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PPAR γ and its ligands: therapeutic implications in cardiovascular disease

Luis Villacorta¹, Francisco J. Schopfer², Jifeng Zhang¹, Bruce A. Freeman², and Y. Eugene Chen^{1,†}

¹Cardiovascular Center, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI 48109, USA.

²Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA.

Abstract

The relevance of peroxisome proliferator-activated receptor- γ (PPAR γ) as an important therapeutic target for the treatment of diabetes arises from its hypoglycemic effects in diabetic patients and also from the critical role in the regulation of cardiovascular functions. From a clinical perspective, differences between currently FDA-approved PPAR γ drugs have been observed in terms of atherosclerosis, cardiac and stroke events. The adverse effects of PPAR γ -specific treatments that hamper their cardiovascular protective roles, affirm the strong need to evaluate the efficacy of the current drugs. Therefore, active research is directed towards high-throughput screening and pharmacologic testing of a plethora of newly identified natural or synthetic compounds. Here we describe the rationale behind drug design strategies targeting PPAR γ , based on current knowledge regarding the effects of such drugs in experimental animal models as well as in the clinical practice. Regarding endogenous PPAR γ ligands, several fatty acid derivatives bind PPAR γ with different affinity, though the physiological relevance of these interactions is not always evident. Recently, nitric oxide-derived unsaturated fatty acids were found to be potent agonists of PPARs, with preferential affinity for PPAR γ , compared to oxidized fatty acid derivatives. Nitroalkenes exert important bioactivities of relevance for the cardiovascular system including anti-inflammatory and anti-platelet actions and are important mediators of vascular tone. A new generation of insulin sensitizers with PPAR γ function for the treatment of diabetes, may serve to limit patients from the increased cardiovascular burden of this disease.

Keywords

peroxisome proliferator-activated receptor- γ ; diabetes; cardiovascular disease; drug design; nitroalkenes; fatty acids

INTRODUCTION

Obesity is a major risk factor for a number of chronic diseases, including diabetes, cardiovascular disease (CVD) and cancer. Once considered a problem only in high income countries, obesity is now booming in low- and middle-income countries, particularly in urban settings [1]. Diabetes is a chronic, progressively worsening disease caused primarily by loss of insulin sensitivity to glucose in the peripheral tissues. Obesity and/or dyslipidemia are two key factors in the development of insulin resistance (IR). According to the World Health Organization, diabetes is responsible for 5% of all deaths globally each year. Following current

[†]To whom correspondence should be addressed: echenum@umich.edu

projections, deaths due to diabetes are likely to increase by more than 50% in the next 10 years (<http://www.who.int/diabetes/en/>). Cardiovascular complications are the major causes of mortality in the diabetic population and they account for almost 75% of all deaths only in the USA [2]. Thus, a major goal in terms of therapeutic interventions for diabetic patients has been the maintenance of glycemic levels within the non-diabetic range.

Several drugs are available for the management of type 2 diabetes used either in monotherapy or in combination [3]. These include insulin, insulin secretagogues such as sulfonylureas and metiglidides, α -glucosidase inhibitors, biguanides such as metformin, DPP-4 inhibitors such as sitagliptin and vildagliptin, incretins such as exenatide, and thiazolidinediones (TZDs) such as rosiglitazone and pioglitazone. So far, all oral antidiabetic drugs lower glucose and glycosylated hemoglobin (HbA1c) levels, although the effects of these agents differ in terms of cardiovascular outcome [4]. In fact, the question whether glucose lowering reduces the risk of CVD is conflicting. The recent multicenter clinical trial (ACCORD) has challenged this notion since intensive therapy aimed to lower HbA1c below 6% resulted in an increased mortality within 3.5 years of follow-up compared to a standard therapy with HbA1c between 7% and 7.9% [5]. A consensus statement from both the American Diabetes Association and the European Association for the Study of Diabetes has established an algorithm for the management of diabetic patients in terms of choice of either sulfonylureas, insulin, or TZDs on top of metformin treatment and life-style intervention for appropriate management of glucose and HbA1c levels [6]. Of those, TZDs are ligands of the peroxisome proliferator-activated receptor- γ (PPAR γ), which appear to have a great prospect for serving as a high impact therapeutic target for CVD, since the beneficial effects of PPAR γ agonists go beyond controlling hyperglycemia [7]. On the other hand, TZDs display adverse side effects including weight gain, fluid retention, and hepatotoxicity [8], which might be responsible for the associated heart risks of rosiglitazone and pioglitazone [9,10]. Nevertheless, it has been generally accepted that the activation of PPAR γ leads to inhibition of manifestations of vascular disease in diabetic patients.

