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The Positive Regulation of eNOS Signaling by PPAR Agonistsin Cardiovascular Diseases

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Abstract Increasing evidence shows that activation of peroxisome proliferator-activated receptors (PPARs) plays an essential role in the regulation of vascular endothelial function through a range of mechanisms, including non-metabolic. Among these, the PPAR-mediated activation of endothelial nitric oxide synthase (eNOS) appears to be of considerable importance. The regulated and sustained bioavailability of nitric oxide (NO) in the endothelium is essential to avoid the development of cardiovascular diseases such as hypertension or atherosclerosis. Therefore, a deeper understanding of the different effects of specific PPAR ligands on NO bioavailability could be useful in the development of novel or multi-targeted PPAR agonists. In this review, we report the most meaningful and up-to-date in vitro and in vivo studies of the regulation of NO production performed by different PPAR agonists. Insights into the molecular mechanisms of PPAR-mediated eNOS activation are also provided. Although findings from animal studies in which the activation of PPARa, PPARb/d, or PPARc have provided clear vasoprotective effects have been promising, several benefits from PPAR agonists are offset by unwanted outcomes. Therefore, new insights could be useful in the development of tissue-targeted PPAR agonists with more tolerable side effects to improve treatment options for cardiovascular diseases.

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Key Points

- Peroxisome proliferator-activated receptor (PPAR) activity is downregulated in some cardiovascular dysfunctions.
- Low levels of nitric oxide (NO) are a major cause of cardiovascular disease.
- Strong evidence demonstrates that many PPAR agonists are able to stimulate the production of nitric oxide by endothelial nitric oxide synthase (eNOS) through a range of pathways.
- An understanding of the signaling system involved in PPAR-mediated eNOS activation and therefore the beneficial role of PPAR agonists in improving cardiac function is useful in research into novel and safer ligands.

1 Introduction

Cardiovascular disease is a major cause of death worldwide, and metabolic disorders such as obesity, dyslipidemia, hypertension, insulin resistance, increased systemic inflammation, and hypercoagulability are important risk factors.

While the pathophysiological role of peroxisome proliferator-activated receptor (PPAR) nuclear receptors in glucose and lipid metabolism is well defined, their involvement in the vascular system is receiving increasing attention. Indeed, evidence is growing that PPAR activation in the vasculature has a protective role against

endothelial dysfunction, lipotoxicity, and oxidative stress [1]. PPARs have broad expression in several tissues: PPARa is predominantly localized in tissue exhibiting high catabolic rates of fatty acids (liver, skeletal muscle); PPARb/d are ubiquitous, although the major expression is in the liver, esophagus, intestine, kidneys, and skeletal muscle. Although PPARc is mainly expressed in white and brown adipose tissue, it is not abundantly expressed under normal physiological circumstances [2]. All of the isoforms are also expressed in the heart, as well as in endothelial cells and vascular smooth muscle cells (VSMCs), and alterations in cardiac PPAR levels or functionality cause disorders in glucose and fatty acid metabolism that trigger several cardiac and endothelial dysfunctions [3–7]. All PPARs can be activated by numerous endogenous ligands, such as saturated and unsaturated fatty acids, as well as by different classes of drugs [8]. Ligands bind to the PPAR ligand-binding domain (LBD) in the cytoplasm, and then the activated receptor translocate to the cell nucleus. Once within the nucleus, PPAR forms a heterodimer with retinoid X receptor (RXR) and binds to specific DNA-response elements known as PPAR response elements (PPREs), increasing gene transcription [9].

