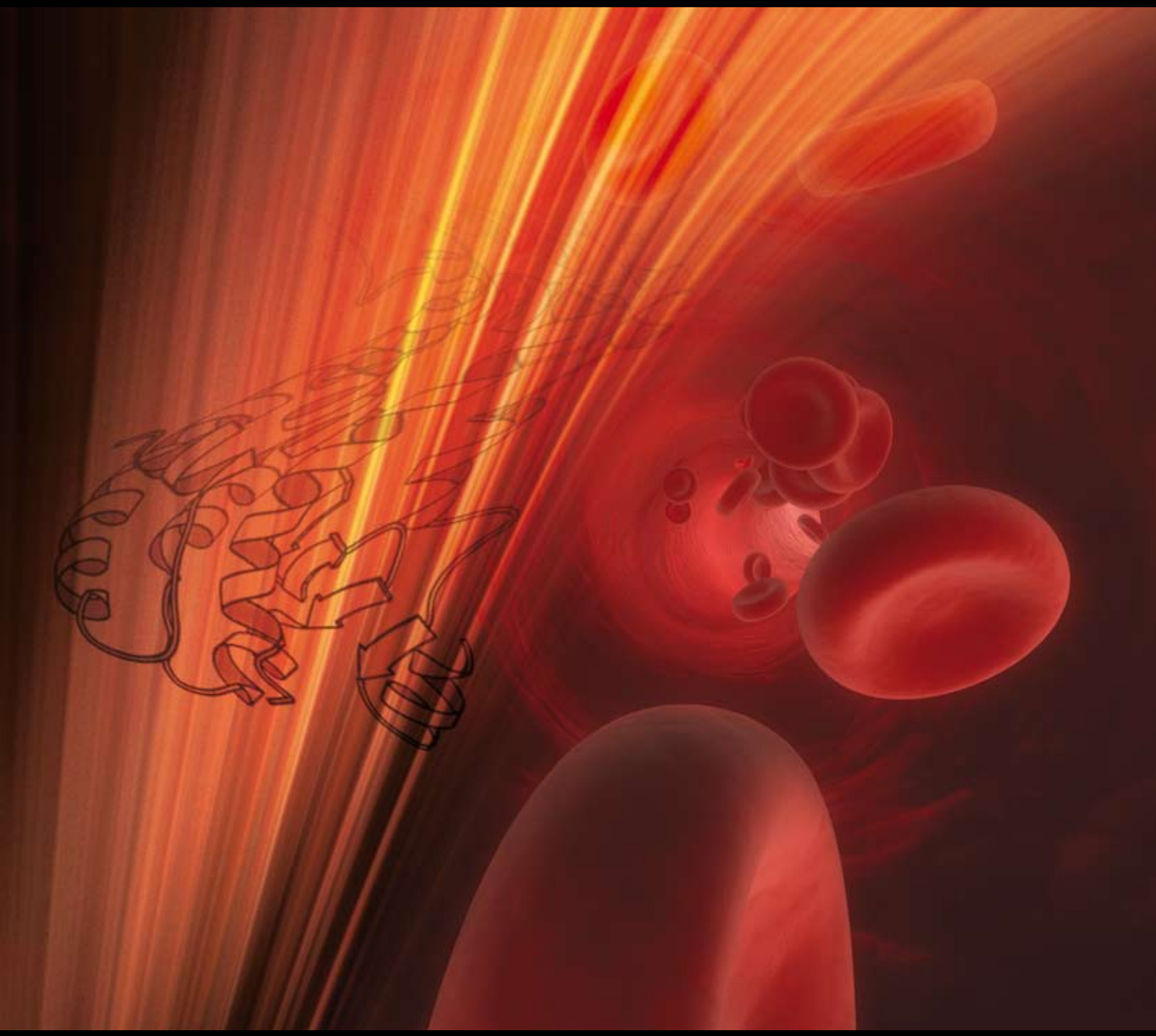
PPAR Research

PPARs/RXRs in Cardiovascular Physiology and Disease

Guest Editors: Brian N. Finck, Giulia Chinetti, and Bart Staels



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Editorial
PPARs/RXRs in Cardiovascular Physiology and Disease

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The PPAR family of nuclear receptor transcription factors are important regulators of cardiovascular function and metabolism. Because of this, PPARs are potentially interesting pharmacologic targets for treating cardiometabolic disease. The reviews in this series discuss the regulatory functions of PPARs in maintaining metabolic and physiologic homeostasis in a variety of cells and tissues. Additionally, the therapeutic potential and mechanisms of action of ligands of the different PPAR isotypes are discussed.

The review series is started by an examination of the effects of PPARs on lipoprotein metabolism. This is one of the first identified functions of PPARs. Indeed, ligands for PPARα were in clinical use as lipid-lowering agents even before their pharmacological target, PPARα, were known. The second review evaluates the important anti-inflammatory effects of PPARs in platelets, which is emerging as an important mechanism of their beneficial effects. Next, the critical role that PPARα and its transcriptional coactivator protein PGC-1α play in regulating energy metabolism and function of the myocardium is discussed. Then, a series of reviews focuses on the potentially beneficial effects of PPARγ agonists on the cardiovascular system. Several aspects are presented. The effects of PPAR activation on the cardiovascular system as a whole, on the vascular smooth muscle cell, and in the context of diabetic cardiovascular disease are each discussed at length. A review by Demers et al. also discusses the potential input of the hexarelin signaling pathway in regulating PPARγ activity and its potential impact on cardiometabolic disease. The genes encoding the PPARs are rich with genetic variation and the impact of these polymorphisms and haplotypes on the response to PPAR activators is only beginning to be understood. Thus, the "pharmacogenomics" of PPARs are discussed in a review by Dr. Sharon Cresci. Finally, the potential toxicity and adverse outcomes of PPAR agonism are summarized in detail by Jennifer Robinson. The timeliness of this discussion is outstanding given the recent reports of increased cardiovascular morbidity associated with use of rosiglitazone and the failure of PPAR dual agonists at different stages of development. Several of the other reviews in this series also touch this controversial issue at least briefly.

We are also pleased to present two original research reports. The first report found associations between PPARγ gene polymorphisms and several cardiometabolic indices, but found no link with cardiovascular morbidity and mortality. Second, Buroker et al. report an important role for PGC-1α in postnatal metabolic maturation. This preprogrammed burst in cardiac oxidative metabolism is an important developmental response that also has implications for other physiologic states wherein the demand for ATP production is rapidly induced.

We hope that you will find this issue enjoyable and informative.

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Review Article

Peroxisome Proliferator Activated Receptors and Lipoprotein Metabolism

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Recommended by Giulia Chinetti

Plasma lipoproteins are responsible for carrying triglycerides and cholesterol in the blood and ensuring their delivery to target organs. Regulation of lipoprotein metabolism takes place at numerous levels including via changes in gene transcription. An important group of transcription factors that mediates the effect of dietary fatty acids and certain drugs on plasma lipoproteins are the peroxisome proliferator activated receptors (PPARs). Three PPAR isotypes can be distinguished, all of which have a major role in regulating lipoprotein metabolism. PPARα is the molecular target for the fibrate class of drugs. Activation of PPARα in mice and humans markedly reduces hepatic triglyceride production and promotes plasma triglyceride clearance, leading to a clinically significant reduction in plasma triglyceride levels. In addition, plasma high-density lipoprotein (HDL)-cholesterol levels are increased upon PPARα activation in humans. PPARγ is the molecular target for the thiazolidinedione class of drugs. Activation of PPARγ in mice and human is generally associated with a modest increase in plasma HDL-cholesterol and a decrease in plasma triglycerides. The latter effect is caused by an increase in lipoprotein lipase-dependent plasma triglyceride clearance. Analogous to PPARα, activation of PPARβ/δ leads to increased plasma HDL-cholesterol and decreased plasma triglyceride levels. In this paper, a fresh perspective on the relation between PPARs and lipoprotein metabolism is presented. The emphasis is on the physiological role of PPARs and the mechanisms underlying the effect of synthetic PPAR agonists on plasma lipoprotein levels.

1. INTRODUCTION

Plasma lipoproteins are responsible for carrying triglycerides and cholesterol in the blood and ensuring their delivery to target organs. Extensive research over the past few decades has demonstrated that elevated plasma levels of cholesterol-rich low-density lipoproteins (LDLs) are associated with increased risk for coronary heart disease, whereas elevated levels of high-density lipoproteins (HDLs) have a protective effect. Accordingly, raising HDL levels and especially lowering LDL levels has become the cornerstone for the nutritional and pharmacological prevention and treatment of coronary heart disease. While lowering of plasma LDL can be efficiently and adequately achieved by treatment with statins, limited pharmacological treatment options are available for efficiently raising HDL levels. Hence, the quest for effective and safe drugs that raise HDL levels and/or decrease the atherogenic properties of plasma lipoproteins continues. A group of proteins that plays a major role in the regulation of lipoprotein metabolism and can be considered as major drug targets for correcting abnormal plasma lipoprotein levels are the nuclear receptors [1]. Nuclear receptors are ligand-activated transcription factors that alter gene transcription by direct binding to specific DNA response elements in target genes [2]. In addition, they modulate transcription by interfering with specific intracellular signaling pathways, thereby impairing transcriptional activation by other transcription factors. Nuclear receptors share a common modular structure that includes a relatively well-conserved central DNA-binding domain and a C-terminal ligand binding domain (LBD) [2]. Several nuclear receptors have been shown to be involved in the regulation of plasma lipoprotein metabolism, including the estrogen receptors (ERs), the oxysterol receptors (LXRs), the bile acid receptor (FXR), and the fatty acid receptors (PPARs). Here, the emphasis will be on the role of PPARs.

The PPAR family includes three members encoded by distinct genes: α, β/δ, and γ [3]. Since the initial discovery of the PPARα isotype in 1990 [4], an impressive amount of literature on these receptors has accumulated. PPARs mainly operate by governing the expression of specific sets of genes. Analogous to many other nuclear receptors, PPARs bind to DNA and regulate transcription as a heterodimer with the retinoid X receptor (RXR) [5]. The genomic sequence recognized by PPARs, referred to as PPAR response element or PPRE, consists of a direct repeat of the consensus hexameric motif AGGTCA interspaced by a single nucleotide. Functional PPREs have been identified in genes involved in a variety of biological processes including lipid and glucose metabolism, detoxification, and inflammation [6]. Activation of transcription by PPARs is achieved by binding of specific ligands to the LBD, followed by recruitment of coactivator proteins and dissociation of corepressors. Coactivator recruitment generally leads to an increase in enzymatic activity of histone acetyltransferases, histone methyltransferases, and subsequent nucleosome remodeling, activities which are essential to initiate transcription of PPAR target genes. X-ray crystallographic analysis of the LBD of PPARs has revealed an exceptionally spacious ligand binding pocket that can be occupied by a wide variety of synthetic and natural agonists, including numerous fatty acids and fatty acid-derived eicosanoids [7, 8].

The three PPARs are distinguishable by specific tissue and developmental patterns of expression and by their activation by distinct, yet overlapping, ligands [9]. The PPARα isotype is well expressed in tissues such as liver, heart, and small intestine and regulates a variety of target genes involved in cellular lipid metabolism ranging from mitochondrial, peroxisomal, and microsomal fatty acid oxidation to fatty acid uptake and binding, lipolysis, lipogenesis, and glycerol metabolism [6]. In contrast, PPARγ, which is highly expressed in brown and white adipose tissue, directs the expression of genes involved in adipocyte differentiation and fat storage. In addition, PPARγ governs glucose uptake and storage [10]. Much less is known about the ubiquitously expressed PPARβ/δ, although recent evidence suggests an involvement in wound healing [11], fatty acid oxidation [12], and lipoprotein metabolism [13].

Here we present an overview of the literature on PPARs and lipoprotein metabolism. The emphasis is on physiological role of PPARs and the mechanisms underlying the effect of synthetic PPAR agonists on plasma lipoproteins.

2. PPARα AND PLASMA TRIGLYCERIDE METABOLISM

The seminal evidence that placed PPARα at the center of lipoprotein metabolism was the demonstration that fibrates, which had been used clinically for many years to treat dyslipidemia, act by binding to PPARα and induce PPARα-dependent gene transcription [4, 14]. The role of PPARα in lipoprotein metabolism could thus be extrapolated retrospectively by analyzing the reported effect of fibrates. The availability of PPARα null mice further spurred progress in elucidating PPARα function and has resulted in an extensive picture of the role of PPARα in lipoprotein metabolism [15].

Numerous clinical studies in humans have provided ample evidence that fibrates, which include clofibrate, bezafibrate, fenofibrate, and gemfibrozil, effectively lower fasting plasma triglycerides (TG) [16–19]. The plasma TG lowering effect of fibrates can be reproduced in mice [20, 21]. Conversely, plasma TG levels are elevated in mice lacking PPARα [22]. Since in the fasted state plasma TG are carried mainly in the form of very low-density lipoproteins (VLDL), this suggests that PPARα suppresses VLDL production in liver and/or stimulates clearance of VLDL triglycerides in peripheral tissues.

3. PPARα AND VLDL PRODUCTION

Limited data are available on the effect of fibrates on production and secretion of VLDL in humans. In one study, the PPARα agonist gemfibrozil decreased production of VLDL-TG, while clofibrate had no effect [23]. In mice, PPARα has been shown to have a major impact on hepatic TG secretion. Indeed, deletion of PPARα is associated with a significant increase in VLDL-TG production in liver [24, 25]. In contrast, activation of PPARα using Wy14643 dramatically lowers VLDL-TG production (Figure 1). Furthermore, activation of PPARα suppresses TG secretion from primary rat hepatocytes [26].

VLDL is synthesized by the stepwise lipidation of the structural component apolipoprotein B through the action of microsomal triglyceride transfer protein (MTTP), resulting in the gradual formation of a mature TG-rich VLDL1 particle [27]. It may be expected that elevated hepatic TG levels increase VLDL secretion, on the one hand by targeting apolipoprotein B away from degradation toward secretion, thus increasing VLDL particle number, and on the other hand by increasing the amount of TG that becomes incorporated into VLDL, thus increasing VLDL particle size [27–29]. However, a positive correlation between hepatic TG and VLDL production is not always evident, as illustrated by the lack of change in hepatic VLDL production in ob/ob mice despite massive steatosis [30]. These data feed a growing recognition that the relation between hepatic TG storage and VLDL production is dependent on where the excess TG are stored. This argument holds both at the tissue level, as only excess TG stored in the periportal area may promote VLDL formation, and at the cellular level, as TG incorporated into VLDL are likely drawn from specific intracellular lipid compartments.

Numerous studies have shown that PPARα activation lowers liver TG levels, especially in the context of a fatty liver [31–36]. Conversely, deletion of PPARα is associated with elevated hepatic TG stores, which is evident under normal fed conditions but becomes considerably more pronounced after prolonged fasting and chronic high fat feeding [22, 37–40]. The potent effect of PPARα activation and deletion on hepatic TG levels is illustrated in Figure 2. Remarkably, treatment of wildtype but not PPARα null mice with Wy14643 for 10 days can completely prevent the fasting-induced increase in hepatic TG, most likely by stimulating fatty acid oxidation. Indeed, probably the best understood property of PPARα is its ability to stimulate fatty acid oxidation by upregulating

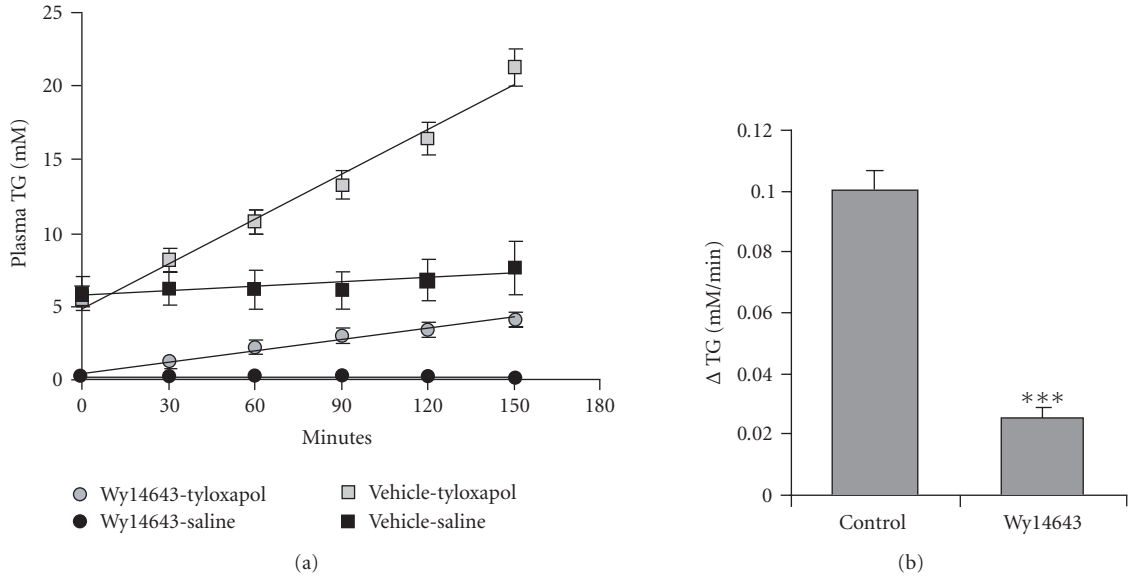

Figure 1: The PPARα agonist Wy14643 dramatically lowers VLDL production in a mouse model of hypertriglyceridemia. Male Angptl4-transgenic mice ($n = 7$ per group) were given vehicle or Wy14643 for 10 days (0.1% mixed in their food). After a 24-hour fast, the LPL-inhibitor tyloxapol (Triton WR1339, 500 mg/kg bodyweight as 15% solution in saline) or saline were injected intraorbitally. (a) Plasma triglyceride concentration was measured every 30 minutes to determine the VLDL production rate. (b) Mean rate of increase of plasma TG concentration in mice that received tyloxapol. Differences were evaluated by Student's t-test (***$P < .001$). Error bars represent SEM.

almost every single gene within the mitochondrial, peroxisomal, and microsomal fatty acid oxidation pathway, including carnitine palmitoyl transferase 1 and 2, acyl-CoA oxidase, acyl-CoA dehydrogenases, and numerous others [6]. Many of these genes have been identified as direct PPARα targets characterized by the presence of a functional PPRE. Accordingly, the most plausible explanation for the hepatic TG lowering effect of PPARα activation is that by promoting fatty acid oxidation, PPARα shifts fatty acids away from esterification and storage. While its effect on fatty acid oxidation likely accounts for the major share of its antisteatotic action, regulation of other genes and pathways by PPARα may contribute to some extent as well.

Suppression of VLDL production by PPARα agonists is generally attributed to lowering of hepatic TG stores, despite uncertainties surrounding the relationship between hepatic TG storage and VLDL production. In addition to its role in fatty acid catabolism, PPARα impacts on multiple aspects of intracellular lipid trafficking and metabolism, some of which may oppose hepatic TG lowering, including induction of genes involved in fatty acid synthesis and fatty acid elongation/desaturation [41–44]. Furthermore, expression of MTTP, which is involved in the lipidation of apoB100 to form a nascent VLDL particle, has recently been shown to be increased by PPARα [21]. Upregulation of MTTP may promote apoB100 secretion, which together with a decreased TG availability may favor the targeting of apoB100 to IDL and LDL rather than VLDL [21]. Interestingly, a recent study points to adipose differentiation-related protein (ADRP), which is a direct target gene of PPARα [45], as a potential mediator of the effect of PPARα on VLDL production. Using cultured cells it was shown that an increase in ADRP prevents the formation of VLDL by diverting fatty acids from the VLDL assembly pathway into cytosolic lipid droplets [46]. It can be expected that as the process of VLDL assembly and secretion becomes better understood and the role of PPARα in this process is further clarified, the general view on the mechanism underlying the effect of PPARα on hepatic VLDL secretion may change.

4. PPARα AND VLDL-TG CLEARANCE

Several studies have examined the impact of PPARα on clearance of TG-rich lipoproteins in humans, all of which show increased clearance after treatment with PPARα agonists [23, 47–49]. Clearance of VLDL-TG from plasma is mediated by the enzyme lipoprotein lipase (LPL) which thus has a critical role in determining plasma TG concentrations. LPL is synthesized mainly by adipocytes and myocytes, and after translocation to capillary endothelial cells it is anchored into the vessel wall via heparin-sulphate proteoglycans. Treatment of human subjects with PPARα agonists is associated with a significant increase in postheparin total LPL activity, suggesting that stimulation of plasma TG clearance by PPARα agonists can be attributed to enhanced LPL activity [49–51].

Theoretically, changes in LPL activity can be achieved by altering the production of LPL itself, or by altering the production of proteins that assist with LPL function or modulate its enzymatic activity. The latter group includes apolipoproteins such as APOC3, APOC2, and APOA5, as well as angiopoietin-like proteins 3 and 4. While it is clear that expression of LPL is upregulated by PPARα in liver [52], no evidence is available indicating a role for PPARα in governing LPL expression in heart and skeletal muscle. According

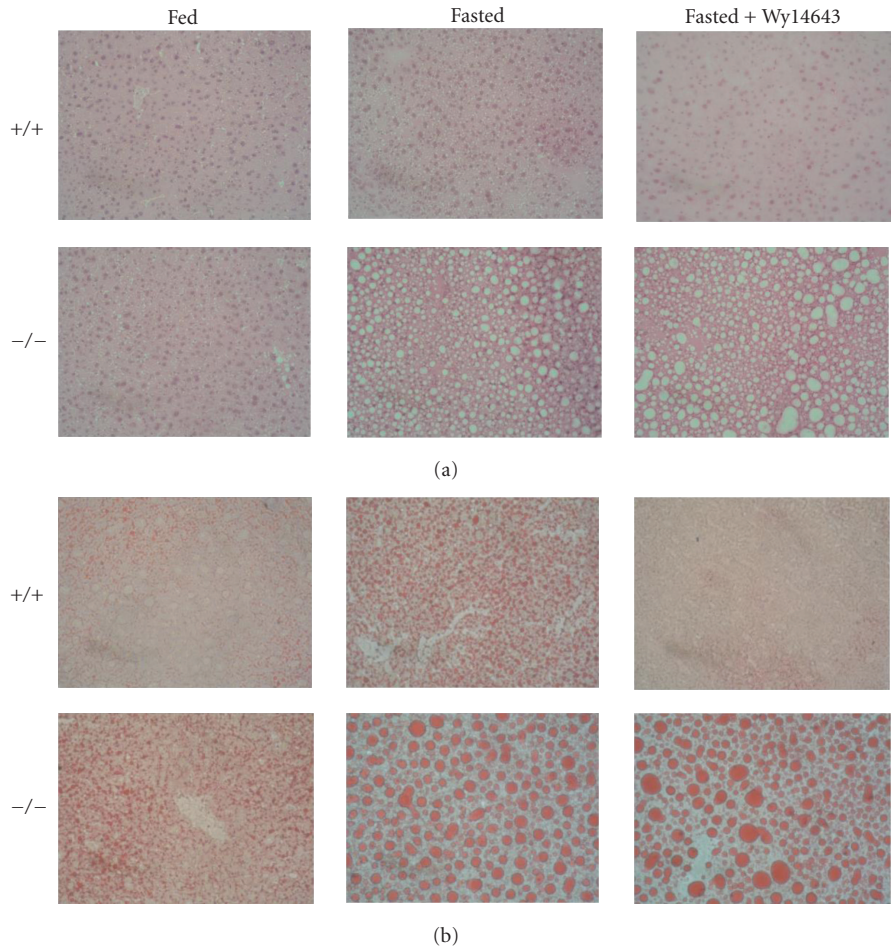

Figure 2: The PPARα agonist Wy14643 prevents the fasting-induced increase in liver TG levels. Male wild-type and PPARα null mice ($n = 5$ per group) were given vehicle or Wy14643 for 10 days (0.1% mixed in their food). After a 24-hour fast, livers were dissected and stained histochemically using hematoxylin/eosin (a) or oil Red O (b). Representative livers sections are shown. Differences visualized by histochemistry were perfectly confirmed by quantitative measurement of hepatic TG levels.

to our unpublished microarray data, neither PPARα deletion nor 5-day treatment with Wy14643 had any influence on LPL mRNA expression in mouse heart. It thus appears that rather than by regulating expression of LPL itself, PPARα agonists stimulate plasma TG clearance by altering the hepatic expression of inhibitors or activators of LPL activity. In both mouse and human, hepatic mRNA expression and plasma levels of APOC3, which inhibits LPL activity, are lowered by PPARα agonists [53–56]. Several mechanism have been put forward to explain downregulation of APOC3 expression by PPARα, involving the transcription factors Rev-erbα, HNF4α, or FOXO1 [57–60]. In contrast to APOC3, PPARα agonists increase hepatic expression and plasma levels of APOA5, an activator of LPL [61]. A functional PPAR responsive element has been identified in the promoter of the human APOA5 gene, classifying APOA5 as a direct PPARα target gene [62, 63].

It can be hypothesized that the stimulatory effect of PPARα on clearance of TG-rich lipoproteins may be counterbalanced by PPARα-dependent upregulation of the LPL inhibitor Angptl4 [64, 65]. Plasma levels of Angptl4 are increased by fenofibrate treatment [66]. Data obtained from various transgenic mouse models and from human genetic studies indicate that Angptl4 inhibits the clearance of TG-rich lipoproteins, likely by stimulating the conversion of catalytically active dimeric LPL to catalytically inactive monomeric LPL [67–72]. It can be speculated that upregulation of Angptl4 may explain the inhibitory effect of PPARα agonists on LPL activity in macrophages, adipose tissue, and cardiomyocytes [73–76].

5. PPARα AND HDL METABOLISM

In addition to their plasma TG-lowering effect, fibrates are used clinically for their ability to raise plasma HDL-cholesterol (HDLc) levels. A recent meta-analysis of 53 clinical trials indicates that on average, fibrates elevate plasma HDLc levels by about 10%, which translates into a 25% reduction in risk for major coronary events [77]. Remarkably, this effect is not observed in rodents, which seriously complicates study of the molecular mechanisms underlying the effect of PPARα agonists on HDL. In mice, plasma

total cholesterol and HDLc levels are reduced by treatment with synthetic PPARα agonists [78], whereas levels are increased in mice lacking PPARα [20]. The differential effects of PPARα on plasma HDL between mouse and human is likely due to species-specific regulation of apolipoprotein A-I (APOA1), the core apolipoprotein of HDL. Whereas PPARα activation increases plasma levels and hepatic mRNA expression of APOA1 in human, as supported by studies using human APOA1 transgenic mice and human hepatocytes [79], the opposite effect is observed in rodents [78]. The lack of upregulation of APOA1 gene expression by PPARα in rat was attributed to 3 nucleotide differences between the rat and the human APOA1 promoter A site, rendering a positive PPAR-response element in the human APOA1 promoter nonfunctional in rats [80]. In addition to APOA1, plasma levels and hepatic mRNA expression of APOA2, another major apolipoprotein component of HDL, are also increased by fibrates in humans [51, 81]. In contrast, in rodents both a reduction and induction of APOA2 expression after treatment with fibrates have been observed [20, 78].

In recent years, our knowledge regarding the mechanisms and location of HDL formation has improved considerably. Recent evidence suggests that the intestine and liver are responsible for the major share of HDL synthesis [82, 83]. It is generally believed that HDL is formed by lipidation of lipid poor APOA1 mediated by the cholesterol efflux transporter ABCA1. The importance of ABCA1 in HDL synthesis is illustrated by the almost complete absence of HDL from plasma of patients with a dysfunctional ABCA1 gene [84]. This metabolic abnormality is reproduced in mice that lack ABCA1 [85, 86]. Importantly, the expression of ABCA1 is increased by PPARα in intestine and macrophages [87, 88]. No PPRE has yet been identified in the human or mouse ABCA1 gene, suggesting that ABCA1 may not be a direct PPARα target. Instead, upregulation of ABCA1 mRNA by PPARα agonists in macrophages likely occurs via PPARα-dependent upregulation of LXR, which is a transcriptional activator of ABCA1 [88, 89]. Whether the same mechanism operates in intestine remains unclear.

Recently, ABCG1 was identified as the transporter responsible for cellular efflux of cholesterol towards mature HDL [90]. So far no evidence is available that suggests regulation of ABCG1 by PPARs.

Several proteins are involved in HDL remodeling including lecithin cholesterol acyltransferase (LCAT), phospholipid transfer protein (PLTP), and cholesteryl ester transfer protein (CETP). In mice, fibrates decrease plasma LCAT activity and hepatic LCAT mRNA expression [91]. Hepatic expression and plasma activity of PLTP, which increases HDL particle size by catalyzing the transfer of phospholipids from VLDL/IDL to HDL, are increased by PPARα in wild-type but not PPARα null mice. Accordingly, upregulation of PLTP may account for the observed increase in HDL particle size in mice treated with fibrates [92]. Since CETP is absent from mice, the role of PPARα in the regulation of CETP activity has largely remained elusive. Interestingly, in a recent study using hCETP-transgenic mice on an apoE3 Leiden background, it was found that fenofibrate markedly reduced CETP activity in parallel with an increase in plasma HDLc levels [93]. These data imply that fibrates reduce CETP activity in humans, suggesting that the effect of fibrates on plasma HDL levels in humans may be partially achieved by suppressing CETP activity. In addition, it can be speculated that as PPARα activation decreases plasma VLDL levels, the acceptor pool for the CETP-catalyzed exchange of cholesterol-esters with HDL will be diminished, resulting in increased HDL size.

HDL cholesterol can also be cleared by the SCARB1-mediated selective removal of cholesterol from the HDL particle, or by endocytic uptake and degradation of the whole particle, called holoparticle HDL uptake. A possible mechanism by which fibrates may impair HDL clearance is by downregulating hepatic SCARB1 gene expression in a PPARα dependent manner [94].

6. PPARβ/δ AND LIPOPROTEIN METABOLISM

While the role of PPARα in the regulation of lipoprotein metabolism is relatively well characterized, much less is known about PPARβ/δ. Initial studies in mice showed that selective PPARβ/δ agonists raise plasma HDLc levels [13, 95]. The HDLc-raising effect is also evident in rhesus monkeys [96], and, according to a recent report, in human subjects [97]. In monkey and human, but seemingly not in mice, PPARβ/δ agonists decrease plasma TG levels as well. The mechanism behind the HDLc-raising effect of PPARβ/δ agonists remains obscure, although a role for ABCA1, which is upregulated by PPARβ/δ, has been proposed [96].

In line with the plasma TG-lowering effect of PPARβ/δ agonists observed in primates, plasma TG levels are elevated in PPARβ/δ null mice [98]. In contrast, plasma total cholesterol and HDLc remain unchanged. It was proposed that the elevated plasma TG levels in PPARβ/δ null mice are caused by a combination of increased VLDL production and decreased plasma TG clearance, as evidenced by a decrease in postheparin LPL activity and increased hepatic expression of LPL inhibitors Angptl3 and 4. Overall, insight into the molecular mechanisms that may underlie the observed changes in plasma lipoproteins is lacking, which is partly due to the fact that very few PPARβ/δ specific or selective target genes are known. Since PPARα agonists also increase plasma HDLc levels, it might be hypothesized that PPARβ/δ agonists might act via common molecular targets. However, at least in mice, PPARα and PPARβ/δ agonists display divergent effect on plasma TG levels, suggesting a different mode of action.

7. PPARγ AND PLASMA TRIGLYCERIDE METABOLISM

Synthetic PPARγ agonists are prescribed for their ability to promote insulin sensitivity and lower plasma glucose levels in patients suffering from type 2 diabetes mellitus (T2DM). On top of an insulin-sensitizing action, numerous studies in mice and humans have shown that use of PPARγ agonists leads to a reduction in fasting and postprandial plasma TG levels [99–103]. Some variability in the plasma TG lowering effect is observed between different PPARγ agonists, and in mice between various disease models. Indeed, no effect of PPARγ agonists on plasma TG is observed in the two mouse

models most commonly used for atherosclerosis research, which are the LDL receptor knock-out and apoE knock-out mice [104]. In humans rosiglitazone seems to specifically lower postprandial but not fasting TG levels [105, 106]. Evidence has been provided that the plasma TG lowering effect of PPARγ agonists may be connected to their insulin sensitizing action by suppressing adipose tissue lipolysis and plasma FFA levels, which is expected to lead to decreased hepatic VLDL-TG production [106]. However, no relationship exists between the insulin-sensitizing potency of PPARγ agonists and plasma TG lowering [107]. Furthermore, in a recent study, treatment of type 2 diabetic subjects with pioglitazone did not result in any change in hepatic VLDL-TG production [108]. Thus, whereas PPARα agonist lowers plasma TG by a combination of suppressing hepatic VLDL production and stimulating plasma TG clearance, PPARγ agonists seem to lower plasma TG exclusively by enhancing plasma TG clearance [100, 108].

The stimulatory effect of PPARγ agonists on plasma TG clearance is achieved by upregulating LPL expression and activity in adipose tissue [52, 100, 106, 109, 110], which is associated with an increase in postheparin plasma LPL mass/total activity [101, 102]. As a consequence, LPL-mediated lipolysis and the fractional clearance rate of VLDL-TG are elevated [108]. Besides directly regulating LPL production, PPARγ agonists may influence LPL-mediated lipolysis by decreasing plasma levels of APOC3, a potent inhibitor of LPL [108].

Interestingly, in rats induction of LPL activity and gene expression by PPARγ agonist was observed in inguinal but not retroperitoneal adipose tissue [111]. This type of adipose depot-specific regulation of LPL by PPARγ likely accounts for the redistribution of stored fat from visceral towards subcutaneous adipose tissue upon treatment with PPARγ agonists [112]. Also, no induction of LPL expression by PPARγ was observed in murine skeletal muscle [113].

In contrast to what is observed in vivo, PPARγ agonists decrease LPL activity in primary rat and mouse 3T3 adipocytes [100, 114]. It can be hypothesized that the inhibition of LPL activity may be mediated by upregulation of Angptl4, similar to what was discussed for the suppression of LPL activity in various cell types after treatment with PPARα agonist. In light of the recent finding that rosiglitazone raises plasma Angptl4 levels in human subjects [115], and that Angptl4 increases abundance of monomeric LPL in preheparin plasma (our unpublished data), it can be speculated that upregulation of Angptl4 may also account for the observed increase in plasma preheparin LPL levels in subjects treated with pioglitazone [108].

Use of gene targeting to study of the role of PPARγ in regulation of lipoprotein metabolism has been complicated by the lethality of PPARγ null mice. However, mice with a specific ablation of the PPARγ2 isoform are viable and, opposite to the effect of PPARγ agonists, show elevated plasma TG levels, especially on a leptin-deficient background [116]. A similar elevation of plasma TG was observed in mice in which PPARγ was specifically deleted in adipose tissue [117].

Apart from LPL, very few PPARγ target genes that impact on TG-rich lipoproteins are known. It has been shown that LDL-receptor-related protein 1 (LRP-1), which is involved in clearance of cholesteryl-esters from chylomicron remnants and possibly HDL, is a direct target gene of PPARγ in human adipocytes [118]. These data suggest that upregulation of LRP-1 may contribute to the stimulatory effect of PPARγ agonists on clearance of TG-rich lipoproteins.

8. PPARγ AND HDL METABOLISM

Although PPARγ agonists are best known for their ability to lower plasma glucose and TG levels, depending on the type of PPARγ agonist and the type of animal species/model, plasma levels of cholesterol and specific lipoprotein subclasses may be altered as well [104, 119]. Recently, the results of two large clinical trials involving either rosiglitazone or pioglitazone were reported. In the ADOPT trial, 4360 subjects recently diagnosed with T2DM were randomly assigned to treatment with metformin, glyburide, or rosiglitazone. After 4 years, plasma HDLc levels were modestly higher in the rosiglitazone-treated patients [120]. In the proactive trial, 5238 patients with type 2 diabetes received either pioglitazone or placebo. Again, a significant increase in plasma HDLc levels was observed in the patients treated with pioglitazone [121]. The small but reproducible increase in plasma HDLc upon treatment with PPARγ agonists was substantiated in a meta-analysis summarizing the effects of thiazolidinediones from a large number of randomized controlled trails [122]. In addition, treatment with PPARγ agonists is associated with an increase in LDL size [101, 103, 119]. It has been reported that the relative efficacy of pioglitazone towards ameliorating plasma lipid levels is more favorable compared to rosiglitazone [119].

Presently, the mechanism(s) behind the HDLc raising effect of PPARγ agonists remains elusive. Possibly, PPARγ agonists may carry minor agonist activity towards PPARα. However, in contrast to PPARα agonists, PPARγ agonists do not appear to have any effect of APOA1 and APOA2 syntheses [100, 108]. The observation that plasma HDLc levels do not respond to PPARγ agonist treatment in rodents complicates study of the underlying mechanisms [100]. It is conceivable that the modest increase in HDLc following PPARγ agonist treatment is due to reduced CETP-mediated exchange of VLDL TGs for HDL cholesterol, concomitant with a drop in VLDL-TG levels. Finally, PPARγ has been shown to upregulate expression of ABCA1 in macrophages [88, 123]. As ABCA1 is required for the flux of cholesterol from cells onto APOA1 to form nascent HDL, upregulation of ABCA1 by PPARγ may contribute to the HDLc-raising effect of PPARγ. However, it still needs to be demonstrated that expression of ABCA1 is under control of PPARγ in tissues responsible for the major share of HDL synthesis, which are intestine and liver.

9. CONCLUSION

PPARs have a major impact on levels of lipoproteins in plasma by governing the expression of numerous genes involved in the synthesis, remodeling, and clearance of plasma lipids and lipoproteins. The changes in plasma lipoprotein

levels associated with treatment with PPAR agonists, characterized by decreased plasma TG levels, increased HDLc, and an increase in LDL size, are expected to decrease the risk for cardiovascular disease. In recent years, several new proteins that play a role in lipoprotein metabolism have been identified. In addition, the functional characterization of other proteins involved in lipoprotein metabolism has advanced significantly. As progress is made in PPAR-dependent gene regulation, especially in human, our insight into the molecular mechanisms underlying the effects of PPARs on plasma lipoproteins will further continue to improve.

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Review Article

The PPAR-Platelet Connection: Modulators of Inflammation and Potential Cardiovascular Effects

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Historically, platelets were viewed as simple anucleate cells responsible for initiating thrombosis and maintaining hemostasis, but clearly they are also key mediators of inflammation and immune cell activation. An emerging body of evidence links platelet function and thrombosis to vascular inflammation. Peroxisome proliferator-activated receptors (PPARs) play a major role in modulating inflammation and, interestingly, PPARs (PPARβ/δ and PPARγ) were recently identified in platelets. Additionally, PPAR agonists attenuate platelet activation; an important discovery for two reasons. First, activated platelets are formidable antagonists that initiate and prolong a cascade of events that contribute to cardiovascular disease (CVD) progression. Dampening platelet release of proinflammatory mediators, including CD40 ligand (CD40L, CD154), is essential to hinder this cascade. Second, understanding the biologic importance of platelet PPARs and the mechanism(s) by which PPARs regulate platelet activation will be imperative in designing therapeutic strategies lacking the deleterious or unwanted side effects of current treatment options.

1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality world-wide. In part, this is due to social and economic changes that lead to atherosclerosis, obesity, hypertension, dyslipidemia, and type 2 diabetes mellitus (T2DM) [1–5]. Life-style factors such as exercise, healthy diet, and avoidance of smoking are crucial to prevent disease or reduce cardiovascular risk factors. While it is important to educate individuals about healthy life-style decisions, it is also imperative to develop therapeutic strategies to attenuate the chronic inflammatory pathways linked to vascular disease [4–6]. Recently, platelets have been implicated as key contributors to the chronic inflammation that leads to CVD [5].

While platelets are essential for hemostatic regulation, new studies reveal an expanded role for platelets in thrombosis, immune cell activation, and inflammatory processes creating an obvious link between thrombosis and vascular inflammation. Platelet hyperactivity is implicated in a variety of conditions including atherosclerosis, peripheral arterial disease (PAD), T2DM, and inflammatory bowel disease (IBD) [7–10]. Although activated platelets release many proinflammatory mediators such as CD40 ligand (CD40L, CD154) and thromboxane A_2 (TXA_2), they also release membrane vesicles and platelet microparticles (PMPs), which influence the activities of other cell types both regionally and systemically. Since PMPs contain proteins important for both hemostasis and inflammation, they may amplify or sustain

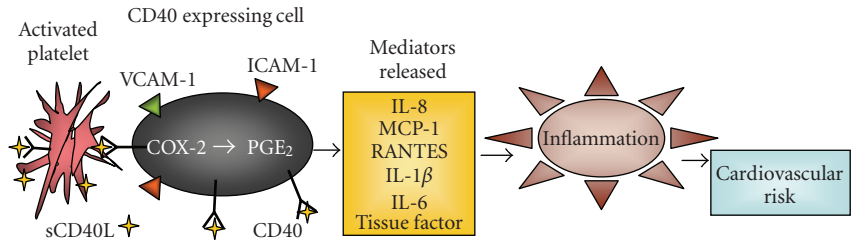

Figure 1: Platelets promote inflammation. CD40 expressing cells, such as endothelial cells or fibroblasts, can be activated by platelet-derived CD40L. CD40 signaling upregulates bioactive mediators in these cells; therefore, potentiating inflammation and increasing the risk for CVD.

inflammation and thrombosis contributing to a chronic inflammatory state. Moreover, higher than normal levels of platelet-released microparticles are present in individuals with atherosclerosis, T2DM, stroke, and PAD [9, 11–13].

Proteomic studies are beginning to reveal the remarkable diversity of platelet proteins and have identified proteins not known to be expressed in or released from platelets [14–16]. While lacking a nucleus, platelets contain transcription factors, notably the peroxisome proliferator-activated receptors (PPARS). PPARs are key regulators of metabolism and inflammation, and thus are poised to play an important role in processes that govern chronic inflammatory diseases [17]. Accumulating evidence suggests that PPAR activation is beneficial in the prevention of stroke and myocardial infarction (heart attack) [17, 18]. However, other studies show that some PPAR activating drugs may increase the risk of cardiovascular events [19]. Despite the lack of definitive information on the risk and benefits of taking PPAR-targeting drugs, it is clear that PPARs remain a promising target for treating CVD and more importantly, that dampening unwanted platelet activation will reduce the risk of CVD and/or improve disease outcome.

2. PLATELETS ARE MODULATORS OF INFLAMMATION AND THROMBOSIS

Platelets are anuclear cells released from megakaryocytes, a hematopoietic cell that differentiates and undergoes endomitosis [20]. The platelet's composition is a product of specific packaging by the megakaryocyte and the acquisition by endocytosis of blood components. Platelets contain classical cellular organelles including mitochondria and lysosomes, a complex cytoskeleton, specific platelet granules, and an open canalicular system, a complex structure of internal membranes that serves as a conduit for the movement and release of platelet contents. Despite the lack of a nucleus, platelets contain mRNA and spliceosomal components for mRNA processing, as well as the translational machinery for protein synthesis [21–23]. The recent discovery of *de novo* synthesis by platelets of mRNAs, including Bcl-3, interleukin-1β (IL-1β), plasminogen activator inhibitor-1 (PAI-1), and tissue factor (TF), exemplifies the complexity of platelet signaling and underscores their role as formidable players in regulating coagulant and inflammatory pathways [24–29].

Platelets contain vast stores of bioactive mediators including thromboxanes, prostaglandins, chemokines, and cytokines that promote clot formation and incite inflammation. Upon activation, platelets produce high levels of proinflammatory mediators such as CD40L, intercellular adhesion molecule-1 (ICAM-1), tissue factor, and C-reactive protein (CRP). These mediators enhance inflammatory responses and recruitment of immune cells. Recently, it was shown that plasma levels of soluble CD40L (sCD40L) are high at birth and remain so throughout childhood [30]. The reason for the developmental change is not yet understood. In contrast, higher than normal adult levels of sCD40L in the adult bloodstream are linked with increased risk for ischemia, stroke, and myocardial infarcts due to thrombosis [4, 31]. Based on these studies, much interest has been generated in CD40L as a possible biomarker and major factor in the progression of CVD [32–34].

2.1. *CD40L is a major contributor to chronic inflammation*

A surprising and important finding was that CD40L, a member of the tumor necrosis factor (TNF) receptor superfamily and a key mediator of both innate and adaptive immunity [4, 5, 35, 36], is released by activated platelets [31, 33, 35]. Shortly after platelets become activated, they express CD40L on their surface which is subsequently enzymatically cleaved releasing soluble bioactive CD40L into the bloodstream. This is highly significant for the following two reasons. First, platelets contain approximately 95% of the CD40L found in human beings, and thus are a crucial link in the regulation of the CD40/CD40L pathway, as many cells express its receptor, CD40. These cells include fibroblasts, endothelial, epithelial, monocytes, neutrophils, B cells, and dendritic cells. CD40L is found in abnormally high levels in the blood of patients with chronic inflammatory diseases such as diabetes, atherosclerosis, as well as some recipients of platelet transfusions [33, 37–40]. Disruption of CD40/CD40L pathway can blunt chronic inflammation, retard atherosclerosis, and transplant rejection [33, 35, 41]. Further, recent exciting research demonstrates that CD40L is crucial for stabilizing thrombi, for normal platelet responses to sheer stress, and for platelet activation through the RGD domain of sCD40L which binds to platelet αIIbβ3, a receptor critical for platelet activation and aggregation [42, 43]. Collectively, these data strongly support the importance of CD40L as a primary agonist for platelets and is considered a prototypical mediator with roles in both hemostasis and inflammation (Figure 1

summarizes CD40 activation by platelet CD40L). Therefore, the platelet is a crucial link in the CD40/CD40L pathway and sCD40L release alone or in combination with other proinflammatory mediators may increase the risk for cardiovascular effects promoting atherosclerosis, hypertension, and dyslipidemia to list a few.

