotrienes by L-γ-glutamyl-transpeptidase and dipeptidase from human polymorphonuclear granulocytes," *Immunology*, vol. 55, no. 1, pp. 135–147, 1985.

[5] A. H. Dantzig, R. L. Shepard, K. L. Law, et al., "Selectivity of the multidrug resistance modulator, LY335979, for P-glycoprotein and effect on cytochrome P-450 activities," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 290, no. 2, pp. 854–862, 1999.

[6] G. W. James, "The use of the *in vivo* trachea preparation of the guinea-pig to asses drug action on lung," *Journal of Pharmacy and Pharmacology*, vol. 21, no. 6, pp. 379–386, 1969.

[7] C. E. Heise, B. F. O'Dowd, D. J. Figueroa, et al., "Characterization of the human cysteinyl leukotriene 2 receptor," *The Journal of Biological Chemistry*, vol. 275, no. 39, pp. 30531–30536, 2000.

[8] K. R. Lynch, G. P. O'Neill, Q. Liu, et al., "Characterization of the human cysteinyl leukotriene $CysLT_1$ receptor," *Nature*, vol. 399, no. 6738, pp. 789–793, 1999.

[9] B. Dahlén, E. Nizankowska, A. Szczeklik, et al., "Benefits from adding the 5-lipoxygenase inhibitor zileuton to conventional therapy in aspirin-intolerant asthmatics," *American Journal of Respiratory and Critical Care Medicine*, vol. 157, no. 4, pp. 1187–1194, 1998.

[10] T. F. Reiss, C. A. Sorkness, W. Stricker, et al., "Effects of montelukast (MK-0476), a potent cysteinyl leukotriene receptor antagonist, on bronchodilation in asthmatic subjects treated with and without inhaled corticosteroids," *Thorax*, vol. 52, no. 1, pp. 45–48, 1997.

[11] J. E. Fish, J. P. Kemp, R. F. Lockey, M. Glass, L. A. Hanby, and C. M. Bonuccelli, "Zafirlukast for symptomatic mild-to-moderate asthma: a 13-week multicenter study," *Clinical Therapeutics*, vol. 19, no. 4, pp. 675–690, 1997.

[12] N. C. Barnes and J.-C. Pujet, "Pranlukast, a novel leukotriene receptor antagonist: results of the first European, placebo controlled, multicentre clinical study in asthma," *Thorax*, vol. 52, no. 6, pp. 523–527, 1997.

[13] M. Yamashita, "Peroxisome proliferator-activated receptor α-independent effects of peroxisome proliferators on cysteinyl leukotriene production in mast cells," *European Journal of Pharmacology*, vol. 556, no. 1–3, pp. 172–180, 2007.

[14] C. J. Nicol, M. Yoon, J. M. Ward, et al., "PPARγ influences susceptibility to DMBA-induced mammary, ovarian and skin carcinogenesis," *Carcinogenesis*, vol. 25, no. 9, pp. 1747–1755, 2004.

[15] G. Krey, O. Braissant, F. L'Horset, et al., "Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay," *Molecular Endocrinology*, vol. 11, no. 6, pp. 779–791, 1997.

[16] N. Latruffe and J. Vamecq, "Peroxisome proliferators and peroxisome proliferator activated receptors (PPARs) as regulators of lipid metabolism," *Biochimie*, vol. 79, no. 2-3, pp. 81–94, 1997.

[17] M. Yamashita, M. Kushihara, N. Hirasawa, et al., "Inhibition by troglitazone of the antigen-induced production of leukotrienes in immunoglobulin E-sensitized RBL-2H3 cells," *British Journal of Pharmacology*, vol. 129, no. 2, pp. 367–373, 2000.

[18] K. Okuyama, M. Yamashita, Y. Kitabatake, S. Kawamura, M. Takayanagi, and I. Ohno, "Ciglitazone inhibits the antigen-induced leukotrienes production independently of PPARγ in RBL-2H3 mast cells," *European Journal of Pharmacology*, vol. 521, no. 1–3, pp. 21–28, 2005.

[19] J. T. Huang, J. S. Welch, M. Ricote, et al., "Interleukin-4-dependent production of PPAR-γ ligands in macrophages by 12/15-lipoxygenase," *Nature*, vol. 400, no. 6742, pp. 378–382, 1999.

[20] H. N. Sørensen, E. Hvattum, E. J. Paulssen, K. M. Gautvik, J. Bremer, and Ø. Spydevold, "Induction of peroxisomal acyl-CoA oxidase by 3-thia fatty acid, in hepatoma cells and hepatocytes in culture is modified by dexamethasone and insulin," *Biochimica et Biophysica Acta*, vol. 1171, no. 3, pp. 263–271, 1993.

[21] J. D. Tugwood, I. Issemann, R. G. Anderson, K. R. Bundell, W. L. McPheat, and S. Green, "The mouse peroxisome proliferator activated receptor recognizes a response element in the 5′ flanking sequence of the rat acyl CoA oxidase gene," *The EMBO Journal*, vol. 11, no. 2, pp. 433–439, 1992.

[22] T. Helledie, L. Gróntved, S. S. Jensen, et al., "The gene encoding the acyl-CoA-binding protein is activated by peroxisome proliferator-activated receptor γ through an intronic response element functionally conserved between humans and rodents," *The Journal of Biological Chemistry*, vol. 277, no. 30, pp. 26821–26830, 2002.

[23] B. L. Diaz, H. Fujishima, Y. Kanaoka, Y. Urade, and J. P. Arm, "Regulation of prostaglandin endoperoxide synthase-2 and IL-6 expression in mouse bone marrow-derived mast cells by exogenous but not endogenous prostanoids," *The Journal of Immunology*, vol. 168, no. 3, pp. 1397–1404, 2002.

[24] A. Tautenhahn, B. Brüne, and A. von Knethen, "Activation-induced PPARγ expression sensitizes primary human T cells toward apoptosis," *Journal of Leukocyte Biology*, vol. 73, no. 5, pp. 665–672, 2003.

[25] K. Maeyama, M. Emi, and M. Tachibana, "Nuclear receptors as targets for drug development: peroxisome proliferator-activated receptor γ in mast cells: its roles in proliferation and differentiation," *Journal of Pharmacological Sciences*, vol. 97, no. 2, pp. 190–194, 2005.

[26] J. E. Ward and X. Tan, "Peroxisome proliferator activated receptor ligands as regulators of airway inflammation and remodelling in chronic lung disease," *PPAR Research*, vol. 2007, Article ID 14983, 12 pages, 2007.

[27] S. Paruchuri, Y. Jiang, C. Feng, S. A. Francis, J. Plutzky, and J. A. Boyce, "Leukotriene E_4 activates peroxisome proliferator-activated receptor γ and induces prostaglandin D_2 generation by human mast cells," *The Journal of Biological Chemistry*, vol. 283, no. 24, pp. 16477–16487, 2008.

[28] X. Zhang, N. Tanaka, T. Nakajima, Y. Kamijo, F. J. Gonzalez, and T. Aoyama, "Peroxisome proliferator-activated receptor α-independent peroxisome proliferation," *Biochemical and Biophysical Research Communications*, vol. 346, no. 4, pp. 1307–1311, 2006.

[29] C. Banfi, J. Auwerx, F. Poma, E. Tremoli, and L. Mussoni, "Induction of plasminogen activator inhibitor I by the PPARα ligand, Wy-14,643, is dependent on ERK 1/2 signaling pathway," *Thrombosis and Haemostasis*, vol. 90, no. 4, pp. 611–619, 2003.

[30] C. J. Pauley, B. J. Ledwith, and C. Kaplanski, "Peroxisome proliferators activate growth regulatory pathways largely via peroxisome proliferator-activated receptor α-independent mechanisms," *Cellular Signalling*, vol. 14, no. 4, pp. 351–358, 2002.

[31] A. M. Lennon, M. Ramaugé, A. Dessouroux, and M. Pierre, "MAP kinase cascades are activated in astrocytes and preadipocytes by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and the thiazolidinedione ciglitazone through peroxisome proliferator activator receptor γ-independent mechanisms involving reactive oxygenated species," *The Journal of Biological Chemistry*, vol. 277, no. 33, pp. 29681–29685, 2002.

[32] S. Goetze, X.-P. Xi, Y. Kawano, et al., "TNF-α-induced migration of vascular smooth muscle cells is MAPK dependent," *Hypertension*, vol. 33, no. 1, pp. 183–189, 1999.

[33] M. Caivano, C. Rodriguez, P. Cohen, and S. Alemany, "15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 regulates endogenous cot MAPK kinase kinase 1 activity induced by lipopolysaccharide," *The Journal of Biological Chemistry*, vol. 278, no. 52, pp. 52124–52130, 2003.

[34] O. Werz, J. Klemm, B. Samuelsson, and O. Rådmark, "5-lipoxygenase is phosphorylated by p38 kinase-dependent MAPKAP kinases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 10, pp. 5261–5266, 2000.

[35] Z.-Z. Shi, B. Han, G. M. Habib, M. M. Matzuk, and M. W. Lieberman, "Disruption of γ-glutamyl leukotrienase results in disruption of leukotriene D_4 synthesis *in vivo* and attenuation of the acute inflammatory response," *Molecular and Cellular Biology*, vol. 21, no. 16, pp. 5389–5395, 2001.

[36] G. M. Habib, Z.-Z. Shi, A. A. Cuevas, Q. Guo, M. M. Matzuk, and M. W. Lieberman, "Leukotriene D_4 and cystinyl-bis-glycine metabolism in membrane-bound dipeptidase-deficient mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 9, pp. 4859–4863, 1998.

[37] R. S. Peebles and H. P. Glauert, "Effect of phenobarbital on hepatic eicosanoid concentrations in rats," *Archives of Toxicology*, vol. 71, no. 10, pp. 646–650, 1997.

[38] Y. Fujimura, H. Tachibana, and K. Yamada, "Peroxisome proliferator-activated receptor ligands negatively regulate the expression of the high-affinity IgE receptor FcεRI in human basophilic KU812 cells," *Biochemical and Biophysical Research Communications*, vol. 297, no. 2, pp. 193–201, 2002.

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

[40] N. Kubota, Y. Terauchi, H. Miki, et al., "PPARγ mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance," *Molecular Cell*, vol. 4, no. 4, pp. 597–609, 1999.

[41] S. S. Deeb, L. Fajas, M. Nemoto, et al., "A Pro12Ala substitution in PPARγ 2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity," *Nature Genetics*, vol. 20, no. 3, pp. 284–287, 1998.

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Research Article

Cross-Species Differential Plasma Protein Binding of MBX-102/JNJ39659100: A Novel PPAR-γ Agonist

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Drug binding to plasma proteins restricts their free and active concentrations, thereby affecting their pharmacokinetic properties. Species differences in plasma protein levels complicate the understanding of interspecies pharmacodynamic and toxicological effects. MBX-102 acid/JNJ39659100 is a novel PPAR-γ agonist in development for the treatment of type 2 diabetes. Studies were performed to evaluate plasma protein binding to MBX-102 acid and evaluate species differences in free drug levels. Equilibrium dialysis studies demonstrated that MBX-102 acid is highly bound (>98%) to human, rat and mouse albumin and that free MBX-102 acid levels are higher in rodent than in human plasma. Interspecies differences in free drug levels were further studied using PPAR-γ transactivation assays and a newly developed PPAR-γ corepressor displacement (biochemical) assay. PPAR-γ transactivation and corepressor displacement by MBX-102 acid was higher in rat and mouse serum than human serum. These results confirm the relevance of interspecies differences in free MBX-102 acid levels.

1. INTRODUCTION

MBX-102/JNJ39659100 is a compound in development for the treatment of type 2 diabetes. It is a single enantiomer of halofenate, a drug that was tested clinically in the 1970s as a hypolipidemic and hypouricemic agent [1–6]. Although developed for lipid lowering, studies with halofenate in diabetic patients also demonstrated significant effects on plasma glucose and insulin both in monotherapy [7, 8] and in combination with other oral hypoglycemic agents [9–11]. Two decades later, it was discovered that both halofenate and MBX-102/JNJ39659100 are selective partial PPAR-γ agonists [12, 13] thereby offering an explanation for its antidiabetic properties.

Translational medicine is important for studying the action and safety of drugs. Studies in animals allow for interventional procedures that are not appropriate for humans. Key to interpreting these studies is to understand the relationship of the pharmacologically active form, (i.e., free drug) to the pharmacodynamic effects in each species studied.

Connecting preclinical pharmacology and safety studies in different species to the likely human experience therefore requires an understanding of the action of the drug at the target from these different species as well as the relationship of the free, pharmacologically active form to total drug concentration in these species.

For drugs with high serum protein binding this is particularly important. High serum protein binding appears to be a common feature of PPAR-γ agonists such as rosiglitazone, pioglitazone, and others [14–16] and previous data suggest that it may also be a feature of halofenate [17, 18] and therefore, also of MBX-102/JNJ39659100. Accurately determining free levels of highly plasma protein-bound drugs is technically challenging, making comparisons between species for these drugs extremely difficult. In the results reported herein, methods were used that allow for comparison between mouse, rat, and human plasma protein binding. This allowed for the appropriate interpretation of the pharmacology and potential for human risk of MBX-102/JNJ39659100. This study provides an approach that could be applied to the translational medicine and safety assessments for other PPAR agonists.

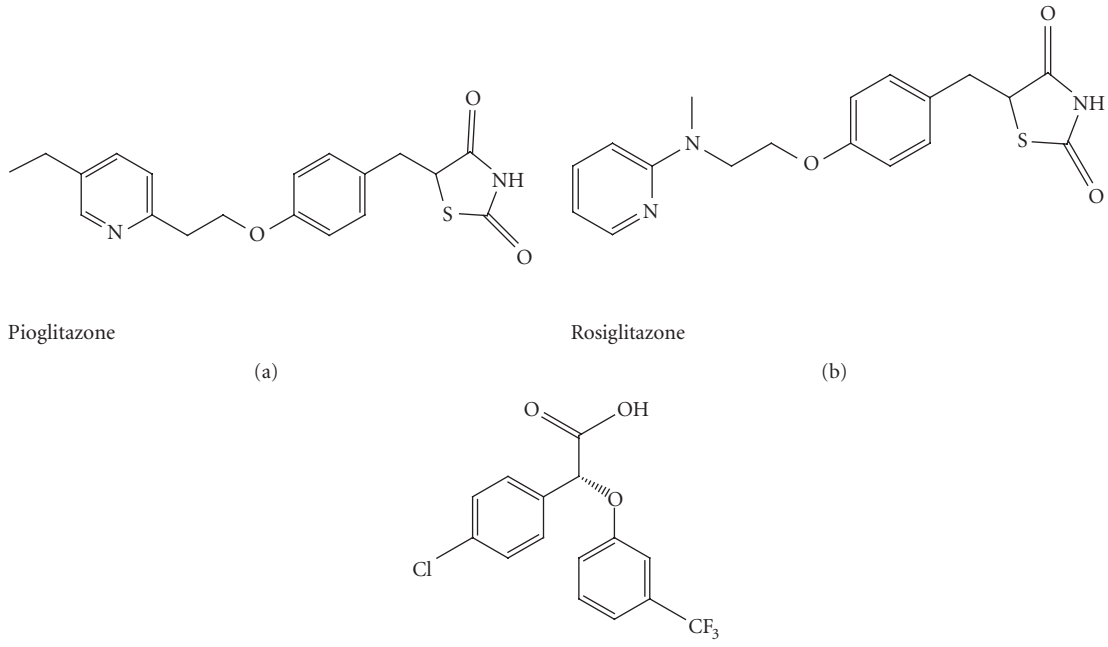

Figure 1: Structure of rosiglitazone, pioglitazone, and MBX-102 acid (active form).

2. MATERIALS AND METHODS

[^{3}H] MBX-102 acid (740 GBq/mmol, 20 Ci/mmol) was synthesized by Amersham Biosciences (Buckinghamshire, UK). MBX-102 acid was synthesized at IRIX Pharmaceuticals (Florence, SC, USA). The structure of MBX-102 acid is shown in Figure 1 in comparison to the full agonists, rosiglitazone and pioglitazone. For radiolabeled binding studies, pooled frozen plasma from either Sprague Dawley rats, CD-1 mice, or humans were purchased from Bioreclamation, Inc. (Hicksville, NY, USA). For the competitive equilibrium dialysis experiments, fresh pooled mixed gender plasma from either CD-1 mouse, Sprague-Dawley rat, or humans obtained from Bioreclamation, Inc. (Hicksville, NY) were used. Human, mouse, and rat serum albumins, and human alpha-1-acid glycoprotein were purchased from Sigma (St. Louis, Mo, USA). Charcoal stripped and delipidated sera from either human males, CD-1 male mice, or Sprague Dawley male rats were purchased from Biochemed (Winchester, Va, USA). FDG (Fluorescein di-β-D-galactopyranoside) was purchased from Invitrogen (Carlsbad, Calif, USA). Lanthascreen TR-FRET PPAR-gamma Coactivator Assay Kit and fluorescently labeled NCOR peptide (Fluor-DPASNLGLEDIIRKALMGSFDDK) were purchased from Invitrogen (Carlsbad, Calif). Steady Glo reagent was purchased from Promega (Madison, Wis, USA). DMEM culture media, Lipofectamine, Optimem, and Penicillin-Streptomycin were purchased from Invitrogen (Carlsbad, Calif). Bovine Insulin, isobutylmethylxanthine, and dexamethasone were purchased from Sigma (St. Louis, Mo). HEK 293T cells were obtained from ATCC (Manassas, Va, USA). Pro 293-Culture defined media was purchased from Cambrex (East Rutherford, NJ, USA).

2.1. Formulation of [^{3}H] MBX-102 acid

Radiolabeled MBX-102 acid was prepared as a 1 mL ethanol solution at a concentration of 50 μM (1 mCi total). Stock MBX-102 acid dosing solutions (100-fold of final concentration) were prepared with unlabeled MBX-102 acid in dimethyl sulfoxide (DMSO) and spiked with 1 μL/mL (0.05 μM) of [^{3}H] labeled MBX-102 acid so that the final evaluated concentrations of MBX-102 acid were 400 μM, 600 μM, 1000 μM, 1500 μM, and 2000 μM. Final solvent concentrations were 1% of the total volume.

2.2. Determination of plasma protein binding of MBX-102 acid by equilibrium dialysis

Plasma was stored at -20°C. Prior to use, it was thawed and spun at approximately 2000 rpm for 5 minutes to remove any precipitated material. The pH was adjusted to pH 7.4 by careful addition of NaH_2PO_4. A 1 mL sample of spiked plasma was prepared by direct dilution of [^{3}H]-MBX-102 acid stock solution into plasma and then added to one side of an equilibrium dialysis chamber. The other chamber was filled with 1 mL of 0.01 M phosphate buffered saline (PBS). The dialysis apparatus was placed in a water bath at 37°C

Table 1: *Binding of MBX-102 acid to rat, mouse, and human plasma determined by equilibrium dialysis.* Binding of [^{3}H] MBX-102 acid to plasma was conducted by equilibrium dialysis against PBS buffer at 37°C and the percentage of total radiolabel bound to plasma was determined by dividing the amount of sample in the plasma compartment by the combined total amounts in the plasma and PBS buffer compartments. Values represent the result of a representative experiment and are the mean ± SD of triplicate determinations.

MBX-102 acid (μM)	%Protein Binding ± SD		
	Human	Mouse	Rat
400	99.8 ± 0.1	99.8 ± 0.0	99.8 ± 0.1
600	99.8 ± 0.1	99.7 ± 0.0	99.8 ± 0.1
1000	99.7 ± 0.1	99.5 ± 0.1	99.7 ± 0.0
1500	100 ± 0.1	99.8 ± 0.1	99.8 ± 0.2
2000	99.8 ± 0.1	99.5 ± 0.1	99.5 ± 0.1

and rotated at 20 rpm. Preliminary studies indicated that equilibrium is achieved within 5 hours (data not shown). Once equilibrium was established, the contents of the cell chambers were removed and analyzed by liquid scintillation counting. The chambers were sampled in triplicate. Nonspecific binding, in the absence of plasma, was determined to be 5.3 +/− 3.9% (mean +/− SD, $n = 3$). The mean recovery of [^{3}H] MBX-102 acid was determined in triplicate by sampling of both dialysis chambers at each concentration of MBX-102 acid. The recovery percentage was found not to vary with MBX-102 acid concentration. The mean +/− SD % recoveries across all MBX-102 acid concentrations for each species were 83.9 +/− 6.7%, 84.4 +/− 2.4%, and 85.8 +/− 2.6% for human, rat, and mouse plasma, respectively.

2.3. Determination of protein binding of MBX-102 acid to selected human plasma proteins

Stock solutions of human serum albumin and alpha-1-acid glycoprotein were prepared in PBS buffer. Human serum albumin (40 mg/mL, ~600 μM) and human alpha-1-acid glycoprotein (22.5 μM) were spiked with [^{3}H] MBX-102 acid. The spiked protein solution (175 μL) was added to one side of an equilibrium dialysis chamber, and an equal volume of PBS buffer was added to the other chamber. Dialysis was allowed to reach equilibrium and the binding to protein was determined by liquid scintillation counting of samples from both chambers as described above. The percent recovery of [^{3}H] MBX-102 acid with both serum proteins was between 95.7% and 98.5%.

2.4. Determination of MBX-102 acid binding to albumin by surface plasmon resonance (SPR)

The characterization of the binding of MBX-102 acid against human, mouse, and rat albumin was performed using SPR-based biosensors (Biosensor Tools, Salt Lake City, Utah, USA). The assay methods used to assess the binding of MBX-102 acid to human, mouse, and rat albumins have been described previously [19]. Briefly, each albumin was immobilized onto a CM5 sensor chip using standard amine coupling. Immobilization densities were between 10 000 and 13 000 RU. The test compound was run in a twofold dilution series with the highest concentration of 200 μM. Each of the 16 different concentrations was tested in duplicate. The running buffer contained 53 mM Na_2HPO_4, 12.5 mM KH_2PO_4, 70 mM NaCl at pH 7.4, and 5% DMSO. All binding data were collected at 37°C. The binding response profile of MBX-102 acid over the three different albumin surfaces was evaluated and the binding constants for the high-affinity site were determined using a two-independent-site model. Conversion from K_D to %bound was performed as previously described [19].

2.5. Determination of species differences in protein binding of MBX-102 acid by competitive equilibrium dialysis

A comparison of the binding to plasma from different species was performed essentially by the method described below. Briefly, [^{3}H] MBX-102 acid spiked plasma samples were formulated as described above with the exception that pH was not adjusted to 7.4 and the final DMSO concentration was 0.6%. A 1 mL sample of spiked human plasma was applied to one side of the dialysis membrane and 1 mL of spiked animal plasma was applied to the other side. The samples were dialyzed by rotation at 20 rpm for up to 120 hours in a 37°C incubator. The ratio of free drug in plasma was calculated according to the equation: ratio of free drug (animal versus human) = (total cpm in human plasma)/(total cpm in animal plasma).

2.6. Cell culture

HEK 293T cells (ATCC) were cultured in 15-cm dishes at subconfluence (approx. cell density was 14 000/cm^2) in DMEM (high glucose), and 10% (v/v) fetal bovine serum (FBS) supplemented with 1% (v/v) Penicillin-Streptomycin. All cells were maintained at 37°C in a humidified atmosphere of 8% CO_2 in air.