Nitric oxide (NO) is a diatomic, nonpolar, free radical that can interact with soluble guanylyl cyclase (sGC). The resultant conversion of guanosine 5^0-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) regulates several biochemical and physiological pathways in the vasculature[10, 11]. In physiological conditions, NO is biosynthesized inthe cardiovascular system by the endothelial NO synthase (eNOS). The eNOS-derived NO possesses multiple anti-atherosclerotic properties and is an antihypertensive and antithrombotic factor. Essentially, although NO is considered to be protective of the endothelium, if NO is dysregulated it could be detrimental for tissues [12–14]. In response to multiple stimuli, eNOS can be activated through phosphorylation at Ser^{1177} and Ser^{633} sites by different protein kinases (PKs), such as PKB, PKA, PKC, AMP-activated protein kinase (AMPK), or through dephosphorylation at Thr^{495} by phosphatases such as protein phosphatase (PP)-1 and PP2A [15]. Moreover, bioavailability of the natural substrate L-Arg and of the tetrahydrobiopterin (H_4B) cofactor is crucial for a

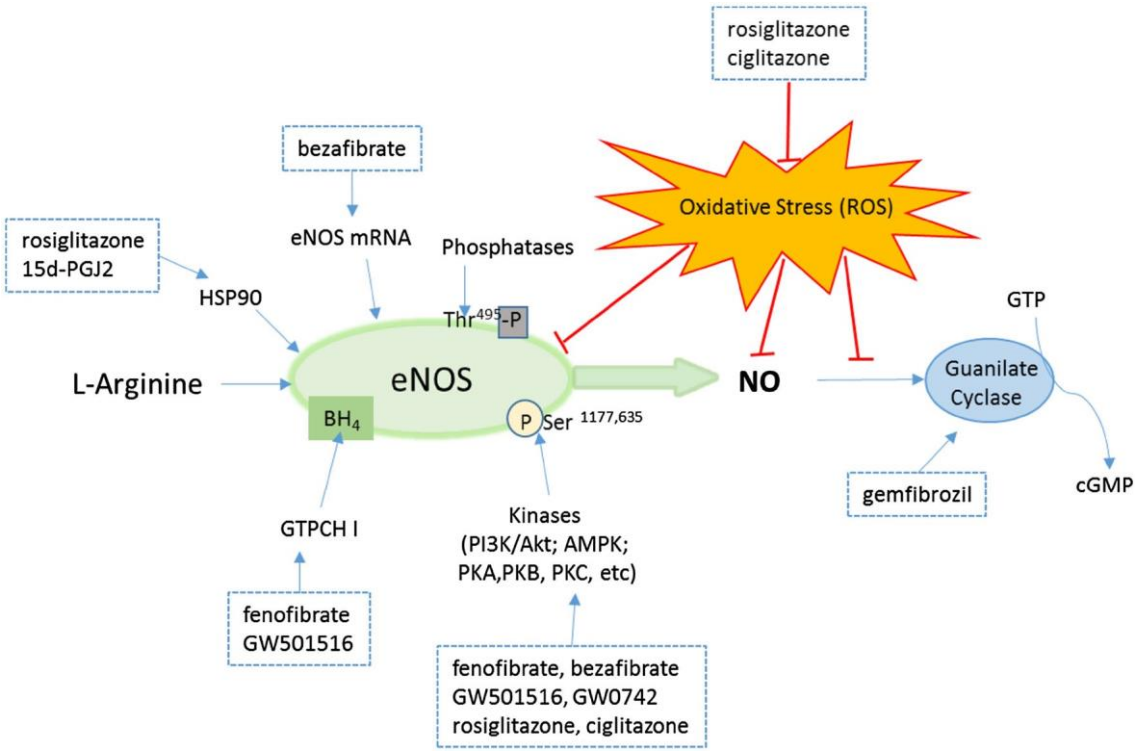

Fig. 1 Regulation of endothelial nitric oxide (NO) synthase (eNOS). NO production by eNOS depends on the extent of protein activation, which is due to several mechanisms including eNOS messenger RNA (mRNA) or protein level; substrate availability; cofactor BH_4 availability; phosphorylation at $Ser^{1177,635}$; dephosphorylation atThr^{495}; and interaction with heat shock protein (HSP)-90. Incardiovascular diseases, both eNOS activation and NO bioavailability are compromised, principally due to reactive oxygen species (ROS) and oxidative stress. Several peroxisome proliferator-activated receptor (PPAR) agonists stimulate eNOS activation through different pathways. *AMPK* AMP-activated protein kinase, *cGMP* cyclic guanosine monophosphate, *eNOS* endothelial NO synthase, *GTP* guanosine 5^0-triphosphate, *GTPCH* guanosine triphosphate cyclohydrolase, *HSP* heat shock protein, *mRNA* messenger RNA, *NO* nitric oxide, *PI3 K* phosphatidylinositol 3-kinase, *PK* protein kinase, *PPAR* peroxisome proliferator-activated receptor, *ROS* reactive oxygen species