PPAR γ IN THE CARDIOVASCULAR SYSTEM

During the past decade, extensive studies have demonstrated that PPAR γ plays an important role in the cardiovascular system [11-13]. It is viewed that despite a strong correlation with its insulin sensitizing action, the vascular protection observed with PPAR γ and its ligands is independent from improvements in metabolic control [14]. All of the major cells in the vasculature express PPAR γ . Thus, it is plausible that PPAR γ is a key mediator of signaling events that can influence the pathogenesis of CVD. It is well-established that PPAR γ expression is up-regulated in intimal vascular smooth muscle cells (VSMC) and macrophages in early human atheromas [15-18]. In an animal model of vascular injury, PPAR γ levels are substantially elevated in the neointima that develops after mechanical injury to the endothelium [15]. Cell culture studies have shown that TZD PPAR γ ligands inhibit both the proliferation and migration of VSMC, and induce VSMC apoptosis [17,19]. These anti-atherogenic activities of PPAR γ also occur *in vivo*, as TZDs inhibit lesion formation in several animal models [20-23]. In patients with type 2 diabetes, pioglitazone treatment also resulted in regression of carotid intimal media thickening, which is a strong predictor for myocardial infarction and stroke [14]. A number of PPAR γ target genes have been documented to be linked with the PPAR γ -mediated biological actions in the cardiovascular system (for details see [13]). For example, PPAR γ activation in vascular cells inhibits the production of cytokines such as tumor necrosis factor- α (TNF- α), and monocyte chemoattractant protein-1 (MCP-1) [23-25], reduces the production of matrix metalloproteinases [17] and down-regulates the expression of several growth factor receptors including angiotensin II receptor-1a (AT1R) (Figure 1) [26]. Both pioglitazone and rosiglitazone improve endothelial function and restore impaired acetylcholine-induced relaxation in angiotensin II-infused rats. TZDs reduce vascular

DNA synthesis, vascular cell adhesion molecule-1, and platelet and endothelial cell adhesion expression in mesenteric arteries [27]. Further evidence is provided by the increased re-endothelization capacity of endothelial progenitor cells (EPCs) upon rosiglitazone treatment. Rosiglitazone therapy in patients with type 2 diabetes normalizes NAD(P)H oxidase activity, a downstream effector of angiotensin II in the vasculature, restores nitric oxide (NO) bioavailability, and improves *in vivo* re-endothelization capacity of EPCs [28].

TZDs have been associated with a mild reduction in blood pressure [29] before TZDs were even identified as PPAR γ ligands [30]. A role of PPAR γ in hypertension is indicated by a provocative report by Barroso et al. [31]. This group has identified subjects in which family members who are heterozygous for a dominant-negative PPAR γ allele exhibit severe insulin resistance, diabetes and early-onset hypertension [31]. Transgenic mice expressing one of those mutant alleles identified in humans have a hypertensive phenotype [32]. These “loss-of-function” experiments suggest that PPAR γ may normally function as an endogenous inhibitor of insulinresistance and hypertension susceptibility. Some studies have confirmed that PPAR γ activation tends to promote a decrease in blood pressure in hypertensive animals [33-35]. Also, patients with diabetes present an up-regulation of the renin angiotensin system (RAS), most likely by the direct action of increased circulating insulin levels [36,37]. Thus, reduction of the RAS by PPAR γ treatment is a consequence of improved insulin sensitivity, although it was also shown that PPAR γ down-regulates angiotensin II type 1 receptor (AT1R) in VSMC at the transcriptional level [26]. As for cardiovascular function, it has been suggested that addition of AT1R blockers to anti-diabetic medications may improve the remodeling of resistance arteries in diabetic hypertensive patients [38]. These findings support the notion of a role for PPAR γ in mediating a link between determinants of blood pressure regulation/vascular remodeling and the regulation of energy metabolism/insulin resistance (Figure 1).

TZDs reduce a prothrombotic state in type 2 diabetic patients. A comparative analysis of the effect of rosiglitazone *versus* sulfonylurea indicates that a reduction in the circulating levels of the prothrombotic factor, plasminogen activator inhibitor (PAI-1) is a consequence of elevated adiponectin expression in response to rosiglitazone treatment [39]. Recent evidence indicates that TZDs significantly reduce atherosclerotic lesion surface area induced by visceral adipose-related inflammation [40]. Visceral *versus* subcutaneous fat transplants produced increased leptin, resistin, and MCP-1 and develop more atherosclerotic lesions in apoE knockout mice. Plasma adiponectin levels were higher upon treatment with pioglitazone. Reduction of inflammation was evidenced by the reduced levels of MCP-1 in the visceral adipose tissue and increased expression of the anti-inflammatory cytokine, interleukin-10, without an apparent change in macrophage infiltration [40].

TZDs also induce the tissue factor pathway inhibitor (TFPI), which is remarkably altered in unstable carotid atheromas [41]. Consequently, the vascular effects of PPAR γ ligands on these thrombotic pathways indicates that TZDs play an important role in neuroprotection and in particular against focal ischemic brain injury [42,43].

PPAR γ TISSUE SPECIFIC ANIMAL MODELS

The molecular mechanisms involved in PPAR γ -mediated insulin sensitivity remain elusive. In terms of expression of the PPAR γ isotypes, it is normally recognized that the γ_2 isoform, which is preferentially expressed in adipose tissue, is most likely responsible for PPAR γ -mediated insulin sensitivity. Selective disruption of PPAR γ_2 in mice presents a phenotype of an overall reduction in white adipose tissue, less lipid accumulation, and decreased expression of adipogenic genes in adipose tissue. In addition, insulin sensitivity is impaired in male PPAR γ_2 knockout mice, with a decreased expression of insulin receptor substrate 1 and glucose transporter 4 in skeletal muscle [44].