2.7. PPAR-γ reporter gene assays

HEK-293T cells were cultured as described above. Prior to use, the cells were trypsinized using 0.25% trypsin/1 mM EDTA and resuspended in DMEM, 10% (v/v) FBS lacking Penicillin-Streptomycin. For a pool sufficient to supply 100 wells, 6 million cells were diluted into medium for a total

volume of 9 mL. The DNA-Lipofectamine 2000 mixture was prepared as per manufacturer's instructions. For a pool sufficient to supply 100 wells, 5 μg Gal 4-Mouse PPAR-γ LBD, 5 μg pFR-Luciferase, and 500 ng Lac-z plasmids were mixed with 40 μL of Lipofectamine 2000 in Optimem medium in a total volume of 1 mL. The cell suspension was mixed with 1 mL of the DNA-Lipofectamine 2000 mixture. The mixture was plated into a 96-well plate and incubated for 4 hours at which time the transfection medium was removed and replaced with 100 μL DMEM, 10% (v/v) FBS and cultured overnight. The culture medium was then removed from the plates and replaced with 50 μL Pro293A medium. Compounds and charcoal stripped/delipidated serum or serum albumin, *or* alpha-1 acid glycoprotein stock solutions were prepared at 2X final concentration in Pro293A medium and mixed together prior to addition of 50 μL to the transfected cells and incubated for an additional 24 hours. Measurement of luciferase and fluorescence activity was performed according to the manufacturer's instructions. Briefly, after removal of media, cells were incubated for 10 minutes in 100 μL of Steady-Glo reagent. An 80 μL lysate aliquot was transferred to opaque white well plates and the luminescence measured. The 80 μL aliquot was then transferred back to the original plate. The fluorescence emission (excitation 485 nm, emission 535 nm) was measured after the addition of 100 μL of 10 μM fluorescein di-β-D-galactopyranoside in assay buffer (2.1 mM KH_2PO_4, 310.3 mM NaCl, 5.9 mM Na_2HPO_4-$7H_2O$, 20 mM KCl, 2 mM $MgSO_4$, 0.2% triton-X100). Each experimental condition was assessed in quadruplicate. The data were normalized for each well by dividing the luminescence measurement by the fluorescence measurement. Dose-response curves were generated and EC_{50} values were calculated using Prism Graphpad version 5.1.

2.8. Lanthascreen corepressor displacement assay

Assays were performed according to the manufacturer's instructions. Briefly, GST-PPARγ-LBD (5 nM), Tb-labeled anti-GST antibody (5 nM), and fluorescent-peptide (125 nM) were diluted together in kit assay buffer with 5 mM DTT and 10 μL/well of this solution was added to 384-well black plates (Costar, Corning Inc. Life Science, Lowell, Mass, USA). Ligands were prepared as stock solutions in DMSO at 100-fold their final concentration followed by dilution to 2X concentration in kit assay buffer with 5 mM DTT containing a 2X concentration of serum albumin or charcoal stripped/delipidated serum prior to addition of 10 μL/well to the assay plate. The plate was covered and incubated for 4 hours at room temperature. The time resolved fluorescence resonance energy transfer (TR-FRET) signal was measured using a Pherastar fluorescence counter (BMG labtech, Offenburg, Germany). The ratio of the emission intensity of the acceptor (Fluorescein: λ = 520 nm) divided by the emission intensity of the donor (Tb: λ = 490 nm) was then calculated to determine the degree of NCOR binding. Each measurement was performed in quadruplicate. Dose-response curves were generated and IC_{50} values were calculated using Prism Graphpad version 5.01.

Table 2: *Binding of MBX-102 acid to rat, mouse, and human albumin determined by plasmon resonance-based biosensors.* The binding constants for the high-affinity site were determined at 37°C. Values represent the mean of duplicate determinations (HSA: human serum albumin, MSA: mouse serum albumin, RSA: rat serum albumin).

Interaction	K_D (μM)	%Bound
HSA:MBX-102	5.8	99.1
MSA:MBX-102	5.5	99.2
RSA:MBX-102	12.8	98.1

2.9. Statistics

To compare logEC50 (or logIC50), ANOVA model of randomized block design was used. If block effect (experiment effect) was not significant, the data were reanalyzed by a reduced ANOVA model. Tukey's test was used for multiple comparisons (SAS). Differences were considered significant at a P value <.05.

3. RESULTS

3.1. Interspecies protein binding of MBX-102 acid

MBX-102 is a selective partial PPAR-γ modulator which is structurally distinct from the full PPAR-γ agonists, rosiglitazone and pioglitazone (see Figure 1). In order to understand the relationship between free drug levels and the efficacy of the selective partial PPAR-γ agonist MBX-102 acid in different species, the plasma binding properties of MBX-102 acid were determined. Pooled, mixed sex plasma obtained from humans, Sprague Dawley rats, and CD-1 mice were spiked with MBX-102 acid and the % MBX-102 acid bound to protein was determined by equilibrium dialysis. The data shown in Table 1 reveal that MBX-102 acid is 99.5%–100% bound to plasma proteins from humans, rats, and mice. The high degree of binding observed was also independent of MBX-102 acid concentration. To identify potential MBX-102 acid binding proteins in humans, equilibrium binding studies were performed using purified human serum albumin and human alpha 1-acid glycoprotein. A high level of MBX-102 acid binding (>98%) to human serum albumin was observed. In comparison, the binding to human alpha 1-acid glycoprotein was very low (<5%) (data not shown). These studies indicate that the selective partial PPAR-γ agonist MBX-102 acid is highly protein-bound in plasma across different species and identifies serum albumin as a protein that binds MBX-102 acid.

To further characterize the binding of MBX-102 acid to albumin, we used surface plasmon resonance (SPR), a label-free technique that can be used to provide information on the kinetics and affinity of complex formation for drugs that are highly bound to albumin [19, 20]. The binding constants (KD) and the bound percentage for human, mouse, and rat albumin are reported in Table 2. In full agreement

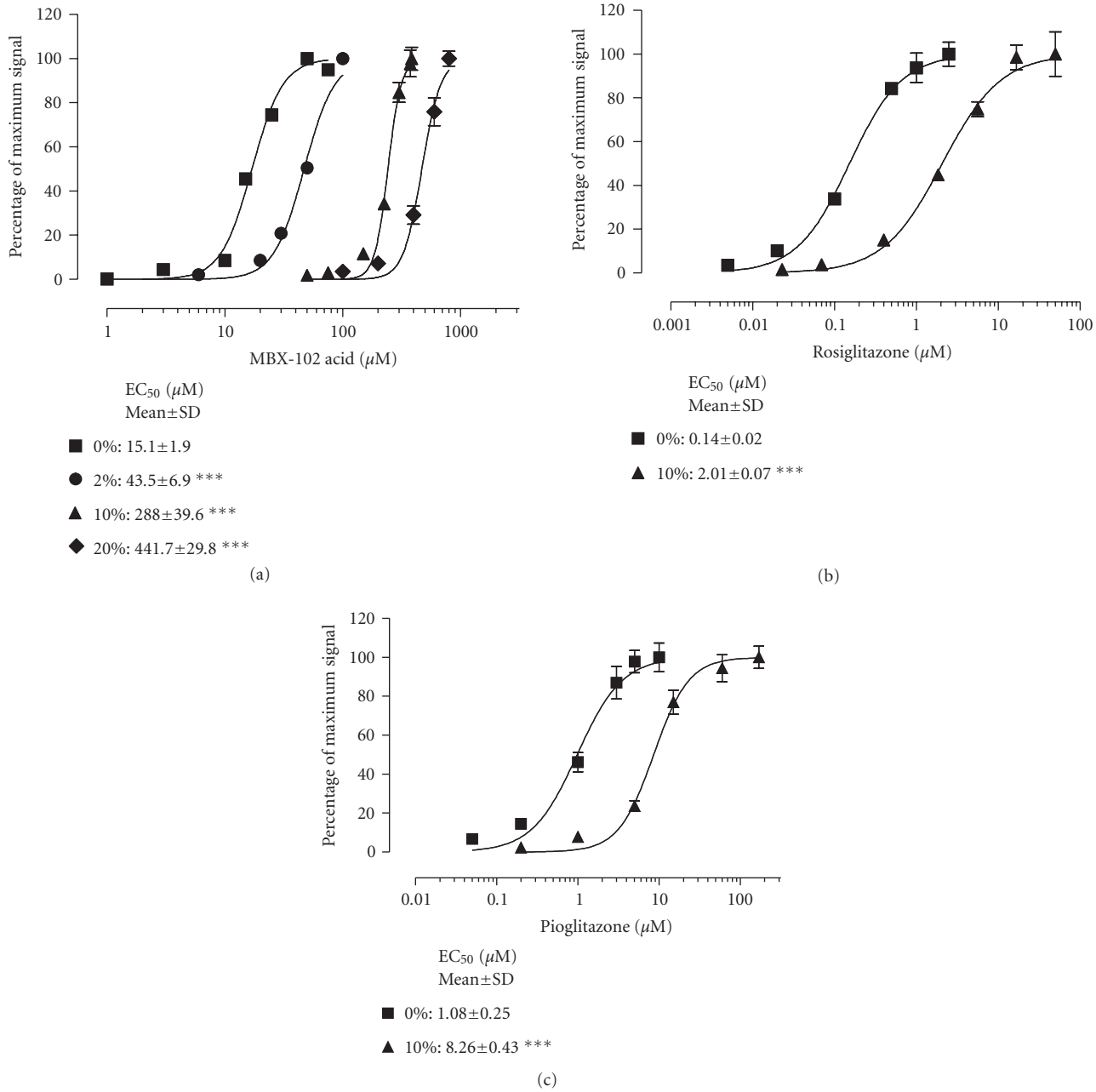

Figure 2: *PPAR-γ activation by (a) MBX-102 acid, (b) rosiglitazone, and (c) pioglitazone in the presence of increasing human serum.* Normalized reporter assay data were calculated as the percentage of maximum signal by expressing each data point as a percentage of the mean for the maximum signal. The percentage of maximum signal for the curves representing 0%, 2%, 10%, and 20% (v/v) serum was calculated independently. The dose-response curves shown are from a representative experiment. Values are EC_{50} (μM) determined from 3 experiments and shown as the mean ± SD.

with the studies reported above, MBX-102 acid binding to albumin was >98%. This high degree of protein binding precluded any further analysis of differential binding of MBX-102 acid to plasma proteins across species because the absolute binding could not be determined accurately by any of the two methodologies used. Therefore, competitive equilibrium dialysis (CED) was used to address the question of differences in the binding of MBX-102 acid to plasma proteins among species. CED utilizes competition dialysis between the plasma of two species to accurately determine the ratios of the free drug fractions in these species [21]. Using this technique, the ratio of the free fractions is inversely related to the fold accumulation of total drug in the plasma of each species plasma at equilibrium. The ratios of rat-to-human and mouse-to-human free fraction were determined over several concentrations of MBX-102 acid. The data shown in Table 3 indicate that the free MBX-102 acid in rat plasma is 1.7 to 2.3 fold higher than in human plasma and

Table 3: *Interspecies free MBX-102 acid ratios determined by competitive equilibrium dialysis.* [^{3}H] MBX-102 acid distribution between either mouse and human plasma or rat and human plasma was conducted by competitive equilibrium dialysis at 37°C. Values represent mean ± SD for 5 independent experiments.

MBX-102 Acid (μM)	Free Fraction Ratio ($n = 5$ ±SD)	
	Rat:Human	Mouse:Human
100	2.3 ± 0.6	10.5 ± 5.5
300	2.3 ± 0.6	5.9 ± 3.6
700	2.0 ± 0.3	3.7 ± 1.9
1000	1.8 ± 0.2	2.6 ± 1.1
1300	1.7 ± 0.2	2.3 ± 0.7

that the free MBX-102 acid concentration in mouse plasma is 2.3 to 10.5 fold higher than in human plasma. Interestingly, both the rat-to-human and the mouse-to-human free drug ratios were found to decrease with total drug concentration possibly due to saturation of weak binding sites on human binding proteins. These findings predict that at a fixed total drug level of MBX-102 acid, the relative free drug levels across species will be in the order mouse > rat > human.

3.2. Activation of PPAR-γ by free drug in the presence of human serum

The finding that the partial PPAR-γ agonist MBX-102 acid is differentially bound to plasma proteins across species suggested that the free levels, putatively responsible for pharmacodynamic effects of MBX-102 acid, could lead to a different dependence on total drug levels amongst the different species. In order to fully interpret the impact of different levels of free MBX-102 acid between species, it is essential to confirm that free drug level is responsible for the action at the receptor and to know if there are any intrinsic interspecies differences in PPAR-γ activity of MBX-102 acid. PPAR-γ reporter gene assays demonstrated that there were no intrinsic differences in the ability of MBX-102 acid to activate human, mouse, or rat PPAR-γ (data not shown). To understand the effect of serum on the activation of PPAR-γ by MBX-102 acid, the ability of MBX-102 acid to transactivate PPAR-γ was determined in a cell-based assay in the presence of increasing concentrations of human serum. As illustrated in Figure 2(a), MBX-102 acid induced PPAR-γ activity in a dose-dependent manner in the absence of serum. In the presence of increasing concentrations of human serum, there was a pronounced and serum concentration-dependent rightward shift of the dose-response curve for MBX-102 acid. The fold changes in mean EC_{50} values relative to no serum were 3, 19-, and 29-fold for 2%, 10%, and 20% human serum, respectively. At higher human serum concentrations, there was a decrease in the window of activation precluding an analysis of serum concentrations above 20%. Similar studies were performed for the full PPAR-γ agonists, rosiglitazone and pioglitazone (see Figures 2(b) and 2(c)). For both compounds, as was seen for MBX-102 acid, a rightward shift in the dose-response curve for PPAR-γ activation was observed in the presence of 10% human serum compared to serum free. For rosiglitazone, there was a 14-fold increase in EC_{50}, and for pioglitazone, there was an 8-fold increase in EC_{50}. Serum protein binding therefore affects the degree to which PPAR-γ can be activated by agonists in a cellular environment. Similar studies were performed for all three PPAR-γ agonists in the presence of human serum albumin. As expected, the EC_{50}s for activation of PPAR-γ were rightward shifted in the presence of human serum albumin for all three PPAR-γ agonists (see Figures 3(a), 3(b), and 3(c)). Concentrations of serum albumin greater than 0.08% caused interference in the reporter assay precluding an analysis of the effect of higher and more physiologically relevant albumin concentrations. To further confirm the selectivity of the albumin effect, the EC_{50} for activation of PPAR-γ was also evaluated in the presence of alpha 1-acid glycoprotein. As anticipated, no shift in EC_{50} was detected even in the presence of the highest concentration of alpha 1-acid glycoprotein tested (0.14%, data not shown).

3.3. Differential activation of PPAR-γ across species

On the basis of the finding that MBX-102 acid is differentially bound to serum proteins from human, mouse, and rat, and the confirmation that free drug levels determine the ability of MBX-102 acid to activate PPAR-γ, it is predicted that MBX-102 acid should differentially activate PPAR-γ in the presence of serum from different species. As illustrated in Figure 4, this was found to be the case. In the presence of 10% human, rat, or mouse serum, MBX-102 acid activated PPAR-γ with EC_{50}s of 260 μM, 196 μM, and 170 μM, respectively. These differences in EC_{50} were found to be highly statistically significant. Similar studies were also performed with the full PPAR-γ agonists, rosiglitazone and pioglitazone. As summarized in Table 4, MBX-102 acid activation of PPAR-γ was affected differently in the presence of 10% serum from different species compared to the effects seen with rosiglitazone and pioglitazone. For MBX-102 acid, the EC_{50} in the presence of mouse and rat serum occurred at lower concentrations than in human serum, whereas for both rosiglitazone and pioglitazone the opposite effect was observed, namely, that higher concentrations were needed in the presence of rat and mouse serum. These data suggest that the differential effect of serum on PPAR-γ activation observed with MBX-102 acid is a property of MBX-102 acid and not of the serum proteins.

3.4. Differential corepressor displacement from PPAR-γ across species

The cell-based PPAR-γ reporter assay is adversely affected by mouse serum concentrations greater than 10% precluding analysis of cross-species differential serum binding at serum concentrations closer to physiological levels. An alternate in vitro assay was developed that allowed the assessment of the effect of much higher and more physiologically relevant serum concentrations on MBX-102 acid action. The data shown in Figure 5 demonstrate that a peptide

Table 4: *Differential activation of PPAR-γ by PPAR-γ agonists in the presence of 10% of human, rat, and mouse serum.* Values are EC_{50} (μM) determined from 3 experiments and shown as the mean ± SD. FC is the ratio of EC_{50}s for human: rat or human: mouse ($* = P < .05$, $** = P < .01$, $*** = P < .001$ by ANOVA with Tukey post hoc test).

PPAR agonist	Mean EC_{50} (μM) ±SD			Fold Change in EC_{50}	
	Human	Rat	Mouse	Human:Rat	Human:Mouse
MBX-102 acid	260 ± 16.9	196 ± 18	169 ± 5.2	1.33**	1.53***
Rosiglitazone	2.0 ± 0.1	5.2 ± 0.3	4.5 ± 0.3	0.39***	0.45***
Pioglitazone	8.3 ± 0.4	11.4 ± 1.2	9.7 ± 1.4	0.73^{NS}	0.86^{NS}

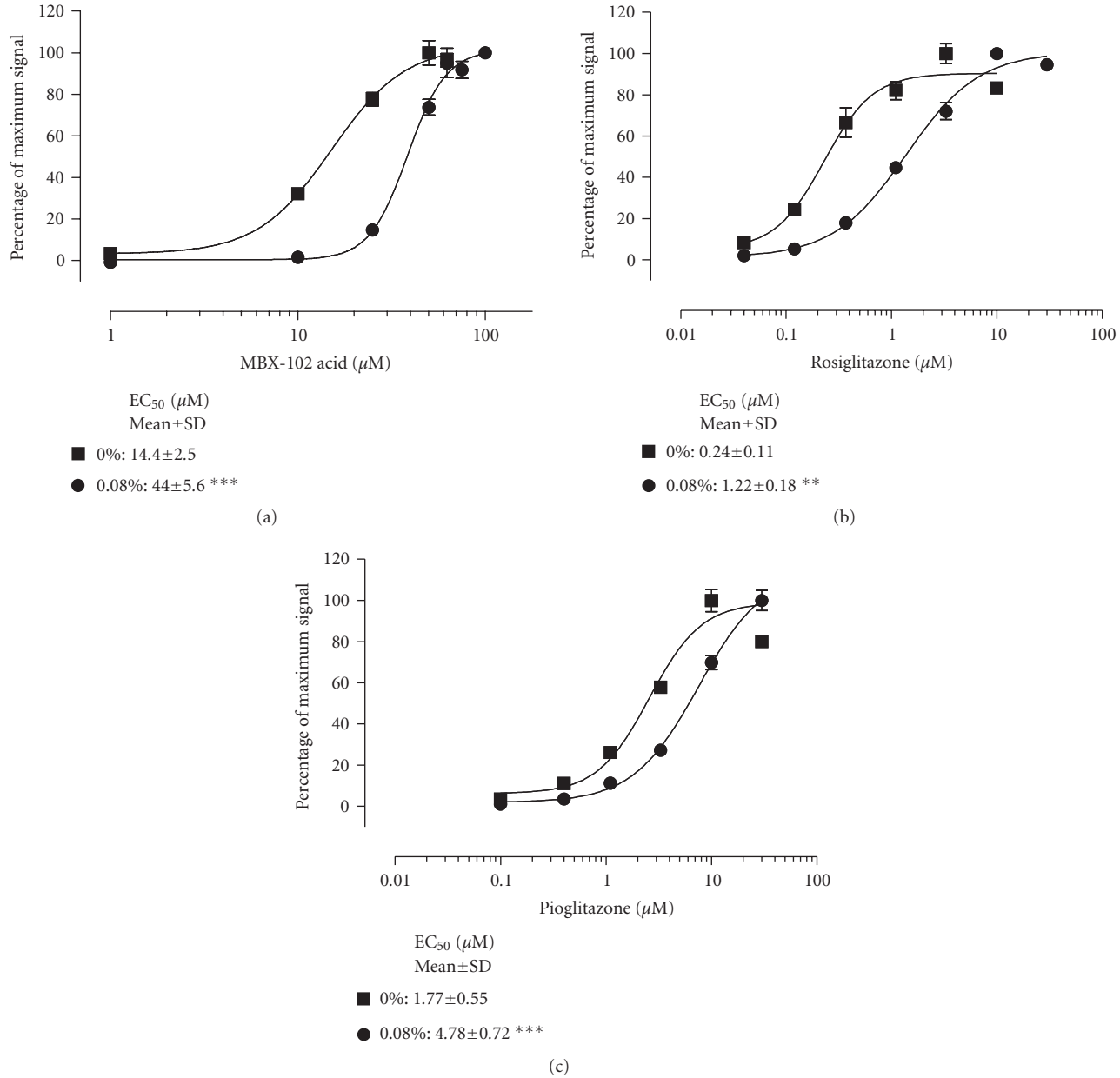

Figure 3: *PPAR-γ activation by (a) MBX-102 acid, (b) rosiglitazone, and (c) pioglitazone in the presence of increasing human serum albumin.* Normalized reporter assay data were calculated as the percentage of maximum signal as described in Figure 2. The percentage of maximum signal for the curves representing 0 and 0.08% serum albumin was calculated independently. The dose-response curves shown are from a representative experiment. Values are EC_{50} (μM) determined from 2–6 experiments and shown as the mean ± SD.

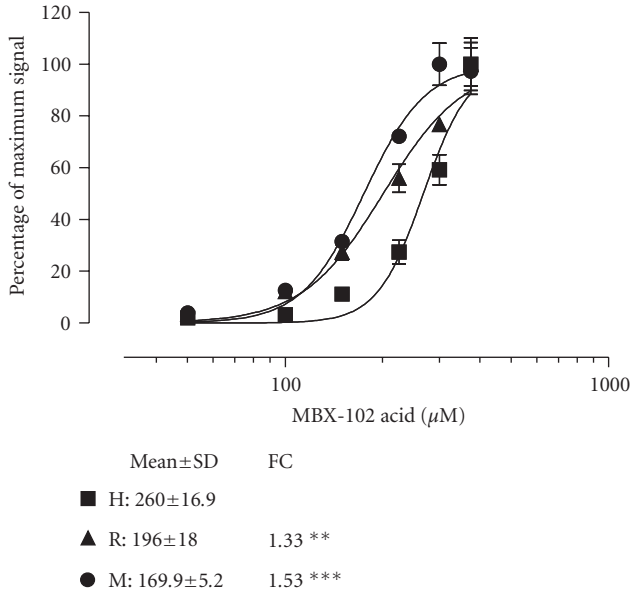

Figure 4: *Activation of PPAR-γ by MBX-102 acid in the presence of human serum compared to mouse and rat serum.* Normalized reporter assay data are expressed as the percentage of maximum signal as described in Figure 2. The dose-response curves shown are from representative experiments. MBX-102 acid activation of PPAR-γ in the presence of 10% (v/v) human (H), mouse (M), or rat (R) serum. The dose-response curves shown are from a representative experiment. Values are EC_{50} (μM) determined from 3 experiments and shown as the mean ±SD. FC is the ratio of EC_{50}s for human: rat or human: mouse (∗ = $P < .05$, ∗∗ = $P < .01$, ∗∗∗ = $P < .001$ by ANOVA with Tukey post hoc test).

