DISCOVERY OF BOTANICAL FLAVONOIDS AS DUAL PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR (PPAR) LIGANDS AND FUNCTIONAL CHARACTERIZATION OF A NATURAL PPARα POLYMORPHISM THAT ENHANCES INTERACTION WITH NUCLEAR COREPRESSOR

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TABLE OF CONTENTS

CHAPTER 4 DISCUSSION

SUMMARY

Peroxisome Proliferator Activated Receptors (PPAR), part of the 48 member nuclear/steroid receptor superfamily of transcription factors, have critical roles in lipid and carbohydrate metabolism. While PPARγ regulates glucose levels and adipogenesis, PPARα is highly expressed in tissues involved in fatty acid metabolism where it regulates several key proteins in fatty acid oxidation and ketogenesis. Compounds that target PPARα and PPARγ are used extensively in the clinical setting to correct dyslipidemia and to restore glycemic balance in diabetes and atherosclerosis. However many of the drugs in current use have significant adverse effects. Therefore, there is a need for the discovery of more PPAR-active compounds with beneficial efficacy/risk profiles.

Recently, natural variants of PPAR have been shown to be functionally significant and are important determinants of cardiovascular and metabolic health. In particular, a non-synonymous variant at the *PPARA* locus encoding a substitution of valine for alanine at residue 227 (V227A) in the hinge region of the PPARα has been observed in Singapore and other East-Asian populations with relatively high allelic frequencies. This variant was associated with perturbations in plasma lipid levels and modulated the association between dietary polyunsaturated fatty acids and high density lipoprotein cholesterol. The impact of this variant on the function of PPARα is unknown.

To address the above issues, the objectives of this study were:

1) To identify, isolate and structurally characterize PPAR active compounds from an anti-diabetic botanical, *Pueraria Thomsonii* (PT), and to characterize their functional effects in relevant cell models.

2) To examine the effects of the V227A variant on PPARα function and to elucidate the molecular mechanisms for any observed effects.

Firstly, we demonstrated that extracts of PT can activate PPARα and PPARγ. Repeated bioassay guided fractionation resulted in the identification and isolation of the isoflavones, daidzin, daidzein, genistin, puerarin and 2'hydroxydaidzein, as bioactive compounds of PT. We characterized the effects of daidzein from PT and other isoflavones, calycosin, formononetin, genistein and biochanin A, using chimeric and full-length PPAR constructs *in vitro*. Biochanin A and formononetin were potent activators of both PPAR receptors (EC50=1-4 μM) with PPARα/PPARγ activity ratios of 1:3 in the chimeric and almost 1:1 in the full length assay, comparable to that observed for synthetic dual PPAR-activating compounds under pharmaceutical development. There was a subtle hierarchy of PPARα/γ activities with biochanin A, formononetin and genistein being more potent than calycosin and daidzein in chimeric as well as full length receptor assays. At low doses only biochanin A and formononetin, but not genistein, calycosin or daidzein, activated PPARγ-driven reporter gene activity and induced differentiation of 3T3-L1 preadipocytes. Our data suggest the potential value of isoflavones, especially biochanin A, and their parent botanicals as anti-diabetic agents and for use in regulating lipid metabolism.

Secondly, the functional significance of the V227A substitution was explored. The polymorphism significantly attenuated PPARα mediated transactivation of the CYP4A6 and mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2) genes, with polyunsaturated fatty acids and the fibrate, WY14,643, in a dominant-negative manner. Screening of a panel of PPARα coregulators revealed that V227A

enhanced recruitment of the nuclear corepressor, NCoR. Weaker transactivation activity of V227A can be restored by silencing NCoR, or by inhibition of its histone deacetylase activity. Deletion studies indicate that PPARα interacts with NCoR receptor-interacting domain 1 (ID1), but not ID2 or ID3. These interactions were dependent on the intact consensus nonapeptide nuclear receptor interaction motif in NCoR ID1, and were enhanced by the adjacent 24 N-terminal residues. Novel corepressor interaction determinants involving PPARα helices 1 and 2 were identified. The V227A substitution stabilized PPARα/NCoR interactions in the unliganded state, and caused defective corepressor/coactivator exchange in the presence of ligands, on the HMGCS2 promoter in hepatic cells. These results provide the first indication that defective function of a natural PPARα variant was due to increased corepressor binding.

In all, our data suggest that the PPARα/NCoR interaction is physiologically relevant, and can produce a discernable phenotype when the magnitude of the interaction is altered by a naturally occurring variation. Our detailed mechanistic study of the PPARα V227A variant allows for the design of future human studies to identify other benefits and risks associated with this mutation. Furthermore, the identification and characterization of isoflavones, and their parent botanicals, with different PPARα/γ potencies suggest their value in the management of the epidemic of diabetes, dyslipidemia and the metabolic syndrome.

LIST OF TABLES

LIST OF FIGURES

LIST OF PUBLICATIONS

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ABBREVIATIONS

15dPGJ2	15-deoxy-$\Delta^{12,14}$-Prostaglandin J2
$Å^3$	cubic angstrom
ABCA1	adenosine triphosphate-binding cassette transporter A1
ACO	acyl-CoA oxidase
Acrp30	adipocyte complement-related protein of 30 kDa
AD	activation domain
AF-1	activation function-1, ligand independent
AF-2	activation function-2, ligand dependent
AM	*Astragalus membranaceus*
aP2	adipocyte fatty acid binding protein
apoA-I	Apolipoprotein A-I
apoA-II	Apolipoprotein A-II
apoA-V	apolipoprotein A-V
apoC-III	apolipoprotein C-III
AR	androgen receptor
bp	base pair
Bio	biochanin A
Cal	calycosin
CAP350	centrosome-associated protein 350
CARM-1	coactivator-associated arginine methyltransferase 1
CBP	CREB-binding protein
cDNA	complementary DNA
CD36	fatty acid translocase
C/EBP	CCAAT/enhancer binding protein
ChIP	chromatin immunoprecipitation
CITED-2	CBP/p300 interacting transactivator with ED-rich tail 2
CPT1A	carnitine palmitoyl transferase 1A
Co-IP	co-immunoprecipitation
COOH	carboxyl group
CoRNR	core consensus nonapeptide motif (LXXI/HIXXXI/L)
COUP-TFII	COUP transcription factor II
CVD	cardiovascular heart disease
CYP4A	cytochrome P450 4A
Dai	daidzein
DBD	DNA-binding domain
DHT	dihydrotestosterone
DM	diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DR	direct repeat eg. DR1 is direct repeat 1
DRIP	vitamin D3 receptor interacting protein
EC50	50% effective concentration
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor

FA	fatty acid
FATP-1	fatty acid transport protein
FDA	U.S. Food and Drug Administration
FAO	fatty acid oxidation
For	formononetin
Gen	genistein
GPS2	G protein pathway suppressor 2
GR	glucocorticoid receptor
GST	glutathione-S-transferase
GyK	glycerol kinase
h	hour
H	helix
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDL	high density lipoprotein
HDL-c	high density lipoprotein-cholesterol
HETE	hydroxyeicosatetraenoic acid
HMGCS1	cytosol HMG-CoA synthase
HMGCS2	mitochondria HMG-CoA synthase
HMT	histone methyltransferase
HNF4	hepatocyte nuclear factor 4
HODE	hydroxyoctadecadienoic acid
HPLC	high performance liquid chromatography
HRE	hormone response element
hsp90	heat shock protein 90
IC50	50% inhibitory concentration
ID	interaction domain
IF	immunoflourescence
IP	immunoprecipitation
IR	inverted repeat
kDa	kilodalton
LBD	ligand-binding domain
LC-MS	liquid chromatography-mass spectrometry
LCoR	ligand-dependent corepressor
LDL	low density lipoprotein
LDL-c	low density lipoprotein cholesterol
L-FABP	liver fatty acid binding protein
LPL	lipoprotein lipase
Luc	luciferase
MAPK	mitogen-activated protein kinase
Med	mediator
mg	milligram
min	minute
mM	millimolar
ml	millilitre
μg	microgram

μM	micromolar
μl	microlitre
MODY	maturity-onset diabetes of the young
mP	polarization value
MPLC	medium pressure liquid chromatography
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NCoR	nuclear receptor corepressor
ng	nanogram
nM	nanomolar
NMR	nuclear magnetic resonance
NR	nuclear receptor
NS	not studied
NSA	no significant activity
OLR1	oxidized LDL receptor 1
PBP	PPARγ binding protein
PCR	polymerase chain reaction
PDK1	pyruvate dehydrogenase kinase isoform 1
PDK4	pyruvate dehydrogenase kinase isoform 4
PEPCK	phosphoenol pyruvate carboxykinase
PG	prostaglandins
PGC-1α	PPARγ coactivator-1α
PIMT	PRIP-interacting protein with methyltransferase domain
PKA	protein kinase A
PKC	protein kinase C
PLTP	phospholipid transfer protein
Pol II	RNA polymerase II
Pos	positive control
PPARA	locus encoding for PPARα
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response element
PR	progesterone receptor
PRIC	PPARα interacting complex
PRIC285	PPARα interacting cofactor complex 285
PRIP	PPARγ interacting protein
PRMT-1	protein arginine methyltransferase 1
PRMT-2	protein arginine methyltransferase 2
PT	*Pueraria thomsonii*
PUFA	polyunsaturated fatty acid
RAR	retinoid acid receptor
RCT	reverse cholesterol transport
RD	repression domain
RIP140	receptor interacting protein 140
RNA	ribonucleic acid
ROR	retinoid- related orphan receptor

rpL11	ribosomal protein regulator of p53
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
RXR	retinoid X receptor
SEM	standard error of mean
sh	short hairpin
siNCoR	short hairpin sequences complementary to NCoR
siScram	short hairpin sequences complementary to a randomized sequence
SMRT	silencing mediator of retinoid and thyroid receptors
SNP	single nucleotide polymorphism
SPPARM	selective PPAR modulating
SR-BI	scavenger receptor BI
SRC-1	steroid receptor coactivator-1
SUMO	small ubiquitin-like modifiers
SWI/SNF	switch/sucrose non-fermenting complex
T3	triiodothyronine
TAD	transactivation domain
TBL-1	transducin-like protein 1
TCM	Traditional Chinese Medicine
TG	triglyceride
TIF2	transcriptional intermediate factor 2
TK	thymidine kinase
TNFα	tumour necrosis factor α
TR	thyroid receptor
T3Rβ	thyroid receptor β
TRAP	thyroid hormone receptor associated proteins
TRB3	mammalian homolog of Drosophila tribbles
TSA	trichostatin A
TZD	thiazolidinedione
UASG	upstream activating sequence of Gal4
VDR	vitamin D receptor
VLDL	very-low-density-lipoprotein
WHO	World Health Organization
WT	wild type
XAP2	hsp90 accessory protein

CHAPTER 1: INTRODUCTION

1.1 The Peroxisome Proliferator Activated Receptor

1.1.1 The Nuclear Receptor Superfamily

Nuclear receptors (NR) are members of a large superfamily of evolutionarily related DNA-binding transcription factors which have diverse, crucial roles in the regulation of growth, development and homeostasis (Chambon 2005; Evans 2005; Germain et al. 2006). Since the isolation of cDNA encoding the Glucocorticoid Receptor (GR) (Hollenberg et al. 1985) and the Estrogen Receptor α (ERα) (Green et al. 1986; Greene et al. 1986), the sequencing of the human genome has so far led to the identification of 48 nuclear receptors (Perissi and Rosenfeld 2005).

Members of this superfamily can be broadly divided into subgroups on the basis of their pattern of dimerization (Germain et al. 2006) (Table 1.1). One group consists of the steroid receptors, including the Androgen Receptor (AR), the Mineralocorticoid Receptor (MR), the GR, the ERα and ERβ (Table 1.1). These steroid receptors function as homodimers and bind to a degenerate set of hexameric half-sites separated by 3 base pairs of spacer (IR3) on the DNA (Glass 1994). These specific DNA sequences are called hormone response elements (HRE) (Kumar et al. 1986). Except ER, the steroid receptors recognize the consensus DNA half-site sequence 5'-AGAACA-3'. ER binds in similar symmetric sites but with the consensus half-site sequence of 5'-AGGTCA-3'.

Nearly all non-steroidal receptors recognize one or two copies of the consensus DNA half-site sequence 5'-AGGTCA-3' that can be configured into a variety of structured motifs (Mangelsdorf and Evans 1995; Germain et al. 2006). Among these receptors, a major group consists of receptors that form heterodimers with the Retinoid X

Receptor (RXR) (Table 1.1). The various RXR heterodimers can bind to direct repeats (DRs) with one to five base pairs of spacing, referred to as DR1 to DR5. The Peroxisome Proliferator Activated Receptor (PPAR), the Retinoid Acid Receptor (RAR), the Vitamin D Receptor (VDR) and the Thyroid Receptor (TR) heterodimerize with RXR on DR1 to DR4 respectively. RAR can also heterodimerize with RXR on DR5 (Mangelsdorf and Evans 1995). In addition, some NRs such as Rev-erb and Retinoid- related Orphan Receptor (ROR) binds DNA efficiently as monomers (Giguere et al. 1995; Harding and Lazar 1995).