The important effects of PPAR γ , not only as an insulin sensitizer but also on the cardiovascular system, have been elegantly demonstrated in studies of mice with tissue-specific targeted deletion of the PPAR γ gene. In this way, genetic manipulation using the *Cre/Lox(P)* system has provided insight into the functional and tissue specific roles of PPAR γ on metabolism. PPAR γ specific disruption in pancreatic β -cells indicates a critical role of PPAR γ on beta-cell mass and proliferation [45]. Also, PPAR γ directly regulates insulin secretion from pancreatic β -cells since glucose-mediated insulin secretion is abrogated in PPAR γ targeted deletion upon TZD treatment. On the other hand, administration of rosiglitazone to insulinresistant mice due to a high-fat diet, indicates that PPAR γ targeted disruption in β -cells is not required for the anti-diabetic actions [45], although the adaptive response of β -cells to insulin resistance is impaired by further metabolic challenge as demonstrated by disruption of PPAR γ 2 in ob/ob mice [46].

Targeted deletion of PPAR γ in the adipose tissue has been achieved by studying PPAR γ -*loxP* mice crossed with mice carrying adipose specific *Cre* under the control of the aP2 gene promoter [47]. Mice deficient for PPAR γ in fat developed white adipose tissue (WAT) lipodystrophy, subcutaneous adipocyte loss and fibrosis, and an increased rate of macrophage infiltration. Plasma free fatty acid (FFA) and triglyceride (TG) levels are remarkably elevated in these mice. Evidence of IR was demonstrated with hyperinsulinemic-euglycemic clamp experiments which did not restore normal FFA levels, indicating IR in the adipose tissue. However, no differences were observed in the systemic insulin sensitivity due to normal insulin-stimulated glucose uptake in skeletal muscle. Notwithstanding, PPAR γ -deficient mice in the adipose tissue developed a fatty liver, reduced glucose production in response to insulin and increased gluconeogenesis, phenotypes that were otherwise reversed by TZD treatment, indicating a direct effect of TZDs in the liver.

A similar genetic approach was used to study the effect of a PPAR γ targeted deletion in skeletal muscle by two different groups [48,49]. Muscle-specific PPAR γ knockout mice showed increased whole body IR with conflicting results in insulin sensitivity in skeletal muscle. TZD treatment was ineffective towards insulin sensitivity in the muscle, although the response was observed in terms of a decrease in the circulating glucose, insulin, TG and FFA levels, again indicating that TZD treatment was effective in other tissues. Overall, the data from these two groups showed contrasting results that could be explained by strain differences or the age of animals used in each experimental design [50].

Hyperglycemia and reduced insulin sensitivity is also observed in liver-specific PPAR γ deficient mice. However, high glucose levels are improved by TZDs, suggesting that reduction of hyperglycemia by TZDs is not primarily the result of PPAR γ in the liver [51]. In contrast, the importance of PPAR γ in the liver is revealed in a mouse model of lipodystrophy. Inactivation of hepatic PPAR γ in AZIP mice abolished the hypoglycemic and hypolipidemic effects of rosiglitazone, demonstrating that, in the absence of adipose tissue, the liver is a major site of TZD actions [52].

In a similar fashion, several studies have been conducted to assess the effect of PPAR γ in the cardiovascular system. For instance, transgenic mice with cardiac overexpression of PPAR γ develop dilated cardiomyopathy, due to increased expression of fatty acid oxidation genes and exacerbated TG uptake [53]. On the other hand, using the α -myosin heavy chain promoter to drive *Cre* expression, Duan et al. showed the development of cardiac hypertrophy in cardiac-specific PPAR γ knockout mice [54]. Interestingly, rosiglitazone treatment also displayed a cardiac hypertrophy phenotype that seemed partially independent of PPAR γ , though there were some differences noted [54]. Whereas PPAR γ deficiency in cardiomyocytes showed a cardiac hypertrophy-associated gene expression profile (increased ANP and β -MHC), and NF- κ B activation, no such effect was observed upon rosiglitazone treatment, despite the presence of

a hypertrophic phenotype due to TZD treatment. Additionally, cardiac-specific PPAR γ knockout mice die due to dilated cardiomyopathy with remarkable mitochondrial abnormalities, an effect that can be partially explained by reduced superoxide dismutase (SOD) expression, despite a lack of changes in apparent superoxide production in these animals [55]. Using ventricular myosin light chain-2 (MLC2v) *cre* mice to target PPAR γ in cardiomyocytes, Caglayan et al. also describe cardiac hypertrophy both in cardiac-specific PPAR γ knockout mice and upon treatment with pioglitazone, though only in wild-type mice [55]. Thus, the increased ventricular heart-to-body ratio after pioglitazone treatment is a cardiac PPAR γ -dependent effect. Pioglitazone attenuates angiotensin II-induced cardiac fibrosis and proinflammatory gene expression in a macrophage PPAR γ dependent manner instead of a cardiomyocyte-dependent [55]. It is important to note that cardiac hypertrophy after PPAR γ agonist treatment occurs in the absence of myocardial insulin signaling and it might be also secondary to plasma volume expansion [56]. This constitutes one of the most relevant side-effects of the current PPAR γ agonists currently in the clinical marketplace (see below).

