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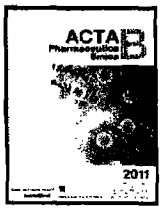

REVIEW

Challenges in drug discovery for thiazolidinedione substitute

Jian-ping Ye

Antioxidant and Gene Regulation Lab, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70808, USA

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KEY WORDS

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Abstract Thiazolidinedione (TZD) is a powerful insulin sensitizer in the treatment of type 2 diabetes. It acts as a ligand to the nuclear receptor PPARγ (peroxisome proliferator-activated receptor-gamma) and induces transcription of PPARγ-responsive genes. TZD controls lipid synthesis and storage in adipose tissue, liver and many other tissues through PPARγ. Derivatives of TZD, such as rosiglitazone (Avandia) and pioglitazone (Actos), are more powerful than metformin or berberine in insulin sensitization. Although they have common side effects such as weight gain and edema, these did not influence their clinical application in general. However, recent findings of risk for congestive heart failure and bladder cancer have significantly impaired their future in many countries. European countries have prohibited those drugs, and US will terminate application of rosiglitazone in clinics and hospitals. The multiple country actions may mark the end of TZD era. As a result, there is a strong demand for identification of TZD substitute in the treatment of type 2 diabetes. In this regard, literature about PPARγ ligands and potential TZD substitute are reviewed in this article. Histone deacetylase (HDAC) inhibitor is emphasized as a new class of insulin sensitizer here. Regulators of SIRT1, CREB, NO, p38, ERK and Cdk5 are discussed in the activation of PPARγ.

E-mail address: yej@pbrc.edu

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1. Introduction

Thiazolidinedione (TZD) is a powerful insulin sensitizer in the treatment of type 2 diabetes, a disease that is associated with obesity and aging through insulin resistance. With prevalence of obesity and increased lifespan in industrial countries, incidence of type 2 diabetes is increased quickly worldwide. In the treatment of type 2 diabetes, restoration of insulin sensitivity is a major strategy. Currently, there are several classes of drugs available in the treatment of insulin resistance. They are metformin, TZDs (Avandia and Actos), berberine, meglitinides, dipeptidyl peptidase-4 (DPP-4) inhibitors, and glucagon-like peptide-1 receptor agonists. Among those, TZD-based drugs are the most powerful medicines among these insulin sensitizers. There are two widely used commercial products derived from TZD, rosiglitazone (Avandia), and pioglitazone (Actos). The two medicines have common side effects such as weight gain and edema. However, rosiglitazone and muraglitazone have recently been reported to increase risk for congestive heart failure and risk for bone fractures[1]. The heart attack is lethal and not acceptable in the treatment of type 2 diabetes. As a result, the European Medicines Agency (EMA) recommended in September 2010 to suspend rosiglitazone (Avandia) from the European market. In US, rosiglitazone will be removed from the retail pharmacy stores by November 18th in 2011. In this case, pioglitazone will be the only TZD-based drug available in the US and European markets. However, pioglitazone has been reported to increase risk for bladder cancer in two recent reports[2,3]. In response, application of rosiglitazone has been suspended in the treatment of type 2 diabetes in some European counties (France and Italy) in June 2011. FDA in US has issued a warning to doctors and patients about the risk of bladder cancer from pioglitazone. Pioglitazone is the fourth drug in the thiazolidinedione class that has significant adverse clinical events: troglitazone (Rezulin) is the first with massive hepatic necrosis; muraglitazone (Pargluva, not marketed) and rosiglitazone (Avandia) are the second and third with increased cardiovascular events; and now pioglitazone is the fourth with bladder cancer. The risk of blander cancer may restrict pioglitazone application in the treatment of type 2 diabetes worldwide soon. It is now clear that a substitute for TZD is in demand for correction of insulin resistance in the treatment of type 2 diabetes. In this aspect, I like to provide this review to facilitate our search for TZD substitute.