functional enzyme (Fig. 1). Under atherosclerosis and vascular disease conditions, endothelial protection by eNOS-derived NO is compromised, principally because of the high levels of reactive oxygen species (ROS), such as superoxide ion ($O_2^{\cdot -}$), which reduce NO bioavailability in the vasculature. Indeed, ROS leads to both NO chemical inactivation and eNOS dysfunction and uncoupling. The uncoupled eNOS is an $O_2^{\cdot -}$-producing enzyme and is responsible for the potentiation of the pre-existing oxidative stress. In this damaged framework, the reduction in NO bioavailability is detrimental and contributes significantly to atherogenesis and vascular diseases [16, 17]. Therefore, attempts to increase levels of endogenous NO and restore eNOS activity in the treatment of cardiovascular diseases have attracted great pharmaceutical interest [18].

In recent years, several studies have demonstrated the key role of PPARs in the regulation of vascular endothe- lium functions, mainly through the reduction of endothelial oxidative stress and the consequent increase of NO bioavailability. However, increasing evidence indicates that the activation of PPARs can also lead to eNOS stim- ulation. The aim of this review was to summarize updated findings about the positive modulation exerted by PPAR agonists on eNOS levels and activity, strengthening their value as potential therapeutics for the treatment of car- diovascular diseases associated with metabolic disorders.

2 Peroxisome Proliferator-Activated Receptor (PPAR)-a–Endothelial Nitric Oxide Synthase (eNOS)

PPARa plays a pivotal role in lipid metabolism by decreasing the levels of both serum triglycerides and free fatty acids and increasing levels of high-density lipoprotein (HDL). Indeed, fibrates exert their most potent effects within hepatocytes and adipocytes by increasing the synthesis of apolipoprotein AI and AII and stimulating lipase activity [19]. However, strong evidence supports the importance of the biological functions of PPARa in the cardiovascular system. For example, PPARa knock-out (KO) mice progressively develop cardiac fibrosis, with irregular mitochondria and myofibrils [20], cardiomyocyte hypertrophy [21], and impaired myosin [22]. Moreover, in pressure overload-induced cardiac hypertrophy, PPARa gene expression is downregulated, leading to cardiac lipotoxicity [23, 24]. It was recently demonstrated that low levels of PPARa contribute to the early phase of essential hypertension; therefore, the induction of this ligand-activated nuclear receptor could confer protection against this pathological condition [25]. Moreover, PPARa deactivation compromises the normal equilibrium between oxidant production and antioxidant defenses, contributing to cardiac dysfunction [26].

Both metabolic syndrome and aging are responsible for the impairment of PPARa levels in the cardiovascular system [27, 28], and pharmacological activation can be restored through fibrates (Fig. 2), a class of lipid-lowering drugs used clinically since the 1960 s. The chemical structures of synthetic PPARa agonists show a general pharmacophore, including a carboxylic head, an aromatic ring, and a lipophilic tail, connected by linkers; the nature of these fragments affects the potency and subtype selec- tivity [29–32]. Although bezafibrate, ciprofibrate, fenofi- brate, and gemfibrozil are currently available in several countries for the treatment of hyperlipidemia associated with metabolic syndrome, the US FDA has approved only the last two for clinical use.

In the last 10 years, different studies have shown that treatment with fibrates generally provides beneficial effects in the therapy of cardiac dysfunction through multiple pathways. For example, PPARa activation is responsible for inhibition of nuclear factor (NF)-jB and activator protein (AP)-1, limiting inflammatory responses in the vasculature. Depending on the agonist used, an important