In macrophages, PPAR γ inactivation leads to glucose intolerance and impaired systemic insulin function as determined in the adipose tissue, liver and muscle as well as in cultured cardiomyocytes [57]. Thus, the anti-inflammatory component of PPAR γ seems indispensable for glucose tolerance and insulin sensitivity. PPAR γ conditional knockout mice in macrophages develop larger atherosclerotic lesions under conditions of mild or severe hypercholesterolemia [58]. In contrast, specific disruption of PPAR γ in endothelial cells displayed a phenotype related to blood pressure regulation [59]. Hypertension in these mice was achieved either the addition of high fat in the diet or with salt-loaded water. Disruption of PPAR γ in the endothelium, by means of a Tie2 promoter-mediated *Cre* recombination, increased systolic blood pressure in mice treated with a high-fat diet, but not in the salt-loaded mice. It is of importance to note that, whereas TZD treatment in these mice resulted in reduced insulin levels in non-fasting conditions, rosiglitazone was not able to alter systolic blood pressure in endothelial-specific knockout mice. This study clearly indicates that the vascular effect of PPAR γ might be independent of the role of PPAR γ and TZD as a systemic insulin sensitizer, thus strengthening the concept of a PPAR γ -dependent vascular protection beyond its role in glycemic control.

As a counterpart for endothelial cells, PPAR γ inactivation in smooth muscle cells has been recently described. These mice develop right ventricular hypertrophy and an elevated right ventricular systolic pressure, both symptomatic of a generalized pulmonary hypertension [60]. Transgenic mice expressing dominant-negative mutations of PPAR γ (V290M and P467L) under the control of smooth muscle myosin heavy chain have also been generated. These mutations elicit early-onset hypertension, IR and type 2 diabetes [31]. Overexpression of either mutation caused impaired aortic vasorelaxation in mice [61]. Strikingly, when analyzing heterozygous knock-in mice expressing the P465L dominant negative mutation in cerebral blood vessels, an increased contractile response to serotonin and endothelin-1, but not acetylcholine, was observed. Vasodilation was impaired as a consequence of elevated superoxide production and associated impaired nitric oxide-dependent endothelial function. Thus, these vessels became hypertrophic as determined by the measurement of wall thickness or by pressure-internal diameter ratios during maximal dilation [62].

ADVERSE SIDE EFFECTS OF CURRENT PPAR γ DRUGS

The most common side-effect related to the administration of PPAR γ drugs for patients with type 2 diabetes is that TZDs sometimes cause peripheral edema, a condition that may be responsible for the exacerbated congestive heart failure observed in clinical studies. This specific side effect is of particular importance because people with diabetes are at increased risk for CVD and many have preexisting heart disease [63]. Thus, the cardiovascular benefits

observed in animal studies as well as in clinical trials are often masked by this and other side-effects of the current TZD (Figure 2). The mechanisms of TZD-mediated fluid retention or edema are the subject of intense current investigation. Targeted deletion of PPAR γ in the collecting duct of the kidney indicates that functional alteration of the endothelial sodium channel ENaC in the nephron is a plausible mechanism explaining elevated fluid retention [64]. In an identical experimental approach, it was additionally confirmed that increased body weight was dependent on peripheral edema, since mice with a targeted deletion of PPAR γ in the collecting duct did not gain weight in response to rosiglitazone treatment, wherein the resulting increased blood volume and decreased plasma aldosterone and hematocrit were completely reversed [65]. Indeed, PPAR γ activation enhances cell ENaC expression *via* upregulation of the serum- and glucocorticoid-inducible protein kinase 1 (SGK1) in human collecting duct cells, which accounts for the sodium reabsorption observed upon TZD treatment [66]. Of interest, simultaneous treatment with amiloride, a selective inhibitor of collecting duct ENaC completely abrogates the fluid accumulation occurring upon TZD treatment [64]. This observation raises the question as to whether the peripheral edema that develops with TZD can be overcome by diuretic administration. In a recent study by the Rosiglitazone Fluid Retention Study Group [67], the efficacy of loop, thiazide or spironolactone diuretics was determined for the management of rosiglitazone-mediated fluid retention. In this study, spironolactone showed the greatest reversal in fluid retention with a significant increase in hematocrit levels and a reduction of extracellular fluid volume. These results also affirm that the weight gain associated with TZD treatment is partially due to fluid retention.

One contributing factor that could account for the elevated vascular permeability in response to TZD administration is the enhanced circulating levels of vascular endothelial growth factor (VEGF) in diabetic patients [68]. Using different rat models of IR and diabetes, the consequences of edema were determined upon rosiglitazone treatment. Increases in water content and epididimal fat weight correlated with vascular permeability in the adipose tissue and the retina [69]. Rosiglitazone increased expression of VEGF, though the upregulation does not appear to be mediated by PKC β . Specific inhibition of PKC β normalized vascular permeability and decreased weight gain, despite persistent VEGF elevation. In female ovariectomized rats, where expression of ENaC is unaltered, pioglitazone induces sodium retention through inhibition of the compensatory effects of renal CYP4A upregulation, which exerts natriuretic action through the production of 20-HETE [70]. As a consequence of peripheral edema and weight gain, vascular permeability was observed upon rosiglitazone treatment in those animals.