2.2. Platelet-released microparticles are elevated in individuals with chronic inflammatory disease

Platelet microparticles (PMPs) are defined as microvesicle particles that measure less than 1 μm in diameter [44]. Platelet agonist stimulation or high shear stress leads to the highly regulated formation and release of PMPs, which are known to regulate a broad spectrum of physiological activities [45–47]. PMPs are an important delivery and cell signaling system in both inflammatory and hemostatic processes. For example, a portion of platelet IL-1β is associated with PMPs and signals endothelial cells, inducing their adhesiveness for neutrophils to elicit an inflammatory response [25]. PMPs signal the expression of specific adhesion molecules and stimulate the production of cytokines and mRNA in endothelial cells and in the monocytic cell line, THP-1 [48]. Notably, a known α-granule component and proinflammatory mediator, regulated on activation, normal T-cell expressed and secreted (RANTES) (CCL5), is delivered to sites of arterial injury and atherosclerotic endothelium via PMP to promote monocyte recruitment [49]. PMPs modulate cell-to-cell interactions by increasing adhesive contacts between monocytes and endothelial cells, an important first step in vascular inflammation [50]. It is also known that platelet-derived tissue factor (TF) is transferred from CD62P positive PMPs to monocytes although the procoagulant role of this particle delivery system has not been established [51]. Elevated numbers of PMPs are present in a variety of diseases including atherosclerosis and other CVDs, T2DM, and cancer [49, 51–54]. PPARs may have a potential role in the regulation of platelet activation and release of platelet contents as will be discussed further below.

3. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS (PPARs) AND PLATELETS

PPARs are ligand-activated transcription factors and members of the nuclear hormone receptor superfamily. These receptors are known to play a role in regulating metabolic risk factors for CVD, such as the vascular inflammation and thrombosis associated with atherosclerosis [55]. There are three PPAR subtypes, PPARα (NR1C1), PPARβ/δ (NUC1, NR1C2), and PPAR (NR1C3), encoded by separate genes and described in several organisms including humans. PPARs are differentially expressed in a variety of tissues and are important in the regulation of lipid and carbohydrate metabolism, energy homeostasis, cellular differentiation and apoptosis, and immune and inflammatory responses [42]. PPARα is highly expressed in brown adipose tissue, liver, kidney, heart, and skeletal muscles [61]. PPARβ/δ has a broad tissue distribution with highest expression in the kidney, gut, and heart [42, 62]. PPARγ is abundant in adipose tissue, colon, retina, and in cells of the immune system [58]. Important for this discussion are PPARβ/δ and PPARγ as they were recently found to be expressed in human platelets, a surprising result considering platelets are anucleate [63, 64]. The impact of this discovery was exemplified upon finding that exposure to PPAR agonists attenuates platelet activation and associated inflammation [63, 64].

Activation of PPARs in nucleated cells occurs by optimal DNA binding to a PPAR DNA response element following ligand binding and conformational changes that facilitate heterodimerization with a second ligand-activated nuclear receptor, retinoic X receptor (RXR, 9-cis retinoic acid receptor) [65, 66]. This heterodimer binds to a cis acting DNA element in the promoters of target genes called the peroxisome proliferator response element (PPRE) to induce or repress gene transcription in a cell- and tissue-specific manner, depending on the receptor and a combination of factors, including ligand and accessory molecule binding. The physiological functions of PPARα and PPARγ have been relatively well characterized, whereas the function of PPARβ/δ is poorly understood. A summary of the PPAR subtypes and their potential roles in platelets is discussed below.

3.1. PPARα

PPARα activation affects transcriptional expression of approximately 80–100 genes, the products of which regulate fatty acid oxidation, lipid metabolism, and inflammation [67]. PPARα is expressed in cells of the vasculature and immune system, but has not yet been firmly identified in platelets [68]. The antiinflammatory properties of PPARα are of paramount interest, but there are also reports of proinflammatory effects [69, 70]. For example, it was demonstrated that chronic activation of PPARα is detrimental to cardiac recovery during reperfusion following ischemia [71]. In contrast, it is known that PPARα plays an antiinflammatory role in lung fibrosis although the mechanism is not well understood [72, 73]. It is clear that the intricacies of PPARα function must be discerned to design effective and safe drug strategies. Current PPARα agonists include the fibrates, which are therapeutic agents that increase transcription of high density lipoproteins (HDL) such as ApoAI and ApoAII and are effective at lowering triglyceride levels [74, 75]. PPARα agonists have also been reported to decrease weight gain, as obesity is a contributing factor in atherosclerosis [75].

3.2. PPARβ/δ

PPARβ/δ is suggested to play a role in basic cellular functions such as cellular proliferation and differentiation, and fatty acid catabolism in skeletal muscle where it is most abundant [76, 77]. This receptor has also been implicated in the regulation of inflammation, and shown to slow plaque formation and attenuate the progression of atherosclerosis [78]. Although little is known about the function of PPARβ/δ, especially in platelets, prostacyclin (PGI_2), an important antithrombotic and endogenous platelet hormone, is reported to be a ligand for PPARβ/δ [79, 80]. Several studies have

revealed that PGI_2 synergizes with nitric oxide (NO) to inhibit platelet aggregation in response to a variety of platelet agonists including thrombin, collagen, ADP, and lysophosphatidic acid (LPA) [64, 81–86]. It was previously shown that the synergistic effects of NO and prostacyclin on inhibition of platelet response were due to the simultaneous increase of cyclic nucleotides cGMP and cAMP [81, 87, 88]. The recent discovery that PPARβ/δ ligands and NO inhibit platelet aggregation via PPARβ/δ suggests an alternative signaling mechanism is operative in platelets [64]. This is consistent with a previous study where Ali et al. demonstrated that prostacyclin mimetics exhibited antiproliferative effects that were mediated by PPARβ/δ and not via the prostacyclin receptor in lung fibroblasts [89]. This identified PPARβ/δ as a potential therapeutic target for the treatment of pulmonary hypertension and supports the view that platelet PPARβ/δ may play an important role in thrombosis [64].

3.3. PPARγ

PPARγ is important in adipocyte differentiation, lipid storage, and glucose homeostasis, and has emerged as a key target for new antiinflammatory therapies [6, 90, 91]. There are 3 isoforms of PPARγ (PPARγ1, PPARγ2, and PPARγ3). All are encoded by the same gene, but are the result of differential promoter use and alternative RNA splicing [92]. PPARγ2 differs from PPARγ1 by an additional 30 amino acids at the N-terminus. PPARγ1 is present in adipose tissue, human spleen, liver, intestine, kidney, and platelets, while PPARγ2 is abundantly expressed only in adipose tissue and liver [93]. PPARγ3 mRNA has been detected in mouse macrophage cells, however its function remains unknown [94].

PPARγ is expressed in many cell types including fibroblasts, endothelial cells, dendritic cells, macrophages, T cells, B cells, and most recently we identified PPARγ in human platelets [59, 63, 91, 95–98]. Our laboratory recently discovered that human platelets express PPARγ and that PPARγ ligands attenuate platelet release of the proinflammatory and procoagulant mediators, sCD40L and TXA_2, a cyclooxygenase (COX) product that enhances platelet activation [63]. Platelets can respond to at least two natural PPARγ ligands: lysophosphatidic acid (LPA) which they produce, and 15d-PGJ_2 which has potent antiinflammatory properties and is a metabolite of PGD_2 [91, 99, 100]. Additionally, there are several synthetic ligands in development and clinical use that are specific and potent agonists for PPARγ including the antidiabetic thiazolidinedione drugs (TZDs) (e.g., rosiglitazone (Avandia) and pioglitazone (Actos) both in clinical use) [91, 99]. These will be discussed in greater detail in Section 5.

Interestingly, human platelets also contain the PPARγ binding partner RXR, and PPARγ is able to bind DNA suggesting that it can form an active PPARγ/RXR heterodimer, and thus may be capable of biologic activity within the platelet. It is therefore possible that PPARγ agonists interact directly with platelets to alter platelet activation and hemostatic function. While PPARγ was first thought to be located only in the nucleus to regulate transcription, we and others have demonstrated that PPARγ can be found in the cytoplasm of eukaryotic cells [91, 101]. There is increasing evidence suggesting that PPARγ binds proteins in the cytoplasm of cells separate from its transcriptional role. For example, it was recently reported that PPARγ ligands, via a PPARγ-dependent mechanism, block PKCα translocation to the membrane attenuating inflammatory responses in monocytes/macrophages [101]. Additionally, cytoplasmic PPARγ can repress the transcriptional activity of the proinflammatory mediator, nuclear factor–κB (NF-κB), preventing its translocation to the nucleus [92, 102]. NF-κB is involved in regulating many aspects of cellular activity, including the immune response and has a well established role in the pathological progression of chronic inflammatory diseases [103]. Interestingly, it has also been shown in platelets that the PPARγ binding partner, RXR, signals through the Gq-protein receptor in a ligand-dependent manner to inhibit platelet activation [104].

Intriguingly, our group has discovered that PPARγ is released in a PMP-associated form and some PPARγ is expelled from activated platelets as a functional PPARγ/RXR heterodimer [105]. Moreover, the released PPARγ is taken up by a promonocytic cell line (THP-1) [105]. Thus, it is possible that other cells also take up platelet-released PPARγ, quickly elevating PPARγ levels in recipient cells. This potential transcellular mechanism for PPARγ would then influence the recipient cell's susceptibility to PPARγ ligands and may represent a novel antiinflammatory mechanism. For example, PPARγ and its ligands are known to reduce VCAM-1 and ICAM-1 expression, and increase nitric oxide synthase expression on endothelial cells which is important for inhibiting platelet activation [106, 107]. These expanded antiinflammatory roles for PPARγ provide new avenues to pursue novel drug strategies.

4. PLATELETS AND CARDIOVASCULAR DISEASE

Cardiovascular disease comprises a broad spectrum of illnesses, such as hypertension, dyslipidemia, and myocardial infarction and stroke that affect the heart and the blood vessels. These conditions have similar causes (obesity, smoking, diabetes, sedentary lifestyle, and age) and platelets play a complex role in CVD, triggering early events that lead to endothelial dysfunction, to progression of vascular damage, to plaque production, and to formation of thrombi that can result in myocardial infarcts and stroke.

4.1. Metabolic syndrome

Platelets and their PPARs play putative roles in several manifestations of the dyslipidemia-associated "metabolic syndrome" or "syndrome X," which includes hyperglycemia, insulin resistance, obesity, hypertension, and atherosclerosis [77, 108–113]. Dyslipidemia, an increasingly common consequence of a high-fat diet, is characterized by increased serum triglycerides, low levels of antiatherogenic high density lipoprotein cholesterol (HDL) and prevalence of proatherogenic low density lipoprotein particles (LDL). Considering the imbalance between pro- and antiatherogenic factors, it is not surprising that dyslipidemia is associated with a high risk of atherosclerosis in afflicted patients [77]. HDL

protects against atherosclerosis by driving the reverse transport of cholesterol from peripheral cells to the liver for excretion [77, 113]. The contribution of LDL particles to the development of atherosclerosis is closely connected to platelet function and may be modulated by PPARs, as described below.

4.2. Atherosclerosis

Atherosclerosis is a chronic inflammatory disease characterized by plaque development within the arterial intima [5, 114]. These atherosclerotic plaques may erode or rupture over time, triggering thrombogenesis, and possible myocardial infarction or stroke [5, 115]. Platelets are famous for their role in clot formation during the final stages of atherosclerosis, but it has become clear from studies in both humans and animal models that the early stages of plaque formation are also platelet-mediated [5, 115–120]. Atherosclerosis is initiated when inflammatory processes activate vascular endothelial cells, resulting in platelet adhesion to the arterial wall [115, 121–123]. When platelets adhere to the endothelial surface, they are activated, causing them to release mediators that attract and activate other cell types, including neutrophils, monocytes, and bone-marrow-derived progenitor cells [5, 115]. Monocytes cross the endothelial monolayer and enter the arterial intima by extravasation [115]. There they differentiate first into macrophages, and then, into cholesterol-laden foam cells, a critical step in atherosclerotic plaque formation [77, 115, 118]. Platelets regulate the differentiation of bone-marrow-derived progenitor cells and macrophages into foam cells [5, 115, 118, 119, 124]. Studies using fluorochrome-modified LDL have shown that platelets take up LDL and store it in dense granules [115, 118]. These platelets can then be internalized by macrophages, a critical step in foam cell differentiation and plaque formation [115, 118, 125, 126].

One platelet-derived mediator with a clear link to atherogenesis is platelet factor 4 (PF4) which both inhibits LDL degradation by the LDL receptor and promotes monocyte-to-foam cell differentiation [115, 127]. Activated platelets also release CD40L and interleukin-1β which further activate the vascular endothelium, causing it to produce chemoattractants and adhesion molecules that act to recruit neutrophils and monocytes into the arterial intima [5, 115, 118, 128, 129]. Matrix metalloproteinases (MMPs) are also expressed by activated platelets, monocytes, and endothelial cells in response to CD40L; these are important in foam cell generation and the physical remodeling of the normal arterial wall to an atherosclerotic plaque [115, 118, 130–136]. Smooth muscle cell proliferation, promoted by platelet release of transforming growth factor-β, platelet-derived growth factor, and serotonin, is also critical to this process [115].

PPARs appear to play a major role in the regulation of atherogenesis by countering the inflammation-provoking action of platelet adhesion and activation [5]. In vitro incubation of platelets with PPARγ agonists inhibits their ability to express CD40L and to aggregate in response to thrombin [63, 137]. Pioglitazone, a PPARγ-specific ligand, decreases platelet aggregation and delays arterial thrombus formation in male LDL receptor-deficient mice [5, 138]. Other PPARγ ligands, including rosiglitazone and *c*9, *t*11-conjugated linoleic acid, inhibit atherosclerotic progression in this model and in the apoE$^{-/-}$ mouse [139, 140], possibly through their ability to inhibit platelet deposition, monocyte recruitment, macrophage differentiation, LDL uptake, foam cell formation, MMP expression, and vascular smooth muscle cell migration within atherosclerotic plaques [115, 118, 137, 138, 141, 142]. Studies in human patients with atherosclerosis have shown that certain TZD type PPARγ agonists reduce both platelet and endothelial cell activation, inhibit plaque progression, improve flow-mediated vasodilation, and remarkably promote regression of existing atherosclerotic plaques [5, 115, 143]. Since phagocytosis of platelets (and their internalized LDL) by macrophages is critical to foam cell formation and atherosclerotic progression, platelet-derived PPARγ may be of paramount importance to the antiatherosclerotic actions of these drugs [115, 118, 125, 126]. Packaging of PPARγ into platelets and/or its release in PMPs may be a convenient mechanism by which this transcription factor is delivered to endothelial lesions where it may act to attenuate pathological remodeling of the arterial wall. The potential benefits of PPAR signaling are not limited to atherosclerosis, but may extend to "metabolic syndrome" as a whole. Rosiglitazone therapy reduces the systemic inflammation characteristic of "metabolic syndrome," as evidenced by decreases in serum levels of IL-6 and TNFα [5, 144]. PPARβ/δ ligands have been shown to ameliorate dyslipidemia in both mice and insulin-resistant obese rhesus monkeys [113, 145, 146]. Current data suggest that PPARs will prove to be premium targets for the development of drugs to combat both dyslipidemia and atherosclerosis.

4.3. Thrombosis

As was discussed above, endothelial dysfunction in blood vessels is one of the earliest events that contribute to disease development triggering a chain reaction, which results in formation of atherosclerotic plaques and rupture in the blood vessel walls. A major function of platelets is to "plug" these holes by changing their shape, adhering to subendothelial surfaces, secreting the contents of intracellular organelles, and aggregating to form a thrombus in response to stimuli generated in endothelia of damaged blood vessels [147]. Several mediators are involved in platelet aggregation, such as thrombin, collagen, epinephrine (exogenous to the platelet); agents such as ADP (secreted from platelet storage granules); and thromboxane A_2 (synthesized by the platelets during activation) [148]. As was mentioned above, the PPARγ agonists rosiglitazone and pioglitazone dampened platelet release of key proinflamatory and proatherogenic mediators such as CD40L and TXA_2 [63]. The PPARγ agonist troglitazone has also been shown to decrease platelet aggregation in response to ADP, collagen, and arachidonic acid [149]. The mechanism whereby the vascular endothelium defends against thrombus formation involves the generation of the potent vasodilator nitric oxide (NO). NO interferes with platelet aggregation and is generated from L-arginine by the enzyme

nitric oxide synthase (NOS) which is constitutively expressed in endothelium [150]. In experiments where rats received pioglitazone, it was found that aortic cNOS and thrombomodulin expression was upregulated and thrombus formation was delayed [151]. Pioglitazone had similar effects in the human monocyte/macrophage cell line (THP-1) where dose-dependently upregulated thrombomodulin expression was seen [152]. Other PPARγ ligands, such as rosiglitazone, also upregulate cNOS gene expression [153, 154].

4.4. Myocardial infarction and stroke

Myocardial infarction occurs when the blood supply to the heart is interrupted causing damage and possible death of the heart tissue. One of the major causes of myocardial infarction is rupture of the atherosclerotic plaque and formation of a platelet-rich thrombus. PPARγ is present in heart tissue, but there is limited data about its function there. The PPARγ activator rosiglitazone does inhibit TNF-α gene expression in cultured myocytes [155]. Additionally, Rosiglitazone treatment of male Lewis rats following myocardial ischemia and reperfusion injury showed a dramatic protection against myocardial infarction, and also improved cardiac function [156]. Ischemia/reperfusion injury is characterized by an inflammatory response. Activated neutrophils release a variety of cytotoxic substances, such as oxygen-derived free radicals and proteases and activated monocytes/macrophages synthesize inflammatory cytokines [157]. Activated platelets can upregulate these responses in neutrophils and monocytes/macrophages. Together, these mediators directly participate in the amplification of an inflammatory response and, therefore, in vascular endothelial dysfunction that can lead to myocardial injury. PPARγ is present in monocytes/macrophages, neutrophils, and platelets, which suggests a role for PPARγ in negatively regulating expression of proinflammatory genes and thus, myocardial infarction [158].

Thrombus can also form in the cerebral arteries blocking the normal blood flow and causing a cerebrovascular accident (stroke). Stroke is a complex process in which several pathways are involved and successful prevention of a stroke will require drugs with pleiotropic effects. Resveratrol, found in the seeds and skin of grapes, was found to have neuroprotective effects [159] and shown to be a dual PPARα/γ activator [18]. Experiments in a rat model have shown that pretreatment with fenofibrate and/or Wy-14643, which are PPARα activators, and resveratrol reduced brain infarct size after permanent focal cerebral ischemia [18]. PPARβ/δ is found in numerous brain areas whereas PPARα and PPARγ have a more localized expression. Inflammation and oxidative stress induce apoptotic and necrotic neuronal death and NF-κB is one of the culprits [160]. It is thought that PPARs have a neuroprotective function due to their interaction with NF-κB. For example, PPARγ binds to NF-κB complexes and facilitates its translocation out of the nucleus [102]. Due to their wide distribution in the neurovascular-glial compartments and their complex function, PPAR agonists offer hope in the prevention of stroke [161]. It will be of major importance to dampen platelet activity in the case of both myocardial infarction and stroke as ultimately, hyperactive platelets will be the major culprits in the occlusion or rupture of an artery.

4.5. Diabetes mellitus

Type 2 diabetes mellitus (T2DM), primarily characterized by hyperglycemia and insulin resistance, is often part of a "metabolic syndrome" which comprises hypertension, dyslipidemia, decreased fibrinolysis, and increased procoagulant factors (discussed above) [162]. Thrombocytopathia (any qualitative modification of platelets) in diabetes includes: increased platelet aggregation and adhesiveness, increased platelet number, and enhanced expression of activation-dependent adhesion molecules [10]. Platelet hyperaggregability and adhesiveness in diabetes has several causes. Prostacyclin and the endothelium-derived relaxing factor nitric oxide (NO) are released by intact vascular endothelium and antagonize the effects of proaggregants so that thrombi do not form in blood vessels [163]. Platelets from diabetic patients produce less prostacyclin and NO and, in addition, they are less sensitive to PGI_2 and nitric oxides inhibitory effects [164–166]. Insulin can target platelets directly through the platelet insulin receptor, which binds insulin and undergoes autophosphorylation [167]. Insulin reduces platelet responses to the agonists ADP, collagen, thrombin, arachidonate, and platelet-activating factor [168]. However, in T2DM platelets express fewer insulin receptors and a decreased affinity for insulin [169]. Insulin has a direct effect on platelets and is important for maintaining platelet PGI_2 sensitivity by increasing the PGI_2 binding sites and as a consequence, augments cAMP response to PGI_2 [170]. Numerous studies support the fact that there is an association between diabetes and oxidative stress [171]. A higher production of reactive oxygen species is thought to play an important role in diabetes complications and has been attributed to protein glycation and/or autoxidation caused by a hyperglycemic environment, and lipid peroxidation of cellular structures [172].

Oxidative defense is provided by vitamins, such as vitamin E, and by a number of enzymes, such as glutathione peroxidases. Platelets contain two glutathione peroxidases: cytosolic glutathione peroxidase (cGPx) and phospholipid hydroperoxide glutathione peroxidase (PHGPx). CGPx is involved in oxidative stress protection and in formation of eicosanoids [173, 174]. Vitamin E is decreased in plasma of type 1 and type 2 diabetic patients [175]. In type 2 diabetics, platelet cGPx activities were found to be lower and can lead to a relative accumulation of 12-hydroperoxyeicosatetraenoic acid (12-HpETE), the main hydroperoxide formed from arachidonic acid [175]. Thus, increase in 12-HpETE could activate signal transduction pathways leading to arachidonic acid release, and amplification of platelet activation [176]. Platelet PHGPx activity was also measured for the first time in diabetic patients and was decreased in type 2 diabetics [175]. Thus, in diabetes there is an increase in free radical production and a decrease in mechanisms responsible for antioxidant defense which give rise to an environment that favors generation of radical species.

Type 1 and 2 diabetic patients exhibit increased expression of activation-dependent adhesion molecules, such as activated $\alpha IIb\beta_3$, lysosomal Gp53, thrombospondin, and P-selectin (CD62P) [177]. The increased expression of $\alpha IIb\beta_3$ is consistent with the enhanced fibrinogen binding and aggregability seen in platelets from diabetic subjects [178]. Arachidonic acid metabolism, which leads to TXA_2 production, is increased in diabetes and may cause platelet sensitivity [179, 180]. Because diabetes is accompanied by CVD development, drugs that can reduce hyperglycemia and inhibit the progression of cardiovascular complications are desirable. PPAR$\alpha/\gamma/\beta$ pan agonists may offer new options for treatment of diabetic complications. The blood of both type 1 and 2 diabetics shows elevated levels of CD40L [39]. PPARγ ligands can reduce platelet activation and thrombosis by reducing CD40L from platelets. Treatment of diabetic patients with TZD-type drugs decreased circulating CD40L blood levels [181].

4.6. Obesity

Obesity represents a major health threat and, in recent years, it has become clear that obesity and inflammation are linked [109–111, 182]. Obese individuals show persistent platelet activation and subsequent increased plasma levels of several proinflamatory cytokines [183]. TNFα, adiponectin, leptin, and monocyte chemoattractant protein-1, all can originate from fat, have immunomodulating functions and show an altered profile during obesity [184]. Furthermore, PPARβ/δ has been linked to the development of obesity. Its activation decreases adipose mass in mouse and increases fatty acid oxidation in the heart, improving muscle contraction [76]. Thus dampening platelet activation may be a means of reducing an inflammatory cascade that leads to further vascular damage and CVD.

5. PPAR AGONISTS AS PLATELET THERAPEUTICS

Platelets are an important pharmacological target because the thrombi developed during CVD that lead to morbidity and mortality are platelet-rich in content. Nonsteroidal anti-inflammatory drugs, including aspirin, are among the most widely used drugs around the world [185]. Aspirin's primary action is to inhibit arachidonate-cyclooxygenase activity in platelets and ultimately, TXA_2 release thereby, attenuating thrombus formation. Recent reports show that a subset of patients is aspirin-resistant and that aspirin may not be as effective in women. This, coupled with the fact that the cyclooxygenase pathway plays only a minor role in the action of many platelet agonists, has lead to the development of new antiplatelet therapies that complement aspirin's therapeutic effects [186–189].

There are two groups of antiplatelet agents used in conjunction with aspirin: the thienopyridines (ticlopidine and clopidogrel) and the glycoprotein (GP) IIb/IIIa ($\alpha IIb\beta_3$) receptor antagonists (abciximab and eptifibatide). The thienopyridines are adenosine 5′-diphosphate (ADP) receptor antagonists which block ADP from binding, thereby, inhibiting platelet activation, aggregation, and degranulation.

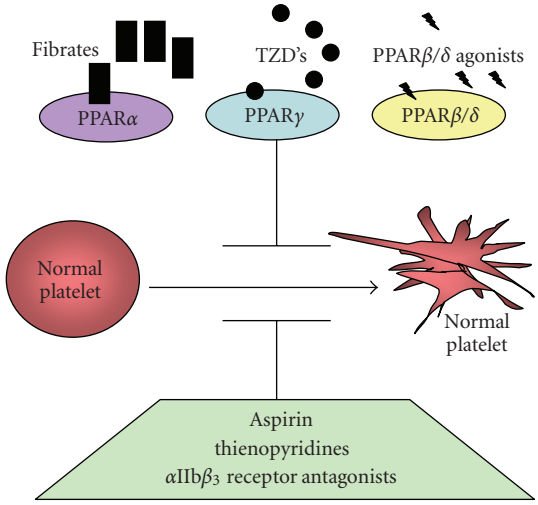

Figure 2: Possible role of PPAR agonists in dampening inflammation and reducing cardiovascular events. PPAR agonists may reduce the risk for thrombosis. Besides playing a role in adipogenesis, lipid metabolism, and insulin sensitivity, PPARs may dampen inflammation by attenuating platelet activation.

While for the most part, thienopyridines are efficacious for reducing ischemic events, it is unclear as to whether or not clopidogrel and aspirin together are more effective than aspirin alone [190, 191]. In rare cases, thienopyridines may cause neutropenia or thrombotic thrombocytopenia purpura [192, 193].

$\alpha IIb\beta_3$ is the most important platelet membrane receptor for aggregation because it is found in high concentrations on the cell surface and binds both fibrinogen and von Willebrand factor. Blocking this receptor reduces thrombotic risks associated with acute coronary syndromes and diabetes. Unfortunately, $\alpha IIb\beta_3$ receptor antagonists have to be administered intravenously because oral therapy causes excessive bleeding [194]. Moreover, a meta analysis of four $\alpha IIb\beta_3$ receptor antagonist trials showed an overall increase in mortality with drug use [195].

Clearly, there is a need to develop new therapeutics that are easily administered and can dampen platelet function with fewer adverse side effects. Adding complexity to function, platelets activate and release many proinflammatory mediators and interact with not only each other, but also with many other cell-types as described in previous sections. Targeting this action of platelets could be effective in not only reducing platelet aggregation and thrombus formation, but also in attenuating chronic inflammation and, therefore, slowing disease progression.

PPAR agonists are a class of potential antiplatelet drugs that are easily administered and have the ability to impact this new physiology of platelet function. Even though PPAR agonists are primarily prescribed for the treatment of metabolic disorders, some possess the secondary benefit of inhibiting cardiovascular complications associated with hyperlipidemia and hyperglycemia. PPARα agonists, fibrates, are prescribed for hyperlipidemia. They potently diminish

blood cholesterol and triglyceride levels while raising plasma HDL levels (platelet agonists that dampen platelet activation are summarized in Figure 2).

The effect of PPARα agonists on cardiovascular risk during clinical studies show mixed results. The Veterans Affair High-Density Lipoprotein Cholesterol Intervention Trial study (VA-HIT) demonstrated that the fibrate, gemfibrozil, significantly reduced nonfatal myocardial infarction and death in men with coronary cardiopathy [196]. Disappointingly, results from the recent Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial showed no reduction in risk for the primary end-point (coronary heart disease death and nonfatatal myocardial infarction) in coronary events with fenofibrate therapy [197]. There are many explanations for these results, including the use of a low cardiovascular risk diabetic population, but it is clear that more investigation is needed to understand the clinical relevance of fibrates for treating CVD. Since platelets may lack PPARα, these drugs may not have a direct effect on platelet function, but may be useful in conjunction with other PPAR agonists to target multiple pathways involved in cardiovascular pathophysiology (see below).

Perhaps more promising is the use of PPARγ TZD agonists as antiplatelet agents. TZDs are mainly used in the treatment of T2DM because they improve insulin sensitivity by decreasing TNF-α and IL-6 expression and increasing adiponectin expression [198, 199]. Troglitazone was the first PPARγ agonist marketed, but was withdrawn in 2000 for causing hepatotoxicity [200, 201]. Rosiglitazone and pioglitazone are the current TZDs prescribed in T2DM and have been shown to reduce the risk of myocardial infarction and stroke [202]. As was discussed in Section 3, our laboratory demonstrated that rosiglitazone attenuates CD40L surface expression and sCD40L release from thrombin-activated platelets [63]. Downregulating the CD40/CD40L system would likely provide great clinical benefit for patients with CVD. Furthermore, 15d-PGJ_2 was found to attenuate TXA_2 and CD40L from thrombin-activated platelets, and prevent ATP release and ADP-induced aggregation [63]. This correlates with data from a mouse model of atherosclerosis showing that pioglitazone decreases platelet activation and delays arterial thrombus formation [138]. The PROspective pioglitAzone Clinical Trial (PROACTIVE) demonstrated that pioglitazone is protective against macrovascular events in diabetic patients [203]. Rosiglitazone was also shown to reduce serum levels of matrix metalloproteinase-9 (MMP-9), implicated in atherosclerotic plaque rupture, and the proinflammatory marker CRP in patients with T2DM [204]. Conversely, some recent studies, A Diabetes Outcome Progression Trial (ADOPT) and Diabetes Reduction Assessment ramipril and Rosiglitazone Medication (DREAM), demonstrated that rosiglitazone was associated with an increase in cardiovascular risks when compared with placebo [205, 206]. As a consequence of these recent reports that rosiglitazone may increase the incidence of myocardial infarction, a randomized, prospective, open-label trial (RECORD) was performed to assess the effects of rosiglitazone on CVD [207]. The results of this study showed a significant increase in the risk of congestive heart failure in patients taking rosiglitazone, but no significant differences in cardiovascular-related hospitalization or death. There are many limitations to the recent studies on the cardiovascular effects of TZDs, such as small sample sizes and short trials, which clearly need to be resolved before an accurate interpretation of the data can be made. In the short term, it appears that the use of rosiglitazone and pioglitazone in patients that are not at high risk for congestive heart failure is warranted [19]. However, a better understanding of the biological effects of PPARs and the cogent design of selective therapeutics without adverse effects are imperative.

One alternative may lie in a promising new class of PPARγ ligands known as selective PPAR modulators (SPPARMs) that have been designed as partial PPARγ agonists, retaining insulin sensitization but lacking the fat-accumulating properties of the classical TZD PPARγ ligands [208, 209]. Given the success with SPPARMs in targeting insulin resistance, one can speculate that other properties of PPARγ could be targeted for partial agonist design in the future to have specific antiinflammatory activity without interference of normal thrombotic benefits or risk of potential negative cardiac effects.

There are also many other PPAR candidate drugs under investigation for the treatment of metabolic syndrome. PPAR dual agonists and PPAR pan agonists are new classes of drugs that target multiple PPAR isoforms at once to produce synergistic antidiabetic and cardioprotective effects. These drugs have the potential to improve insulin sensitivity and lower triglycerides while reducing the unwanted side effects of weight gain and edema associated with the administration of fibrates and TZDs. A novel group of dual agonists have been discovered that appear to be potent agonists of both PPARα and PPARγ. These compounds known as alkoxybenzylglycines are synthetic tertiary amino acids, one of which has been demonstrated to have beneficial oral antidiabetic and antidyslipidemic efficacy in vivo [210, 211]. However, the therapeutic efficacy of dual and pan agonists in diabetes-associated cardiovascular risks is unknown.

PPARβ/δ agonists are being developed for their ability to treat hyperlipidemia and they have the potential to exert antithrombotic effects. It was recently published that platelets express PPARβ/δ a putative receptor for PGI_2 whose activation inhibits platelet aggregation [64, 212–214]. Clearly, further studies are needed to address the effects that all PPAR agonists have on not only cardiovascular risks, but also on platelet activity. It appears that TZDs have potentially beneficial effects on overall cardiovascular risk. Understanding how targeting PPAR with pharmacological agents influences platelet biology will provide insight into the function of PPARs in platelets and help in designing drugs with better specificity and fewer adverse side effects.

6. CONCLUSION

The studies described herein illustrate a connection between PPARs and platelets that is significant in the pathophysiology of CVD. Platelets are emerging as potent immune and inflammatory mediators that both initiate early responses in the vasculature and elicit protracted responses that lead to

the development of chronic inflammatory disease. Platelets contain PPARβ/δ and PPARγ, nuclear receptors with known antiinflammatory functions. Thus, platelets are important contributors to CVD processes and PPARs have the ability to attenuate these processes. Platelet-derived PPARs are likely to play an important role in controlling the magnitude of a platelet-driven inflammatory response. Treatment of platelets with PPAR agonists dampens the risk of thrombus formation and attenuates increased blood levels of proinflammatory mediators such as CD40L and TXA_2. These functions of PPARs can be exploited for the development of drugs to combat such prevalent and devastating conditions as dyslipidemia, atherosclerosis, and diabetes. Understanding the specific role of platelet-derived PPARs in the process of platelet activation attenuation is essential for intelligent prevention and management of these disease states.

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Review Article

The PPARα-PGC-1α Axis Controls Cardiac Energy Metabolism in Healthy and Diseased Myocardium

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The mammalian myocardium is an omnivorous organ that relies on multiple substrates in order to fulfill its tremendous energy demands. Cardiac energy metabolism preference is regulated at several critical points, including at the level of gene transcription. Emerging evidence indicates that the nuclear receptor PPARα and its cardiac-enriched coactivator protein, PGC-1α, play important roles in the transcriptional control of myocardial energy metabolism. The PPARα-PGC-1α complex controls the expression of genes encoding enzymes involved in cardiac fatty acid and glucose metabolism as well as mitochondrial biogenesis. Also, evidence has emerged that the activity of the PPARα-PGC-1α complex is perturbed in several pathophysiologic conditions and that altered activity of this pathway may play a role in cardiomyopathic remodeling. In this review, we detail the current understanding of the effects of the PPARα-PGC-1α axis in regulating mitochondrial energy metabolism and cardiac function in response to physiologic and pathophysiologic stimuli.

1. INTRODUCTION

The myocardium has an enormous and steady demand for energy that is met through high-level mitochondrial oxidative metabolism. Glucose, lactate, and fatty acids are all oxidized in the mitochondrion to produce reducing equivalents required for ATP synthesis in the process of oxidative phosphorylation (OXPHOS). Much of the mitochondrial-derived ATP is then transported to the cytoplasm, making energy available for cellular work, which includes its crucial role in cardiac myocyte contraction. Acute changes in flux through these metabolic pathways are mediated by changes in substrate concentrations and covalent or allosteric modification of enzymes catalyzing these reactions. However, the capacity for mitochondrial oxidative metabolism is also mediated at the level of gene transcription [1].

Work in several labs has demonstrated that the three PPAR isoforms (PPARα, β/δ, and γ) are expressed, to varying degrees, in the myocardium and play important roles in the transcriptional regulation of cardiac metabolism and function. The ability to modulate PPAR activity with specific activating ligands as well as genetic activation or deactivation in mice has enriched our understanding of the importance of each of the various PPAR isoforms in determining cardiac metabolism, structure, and function. However, given the limited space available in this review, we will focus our attention on the PPARα isoform and its coactivator protein PGC-1α.

2. PPARα AND MYOCARDIAL FATTY ACID METABOLISM

The PPARα isoform is robustly expressed in the parenchymal cells of the adult heart and plays an important role in regulating cardiac myocyte metabolism [2, 3]. In the myocardium, PPARα activation induces the expression of genes encoding nearly every step in the cellular fatty acid utilization pathway including (i) fatty acid transport proteins that facilitate fatty acid entry into the cell, (ii) acyl-CoA synthetases that esterify fatty acids to coenzyme A and prevent their efflux, (iii) fatty acid binding proteins that shuttle fatty acids to various cellular compartments, (iv) proteins that catalyze the import of

fatty acids into the mitochondrion, (v) every enzyme in the mitochondrial fatty acid β-oxidation spiral, and (vi) various accessory components of fatty acid metabolism (e.g., uncoupling proteins).

Administration of PPARα ligand to rodent models results in a robust activation of PPAR target genes in liver, but the effects of in vivo ligand administration on cardiac gene expression is minimal [4]. Indeed, PPARα agonist administration to diabetic mice actually leads to diminished cardiac fatty acid utilization [5, 6], possibly by reducing the exposure of the heart to triglyceride-rich lipoproteins or endogenous fatty acid ligands. It is unclear whether PPARα ligand administration targets the heart directly in humans; and there are likely differences in the PPAR response between the species. Due to the hepatic specific effects of PPARα ligands in rodents, much of our knowledge regarding the target pathways of PPARα in myocardium is based on studies with genetic alterations in PPARα activity. Mice with constitutive deletion (in all tissues) of the gene encoding PPARα (PPARα null mice) exhibit diminished rates of cardiac fatty acid oxidation (FAO) and increased reliance on glucose utilization pathways [7–9]. This shift is mediated, at least in part, by diminished expression of several genes involved in FAO [10] and a concomitant increase in the expression of genes encoding proteins involved in glucose uptake and utilization [7]. At the other end of the metabolic spectrum, we have characterized transgenic mice overexpressing PPARα in a cardiac-restricted manner (MHC-PPARα mice) [8, 11–16]. The expression of many genes involved in fatty acid uptake and utilization is upregulated in MHC-PPARα mice, while the expression of glucose transporter and glycolytic enzymes is strikingly suppressed [11]. Consistent with this pattern of metabolic gene expression, MHC-PPARα mice rely almost exclusively on FAO and use very little glucose [8, 9, 11]. In summary, the opposing metabolic phenotypes of these transgenic models with activation or deactivation of PPARα support an important role for PPARα in regulating cardiac energy metabolism.

3. THE PGC-1α TRANSCRIPTIONAL COACTIVATOR AND THE CONTROL OF CARDIAC ENERGY METABOLISM

Transcriptional coactivators are a group of proteins that control gene expression via protein-protein interactions with DNA-bound transcription factors, including PPARα (Figure 1). Although several transcriptional coactivators are known to interact with PPARα, in the heart, the physical and functional interaction with PPARγ coactivator 1α (PGC-1α) has been best described. PGC-1α was originally discovered in a yeast two-hybrid screen for proteins that interacted with the PPARγ isoform and that were enriched in a brown adipocyte library [17]. Based on sequence homology in some highly conserved regions, two additional PGC-1 family members have now been identified (PGC-1β and PGC-related coactivator (PRC)) [18, 19].

Coactivators are broadly categorized into two classes. Class I coactivators regulate genetranscription through enzymatic modification of chromatin (e.g., acetylation and methylation), which facilitates DNA unwinding and enhances the probability that a gene will be transcribed by the RNA polymerase II complex. Class II coactivators work by interacting with the RNA polymerase machinery (e.g., RNA polymerase II or the TRAP/DRIP complex) [20, 21]. PGC-1α functions as a Class II coactivator since it does not possess intrinsic chromatin modifying activity and interacts directly with the TRAP/DRIP complex to link with RNA polymerase II (Figure 1) [20]. PGC-1α also recruits Class I coactivators with histone acetyltransferase activity to chromatin in the target gene promoter [20, 22] and docks with a protein called ménage-à-trois 1, which phosphorylates RNA polymerase II to modulate its activity (Figure 1) [23]. Finally, PGC-1α possesses an RNA processing domain that may also contribute to its transcriptional regulatory function [24].

PGC-1 interacts with and coactivates a broad array of transcription factors to transduce developmental, nutritional, and physiological stimuli to the control of diverse cellular energy metabolic pathways [25, 26]. In heart, PGC-1α has thus far been linked with 3 families of transcription factors: (i) the PPAR family, (ii) the estrogen-related receptor (ERR) family, and (iii) nuclear respiratory factor 1 (NRF-1). The interaction between PGC-1α and PPARα serves to control the expression of enzymes involved in fatty acid uptake and oxidation [27] and possibly proteins involved in the process of mitochondrial biogenesis [15]. The ERR family (ERRα, β, γ) of orphan nuclear receptors is also an important cardiac PGC-1α target that drives increased expression of genes encoding FAO and OXPHOS enzymes [28–31]. Finally, NRF-1 is a nuclear-encoded transcription factor that is coactivated by PGC-1α to regulate transcription of genes involved in mitochondrial OXPHOS, mtDNA transcription and replication, and mitochondrial biogenesis [32–35]. Additional details regarding PGC-1-mediated control of energy metabolism through ERRα and NRF-1 can be found in other recent reviews [26, 35–37].

Several genetically-engineered mouse models have been used to probe the role of PGC-1α in regulating cardiac metabolism. Mice that constitutively overexpress PGC-1α in the myocardium exhibit profound mitochondrial proliferation, cardiomyopathy, and early death secondary to heart failure [33]. The severity of the cardiomyopathy in this model precluded a full investigation of the pathologic mechanisms that contribute to cardiac dysfunction. To address this issue, a second model evaluated overexpression of PGC-1α in the heart using a tetracycline-inducible system [38]. This model revealed dramatic mitochondrial proliferation when PGC-1α was overexpressed in the neonatal phase, without overt effects on cardiac function. In contrast, overexpression of PGC-1α in adult mice provoked only modest mitochondrial proliferation, but led to abnormal mitochondrial and myofibril architecture and severe cardiac dysfunction [38]. Interestingly, cardiomyopathy in these mice was completely reversible by discontinuing PGC-1α overexpression [38]. These gain-of-function strategies indicate that PGC-1α plays important roles in regulating multiple aspects of myocardial metabolism and is a strong stimulus for the process of mitochondrial biogenesis.

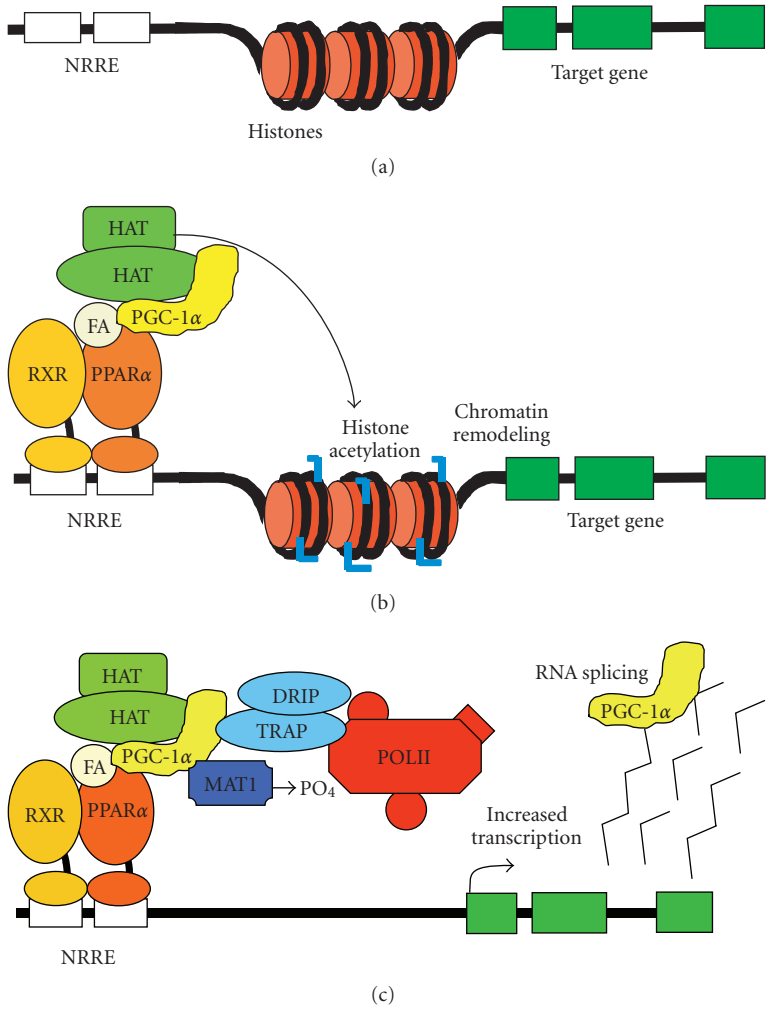

Figure 1: *Mechanisms of PPARα activation and PGC-1α coactivator activity.* Depiction of a potential PPARα target gene and nuclear receptor response element (NRRE) within the promoter region in the nonactivated state *(top)*. PPARα activation by fatty acid (FA) ligand leads to binding to the NRRE with its heterodimeric partner RXRα; and its coactivator PGC-1α. PGC-1α recruits additional coactivators with histone acetyltransferase (HAT) activity, which promotes chromatin unwinding and increases RNA polymerase II (POL II) access to the target gene promoter *(middle)*. PGC-1α also interacts with the TRAP/DRIP complex and with ménage-à-trois 1 (MAT1) which phosphorylates POL II to increase the probability of gene transcription. In addition, PGC-1α plays a role in RNA splicing via an RNA processing domain in its C-terminus *(bottom)*.