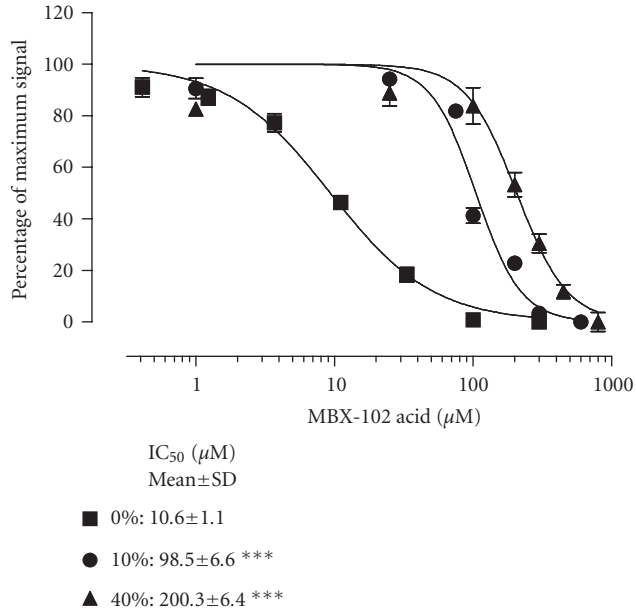

Figure 5: *Displacement of NCOR corepressor peptide from PPAR-γ by MBX-102 acid in the presence of human serum.* MBX-102 acid induced displacement of NCOR corepressor peptide from the human PPAR-γ ligand-binding domain in the presence of human serum at 0, 10%, or 40% (v/v). Normalized FRET assay data are expressed as the percentage of maximum signal (as described in Figure 2). The dose-response curves shown are from a representative experiment. Values are IC_{50} (μM) determined from 3 experiments and shown as the mean ± SD.

derived from the corepressor NCOR is constitutively bound to the ligand-binding domain of PPAR-γ and can be fully displaced by MBX-102 acid with an IC_{50} of 11 μM. Increasing concentrations of human serum caused a rightward shift of the dose-response curve resulting in up to a 19-fold shift in the IC_{50} at 40% human serum. Differential displacement of NCOR by MBX-102 acid was assessed at 40% serum for human, rat, and mouse (see Figure 6). The fold changes in IC_{50} for human-to-rat serum and human-to-mouse serum were 4 and 7, respectively. These data are very consistent with the relative free drug ratios predicted by the competitive equilibrium dialysis studies.

4. DISCUSSION

The data presented here demonstrate that MBX-102/JNJ39659100 is highly protein-bound, as had been suggested by previous studies with halofenate, and that at least one of the MBX-102 acid binding proteins is serum albumin. Our goal was to understand the serum binding properties of MBX-102 acid across species and to use this information in interpreting the pharmacodynamic and toxicological effects across species. The use of competitive equilibrium dialysis studies successfully demonstrated that MBX-102 acid is indeed differentially bound to plasma with the order of tightness of binding being human > rat > mouse. The studies performed using the cell-based PPAR-γ reporter assay confirmed, at least qualitatively, our hypothesis that the pharmacodynamic effects of MBX-102 acid are dictated by free drug levels and, further, that the differential binding of MBX-102 acid to serum proteins across species also results in a predictable and highly reproducible effect on pharmacodynamics. From these studies, the order of binding of MBX-102 acid to serum across species is predicted to be human > rat > mouse, which is in agreement with the data from the CED studies. Although we observed good qualitative correlations with the reporter assay and the CED assay, the magnitude of shifts in EC_{50} in the reporter assay was much smaller than those seen with the CED assay. One limitation of these reporter assay studies was the inability to investigate the effect of serum concentrations higher than 10% which could possibly explain the quantitative differences observed between these two assays. For this reason, we developed a new assay for measuring PPAR-γ activity in vitro that was able to tolerate serum concentrations as high as 40%. The data from this new assay confirmed the predicted order of binding for MBX-102 acid to serum across species as human > rat > mouse and also provided quantitatively very similar fold changes to the CED assay. The basis of the differential binding of MBX-102 to serum albumin from different species is unknown. Although at the protein level, mouse and rat albumins are highly conserved (~90% homology),

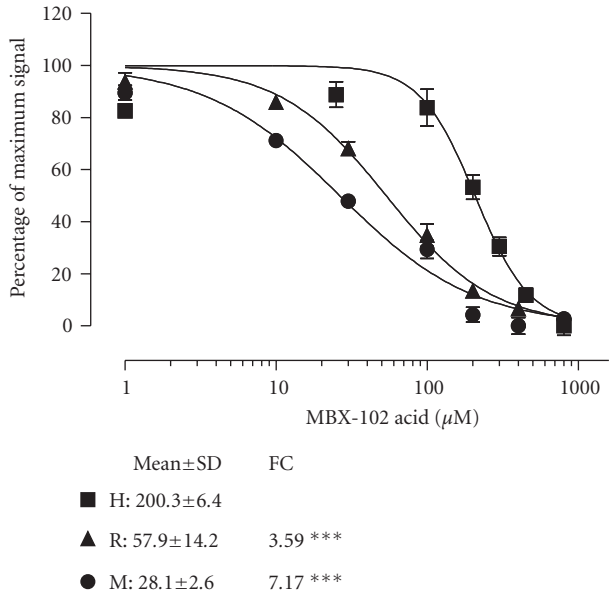

Figure 6: *Displacement of NCOR corepressor peptide from PPAR-γ ligand-binding domain by MBX-102 acid in the presence of human serum compared to mouse and rat serum.* MBX-102 acid induced displacement of NCOR corepressor peptide from human PPAR-γ ligand-binding domain in the presence of 40% (v/v) human (H), mouse (M), or rat (R) serum. Normalized FRET assay data are expressed as the percentage of maximum signal ("percentage of maximal signal," as described in Figure 2(a)). The dose-response curves shown are from a representative experiment. Values are IC_{50} (μM) determined from 3 experiments and shown as the mean ± SD. FC is the IC_{50} fold change of mouse or rat compared to human ($*** = P < .001$ by ANOVA with Tukey post hoc test).

the degree of conservation is much lower between human and mouse (~72%) and human and rat (~73%). Such differences may, at least in part, be responsible for the differential binding observed between species.

The approaches described here will be generally useful for interpreting preclinical pharmacology data in different species as well as toxicology studies and how these will relate to the human experience. Whilst confined initially to PPAR-γ, the approaches could easily be adapted for PPAR-α and PPAR-δ and indeed to virtually any other ligand-modulated receptor.

REFERENCES

[1] W. S. Aronow, P. R. Harding, M. Khursheed, J. S. Vangrow, and N. P. Papageorge's, "Effect of halofenate on serum uric acid," *Clinical Pharmacology & Therapeutics*, vol. 14, no. 3, pp. 371–373, 1973.

[2] W. S. Aronow, P. R. Harding, M. Khursheed, J. S. Vangrow, N. P. Papageorge's, and J. Mays, "Effect of halofenate on serum lipids," *Clinical Pharmacology & Therapeutics*, vol. 14, no. 3, pp. 358–365, 1973.

[3] W. S. Aronow, J. Vangrow, J. Pagano, M. Khemka, M. Vawter, and N. P. Papageorge's, "Long-term effect of halofenate on serum lipids," *Current Therapeutic Research: Clinical and Experimental*, vol. 16, no. 9, pp. 897–903, 1974.

[4] W. S. Aronow, J. S. Vangrow, W. H. Nelson, et al., "Halofenate: an effective hypolipemia- and hypouricemia-inducing drug," *Current Therapeutic Research: Clinical and Experimental*, vol. 15, no. 12, pp. 902–906, 1973.

[5] W. S. Aronow, M. D. Vicario, K. Moorthy, J. King, M. Vawter, and N. P. Papageorge's, "Long-term efficacy of halofenate on serum triglyceride levels," *Current Therapeutic Research: Clinical and Experimental*, vol. 18, no. 6, pp. 855–861, 1975.

[6] C. A. Dujovne, D. L. Azarnoff, P. Pentikainen, C. Manion, A. Hurwitz, and K. Hassanein, "A two-year crossover therapeutic trial with halofenate and clofibrate," *The American Journal of the Medical Sciences*, vol. 272, no. 3, pp. 277–284, 1976.

[7] E. B. Feldman, F. B. Gluck, and A. C. Carter, "Effects of halofenate on glucose tolerance in patients with hyperlipoproteinemia," *The Journal of Clinical Pharmacology*, vol. 18, no. 5-6, pp. 241–248, 1978.

[8] L. H. Krut, H. C. Seftel, and B. I. Joffe, "Comparison of clofibrate with halofenate in diabetics with hyperlipidaemia," *South African Medical Journal*, vol. 51, no. 11, pp. 348–352, 1977.

[9] A. K. Jain, J. R. Ryan, and F. G. McMahon, "Potentiation of hypoglycemic effect of sulfonylureas by halofenate," *The New England Journal of Medicine*, vol. 293, no. 25, pp. 1283–1286, 1975.

[10] E. A. Kohl, J. A. Magner, S. T. Persellin, G. M. Vaughan, D. J. Kudzma, and S. J. Friedberg, "Improved control of non-insulin-dependent diabetes mellitus by combined halofenate and chlorpropamide therapy," *Diabetes Care*, vol. 7, no. 1, pp. 19–24, 1984.

[11] D. J. Kudzma and S. J. Friedberg, "Potentiation of hypoglycemic effect of chlorpropamide and phenformin by halofenate," *Diabetes*, vol. 26, no. 4, pp. 291–295, 1977.

[12] T. Allen, F. Zhang, S. A. Moodie, et al., "Halofenate is a selective peroxisome proliferator-activated receptor γ modulator with antidiabetic activity," *Diabetes*, vol. 55, no. 9, pp. 2523–2533, 2006.

[13] F. Zhang, B. E. Lavan, and F. M. Gregoire, "Selective modulators of PPAR-γ activity: molecular aspects related to obesity and side-effects," *PPAR Research*, vol. 2007, Article ID 32696, 7 pages, 2007.

[14] P. A. Krieter, A. E. Colletti, G. A. Doss, and R. R. Miller, "Disposition and metabolism of the hypoglycemic agent pioglitazone in rats," *Drug Metabolism and Disposition*, vol. 22, no. 4, pp. 625–630, 1994.

[15] Z. J. Lin, D. Desai-Krieger, and L. Shum, "Simultaneous determination of glipizide and rosiglitazone unbound drug concentrations in plasma by equilibrium dialysis and liquid chromatography-tandem mass spectrometry," *Journal of Chromatography B*, vol. 801, no. 2, pp. 265–272, 2004.

[16] P. Yi, C. E. Hadden, W. F. Annes, et al., "The disposition and metabolism of naveglitazar, a peroxisome proliferator-activated receptor α-γ dual, γ-dominant agonist in mice, rats, and monkeys," *Drug Metabolism and Disposition*, vol. 35, no. 1, pp. 51–61, 2007.

[17] M. D. Greenspan, J. I. Germershausen, and R. Mackow, "Effect of halofenate and clofibrate on lipid synthesis in rat adipocytes," *Biochimica et Biophysica Acta*, vol. 380, no. 2, pp. 190–198, 1975.

[18] W. D. Wosilait and P. Nagy, "The distribution of halofenate in plasma: a comparative analysis using Scatchard vs. stepwise association constants," *Research Communications in Chemical Pathology and Pharmacology*, vol. 14, no. 1, pp. 75–81, 1976.

[19] R. L. Rich, Y. S. N. Day, T. A. Morton, and D. G. Myszka, "High-resolution and high-throughput protocols for measuring drug/human serum albumin interactions using BIACORE," *Analytical Biochemistry*, vol. 296, no. 2, pp. 197–207, 2001.

[20] Y. S. N. Day and D. G. Myszka, "Characterizing a drug's primary binding site on albumin," *Journal of Pharmaceutical Sciences*, vol. 92, no. 2, pp. 333–343, 2003.

[21] J. M. Collins and R. W. Klecker Jr., "Evaluation of highly bound drugs: interspecies, intersubject, and related comparisons," *The Journal of Clinical Pharmacology*, vol. 42, no. 9, pp. 971–975, 2002.

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Review Article

Peroxisome Proliferators-Activated Receptor (PPAR) Modulators and Metabolic Disorders

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Overweight and obesity lead to an increased risk for metabolic disorders such as impaired glucose regulation/insulin resistance, dyslipidemia, and hypertension. Several molecular drug targets with potential to prevent or treat metabolic disorders have been revealed. Interestingly, the activation of peroxisome proliferator-activated receptor (PPAR), which belongs to the nuclear receptor superfamily, has many beneficial clinical effects. PPAR directly modulates gene expression by binding to a specific ligand. All PPAR subtypes (α, γ, and δ) are involved in glucose metabolism, lipid metabolism, and energy balance. PPAR agonists play an important role in therapeutic aspects of metabolic disorders. However, undesired effects of the existing PPAR agonists have been reported. A great deal of recent research has focused on the discovery of new PPAR modulators with more beneficial effects and more safety without producing undesired side effects. Herein, we briefly review the roles of PPAR in metabolic disorders, the effects of PPAR modulators in metabolic disorders, and the technologies with which to discover new PPAR modulators.

1. OBESITY, ADIPOCYTES, AND ADIPOKINES

Obesity, which is defined as excess adiposity for a given body size, results from an imbalance between energy intake and energy expenditure. Body mass index (BMI), measured as body weight in kilograms over the square of the height in meters (kg/m^2), represents a widely accepted measure of adiposity. Wealth in industrialized societies, combined with an often-sedentary lifestyle and plentiful, high-calorie diets, creates irreversible weight gain. This social phenomenon can adversely impact well-being. Due to explosive concern for health and well-being, genes associated with human obesity are currently being defined, and whole genome scans will soon unveil its underlying genetic loci. The various causes of obesity are grouped according to behavioral (activity levels, nutrition, smoking status, and socioeconomic status), metabolic (physiological endocrine factors), and biological (genetic, racial, gender, age, and pregnancy status) influences [1]. Obesity has been recognized as a chronic disease since the National Institutes of Health Consensus conference in 1985 [2]. The increase in the prevalence of obesity has led the World Health Organization (WHO) to recently refer to the obesity issue as a "global epidemic".

Chronic disruption of the energy balance due to exceeding energy intake causes hypertrophy and hyperplasia of fat cells, and this is representative of the pathology of obesity. When the intake of energy chronically exceeds energy expenditure, most of the excess energy is stored in the form of triglyceride in adipose tissue (from Greek *adip-* or *adipo*, mean fat). Increased adipose tissue mass can arise through an increase in cell size, cell number, or both. Adipocytes are remarkably variable in size, which reflects the amount of stored triglyceride. Mild obesity mainly reflects an increased adipose cell size (hypertrophic obesity), while more severe obesity or obesity arising in childhood typically also involves an increased number of fat cells (hyperplastic obesity) [3]. As a key part of the homeostatic system that controls energy balance, the molecular mechanisms that regulate preadipose cell growth (proliferation), adipose differentiation (adipogenesis), and lipogenesis have been subject to extensive scrutiny. An overview of cell types and molecular events that occur during adipogenesis is presented in Figure 1. Preadipocytes undergo growth arrest, postconfluent mitosis, and clonal expansion following appropriate environmental and gene expression cues. The committed preadipocytes must then withdraw from the cell cycle before

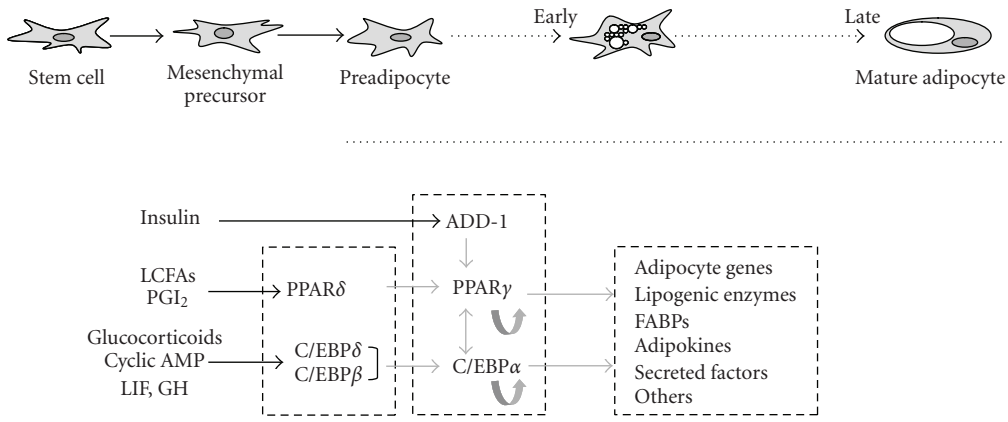


Figure 1: Adipocyte differentiation (adipogenesis) and transcriptional events in adipogenesis. A pluripotent stem cell precursor gives rise to a multipotential mesenchymal precursor cell with a potential to differentiate into an adipocyte. The preadipocyte enters the adipogenesis stage via environmental and gene expression signals. In an early stage of adipogenesis, major transcriptional factors such as PPARγ and C/EBPα are expressed, and these factors strongly regulate the expressions of adipogenesis-related genes. The adipocyte secretes various factors, including adipokines, and the secreted factors play an important role in glucose and lipid metabolism, immune system, appetite regulation, and vascular disease. LCFAs: long-chain fatty acids; PGI_2: prostacyclin; LIF: leukemia inhibitory factor; GH: growth hormone; ADD-1: adipocyte determination and differentiation factor-1; FABPs: fatty acid-binding proteins.

adipose conversion. During the differentiation of adipocytes, the adipocyte phenotypes are characterized by sequential changes in the expression of numerous genes [4, 5]. The study of the cellular and molecular events of adipogenesis was facilitated by the establishment of preadipose cell lines. Among these cell lines, some are derived from embryonic cells such as the 3T3-L1 and 3T3-F442A cell lines, and others such as the Ob17 cell line and its subclones, which originated from adult animals [6]. When maintained in appropriate culture conditions, these cells undergo an adipose conversion characterized by the transcriptional activation of numerous genes. The process of adipose conversion is controlled by external signals, and it has been found that adipogenic cocktails are different depending on the cell systems used. For instance, the 3T3-L1 preadipose cells are induced to differentiate by a treatment with high concentrations of cyclic AMP, dexamethasone, and insulin at the preadipose stage. Hormones such as insulin, triiodothyronine, glucocorticoides, and growth hormone exert positive actions on the differentiation of adipose cells. Prostaglandins such as prostacyclin (PGI_2), prostaglandin D_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) have also been found to be strong activators of adipogenesis. Several transcription factors have been shown to act cooperatively and sequentially to control adipogenesis. These include members of the transcription factors, such as CCAAT/enhancer-binding protein α (C/EBPα) [7], peroxisome proliferator-activated receptor-γ (PPAR-γ), and adipocyte determination, as well as differentiation factor-1 (ADD-1). The last stage of terminal differentiation corresponds to the activation of several genes, including those for proteins involved in triglyceride metabolism [8].

Adipose tissue is partitioned into a few large depots (subcutaneous and visceral locations), and many small depots (heart, epicardium, pericardium, large blood vessels, major lymph nodes, bone marrow, kidney, adrenal glands, and the brain) [9]. All adipocytes secrete a large number of multifunctional molecules, including cytokines, growth factors, enzymes, hormones, complement factors, matrix proteins, and so forth. The proteins that are secreted from adipocytes are designated "adipokines" or "adipocytokines". Since the isolation of the first-known adipocyte-secreted protein (the serine protease adipsin) in 1987 [10], the list of adipokines has been greatly extended. Leptin (from Greek *leptos*, means thin), encoded by the obese (*ob*) gene [6, 11], adiponectin (also called Acrp30) [12, 13], Interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) [14], resistin [15], and visfatin [16] are candidates of great interest among the growing number of factors found to be secreted by adipocytes. It has recently been shown that adipokines that are secreted from adipocytes contribute to the development of obesity-associated metabolic disorders, including insulin resistance, cardiovascular disease, and cancer [17].

2. METABOLIC DISORDERS AND THERAPEUTIC TARGETS

Overweight and obesity lead to increased risk for noninsulin-dependent diabetes, hypertension, coronary artery disease, dyslipidemia, gallstones, osteoarthritis, sleep apnea, certain forms of cancer, and degenerative arthritis. As the prevalence of obesity has increased, the heterogeneous clinical disorder strongly associated with abdominal obesity and insulin resistance has been identified as a major risk factor for atherosclerotic cardiovascular disease. This disorder, previously termed "syndrome X" by Reaven, and "insulin-resistance syndrome" by others, is now considered to be metabolic syndrome or metabolic disorder [18]. This disorder shares similar cardiovascular risk factors, including abdominal obesity, impaired glucose regulation/insulin resistance, dyslipidemia, and hypertension. Accordingly, these factors define the

clustering of findings typical of the metabolic disorders, and establish diagnostic criteria. A number of studies have shown that the excess body fat that is stored in the deep abdominal area is associated with metabolic complications [19]. Recently, several molecular drug targets with potential to prevent or treat metabolic disorders have been revealed.

The excess glucocorticoid action by the enzyme 11β-HSD (hydroxysteroid dehydrogenase) type 1 induces obesity and features of metabolic disorders. Transgenic mice which are selectively over-expressing 11β-HSD1 in adipose tissue lead to increased food intake and body weight, as well as the development of visceral obesity. In addition, insulin-resistant diabetes, hyperlipidemia, and hyperphagia were observed in 11β-HSD1 transgenic mice [20]. On the other hand, 11β-HSD1 deficiency causes favorably altered fat distribution and adipose insulin sensitization. Even with high-fat and cholesterogenic diets, lipid profiles are also improved [21]. 11β-HSD1 inhibitors might have beneficial consequences in metabolic disorder. For instance, carbenoxolone, an 11β-HSD1 inhibitor, reduced total cholesterol in healthy subjects, and decreased the glucose production rate during hyperglucagonemia in diabetic patients [22]. AMP-activated protein kinase (AMPK) is a major regulator of lipid and glucose metabolism, and AMPK activation appears as a benefit of exercise in diabetic patients. Activation of AMPK by metformin decreased the level of plasma glucose and plasma triglycerides by promoting muscle glucose uptake and inhibiting hepatic glucose output [23]. SCD-1 (stearoyl CoA desaturase-1) is required for the biosynthesis of the monounsaturated fatty acids from saturated fatty acids, and SCD-1-deficient mice appear visibly lean compared to their littermates. SCD-1 deficiency in *ob/ob* mice ameliorates obesity and completely corrects the excessive hepatic lipid storage and VLDL production of the hypometabolic phenotype in leptin deficiency [24]. An SCD-1 inhibitor that reduces SCD-1 activity may serve as a therapeutic strategy for metabolic disorders, but very few reports are available for the use of SCD-1 inhibitor. I*k*B kinase β (IKKβ) plays a key role in the activation of NF-*k*B by phosphorylating I*k*Bα. It has recently been reported to act as a key role in obesity-linked insulin resistance. In obese rodents, increased IKK activity or overexpressed IKK promotes insulin resistance, whereas reduction of IKK activity or IKKβ expression improves insulin sensitivity. In addition, high doses of IKKβ inhibitors such as aspirin and salicylate reverse insulin resistance by sensitizing insulin signaling in obese rodents [25]. Protein tyrosine phosphatase 1B (PTP1B) is closely associated with insulin signaling through the dephosphorylation of activated insulin receptor or insulin receptor substrates. PTP1B deficiency and its heterozygote significantly reduce glucose concentrations in the blood, and PTP1B deficiency causes a significant reduction of circulating insulin concentration compared to wild-type mice. When on a high-fat diet, PTP1B-deficient mice were resistant to diet-induced weight gain, and remained insulin-sensitive [26]. Because PTP1B inhibition provides attractive therapies against metabolic disorders, various studies for the inhibition mechanism of inhibitors against PTP1B, the structure-activity relationship, and synthetic and pharmacological materials have been performed by different groups. Acetyl-CoA carboxylase (ACC) is a key determinant of energy homeostasis because increased malonyl-CoA by ACC activation inhibits mitochondrial fatty acid uptake and oxidation. A lack of malonyl-CoA in the muscle and heart of ACC2-deficient mice show increased oxidation of fatty acid, decreased fat in adipose and liver tissue, and decreased the storage of glycogen in the liver [27]. CP-640186, an isozyme-nonselective ACC inhibitor, inhibits fatty acid and TG synthesis in HepG2 cells, as well as fatty acid synthesis in obese rodents. CP-640186 also stimulates fatty acid oxidation in C2C12 cells [28]. These effects of the ACC inhibitor may provide novel therapeutic potential for treatment of the metabolic disorder. Interestingly, the activation of PPARs by their ligands has many beneficial effects in the improvement of glucose homeostasis and lipid homeostasis.

