Hiroshi Kuriyama Award 2007 Memorial Review

Relationships among ET-1, PPARγ, oxidative stress and endothelial dysfunction in diabetic animals

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Abstract

Macro- and microvascular disorders currently represent the principal causes of morbidity and mortality in patients with diseases involving the cardiovascular system, such as atherosclerosis, hypertension, stroke, and diabetes. Abnormal vasomotor responses and impaired endothelium-dependent vasodilation have been demonstrated in a number of vessels in a variety of animal models and in humans with such diseases. Endothelial dysfunction plays a key role in the development of these diseases, yet the genesis of this endothelial dysfunction and its associated vasomotor abnormalities remain poorly understood. Peroxisome proliferator-activated receptor (PPARγ) is a nuclear receptor and transcription factor in the steroid superfamily, and PPARγ agonists (the thiazolidinediones) are used clinically to treat type 2 diabetes. Recent studies have revealed that as well as being involved in adipogenesis and in increased sensitivity to insulin, PPARγ plays critical roles in the vasculature. In the present review, we discuss the beneficial effects of PPARγ agonists on vasomotor activities, focusing in particular on endothelium-dependent relaxation in vessels affected by cardiovascular diseases.

Key words: cardiovascular disease, endothelin, diabetes, oxidative stress, thiazolidinedione

Introduction

The peroxisome proliferator-activated receptors (PPARs), which are members of the nuclear receptor family, acts as transcription factors, regulating the expression of genes. Each PPAR heterodimerizes with the retinoid X receptor (RXR) and is rendered transcriptionally active by binding to a specific DNA sequence element termed a PPAR response element (PPRE) (Berger and Moller, 2002; Marx et al., 2004; Michalik et al., 2006). The three PPAR subtypes, PPARγ, PPARα and PPARδ (also known as PPAR/δ), constitute a subfamily of nuclear receptors (Berger and Moller, 2002; Blaschke et al., 2006ab). PPARγ controls adipocyte differentiation and lipid storage, and accordingly is heavily expressed in adipose tissue. Through its effects on

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adipose tissue and skeletal muscle, PPARγ regulates the action of insulin (Berger et al., 2005; Rangwala and Lazar, 2004; Sharma and Steals, 2007; Willson et al., 2001), and is therefore relevant to diabetes research.

The deployment of genetic approaches, such as gene targeting and human molecular genetics, has led to major advances in our knowledge of the physiological roles and medical importance of PPARγ. Null mice for PPARγ normally die as embryos (Barak et al., 1999, Kubota et al., 1999), while PPARγ null embryos that are rescued to term have no visible white adipose tissue and possess a fatty liver (Barak et al., 1999). Moreover, embryonic stem cells lacking PPARγ are unable to differentiate into adipocytes in vitro (Rosen et al., 1999). These two results reveal the contribution of PPARγ to adipogenesis. Further, recent studies using conditional knock-out strategies in mice have revealed the roles performed by PPARγ in individual tissues. Adipocyte PPARγ is required for normal adiposity (He et al., 2003; Imai et al., 2004) as well as for insulin sensitivity at fat and liver, but not in muscle (He et al., 2003). Myocyte PPARγ has been reported variously to be required to maintain whole-body insulin sensitivity (Hevener et al., 2003) or liver insulin sensitivity alone (Norris et al., 2003). Hepatic PPARγ is required to maintain insulin sensitivity, particularly in older animals or in animals with genetically diabetic backgrounds (Gavriloja et al., 2003; Matsusue et al., 2003), while endothelial PPARγ is important in the regulation of diet-induced hypertension (Nicol et al., 2005). Thus, PPARγ is a key regulator of glucose homeostasis and adipogenesis, and plays important roles in physiological and pathophysiological states (Berger et al., 2005; Rangwala and Lazar, 2004; Sharma and Steals, 2007).