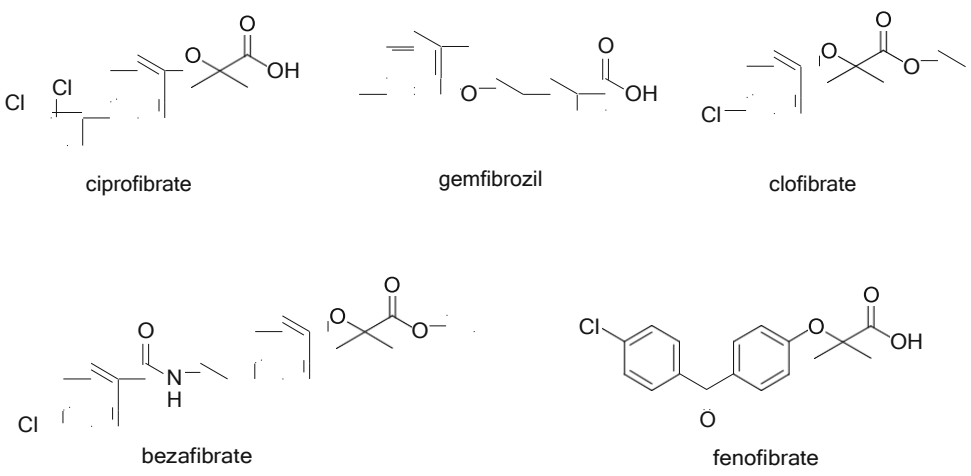

Fig. 2 Molecular structures of peroxisome proliferator-activated receptor (PPAR)-a agonists

consequence of PPARa activation in the endothelium is also the enhancement of NO production through stimulation of eNOS expression and activity. For example, in hypertensive rats, clofibrate significantly increased eNOS protein expression and enzymatic activity in the left ventricle, improving endothelium-dependent vasodilation and resulting in appropriate cardiac performance [33]. Another study demonstrated that bezafibrate upregulated both eNOS expression and NO production in a dose-dependent manner. Indeed, this drug both extended the half-life of eNOS messenger RNA (mRNA) and activated the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways, increasing eNOS activation through phosphorylation at Ser^{1177} [34].

Fenofibrate was found to increase eNOS expression in endothelial cells [35]; this effect is due to the activation of the AMPK, which in turn stimulated eNOS through phosphorylation at Ser^{1177} and inhibited NF-jB [36]. Consistent with these findings, a clinical study in healthy normolipidemic middle-aged adults reported that fenofibrate improved vascular endothelial function, an effect that was connected to the increased eNOS protein levels in endothelial cells after 7 days of treatment [37]. Additionally, fenofibrate seemed to prevent endothelial dysfunction by also upregulating levels of H_4B, and decreasing production of ROS. Indeed, low levels of the tetrahydrobiopterin cofactor are responsible for the eNOS uncoupling, with subsequent reduction in NO levels, nitrosative stress enhancement, and endothelial dysfunction[38]. Therefore, fenofibrate may protect against atherosclerosis and endothelial disorders by promoting the re-coupling of eNOS [39].

Interestingly, clinical trials of gemfibrozil, such as the Helsinki Heart Study and the Veterans Affairs HDL Cholesterol Intervention Trial [40, 41], showed that gemfibrozil provides the most pronounced cardiovascular preventive benefits, with a decreased incidence of coronary events over other fibrates. However, the vasorelaxation and antiplatelet properties of gemfibrozil are not due to the stimulation of NO biosynthesis. In fact, gemfibrozil does not induce a significant increase in eNOS mRNA levels but instead behaves as a heme-independent sGC activator, promoting the release of cGMP [42, 43].

3 PPARb/d–eNOS

Activation of PPARb/d has therapeutic value in the dyslipidemias and glucose metabolism dysfunctions, and studies have shown it to be an important repressor of inflammatory pathways [44–46]. The first selective PPARbd agonists identified were GW501516 and GW0742 (Fig. 3).

These compounds show high potency and a 1000-fold selectivity for PPARd over the other subtypes, without activity on other nuclear or non-nuclear receptors [47, 48]. They contain an unsubstituted phenoxyacetic acid group that complements the narrow PPARb/d ligand-binding pocket [49]. Treatment of moderately obese men with these agents promoted reversal of several metabolic abnormalities, reduced oxidative stress, and increased fatty acid oxidation; however, no PPARb/d ligand has yet been approved for clinical use because side effects are unacceptable.