3. PPARs AND METABOLIC DISORDERS

3.1. PPARs as a nuclear receptor family

Peroxisomes are subcellular organelles that perform diverse metabolic functions, including H_2O_2-derived respiration, β-oxidation of fatty acids, and cholesterol metabolism. Rodents exposed to peroxisome proliferators lead to hepatocellular hypertrophy, hyperplasia, and transcriptional induction of fatty acid-metabolizing enzymes that are regulated in parallel with peroxisome proliferation [29]. Peroxisome proliferators may activate PPARs by binding directly to the receptors, and the activated PPARs may regulate the expression of genes involved in lipid metabolism and peroxisome proliferation. Recent research on PPARs has moved toward their pivotal roles comprising one family of nuclear receptors [30]. Nuclear receptors, which are present in multicellular organisms, directly control the expression of genes in response to a wide range of developmental, physiological, and environmental signals.

The PPARs of nuclear receptors mainly consist of three subtypes (PPARα, PPARγ, and PPARδ/β). All three PPAR isoforms possess similar structural and functional features. Principally, four functional domains have been identified, and are referred to as A/B, C, D, and E/F. The N-terminal A/B domain contains ligand-independent activation function 1 (AF-1). The ligand-independent activation region can confer constitutive activity on the receptor, and is negatively regulated by phosphorylation [31]. The DNA-binding domain (DBD) or C domain consists of two zinc fingers, and is directly involved with the binding of PPAR to the peroxisome proliferator response element (PPRE) in the promoter regions of target genes. PPREs are direct repeat (DR)-1 elements consisting of two hexanucleotides with the AGGTCA consensus sequence separated by a single nucleotide spacer. Such a sequence, or a similar one, has been found in numerous PPAR-inducible genes, including acyl-CoA oxidase (ACO) and adipocyte fatty acid-binding protein (aP2) [32]. The D site is a hinge region and a docking domain for corepressors. The E/F domain or ligand-binding domain (LBD) is responsible for ligand specificity and the activation of PPAR binding to the PPRE, which increases the expression

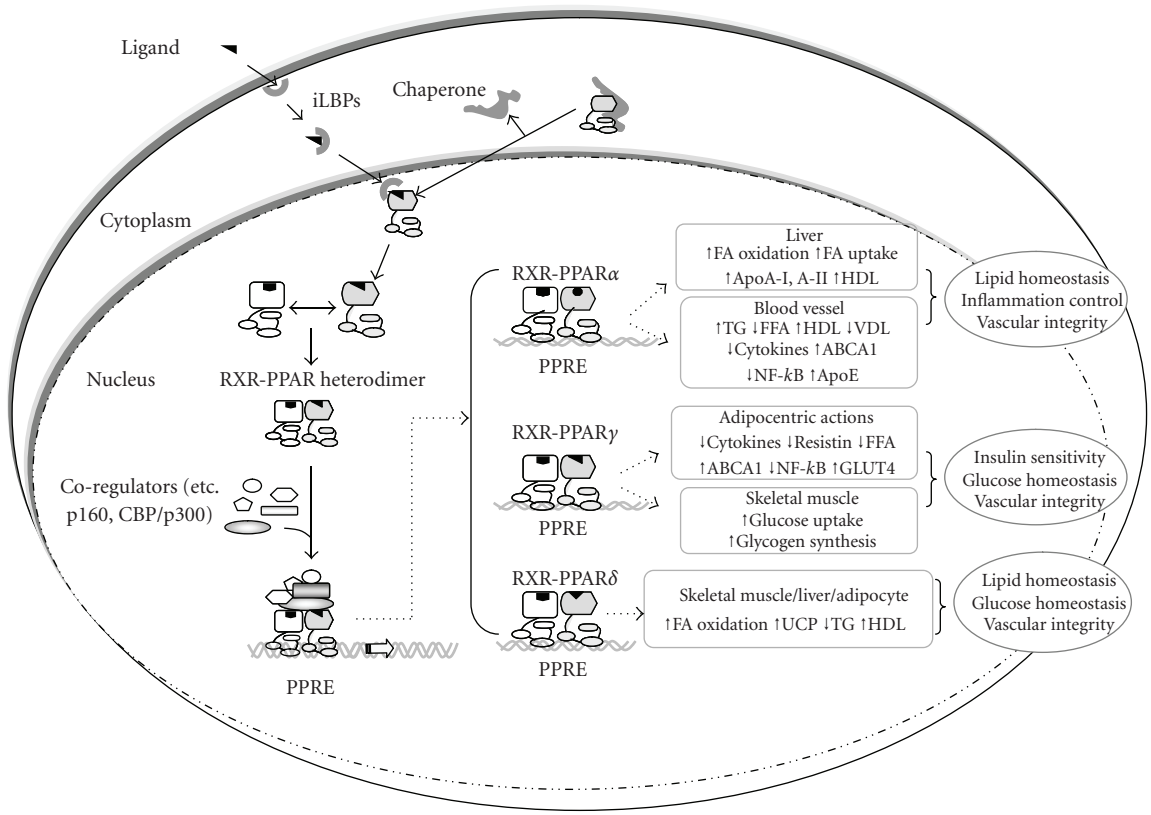


Figure 2: Signaling pathways activating PPAR and regulating the biological effects of PPAR in different organs. PPAR activity can be regulated by the direct binding of small lipophilic ligands. Ligand-unbound PPAR in the cytosol is associated with chaperons, and the association changes the conformation of PPAR that allows for high-affinity binding to the ligand. Ligand-bound PPAR forms a heterodimer with RXR, and the PPAR-RXR heterodimer constructs the transcriptional machinery through the recruitment of coregulators. The transcriptional machinery regulates gene expression by binding to specific DNA sequence elements, termed PPAR response elements (PPRE). PPARα is strongly expressed in the liver, heart, and blood vessels, and regulates the expressions of genes related to lipid metabolism and inflammation control. PPARγ exerts its effects on insulin sensitivity and glucose homeostasis in adipocytes and skeletal muscles. PPARδ is expressed ubiquitously, and controls the expressions of genes that are involved in glucose and lipid metabolism. FA: fatty acid; HDL: high-density lipoprotein; VDL: very low-density lipoprotein; ABCA1: ATP-binding cassette transporter A1; UCP: uncoupling protein; TG: triglyceride.

of the targeted gene. Upon the binding of a specific ligand to LBD of the E/F domain, the conformation of a PPAR is altered and stabilized. The ligand-bound LBD results in the recruitment of transcriptional coactivators, resulting in gene transcription. Although three of the PPAR isoforms possess similar structures, it is clear that these receptors perform distinct functions according to the specific ligands and their expression patterns in the tissues.

3.2. PPARs and their ligands

Ligand-induced activation of the PPAR, by means of low-affinity binding to natural lipid ligands, stimulates an array of molecular responses that aim at maintaining lipid and glucose homeostasis. Ligand-unbound PPAR is associated with chaperon in the cytosol, and the association induces the PPAR to be held in a conformation that allows for high-affinity binding of the ligand [33]. The translocation of a hydrophobic ligand into the cell is facilitated in intra- and extracellular fluids by intracellular lipid-binding proteins (iLBPs) that are members of the family of fatty acid-binding proteins (FABPs). The iLBPs with relatively small sizes (15-16kDa) play important roles in the solubilization and protection of ligands in aqueous spaces. Ligand-loaded iLBP in the cytosol translocates into the nucleus by free diffusion, and they form a short-lived complex with PPAR [34–36]. Ligand is then transferred to the PPAR, and the ligand-bound PPAR forms a heterodimer with the partner nuclear receptor, retinoid X receptor (RXRα). Upon binding to a ligand, the conformation of PPAR is altered and stabilized, and the PPAR-RXR heterodimer then recruits transcriptional coactivators [37–39]. The transcription machinery is bound to PPRE, and directly controls the expression of the target gene (Figure 2) [40].

PPARα was first cloned from the rodent liver in 1990 [41], and PPARβ and PPARγ were first identified in *Xenopus* [42]. Several groups subsequently reported the cloning of mammalian orthologs of PPARα, PPARβ, and PPARγ. Although PPARα and PPARγ are highly conserved across species, PPARβ varies considerably between *Xenopus* and

mammals. The murine clone was named PPARδ because of this divergence [43]. PPARα is predominantly expressed in the liver, and is involved in peroxisome proliferation and regulation of fatty acid catabolism. The expression of PPARδ is ubiquitous and abundant in the brain, intestine, skeletal muscle, spleen, macrophages, lung, and adrenals [44]. PPARδ is activated by a large variety of ligands, and has been implicated in developmental and metabolic regulation in several tissues. PPARγ is expressed in adipose tissue, promoting adipogenesis and increasing lipid storage. PPARγ has at least two promoters, and results in the production of two isoforms, 1 and 2. These isoforms are expressed in a tissue-specific pattern. The PPARγ1 isoform is expressed in the spleen, intestine, and white adipose tissue, while the PPARγ2 is preferentially expressed in white and brown fat. PPARγ2 is most abundantly expressed in fat cells, and plays a pivotal role in fat cell differentiation and lipid storage [45]. The distinct physiological roles of each subtype have been shown to be determined by binding to a discrete set of ligands. Although fatty acids could activate PPARs, PPARα activity was induced by eicosanoids [46], cabaprostacyclin [47], and nonsteroidal anti-inflammatory drugs (NSAIDs) [48]. PPARδ was activated by several polyunsaturated fatty acids [49] and eicosanoids [50]. PPARγ specifically binds to thiazolidinediones (TZDs), a class of antidiabetic drugs. Other PPARγ ligands include the natural prostaglandin metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2(PGJ_2), polyunsaturated fatty acids, and NSAIDs such as ibuprofen and indomethacin [51, 52].

3.3. Post-translational regulation of PPARs

PPARs and other nuclear receptors modulate their transcriptional activity via phosphorylation by various kinases, including the mitogen-activated protein kinase (ERK MAPK and p38 MAPK), protein kinase A and C (PKA and PKC), AMP kinase (AMPK), and glycogen synthase kinase 3 (GSK3) [53]. Several mechanisms have been described to explain the modulation of PPAR transcriptional function. First, phosphorylation modulates the affinity of PPARs for their ligand, as well as the coactivator recruitment abilities of PPARs. Although the main phosphorylation site of PPARγ (Ser 112) is located far from the ligand-binding domain, mutated PPARγ (S112D) exhibits a decreased ligand-binding affinity and decreased coactivator recruitment [54]. Second, the phosphorylation of PPARs modulates binding to PPRE. In gel retardation experiments, PPARα phosphorylation via PKA enhances gene expression due to the stabilization of the binding of PPARα to DNA [55]. Finally, phosphorylation plays an important role in ubiquitination and proteasomal catabolism of PPARs. Phosphorylation of the PPARγ AF-1 domain by IFNγ-ERK-regulated serine phosphorylation promotes the degradation of PPARγ by the ubiquitin-proteasome-dependent degradation in response to ligand activation [56]. PPARα is also degraded by the ubiquitin-proteasome-dependent degradation. However, in contrast to PPARγ, phosphorylation of PPARα induces the stabilization of PPARα by reducing ubiquitination. The phosphorylation and interaction of PPARα with a corepressor stabilize PPARα protein by decreasing its ubiquitination in order to keep a pool of PPARα available for ligand binding and activation [57].

SUMOylation consists of the covalent and reversible conjugation of small ubiquitin-related modifiers (SUMOs) to target protein and regulate biological processes. The number of known SUMO targets is growing, and SUMOylation of PPARγ has recently been reported. SUMOylation of PPARγ mainly occurs at a lysine residue within a ligand-independent activating function domain (AF-1). PPARγ is SUMOylated by SUMO-1 and PIAS proteins that function as E3 ligases [58]. Potential SUMOylation sites of PPARγ include K77 (equivalent to K107 of PPARγ2) and K365. SUMOylation of PPARγ at K77 and K365 occurs in a ligand-dependent manner. SUMOylation of K107 inhibits PPARγ-dependent gene induction, but does not affect transrepression, whereas mutation of K365 eliminates the ability of agonist-activated PPARγ to repress iNOS and to be recruited to its promoter [59]. Phosphorylation at S112 of PPARγ2 promotes K107 SUMOylation and exerts more potent repressive effects. The SUMOylation-defective mutation of PPARγ at K77R promotes adipocyte differentiation. The potential SUMOylation site of PPARα has one K185 within the D region, and PPARδ/β has one K104 in the C region, but in vivo SUMOylation is specific for PPARγ among the PPARs [60]. Relatively few studies of post-translational regulation of PPARs have been reported.

3.4. Role of PPAR ligands in metabolic disorders

The activation of PPARα upregulates the expressions of several catabolic enzymes that are involved in mitochondrial and peroxisomal β-oxidation and microsomal ω-oxidation, as well as in the transcriptional regulation of genes that are necessary for the maintenance of the redox balance during the oxidative catabolism of fatty acids. The derivatives from fatty acids and fibrates, including gemfibrozil, fenofibrate, cofibrate, bezafibrate, and ciprofibrate, can activate PPARα. These fibrates are used in the treatment of hypertriglyceridemia. PPARα agonists fundamentally regulate β-oxidation of fatty acids, and promote the expression of cytochrome P450 enzymes, which catalyze the ω-hydroxylation of fatty acid [61]. WY14,643, a well-known specific PPARα agonist, increases fatty acid oxidation by increasing the expressions of peroxisomal and mitochondrial fatty acid β-oxidation enzymes. WY14,643 reduces liver insulin resistance more efficiently than muscle insulin resistance by normalizing the circulating triglyceride levels and blood glucose levels in lipoatrophic mice [62]. PPARα agonists also activate the expression of apolipoprotein A-1 (ApoA-1) and ATP-binding cassette transporter A1 (ABCA1) [63, 64]. The increased ApoA-1 and ABCA1 proteins enhance cholesterol efflux by the reverse cholesterol transport (RCT) pathway. In addition, PPARα agonists have anti-inflammatory effects in vascular cells. WY14,643 or bezafibrate induces PPARα-mediated inhibition of osteopontin (OPN) expression in human macrophages of atherosclerotic lesions, where they are abundantly synthesized. Bezafibrate significantly decreases OPN plasma levels in type 2 diabetic

patients [65]. Therefore, the PPARα agonist reduces the progression of atherosclerosis and decreases the incidence of coronary heart disease [66]. However, fibrates are contraindicated in patients with renal insufficiency, gallstones, abnormal liver function tests, and pregnancy [67].

The activation of PPARγ promotes the storage of fat by increasing adipocyte differentiation and enhancing the transcription of genes that are important for lipogenesis. The activation of either PPARα or PPARγ in macrophages promotes the cellular efflux of phospholipids and cholesterol in the form of high-density lipoproteins by upregulating the expression of the liver X-receptor (LXR), an oxysterol-activated nuclear hormone receptor that increases expression of the lipid transporter ABCA1 (ATP-binding cassette, subfamily A, member 1) [68]. PPARγ has been the focus of intense research during the past decade because ligands for this receptor have emerged as potent insulin sensitizers that can be used in the treatment of type 2 diabetes [69]. Increased levels of circulating free fatty acids and lipid accumulation in non-adipose tissue have been implicated in the development of insulin resistance. This situation is improved by the PPARγ agonist, which promotes fatty acid storage in fat depots and regulates the expression of adipocyte-secreted hormones that impact glucose homeostasis [70]. The net result of the pleiotropic effects of the PPARγ agonist is improvement of insulin sensitivity, although undesired side effects limit the utility of this therapy. In fact, TZD, a synthetic agonist of PPARγ, appears to be ideally suited for the treatment of this cluster of metabolic abnormalities, which has been termed the insulin resistance or cardiovascular dysmetabolic syndrome as a whole [71]. Two compounds in this class are currently approved for use in the United States. They are Rosiglitazone (Avandia), approved by the US Food and Drug Administration (FDA) in May 1999, and Pioglitazone (Actos), which was approved in July 1999. Historically, the first agent in this class, Troglitazone (Rezulin), was marketed in the United States from March 1997 to March 2000. Troglitazone was banned because the FDA determined that the risk of idiosyncratic hepatoxicity associated with Troglitazone therapy outweighed its potential benefits [72, 73].

The activation of PPARδ in macrophages also upregulates the expression of the ABCA1 transporter. Recent evidence indicates that PPARδ can also promote cellular lipid accumulation by increasing the expressions of genes that are involved in lipid uptake, and by repressing key genes that are involved in lipid metabolism, inflammation, atherosclerosis, obesity, fertility, and cancer [74, 75]. Several 14- to 18-carbon saturated fatty acids as well as 16- to 20-carbon polyunsaturated fatty acids are screened as PPARδ-binding chemicals in ligand screening and competition binding assays [50, 76, 77]. As physiological ligands of PPARδ, these fatty acids or eicosanoids are unsettled. However, Chawla et al. hypothesized that PPARδ acts as a lipid sensor, where fatty acids derived from very-low-density lipoprotein (VLDL) can activate PPARδ [78]. A PPARδ-specific agonist, GW501516, decreases plasma triglyceride levels in obese monkeys, raises high-density lipoprotein levels, and prompts the initiation of clinical trials to assess its efficacy in hyperlipidemic patients [79]. GW501516 also attenuates weight gain and insulin resistance in mice fed high-fat diets by increasing the expressions of genes that promote lipid catabolism and mitochondrial uncoupling in skeletal muscle, thereby increasing β-oxidation of the fatty acids in skeletal muscle [80]. PPARδ agonists also have anti-inflammatory properties. The PPARδ agonist inhibits LPS-inducible genes, such as COX-2 and iNOS in murine peritoneal macrophages [81]. These reports indicate that the PPARδ-specific agonist is a potential therapeutic interest for the treatment of metabolic disorder.

4. VARIOUS STRATEGIES FOR SAFER PPAR MODULATORS

Each PPAR subtype regulates a distinct metabolic pathway, and the agonists of each of the PPAR subtypes have distinct effects with undesired side effects such as weight gain, hepatotoxicity, and heart failure. In the case of the TZD class as PPARγ agonists, the major side effect is weight gain. A Pro12Ala substitution in PPARγ2 decreases PPARγ activity, BMI, and insulin resistance [82]. Because of these undesirable effects caused by PPARγ agonists, new therapeutic solutions have been investigated in order to reduce their side effects. Various compounds have been reported to be PPAR antagonists, including Bisphenol A diglycidyl ether (BADGE), PD068235, LG100641, GW9662, SR-202, GW6741, and Compound A and B [83]. A potent selective PPARγ antagonist, GW9662, does not recruit PPAR coactivators such as SRC-1 and p300, and it suppresses rosiglitazone-induced adipocyte differentiation in 3T3-L1 adipocytes. GW9662 prevents high-fat diet-induced obesity without affecting food intake, and has no effect on high-fat diet-induced glucose intolerance [84]. The phosphonophosphate SR-202, a PPARγ antagonist, inhibits BRL 49653-mediated recruitment of SRC-1 and troglitazone-induced transcriptional activity. SR-202 inhibits PPARγ-induced adipocyte differentiation of 3T3-L1 and prevents weight gain and adipose tissue deposition in mice given a standard diet or high-fat diet. In addition, SR-202 markedly reduces hyperglycemia and hyperinsulinemia in ob/ob mice [85]. A few PPARα antagonists have been reported, but in vivo data have not been disclosed. Several PPARγ antagonists may have therapeutic availability as antiobesity drugs. However, further studies of the molecular effects of PPARγ antagonists are necessary.

The combination agonist strategy, which uses a combination of agonists, has been designed to activate each receptor subtype. In terms of its pharmacological aspects, this strategy may provide more efficacious effects and more safety for undesired side effects. The possible combinations are PPARα/γ dual agonist, PPARγ/δ dual agonist, PPARα/δ dual agonist, and PPARpan (PPAR$\alpha/\gamma/\delta$) agonist. The initial combination agonist strategy was focused on the development of PPARα/γ dual agonists. PPARγ agonists such as rosiglitazone and pioglitazone provide undesired side effects of TZDs, including weight gain. By contrast, PPARα agonists such as fibrate decreased adiposity through the stimulation of lipid oxidation. Dual PPARα/γ stimulation with a combination

of rosiglitazone and fenofibrate in type 2 diabetic patients effectively improved the atherogenic dyslipidemic profile, which plays a key role in the occurrence of cardiovascular mortality [86]. Applications of structurally various PPARα/γ dual agonists have recently been reported. Among these dual PPARα/γ agonists, compounds belonging to the glitazar class have been advanced to clinical development (Phases II and III). These PPARα/γ dual agonists commonly reduce triglycerides and total cholesterol, increase HDL levels, and consequently improve insulin sensitivity. However, the use of a few PPARα/γ dual agonists, including muraglitazar, tesaglitazar, ragaglitazar, farglitazar, TAK559, and KRP297, has been discontinued due to various safety liabilities compared to selective agonists. All glitazars significantly increase weight gain and edema, because of higher PPARγ affinity than PPARα affinity although their affinity for PPARα is higher than fibrates. Muraglitazar increases cardiovascular risks, tesaglitazar impairs glomerular filteration rate, and some have carcinogenic effects in mice [87]. The safety liabilities may be the result of their imbalanced activities on PPARγ and PPARα. Therefore, the best solution would be to screen candidates with appropriate affinity for PPARα and selective PPARγ-modulating activity [88].

Both PPARγ and PPARδ play important roles in glucose and lipid metabolism. A PPARγ/δ dual agonist with a properly controlled γ/δ ratio could attenuate undesired weight gain, improve insulin sensitivity, and stimulate fatty acid oxidation. The dual PPARγ/δ agonist (R)-3-{2-ethyl-4-[3-(4-ethyl-2-pyridin-2-yl-phenoxy)-butoxy]-phenyl}-propionic acid has been shown to lower the glucose level and cause less weight gain than rosiglitazone in hyperglycemic male Zucker diabetic fatty (ZDF) rats [89]. The other dual PPARγ/δ agonist, (R)-3-{4-[3-(4-chloro-2-phenoxy-phenoxy)-butoxy]-2-ethyl-phenyl}-propionic acid, improves insulin sensitivity and reverses diabetic hyperglycemia with less weight gain relative to rosiglitazone in female ZDF rats [90]. PPARα/δ dual agonists (T659 and Compound 24) have recently been reported. T659 has had beneficial effects on HDL-C in experimental primates [91]. Compound 24 has also shown significant effects on HDL-C, TG, and FFA levels in male hApoA1 transgenic mice [92]. PPARα/δ dual agonists may improve hyperlipidemia, insulin resistance, and risk of atherosclerosis. The development of PPARα/δ and PPARγ/δ dual agonists is currently being pursued.

Another strategy to reduce the adverse effects of previous PPARγ agonists is the identification of partial agonists, also referred to as selective PPARγ modulators (SPPARγMs). SPPARγMs are PPARγ ligands with insulin-sensitizing activity and lower stimulation of adipogenesis. Because SPPARγMs bind to the ligand-binding pocket of the PPARγ receptor in distinct manners, SPPARγM-bound PPARγ induces the displacement of the differential cofactor and the specific gene expression in a tissue-specific manner. Although several PPARγ agonists have been classified as SPPARγMs, the majority of these synthetic ligands remain to be characterized at the molecular level, and need to be evaluated in in vivopreclinical models to assess their relationships with weight gain [93]. Halofenate (HA) and PA-082, new SPPARγMs, were recently developed. HA causes displacement of corepressors (N-CoR and SMRT), but does not cause efficient recruitment of coactivators (p300, CBP, and TRAP 220). Moreover, HA selectively regulates the expression of multiple PPARγ responsive genes in 3T3-L1 adipocytes, and has acute antidiabetic properties in diabetic *ob/ob* mice [94]. The isoquinoline derivative PA-082, a prototype of a novel class of non-TZD partial PPARγ agonists, causes preferential recruitment of PPARγ-coactivator-1α (PGC1α) to the receptor compared with rosiglitazone. PA-082 antagonizes rosiglitazone-driven transactivation and TG accumulation in C2H10T1/2 mesenchymal stem cells. However, PA-082 induces mRNAs of genes that encode components of insulin signaling pathways. It also facilitates glucose uptake and insulin signaling in mature adipocytes [95]. The functional study of SPPARγMs will provide more information about effective antidiabetic agents to reduce the side effects of weight gain.