Substantial evidence has accrued to indicate that PPARγ are expressed in all the major cells found in vascular beds, and that this receptor is activated not only by natural ligands such as free fatty acids and eicosanoids, but also by the insulin-sensitizing, thiazolidinedione (TZD) class of agents (including troglitazone, cigitazone, rosiglitazone, and pioglitazone) (Bishop-Bailey, 2000; Blaschke et al., 2006a; Li and Palinski, 2006; Schiffrin, 2005; Touyz and Schiffrin, 2006; Walcher and Marx, 2004). The TZDs are currently used in the treatment of diabetic hyperglycemia (Stumvoll and Haring, 2002), although these compounds may have direct beneficial effects on cardiovascular risk that are independent of their hypoglycemic actions (Martens et al., 2002).

Several lines of evidence suggest that endothelial dysfunction plays a key role in the development of both macro- and microangiopathy in patients with inflammatory-associated diseases as atherosclerosis, hypercholesterolemia, hypertension, stroke, and diabetes, as well as in animal models of these diseases (Cai and Harrison, 2000; Kobayashi et al., 2000, 2004, 2005, Landmesser et al., 2004; Matsumoto et al., 2003, 2004b, 2005, 2006ab, 2007abc; Triggle et al., 2003). Although the genesis of this endothelial dysfunction and its associated vasomotor abnormalities remains poorly understood, there have been recent demonstrations that in cardiovascular diseases, activation of PPARγ not only has cardio-protective effects, but also restores vascular structure and corrects endothelial dysfunction (Bishop-Bailey, 2000; Li and Palinski, 2006; Schiffrin, 2005; Touyz and Schiffrin, 2006; Walcher and Marx, 2004). Because of these beneficial effects, a given activator of PPARγ may have a therapeutic potential for the prevention of cardiovascular diseases that lies beyond its actions on carbohydrate and lipid
metabolism. In the present review, we shall discuss the possible effects of PPARγ agonists (especially TZDs) on vascular tone, as well as their regulation and the alterations in cellular signaling that occur in cardiovascular diseases.

**PPARγ in vascular cells**

PPARγ is reportedly present in the vasculature, and indeed this receptor has been demonstrated in endothelial cells (ECs) (Satoh *et al.*, 1999), vascular smooth muscle cells (VSMCs) (Diep and Schiffrin, 2001; Law *et al.*, 2000), and monocyte/macrophages (Ricote *et al.*, 1998). Evidence concerning the role of this nuclear factor in the vasculature has forthcoming over the last few years: for example, PPARγ agonists can inhibit the activities of several transcription factors [such as nuclear factor-kappa B (NF-κB), AP-1, STAT-1, NFAT, Erg-1, GATA-3, and Jun] within cells. Furthermore, PPARγ activation leads to the following responses within the vasculature: (a) in ECs, it inhibits endothelial inflammation by suppressing inflammatory gene expression, (b) in VSMCs, it inhibits proliferation and migration, and promotes apoptosis, and (c) in macrophages, it suppresses inflammation by regulating gene expression and increases cholesterol uptake and efflux. The literature on these beneficial effects of PPARγ activation has been extensively reviewed elsewhere (Bishop-Bailey, 2000; Duan *et al.*, 2008; Li and Palinski, 2006; Marx *et al.*, 2004; Schiffrin, 2005; Touyz and Schiffrin, 2006; Walcher and Marx, 2004).