However, these drugs have also shown non-metabolic effects, with a favorable cardiovascular profile. Selective PPARb/d ligands improved cardiac hypertrophy in vitro [50], protected human umbilical vein endothelial cells (HUVECs) from hydrogen peroxide-induced apoptosis [51], inhibited VSMC proliferation and migration, and produced fast, concentration-dependent relaxant effects in rat vascular tissue [52].

Cardiovascular effects of the selective agonist GW0742 are mostly endothelium and NO dependent, although they are not strictly related to the classic $Ca^{2?}$/calmodulin pathway for eNOS activation, being instead due to the stimulation of eNOS phosphorylation via the PI3K-Akt pathway [53]. This was also confirmed in streptozotocin (STZ)-induced diabetic rats, where GW0742 does not upregulate eNOS expression, while increasing the levels of phosphorylation in protein. In line with these results, the potent and selective PPARb/d agonist GW501516increased eNOS expression and Ser^{1177} phosphorylation in cultured human endothelial progenitor cells, enhancing their regenerative capacity [54].

Activation of PPARd could also be responsible for the increase of intracellular concentrations of the eNOScofactor H_4B. This effect is caused by an AKT-dependent increase in expression and activity of guanosine

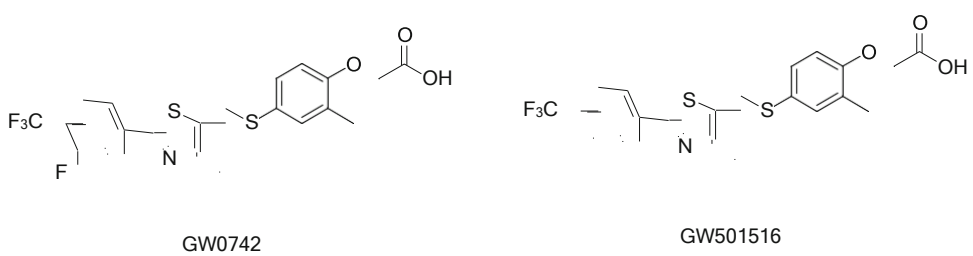

Fig. 3 Molecular structures of peroxisome proliferator-activated receptor (PPAR)-b/d agonists

triphosphate cyclohydrolase (GTPCH)-I, a rate-limiting enzyme in the production of H_4B. This was observed in GW501516-treated Tg2576 mice, which showed augmented expressions of manganese superoxide dismutase (MnSOD) and catalase, together with an increased bioavailability of H_4B, of eNOS uncoupling [55].

4 PPARc–eNOS

PPARc activation enhances insulin sensitization and therefore plays an important role in glucose metabolism. Besides the natural PPARc ligand 15d-PGJ2, the thiazolidinediones are a well-known class of PPARc agonists used in the therapy of type 2 diabetes mellitus (Fig. 4).