2. PPARγ ligand

2.1. Synthetic ligand

TZD is a synthetic ligand of the nuclear receptor PPARγ (peroxisome proliferator-activated receptor gamma)[4] (Fig. 1). This class of insulin sensitizers include rosiglitazone (commercial name "Avandia" from GlaxoSmithKline, GSK), pioglitazone (Actos from Takeda Pharmaceuticals), muraglitazone, englitazone, ciglitazone and troglitazone[5]. Rosiglitazone binds to PPARγ with a high affinity (K_d of ~40 nmol/L), whereas pioglitazone, englitazone, and ciglitazone were less potent ligands of PPARγ. *In vivo*, activation of PPARγ by these compounds is required for the insulin-sensitizing actions of the TZD-based drugs. Gene knockout studies in mice suggest that the PPARγ activation in adipose tissue, skeletal muscle and macrophages are involved in the therapeutic activities of TZD for insulin sensitization. GW2570 is a very potent non-TZD PPARγ-selective agonist that was recently shown to have antidiabetic efficacy in humans[5].

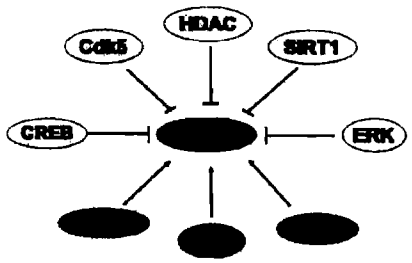

Figure 1 Regulation of PPARγ function in cells.

2.2. Fatty acid ligand

Fatty acid and their derivatives are PPARγ ligands (Fig. 1). The search for natural ligands for PPARγ begins with fatty acids and eicosanoids. Cell-based transactivation assays and direct binding studies are routinely used to characterize the endogenous activator of PPARγ. Fatty acids and eicosanoid derivatives activate PPARγ at micromolar concentrations. PPARγ prefers polyunsaturated fatty acids, including the fatty acids lauric, palmitic, oleic, and essential fatty acids linoleic acid, linolenic acid, arachidonic acid, 15d-PGJ2 (15-deoxy-D12,14-prostaglandin J2) and eicosapentaenoic acid. Those fatty acids activate PPARγ directly or act through a derivative such as nitrolinoleic acid[6]. Nitroalkene derivatives of linoleic acid (nitrolinoleic acid, LNO2) are formed via nitric oxide-dependent oxidative reactions and are found at concentrations of 500 nmol in the blood of healthy individuals. LNO2 is able to bind to PPARγ directly, and is more robust to activate PPARγ than other endogenous PPARγ ligands. LNO2 induces PPARγ-dependent macrophage CD-36 expression, adipocyte differentiation, and glucose uptake at a potency rivaling TZDs. These observations reveal that NO signal can be transduced by fatty acid nitration products and PPAR-dependent gene expression.

15d-PGJ2, a lipid metabolite, was the first endogenous ligand for PPARγ discovered in laboratory. Although 15d-PGJ2 is the most potent natural ligand of PPARγ *in vitro*, its effects remain to be determined *in vivo*. Two components of oxidized low density lipoprotein (ox-LDL), the 9-hydroxy and 13-hydroxy octadecadienoic acids (HODE), are also potent endogenous activators of PPARγ. Activation of 12/15-lipoxygenase induced by interleukin (IL)-4 also produced endogenous ligands, 15-hydroxyeicosatetraenoic acid (15-HETE) and 13-hydroxyoctadecadienoic acid (13-HETE) for PPARγ. However, it remains unknown if these natural ligands act as physiological PPARγ ligands *in vivo*.