The PPARpan agonists can activate all three PPAR subtypes, and they can potentially exert various effects on metabolic disorders such as insulin resistance, obesity, dyslipidemia, and hypertension. The well-known lipid-lowering bezafibrate is the first clinically-tested PPARpan agonist. Though bezafibrate is a PPAR ligand with a relatively low potency, it considerably raises HDL cholesterol, reduces triglycerides, improves insulin sensitivity, and reduces blood glucose levels [96]. GW677954, a novel PPARpan agonist, is being investigated in Phase II trials for the treatment of metabolic disorders [97]. PLX-204 and GW-625019 are also progressing in Phase I trials for the treatment of metabolic disorders. In addition, LY-465608, DRF-11605, CS-204, and DRL-11605 are under investigation, and may be potent therapeutic agents for the treatment of metabolic disorders [88].

5. TECHNOLOGIES TO DISCOVER NEW PPAR MODULATORS

The development of new technology to discover PPAR modulator is significant in functional study of the nuclear receptors and new potent drug discovery. In general, transactivation and chimeric receptor transactivation assays have been used as cell-based methods employing mammalian cells for the screening of new PPAR modulators. Cell-based assays provide a more physiological relevance, but these assays are costly, time-consuming, and difficult to apply to automated systems used for high-throughput screening (HTS). Recently, Chen et al. introduced a yeast-based method for screening PPAR modulators [98]. Cell-free assays for the screening of PPAR modulators have been developed in numerous forms. The X-ray crystal structure study revealed that the human apo-PPARγ ligand-binding domain (LBD) has a large binding pocket, which may explain the diversity of the PPARγ ligands [99]. When binding to specific ligands in LBD, PPAR changes its conformation. Glutamate and lysine residues that are highly conserved in LBDs of PPAR form a "charge clamp" that contacts the backbone atoms of the LXXLL helices of coactivators such as steroid receptor coactivator-1 (SRC-1). In the case of SRC-1, four consecutive LXXLL motifs make identical contacts with both subunits of a PPAR-RXR heterodimer [100]. Such allosteric conformational changes

promote the recruitment of nuclear receptor coactivators and effectively stimulate the transcription of their target genes. Different PPAR ligands may elicit distinct downstream biological effects due to unique conformational changes in the nuclear receptor.

A cell-free competition radioreceptor assay using competitive interaction between a recombinant PPAR protein and a radioisotope-labeled ligand in the presence of competitor ligands has been reported previously (Figure 3(a)) [101, 102]. The coactivator-dependent receptor ligand assay (CARLA) has been reported as a cell-free assay based on the interaction between PPAR and the coactivator. CARLA is based on the recruitment of a transcriptional coactivator by changes in the conformation of ligand-bound PPAR (Figure 3(b)). In presence of PPAR ligands,^{35}S-labeled SRC-1 has stronger interaction with GST-fused PPAR proteins immobilized on glutathione Sepharose beads. Autoradiograms and the quantification of the effect of candidates are dependent on the retention of SRC-1 by the GST-PPAR LBD [76]. The major advantage of CARLA is that it does not require radioactive labeling of candidate modulators, which makes it possible to screen a large number of compounds with this assay; this has simultaneous economic advantages in terms of materials and time.

Scintillation proximity has been developed as a tool for measuring the interaction between a receptor and a ligand. The scintillation proximity assay (SPA) bead is impregnated with a scintillant and coated with a capture molecule such as streptavidin. After preincubation of SPA beads and biotinylated PPAR LBD, radiolabeled ligands were added to a complex of SPA bead-PPAR LBD. Unbound free ligands were eliminated from the SPA-PPAR complex. When SPA-bead and radiolabeled ligands come into close proximity, radioactive counts are determined by the β emission from the radioisotope to be absorbed by the scintillant, which will then shift this energy to produce light (Figure 3(c)) [103, 104]. The advantages of the SPA are as follows: low cost, high sensitivity, high reliability, and simplicity, that is, no separation step is required. The simplicity of SPA is an important benefit in its application to HTS. However, unsuitability for kinetic determination and the limited number of useful isotopes were perceived as potential disadvantages.

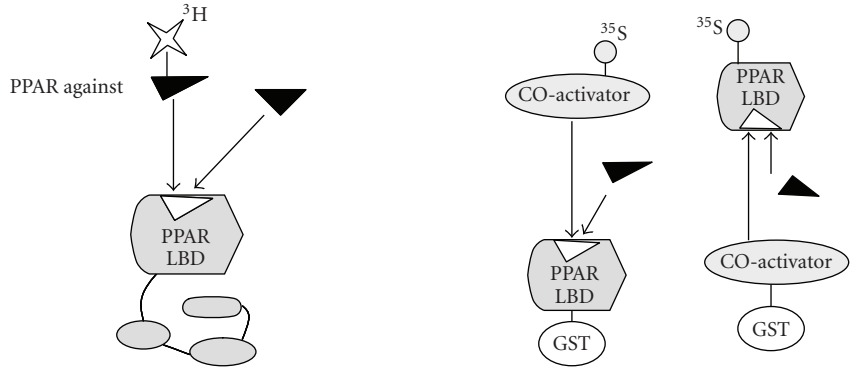
Fluorescence has been considered as an analytical technique with which to study for the detection and quantitation of interacting molecules. There are several advantages to this technique, including high sensitivity, the relative ease of handling and disposal compared to radioactivity, and the diversity of available fluorophores. Thus, fluorescence resonance energy transfer (FRET) has been applied in the probing of molecular interactions [105]. As shown in Figure 3(d), GST-PPAR LBD was indirectly linked to Eu(K) through an anti-GST antibody, which was covalently linked to Eu(K). Coactivator was also indirectly linked to XL665 through a streptavidin (SA)-biotin adapter. The conformational change of PPAR by the PPAR agonist induces the recruitment of coactivator, and the interaction between PPAR and a coactivator will result in the close proximity of the fluorescence donor and acceptor. Consequently, the fluorescence donor (anti-GST-Eu(K)) is excited, and inputted energy will be transferred to the acceptor (streptavidin-XL665). Homogenous time-resolved fluorescence (HTRF) energy transfer technology takes advantage of fluorescence, as well as the homogenous and time-resolved detection mode. These specificities of HTRF enable it to overcome most of the drawbacks encountered in FRET [106].

Previous cell-free methods with which to screen PPAR agonists have used isotope or fluorescence labeling agonists or proteins. We established very simple ELISA systems based on the ligand-dependent binding between PPAR and coactivators. In brief, the purified recombinant LXXLL motif of coactivators was applied into a 96-well plate, and *E. coli* lysates containing recombinant PPAR proteins were then added with candidate PPAR agonists. The complex consisting of PPAR and coactivator was then identified with the anti-PPAR antibody (Figure 3(e)). Major advantages of this simple method are its simplicity and its low cost, as these systems do not require any labeling of candidate modulators and proteins. This makes it possible to screen a large number of compounds, with simultaneous economic advantages in terms of materials and time. On the other hand, this method has relatively low sensitivity and has to use a suitable anti-PPAR antibody [107–109].

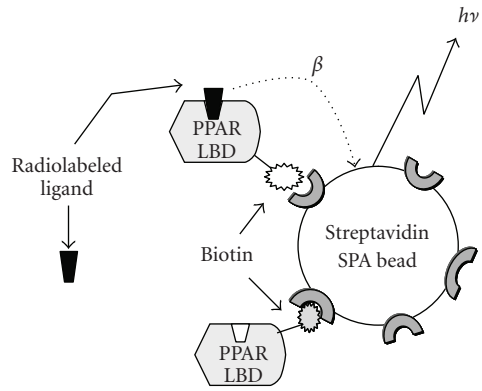
In the 1980s, surface plasmon resonance (SPR) and related techniques that exploited evanescent waves were applied for the study of biological and chemical interactions. SPR technology has also been successfully employed to study the interactions between ligands and nuclear receptors [110–112], the effects of ligand-binding on nuclear receptor dimerization [113], and ligand screening based on interactions between ligand-bound nuclear receptors and coactivators [114, 115] (Figure 3(f)). In the interaction analysis between PPAR LBD and ligand, PPAR LBD is immobilized on the sensor chip by a standard primary amine-coupling reaction, and the ligand is injected over the immobilized PPAR LBD. In the investigation of ligand binding on receptor dimerization, the partner nuclear receptor is immobilized on the sensor chip by the standard amine-coupling reaction, and the nuclear receptor pre-incubated with its ligand is injected over the immobilized partner nuclear receptor. In ligand screening based on interactions between ligand-bound nuclear receptors and coactivator, the coactivator or LXXLL peptide is immobilized on the sensor using same methods, and the nuclear receptor that was preincubated with a candidate chemical is injected over the immobilized coactivator peptide. The association (k_a) and dissociation (k_d) rate constants and the dissociation equilibrium constants (k_Ds) for the bindings were determined using the Biacore biosensor. The binding responses in resonance units (RUs) were continuously recorded, and were presented graphically as a function of time. SPR technology has the advantages in that it requires no labeling, can be performed in real-time, and utilizes noninvasive measurements.

6. SUMMARY

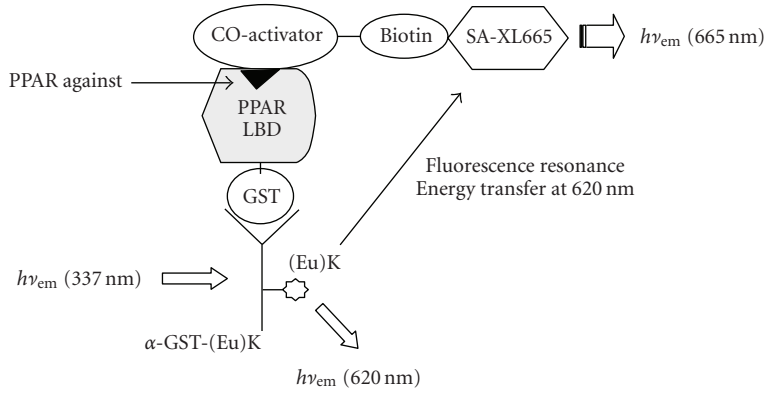
Obesity mainly reflects an increased adipose cell size, an increased adipocyte cell number, and an imbalance between



(a) (b)

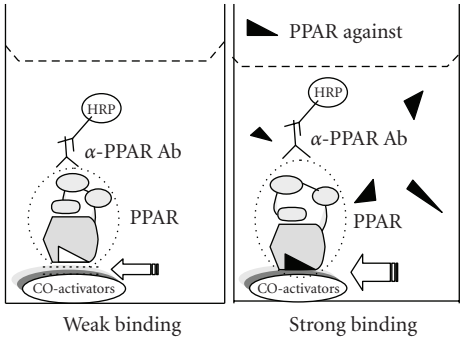


(c)



Readout (ratio) = (665 nm/620 nm) × 10,000

(d)



(e)

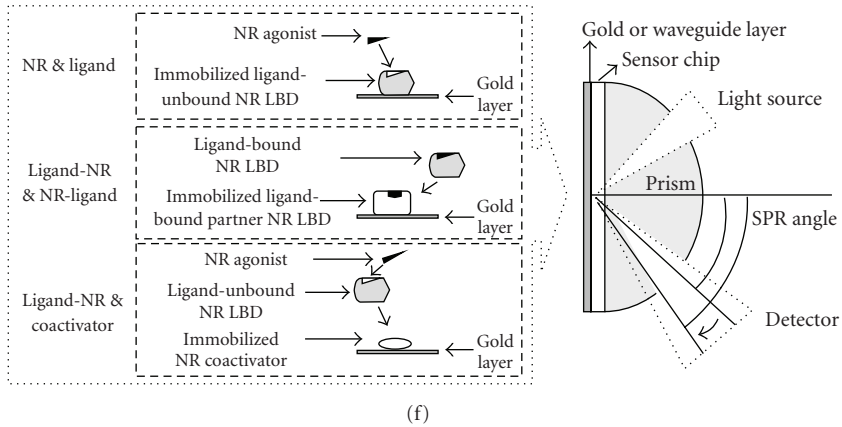


Figure 3: Various cell-free assays to discover PPAR modulator. A. Competition radioreceptor assays are performed by incubating recombinant PPAR protein and radioisotope-labeled ligand in the presence of competitor ligands. Bound ligands are separated from free forms by filtration. The amount of bound radioisotope-labeled ligand is determined by liquid scintillation counting. B. Coactivator-dependent receptor ligand assays (CARLAs) based on the recruitment of a coactivator due to a conformational change of specific ligand-bound PPAR. CARLA is carried out by incubating GST-PPAR and radioisotope-labeled coactivator with a ligand candidate or by incubating radioisotope-labeled PPAR and GST-coactivator with ligand candidates. The amount of ligand-bound PPAR-coactivator complex is determined by pull-down assay. C. In the scintillation proximity assay (SPA), the receptor-ligand complex is bound to the SPA bead through interaction between the biotinylated receptor and the streptavidin moiety located on the surface of the SPA bead. Because no separation step is required, SPA has benefits in its application to HTS. D. In the fluorescence resonance energy transfer (FRET)-based in vitro recruitment assay, GST-PPAR LBD proteins are indirectly linked to EU cryptate, (Eu)K, through (Eu)K-labeled anti-GST antibody, α-GST-(Eu)K. Purified recombinant coactivator is biotinylated, and is indirectly linked to XL665, which is produced only when there is a ligand-induced change in receptor conformation that results in binding to the coactivator. The extent of the FRET is measured as a ratio of 665 nm/620 nm X 10,000. E. A simple ELISA based on binding between PPAR and coactivators. The ligand unbound-PPAR weakly binds to the LXXLL motifs of coactivator, whereas ligand loaded-PPAR strongly binds to the LXXLL motifs of coactivator due to the conformational change of PPAR by specific agonists. This binding is detected by a specific anti-PPAR antibody, followed by horseradish peroxidase-conjugated secondary antibody. F. Schematic representation of SPR technology. One of the interacting partners is immobilized on a gold or waveguide layer of the sensor chip using the standard amine-coupling protocol. The other flows over the surface of the sensor chip, allowing interaction with the immobilized interacting partners. The interaction of immobilized partners with interacting molecules gives rise to an increase in mass. The refractive index and the angle of reflected light is thereby changed. As soon as the injection is stopped, the complex is washed with a washing buffer. The interacting molecules are dissociated from the immobilized interacting partner, resulting in a decrease in the signal due to a shift in the angle of the reflected light to its original position.

energy intake and energy expenditure. Excess body fat is associated with metabolic disorders. As a molecular drug target for metabolic disorders, the activation of PPAR by specific ligands has many beneficial clinical effects in the improvement of glucose and lipid homeostasis. The PPARs mainly consist of three subtypes (PPARα, PPARγ, and PPARδ), and all three PPAR isoforms possess similar structural and functional features involving glucose metabolism, lipid metabolism, and energy balance. PPARs directly modulate gene expression upon binding to specific ligands transferred into PPAR via iLBP-mediated translocation. PPAR agonists play an important role in therapeutic aspects of metabolic disorders, whereas undesired effects for the existing PPAR agonists prescribed as therapeutic agents have been reported. To discover new PPAR modulators with more efficacious effects and more safety against undesired side effects, a novel PPAR antagonist or the combination of agonists such as PPARα/γ dual agonist, PPARγ/δ dual agonist, PPARα/δ dual agonist, and PPARpan (PPAR$\alpha/\gamma/\delta$) agonist has been applied to activate each receptor subtype and selective PPARγ modulators (SPPARγMs). In addition, various technologies have been developed in attempts to discover PPAR modulators as therapeutic agents for the treatment of metabolic disorders. Because cell-based assays have more physiological relevance, the transactivation assay, chimeric receptor transactivation, and yeast two-hybrid methods have also been used. Since cell-free assays are based on direct interaction between PPAR and their specific ligands, a new concept for competing radioreceptor assays has been developed by making the best use of competitive interactions between recombinant PPAR protein and radioisope-labeled ligands. Later, cell-free assays (CARLA and SPA) were developed based on conformational changes in PPARs caused by their ligands, and the simplicity of SPA permitted application to high-throughput screening (HTS). Radioisotope-free assays like FRET (HTRF), ELISA, and SPR methods are relatively simple in terms of handling and disposal. Thus, HTRF and SPR assays can be applied to a homogenous and time-resolved detection mode.

Interestingly, prior to the discovery of the PPARα, it was reported that Wy-14,643, a well-known synthetic agonist of PPARα, decreased serum cholesterol and triglyceride levels in mice [116]. TZD derivatives, well-known synthetic agonists of PPARγ, were reported as antidiabetes agents prior to the discovery of the PPARγ [117, 118]. An antidiabetes agent,

pioglitazone, a TZD derivative, has recently been shown to bind specifically to a protein named mitoNEET [119]. These studies of PPAR, PPAR modulators, and technologies to discover PPAR modulators will elicit the development of drugs with more efficacious effects and more safety for the treatment of metabolic disorders.

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REFERENCES

[1] E. Ravussin and C. Bouchard, "Human genomics and obesity: finding appropriate drug targets," *European Journal of Pharmacology*, vol. 410, no. 2-3, pp. 131–145, 2000.

[2] T. B. Van Itallie, "Health implications of overweight and obesity in the United States," *Annals of Internal Medicine*, vol. 103, no. 6, part 2, pp. 983–988, 1985.

[3] M. Lafontan, "Fat cells: afferent and efferent messages define new approaches to treat obesity," *Annual Review of Pharmacology and Toxicology*, vol. 45, pp. 119–146, 2005.

[4] F. M. Gregoire, C. M. Smas, and H. S. Sul, "Understanding adipocyte differentiation," *Physiological Reviews*, vol. 78, no. 3, pp. 783–809, 1998.

[5] E. D. Rosen and B. M. Spiegelman, "Molecular regulation of adipogenesis," *Annual Review of Cell and Developmental Biology*, vol. 16, pp. 145–171, 2000.

[6] C.-S. Hwang, T. M. Loftus, S. Mandrup, and M. Daniel Lane, "Adipocyte differentiation and leptin expression," *Annual Review of Cell and Developmental Biology*, vol. 13, pp. 231–259, 1997.

[7] Q.-Q. Tang, J.-W. Zhang, and M. Daniel Lane, "Sequential gene promoter interactions by C/EBPβ, C/EBPα, and PPARγ during adipogenesis," *Biochemical and Biophysical Research Communications*, vol. 318, no. 1, pp. 213–218, 2004.

[8] P. A. Grimaldi, "Fatty acid regulation of gene expression," *Current Opinion in Clinical Nutrition & Metabolic Care*, vol. 4, no. 5, pp. 433–437, 2001.

[9] B. M. Spieglelman and J. S. Flier, "Adipogenesis and obesity: rounding out the big picture," *Cell*, vol. 87, no. 3, pp. 377–389, 1996.

[10] K. S. Cook, H. Y. Min, D. Johnson, et al., "Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve," *Science*, vol. 237, no. 4813, pp. 402–405, 1987.

[11] M. Otero, R. Lago, F. Lago, et al., "Leptin, from fat to inflammation: old questions and new insights," *FEBS Letters*, vol. 579, no. 2, pp. 295–301, 2005.

[12] A. H. Berg, T. P. Combs, and P. E. Scherer, "ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism," *Trends Endocrinology and Metabolism*, vol. 13, no. 2, pp. 84–89, 2002.

[13] M. Fasshauer, R. Paschke, and M. Stumvoll, "Adiponectin, obesity, and cardiovascular disease," *Biochimie*, vol. 86, no. 11, pp. 779–784, 2004.

[14] J. N. Fain, A. K. Madan, M. L. Hiler, P. Cheema, and S. W. Bahouth, "Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans," *Endocrinology*, vol. 145, no. 5, pp. 2273–2282, 2004.

[15] C. M. Steppan and M. A. Lazar, "Resistin and obesity-associated insulin resistance," *Trends in Endocrinology and Metabolism*, vol. 13, no. 1, pp. 18–23, 2002.

[16] C. Hug and H. F. Lodish, "Visfatin: a new adipokine," *Science*, vol. 307, no. 5708, pp. 366–367, 2005.

[17] G. N. Chaldakov, I. S. Stankulov, M. Hristova, and P. I. Ghenev, "Adipobiology of disease: adipokines and adipokine-targeted pharmacology," *Current Pharmaceutical Design*, vol. 9, no. 12, pp. 1023–1031, 2003.

[18] D. E. Moller and K. D. Kaufman, "Metabolic syndrome: a clinical and molecular perspective," *Annual Review of Medicine*, vol. 56, pp. 45–62, 2005.

[19] A. Tremblay and E. Doucet, "Obesity: a disease or a biological adaptation?" *Obesity Review*, vol. 1, no. 1, pp. 27–35, 2000.

[20] H. Masuzaki, J. Paterson, H. Shinyama, et al., "A transgenic model of visceral obesity and the metabolic syndrome," *Science*, vol. 294, no. 5549, pp. 2166–2170, 2001.

[21] N. M. Morton, J. M. Paterson, H. Masuzaki, et al., "Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11 β-hydroxysteroid dehydrogenase type 1-deficient mice," *Diabetes*, vol. 53, no. 4, pp. 931–938, 2004.

[22] R. C. Andrews, O. Rooyackers, and B. R. Walker, "Effects of the 11 β-hydroxysteroid dehydrogenase inhibitor carbenoxolone on insulin sensitivity in men with type 2 diabetes," *Journal of Clinical Endocrinology & Metabolism*, vol. 88, no. 1, pp. 285–291, 2003.

[23] G. Zhou, R. Myers, Y. Li, et al., "Role of AMP-activated protein kinase in mechanism of metformin action," *Journal of Clinical Investigation*, vol. 108, no. 8, pp. 1167–1174, 2001.

[24] P. Cohen, M. Miyazaki, N. D. Socci, et al., "Role for stearoyl-CoA desaturase-1 in leptin-mediated weight loss," *Science*, vol. 297, no. 5579, pp. 240–243, 2002.

[25] M. Yuan, N. Konstantopoulos, J. Lee, et al., "Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkβ," *Science*, vol. 293, no. 5535, pp. 1673–1677, 2001.

[26] M. Elchebly, P. Payette, E. Michaliszyn, et al., "Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene," *Science*, vol. 283, no. 5407, pp. 1544–1548, 1999.

[27] L. Abu-Elheiga, M. M. Matzuk, K. A. Abo-Hashema, and S. J. Wakil, "Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2," *Science*, vol. 291, no. 5513, pp. 2613–2616, 2001.

[28] H. J. Harwood Jr., S. F. Peteras, L. D. Shelly, et al., "Isozyme-nonselective *N* -substituted bipiperidylcarboxamide acetyl-CoA carboxylase inhibitors reduce tissue malonyl-CoA concentrations, inhibit fatty acid synthesis, and increase fatty acid oxidation in cultured cells and in experimental animals," *Journal of Biological Chemistry*, vol. 278, no. 39, pp. 37099–37111, 2003.

[29] E. A. Lock, A. M. Mitchell, and C. R. Elcombe, "Biochemical mechanisms of induction of hepatic peroxisome proliferation," *Annual Review of Pharmacology and Toxicology*, vol. 29, pp. 145–163, 1989.

[30] V. Giguère, "Orphan nuclear receptor: from gene to function," *Endocrinology Review*, vol. 20, no. 5, pp. 689–725, 1999.

