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THE MECHANISMS OF ACTION OF PPARS

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■ **Abstract** The peroxisome proliferator-activated receptors (PPARs) are a group of three nuclear receptor isoforms, PPARγ, PPARα, and PPARδ, encoded by different genes. PPARs are ligand-regulated transcription factors that control gene expression by binding to specific response elements (PPREs) within promoters. PPARs bind as heterodimers with a retinoid X receptor and, upon binding agonist, interact with co-factors such that the rate of transcription initiation is increased. The PPARs play a critical physiological role as lipid sensors and regulators of lipid metabolism. Fatty acids and eicosanoids have been identified as natural ligands for the PPARs. More potent synthetic PPAR ligands, including the fibrates and thiazolidinediones, have proven effective in the treatment of dyslipidemia and diabetes. Use of such ligands has allowed researchers to unveil many potential roles for the PPARs in pathological states including atherosclerosis, inflammation, cancer, infertility, and demyelination. Here, we present the current state of knowledge regarding the molecular mechanisms of PPAR action and the involvement of the PPARs in the etiology and treatment of several chronic diseases.

INTRODUCTION

The peroxisome proliferator-activated receptors (PPARs) form a subfamily of the nuclear receptor superfamily. Three isoforms, encoded by separate genes, have been identified thus far: PPARγ, PPARα, and PPARδ. The PPARs are ligand-dependent transcription factors that regulate target gene expression by binding to specific peroxisome proliferator response elements (PPREs) in enhancer sites of regulated genes. Each receptor binds to its PPRE as a heterodimer with a retinoid X receptor (RXR). Upon binding an agonist, the conformation of a PPAR is altered and stabilized such that a binding cleft is created and recruitment of transcriptional coactivators occurs. The result is an increase in gene transcription.

The first cloning of a PPAR (PPARα) occurred in the course of the search for the molecular target of hepatic peroxisome proliferating agents in rodents. Since then, numerous fatty acids and their derivatives, including a variety of eicosanoids and prostaglandins, have been shown to serve as ligands of the PPARs. It has therefore been suggested that these receptors play a central role in sensing nutrient levels and in modulating their metabolism. Recently, it has been demonstrated that

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the PPARs are the primary targets of numerous classes of synthetic compounds used in the successful treatment of diabetes and dyslipidemia. As such, a significant understanding of the molecular and physiological characteristics of these receptors has become extremely important to those engaged in the development or utilization of drugs used to treat metabolic disorders. In addition, owing to the great interest within the research community, additional putative roles for the PPARs have been proposed. Various researchers have put forth data supporting regulatory roles for PPARγ and PPARα in a wide range of events involving the vasculature, including atherosclerotic plaque formation and stability, vascular tone, and angiogenesis. PPARγ has also demonstrated significant anti-inflammatory action in models of colon inflammation. PPARδ, γ, and α have each been implicated in regulating both normal cellular differentiation and the pathophysiology of carcinogenesis. Another potentially exciting area of research is the central nervous system (CNS), where PPARδ has been linked to myelinogenesis and glial cell maturation. Finally, PPARδ has been shown to affect embryo implantation and therefore fertility. Such observations, discussed in greater detail below, may eventually lead to important new therapeutic uses for PPAR ligands.

RECEPTOR STRUCTURE

PPARs, like other nuclear receptors, possess a modular structure composed of functional domains (1). The DNA binding domain (DBD) and the ligand binding domain (LBD) are the most highly conserved regions across the receptor isoforms. The DBD consists of two zinc fingers that specifically bind PPREs in the regulatory region of PPAR-responsive genes. The LBD, located in the C-terminal half of the receptor, has been shown by crystallographic studies to be composed of 13 α-helices and a small 4-stranded β-sheet (Figure 1). The ligand binding "pocket" of PPARs appears to be quite large in comparison with that of other nuclear receptors (2, 3). This difference may allow the PPARs to interact with a broad range of structurally distinct natural and synthetic ligands. Located in the C terminus of the LBD is the ligand-dependent activation domain, AF-2. This region is intimately involved in the generation of the receptors' coactivator binding pocket (2). A ligand-independent activation function, AF-1, is found in close proximity to the N terminus of the receptor in the A/B domain (4).

RXR AND HETERODIMERIZATION

Unlike the steroid hormone receptors, which function as homodimers, PPARs form heterodimers with the retinoid X receptor (RXR) (5). Like PPARs, RXR exists as three distinct isoforms: RXRα, β, and γ, all of which are activated by the endogenous agonist 9-*cis* retinoic acid (6). No specific roles have yet been elaborated for these different isoforms within the PPAR:RXR complex. However, synthetic RXR agonists ("rexinoids") can activate the complex and thereby obtain

antidiabetic outcomes similar to those seen with PPAR agonists in mouse models of type 2 diabetes (7).

PEROXISOME PROLIFERATOR RESPONSE ELEMENT

Peroxisome proliferator response elements (PPREs) are direct repeat (DR)-1 elements consisting of two hexanucleotides with the consensus sequence AGGTCA 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 and adipocyte fatty acid-binding protein (8). *Cis* elements adjacent to the PPRE core site (especially 5′) appear to play a role in defining the binding selectivity of these response elements (8). Interestingly, PPAR:RXR binds the PPRE with a reverse polarity in comparison with vitamin D receptor (VDR):RXR and thyroid receptor (TR):RXR heterodimers on DR-3 and DR-4 elements, respectively (9).

COACTIVATORS

Several cofactor proteins, coactivators, and corepressors that mediate the ability of nuclear receptors to initiate (or suppress) the transcription process were recently identified (10). Coactivators interact with nuclear receptors in an agonist-dependent manner through a conserved LXXLL motif (where X is any amino acid) (11, 12). This coactivator domain is oriented by a "charge clamp" formed by residues within helix 3 and the AF-2 of helix 12 of the LBD. It can then bind to a hydrophobic cleft in the surface of the receptor formed by helices 3, 4, and 5 and the AF-2 helix (2). Agonist-induced alterations in the conformation of PPAR have been demonstrated by comparing the protease digest patterns of the apo- and agonist-bound receptor (13). Several coactivators, including CBP/p300 and steroid receptor coactivator (SRC)-1 (14), possess histone acetylase activity that can remodel chromatin structure. A second group, represented by the members of the DRIP/TRAP complex such as PPAR binding protein (PBP)/TRAP220 (15), form a bridge between the nuclear receptor and the transcription initiation machinery. The precise role of a third group, including PGC-1 (16), RIP140 (17), and ARA70 (18), is not well understood at the molecular level. At its most simple, a sequence of events can be envisioned in which coactivators with histone acetylase activity complex with liganded, PPRE-bound PPAR/RXR receptors, disrupt nucleosomes, and "open-up" chromatin structure in the vicinity of the regulatory region of a gene (Figure 2). Complexes such as DRIP/TRAP are then recruited and provide a direct link to the basal transcription machinery. As a result, initiation of transcription is induced.