The cardiac phenotype of two separate lines of mice with constitutive PGC-1α deficiency also support an important role for PGC-1α in cardiac metabolism and function [39–41]. Both lines of PGC-1α-deficient mice exhibit impaired mitochondrial OXPHOS function and decreased expression of many genes encoding enzymes in mitochondrial metabolic pathways. PGC-1α deficiency also leads to cardiac dysfunction, especially in the context of pathophysiologic stimuli like pressure overload-induced cardiac hypertrophy [40, 41]. Interestingly, the severity of the cardiac functional phenotype varies between the two lines of knockout mice. One line exhibits age-associated cardiac dysfunction that is manifested by 7-8 months old as left ventricular chamber dilatation, diminished fractional shortening, and an activation of gene markers of cardiomyopathy [41]. Conversely, the other line of knockout mice exhibits no signs of cardiac dysfunction, but displays diminished chronotropic capacity in response to a β-adrenergic stimulus [39]. The mechanistic basis for this disparity in the two mouse mouse models is unknown. Collectively, these gain- and loss-of-function studies demonstrate that PGC-1α has a critical role in control of cardiac energy metabolism.

4. PPARα-PGC-1α-MEDIATED CONTROL OF METABOLISM IN RESPONSE TO DEVELOPMENTAL OR PHYSIOLOGIC CUES

Myocardial energy substrate preference is remarkably pliant and the heart can rapidly modulate fuel utilization depending upon the developmental stage, nutritional context, or disease state [42]. The PPARα-PGC-1α complex plays an important role in catalyzing these changes. For example,

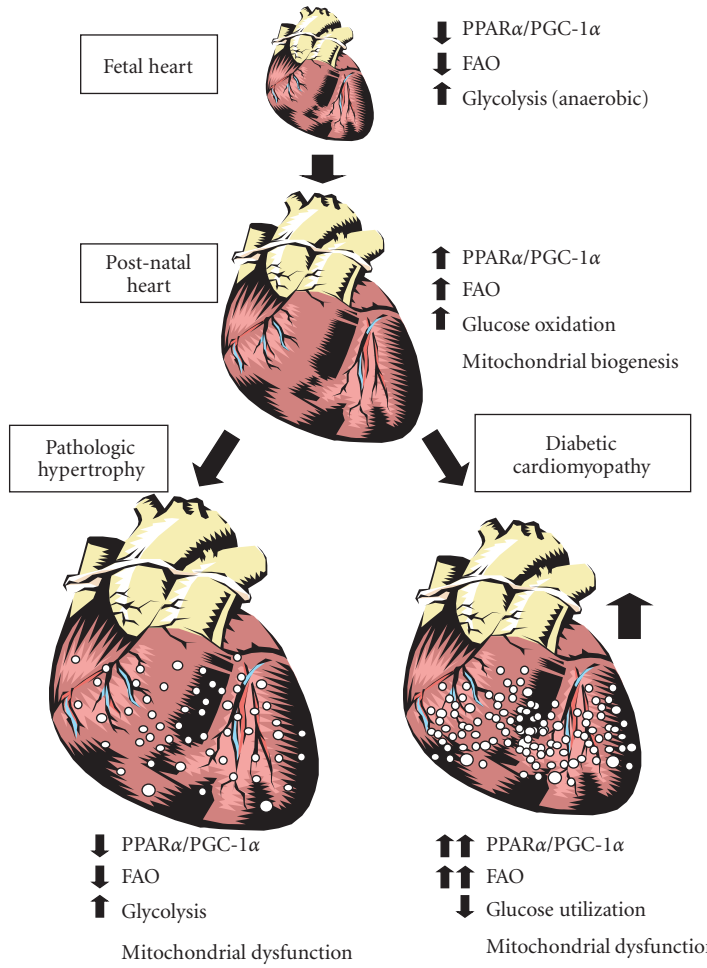

Figure 2: *Dynamic regulation of PPARα-PGC-1α complex activity in developing, failing, and diabetic heart.* Physiological cardiac growth resulting from postnatal maturation is associated with increased PPARα and PGC-1α expression and marked expansion of mitochondrial volume density and oxidative capacity. Conversely, pathologic hypertrophy is linked to decreased PPARα-PGC-1α expression and/or activity and diminished reliance on oxidative mitochondrial metabolism often leading to intramyocellular lipid accumulation. Finally, in the diabetic heart, PPARα-PGC-1α complex activity is increased along with the cardiac reliance on FAO. Despite of high-level FAO, the cardiac lipid accumulation is a hallmark of the diabetic heart and lipotoxicity may play a key role in the development of diabetic cardiomyopathy.

the fetal heart utilizes predominantly anaerobic glucose metabolism to fulfill its energy needs. However, almost immediately after birth, a rapid and profound developmental shift occurs. The workload of the heart is increased and the availability of fatty acids and oxygen for fuel becomes much greater (Figure 2). In response to these changes, the myocardium increases its reliance on mitochondrially derived ATP as an energy source through a coordinated induction of mitochondrially and nuclear-encoded genes involved in mitochondrial metabolism, structure, and function [43–45]. This developmental shift is accompanied by a robust activation of the PPARα-PGC-1α system [33, 43]; and it is likely that these two factors play a crucial role in this developmental switch.

Fasting is another physiologic context associated with a marked increase in PPARα-PGC-1α activity. To "spare" glucose for other organs that lack the capacity to catabolize fatty acids, the heart markedly increases its use of fatty acids under conditions of food deprivation [42]. Although the expression of the gene encoding PPARα is unaltered, the expression of PGC-1α is strongly induced [33]. Together with heightened availability of fatty acids that act as endogenous ligands for PPARα, this serves to rapidly amplify PPARα transcriptional activity. In fact, the expression of the broad program of myocardial FAO enzymes is markedly induced by food deprivation and this response is significantly blunted in mice lacking PPARα [10]. In sum, the PPARα-PGC-1α complex serves to regulate the capacity for FAO in response to physiologic cues that signal an increased need for mitochondrial fatty acid utilization.

5. ALTERED PPARα-PGC-1α SIGNALING IN THE FAILING HEART

Cardiac energy substrate metabolism is perturbed in the hypertrophied and failing heart, reverting to a program of

energy substrate metabolism similar to the "fetal" profile (Figure 2). Specifically, the myocardium shifts from dependence on FAO towards glucose utilization; primarily anaerobic glycolysis [46–49]. Importantly, this switch in energy substrate preference detected in various experimental models is also observed in humans with idiopathic dilated cardiomyopathy [50]. These changes in energy substrate preference are mediated, at least in part, by a downregulation of the genes encoding enzymes involved in FAO, OXPHOS, and the PPARα-PGC-1α complex [3, 48, 51–60]. The expression of the genes encoding PPARα and PGC-1α is known to be diminished in several rodent models of pressure overload or hypertensive heart disease [3, 40, 61], pacing-induced heart failure [62, 63], hypoxia [52], ischemic heart disease [55, 58, 59, 64], as well as genetically engineered models of heart failure [65–67]. The molecular mechanisms whereby pathologic stimuli lead to a transcriptional downregulation of PPARα and PGC-1α are not well understood, but may involve reactive oxygen species generation [64]. In addition, under pathologic conditions, PPARα activity is inhibited post-translationally through lower levels of the obligate heterodimeric partner of PPARα, retinoid X receptor (RXR) [57], and direct phosphorylation by the extracellular signal-related kinase and mitogen-activated protein kinase (ERK-MAPK) pathway [3]. These findings suggest that deactivation of the cardiac PPARα-PGC-1α axis in failing heart is a key component of the observed shift in energy metabolism. In support of this, reactivation of PPARα or PGC-1α prevents the downregulation of oxidative gene expression that occurs in cardiac myocytes challenged with pathologic stimuli [61, 63–65, 68, 69]. Experimental models have found altered metabolism and gene expression in both the hypertrophied and the overtly failing heart, but longitudinal evaluation of progressive changes in the PPARα-PGC-1α axis has not been done. Studies to evaluate the sequence of events will be crucial to understanding the role of altered metabolic regulation in disease progression.

One point that remains to be addressed is whether deactivation of oxidative metabolism and the PPARα-PGC-1α complex in the hypertrophied and failing heart is adaptive or maladaptive. The shift towards glycolysis allows continued ATP production with less oxygen consumption, and thus would appear to be an adaptive response. Indeed, overexpression of the GLUT1 glucose transporter prevented cardiac dysfunction in response to pressure overload [70]. Partial inhibitors of FAO also produce positive inotropic effects in patients with ischemic and nonischemic heart disease [71–76]. Ligand-mediated activation of PPARα in models of pressure overload [61] or ischemia [64] exacerbated ventricular dysfunction and pathologic remodeling. However, other reports show no ill effects of PPARα agonism or increased FAO in pathologic conditions [68, 69, 77]. Moreover, there is abundant evidence that chronic shifts towards glycolysis are maladaptive. Most reports suggest that PPARα agonists are beneficial in the response to ischemia [78–80] and various models of heart failure [63, 81–83]. Similarly, PGC-1α overexpression rescued the cardiac myocyte dysfunction and apoptosis in a mouse model of cardiomyopathy [65]. Mice with chronic reliance on glucose metabolism due to loss of cardiac lipoprotein lipase develop cardiac dysfunction with age and demonstrate significant mortality associated with the stress of aortic banding [84]. Finally, PPARα deficient animals that shift metabolism predominantly towards glucose oxidation exhibit age-associated cardiac fibrosis [85] and were unable to respond to increased workload and developed energy depletion [86].

The concept that the myocardium must maintain metabolic flexibility and a balance of substrate utilization during pathologic remodeling has recently pushed to the forefront. However, the biologic basis for this concept is unclear. It may be that chronic reliance on glucose as the predominant substrate is insufficient for ATP production in failing heart. Compared to FAO, glycolysis produces much less ATP per mole of substrate and there is evidence that long-term reliance on glycolysis leads to ATP deficiency in failing heart. Indeed, the phospho-creatine/ATP ratio is reduced in failing heart [49, 87–89] and a decline in this ratio is predictive of impending mortality in human heart failure patients [90]. The idea that energy starvation plays a significant role in the development of heart failure is also supported by severe cardiomyopathies in animal models with deletions in FAO enzymes [91, 92] or enzymes involved in mitochondrial ATP production [93–95]. Moreover, humans with inborn errors in these pathways often present with cardiomyopathy [96]. It is also possible that impairments in rates of FAO in failing heart are maladaptive because they lead to myocardial lipid accumulation (lipotoxicity) [97], which is linked to cardiac dysfunction [98–100]. Alternatively, or in addition, the inability to switch energy substrate preference in the context of changes in substrate availability could also contribute to pathologic remodeling.

6. PPARα AND PGC-1α IN THE DIABETIC HEART

Cardiovascular disease is exceptionally prevalent in patients with diabetes. Although the prevalence of dyslipidemias and hypertension certainly contributes to cardiovascular risk in diabetic subjects, cardiomyopathy is highly prevalent independent of these risk factors. Cardiomyopathy in diabetic subjects that occurs in the absence of known risk factors is often termed "diabetic cardiomyopathy" [101–104]. Unfortunately, the etiology of diabetic cardiomyopathy is poorly understood.

Evidence has emerged that abnormalities in myocardial energy metabolism play a significant role in the pathogenesis of diabetic cardiomyopathy. Indeed, in experimental models of uncontrolled diabetes (type 1 or 2), cardiac energy substrate flexibility becomes constrained and the diabetic heart relies almost exclusively on mitochondrial FAO for its ATP requirements [105–108]. Recently, these metabolic observations from animal models have also been confirmed in human subjects with type 1 diabetes [109]. The expression of PPARα, PGC-1α, and many target genes involved in FAO are increased in the murine insulin-resistant [15] and diabetic heart (type 1 and type 2) [11, 110, 111] and may play a key role in the observed metabolic switch to FAO. PPARα deficiency in the setting of insulin resistance [15] or

diabetes [110] blunts activation of FAO gene expression, suggesting that activation of the PPARα-PGC-1α regulatory network is critical for the increased FAO rates and lipid uptake seen in the diabetic heart. Consistent with this, transgenic mice that overexpress PPARα exclusively in the heart (MHC-PPARα mice) have a cardiac metabolic phenotype similar to that observed in diabetic heart, including accelerated rates of FAO, a striking diminution in glucose uptake and utilization, and a mitochondrial biogenic response [11, 15]. We have also observed that high-level fatty acid utilization in hearts of MHC-PPARα mice leads to the development of cardiac hypertrophy and dysfunction [11, 12]. We believe that sustained activation of the PPARα-PGC-1α complex in the insulin-resistant and diabetic heart promotes a state of metabolic inflexibility that leads to cardiomyopathic remodeling.

Despite high rates of FAO, myocardial lipid accumulation is a hallmark of the diabetic heart [112–116]. Prolonged accumulation of fats in the myocardium is believed to be highly toxic and is linked to the development of insulin resistance and cardiac dysfunction [12, 98–100, 114]. Our data suggest that PPARα drives this lipotoxic response in diabetic heart. The cardiomyopathic phenotype is relatively mild in unchallenged MHC-PPARα mice, but when the transgenic mice were given a high-fat diet, the cardiomyopathic phenotype was strikingly exacerbated; and mice exhibited clinical signs of heart failure, including depressed fractional shortening and ventricular chamber dilatation [12]. Pathologic remodeling in MHC-PPARα mice was accompanied by marked cardiac lipid accumulation. Moreover, genetic ablation of the fatty acid transporter CD36 in the context of PPARα overexpression prevents high-fat diet-induced cardiac lipid accumulation and dysfunction [16]. Finally, ligand-mediated activation of PPARα also drives lipid accumulation and an adverse outcome following ischemic insult [64]. These findings suggest that PPARα-driven lipotoxicity could be an important mechanism in cardiomyopathic remodeling of the diabetic heart.

Other components of the metabolic derangements in diabetic heart are abnormalities in mitochondrial ultrastructure and function [15, 111, 117–120]. Mitochondria isolated from diabetic rodents exhibit depressed rates of OXPHOS [117, 118] and diminished efficiency in ATP synthesis [120, 121], likely due to increased uncoupled respiration [121]. Mitochondrial proliferation is common in hearts of diabetic rodents [15, 119, 121, 122]. However, mitochondria from both type 1 and type 2 diabetic hearts often exhibit ultrastructural abnormalities, including degenerative cristae [15, 119]. The literature regarding the effects of insulin resistance and diabetes on mitochondrial gene expression is mixed with some reports showing an activation [15, 119] and others showing deactivation [123, 124]. We recently found that mitochondrial biogenesis and OXPHOS gene expression are increased in a mouse model of obesity-related insulin resistance [15]. These effects of insulin resistance were blunted in PPARα null mice and recapitulated in MHC-PPARα mice, suggesting that PPARα is involved in mitochondrial biogenesis in the myocardium in the context of insulin resistance, which was previously not well-appreciated.

7. CONCLUSIONS

In summary, the heart requires a continuous and abundant source of substrate to meet it high-energy demands. In situations where energy needs change, such as heart failure, the heart must adapt and will utilize the most efficient source of substrate (glucose) to meet its needs. Similarly, when glucose availability becomes limited, as it does in fasting or diabetes, the heart will adapt and use fatty acid to meet its ATP requirements. PPARα and PGC-1α play a central role in this metabolic flexibility by driving robust changes in gene expression of key components of mitochondrial biogenesis and metabolism. However, it is still not entirely clear whether long-term PPARα-PGC-1α-mediated alterations in energy metabolism are adaptive versus maladaptive changes for both heart failure and diabetic cardiomyopathy.

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Review Article

PPAR-γ in the Cardiovascular System

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Peroxisome proliferator-activated receptor-γ (PPAR-γ), an essential transcriptional mediator of adipogenesis, lipid metabolism, insulin sensitivity, and glucose homeostasis, is increasingly recognized as a key player in inflammatory cells and in cardiovascular diseases (CVD) such as hypertension, cardiac hypertrophy, congestive heart failure, and atherosclerosis. PPAR-γ agonists, the thiazolidinediones (TZDs), increase insulin sensitivity, lower blood glucose, decrease circulating free fatty acids and triglycerides, lower blood pressure, reduce inflammatory markers, and reduce atherosclerosis in insulin-resistant patients and animal models. Human genetic studies on PPAR-γ have revealed that functional changes in this nuclear receptor are associated with CVD. Recent controversial clinical studies raise the question of deleterious action of PPAR-γ agonists on the cardiovascular system. These complex interactions of metabolic responsive factors and cardiovascular disease promise to be important areas of focus for the future.

1. INTRODUCTION

Cardiovascular disease (CVD) is the major cause of morbidity and mortality in developed countries [1]. Searching for the underlying risk factors has revealed that a cluster of contributors is often present simultaneously. This risk factor clustering, most notably the core trio of insulin resistance, dyslipidemia, and hypertension, has been called by a number of different names including metabolic syndrome (MetS), insulin resistance syndrome, the deadly quartet, and Syndrome X [1–7]. Although somewhat controversial, the usefulness of clustering this syndrome remains clear. The mechanistic connections among the trio are not completely understood.

A major focus is to understand the biological and molecular mechanisms underlying this syndrome and to develop better treatment. One class of molecules that are proposed to be important in the etiology of MetS is the nutrient-sensing nuclear transcription factors, peroxisome proliferator-activated receptors (PPARs), and the related liver X receptors (LXRs) [7]. Among these nuclear receptors, PPAR-γ is of intense interest, not only because its ligands, thiazolidinediones (TZDs), are clinically used for T2DM, but also because it may be a nexus that connects metabolic disorders to CVD [1, 4–9]. In addition to its important roles in insulin sensitivity and glucose homeostasis, PPAR-γ is also associated with CVD such as coronary heart disease, atherosclerosis, and stroke [7, 10]. MetS is also linked to cardiac hypertrophy because populations with MetS have higher prevalence of cardiac hypertrophy [11–14]. However, action of PPAR-γ agonists is not only of metabolism in insulin responsive tissues, but also more directly in the inflammatory, cardiac, and vascular cells. The components of MetS are common risk factors to CVD [1]. In this review, we will focus on PPAR-γ in the cardiovascular (CV) system, including its expression, gain, and loss of function, and the mechanisms by which it functions in cardiovascular cells.

2. PPAR-γ GENE AND ITS EXPRESSION OF CV-RELEVANT TISSUES

PPAR-γ is the most extensively studied PPAR, even though the cloning of this receptor came four years later than that of PPAR-α [15]. The PPAR-γ gene extends over more than 100 kb of genomic DNA. It includes six common coding exons: one exon for the N-terminal A/B domain, two exons for the DNA binding domain, with each one encoding one

of the two zinc fingers, one exon for the hinge region, and two exons for the ligand binding domain in the C-terminal region [16, 17]. There are two major splice isoforms in the mouse, PPAR-γ1 and PPAR-γ2, whereas at least two other isoforms, PPAR-γ3 and PPAR-γ4, have also been identified in other species including humans [16]. PPAR-γ1 is encoded by eight exons, comprising two γ1-specific exons, A1 and A2, that constitute the 5′-untranslated region, and the six coding exons that are common to both γ1 and γ2 mRNAs. The PPAR-γ1 protein consists of 477 amino acids [16]. The PPAR-γ2 mRNA is composed of seven exons, the additional one, exon B, comprising the γ2 5′-untranslated region and an additional N-terminal amino acid sequence specific for γ2. As a result, PPAR-γ2 is a larger protein, consisting of 505 amino acids [16].

The function PPAR-γ was initially recognized in adipose tissue [18], although its expression was first identified in other tissues [15]. It is well expressed in cardiovascular-system-relevant tissues such as heart, endothelium, vascular smooth muscle, kidney, and macrophages [10, 19–23]. PPAR-γ2 is mainly expressed in adipocytes while PPAR-γ1 is more widely expressed [23].

3. PPAR-γ LIGANDS

Natural ligands

Several polyunsaturated fatty acids and their metabolites have been identified as PPAR-γ ligands although no ligand has clearly been identified as a critical physiologic ligand. Endogenous ligands including 15-deoxy-$\Delta^{12,14}$ prostaglandin J2 (15-d-PGJ2) [24]. 15-d-PGJ2 had been one of the most promising candidates for the endogenous PPAR-γ ligand. It binds to PPAR-γ with a dissociation constant (K_d) in the low-micromolar range and can activate PPAR-γ target genes at concentrations at or near the K_d [25]. However, it has never been definitively proven to exist in vivo, nor are its effects that are specific to PPAR-γ [25]. Other natural ligands of PPAR-γ include 9- and 13-hydroxyoctadecadienoic acid (HODE), which are components of oxidized low-density lipoprotein [26], and 12- and 15-hydroxyeicosatetraenoic acid (HETE) [27]. Some researchers have argued that fatty acids are the important natural ligands although they are fairly low affinity ligands. A recent class of high-affinity ligands, the nitrolipids, has been identified, but their physiologic function and the role of PPAR-γ in their effects have not yet been fully delineated [28].

Synthetic ligands

TZDs, or "glitazones," are a class of pharmaceutical compounds used clinically as insulin sensitizers in patients with T2DM [17]. The first clinically used agent in this class, troglitazone (Rezulin), was removed from the market because of rare but life-threatening hepatic toxicity. Fortunately, its successors, rosiglitazone (Avandia), and pioglitazone (Actos), have not been linked to this side effect [23]. More than 15 million prescriptions for these TZDs are dispensed annually in the United States alone. However, adverse effects such as edema and weight gain have been problematic [23]. Another side effect of TZDs in animals is cardiac hypertrophy, which has limited the approved doses of these drugs for clinical use [29–33]. More recently, increased risks of myocardial infarction and possibly death from cardiovascular causes have been reported to be associated with rosiglitazone (Avandia) treatment [34], although the result is controversial [35].

TZDs' "on-target" and "off-target" effects

Compelling evidence has shown that PPAR-γ is the main target of TZDs. PPAR-γ mediates the insulin sensitizing effects of TZDs in fat, skeletal muscle, and liver [36–39]. TZDs' effects on fluid retention and weight gain are also dependent on PPAR-γ [40, 41]. However, several studies demonstrate that some of TZDs' effects are independent of PPAR-γ or "off-target." In macrophages, it has been recognized that although TZDs modulate lipid metabolism through PPAR-γ, some of TZDs' anti-inflammatory effects are independent of it [42] although only at higher doses. Some of the antiproliferative effects of TZDs in embryonic stem cells [43] or cancer cell lines [44, 45] are independent of PPAR-γ. Further, PPAR-γ in skeletal muscle and liver may not be mediating TZDs' insulin sensitizing effects under different conditions or in different models [38, 39, 46]. Loss-of-function studies have provided additional insight on the possible "off-target" effects of TZDs.

Understanding which TZD effects are PPAR-γ independent is an important issue for designing more specific PPAR-γ agonists with fewer side effects. TZDs induce cardiac hypertrophy in animals [29–33] independent of cardiac PPAR-γ [47]. TZDs also increase the incidence of congestive heart failure [48] presumably due to fluid retention caused by PPAR-γ activation in the kidney [40, 41]. Myocardial infarction incidence is increased in meta-analysis of clinical trials [34], but it is not known whether this side effect is mediated by PPAR-γ or whether this finding will be confirmed in a prospective study [35]. Further, defining the role of PPAR-γ in these effects would provide guidance for the design of the next generation of TZDs.

4. GAIN AND LOSS OF PPAR-γ FUNCTION IN THE CV SYSTEM

Although originally found to be critical in adipogenesis and regulating insulin signaling, PPAR-γ is also important in CV system [16, 17, 49, 50]. Human genetic studies have revealed that PPAR-γ mutation in humans can result in either gain-of-function or loss-of-function [51]. In animals, gain-of-function studies of PPAR-γ have mostly utilized agonists, particularly synthetic ones (TZDs); Loss-of-function studies have used knockdown or knockout and transgenic mouse models of mutant PPAR-γ, which are powerful tools to study physiological mechanisms. The outcome of these approaches in studying PPAR-γ in CV system has been fruitful and sometimes surprising.

Human mutations

Pro12Ala mutation is a loss-of-function mutation and has been reported to be associated with not only increased protection against insulin resistance and type-2 diabetes [52–56], but also a decreased incidence of myocardial infraction [57] and lower diastolic blood pressure [58]. These cardiovascular effects are likely independent of metabolic impact of this mutation [57, 58].

Pro467Leu, Val290Met, Phe388Leu, and Arg425Cys are all loss-of-function mutations (dominant negative) and have been associated with partial lipodystrophy, insulin resistance, diabetes, and hypertension [59–62], although it is not known whether the elevated blood pressure is due to impaired insulin sensitivity.

C161T mutation is a silent polymorphism and has been reported to be associated with reduction in coronary artery disease, likely independent of obesity and of lipid abnormalities, possibly through direct effects on local vascular wall, implicating the protective role of PPAR-γ in atherogenesis [63].

However, ligand binding domain mutants of PPAR-γ with dominant negative actions have been shown to be promiscuous, stimulate associations with nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid receptors (SMRT), and inhibit activities of all three wild-type PPARs [64]. These less specific properties of the mutants need to be explored in the human mutations to determine which effects on metabolic syndrome are mediated through PPAR-γ or the PPARs, PPAR-α or PPAR-δ.

Germline gene inactivation

Homozygous germline PPAR-γ knockout mice die at E10 due to defects in trophoblast [65, 66]. Heterozygous mutations are viable, have less adipose tissue, and are more insulin sensitive than wild-type counterparts [67, 68]. This illustrates the complex response to gene dosage of PPAR-γ.

PPAR-γ and hypertension

PPAR-γ agonists can lower blood pressure and this effect may be at least partially independent of their insulin-sensitizing effects [17, 49, 50]. Dominant negative mutation of PPAR-γ (Pro467Leu) in mice results in hypertension and fat redistribution but not insulin resistance or diabetes seen in the same mutation in humans [69]. Another line of dominant negative PPAR-γ mutant mice (Leu466Ala) have hypertension (female only) and insulin resistance [70]. As discussed above, dominant negative PPAR-γ mutants can be more promiscuous, inhibiting activity of other PPARs, so we cannot at this point conclude that these mutants strictly act by altering only PPAR-γ activity [64]. Therefore, comparison to loss of function mutants and knockouts is critical.

Embryonic lethality of the germline PPAR-γ knockout has been rescued by breeding Mox2-Cre, in which Cre recombinase is expressed in epiblast-derived tissue but not other tissues, to floxed PPAR-γ mice [71]. The generalized PPAR-γ knockout mice have lipodystrophy and insulin resistance as expected. Surprisingly, they have hypotension rather than hypertension. This is paradoxical because PPAR-γ agonists lower blood pressure [72–74]. Knockout and agonist having the same phenotype may be resolved by testing the hypothesis that PPAR-γ suppresses certain key gene expression to control blood pressure and that both agonist and knockout can relieve the suppression. Further, the phenotypes in the generalized PPAR-γ knockout mice suggest that hypertension is separable from lipodystrophy or insulin resistance, even though they are highly associated in humans [59, 61] and in A-ZIP mice [71]. Mechanistically, the vasculature from these generalized PPAR-γ knockout mice has defects in both relaxation and contraction, contributing to the hypotension.

It has not been completely determined whether the hypotension phenotype seen in the generalized PPAR-γ knockout mice is attributable to PPAR-γ deficiency in vascular endothelium or smooth muscle or both. Endothelium-specific PPAR-γ knockout mice are reported to be not having any phenotype at baseline, although this study only used tail cuff to measure the blood pressure [75]. When fed with high fat diet, they have higher blood pressure and heart rate than their wild type control mice. Rosiglitazone does not affect the diet-induced hypertension in these knockout mice, although the decrease of blood pressure typically seen in treated wild-type control mice was not reported [75]. Smooth muscle-specific PPAR-γ knockout mouse model may clarify the role of smooth muscle PPAR-γ in blood pressure regulation.

The kidney is an important organ in controlling blood pressure and PPAR-γ is expressed in this organ, although PPAR-γ deficiency in collecting duct does not alter blood pressure in mice [40, 41]. The knockout does block weight gain, fluid retention, and blood volume expansion caused by TZDs [40, 41]. These results indicate that PPAR-γ deficiency in collecting duct is unlikely to contribute to the hypotension phenotype seen in the generalized PPAR-γ knockout mice [71].

PPAR-γ and cardiac hypertrophy

PPAR-γ agonists have been shown to inhibit hypertrophy of cultured neonatal rat ventricular cardiomyocytes induced by mechanical stress or angiotensin II, and cardiac hypertrophy induced by aortic constriction in rats and mice [76–78]. The inhibition on hypertrophy was accompanied by the inhibition on expression of embryonic genes, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), skeletal α-Actin, as well as that of endothelin-1 that can induce cardiac hypertrophy [76–78]. Aortic constriction causes more profound cardiac hypertrophy in heterozygous PPAR-γ knockout mice than wild-type controls, further indicating the involvement of PPAR-γ in cardiac growth [77]. Nuclear Factor-Kappa Beta (NF-κB) pathway is at least partially mediating the inhibition on hypertrophic growth in vitro [76]. More recently, pioglitazone has been reported to inhibit the gene expression of inflammatory cytokines such as interleukin (IL)-1β, IL-6 [79], suggesting the possible involvement of PPAR-γ's anti-inflammatory activity in cardiac

hypertrophy. Paradoxically, TZDs also induce cardiac hypertrophy in mice, rats, and dogs [30–33].

The cardiomyocyte-specific PPAR-γ knockout mice have age-progressive cardiac hypertrophy with preserved systolic cardiac function [47]. Rosiglitazone induce cardiac hypertrophy in both knockout mice and wild-type littermate control mice, demonstrating that this TZD's hypertrophic effect is at least partially independent of PPAR-γ in cardiomyocytes [47]. Whether the cardiac hypertrophy caused by rosiglitazone occurs through PPAR-γ independent effects in cardiomyocytes, PPAR-γ in nonmyocyte cells, or blood volume expansion [33, 48], remains to be further determined. Studying these knockout mice under pathological stimulations such as pressure overload may yield more meaningful results to understand the function of PPAR-γ in the heart.

Rosiglitazone and cardiomyocyte-specific PPAR-γ knockout activate distinctly different hypertrophic pathways [47]. Rosiglitazone increases phosphorylation of both p38 mitogen-activated protein kinase (p38-MAPK) and extracellular signal-related kinase (ERK) 1/2 in the heart. The activation of p38-MAPK is independent of cardiomyocyte PPAR-γ and that of ERK1/2 is dependent. The activation of either ERKs or p38-MAPK is sufficient to induce hypertrophy [80, 81] and may therefore contribute to the cardiac hypertrophy induced by rosiglitazone.

The cardiomyocyte-specific PPAR-γ knockout mouse hearts were found to have increased expression of cardiac embryonic genes ANP and β-myosin heavy chain (β-MHC), and elevated NF-κB activity. Embryonic gene expression in adult hearts is a one of the characteristics of pathological cardiac hypertrophy [82, 83]. NF-κB is both necessary and sufficient for hypertrophic growth of cardiomyocytes [84]. Therefore, NF-κB activation is likely to be part of the mechanisms that PPAR-γ deficiency in cardiomyocytes induces cardiac hypertrophy. However, the interaction between these two transcription factors needs to be further characterized.

Another cardiomyocyte-specific PPAR-γ knockout mouse line also has progressive cardiac hypertrophy and accompanied elevation of cardiac gene expression (ANP and skeletal α-actin) [85]. However, these mice also have dilated cardiomyopathy, heart failure, and mitochondrial oxidative damage. Increased myocardial superoxide content, instead of NF-κB activation, seems to be mediating the severe cardiac phenotype [85]. Similarly, when different floxed PPAR-γ mice were used to delete this receptor in skeletal muscle specifically, one mouse line [37] had more severe phenotypes than the other [46]. It is not clear whether these phenotypic differences are because of the different genetic design for the deletion or purely genetic background differences.

PPAR-γ, inflammation, and atherosclerosis

PPAR-γ is not only expressed in macrophages, endothelial cells, and smooth muscle cells in normal vasculature [10, 19–23], but also in atherosclerotic lesions [86, 87]. PPAR-γ agonists reduce atherosclerosis in human patients and animal models [88–92] even though there were concerns that these compounds could be proatherogenic because they may promote the macrophages uptake of lipids and speed the foam cell formation [26]. These antiatherogenic effects can be independent of their beneficial effects on metabolism [93, 94]. More direct antiatherogenic function of PPAR-γ was demonstrated by the result that transplantation of PPAR-γ deficient bone marrow to low-density lipoprotein (LDL) receptor null mice led to a significant increase in atherosclerosis [95]. The beneficial effects are largely attributable to PPAR-γ's anti-inflammation activity and its role in modulating lipid homeostasis in macrophages.

Vascular inflammation has been increasingly appreciated as an important factor in the pathogenesis of atherosclerosis [96–99]. The importance of macrophage PPAR-γ in CVD has begun to be appreciated since foam cells in atherosclerotic lesions were found to have high level of PPAR-γ expression [86, 87]. PPAR-γ activation decreases inflammatory cytokines (e.g., tumor necrosis factor-α, IL-6, and IL-1β) produced by macrophages [100, 101]. By inducing the expression of LXR-α and ATP-binding cassette A1, PPAR-γ activation promotes cholesterol efflux from macrophages resulting in inhibition of foam cell formation [95]. Consistently, macrophage-specific PPAR-γ knockout mice have reduced basal cholesterol efflux, most likely because of decreased expression of lipoprotein lipase, scavenger receptor CD36, LXR-α, and ATP-binding cassette G1 [21]. More profound effects on macrophages by PPAR-γ are also possible since it has been recently shown that PPAR-γ controls alternative activation of macrophages and can thereby improve insulin resistance [102]. It is likely that this effect on differentiation of macrophages is also important in effects of CVD.

Endothelial cells play a key role in the inflammatory process of vasculature responding to injuries [96–99]. TZDs have been shown to reduce superoxide generation and inhibit the expression of vascular cell adhesion molecule-1, intercellular cell adhesion molecule-1, and lectinlike oxidized LDL receptor, and hence inhibit inflammation in endothelial cells [103–106], suggesting an important role of endothelial PPAR-γ in the development of atherosclerosis. The existing endothelium-specific PPAR-γ knockout mice [75] and generalized PPAR-γ knockout mice [71] can be useful tools to study the function of endothelial PPAR-γ in atherosclerosis.

Different cell types are reported to have different mechanisms for PPAR-γ to inhibit inflammation. In intestinal Caco-2 cells, PPAR-γ inhibits inflammation by binding to NF-κB and facilitating its nuclear export [107]. In macrophages, PPAR-γ prevents corepressor complex N-CoR dissociation from the promoters of NF-κB responsive inflammatory gene inducible nitric oxide synthase and therefore represses its expression [108]. It remains to be determined whether PPAR-γ in endothelial or other vascular cells uses one of these pathways or an entirely different mechanism.

The growth and movement of vascular smooth muscle cell within neointima is one of the key steps leading to the formation of atherosclerotic plaque [96]. PPAR-γ agonists have been shown to block the proliferation and increase the apoptosis of vascular smooth muscle cells, suggesting more beneficial effects of PPAR-γ activation in vasculature [109, 110].

Table 1: Cardiovascular phenotypes in gain and loss of PPAR-γ function.

			CH		MI		BP		CAD		AS	
TZDs	Agonism	Gain of function	↑ *	[30–33, 47]	↑ **	[34]	↓	[17, 49, 50]			↓	[87–91]
Pro12Ala	Human mutation	Loss of function			↓	[57]	↓	[58]				
Pro467Leu	Human mutation	Loss of function					↑	[59, 60]				
Val290Met	Human mutation	Loss of function					↑	[60]				
Phe388Leu	Human mutation	Loss of function					↑	[61]				
Arg425Cys	Human mutation	Loss of function					↑	[62]				
C161T	Human mutation	Loss of function							↓	[63]		
Pro467Leu	Mouse mutation	Loss of function					↑	[69]				
Leu466Ala	Mouse mutation	Loss of function					↑	[70]				
Generalized KO	Transgenic mouse	Loss of function	↑	[71]			↓	[71]				
Cardiac KO	Transgenic mouse	Loss of function	↑	[47]								
Endothelial KO	Transgenic mouse	Loss of function					→	[75]				
Collecting duct KO	Transgenic mouse	Loss of function					→	[40, 41]				

TZDs: thiazolidinediones; KO: knockout; CH: cardiac hypertrophy;
MI: myocardial infarction; BP: blood pressure; CAD: coronary artery disease; AS: atherosclerosis
*: in animals only; **: rosiglitazone only
Numbers in square brackets are the reference numbers.

PPAR-γ and cardiac remodeling

Cardiac remodeling after ischemic injury is one of the major causes that lead to heart failure [111, 112]. The remodeling process is characterized by myocyte hypertrophy and cardiac fibrosis [111, 112]. PPAR-γ agonists attenuate this remodeling process after ischemia in experimental animals [113]. Recent in vitro studies on PPAR-γ in cardiac fibroblasts, a major source of fibrillar collagens that lead to fibrosis [111, 112], have revealed more mechanistic insights.

Pioglitazone reduces cell growth, synthesis of collagen type I, and expression of matrix metalloproteinase-1 in cardiac fibroblasts undergone anoxia-reoxygenation or treated with angiotensin II, likely through inhibition of reactive oxygen species generation and NF-κB activation [114, 115]. Brain natriuretic peptide has been implicated in these effects [116]. In cultured cardiac fibroblasts, PPAR-γ agonists induce the expression of vascular endothelial growth factor, a crucial player in the infarcted/ischemic heart, further indicating the beneficial effects of PPAR-γ agonists in cardiac remodeling [117]. However, all of these studies are based upon gain-of-function results. Further investigation using loss-of-function studies would advance our understanding the role of PPAR-γ in cardiac fibroblasts and cardiac remodeling and provide more therapeutic guidance.

PPAR-γ in cardiovascular side effects of TZDs

Despite the obviously beneficial effects that TZDs have in CV system [118], these compounds have some cardiovascular side effects that are dangerous to be overlooked. As mentioned above, TZDs induce cardiac hypertrophy in animals, a limitation to the dosages in their clinic use.

Congestive heart failure remains to be one of the major contraindications to the clinical use of TZDs [48]. This is presumed to be secondary to the fluid retention caused by activation of PPAR-γ in the kidney, likely in the collecting duct [40, 41]. Collecting duct knockouts of PPAR-γ is able to excrete salt loads more easily although there is no end effect on blood pressure on normal salt diets [40, 41]. PPAR-γ knockout blocked the effect of TZD on mRNA expression of the sodium channel ENaC-γ although the baseline level in the knockout was higher [40].

One recent report regarding the association between rosiglitazone treatment and significantly increased risk in myocardial infarction as well as an increased risk with borderline significance in death from cardiovascular causes has brought a lot of attention to the safety of this drug [34]. The findings were based on limited access to the original data, and meta-analysis used to reach the conclusions is always considered less convincing than a large prospective trial designed to assess the outcome of interest. Such a prospective study is indeed ongoing and the investigators performed an interim analysis and found that rosiglitazone was not significantly associated with increased risk of myocardial infarction and death from cardiovascular causes, although the findings were inconclusive because of the incompleteness of the study [35]. One side effect of rosiglitazone this interim analysis did confirm is the increased incidence of heart failure [35].

Although the findings need to be confirmed, the possible adverse effects of rosiglitazone in myocardial infarction and death from cardiovascular causes are worrisome due to the fact that diabetic patients are already at higher risk for cardiovascular diseases. The mechanisms of the possible adverse effects are uncertain, and could involve myocardial as well as vascular changes. Pioglitazone, another member in the same TZD class, does not seem to have these side effects [119]. In comparison to rosiglitazone, pioglitazone appears to have more beneficial effects on lipid profile [120], which may be one of the contributors to these side effects. However, the exact mechanisms and molecular basis are yet to be explored. In order to ultimately understand this drug and help

new drug design, it is critical to address questions such as whether PPAR-γ is mediating these effects of rosiglitazone and whether heart (cardiomyocytes, cardiac fibroblasts, endothelial cells, or smooth muscle cells) is the direct target.

The cardiovascular phenotypes of these gain- or loss-of function studies are summarized in Table 1.

5. CONCLUSIONS

PPAR-γ is now firmly established as an important player in cardiovascular diseases. Understanding the mechanisms of PPAR-γ action in heart and vascular cells where action on NF-κB appears to be important in controlling growth and inflammation may lead to improved targeting of the PPAR-γ activity in these cells. The interactions of PPAR-γ with other nuclear transcription factors which have partially overlapping effects such as the PPAR-α, PPAR-δ, and LXR will likely reveal a complex control system of inflammatory and growth responses to nutrient signaling.

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Review Article

Transcriptional Control of Vascular Smooth Muscle Cell Proliferation by Peroxisome Proliferator-Activated Receptor-γ: Therapeutic Implications for Cardiovascular Diseases

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Proliferation of vascular smooth muscle cells (SMCs) is a critical process for the development of atherosclerosis and complications of procedures used to treat atherosclerotic diseases, including postangioplasty restenosis, vein graft failure, and transplant vasculopathy. Peroxisome proliferator-activated receptor (PPAR) γ is a member of the nuclear hormone receptor superfamily and the molecular target for the thiazolidinediones (TZD), used clinically to treat insulin resistance in patients with type 2 diabetes. In addition to their efficacy to improve insulin sensitivity, TZD exert a broad spectrum of pleiotropic beneficial effects on vascular gene expression programs. In SMCs, PPARγ is prominently upregulated during neointima formation and suppresses the proliferative response to injury of the arterial wall. Among the molecular target genes regulated by PPARγ in SMCs are genes encoding proteins involved in the regulation of cell-cycle progression, cellular senescence, and apoptosis. This inhibition of SMC proliferation is likely to contribute to the prevention of atherosclerosis and postangioplasty restenosis observed in animal models and proof-of-concept clinical studies. This review will summarize the transcriptional target genes regulated by PPARγ in SMCs and outline the therapeutic implications of PPARγ activation for the treatment and prevention of atherosclerosis and its complications.

1. INTRODUCTION

Most cardiovascular diseases result from complications of atherosclerosis, which is a multifactorial process characterized by chronic inflammation, lipid accumulation, and the formation of a complex atherosclerotic lesion [1]. Recruitment of monocytes, their differentiation into macrophages, and uptake of LDL-derived cholesterol are the major cellular events contributing to early fatty streak formation [2, 3]. Continued intracellular cholesterol accumulation results in the generation of endogenous inducers of inflammatory and proliferative gene expression and a broad range of cellular and humoral responses contributing to lesion initiation and progression [4]. The resulting chronic inflammatory state and the enrichment of lipid-laden macrophages ultimately lead to the formation of a complex atherosclerotic lesion [5].

During the course of atherosclerotic lesion formation, secreted growth factors and cytokines promote the migration and proliferation of vascular smooth muscle cells (SMCs) to contribute to neointima formation [6]. This chronic proliferative response of SMCs promotes further lesion development through the production of proinflammatory mediators and the synthesis of extracellular matrix molecules, which is required for the retention of lipoproteins and often constitutes the majority of the protein content of the advanced lesion responsible for luminal obstruction [1]. However, SMC proliferation within the developing lesion may also exert beneficial effects by forming a fibrous cap covering the advanced atherosclerotic lesion, an important mechanism for the stability of the plaque [7]. The result of this chronic process is the development of an advanced atherosclerotic lesion, which may ultimately cause luminal obstruction and ischemic complications.

Once occlusive atherosclerotic disease has developed, the standard of care may include angioplasty, coronary artery bypass grafting, or cardiac transplantation. However, all current treatment approaches are limited by a varying degree of treatment failure and reocclusion of the arterial lumen.

Among the cellular mechanisms responsible for this failure of the current interventional procedures used to treat occlusive atherosclerotic diseases, such as postangioplasty restenosis, transplant vasculopathy, and coronary artery bypass graft failure, SMC proliferation constitutes a prime mechanism [6]. In the past decade, elegant progress in interventional cardiology has provided the introduction of drug-eluting stents delivering rapamycin or paclitaxel into the vessel wall that target SMC proliferation [8]. However, despite initial enthusiasm, the complete inhibition of the healing response using these approaches may leave a thrombogenic vessel surface at risk of in-stent thrombosis and vessel occlusion [9]. Thus, despite these advances, ideal therapy for occlusive vascular disease is still far from established.

In an era marked by the increasing prevalence of obesity, diabetes, and cardiovascular disease, members of the nuclear hormone receptor superfamily have emerged as transcription factors that regulate diverse aspects of metabolism [10, 11]. In addition to their function to act as molecular sensors of lipid and carbohydrate homeostasis, several members of the nuclear hormone receptor family, including the peroxisome proliferator activated receptor (PPAR) γ, also exert beneficial pleiotropic effects to reduce atherosclerosis and its complications [12, 13]. PPARγ is the molecular target for the synthetic thiazolidinediones (TZD), such as rosiglitazone and pioglitazone, clinically used as insulin sensitizers in patients with type 2 diabetes [14]. Over the last decade, a wealth of evidence has supported a beneficial role for TZD PPARγ agonists in the regulation of vascular gene expression programs [12, 13]. While PPARγ expression itself is increased in response to vascular injury [15–17], its activation by TZD suppresses SMC proliferation through several mechanisms involving the regulation of genes encoding proteins in SMC migration [15], proliferation [15], differentiation [18], senescence [19], and apoptosis [16]. In the following review, we will discuss the role of PPARγ in vascular biology with respect to the control of proliferative gene expression programs that underlie SMC proliferation and the development of cardiovascular diseases.

2. PPARγ: A LIGAND-ACTIVATED TRANSCRIPTON FACTOR EXPRESSED IN VASCULAR CELLS

The detailed structure and molecular biology of PPARγ have previously been outlined in excellent review articles [11, 20]. Briefly, the PPAR subfamily of nuclear receptors consists of 3 isoforms, that is, PPARα (NR1C1), PPARβ (also known as δ, NR1C2), and PPARγ (NR1C3). PPARs regulate gene expression upon heterodimerization with the retinoid X receptor (RXR, or NR2B) and subsequent binding to specific response elements located in the promoter regions of target genes. Although presently there are no proven pathways for endogenous ligands in vivo, all PPARs are activated in vitro by fatty acids (FAs). PPARγ is activated by the prostaglandin D2 derivative 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2) [21] and forms of oxidized linoleic acid, 9- and 13(S)-HODE [22]. Synthetic PPARγ ligands include TZD, such as troglitazone, rosiglitazone, and pioglitazone, as well as non-TZD derivates. PPARγ is predominantly expressed in adipose tissue and has been characterized as an important regulator of adipocyte differentiation and glucose homeostasis [14]. Based on their efficacy to improve insulin sensitivity, the TZD PPARγ ligands rosiglitazone and pioglitazone are currently being utilized in clinical practice to treat insulin resistance in patients with type 2 diabetes [23, 24].