Table 1.1 DNA binding properties of homodimers and RXR heterodimers of nuclear receptors

Dimerization	Receptor	Response element	Consensus sequence
Homodimer	AR PR GR MR	IR-3	AGAACA-NNN-TGTTCT
	ER	IR-3	AGGTCA-NNN-TGACCT
RXR Heterodimer	PPAR	DR-1	AGGTCA-N-AGGTCA
	RAR	DR-2	AGGTCA-NN-AGGTCA
	VDR	DR-3	AGGTCA-NNN-AGGTCA
	TR	DR-4	AGGTCA-NNNN-AGGTCA
	RAR	DR-5	AGGTCA-NNNNN-AGGTCA

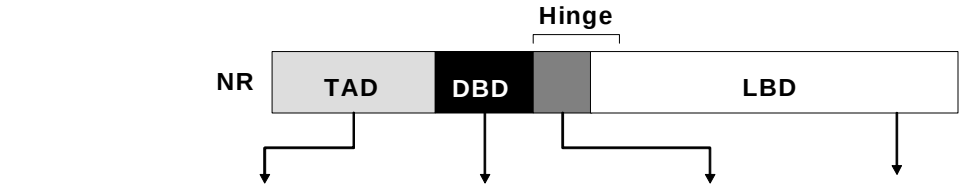
Members of the NR superfamily share a common structural organization that is well-defined and has specific functions (Fig 1.1). The N-terminal transactivation domain (TAD) contains at least one ligand-independent activation function (AF-1) and is the least conserved among NR both in terms of length and sequence (Robinson-Rechavi et al. 2003). So far, no crystal structure of the TAD has been elucidated (Germain et al. 2006).

The most conserved region is the central DNA Binding Domain (DBD). The DBD allows the binding of ligand activated NR on HREs to elicit a biological response. Within the DBD, several sequence elements have been shown to determine HRE specificity, dimerization and facilitate contacts with the DNA backbone (Umesono and Evans 1989). The crystal structure of the GR homodimer on its cognate DNA (Luisi et al. 1991) and subsequent studies revealed that this DBD consists of a highly conserved 66 residues core made up of two typical cysteine-rich zinc finger motifs, two α helices and a COOH terminal (Gronemeyer et al. 2004).

Between the DBD and the Ligand Binding Domain (LBD) is a less conserved region that behaves as a flexible Hinge between the two domains and may overlap onto the LBD (Robinson-Rechavi et al. 2003). Being the least studied, this region is thought to allow rotation of the DBD and permits the DBD and the LBD to adopt different conformations without creating steric hindrance (Germain et al. 2006).

The largest domain is the moderately conserved LBD whose secondary structure of 12 α-helices is better conserved than the primary sequence (Robinson-Rechavi et al. 2003). The LBD contains four structurally distinct but functionally linked surfaces (Germain et al. 2006): 1) a dimerization surface, which mediates interaction with partner LBDs, 2) a ligand binding pocket, which interacts with diverse lipophilic small molecules,

Figure 1.1 Schematic illustration of the general structural and functional organization of a nuclear receptor (NR)



General function	Ligand independent AF-1	- Specific DNA binding to HRE - Dimerization	- Flexibility prevents steric hindrance between DBD and LBD	-Dimerization -Ligand binding -Coregulator recruitment -Ligand dependent AF-2
Homology between NR	Least conserved in length & sequence	Most conserved	Less conserved than DBD	Secondary structure is more conserved than primary
Crystal	No crystal structure elucidated	2 cysteine rich zinc fingers + 2 α-helices + a carboxyl terminal	Hinge may overlap into the N-terminal of LBD	Anti-parallel 'sandwich' of 12 α-helices organized in 3 layers with a central hydrophobic ligand binding pocket

3) a coregulator binding surface which binds regulatory protein complexes that modulate positively (by coactivators) or negatively (by corepressors) transcriptional activity, and 4) an activation function helix (AF-2) which mediates ligand dependent transactivation. NR crystal structures resolved show that the LBDs of these receptors form an anti-parallel α-helical sandwich of 12 helices organized in three layers with a central hydrophobic ligand binding pocket (Nolte et al. 1998). Ligand binding to this pocket induces a structural change which favours the closure of the ligand binding pocket by helix 12 like a lid in a 'mouse trap' model of ligand dependent activation. (Moras and Gronemeyer 1998). Despite the conserved fold of LBDs, the shape and size of the ligand binding pocket can vary greatly from receptor to receptor. This allow for selectivity of specific ligands for each receptor in the NR Superfamily (Germain et al. 2006).

Collectively, these properties allows transactivation by members of the NR superfamily, especially homodimers, to occur in 5 major steps (Lefebvre et al. 2006): 1) ligand binding; 2) stable binding of liganded NR to HRE; 3) corepressors dismissal and

coactivators recruitment; 4) activation of transcription; and 5) either shut down or re-initiation of transcription.

1.1.2 The Peroxisome Proliferator Activated Receptor

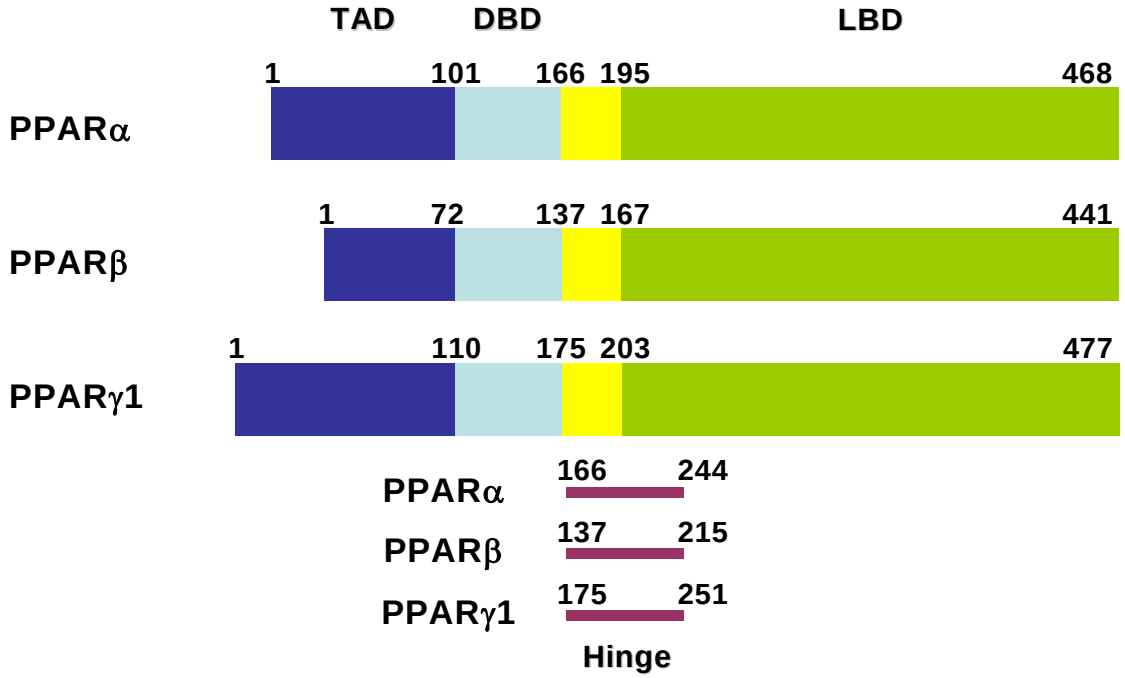
Peroxisome Proliferator Activated Receptors (PPARs) are members of the NR Superfamily. PPARs are transcriptional regulators involved in the regulation of key metabolic pathways in lipid metabolism, adipogenesis, and insulin sensitivity (Brown and Plutzky 2007).

PPARα was first described as a receptor that is activated by peroxisomes proliferators in rodent hepatocytes (Issemann and Green 1990). Two additional related isotypes, PPARβ (also known as PPARδ) and PPARγ, have since been identified and characterized (Dreyer et al. 1992) (Fig 1.2). Due to different promoter usage within the same gene and subsequent alternative RNA splicing, there are two isoforms of PPARγ (Elbrecht et al. 1996). PPARγ2 has 28 amino acids more than the PPARγ1 isoform at the N-terminal domain.

PPARs exhibit a broad but isotype specific tissue expression pattern that can account for the variety of cellular functions they regulate (Kliewer et al. 1994; Braissant et al. 1996). PPARα is expressed in tissues with high fatty acid catabolism such as the liver, the heart, the brown adipose tissue, the kidney, and the intestine (Mandard et al. 2004). Of the three isotypes, PPARβ exhibits the broadest expression pattern (Feige et al. 2006). Nonetheless, higher levels of PPARβ were noted in the brain, adipose and skin (Amri et al. 1995; Braissant et al. 1996). PPARγ2 is expressed primarily in adipose tissues while PPARγ1 is expressed in a broad range of tissue such as the gut, brain,

Figure 1.2 General structure of human PPAR

Illustration of the four domain structures of PPAR with coordinates of each domain boundary given according to Desvergne and Wahli (1999). Purple bar represents the hinge which starts from the termination of DBD and extends into helix 2 of the LBD.



vascular cells, and specific kinds of immune and inflammatory cells (Dreyer et al. 1992; Chawla et al. 1994; Tontonoz et al. 1994a; Tontonoz et al. 1994b; Zhu et al. 1995).

Consistent with their distribution in tissues, PPARα is important in the transcription modulation of liver and skeletal muscle lipid metabolism (Lefebvre et al. 2006). PPARβ, the least described PPAR isotype, enhances fatty acid catabolism and energy uncoupling in adipose tissue and muscle, and suppresses macrophage-derived inflammation (Barish et al. 2006). PPARγ is pivotal in adipose tissue differentiation, adipocyte specific functions and glucose metabolism (Michalik et al. 2006).

PPARs function as a ligand activated transcription factor which control gene expression by binding to specific DNA sequence, called PPAR response elements (PPRE), as heterodimers with RXR in a ligand dependent manner. The first natural PPRE

identified was in the promoter of the acyl-CoA oxidase gene (Tugwood et al. 1992) which contains a direct repeat of two core recognition motifs AGGTCA spaced by one nucleotide (DR1). Since then, sequence comparison of other natural PPREs has broadened the definition of a PPRE to include the following properties: an extended 5' half site, an imperfect core DR1 and an adenine as the spacing nucleotide (Palmer et al. 1995; IJpenberg et al. 1997; Juge-Aubry et al. 1997; Osada et al. 1997).

Like members of the NR Superfamily, the first step of transactivation by PPARs involves ligand binding. PPARs are activated by a wide range of naturally occurring or metabolized lipids that are derived from the diet or from intracellular signaling pathways (Feige et al. 2006). These include saturated and unsaturated fatty acids and fatty acid derivatives such as prostaglandins and leukotrienes (Forman et al. 1995; Forman et al. 1997; Kliewer et al. 1997; Krey et al. 1997). Synthetic ligands such as fibrates, the plasma lipid-lowering drug used in the treatment of hyperlipidaemia, activate PPARα (Forman et al. 1997) while thiazolidinediones, the insulin sensitizing drug for the treatment of type 2 diabetes, activate PPARγ (Lehmann et al. 1995).

Crystal structure analyses of the PPAR LBD have revealed a three dimensional fold that is similar to other NRs (Nolte et al. 1998; Uppenberg et al. 1998; Xu et al. 1999; Xu et al. 2001). The PPAR LBD consists of 12 α-helices that form the characteristic three layer anti-parallel α-helical sandwich with a small four stranded β-sheet. However, some distinct differences are apparent (Nolte et al. 1998; Uppenberg et al. 1998). The core AF2 in the apo-PPAR (unliganded) is folded against the ligand binding pocket in a conformation similar to that in the holoforms (liganded) of PPAR and other nuclear receptors. Unlike other NRs, PPAR contains an additional helix 2' which is found

between the first β strand and helix 3 (Gampe et al. 2000). This, together with a placement of helix 2 that differs from other NR tertiary structure, provides easy access to the hydrophobic pocket for ligands. The region between helix 2' and helix 3 that corresponds to the Ω loop in RAR is extended, most thermally mobile and participates in the structural changes occurring upon ligand binding. Together, these structures define a large Y-shape hydrophobic ligand binding pocket which is larger in PPAR than in other receptors. While the ligand binding pocket is particularly large (~1300Å^3), ligands only occupy 30-40% of this space. It is thus larger and more accessible than other known LBDs such as the TR which ~600Å^3 cavity is largely occupied by its ligand, T3 of ~530Å^3 (Wagner et al. 1995). Collectively, these differences may contribute to the ability of PPAR to bind a wide range of synthetic and natural ligands at micromolar concentrations (Michalik et al. 2006).

In the next two sections, focus will be on the detailed physiological roles of PPARα and PPARγ; and the synthetic and natural ligands which control them. This will be followed by a section which concentrates on the molecular mechanisms of transcription regulation in PPAR. Discussion in that section will be largely centered on PPARα although reference to PPARγ and PPARβ will be made where relevant. After which, a summary of PPARα polymorphisms will follow. Finally, this chapter will end with an outline on flavonoids and their botanical sources.

1.2 Physiological Aspects of PPAR

1.2.1 PPARα

PPARα controls intracellular lipid metabolism, lipoprotein metabolism and glucose homeostasis through direct transcriptional control of genes involved in fatty acid oxidation pathways (FAO) and fatty acid (FA) uptake; lipoprotein assembly and transport; and glucose homeostasis (Lefebvre et al. 2006).