The binding of a partial agonist to PPARγ was recently shown to cause the receptor to interact with CBP or SRC-1 in a less efficacious manner than a full agonist (19). Such distinctive PPAR:cofactor interactions may be a critical element in transmitting signals that result in unique gene regulatory activity and could

therefore prove useful in identifying and characterizing selective PPAR modulators with novel physiological actions.

LIGAND SCREENING ASSAYS

Several assays have been developed to identify and characterize PPAR ligands (Figure 2). Transactivation assays involve cotransfection of cells with a PPAR expression vector and a reporter construct containing a PPRE-driven gene reporter (20). An agonist will increase the reporter gene signal in such assays. Alternatively, chimeric receptors consisting of the PPAR LBD and the yeast transcription factor Gal4 DBD have been utilized with a Gal4-responsive reporter plasmid (21). Radiolabeled thiazolidinediones (TZDs) and subsequently developed non-TZDs have been used in competitive PPAR ligand binding assays (13, 20). PPAR scintillation proximity assays (SPAs), using receptor LBDs attached to scintillant-containing beads, allowed for high-throughput screening for ligands (22). Most recently, a novel fluorescent energy transfer assay was implemented to evaluate the ability of ligands to induce PPAR-cofactor interaction in a rapid, cell-free format (23).

NATURAL LIGANDS

Owing to the critical role PPARs play in lipid metabolism, the search for natural ligands began with fatty acids and eicosanoids. In fact, such metabolites have been identified as bona fide natural ligands of the PPARs. Cell-based transactivation assays and, more recently, direct binding studies have been used to characterize these endogenous receptor effectors.

Fatty acids and eicosanoid derivatives bind and activate PPARγ at micromolar concentrations. PPARγ clearly prefers polyunsaturated fatty acids, including the essential fatty acids linoleic acid, linolenic acid, arachidonic acid, and eicosapentaenoic acid (3). The micromolar affinity of these metabolites is in line with their serum levels. However, their intracellular concentration ranges are unknown. Conversion of linoleic acid to 9-HODE and 13-HODE by 15-lipoxygenase can provide additional micromolar PPARγ agonists (24). A PGD2-derivative, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ2), was demonstrated to be a relatively weak (2–5 μM) PPARγ ligand and agonist (25, 26), although the physiological relevance of this ligand is unclear because cellular concentrations cannot be accurately determined. More recently, an oxidized alkyl phospholipid, hexadecyl azelaoyl phosphatidylcholine, was shown to bind PPARγ with a K_d of $\sim$40 nM; it activated the receptor with a similar EC_{50} (27). These affinities, which are the highest thus far reported for a natural PPAR ligand, are similar to those of the potent synthetic ligand rosiglitazone. This work provides a new and perhaps important link between oxidized low-density lipoproteins, PPARγ activation, and the physiology of atherosclerotic plaques.

PPARα can be activated by a wide variety of saturated and unsaturated fatty acids, including palmitic acid, oleic acid, linoleic acid, and arachidonic acid (28). A

number of fatty acids have been found to bind the receptor directly with micromolar affinities (29, 30). As discussed above, it is unclear whether the concentrations at which binding has been noted are physiologically relevant. The lipoxygenase metabolite 8(S)-HETE was identified as a submicromolar ligand for PPARα (31) but is apparently not present at high enough levels in the cell to be classified as a true natural ligand. In lieu of high-affinity endogenous ligands, it is plausible that PPARα functions primarily as a sensor of free fatty acid levels in the tissues where it is expressed.

Like other PPARs, PPARδ interacts with saturated and unsaturated fatty acids; its ligand selectivity is intermediate between that of PPARγ and PPARα (3). Notably, the polyunsaturated fatty acids dihomo-γ-linolenic acid, EPA, and arachidonic acid had low micromolar affinities for PPARδ (30). Palmitic acid and its metabolically stable analogue, 2-bromopalmitic acid, were also identified as PPARδ agonists (32). A number of eicosanoids, including PGA1 and PGD2, have been shown to activate PPARδ (31). Carbaprostacyclin, a semisynthetic prostaglandin, is also a micromolar PPARδ agonist (30). The physiological levels of its naturally occurring precursor, prostacyclin, however, are unknown because of its metabolic instability.

SYNTHETIC LIGANDS

Several key observations made in the mid-1990s regarding thiazolidinedione (TZD) antidiabetic agents have allowed researchers to determine their primary molecular site of action. Such compounds had been developed over the preceding 15 years on the basis of their insulin-sensitizing effects in pharmacological studies in animals. TZDs were found to induce adipocyte differentiation and increase expression of adipocyte genes, including the adipocyte fatty acid-binding protein aP2 (33, 34). Independently, Spiegelman and colleagues reported that PPARγ interacted with a regulatory element within the 5′ flanking region of the *aP2* gene that controlled its adipocyte-specific expression (35). These seminal observations were the precursor to additional experiments, which determined that TZDs such as rosiglitazone, pioglitazone, englitazone, and ciglitazone were, in fact, PPARγ ligands and agonists (13, 20, 36). Rosiglitazone was shown to bind the receptor with a high affinity (K_d of $\sim$40 nM), whereas pioglitazone, englitazone, and ciglitazone were less potent ligands. Such characterization of these antidiabetic agents also demonstrated a definite correlation between the in vivo PPARγ binding and agonist activities of these compounds and their in vivo insulin-sensitizing actions (13, 36).

TZDs were developed primarily to improve the antidiabetic actions of the fibrate hypolipidemic agents. Several TZDs, including troglitazone, rosiglitazone, and pioglitazone, have insulin-sensitizing and antidiabetic activity in humans with type 2 diabetes or impaired glucose tolerance (37, 38). AL-294, the first significant lead compound, evolved into both the TZDs and the parallel α-alkoxyphenylproprionates (39). Select compounds of this latter class have shown potent PPARγ activity as well as significant PPARα activity. TZDs have also been identified that are

dual PPARγ/α agonists; KRP-297 is representative of this compound class (40). Previously, we presented a novel class of phenylacetic acid derivatives, such as L-796449, which are potent agonists of all three PPARs, and L-805645, which is a PPARγ selective compound (21, 41). GW2570 is a very potent non-TZD PPARγ-selective agonist that was recently shown to have antidiabetic efficacy in humans (38). In addition to these potent PPARγ ligands, a subset of the non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin, fenoprofen, and ibuprofen, have displayed weak PPARγ and PPARα activities (42). The PPARγ antagonist GW0072 was recently reported to interact with different amino acid residues within the LBD of the receptor versus full agonists; in cell culture experiments, the antagonist blocked adipocyte differentiation (19).