**Effect of PPARγ agonists on endothelium-dependent relaxation**

ECs actively regulate both basal vascular tone and vascular reactivity in physiological and pathophysiological conditions. They do this by responding to mechanical forces and neurohumoral mediators with the release of a variety of contracting [endothelium-derived contracting factors (EDCFs)] and relaxing factors [endothelium-derived relaxing factors (EDRFs)] (Cohen, 2005; Kamata *et al.*, 1989; Kobayashi *et al.*, 2005; Matsumoto *et al.*, 2004a, 2006a; Pieper, 1998; Triggle *et al.*, 2003; Vanhoutte *et al.*, 2005). A balanced release of these bioactive factors facilitates vascular homeostasis. The activity of the endothelium extends far beyond the control of vascular tone and reactivity, and the release of vasodilator mediators clearly reflects only one aspect of the homeostatic and protective role of the endothelium. Importantly, a healthy endothelium inhibits platelet and leukocyte adhesion to the vascular surface, and also maintains the balance between profibrinolytic and prothrombotic activities (Landmesser *et al.*, 2004). Several common conditions carrying a predisposition to atherosclerosis (such as hypercholesterolemia, hypertension, diabetes, and stroke) are associated with endothelial dysfunction, leading to an endothelium with a proinflammatory and prothrombotic phenotype (Landmesser *et al.*, 2004). In practice, endothelial function has been assessed primarily in terms of endothelium-dependent vasomotion, largely on the assumption that impaired endothelium-dependent vasomotion reflects alterations of other functions of the endothelium as well.

There is an accumulating body of evidence to show that in several vessels, the endothelium-dependent relaxation responses are weaker in experimental models of cardiovascular diseases.
and in patients with such diseases (De Vries et al., 2000; Kamata et al., 1989; Kobayashi et al., 2000, 2005; Poston and Taylor, 1995; Sekiguchi et al., 2004; Triggle et al., 2003). Further, improvements in such impaired relaxation responses have been reported following treatment with a PPARγ agonist. For example, an improvement in acetylcholine (ACh)-induced endothelium-dependent vasodilation has been seen in such animal models as Zucker Fatty rats (Walker et al., 1999) and Ang II-induced rats (Diep et al., 2002), while rosiglitazone improves the impaired ACh-induced relaxation of carotid arteries (and lowers blood pressure) seen in hypertensive transgenic mice expressing both human renin and human angiotensinogen transgenes (Ryan et al., 2004). Moreover, endothelium-dependent vasodilation of the brachial artery (as evaluated by flow-mediated dilation) has been shown to be improved (a) by pioglitazone both in nondiabetic patients with essential hypertension (Horio et al., 2005) and in newly diagnosed type 2 diabetes mellitus (T2DM) patients with coronary artery disease (Sourij et al., 2006), and (b) by rosiglitazone in T2DM patients (Pistrosch et al., 2004). In addition, short-term treatment with pioglitazone protects completely against the TNFα-induced depression of endothelium-dependent vasodilation in T2DM patients (Martens et al., 2006). Although the mechanisms underlying the TZD-induced improvements in endothelium-dependent relaxation are not fully understood, such improvements might be attributable not to their anti-diabetic actions, but rather to direct effects on vascular cells (see below).

**PPARγ agonists and NO signaling**

Endothelium-derived nitric oxide (NO) is a key molecule in vascular biology since it is capable of reducing vascular tone, SMC proliferation, leukocyte adhesion, and platelet aggregation (Landmesser et al., 2004; Matsuda and Hattori, 2007; Pieper, 1998; Tanaka et al., 2004). Indeed, impaired endothelial NO production participates in the pathogenesis of a number of cardiovascular diseases (Kobayashi et al., 2005; Landmesser et al., 2004; Pieper, 1998). In ECs, endothelial NO synthase (eNOS) produces NO from the amino acid L-arginine, and eNOS itself is regulated not only at the level of expression (Drummond et al., 2000), but also post-translationally [by mechanisms including interactions of eNOS with other proteins (Garcia-Cardenas et al., 1998; Murata et al., 2002; Sessa, 2005) and eNOS phosphorylation (Du et al., 2001; Kobayashi et al., 2005)]. For example, specific stimuli including histamine, vascular endothelial growth factor (VEGF), and shear stress have been shown to activate eNOS by promoting its interaction with heat shock protein 90 (hsp90), a molecular chaperone protein (Garcia-Cardenas et al., 1998). Hsp90 has been shown to increase eNOS activity by: 1) recruiting Akt (serine protein kinase B) to phosphorylate eNOS at Ser1177 (Fontana et al., 2002), 2) facilitating the displacement of eNOS from inhibitory interactions with caveolin (Gratton et al., 2000), and 3) increasing the affinity of eNOS for calmodulin (Takahashi and Mendelsohn, 2003). In addition to protein-protein interactions, several specific phosphorylation sites regulate eNOS activity. For example, phosphorylation of eNOS at Ser1177 increases electron flux from the reductase to the oxygenase domain of eNOS and increases enzyme activity (Shi et al., 2002).