In addition to the metabolic effect of PPARγ, the receptor is expressed in atherosclerotic lesions [15, 25] and in all vascular cell types including endothelial cells (EC) [26], macrophages [27], T lymphocytes [28], and SMCs [29]. In EC, PPARγ is activated in response to atheroprotective laminar flow [30]. Ligand-induced activation of PPARγ in these cells suppresses the expression of genes responsible for the adhesion of monocytes to the endothelium (i.e., VCAM-1 [31, 32], ICAM-1 [33]) and their transendothelial migration [34], which are both crucial early processes for the subsequent development of atherosclerosis. In macrophage biology, PPARγ has been demonstrated to suppress inflammatory gene expression and to decrease intracellular lipid accumulation and foam-cell formation [35, 36]. Finally, increased PPARγ expression has been demonstrated in neointimal layers during atherosclerotic lesion development [15, 25]. Concomitant with the phenotypic shift from quiescent SMCs resident in the uninjured vessel wall to proliferating SMCs in the neointima, PPARγ expression is induced in the neointima following vascular injury [15, 16]. Considering the importance of SMC proliferation during atherosclerosis and its complications [6], this increased expression of PPARγ in neointimal SMCs has provided an important rationale to further exploit the role of PPARγ for the proliferative response that underlies the development of neointima formation and atherosclerotic cardiovascular diseases.

3. TRANSCRIPTIONAL REGULATION OF SMOOTH MUSCLE CELL PROLIFERATION BY PPARγ LIGANDS

The physiological state of the SMCs in the arterial wall is determined by endogenous and exogenous signals, and often the endpoint that interpretates these signals is gene transcription [37]. Emerging evidence has implicated PPARγ as a key transcriptional modulator of SMC function. In the following section, we outline the role of PPARγ in the regulation of diverse SMC processes including cell proliferation, cell-cycle progression, senescence, and apoptosis (see Figure 1).

3.1. Regulation of SMC proliferation and cell-cycle progression by PPARγ agonists

Mitogenic growth factors secreted during vascular injury converge into a final common signaling pathway regulating the proliferative response of SMCs: the cell-cycle [6] (see Figure 2). While SMCs are in a quiescent state (G_0) in the uninjured artery, they transit in response to mitogenic stimulation through the G_1 phase of the cell-cycle and ultimately enter S phase to undergo replication [38]. Cell-cycle progression is under the control of cyclins and cyclin-dependent kinases (CDKs), which phosphorylate the retinoblastoma gene product (pRB) [39]. pRB

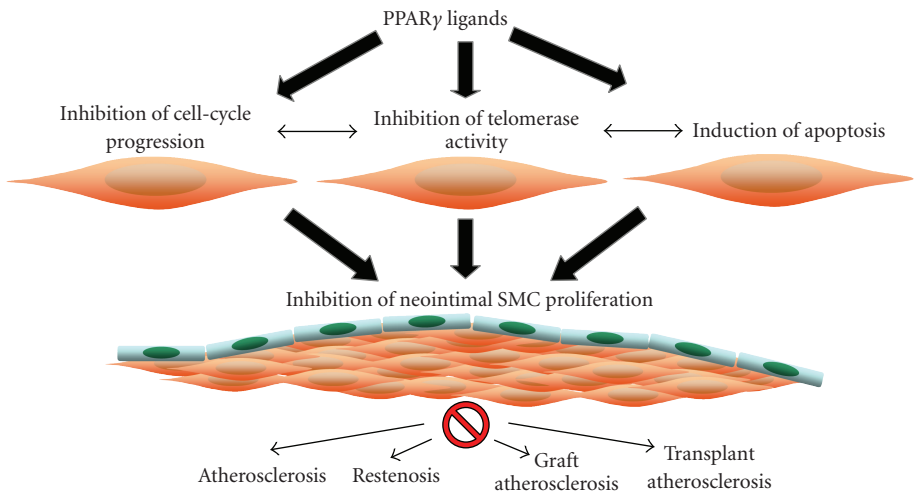

Figure 1: Cellular mechanisms involved in the inhibition of SMC proliferation by PPARγ during cardiovascular diseases. PPARγ regulates genes encoding proteins involved in diverse SMC processes including cell proliferation, cell-cycle progression, senescence, and apoptosis.

phosphorylation represents the critical checkpoint of the $G_1 \rightarrow S$ phase transition and increased pRB phosphorylation correlates with the induction of SMC proliferation in injured vessels [40, 41]. Consistent with this, maintenance of high levels of phosphorylated pRB is required for the development of intimal hyperplasia. Upon pRB phosphorylation, sequestered E2F transcription factors are released to induce the transcription of genes involved in the regulation of S phase DNA synthesis [42]. Through CDK-inhibitors (CDKI), including $p27^{Kip1}$, the activity of cyclin/CDK complexes in quiescent SMCs is inhibited providing a second layer of regulation [43, 44]. In response to mitogens, $p27^{Kip1}$ undergoes ubiquitination and degradation through the proteasome pathway allowing CDK/cyclin complexes to phosphorylate pRB [45]. Therefore, mitogen-induced degradation of $p27^{Kip1}$ is an initial requirement for pRB phosphorylation and subsequent $G_1 \rightarrow S$ cell-cycle progression [46].

PPARγ ligands have been demonstrated in various studies to prevent mitogen-induced SMC proliferation and the mechanisms by which this inhibition of proliferation occurs appear to involve an arrest in the G_1 phase of the cell cycle [47–49]. The growth-inhibitory effects of PPARγ agonists were first associated with their ability to prevent mitogen-induced degradation of the CDKI cyclin-dependent kinase inhibitor (CDKI) $p27^{Kip1}$, which inhibits the activity of cyclin/CDK and consequently reduces the cellular levels of phosphorylated pRB [47]. Since in vivo gene transfer of $p27^{Kip1}$ significantly inhibits neointimal cell proliferation [43], $p27^{Kip1}$ likely constitutes an important target for the anti-proliferative effects of PPARγ activation. Consistent with its function to suppress the activity of cyclin/CDK-complexes, stabilization of $p27^{Kip1}$ by PPARγ ligands has been demonstrated to inhibit cyclin/CDK activity, an effect that ultimately translates into a prevention of mitogen-induced pRB phosphorylation [47].

DNA microarray analysis further identified that minichromosome maintenance proteins (MCM) 6 and 7 are inhibited by PPARγ ligands in SMCs [50]. MCM proteins represent bona fide E2F target genes [51] and play a central role in the regulation of the initiation of DNA replication ensuring that DNA replicates only once during cell cycle (for review see [52]). In eukaryotes, MCM2–MCM7 are recruited onto replication origins during the G_1 phase of the cell cycle and assembled into a heteromeric hexamer. Formation of this prereplication complex, a process often referred to as "replication licensing", establishes the competence of this origin for the initiation of DNA replication in the subsequent S phase. Therefore, the inhibition of MCM gene expression by PPARγ ligands provides evidence that the inhibitory effects of PPARγ ligands on $G_1 \rightarrow S$ transition are the result of targeting the pRB/E2F/MCM pathway.

3.2. PPARγ activation and induction of apoptosis in SMCs

In addition to the role of TZD in the regulation of $G_1 \rightarrow S$ cell-cycle progression, several studies have demonstrated that TZD induce apoptosis in SMCs [16, 53, 54]. Among the regulated target genes mediating PPARγ-induced apoptosis is the growth-arrest and DNA damage-inducible gene 45 (GADD45) [53]. Molecular analyses demonstrated that PPARγ-induced GADD45 gene transcription is mediated through an Oct-1-dependent mechanism [53]. Although the exact function of GADD45 remains unclear, GADD45 has been implicated in growth suppression [55] and apoptosis [56, 57]. Through its association with Cdc2, GADD45 disrupts the interactions of Cdc2 with cyclin B1 and, thus, may induce G_2/M arrest [58]. The GADD45 gene, therefore, may represent a unique target for drugs that induce cell-cycle arrest, apoptosis, and differentiation such as PPARγ ligands.

The second pathway that has been demonstrated to induce apoptosis by PPARγ ligands involves the induction of transforming growth factor (TGF)-β by PPARγ [54]. TGF-β is an essential cytokine involved in the control of the balance

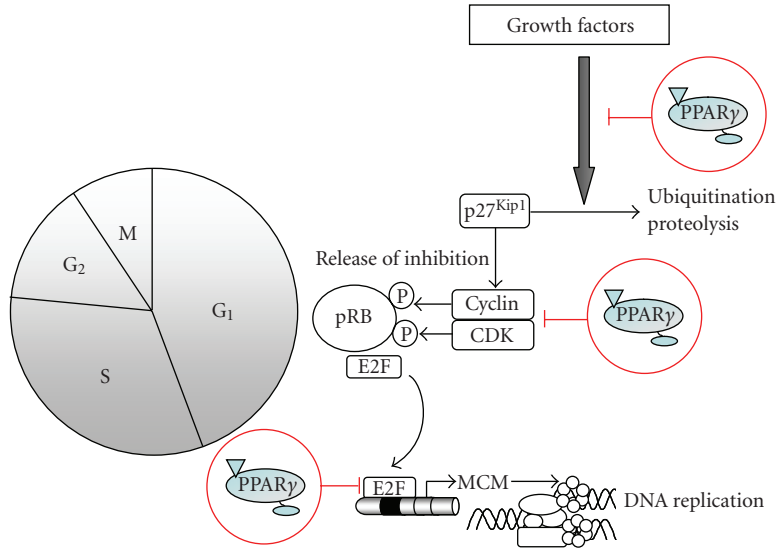

Figure 2: PPARγ targets cell-cycle progression. Phosphorylation of the retinoblastoma gene product (pRB) by specific G_1 CDKs represents the critical checkpoint of the G_1/S transition of the cell cycle. pRB phosphorylation releases E2F allowing the expression of genes required for DNA synthesis. By preventing the degradation of the CDK inhibitor (CDKI) $p27^{Kip1}$, PPARγ ligands inhibit mitogen-induced pRB phosphorylation and downstream expression of key E2F-regulated genes (i.e., MCM genes) responsible for the initiation of DNA replication.

between proliferation and apoptosis in SMCs [59]. Previously, TZD-induced apoptosis of SMCs has been suggested to depend on the induction of TGF-β and subsequent downstream nuclear recruitment of phospho-Smad2 [54]. Interestingly, TGF-β-induced apoptosis is partly mediated by Smad-dependent expression of GADD45 [60]. Therefore, it is possible if not likely that GADD45 constitutes a key downstream mediator of apoptosis induced by PPARγ activation.

A third mechanism that has been implicated in PPARγ-induced SMC apoptosis involves the transcriptional induction of the interferon regulatory factor-1 (IRF-1), a transcriptional factor with anti-proliferative and proapoptotic properties. Lin et al. recently demonstrated that both TZD and PPARγ overexpression upregulate IRF-1 expression in SMCs [61]. Reducing IRF-1 expression by antisense approaches attenuated PPARγ-induced SMC apoptosis suggesting that the PPARγ-regulated IRF-1 pathway contributes to the proapoptotic effects observed with TZD.

3.3. Regulation of SMC telomerase and senescence by PPARγ ligands

Telomerase has been linked to multiple developmental processes including cell proliferation, senescence, and aging [62–64]. Telomeres, the DNA-protein complexes at the ends of chromosomes, are stabilized by the ribonucleoprotein telomerase reverse transcriptase (TERT) to serve as protective capping and to prevent cellular senescence [65, 66]. In most adult cells TERT expression and telomerase activity are repressed and telomeres shorten during tissue renewal [67], and it has been proposed that this telomere exhaustion is rate limiting for lifespan [68]. Loss of telomere length beyond a critical threshold results in cellular senescence [59], a state in which cells are unresponsive to mitogenic stimuli [69]. These molecular features of telomerase to prevent senescence are highly conserved among eukaryotes and act on somatic cells as biological clock to ultimately result in permanent growth arrest and entry into replicative senescence [70].

In SMCs, telomerase activity is required for cell proliferation, and disruption of telomerase activity reduces atherosclerosis and neointima formation [71–73]. TERT is the limiting factor for telomerase activation in response to mitogenic stimuli and TERT antisense oligonucleotides inhibit SMC proliferation [71, 72]. This suggests that TERT may play an important role in the regulation of SMC proliferation and neointima formation. A recent study demonstrated that mitogen-induced telomerase activity in SMCs is inhibited by ligand-induced and constitutive PPARγ activation [19]. The transcriptional mechanisms responsible for the suppression of telomerase activity by PPARγ ligands involve an inhibition of Ets-1-dependent transactivation of the TERT promoter [19]. Ets-1 is an early response gene that mediates a variety of growth signals in neointimal SMC proliferation [74]; and atherosclerosis [75] and PPARγ ligands have been reported to inhibit Ets-1 expression [76]. The relevance of telomerase as target for PPARγ was further demonstrated in SMCs overexpressing telomerase, in which the efficacy of PPARγ ligand pioglitazone to inhibit cell proliferation is lost [19]. These studies indicate that telomerase constitutes an important molecular target for the antiproliferative effects of PPARγ activation in SMCs.

3.4. Ligand-receptor relationship and specificity: is TZD-regulated gene expression in SMCs PPARγ-dependent?

Although the above-described evidence outlines the ability of TZD to suppress SMC proliferation and induce apoptosis,

it remains controversial whether the cell-cycle-inhibitory effects of TZD occur through a ligand-dependent activation of PPARγ. Several experimental approaches have been used by different investigators to specifically address this question, including PPARγ-deficient cells [48, 77], overexpression of either dominant-negative or constitutively-active PPARγ mutants [19, 50, 53, 78], or pharmacologic inhibition of PPARγ [16, 53, 54, 61]. In PPARγ-deficient embryonic stem cells, TZD have been demonstrated to inhibit cell proliferation, which indicated that this effect might occur independent of their binding and activation of PPARγ [77]. In contrast to these earlier studies in stem cells, overexpression of a dominant-negative PPARγ mutant has been demonstrated to increase SMC proliferation in vitro and neointima formation in vivo (discussed in Section 4.1) pointing to a role of PPARγ to function as an endogenous repressor of SMC proliferation [78]. Complementary to these observations, overexpression of a constitutively-active PPARγ induces SMC apoptosis in the absence of ligand [53] while pharmacologic inhibition of PPARγ prevents rosiglitazone-induced apoptosis of neointimal SMCs [16]. In addition, many of the target genes, thought to be involved in the regulation of SMC proliferation/apoptosis by PPARγ ligands, have been demonstrated to be either directly regulated by overexpression of PPARγ or the ligand effect is reversed following pharmacologic inhibition of PPARγ [16, 19, 48, 50, 53, 54, 61, 78, 79]. These studies in concert support the concept that the antiproliferative activity of PPARγ ligands against SMC stems at least in part from a ligand-dependent activation of the receptor. However, further studies including in particular SMC-specific PPARγ-deficiency or overexpression are warranted to further support this notion.

A second important question that arises from this discussion relates to ligand specificity and whether the inhibition of SMC proliferation by agonists for PPARγ is exclusively mediated through this receptor or whether PPARγ ligands may also activate PPARα or δ. Approximately 80% of the 34 residues defining the ligand binding cavity of PPARγ are conserved across the three receptor isotypes [11, 20]. In addition, all three isoforms possess unusually large binding pockets, compared to other nuclear receptors, which accommodate a diverse set of lipophilic acids as ligands [80]. Furthermore, anti-proliferative effects of PPARγ ligands are observed at concentrations considerably higher than their EC_{50} for transcriptional activation in cell-based transfection assays or in in vitro binding assays with isolated ligand-binding domain fragments [15, 81]. Considering this knowledge, at high concentrations spillover of PPARγ-selective ligands to PPARα and/or PPARδ is theoretically possible and the antiproliferative activity of TZD observed in PPARγ-deficient cells could be explained by their binding to and activation of PPARα or PPARδ. Indeed, activation of PPARα represses SMC proliferation [82], while PPARδ activation has been reported to stimulate rather than inhibit growth of SMCs [83] and keratinocytes [84]. Although very few studies have directly compared the effects of PPARγ, PPARα, and PPARδ ligands on SMC function, Lin et al. recently identified that the above-described IRF-1-dependent apoptosis induced by PPARγ ligands is selective and not observed with PPARα or PPARδ ligands [61]. This study supports ligand selectivity for PPARγ in SMCs, although detailed studies are required to further address this question.

4. TZD IN THE TREATMENT OF CARDIOVASCULAR DISEASE

4.1. Lessons from animal models

TZD PPARγ ligands have been demonstrated to prevent the development of atherosclerosis in several murine atherosclerosis models including the low-density lipoprotein receptor-deficient ($LDLR^{-/-}$) and the apolipoprotein E deficient mouse model ($apoE^{-/-}$) [85–88]. This preventive effect on hyperlipidemia-induced atherosclerosis occurs independently of changes in circulating lipids, blood pressure, glucose, or insulin, implicating direct pleiotropic effects on the vascular wall. Inhibition of atherosclerosis by TZD ligands in these models appears to be also independent of their efficacy to improve insulin sensitivity as the prevention of atherosclerosis is observed in both insulin-sensitive and insulin-resistant models· [85–88]. The mechanisms responsible for the prevention of atherosclerosis by TZD in these murine atherosclerosis models likely involve macrophage-driven processes contributing to atherosclerosis since conditional deletion of PPARγ in macrophages accelerates atherosclerosis [89]. In addition, specific deletion of PPARγ in EC has recently been demonstrated to increase blood pressure in mice suggesting that PPARγ in EC is an important regulator of hypertension, which may contribute to the prevention of atherosclerosis in murine models [90].

Consistent with the observations that TZD PPARγ ligands limit SMC proliferation in vitro, Law et al. demonstrated over a decade ago that the TZD ligand troglitazone reduces intimal hyperplasia in a rat carotid artery balloon injury model [91]. Subsequent studies confirmed these observations and demonstrated that TZD inhibit intimal hyperplasia in models of restenosis in both insulin-resistant and insulin-sensitive animals [92–95]. Similarly, Joner et al. recently demonstrated the prevention of in-stent restenosis by TZD ligands using a hypercholesterolemic rabbit atherosclerosis model [96]. Additional beneficial effects of TZD in the process of neointima formation include accelerated reendothelialization, which is mediated through an enhanced differentiation of angiogenic progenitor cells into mature endothelial cells [97, 98]. As detailed above, the question as to whether the prevention of neointima formation by TZD involves a receptor-dependent pathway has been addressed in a recent study using overexpression of PPARγ. While in vivo transfer of an adenoviral vector expressing wild-type PPARγ inhibited SMC proliferation and reduced neointima formation after balloon injury, overexpression of a dominant-negative PPARγ mutant increased neointima formation [78]. These studies have provided the first in vivo evidence to support a direct role of PPARγ in suppressing the proliferative response following vascular injury.

4.2. Clinical evidence for vascular protection by TZD

4.2.1. Carotid artery intima/media thickness

Carotid artery intima/media thickness (CIMT) is a well-described surrogate marker for cardiovascular risk and correlates not only with the presence of cardiovascular risk factors but also with the risk of future macrovascular events [99, 100]. The first study that used CIMT to assess whether TZD treatment prevents the progression of atherosclerosis was performed 10 years ago. In this study 57 patients with type 2 diabetes were treated with 400 mg troglitazone, which resulted in a significant decline in CIMT after 3 months of treatment [101]. This reduction in CIMT with troglitazone has been confirmed in a recent cohort of patients with insulin-requiring type 2 diabetes [102]. A similar decline in CIMT was observed a few years later in two independent studies performed with pioglitazone [103, 104]. The recently reported CHICAGO trial (Carotid Intima-Media Thickness in Atherosclerosis Using Pioglitazone) was a randomized, double-blind, comparator-controlled, multicenter trial in patients with type 2 diabetes assessing the effect of pioglitazone versus the sulfonylurea glimepiride on CIMT progression [105]. In this study of 462 patients the primary endpoint of progression of mean CIMT was less with pioglitazone versus glimepiride after 72 weeks. Notably, the beneficial effect of pioglitazone on mean CIMT was similar across prespecified subgroups based on age, sex, systolic blood pressure, duration of type 2 diabetes, body mass index, HbA(1c) value, and statin use. The fourth CIMT study performed with pioglitazone compared the effects of pioglitazone (45 mg/d) and glimepiride (2.7 +/− 1.6 mg/d) in a randomized controlled study of 173 patients with type 2 diabetes [106]. In this study, CIMT was reduced only in the pioglitazone group and not in patients treated with glimepiride and this effect was independent of glycemic control.

Comparable results on CIMT progression have been obtained with rosiglitazone. Sidhu et al. analyzed the effect of rosiglitazone on CIMT progression in a double-blind, placebo-controlled randomized study in 92 non-diabetic patients with documented coronary artery disease [107]. In this study, rosiglitazone therapy revealed a reduced progression in CIMT after 48 weeks of treatment. The Rosiglitazone Atherosclerosis Study analyzed the effect of TZD treatment on CIMT progression in a mixed patient cohort of 555 subjects with type 2 diabetes or insulin resistance [108]. Although in this study there was no effect of rosiglitazone treatment in the mixed population of type 2 diabetes and insulin resistance, in the subanalysis of type 2 diabetic patients there was a reduced progression of CIMT. A third study reported by Stocker et al. analyzed whether rosiglitazone compared to metformin decreased CIMT in 93 subjects with type 2 diabetes [109]. In this study, metformin and rosiglitazone treatment led to similar improvement in glycemic control; however, CIMT progressed in the metformin group while regression of maximal CIMT was observed in the rosiglitazone group.

4.2.2. Postangioplasty restenosis

Takagi et al. [110–112] first demonstrated that troglitazone reduced neointimal tissue proliferation after coronary stent implantation in patients with type 2 diabetes mellitus. Following the withdrawal of troglitazone from the market, it was subsequently demonstrated that pioglitazone has similar effects and significantly reduces neointimal tissue proliferation in patients with type 2 diabetes mellitus [113]. In this study, 44 patients with type 2 diabetes and 44 stented lesions were randomized to either pioglitazone therapy or control. Intravascular ultrasound demonstrated that the neointimal index in the pioglitazone group was significantly smaller than that in the control group. Similarly, Nishio et al. observed that the late luminal loss and in-stent restenosis were significantly less in patients treated with pioglitazone [114]. A third study performed with pioglitazone demonstrated in a randomized, placebo-controlled, double-blind trial that pioglitazone significantly reduced neointima volume after coronary stent implantation in patients without diabetes [115].

Comparable results have been obtained with rosiglitazone in a prospective, randomized, case-controlled trial involving 95 diabetic patients with coronary artery disease, which demonstrated that the in-stent restenosis rate was significantly reduced in the rosiglitazone group compared with the control group [116]. However, a second study of a smaller cohort of sixteen patients did not observe a significant decrease in in-stent luminal diameter stenosis measured by quantitative coronary angiography intravascular ultrasound [117]. Finally, the third study performed by Wang et al. suggested that the occurrence of coronary events following angioplasty in 71 patients was significantly decreased in the rosiglitazone group at 6-month follow-up [118]. These studies in concert suggest that TZD therapy in patients undergoing coronary stent implantation may be associated with less in-stent restenosis and repeated revascularization. This notion is further supported by two recent meta-analyses [119, 120]. However, decisions on clinical use of an adjunctive TZD therapy following coronary interventions must await larger double-blind clinical trials.

4.2.3. Cardiovascular outcome studies

The beneficial vascular effects observed with TZD provided the rationale for larger cardiovascular trials and the first results from these studies are beginning to emerge. The Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROactive) trail is a prospective, randomized controlled trial in 5238 patients with type 2 diabetes who had evidence of macrovascular disease [121]. This study tested theeffects of pioglitazone or placebo in addition to their glucose-lowering drugs and other medications on a combined vascular endpoint in patients with known vascular disease. The broad primary endpoint (the composite of all-cause mortality, nonfatal myocardial infarction (including silent myocardial infarction), stroke, acute coronary syndrome, endovascular or surgical intervention in the coronary or leg arteries, and amputation above the ankle) was not statistically different between the pioglitazone and placebo

arm of the study. However, the study demonstrated a significant 16% reduction of the main cardiovascular secondary endpoint of all-cause mortality, myocardial infarction, and stroke in type 2 diabetic patients treated with pioglitazone. A recently published subanalysis out of this study further reported the effect of pioglitazone on recurrent myocardial infarction in 2,445 patients with type 2 diabetes and previous myocardial infarction [122]. In this prespecified endpoint, pioglitazone had a statistically significant beneficial effect on fatal and nonfatal MI (28% risk reduction) and acute coronary syndrome (37% risk reduction). A second subanalysis from the PROactive trial in patients with previous stroke ($n = 486$ in the pioglitazone group and $n = 498$ in the placebo group) further reported that pioglitazone reduced fatal or nonfatal stroke by 47% [123]. Consistent with the reported side-effect profile for TZD, the PROactive trial confirmed an increased rate of edema and heart failure in patients treated with pioglitazone [121]. However, in this context it is important to note that heart failure was a nonadjudicated event and mortality due to heart failure was not increased compared to the placebo group.

Currently, trials with rosiglitazone are being performed to determine whether rosiglitazone affects cardiovascular outcomes. Three clinical trials are currently testing approaches that use rosiglitazone to reduce cardiovascular disease in patients with diabetes: the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial [124], the Bypass Angioplasty Revascularization Investigation 2 Diabetes (BARI 2D) trial [125], and The Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycemia in Diabetes (RECORD) trial [126]. A recent meta-analysis of trials performed with rosiglitazone reported an association with a significant increase in the risk of myocardial infarction and with a nonsignificant increase of the risk of death from cardiovascular causes [127]. However, the authors of this meta-analysis acknowledged considerable limitations of their analysis and the National Institutes of Health (supporting the ACCORD and BARI 2D trials) found no evidence in this analysis to require discontinuing the use of rosiglitazone in the trials or to revise the study protocols [128]. Similarly, an interim analysis of the RECORD trial did not show a statistically significant difference between the rosiglitazone group and the control group for the endpoints acute myocardial infarction and death from cardiovascular causes, although patients treated with rosiglitazone were at increased risk to develop heart failure [129]. Therefore, completion of these studies will enable the determination whether rosiglitazone provides a similar reduction in cardiovascular outcomes as seen with pioglitazone and will aid to determine the most appropriate combination therapies for patients with type 2 diabetes.

5. SUMMARY AND CONCLUSIONS

Research performed over the last decade has highlighted an important role for TZD-induced PPARγ activation in vascular cells. TZD exert a broad spectrum of anti-inflammatory and anti-proliferative on all cell types participating in the development of cardiovascular diseases. A wealth of evidence from preclinical and clinical studies supports that these pleiotropic effects of TZD translate into reduced atherosclerosis and failure of coronary angioplasty as the primary approach to treat luminal obstruction. The PROactive trial was the first cardiovascular outcome trial to demonstrate that pioglitazone decreases all-cause mortality, myocardial infarction, and stroke in patients with type 2 diabetes. Further studies including the ACCORD, RECORD, and BARI 2D trials will determine whether similar effects are seen with rosiglitazone and outline ideal treatment strategies to reduce cardiovascular disease in patients with type 2 diabetes.

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Review Article

Hexarelin Signaling to PPARγ in Metabolic Diseases

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Investigating the metabolic functions of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) has been extremely rewarding over the past years. Uncovering the biologic roles of PPARγ and its mechanism of action has greatly advanced our understanding of the transcriptional control of lipid and glucose metabolism, and compounds such as thiazolidinediones which directly regulate PPARγ have proven to exhibit potent insulin-sensitizer effects in the treatment of diabetes. We review here recent advances on the emerging role of growth hormone releasing peptides in regulating PPARγ through interaction with scavenger receptor CD36 and ghrelin GHS-R1a receptor. With the impact that these peptides exert on the metabolic pathways involved in lipid metabolism and energy homeostasis, it is hoped that the development of novel approaches in the regulation of PPAR functions will bring additional therapeutic possibilities to face problems related to metabolic diseases.

1. INTRODUCTION

Vascular diseases impose the greatest burden upon health care systems and are predicted to remain the leading cause of death and disability in industrialized countries. The identification of excess body weight as a major risk factor, the epidemic of obesity and diabetes in Western societies and their increasing prevalence in children indicate that pathologies associated to the metabolic syndrome will continue to impact the health of individuals. Insulin resistance is a recurrent trait associated with increased adiposity, and despite the amplitude of health problems related to metabolic disorders, the mechanisms underlying excessive fat storage by adipocytes remain largely undefined.

The adipocyte is the major site of fatty acid storage in the body and plays a critical role in maintaining normal glucose and lipid homeostasis. If the capacity of the adipocyte to store lipids is exceeded, it can no longer regulate normally the release of fatty acids into the circulation, which ultimately leads to the abnormal accumulation of lipids in fat tissues and nonadipose depots. Such buildup of lipids in fat, liver, pancreatic islets, and muscle cells is associated to metabolic dysregulation of these tissues, resulting in many pathologic states of the metabolic syndrome, such as central obesity, atherosclerosis, type 2 diabetes, and insulin resistance [1, 2]. Over the recent years, with the unveiling of their ability to behave as master regulators of an array of genes that coordinate numerous pathways in lipid, glucose, and energy metabolism, the peroxisome proliferator-activated receptors (PPAR) have been considered important targets in the therapeutic management of metabolic disorders.

2. THE PPARs, FATTY ACID SENSORS

The PPARs consist of three isoforms, PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3), all of which are *bona fide* members of the nuclear receptor family. Upon ligand activation, the PPARs act as transcription factors by directly binding DNA as obligate heterodimers with retinoid X receptor RXR (NR2B) to a peroxisome proliferator response element (PPRE) contained in the promoters of target genes. With identified ligands such as mono- and polyunsaturated fatty acids, and derivatives such as eicosanoids, the PPARs have been recognized as physiologic sensors for fatty acids that control the transcription of many genes governing lipid metabolism [3–5].

PPARα is predominantly expressed in the liver, where it activates a broad range of genes involved in fatty acid uptake,

glycerol metabolism, β- and ω-oxidation of unsaturated fatty acids, and their transport into peroxisomes [6]. PPARα deficiency results in hypoglycemia and hypoketonemia, fatty liver, and elevated plasma fatty acids, revealing its importance in the hypoglycemic response [7, 8]. When fed a high-fat diet, PPARα-null mice are unable to catabolize fatty acids and develop severe hypertriglyceridemias without apparent obesity [9]. It is therefore predicted that fibrates, which selectively activate PPARα, are effective in treating hyperlipidemias [10]. PPARβ/δ is expressed ubiquitously and while biochemical and genetic evidence has linked PPARβ/δ to aspects of the metabolic syndrome [11–13], its emerging role in lipid metabolism remains to be further ascertained. Although the benefit of targeting PPARα and/or PPARβ/δ in lipid disorders is not excluded, the current review specifically emphasizes on PPARγ and its metabolic control by growth hormone releasing peptides.

3. PPARγ, A METABOLIC REGULATOR OF INSULIN RESISTANCE

Insulin resistance is marked by hyperinsulinemia, enhanced hepatic gluconeogenesis, and impaired insulin-stimulated glucose uptake into skeletal muscle and fat. Elevated levels of circulating fatty acids, associated with obesity and insulin resistance, increase fat accumulation in insulin target tissues and contribute to defective insulin action. In addition, obese adipose tissue-derived inflammation and altered secretion of adipocyte proteins, also known as adipokines or adipocytokines, can also impair insulin signals and affect systemic metabolism [14, 15]. The resulting hyperglycemia, dyslipidemia, and hypertension of the metabolic syndrome cause endothelial dysfunction and hasten vascular diseases.

Over the recent years, a number of adipokines, some of which being adipocyte-specific while others are not, have been identified to be produced and secreted by mature adipocytes. Adipokines, such as adiponectin and leptin which exhibit insulin-sensitizing effects, or resistin, tumor necrosis factor α(TNFα), and interleukin-6 (IL-6) which act as insulin resistance factors, all share autocrine, paracrine, or endocrine activity that regulates insulin sensitivity, therefore, establishing a role for the adipose tissue to function as an endocrine organ [14, 16, 17].

Remarkably, the thiazolidinediones (TZDs), which have been described as high-affinity ligands for PPARγ [18, 19], can modulate in a beneficial manner the expression of many if not all of these adipokines at the gene level, thereby correlating adipokine production with PPARγ activation. Originally discovered because of their potent insulin-sensitizing and glucose-lowering effects, TZDs are being used in clinics to correct abnormalities of lipid and glucose homeostasis, such as in type 2 diabetes, by reducing tissue insulin resistance [20]. For example, TZDs enhance adiponectin gene expression and circulating protein levels [21, 22], and decrease resistin [23, 24], TNFα [25], and IL-6 [26]. This suggests that the effect by which TZDs enhance insulin sensitivity likely resides in their ability to promote a beneficial profile of hormones secreted by adipocytes, which can then influence glucose disposal by the liver and muscle.

However, the mechanism by which TZD activation of adipocyte PPARγ leads to insulin sensitivity is not completely understood. Adipocyte-derived leptin is a circulating regulator of appetite and energy expenditure, whose increased levels reduce food intake and minimize ectopic lipid deposition by promoting fatty acid oxidation in peripheral tissues [27]. These effects contribute to the insulin-sensitizing properties of leptin, but its expression was found downregulated by PPARγ ligands [28, 29]. TZDs were also found to stimulate adipogenesis by upregulating many PPARγ target genes involved in fatty acid metabolism and storage [30]. Studies in rodent models and in humans have shown that TZD treatment causes weight gain [31, 32], an unwanted side effect that limits TZD efficacy on insulin sensitivity by increasing adiposity. This paradox remains largely unexplained, and among the likely hypotheses raised are a selective unequal accumulation of subcutaneous fat compared to visceral depots, and a possible activation of distinct yet overlapping adipogenic/antidiabetic gene programs in the adipocyte induced by TZDs [20, 33].

The use of genetic mouse models including tissue-specific deletion of the *Pparg* gene has enabled the identification of fat tissue as the primary target for TZDs but also revealed that other insulin-sensitive organs, such as liver and muscle, albeit expressing lower levels of PPARγ compared to fat, were also responsive to some extent to TZDs. Mice lacking white adipose fat, resulting in a phenotype similar to that of humans with lipoatrophic diabetes, fatty liver, hyperglycemia, and insulin resistance [31], or mice lacking adipose PPARγ, which also exhibit an insulin resistance phenotype [34], were refractory to the antidiabetic, but not the hypolipidemic effect of TZDs. In addition, these mice were highly predisposed to hepatic steatosis, an effect mainly attributed to liver PPARγ [35, 36]. TZDs also retained their glucose-lowering effects in liver- and muscle-specific PPARγ knockout mice [37, 38], arguying for a predominant role of adipose PPARγ in the insulin-sensitizing effects of TZDs, although another study reported that muscle PPARγ contributes to some extent to insulin resistance which was not improved by TZDs [39]. The kidney also appears as a target for TZDs in which however, renal PPARγ activation lead to fluid retention by inducing the Na^+ transporter ENaC in the collecting duct [40, 41]. This adverse effect of TZDs is viewed as a serious complication for patients with preexisting congestive heart failure [42]. In addition, the prototype TZD troglitazone was withdrawn from clinics due to life-threatening hepatic toxicity, whereas the other two TZDs, rosiglitazone and pioglitazone, are still being used in large-scale clinical practice. Hence, the crucial benefit of TZDs to consistently lower fasting and postprandial glucose concentrations as well as free fatty acid concentrations in clinical studies is clearly established, but also tempered by other effects, mostly undesired, therefore adding complexity in our understanding of the systemic response to PPARγ ligands [43]. It thus becomes essential and of fundamental interest that other ways need to be identified in order to avoid the adverse effects of TZDs while keeping the benefits of correcting whole body glucose and fatty acid dysfunctions.

4. THE GHRP-PPARγ PATHWAY IN MACROPHAGES

One critical step initiating fatty streak formation in atherosclerosis consists in the accumulation of oxidized lipoprotein particles, mainly oxLDL, into the intima and their subsequent uptake by monocyte-derived macrophages, leading to the formation of cholesterol-loaded foam cells. Many lines of evidence suggest that the endocytosis of oxLDL by macrophages is mainly dependent upon their interaction with CD36, a member of the class B scavenger receptor family [44–47]. Studies in macrophages have shown that oxLDL uptake through CD36 provides a source of oxidized fatty acids and oxysterols that activate, respectively, PPAR and LXR (liver X receptor; NR1H3), thereby inducing a metabolic cascade resulting in enhanced expression of downstream genes, such as apolipoprotein E and ABC sterol transporters, and ultimately in cholesterol efflux to high density lipoproteins (HDL) [48]. However, these apparent beneficial effects are opposed by a positive feedback loop in which PPARγ activation by internalized fatty acids enhances the expression of CD36, a process shown to mediate foam cell formation [49–53].

CD36 is an 88 kDa glycoprotein originally identified as a platelet receptor and also known as fatty acid translocase, which is expressed in numerous cell types including monocytes/macrophages, platelets, endothelial cells, and adipocytes [53–55]. CD36 is a multiligand receptor that is recognized by fatty acids, anionic phospholipids, thrombospondin, and oxidized lipoproteins. It is this latter property of scavenging (e.g., clearing) oxLDL which implicates CD36 in the initial steps of atherogenesis, as evidenced with studies in mice [53, 56] and humans [57].

The findings that growth hormone releasing peptides (GHRPs) serve as ligands for CD36 [58, 59] led to the evaluation of their potential role in regulating cholesterol metabolism in macrophages. The GHRPs belong to a class of small synthetic peptides known to stimulate growth hormone release through binding to the GH secretagogue-receptor 1a (GHS-R1a), a G-protein-coupled receptor originally identified in hypothalamus and pituitary [60] and later recognized as the receptor for ghrelin [61]. The peripheral distribution of the ghrelin GHS-R1a receptor in tissues, such as heart, adrenals, fat, prostate, and bone, has supported physiological roles of ghrelin and GHRPs not exclusively linked to GH release. For example, GH-independent effects on orexigenic properties, fat metabolism, bone cell differentiation, and hemodynamic control have been reported for ghrelin and GHRPs [62, 63]. Also, in conditions in which GH release was not promoted or in GH-deficient animals, the GHRP hexarelin was shown to feature cardioprotective effects by preventing ventricular dysfunction [64, 65], and by protecting the heart from damages induced by postischemic reperfusion [66]. These studies suggest that part of the beneficial effects of hexarelin may not involve GH release.

To evaluate the potential of hexarelin to regulate cholesterol metabolism in vivo, apolipoprotein E (apoE)-null mice maintained on a long-term high-fat and high-cholesterol diet, a condition known to promote atherosclerosis, showed a significant regression in plaque formation when treated with hexarelin compared to saline-treated controls [67]. These beneficial effects were observed in conditions in which GH was not upregulated by hexarelin [67], and also using EP80317, an hexarelin derivative with no GH release activity [68], supporting a GH-independent role for GHRPs.

To address the mechanism by which hexarelin exerts these beneficial effects, treatment of differentiated THP-1 macrophages or mouse peritoneal macrophages with hexarelin resulted in an increase in cholesterol efflux, which correlates with an enhanced expression of LXRα, apoE, and sterol transporters ABCA1 and ABCG1, all involved in promoting the high density lipoprotein (HDL) pathway (see Figure 1). In addition, these effects were severely impaired in treated peritoneal macrophages isolated from PPARγ heterozygote mice, implying an essential role for PPARγ in mediating the response to hexarelin [67]. We further showed using cell reporter assays that the interaction of hexarelin with CD36 or with ghrelin receptor resulted in an enhanced transcriptional activation of PPARγ, suggesting that both receptors signal to PPARγ [67]. These studies have helped to define that the beneficial effects of hexarelin involved the activation of the PPARγ-LXRα-ABC metabolic cascade, thereby causing macrophages to mobilize excess cholesterol into the HDL cholesterol reverse pathway [67]. These findings therefore support a novel regulatory pathway by which CD36 and possibly ghrelin receptor may impact PPARγ-regulated functions. Consequently, a detailed knowledge of the concerted modulation of CD36 and ghrelin receptor signaling pathways may help to provide additional strategies in pathologic conditions such as atherosclerosis.

5. A GHRP-PPARγ PATHWAY IN ADIPOCYTES

Based on our observations that hexarelin promotes PPARγ activation through CD36 and ghrelin receptors in macrophages [67], we wanted to address whether hexarelin could exert activation of PPARγ and subsequent downstream effects in adipocytes. PPARγ is considered a master regulator of fatty acid metabolism in fat through its direct role in regulating the expression of a broad range of genes involved in fatty acid and glucose metabolism. Among the genes upregulated by PPARγ are found genes related to fatty acid uptake (fatty acid transport protein FATP, CD36), glucose uptake (GLUT4), β-oxidation (acyl-CoA dehydrogenase, carnitine palmitoyltransferase CPT-1, acyl CoA oxidase), gluconeogenesis (phosphoenolpyruvate carboxykinase PEPCK), and lipid storage (adipophilin) ([69, 70], and references therein). Increased expression of many of these genes might result in a net influx and trapping of fatty acids into adipocytes, which is considered a mechanism by which TZDs consistently reduce circulating free fatty acids.

Mature adipocytes are known to express CD36 but not the other hexarelin receptor GHS-R1a ([71, 72], and data not shown). Whereas the role of CD36 in mediating oxLDL-derived cholesterol and fatty acid uptake by macrophages is recognized, the mechanisms by which CD36 may impact the overall metabolic activity of fat storage and mobilization by adipocytes is not completely understood. With these considerations and the central role of PPARγ in

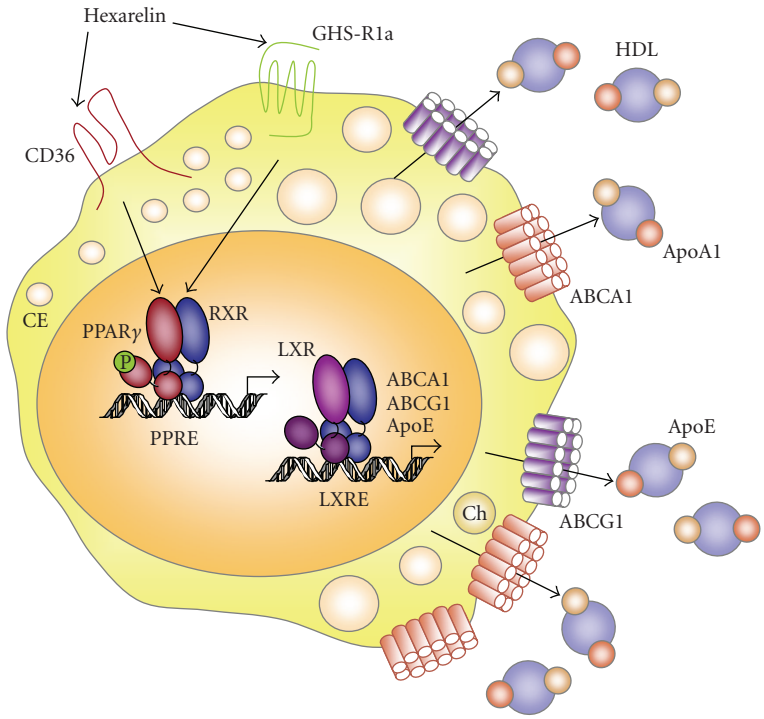

Figure 1: *A GHRP-PPARγ pathway in macrophages.* Overview of the effects of hexarelin which by interacting with scavenger receptor CD36 and GHS-R1a ghrelin receptor promotes the transcriptional activation of PPARγ. LXRα which is a target of PPARγ is then upregulated with the subsequent increase in apolipoprotein E (apoE) and sterol transporters ABCA1 and ABCG1 expression. Activation of the PPARγ-LXRα-ABC metabolic pathway in response to hexarelin favors cholesterol efflux by macrophages through high density lipoproteins (HDLs). Adapted from [52].

regulating many aspects of fatty acid metabolism, it was expected that hexarelin may impact PPARγ-regulated events in adipocytes.

As such, we recently reported the ability of hexarelin to regulate PPARγ-dependent downstream events in cultured adipocytes and in fat tissues from treated mice [73], thereby providing evidence that hexarelin may target different PPARγ expressing tissues. In these studies, we observed that treatment of differentiated 3T3-L1 adipocytes with hexarelin resulted in a depletion in triglyceride cellular content, accompanied by profound changes in the gene expression profile of key markers of fatty acid metabolism [73]. Interestingly, many of these genes were shared with TZD troglitazone treatment, indicating that PPARγ may be considered as a common regulator in both responses. Consistent with this, among the genes upregulated by hexarelin, we found many established PPARγ targets, such as nuclear receptor LXRα, FATP1 (fatty acid transport protein), and F_1-ATP synthase (see Figure 2). Other genes involved in various aspects of entry, transport, synthesis, and mobilization of fatty acids, such as hormone-sensitive lipase (HSL), fatty acid synthase (FAS), and acetyl-CoA synthase (ACS) among others, were also upregulated, whereas glycerol-3-phosphate acyltransferase (GPAT), which catalyzes the initial and committing step in glycerolipid biosynthesis, was downregulated by hexarelin [73]. All together, this type of profile is strongly suggestive of an increase in the cellular mobilization of free fatty acids in response to hexarelin.