2.3. NSAIDs and PPARγ

Non-steroidal anti-inflammatory drugs (*NSAID*) including indomethacin, ibuprofen, and fenoprofen have PPARγ

agonist activity at high drug concentrations[7]. Docosahexaenoic, a fish oil component, and 15d-PGJ2 are two natural PPARγ agonists[8]. These agents may inhibit inflammation through activation of PPARγ as their blood concentration is sufficient to inhibit production of inflammatory cytokines, such as TNF-α, IL-1 and IL-6[9]. These agents were tested in fresh human blood monocytes. They suppress expression of pro-inflammatory cytokines in macrophages. They inhibit PMA or okadaic acid-induced, but not LPS-induced production of inflammatory cytokines. For inhibition of okadaic acid-induced cytokine production, potency and IC_{50} of these NSAIDs are 15d-PGJ2 (2 μmol/L) > troglitazone (10 μmol/L) > indomethacin (47 μmol/L) > fenoprofen (133 μmol/L) > ibuprofen (142 μmol/L). These IC_{50} concentrations are in the range of plasma concentrations under high-dose NSAID therapy, such as indomethacin (10 μmol/L), ibuprofen (300 μmol/L) and fenoprofen (250 μmol/L)[9]. Since PPARγ may inhibit inflammatory cytokine in PPARγ dependent and independent manners, the information suggests that PPAR may mediate NSAID activity to decrease inflammatory response. Inhibition of PG production by suppressing COXII, a NF-κB target gene, will leads to pain relief. This hypothesis is supported by a recent study that NF-κB interacts with PPARγ through protein–protein interaction and leads to inhibition of PPARγ function[10]. These anti-inflammatory agents may activate PPARγ indirectly by suppression of co-repressor[11].

3. Potential TZD substitutes

3.1. *HDAC inhibitor*

PPARγ function is inhibited by nuclear co-repressor in the absence of ligand (Fig. 2). Upon ligand binding, the co-repressor complex is replaced by coactivators leading to activation of PPARγ. The corepressor contains catalytic subunit HDAC3 and regulatory subunit SMRT. In the study of inflammation in insulin resistance, we found that HDAC3 activity is enhanced in the nucleus by TNF-α for PPARγ inhibition[11]. To block the HDAC3 activity, we used a HDAC inhibitor, sodium butyrate, in a mouse study to prevent inflammation-induced PPAR inhibition. We found that the HDAC inhibitor protects the mice from diet-induced insulin resistance[12]. The mechanism is related to stimulation of energy expenditure by the HDAC inhibitor in mice. The same effects were observed for the classical HDAC inhibitor TSA in our study. The results suggest that HDAC inhibitor may be a new class of insulin sensitizer to substitute TZD (Fig. 1). Compared to TZD, HDAC inhibitor is not specific to a transcription factor. It is able to activate many transcription factors including PPARγ, PPARα, CREB and thyroid hormone receptor by inhibiting HDACs. This broad activity may be an advantage over a specific activator of PPARγ. For example, the side effect of rosiglitazone in heart is associated with its high affinity to PPARγ. Pioglitazone has less affinity to PPARγ compared to rosiglitazone. Pioglitazone has fewer side effects in heart. These results suggest that a drug with low specificity to PPARγ may be good alternative in the treatment of type 2 diabetes. An extension of this possibility is that a drug with multiple targets may be better than those with a single target. This possibility is supported by the HDAC inhibitor activity in the improvement of insulin sensitivity in mice[12].

3.2. *SIRT1 inhibitor*

SIRT1, a class III HDAC, was shown to inhibit PPARγ transcriptional activity at the target gene promoter[13]. Upon food withdrawal SIRT1 protein binds to the PPARγ-responsive genes, including those for fat synthesis, uptake and storage. SIRT1 represses PPARγ by docking with its cofactors NCoR (nuclear receptor co-repressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors). Mobilization of fatty acids from white adipocytes upon fasting is compromised in SIRT1+/− mice. Repression of PPARγ by SIRT1 is also evident in 3T3-L1 adipocytes, where overexpression of SIRT1 attenuates adipogenesis, and RNA interference of SIRT1 enhances it. In differentiated fat cells, up-regulation of SIRT1 triggers lipolysis for loss of fat content. According to this observation, inhibition of SIRT1 will enhanced PPARγ function, and improves insulin sensitivity thereafter. However, this rational is challenged by the beneficial activities of SIRT1 in others area, such as aging and liver steatosis[14]. Inhibition of SIRT1 activity may reduce lifespan and increase risk of fatty liver. It remains to be tested if SIRT1 inhibitor is a good candidate for a new class of insulin sensitizer (Fig. 1).