Current evidence suggests that PPARγ agonists may modulate eNOS activity in ECs (Fig. 1). Indeed, Calnek et al. (2003) demonstrated that the PPARγ ligands 15d-PGJ2 and ciglitazone
increased NO release from porcine and human aortic ECs, although PPARγ activation did not increase eNOS expression. They also found that overexpression of PPARγ or treatment with 9-cis-retinoic acid (a ligand for the PPAR heterodimer RXR) enhanced NO release too, while neither 15d-PGJ2 nor ciglitazone altered eNOS mRNA. Thus, PPARγ ligands stimulate NO release from ECs through a transcriptional mechanism unrelated to eNOS expression. Polikandriotis et al. (2005) further demonstrated that rosiglitazone and 15d-PGJ2, but not ciglitazone, increased both hsp90-eNOS interaction and eNOS Ser1177 phosphorylation, and that individual ligands exert their effects through distinct PPARγ-dependent mechanism. Moreover, Cho et al. (2004) found that troglitazone increased endothelial NO production in bovine aortic ECs through at least two distinct signaling pathways: PPARγ-dependent, VEGF-KDR/Flk-1-Akt-mediated eNOS Ser1177 phosphorylation and PPARγ-independent, eNOS Ser116 dephosphorylation, while dephosphorylation of eNOS Ser116 by protein phosphatase 2B increased eNOS activity (Kou et al., 2002). These findings provide further evidence that PPARγ agonists have the potential both to induce direct modifying of EC function and to modulate the production of NO, a critical mediator in the maintenance of normal vascular physiology.

**PPARγ agonists and oxidative stress in ECs**

To date, although the mechanisms responsible for mediating endothelial dysfunction in
cardiovascular diseases remain in completely defined, a considerable body of evidence suggests that the impairment of endothelium-dependent relaxation seen in many cardiovascular diseases involves not only decreased NO production, but also decreased NO bioavailability [e.g., inactivation of NO by superoxide (De Vriese et al., 2000; Jay et al., 2005; Kamata and Kobayashi, 1996; Pieper, 1998)]. This implicates oxidative stress as an important pathogenic element in the development of endothelial dysfunction (De Vriese et al., 2000; Jay et al., 2005; Matsumoto et al., 2004d, 2007c; Pieper, 1998).

Oxidative stress, defined as an increase in the steady-state levels of reactive oxygen species (ROS), may occur as a result of increased free-radical generation — an event attribute to a large extent to activation of NAD(P)H oxidase within the vascular system (Cai and Harrison, 2000; Jay et al., 2005; Sonta et al., 2004) — and/or to a decrease in antioxidant defense mechanisms, such as superoxide dismutase (SOD) (Kamata and Kobayashi, 1996; Sekiguchi et al., 2004). For instance, in diabetic animals endothelium-dependent relaxation may be impaired by an excess generation of superoxide, which destroys NO (De Vriese et al., 2000; Pieper, 1998). To judge from several reports that have appeared in recent years, PPARγ agonists may improve endothelium-dependent relaxation in animals and patients with any of a number of cardiovascular diseases via reduced oxidative stress (Bagi et al., 2004; Caballero et al., 2003; De Ciuceis et al., 2007; Howarth et al., 2006; Maegawa et al., 2007; Matsumoto et al., 2007d; Namikoshi et al., 2007; Ryan et al., 2004; Stakos et al., 2003). Further, it has been found that in ECs in culture, activation of PPARγ enhances the expression of Cu/Zn-SOD (Inoue et al., 2001), and also that PPARγ agonists stimulate the activity and protein expression of Cu/Zn-SOD in ECs (Hwang et al., 2005) (Fig. 1). Although the molecular basis for the PPARγ agonist-induced increases in SOD expression has not been defined, the identification of a functional PPRE in the Cu/Zn-SOD promoter suggests that Cu/Zn-SOD gene expression may be stimulated directly by PPARγ activation (Kim et al., 1996; Yoo et al., 1999). In addition, it has been suggested that rosiglitazone may reduce vascular oxidative stress by enhancing catalase expression and activity (Bagi et al., 2004). Indeed, a functional PPARγ response element has been identified in the rat catalase promoter of brain microvascular ECs (Girnum et al., 2002). All this indicates that PPARγ regulates the expression and/or activity of antioxidant components within ECs.