1.2.1.1 Lipid metabolism

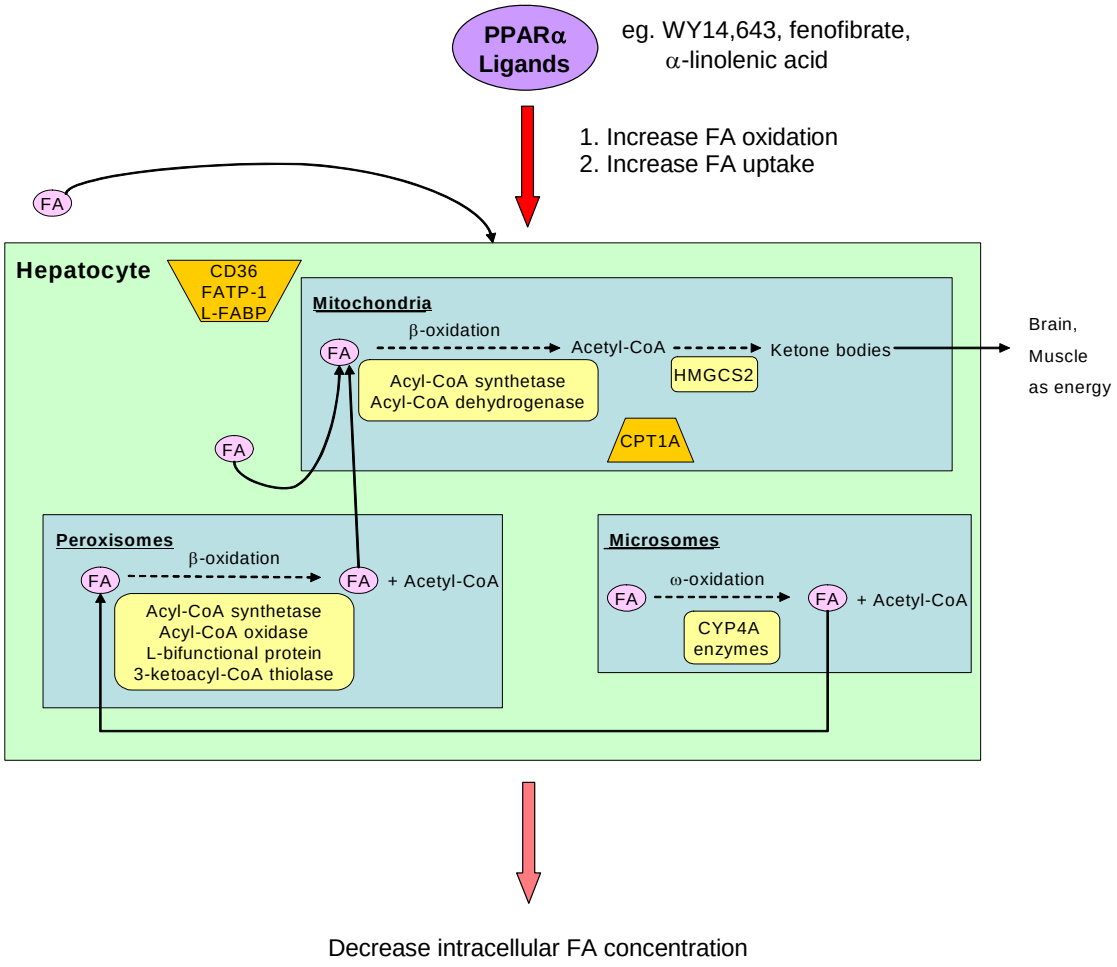
PPARα acts in the liver to reduce FA concentration through the control of key enzymes in FAO and FA uptake. Major enzymes of FA β-oxidation (peroxisomal and mitochondrial) and FA ω-oxidation (microsomal), together with proteins involved in the transport of FA, are increased in response to PPARα (Fig 1.3).

In the peroxisomes, the β-oxidation pathway breaks down very-long-chain FA (of carbon atoms more than 20, >C20), as well as of other lipid derivatives such as eicosanoids or branched FAs, for further β-oxidation in the mitochondria. Major enzymes of the peroxisomal β-oxidation pathway, acyl-CoA synthetase (very-long and long chain FA) (Schoonjans et al. 1995), acyl-CoA oxidase (ACO) (short chained and branched FA) (Dreyer et al. 1992; Tugwood et al. 1992), L-bifunctional protein (Marcus et al. 1993) and 3-ketoacyl-CoA thiolase (Zhang et al. 1993) are regulated by PPARα.

In the mitochondria, the β-oxidation pathways breaks down short-chain (<C8), medium-chain (C8-C12), and long-chain (C13-20) FA for energy in cellular processes through progressive shortening of FA into acetyl-CoA subunits. The acetyl-CoA subunits

Figure 1.3 PPARα in lipid metabolism

Role of PPARα in lipid metabolism. Upon ligand activation, PPARα upregulates key enzymes (group of enzymes depicted by yellow squares) involved in the fatty acid (FA) β-oxidation (peroxisomal and mitochondrial) and ω-oxidation (microsomal) pathways. During fasting, final products of FA, the acetyl-CoA subunits, are converted to ketones by PPARα controlled HMGCS2 and other enzymes. PPARα also upregulates proteins of FA uptake (group of enzymes depicted by orange trapezium). The collective effect of increased FA oxidation and uptake by PPARα regulated genes lead to a decrease in intracellular FA concentration. Solid lines represent transport, broken lines represent enzymatic conversion which usually involves several steps.



may be condensed into ketone bodies that serve as oxidizable energy substrates for extrahepatic tissues especially during starvation. Carnitine palmitoyl transferase 1A (CPT1A), the rate limiting enzyme that controls FA import into the mitochondria is regulated by PPARα in liver (Mascaro et al. 1998). Major enzymes of the mitochondria β-oxidation pathway, acyl-CoA synthetase (long chain FA) (Schoonjans et al. 1995) and very-long and medium-chain acyl-CoA dehydrogenase (Gulick et al. 1994; Aoyama et al. 1998), are PPARα regulated. During fasting or diabetes, the breakdown products of FA in the mitochondria, acetyl-CoA, are converted into ketone bodies. PPARα controls the expression of mitochondrial HMG-CoA synthase (HMGCS2), the key step in ketone body generation (Rodriguez et al. 1994).

The CYP4A subclass of cytochrome P450 enzymes catalyze the ω-oxidation of FA in microsomes, a pathway that is particularly active in the fasted and diabetic states (Berger and Moller 2002), through hydroxylation of long chain saturated and unsaturated FAs for further β-oxidation in the peroxisome. Fibrates have been shown to activate expression of CYP4As and functional PPREs have been found in the promoters of CYP4A genes. (Aldridge et al. 1995; Kroetz et al. 1998)

In FA transport, fatty acid translocase, (CD36), a glycoprotein that controls FA uptake in multiple cell types, is regulated by PPARα in the liver (Motojima et al. 1998). Similarly, the expression of the fatty acid transport protein (FATP-1) (Martin et al. 1997) and liver fatty acid binding protein (L-FABP) (Poirier et al. 2001), important proteins in the transport of FA across cell membrane, are upregulated by PPARα activation in hepatocytes.

Collectively, PPARα acts in the liver to reduce intracellular FA concentrations and likely contributing to a decrease in very-low-density-lipoprotein (VLDL) particle production and plasma triglyceride (TG) levels (Lefebvre et al. 2006).

1.2.1.2 Lipoprotein metabolism

In lipoprotein metabolism, VLDL and TG levels are reduced while high density lipoprotein (HDL) is increased upon PPARα activation by the lipid-lowering drug ligand of PPARα, fibrates.

In TG breakdown, PPARα activation upregulates the expression of lipoprotein lipase (LPL) (Schoonjans et al. 1996a). LPL is a triacylglycerol hydrolase which hydrolyze TG from lipoprotein particles to FA. PPARα also controls TG degradation through the regulation of various apolipoprotein levels (Lefebvre et al. 2006). PPARα downregulates apolipoprotein C-III (apoC-III) in hepatocytes (Staels et al. 1995; Peters et al. 1997). apoC-III inhibits LPL activity and VLDL lipolysis. On the other hand, apolipoprotein A-V (apoA-V), a potent activator of lipolysis, is upregulated by PPARα (Vu-Dac et al. 2003).

Apolipoprotein A-I (apoA-I) and A-II (apoA-II) are major components of HDL. HDL is protective against atherosclerotic vascular disease and are the main vehicle for reverse cholesterol transport (RCT) (Lefebvre et al. 2006). apoA-I and apoA-II gene expression is under direct transcriptional control of PPARα *in-vitro* (Vu-Dac et al. 1994; Hennuyer et al. 1999) and in humans (Vu-Dac et al. 1995; Watts et al. 2003). Interestingly, the murine apoA-I gene is negatively regulated by PPARα

agonists (Vu-Dac et al. 1998). The murine apoA-I lacks a functional PPRE in its promoter. Furthermore, PPARα agonists upregulate the expression of Rev-erb, an orphan receptor, which binds to the promoter of murine apoA-I to downregulate apoA-I expression.

HDL particle size and lipid composition are modulated by the PPARα controlled phospholipid transfer protein (PLTP) (Tu and Albers 2001). Increase in PLTP and LPL also increase pre-β-HDL (Tu and Albers 2001; Fruchart and Duriez 2006). pre-β-HDL, is a key acceptor of cholesterol from peripheral cells during RCT (Fruchart 2001). Furthermore, PPARα agonists also induce the expression of adenosine triphosphate-binding cassette transporter A1 (ABCA1) (Chinetti et al. 2001) and scavenger receptor BI (SR-BI) (Chinetti et al. 2000). ABCA1 is an exporter of cholesterol from cells (eg. macrophage) while SR-BI are hepatic cell surface receptors that bind HDL with high affinity and mediate the selective uptake of cholesteryl esters from HDL into the liver (Fruchart 2001). Together, interaction of HDL with SR-BI and ABCA1 triggers cholesterol efflux from peripheral tissues and HDL particles direct cholesterol for hepatic excretion into the bile (Eriksson et al. 1999).

Collectively, PPARα activation reduces TG levels through increased lipolysis and promotes HDL metabolism by increasing HDL formation to aid in the transport of cholesterol from peripheral tissues to the liver for breakdown.

1.2.1.3 Glucose metabolism

Severe hypoglycemia and hyperinsulinemia exhibited in PPARα null mice after fasting suggests a role for PPARα in glucose homeostasis (Kersten et al. 1999). Plasma glucose levels during fasting are affected by a combination of glucose synthesis, glycogen breakdown and glucose utilization (Mandard et al. 2004).

PPARα activation reduces TG level. This causes a reduction in glucose synthesis because TG provides the pathway with the essential substrate, glycerol. Rate-limiting enzymes of glucose synthesis are phosphoenol pyruvate carboxykinase (PEPCK) and pyruvate carboxylase. While PEPCK contains a PPRE shown to be functional in adipocytes (Tontonoz et al. 1995), there is no observable difference in hepatic PEPCK expression between wild type and PPARα null mice, regardless of nutritional status (Kersten et al. 1999; Xu et al. 2002b). In contrast, pyruvate carboxylase is reduced in fasting PPARα null mice (Mandard et al. 2004). However, no PPRE has yet been identified on its promoter.

Fasting induces the breakdown of glycogen into glucose through the induction of several hepatic enzymes such as glycerol-3-phosphate dehydrogenase and glycerol kinase. The expression of these enzymes, and of the glycerol transporters aquaporins 3 and 9, are PPARα dependent too (Patsouris et al. 2004).

The pyruvate dehydrogenase kinase isoform 4 (PDK4) is an inhibitor of glucose utilization and is PPARα activated (Wu et al. 2001). During fasting, low levels of PDK4 in PPARα null mice leads to an increase in its substrate, pyruvate dehydrogenase, and an increase in glucose utilization.

Response to fasting is also dependent on the pancreas, and PPARα null mice exhibit hyperinsulinemia due to inefficient suppression of insulin secretion upon fasting (Gremlich et al. 2005). PPARα activation in pancreatic islet β cells also increases pancreatic FAO and potentiates glucose-induced insulin secretion, suggesting that PPARα activation protects pancreatic islets from lipotoxicity (Ravnskjaer et al. 2005), a major causative factor for the development of type 2 diabetes mellitus (Lefebvre et al. 2006).

While PPARα activation of TRB3, an inhibitor of Akt/protein kinase B, negatively impacts on liver insulin signaling and perturbs glucose homeostasis (Koo et al. 2004), the general marked hypoglycemic and hyperinsulinemic phenotype exhibited by PPARα null mice upon fasting indicates the role of PPARα as a key player in glucose homeostasis.

1.2.3.4 PPARα null mice

In rodents, PPARα activation leads to peroxisome proliferation and hepatocarcinoma, a property intrinsic to mouse PPARα but not observed in humans (Cheung et al. 2004), due in part to the ten fold lower expression of PPARα in human liver (Palmer et al. 1998; Berger and Moller 2002). The phenotype of PPARα null mice fed on a normal diet is mild (Lee et al. 1995). However, fasting or inhibition of mitochondrial FA import severely impairs FA uptake and FAO, leading to sex-specific liver steatosis and cardiac lipid accumulation in male mice, hypoglycemia and hypothermia (Costet et al. 1998; Djouadi et al. 1998; Kersten et al. 1999). The induction of satiety in mice through

PPARα activation also suggest a role for PPARα in body weight control and indirectly supports the use of PPARα agonists to treat obesity (Fu et al. 2003).