However, the response to hexarelin was not totally mimicked by troglitazone as other described PPARγ targets, such as adipocyte fatty acid binding protein FABP4 (also referred to as aP2) and lipid droplet-associated protein adipophilin remained mostly unchanged upon treatment with hexarelin [73]. It is also important to note that gene expression and protein levels of CD36, a well-known target of PPARγ [49, 50], were not changed by hexarelin, as opposed to troglitazone which significantly induced both in treated adipocytes. Similar results were also found in macrophages, indicating that this regulation is not cell-specific [67], and may prevent any undesired increase in macrophage CD36, a situation that correlates with proatherosclerotic events [55, 74]. Also, as opposed to troglitazone which decreased PPARγ expression, hexarelin contributed to maintain expression and steady-state levels of PPARγ in adipocytes and macrophages [67, 73]. The exact mechanism(s) by which hexarelin exerts such gene-specific regulation compared to TZDs are not clearly understood, but differences in PPARγ occupancy of targeted promoters and/or posttranslational modifications of PPARγ are certainly among the likely possibilities to consider in the response of PPARγ to hexarelin ([67], see below).

6. HEXARELIN PROMOTES MITOCHONDRIAL ACTIVITY AND BIOGENESIS

Uptake of fatty acids and glucose by muscle and fat tissues is an important component regulating energy expenditure

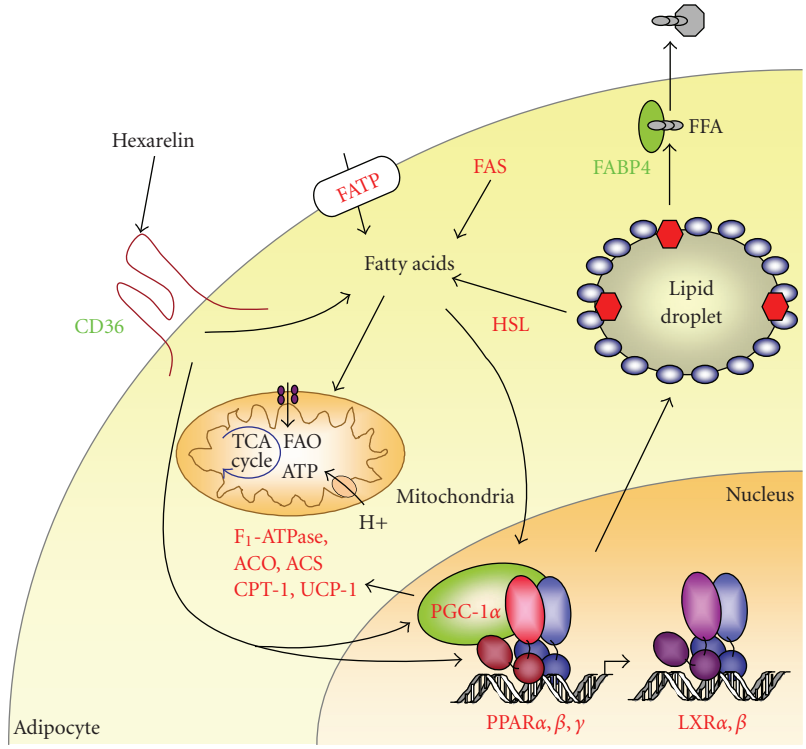

Figure 2: *Hexarelin promotes mitochondrial activity in adipocytes.* Scheme of gene expression analysis of fatty acid metabolic regulators in 3T3-L1 adipocytes. Shown are a subset of genes identified as upregulated (red) or downregulated (green) by hexarelin compared to untreated cells. These effects of hexarelin require CD36 which is expressed in adipocytes as opposed to GHS-R1a receptor; FAO, fatty acid oxidation; FABP, fatty acid binding protein; FAS, fatty acid synthase; HSL, hormone-sensitive lipase; ACO, acyl CoA oxidase; ACS, and acyl CoA synthase. Other abbreviations appear in text.

and defects in CD36 have been associated with impaired fatty acid and glucose homeostasis in humans [75, 76]. However, the role of CD36 in regulating energy metabolism in adipocytes remains an unresolved issue.

By transposing the ability of hexarelin to promote PPARγ activation to adipocytes, it was interesting to observe that many genes upregulated by hexarelin were characteristic of an enhanced profile of fatty acid oxidation and mitochondria morphology [73]. More specifically, among the genes upregulated were found acetyl CoA acyl transferase, CPT-1, and several subunits of the ATP synthase and of the cytochrome c oxidase complexes, all suggesting an increased fatty acid mobilization towards the mitochondrial oxidative phosphorylation pathway [73].

Enhanced mitochondrial oxidative potential is required to supply adequate ATP production in high energy-demanding processes, such as adaptation to cold in brown fat, heart and skeletal muscle contraction, and liver gluconeogenesis in response to fasting. Such mitochondrial energy-producing capacity correlates with active β-oxidation of fatty acids and increased expression of PPARγ coactivator-1 (PGC-1) in these tissues [77–82]. PGC-1α is a coactivator of most nuclear receptors that was discovered as a molecular switch that turns on several key components of the adaptive thermogenic program in brown fat, including the stimulation of fuel intake, mitochondrial fatty-acid oxidation, and heat production [83, 84]. These metabolic changes are supported by the ability of PGC-1 to upregulate the expression of UCP-1, a biological uncoupler of mitochondrial oxidative phosphorylation, and of genes of gluconeogenesis, such as PEPCK and glucose-6-phosphatase (reviewed in [84, 85]). Thus, modulating the relative activity of PGC-1 within a particular tissue may lead to a fine tuning of mitochondrial function in fatty acid oxidation and energy balance. Interestingly, hexarelin induced an increase in PGC-1α and UCP-1 in 3T3-L1 adipocytes as well as in epididymal fat of treated mice, indicating a potential fat burning phenotype taking place in white fat in response to hexarelin [73]. Consistent with these changes, electron microscopy of hexarelin-treated 3T3-L1 adipocytes showed an intense and highly organized cristae formation that spans the entire width of mitochondria compared to untreated cells, accompanied with an increase in cytochrome c oxidase activity, two features characteristic of highly oxidative tissues [73]. A similar mitochondrial phenotype and gene expression profile was detected in epididymal white fat of mice treated with hexarelin, and shown to be dependent on CD36, indicating that the ability of hexarelin to promote a fat burning-like phenotype was maintained in vivo [73]. These studies therefore support a functional GHRP-PPARγ signaling cascade in adipocytes, which provides a potential role for CD36 to impact the overall metabolic activity of fatty acid usage and mitochondrial

biogenesis in fat. These aspects are particularly relevant to the emerging association of mitochondrial dysfunction with insulin resistance and type 2 diabetes [86].

7. HEXARELIN INCREASES PPARγ PHOSPHORYLATION

The exact mechanism(s) by which PPARγ activity is modulated in response to hexarelin remains to be clearly defined. In an attempt to partly characterize such a response, we found that PPARγ was highly phosphorylated in macrophages treated with hexarelin, therefore providing a basis on how PPARγ can respond to hexarelin signaling [67]. Although macrophages do express both receptors recognized by hexarelin, our observation that GHS-R1a activation by hexarelin enhanced PPARγ activity in transfected heterologous cells may therefore suggest that GHS-R1a signals to activate PPARγ [67]. Consistent with this, the activation of GHS-R1a receptor by hexarelin or its natural ligand ghrelin leads to the phosphorylation of PPARγ in macrophages, while a GHRP selective for CD36 did not ([67] and unpublished observations). These findings rather implicate GHS-R1a signaling in the phosphorylation of PPARγ, at least in macrophages.

The effects of phosphorylation on PPARγ activity have been reported to vary, often in opposite directions, depending on the cellular and promoter context [87]. In that respect, it is interesting to note that while PPARγ ligands of the TZD family are recognized to upregulate CD36 gene expression [49, 50], no significant changes in CD36 expression were measured in response to GHRPs despite PPARγ activation [67, 68, 73]. In order to further investigate the mechanism by which this unexpected regulation of CD36 by hexarelin may result, chromatin immunoprecipitation assay has revealed that the relative occupancy of the CD36 promoter region by PPARγ remained mostly unchanged, whereas that of nuclear receptor LXRα, also a known target of PPARγ [88], was occupied by PPARγ in a greater extent in macrophages treated with hexarelin, indicating that LXRα upregulation by hexarelin may result from a preferred recruitment of PPARγ to the LXRα promoter, as opposed to CD36 [67]. Whether PPARγ phosphorylation may discriminate for promoter usage is not yet known but interestingly, it was reported that PPARγ phosphorylation could decrease CD36 transcription in macrophages [53]. Given the ability by which posttranslational modifications such as phosphorylation could regulate PPARγ transcriptional activity and that ligand-independent recruitment of transcriptional coregulators is favored by nuclear receptor phosphorylation [87, 89–91], it is predicted that such mechanism may contribute in the cellular response to hexarelin by selectively regulating PPARγ-targeted genes. These aspects need to be further investigated in order to ascertain such selectivity.

8. CONCLUDING REMARKS

Although the exact mechanisms by which GHRPs promote their metabolic response are not fully understood, it becomes clear that interacting with CD36 and/or GHS-R1a receptors induces profound changes in metabolic activities of target tissues, especially regarding PPARγ-regulated events. However, it is important to note that the sole activation of PPARγ may not be exclusive in translating the signal by hexarelin or other GHRPs. Indeed, in view that hexarelin can also promote PPARα and PPARβ/δ activation [67], and with the propensity of PGC-1α to coactivate other nuclear receptors besides PPARγ, such as thyroid hormone receptor TRα, retinoic acid receptor RARα, estrogen-related receptor ERRs, and PPARα [83], it is expected that these pathways may also be affected by hexarelin. So clearly, the mechanism(s) by which hexarelin exerts its metabolic effects represents a promising avenue which deserves further investigation to face problems related to multipathological states associated with metabolic syndrome.

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Review Article

PPAR Genomics and Pharmacogenomics: Implications for Cardiovascular Disease

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The peroxisome proliferator-activated receptors (PPARs) consist of three related transcription factors that serve to regulate a number of cellular processes that are central to cardiovascular health and disease. Numerous pharmacologic studies have assessed the effects of specific PPAR agonists in clinical trials and have provided insight into the clinical effects of these genes while genetic studies have demonstrated clinical associations between PPAR polymorphisms and abnormal cardiovascular phenotypes. With the abundance of data available from these studies as a background, PPAR pharmacogenetics has become a promising and rapidly advancing field. This review focuses on summarizing the current state of understanding of PPAR genetics and pharmacogenetics and the important implications for the individualization of therapy for patients with cardiovascular diseases.

1. INTRODUCTION

PPAR-alpha (PPARα), PPAR-beta/delta (PPARβ/δ), and PPAR-gamma (PPARγ) are nuclear hormone receptor transcription factor proteins encoded by similarly named genes (*PPARA*; *PPARD*; *PPARG*) [1, 2]. Each of the PPARs has multiple promoters and more than one isoform, resulting from alternate splicing, alternative transcription start sites or both [3–5]. The PPARs have distinct, but overlapping, tissue expression patterns and act to coordinately regulate multiple metabolic pathways [1, 2].

PPARα is highly expressed in the heart, liver, and skeletal muscles [2]. In these tissues, PPARα is the central regulator of genes involved in fatty acid metabolism and appears to mediate the balance between cellular fatty acid and glucose metabolism, particularly at times of metabolic or physiologic stress, such as myocardial ischemia, hypertrophy, heart failure, and insulin resistance [6–15]. In addition, PPARα is involved in the energy substrate and fiber-type switches that occur in skeletal muscle as a result of conditioning [16] and is involved in the inflammatory response during vascular atherosclerosis [17–19].

PPARγ is highly expressed in both white and brown adipocytes [2, 20, 21]. PPARγ controls adipocyte lipid storage and release and is an important mediator of insulin sensitivity [22, 23]. In addition, PPARγ regulates adipocyte release of adipokines including tumor necrosis factor alpha (TNFα), angiotensinogen (AGT), interleukin-6 (IL-6), and plasminogen activator inhibitor type 1 (PAI-1) [24].

PPARβ/δ, also known as nuclear hormone receptor 1 (NUC 1) or fatty acid-activated receptor (FAAR), is ubiquitously expressed but is expressed at higher levels in the brain, adipose tissue, and skin [2, 25]. PPARβ/δ is thought to be critically important in adipocyte and skeletal muscle fatty acid oxidation and is another important mediator of insulin sensitivity [26–28]. PPARβ/δ appears to also be involved in obesity [26–28] and in preventing myocardial hypertrophy via NF-κB inhibition [29–31].

The PPARs are able to bind many different ligands including metabolic intermediates (fatty acids), pharmacologic agents (fibrates, thiazolidinediones), and natural herbs (green tea) [32–36]. In the presence of ligand, PPARs bind to their cognate regulatory elements as a heterodimer with retinoid X receptor α [37]. Ligand binding causes a conformational change that results in the recruitment of coactivators and increased transcriptional activation of target genes [34, 35, 38, 39].

There is considerable clinical association data linking polymorphisms of *PPARA*, *PPARD*, and *PPARG* with cardiovascular disease (coronary and carotid atherosclerosis, left

Table 1

	SNP	rs number
PPARA	Leu162Val	rs1800206
	Val227Ala	rs1800234
	IVS7 2498	rs4253778
	IVS7 1343	rs4253776
PPARG	Pro12Ala	rs1801282
	25,506 C > T	rs2028759
	54,347 C > T	rs3856806
PPARD	−87 T > C	rs9658134
	−4,401 C > T	rs2038068
	−48,444 C > T	rs6902123

ventricular hypertrophy) and cardiovascular risk factors (incidence of type 2 diabetes mellitus (DM), obesity, insulin resistance, and abnormal lipid profiles) in populations of diverse ethnicity. There is less data on PPAR pharmacogenetics, but the field is rapidly growing and of considerable interest to many investigators. PPAR pharmacogenetics of fibrates (gemfibrozil, fenofibrate, and bezafibrate), thiazolidinediones or glitazones (troglitazone, pioglitazone, and rosiglitazone), statins, and acarbose have particular relevance to cardiovascular disease.

This review will discuss several significant PPAR genetic and pharmacogenetic associations that have been observed with respect to cardiovascular disease (Table 1 provides the rs number for each SNP discussed in this review). Understanding the current state of PPAR genetics and pharmacogenetics may have important implications for the future individualization of therapy for patients with cardiovascular disease.

2. *PPARA*

2.1. *PPARA Leu162Val genetic associations*

2.1.1. *Dyslipidemias*

PPARA Leu162Val is a polymorphism located in the DNA binding region of PPARα that confers differential ligand-mediated activation of PPARα in vitro [40, 41]. Investigators from several clinical studies have observed that carriers of the *PPARA* Val162 allele, compared to *PPARA* Leu162 homozygotes, have significantly higher concentrations of serum triglycerides, total cholesterol, LDL cholesterol, and apolipoprotein (apo) B and apoC-III. However, there have been exceptions, and not all studies have found an association with all five serum lipids [41–45]. The larger trial findings, as well as the studies that have negative findings, will be discussed here.

Recently, the association of the *PPARA* Leu162Val polymorphism with serum lipid levels was investigated in 5799 individuals from the Inter99 cohort, a Danish cohort targeted for identifying parameters affecting participation in a diet and exercise intervention in the general population [46]. In this cohort, individuals homozygous for the *PPARA* Val162 allele, compared to PPARA Leu162 allele carriers, demonstrated a 70% greater mean fasting serum triglyceride level (2.2 mmol/L (195 mg/dL) versus 1.3 mmol/L (115 mg/dL), resp.; $P = .007$) and a greater mean fasting serum total cholesterol levels (6.2 mmol/L (240 mg/dL) versus 5.5 mmol/L (213 mg/dL), resp.; $P = .01$) [45].

These findings confirmed previous observations in 2373 participants of the Framingham Offspring Study. When the association of the *PPARA* Leu162Val polymorphism with variation in lipid levels was investigated in these subjects, *PPARA* Val162 carriers, compared to *PPARA* Leu162 homozygotes, had significantly increased serum concentrations of total cholesterol in men ($P = .0012$), LDL cholesterol in men ($P = .0004$), apoC-III in men ($P = .009$), and apoB in men and women ($P = .009$ and .03, resp.) [44]. These same investigators went on to demonstrate that the association of the *PPARA* Leu 162Val polymorphism on plasma triglycerides and apoC-III concentrations was more complex and depended on the person's regular dietary polyunsaturated fatty acid intake. PPARA Val162 allele carriers that had a low polyunsaturated fatty acid intake (<6% of calories) had greater serum triglyceride and apoC-III concentrations,compared to PPARA Leu162 homozygotes, whereas PPARA Val162 allele carriers that had a high polyunsaturated fatty acid intake had lower triglyceride and apoC-III concentrations, compared to *PPARA* Leu162 homozygotes [47].

Other studies have also investigated the association of the *PPARA* Leu162Val polymorphism with serum lipid response to diets of different fat composition. Tanaka et al. studied 59 healthy male students fed a single high-fat meal (60% calories as fat (63% saturated fatty acids, 33% monounsaturated fatty acids, and 4% polyunsaturated fatty acids); 15% calories as protein; and 25% calories as carbohydrate) following a 12-hour fast [48]. *PPARA* Val162 allele carriers had significantly higher fasting (baseline) total cholesterol, LDL cholesterol, and apoB levels, compared to Leu162 homozygotes and this variation in serum lipids was maintained after the high-fat meal [48]. No significant association of the *PPARA* Leu162Val polymorphism with serum triglyceride concentrations (either fasting or postprandial) was observed (apoCIII was not measured) [48]. Paradis et al. investigated the association of the *PPARA* Leu162Val polymorphism with serum lipid response in ten PPARA Val162 allele carriers and ten age and body mass index-matched PPARA Leu162 homozygotes subjected to a high polyunsaturated fat followed by a low polyunsaturated fat diet [49]. At baseline, the *PPARA* Leu162Val polymorphism was not associated with variation in serum lipids [49]. After the high polyunsaturated fat diet, *PPARA* Val162 allele carriers had a significant decrease in plasma apoA-I levels, total cholesterol, and LDL cholesterol (small particles), compared to the *PPARA* Leu162 homozygotes (who demonstrated an increase in plasma apoA-I levels, total cholesterol, and LDL cholesterol (small particles): $P = .02$, $P = .07$ and $P = .08$, resp.) [49].

In contrast to the aforementioned studies, when the association of the *PPARA* Leu162Val polymorphism with variations in serum lipids was investigated in 3012 healthy middle-aged men in the second Northwick Park Health Study (NPHS2, Northwick, UK), no association of the *PPARA* Leu162Val polymorphism with serum lipids at

baseline, or in response to therapy, was found [50]. Although it was a smaller study, the Lopid Coronary Angiography Trial (LOCAT), a clinical trial of 395 postcoronary bypass men, with an HDL cholesterol ≤1.1 mmol/L and LDL cholesterol ≤4.5 mmol/L that investigated the progression of coronary atherosclerosis in response to lipid lowering therapy with gemfibrozil, [51, 52] also found no association between the *PPARA* Leu162Val polymorphism and serum lipids either at baseline, or in response to therapy [50].

2.1.2. Coronary atherosclerosis

As discussed above, LOCAT found no association of the *PPARA* Leu162Val polymorphism with variations in serum lipids [50]. However, this study did observe that carriers of the *PPARA* Val162 allele showed significantly less progression of atherosclerosis in both gemfibrozil-treated and untreated groups [50]. No pharmacogenetic (i.e., treatment by genotype) interaction was found [50].

2.2. PPARA Leu162Val pharmacogenetic associations

2.2.1. Response to gemfibrozil

The Helsinki Heart Study (Helsinki, Finland) was a primary prevention trial that demonstrated that randomization to treatment with gemfibrozil resulted in a 34% reduction in cumulative cardiac events and a 26% reduction in cardiac mortality [53, 54]. Subgroup analysis demonstrated that overweight men with body mass index between 27–40 kg/m^2 had the largest reduction in cardiac events in response to gemfibrozil in the Helsinki Heart study [55]. Given that the greatest response to gemfibrozil was observed in this group, the association between genetic variation in the *PPARA* Leu162Val polymorphism and the response to gemfibrozil was investigated in 63 abdominally obese men in a randomized placebo-controlled trial [56]. After 6 months of treatment, carriers of the *PPARA* Val162 allele demonstrated a 50% increase in HDL_2 cholesterol compared to *PPARA* Leu162 allele homozygotes who only had a 5.5% increase (P = .03) [56]. The *PPARA* Leu162Val was responsible for 7% of the variance of the change in HDL_2 cholesterol and there was a significant genotype-by-treatment interaction between the *PPARA* Leu162Val polymorphism and the increase in HDL_2 cholesterol [56].

The Veterans Affairs High-Density Lipoprotein Intervention Trial (VA-HIT) study of patients with known ischemic heart disease, selected for low levels of HDL cholesterol (mean of 32 mg/dL), demonstrated that randomization to gemfibrozil therapy resulted in a 22% reduction in relative risk of coronary events and a 31% reduction in cerebral vascular events [57–59]. In VA-HIT, the subgroup that benefited the most in reduction of cardiovascular events in response to gemfibrozil were those patients that had DM or insulin resistance [60, 61]. Given that this group had demonstrated the greatest response, the association between genetic variation in the *PPARA* Leu162Val polymorphism and the response to gemfibrozil was investigated [62]. VA-HIT patients with DM or insulin resistance treated with gemfibrozil who were *PPARA* Leu162 homozygotes had a greater absolute reduction in cardiovascular events (12.1% reduction compared to treatment with placebo; P = .06) compared to carriers of the *PPARA* Val162 allele who had a nonsignificant reduction (9.9% compared to treatment with placebo; P = .28) [62]. Furthermore, in VA-HIT patients without DM or insulin resistance, carriers of the Val162 allele had a significant increase in cardiovascular events in response to gemfibrozil (7% increase compared to treatment with placebo; P = .01) [62].

2.2.2. Response to fenofibrate

The Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study investigated the response to fenofibrate (160 mg) for ≥21 days in 791 men and women enrolled in The Family Heart Study (FHS, a multicenter, family pedigree study aimed to identify genetic and environmental risk factors of cardiovascular disease) [63]. Overall, there was a 37 mg/dL reduction in fasting serum triglyceride levels after treatment with fenofibrate (the average of two separate measurements obtained prior to treatment and at the end of treatment were used). Although only reported in abstract form to date, variation in *PPARA* Leu162Val polymorphism was significantly associated with fasting triglyceride level response to fenofibrate treatment [64]. Individuals homozygous for the *PPARA* Val162 allele had a 73 mg/dL reduction in their fasting triglyceride response to fenofibrate compared to *PPARA* Leu162Val heterozygotes (46 mg/dL reduction) and *PPARA* 162Leu homozygotes (53 mg/dL reduction; P < .0001) [64].

2.3. PPARA Val227Ala genetic associations

2.3.1. Dyslipidemias

PPARA Val227Ala is a polymorphism located between the DNA binding and ligand binding domain of PPARα. This region is thought to be important in heterodimerization but in vitro experiments confirming a functional difference in alleles have not yet been performed [65]. The association of the *PPARA* Val227Ala polymorphism with serum lipid levels was investigated in a study of 401 healthy Japanese individuals presenting to medical clinic for routine health care [65]. After adjustment for age and body mass index, female carriers of the Val227 allele had significantly lower serum total cholesterol (P = .046) and triglyceride levels (P = .038) compared to Ala227 homozygotes [65]. Male carriers of the Val227 allele also had lower serum total cholesterol and triglyceride levels compared to Ala227 homozygotes, but the differences were not significant (P = .30 and .54, resp.) [65].

Recently, the finding of this small study was confirmed in 2899 Chinese individuals from the 1998 Singapore National Health Survey (NHS98) [66]. Women *PPARA* Ala227 allele carriers had significantly lower serum total cholesterol (P = .047) and triglyceride levels (P = .048), compared to *PPARA* Val227 homozygotes, and men had lower levels that were, again, not significant (P = .65 and .12, resp.) [66]. In addition to these findings, this study also found a significant interaction between the *PPARA* Val227Ala polymorphism and

serum HDL cholesterol levels in response to dietary polyunsaturated fatty acid intake in women suggesting a gene-environment interaction (P-value for interaction = .049) [66]. Specifically, the authors found that, in women who were *PPARA* Ala227 allele carriers, increasing dietary polyunsaturated fatty acid intake resulted in lower serum HDL cholesterol levels. This result was in contrast to male *PPARA* Ala227 allele carriers, who had an increase in serum HDL cholesterol levels, and women who were *PPARA* Val227 homozygotes, who demonstrated less lowering [66].

2.4. *PPARA IVS7 2498 G* > *C genetic associations*

2.4.1. Coronary atherosclerosis

PPARA IVS7 2498 is a polymorphism located in intron 7 of *PPARA*. The functional significance of this polymorphism has remained elusive but significant clinical associations have been found with this polymorphism. In LOCAT, *PPARA* IVS7 2498 (designated "*PPARA* intron 7 G/C polymorphism" in the publication) C allele carriers had a significantly greater progression of coronary atherosclerosis compared with GG homozygotes [50]. No pharmacogenetic interaction was noted [50]. When the association of *PPARA* IVS7 2498 polymorphism with coronary atherosclerosis was investigated in 3,012 healthy middle-aged men in NPHS2, *PPARA* IVS7 2498 CC homozygotes showed a trend toward greater incidence of ischemic events (myocardial infarction (MI) or coronary revascularization) (HR 1.83; 95% CI 0.96–3.51; P = .07) compared to *PPARA* IVS7 2498 CG heterozygotes and *PPARA* IVS7 2498 GG homozygotes [50].

2.4.2. Left ventricular hypertrophy

The *PPARA* IVS7 2498 (designated "*PPARA* intron 7 G/C polymorphism" in the publication) has also been associated with physiologic left ventricular hypertrophy in 144 young male British army recruits undergoing a rigorous ten-week exercise program (mixed upper and lower body strength and endurance training) [67]. This polymorphism has also been associated with pathologic left ventricular hypertrophy in 1148 hypertensive men and women enrolled in an echocardiography substudy of the third monitoring trends and determinants in cardiovascular disease (MONICA) Augsburg study [67]. In both studies, the *PPARA* IVS7 2498 C allele was significantly associated with increased LV mass index [67].

2.5. *PPARA IVS7 2498 G* > *C pharmacogenetic associations*

2.5.1. Response to fenofibrate

The Diabetes Atherosclerosis Intervention Study (DAIS) was designed to investigate if fenofibrate treatment of relatively mild dyslipidemia in 418 patients with type 2 DM would be associated with less progression of coronary atherosclerosis after treatment for at least 3 years with fenofibrate [68]. DAIS found that fenofibrate reduced the progression of angiographic coronary artery disease [69], the progression of microalbuminuria (an early marker of diabetic nephropathy, and an independent risk factor for cardiovascular disease) [70]; and although not powered to examine clinical events, there were fewer in the fenofibrate group compared to the placebo group [69]. Given these findings, the association between genetic variation in the *PPARA* IVS7 2498 polymorphism (designated "*PPARA* intron 7 G/C polymorphism" in the publication) and response to fenofibrate in DAIS was investigated [71]. DAIS subjects were divided into high responders (greater than 30% reduction, chosen because 30% was the mean reduction in DAIS) and low responders (less than 30% reduction) in their plasma triglyceride levels and the prevalence of *PPARA* IVS7 2498 genotype in the two groups was assessed [71]. Of the 85 high responders (55% of population), there was a significantly different prevalence of *PPARA* IVS7 2498 GG homozygotes (84.7%) when compared to the low responders (68.6%; $P < .05$) [71]. In stepwise logistic regression analysis, the best independent predictors of response to fenofibrate treatment were baseline triglyceride level and *PPARA* IVS7 2498 genotype (*PPARA* IVS7 2498 GG versus C allele carriers response to fenofibrate: OR 3.1; 95% CI 1.28–7.52; P = .012) [71].

2.5.2. Response to acarbose

Investigators from the STOP-NIDDM trial were interested in whether *PPARA* polymorphisms would be associated with the conversion to type 2 DM in response to acarbose in patients with impaired glucose tolerance [72, 73]. They investigated this association with 11 SNPs located from exon 1 to exon 8 of *PPARA* and found that in the acarbose-treated group, *PPARA* IVS7 2498 (designated "rs4253778" in the publication) CC homozygotes had a 2.7-fold risk of developing type 2 DM (95% CI 1.14–6.79; P = .03) [74]. *PPARA* IVS7 1343 (designated "rs4253776" in the publication), a SNP located1,155 nucleotides upstream of *PPARA* IVS7 2498 and in moderate LD with *PPARA* IVS7 2498 (r^2 of 0.565 in this population), also had an association with the development of type 2 DM [74]. *PPARA* IVS7 1343 G allele carriers had a 1.7-fold increased risk of developing type 2 DM (95% CI 1.04–2.88; P = .04) and a significant treatment by genotype interaction was observed [74].

3. *PPARG*

3.1. *PPARG Pro12Ala genetic associations*

3.1.1. Metabolic traits and the development of type 2 DM

The *PPARG* Pro12Ala polymorphism is in exon B of *PPARG* which is specific to PPARγ2, the PPARγ isoform restricted to adipose tissue [75]. In vitro experiments have demonstrated that, compared to the *PPARG* Pro12 variant, the *PPARG* Ala12 variant has lower binding affinity for a PPAR responsive element and decreased PPARγ-activation of a reporter construct in response to ligand [75]. The *PPARG* Pro12Ala polymorphism has been the most investigated PPAR polymorphism.

The association of the *PPARG* Pro12Ala polymorphism with metabolic traits and the risk/development of DM has been investigated in individuals of all ages and of different ethnicities including Chinese and Japanese individuals in the Hypertension and Insulin Resistance (SAPPHIRe) study, [76], Iranian individuals [77], obese Italian children, [78] middle-aged and elderly Finns, [75] and Spanish women [79]. Although most of these studies (including the ones mentioned here) report that *PPARG* Ala12 allele carriers have increased insulin sensitivity compared to *PPARG* Pro12 homozygotes, a recent meta-analysis of 57 studies reported that this association only held for certain subgroups [80]. When *PPARG* Ala12 allele carriers were compared to *PPARG* Pro12 homozygotes, only the obese subgroup demonstrated increased insulin sensitivity [80]. However, when *PPARG* Ala12 homozygotes were compared to *PPARG* Pro12 homozygotes (full genotype information that allowed this analysis was only available in 12 of the 57 studies), the association of the *PPARG* Ala12 allele with increased insulin sensitivity was more evident in all groups [80].

More recently, the association of the *PPARG* Pro12Ala polymorphism with metabolic traits and the risk of developing hyperglycemia over 6 years was investigated in 3,914 French Caucasians in the Data From an Epidemiological Study on the Insulin Resistance Syndrome (DESIR) cohort (of note, this study was not included in the meta-analysis as it was published after the meta-analysis was submitted) [81]. At baseline, *PPARG* Ala12 allele carriers had significantly lower fasting insulin and insulin resistance as determined by homeostasis model assessment of insulin resistance ($P = .001$, compared to *PPARG* Pro12 homozygotes) [81]. After 6 years of follow up, *PPARG* Ala12 allele carriers had significantly less increase in fasting insulin ($P = .007$, compared to *PPARG* Pro12 homozygotes) and insulin resistance ($P = .018$, compared to *PPARG* Pro12 homozygotes) [81]. In addition, after 6 years of follow up, *PPARG* Ala12 allele carriers who were normoglycemic at baseline ($n = 3{,}498$) had significantly less hyperglycemia, compared to compared to *PPARG* Pro12 homozygotes [81].

This data, as well as very recent data from 3,548 individuals in the diabetes prevention program (DPP) [82] confirmed two earlier meta-analyses (of the literature available at time of each meta-analysis publication) [83, 84]. This large study reported that *PPARG* Pro12 homozygotes had a 1.2-fold increased risk of developing type 2 DM (95% CI 0.99–1.57; $P = .07$) compared to *PPARG* Ala12 allele carriers [85]. This relative risk matched the 1.2-fold risk found in both meta-analyses ($P = .002$ in the meta-analysis performed by Altshuler et al.) [83, 84].

3.1.2. Coronary and carotid atherosclerosis

Several studies have investigated the association of the *PPARG* Pro12Ala polymorphism with coronary artery disease and/or myocardial ischemic events, however, some have yielded contradictory results [86–88]. 14,916 men enrolled in the Physicians' Health Study [89] were followed for a mean of 13.2 years and the association between *PPARG* Pro12Ala polymorphism and MI was assessed [88]. *PPARG* Pro12Ala genotype was compared in 523 individuals who developed an MI, and 2,092 who did not show evidence of an MI [88]. Of those individuals who developed an MI, the frequency of *PPARG* Ala12 allele carriers was significantly less than in the controls, with a decreased risk of subsequent MI (hazard ratio HR = 0.77; 95% CI 0.60–0.98; $P = .034$) [88]. This relationship held even after controlling for traditional cardiac risk factors.

In contrast, a study of 2,016 patients with type 2 DM from the genetic portion of the continually updated dataset known as the Diabetes Audit and Research in Tayside Scotland database (Go-DARTS) [87], a borderline, nonsignificant association of the *PPARG* Ala12 allele carriers with nonfatal MI or revascularization (HR 0.54; 95%CI 0.27–1.08; $P = 0.08$, compared to *PPARG* Pro12 homozygotes) was observed for the entire group. Subgroup analysis demonstrated a significant association if patients younger than 70 years old at time of enrollment were assessed separately (HR 0.43; CI 0.18–0.99; $P = .05$) or if patients younger than 70 year old at time of enrollment with no prior history of stroke, MI, or revascularization were evaluated for time to first event (HR 0.21; CI 0.06–0.69; $P = .01$) [87].

When the association of *PPARG* Pro12Ala polymorphism with the risk of coronary artery disease was assessed prospectively in women enrolled in the Nurses' Health Study (8 years mean follow up) and in men (6 years mean follow up) enrolled in the Health Professionals Follow-Up Study (HPSF) [86], carriers of the *PPARG* Ala12 allele again had an increased risk of MI [86]. 249 women and 266 men with MI were compared to nested case-controls and matched for age, smoking status, and phlebotomy date [86]. Men carriers of the *PPARG* Ala12 allele had an increased risk of MI or cardiac death (RR = 1.44; CI 1.00–2.07; $P = .05$) [86]. There was no statistical difference in nonfatal MI or cardiac death in women carriers of the *PPARG* Ala12 allele (RR = 1.17; CI 0.82–1.68; $P = .39$) [86]. When data were pooled for men and women, carriers of the *PPARG* Ala12 allele had an increase risk of MI or cardiac death (RR = 1.30; CI 1.00–1.67; $P = .05$) and, when stratified by body weight, men and women with a body mass index $\geq 25\ \text{kg/m}^2$ had a 1.68-fold increase in risk (CI 1.13–2.50; $P = .01$) [86].

A study of 267 Korean individuals (158 males and 109 females) referred for coronary angiography for chest pain, found no significant association between the *PPARG* Pro12Ala polymorphism and prevalence or severity of coronary artery disease [90]. While the results from these studies may seem contradictory, there are obvious differences in study design, patient cohorts, primary end-points, and power. In addition, it is possible that geographic and ethnic differences in allele frequencies may contribute to variability in the study findings.

An association has also been observed between the *PPARG* Pro12Ala polymorphism and carotid intima media thickness [91, 92]. In two studies involving over 300 patients, carriers of the *PPARG* Ala12 allele had less carotid intima media thickness measured by B-mode ultrasound [91, 92].

3.2. PPARG Pro12Ala pharmacogenetic associations

3.2.1. Response to rosiglitazone

The *PPARG* Pro12Ala polymorphism resides in the ligand binding domain of PPARγ and could therefore result in different affinity to bind TZDs. Variation in the *PPARG* Pro12Ala polymorphism and response to rosiglitazone was investigated in 198 men and women with type 2 DM (HbA_{1C} values between 7.5–11.5% and fasting glucoses between 140–250 mg/dL) treated with rosiglitazone for 12 weeks [93]. The decrease in fasting glucose in response to the drug was significantly greater in carriers of the *PPARG* Ala12 allele compared to *PPARG* Pro12 homozygous patients [93]. Improvement in HbA_{1C} was also significantly better in carriers of the *PPARG* Ala12 allele compared to *PPARG* Pro12 homozygous patients [93]. In addition, 86.67% of *PPARG* Ala12 allele carriers responded to rosiglitazone (defined by a greater than 15% decrease in HbA_{1C} levels and/or a greater than 20% decrease in fasting glucose level) compared to 43.72% of *PPARG* Pro12 homozygous patients ($P = .002$) [93].

3.2.2. Response to acarbose

Investigators from the STOP-NIDDM trial were interested in whether PPAR polymorphisms would be associated with the conversion to type 2 DM in response to acarbose in patients with impaired glucose tolerance [72, 73]. They found that women treated with acarbose homozygous for the *PPARG* Pro12 allele had increased risk of developing type 2 DM compared to *PPARG* Ala12 allele carriers treated with acarbose (OR 2.89; 95% CI 1.20–6.96; $P = .018$) but found no significant difference in the men [72]. The authors did not provide an explanation for the gender differences.

3.3. PPARG 54,347 C > T genetic associations

3.3.1. Coronary atherosclerosis

The *PPARG* 54,347 C > T polymorphism (also referred to as *PPARG* 161 C > T and *PPARG* 14,311 C > T) is a silent C > T substitution (i.e., does not cause an amino acid change in the protein) in nucleotide 161 of exon 6 [94]. No functional information on this polymorphism is available to date. The *PPARG* 54,347 C > T polymorphism has been associated with the extent of coronary artery disease by angiography [95], carotid intima media thickness [92], and incidence of MI among individuals younger than age 50 [96].

3.4. PPARG 54,347 C > T pharmacogenetic associations

3.4.1. Response to fluvastatin

The Lipoprotein and Coronary Atherosclerosis Study (LCAS) was a randomized, placebo-controlled study of 429 subjects, 35–70 years old, with at least one 30–75% diameter stenosis on coronary angiography and LDL cholesterol of 115–190 mg/dL designed to assess the regression in coronary atherosclerosis (as measured by within-patient per-lesion change in minimal lumen diameter by quantitative coronary angiography) in response to fluvastatin [97, 98]. After 2.5 years of treatment with fluvastatin, mean LDL cholesterol was reduced by 23.9%, and change in minimal lumen diameter by quantitative coronary angiography was significantly less in the fluvastatin-treated group (0.028 mm decrease in diameter in the fluvastatin-treated group compared to 0.100 mm decrease in diameter in the placebo group; $P < .01$) [99]. Clinical event rates had a trend towards benefit in the fluvastatin-treated group but were not statistically significant [99].

Genetic variation of *PPARG* 54,347 C > T (designated "*PPARG* 161 C > T" in the publication), *PPARG* Pro12Ala, and *PPARG* 25,506 C > T as well as the association with baseline lipid parameters and response to fluvastatin was assessed in 372 individuals from LCAS [100]. *PPARG* haplotype was associated with the degree of coronary atherosclerosis (mean number of coronary lesions; $P = .026$) and changes in minimum lumen diameter ($P = .022$) in response to fluvastatin [100]. *PPARA* and *PPARD* polymorphisms were also assessed: no associations were found with *PPARA* genotype or haplotype; *PPARD* associations are discussed below [100].

3.5. PPARG haplotype pharmacogenetic associations

3.5.1. Response to troglitazone

The Troglitazone in the Prevention of Diabetes (TRIPOD) study was a placebo-controlled trial designed to test if TZD therapy could prevent the development of type 2 DM in Hispanic women with previous gestational DM [101, 102]. In this trial, the incidence of type 2 DM was decreased by 55% in the troglitazone-treated group (coincident with improvement in insulin sensitivity) compared to placebo [102]. Interestingly, 8 months after discontinuation of treatment, there remained a statistically significant difference in the development of type 2 DM between those treated with troglitazone and placebo (2.3% versus 15%; $P = .03$) [102].

In TRIPOD, 30% of women were classified as nonresponders as they were in the lowest tertile of 3 month improvement in insulin sensitivity and did not gain any protection from development of type 2 DM [102]. Although there was no association of the common, functional *PPARG* Pro12Ala polymorphism with response to troglitazone [103], there was an individual association of eight other *PPARG* polymorphisms with troglitazone response [104]. In addition, three hapolotypes blocks were defined that were independently, or jointly, involved in mediating the response to troglitazone [104]. Specifically, individuals with the most common haplotype within a haplotype block starting in intron 1, containing the A2 promoter and ending within intron 2 (designated "Block 1" in the publication) had an odds ratio of 2.22 for nonresponse to troglitazone ($P = .032$), and the most common haplotype within a haplotype, located completely within intron 2 (designated "Block 2" in the publication), had an odds ratio of 4.18 for nonresponse ($P = .012$) [104]. In addition, the most common haplotype within a haplotype located in the 3′ untranslated region of

PPARG (designated "Block 5" in the publication) had a borderline significant odds ratio of 0.51 for response ($P = .049$) [104].

4. *PPARD*

4.1. *PPARD −87 T > C genetic associations*

4.1.1. *Dyslipidemias*

The *PPARD* −87 T > C (designated "*PPARD* 294 T > C" polymorphism in the publication) was one of four polymorphisms identified by direct sequencing of the 5′ untranslated region of *PPARD* in 20 unrelated healthy subjects [105]. This polymorphism is located 87 base pairs upstream of the translation start site and 294 base pairs downstream from the transcription start site. In vitro experiments have demonstrated functional differences of the two variants and have implicated the transcriptional corepressor SP1 in contributing to the differences [106].

When the association of the *PPARD* −87 T > C polymorphism with variation in plasma lipid levels was investigated in 543 healthy men (and validated in an independent cohort of 282 healthy men), *PPARD* −87 CC homozygotes had increased plasma LDL cholesterol compared to *PPARD* −87 TT homozygotes [106].

4.1.2. *Coronary atherosclerosis and cardiac events*

Skogsberg et al. investigated whether the *PPARD* −87 T > C polymorphism (designated "*PPARD* 294 T > C" polymorphism in the publication) was associated with increased plasma-LDL cholesterol levels and/or increased risk of having cardiac events. In the West Of Scotland Coronary Prevention Study (WOSCOPS), a randomized, double-blind, placebo-controlled trial with the primary goal of investigating the effect of pravastatin in preventing cardiac events in patients with mild-to-moderate hypercholesterolemia (LDL cholesterol between 4.5 and 6.0 mmol/L) [107]. Although carriers of the *PPARD* −87 C allele had a significantly lower HDL cholesterol compared with the PPARD −87 TT homozygotes, there was no association of this polymorphism with cardiac events and no genotype-by-treatment interaction [107].

4.2. *PPARD haplotype pharmacogenetic associations*

4.2.1. *Response to fluvastatin*

Genetic variation of *PPARD* −87 T > C (designated "*PPARD* 294 T > C" in the publication) and *PPARD* −4401 C > T as well as the association with baseline lipid parameters and response to fluvastatin was assessed in 372 individuals from LCAS [100]. *PPARD* haplotype was associated with the degree of coronary atherosclerosis (mean number of coronary lesions) and changes in triglyceride ($P = .01$) and apoC-III ($P = .047$) levels in response to fluvastatin [100].

4.2.2. *Response to acarbose*

Genetic variation in six SNPs in *PPARD* in patients with impaired glucose tolerance and association with the conversion to type 2 DM in response to acarbose was investigated in the STOP-NIDDM trial [72, 73]. Women treated with acarbose carrying the C allele of *PPARD* −48,444 C > T (designated "rs6902123" in the publication) had increased risk of developing type 2 DM compared to TT homozygous women treated with acarbose (OR 2.70; 95% CI 1.44–5.30; adjusted $P = .002$) [73].

5. CONCLUSIONS

With their pleiotropic effects on lipid metabolism, glucose homeostasis, myocardial energetics, and responses to ischemia, as well as the considerable evidence linking genetic polymorphisms identified within the PPAR complex to common cardiovascular diseases, the PPAR family of transcription factors is central to the regulation of a number of key cellular pathways that impact on normal and pathologic cardiovascular physiology and thus represent very promising targets for further advances in pharmacologic intervention. Early pharmacogenetic investigations into the associations of a select few of these polymorphisms with patient responses to drug therapy have yielded important clues to commonly observed variability in both response and outcomes. Given the central role of the PPARs in critical metabolic pathways, this experience points the way to a future where knowledge of relevant PPAR genotype might be utilized to guide more appropriately tailored and individualized therapy.

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Review Article

PPAR Agonists and Cardiovascular Disease in Diabetes

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Peroxisome proliferators activated receptors (PPARs) are ligand-activated nuclear transcription factors that play important roles in lipid and glucose homeostasis. To the extent that PPAR agonists improve diabetic dyslipidaemia and insulin resistance, these agents have been considered to reduce cardiovascular risk. However, data from murine models suggests that PPAR agonists also have independent anti-atherosclerotic actions, including the suppression of vascular inflammation, oxidative stress, and activation of the renin angiotensin system. Many of these potentially anti-atherosclerotic effects are thought to be mediated by transrepression of nuclear factor-kB, STAT, and activator protein-1 dependent pathways. In recent clinical trials, PPARα agonists have been shown to be effective in the primary prevention of cardiovascular events, while their cardiovascular benefit in patients with established cardiovascular disease remains equivocal. However, the use of PPARγ agonists, and more recently dual PPARα/γ coagonists, has been associated with an excess in cardiovascular events, possibly reflecting unrecognised fluid retention with potent agonists of the PPARγ receptor. Newer pan agonists, which retain their anti-atherosclerotic activity without weight gain, may provide one solution to this problem. However, the complex biologic effects of the PPARs may mean that only vascular targeted agents or pure transrepressors will realise the goal of preventing atherosclerotic vascular disease.