The fibrates, amphipathic carboxylic acids that have been proven useful in the treatment of hypertriglyceridemia, are PPARα ligands. Clofibrate is a prototype for this class, which was developed before PPARs were identified, using in vivo assays in rodents to assess lipid-lowering efficacy (43). This compound was later found to induce peroxisome proliferation in rodents (44). Since the identification of clofibrate, research efforts have expanded considerably, and this class of lipid-lowering agents has been further characterized. Clofibrate and fenofibrate have been shown to activate PPARα with a tenfold selectivity over PPARγ (38). Bezafibrate acted as a pan-agonist that showed similar potency on all three PPAR isoforms. WY-14643, the 2-arylthioacetic acid analogue of clofibrate, was a potent murine PPARα agonist as well as a weak PPARγ agonist. In humans, fibrates must all be used at high doses (300–1200 mg/day) to achieve efficacious lipid-lowering activity. Recently, the ureidofibrate, GW2331, was found to be a nanomolar PPARα and PPARγ ligand (45), whereas the closely related GW9578, a ureidobutyric acid, was reported to be a potent PPARα-selective agonist with robust hypolipidemic activity in vivo (46).

In order to define the physiological role of PPARδ, efforts have been made to develop novel compounds that activate this receptor in a selective manner. Among the α-substituted carboxylic acids described previously (21), the potent PPARδ ligand L-165041 demonstrated ~30-fold agonist selectivity for this receptor over PPARγ; additionally, it was inactive on murine PPARα. This compound was found to increase high-density lipoprotein levels in rodents (47). Recently, Oliver et al. reported that GW501516 was a potent, highly selective PPARδ ligand and agonist (48). In obese, insulin-resistant rhesus monkeys, this compound afforded beneficial changes in serum lipid parameters.

PPARγ

Cloning and Characterization

Three homologous PPARs, classified as PPARα, β (δ), and γ, were cloned from a *Xenopus* cDNA library in 1992 (49). PPARγ was subsequently cloned from several mammalian species including human (50). Two PPARγ isoforms are expressed at

the protein level in mouse (51) and human (52), $\gamma 1$ and $\gamma 2$. These differ only in that $\gamma 2$ has 30 additional amino acids at its N terminus due to differential promoter usage within the same gene and subsequent alternative RNA processing. PPAR$\gamma 2$ is expressed primarily in adipose tissue (53). PPAR$\gamma 1$ is expressed in a broad range of tissues including heart, skeletal muscle, colon, small and large intestines, kidney, pancreas, and spleen.

Physiologic Effects and Mechanisms of Insulin Sensitization

PPARγ is necessary and sufficient to differentiate adipocytes. It was first shown to interact directly with the *cis* element that regulates adipocyte-specific expression of the fatty acid-binding protein aP2 (54). Introduction of PPARγ into fibroblasts in the presence of weak PPAR ligands induced differentiation of the cells into adipocytes (55). Recently, several groups of researchers reported that PPARγ heterozygous null mice had reduced amounts of adipose tissue (56–58). Barak et al. (56) described a homozygous null mouse that exhibited extreme lipodystrophy. PPARγ dominant-negative mutants have been generated (59–61). When expressed in 3T3-L1 cells, such mutants inhibited their differentiation into adipocytes (59, 60). In adipocytes, PPARγ regulates the expression of numerous genes (Table 1) involved in lipid metabolism, including aP2 (35), PEPCK (62), acyl-CoA synthase (63), and LPL (64). PPARγ has also been shown to control expression of FATP-1 (65) and CD36 (66), both involved in lipid uptake into adipocytes. These genes have all been shown to possess PPREs within their regulatory regions.

PPARγ also regulates genes that control cellular energy homeostasis (Table 1). It has been shown to increase expression of the mitochondrial uncoupling proteins, UCP-1, UCP-2, and UCP-3 in vitro and in vivo (67). The physiological outcomes of these alterations are not yet understood. In contrast to its positive action on the UCPs, PPARγ downregulates leptin, a secreted, adipocyte-selective protein that inhibits feeding and augments catabolic lipid metabolism (68, 69). This receptor activity might explain the increased caloric uptake and storage noted in vivo upon treatment with PPARγ agonists.

PPARγ has been associated with several genes that affect insulin action. TNFα, a pro-inflammatory cytokine that is expressed by adipocytes, has been associated with insulin resistance (70) and diminished insulin signal transduction (71). PPARγ agonists inhibited expression of TNFα in adipose tissue of obese rodents (72) and TNFα-induced insulin resistance (73). They also ablated the actions of TNFα in adipocytes in vitro (74). Activation of PPARγ has been shown to increase expression of c-CBL-associated protein in cultured adipocytes (75). This protein, which appears to play a positive role in the insulin signaling pathway, contains a functional PPRE within the 5′ regulatory region of its gene (76). Expression of IRS-2, a protein with a proven role in insulin signal transduction in insulin-sensitive tissue, was also increased in cultured adipocytes and human adipose tissue incubated with PPARγ agonists (77). Recently, we have demonstrated that PPARγ

TABLE 1 Genes regulated in vivo by PPARγ agonists*

Gene	Regulation	Potential function(s)
aP2—adipocyte fatty acid binding protein	↑ WAT	Intracellular fatty acid binding
Acyl-CoA synthetase	↑ WAT	Lipogenesis and/or catabolism
PEPCK—phosphoenolpyruvate carboxykinase	↑ WAT	Glycerol synthesis (for triglycerides)
LPL-lipoprotein lipase	↑ WAT	Hydrolysis of triglyceride-containing particles
CD36	↑ WAT	Cell surface fatty acid transporter
FATP-1	↑ WAT ↓ muscle	Cell surface fatty acid transporter
Uncoupling protein 1—UCP1	↑ BAT ↑ WAT	Uncouple mitochondrial respiration
UCP3 (+/−UCP2)	↑ WAT	Uncouple mitochondrial respiration
Carnitine palmitoyl transferase1 CPT1	↑ WAT	Translocation of fatty acids into mitochondria
c-CBL-associated protein	↑ WAT	Insulin signaling toward glucose transport
Insulin receptor substrate-2—IRS-2	↑ WAT	Insulin receptor-mediated signaling
Pyruvate dehydrogenase kinase 4—PDK4	↑ WAT ↓ muscle	Inhibition of pyruvate dehydrogenase (inhibition of glucose oxidation)
Adipocyte complement-related factor 30—Acrp30	↑ WAT	Fat-specific secreted protein; beneficial metabolic effects on liver/muscle (?)
TNFα	↓ WAT	Pro-inflammatory cytokine; potential mediator of insulin resistance
Leptin	↓ WAT	Fat-derived hormone that inhibits food intake
11-β hydroxysteroid dehydrogenase 1—11β-HSD-1	↓ WAT (↓ liver)	Controls intracellular conversion to active cortisol