Hepatic steatosis was the first adverse symptomatic feature of TZDs that was detected, and as a consequence troglitazone, the first approved TZD drug, was removed from the market. Whereas currently-available TZDs display a considerably reduced incidence of hepatic steatosis, there are still reasonable concerns regarding TZD-induced liver toxicity. In the mouse model of lipodystrophy (AZIP mice) mentioned before, inactivation of liver PPAR γ significantly altered the AZIP phenotype [52]. The increased body and liver weights of the AZIP mice were reduced by liver PPAR γ ablation. This was associated with a decrease in liver triglyceride levels and secretion rates, along with a reduction of the mRNA levels of several genes involved in fatty acid synthesis. Pharmacogenomic analysis of outcrossed mice strains derived from both non-obesity and obesity-associated diabetes has identified certain strains with particular susceptibility to hepatic steatosis in response to TZD treatment [71]. Metabolic analysis of these murine tissues and plasma suggested that TZD treatment lead to a hypolipidemic shift in plasma *versus* the liver, with specific decreases in circulating TG, phosphatidyl choline (PC), and cholesterol esters [72]. Similarly, as for the AZIP mice, in this experimental model rosiglitazone treatment resulted in an elevated lipid uptake and fatty acid biosynthesis, which additionally suggests a deficient TG export from the liver, thus exacerbating hepatic steatosis. The mechanism underlying the elevated hepatic steatosis came

from the observation of increased lipid uptake from the plasma into the liver and the increased expression of adipsin, fatty acid binding protein 4 (aP2) and lipoprotein lipase in the liver.

TZDs administration is associated with increased risk of bone fracture in women, and, in the case of rosiglitazone, more rapid bone loss [73]. Indeed, an important role for PPAR γ in bone homeostasis was initially determined in animal models. The congenic PPAR $\gamma^{\text{hyp/hyp}}$ mice, which lack PPAR γ in the WAT, and thus develop severe lipodystrophy, have increased bone mineral density [74] and haploinsufficient PPAR γ mice have increased osteogenesis [75]. Thus, the inverse correlation between adipogenesis and osteogenesis in bone marrow cells is strongly dependent on PPAR γ function and could account for the negative skeletal effects of TZDs. Moreover, it was recently shown an additive component on the skeletal function of TZDs, not only as a result of the above mentioned inhibition of osteoblast differentiation but also through an active stimulation of bone resorption [76].

Recent meta-analyses of randomized clinical trials have addressed the risk of cardiovascular events with TZD treatment. Rosiglitazone therapy is associated with an increased risk of myocardial infarction and mortality due to CVD [8]. Pioglitazone, in contrast, significantly reduces the myocardial infarction and mortality risks, though it might be also associated with the enhanced progression of heart failure and allied events in type 2 diabetic patients [8]. The mechanisms responsible for such a difference in clinical outcomes, although still elusive, may be related to a lower affinity of pioglitazone *versus* rosiglitazone for PPAR γ binding [8], differences in as of yet undefined “off-target” effects of each drug [8] or benefits stemming from the partial affinity of pioglitazone for other PPAR receptors [8] (see below). To date, what has become clear is that pioglitazone may result in a better net clinical outcome, compared to rosiglitazone, for the management of dyslipidemia in type 2 diabetic patients [8].

RATIONAL DRUG DESIGN FOR PPAR γ EFFECTORS

Several lines of research are being pursued to improve the actions of synthetic PPAR γ agonists over those already in the clinical marketplace. First, it is useful to distinguish the different drug design rationales being employed (Figure 3).

1. *Drugs targeting different PPAR isotypes*, such as dual agonists for PPAR γ and α , or the non specific PPAR agonists (pan-agonists). The rationale for the design of such drugs is to broaden the cardiovascular benefits observed for each individual PPAR isozyme during the treatment of type 2 diabetes. Considerable attention has been directed towards the dual- or pan-agonists since some existing PPAR γ agonists may also have some benefits that are additionally derived through activation of PPAR α . Fibrates, as PPAR α ligands, reduce high fat diet-induced increases of body weight and improve glucose utilization in experimental models of insulin resistance [77]. The cardiovascular benefits derived from activation of PPAR α arise from its role as modulator of fatty acid catabolism and the lipid-lowering activity of the fibrates [78]. A promising class of dual agonists, the phenylpropanoic acid-based agonists (the glitazar group), has already been evaluated in the clinic. Contrary to what was expected, this strategy so far has been disappointing in several glitazar-based drugs, since other adverse effects were added to the above mentioned side-effects of PPAR γ agonists, including toxicology issues and additional cardiovascular events that resulted in increased mortality [79]. Safety issues with bezafibrate, which is considered more of a pan-PPAR agonist activator, are not observed clinically. Although the effects of bezafibrate on individuals with diabetes have not been described, it reduces triglycerides, enhances insulin sensitivity and reduces blood glucose levels, delaying the onset of diabetes in patients with coronary artery disease [80]. For an excellent and comprehensive review on the dual- and pan-agonists for the management of type 2 diabetes see [81]. Recently, it was also demonstrated that

retinoic acid, an RAR nuclear receptor agonist, also has PPAR β/δ receptor-activating properties. This selectivity that depends on differential transport through CRABP II or fatty acid binding protein 5 [82]. Similarly, inhibition of RAR by Ro 41-5253 enhanced differentiation of mouse and human preadipocytes and activated PPAR γ target genes in mature adipocytes. [83].