Collectively, the general positive effects of PPARα on energy metabolism reflects its ability to improve symptoms of the metabolic syndrome (obesity, insulin resistance and dyslipidemia) and also suggest that PPARα may be beneficial in the prevention or treatment of type 2 diabetes mellitus and its associated complications (Lefebvre et al. 2006).

1.2.2 PPARγ

As a master modulator of adipocyte differentiation, PPARγ is required for the accumulation of adipose tissue and hence contributes to obesity (Lehrke and Lazar 2005). Crucial indication of the importance of PPARγ in human metabolism stemmed from its identification as the cognate receptor for the thiazolidinedione (TZD) class of insulin sensitizing drugs (Lehmann et al. 1995). Clinical studies involving TZDs such as pioglitazone and rosiglitazone suggest that the direct effects of these glucose-lowering agents on adipose tissue can contribute to improvements in hepatic and peripheral insulin sensitivity in patients with type 2 diabetes (Maeda et al. 2001; Yamauchi et al. 2001; Yu et al. 2002; Bajaj et al. 2004; Miyazaki et al. 2004), a disease of which insulin resistance is a hallmark (Gervois et al. 2007).

1.2.2.1 Insulin sensitization

PPARγ regulation of insulin sensitivity involves the primary effects of this receptor on gene transcription in adipose tissue, where it is abundantly expressed (Mukherjee et al. 1997b). In adipocytes, PPARγ regulates the expression of numerous genes such as adipocyte fatty acid binding protein (aP2) (Tontonoz et al. 1994b), PEPCK (Tontonoz et al. 1995), acyl-CoA synthetase (Schoonjans et al. 1995) and LPL (Schoonjans et al. 1996b). PPARγ also increases lipid uptake by adipocytes through upregulation of FATP-1 (Martin et al. 1997) and CD36 (Motojima et al. 1998). The collective effect of PPARγ upregulation is an increase in FA uptake and a decrease in lipolysis in adipocytes; and a reduction of free FA in the peripheral tissues.

PPARγ also upregulates the secretion of adipocyte-specific proteins (adipokines) which either increase or decrease insulin sensitivity. Insulin sensitizing adipokines such as Acrp30/ adiponectin (Berger and Moller 2002) (Iwaki et al. 2003) decreases glucose, TG, and free FA (Berger and Moller 2002).

In contrast, PPARγ inhibits the expression of insulin resistance adipokine such as tumour necrosis factor α (TNFα) (Hofmann et al. 1994). PPARγ also downregulates leptin, an adipokine that inhibits feeding and augments catabolic lipid metabolism (De Vos et al. 1996; Kallen and Lazar 1996).

Selective PPRE-containing genes that are induced in adipose tissue are suppressed in skeletal muscle or liver (Berger and Moller 2002). For example, PPARγ mediated downregulation of PDK4 in muscle produces a net increase in glucose oxidation while selectively exerting a net decrease in glucose oxidation in the adipose tissue through upregulation of PDK4 there (Way et al. 2001).

Indeed, the net efficacy of PPARγ agonists involves direct actions on adipose cells, with secondary effects on key insulin responsive tissues such as skeletal muscle and liver (Berger and Moller 2002). Collectively, the beneficial metabolic effects of PPARγ is likely to involve a combination of enhance insulin mediated adipose tissue uptake, storage and breakdown of FA (Oakes et al. 2001); an increase in circulating levels and/or action of insulin sensitizing adipokine (eg. Arcp30) and a decrease in insulin resistance causing adipokines (eg. TNFα, leptin, resistin (Steppan et al. 2001)).

1.2.2.2 PPARγ null mice

PPARγ null mice are not viable due to defects in placenta formation (Barak et al. 1999) while heterozygous PPARγ mice have reduced body size and weight, reduced insulin resistance and smaller adipocytes and fat depots (Kubota et al. 1999; Jaradat et al. 2001; Rieusset et al. 2004). In conditional knockout of PPARγ in specific tissues, mice lacking expression of PPARγ in adipose tissue have raised plasma levels of lipids, increased gluconeogenesis and developed insulin resistance (He et al. 2003).

1.3 Ligands of PPAR

PPAR modulate metabolic and inflammatory pertubations that predispose one to cardiovascular diseases and type 2 diabetes. Indeed, hypolipidemic fibrates and the anti-diabetic TZDs are drugs used in clinical practice that act via PPARα and PPARγ respectively. The pleiotropic actions of PPARs and the fact that chemically diverse PPAR agonists may induce distinct pharmacological responses have led to new concepts for drug design (Gervois et al. 2007).

Several assays have been developed to identify and characterize PPAR ligands. Transactivation assays involve cotransfection of cells with a PPAR expression vector and a reporter construct containing a PPRE-driven gene reporter. However, in such assays, PPAR forms obligate heterodimers with RXR and activation of this heterodimeric complex might be due to ligands activating either RXR or PPAR. Thus, specific screening for PPAR ligand cannot be achieved. Alternatively, chimeric receptors consisting of the PPAR LBD and the yeast transcription factor Gal4 DBD have been utilized with a Gal4-responsive reporter plasmid. Activation of this chimeric reporter gene assay will more likely indicate the presence of a specific PPAR ligand. Recently, cell lines stably expressing this system has been reported and provides an additional tool for high throughput, cell based screening of PPAR ligands (Seimandi et al. 2005).

Radiolabelled TZD and subsequently developed non-TZDs have been used in competitive PPAR ligand binding assays (Lehmann et al. 1995; Berger et al. 1996). PPAR scintillation proximity assays, using receptor LBDs attached to scintillant-containing beads, allowed for high-throughput screening for ligands (Elbrecht et al. 1999). Most recently, a fluorescent energy transfer assay was implemented to evaluate the

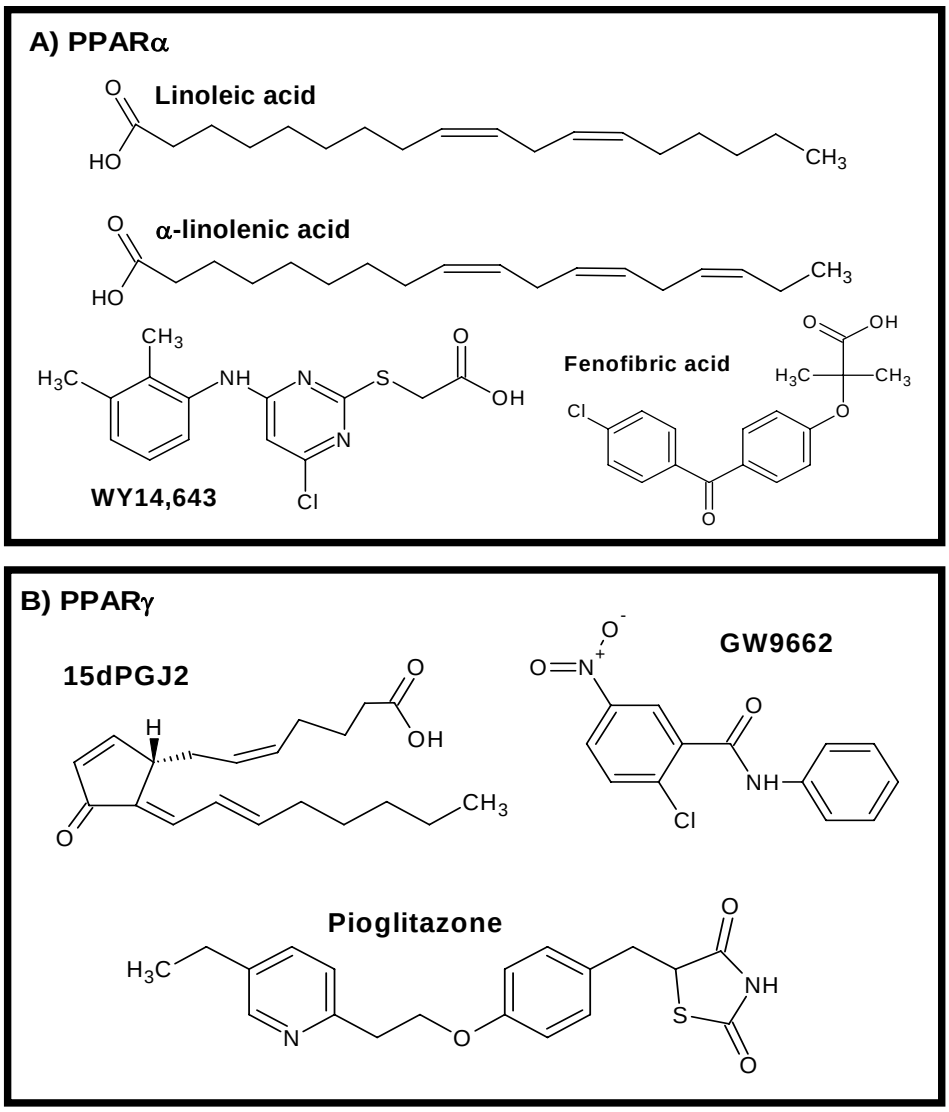
ability of ligands to induce PPAR-cofactor interaction in a rapid, cell-free format (Zhou et al. 1998a).

The prevalent point of view today is that PPARs act as lipid sensors that translate changes in lipid/fatty acid levels from the diet or from food deprivation into metabolic activity, leading to either fatty acid catabolism or lipid storage (Michalik et al. 2006). The endogenous ligands or mediators of these changes have not been characterized but are probably generated by fatty acid metabolism. Thus, the quest for "the" endogenous PPAR ligand is still ongoing (Lefebvre et al. 2006).

1.3.1 PPARα ligands

PPARα is activated naturally by a wide variety of saturated (palmitic acid), monounsaturated FA (oleic acid) and polyunsaturated FA (PUFA) (linoleic acid, linolenic acid and arachidonic acid) (Gottlicher et al. 1992; Keller et al. 1993; Forman et al. 1997; Kliewer et al. 1997; Xu et al. 1999) (Fig 1.4A). Notably, PPARα has the highest affinity for unsaturated FA among the subtypes and is also the only subtype that binds to a wide range of saturated FA (Xu et al. 1999). Comparison of the ligand-binding pocket of the three PPAR isotypes has revealed the following interesting characteristics. The PPARβ ligand binding pocket is significantly smaller than the pockets of PPARα and PPARγ. The pockets of PPARα and PPARγ are similar to each other in shape and size. This difference might explain why PPARβ binds to less and more specific ligands than PPARα and PPARγ. The PPARα pocket is more lipophilic than the two others, and

Figure 1.4 Selected PPAR ligands



Note: While the ligands shown here are mostly agonists, GW9662 is a PPARγ antagonist.

suggests a possible explanation that certain potent PPARγ ligands do not bind PPARα; and that PPARα can bind the more lipophilic saturated FA.

All these FA bind to PPARα directly with micromolar affinities. However, it is unclear whether the concentrations at which binding has been noted are physiologically relevant due to the variety, distribution pattern and affinity of FA and FA derivatives for PPAR in the body (Michalik et al. 2006). Eicosanoids are a class of FA that are mainly

derived from arachidonic acid, either via the lipoxygenase pathway leading to the formation of leukotrienes and hydroxyeicosatetraenoic acids (HETEs) or via the cyclooxygenase pathway producing prostaglandins (PG) (Desvergne et al. 2006). The lipoxygenase metabolite 8(S)-HETE (Yu et al. 1995) and leukotriene B4 (Devchand et al. 1996) were also identified as a submicromolar ligand for PPARα.

Fibrates such as fenofibrate and clofibrate are lipid lowering drugs which are synthetic ligands of PPARα (Willson et al. 2000) (Fig 1.4A). WY14,643, the 2-arylthioacetic acid analogue of clofibrate, is also a potent PPARα agonist (Berger and Moller 2002). In humans, fibrates are used at high doses (200–1200 mg/day) to achieve efficacious lipid-lowering activity at micromolar levels *in vivo* (Fruchart and Duriez 2006).

The ureidofibrate, GW2331, however, was found to be a nanomolar PPARα and PPARγ ligand (Brown et al. 1997), whereas the closely related ureidobutyric acid, GW9578 (Brown et al. 1999) and GW7647 (Brown et al. 2001) were found to be nanomolar PPARα agonist with potent PPARα-selective hypolipidemic activity *in vivo*.

1.3.2 PPARγ ligands

Unlike PPARα, PPARγ does not bind to saturated FA. Instead, PPARγ preferentially binds PUFA, including the essential FA linoleic acid, linolenic acid, arachidonic acid, and eicosapentaenoic acid at micromolar concentrations (Xu et al. 1999). Conversion of linoleic acid to 9-HODE and 13-HODE by 15-lipoxygenase can provide additional micromolar PPARγ agonists (Nagy et al. 1998). A prostaglandin derivative, 15-deoxy-$\Delta^{12,14}$-Prostaglandin J2 (15dPGJ2) was also demonstrated to be a PPARγ agonist (Forman

et al. 1995; Kliewer et al. 1995) (Fig 1.4B). However, the physiologic relevance of this ligand is questionable since cellular concentrations cannot be accurately determined (Berger and Moller 2002).