*Increases or decreases in mRNA expression are noted in white (WAT) or brown (BAT) adipose tissue and skeletal muscle. See text for details and references.

agonists inhibit expression of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD-1) in adipocytes and adipose tissue of type 2 diabetes mouse models (41). This enzyme, which is highly expressed in adipocytes and hepatocytes, converts cortisone to the glucocorticoid agonist cortisol. Because hypercorticosteroidism exacerbates insulin resistance (78) and 11β-HSD-1 null mice are resistant to diet-induced diabetes (79), our results suggest that some of the insulin-sensitizing actions observed after activation of PPARγ may result from a decrease in adipose 11β-HSD-1 levels.

Adipocyte-related complement protein (Acrp)30 is a secreted adipocyte-specific protein that was recently shown to have in vivo effects including decreased glucose, triglycerides, and free fatty acids (80, 81). Treatment of diabetic mice with PPARγ agonists normalized low mRNA levels and increased plasma levels of Acrp30 (82). Compared with normal human subjects, patients with type 2 diabetes have reduced plasma levels of Acrp30 (83). Increases in Acrp30 plasma levels were seen in human subjects treated with rosiglitazone but not the PPARα agonist fenofibrate (82). Induction of Acrp30 by PPARγ agonists might therefore also play a key role in the mechanism of PPARγ agonist-mediated amelioration of the metabolic syndrome.

Given that PPARγ is expressed predominantly in adipose tissue, the prevailing hypothesis regarding the net in vivo efficacy of PPARγ agonists involves direct actions on adipose cells, with secondary effects in key insulin-responsive tissues such as skeletal muscle and liver. The lack of glucose-lowering efficacy of rosiglitazone in a mouse model of severe insulin resistance where white adipose tissue was essentially absent supports this notion (84). Although low levels of PPARγ are expressed in muscle, in vivo treatment of insulin-resistant rats produced acute ($<$24 h) normalization of adipose tissue insulin action, whereas insulin-mediated glucose uptake in muscle was not improved until several days after the initiation of therapy (85). This is consistent with the fact that PPARγ agonists can produce an increase in adipose tissue insulin action after direct in vitro incubation (86), whereas no such effect could be demonstrated using isolated in vitro incubated skeletal muscle (85). In addition, recent analysis of tissue mRNA expression reveals that selected PPRE-containing genes that are induced in adipose tissue are actually suppressed in skeletal muscle. An example is pyruvate dehydrogenase kinase 4 (87). In vivo, PPARγ-mediated suppression of this gene in muscle would be expected to produce a net increase in glucose oxidation. Therefore, as depicted in Figure 3, mediators of the beneficial metabolic effects of PPARγ agonists on distant tissues (muscle and liver) are likely to involve a combined effect to (*a*) enhance insulin-mediated adipose tissue uptake, storage (and potentially catabolism) of free fatty acids (88); (*b*) induce the production of adipose-derived factors with potential insulin-sensitizing activity (e.g., Acrp30); and (*c*) suppress the circulating levels and/or actions of insulin resistance-causing adipose-derived factors such as TNFα or "resistin" (89).

Inflammation

The inhibitory effects of PPARγ activation on TNFα action discussed above led several research groups to examine the anti-inflammatory properties of PPARγ agonists. Monocytes and macrophages play an important part in the inflammatory process through the release of inflammatory cytokines such as TNFα and IL-6 and the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS). Expression of PPARγ was robustly upregulated upon the differentiation of monocytes into macrophages (90). In vitro treatment of rodent macrophages with PPARγ

agonists downregulated NO production (91). Such ligands were also found to block PMA-induced synthesis of IL-6 and TNFα in primary human monocytes in spite of the low level of expression PPARγ in these cells (92). Note that the agonist concentrations used in the two aforementioned experiments did not correlate with the reported affinities of the compounds for the receptor. Furthermore, in contrast to the results described above, TZD and non-TZD PPARγ agonists, with the exception of the natural ligand 15d-PGJ2, do not inhibit LPS-induced cytokine production in cultured macrophages and db/db mice treated in vivo (93). We concluded that activation of PPARγ was not a major mechanism by which to inhibit activation of monocytic cells. In general accordance with this conclusion, the Evans group recently utilized murine PPARγ null macrophages to demonstrate that previously reported inhibitory actions of PPARγ agonists on macrophage cytokine production occur via a receptor-independent mechanism (94).

In contrast to the results above, Chinetti et al. demonstrated that rosiglitazone induced apoptosis of cultured macrophages by altering NFκB signaling at concentrations that paralleled its known affinity for PPARγ (90). This ligand has also been shown to block inflammatory cytokine synthesis in colonic cell lines by inhibiting activation of the NFκB pathway (95). This latter observation offers a possible mechanistic explanation for the observed anti-inflammatory actions of TZDs in rodent models of colitis (95).

Cancer

The interest in studying the effects of PPARγ activation on various forms of cancer is derived from previous results suggesting that PPARγ ligands inhibited cell proliferation when inducing adipocyte differentiation. For example, activation of PPARγ caused logarithmically growing fibroblasts and virally transformed HIB1B adipocytes to withdraw from the cell cycle (96). Activation of PPARγ by pioglitazone blocked the cell cycle and caused differentiation of primary liposarcoma cells in culture (97). In human subjects, the PPARγ agonist troglitazone caused differentiation of advanced liposarcomas (98). Such results support a therapeutic role for PPARγ ligands in the treatment of this often recalcitrant form of cancer. PPARγ has been shown to be expressed at significant levels in human mammary adenocarcinomas, and PPARγ agonists have been reported to reduce growth and induce differentiation of malignant breast epithelial cells (99). Such ligands have also inhibited tumor growth in mouse models of mammary carcinoma (100).

PPARγ is expressed at high levels in primary colon tumors and colon cancer cell lines (101). Incubating such transformed cells with PPARγ agonists caused them to withdraw from the cell cycle, decrease their growth rate, and demonstrate changes in morphology indicative of increased differentiation (102). Inhibitors of cyclooxygenases (COXs) have been shown to be effective in reducing the risk of colon cancer. Since the COXs metabolize fatty acids to prostaglandins and eicosanoids, it was suggested that they might promote carcinogenesis by generating PPARγ ligands. In support of this hypothesis, $APC^{min/+}$ mice (a model of inherited

polyposis) treated with high doses of two TZDs displayed a small but statistically significant increase in the number of colon polyps (103, 104). However, others have found that treating mice with troglitazone inhibited growth of transplanted human colon tumors (102). In light of the above contradictory results, it is not presently possible to conclude what role PPARγ plays in the pathophysiology of colon cancer.