In addition, several reports have suggested that activation of PPARγ can suppress superoxide generation. For example, activation of PPARγ was found (a) to decrease both the mRNA for NAD(P)H oxidase subunit p22phox and the p47phox protein level in human aortic ECs (Inoue et al., 2001), (b) to reduce the protein expression of p22phox and Rac1 in the aorta (Nakamura et al., 2008; Namikoshi et al., 2007), and (c) to suppress Nox homolog expression in both vascular ECs in vitro and in the diabetic mouse aorta (Hwang et al., 2005, 2007). Moreover, rosiglitazone reportedly decreased NAD(P)H oxidase activity both in a rat model of hypertension (Iglarz et al., 2003a) and in a mouse model of diabetes (Bagi et al., 2004). Collectively, these results indicate that NAD(P)H oxidase constitutes an important target for PPARγ signaling within the vascular wall that is independent of the metabolic effects of PPARγ agonists. Furthermore, we recently demonstrated, in aortas from streptozotocin (STZ)-induced diabetic rats, that chronic rosiglitazone treatment improved ACh-induced endothelium-dependent relaxation, cGMP signaling (i.e., cGMP accumulation and increased phosphorylation
at Ser239 of the cGMP-dependent protein kinase substrate vasodilator-stimulated phosphoprotein), Cu/Zn-SOD expression, and SOD activity, while suppressing superoxide generation, nitrotyrosine expression, and NAD(P)H oxidase activity (Matsumoto et al., 2007d). The above findings suggest that PPARγ agonists normalize endothelium-dependent relaxation by increasing NO bioavailability via a reduction in the level of vascular ROS (Fig. 1).