1. INTRODUCTION

Cardiovascular complications are the leading cause of premature mortality in patients with diabetes [1]. While classical risk factors for cardiovascular disease (CVD), such as smoking, cholesterol, and hypertension, operate in persons both with and without diabetes, the absolute risk of death is 2–4 times greater in patients with diabetes [2] and progressively larger with each additional risk factor [3]. Moreover, CVD, cerebrovascular diseases, and peripheral vascular diseases significantly contribute to the morbidity in individuals with diabetes [1]. Ultimately, these macrovascular complications will develop in more than half of the diabetic population [1]. In primary care, over a third of all patients presenting with type 2 diabetes have an overt history of CVD, with a similar number again likely to have undiagnosed macrovascular disease [4]. Consequently, a key component (and some would argue the most important component) in the management of diabetes is the primary and secondary prevention of cardiovascular events.

Diabetes is said to act as an amplifier of cardiovascular risk leading to the increased incidence, size, and complexity of atherosclerotic plaques [5, 6]. A number of components contribute to accelerated atherosclerosis in diabetes. Diabetic dyslipidaemia and insulin resistance significantly contribute to the development and progression of macrovascular disease in diabetes. In addition, inflammation, oxidative stress, enhanced matrix metalloproteinase activity, activation of the local renin angiotensin system (RAS), and the accumulation of advanced glycation end-products (AGEs) in the diabetic vasculature also act to enhance atherogenesis and impair plaque stability. Significantly, each of these pathways may be modified by the activity of peroxisome proliferator-activated receptors (PPARs), ligand-activated nuclear transcription factors with a diverse range of metabolic functions [7–11]. This review will examine the actions of PPARs in diabetes-associated atherosclerosis and explore the recent controversies surrounding the actions of PPAR agonists on CVD in patients with diabetes.

2. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS (PPARs)

PPARs are nuclear transcription factors that have complex biological effects, resulting from the transactivation or

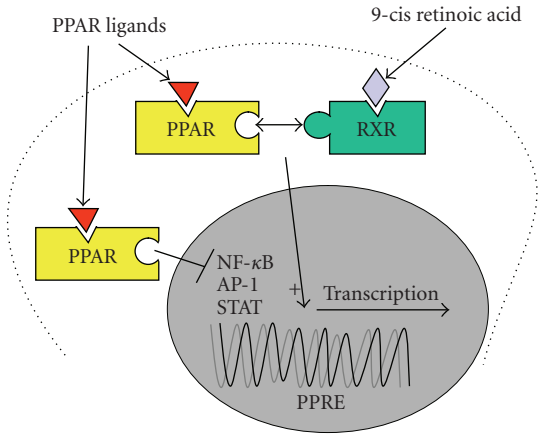

Figure 1: Transactivation and transrepression effects of peroxisome proliferator-activated receptors.

transrepression of dozens of genes that play important roles in glucose and lipid homeostasis [12]. Transactivation effects require ligand-activated dimerisation of PPAR with the retinoid X receptor (RXR), followed by translocation of the PPAR : RXR heterodimer complex to the nucleus, whereupon it binds to PPAR response elements of target genes and induces their expression [12]. *Transrepression* effects are mediated via interference with transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) [13] (see Figure 1). In addition, conformational remodelling of the PPAR receptor that follows ligand binding results in the release of co-repressor molecules. The relative importance of activation versus repression pathways for the *in vivo* actions of PPAR agonists remains to be established. Moreover, there is evidence that all PPAR ligands do not stimulate transactivation and transrepression pathways to a similar extent, meaning that different agents of the same class may have potentially disparate effects [14, 15].

Three different PPAR isoforms have been identified in humans. These share similar structural organization and sequence homology. However, these isoforms possess distinct functions, and vary in their ligand affinity, expression, and activity in different metabolic pathways.

3. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA (PPARα)

PPARα is highly expressed in the vasculature, including the endothelial cells [16, 17], smooth muscle cells [18], and macrophages [19]. Activation of PPARα leads to modulation of lipid metabolism, including transcription of apolipoprotein A1 (apoA1) [20] and apolipoprotein AII [21], resulting in increased levels of "cardioprotective" high-density lipoprotein (HDL) cholesterol. Uptake of HDL cholesterol is also increased via the upregulation of CLA-1/SR-B1 [22]. β-oxidation and lipoprotein lipase (LPL) activity are also stimulated following activation of PPARα. This leads to a decrease in triglycerides and free fatty acids, and levels of apolipoprotein CIII, which inhibits LPL-mediated breakdown of triglycerides, further resulting in lower triglyceride levels [23]. Finally, low-density lipoprotein (LDL) cholesterol particles are shifted from a small, dense to a large, buoyant form to create particles that are less atherogenic and more easily cleared [24]. The natural ligands of PPARα include prostaglandins, leukotrienes, and medium- and long-chain free fatty acids such as eicosapentaenoic acid and docosahexenoic acid [25, 26]. Synthetic ligands of this receptor are utilized in the management of dyslipidaemia [27], and include members of the fibrate drug class (e.g., gemfibrozil, clofibrate, fenofibrate, and bezafibrate).

4. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARγ)

PPARγ is largely expressed in adipose tissue as well as in skeletal muscle, sites where this PPAR isoform exerts much of its metabolic actions [28]. However, PPARγ is also expressed locally in the vasculature, including the endothelial cells [17], smooth muscle cells [29], and macrophages [13, 19]. Broadly, PPARγ activation results in increased sensitivity to the metabolic actions of insulin by reversing lipotoxicity-induced insulin resistance. PPARγ activation has also been shown to rejuvenate pancreatic β-cell function resulting in their improved function [30]. In adipose tissue, activation of PPARγ leads to differentiation of adipocytes, making them more able to uptake fatty acids, in turn, sparing other metabolic tissue such as skeletal muscle and liver [28]. In addition, PPARγ agonists increase the expression and activity of glucose transporter-4 and phosphatidyl-3-kinase [31, 32]. The natural ligands of PPARγ include prostaglandins, such as 15-deoxy-(12,14)-prostaglandinJ_2, and fatty acids including linoleic and arachidonic acids [32]. Synthetic ligands of PPARγ include the thiazolidinedione drug class (e.g., rosiglitazone and pioglitazone). Some other drugs also have partial agonist activity at the PPARγ receptor, including the AT_1 receptor antagonist, telmisartan [33].

5. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR DELTA (PPARβ/δ)

The PPAR delta isoform (also known as beta) is the most widely distributed of the PPARs, with expression seen in most tissues including the vasculature [34]. Unlike the other PPARs, PPARβ/δ-RXR heterodimers bind to consensus PPAR DNA response elements in the absence of a ligand, and repress target gene expression indirectly by recruiting co-repressors [35]. Following ligand activation, the co-repressor complex is disrupted, leading to enhanced PPARβ/δ target gene expression by both ligand-induced transcriptional activation and transcriptional derepression. In addition, repressor molecules, such as BCL-6, are liberated on ligand binding, leading to the repression of other pathways, such as inflammation and the transcriptional activity of PPARα and PPARγ [36, 37]. Because of its wide tissue expression, it was initially suggested that PPARβ/δ might simply serve a house-keeping role. However, more recent data suggest that PPARβ/δ can play an important role in wound healing, inflammatory responses, and lipid metabolism [34, 36]. For

example, PPARβ/δ activation has been shown to increase HDL cholesterol levels like PPARα ligands, as well as mediate insulin-sensitising and glucose lowering effects like PPARγ [34, 38]. PPARβ/δ deficient macrophages also show reduced recruitment, which may be particularly important for plaque stability [36] (see below). PPARβ/δ is activated by a large variety of ligands, such as fatty acids and eiconsanoids including prostaglandin A1, although the major natural ligand remains to be established [25]. Synthetic agonists with nanomolar affinities for PPARβ/δ have also been generated, although none are currently used in clinical practice. Interestingly, the physiological action of these agonists in experimental atherosclerosis is similar to the phenotype observed in PPARβ/δ knockout mice [36, 39], consistent with the important actions of transcriptional de-repression following activation of PPARβ/δ.

6. PPAR AGONISTS AND DIABETIC DYSLIPIDAEMIA

Diabetic dyslipidaemia is a major reversible risk factor for the prevention of CVD in individuals with diabetes [40]. A range of quantitative and qualitative lipid and lipoprotein abnormalities are observed in patients with diabetes [41]. The main components of diabetic dyslipidaemia are excessive postprandial lipaemia associated with increased plasma triglyceride [42], due to the accumulation of very low-density lipoprotein (VLDL), chylomicron remnants, and intermediate density lipoprotein particles in the plasma. This is thought to reflect both the overproduction of triglyceride-rich VLDL (due to increased flux of free fatty acids and hepatic resistance to the effects of insulin), together with reduced catabolism (associated with reduced LPL activity) [43]. HDL cholesterol levels are invariably reduced in patients with type 2 diabetes, reflecting increased catabolism of HDL particles [44]. In addition, HDL particles become enriched with triglyceride, in an attempt to cope with an increased VLDL burden. Although LDL cholesterol levels in patients with type 2 diabetes are often within the normal range, there remain significant disturbances in LDL metabolism in diabetes. For example, LDL production is significantly reduced, while impaired turnover of LDL particles promotes glycoxidative modification of lipoprotein particles and cholesterol deposition in the arterial wall [45–47]. Diabetes is also associated with the accumulation of small dense, triglyceride-rich, LDL particles that have an increased atherogenic potential [24].

As noted above, both PPAR agonists are able to significantly modify circulating lipid levels, and therein reduce cardiovascular risk in patients with diabetes [27, 48]. In particular, the use of fibrates in patients with diabetes increases HDL cholesterol, decreases triglyceride levels, and shifts LDL cholesterol distribution toward larger, less atherogenic particles [24, 27, 49]. PPARγ agonists also stimulate reverse cholesterol transport [50, 51] and have beneficial effects on the low HDL cholesterol levels and elevated triglyceride levels that characterize diabetic dyslipidaemia. However, thiazolidinediones can also modestly increase LDL cholesterol levels in some patients [48]. PPARβ/δ agonists are able to increase HDL cholesterol levels and improve postprandial triglyceride clearance [52].

7. PPAR AGONISTS AND INSULIN RESISTANCE

While glycemic control is important for the prevention of microvascular complications, its role in the development of atherosclerotic vascular disease is less clear [53]. For example, in the UKPDS study, macrovascular outcomes were not correlated with HbA_{1c}. However, CVD was reduced in patients that received the insulin sensitizer, metformin, when compared to equivalent glycemic control achieved by sulphonylureas or insulin therapy [54]. This led to the hypothesis that insulin sensitivity may itself play an important role in the development of macrovascular disease, and that agents that reduce insulin resistance, such as metformin and PPARγ agonists, by extension, may have particular benefits in the management of type 2 diabetes [55]. Certainly, resistance to the actions of insulin is strongly associated with CVD in patients with diabetes. To the extent that insulin resistance is linked to chronic hyperglycaemia, dyslipidaemia, inflammation, and hypertension as part of the metabolic syndrome, this association is not surprising. However, it is now clear that insulin also has direct actions in the vasculature that influence the development and progression of atherosclerotic disease. For example, in diabetic tissues, selective insulin resistance in the PI-3-kinase signaling pathway leads to reduced synthesis of nitric oxide, impaired metabolic control, and compensatory hyperinsulinaemia. At the same time, insulin signaling, via extracellular signal regulated kinase- (ERK) dependent pathways, is relatively unaffected in diabetes, meaning that hyperinsulinaemia is able to stimulate the expression of endothelin and other pathogenic mediators, tipping the balance of insulin's actions in favor of abnormal vasoreactivity, angiogenesis, and other pathways implicated in atherosclerosis [56, 57]. In addition, preferential impairment of non-oxidative glucose metabolism in diabetes leads to increased intracellular formation of AGEs and oxidative stress. Nonetheless, while it is conceivable that improvements in insulin sensitivity may have beneficial vascular effects in diabetes, the fact that PPAR agonists retain their anti-atherosclerotic activity in the absence of insulin [9] suggests that other (direct) actions may also be important for their anti-atherosclerotic activities.

8. THE POTENTIAL DIRECT ANTI-ATHEROSCLEROTIC ACTIONS OF PPAR AGONISTS

While improvements in metabolic control and the lipid profile have important effects on CVD in patients with diabetes, it is becoming increasingly clear that PPAR agonists have a range of independent actions on the vascular wall which impact on atherogenesis. In particular, pre-clinical studies demonstrate ligand-dependent PPAR activation is able to reduce the development and progression of atherosclerotic lesions in a range of experimental models, without needing to normalise dyslipidaemia and hyperglycaemia, or improve insulin resistance [58]. For example, studies from our group demonstrated that treatment with the PPARα

agonist, gemfibrozil, was able to prevent the accumulation of atherosclerotic plaque in apolipoprotein E (apoE) knockout (KO) mice, a model in which PPARα agonists have no effect on severe dyslipidaemia [8]. Similarly, treatment with the PPARγ agonist, rosiglitazone, in insulinopenic diabetic apoE KO mice was also associated with a reduction in aortic atherosclerosis [9], in the absence of insulin sensitization or improvement in glucose levels. Finally, treatment with a PPARβ/δ agonist, GW0742X, has also been shown to attenuate atherosclerosis in LDL receptor KO mice, in the absence of changes in plasma lipids [39]. Taken together, these studies point to possible direct effects of PPAR agonists on the vasculature that impedes pathogenic pathways implicated in the development of atherosclerosis, including inflammation, oxidative stress, metalloprotease activity, AGE accumulation, and activation of the RAS.

9. PPAR AGONISTS AND VASCULAR INFLAMMATION

Inflammation plays a key role in the development and progression of atherosclerotic vascular disease. Inflammatory cells are a major component of early atherosclerotic lesions, and inflammatory cytokines and chemokines accelerate plaque accumulation. Some of the earliest changes involve the activation of endothelial cells, which then express adhesion molecules such as vascular-cell adhesion molecule 1 (VCAM-1) [59], encouraging leucocyte recruitment, the production of chemokines, and further inflammation. Activation of PPAR receptors has also been strongly linked to this early inflammatory response. PPARα,γ and β/δ agonists reduce the expression of adhesion molecules, such as VCAM-1, on the surface of cytokine-activated endothelial cells, as well as reduce macrophage infiltration within atherosclerotic plaque [8, 9, 60, 61]. PPAR agonists also reduce the production of inflammatory cytokines including tumour necrosis factor (TNF)-α, IL-6, and IL-1β [7, 18]. PPARα activation indirectly modulates inflammatory components in HDL, such as apoA1, serum amyloid A, and paraoxonase-1 [62]. Thiazolidinediones are also able to inhibit endothelial cell activation [63] and indirectly alter systemic inflammation by actions in adipose tissue, reducing the production of pro-atherogenic adipokines including TNF-α and resistin [64]. PPARβ/δ may also have important anti-inflammatory actions. For example, in LDLR KO mice treatment with the PPARβ/δ agonist, GW0742X, was associated with a marked attenuation of atherosclerosis, with a concomitant decrease in monocyte chemoattractant protein (MCP)-1 and intercellular adhesion molecule (ICAM)-1 [39].

10. PPAR AGONISTS AND OXIDATIVE STRESS

Oxidative stress is thought to be a key mediator of atherosclerosis, contributing to the upregulation of adhesion molecules [65], acceleration of foam cell formation, and a reduction in plaque stability [66]. PPAR agonists are also able to modulate oxidative stress in vascular tissues. PPARα activation reduces the expression of the pro-oxidant NAD(P)H subunit p22phox, and increases endothelial expression of the anti-oxidant, CuZn superoxide dismutase [67]. PPARγ agonists also have potent anti-oxidant activity in human endothelial cells [67], hypercholesterolemic rabbits [68], and obese subjects [69]. Studies from our laboratory have shown that treatment of diabetic animals with either a PPARα or a PPARγ agonist is associated with a reduction in vascular superoxide production, together with reduced gene expression of the NAD(P)H oxidase subunits p47phox and gp91phox observed in the aorta of diabetic apoE KO mice [8, 9] (see Figure 3).

11. PPAR AGONISTS AND MATRIX METALLOPROTEINASES

Atherosclerotic plaque rupture, with subsequent occlusive thrombosis, is the underlying cause of sudden cardiac events. Matrix metalloproteinases (MMPs) are thought to mediate the progression of atherosclerotic lesions to an unstable phenotype that is more prone to rupture, through the destruction of the overlying fibrous cap. PPAR agonists may promote plaque stability by reducing the production of MMPs from monocytes/macrophages and vascular smooth muscle cells [70]. Our group has recently demonstrated that gemfibrozil treatment was associated with attenuation of diabetes-associated MMP-2 and MMP-9 gene expression in aorta of diabetic apoE KO mice [8]. Furthermore, studies in patients with type 2 diabetes and CVD have shown that treatment with a PPARγ agonist is associated with a reduction in plasma MMP-9 levels [11].

12. PPARs AND ADVANCED GLYCATION END-PRODUCTS

The accumulation of AGEs, as a result of hyperglycaemia, dyslipidaemia, and oxidative stress in diabetes, contributes to the development and progression of vascular disease in diabetes [71, 72]. AGEs accumulate in many diabetic tissues [73], including in atherosclerotic plaques [71]. Their importance as downstream mediators of hyperglycaemia in diabetes has been amply demonstrated by animal studies using inhibitors of advanced glycation to retard the development of atherosclerotic vascular disease without directly influencing plasma glucose levels [71, 74]. Furthermore, dietary excess of AGEs has been shown to accelerate atherosclerosis without affecting glycemic control [75]. Recent studies suggest that, in addition to lowering glucose levels, PPARγ agonists are able to inhibit the formation of AGEs [76]. The mechanism by which PPARγ agonists might reduce AGEs remains to be established, although their anti-oxidant and lipid lowering activities may be relevant to AGE formation and the advanced glycation pathway [10].

13. PPARs AND THE RENIN ANGIOTENSIN SYSTEM

The RAS has an important role in the development and progression of diabetic atherosclerosis. For example, our group has demonstrated clear anti-atherosclerotic activity of RAS blockade with an AT_1 receptor antagonist or an angiotensin converting enzyme (ACE) inhibitor in diabetic apoE KO mice [77, 78]. PPAR activators are known to be negative

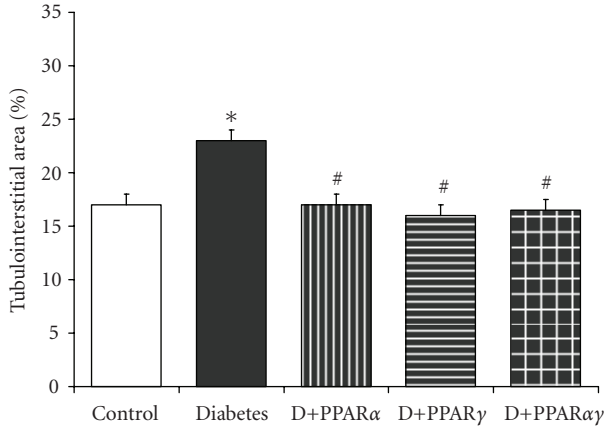

Figure 2: An increase in renal tubulointerstitial area associated with streptozotocin diabetes in apoE KO mice is attenuated following treatment with PPARγ agonist, rosiglitazone, PPARα agonist, gemfibrozil, or the dual PPARα/γ agonist, ragaglitazar.

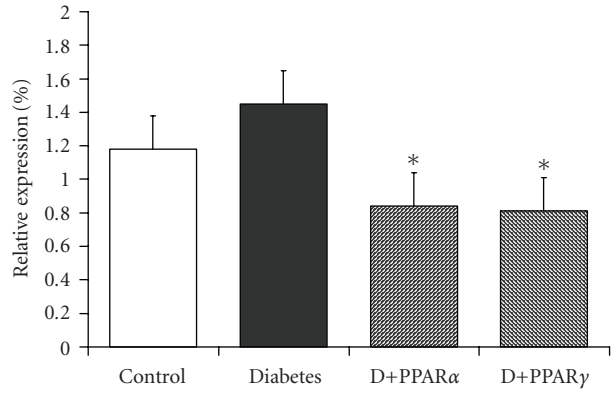

Figure 4: Gene expression of the angiotensin II subtype 1 receptor as assessed by real-time RT-PCR in aorta from apoE knockout mice treated with the PPARγ agonist, rosiglitazone or the PPARα agonist, gemfibrozil for 20 weeks. $P < .05$ versus diabetic mice.

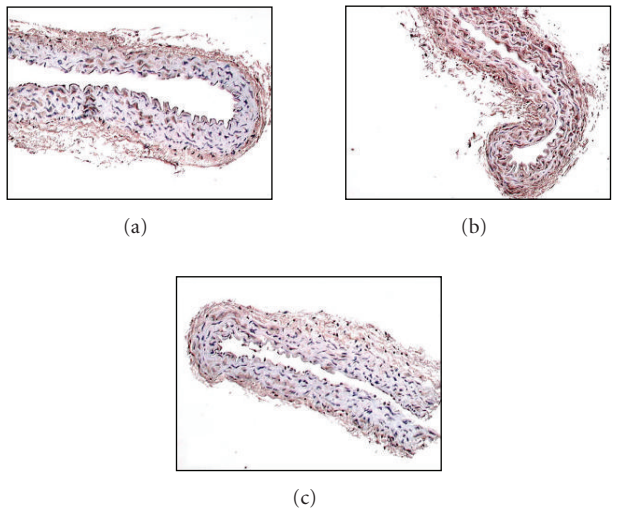

Figure 3: Cross-sections of aorta stained for the NAD(P)H oxidase subunit, p47phox. ApoE KO mouse aorta from (a) control, (b) diabetic, and (c) diabetic + rosiglitazone treated mice.

regulators of the AT_1 receptor gene. For example, our studies with either rosiglitazone or gemfibrozil resulted in a significant reduction in the vascular expression of the AT_1 receptor in diabetic apoE KO mice (see Figure 4). At least in this model, this repression of AT_1 receptor expression by PPAR agonists may function, in terms of atherogenesis, in an equivalent manner to angiotensin receptor blockade.

14. PPARs AND THE DIABETIC KIDNEY

Chronic kidney disease is a major risk factor for cardiovascular disease in patients with diabetes. For example, myocardial infarction and stroke are 10 times more common in type 1 diabetic patients with kidney disease than those without renal disease [79]. Below the age of 50 years, the excess of mortality from cardiovascular disease is almost entirely confined to patients with diabetic nephropathy [80]. Equally, in patients with type 2 diabetes, the risk of developing cardiovascular disease is 2-3 times higher in others with microalbuminuria compared to normal albumin excretion. In patients with proteinuria, the risk is increased at least 10-fold [81].

PPAR agonists have a number of important actions in the diabetic kidney, which may attenuate renal injury and therein (indirectly) reduce cardiovascular risk. For example, we have shown that albuminuria in streptozotocin-diabetic mice is reduced by treatment with the PPARα agonist, gemfibrozil, the PPARγ agonist, rosiglitazone, or the dual PPAR agonist, compound 3q [82]. In addition, glomerulosclerosis, tubulointerstitial expansion (see Figure 2), and collagen deposition were significantly attenuated. PPARγ agonists may also have beneficial actions on renal hypertrophy in models of experimental diabetes [83–85]. Notably, these renoprotective effects are observed in the absence of changes in glucose or lipid levels, insulin sensitivity, or a reduction in blood pressure, and taken together suggest some independent renoprotective action. Moreover, the finding of similar beneficial effects of PPARα and PPARγ agonists, as well as thiazolidinedione and non-TZD dual agonist compounds, raises the possibility that neither of these agents are working through conventional PPARα and γ pathways in this model, but through the transrepression of other transcription factors implicated in diabetic kidney disease including AP-1, signal transducers and activators of transcription 1 (STAT-1) and NF-κB, even in the absence of PPAR receptors [86]. Importantly, these renal benefits have also been observed in clinical trials with PPAR agonists, including the recently completed FIELD trial where a reduction in microalbuminuria was observed in patients treated with fenofibrate [87]. Similarly, in the Diabetes Atherosclerosis Interventional Study, fenofibrate therapy was associated with reduced progression from normal urinary albumin excretion to microalbuminuria in patients with type 2 diabetes [88]. Several previous studies have also demonstrated that thiazolidinediones are able to improve markers

of renal structure and function in patients with diabetes [89–91]. However, the cardioprotective benefits of long-term renoprotection observed in these studies remain to be established.

15. CLINICAL TRIALS WITH PPARα AGONISTS

A number of clinical studies have shown that treatment with lipid lowering agents is able to prevent adverse CVD outcomes in patients with diabetes. Yet while PPARα agonists are able to reduce lipid levels in patients with diabetes, their clinical efficacy remains controversial, with a number of both positive and equivocal results reported in clinical trials. For example, in the Veterans Affairs High-Density Lipoprotein Intervention Trial (VA-HIT), patients with diabetes treated with gemfibrozil had a reduced risk of a composite end point of coronary heart disease (CHD) death, stroke, or myocardial infarction by 32% and reduced CHD deaths by 41% compared to those with diabetes receiving standard care [92]. Moreover, the clinical benefit derived from fibrates exceeded that attributable to changes in the lipid profile. The Diabetes Atherosclerosis Intervention Study (DAIS) showed that 3 years of treatment with fenofibrate resulted in significant reductions in angiographic progression of atherosclerosis and stenosis ($P \leq .03$) [27]. Ciprofibrate therapy has also been associated with an increase in flow-mediated dilation in association with an improvement in lipid profile in people with type 2 diabetes [49]. However, in the Helsinki Heart Study, although gemfibrozil reduced the incidence of primary CHD compared with placebo among patients with diabetes (3.4 versus 10.5%), this difference was not statistically significant [93]. Similarly, the recently published Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study demonstrated a non-significant 11% reduction in the primary end point (coronary artery disease (CAD) death or non-fatal myocardial infarction (MI); $P = .16$) and an 11% reduction in total cardiovascular events ($P = .035$) [87]. However, a 25% reduction in total CVD events and coronary heart disease events was observed in patients without a history of CVD ($P = .014$). This possibly suggests that early and primary therapy with PPARα agonists, comparable to the strategy employed in animal models and shown to be definitively antiatherosclerotic, may also be beneficial in the clinical setting. In addition, improvements in microvascular outcomes, including a reduction in microalbuminuria in the FIELD study, would be expected to have long-term macrovascular benefits.

16. CLINICAL TRIALS WITH PPARγ AGONISTS

Thiazolidinediones have been shown to have a range of positive effects on vascular function in clinical studies. For example, small clinical studies have demonstrated positive effects of thiazolidinediones on cardiovascular parameters such as acetylcholine-mediated dilation [94] and pulse wave velocity [11, 95–97]. Whether such benefits translate to a reduction in cardiovascular risk has been tested in several recent and ongoing clinical trials, although these short-term studies may be inadequate to assess a process like atherosclerosis that takes many decades to evolve.

The Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROACTIVE trial) examined the effect of pioglitazone, taken in addition to conventional therapy for three years, on all-cause mortality, non-fatal MI, stroke, acute coronary syndrome, leg amputation, and coronary or leg revascularisation [98]. While there was a non-significant 10% reduction in this primary outcome $P = .09$, the main secondary endpoint (composed of all-cause mortality, non-fatal MI, and stroke) was reduced by 16% ($P = .03$). However, heart failure and symptomatic oedema due to fluid retention due to PPARγ agonists may have masked any benefit from actions on atherogenesis.

A recent meta-analysis has also been performed to examine the cardiovascular effects of the PPARγ agonist, rosiglitazone, which includes outcome data from 35 trials, such as the large Diabetes Reduction Assessment with Ramipiril and Rosiglitazone Medication (DREAM) trial and the A Diabetes Outcome Prevention Trial (ADOPT) [99–101]. This meta-analysis demonstrated that treatment with rosiglitazone increased the risk for MI by 43% ($P = .03$), and death from cardiovascular causes by 64% ($P = .06$). Whether this finding also reflects increased fluid retention remains to be established.

17. THE PROMISE OF DUAL α/γ PPAR AGONISTS

The apparent efficacy of PPARα and PPARγ agonists individually on metabolic control, led to the development of dual PPARα/γ agonists, offering the potential of optimising the metabolic and anti-atherosclerotic actions arising from activating both receptors. In general, these agents proved to be more potent agonists of PPARγ than conventional thiazolidinediones and highly effective at improving metabolic parameters. For example, ragaglitazar was more effective at improving glycemic control and attenuating plasma lipid levels than single agonists such as rosiglitazone [102]. Similarly, treatment with muraglitazar in *db/db* mice was more effective at reducing plasma glucose levels than rosiglitazone [103]. Yet despite improved metabolic outcomes, the effects on atherogenesis have been less clear. For example, we found that treatment with the dual PPARα/γ agonist, *compound 3q,* was associated with a marked increase in atherosclerosis in control apoE KO mice [104] (see Figure 5), while PPARγ and α agonists used alone in this model were protective [8, 9]. This increase in atherosclerotic plaque was observed in control animals despite an improvement in glycemic control and an improvement in lipid profile [104]. Furthermore, plaque accumulation in mice treated with the dual PPAR compound was also associated with a concomitant increase in aortic gene expression of the pro-inflammatory molecules, P-selectin, CD36, VCAM-1, and MCP-1 and increased macrophage infiltration, an effect not seen with the single PPAR agonists, rosiglitazone or gemfibrozil [104]. By contrast, Claudel and colleagues demonstrated that treatment with the dual PPARα/γ compound, GW2331, for 11 weeks was more effective at attenuating atherosclerosis in female apoE KO mice than rosiglitazone alone [105]. Similarly, Zuckerman et al. found that LY465608 reduced atherosclerosis in male apoE mice fed a high-fat diet in the absence

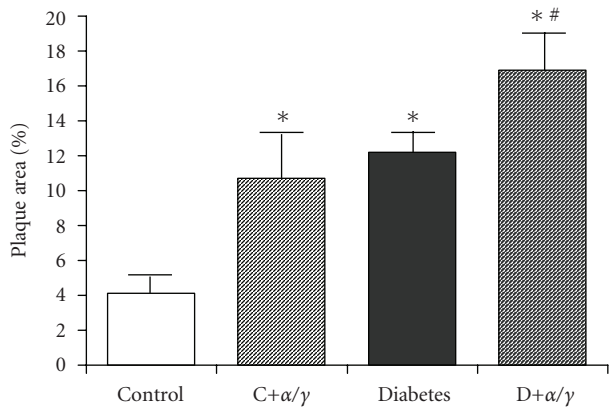

Figure 5: Total aortic plaque area as assessed by an en face approach in apoE knockout mice treated with the dual PPARα/γ agonist, *compound 3q* for 20 weeks. $P < .05$ versus control mice.

of changes in plasma total cholesterol levels [106]. More recently, the anti-atherosclerotic actions of tesaglitazar on vascular disease have also been investigated. In apoE*Leiden mice fed a high-fat diet, tesaglitazar was associated with a 92% reduction in aortic atherosclerosis in association with a reduction in macrophages and collagen in lesions [107]. In high-fat fed LDL receptor KO mice treatment with tesaglitazar for 12 weeks was associated also with a decrease in atherosclerosis in female mice in the absence of alterations in cholesterol or triglyceride levels or a reduction in the inflammatory markers serum amyloid A and serum amyloid *P* [108]. The reasons for these conflicting results are unclear. However, it is possible that the different balance of activation of PPARα and γ with each of these agents, as well as differential effects on transrepression may have contributed to these disparate findings.

18. CLINICAL STUDIES WITH DUAL PPAR AGONISTS

Despite their clear actions as PPARγ and PPARα agonists, and clinical efficacy in terms of lipid and glycemic control [109–116], which were comparable or better than achieved by PPAR agonist alone, recent reports have suggested that dual PPARα/γ agonists may also be associated with an increased risk of adverse cardiovascular events when used by individuals with diabetes [116]. In particular, the risk of death, myocardial infarction, stroke, transient ischaemia attack, or CHF was increased by over two-fold (RR 2.62; 95% CI 1.36 to 5.05) in patients with type 2 diabetes receiving the dual agonist, muraglitazar, compared to those receiving a PPARγ agonist (pioglitazone) alone, despite comparable effects on glycemic control [116]. Whether this increase in events is due to an augmentation of atherogenesis, as observed in our pre-clinical models, or the by-product of augmented fluid retention in patients with a stiff vasculature, remains to be established. Certainly, the more potent activation of the PPARγ receptor achieved by dual agonists may lead to clinically important fluid retention in some patients, particularly at high doses or in patients with established congestive heart failure. Nonetheless, even when patients with NYHA III/IV heart failure were excluded from these trials, the muraglitazar treated group still had 13 adjudicated cases of heart failure compared with only one patient in the control group. More recently, the development tesaglitazar has been discontinued due to concerns about increased serum creatinine and decreased glomerular filtration rate [117]. Taken together with reports of toxicity and carcinogenic effects with some of the dual PPAR agonists in pre-clinical studies [118–120], these finding have meant that ongoing evaluation of this class of drug has been delayed and largely superseded by the pan-PPAR agonists (detailed below).

19. THE DEVELOPMENT OF PAN-PPAR AGONISTS

The clinical efficacy of PPAR agonists individually have led to the development of chemical ligands with activity across all three receptor isoforms. The potential advantage of such a combination rests in the finding that these so-called "pan-PPAR" agonists retain their broad metabolic activity, without the weight gain associated with PPARγ agonists [121, 122]. Cell culture and pre-clinical studies have also demonstrated the efficacy of pan PPAR agonists in modulating various pathways linked to the development of atherosclerosis [67, 123, 124].

20. CLINICAL STUDIES WITH PAN PPAR AGONISTS

There are a small number of pan-PPAR agonists now in the early stages of clinical trials including GW766954, GW625019, PLX-204, and netoglitazone (MCC-555) [125]. These agents have been shown to improve glycaemic and lipid control in a range of settings. While such benefits should confer some cardiovascular benefit, the actions of agents of this class on the development and progression of atherosclerosis in diabetes remain to be established. However, some insight into the possible efficacy of pan PPAR agonists may be inferred from clinical studies using bezafibrate. Although originally classed as a fibrate, bezafibrate is now considered a pan-PPAR agonist, albeit of low potency. Nonetheless, like other PPAR agonists, treatment with bezafibrate significantly raises HDL cholesterol levels, reduces triglycerides, and improves insulin sensitivity in patients with diabetes [126]. In the Bezafibrate Coronary Atherosclerosis Intervention Trial (BECAIT) of dyslipidemic males under the age of 45 who have experienced a previous MI, bezafibrate improved dyslipidaemia, reduced the cumulative coronary event rate ($P = .02$) and slowed the progression of focal coronary atherosclerosis. The St Mary's, Ealing, Northwick Park Diabetes Cardiovascular Disease Prevention (SENDCAP) trial also demonstrated a reduction in the combined incidence of ischemic change on resting ECG and documented MI [127]. Despite this, they were unable to see any effect of bezafibrate on the progression of coronary of femoral atherosclerosis over the 3 years of the study. By contrast, the Bezafibrate Infarct Prevention (BIP) study demonstrated no significant effect of bezafibrate on fatal or nonfatal MI in those with diabetes [128]. Whether newer and more potent pan-PPAR ligands with differential activation of the various PPAR isoforms will prove to be more beneficial

with respect to cardiovascular outcomes remains to be established. In addition, given the actions of PPAR in the transcriptional regulation of an enormous range of genes and pathways, the potential adverse impact of such pan-PPAR activity needs to be carefully studied.

21. CONCLUDING REMARKS

Agonists of the PPAR family have represented the most important development in the management of diabetes over the last decade. Despite the promise of improved insulin sensitivity and better lipid control, these agents have not achieved the cardiovascular benefits expected of them. There is little doubt that in experimental models, PPAR agonists have clear and independent anti-atherosclerotic actions, including the suppression of vascular inflammation, oxidative stress, and activation of the renin angiotensin system. Why this has not translated into clinical benefit remains to be fully established. It may be that longer-term follow up of clinical studies will reveal statistically significant results, as the long-term benefits of improved metabolic control are realized. Equally, the complex biological effects of the PPARs in a range of organs may mean that any benefits are offset by unwanted actions that impact on CVD, such as fluid retention, malignancy, renal impairment, or increases in LDL cholesterol. Whether more organ-targeted agonists or pan-PPAR agonists will prove more effective remains to be seen.

However, the fact that many of the potentially useful vascular effects are thought to be mediated by transrepression of pro-atherogenic signalling pathways, should lead in the future to the development of more selective transrepressors for the prevention and management of cardiovascular disease in diabetes.

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Review Article

Should We Use PPAR Agonists to Reduce Cardiovascular Risk?

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Trials of peroxisome proliferator-activated receptor (PPAR) agonists have shown mixed results for cardiovascular prevention. Fibrates are PPAR-α agonists that act primarily to improve dyslipidemia. Based on low- and high-density lipoprotein cholesterol (LDL and HDL) effects, gemfibrozil may be of greater cardiovascular benefit than expected, fenofibrate performed about as expected, and bezafibrate performed worse than expected. Increases in both cardiovascular and noncardiovascular serious adverse events have been observed with some fibrates. Thiazolidinediones (TZDs) are PPAR-γ agonists used to improve impaired glucose metabolism but also influence lipids. Pioglitazone reduces atherosclerotic events in diabetic subjects, but has no net cardiovascular benefit due to increased congestive heart failure risk. Rosiglitazone may increase the risk of atherosclerotic events, and has a net harmful effect on the cardiovascular system when congestive heart failure is included. The primary benefit of TZDs appears to be the prevention of diabetic microvascular complications. Dual PPAR-α/γ agonists have had unacceptable adverse effects but more selective agents are in development. PPAR-δ and pan-agonists are also in development. It will be imperative to prove that future PPAR agonists not only prevent atherosclerotic events but also result in a net reduction on total cardiovascular events without significant noncardiovascular adverse effects with long-term use.

1. INTRODUCTION

Drugs affecting peroxisome proliferator-activated receptors (PPARs) are of intense interest for regulating disorders of glucose and fatty acid metabolism [1].As an end-stage manifestation of insulin resistance and glucose intolerance, diabetes confers a 2-to-8-fold higher risk of coronary heart disease (CHD), stroke, and mortality [2].Impaired glucose tolerance also contributes to the development of atherogenic dyslipidemia, which is characterized by elevated triglycerides, low high-density lipoprotein (HDL) cholesterol, small dense low-density lipoprotein (LDL) cholesterol, and elevated LDL particle number. Independent of insulin resistance and glucose levels, atherogenic dislipidemia imparts a risk for CHD at least equal to that of the well-characterized risk of isolated, moderate hypercholesterolemia [3].

Agonists of PPAR-α and PPAR-γ have been evaluated for the long-term prevention of cardiovascular events. Fibrates are low-affinity PPAR-α agonists which lower triglycerides by increasing lipolysis and β-oxidation of fatty acids [4]. Fibrates also mildly raise HDL and, in some cases, lower LDL. Pharmacologic activation of PPAR-γ also lowers triglyceride levels by promoting fatty acid storage [5]. The main benefits of PPAR-γ agonists, however, are improvements in glucose homeostasis. Thiazolidinediones (TZDs), or glitazones, are primarily PPAR-γ agonists that promote fatty acid oxidation and insulin sensitivity in liver and muscle [1]. These beneficial effects appear to be mediated, at least in part, through inhibition of the release of signaling molecules from adipose tissue that promote insulin resistance, including inflammatory factors such as tumor necrosis factor-α (TNF-α) and resistin, and stimulating the release of adiponectin. PPAR-γ agonism may additionally lower plasma glucose levels via decreased hepatic glucose production. Dual PPAR-α and PPAR-γ agonists have also been developed. Drugs affecting the more recently identified PPAR-δ (also called β) are in the early stages of development. PPAR-δ is also a powerful regulator of fatty acid catabolism and energy homeostasis and has been shown to prevent weight gain, dyslipidemia, and fatty liver in animals fed high-calorie diets [6, 7]. Given the central role of PPARs in lipid and glucose metabolism, has the promise of PPAR modulation translated into a significant

cardiovascular risk reduction benefit from these agents? Several recently completed large trials addressing this question have had mixed results.

2. PPAR-α AGONISTS: FIBRATES

Randomized, placebo-controlled trials have shown that gemfibrozil significantly reduces the risk of CHD in primary and secondary prevention populations of dyslipidemic men, with evidence of a trend toward a decrease in stroke (Table 1) [8, 9]. Less robust results were observed for bezafibrate in subjects with CHD, and for fenofibrate in subjects with diabetes [10, 11]. The cardiovascular benefits of gemfibrozil appear to be greater than expected from changes in LDL and HDL. In the Veterans Affairs HDL Intervention Trial (VA-HIT), a >20% reduction in CHD and stroke occurred despite no effect on LDL and only a 6% increase in HDL. This reduction in risk was also found to be independent of changes in triglycerides and was largely attributable to the use of gemfibrozil itself [12]. The only other long-term trial with gemfibrozil, the Helsinki Heart Study, also reported a greater reduction in cardiovascular risk than have been expected on the basis of changes in LDL and HDL. Figure 1 is based on the assumption that each 1% decrease in LDL and each 1% increase in HDL are additive and would therefore result in a 2% reduction in cardiovascular risk. Data supporting this assumption comes from clinical trials where each 1% reduction in LDL results in approximately a 1% reduction in the risk of CHD and stroke, regardless of the method by which LDL is lowered [13]. The VA-HIT study found that a 5 mg/dl increase in HDL (16%) reduced risk by 11% [12]. This is consistent with epidemiologic data in which each 1 mg/dl (0.03 mmol, or about a 2-3%, depending on baseline HDL level) increase in HDL is associated with a 2–4% reduction in the risk of CHD events, independent of LDL-C cholesterol levels [14]. It is assumed, but not proven, that raising HDL results in risk reduction additive to that of lowering LDL.

In contrast to the 2 trials with gemfibrozil, the 11% reduction in cardiovascular risk observed in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial was similar to that expected (about 12%) from average changes in LDL (−9%) and HDL (+3%) between 4 months and the end of the study (Figure 1; Table 2) [11]. The midpoint of the study was chosen due to crossover to statin treatment in both treatment arms. By the end of the trial, 17% of the placebo group and 8% of the fenofibrate group started lipid-lowering therapy, mainly with statins. As a consequence, the lipid parameters for the 2 treatment groups became more similar over time.

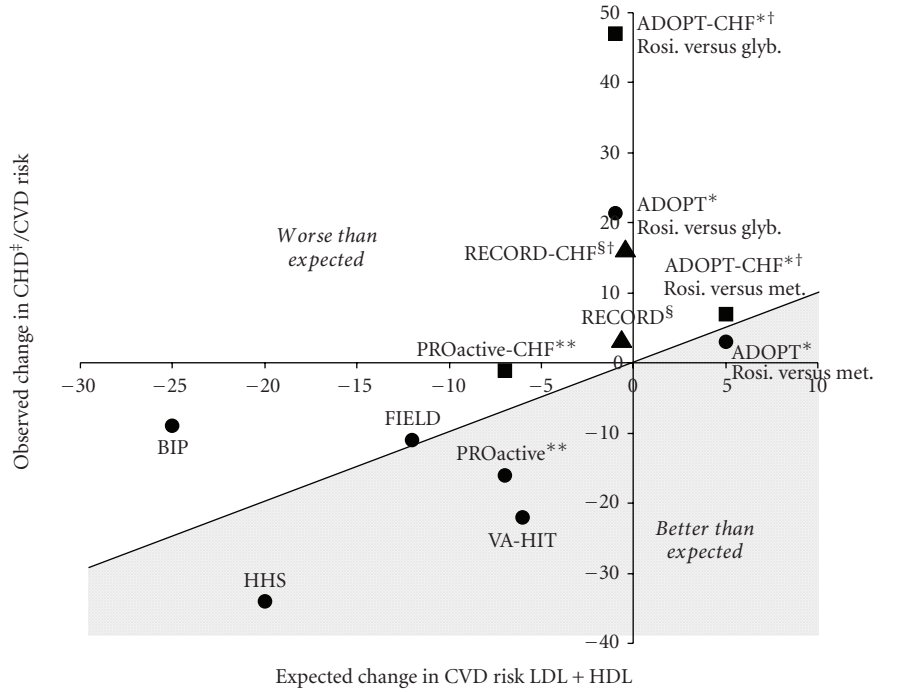
The Bezafibrate Infarction Prevention (BIP) study showed a nonsignificant reduction in cardiovascular events of only 9% despite greater changes in LDL and HDL than those observed in FIELD or VA-HIT (Table 2) [8, 10, 11]. Indeed, bezafibrate performed substantially worse than expected from the LDL and HDL changes (Figure 1), suggesting that bezafibrate may have vascular toxicity that counteracts its beneficial lipid changes. This may be due to bezafibrate acting as a pan-PPAR activator, as discussed below [15].

It has been argued that the lesser cardiovascular benefit observed in FIELD and BIP was due to inclusion of less dyslipidemic subjects than in the gemfibrozil trials. A post hoc subgroup analysis of BIP found a significant (40%) reduction in CHD in those with triglycerides ≥200 mg/dl [10]. VA-HIT found a similar trend toward increasing risk reduction with triglyceride levels ≥180 mg/dl [12]. In FIELD, fenofibrate, there were similar with reductions in cardiovascular risk in subjects with triglycerides less than and greater than 150 mg/dl. On the other hand, the Diabetes Atherosclerosis Intervention Study (DAIS) found that fenofibrate reduced angiographic progression of coronary atherosclerosis in a more markedly hypertriglyceridemic diabetic population [triglycerides 229 mg/dl (2.59 mmol/L); HDL 39 mg/dl (1.01 mmo/L); LDL 130 mg/dl (3.38 mmol/L)] [16]. However, when looking at the mean lipid levels across the studies, the case is less clear. The triglyceride levels in FIELD (172 mg/dl) were similar to those in the Helsinki Heart Study (178 mg/dl), but somewhat higher than in VA-HIT (160 mg/dl) and BIP (145 mg/dl) (Table 2). HDL levels were markedly lower in BIP (35 mg/dl) and in VA-HIT (32 mg/dl) than in either FIELD (43 mg/dl) or the Helsinki Heart Study (47 mg/dl). Taken as a whole, these findings may suggest that gemfibrozil may have a greater impact on cardiovascular risk than fenofibrate, regardless of the population studied.