Hypertension

Hypertension is a complex disorder of the cardiovascular system that is associated with insulin resistance. Type 2 diabetes patients demonstrate a 1.5- to 2-fold increase in hypertension in comparison with the general population (105). Troglitazone therapy has been shown to decrease blood pressure in diabetic patients (106) as well as in obese, insulin-resistant persons (107). Since such reductions in blood pressure correlate with decreases in insulin levels (106), they may be mediated, at least in part, by an improvement in insulin sensitivity.

Genetic Variation

Several groups have reported nucleotide sequence polymorphisms within the coding exons of the PPARγ gene (108–111); however, there are no known spontaneous mutations affecting PPARγ in nonhuman species. A silent polymorphism (C $\rightarrow$ T) in the sixth exon common to PPARγ1 and PPARγ2 (109, 111) was suggested to distinguish the relationship between body mass index (BMI) and plasma leptin levels in subjects with the CC genotype versus those with the T allele (CT or TT). Thus, genetic variation in or near the PPARγ locus could modulate leptin levels in response to variable degrees of body adiposity.

More important was the discovery of a polymorphism encoding the substitution of Ala for Pro at amino acid 12, as initially reported by Yen et al. (109). The Ala^{12} allele frequency varies from 0.03 to 0.12 in several populations and was initially shown to be associated with increasing degrees of obesity (112). In several additional studies, the Ala^{12} allele was associated with lower BMI, improved insulin sensitivity, and reduced incidence of type 2 diabetes (108, 113). In one large study, the more common Pro^{12} allele was associated with a 1.25-fold increase in risk of type 2 diabetes (113). In contrast, other groups failed to detect an association of Ala^{12} with altered metabolic parameters (114, 115). Importantly, the recombinant receptor bearing this single amino acid change was apparently defective with respect to DNA binding and its ability to mediate ligand-stimulated transactivation in transfected cells (108). Because this variant is relatively prevalent, it may contribute to altered physiology of fat metabolism in humans.

A second PPARγ polymorphism, encoding a $Pro^{115} \rightarrow$ Gln substitution, was present in 4 of 121 obese German subjects (mean BMI 33.9) but was absent in each of 237 normal-weight controls (mean BMI 25) (110). Interestingly, this polymorphism is adjacent to Ser^{114}, which may be an important site of negative regulation via growth factor-mediated phosphorylation (116). Thus, like an artificial

$Ser^{114} \rightarrow$ Ala mutant (116), the naturally occurring Gln^{115} mutant resulted in a greater degree of adipogenesis than wild-type PPARγ when studied in overexpressing cells (110).

In contrast to the more subtle potential effects of the Ala^{12} or Gln^{115} polymorphisms, Barroso et al. recently reported on two families with a phenotype of severely insulin-resistant type 2 diabetes in association with heterozygous PPARγ mutations—either $Pro^{467} \rightarrow$ Leu or $Val^{290} \rightarrow$ Met (117). Interestingly, hypertension was reported as an additional associated phenotype. Importantly, in both families, these mutant receptors were shown to have severely impaired function with potential dominant-negative effects when studied in transfected cells.

PPARα

Cloning and Characterization

Murine PPARα was the first member of this nuclear receptor subclass to be cloned (118). It has subsequently been cloned from frog (49), rat (119), rabbit (120), and human (121). Human PPARα has been mapped to chromosome 22 adjacent to the region 22q12-q13.1 (121). In rodents and humans, PPARα is expressed in numerous metabolically active tissues including liver, kidney, heart, skeletal muscle, and brown fat (122, 123). It is also present in monocytic (90), vascular endothelial (124), and vascular smooth muscle cells (125).

PPARα serves as the receptor for a structurally diverse class of compounds, including hypolipidemic fibrates, that induce hepatic peroxisome proliferation, hepatomegaly, and hepatocarcinogenesis in rodents (118). Remarkably, these toxic effects are lost in humans, although the same compounds activate PPARα across species (126). Several explanations have been proffered for the differential effects of PPARα agonists. Hepatic expression of wild PPARα is expressed at levels tenfold higher in rodent liver than in human liver (127). The PPREs of genes involved in peroxisome proliferation, including acetyl CoA oxidase (ACO), have been shown to differ between rodents and humans. The human enhancer sequence of ACO could not be activated by PPARα in transactivation experiments (128).

PPARα has been shown to play a critical role in the regulation of cellular uptake, activation, and β-oxidation of fatty acids. PPARα induces expression of the fatty acid transport protein (FATP) (65) and FAT (129), two proteins that transport fatty acids across the cell membrane. Activation of PPARα also directly upregulates transcription of long chain fatty acid acetyl-CoA synthase (63) as well as ACO (49, 130), enoyl-CoA hydratase/dehydrogenase multifunctional enzyme (131), and keto-acyl-CoA thiolase (132) enzymes in the peroxisomal β-oxidation pathway. Carnitine palmitoyltransferase I (CPT I) catalyzes the rate-limiting step in the translocation of activated fatty acids into the inner membrane of the mitochondria where the most productive step in their catabolism occurs. This enzyme is strongly induced by PPARα ligands (133), and a functional PPRE has been identified in the 5′ flanking region of its gene (134–136). Other PPARα-responsive genes in this

mitochondrial metabolic pathway have also been reported, including various acyl-CoA dehydrogenases (137, 138) and hydroxymethylglutaryl-CoA synthase (139). The CYP4A subclass of cytochrome P450 enzymes catalyzes the ω-hydroxylation of fatty acids, a pathway that is particularly active in the fasted and diabetic states. Fibrates and other peroxisome proliferators activate expression of the CYP4As, and functional PPREs have been found in the regulatory regions of CYP4A genes (140, 141). In sum, PPARα is an important lipid sensor and regulator of cellular energy-harvesting metabolism. Potent genetic proof for this conclusion is offered by Lee et al., who reported that PPARα null mice had depressed levels of numerous fatty acid metabolizing enzymes and were unresponsive to the actions of peroxisome proliferating agents (142).