TZDs such as rosiglitazone and pioglitazone (Fig 1.4B) are insulin sensitizing drugs and are PPARγ agonists (Lehmann et al. 1995; Berger et al. 1996; Willson et al. 1996). GW2570 is a very potent non-TZD PPARγ-selective agonist that was shown to have antidiabetic efficacy in humans (Willson et al. 2000). In contrast, GW9662 is a PPARγ antagonist and also a PPARα partial agonist which binds irreversibly to the LBD of PPAR through covalent modification of cysteine (Leesnitzer et al. 2002) (Fig 1.4B).

Unfortunately, PPARγ agonists can have undesirable clinical effects too. These include weight gain due to increased adiposity, edema, hemodilution, and plasma-volume expansion, which preclude their clinical application especially in patients with heart failure (Arakawa et al. 2004; Rangwala and Lazar 2004; Staels 2005).

1.3.2 Dual PPARα/PPARγ ligands

In general, diabetic patients suffer from both hyperglycemia and dyslipidemia with their associated complications, such as peripheral neuropathy, kidney failure, retinopathy, and atherosclerosis, culminating in myocardial infarction and stroke (Plutzky 2003; Michalik et al. 2006). Thus, despite the negative effects of PPARγ agonist, positive clinical data on the use of PPARγ agonists for the treatment of diabetes and the use of PPARα agonists for the treatment of dyslipidemia and coronary heart disease makes it likely that dual PPARα and PPARγ agonists might provide broadly beneficial metabolic effects on these

patients through a simultaneous treatment of hyperglycemia, dyslipidemia and the associated cardiovascular risk factors (Staels and Fruchart 2005; Tenenbaum et al. 2005).

Among the dual PPARα/PPARγ agonists developed, compounds that belong to the glitazar class were on the most advanced stage of development (Fievet et al. 2006). However, ragaglitazar (Lohray et al. 2001) was halted due to the detection of bladder tumors on long term carcinogenicity studies in rodents. Strikingly, a major concern of those attempting to develop novel PPAR targeted drugs is to avoid agents similar to present compounds that are carcinogenic in rodents (Michalik et al. 2006). Indeed, the U.S. Food and Drug Administration (FDA) has issued guidelines requiring that PPAR ligand clinical trials exceeding 6 months must be preceded by the successful completion of a 2 year carcinogenicity tests in rodents.

Besides carcinogenicity, adverse effects of glitazar administration include weight gain, oedema, fluid retention, fatty infiltration in bone marrow, decrease in white blood cell count, anemia and raised levels of hepatic enzymes (Fievet et al. 2006). Indeed, while tesaglitazar was generally well tolerated in Phase II studies (Fagerberg et al. 2005), its development was recently halted due to the induction of impaired renal function (Fievet et al. 2006). Similarly, termination of muraglitazar recently was due to the existence of significant cardiovascular risks to treatment (Rubenstrunk et al. 2007). Collectively, the glitazars were associated with an excess incidence of the composite end-point death, major adverse cardiovascular events and congestive heart failure (Nissen et al. 2005).

Challenged by today's global epidemic of obesity, research on PPAR modulators continues vigorously. Indeed, the ideal PPAR modulator remains highly anticipated.

1.4 Molecular mechanisms of PPAR activity

There are several levels at which PPAR activity can be controlled especially in regards to PPARα. Besides the nature of the ligand used, transcriptional regulation can be affected by the action of AF-1 and AF-2, the dimerization of RXR on the PPRE, the regulation of PPAR expression, the post-translational modifications of PPAR; and the association with coregulators.

1.4.1 Action of AF-1 and AF-2

The AF-1 at the N-terminal of the PPAR TAD has been characterized. Interestingly, only the AF-1 of PPARα and PPARγ is ligand independent (Adams et al. 1997; Werman et al. 1997; Juge-Aubry et al. 1999). Indeed, a truncated PPARα lacking the TAD had 60-70% lower transactivation function compared to the full length regardless of the presence of agonists (Hi et al. 1999). The AF-1 has been identified on residues 1-92 of PPARα (Juge-Aubry et al. 1999). This AF-1 region on PPARα is acidic and the minimally essential region (residues 15-44) contains a helical motif that is implicated in transactivation function (Hi et al. 1999). In addition, the N-terminal domain is a major phosphorylation site (Gelman et al. 2005) and is thus unclear whether the AF-1 is an autonomous activation function or whether it primarily serves as a modulator of receptor activity by integrating various intracellular signaling pathways through post-translational modifications such as phosphorylation.

Several PPARα and PPARγ AF-2 mutants showed reduced activity in the absence of ligand but maintained or increased ligand dependent activity. This observation

suggests a key role for the AF-2 domain in mediating the high basal activity of PPARs (Molnar et al. 2005). Interestingly, this constitutive activity which occurs in the absence of ligand is due to the interaction of PPARs with coactivators through intra- and inter-molecular stabilization of helix 12 by four groups of residues (Molnar et al. 2005). The four different amino acid groups on PPARγ which contribute to the ligand independent stabilization of helix 12 in the LBD are: (i) Lys329 and Glu499, mediating a charge clamp-type stabilization of helix 12 via a coactivator bridge; (ii) Glu352, Arg425, and Tyr505, directly stabilizing the helix via salt bridges and hydrogen bonds; (iii) Lys347 and Asp503, interacting with each other as well as contacting the coactivator; and (iv) His351, Tyr355, His477, and Tyr501, forming a hydrogen bond network. Except for His351 and Tyr355 that are responsible for ligand specific effects of PPARγ, these amino acids are highly conserved within the PPAR subfamily, suggesting that the same mechanism may apply for all three PPARs. Accordingly, the corresponding residues for PPARα are: (i) Lys292 and Glu462 (ii) Glu315, Arg388 and Tyr468 (iii) Lys310 and Asp464 (iv) His440 and Tyr464. Consistently, a phylogenetic analysis revealed that the helix 12 sequences from the three PPAR isotypes clustered with the homologous sequence from the constitutive androstane receptor, an orphan nuclear receptor with high constitutive activity which stabilizes its helix 12 in an active conformation using similar groups of residues.

Enhancement of gene transcription in a ligand dependent manner is the most prominent feature of PPAR. However, recent studies have unveiled important insights regarding the high basal activity of the receptor in the absence of ligand. Nonetheless, it still remains debatable that the moderate PPAR activity in the absence of exogenous

ligand actually reflects a true ligand-independent action or a result of a weak concentration of agonist from either cellular metabolism or the culture medium (Feige et al. 2006).

1.4.2 RXR dimerization

It has been demonstrated recently, through live cell imaging, that the association of PPARs with RXR occurs independently of ligand binding and does not require DNA binding (Feige et al. 2005; Tudor et al. 2007). Although all three RXR isotype are reported to dimerize with PPARs, specific association with each RXR isotype seems to influence the recognition of target gene promoters (Juge-Aubry et al. 1997). PPAR prefers to bind RXRα and RXRγ on weak and strong binding elements respectively. The binding strength of the element is not dependent on the core DR1 sequence, which has a relatively uniform degree of conservation, but correlates with the extent of similarity of the 5'-flanking nucleotides and core sequence with respect to a consensus element. This 5'-flanking sequence is essential for PPARα binding and thus contributes to subtype specificity. However, very little is known on the specificity of RXR isotype utilization by the different PPARs *in vivo*.

Interestingly, PPAR/RXR heterodimers can induce transcription on PPAR target genes in response to either PPAR or RXR agonist alone. Moreover, treatment with PPAR and RXR agonists together potentiate the effects observed with each ligand alone (Kliewer et al. 1992; Gearing et al. 1993; Keller et al. 1993). This convergence of PPAR and RXR mediated effects was illustrated *in vivo* by the sensitization of diabetic mice to insulin by RXR agonists, an effect similar to the anti-diabetic action of PPARγ agonists

(Mukherjee et al. 1997a). However, the molecular mechanisms underlying transcriptional permissivity and synergy are not well understood in terms of cofactor recruitment by each partner of the heterodimers (Feige et al. 2006). Indeed, mutation of the PPARγ AF2 abolishes the ability of RXR/PPARγ heterodimers to respond to ligands specific for either subunit. In contrast, the permissivity of PPAR/RXR to either of their ligands was not affected by mutation of RXR AF-2. This indicates that binding of ligands to RXR may alter the conformation of the dimerization partner, PPAR, and modulate the activity of the heterodimer in a manner independent of the RXR ligand dependent AF-2 (Schulman et al. 1998). The interplay between PPARα AF-2 and RXR AF-2 together with coregulators is unclear.

RXR homodimers have been shown to activate the PPRE of the malic enzyme, PDK1, acyl-CoA oxidase and the bifunctional gene (IJpenberg et al. 2004). Moreover, in PPARα null mice, RXR homodimers can substitute for PPAR/RXR heterodimers to overcome the drastic effects of fasting observed in these animals. Indeed, in the presence of RXR agonists, coactivators (SRC-1 and TIF2) stabilizes the binding of RXR homodimers to PPREs. In contrast, p300 is unable to interact with the DNA-bound RXR homodimer, but readily forms a complex with PPAR/RXR regardless of the presence of ligand. This indicates that RXR homodimer formation onto PPREs requires selective and ligand-dependent interaction with specific coregulators. Although simplified with respect to the diversity of PPAR and RXR coregulators, this model reflects that relative levels of coregulator expression are important determinants mediating the specificity of the physiological response to PPAR or RXR agonists.

1.4.3 Protein regulation

Human PPARα promoter activity is induced by PPARα itself and hepatocyte nuclear factor 4 (HNF4), a major regulator of gluconeogenesis. In contrast, the orphan receptor COUP-TFII antagonizes this action (Pineda Torra et al. 2002). Temporal expression of PPARα is also controlled by the circadian clock (Lemberger et al. 1996) through positive control by glucocorticoids (Lemberger et al. 1994; Steineger et al. 1994) and the clock gene Bmal1 (Oishi et al. 2005). Insulin downregulates PPARα expression (Steineger et al. 1994) while leptin upregulates it (Zhou et al. 1998b). Glucose decreases PPARα expression in the pancreas, leading to diminished FA oxidation and TG accumulation (Roduit et al. 2000). Fasting in mice also induces PPARα expression (Kersten et al. 1999; Leone et al. 1999). Interestingly, male rats had higher levels of hepatic PPARα mRNA and protein than female rats and this is regulated by sex hormones (Jalouli et al. 2003). However, fasting increased hepatic PPARα mRNA levels to a similar degree in both sexes. Finally, synthetic PPAR ligands such as WY14,643 or fibrates increase the half-life of the PPARα by preventing its ubiquitination and its subsequent degradation via the proteasome (Blanquart et al. 2002).

1.4.4 Post-translational modification

Post-translational modifications such as phosphorylation, ubiquitylation and sumoylation can affect transcription. Thus, a comprehensive study integrating the influence of such modifications would benefit the understanding of molecular mechanisms in PPAR-mediated transcription.

PPAR activity can be modulated through phosphorylation by major intracellular signaling cascades such as the mitogen-activated protein kinase (MAPK) or the protein kinases A and C (PKA and PKC) pathways (Feige et al. 2006). Several phosphorylation sites spanning all across the receptor have been identified and characterized. Collectively, the effects of PPAR phosphorylation mediate different mechanisms which include changes in the affinity for ligands, RXR, coregulators and target genes.

Phosphorylation of PPARγ2 serine 112 by MAPK induces conformational changes which are transmitted to the LBD and result in weakened affinity for ligands (Shao et al. 1998), resulting in lower transcriptional activity (Hu et al. 1996). The interaction between PPARα and RXR is modulated by phosphorylation in the hinge region as mutation of the PKC phosphorylation sites T129 and S179 of PPARα reduces heterodimerization (Gray et al. 2005). Mutation of T129 also prevented PPARα from binding to DNA *in vitro*. The effects of phosphorylation on interactions with cofactors are illustrated by the MAPK-mediated phosphorylation of the AF-1 (at ser12 and ser21 by p42 and p44 of MAPK) which activates PPARα in response to insulin, probably by relieving inhibition by corepressors (Juge-Aubry et al. 1999). Intriguingly, both these residues must be phosphorylated in order to activate transcription. This is in contrast to PPARγ2, which was mentioned previously to be phosphorylated at a single site that is not homologous to the sites described in PPARα. Phosphorylation by p38 of MAPK on serine residues of the TAD of PPARα specifically enhances coactivation by the PPARγ coactivator-1α (PGC-1α) (Barger et al. 2001), which interacts with the PPARα AF-2 (Vega et al. 2000). Phosphorylation of the PPARα DNA binding domain, where PKA primarily acts on, regulate binding to response elements (Lazennec et al. 2000).

Finally, besides these direct effects of phosphorylation, PPAR activity can also be modulated by changing the expression level of the receptor. For example, the PKC and stress-associated kinase pathways induce the expression of the PPARα (Yaacob et al. 2001). Other means of regulating PPAR activity indirectly include the phosphorylation of RXR (Solomon et al. 1999).