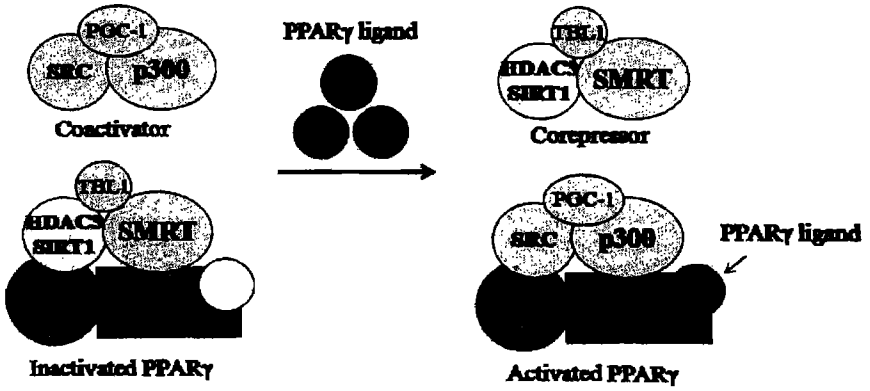

Figure 2 Mechanism of PPARγ activation.

3.3. *CREB inhibitor*

A reduction in PPARγ expression will decrease the transcriptional activity of PPARγ. PPARγ expression is inhibited by CREB (cAMP response element binding protein). Herzig et al. reported that CREB inhibited PPARγ expression as CREB knockdown *in vivo* leads to 4–5 fold induction of PPARγ mRNA[15]. Mechanism analysis suggests that CREB inhibits PPARγ through a transcriptional repressor, Hairy Enhancer of Split (HES-1). CREB induces HES-1 and HES-1 binds to the gene promoter of PPARγ leading to the inhibition. According to this observation, inhibition of CREB should increase PPARγ expression and PPARγ function thereafter. However, CREB regulates many other genes that are required for metabolic homeostasis. CREB controls mitochondrial biogenesis through induction of PGC-1α expression[16], and hepatic gluconeogenesis through PEPCK expression. Inhibition of CREB may have multiple effects in the body. It is not known if a CREB inhibitor can improve insulin sensitivity *in vivo* (Fig. 1).