Dyslipidemia and Atherosclerosis

Atherosclerosis is a very prevalent disease in westernized societies. In addition to a strong association with elevated LDL cholesterol, dyslipidemia characterized by elevated triglyceride-rich particles and low levels of HDL cholesterol is commonly associated with other aspects of a metabolic syndrome that includes obesity, insulin resistance, type 2 diabetes, and an increased risk of coronary artery disease (143). Thus, in 8500 men with known coronary artery disease, 38% were found to have low HDL ($<$35 mg/dL) and 33% had elevated triglycerides ($>$200 mg/dL) (144). Treatment of these patients with fibrates such as gemfibrozil and fenofibrate, which are weak PPARα agonists, resulted in substantial triglyceride lowering and modest HDL-raising efficacy (145). More importantly, a recent large prospective trial proved that treatment with gemfibrozil produced a 22% reduction in cardiovascular events or death (145, 146). Thus PPARα agonists can effectively improve cardiovascular risk factors and have a net benefit to improve cardiovascular outcomes.

Mechanisms by which PPARα activation causes triglyceride lowering are likely to include the effects of agonists to suppress hepatic apo-CIII gene expression while also stimulating LPL gene expression (64, 147). Moreover, the triglyceride-lowering activity of fibrates and related compounds is ablated in PPARα null mice (148). The effect of fibrates to increase HDL levels has been suggestively associated with an increase in apo-AI gene expression, although this finding is not universally observed (149); thus, additional mechanisms may be involved (discussed below).

The presence of PPARα and/or PPARγ expression in vascular cell types including macrophages, endothelial cells, and vascular smooth muscle cells suggests that direct vascular effects might contribute to potential antiatherosclerosis efficacy (143). As discussed above, PPARγ agonists have been reported to produce variable antiinflammatory effects in monocyte-macrophages. In addition, several lines of evidence have shown that PPARα agonists have potentially relevant local or systemic antiinflammatory effects, particularly in vascular smooth muscle cells (see below). A particular effect of either PPARα (150) or PPARγ (151–153) activation to inhibit cytokine-induced vascular cell adhesion and to suppress

monocyte-macrophage migration has also been recently reported as a possible mechanism of antiatherosclerosis efficacy.

Two recent studies have suggested that either PPARα (154) or PPARγ (154, 155) activation in macrophages can induce the expression of a cholesterol efflux "pump" known as ABC-A1. Since ABC-A1 is a target gene for the liver-X-receptor (LXR), these investigators also showed a modest induction of LXR expression, which may represent the indirect mechanism by which PPAR activation can upregulate ABC-A1.

Although the net effect of fibrates to reduce cardiovascular risk in humans is now well accepted, the potential for an antiatherosclerosis effect of PPARγ agonists (e.g., TZDs) remains unexplored in humans. Several recent studies have shown that PPARγ-selective compounds have the capacity to reduce arterial lesion size in animal models of atherosclerosis. In LDL-receptor null mice, rosiglitazone, troglitazone, and a potent non-TZD PPARγ agonist were shown to inhibit lesion formation (156, 157). In addition, troglitazone was shown to suppress lesion formation in atherosclerosis-prone apo-E null mice (158) and in Wantanabe hyperlipidemic rabbits (159). Furthermore, troglitazone treatment of apo-E–deficient mice for 7 days was sufficient to attenuate monocyte-macrophage homing to arterial lesions in vivo (153). Thus, via multifactorial mechanisms including improvements in circulating lipids, systemic and local anti-inflammatory effects, and, potentially, inhibition of vascular cell proliferation, both PPARα and PPARγ agonists show strong promise for use in the treatment or prevention of atherosclerosis.

Inflammation

PPARα was first proposed to be a modulator of inflammation when leukotriene B4 (LTB4), a potent chemotactic agent, was found to be a ligand and agonist for the receptor (160). It was suggested that activation of PPARα inhibited the inflammatory action of such eicosanoids by augmenting expression of hepatic enzymes involved in their metabolism. This argument was fortified when it was observed that PPARα null mice have more extended inflammatory responses than their wild-type littermates in response to LTB4 or its precursor arachidonic acid.

Other, nonhepatic, anti-inflammatory mechanisms have been described for PPARα ligands that may be important in maintaining vascular health. Treatment of cytokine-activated human macrophages with PPARα agonists induced apoptosis of the cells by interfering with the antiapoptotic NFκB signaling pathway (90). Staels et al. reported that PPARα but not PPARγ agonists inhibited activation of aortic smooth muscle cells in response to inflammatory stimuli by repressing NFκB signaling (125). In hyperlipidemic patients, fenofibrate treatment decreased the plasma concentrations of the inflammatory cytokine interleukin-6 (125). Additional work showed that IκBα levels were induced in vascular smooth muscle cells by fibrates, thereby offering another anti-inflammatory mechanism for PPARα agonists (161). In contrast with these results, increased plasma TNFα levels were observed in fibrate-treated endotoxemic mice (162). This undesirable effect may

be associated with PPARα-induced hepatic peroxisome proliferation. Clearly, additional research is needed to further investigate these provocative results and to deepen our knowledge of PPARα's role in the physiopathology of the inflammatory process, especially as it affects the vascular system.

Genetic Variation

The existence of a few sequence variants in the human PPARα gene was first reported by Tugwood; these included $Thr^{71} \rightarrow$ Met, $Lys^{123} \rightarrow$ Met, $Ala^{268} \rightarrow$ Val, $Gly^{296} \rightarrow$ Ala, and $Val^{444} \rightarrow$ Ala (163). One particular allele with Met at positions 71 and 123 as well as the Ala^{444} substitution was shown to undergo normal RXR dimerization and DNA binding but was inactive in a cell-based transactivation assay (163). Subsequently, another potentially important hPPARα polymorphism was described that lacks 203 basepairs encoding residues 508–712 at the C-terminal end of the DNA binding domain (further described above). More recently, a $Leu^{162} \rightarrow$ Val variant was shown to be associated with higher total and HDL cholesterol in a relative small cohort of human subjects (164). This polymorphism apparently has greater transcriptional activity when studied in transfected cells. In an additional study, the $Leu^{162} \rightarrow$ Val allele was associated with higher LDL and apoB levels, suggesting that it conferred increased atherosclerosis risk. Therefore, the existence of PPARα genetic variants with clear-cut functional effects and a bona fide causal relationship to metabolic alterations has yet to be discovered.

PPARδ

Cloning and Characterization

Human (165) and *Xenopus* (49) PPARδ cDNAs were cloned in the early 1990s. The receptor was subsequently cloned from mouse (166) and rat (167). Human PPARδ has been localized to chromosome 6p21.1–p21.2 (168) whereas the murine gene has been mapped to chromosome 17 (169). PPARδ is expressed in a wide range of tissues and cells, with relatively higher levels of expression noted in brain, adipose, and skin (122, 170). Thus far, no PPARδ-specific gene targets have been identified.