2. *Drugs that serve as partial agonists of PPAR γ .* The rationale for this approach is to identify novel compounds with sufficient affinity for PPAR γ and to preserve the insulin sensitizing effects, but are unable to saturate PPAR γ activity, in order to diminish the side effects associated with the TZDs. They have been collectively named selective PPAR γ modulators (SPPARM) including metaglidase, halofenate [84], FMOC-leucine [85], and non-TZD partial agonist (nTZDpa) [86], among others. Some of these drugs are at different stages of preclinical and clinical study, while others have been discontinued (for a review, see [87]). A common chemical feature of some of these SPPARM is the presence of an indole moiety characteristic of non-steroidal anti-inflammatory drugs (NSAID) [88]. For instance, the non-TZD partial agonist (nTZDpa), which contains an indole moiety, improved insulin sensitivity in obese mice with diminished adverse side-effects on weight gain, adiposity, and cardiac hypertrophy [86]. Similar NSAID derivatives have been tested as partial PPAR γ agonists *in vitro*, their pharmacokinetics studied in rats and then tested in insulin resistant mouse models. These are the benzoyl 2-methyl or carboxylic acid indoles [89,90]. Similarly, SPPARM5 also acts as a partial agonist of PPAR γ , with a reduced capability to induce adipose gene expression while at the same time preserving the insulin sensitizing properties. SPPARM5 was additionally tested in Zucker rats, in comparison with rosiglitazone, for an impact on plasma and extracellular volume, heart weight, and fluid retention [91]. Another SPPARM, FK614 has been shown to have significant antidiabetic activity in mice with diminished hemodilution and cardiac hypertrophy in rats [92]. A plethora of novel compounds are being tested and their pharmacological activity determined, analyzing the structure-activity relationship (SAR) as a drug design method. In this way, certain compounds derived from the benzalolonic heterocycles (JTT-501) [93] have already been tested in animal models, with the common presentation of acting as partial PPAR γ agonists and the maintenance of the hypoglycemic affects [94,95]. Other novel TZD compounds, hybrids of α -lipoic acid derivatives, are being experimentally tested in the IR Zucker fatty (fa/fa) rat [96]. Despite the hypolipidemic effects and a significant reduction in serum insulin levels, no glucose lowering effect was observed for some of these compounds. Other SPPARM, such as PA-082, have been to date only compared to PPAR γ agonists *in vitro* for adipogenesis differentiation in C3H10T1/2 mesenchymal stem cells. This compound displays a reduced capacity to induce lipid accumulation during *in vitro* adipogenesis yet promotes glucose uptake [97].
3. *Antagonists of PPAR γ .* The rationale for the development of PPAR γ antagonists comes from the still unexplained paradoxical observations that reduction of PPAR γ activity also improves insulin sensitivity [98]. Thus, different compounds with PPAR γ antagonistic effects are being tested. Some of those compounds have been analyzed in the well-established model of 3T3-L1 adipocyte differentiation. For instance, the bisphenol A diglycidyl ether (BADGE), lacks of clinical utility; while LG 100641 or PD 068235 blocks adipocyte differentiation and stimulates glucose uptake. To date, few antagonists so far have been tested *in vivo*. SR-202 protects against high-fat diet induced insulin resistance and improves insulin sensitivity in ob/ob mice [99]. Similarly, using diabetic KKAY mice, the inhibitor T2384 was tested to verify the prevention of obesity and reduction of insulin resistance [100]. T2384 improved the glucose disposal rate in diabetic KKAY mice and opposed the

rosiglitazone-induced effects on weight gain, plasma volume expansion or anemia in this experimental context.

4. Drugs serving simultaneously as PPAR γ agonists and AT1R blockers (ARBs). The rationale for this drug class is similar to above with respect to targeting different PPAR isozymes. The additional cardiovascular benefit that could account for the effectiveness of certain “sartans”, such as telmisartan and irbesartan is derived from their ability to simultaneously block AT1R-dependent vascular dysfunction [101] and allied pro-inflammatory, pro-atherogenic pathways [102], together with their action as partial agonists of PPAR γ . Both telmisartan and irbesartan display a more pronounced induction of aP2 expression in lower, pharmacologically relevant concentrations compared with, for instance to losartan [103]. As for telmisartan, its role as an active SPPARM was demonstrated in diet-induced obese mice by improving insulin sensitivity in the absence of weight gain [104]. Thus, telmisartan may display an overall improved cardiovascular protection role by dually targeting AT1R and acting as a SPPARM. In this latter function, it was shown that telmisartan efficiently reduces C-reactive protein production mediated by advance glycation end products (AGE) [105] and it increases nitric oxide (NO) formation in endothelial cells by regulating the asymmetrical dimethylarginine (ADMA)-dimethylarginine dimethylaminohydrolase (DDAH)-system during aging [106]. Irbesartan also mediates adiponectin upregulation, an effect that was found to be independent of AT1R blockade [107].