Also of concern, some fibrates used alone may potentially increase the risk of cardiovascular and noncardiovascular mortality, and of serious adverse events (Table 1). Clofibrate, the earliest fibrate studied, is rarely used due to a consistent increase in mortality when compared to placebo, which occurred despite a substantial reduction in CHD events [17, 18]. In BIP, more cases of CHD mortality were reported for the bezafibrate group compared to placebo, although the difference was not statistically significant (Table 1) [10]. In FIELD, there were also more adverse events and deaths among those receiving fenofibrate compared to placebo [11]. The reduction in nonfatal coronary events and stroke in FIELD was counterbalanced by an 11% increase in cardiovascular deaths (due to a 19% increase in CHD death) and total mortality that did not reach statistical significance. The excess in deaths was due to a variety of causes: sudden cardiac death (70 versus 54, resp.), heart failure (13 versus 11), noncoronary cardiac (8 versus 4), and pulmonary embolism (4 versus 1, $P = .22$). Although a lower rate of cardiac events in the statin-treated placebo group is one possible explanation for the unexpected increase in cardiac deaths, a 30% excess of sudden death in the fenofibrate group is hard to explain if only an excess 9% of the placebo group received a statin. In contrast, fewer deaths occurred in the secondary-prevention population studied in the Veterans Affairs HDL Intervention Trial (VA-HIT) and in the primary-prevention Helsinki Heart Study [8, 9]. The secondary-prevention component of the Helsinki Heart Study reported a nonsignificant increase in CHD deaths with gemfibrozil compared to placebo in a much smaller sample ($N = 628$, HR 2.2% (95% CI 0.94–5.05)) [19]. It is important to note that no excess of harm has emerged in any statin trial. A meta-analysis of statin therapy in over 90,000 participants in 14 event trials found a 19% reduction in

Table 1: Selected morbidity and mortality outcomes in large, long-term fibrate trials. CHD = coronary heart disease, CVD = cardiovascular disease, MI = myocardial infarction, NR = not reported, ns = reported as "not significant," RR = Crude relative risk calculated form reported number of events; hazard ratio was not reported.

	Event rates							
Study treatment	Nonfatal MI	CHD mortality	Nonfatal MI or CHD death	Total stroke	Cancer	Total mortality	Hospitalized CHF	
Helsinki Heart [9] Mean F/U 5.0 years Primary prevention Dyslipidemia High LDL								
Placebo $N = 2030$	3.5%	0.64%	4.1%	NR	1.3%	2.1%		
Gemfibrozil $N = 2051$	2.2%	0.53%	2.7%	NR	1.5%	2.2%		
Hazard ratio (95% CI)	RR 0.63 $P < .02$	RR 0.83 $p = NR$	0.66 $P < .02$	NR	RR 1.15 $p = NR$	RR 1.05 $p = NR$		
VA-HIT [8] Mean F/U 5.1 years CHD HDL < 40 mg/dl LDL < 140 mg/dl								
Placebo $N = 1267$	14.5%	9.3%	21.7%	6.0%	10.9%	17.4%	13.3%	
Gemfibrozil $N = 1264$	11.6%	7.4%	17.3%	4.6%	9.9%	15.7%	10.6%	
Hazard ratio (95% CI)	0.77 (0.62–0.96) $P < .02$	0.78 (0.59–1.02) $P = .07$	0.78 (0.65–0.93) $P = .006$	0.75 (0.53–1.06) $P = .10$	RR 0.91	0.89 (0.73–1.08) $P = .23$	0.78 (0.62–0.98) $P = .04$	
BIP [**10**] Mean F/U 6.2 years CHD Dyslipidemia					**Noncardiac death**			
Placebo $N = 1542$	11.2%	5.7%	15.0%	5.0%	5.9%	4.2%		
Bezafibrate $N = 1548$	9.7%	6.1%	13.6%	4.6%	5.5%	4.3%		
Hazard ratio (95% CI)	0.87 $P = .18$	RR 1.07 $P = .61$	0.91 $P = .26$	RR 0.92 $P = .66$ ns	RR 0.93	RR 1.02 $P = .87$		
FIELD [11] Mean F/U 5 years Type 2 diabetes Dyslipidemia Low LDL							**Laser therapy**	**Albuminuria not progressing/ regressing**
Placebo $n = 4900$	4.2%	1.9%	6%	3.6%	8%	6.6%	5.2%	
Fenofibrate $N = 4895$	3.2%	2.2%	5%	3.2%	8%	7.3%	3.6%	
Hazard ratio (95% CI)	0.76 (0.62–0.94) $P = .01$	1.19 (0.90–1.57) $P = .22$	0.89 (0.75–1.05) $P = .16$	0.90 (0.73–1.12) $P = .36$	RR 1.0	1.11 (0.95–1.29) $P = .18$	0.70 (0.58–0.85) $P = .0003$	RR 1.15 $P = .002$



* Nonfatal and fatal myocardial infarction (MI), and stroke
† Congestive heart failure (CHF) added to CHD/CVD endpoint
** MI, stroke, any death
‡ Nonfatal MI, coronary heart disease (CHD) death unless otherwise specified
§ MI, stroke, and CVD death
▲ Lipid changes in RECORD estimated to be 50% of those of pioglitazone based on Goldberg et al. [2005]

Glyb = glyburide
Met = metformin
Rosi = rosiglitazone

Figure 1: Approximate expected cardiovascular (CVD) risk reduction from percent changes in LDL and HDL versus observed percent reduction in coronary heart disease (CHD) or CVD. Above the slope = 1 line, CVD risk reduction was worse than expected based on lipid changes; below the slope = 1 line, CVD risk reduction was greater than expected based on the lipid changes.

CHD mortality and a 12% reduction in all-cause mortality [20].

Although more malignancies were initially reported with clofibrate and gemfibrozil in 5-year primary-prevention trials, with long-term followup there were no significant increases in cancer incidence or mortality with gemfibrozil, even with followup as long as 18 years in the Helsinki Heart Study [8, 21, 22]. Cancer incidence was similar for both the fenofibrate and placebo groups (8%) in FIELD [11].

Also of concern in FIELD, cardiovascular events, including revascularizations, were significantly reduced only in those without previous cardiovascular disease and in those <65 years of age (19% and 20%, resp.; $P < .005$), with no benefit (0%) observed in those with previous cardiovascular disease or who where ≥ age 65 years at baseline. These finding are in clear contradiction to the findings of the VA-HIT study where men with both diabetes and CHD experienced a 32% (95% CI 12–47, $P = .004$) reduction in cardiovascular events from gemfibrozil treatment [23]. The analysis has not been published to determine whether the explanation for the FIELD findings lies in the higher rate of crossover to other lipid-treatments in those with previous cardiovascular disease. In those with previous cardiovascular disease, 23% of the placebo group and 14% of the fenofibrate group crossed over to lipid-lowering therapy. In comparison, in those without previous cardiovascular disease, 16% of placebo and 7% of fenofibrate groups crossed over to statin therapy. On-treatment lipid values of the various groups were not reported so it is difficult to estimate whether the lack of benefit in those with previous cardiovascular disease and those ≥ age 65 years was due to crossover to active treatment or to other factors.

In FIELD, the fenofibrate group also experienced a nonsignificant increase in deep venous thrombosis [67 (1.4%) versus 48 (1.0%); $P = .74$)]. No clear explanations for the nonsignificant higher rates of sudden death, venous thrombosis, and pulmonary embolism in FIELD are readily apparent. It is not known whether the increased risk of thrombosis was due to higher homocysteine levels in the fenofibrate group. Gemfibrozil may raise homocysteine levels less than

Table 2: Selected laboratory data from fibrate endpoint trials.

Mean baseline level (mg/dL (mmol/L))		Percent difference between treatment groups		
Helsinki Heart [76]		Gemfibrozil versus placebo		
		1 year	3 years	5 years
Total cholesterol	269 (6.98)	−11%	−10%	−9%
LDL	189 (4.90)	−11%	−10%	−9%
HDL	47 (1.22)	11%	10%	7%
Triglycerides	178 (2.01)	−39%	−37%	−33%
Non-HDL	222 (5.76)	−15%	−14%	−13%
VA-HIT [12]		Gemfibrozil versus placebo		
		1 year		
Total cholesterol	175 (4.53)	−4%		
LDL	112 (2.90)	0%		
HDL	32 (0.83)	6%		
Triglycerides	160 (1.81)	−31%		
BIP [10]		Bezafibrate versus placebo		
		1 year		
Total cholesterol	212 (5.49)	−5%		
LDL	148 (3.83)	−7%		
HDL	34.6 (0.90)	18%		
Triglycerides	145 (1.64)	−21%		
FIELD [11]		Fenofibrate versus placebo		
		4 months		End-of-study
Total cholesterol	194 (5.04)	−11%		−7%
LDL	119 (3.07)	−12%		−6%
HDL	42.5 (1.10)	5%		1%
Triglycerides	172 (1.94)	−29%		−22%

fenofibrate [24]. It is not known whether the increased homocysteine levels resulted from the reversible increases in creatinine observed with fenofibrate, and also bezafibrate, and less commonly gemfibrozil [25]. Fenofibrate is known to raise homocysteine through a PPAR-α mediated mechanism [26]. Folic acid appears to lower fenofibrate-induced homocysteine elevations [27]. However, since clinical trials of folic acid supplementation to lower homocysteine have not demonstrated a reduction in cardiovascular events [28], the clinical importance of fenofibrate-induced homocysteine elevations remains to be established.

Nor is it clear that the increase in creatinine levels with fibrates increases cardiovascular risk since preliminary studies have shown that fenofibrate increases creatinine production rather than decreasing the glomerular filtration rate [25, 29]. In FIELD, progression of proteinuria and renal failure were less frequent in those receiving fenofibrate (Table 2) [11, 25]. No cases of renal failure were reported with gemfibrozil in the Helsinki Heart Study or in VA-HIT [8, 9].

All fibrates are known to increase biliary cholesterol saturation with clofibrate having the greatest effect and gemfibrozil the least effect [25]. In the World Health Organization (WHO) clofibrate primary prevention study, the excess mortality in the clofibrate group was due to a 33% increase in noncardiovascular mortality, including malignancy, postcholecyctomy complications, and pancreatitis [18]. Cholelithiasis and cholecystectomy rates were also higher in the Coronary Drug Project clofibrate arm and with gemfibrozil in the Helsinki Heart Study [17, 22]. In FIELD, although the rate of cholecystectomy was not reported, more cases of pancreatitis occurred in those receiving fenofibrate than placebo [40 (0.8%) versus 23 (0.5%), resp.; $P = .31$] [11].

Therefore, for a number of efficacy and safety reasons, fibrates should not be used indiscriminately for cardiovascular risk reduction. Furthermore, the role of fibrates for cardiovascular prevention is not clearly defined in the era of statin therapy. Statins are first-line therapy based on an extensive record of safety and efficacy in over 100,000 subjects to date, regardless of LDL or HDL level [30]. Whether adding a fibrate to statin therapy will reduce cardiovascular risk beyond that of statin monotherapy remains to be proven in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study to be completed in 2010 [31]. This trial will also evaluate the safety of adding fenofibrate to simvastatin therapy. In a corrected post hoc analysis of FIELD, when adjusting for the use of other lipid-lowering therapy, fenofibrate reduced major cardiovascular events by only 4% (95% CI −7 to 14,

$P = .45$) [32]. It should be noted that this degree of risk reduction could simply be achieved by doubling the statin dose, which would lower LDL an additional 5–7% [33].

Safety is the other main concern with combination fibrate-statin therapy. There is consistent evidence that fibrates increase the risk of myopathy when used in combination with currently marketed statins. Fenofibrate is considered the fibrate of choice for those requiring statin therapy due to the lesser impact of fenofibrate on statin pharmacokinetics compared with gemfibrozil [25]. The risk of myopathy with gemfibrozil-statin therapy is about 30-fold higher than for fenofibrate-statin therapy [34]. When a gemfibrozil-statin combination is used in the highest-risk patients who are most likely to benefit (age ≥ 65 years with CHD and diabetes) the risk of rhabdomyolysis is almost 50-fold higher (1 in 484) than for statin monotherapy in unselected hospitalized patients [35].

Until more data become available, the addition of a fibrate to statin therapy should be reserved for patients at the highest near-term risk of cardiovascular death with elevated triglycerides and/or low HDL. In these patients, the reduction in deaths from cardiovascular causes by far outweighs any excess risk of death from noncardiovascular causes or of serious adverse events. This would include patients identified as very high risk by the U.S. National Cholesterol Education Program Adult Treatment Panel, such as those with cardiovascular disease with additional high risk characteristics, such as diabetes or metabolic syndrome, smokers, multiple risk factors, or those with diabetes and multiple poorly controlled risk factors, including smoking [30]. However, given the modest incremental benefit beyond that expected from its degree of LDL-lowering, the FIELD results may dampen enthusiasm for combination fenofibrate-statin therapy for the treatment dyslipidemia in the absence of severe hypertriglyceridemia (defined as ≥500 mg/dl [36]).

Even though gemfibrozil may be more effective for reducing cardiovascular events than fenofibrate, at least when used as monotherapy, concomitant use of gemfibrozil with a statin carries a much higher risk of myopathy than the fenofibrate statin combination. There were no cases of rhabdomyolysis in the 1000 subjects receiving both fenofibrate and statin therapy in FIELD [11]. Whether gemfibrozil is actually safer than fenofibrate would depend on the results of a head-to-head trial, although such a trial is unlikely to be performed. Marine omega-3 oils might prove to be a superior choice in terms of safety for the treatment of severe hypertriglyceridemia in patients requiring a statin therapy, especially in patients with impaired renal function since both fenofibrate and gemfibrozil have significant renal excretion [25]. Doses of omega-3 fatty acids of 3.4 grams or greater offer similar triglyceride-lowering efficacy to fibrates in some patient populations [37]. Although yet to be proven in a clinical trial in a population without high fish consumption, omega-3 fatty acids may also provide the added benefit of sudden death prevention and lower risk of total mortality [38].

Fibrates may also be reasonably considered for cardiovascular prevention in statin intolerant patients with dyslipidemia (for which gemfibrozil may be preferred). Fenofibrate has been shown to produce incremental improvements in triglycerides, HDL, and non-high-density lipoprotein (non-HDL) cholesterol used in combination with ezetimibe [39]. Fibrates are considered first-line drug therapy for the treatment of severe hypertriglyceridemia to prevent pancreatitis. Although clinical trials have not been performed to establish the morbidity and mortality benefits of treating severe hypertriglyceridemia, fibrates are very effective for treating triglyceride levels >500 mg/dl [36]. It is not clear whether the small increase in pancreatitis risk with fenofibrate will increase the overall risk of pancreatitis in severely hypertriglyceridemic patients.

3. PPAR-γ AGONISTS: THIAZOLIDINEDIONES

Four large trials of TZDs with cardiovascular endpoints have now been reported. The first cardiovascular endpoint trial, the PROspective pioglitAzone Clinical Trial In macroVascular Events (PROACTIVE) study, enrolled over 5200 subjects with both diabetes and clinical CHD or peripheral arterial disease [40]. When acute coronary syndromes, revascularization, and amputation were included along with the accepted "hard" endpoints of nonfatal myocardial infarction, stroke, and total mortality in the primary endpoint, pioglitazone was not of significant benefit [HR 0.90% (95% CI 0.80 to 1.02), $P = .095$] (Table 3). However, for the secondary endpoint of nonfatal myocardial infarction, stroke, and total mortality, those receiving pioglitazone experienced a significant 16% reduction over the 3 years of the trial. The 16% reduction in ischemic events and death appears to be better than expected for the degree of lipid changes (Figure 1). The approximate 9% decrease in risk from the increase in HDL with pioglitazone might have been counterbalanced by the 2% increase in risk due to the 2% increase in LDL (Table 4) for a net expected cardiovascular risk reduction of 7%. Based on a meta-analysis, the 0.5% absolute decrease in hemoglobin A1c (HbA1c) would be expected to result in a 6-7% decrease in cardiovascular risk [41]. Thus, it appears that the reduction in cardiovascular risk observed with pioglitazone is similar to the expected 14% reduction from the combined changes in HDL, LDL, and HbA1C.

The US Food and Drug Administration recently required that a "black box" warning for congestive heart failure be placed on the labels of both currently available TZDs, pioglitazone and rosiglitazone [42]. TZDs, as a class, are well known to increase fluid retention through unknown mechanisms, which appear to be the primary contributor to the increased risk of congestive heart failure with TZDs [43, 44]. Fluid retention or edema occurs in 3–5% of patients with diabetes started on TZDs and upto 15% of patients treated with both TZDs and insulin [45, 46]. In PROactive, more cases of congestive heart failure occurred with pioglitazone (11%) compared to placebo (8%; $P < .0001$). The additional 56 cases of heart failure in the pioglitazone group directly counterbalanced the 55 fewer primary event endpoints (excluding silent myocardial infarctions). Despite 25 of the 47 cases of fatal heart failure occurring in the pioglitazone group, those receiving pioglitazone still had fewer deaths, 177 versus 186, although this was not statistically significant. In the Figure 1, when the increased risk of congestive heart failure is

Table 3: Selected morbidity and mortality outcomes in large, long-term trials of PPAR-γ agonists. CHD = coronary heart disease, CVD = cardiovascular disease, MI = myocardial infarction, NR = not reported.

	Event rates						
PROACTIVE [40] Mean F/U 2.9 years Type 2 diabetes	**Nonfatal MI**		**Stroke**	**Nonfatal MI/stroke/ any death**	**Total mortality**	**Hospitalized CHF**	**Cancer**
Placebo $N = 2633$	5.5%		4.1%	13.6%	7.1%	4%	4%
Pioglitazone $N = 2605$	4.6%		3.3%	11.6%	6.8%	6%	4%
Hazard ratio (95% CI)	0.83 (0.65–1.06)		0.81 (0.61–1.07)	0.84 (0.72–0.98) $P = .03$	0.96 (0.78–1.18)	RR* 1.5 $P = .007$	RR* 1.0
DREAM [50] Median F/U 3.0 years Glucose intolerance	**All MI**	**CVD death**	**Stroke**	**Nonfatal MI/stroke/ CVD death**	**Total mortality**	**CHF**	**Diabetes**
Placebo $N = 2634$	0.3%	0.4%	0.2%	0.9%	1.3%	0.1%	25%
Rosiglitazone $N = 2365$	0.6%	0.5%	0.3%	1.2%	1.1%	0.5%	10.6%
Hazard ratio (95% CI)	1.66 (0.73–3.80) $P = .2$	1.20 (0.52–2.77) $P = .7$	1.39 (0.44–4.40) $P = .6$	1.39 $P = .2$	0.91 (0.55–1.49) $P = .7$	7.03 (1.60–30.9) $P = .01$	0.38 (0.33–0.44) $P < .0001$
ADOPT [49] Median F/U 4.0 years Type 2 diabetes	**All MI**		**Stroke**	**MI/stroke**		**CHF**	
Metformin (M) $N = 1454$ 38% drop-out rate	1.5%		1.3%	2.8%		1.3%	
Glyburide (G) $N = 1441$ 37% drop-out rate	1.2%		1.2%	2.4%		0.6%	
Rosiglitazone (R) $N = 1456$ 44% drop-out rate	1.8%		1.1%	2.9%		1.5%	
Hazard ratio (95% CI)	R versus M RR* 1.2 R versus G RR* 1.5		R versus M RR* 0.85 R versus G RR* 0.92	R versus M RR* 1.03 R versus G RR* 1.21		R versus M 1.22 (0.66–2.26, $P = .52$) R versus G 2.20 (1.01–4.79, $P = .05$)	
RECORD [51] interim analysis Mean F/U 3.75 years Type 2 diabetes	**All MI**	**CVD death**		**Nonfatal MI/stroke/ CVD death**	**Total mortality**	**CHF**	
Metformin/sulfonylurea $N = 2227$ 10% drop-out rate	1.8%	2.1%		5.1%	3.6%	1.0%	
Rosiglitazone added on to metformin/sulfonylurea $N = 2220$ 10% drop-out rate	2.2%	1.7%		4.9%	3.3%	2.1%	

Table 3: Continued.

Event rates					
Hazard ratio (95% CI)	1.23 (0.81–1.86) $P = .34$	0.80 (0.52–1.24) $P = .32$	0.96 (0.74–1.24) $P = .74$	0.93 (0.67–1.27) $P = .63$	2.15 (1.30–3.57) $P = .003$

* RR = Crude relative risk; hazard ratio not reported.

Table 4: Selected laboratory data from endpoint trials of PPAR-γ agonists.

	Mean baseline level [mg/dL (mmol/L)]	Difference between treatment groups End-of-study	
PROACTIVE [40]			
HbA1c	7.9%	−6%	
LDL	112 (2.9)	2%	
HDL	42 (1.1)	9%	
Triglycerides	159 (1.8)	−13%	
DREAM [50]	HbA1c and lipids not reported		
ADOPT [49]	Median baseline level [mg/dL (mmol/L)]	Rosiglitazone versus Metformin	Rosiglitazone versus Glyburide
Glycated Hgb	7.4%	−2%	−6%
Total cholesterol	204 (5.28)	NR	NR
LDL	120 (3.11)	8%	5%
HDL	47 (1.22)	3%	6%
Triglycerides	161 (1.82)	−2%	−5%
RECORD [51, 77]	Mean baseline level [mg/dL (mmol/L)]		
Glycated Hgb	7.9%	NR	
LDL	127 (3.29)	NR	
HDL	46 (1.20)	NR	
Triglycerides	202 (2.28)	NR	

combined with the reduction in nonfatal MI, stroke, and death, pioglitazone performs worse than expected based on the lipid changes and appears to obviate the reduction in risk from improved glucose control. Taken together, these findings suggest that overall cardiovascular prevention is not a significant benefit of pioglitazone. There is a suggestion, however, that pioglitazone may have a net cardiovascular benefit over a period as short at 3 years if a method to prevent the fluid retention of TZDs is found.

On the other hand, rosiglitazone may not provide any clear cardiovascular benefits, and indeed there is concern that rosiglitazone may increase CHD risk. In a recent meta-analysis of 42 trials of at least 24 weeks duration, Nissen and Wolski found that those receiving rosiglitazone had a 43% higher risk of myocardial infarction and a 64% higher risk of cardiovascular death [47]. However, substantial methodologic limitations prevent definitive conclusions from being drawn regarding the safety of rosiglitazone from this analysis [48]. In the 3 large, long-term trials of rosiglitazone reported to date, findings have been mixed regarding its benefits [49–51]. Two trials were performed in subjects with type 2 diabetes, and 1 trial was for diabetes prevention. In all 3 trials, nonsignificant increases in nonfatal and fatal myocardial infarctions occurred in the rosiglitazone compared to control groups (Table 3). However, in all 3 trials, total mortality was lower in the rosiglitazone-treated groups, albeit again not achieving statistical significance. Since myocardial infarction, stroke, and death rates were low over the 3-4 years of observation in these trials, they were not powered to detect a difference in macrovascular events or mortality. As expected, all trials observed an increase in congestive heart failure, which further exacerbated the lack of cardiovascular benefit for rosiglitazone compared to control.

Both currently approved TZDs lower HbA1c by 1% when used alone or in combination in patients with poorly controlled diabetes [45, 46]. Both TZDs modify lipids to a lesser degree than fibrates. Rosiglitazone, however, appears to increase HDL half as much and LDL twice as much as pioglitazone [52]. The only TZD endpoint trial reporting both baseline and end-of-study laboratory values was the A Diabetes Outcome Progression Trial (ADOPT), comparing rosiglitazone to metformin or glyburide [Table 4] [49]. About 35% of subjects dropped out of the rosiglitazone and metformin groups during the trial, and over 45% dropped out of the glyburide group, limiting conclusions that can be drawn regarding the relative cardiovascular effects of these

agents. Acknowledging this limitation, in Figure 1, rosiglitazone performed about as well in terms of a reduction in cardiovascular events, even if congestive heart failure events were included, as would be expected from the lipid changes when compared to metformin. It is perhaps surprising that rosiglitazone performed much worse than expected when compared to glyburide. An analysis of a large insurance database suggested that the risk of cardiovascular events with rosiglitazone was higher than with metformin, but lower than with sulfonylureas [53]. Another analysis of a large Veterans Health Administration database, however, suggested no differences in overall mortality for those receiving metformin, sulfonylureas, or TZDs [54].

Only baseline lipids were reported for the Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycaemia in Diabetes (RECORD) trial [51]. Extrapolating the relative degree of lipid changes observed in a head-to-head comparison of rosiglitazone to pioglitazone [52], it can be seen in Figure 1 that the cardiovascular event rates in RECORD was about what was expected from the extrapolated lipid changes (4.5% increase in HDL and 4% increase in LDL, or a 1% expected decrease in cardiovascular risk). Rosiglitazone has a net cardiovascular harm when congestive heart failure is added to myocardial infarctions and strokes (131 events versus 113 events, crude relative risk 1.16). Unfortunately, neither lipids nor HbA1c were reported for the Diabetes REduction Assessment with ramipril and rosiglitazone Medication (DREAM) trial, which evaluated the effect of rosiglitazone for the prevention of type 2 diabetes in 5269 adults at high risk on the basis of impaired fasting glucose and/or impaired glucose tolerance [50].

Taken as a whole, these findings may suggest that rosiglitazone has adverse effects on both heart failure and non-heart failure cardiovascular events that outweigh any beneficial changes in HbA1c. It is possible that a period of treatment longer than 3-4 years is needed to demonstrate a reduction in cardiovascular events, and ongoing trials of rosiglitazone will help to address this question, the Bypass Angioplasty Revascularization Investigation 2 Diabetes (BARI 2D) Trial, Veterans Affairs Diabetes Trial (VADT), and ACCORD [31, 55, 56]. However, it should be noted that pioglitazone already appears to perform better than expected from its lipid-modifying effects over a period of 3 years. Pioglitazone has been shown to reduce inflammation additive to that of simvastatin therapy, an effect that appears to be related to improvements in insulin resistance [57]. As for fibrates, it remains to be established whether adding pioglitazone to statin therapy will provide additional cardiovascular risk reduction. Some data regarding this question may emerge from ACCORD if pioglitazone replaces rosiglitazone as part of the diabetes management regimen [58].

Safety concerns in addition to congestive heart failure have emerged for TZDs. Both pioglitazone and rosiglitazone have an increased fracture risk [46, 59]. This may influence net benefits in women, and in older men, with long-term use. Cancer rates were reported only for PROactive among the longer-term TZD trials. Rates were similar in both treatment groups, with the exception of bladder cancer which was more frequent in the pioglitazone group [40]. Once bladder cancers occurring within the first year of the study were excluded from the analysis, 6 of the 9 cases were in the pioglitazone group and the imbalance was not felt to be related to pioglitazone treatment by the investigators. There have not yet been sufficient long-term follow-up studies to confirm if this finding is other than chance. Given the short duration of the study, this finding could eventually be of importance since rodents have shown an excess of bladder cancers with pioglitazone despite in vitro antineoplastic effects [45, 60].

In sum, PROACTIVE demonstrated that pioglitazone can be used without a net excess of serious adverse cardiovascular effects to manage hyperglycemia in a population of patients with diabetes and advanced cardiovascular disease. Piogltiazone may have benefits other than cardiovascular prevention, including its use in combination with other agents to control glucose and prevent microvascular events in properly selected patients. Piogltiazone should be used with caution in patients with New York Heart Association (NYHA) functional class 1 and 2 heart failure and are contraindicated in patients with class 3 or 4 heart failure. [43]. There were consistently fewer atherosclerotic CHD and stroke events in those who received pioglitazone who had history of either CHD or stroke at baseline and the risk of congestive heart failure with pioglitazone was similar in those with and without CHD and with and without stroke [61, 62].

However, in PROactive, in addition to hospitalized and unhospitalized heart failure, 1 out of 10 patients experienced discomfort and concern from fluid retention not requiring hospitalization [221 excess cases of edema without heart failure, number needed to treat (NNT) = 12]. These findings confirm that pioglitazone should remain second- or third-line therapy for the treatment of diabetes in patients [63]. Given the suggestion that rosiglitazone may carry an excess of cardiovascular events beyond the expected increase in congestive heart failure, until more data from long-term studies are available, rosiglitazone should be avoided and pioglitazone used preferentially for glucose management if indicated. Long-term event trials will be needed necessary to establish both efficacy and safety of any future PPAR-γ agonists, especially in light of the earlier withdrawal of troglitazone due to excess hepatic toxicity the emerged in postmarketing experience.

4. DUAL AGONISTS

The dual PPAR-α/γ agonists, or glitazars, developed to date display significantly higher PPAR-γ affinity than PPAR-α affinity, although their affinity for PPAR-α is higher than that of clinically used fibrates [64]. The dual PPAR-α/γ agonists have also been a disappointment in terms of cardiovascular prevention. Muraglitazar came the furthest along in development, and appears to have compounded the worst properties of the PPAR-α and PPAR-γ agonists used separately. In another review by Nissen et al. of Phase 2 and 3 trials ranging from 24 weeks to 2 years in duration, muraglitazar had a more than 2-fold incidence of CHD and stroke over placebo [65]. The adverse impact on cardiovascular risk occurred despite superior glucose-lowering and HDL-raising over pioglitazone [66]. Despite some suggestion that

fenofibrate may attenuate fluid retention from rosiglitazone [67], fluid retention with muraglitazar occurred at a rate significantly higher than placebo. Development of tesaglitazar, another dual PPAR-α/γ agonist, was also terminated in Phase 3 development due to impairments of renal function [25, 68]. Bezafibrate is a pan-PPAR activator [15] and was associated with increased cardiovascular mortality in the Bezafibrate Infarction Prevention study, despite a large increase in HDL and improvements in LDL and triglyceride levels [10]. A number of other glitazars, including ragaglitazar, farglitazar, and imiglitazar, some with even more impressive effects on HDL and LDL than muraglitazar, have been terminated in late stage clinical trials due to safety concerns including carcinogenic effects, liver function test abnormalities, anemia, and decreased blood counts in part due to fatty infiltration of the bone marrow, in addition to fluid retention [64, 69].

5. PPAR AGONISTS AND CARDIOVASCULAR PREVENTION—WHAT NEXT?

In regard to pioglitazone, and perhaps other drugs activating PPAR-γ, if the mechanism underlying excess fluid retention can be addressed, the benefits should begin to outweigh adverse effects when used in high-risk populations. In the absence of such atherapeutic advance, a gene strongly predicting fluid overload with PPAR-γ and dual PPAR α/γ has been identified. If replicated in larger populations, this genetic polymorphism may identify which patients are least likely to experience fluid overload, which should result in a net cardiovascular benefit, at least for pioglitazone [70].

Research into other dual PPARα/γ agonists with an improved safety margin is ongoing [64]. Selective modulation has been described for both PPAR-α [71] and PPAR-γ [72] and could explain the variation in biologic activity of various PPAR ligands within the same pharmacologic class. Since PPARs control numerous genes, beyond those influencing lipid and glucose metabolism, it is not surprising that the diverse origins adverse effects with PPAR agonists appear to be compound-specific, rather than a result of activation of more than one PPAR. The selective PPAR modulator (SPPARM) approach has been proposed as a method for developing ligands that differentially regulate genes specific for desirable biological effects but devoid of adverse effects. Several selective dual PPAR agonists in development do not appear to have adverse effects on fat accumulation and edema [64]. Metaglidasen is one such compound [73]. To further enhance safety, partial selective agonists appear to be more desirable than potent agonists. For example, potent PPAR-α activators may increase insulin resistance, induce cardiac hypertrophy, and reduce cardiac function [74]. Since gemfirbrozil appears to be of greater benefit for cardiovascular prevention while fenofibrate appears to be safer, a potentially fruitful avenue of investigation may be using the SPPARM approach to characterize the differential patterns of gene activation in various tissues for these 2 drugs.

The more recently discovered PPAR-δ has also been found to be a powerful regulator of fatty acid catabolism and energy homeostasis [6]. PPAR-δ agonism has been shown to prevent weight gain, dyslipidemia, and fatty liver in animals fed high-calorie diets [7]. A synthetic PPAR-δ agonist, GW501516, has been shown to modestly increase HDL-C levels and enhance serum fat clearance in an early human study [75]. Pan PPAR-α, δ, γ agonists have the potential to address multiple aspects of the metabolic syndrome with a single medication. One such pan-agonist, netoglitazone, has improved cell and tissue selectivity and is undergoing Phase II and III trials [73].

As our understanding of the effects modulating genetic expression in a variety of tissues continues to develop, safe and effective drugs to prevent the complications of obesity and diabetes should emerge. Clearly, all such drugs will need to undergo rigorous evaluation in long-term morbidity/mortality trials early in their development. Appropriate composite endpoints in these trials will be needed to evaluate the net benefits of PPAR activating drugs.

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Research Article

Genetic Polymorphisms of Peroxisome Proliferator-Activated Receptors and the Risk of Cardiovascular Morbidity and Mortality in a Community-Based Cohort in Washington County, Maryland

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The primary aim of this study was to examine prospectively the associations between 5 peroxisome proliferator-activated receptor (*PPAR*) single nucleotide polymorphisms (SNPs) and cardiovascular morbidity and mortality in a community-based cohort study in Washington County, Maryland. Data were analyzed from 9364 Caucasian men and women participating in CLUE-II. Genotyping on 5 *PPAR* polymorphisms was conducted using peripheral DNA samples collected in 1989. The followup period was from 1989 to 2003. The results showed that there were no statistically significant associations between the *PPAR* SNPs and cardiovascular deaths or events. In contrast, statistically significant age-adjusted associations were observed for PPARG rs4684847 with both baseline body mass and blood pressure, and for PPARG rs709158, PPARG rs1175543, and PPARD rs2016520 with baseline cholesterol levels. Future studies should be conducted to confirm these findings and to explore the associations in populations with greater racial and ethnic diversity.

1. INTRODUCTION

The peroxisome proliferator-activated receptors (*PPAR*s) are part of a superfamily of ligand-activated transcription factors involved in fatty acid oxidation and lipid metabolism [1]. Three distinct isoforms of *PPAR*s that are encoded by separate genes have been identified: *PPAR-α*, *PPAR-γ*, and *PPAR-δ* [2]. The three isoforms play distinct physiological roles depending on their tissue distribution. *PPAR-α*, which is expressed in the liver, heart, skeletal muscle, and kidney, regulates lipid and lipoprotein metabolism. *PPAR-γ* is expressed in white and brown adipose tissue and is involved in adipocyte differentiation, lipid storage, and glucose metabolism. *PPAR-δ* is expressed in many tissues and stimulates fatty acid oxidation [2, 3]. Beyond these major roles, *PPAR*s also have been shown to play a role in other biological processes, including the regulation of inflammatory and oxidative pathways [2].

*PPAR*s are found in endothelial and vascular smooth muscle cells and have been shown to influence inflammatory, fibrotic, and hypertrophic responses in the heart and vascular wall [4]. Because of their location and their involvement in fatty acid oxidation, lipid metabolism, and inflammation, the role of *PPAR*s in cardiovascular disease and risk factors of cardiovascular disease has been of great interest. In general, activation of the *PPAR*s, both naturally and synthetically, is considered beneficial for cardiovascular health [2]. Both PPAR-α and PPAR-γ play a role in modulating

atherosclerosis; for example, PPAR-γ activation may promote monocyte apoptosis, contributing to the stabilization of atherosclerotic lesions [5, 6]. Further, clinical trials have shown that the use of pharmacological *PPAR* agonists such as fibrates (*PPAR-α* agonist) is antiatherogenic. Fibrates elevate high-density lipoprotein (HDL) levels, decrease low-density lipoprotein (LDL) and triglyceride levels, and reduce an individual's risk of experiencing a cardiac event [7].

A number of *PPAR* polymorphisms have been identified within the 3 *PPAR* isoforms and there is a considerable amount of literature on the associations between these polymorphisms and cardiovascular risk factors (reviewed in Cresci [7]). There are less data, however, on the associations between the *PPAR* polymorphisms and cardiovascular disease events (e.g., myocardial infarction (MI), cardiovascular-related death). Further, findings with regard to these associations have been inconsistent. For example, a case-control study nested within the Physician's Health Study suggested that the *PPARG* Pro12Ala polymorphism, located in exon B of *PPAR-γ*, is associated with a reduced risk of MI [8]. In contrast, a recent publication using data from the Health Professionals Followup Study showed that male carriers of the Ala12 allele had an increased risk of MI or cardiac death [9] while other studies have observed no statistically significant association between *PPARG* Pro12Ala and cardiovascular events or death [9, 10]. Thus, additional studies of these polymorphisms are necessary to help us better understand the role of *PPAR* genetics in cardiovascular disease especially in light of available pharmacological *PPAR* targeted agents.

The primary aim of this study was to examine prospectively the associations between 5 *PPAR* polymorphisms (4 in *PPAR-γ*: rs4684847, rs709158, rs1175543, and rs1801282; and 1 in *PPAR-δ*: rs2016520) and cardiovascular morbidity and mortality in a community-based cohort study in Washington County, Maryland. As a secondary aim, we also examined the associations between the *PPAR* polymorphisms and cardiovascular risk factors.

2. METHODS

2.1. Study sample

In 1974 and 1989, two cohorts named CLUE I and CLUE II ("*Give us a Clue to Cancer and Heart Disease*") were established in Washington County, Maryland. CLUE I and CLUE II enrolled 20 305 and 25 081 Washington County residents, respectively. At baseline for both cohorts, participants provided informed consent, completed a brief questionnaire, and donated a blood sample. The questionnaire ascertained data on age, gender, marital status, education, height and weight (CLUE II only), cigarette smoking, and medication and vitamin supplement use within the 48 hours prior to blood donation. In addition, in both 1974 and 1989, blood pressure was measured by a study nurse with a blood pressure cuff while the participant was in a seated position. Blood pressure was assessed three times in succession and the third blood pressure value was recorded. In 1989, total cholesterol (nonfasting) was assayed. Individuals who donated blood to both CLUE I and CLUE II constitute the Odyssey cohort (N = 8394) [11, 12].

In addition to the Odyssey cohort, a CLUE II subcohort was selected for case-cohort studies that would be conducted using the CLUE II cohort data. The subcohort was identified by taking an approximate 10% age- and sex-stratified random sample of CLUE II participants who donated a blood specimen and were adult residents of Washington County, Maryland. Of the 2460 participants identified for the subcohort, 807 were also in the Odyssey cohort. Therefore, 10 047 unique participants were part of either the Odyssey cohort or the randomly selected CLUE II subcohort.

Of the participants in the Odyssey Cohort and the CLUE II subcohort, DNA was successfully extracted from the buffy coat samples of 9960 individuals (99.1%). DNA from these participants was genotyped for polymorphisms in genes controlling biological processes such as inflammation that have been associated with multiple diseases. For the study presented here, 5% of the Odyssey and subcohort participants who had no data on all of the chosen *PPAR* SNPs (n = 475) were excluded from the analysis. Further, all non-Caucasians (n = 121) were excluded from the analysis because previous studies have shown that race is an important effect modifier in investigations of polymorphisms and disease and there were not a sufficient number of non-Caucasians in the cohort to analyze the associations among this group. With the exception of race, excluded and included participants did not differ with respect to baseline characteristics. This study was approved by the Institutional Review Board of the Johns Hopkins Bloomberg School of Public Health.

2.2. Outcome assessment

Mortality

All participants were followed from the date of blood draw to the date of death or the end of follow up (August 31, 2003), whichever came first. In the CLUE cohorts, deaths are identified through daily searches of obituaries, cross-linkage with death certificates for Washington County, and through searches of the Social Security Administration for individuals aged 65 or older and the National Death Index. Cause of death is ascertained from the underlying cause on Maryland State death certificates as coded by state nosologists. Of specific interest in this study were cardiovascular disease deaths, for which the underlying cause was coded as ICD-9 390–459 or ICD-10 I00–I99. During the followup period, 2159 deaths were documented in the Odyssey cohort and the CLUE II subcohort, and of these, 791 (36.6%) were cardiovascular deaths. Approximately 4% (n = 334) of the Odyssey cohort and the CLUE II subcohort participants were lost to follow up. Since these individuals were not documented to have died during the followup period, they were considered alive at the end of follow up and censored at August 31, 2003.

Morbidity

Information on cardiovascular events was obtained using participant self-report beginning with questionnaires

administered in 1996 and about every 2 years thereafter. On these questionnaires, participants were asked whether a "doctor had told them they ever had" a specific condition and at what age the condition was first diagnosed. The cardiovascular-related outcomes queried were: diabetes, high blood pressure, high cholesterol, heart attack (MI), angina pectoris, stroke, transient ischemic attack, peripheral artery disease, arrhythmia, and blood clots. Data were examined across the questionnaires for consistency; 5% of the participants had inconsistent data with regards to self-reported events. However, exclusion of these participants did not change the results and, therefore, these participants were not excluded. For this analysis, we examined any self-reported nonfatal cardiovascular event as an outcome. A nonfatal cardiovascular event was defined as consistent reporting from 1996 to 2003 of any one of the following cardiovascular conditions: MI, angina, stroke, transient ischemic attack, peripheral arterial disease, arrhythmia, or blood clots. We also considered a composite variable including only MI, stroke, transient ischemic attack, and peripheral arterial disease; however, the results were similar to the composite variable including all seven outcomes and, therefore, the seven outcome variable was used in all analyses. Individual diagnoses were also examined separately. Because of the inconsistency in the collection of data on the age at which a condition occurred as well as the large amount of missing data for the age variables, age at diagnosis data were not used in the analysis.

2.3. Genotyping

The *PPAR* SNPs analyzed in this study were a part of a larger group of 210 SNPs selected for investigation within the Odyssey cohort. SNPs were selected based on the following criteria: (a) the minor allele frequency was estimated to be ≥5% among Caucasians in the published literature or databases; (b) the polymorphism was in a gene of known or of promising importance in the development of cancer, cardiovascular diseases, and/or longevity; and (c) the polymorphism was either known to be functional or was likely to alter function based on the published literature. *PPAR* polymorphisms selected for analysis were rs2016520 (*PPARD* Ex4 + 15C > T), rs709158 (*PPARG* IVS9 + 4523A > G), rs1175543 (*PPARG* IVS9 + 7780A > G), rs1801282 (*PPARG* Pro12Ala), and rs4684847 (*PPARG* IVS3-6622C > T). To note, none of the other 210 SNPs selected for investigation in the Odyssey cohort were located in PPAR-α, PPAR-γ, or PPAR-δ.

DNA extracted from the preserved buffy coat samples collected in 1989 were used for genotyping. Within 6 hours of collection, the heparinized blood sample was centrifuged at 1500g for 30 minutes at room temperature. Blood samples were separated into plasma, buffy coat, and red blood cells and frozen at −70°C within 24 hours of collection. The buffy coat remained frozen until DNA extraction was performed. The DNA extraction procedures used the alkaline lysis method [13]. Genotyping was performed by Celera Genomics Co. (Rockville, Md, USA) for rs4684847, rs709158, and rs1175543 and by Applied Biosystems Inc. (Foster City, Calif, USA) for rs2016520 and rs1801282. All polymorphisms were genotyped using TaqMan technology. Laboratory technicians were masked to disease status. Of the 9,364 participants in the analytic cohort, approximately 90% had data on all five genotypes; 6.7% had data on four, 2.4% had data on three, 0.7% had data on two, and 0.07% had data on only one.

2.4. Statistical analysis

The Hardy-Weinberg equilibrium for each SNP was tested by a goodness-of-fit approach. As reported in separate publication, all of the *PPAR* SNPs were in Hardy-Weinberg equilibrium [12]. The cohort characteristics were stratified by gender and compared using chi-square tests or student t-tests. Blood pressure at baseline was categorized into 3 groups independent of antihypertensive medication use as follows: normal, individuals with a systolic pressure less than 120 and diastolic pressure less than 80; hypertensive, those with systolic pressure greater than 140 or diastolic pressure greater than 90; and prehypertensive, those with a systolic pressure between 120 and 140 or diastolic pressure between 80 and 90. The age-adjusted associations between the *PPAR* SNPs and cardiovascular risk factors (i.e., baseline BMI, cholesterol levels, blood pressure) were examined using logistic regression models. Age was adjusted for in all analyses as there were statistically significant age differences for several of the SNPs. Gender was not adjusted for in these analyses because it was not associated with SNP prevalence. Cox-proportional hazard ratios were calculated for both all-cause and cardiovascular mortality after adjustment for age. Since the nonfatal cardiovascular outcomes (including MI), followed a Poisson distribution, and age at diagnosis data were not used in the analysis, age-adjusted relative risks for nonfatal cardiovascular events and for only MI were obtained using Poisson regression methods; this type of analysis was also used when analyzing fatal and nonfatal outcomes combined. Premature death (both overall and due to cardiovascular disease) was also examined as an outcome variable and defined as death prior to the age of 65. All analyses were done separately for the 5 SNPs and stratified by gender, diabetes diagnosis, and body mass index (BMI) at baseline. No differences in the risk estimates were observed in these strata and, therefore, only results for the entire cohort are presented.

To address the issue of multiple testing in this study, P values for the associations between SNPs and the cardiovascular risk factors were adjusted for the false discovery rate utilizing Fisher's combination method using bootstrap resampling. All statistical analysis was carried out using SAS software, version 9.1 (SAS Institute, Inc., Cary, NC, USA). A two-sided P value ≤.05 was considered statistically significant.

3. RESULTS

Baseline characteristics of the study sample, overall and by gender, are shown on Table 1. In 1989, males were significantly more likely than females to have some college education, to report being a current or former smoker, and to be categorized as prehypertensive or hypertensive. In addition, males had a significantly higher mean BMI than females. In

Table 1: Characteristics of study sample by gender, $N = 9364$, P value derived from $\chi 2$ test for categorical variables, Student's t-test for continuous variables.