Ubiquitin is an 8 kDa protein which covalently binds to proteins to target them for degradation by the 26S proteasome. Ubiquitylation of proteins occurs on lysine residues. Surprisingly, little information is available on the mechanisms regulating PPAR degradation (Feige et al. 2006). Both PPARα and PPARγ can be targeted for degradation by ubiquitylation. The degradation of PPARγ is enhanced by ligand binding through a mechanism that requires an intact AF-2 structure and probably an active conformation of the receptor (Hauser et al. 2000). In contrast, PPARα agonists stabilize the PPARα and decreased its ubiquitylation and subsequent degradation (Hirotani et al. 2001; Blanquart et al. 2002).

Together with the observations that phosphorylation events may influence ubiquitylation and degradation of PPARs (Floyd and Stephens 2002; Tsao et al. 2005), these results highlight the need for further integrated analyses linking ubiquitylation and proteasomal degradation to transcriptional activity.

Small ubiquitin-like modifiers (SUMO) comprise a family of three 11 kDa proteins homologous to ubiquitin which can be reversibly conjugated to proteins through covalent binding to regulate various cellular mechanisms including transcriptional repression. Sumoylation occurs on lysine residues located in ΨKXE/D motifs (Ψ being a large hydrophobic residue and X any residue) (Feige et al. 2006). Till now, no detailed

work on sumoylation of PPARα has been carried out. In contrast, much of the current understanding of sumoylation on PPAR signaling is derived from work with PPARγ. PPARγ can be sumoylated on two different sites at lysines 77 and 365 (107 and 395 in PPARγ2) (Pascual et al. 2005). K77 sumoylation occurs in a ligand-independent manner and exerts inhibitory effects on the activity of the receptor (Ohshima et al. 2004; Yamashita et al. 2004; Pascual et al. 2005). In contrast, sumoylation at lysine 365 is ligand-dependent and leads to complex transrepressive effects on the iNOS promoter by promoting the interaction with the corepressor NCoR (Pascual et al. 2005).

1.5 Molecular mechanisms of PPAR activity- Coregulators

Coregulators are proteins which can repress (corepressors) or enhance (coactivators) nuclear receptor transcriptional activity by bridging transcription factors to the basic transcription machinery and by specifically modifying chromatin structure. While chromatin compaction and histone hypoacetylation mainly repress gene activation, remodeling and repositioning of nucleosomes allows binding of transcription factors and formation of the pre-initiation complex (McKenna and O'Malley 2002; Feige et al. 2006). Indeed, the regulation of a general transcription unit by nuclear receptors requires a vast number of coregulatory complexes that have various functions and enzymatic activities (Perissi and Rosenfeld 2005). The influence of coregulators on nuclear receptor signaling has been widely investigated, including those related to PPAR action.

1.5.1 Corepressor

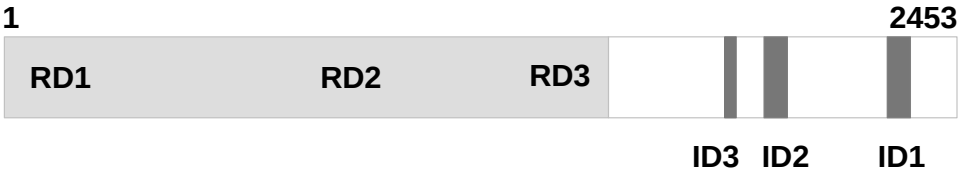
The nuclear receptor corepressor (NCoR) (Horlein et al. 1995) and the silencing mediator of retinoid and thyroid receptors (SMRT) (Chen and Evans 1995) were initially isolated as proteins mediating active repression by the thyroid hormone and retinoic acid receptors. NCoR and SMRT repress the activity of nuclear receptors in the absence of ligand or upon antagonist treatment until agonist binding induces their exchange for a coactivator complex to induce transcriptional activation (Privalsky 2004; Feige et al. 2006).

NCoR (Fig 1.5) and SMRT are paralogs of one another and function in a similar fashion (Ordentlich et al. 2001). NCoR is divided into a N-terminal portion having three distinct transcriptional repression domains (RD) and a C-terminal portion composed three nuclear receptor interaction domains (ID) (Privalsky 2004). Similarly, SMRT has fours RDs and two IDs. The RDs are docking surface that recruit additional components of the receptor complex, including histone deacetylases (HDACs), transducin-like protein 1 (TBL-1), G protein pathway suppressor 2 (GPS2) and Sin3 (Goodson et al. 2005).

While their repressive effects are essentially mediated through the recruitment of HDACs, like HDAC3 (Heinzel et al. 1997), interactions with the basal transcriptional machinery might also play a role (Muscat et al. 1998). Indeed, the N-terminal RD and the C-terminal receptor ID of NCoR are also part of the contact interface that directly mediates interaction with general transcription factors, and mediate signals from repressors to the basal machinery. This suggests that NCoR interacts with the central components of the transcriptional initiation process and locks them into a non-functional complex or conformation that is not conducive to transcription (Privalsky 2004).

Figure 1.5 A simplified schematic of the nuclear corepressor, NCoR

A simplified representation of full length murine NCoR (2453 residues). The N-terminal of NCoR comprises three distinct transcriptional repression domains (RD) and the C-terminal comprises three nuclear receptor interaction domains (ID). Dark grey boxes represent unique CoRNR box sequences within each ID, residues 2277-2285, 2073-2081, 1949-1953 for ID1, ID2 and ID3 respectively.



The interaction between PPARs with NCoR and SMRT in solution was documented by the isolation of NCoR as a PPARα and PPARβ interacting protein in yeast-two hybrid screens and by direct interactions with the three PPAR isotypes mostly in *in vitro* interaction assays (Dowell et al. 1999; Hu and Lazar 1999; Gurnell et al. 2000; Krogsdam et al. 2002; Stanley et al. 2003). Among the three PPAR isotypes, repression by PPARβ seems to play an important role since its association with NCoR and SMRT results in the strongest effects in transactivation (with PPARα being the weakest) (Krogsdam et al. 2002) but also because PPARβ can inhibit PPARα and PPARγ activation through corepressor and HDAC binding (Shi et al. 2002). Nonetheless, the physiological relevance of corepressor action on PPAR signaling remained controversial, especially for PPARα (Semple et al. 2005; Feige et al. 2006). While information on the action of corepressors on specific PPARα target gene is clearly lacking (Feige et al. 2006), a clear role of corepressors on PPARγ mediated adipogenesis was only recently demonstrated (Guan et al. 2005; Yu et al. 2005).

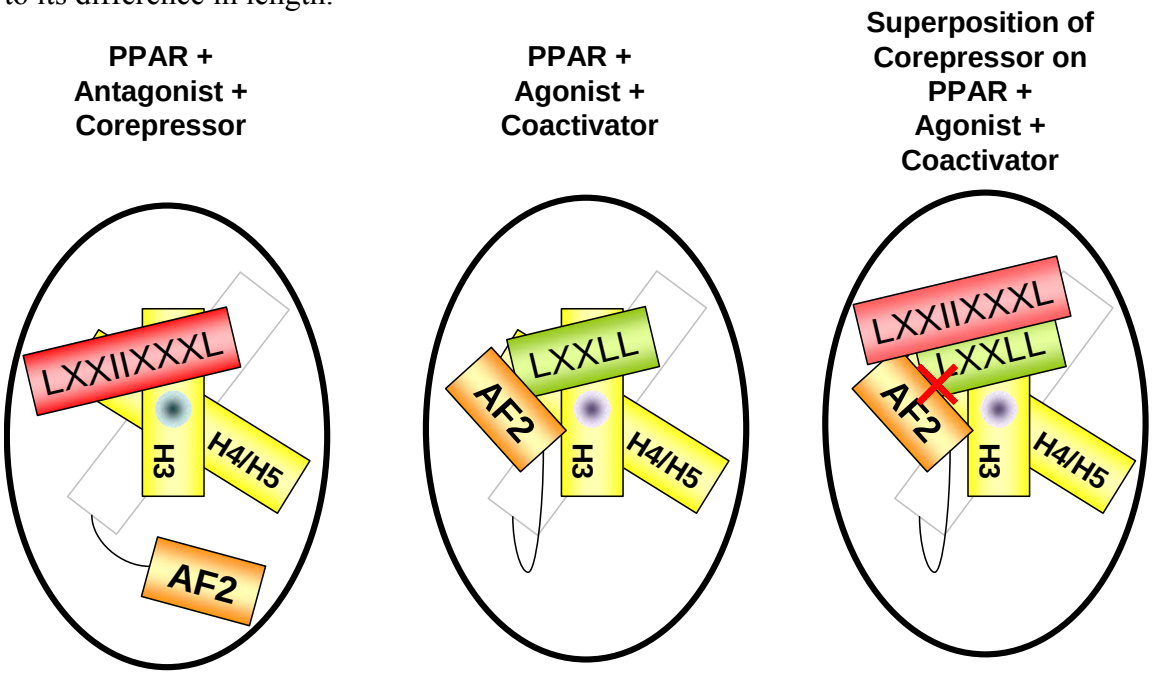
Interestingly, the action of corepressors on endogenous PPARγ target genes is promoter specific. NCoR and SMRT were associated with the glycerol kinase (GyK) and oxidized LDL receptor 1 (OLR1) promoters in complex with PPARγ but absent from the aP2 promoter (Chui et al. 2005; Guan et al. 2005). On the aP2 promoter, the PPARγ/RXR heterodimer was constitutively activated by the association of coactivators, such as SRC-1, even in the absence of exogenous ligand (Guan et al. 2005). The functional role of this corepressor-PPARγ interaction was further demonstrated in adipogenesis (Yu et al. 2005). Here, the significance of corepressors on PPARγ target genes during adipogenesis was demonstrated by the upregulation of adiponectin, perilipin and C/EBPα after NCoR and SMRT knockdown (Yu et al. 2005). Collectively, corepressors could provide a molecular switch to specifically silence PPAR target genes that do not require activation during key metabolic processes such as adipogenesis (Feige et al. 2006). Since this target gene specificity was observed within the same cell type (3T3-L1 cells), these results suggest that DNA binding may impose specific receptor conformations which determine affinity for corepressors and therefore possibly explain why corepressor binding was not observed *in vitro* on the ACO PPRE (Zamir et al. 1997; Feige et al. 2006).

An important aspect of corepressor action is the response to antagonist binding as pharmacological inhibition of PPARs promises interesting therapeutic applications. To this respect, the crystal structure of the PPARα LBD bound to the antagonist GW6471 and to a SMRT corepressor motif is particularly interesting as it revealed that antagonists promote a conformation of the receptor which prevents the correct positioning of helix 12 and subsequent coregulator recruitment (Xu et al. 2002a).

Each ID in NCoR and SMRT contains a CoRNR box motif (or LXXI/HIXXXI/L) (where X is any residue) (Fig 1.5 and 1.6) that forms the core contact surface between corepressor and nuclear receptor (Hu and Lazar 1999; Perissi et al. 1999; Hu et al. 2001; Xu et al. 2002a). The CoRNR motif forms an extended α-helical domain that docks into a complementary groove formed by helices 3/4/5 in the LBD of the nuclear receptor. In contrast, the SRC-1 α-helical domain which docks in the similar groove is shorter (Xu et al. 2001). Differences in the CoRNR box motifs, in adjacent amino acids, and in the sequence of the helix 3/4/6 regions of the nuclear receptors contribute to the different affinities of various receptors for the different corepressor IDs (Cohen et al. 2001; Makowski et al. 2003). Helix 12, located at the very C terminus, also plays an important but indirect role in the receptor/corepressor interaction (Privalsky 2004). The helix 12 of most nuclear receptors can form an extended conformation in the absence of hormone that allows access of corepressor to its docking site on helices 3/4/5. The binding of hormone agonist can reorient this helix 12 to a sequestered position that occludes the corepressor docking surface, resulting in release of corepressor (Lin et al. 1997; Nagy et al. 1999; Zhang et al. 1999; Xu et al. 2002a). Interestingly, this same reorientation of helix 12 forms a new binding surface, made up of portions of helix 3/4/5 and 12, that can recruit the LXXLL motifs found in many coactivators (Darimont et al. 1998; Shiau et al. 1998; Mak et al. 1999). Helix 12 therefore operates as a hormone-operated molecular toggle switch that regulates the equilibrium between corepressor recruitment and coactivators recruitment (Privalsky 2004).

Figure 1.6 Illustration of antagonist bound PPAR with corepressor and its comparison with agonist bound PPAR/coactivator

Position of the AF-2 helix (orange bar) with antagonist (left panel) is different from that with agonist (middle panel). Both receptor interaction core consensus motif in corepressor (eg. NCoR) (red bar) and coactivator (SRC-1) (green bar) bind to the same groove of PPAR LBD formed by helices 3,4,5 (yellow bar). Superposition of the corepressor motif on the PPAR/agonist/coactivator complex (right-most panel) represents competition by the corepressor motif with the coactivator motif for AF-2 interaction due to its difference in length.



Although not corepressors *per se*, heat shock protein 90 (hsp90) (Sumanasekera et al. 2003a; Sumanasekera et al. 2003b); hsp90 accessory protein XAP2 (Sumanasekera et al. 2003b) and ribosomal protein regulator of p53, rpL11, (Gray et al. 2006) have also been reported to repress PPARα activity, and interestingly, through interaction with the PPARα hinge.