PPAR γ AND ITS ENDOGENOUS LIGANDS

It is viewed that a more rational drug design, based on endogenous PPAR γ ligands, will lead to development of better PPAR γ drugs, yet little is known about the physiological ligands of PPAR γ . Presently-reported endogenous PPAR γ agonists include free fatty acids, components of oxidized plasma lipoproteins (9- and 13-oxoODE, azelaoyl phosphatidylcholine (azPC)) [108], conjugated linoleic acid derivatives (CLA1 and CLA-2), products of phospholipase hydrolysis of complex lipids (LPA), platelet activating factor (PAF) and eicosanoid derivatives such as the dehydration product of PGD₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) [108-110]. While a number of such endogenous lipophilic species are generally considered to be PPAR γ ligands, their intrinsically low binding affinities and low *in vivo* concentrations do not support a capability to serve as physiologically-relevant signaling mediators. A dilemma exists in that some putative endogenous PPAR γ agonists have only been generated by aggressive *in vitro* oxidizing conditions, for instance, through CuSO₂-mediated LDL oxidation [108]. Other lipid derivatives proposed as PPAR γ ligands are present in <100 nM tissue concentrations, orders of magnitude below their binding affinities (1-15 μ M) and are not expected to result in significant receptor occupancy and activation *in vivo*. This latter category includes free fatty acids, eicosanoids, 9- and 13-oxoODE, PAF-phospholipids and 15d-PGJ₂. Indeed, a recent study documented that 15d-PGJ₂ is not the endogenous ligand for PPAR γ activation in adipogenesis [111]. It is also viewed that production of such endogenous ligands is regulated by intracellular signaling and the subsequent generation of lipophilic byproducts with signaling properties which ultimately bind and activate PPAR γ . For example, assuming 15d-PGJ₂ as the paradigm of an endogenous PPAR γ ligand, intracellular production of 15d-PGJ₂ has been shown to be cyclooxygenase-2 (Cox-2) dependent [112]. In this way, one of the pleiotropic effects of statins recently described is the Cox-2-dependent production of 15d-PGJ₂, thereby activating PPAR γ . In an elegant experimental approach, Tzameli et al. [111] created a line of 3T3-L1 preadipocytes that carry a β -galactosidase-based PPAR γ ligand-sensing vector system. In this model, induction of adipogenesis resulted in elevated β -galactosidase activity implying endogenous mediators that activated PPAR γ *via* its ligand-binding domain. Indeed, factors that may serve as endogenous PPAR γ ligands were readily generated at the early stages of adipocyte differentiation and were produced in response to

increases in cAMP [111]. On the contrary, and using a sensitive ELISA for 15d-PGJ₂, the ability of adipocytes to synthesize increased levels of endogenous 15d-PGJ₂ occurred only during the later maturation phase [113]. Previous studies could not determine changes in 15d-PGJ₂ production during adipocyte differentiation by liquid chromatography-mass spectrometry, thus not supporting 15d-PGJ₂ as an endogenous PPAR γ ligand [109]. In macrophages, 13-HODE and 15-HETE can be generated from linoleic and arachidonic acids, respectively, by a 12/15-lipoxygenase that is up-regulated by interleukin-4 [114]. In the vasculature, laminar flow-mediated activation of phospholipase A₂ and cytochrome P450 epoxygenases, generates epoxyeicosatrienoic acids (EETs) [115], which exert anti-inflammatory and athero-protective effects by serving as putative endogenous ligands of PPAR γ [116]. In the endothelium, fluid shear stress also increases the production of 15d-PGJ₂, by increasing the expression of the lipocalin-type PGD(2) synthase and the subsequent release of its metabolic precursor PGD(2) [117].

Based on the *in vitro* 3T3-L1 adipogenesis model, Christianson et al. [118] performed Affimetrix GeneChip analysis to identify differential expression in the genes related to fatty acid metabolism. In addition, they contrasted their *in vitro* data with primary adipocytes from mice on normal chow *versus* a high fat diet. The analysis included clustered genes involved in α -, β - and ω -oxidation, desaturation and elongation, among others [118]. In this approach, they also conducted small-interfering RNA-screens to identify critical fatty acid metabolizing enzymes responsible for adipocyte differentiation. The stearoyl-CoA desaturase-2 (SCD2) was critical for this differentiation process. Whether any putative product derived from SCD2 enzymatic activity may serve as a ligand to activate PPAR γ in transactivation assays is not known, although the study showed that SCD2 plays an important role in PPAR γ protein synthesis.

In the past six years, several groups have performed extensive screening assays in an attempt to identify either endogenous or natural products that activate PPAR γ . Various candidates have already been proposed. In an effort to find bioactive molecules based on *in vitro* adipocyte differentiation assays, a high-throughput screening analysis of natural compounds was performed. In this way a β -carboline alkaloid, harmine, was identified and its role in adipocyte differentiation determined [119]. Harmine increased expression of PPAR γ through a mechanism involving the Wnt signaling pathway, but it did not directly activate PPAR γ in transactivation analyses. In diabetic mice, harmine reduced adiposity and improve insulin sensitivity.