[31] A. Werman, A. Hollenberg, G. Solanes, C. Bjørbaek, A. J. Vidal-Puig, and J. S. Flier, "Ligand-independent activation domain in the N terminus of peroxisome proliferator-activated receptor γ (PPARγ). Differential activity of PPARγ1

and -2 isoforms and influence of insulin," *Journal of Biological Chemistry*, vol. 272, no. 32, pp. 20230–20235, 1997.

[32] R. A. Graves, P. Tontonoz, K. A. Platt, S. R. Ross, and B. M. Spiegelman, "Identification of a fat cell enhancer: analysis of requirements for adipose tissue-specific gene expression," *Journal of Cellular Biochemistry*, vol. 49, no. 3, pp. 219–224, 1992.

[33] W. K. Sumanasekera, E. S. Tien, J. W. Davis II, R. Turpey, G. H. Perdew, and J. P. Vanden Heuvel, "Heat shock protein-90 (Hsp90) acts as a repressor of peroxisome proliferator-activated receptor-α (PPARα) and PPARβ activity," *Biochemistry*, vol. 42, no. 36, pp. 10726–10735, 2003.

[34] A. W. Zimmerman and J. H. Veerkamp, "New insights into the structure and function of fatty acid-binding proteins," *Cellular and Molecular Life Sciences*, vol. 59, no. 7, pp. 1196–1116, 2002.

[35] N.-S. Tan, N. S. Shaw, N. Vinckenbosch, et al., "Selective cooperation between fatty acid binding proteins and proxisome proliferator-activated receptors in regulating transcription," *Molecular and Cellular Biology*, vol. 22, no. 14, pp. 5114–5127, 2002.

[36] A. Adida and F. Spener, "Adipocyte-type fatty acid-binding protein as inter-compartmental shuttle for peroxisome proliferator activated receptor γ agonists in cultured cell," *Biochimica et Biophysica Acta*, vol. 1761, no. 2, pp. 172–181, 2006.

[37] Y. Zhu, C. Qi, M. S. Rao, and J. K. Reddy, "Cloning and identification of mouse steroid receptor coactivator-1(mSRC-1), as a coactivator of peroxisome proliferators-activated receptor γ," *Gene Expression*, vol. 6, no. 3, pp. 185–195, 1996.

[38] J. J. Voegel, M. J. Heine, C. Zechel, P. Chambon, and H. Gronemeyer, "TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors," *The EMBO Journal*, vol. 15, no. 14, pp. 3667–3675, 1996.

[39] J. Torchia, D. W. Rose, J. Inostroza, et al., "The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function," *Nature*, vol. 387, no. 6634, pp. 677–684, 1997.

[40] B. P. Kota, T. H.-W. Huang, and B. D. Roufogalis, "An overview on biological mechanisms of PPARs," *Pharmacological Research*, vol. 51, no. 2, pp. 85–94, 2005.

[41] I. Issemann and S. Green, "Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators," *Nature*, vol. 347, no. 6294, pp. 645–650, 1990.

[42] C. Dreyer, G. Krey, H. Keller, F. Givel, G. Helftenbein, and W. Wahli, "Control of the peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors," *Cell*, vol. 68, no. 5, pp. 879–887, 1992.

[43] S. A. Kliewer, B. M. Forman, B. Blumberg, et al., "Differential expression and activation of a family of murine peroxisome proliferators-activated receptors," *Proceedings of the National Academy of Science of the United States of America*, vol. 91, no. 15, pp. 7355–7359, 1994.

[44] O. Braissant, F. Foufelle, C. Scotto, M. Dauca, and W. Wahli, "Differential expression of peroxisome proliferator-activated receptor (PPARs): tissue distribution of PPAR-α, -β, and -γ in the adult rat," *Endocrinology*, vol. 137, no. 1, pp. 354–366, 1996.

[45] P. Tontonoz, E. Hu, R. A. Graves, A. I. Budavari, and B. M. Spiegelman, "mPPARγ 2: tissue-specific regulator of an adipocyte enhancer," *Genes & Development*, vol. 8, no. 10, pp. 1224–1234, 1994.

[46] K. Yu, W. Bayona, C. B. Kallen, et al., "Differntial activation of peroxisome proliferator-activators by eicosanoids," *Journal of Biological Chemistry*, vol. 270, no. 41, pp. 23975–23983, 1995.

[47] R. Hertz, I. Berman, D. Keppler, and J. Bar-Tana, "Activation of gene transcription by prostacyclin analogues is mediated by the peroxisome-proliferators-activated receptor (PPAR)," *European Journal of Biochemistry*, vol. 235, no. 1-2, pp. 242–247, 1996.

[48] J. M. Lehmann, J. M. Lenhard, B. B. Oliver, G. M. Ringold, and S. A. Kliewer, "Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs," *Journal of Biological Chemistry*, vol. 272, no. 6, pp. 3406–3410, 1997.

[49] A. Schmidt, N. Endo, S. J. Rutledge, R. Vogel, D. Shinar, and G. A. Rodan, "Identification of a new member of the steroid hormone receptor superfamily that is activated by a proxisome proliferators and fatty acids," *Molecular Endocrinolgoy*, vol. 6, no. 10, pp. 1634–1641, 1992.

[50] B. M. Forman, J. Chen, and R. M. Evans, "Hypolipidemic drugs, polyunsaturated fatty acid, and eicosanoids are ligands for peroxisome proliferators-activated receptors α and δ," *Proceedings of the National Academy of Science of the United States of America*, vol. 94, no. 9, pp. 4312–4317, 1997.

[51] B. Desvergne and W. Wahli, "Peroxisome proliferators-activated receptors: nuclear control of metabolism," *Endocrinology Review*, vol. 20, no. 5, pp. 649–688, 1999.

[52] J. C. Corton, S. P. Anderson, and A. Stauber, "Central role of peroxisome proliferators-activated receptors in the actions of peroxisome proliferators," *Annual Review of Pharmacology and Toxicology*, vol. 40, pp. 491–518, 2000.

[53] C. Diradourian, J. Girard, and J.-P. Pégorier, "Phosphorylation of PPARs: from molecular characterization to physiological relevance," *Biochimie*, vol. 87, no. 1, pp. 33–38, 2005.

[54] D. Shao, S. M. Rangwala, S. T. Bailey, S. L. Krakow, M. J. Reginato, and M. A. Lazar, "Interdomain communication regulating ligand binding by PPAR-γ," *Nature*, vol. 396, no. 6709, pp. 377–380, 1998.

[55] G. Lazennec, L. Canaple, D. Saugy, and W. Wahli, "Activation of peroxisome proliferator-activated receptors (PPARs) by their ligands and protein kinase A activators," *Molecular Endocrinology*, vol. 14, no. 12, pp. 1962–1975, 2000.

[56] Z. E. Floyd and J. M. Stephens, "Interferon-γ-mediated activation and ubiquitin-proteasome-dependent degradation of PPARγ in adipocytes," *Journal of Biological Chemistry*, vol. 277, no. 6, pp. 4062–4068, 2002.

[57] C. Blanquart, R. Mansouri, J.-C. Fruchart, B. Staels, and C. Glineur, "Different ways to regulate the PPARα stability," *Biochemical and Biophysical Research Communications*, vol. 319, no. 2, pp. 663–670, 2004.

[58] T. Ohshima, H. Koga, and K. Shimotahno, "Transcriptional activity of peroxisome proliferator-activated receptor γ is modulated by SUMO-1 modification," *Journal of Biological Chemistry*, vol. 279, no. 28, pp. 29551–29557, 2004.

[59] G. Pascual, A. L. Fong, S. Ogawa, et al., "A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-γ," *Nature*, vol. 437, no. 7059, pp. 759–763, 2005.

[60] D. Yamashita, T. Yamaguchi, M. Shimizu, N. Nakata, F. Hirose, and T. Osumi, "The transactivating function of peroxisome proliferator-activated receptor γ is negatively regulated by SUMO conjugation in the amino-terminal domain," *Genes to Cells*, vol. 9, no. 11, pp. 1017–1029, 2004.

[61] S. Yu, S. Rao, and J. K. Reddy, "Peroxisome proliferators-activated receptors, fatty acid oxidation, steatohepatitis and hepatocarcinogenesis," *Current Molecular Medicine*, vol. 3, no. 6, pp. 561–572, 2003.

[62] C. J. Chou, M. Haluzik, C. Gregory, et al., "WY14,643, a peroxisome proliferators-activated receptor α (PPARα) agonist, improves hepatic and muscle steatosis and reverses insulin resistanc in lipoatrophic A-ZIP/F-1 mice," *Journal of Biological Chemistry*, vol. 277, no. 27, pp. 24484–24489, 2002.

[63] N. Vu-Dac, S. Chopin-Delannoy, P. Gervois, et al., "The nuclear receptors peroxisome proliferator-activated receptor α and Rev-erbα mediate the species-specific regulation of apolipoprotein A-1 expression by fibrates," *Journal of Biological Chemistry*, vol. 273, no. 40, pp. 25713–25720, 1998.

[64] B. L. Knight, D. D. Patel, S. M. Humphreys, D. Wiggins, and G. F. Gibbons, "Inhibition of cholesterol absorption associated with a PPARα-dependent increase in ABC binding cassette transporter A1 in mice," *Journal of Lipid Research*, vol. 44, no. 11, pp. 2049–2058, 2003.

[65] T. Nakamachi, T. Nomiyama, F. Gizard, et al., "PPARα agonists suppress osteopontin expression in macrophages and decrease plasma levels in patients with type 2 diabetes," *Diabetes*, vol. 56, no. 6, pp. 1662–1670, 2007.

[66] G. Chinetti, F. G. Gbaguidi, S. Griglio, et al., "CLA-1/SR-BI is expressed in atherosclerotic lesion macrophages and regulated by activators of peroxisome proliferator-activated receptors," *Circulation*, vol. 101, no. 20, pp. 2411–2417, 2000.

[67] A. C. Li and W. Palinski, "Peroxisome proliferator-activated receptors: how their effects on macrophages can lead to the development of a new drug therapy against atherosclerosis," *Annual Review of Pharmacology and Toxicology*, vol. 46, pp. 1–39, 2006.

[68] R. A. Daynes and D. C. Jones, "Emerging roles of PPARs in inflammation and immunity," *Nature Reviews Immunology*, vol. 2, no. 10, pp. 748–759, 2002.

[69] R. Pakala, P. Kuchulakanti, S.-W. Rha, E. Cheneau, R. Baffour, and R. Waksman, "Peroxisome proliferators-activated receptor γ: its role in metabolic syndrome," *Cardiovascular Radiation Medicine*, vol. 5, no. 2, pp. 97–103, 2004.

[70] S. M. Rangwala and M. A. Lazar, "Peroxisome proliferators-activated receptor γ in diabetes and metabolism," *Trends in Pharmacological Sciences*, vol. 25, no. 6, pp. 331–336, 2004.

[71] G. J. Murphy and J. C. Holder, "PPAR-γ agonists: therapeutic role in diabetes, inflammation and cancer," *Trends in Pharmacological Sciences*, vol. 21, no. 12, pp. 469–474, 2000.

[72] K. Schoonjans and J. Auwerx, "Thiaolidinediones: an update," *The Lancet*, vol. 355, no. 9208, pp. 1008–1010, 2000.

[73] S. Mudaliar and R. R. Henry, "New oral therapies for type 2 diabetes mellitus: the glitazones or insulin sensitizers," *Annual Review of Medicine*, vol. 52, pp. 239–257, 2001.

[74] A. D. Burdick, D. J. Kim, M. A. Peraza, F. J. Gonzalez, and J. M. Peters, "The role of peroxisome proliferator-activated receptor-β/δ in epithelial cell growth and differentiation," *Cellular Signalling*, vol. 18, no. 1, pp. 9–20, 2006.

[75] G. D. Barish, V. A. Narkar, and R. M. Evans, "PPARδ: a dagger in the heart of the metabolic syndrome," *Journal of Clinical Investigation*, vol. 116, no. 3, pp. 590–597, 2006.

[76] G. Krey, O. Braissant, F. L'Horset, et al., "Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay," *Molecular Endocrinology*, vol. 11, no. 6, pp. 779–791, 1997.

[77] H. Eric Xu, M. H. Lambert, V. G. Montana, et al., "Molecular recognition of fatty acids by peroxisome proliferator-activated receptors," *Molecular Cell*, vol. 3, no. 3, pp. 397–403, 1999.

[78] A. Chawla, C.-H. Lee, Y. Barak, et al., "PPARδ is a very low-density lipoprotein sensor in macrophages," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 3, pp. 1268–1273, 2003.

[79] W. R. Oliver Jr., J. L. Shenk, M. R. Snaith, et al., "A selective peroxisome proliferator-activated receptor δ agonist promotes reverse cholesterol transport," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 9, pp. 5306–5311, 2001.

[80] T. Tanaka, J. Yamamoto, S. Iwasaki, et al., "Activation of peroxisome proliferator-activated receptor δ induces fatty acid β-oxidation in skeletal muscle and attenuates metabolic syndrome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 26, pp. 15924–15929, 2003.

[81] J. S. Welch, M. Ricote, T. E. Akiyama, F. J. Gonzalez, and C. K. Glass, "PPARγ and PPARδ negatively regulate specific subsets of lipopolysaccharide and IFN-γ target genes in macrophages," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 11, pp. 6712–6717, 2003.

[82] S. S. Deeb, L. Fajas, M. Nemoto, et al., "A Pro12Ala substitution in PPARγ2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity," *Nature Genetics*, vol. 20, no. 3, pp. 284–287, 1998.

[83] B. G. Shearer and A. N. Billin, "The next generation of PPAR drugs: do we have the tools to find them?" *Biochimica et Biophysica Acta*, vol. 1771, no. 8, pp. 1082–1093, 2007.

[84] R. Nakano, E. Kurosaki, S. Yoshida, et al., "Antagonism of peroxisome proliferator-activated receptor γ prevents high-fat diet-induced obesity in vivo," *Biochemical Pharmacology*, vol. 72, no. 1, pp. 42–52, 2006.

[85] J. Rieusset, F. Touri, L. Michalik, et al., "A new selective peroxisome proliferator-activated receptor γ antagonist with antiobesity and antidiabetic activity," *Molecular Endocrinology*, vol. 16, no. 11, pp. 2628–2644, 2002.

[86] S. Seber, S. Ucak, O. Basat, and Y. Altuntas, "The effect of dual PPAR α/γ stimulation with combination of rosiglitazone and fenofibrate on metabolic parameters in type 2 diabetic patients," *Diabetes Research and Clinical Practice*, vol. 71, no. 1, pp. 52–58, 2006.

[87] C. Fiévet, J.-C. Fruchart, and B. Staels, "PPARα and PPARγ dual agonists for the treatment of type 2 diabetes and the metabolic syndrome," *Current Opinion in Pharmacology*, vol. 6, no. 6, pp. 606–614, 2006.

[88] P. Balakumar, M. Rose, S. S. Ganti, P. Krishan, and M. Singh, "PPAR dual agonists: are they opening Pandora's Box?" *Pharmacological Research*, vol. 56, no. 2, pp. 91–98, 2007.

[89] I. C. Gonzalez, J. Lamar, F. Iradier, et al., "Design and synthesis of a novel class of dual PPARγ/δ agonists," *Bioorganic & Medicinal Chemistry Letters*, vol. 17, no. 4, pp. 1052–1055, 2007.

[90] Y. Xu, G. J. Etgen, C. L. Broderick, et al., "Design and synthesis of dual peroxisome proliferator-activated receptors γ and δ agonists as novel euglycemic agents with a reduced weight gain profile," *Journal of Medicinal Chemistry*, vol. 49, no. 19, pp. 5649–5652, 2006.

[91] J. M. Wallace, M. Schwarz, P. Coward, et al., "Effects of peroxisome proliferator-activated receptor α/δ agonists on HDL-cholesterol in vervet monkeys," *Journal of Lipid Research*, vol. 46, no. 5, pp. 1009–1016, 2005.

[92] L. Shen, Y. Zhang, A. Wang, et al., "Synthesis and identification of [1,2,4]thiadiazole derivatives as a new series of potent and orally active dual agonists of peroxisome proliferator-activated receptors α and δ," *Journal of Medicinal Chemistry*, vol. 50, no. 16, pp. 3954–3963, 2007.

[93] F. Zhang, B. E. Lavan, and F. M. Gregoire, "Selective modulators of PPAR-γ activity: molecular aspects related to obesity and side-effects," *PPAR Research*, vol. 2007, Article ID 32696, 7 pages, 2007.

[94] T. Allen, F. Zhang, S. A. Moodie, et al., "Halofenate is a selective peroxisome proliferator-activated receptor γ modulator with antidiabetic activity," *Diabetes*, vol. 55, no. 9, pp. 2523–2533, 2006.

[95] E. Burgermeister, A. Schnoebelen, A. Flament, et al., "A novel partial agonist of peroxisome proliferator-activated receptor-γ (PPARγ) recruits PPARγ-coactivator-1α, prevents triglyceride accumulation, and potentiates insulin signaling in vitro," *Molecular Endocrinology*, vol. 20, no. 4, pp. 809–830, 2006.

[96] A. Tenenbaum, M. Motro, and E. Z. Fisman, "Dual and pan-peroxisome proliferator-activated receptors (PPAR) co-agonism: the bezafibrate lessons," *Cardiovascular Diabetology*, vol. 4, article 14, 2005.

[97] U. Ramachandran, R. Kumar, and A. Mittal, "Fine tuning of PPAR ligands for type 2 diabetes and metabolic syndrome," *Mini Reviews in Medicinal Chemistry*, vol. 6, no. 5, pp. 563–573, 2006.

[98] Q. Chen, J. Chen, T. Sun, J. Shen, X. Shen, and H. Jiang, "A yeast two-hybrid technology-based system for the discovery of PPARγ agonist and antagonist," *Analytical Biochemistry*, vol. 335, no. 2, pp. 253–259, 2004.

[99] R. T. Nolte, G. B. Wisely, S. Westin, et al., "Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-γ," *Nature*, vol. 395, no. 6698, pp. 137–143, 1998.

[100] S. Chen, B. A. Johnson, Y. Li, et al., "Both coactivator LXXLL motif-dependent and -independent interactions are required for peroxisome proliferator-activated receptor γ (PPARγ) function," *Journal of Biological Chemistry*, vol. 275, no. 6, pp. 3733–3736, 2000.

[101] B. M. Forman, P. Tontonoz, J. Chen, R. P. Brun, B. M. Spiegelman, and R. M. Evans, "15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ," *Cell*, vol. 83, no. 5, pp. 803–812, 1995.

[102] P. W. Young, D. R. Buckle, B. C. C. Cantello, et al., "Identification of high-affinity binding sites for the insulin sensitizer rosiglitazone (BRL-49653) in rodent and human adipocytes using a radioiodinated ligand for peroxisomal proliferator-activated receptor γ," *Journal of Pharmacology and Experimental Therapeutics*, vol. 284, no. 2, pp. 751–759, 1998.

[103] J. S. Nichols, D. J. Parks, T. G. Consler, and S. G. Blanchard, "Development of a scintillation proximity assay for peroxisome proliferator-activated receptor γ ligand binding domain," *Analytical Biochemistry*, vol. 257, no. 2, pp. 112–119, 1998.

[104] A. Elbrecht, Y. Chen, A. Adams, et al., "L-764406 is a partial agonist of human peroxisome proliferator-activated receptor γ. The role of Cys^{313} in ligand binding," *Journal of Biological Chemistry*, vol. 274, no. 12, pp. 7913–7922, 1999.

[105] G. Zhou, R. Cummings, Y. Li, et al., "Nuclear receptors have distinct affinities for coactivators: characterization by fluorescence resonance energy transfer," *Molecular Endocrinology*, vol. 12, no. 10, pp. 1594–1604, 1998.

[106] G. Zhou, R. Cummings, J. Hermes, and D. E. Moller, "Use of homogeneous time-resolved fluorescence energy transfer in the measurement of nuclear receptor activation," *Methods*, vol. 25, no. 1, pp. 54–61, 2001.

[107] M.-C. Cho, H.-S. Lee, J. H. Kim, et al., "A simple ELISA for screening ligands of peroxisome proliferator-activated receptor γ," *Journal of Biochemistry and Molecular Biology*, vol. 36, no. 2, pp. 207–213, 2003.

[108] H.-S. Lee, M.-C. Cho, T.-W. Baek, et al., "Epitope analysis of PPARγ monoclonal antibody Pγ48.34A and its application for screening PPARγ ligands," *Journal of Immunological Methods*, vol. 296, no. 1-2, pp. 125–134, 2005.

[109] M.-C. Cho, H.-E. Yoon, J.-W. Kang, et al., "A simple method to screen ligands of peroxisome proliferator-activated receptor δ," *European Journal of Pharmaceutical Sciences*, vol. 29, no. 5, pp. 355–360, 2006.

[110] M. A. Cooper, "Optical biosensors in drug discovery," *Nature Reviews Drug Discovery*, vol. 1, no. 7, pp. 515–528, 2002.

[111] B. Cheskis and L. P. Freedman, "Modulation of nuclear receptor interactions by ligands: kinetic analysis using surface plasmon resonance," *Biochemistry*, vol. 35, no. 10, pp. 3309–3318, 1996.

[112] C. Yu, L. Chen, H. Luo, et al., "Binding analyses between human PPARγ-LBD and ligands: surface plasmon resonance biosensor assay correlating with circular dichroic spectroscopy determination and molecular docking," *European Journal of Biochemistry*, vol. 271, no. 2, pp. 386–397, 2004.

[113] L. Yue, F. Ye, C. Gui, et al., "Ligand-binding regulation of LXR/RXR and LXR/PPAR heterodimerizations: SPR technology-based kinetic analysis correlated with molecular dynamics simulation," *Protein Science*, vol. 14, no. 3, pp. 812–822, 2005.

[114] T. Fujino, Y. Sato, M. Une, et al., "In vitro farnesoid X receptor ligand sensor assay using surface plasmon resonance and based on ligand-induced coactivator association," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 87, no. 4-5, pp. 247–252, 2003.

[115] T. Kanayasu-Toyoda, T. Fujino, T. Oshizawa, et al., "HX531, a retinoid X receptor antagonist, inhibited the 9-cis retinoic acid-induced binding with steroid receptor coactivator-1 as detected by surface plasmon resonance," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 94, no. 4, pp. 303–309, 2005.

[116] J. K. Reddy, D. E. Moody, D. L. Azarnoff, and R. M. Tomarelli, "Hepatic effects of some [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (WY-14,643) analogs in the mouse," *Archives Internationales de Pharmacodynamie et de Therapie*, vol. 225, no. 1, pp. 51–57, 1977.

[117] T. Sohda, K. Mizuno, H. Tawada, Y. Sugiyama, T. Fujita, and Y. Kawamatsu, "Studies on antidiabetic agents. I. Synthesis of 5-[4-(2-methyl-2-phenylpropoxy)-benzyl]thiazolidine-2,4-dione (AL-321) and related compounds," *Chemical & Pharmaceutical Bulletin*, vol. 30, no. 10, pp. 3563–3573, 1982.

[118] T. Fujita, Y. Sugiyama, S. Taketomi, et al., "Reduction of insulin resistance in obese and/or diabetic animals by 5-[4-(1-methylcyclohexylmethoxy)benzyl]-thiazolidine-2,4-dione (ADD-3878, U-63,287, ciglitazone), a new antidiabetic agent," *Diabetes*, vol. 32, no. 9, pp. 804–810, 1983.

[119] S. E. Wiley, A. N. Murphy, S. A. Ross, P. van der Geer, and J. E. Dixon, "MitoNEET is an iron-containing outer mitochondrial membrane protein that regulates oxidative capacity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 13, pp. 5318–5323, 2007.