While NCoR and SMRT normally suppress in the absence of a ligand, another class of corepressors is recruited in a ligand dependent manner through LXXLL motifs and repress the activity of nuclear receptors by competing with coactivators and by

recruiting downstream effectors such as HDACs (White et al. 2004). The Receptor Interacting Protein 140 (RIP140) is one of such corepressors which can interact with PPARα and PPARγ (Miyata et al. 1998; Treuter et al. 1998). Furthermore, the recent identification of the ligand-dependent corepressor (LCoR) which represses the activity of several nuclear receptors in the presence of their agonists (Fernandes et al. 2003) suggests that the activity of such corepressors should be considered in the study of PPAR actions (Feige et al. 2006).

1.5.2 Coactivators

In contrast to the action of corepressors, coactivators turn on transcription by NR. The first group of coactivators is the switch/sucrose non-fermenting (SWI/SNF) complexes that change the location or conformation of nucleosomes and remodel chromatin by using energy from ATP hydrolysis (Feige et al. 2006). PPARγ2 has a direct requirement of SWI/SNF complexes on PPARγ target promoters to facilitate pre-initiation complex function in adipogenesis (Salma et al. 2004). Additionally, three members of the SWI/SNF family containing LXXLL motifs were also isolated in a PPARα interacting complex (PRIC) purified from liver extracts using full-length PPARα (Surapureddi et al. 2002). Interestingly, besides gene activation, SWI/SNF has also been associated with gene repression (Perissi and Rosenfeld 2005).

A second group of coactivators comprises factors which covalently modify histone tails by acetylation, methylation, phosphorylation or ubiquitylation. These modifications can occur in a large number of combinations, often in an interdependent

manner, and compose a hypothetical epigenetic code, referred to as histone code, which specifies the fate of the gene (Jenuwein and Allis 2001; Imhof 2003; Feige et al. 2006).

Coactivators with histone acetyltransferase (HAT) activity include the homologous CREB binding protein (CBP) and p300 proteins. Transcriptional activation through CBP/p300 recruitment results in increased histone acetylation but also increased contacts with the basal transcription machinery (Kalkhoven 2004). p300 is also reported to harbor ubiquitin ligase activities (Grossman et al. 2003). PPARα-interacting domain of p300 was mapped to amino acids 39-117 which interacted strongly with PPARα but did not interact with RARγ or RXRα. Amino acids within the carboxyl terminus of PPARα as well as residues within the hinge region were required for ligand-dependent interaction with p300 (Dowell et al. 1997). The importance of CBP and p300 for PPAR action is also highlighted by the high affinity between PPARs and these cofactors. While PPARγ has a greater apparent affinity for CBP than for SRC-1, the steroid receptor ERα interacts preferentially with SRC-1 but very weakly with CBP (Zhou et al. 1998a). PPARα has a higher affinity for CBP than PGC-1α and SRC-1 (Mukherjee et al. 2002). In addition, it has been recently reported that interactions of p300/CBP coactivators with the LBDs of nuclear receptors are not limited to the canonical LXXLL motifs but involves both a longer contiguous segment around the motif and, for certain domains, an additional zone as shown with the LBD of PPARγ and RXRα (Klein et al. 2005).

p160 proteins comprise a family of three related coactivators (SRC-1, TIF2 and SRC-3) (Leo and Chen 2000). These coactivators can also mildly acetylate histones (Chen et al. 1997; Spencer et al. 1997). However, their HAT activity is much weaker than

that of CBP/p300 and interactions with other cofactors suggest that its more important role is to bridge transcriptional complexes to the basal transcriptional machinery.

The interaction of SRC-1 with PPARs is well documented in *in vitro* assays (DiRenzo et al. 1997). However, when the association of p160 proteins to PPARs is considered in the presence of PPAR, RXR and DNA response elements, p160 recruitment occurs in response to RXR ligands but not to PPAR agonists (Yang et al. 2000). Indeed, SRC-1 could be recruited to endogenous PPAR target genes by RXR homodimers in response to retinoid treatment but not by PPAR/RXR heterodimers (IJpenberg et al. 2004). While the strong influence of SRC-1 and TIF2 on obesity and insulin sensitivity indicates that p160 cofactors may modulate PPARγ signaling (Picard et al. 2002), SRC-1 knock-out mice retain normal PPARα signaling as β-oxidation and associated target genes can be induced by PPARα agonists (Qi et al. 1999). Nonetheless, this phenotype may have resulted from a compensation by the other p160 isotypes (Xu and Li 2003). Collectively, these results implies that probably SRC-1 and other p160 members are not bona fide PPAR coactivators but may play an indirect role in PPAR signaling through RXR (Feige et al. 2006).

Histone methyltransferase (HMT) activity provided by proteins such as the coactivator-associated arginine methyltransferase 1 (CARM-1) or the protein arginine methyltransferase 1 (PRMT-1) enhances transcription. However, no study has addressed the influence of CARM-1 or PRMT-1 in PPAR signaling so far (Feige et al. 2006). Nonetheless, the importance of methylation for PPAR activity was briefly illustrated by the isolation of PRMT-2, a methyltransferase that could directly interact with PPARγ and enhance its transcriptional activity (Qi et al. 2002).

The PPARγ coactivator 1α (PGC-1α) is the prototype of another group of cofactors which act as molecular scaffolds to assemble activating complexes (Puigserver et al. 1999) and to couple transcription to mRNA splicing (Monsalve et al. 2000). PGC-1α was isolated as a PPARγ interacting protein but coactivation of PPARα, PPARβ and other nuclear receptors was also reported (Feige et al. 2006). While PGC-1α interaction with PPARα and PPARβ is ligand-dependent and requires the AF-2 domain of the receptor and the first LXXLL motif of PGC-1α (Vega et al. 2000; Wang et al. 2003b), binding to PPARγ occurs in the absence of ligand and relies both on the PGC-1α N-terminus and on a LXXLL motif (Puigserver and Spiegelman 2003; Wu et al. 2003).

The PPARγ interacting protein (PRIP/RAP250) and the PRIP-interacting protein with methyltransferase domain (PIMT) are two coactivators acting as molecular scaffolds which cooperatively enhance PPARγ and RXR-mediated transcription (Zhu et al. 2000; Zhu et al. 2001). PRIP interacts in a ligand-dependent manner with both PPARα and PPARγ (Zhu et al. 2000), while PIMT was subsequently isolated as a PRIP interacting protein (Zhu et al. 2000). PRIP is important in PPAR signaling because PRIP null mouse embryonic fibroblast cannot sustain PPARγ-mediated adipogenesis and ligand-dependent recruitment of coactivators to the aP2 promoter during adipogenesis (Qi et al. 2003).

The last class of cofactors consists of the mediator (Med1) /thyroid hormone receptor associated protein (TRAP220) /vitamin D receptor interacting protein (DRIP205)/ PPARγ binding protein (PBP) multi-subunit complex which plays an important role in bridging nuclear receptors to the general transcriptional machinery and in recruiting and activating Pol II (Lewis and Reinberg 2003; Feige et al. 2006). The PBP subunit which anchors mediator to nuclear receptors is only present in a subset of

mediator complexes which exist with different combinations of subunit arrangements (Zhang et al. 2005). Interestingly, a liver-specific inactivation of PBP mimics the liver phenotype of PPARα null mice (Jia et al. 2004).

Several other PPARα coactivators for which the physiological relevance is still poorly understood have been characterized. Briefly, these coactivators include PRIC285 (Surapureddi et al. 2002) and the CBP/p300 interacting transactivator with ED-rich tail 2 (CITED-2) (Tien et al. 2004). Interestingly, CITED-2 coactivates PPARα and PPARγ but not PPARβ.

In addition to the various roles that groups of coregulators play, an extra layer of command was achieved through the control of phosphorylation status in various coregulators such as PGC-1α (Knutti et al. 2001; Puigserver et al. 2001), PBP (Misra et al. 2002a), SRC-1 and TIF2 (Rowan et al. 2000; Lopez et al. 2001) or SMRT and NCoR (Hermanson et al. 2002; Jonas and Privalsky 2004).

1.5.3 Dynamics of coregulator exchange on chromatin

A key question that underlies the molecular mechanisms of PPAR action is how do multiple cofactors with different activities cooperate to initiate transcription (Feige et al. 2006). Ligand binding is the crucial molecular event that switches the function of nuclear receptors from active repression to transcriptional activation (Perissi and Rosenfeld 2005). A combination of elegant structural and molecular studies of the interactions between nuclear receptors and coregulators has provided the evidence that hormone binding induces a conformational change in the ligand-binding domain of the receptor, which results in reduced affinity for corepressors and, simultaneously, enhanced affinity for

coactivators (Perissi and Rosenfeld 2005) (Fig 1.7A). Similarly, agonist binding to steroid receptors, such as ER, also induces the adoption of a specific conformation that favours coactivator binding, whereas antagonist binding promotes the interaction with corepressors (Perissi and Rosenfeld 2005).

However, the classic model of static receptor/coregulator binding to promoters has recently been challenged because it has become clear that there are additional and unexpected layers of regulation in the molecular events which modulate the nuclear receptor 'switch' from repression to activation (Perissi and Rosenfeld 2005; Feige et al. 2006).

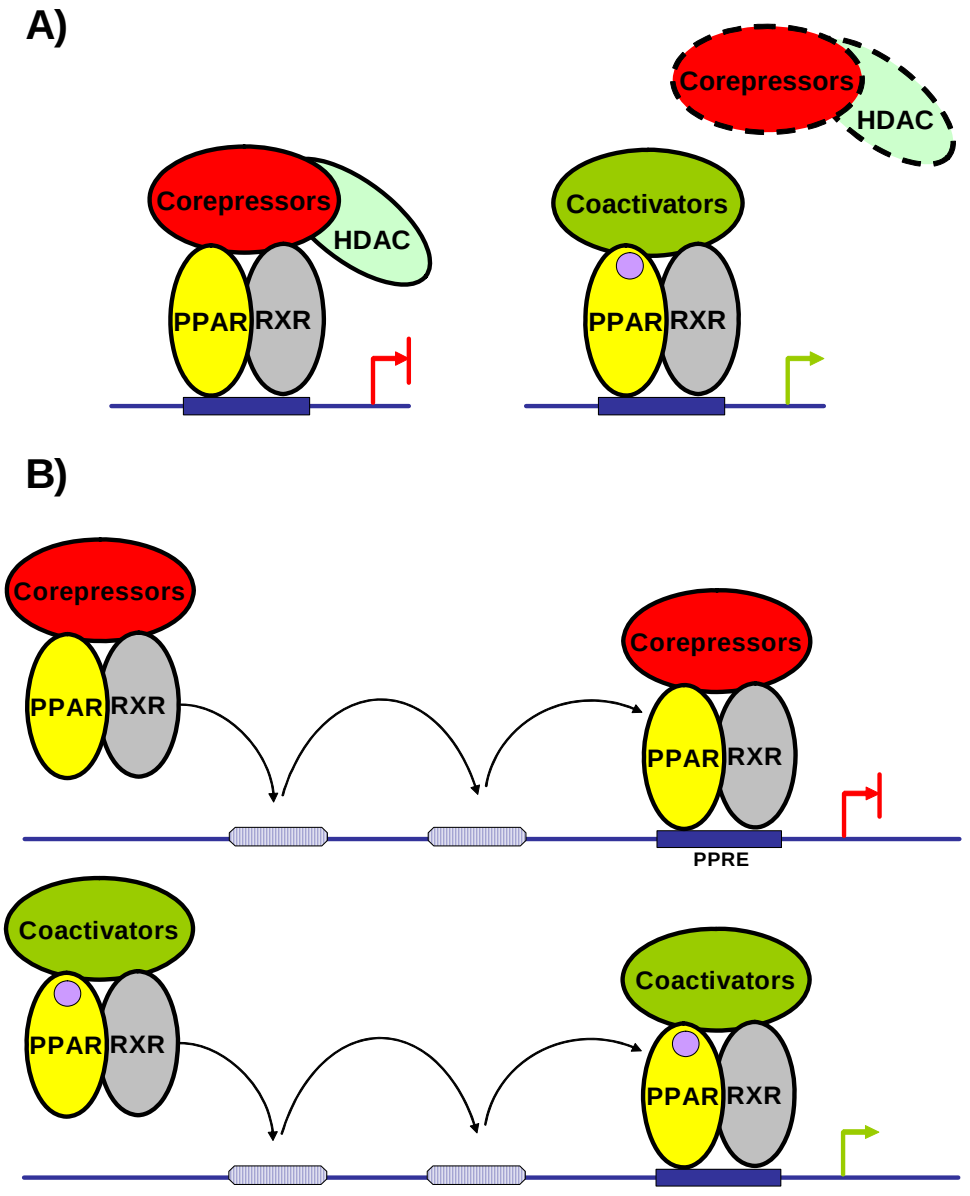
Much of the current paradigm shift has been due to advancements in experimental techniques. Using chromatin immunoprecipitation (ChIP), a technique used to specifically immunoprecipitate complexes of DNA with associated proteins to study chromatin occupancy by different transcription factors and the state of chromatin modification, various studies have shown that coregulator recruitment by NR occurs in an ordered manner and that promoter occupancy by NR is characterized by cycles of recruitment and release in a short time span (Perissi and Rosenfeld 2005).