	Female $N = 5776$ (%)	Male $N = 3588$ (%)	Total (%)	P value
Age, mean(SD)	53.2 (15.4)	52.9 (15.5)	53.1 (15.4)	.3974
Education				.0029
<12	24.5	25.0	24.7	
12	47.3	44.0	46.0	
>12	28.2	31.0	29.3	
Missing, *n*	4	1	5	
BMI, kg/m^2, mean (SD)	26.1 (5.3)	26.6 (4.0)	26.3 (4.9)	<.0001
BMI, kg/m^2				<.0001
<25	48.8	34.0	43.1	
25–30	30.8	48.8	37.7	
>30	20.4	17.3	19.2	
Missing, *n*	11	1	12	
Smoking status				<.0001
Never	62.5	40.3	54.0	
Former	21.4	42.7	29.6	
Current	16.1	17.0	16.4	
Missing, *n*			0	
Cholesterol no Rx, mg/dL				<.0001
≤200	41.3	48.2	43.9	
200–239	37.1	36.9	37.0	
≥240	21.6	14.9	19.1	
Cholesterol with Rx, mg/dL				<.0001
≤200	15.7	36.0	23.1	
200–239	43.9	36.5	41.2	
≥240	40.4	27.5	35.7	
Blood pressure				<.0001
Normal	30.2	15.6	24.6	
Prehypertensive	56.4	67.6	60.7	
Hypertensive	13.4	16.8	14.7	
Missing, *n*	5	6	11	
PPARG rs4684847				.0910
CC	78.8	77.3	78.2	
CT/TT	21.2	22.7	21.8	
Missing, *n*	188	120	308	
PPARG rs709158				.4741
AA	39.6	40.3	39.8	
AG/GG	60.4	59.7	60.2	
Missing, *n*	134	98	232	
PPARG rs1175543				.9218
AA	40.2	40.1	40.2	
AG/GG	59.8	59.9	59.8	
Missing, *n*	136	91	227	
PPARD rs2016520				.5240
CC	64.6	64.0	64.4	
CT/TT	35.4	36.0	35.6	
Missing, *n*	146	104	250	
PPARG rs1801282				.2728
Pro/Pro	78.4	77.5	78.1	
Pro/Ala or Ala/Ala	21.6	22.5	21.9	
Missing, *n*	156	106	262	

Table 2: The associations between PPAR SNPs and cardiovascular risk factors. Goodness of fit likelihood ratio *P* value using logistic regression modeling adjusted for age; *P* value for age based on Student's *t*-test. Combined outcomes had condition at baseline or reported on followup questionnaires (for diabetes, self-reported on followup questionnaires only).

(a)

Risk factors	*PPARG* rs4684847			*PPARG* rs709158			*PPARG* 1175543		
	CC	CT/ TT	*P* value	AA	AG/GG	*P* value	AA	AG/GG	*P* value
Baseline characteristics									
Age, mean(SD)	53.0 (15.5)	53.4 (15.4)	.3571	52.7 (15.4)	53.3 (15.4)	.0780	52.9 (15.5)	53.3 (15.3)	.1910
BMI, kg/m^2, *n* (%)			.0203			.8458			.9777
<25	3097 (79)	815 (21)		1562 (40)	2372 (60)		1575 (40)	2366 (60)	
25–30	2645 (78)	745 (22)		1392 (40)	2048 (60)		1393 (41)	2037 (59)	
>30	1331 (76)	412 (24)		681 (39)	1067 (61)		696 (40)	1059 (60)	
Cholesterol no Rx, mg/dL, *n* (%)			.7144			.0370			.0198
≤200	2956 (79)	791 (21)		1469 (39)	2295 (61)		1481 (39)	2284 (61)	
200–239	2443 (78)	702 (22)		1274 (40)	1897 (60)		1281 (40)	1904 (60)	
≥240	1275 (79)	346 (21)		674 (41)	964 (59)		688 (42)	943 (58)	
Cholesterol with Rx, mg/dL, *n* (%)			.8997			.4595			.3418
≤200	82 (76)	26 (24)		40 (36)	71 (64)		37 (34)	71 (66)	
200–239	141 (74)	50 (26)		78 (39)	121 (61)		79 (40)	119 (60)	
≥240	125 (75)	42 (25)		70 (41)	102 (59)		70 (41)	102 (59)	
Blood pressure, *n* (%)			.0248			.0513			.0509
Normal	1777 (79)	460 (21)		875 (39)	1369 (61)		882 (39)	1362 (61)	
Prehypertensive	4290 (78)	1193 (22)		2209 (40)	3338 (60)		2221 (40)	3322 (60)	
Hypertensive	1005 (76)	320 (24)		549 (41)	782 (59)		561 (42)	779 (58)	
Combined outcomes									
Diabetes, *n* (%)			.7845			.8617			.5499
No	4504 (78)	1243 (22)		2325 (40)	3466 (60)		2350 (41)	3451 (59)	
Yes	821 (78)	225 (22)		420 (40)	643 (60)		418 (39)	648 (61)	
High cholesterol, *n* (%)			.3452			.4527			.5670
No	2502 (78)	706 (22)		1280 (40)	1944 (60)		1296 (40)	1938 (60)	
Yes	2712 (79)	730 (21)		1408 (40)	2081 (60)		1414 (41)	2075 (59)	
High blood pressure, *n* (%)			.6258			.2150			.2724
No	2441 (79)	660 (21)		1287 (41)	1834 (59)		1293 (41)	1829 (59)	
Yes	3379 (78)	969 (22)		1720 (39)	2675 (61)		1741 (40)	2663 (60)	

(b)

Risk factors	*PPARD* rs2016520			*PPARG* rs1801282		
	CC	CT/TT	*P* value	Pro/Pro	Pro/Ala or Ala/Ala	*P* value
Age, mean(SD)	53.0 (15.4)	53.0 (15.5)	.9640	52.9 (15.4)	53.2 (15.3)	.4587
BMI, kg/m^2, *n* (%)			.9138			.1280
<25	2534 (64)	1389 (36)		3085 (79)	832 (21)	
25–30	2190 (64)	1246 (36)		2668 (78)	757 (22)	
>30	1136 (65)	609 (35)		1346 (77)	403 (23)	
Cholesterol no Rx, mg/dL, *n* (%)			.0161			.7401
≤200	2475 (66)	1290 (34)		2953 (78)	811 (22)	
200–239	2011 (63)	1161 (37)		2467 (78)	705 (22)	
≥240	1019 (63)	604 (37)		1261 (78)	354 (22)	
Cholesterol with Rx, mg/dL, *n* (%)			.3821			.6233
≤200	74 (67)	37 (33)		86 (80)	22 (20)	
200–239	135 (69)	62 (31)		148 (76)	48 (24)	
≥240	106 (63)	63 (37)		128 (76)	40 (24)	

(b) Continued.

Risk factors	*PPARD* rs2016520			*PPARG* rs1801282		
	CC	CT/TT	*P* value	Pro/Pro	Pro/Ala or Ala/Ala	*P* value
Blood pressure, *n* (%)			.8076			.2636
Normal	1441 (64)	812 (36)		1769 (79)	483 (21)	
Prehypertensive	3578 (65)	1939 (35)		4312 (78)	1199 (22)	
Hypertensive	842 (63)	491 (37)		1016 (77)	312 (23)	
Combined outcomes						
Diabetes, *n* (%)			.6185			.1278
No	3747 (65)	2058 (35)		4517 (78)	1275 (22)	
Yes	675 (64)	383 (36)		841 (80)	212 (20)	
High cholesterol, *n* (%)			.3178			.3691
No	2060 (64)	1175 (36)		2516 (78)	713 (22)	
Yes	2263 (65)	1225 (35)		2732 (79)	740 (21)	
High blood pressure, *n* (%)			.9412			.8331
No	2006 (64)	1121 (36)		2462 (78)	676 (22)	
Yes	2821 (64)	1564 (36)		3384 (78)	971 (22)	

contrast, females were more likely to have cholesterol levels greater than 200 mg/dL, either with or without cholesterol medication use, than males. There were no gender differences in baseline age or *PPAR* SNP prevalence.

Table 2 shows the age-adjusted associations between the *PPAR* SNPs and cardiovascular risk factors examined at baseline and at follow up. After adjustment for age, BMI at baseline was significantly associated with the *PPARG* rs4684847 SNP such that individuals with at least one of the less common T alleles were significantly more likely to have a higher BMI than individuals carrying the CC genotype. Cholesterol level (without medication use) at baseline was significantly associated with the *PPARG* rs1175543, the *PPARG* rs709158, and the *PPARD* rs2016520 SNPs. Specifically, individuals carrying the *PPARG* rs1175543 AA, the *PPARG* rs709158 AA, or the *PPARD* rs2016520 CT or TT genotypes were significantly more likely to be categorized as having baseline cholesterol levels of 240 mg/dL or greater compared to those with the *PPARG* rs1175543 AG or GG, the *PPARG* rs709158 AG or GG, or the *PPARD* rs2016520 CC genotypes, respectively. Further, participants carrying the *PPARG* rs4684847 CT or TT genotypes were more likely to be categorized as being prehypertensive or hypertensive at baseline compared to participants carrying the CC genotype. No statistically significantly associations were observed between any of the *PPAR* sNPs and high blood pressure, high cholesterol, or diabetes diagnoses over the entire followup period. To note, after adjustment for multiple testing, none of the associations between the SNPs and the cardiovascular risk factors were statistically significant.

There were no statistically significant associations between the *PPAR* SNPs and cardiovascular deaths or events (including nonfatal events combined and MI alone) (see Table 3). A 40% reduction in the risk of premature cardiovascular death was observed for individuals with the *PPARG* rs4684847 CT or TT genotype compared to the CC genotype; however, the confidence interval was wide due to a small number of deaths (see Table 4). There were no statistically significant associations for premature death (all cause and cardiovascular-related) and *PPARG* rs709158, *PPARG* rs1175543, *PPARG* Pro12Ala, and *PPARD* rs2016520.

4. DISCUSSION

In general, the findings from this prospective, community-based cohort study indicate that the selected SNPs in *PPAR* genes are not associated with overall and premature cardiovascular morbidity and mortality. Further, with a few exceptions, we found that the selected *PPAR* SNPs were not associated with risk factors of cardiovascular disease. These findings were consistent among both men and women, among those with a diagnosis of diabetes, and in strata defined by baseline BMI.

Data on *PPAR* polymorphisms and cardiovascular disease and cardiovascular disease risk factors are limited and inconsistent. The most studied *PPAR* polymorphism, *PPARG* Pro12Ala, has been shown to be associated with reduced *PPARG* activity [14], and initial studies reported a lower risk of type 2 diabetes associated with the Ala12 allele [14]. This association was observed in some [15–19], but not all [20–23], subsequent studies. The lack of consistency also extends to studies examining the *PPARG* Pro12Ala polymorphism and cardiovascular disease events: one prospective cohort study examining the *PPARG* Pro12Ala polymorphism and cardiovascular disease suggested a decreased risk of CHD among carriers of the Ala12 allele [8], while others have shown no association [9, 10, 24] or an increase in risk [9, 25]. Although our study was limited by the small number of cardiovascular events, our results are consistent with those previous studies that have shown no association. This finding, as well as the data showing a lack of association between *PPARG* Pro12Ala and cardiovascular risk factors, suggests that this SNP is not involved in the development of cardiovascular disease in this population.

While most of the analyses conducted resulted in null findings, statistically significant age-adjusted associations

Table 3: Age-adjusted hazard ratios (HR) and 95% confidence intervals (95% CI) of all-cause and cardiovascular mortality and nonfatal cardiovascular events for 5 PPAR polymorphisms. Person years: cumulative person years of follow up. RR (95% CI): relative risk using poisson regression.

		All-cause death		CV-related death		Nonfatal CV events		Fatal and nonfatal CV events
SNPs	Person years	*n*	HR (95% CI)	*n*	HR (95% CI)	*n*	RR (95% CI)	RR (95% CI)
PPARG rs4684847								
CC	92827	1705	1.00 (reference)	416	1.00 (reference)	2351	1.00 (reference)	1.00 (reference)
CT/TT	25880	474	0.95 (0.85–1.05)	114	0.90 (0.73–1.11)	679	1.00 (0.73–1.37)	0.88 (0.67–1.18)
PPARG rs709158								
AA	47812	850	1.00 (reference)	205	1.00 (reference)	1202	1.00 (reference)	1.00 (reference)
AG/GG	71972	1328	0.99 (0.91–1.09)	324	1.02 (0.86–1.20)	1843	0.98 (0.75–1.28)	0.98 (0.77–1.24)
PPARG rs1175543								
AA	48172	867	1.00 (reference)	213	1.00 (reference)	1183	1.00 (reference)	1.00 (reference)
AG/GG	71627	1331	1.0 (0.92–1.09)	316	0.99 (0.83–1.18)	1864	1.03 (0.79–1.34)	1.00 (0.79–1.28)
PPARD rs2016520								
CC	77042	1386	1.00 (reference)	342	1.00 (reference)	2027	1.00 (reference)	1.00 (reference)
CT/TT	42593	766	1.03 (0.94–1.12)	178	1.02 (0.85–1.23)	1003	0.91 (0.69–1.19)	0.98 (0.76–1.26)
PPARG rs1801282								
Pro/Pro	93254	1696	1.00 (reference)	408	1.00 (reference)	2350	1.00 (reference)	1.00 (reference)
Pro/Ala or Ala/Ala	26196	456	0.96 (0.86–1.06)	112	0.97 (0.79–1.20)	656	0.98 (0.71–1.35)	0.96 (0.72–1.28)

Table 4: Age-adjusted hazard ratios (HR) and 95% confidence intervals (95% CI) of premature (age <65 years) all-cause and cardiovascular mortality for 5 PPAR SNPs. Person years: cumulative person years of follow up.

		All-cause death		CV-related death	
SNPs	Person years	*n*	HR (95% CI)	*n*	HR (95% CI)
PPARG rs4684847					
CC	43445	231	1.00 (reference)	27	1.00 (reference)
CT/TT	11743	58	1.05 (0.78–1.40)	4	0.60 (0.21–1.72)
PPARG rs709158					
AA	23078	115	1.00 (reference)	13	1.00 (reference)
AG/GG	32822	175	1.05 (0.83–1.33)	18	0.95 (0.47–1.95)
PPARG rs1175543					
AA	22875	115	1.00 (reference)	14	1.00 (reference)
AG/GG	32859	175	1.04 (0.82–1.32)	17	0.83 (0.41–1.68)
PPARD rs2016520					
CC	36389	186	1.00 (reference)	21	1.00 (reference)
CT/TT	19940	99	0.98 (0.77–1.25)	9	0.79 (0.36–1.71)
PPARG rs1801282					
Pro/Pro	44263	236	1.00 (reference)	25	1.00 (reference)
Pro/Ala or Ala/Ala	12247	50	0.90 (0.66–1.23)	5	0.79 (0.30–2.09)

were observed for *PPARG* rs4684847 with both baseline BMI and blood pressure, and for *PPARG* rs709158, *PPARG* rs1175543 and *PPARD* rs2016520 with baseline cholesterol levels. These associations were not statistically significant when self-reported blood pressure or high cholesterol at any time point over the study period were considered as outcome variables. Further, the statistical significance of the associations disappeared after correction for multiple testing. However, this may be due to a lack of statistical power. To our knowledge, these polymorphisms have not been examined in relation to cardiovascular disease or cardiovascular disease risk factors. Further, the functionalities of these polymorphisms are unknown. Although located in intron regions, these polymorphisms may affect enzyme distribution or other physiological functions related to cardiovascular health. Alternatively, it may be that the associations observed with these SNPs in this study are due to the polymorphisms being in linkage disequilibrium with other functional polymorphisms in the respective regions.

Several limitations of this study must be considered when interpreting the results. First, all of the followup data on cardiovascular risk factors and events were based on self-report. Because of this, there were some missing data for those who did not complete any of the followup questionnaires.

Further, among those who did complete the questionnaires, there is the possibility of misclassification; this would likely be nondifferential, resulting in a dilution of the true risk estimate. However, in another investigation of this population, self-report of incidence of MI was compared to data gathered from on ongoing county-wide surveillance of MI events documented by hospital review and the sensitivity and specificity of reports were in excess of 94% (HY Huang, personal communication). A second limitation is that because of the racial homogeneity of the sample we were not able to explore potential racial differences in the associations between the *PPAR* polymorphisms and cardiovascular disease. In a previous publication [12], we did find, however, racial differences in the prevalence of the *PPAR* polymorphisms in this study that are consistent with those published in the SNP500 and dbSNP databases.

5. CONCLUSIONS

The results from this prospective, community-based cohort study showed no association between the selected *PPAR* polymorphisms and cardiovascular morbidity and mortality. In addition, in general, no statistically significant associations were observed between the *PPAR* polymorphisms and cardiovascular risk factors. Future studies should be conducted to confirm these findings and to explore the associations in populations with greater racial and ethnic heterogeneity.

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Research Article

Cardiac PPARα Protein Expression is Constant as Alternate Nuclear Receptors and PGC-1 Coordinately Increase During the Postnatal Metabolic Transition

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Gene expression data obtained in mouse heart indicate that increased expression for the nuclear receptor, peroxisomal proliferator activated receptor α (PPARα), prompts the postnatal transition from predominantly carbohydrate to fatty acid oxidation preference. However, no phenotypic or proteomic data are available to confirm downstream signaling and metabolic transition in mice. We studied the hypothesis that shifts in nuclear receptor expression trigger the newborn metabolic switch in a newborn sheep. This species is well characterized with regards to developmental changes in substrate oxidative metabolism. Heart tissues from fetal (130 days gestation), newborn $\leq$ 24 hours, and 30-day old lambs were evaluated for protein expression from multiple enzymes controlling oxidative metabolism as well as principal nuclear receptors and coactivators. Although muscle and liver type carnitine palmitoyl transferases I showed no significant changes to correspond to the metabolic transition, hexokinase II protein content showed a profound transient drop, and pyruvate dehydrogenase kinase steadily increased. PPARα showed no increases preceding or during the transition, while peroxisomal proliferator activated receptor gamma coactivator-1 (PGC-1) increased approximately 20-fold transiently in newborn heart in conjunction with significant increases in thyroid hormone receptor α1 and retinoid-activated receptor α. These data challenge the paradigm that increases in PPARα prompt the postnatal metabolic switch, and suggest that other nuclear receptors play a major role. As thyroid hormone (TH) modulates PGC-1 expression in sheep during development, these data further suggest that well-characterized perinatal TH surge in sheep contributes to this metabolic switch.

1. INTRODUCTION

The fetal heart uses glucose and lactate as the main oxidative substrate sources, then switches to fatty acids as the predominant fuel shortly after birth [1]. Rapid expansion of mitochondrial pool as well as total oxidative capacity accompanies the perinatal metabolic transition. Initial newborn suckling likely stimulates release of hormonal factors, which trigger modification of substrate preference. Recent data indicate that multiple nuclear receptors and their coactivators initiate transcriptional events controlling both newborn substrate switching and perinatal mitochondrial biogenesis.

The nuclear receptor family includes classical endocrine receptors activated by ligands such a thyroid hormone or steroid hormones. Other more recently identified nuclear receptors (NR) respond to dietary-derived lipid intermediates involved in the metabolism of these activating ligands. In particular, the fatty acid-activated peroxisome proliferator-activated receptors (PPAR) participate as key regulators of cardiac energy metabolism. The hormones or ligands for the nuclear receptors attach to their respective ligand binding domains. These receptors will attach to DNA response elements (REs) of their target genes as monomers, homodimers, or partner as heterodimers [2, 3]. Many NRs involved in metabolic regulation heterodimerize with retinoid X receptor, creating a potential mechanism for regulatory integration. Ligand binding promotes a permissive receptor conformation for coactivator interaction. Coactivators such as PPARγ coactivator-1 (PGC-1) bind the receptor in the process of establishing a transcriptional complex with RNA polymerase-2 to initiate

transcription of target genes [3–5]. PGC-1 enhances transactivation mediated by numerous nuclear receptors involved in energy metabolism and mitochondrial biogenesis, thereby providing a second mechanism for integration of these processes.

Although NR-mediated control of cardiac energy metabolism has been examined with gain or loss of function using transgenic models, developmental regulation of these processes has not been studied in detail. Messenger RNA expression studies have provided the prevailing evidence for the PPAR and PGC-1 triggering of the cardiac metabolic switch after birth. However, multiple NRs are subject to several post-transcriptional processes including alternate heteronuclear RNA splicing and differing translation site initiation, as well as end-product feedback inhibition. For the most part, regulatory patterns have not been confirmed at the protein expression level. Accordingly, we studied developmental integration and coordination of protein expression for three major nuclear receptor families involved in regulation of both cardiac metabolism and mitochondrial biogenesis: thyroid hormone receptors (TRs), PPARs, and RXRs. This study included analyses of PGC-1, as it provides a coactivator function for both TRs and PPARs. Furthermore, previous studies involving NR regulation of developmental metabolic switching have been performed in mice, a poorly characterized species with regard to newborn cardiac metabolism [6]. Since substantial data is available defining developmental cardiac energy metabolism in sheep, we used this species to test the hypothesis that nuclear receptor signaling prompts the newborn metabolic switch. The data obtained in this well-characterized species challenge existing paradigms regarding triggering for the postnatal metabolic transition.

2. MATERIALS AND METHODS

2.1. Animal samples

Domestic sheep (Ovis aries) were used for our study. Heart samples were collected at 130 days into gestation (F), one day after birth (N), and 30 days after birth (C) ($n = 6$, from each group). Heart tissue from the left ventricle was quickly blotted dry, frozen in liquid nitrogen, and stored at −80°C. Our investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The Animal Care Committee of the University of Washington approved all animal protocols.

2.2. Protein isolation

The frozen heart tissue was diced and homogenized at 4°C in a three-fold amount of protein lyses buffer (50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 20 mM HEPES, pH 7.9, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, 100 ug/ml $C_7H_7FO_2S$, 5 ug/ml Aprotinin, and 5 ug/ml Leupeptin). The samples were then incubated on ice for 30 minutes and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was transferred to another microfuge tube and centrifuged one more time. The supernatant was stored at −80°C for immunoblotting analysis.

2.3. Immunoblotting

Fifty micrograms of total protein extracts from sheep heart tissue were electrophoresed along with one lane containing thirty micrograms of human HeLa cells as a positive control and one lane of molecular weight size markers (Chemichrome Western Control, Sigma-Aldrich Co., Mo, USA) in a 4.5% stacking and 7.5, 10, or 12% running SDS-polyacrylamide gel depending on the molecular weight of the protein of interest. The gels were then electroblotted onto polyvinylidene fluoride (PVDF) plus membranes. The western blots were blocked for one hour at room temperature with either a 1% or 5% nonfat milk (depending on the antibodies requirements) in Tris-buffered saline plus Tween-20 (TBST)[10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20], followed by overnight incubation at 4°C with each primary antibody diluted in the appropriate blocking solution as recommended by the supplier. The primary antibodies used in the study are β-actin (SC-1616), HK2 (SC-6521), MTCO3 (SC-23986), PGC-1 (SC-5814), PPARα (SC-9000), and RXRα (SC-553) obtained from (Santa Cruz Biotechnology, Inc., Calif, USA). The primary antibody TRα (PA1-211A) was obtained from (Affinity BioReagents, Inc., CO, USA). The primary antibodies L&M-CPTI and PDK2 were obtained as personal gifts from Gebre Woldegiorgis and Robert Harris, respectively. After two five-minute washes with TBST and one five-minute wash with Tris-buffered saline (TBS), membranes were incubated at room temperature for one hour with the appropriated secondary antibody conjugated to horseradish peroxidase (HRP). The membranes were washed twice for ten minutes with TBST and visualized with enhanced chemiluminescence after exposure to Kodak biomax light ML-2 film. The membranes were stripped by washing them two times for 30 minutes with 200 mM Glycine, 0.1% SDS, and 1% Tween-20 (pH adjusted to 2.2), followed by three ten-minute washes with TBS. The membranes were again blocked for one hour as above, followed by overnight incubation at 4°C with β-Actin antibody diluted 1 : 200 in blocking solution. The next day, the membranes were washed (as above), the appropriate secondary-HRP antibody was applied, and the remaining procedures (as described above) were followed. The β-actin was used to verify protein lane loadings.

2.4. Statistical analysis

The film expression was determined using the ImageJ 1.32j program produced by Wayne Rasband for (the National Institute of Health, Md, USA). The protein expression was standardized against β-Actin and the means and standard errors for the three stages of heart development are displayed in a histogram (Figure 1). Statistical significance was determined with Student's t-test (two tailed) comparison between all stages of development.

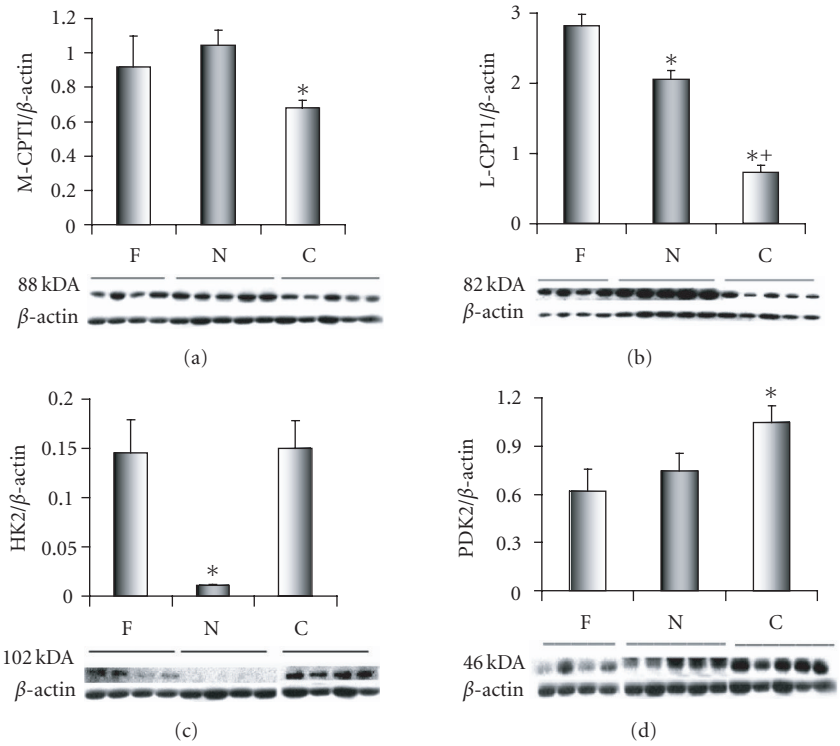

Figure 1: Immunoblots and expression patterns for enzymes controlling substrate oxidation. Data is shown for left ventricle from three development periods (F, N, C) as defined in text. Abbreviations are carnitine palmitoyltransferase I (muscle isoform M-CPTI), carnitine palmitoyltransferase I (liver isoform L-CPTI), hexokinase 2 (HK2), and pyruvate dehydrogenase kinase 2 (PDK2). M-CPTI protein expression decreases occurred between N and C ($^*P < .01$). L-CPTI protein expression differences decreased immediately after birth F ($^*P < .01$) and continued to drop in C ($^+P < 0.001$, versus N). HK2 protein expression exhibited a marked but transient decrease after birth N ($^*P < .05$, versus F & C). PDK2 protein expression increased with significance noted in C compared to F and N ($^*P < .05$, F & N).

3. RESULTS

3.1. Carnitine palmitoyltransferases

The enzyme carnitine palmitoyltransferase I (CPT I; palmitoyl-CoA:L-carnitine O-palmitoyltransferase; EC 2.31.21) is a rate-limiting step in mitochondria transport during FA oxidation. It catalyzes the initial reaction of acyl-CoA and carnitine to acylcarnitine during the mitochondria import of long-chain FAs into the inner mitochondria membrane. PPAR regulates CPTI and turns the gene on during the FA oxidation cascade of events shortly after birth [7, 8]. In mammalian heart, there are two CPTI isoenzymes: a liver, L-CPTI isoenzyme, also known as CPTIα: and a muscle, M-CPTI isoenzyme, also known as CPTIβ [9, 10] In human tissue, the L-CPTI protein is composed of 773 amino acids that corresponds to a molecular weight of 88.3 kDa [P50416]. In sheep heart ,the L-CPTI isoenzyme corresponds to a molecular weight of 82 kDa (Figure 3), which is smaller than that reported in human tissue, but in agreement with what has been reported in fetal and newborn lambs [9]. In human tissue, the M-CPT I protein is composed of 772 amino acids which corresponds to a molecular weight of 87.8 kDa [Q92523]. The adult sheep protein sequence is highly conserved with relation to other mammals with an 89% similarity to humans and 88% similarity to mouse and rat [11]. In sheep heart, the M-CPTI antibody recognizes an 88-kDa isoenzyme consistently expressed in the three study groups (Figure 1). The 88-kDa protein corresponds to the 771 amino acids reported for M-CPTI in sheep [C81315], but is in disagreement with the 80-kDa protein that has been previously reported in sheep [9]. In this study, the M-CPTI (Figure 3(a)) and L-CPTI (Figure 3(b)) protein expression was significantly greater in the day old samples then in the 30-day old samples, while the L-CPTI protein expression was significantly greater in the fetal then in either the day old or 30-day old samples (Figure 1).

3.2. Hexokinase

Hexokinase (HK, E.C.2.7.1.1) in mammalian tissues exist as four isoenzymes (HK1-4) with distinct kinetic properties and tissue distribution [12]. The hexokinases are rate limiting glycolytic enzymes that catalyze the phosphorylation of glucose to glucose-6-phosphate [13]. HK type 2 and HK type 4 are found in heart tissue [14] . In sheep heart, the HK2 antibody detects a 102-kDa protein among the three study groups (Figure 1). The 102-kDa protein corresponds to the molecular weight of the human HK2 isoenzyme with 917 amino acids [P52789]. A 102-kDa protein was also detected by this antibody in a HeLa human cell line obtained from

(Santa Cruz Biotechnology, Inc., Calif, USA) (data not included). In this study, we monitored the appearance of HK2 during the three stages of sheep development and found a significant decrease in HK2 protein expression for the day old samples compared to either the fetal or 30-day old samples. This finding indicates that a reduction in glycolysis at birth due to the onset of FAO.

3.3. Pyruvate dehydrogenase kinase

The isoenzyme pyruvate dehydrogenase kinase 2 (PDK2; pyruvate dehydrogenase [lipoamide] kinase isoenzyme 4; EC 2.7.1.99) is one of four PDK isoenzymes found in mammalian tissues. PDK2 is expressed at high levels in heart tissue [15–17] and is regulated by PPARα [18]. The enzyme is responsible for phosphorylation of pyruvate dehydrogenase (PDH), a mitochondrial multienzyme complex, rendering it inactive. The PDH catalyzes the oxidative decarboxylation of pyruvate, linking glycolysis to the tricarboxylic acid cycle and FA synthesis [18]. Increased levels of PDK in early postnatal life for PDH inactivation are thought to be the result of changes in lipid supply and a switch from glucose to FAs as an energy supply [19]. In addition to hexokinase, PDK is also a rate-limiting step in the glycolytic pathway [20, 21]. In human tissue, the PDK2 isoenzyme is composed of 407 amino acids which corresponds to a molecular weight of 46 kDa for the isoenzyme [Q15119]. PDK2 has its strongest expression in the day old (N) sheep samples. In sheep heart, the PDK2 antibody detects a 46-kDa protein among the three study groups (Figure 1). A 47-kDa protein was also detected by this antibody in a HeLa human cell line obtained from (Santa Cruz Biotechnology, Inc., Calif, USA) (data not included). In this study, PDK2 protein expression levels were significantly greater in the 30-day old (C) samples when compared to either fetal (F) or the one-day old (N) samples; (Figure 1) indicating the switch from glucose to FAO.

3.4. Mitochondrial proteins involved with cardiac energy metabolism

With the beginning of aerobic development at birth there is a rapid deployment of new mitochondria in cardiac cells to handle the FA energy metabolism and ATP output [22]. The mitochondrial genome must be running near full capacity with genome replication for new mitochondria as well as the transcription of mitochondria genes [23] in order to accommodate this expansion. We used protein expression for the cytochrome c oxidase 3 as a reporter for mitochondrial biogenesis. Cytochrome c oxidase 3 (MTCO3; Cytochrome c oxidase polypeptide 3; E.C. 1.9.3.1) is one of three subunits transcribed in the mitochondria from a total of 13 subunits that make up cytochrome c oxidase. The remaining ten subunits are transcribed from nuclear genes [23]. In sheep heart, the MTCO3 antibody detects a 30-kDa molecular weight protein, which represents the cytochrome c oxidase 3 in the three study groups. MTCO3 protein expression is greater in N then either the F or C (Figure 2). We did not see comparable changes for the nuclear cytochrome c oxidase, subunit 4, (NCO4) gene (data not included). This difference between the expression of mitochondria and nuclear cytochrome c oxidase subunits during mitochondria biogenesis has previously been reported [24].

3.5. Peroxisome proliferator-activated receptor-γ co-activator 1 (PGC-1)

In sheep, the PGC-1 antibody recognizes the PGC-1α protein or the 91-kDa isoform, while the PRC and PGC-1β isoforms were not detected among the three study groups (Figure 2). PGC-1α displays a significant increase in protein expression in the day old samples (N) compared with either the fetal (F) or 30-day old samples (C), while the 30-day old samples (C) were found to have a significantly greater protein expression level than the fetal samples (F). In a previous sheep study, we noted that PGC-1 protein expression was near threefold greater in C than in F.

3.6. Peroxisome proliferator-activated receptors (PPARα)

The PPARα antibody detects the 52-kDa nuclear receptor at each stage of development (Figure 3). In human tissues, the PPARα protein is composed of 478 amino acids that reflects a molecular weight of 52 kDa [Q07869], which coincides with the molecular weight observed in sheep. The 52-kDa protein was also detected by this antibody in a HeLa human cell line obtained from (Santa Cruz Biotechnology, Inc., Calif, USA) (data not included). The protein expression of the PPARα nuclear receptor was found to be significantly lower in the 30-day old samples (C) when compared to the Fetal (F) and the day old samples (N) (Figure 3).

3.7. Retinoid X receptors (RXRα)

In sheep heart, the RXRα antibody detects the 51-kDa nuclear receptor at each stage of development (Figure 3) corresponding to the full length RXRα is 462 amino acids, which represents a molecular weight of 51 kDa [P19793]. We noted a small but significant surge in RXRα expression in the one-day old sheep heart (Figure 2(b)).

3.8. Thyroid hormone receptor (TRα_1)

In sheep heart, the TRα1 antibody detects the 47-kDa nuclear receptor among the three study groups (Figure 3). In humans and rodents, the full length of TRα is 410 amino acids, which corresponds to a molecular weight of 47 kDa. In this study, the THRα1 nuclear receptor has a significantly greater level of protein expression in the day old samples (N) compared with either the fetal (F) or the 30-day old samples (C) (Figure 3).

3.9. Other nuclear receptor isoforms and metabolic proteins of interest

Antibodies against other protein expression were used. These include PPARβ, PPARγ, RXRβ, TRα2, TRβ, MYLCD, and PDK4. Unfortunately, a cross reactivity exists between the

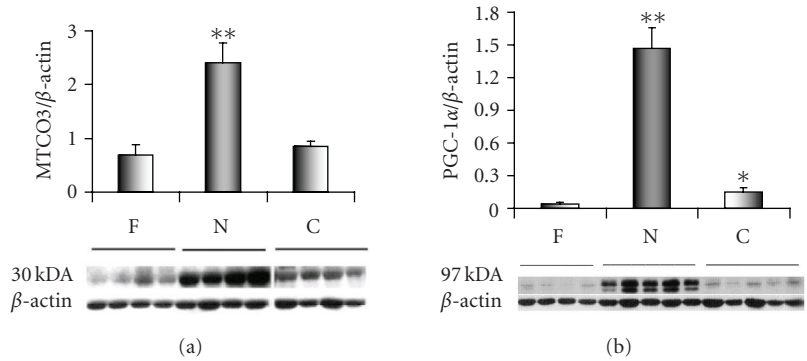

Figure 2: Cytochrome c oxidase 3 expression serves a reporter for the mitochondrial genome (MTCO3; Cytochrome c oxidase polypeptide 3; E.C. 1.9.3.1). MTCO3 increased transiently in the newborn (**$P < .01$, versus F & C). Coordinate changes in protein content occurred for PGC-1α, implicated as a regulator of mitochondrial biogenesis.

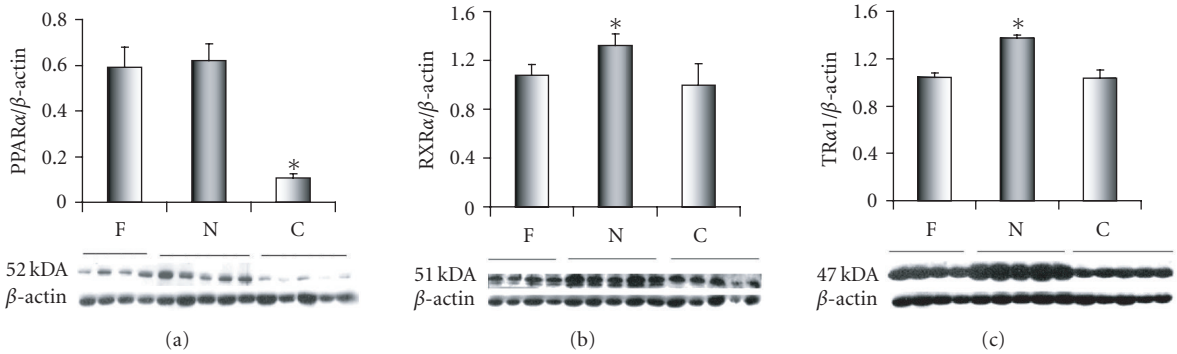

Figure 3: Content for nuclear receptors peroxisome proliferator-activated receptor (PPARα), (b) retinoid X receptor alpha (RXRα), and thyroid hormone receptor (TRα_1) among three stages (F, N, C). Modest transient but significant elevations for RXRα and TRα_1 occurred in N compared to both F and C (*$P < .01$). PPARα expression did not change immediately after birth, but later decreased compared to F and N (*$P < .01$).

secondary antibodies (i.e., those with goat, mouse, and rabbit hosts for the primary antibodies) and the one-day and 30-day old stages of development in our sheep samples. Consequently, we could not get reliable data for these proteins of interest.

4. DISCUSSION

Several investigations have established the time course for maturation of cardiac energy metabolism in the sheep model in vivo. For instance, Bartelds et al. [1] showed that glucose and lactate were the prime energy substrates during ovine fetal life, and the switch to fatty acids as prime oxidative substrate occurred within 2–16 days after birth [1, 25]. Our laboratory has shown that regulation of myocardial oxidative phosphorylation matures within the same age period in parallel with accumulation of the adenine nucleotide translocator protein [26]. Considered in summation, these studies indicate that postnatal transitions in oxidative phosphorylation and substrate oxidation occur coordinately and their regulation is integrated through a unifying signaling mechanism. The nuclear receptors, operating in conjunction with their coactivators, offer a potential mechanism for rapidly integrating these processes shortly after birth.

Fatty acid and carbohydrate metabolism generally exhibit reciprocal type regulatory patterns, where one decreases as the other increases [27]. Prior work in the newborn sheep heart has focused on carnitine palmitoyl transferase I as the pivotal enzyme determining the preferential shift for fatty acid oxidation over carbohydrate utilization. Bartelds et al. [1] detected postnatal increases in CPTI activity and protein content, which were much lower than the increase in the rate of LC-FA oxidation in vivo in the same animals. Furthermore, they found relatively high rates of CPTI activity in fetal lambs [9]. These data led to their contention that substrate supply was the major determinantfor the increase preference for LC-FA oxidation around birth. Similarly, our data show steady decline in L-CPTI immediately after birth with maintained M-CPTI protein levels, and imply that CPTI activity or content does not regulate the postnatal metabolic transition in sheep. Studies in rabbit heart have also shown that the postnatal increases in fatty acid flux do not relate to CPTI-content, isoform pattern or activity. The increase in fatty acid oxidation in the rabbit heart relates directly to a reduction in levels of malonyl-CoA decarboxylase (MYLCD), an inhibitor of CPTI. PDH activity also increases in postnatal rabbit myocardium despite decreases in glucose oxidation.

In our sheep studies, we show for the first time changes in ovine expression for myocardial hexokinase 2 during development. This enzyme catalyzes glucose phosphorylation, a rate limiting step in glucose oxidation and positioned upstream from pyruvate dehydrogenase. Although, glucose transport across the sarcolemma is controlled in part by glucose transporters (GLUT1 and GLUT4), which are expressed abundantly in the fetal sheep heart [28], hexokinase-2 has emerged recently as the rate limiting glucose oxidation step during periods of stress [21, 29]. We also found modest but late postnatal increases for PDK2, which are consistent with inhibition of pyruvate dehydrogenase flux. Thus, our data imply that inhibition of the glucose oxidation pathway contributes to the postnatal metabolic switch with reciprocal and indirect activation of fatty acid oxidation accompanying.

The paradigm of PPARα activation serving as the primary signaling mechanism for the postnatal myocardial metabolic switch has been propagated solely by studies demonstrating increases in PPARα and PGC-1 mRNA levels in mice or rats during development [4, 30]. Supportive developmental expression studies for the corresponding proteins or their activity are lacking in the literature for these species. Furthermore, no published data, that we are aware of, is available regarding the occurrence and timing of the postnatal metabolic switching in either of these species. Extensive literature search reveals no studies regarding myocardial substrate oxidation patterns near or after birth in these species. Rather, mRNA data from rodents has been extrapolated to describe signaling for postnatal phenomenon characterized in larger species such as sheep, rabbit [31], and pigs [32–34]. This interpretative approach is of dubious value, as we have previously shown that mRNA levels for these nuclear receptors and coactivators demonstrate no coordination with protein content in the postnatal period [35]. Results from previous work in sheep imply that steady-state mRNA levels from PPARα and PGC-1 are subject to auto feedback from respective proteins. The dramatic changes in content for the proteins controlling cardiac substrate and oxidative metabolism, such as hexokinase-2, and cytochrome c oxidase, are not preceded by elevation in PPARα protein abundance. Hence, our study indicates that PPARα protein content plays no role in initiating the immediate postnatal upregulation of these metabolic proteins.

Though the data challenge the concept that postnatal increases in PPARα steady-state mRNA coordinates the metabolic shift, they do not eliminate a role for enhanced PPARα-mediated transactivation of target genes. In the current model, PPARα activity might be increased through simultaneous coactivation by PGC-1α, heterodimer formation through increased RXR availability, and enhanced ligand availability generated by newborn suckling and fatty acid intake. However, we have little downstream evidence for increased PPARα transcriptional activity. On the contrary, known PPARα targets, such as LCPTI, MCPTI, and PDK2 are not overtly elevated in terms of protein content during the immediate period after birth. Finally, hexokinase-2, rapidly upregulated by the PPARα antagonist WY14643 in mice [36], shows a marked depression in this model.

PGC-1 remains an attractive candidate as a primary regulator for the postnatal myocardial transition, as this factor also appears to coordinate cross-talk between mitochondrial and nuclear genomes during development [24]. We have previously shown that gene and protein expression for nuclear-encoded adenine nucleotide translocator increases in sheep heart by 28–30 days after birth [26]. In the current study, we demonstrate that the mitochondrial encoded respiratory chain component, MTCO3 increases near four-fold in conjunction with the robust change in PGC-1. As PGC-1 and MTCO3 change coordinately, the data imply that PGC-1 coordinates substrate switching along with mitochondrial membrane expansion by affecting the mitochondrial genome. PGC-1 also closely links to total functional cytochrome c oxidase (Cyt aa_3) and cytochrome c (Cyt c) content and respiratory capacity during postnatal ovine heart development [35]. However, we found no concomitant change in the nuclear-encoded cytochrome c oxidase, subunit 4. This finding suggests that content for nuclear-encoded components are adequate in the fetus, but that rapid postnatal mitochondrial biogenesis depends on PGC-1 promotion of mitochondrial encoded components.

This study was designed to sample three points during sheep development, which would determine the temporal relationship between protein expression and the well-documented late fetal and immediate postnatal surge in circulating thyroid hormone [37]. Since, multiple changes in hormone concentrations and environment occur during this time period, we cannot prove without a doubt that this change in thyroid hormone homeostasis exclusively causes increases in PGC-1 and nuclear receptors. However, thyroidectomy immediately after birth abrogates the postnatal thyroid hormone surge, reduces PGC-1α levels [35], and attenuates expansion of mitochondrial membrane protein content and respiratory capacity [38]. This observation supports the contention that thyroid hormone plays an important role in signaling the postnatal metabolic transition in heart. Our data from prior work and the current study suggest that T_3 simultaneously elevates PGC-1α, and binds as a ligand to the TRs, which also exhibit increased expression, during this critical developmental period. These coordinated events would lead to increased transactivation of metabolic target genes by TRs and PPARs when binding as heterodimers with RXRs.

In summary, our data challenge specific concepts regarding the importance of PPARα protein expression in control of the postnatal metabolic switch. First, we showed that alterations in hexokinase-2, a rate limiting step in glucose oxidation, accompany the transition. These data suggest that inhibition of glucose oxidation reciprocally stimulates fatty acid flux, as opposed to a direct increase in enzyme expression and /or activity of CPTI. Secondly, we demonstrate that no increase in PPARα protein occurs prior to the postnatal metabolic switch in sheep. Thus, transcriptional mediation of this protein does not trigger the change in substrate preference. However, a robust postnatal increase in PGC-1 and the PPARα binding partner, RXRα, does provide conditions for enhanced PPARα activity.

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