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Review Article

The Development of INT131 as a Selective PPARγ Modulator: Approach to a Safer Insulin Sensitizer

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INT131 (formerly T0903131, T131, AMG131) is a potent non-thiazolidinedione (TZD) selective peroxisome proliferator-activated receptor γ modulator (SPPARM) currently in Phase 2 clinical trials for treatment of type-2 diabetes mellitus (T2DM). This new chemical entity represents a second generation SPPARM approach developed after the first generation PPARγ full agonists to address their inherent limitations. INT131 was specifically and carefully designed using preclinical models to exhibit a biological profile of strong efficacy with *de minimis* side effects compared to PPARγ full agonists. As a potent PPARγ modulator, INT131 binds to PPARγ with high affinity. In pharmacology models of diabetes and in early clinical studies, it achieved a high level of efficacy in terms of antidiabetic actions such as insulin sensitization and glucose and insulin lowering, but had little activity in terms of other, undesired, effects associated with TZD PPARγ full agonists such as edema and adipogenesis. Ongoing clinical development is directed at translating these findings into establishing a novel and effective treatment for T2DM patients with an improved safety profile in relation to that currently available.

1. PPARγ FULL AGONISTS

PPARγ full agonists are a mainstay in the treatment of insulin resistance and type-2 diabetes. While the glucose lowering action of thiazolidinediones (TZDs) was well-known as early as 1988 [1], it was not until 1995 that the nuclear receptor PPARγ was identified as their target [2] and that its activation was shown to be responsible for their therapeutic benefits. PPARγ full agonists, including the TZDs rosiglitazone (Avandia) and pioglitazone (Actos) are powerful drugs for the treatment of insulin resistance associated with type-2 diabetes mellitus (T2DM) [3]. Troglitazone (Rezulin) was the first TZD approved for clinical use in the US in 1997, but was subsequently withdrawn from the market in 2000 due to idiosyncratic hepatotoxicity. Rosiglitazone and pioglitazone were approved in the US in 1999. These drugs enabled the beneficial effect of PPARγ activating agents to be recognized in clinical practice globally.

These medications enhance insulin sensitivity and reduce glucose and insulin levels in T2DM patients, and have been shown to have robust and relatively durable benefit for glucose control [4]. Insulin resistance is a key etiologic feature in the onset and subsequent progression of the disease. Furthermore, insulin sensitization comprises a complementary mechanism of action to that of other commonly used therapeutic modalities such as inhibition of gluconeogenesis by metformin, increased insulin secretion by sulfonylureas, and administration of exogenous insulin. The potential to be used in combination with other approaches thus further extends the clinical utility of PPARγ activating agents for glucose control and to treat T2DM. Rosiglitazone and pioglitazone, both in the TZD class, are the only agents currently approved for insulin sensitization as their major mechanism of action.

Realization of PPARγ maximal therapeutic potential by full agonists is limited, however, by associated side effects. PPARγ full agonist binding to PPARγ activates a broad spectrum of PPARγ mediated effects, some of which are undesirable. Thus, use of TZDs is limited by side effects that include weight gain, fluid retention, and decreased

bone density [5]. TZD-induced peripheral edema, which frequently occurs in patients receiving TZD monotherapy, is especially problematic in patients receiving concomitant insulin therapy, and is of special concern for patients who have either clinical or subclinical congestive heart failure (CHF) and thus cannot tolerate the extra fluid volume [6, 7]. In addition, there is strong evidence that activation of PPARγ causes adipocyte differentiation and increased adipose tissue mass, contributing to weight gain [3]. The dose response curve for the therapeutic effects of TZDs overlaps with the dose response for side effects, such that increasing doses produce both greater benefits for glucose control as well as greater incidence and higher degrees of side effects [8]. Thus, doses which would produce the maximal clinical benefit of PPARγ full agonists may not be tolerated by a significant number of patients and the full potential of PPARγ activation for insulin sensitization and glucose control may not be realized at approved clinical doses of rosiglitazone or pioglitazone.

As a consequence of the known safety issues, TZDs are not recommended for patients with New York Heart Association Class 3 and 4 CHF, and the potential clinical impact of cardiovascular side effects prompted the American Heart and the American Diabetes Associations to issue a joint consensus statement advising against the use of TZDs in patients with advanced heart failure [9]. Awareness of the safety issues associated with TZDs was dramatically increased following the publication of a meta-analysis in May of 2007 showing a nonstatistically significant trend towards an increase in macrovascular events in patients taking rosiglitazone [10]. As a result of a detailed examination of the safety record for the TZD class, both rosiglitazone and pioglitazone received black box safety warnings for the increased risk of CHF due to fluid retention. Only rosiglitazone was further implicated for a "possible" risk of increased ischemic cardiovascular events [11] and obtained an additional black box warning, but data suggesting this risk have not been replicated by all studies. Finally, a series of scientific papers has demonstrated an association between TZD use and bone fracture, especially in women [12]. Despite these well-known limitations, Actos and Avandia represent a combined annual global market of more than $5 billion even following a rapid decrease and then stabilization of total sales and a switch from rosiglitazone to pioglitazone or other antidiabetic medications following heightened awareness of safety concerns in 2007. The continued use of the TZDs is a strong testament to the utility of insulin sensitization as a mode of action for treatment of T2DM, but also underscores the need for a safer treatment for insulin resistance.

Historically, the proven therapeutic utility of activating the PPARγ nuclear receptor to reduce glucose and HbA1c led the pharmaceutical industry to focus on a search for greater and broader efficacy through more potent PPARγ full agonists as well as through the development of dual α and γ ("α/γ") PPAR agonists. The latter were intended to combine the insulin sensitizing effects of PPARγ activation with the lipid lowering effects of PPARα activation. Unfortunately, no new agents deriving from these programs have been approved for clinical use. In the case of full PPARγ agonists, efficacy and side effects have been shown to be intrinsically linked, with higher efficacy compounds associated with greater propensity for side effects. Similarly, PPARα/γ dual agonists have been plagued with side effects. For example, muraglitazar, a dual PPARα/γ agonist, was taken through a comprehensive development program and demonstrated remarkable efficacy in lowering HbA1c as well as improving lipid profile in T2DM patients. However, preclinical and clinical safety signals associated with edema, weight gain, and increased cardiovascular events led to a request in 2005 by FDA for outcome studies prior to approval and resulted in abandonment of the program by the sponsor in 2006. In summary, accumulated experience with PPARγ and PPARα/γ ligands has led to an understanding of a spectrum of desirable and undesirable activities, as graphically depicted in Figure 1.

2. SELECTIVE PPARγ MODULATION SEPARATES EFFICACY AND SIDE EFFECT DOSE REPONSE CURVES

A very different approach to leveraging PPARγ antidiabetic therapeutic benefits would focus on minimizing side effects (Figure 1, left) by limiting the spectrum of activation. This approach would require selective PPARγ modulation which by design would minimize side effects while maintaining desired therapeutic benefit.

After the identification of PPARγ as the target for TZDs, the crystal structure of the PPARγ binding pocket as well as its activity relationships were probed, providing an important tool for pursuing selective modulation of the receptor. For example, in the case of the TZD PPARγ full agonists, a key interaction occurs between the ligand and the activation helix (helix 12) of PPARγ [13, 14]. Binding of activating ligands to the nuclear receptor PPARγ leads to conformational changes favoring binding of PPARγ to the RXR nuclear receptor, which is required for PPARγ driven gene transcription, as well as to altered association with cofactors (Figure 2). Different types of PPARγ ligands lead to sufficiently different conformations of the bound receptor heterodimer complex that different combinations and patterns of coactivators and corepressors are recruited for differential transcriptional control [15]. That is, the composition of the protein complex of PPARγ, RXR, and specific cofactors determines the pattern of the ensuing gene transcription and hence the cellular response to the PPARγ ligand. Since the repertoire of cofactors available for recruitment to the PPARγ-RXR complex varies among cell types, PPARγ responses are context-dependent. Thus, full agonists such as TZDs would be expected to lead to a different pattern of cofactor recruitment, gene transcription, and cellular response than a SPPARM.

Theoretically, SPPARMs can be identified or designed which would produce a pattern of cofactor recruitment, gene transcription, and cellular response whereby the dose response curves for desired and undesired effects seen in patients could potentially be sufficiently separated to establish a broad therapeutic window (Figure 3). Is there precedence for the success of a modulator approach for

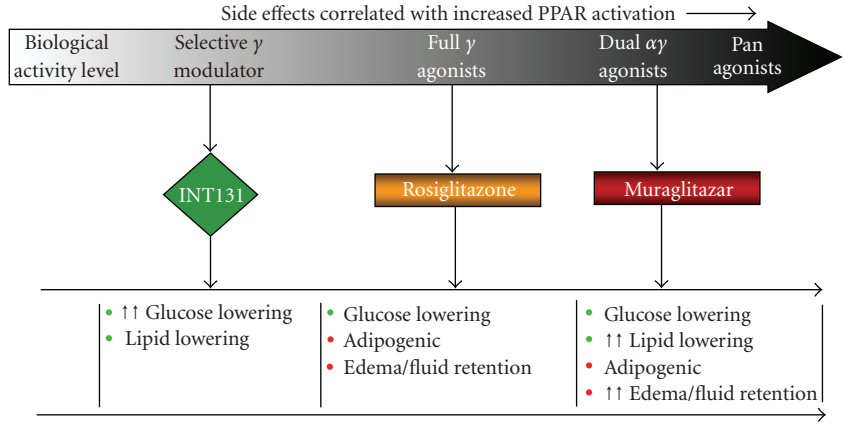


Figure 1: *Spectrum of PPARγ effects*. The range of biological activities, both desired antidiabetic therapeutic effects and undesired effects related to tolerability and safety issues, increases for ligands characterized as antagonists, selective agonists, full agonists, and broad selectivity full agonists.

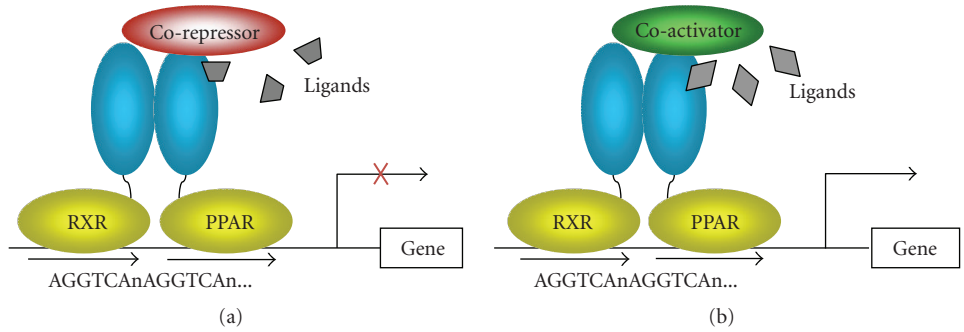


Figure 2: *PPARγ activation*. Upon ligand binding, the nuclear receptor PPARγ associates with nuclear receptor RXR as well as with coactivators and corepressors which are present in a cell type and state specific pattern. This complex binds to PPAR response elements to enhance or repress gene transcription.

another nuclear receptor? Both tamoxifen and its successor raloxifene are selective estrogen receptor modulators (SERMs) which are designed to optimize the therapeutic actions of estrogen receptor activation while minimizing the side effects [16]. A number of SPPARMs have to date been identified by in vitro and preclinical studies and some have entered early clinical studies [11, 14] but no reports have been published on any of these molecules reaching advanced stages of clinical development.

3. INT131 SPECIFIC DESIGN AND DEVELOPMENT FOR MOLECULAR AND IN VITRO SPPARM ACTIVITY

INT131 (formerly T0903131, T131, AMG131) was developed focusing on a strategy to design a SPPARM which would bind to PPARγ with high affinity but could potentially activate only a subset of the full spectrum of activities. Such a specifically designed molecule would thereby retain the antidiabetic actions of full PPARγ agonists such as rosiglitazone and pioglitazone but would have minimal, if any, side effects (including weight gain and fluid retention) caused by these TZDs. In fact, a primary screening assay assed only moieties which *antagonized* roziglitazone induced activity associated with side effects INT131 was thus designed and developed as a non-TZD PPARγ modulator which represents a new chemical class of PPARγ ligands. INT131 binds to PPARγ within the same binding pocket as the TZDs, but occupies a unique space in the pocket and contacts the receptor at distinct points from the TZDs [17]. Importantly, the interaction with the activation helix of PPARγ by INT131 and by TZDs differs. The net result of the different binding by the two types of ligands is alternative conformational change of PPARγ, leading to distinct patterns of association with cofactors by this nuclear receptor, and thus ultimately to unique patterns of gene transcription [15, 17].

INT131 binds to PPARγ and displaces rosiglitazone with a Ki of ~10 nM [17], demonstrating ~20-fold higher affinity than either rosiglitazone or pioglitazone [18], and with greater than 1000-fold selectivity for PPARγ over PPARα, PPARδ, or a set of other nuclear receptors [17]. Characterization beyond binding reveals that selected PPARγ receptor activities are induced by INT131. In a cell-based reporter assay designed to detect full agonist activity, INT131 activates PPARγ with an efficacy of only about 10% of that of rosiglitazone (Figure 4(a)). Similarly, in fluorescence

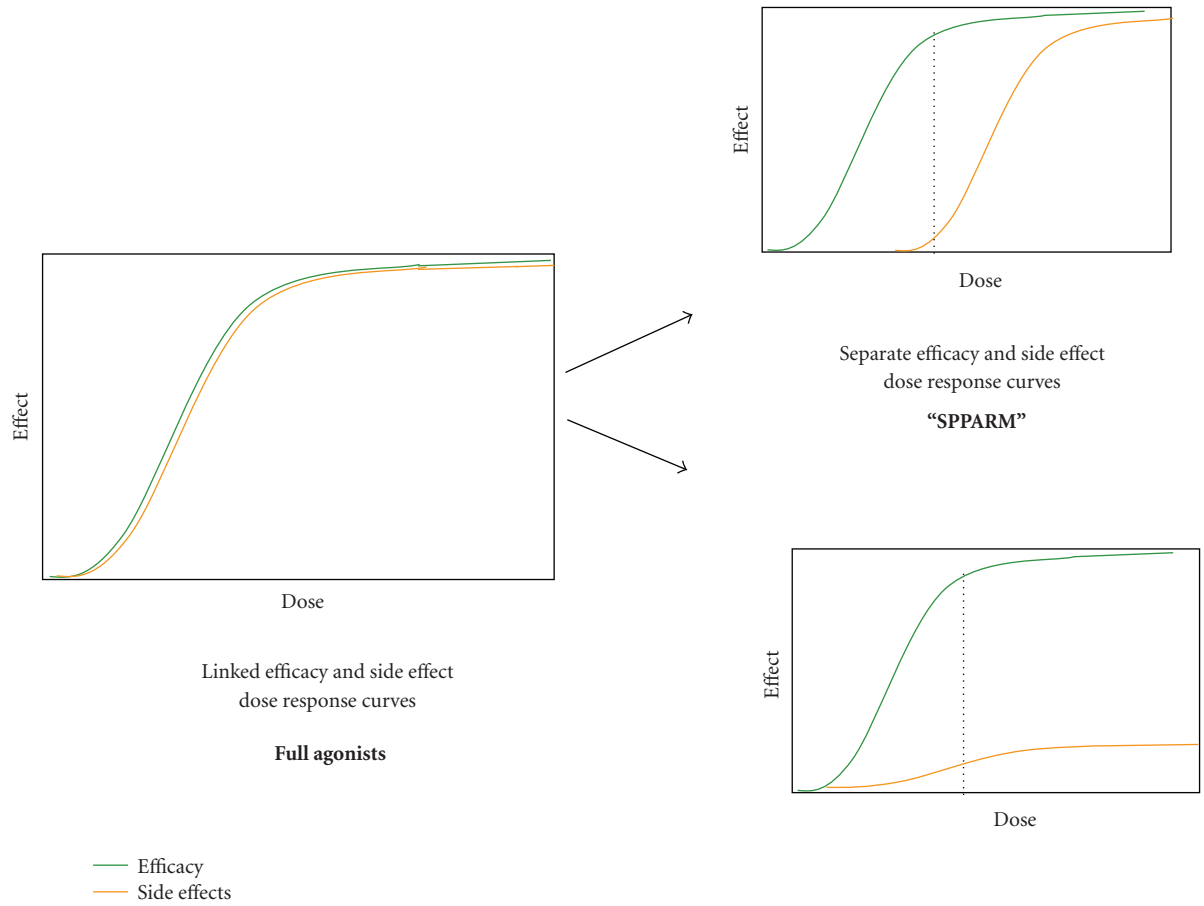


Figure 3: *Selective PPARγ modulation separates dose response curves of different PPARγ effects. Left*: PPARγ full agonists activate the range of receptor responses in a linked fashion. Hence, increasing concentration (or dose) increases responses in concert. In the clinical setting, higher doses of TZDs produce greater efficacy as well as greater side effects. *Right*: selective PPAR modulation is response and context-dependent. Depending on the cellular setting and the response being measured, SPPARM activity may have different potency (top) or different maximal activity (efficacy, bottom) compared to a full agonist. Hence, increasing concentration (or dose) may lead to increases in some responses without linked increases in others. This offers the potential in the clinical setting for separation of antidiabetic efficacy from side effects such as edema and weight gain.

resonance energy transfer assays, INT131 causes recruitment of coactivator DRIP205, which is important for adipocyte differentiation, with an efficacy of about 20–25% of that of a set of full agonists including rosiglitazone, pioglitazone, and troglitazone (Figure 4(b)). Consistent with its high potency, selective activity profile in the full agonist cell-based reporter and FRET assays, INT131 causes little adipocyte differentiation or triglyceride accumulation in cultured mouse (Figure 4(c)) or human preadipocytes [17, 19]. Moreover, INT131 blocks most of the potent effects of rosiglitazone to promote fat cell differentiation [17]. Thus, INT131 shows selectivity among the full spectrum of PPARγ effects and has the desired, nonadipogenic profile.

PPARγ activation by a SPPARM is predicted to be context-dependent. Maximal activity of INT131 is sensitive to cellular environment of PPARγ. That is, using the same reporter construct and assay designed to detect PPARγ full agonist activity, INT131 potency and efficacy may be less than, equal to, or greater than the comparator full agonists rosiglitazone depending on the host-cell type (Figure 5).

4. PHARMACOLOGY OF INT131 IS CONSISTENT WITH SPPARM ACTIVITY

INT131 is potent and highly efficacious in animal models of diabetes, but causes much less weight gain and volume expansion than marketed TZDs. For example, in Zucker fatty rats, a standard rodent model of T2DM, INT131 was more potent than rosiglitazone in reducing serum glucose (Figure 6), insulin, triglyceride, and NEFA concentrations and in improving glucose tolerance [17]. Notably, INT131 increased levels of the adipokine adiponectin in the Zucker fatty rat model and in normal rats with equal or greater potency than does rosiglitazone (Figure 7). Adiponectin levels are suppressed in obesity and in T2DM, and increased adiponectin production is thought to be a key mediator

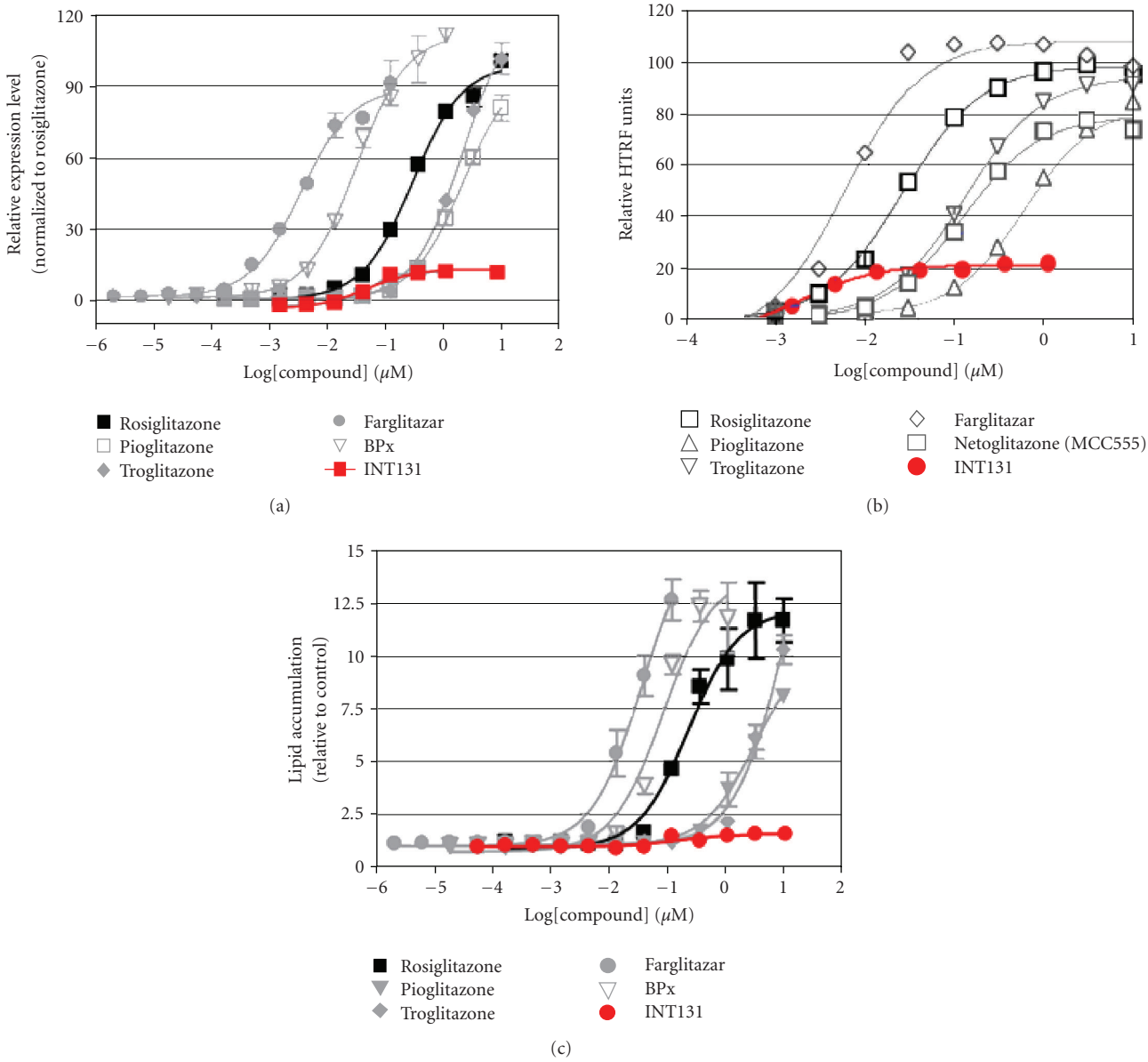


Figure 4: *PPARγ full agonists, but not INT131, activate expression of a full agonist reporter gene, induce recruitment of DRIP205 coactivator peptide to PAPRγ, and cause lipid accumulation*. (a) An expression construct bearing a PPAR response element designed to be activated by PPARγ full agonists was used to detect reporter gene expression. Transfected HEK cells were exposed to a range of concentrations of the indicated PPAR ligands, and expression measured. The maximal expression stimulated by INT131 was about 10% that promoted by rosiglitazone, pioglitazone, troglitazone, farglitazar, and BPx. (b) A homogenous time-resolved fluorescence energy transfer (FRET) assay was used to measure association of a DRIP205 coactivator peptide to PPARγ upon exposure to a range of concentrations of the indicated PPAR ligands. The maximal association stimulated by INT131 was about 20–25% that was promoted by rosiglitazone, pioglitazone, troglitazone, farglitazar, and netoglitazone. (c) Lipid accumulation was measured in murine preadipocytes exposed to a range of concentrations of the indicated PPAR ligands. The maximal lipid accumulation stimulated by INT131 was about 10% that was promoted by rosiglitazone, pioglitazone, troglitazone, farglitazar, and BPx, Data on file.

for the insulin sensitizing and anti-inflammatory effects of PPARγ [20].