**PPARγ agonists and the endothelin system**

Endothelin-1 (ET-1), a potent endothelium-derived vasoconstrictor peptide (Goto et al., 1996; Miyauchi and Masaki, 1999), is widely known to stimulate ET_A receptors in VSMCs (to produce vasoconstriction) and ET_B receptors on ECs (to produce vasodilation) (Goto et al., 1996; Miyauchi and Masaki, 1999). A perturbation of the balance between ET_A and ET_B-receptor activities may contribute to the pathogenesis of vascular disease (Brunner et al., 2006; Goto et al., 1996; Kanie and Kamata, 2002; Matsumoto et al., 2004c; Miyauchi and Masaki, 1999). Some, but not all, experimental models of hypertension, atherosclerosis, and diabetes display high levels of circulating ET-1, and exhibit endothelial dysfunction (Barton et al., 1998; Brunner et al., 2006; Kanie et al., 2003; Makino and Kamata, 1998). ET-1 has been linked to the production of superoxide within the vasculature in association with endothelial dysfunction (Elmarakby et al., 2005; Kamata et al., 2004; Lamplante et al., 2005; Li et al., 2003) (Fig. 1). Interestingly, PPARγ agonists are able to suppress ET-1 secretion from both ECs and VSMCs (Fukunaga et al., 2001; Lepailleur-Enouf et al., 2000; Martin-Nizard et al., 2002; Satoh et al., 1999). In addition, ET-1 production by ECs can be activated by many factors, such as insulin or thrombin, through c-jun fixation on the activator protein-1 (AP-1) site of the prepro-ET-1 promoter (Lee et al., 1991). Furthermore, Delerive et al. (1999) demonstrated in vitro that PPARγ binds c-jun, resulting in an inhibition of ET-1 production. Indeed, PPARγ agonists reduce the endogenous production of ET-1 and have beneficial effects in animals with endothelium-dependent hypertension, such as DOCA-salt rats (Iglarz et al., 2003b). Moreover, we recently studied the relationship among the ET-1 system, endothelium-dependent relaxation, and chronic pioglitazone treatment using aortas from STZ-induced diabetic rats (Matsumoto et al., 2007d). In such animals, we previously reported that: (1) the plasma ET-1 level is increased and that this increase may be due to an overexpression of the mRNA for prepro-ET-1 (Makino and Kamata, 1998; Makino et al., 2001), (2) the overproduction of ET-1 seen in STZ-induced diabetes results from the hyperglycemia, not from any increase in either LDL cholesterol or triglyceride (Makino and Kamata, 2000), (3) the expression of the mRNA for p22phox is increased in STZ-induced diabetes, an increase that is completely preventable by the chronic administration of J-104132 (a potent, orally active, mixed antagonist of ET_A and ET_B receptors) (Kanie and Kamata, 2002), and (4) short-term or prolonged treatment with ET-1 impairs endothelial function in the aorta both in nondiabetic rats (Kamata et al., 2004; Matsumoto et al., 2007d) and in rats with established STZ-induced diabetes (Kamata et al., 2004). Moreover, we have demonstrated that chronic administration of the PPARα agonist bezafibrate to established STZ-induced diabetic rats normalizes both the mRNA for prepro-ET-1 and the plasma concentration of ET-1, while improving endothelium-dependent relaxation (Kanie et al., 2003). In that paper, we also
demonstrated that the expressions of the mRNAs for PPARα and PPARγ were downregulated in aortic segments from STZ-induced diabetic rats (Kanie et al., 2003). In the recent paper referred to above, we found that the elevated plasma ET-1 level seen in such established STZ-induced diabetic rats can be largely suppressed by chronic treatment with pioglitazone, and that the increased expression of aortic c-Jun (an AP-1 component) seen in such rats is greatly reduced by the pioglitazone treatment (Matsumoto et al., 2007d). These results — which seem to be supported by previous findings showing that PPARs agonists regulate prepro-ET-1 gene expression and transcription through AP-1 and NF-κB (Delerive et al., 1999; Okita et al., 2002; Woods et al., 2003) — suggest that downregulation of PPARγ in the aorta may contribute, through the ET-1 system, to the endothelial dysfunction that is evident in diabetic mellitus. Thus, these results strongly suggest that ET-1 plays an important role in the endothelial dysfunction seen in diabetic states, and that PPARγ activation (by pioglitazone) can normalize this dysfunction by its restorative action on the abnormal ET-1 system (Fig. 1).

**Summary and perspectives**

Over the past several years, there has been a considerable improvement in our understanding of the effects of PPARγ agonists on endothelial function in the vasculature in health and cardiovascular diseases. However, significant work remains to be performed to define the underlying molecular mechanisms. Further information is required concerning the mechanisms leading to modulation not only of NO and ET-1, but also of other endothelium-derived factors such as EDHF and EDCF (e.g., COX-derived prostanoids) in pathophysiological states. The requisite studies may provide new insights into the potential of PPARγ agonists as therapeutic targets in the treatment of several vasculopathies.

In conclusion, we believe that manipulation of PPARγ signaling within the vascular system may have considerable potential as a new form of therapy for endothelial dysfunction. Undoubtedly, insights gained from basic research will lead to novel and pertinent clinical research targeted at the prevention and/or treatment a variety of cardiovascular diseases.

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