The X-ray crystal structure of rosiglitazone complexed to PPAR revealed that it binds the large PPARc pocket in a U-shaped conformation while assuming an unfavorable conformation within the PPARa ligand-binding domain [56, 57]. Vascular PPARc is a peripheral regulator of cardiovascular rhythms that controls circadian variations in blood pressure and heart rate through brain and muscle Arnt-like protein (BMAL)-1 [58]. Accordingly, the activation of PPARc in patients with type 2 diabetes mellitus normalizes the circadian rhythm of blood pressure from non-dipper to dipper [59]. Evidence is growing that activation of PPARc plays an essential role in the regulation of vascular endothelial function through different mechanisms, causing eNOS activation and NO generation and leading to a reduction in the risk for cardiovascular diseases, primarily atherosclerosis [60]. One of the first studies about this revealed that treatment of HUVEC cells with PPARc agonists such as 15d-PGJ2, ciglitazone, or rosiglitazone caused an increase in NO release, which was inhibited by co-administration of GW9662, a selective PPARc antagonist [61]. These results clearly indicated a key role for PPARc in the induction of endothelial NO release. From a mechanistic viewpoint, it was found that rosiglitazone and 15d-PGJ2, but not ciglitazone, activated eNOS by stimulating heat shock protein (HSP)-90–eNOS interaction, followed by eNOS phosphorylation at Ser^{1177}. Moreover, treatment with 15d-PGJ2 or ciglitazone decreased relative mRNA levels of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits such as nox-1, gp91phox (nox-2), and nox-4, and enhanced expression of SOD, resulting in a reduction of HUVEC membrane NADPH-dependent superoxide anion generation. Therefore, in addition to stimulating NO release from the endothelium, PPARc activation could also improve NO bioavailability by reducing endothelial superoxide anion generation and oxidative stress [62]. These effects can be further explained given the important interaction between PPARc and the redox-sensitive NF erythroid 2-related factor (NRF)-2, which plays a vital role in cytoprotection against oxidative and electrophilic stress. Indeed, in these conditions, the NRF2 is dissociated from its inhibitor, the Kelch-like enoyl-CoA hydratase-associated protein 1 (KEAP1), and translocates to the nucleus. Here, it regulates the transcription of cytoprotective genes containing in the promoter region the antioxidant response element (ARE), including the *PPAR*c gene [63]. Consequently, PPARc protein expression is enhanced, with a subsequent increase in eNOS phosphorylation and expression [64]. Notably, different studies have demonstrated that PPARc agonists can in turn upregulate NRF2 in a positive feedback loop that sustains the expression of both transcription factors, improving endothelial NO bioavailability [65].

Activation of eNOS was also observed in young male spontaneously hypertensive rats (SHRs) treated with rosiglitazone. Interestingly, SHRs showed significantly reduced expression of PI3K and decreased phosphorylation of PKB–eNOS in vascular tissues. Treatment with rosiglitazone produced an increase of vascular PPARc expression and restored the activation of PI3K/PKB/eNOS signaling, thus improving endothelial function in the young SHRs [66].

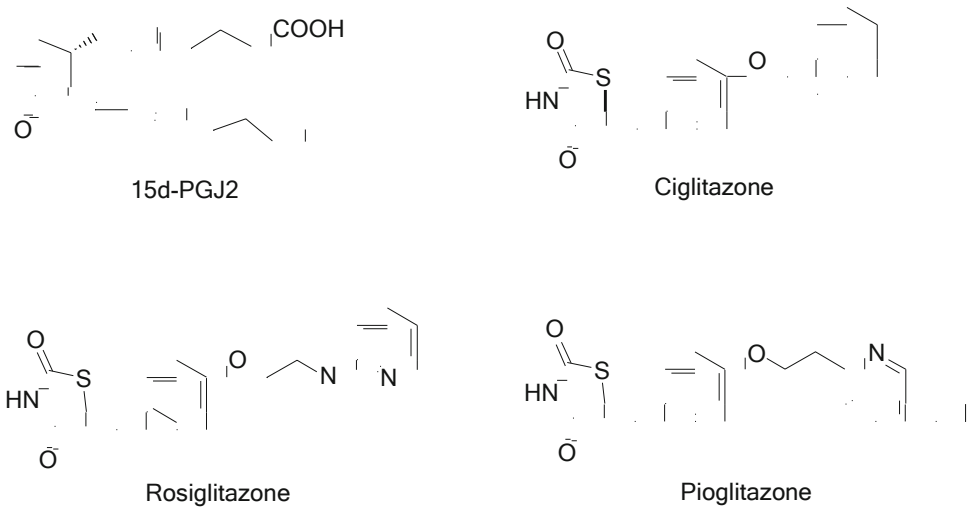

Fig. 4 Molecular structures of peroxisome proliferator-activated receptor (PPAR)-c agonists

Activation of PPARc by rosiglitazone also improved eNOS function in diabetic db/db mice through stimulation of adiponectin, a protein secreted from adipose tissue. Adiponectin activated AMPK/eNOS and cAMP/PKA signaling pathways in the aorta, resulting in a reduction in oxidative stress and enhancement of NO bioavailability[67]. Intriguingly, the authors observed no improvement in endothelial function in diabetic mice lacking adiponectin, suggesting adiponectin could mediate PPARc-induced eNOS activation and NO generation to improve the function of the vascular endothelium. More recently, the potent PPARc agonist ciglitazone was also reported to enhance eNOS activity by stimulating serine phosphorylation through a PPARc-dependent AMPK/AKT signaling pathway, protecting endothelial cells against ox-low-density lipoprotein-induced injury [68].