In a variety of animal models, full agonists cause fluid retention and increased heart weight, probably as a result of the increased cardiac load caused by plasma volume expansion. As expected, administration of rosiglitazone to Zucker diabetic fatty rats for two weeks caused a significant decrease in hematocrit, a marker for increased plasma volume expansion (Figure 8(a)); increase in heart weight (Figure 8(b)); and increased lung weight (Figure 8(c)) consistent with a secondary effect to cardiac hypertrophy and developing CHF. INT131 at the same supratherapeutic dose did not cause these effects. Thus, SPPARM activity is observed in this rodent model of T2DM, and the antidiabetic

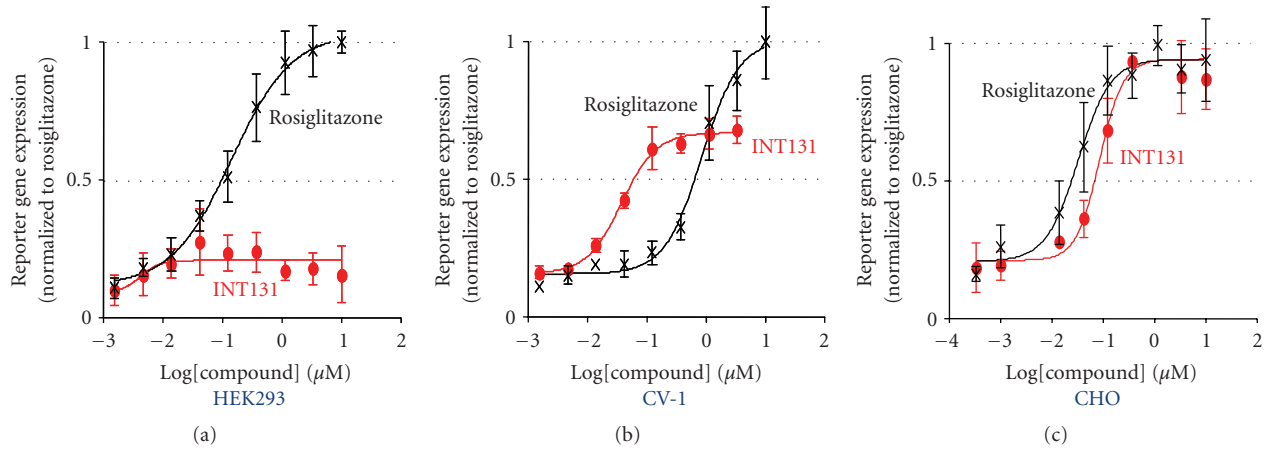


Figure 5: *PPARγ activation by INT131 is cell-type-dependent.* Cell-based reporter assays were performed by transfecting three different cell types (HEK293, CV-1, CHO) with the same reporter construct and stimulating with increasing concentrations of rosiglitazone (black) or INT131 (red). Adapted from [17].

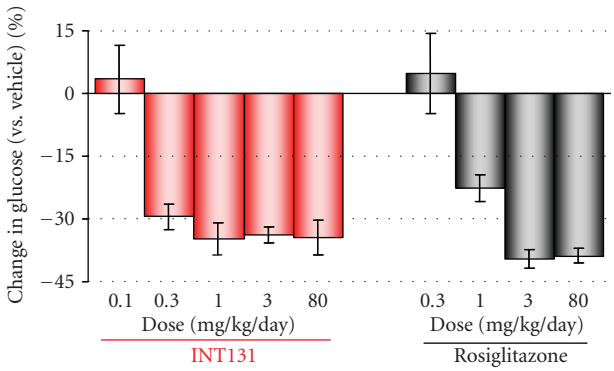
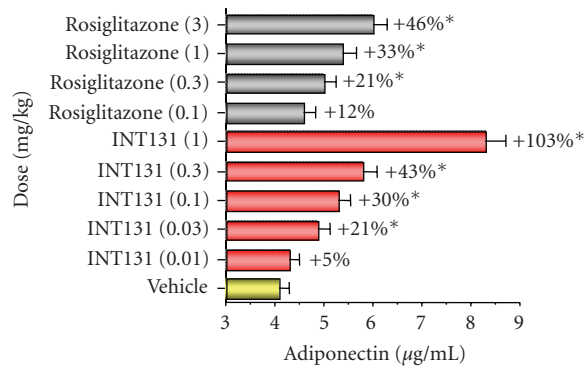


Figure 6: *Glucose level in Zuker fatty rat is reduced in response to either INT131 or rosiglitazone treatment.* Fourteen-day treatment with the indicated daily oral dose of INT131 or rosiglitazone in increasing doses reduce glucose levels. Adapted from [17, 19].



∗ $p < 0.05$ compared to vehicle group

Figure 7: *Adiponectin levels increase in a dose responsive manner following either INT131 or rosiglitazone treatment.* Zucker fatty rats were treated orally with the indicated dose of INT131 or rosiglitazone once daily for 15 days, and plasma adiponectin was measured. $P < 0.05$ compared to vehicle group adapted from [17, 19].

effects of PPAR activation have been separated from fluid retention and adverse cardiac effects.

5. TOXICOLOGY OF INT131 DEMONSTRATES A SAFETY PROFILE DISTINCT FROM TZDs AND CONSISTENT WITH A SPPARM

Preclinical safety experience with PPARγ full agonists has produced a consistent profile of target mediated effects. Prominent among these are: fluid retention as manifested by a drop in hematocrit and related hematological measures of increased plasma volume as well as in edema; weight gain due to increased adipose tissue together with fluid retention; cardiac hypertrophy and heart failure; and fatty infiltration and replacement of bone marrow. Appearance of these adverse effects follows a predictable steep time and dose relationship in multiple species (Figure 9, [21]), and has been predictive of clinical experience. Therefore, preclinical results from subchronic and chronic safety studies take on heightened importance for PPAR ligands in clinical development. Based on experience with many PPAR full agonist programs, the 2008 FDA draft guidance for development of diabetes drugs [22] includes specific recommendations for preclinical studies with PPAR ligands. These include detailed measures to detect cardiac changes, fatty infiltration of organs, and fluid retention. According to the draft guidance, appearance of safety signals in preclinical programs which have been predictive of clinical safety issues for other PPAR ligands could lead to a requirement for more detailed clinical safety studies or outcome studies prior to approval.

INT131 is well tolerated in rats treated for 6 months with doses resulting in up to two to three orders of magnitude greater exposure than exposure attained at efficacious clinical

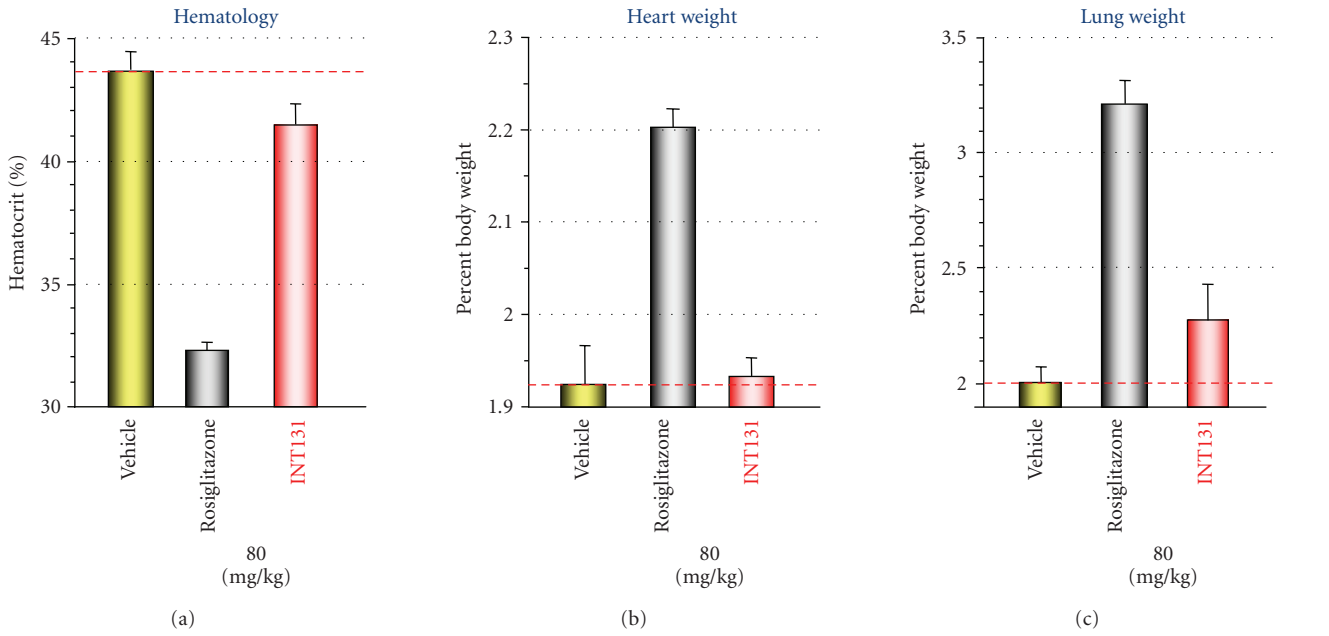


FIGURE 8: *INT131 does not increase plasma volume, heart weight, or lung weight.* Zucker fatty rats were treated orally once daily for 14 days with 80 mg/kg/day of INT131 or rosiglitazone (n = 6/group). (a) Hematocrit, (b) heart weight, and (c) lung weight were measured, and organ weights normalized to body weight. Adapted from [17, 19].

doses in humans. Of particular note was the lack of the toxicities characteristic of PPARγ full agonists, including signs of fluid accumulation or increased heart weight at doses representing these high safety multiples. These adverse effects are typically observed at or near efficacious exposure levels for potent PPARγ full agonists. Thus, the therapeutic window for INT131 is predicted to be significantly greater than it is for the older classes of compounds.

Safety testing of INT131 in cynomolgus monkeys for one and six months at exposures up to >70-fold (highest dose and duration tested) over the exposures expected at the highest dose in the ongoing clinical development program showed that all doses were well tolerated. Confirming the rat safety study results, typical PPAR full agonist effects such as fluid retention, increased adiposity, fatty replacement of marrow, or cardiac changes detected by echocardiography, pathology, or histology were not observed in INT131 treated monkeys.

An additional area of concern for the general PPAR ligand class of compounds is carcinogenicity. In July 2004, FDA provided guidance regarding preclinical and clinical safety assessments for any molecules in clinical development affecting PPAR superfamily members. Cumulative rodent data reviewed by the agency for a number of PPARγ dual α/γ agonists in development had shown an increased incidence of carcinogenicity. Based on these data, the FDA mandated that clinical dosing could not exceed six months with any PPAR ligand (α, γ, δ, α/γ dual, or $\alpha/\gamma/\delta$ pan agonist) unless two-year rodent carcinogenicity studies were completed and satisfactorily reviewed by the agency.

SPPARMs such as INT131 would appear to be at lower risk for demonstrating carcinogenic activity than PPARγ full agonists and dual PPARα/γ agonists (Figure 10) for several reasons. First, many of the PPAR binding molecules that caused tumors in the rodent studies were PPARα/γ dual agonist with which multispecies, multitissue, and both-sex tumor incidence occurred [23]. INT131 is highly selective for

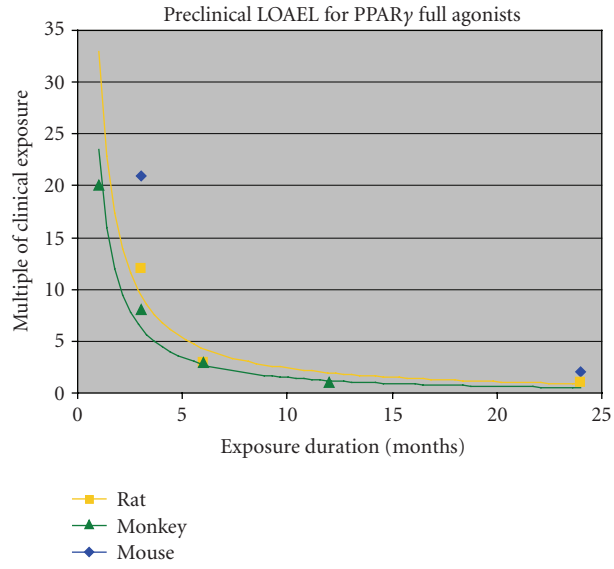


FIGURE 9: *Preclinical LOAEL for PPARγ full agonists follows a steep time dependence.* The lowest observable adverse effect level (LOAEL) at various exposure times in mouse, rat, and monkey is depicted for aggregate data for the class and expressed as multiple of clinical exposure level. Data from [21].

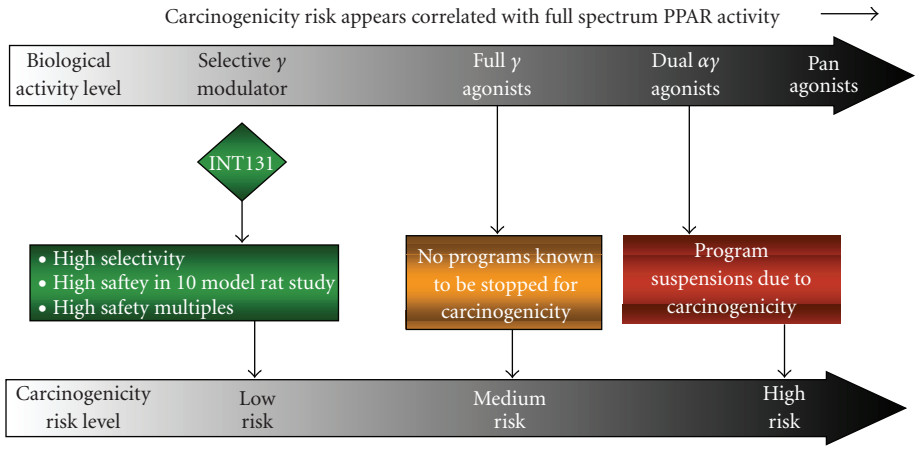


Figure 10: *Carcinogenicity risk in relation to spectrum of PPAR activation.* Propensity for demonstrating carcinogenic activity is predicted to increase with broader PPAR activating activity.

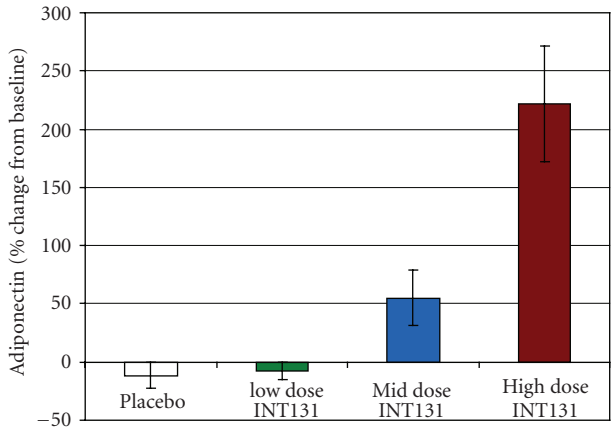


Figure 11: *Adiponectin level is stimulated in human subjects exposed to INT131.* Circulating adiponectin was measured in healthy volunteers exposed to 8 consecutive daily fixed oral doses of INT131. Adapted from [24].

PPARγ, with no binding to PPARα or δ at 10 μM, 1000 fold over the Ki for PPARγ [19].

While carcinogenicity is less of a concern for PPARγ agonists than for PPARα or α/γ dual agonists, the two most prevalent types of tumors associated with PPARγ full agonist molecules which do occur are lipomas and hemangiosarcomas. These cancers derive from adipose tissue and vascular endothelium, respectively. Since INT131 shows little propensity to promote adipocyte differentiation in vitro or adipose proliferation in vivo, it would be reasonable to expect that INT131 would convey minimal, if any, risk for these malignancies. Similarly, the lack of edema in preclinical models suggests a weak activity in the vascular endothelium and thus would be unlikely to invoke the activation associated with hemangiosarcomas at very high doses of full PPARγ agonists. Taken together, it is likely that selectivity of a SPPARM such as INT131 will reduce the potential for carcinogenicity that plague PPAR full agonists, but this remains to be conclusively shown by ongoing studies.

6. EARLY CLINICAL RESULTS WITH INT131 SHOW SEPARATION OF EFFICACY FROM SIDE EFFECTS

Four Phase 1 studies have demonstrated that INT131 besylate is well tolerated and has highly desirable pharmacokinetic and pharmacodynamic properties. The rapid and robust stimulation of adiponectin levels (Figure 11) provides evidence of activation of PPARγ pathways associated with therapeutic efficacy, confirming preclinical pharmacology results [15].

A 4-week Phase 2a multicenter, randomized, double blind, placebo controlled study was conducted to establish the glucose lowering activity of INT131 besylate in subjects with T2DM. INT131 was well tolerated, with no significant safety signals [19]. A reduction in fasting plasma glucose (the primary endpoint of the study) was observed at week 1 and week 4, unusually early for this mechanism of action, and was statistically significant despite the short duration of treatment. Stimulation of adiponectin levels, seen in healthy volunteers in Phase I, was confirmed in the T2DM population in the Phase 2a study. Most notably, the SPPARM activity of INT131 was supported by separation of the observed antidiabetic effects from edema and weight gain, differentiating INT131 from TZD PPARγ full agonists. These results provided the foundation for an ongoing multicenter double blind placebo controlled Phase 2b study of 4 doses of INT131 and pioglitazone comparator in T2DM patients, which is designed to rigorously test the SPPARM activity of INT131 for separation of PPARγ mediated efficacy in treating insulin resistance from TZD side effects.

7. CONCLUSION

The non-TZD selective PPARγ modulator INT131 is the culmination of a molecular target-based strategy to develop an improved insulin-sensitizing drug that does not cause the

weight gain and edema that plague the PPAR full agonists. As predicted by its unique PPARγ profile, INT131 shows potential as a potent and efficacious insulin-sensitizing molecule in T2DM patients that causes little if any weight gain at therapeutically efficacious doses. This emerging clinical profile of efficacy/side-effect separation is consistent with the underlying molecular biology design, the in vitro study data and the robust preclinical data. It thus represents the final part of an accordant continuum testing the hypothesis that selective modulation of PPARγ can create a clinically relevant therapeutic window which is hoped to eventually provide tangible benefits to patients.

REFERENCES

[1] T. Fujiwara, S. Yoshioka, T. Yoshioka, I. Ushiyama, and H. Horikoshi, "Characterization of new oral antidiabetic agent CS-045. Studies in KK and ob/ob mice and Zucker fatty rats," *Diabetes*, vol. 37, no. 11, pp. 1549–1558, 1988.

[2] J. M. Lehmann, L. B. Moore, T. A. Smith-Oliver, W. O. Wilkison, T. M. Willson, and S. A. Kliewer, "An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ)," *The Journal of Biological Chemistry*, vol. 270, no. 22, pp. 12953–12956, 1995.

[3] R. K. Semple, V. K. Chatterjee, and S. O'Rahilly, "PPARγ and human metabolic disease," *The Journal of Clinical Investigation*, vol. 116, no. 3, pp. 581–589, 2006.

[4] H. Yki-Järvinen, "Thiazolidinediones," *The New England Journal of Medicine*, vol. 351, no. 11, pp. 1106–1118, 2004.

[5] H. Yki-Järvinen, "The PROactive study: some answers, many questions," *The Lancet*, vol. 366, no. 9493, pp. 1241–1242, 2005.

[6] S. Mudaliar, A. R. Chang, and R. R. Henry, "Thiazolidinediones, peripheral edema, and type 2 diabetes: incidence, pathophysiology, and clinical implications," *Endocrine Practice*, vol. 9, no. 5, pp. 406–416, 2003.

[7] A. J. Scheen, "Combined thiazolidinedione-insulin therapy: should we be concerned about safety?" *Drug Safety*, vol. 27, no. 12, pp. 841–856, 2004.

[8] R. M. Lago, P. P. Singh, and R. W. Nesto, "Congestive heart failure and cardiovascular death in patients with prediabetes and type 2 diabetes given thiazolidinediones: a meta-analysis of randomised clinical trials," *The Lancet*, vol. 370, no. 9593, pp. 1129–1136, 2007.

[9] D. M. Nathan, J. B. Buse, M. B. Davidson, et al., "Management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: update regarding thiazolidinediones: a consensus statement from the American Diabetes Association and the European Association for the study of diabetes," *Diabetes Care*, vol. 31, no. 1, pp. 173–175, 2008.

[10] S. E. Nissen and K. Wolski, "Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes," *The New England Journal of Medicine*, vol. 356, no. 24, pp. 2457–2471, 2007.

[11] A. B. Goldfine, "The rough road for rosiglitazone," *Current Opinion in Endocrinology, Diabetes and Obesity*, vol. 15, no. 2, pp. 113–117, 2008.

[12] C. E. Murphy and P. T. Rodgers, "Effects of thiazolidinediones on bone loss and fracture," *The Annals of Pharmacotherapy*, vol. 41, no. 12, pp. 2014–2018, 2007.

[13] R. T. Nolte, G. B. Wisely, S. Westin, et al., "Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-γ," *Nature*, vol. 395, no. 6698, pp. 137–143, 1998.

[14] F. Zhang, B. E. Lavan, and F. M. Gregoire, "Selective modulators of PPAR-γ activity: molecular aspects related to obesity and side-effects," *PPAR Research*, vol. 2007, Article ID 32696, 7 pages, 2007.

[15] J. M. Olefsky, "Treatment of insulin resistance with peroxisome proliferator–activated receptor γ agonists," *The Journal of Clinical Investigation*, vol. 106, no. 4, pp. 467–472, 2000.

[16] D. P. McDonnell, "Mining the complexities of the estrogen signaling pathways for novel therapeutics," *Endocrinology*, vol. 144, no. 10, pp. 4237–4240, 2003.

[17] Y. Li, Z. Wang, A. Motani, et al., "T0903131 (T131): a selective modulator of PPARγ," in *Proceedings of the 64th Annual Scientific Sessions of the American Diabetes Association (ADA '04)*, Orlando, Fla, USA, June 2004, Abstract 659-P.

[18] T. M. Willson, P. J. Brown, D. D. Sternbach, and B. R. Henke, "The PPARs: from orphan receptors to drug discovery," *Journal of Medicinal Chemistry*, vol. 43, no. 4, pp. 527–550, 2000.

[19] L. R. McGee, S. M. Rubenstein, J. B. Houze, et al., "Discovery of AMG131: a selective modulator of PPARγ," in *Proceedings of the 231st American Chemical Society National Meeting*, Altanta, Ga, USA, March 2006, Abstract MEDI 20.

[20] T. Kadowaki, T. Yamauchi, N. Kubota, K. Hara, K. Ueki, and K. Tobe, "Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome," *The Journal of Clinical Investigation*, vol. 116, no. 7, pp. 1784–1792, 2006.

[21] J. El Hage, "Peroxisome proliferator-activated receptor agonists: preclinical and clinical cardiac safety considerations," 2005, http://www.fda.gov/ohrms/dockets/ac/05/slides/2005-4169S2_02_02-FDA-ElHage.ppt.

[22] US Department of Health and Human Services Food and drug Administration Center for Drug Evaluation and Research, "Guidance for Industry. Diabetes Mellitus: Developing Drugs and Therapeutic Biologics for Treatment and Prevention," Draft Guidance, 2008, http://www.fda.gov/OHRMS/DOCKETS/98fr/FDA-2008-D- 0118-nad.pdf.

[23] J. El Hage, "Preclinical and clinical safety assessments for PPAR agonists," 2004, http://www.fda.gov/CDER/present/DIA2004/Elhage.ppt.

[24] K. Kersey, L. C. Floren, B. Pendleton, M. J. Stempien, J. Buchanan, and F. Dunn, "T0903131, a selective modulator of PPAR-gamma activity, increases adiponectin levels in healthy subjects," in *Proceedings of the 64th Annual Scientific Sessions of the American Diabetes Association (ADA '04)*, Orlando, Fla, USA, June 2004, Abstract 656-P.