3.4. *Activator of p38 kinase*

Regulation of PPARγ activity by MAP kinase p38 was first indicated in a study when p38 modulators were used in the study of adipogenesis[17]. Later studies confirm that p38 promotes PPARγ transcription activity by several groups using different systems[18–20]. Regarding the molecular mechanism of p38 activity, it was shown that p38 could phosphorylate PPARγ coactivator (PGC-1) leading to enhancement of the transcriptional activity of PPARγ on the UCP-1 gene promoter[18]. p38 activity is required for UCP-1 induction by rosiglitazone and retinoic acid in fetal brown adipocytes[21]. In addition to this possibility, other mechanisms have been proposed to explain p38 effect on the promotion of PPARγ activity, such as induction of PPARγ expression through NFATc4[19] and AFT2 interaction with PPARγ[20]. Bone morphogenetic protein 2 (BMP2) promotes the differentiation of undifferentiated mesenchymal cells into adipocytes. This activity of BMP2 is blocked by p38 kinase inhibitor. The mechanism is that the transcriptional activity of PPARγ induced by BMP is blocked by p38 inhibitor[20]. Activation of p38 kinase by overexpression of TAK1 and TAB1 did not affect PPARγ expression, but led to the up-regulation of transcriptional activity of PPARγ[20]. In discussion, Hata expressed that "the mechanism for the up-regulation of PPARγ by p38 kinase remains unknown". The direct up-regulation of PPARγ by p38 kinase through phosphorylation is unlikely because PPARγ possesses only one consensus phosphorylation site by MAP kinases at serine112, which is shown to be phosphorylated by ERK (extracellular signal-regulated kinase), that inhibits the transcriptional activity of PPARγ[22]. ATF-2 may mediate p38 activity as ATF-2 is regulated by p38 kinase. In indomethacin-treated cells, activation of PPARγ is associated with activation of p38[23], suggesting that p38 may mediate indomethacin signal for activation of PPARγ. Rosiglitazone (Rosi), as well as retinoic acids 9-*cis*-retinoic acid and all-*trans*-retinoic acid have "extragenic" effects on fetal primary brown adipocytes and induce p38 mitogen-activated protein kinase (p38 MAPK) activation[21]. Based on this activity of p38, it is expected that p38 activator

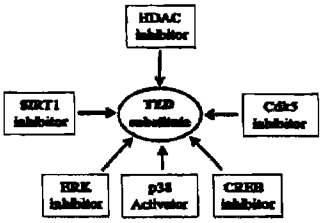

Figure 3 Potential TZD substitutes.

should enhance PPARγ function (Fig. 3). However, p38 activation may have side effects such as risk of atherosclerosis. Activation of the p38 MAP kinase pathway is required for foam cell formation from macrophages exposed to oxidized LDL[24]. Foam cell is the major cell typein the atherosclerosis lesion.

3.5. *ERK inhibitor*

Persistent activation of ERK was shown to inhibit PPARγ activity through a direct phosphorylation[22] (Fig. 1). Persistent increase in ERK activity leads to the inhibition of PPARγ function in the tyrosine kinases-1 (Dok1) KO mice[25]. However, it was shown that transient activation of MEK/ERK signaling promotes PPARγ activity during the differentiation of 3T3-L1 preadipocytes[26]. It is not clear if an ERK inhibitor will be able to enhance PPARγ function *in vivo*. If it does, it may be worth to test its activity in the regulation of insulin resistivity.

3.6. *NO inducer*

NO may enhance PPARγ function through generation of nitrolipid (Fig. 1). Nitrolinoleic acid (LNO2) is a product of linoleic acid after reaction with NO (Nitric Oxide). Nitrolinoleic acid was reported to be a ligand of PPARγ[6]. Additionally, NO may activate PPARγ through activation of cGMP. NO was reported to increase energy expenditure through a signaling pathway of cGMP-PPARγ-PGC-1-UCP1[27]. In the study, $eNOS^{-/-}$ mouse was used and NO effect was observed in cells including 3T3-L1 adipocytes. It was shown that NO can activate the cGMP signaling pathway in 3T3-L1 adipocyte, monocytes and other types of cells[27]. PPARγ activity is enhanced by NO donors *S*-nitrosoacetylpenicillamine (SNAP, 100 μmol/L), *S*-nitroso-*L*-glutathione (GSNO) and cGMP analog 8Br-cGMP (1 mmol/L). The activity is reduced by NO scavenger oxyhemoglobin (50 μmol/L), nitric oxide synthase (NOS) inhibitor L-NAME, and selective guanylate-cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μmol/L). These data suggest that NO inducer may activate PPARγ.