Dyslipidemia and Insulin Resistance

Using relatively selective PPARδ agonists such as L-165041, we determined that such compounds produced minimal, if any, significant glucose- or triglyceride-lowering activity in murine models of type 2 diabetes compared with efficacious PPARγ or PPARα agonists (21). Subsequently, a modest increase in HDL-cholesterol levels was detected with L-165041 in db/db mice (47). More recently, Oliver et al. reported that the potent and selective PPARδ agonist GW-501516 could induce a substantial increase in HDL-cholesterol levels as well as a reduction in triglyceride levels in obese Rhesus monkeys (48). In addition, elevated levels of plasma insulin (a consequence of insulin resistance) were suppressed by

GW-501516 treatment. Although these beneficial metabolic effects in a primate model have yet to be reproduced by others or with other compounds, the results point to an important therapeutic potential for PPARδ -selective compounds.

Fertility

One area in which the role of PPARδ has been examined is fertility. COX-2 null female mice are reported to display decreased fecundity, in part due to decreased blastocyte implantation and decidualization (171). COX-2 catalyzes the rate-limiting step in generating prostaglandins, including prostacyclin, the eicosanoid that appears to serve as the natural agonist for PPARδ. PPARδ was found to be expressed in implantation sites within the uterus, and was strongly upregulated during the decidualization process in a manner similar to COX-2 (172). When COX-2 null mice were treated with carboprostacyclin or our PPARδ-selective agonist L-165041, implantation was restored (172). Such results support the conclusion that PPARδ may play a role in maintaining reproductive capacity in females.

Cancer

Throughout the past decade, researchers have sought to establish the roles of the three PPAR isoforms in the pathophysiology of cancer. In 1999, He et al. identified PPARδ as a target of the tumor suppressor APC in colorectal cancer cells (173). In these cells, which possess inactivation mutations of APC, PPARδ was highly expressed, and transcription factors in the APC signaling pathway, β-catenin/Tcf-4, were found to interact directly with and activate the promoter of PPARδ. Recently, a PPAR$\delta^{-/-}$ colorectal cancer cell line was found to exhibit a greatly decreased ability to form tumors in nude mice in comparison with PPAR$^{+/-}$ cancer cells (174). Although far from conclusive, these results do suggest that PPARδ antagonists might prove beneficial in the treatment of colon cancer.

Central Nervous System

Localization studies have demonstrated that PPARδ is abundantly expressed throughout the rat CNS, with particularly high levels found in the dentate gyrus, hippocampus, telencephalic cortex, cerebellum, and thalamic nuclei (122, 175, 176). Further investigation has shown that PPARδ expression is at its highest level in the embryonic brain (stage E18.5), suggesting that it may play a critical role in regulating the differentiation of cells within the CNS (177).

We have examined the expression of PPARδ in murine brain by in situ hybridization and immunohistochemistry and found it to be expressed widely throughout murine brain but at particularly high levels in the entorhinal cortex, hypothalamus, and hippocampus as well as the corpus callosum and the neostriatum (J.W. Woods, M. Tanen, D.J. Figueroa, C. Biswas, E. Zycband, D.E. Moller, C.P. Austin & J. Berger, unpublished data). Expression of PPARδ in the caudate putamen and corpus callosum suggests its possible involvement in volitional movement

as well as cortical processing of signals from the thalamus (178). PPARδ expression in limbic regions (hypothalamus, hippocampus, and entorhinal cortex) suggests that PPARδ might also play a role in more complex emotional, circadian, and autonomic functions (179). PPARδ was highly expressed within oligodendrocytes of the corpus callosum and neurons but not in astrocytes of the caudate putamen.

High levels of PPARδ expression have recently been reported in cultured murine oligodendrocytes, and PPARδ agonists, bromopalmitate and our L-165041, were found to augment differentiation of and myelinogenesis by these cells (180, 181). It is therefore noteworthy that PPARδ null mice were found to have diminished myelination levels of the corpus callosum (182), an area normally rich in PPARδ-expressing oligodendrocytes (see above). PPARδ has also been found to be the major isotype expressed in cultured rat neurons and to be coexpressed with acyl-CoA synthase 2, an enzyme thought to play an important role in fatty acid utilization within the brain (183). Using L-165041, it was discovered that the *ACS2* gene is transcriptionally regulated by PPARδ. Together, these data suggest a role for PPARδ in myelination, neuronal signaling, and lipid metabolism in the CNS.

CONCLUSIONS AND FUTURE DIRECTIONS

Isoforms of the PPAR family of nuclear receptors are clearly involved in the systemic regulation of lipid metabolism and serve as "sensors" for fatty acids, prostanoid metabolites, eicosanoids, and related molecules. These receptors function to regulate a broad array of genes in a coordinate fashion. Important biochemical pathways that regulate peroxisomal function, lipid oxidation, metabolism of xenobiotics, lipid synthesis, adipocyte differentiation, insulin action, cell proliferation, and inflammation can be modulated by activation (or inhibition) of individual PPAR isoforms. Strong therapeutic effects of PPARα and PPARγ agonists to favorably influence systemic lipid levels, glucose homeostasis, and atherosclerosis risk (in the case of PPARα activation in humans) have recently been discovered. Although specific molecular mechanisms by which PPARα activation can effectively ameliorate dyslipidemia are now well characterized, the multifactorial mechanism by which PPARγ agonists reduce insulin resistance remains to be further elucidated. Recent observations made using PPARδ ligands suggest that this less well characterized isoform may also be an important therapeutic target for selected disorders, including cancer, infertility, and dyslipidemia.

Further assessment of the physiologic effects of individual PPARs, such as can be achieved with the use of tissue-selective knockout mice, should provide better insights into the precise roles of these receptors in individual cell types. In addition, the use of gene microarray and proteomic techniques to carefully monitor the full spectrum of gene expression and protein effects of more selective compounds has yet to be fully exploited. A more complete understanding of the potential utility (and pitfalls) of modulating individual PPAR actions should follow.