PPARc agonism seems to also positively control eNOS activity by means of further mechanisms. It is well known that Rho-kinase, a serine–threonine kinase, plays a critical role in inducing endothelial dysfunction by inactivating eNOS and reducing NO generation [69]. Interestingly, PPARc activation by pioglitazone upregulates the protein tyrosine phosphatase (SHP)-2 in cultured aortic smooth muscle cells of angiotensin-II-treated rats, with subsequent dephosphorylation on tyrosine residues of the Vav protein, a family of guanosine nucleotide exchange factors (GEFs) involved in the activation of Rho/Rac proteins. The resulting inactivation of Rho-kinase restores eNOS function and NO biosynthesis [70].

5 Adverse Effects of PPAR Agonists

PPAR agonists have off-target and side effects that could limit their clinical use. In fact, although PPAR modulators hold great promise, agents launched thus far (e.g., muraglitazar, aleglitazar, etc.), with the possible exception of saroglitazar [71–76], have failed to live up to their potential.

However, safety and tolerability differ between drugs. For example, the PPARa agonist fenofibrate is generally well tolerated, with the most frequent adverse effects being mild gastrointestinal symptoms. Rarely, muscle-related adverse events occur in subjects receiving a combination of fibrates and statins, particularly for severe disease such as myopathy and rhabdomyolysis. Nevertheless, such adverse outcomes are more frequent when statins are combinedwith gemfibrozil than with fenofibrate because of the dif- ferent pharmacokinetic profiles. Increased plasma crea- tinine concentrations appear to be a class effect of fibrates and a consequence of reduced tubular secretion and increased muscle production of creatinine rather than nephrotoxicity or any adverse renal outcome [77–79].

Conversely, the clinical development of PPARb/d agonists was discontinued because of tumorigenic effects in animal studies [80]. While these drugs were well tolerated in short-term treatments, safety concerns, particularly for GW501516, were also related to toxicities in different cell types. However, whether these effects were due to PPAR activation or to off-target interactions was unclear [81].

PPARc activation has also been associated with other adverse effects such as weight gain, fluid retention, and bone fractures [82]. Moreover, thiazolidinediones have been associated with myocardial infarction, heart failure, stroke, and peripheral edema [83], although proarrhythmic effects seem to be PPAR-independent and due to interac- tions between thiazolidinediones and calcium or potassium channel activity [84]. Notably, thiazolidinediones have been the subject of intense controversy for their associationwith an increased risk of bladder cancer. However, clinical trials and meta-analysis could not establish a link between thiazolidinediones and cardiovascular effects or other malignancies, supporting a positive benefit–risk profile [85]. The off-target mechanisms of PPARc agonists have been extensively reviewed recently, highlighting the com- plexity of PPAR regulation and the necessity of improving the on-target actions of PPAR ligands [86].

6 Conclusions

The activation of PPARa, PPARb/d, and PPARc is often associated with clear vasoprotective effects. The stimulation of endothelial NOS is undoubtedly a consequence of PPAR activation, occurring via different pathways, intermediates, and protein post-translational modifications depending on the specific ligand and activated PPAR isoform.

However, full PPAR agonists have been associated with severe adverse events, and the cardiovascular benefits of these drugs are significantly counterbalanced by several side effects and a range of off-target interactions. Never- theless, in light of their effects on cardiac vasculature,submaximal activation of PPARs has recently been pro- posed as having potential for beneficial cardiovascular effects due to eNOS stimulation, avoiding major adverse events [87].

Therefore, a deeper understanding of the different effects of specific PPAR ligands on NO bioavailability is desirable as it could be useful in the development of novel or multi-targeted PPAR agonists with more acceptable sideeffects and thus improved treatments for cardiovasculardiseases.

Compliance with Ethical Standards

Conflicts of interest Dr Maccallini, Professor Mollica, and Professor Amoroso have no conflicts of interest that are relevant to the content of this review.

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