3.7. *Cdk5 inhibitor*

It has been reported recently that Cdk5 (cyclin-dependent kinase 5) inhibits PPARγ function in insulin sensitization (Fig. 1)[28]. Cdk5 is extensively studied in the neuronal cells. Cdk5 is involved in the regulation of various physiological processes of neuronal cells from survival, migration and differentiation, to synaptogenesis, synaptic plasticity and neurotransmission. Dysregulation of Cdk5 has been demonstrated

to play a critical role in the pathogenic process of neurodegenerative disorders, such as Alzheimer's disease. A new study reports that Cdk5 is activated in adipocytes in mice by high-fat diet[28]. In this study, Cdk5 is shown to phosphorylate the nuclear receptor PPARγ at serine 273 leading to dysregulation of a large number of genes in obesity including adiponectin. The phosphorylation of PPARγ by Cdk5 is blocked by rosiglitazone. This inhibition works both *in vivo* and *in vitro*, and is completely independent of gene transcription from PPARγ activation. The inhibition is tightly associated with the anti-diabetic effects of rosiglitazone. These findings suggest that Cdk5-mediated phosphorylation of PPARγ may be involved in the pathogenesis of insulin resistance, and present an opportunity for development of new class of anti-diabetic drugs from Cdk inhibitors. These possibilities remain to be tested *in vivo*.

3.8. PPARγ is required for adipose tissue growth

PPARγ is most abundant in fat tissues, and dysfunction of PPARγ contributes to insulin resistance. The nuclear receptor PPARγ is a member of peroxisome proliferator-activated receptor (PPAR) family that includes PPARα, PPARγ, and PPARδ (PPARβ). There are two isoforms in PPARγ, PPARγ1 and PPARγ2. PPARγ1 is expressed ubiquitously and PPARγ2 is mainly expressed in adipocytes. The biological activities of PPARγ are very broad, but it is generally accepted for a master transcriptional regulator of lipid and glucose metabolism. Activation of PPARγ promotes adipocyte differentiation and triglyceride accumulation in adipocytes. Gene knockout studies consistently suggest that PPARγ is required for adipose tissue development and the tissue growth in response to positive energy balance. Four groups independently made fat-specific knockout (KO) mice and published the metabolic phenotypes of the mice all in PNAS[29–32]. The studies suggest that PPARγ KO in fat prevents obesity and insulin resistance in the mice fed a high-fat diet. In the last report, the mice have an increase in food intake, spontaneous physical activity and energy expenditure[32]. The increased energy expenditure may provide protection against obesity and insulin resistance in the mice. The energy expenditure is likely a result of increased supply of fatty acids to mitochondria since the adipose tissues do not have sufficient room to store the fatty acids. The study suggests that restriction of PPARγ function before obesity may promote energy expenditure and prevent development of dietary obesity leading to preservation of insulin sensitivity. However, activation of PPARγ induces generation of new adipocyte through preadipocyte differentiation to increase storage capacity of adipose tissue. This action in adipose tissue allows PPARγ agonist to improve insulin sensitivity by reducing blood lipids and preventing ectopic fat deposition, which induces lipotoxicity in liver and skeletal muscle in the pathogenesis of insulin resistance. Activation of PPARγ in adipose tissue is the molecular mechanism by which PPARγ ligand improve insulin sensitivity in patients.

4. Summary

Current literature suggests that TZD-based drugs are facing more and more challenges from the severe side effects in the cardiovascular system and bladder cancer. There have been four TZD-based drugs and all of them have life-threatening side effects. Although the side effects are different among the four drugs, the history of TZD-based drugs suggests that this class of insulin sensitizer will reach their end sooner or later. With prevalence of type 2 diabetes and limited number of insulin sensitizers, there is a huge demand for a new class of drug that is better than TZDs in safety. In this review, several approaches are discussed in search for the new class of drugs. These include HDAC inhibitor, SIRT1 inhibitor, Cdk5 inhibitor, ERK inhibitor, CREB inhibitor and p38 activator (Fig. 3). It appears that a HDAC inhibitor holds a strong promise. It improves insulin sensitivity, stimulates energy expenditure and suppresses cancer. This example suggests that a drug for multiple targets may be better than those for a single target in the treatment of insulin resistance and type 2 diabetes.

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