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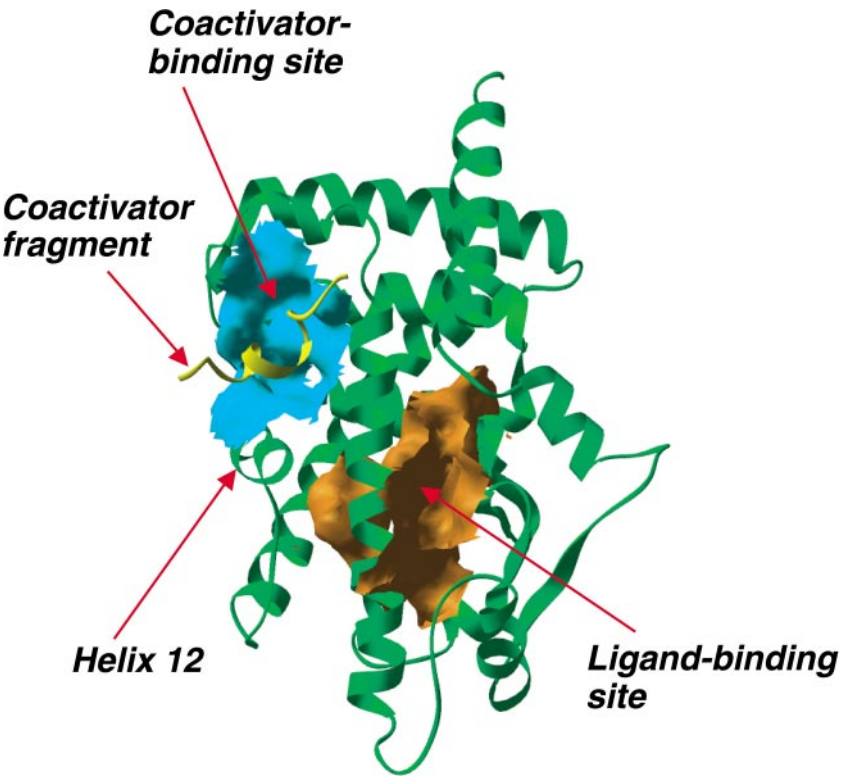

Figure 1 X-ray crystal structure of PPARγ ligand binding domain. Several key α-helices are shown along with the relative location of key functional regions.

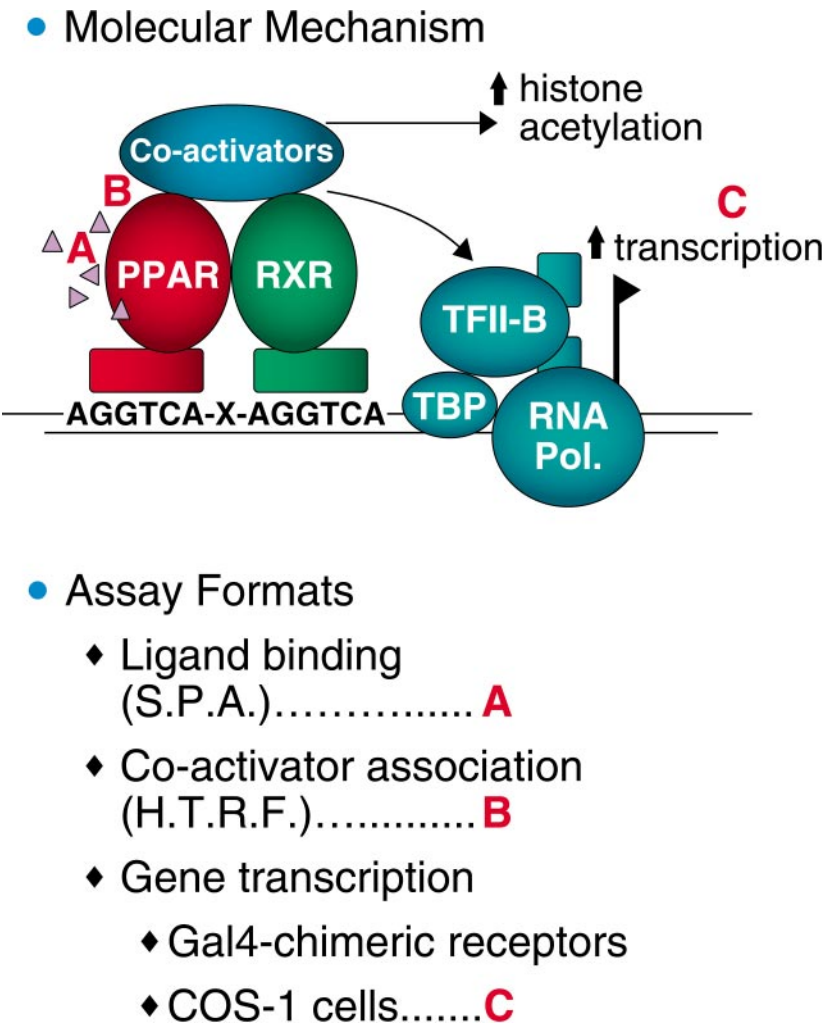

Figure 2 Mechanism of transcriptional activation by PPAR isoforms. Selected molecular components are shown relative to assay formats that can be used to characterize compound activities.

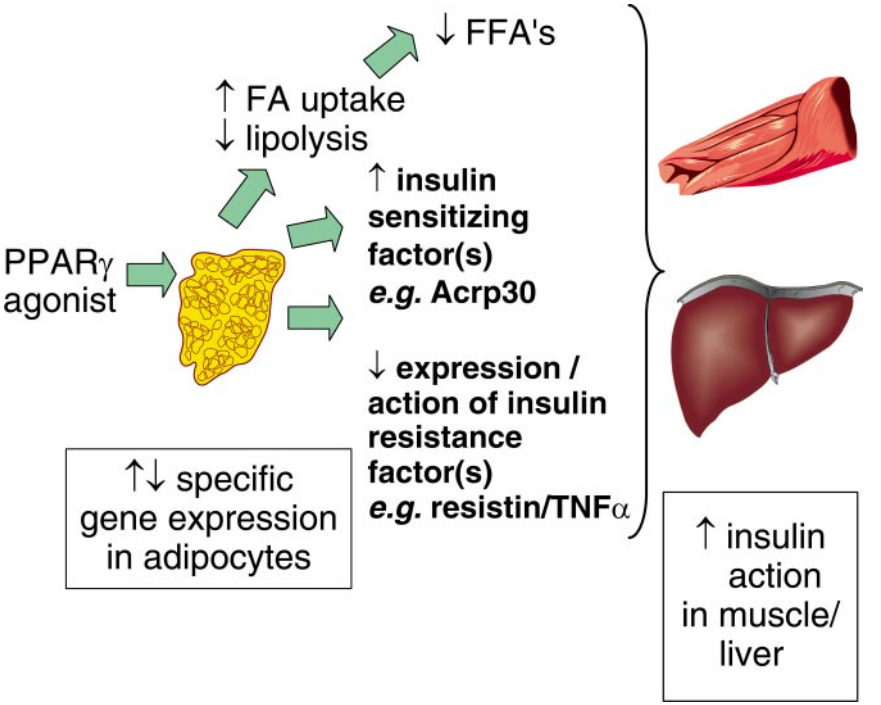

Figure 3 Working hypothesis for PPARγ-mediated increases in insulin sensitivity.