Radioligand competition assays showed that unsaturated-FA displayed a preferential binding to PPAR γ *in vitro*. The poly-unsaturated FAs γ -linolenic (18:3), eicosatrienoic acid (C20:3), dihomo- γ -linolenic (20:3), arachidonic acid (C20:4), and eicosapentaenoic acid (C20:5) interacted most efficiently with PPAR γ compared with their saturated counterparts [120]. A comparative analysis of equimolar concentrations (1-3 μ M) of several putative proposed PPAR γ agonists was performed [121]. These included 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPA 16:0), 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPA 18:1), 1-O-hexadecyl-2-azelaoyl-sn-glycero-3-phosphocholine (AzPC), 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (AzPC ester), $\Delta^{9,11}$ -conjugated LA (CLA1), and $\Delta^{10,12}$ -conjugated LA (CLA2). During this screening approach, nitric oxide (NO)-derived unsaturated FA products, nitroalkenes, were identified as highly potent endogenous PPAR γ ligands [121].

It is becoming increasingly evident that NO-derived reactive species (RNS) mediate diverse cell signaling and pathogenic processes in chronic inflammation and CVD. [122]. RNS react with carbohydrates, DNA bases, protein tyrosine, tryptophan, methionine and cysteine residues [123,124] and of relevance to PPAR signaling, react via multiple mechanisms with unsaturated fatty acids to form relatively stable nitrated products [125]. On one hand, there is a strong

association between elevated levels of nitrated plasma proteins and the incidence of coronary artery disease [126]. On the other, nitro-fatty acids are produced as a consequence of oxidative inflammatory conditions [127]. The identification and quantification of nitro-linoleic acid (LNO₂) in human blood showed free and esterified forms of LNO₂ at a total of ~0.5 μM [128]. Compared with other NO-derived species such as nitrite (NO₂⁻), nitrosothiols (RSNO) and heme-nitrosyl complexes, LNO₂ represents an abundant pool of bioactive oxides of nitrogen in the healthy human vasculature [128], which can be readily detected independently of these other sources of nitric oxide derivatives [129]. A comparative analysis of the lipid profile of blood samples in normolipidemic *versus* hyperlipidemic individuals (>200 mg of cholesterol/dL) demonstrates a significant increase in the amount of nitrated linoleic acid products in the hyperlipidemic group [130]. Several nitrated lipid derivatives have been described, including unsaturated fatty acids, arachidonic acid derivatives and cholesterol esters [130-132]. As a newly discovered class of signaling molecules, one subclass has been collectively termed “nitroalkenes” due to the configuration of the double bond and nitro group. These species display several important bioactivities of relevance for the cardiovascular system including anti-inflammatory signaling, anti-platelet effects and an important role in the regulation of vascular tone. For a recent review of the emerging biological effects of nitroalkenes, see [133].

Whether or not some of the biological functions of nitroalkenes are derived from PPARγ-dependent activation is actively being investigated. Of relevance herein, the electrophilic modification of fatty acids confers further affinity as endogenous PPAR ligands, with preferential binding activity for PPARγ [121,127]. In fact, adduction of the nitro moiety of the lipid by glutathione abrogates nitroalkene-mediated PPARγ activation [134]. Similar to other proposed PPARγ ligands described herein, their physiological relevance is also a matter under current investigation. *In vitro* studies indicate that nitroalkenes induce adipocyte differentiation and favor glucose uptake in differentiated 3T3-L1 adipocytes, suggesting that, as a PPARγ ligand, they display insulin sensitizing properties in cultured adipocytes. Nitro-oleic acid also serves as a pan-PPAR agonist and rivals the potency of synthetic PPAR ligands such as fibrates and TZDs [127].

SUMMARY

The relevance of PPARγ as an important therapeutic target for the treatment of diabetes arises not only from its hypoglycemic effects in diabetic patients but also from its critical roles in the regulation of cardiovascular functions. The emergence of adverse effects, extensively documented by many laboratories and clinical studies, hinder its cardiovascular protective roles and support a strong need to systematically and rationally evaluate the efficacy of the current drugs as well as any novel therapeutic approaches under development. An expanding body of data supports the additional development and study of compounds that partially modulate PPARγ function rather than displaying full-agonism, such as the SPPARMs which can be considered a new generation of insulin sensitizers [87]. Increasing focus on the identification of natural or endogenous PPARγ ligands is changing the research paradigm in the hope of finding promising treatment approaches that would reduce the side-effects of other, less physiological modulators of PPARγ activity. In addition, further studies aimed at uncovering the basic mechanisms of action of PPARγ, in terms of fine-tuning the cardiovascular system, are still needed. With additional insight into these newly identified compounds for the treatment of diabetes, we may ultimately shield patients from the increased cardiovascular burden of this disease.

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Figure 1.
Vascular effects of PPAR γ activation.

Figure 2.

Balance of the systemic and cardiovascular beneficial *versus* adverse effects of PPAR γ .

Figure 3.
Rational drug design strategies targeting PPAR γ .