Using microscopy techniques to study protein behavior in live cells, a new concept on transcription regulation, in complement to the rapid oscillation of transcription factors on promoters, known as 'genome scanning' has emerged (Fig 1.7B). In live cell imaging, PPAR were observed to have low mobility. Indeed, they were not freely diffusing and this low mobility was due to interactions with coregulators (Tudor et al. 2007). Other than coregulator docking, PPARs may also transiently interact with rather immobile components such as chromatin (Feige et al. 2005). Thus, the 'genome

scanning' model was proposed where these interactions reflect a three-dimensional scanning of the genome performed until transcription factors reach a genuine enhancer site where they may reside for longer periods and initiate pre-initiation complex assembly (Phair et al. 2004; Gelman et al. 2006).

Figure 1.7 Models of PPAR action

A) Ligand dependent corepressor/coactivator exchange. Some PPAR target genes are maintained in an inactive state in the absence of ligands by the PPAR/RXR heterodimer bound to corepressor. Presence of ligand (purple circle) allows dissociation and breakdown of corepressors and the recruitment of coactivators for transcriptional activation. B) Genome scanning (Feige et al. 2006; Gelman et al. 2006). Without ligand, PPAR and RXR are heterodimerized, recruit corepressors, and roam the nucleus where they interact transiently with chromatin, both on genuine PPREs and unspecific binding sites. Upon ligand binding, PPAR mobility is reduced due to its AF-2-dependent binding to cofactors. PPAR/cofactor complexes may transiently bind to "non-specific" sites on chromatin, performing a three dimensional-scanning of the genome, until they encounter a genuine response element in a promoter, at which chromatin remodeling and transcription are initiated.



1.6 Natural polymorphisms of the PPARα gene

The gene (*PPARA*) encoding PPARα is located on the long arm of chromosome 22 (Flavell et al. 2000; Lacquemant et al. 2000; Sapone et al. 2000; Vohl et al. 2000; Evans et al. 2001; Flavell et al. 2002; Tai et al. 2002; Tai et al. 2005; Tai et al. 2006). The *PPARA* locus is polymorphic in humans and several of these polymorphisms have been associated with variation in obesity, serum lipid concentrations and coronary artery disease (Naito et al. 2006). To date, 14 polymorphisms of PPARα have been reported (Flavell et al. 2000; Sapone et al. 2000): P22R, R127Q, R131Q, D140Y, D140N, L162V, R178G, V227A, A268V, D304N, G395A, G395E, D409T, Q413L. Among these polymorphisms identified, only a common leucine to valine (L162V) substitution at *PPARA* has been shown to be functional, altering the transcriptional activation associated with fibrate treatment *in vitro* (Flavell et al. 2000; Vohl et al. 2000; Flavell et al. 2002; Tai et al. 2002).

1.6.1 L162V

L162V has been associated with relatively minor and inconsistent changes in the serum concentrations of lipids and lipoproteins (Gouni-Berthold et al. 2004). When examined in relation to plasma lipids, the presence of this polymorphism shows associations with total cholesterol, LDL-c and apolipoprotein B (Lacquemant et al. 2000; Vohl et al. 2000; Tai et al. 2002). However, no association was observed with either plasma triglyceride or HDL-c concentration (Low and Tai 2007).

Evidence is emerging that the benefits of PPARα activation in the prevention of cardiovascular events are dependent on the presence of insulin resistance and/or DM (Tenkanen et al. 1995; Rubins et al. 2002; Robins et al. 2003). Studies showed that the most benefits associated with fibrates such as gemfibrozil (Tai et al. 2006), clofibrate (Anonymous 2000) and bezafibrate (Anonymous 1980) were observed in those with insulin resistance, diabetes mellitus (DM) or features associated with them. When subgroups with and without DM treated with gemfibrozil were compared (Tai et al. 2006), a lower prevalence of atherosclerosis and coronary pathology was seen in carriers of the V162 allele with DM. In contrast, in those without DM, the prevalence of atherosclerosis and coronary events with or without the V162 allele was the same.

Besides the interaction of L162V with fibrates, a gene-diet interaction was also observed for L162V and PUFA consumption in regards to plasma lipoprotein levels. On a low PUFA diet, carriers of L162V have greater TG and apoC-III. Conversely, on a high PUFA diet, carriers of the L162V have lower apoC-III concentrations (Tai et al. 2005). Others examined L162V using a dietary intervention approach to test if plasma lipoprotein and lipid responsiveness modifications in the dietary ratio of polyunsaturated to saturated FA was influenced by this polymorphism. From a high PUFA diet, a gene-diet interaction was observed whereby carriers of L162V had significant lower levels of apoA-I (Paradis et al. 2005).

The L162V polymorphism was first identified in a Caucasian population (Flavell et al. 2000; Vohl et al. 2000) and later, in several populations from the Indian sub-continent (Lacquemant et al. 2000). In a recent study, Chan et al (2005) reported that in Asian Indians in Singapore, the allele frequency remained much lower than amongst

Indians living in the Pondicherry district of India. Furthermore, the L162V polymorphism was found to be very rare in Singapore Chinese and Malays. Interestingly, another polymorphism which has not been described in non-oriental population, the V227A polymorphism, was found in Singapore Chinese with an allele frequency (0.04) similar to that reported in two independent Japanese study (0.04-0.05) (Yamakawa-Kobayashi et al. 2002; Chan et al. 2006; Naito et al. 2006). None of the Japanese studies detected the L162V polymorphism in their population. In all, these studies confirm the differing genetic structure of the *PPARA* locus in various ethnic groups.

1.6.2 V227A

In association studies (Yamakawa-Kobayashi et al. 2002; Chan et al. 2006; Naito et al. 2006), carriers of A227 allele consistently have lower levels of total cholesterol and triglycerides compared to that of non-carriers (Table 1.2). This effect was more obvious in women compared to men. Furthermore, the Singapore study identified a gene-diet interaction between the V227A polymorphism and dietary factors amongst Singapore Chinese women. In these subjects, the A227 allele was shown to modulate the association between dietary PUFA intake and serum HDL concentration. In women who carried the A227 allele, increasing dietary PUFA intake was associated with low HDL-c. This suggested that genetic variation at the *PPARA* locus may determine the lipid response to changes in PUFA intake. In addition, Naito *et al* (2006) reported that while non-alcohol drinkers with the A227 polymorphism have lower total cholesterol levels, this difference was not significant in carriers of the A227 allele who are drinkers. Thus, a gene-diet interaction involving the *PPARA* locus was also demonstrated within these populations.

Subjects with the V227A polymorphism are mostly heterozygous for the A227 allele. Homozygotes of the A227 allele are rare (3-5%) in carriers of the polymorphism.

Table 1.2 Association studies of the V227A polymorphism

Study	Subjects	Results	Comments
Yamakawa-kobayashi et al. 2002	Japanese, healthy Men: 207 Women: 194	Men: No significant difference in serum TG and total cholesterol. Women: Serum TG (27.5% reduction; p=0.038) and total cholesterol (8.3% reduction; p=0.046) were significantly lower in carriers of the A227 variant.	- No significant difference in HDL-c, LDL-c and BMI between carriers and non-carriers of the 227A variant in men and women.
Chan et al. 2006	Singapore Chinese, healthy Men: 1318 Women: 1581	Men: No significant difference in serum TG and total cholesterol. No serum HDL-c association with dietary PUFA. Women: Serum TG (8.4% reduction, p=0.048) and total cholesterol (3.2% reduction, p=0.047) were significantly lower in carriers of the A227 variant. Significant serum HDL-c association with dietary PUFA (p=0.049); where increasing PUFA intake lower HDL-c.	- No significant difference in HDL-c and LDL-c between carriers and non-carriers of the 227A variant in men and women. - Allele frequency of 227A was low in Singapore Malays (0.006) and Singapore Asian Indians (0.003).
Naito et al. 2006	Japanese, healthy Men: 655	Not associated with changes in TG or total cholesterol.	- No significant difference in LDL-c between carriers and non-carriers of the 227A variant. - Significant interaction between V227A polymorphism and drinking.

Unlike L162V, the effect of the A227 allele on cardiovascular risk in subjects with DM is unclear. However, the V227A polymorphism, together with other PPARα polymorphisms, were found at similar frequencies in Japanese subjects with maturity-onset diabetes of the young (MODY) and in nondiabetic Japanese subjects (Hara et al. 2001). This suggests that variation in the coding region of *PPARA* is unlikely to be a major cause of MODY, at least in Japanese.

While L162V was functionally more active than the wild type (WT) on a PPRE, no such equivalent data has been reported for the V227A. Indeed, although the physiological impact of L162V and V227A is steadily emerging, detailed molecular mechanism of action by these polymorphisms, especially pertaining to the functionality of V227A, remains elusive.

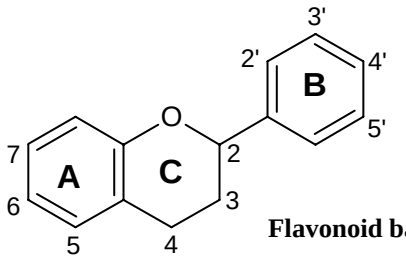
1.7 Flavonoids

Flavonoids are a family of phenolic compounds with strong bioactivity that are present in fruits, vegetables, and herbs. More than 5000 distinct flavonoids have been identified in plants, and several hundreds are known to occur in commonly consumed fruits, vegetables, grains, herbal products, and beverages.

1.7.1 Structure

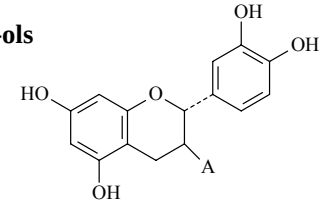
Structurally, flavonoids have a common basic chemical structure that consist of 2 aromatic rings (A and B rings) linked by a 3-carbon chain that forms an oxygenated heterocyclic ring (C ring) (Fig 1.8). Differences in the generic structure of the heterocyclic C ring, as well as the oxidation state and functional groups of the C ring, classify flavonoids as flavonols, flavan-3-ols (flavans), flavanones, flavones, isoflavones and among others (Erdman et al. 2007). Unlike flavones, the isoflavones are characterized by attachment of the B ring at the 3-position instead of the 2-position. The hydroxyl functional groups found on all 3 rings are potential sites for links to carbohydrates. Flavonoids that are bound to 1 or more sugar molecules are known as flavonoid glycosides, whereas those that are not bound to a sugar molecule are called aglycones. With the exception of flavan-3-ols, flavonoids occur in plants and most foods as glycosides. The structural complexity of flavonoids is further increased with the linking of acetyl and malonyl groups to the sugar conjugates. The combination of flavonoid structures, sugars, and acylation contribute to their complexity and the large number of individual molecules that have been identified (Beecher 2003; Erdman et al. 2007).

Figure 1.8 Basic structure of flavonoid and structures of selected flavonoids



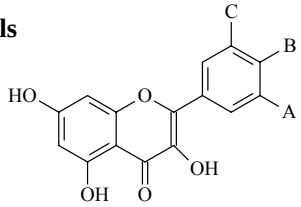
Flavonoid basic structure

Flavan-3-ols



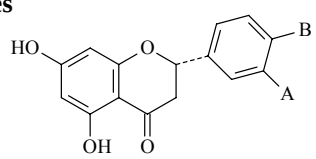
Catechin A= OH
Epicatechin A= OH

Flavonols



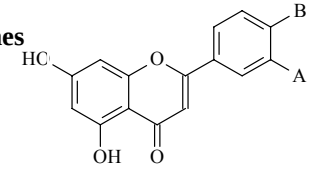
Quercitin A = OH B = OH C = H
Myricetin A = OH B = OH C = OH
Kaempferol A = H B = OH C = H
Isorhamnetin A = OH B = OCH_3

Flavanones



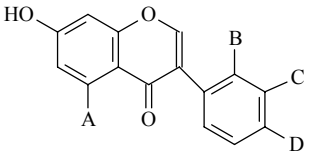
Naringenin A = H B = OH
Hesperetin A = H B = OCH_3
Eriodictyol A = OH B = OH

Flavones



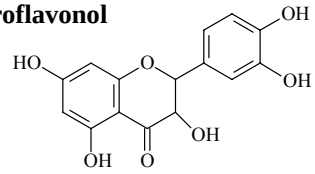
Luteolin A = OH B = OH
Apigenin A = H B = OH
Diosmetin A = OH B = OCH_3

Isoflavones



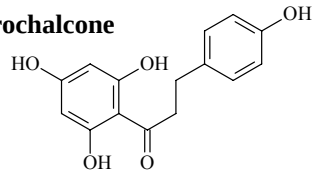
Daidzein A = H B = H C = H D = OH
Genistein A = OH B= H C= H D = OH
Biochanin A A = OH B = H C = H D = OCH_3
2'-hydroxydaidzin A = H B = H C = H D = OH
Formononetin A = H B = H C = H D = OCH_3
Calycosin A = H B = H C = OH D = OCH_3

Dihydroflavonol



Taxifolin

Dihydrochalcone



Phloretin

1.7.2 Source

Flavonols are the most widespread flavonoids in foods, and the most prominent flavonols in food are quercetin and kaempferol (Erdman et al. 2007). Red wine and tea can also contain a significant amount of flavonols. Flavan-3-ols are present in many fruits such as grape products, teas, cocoa, and chocolate. They are either monomers (epicatechin and catechin) or oligomers (e.g., proanthocyanidins). Catechin and epicatechin are the main flavan-3-ols in fruits and cocoa. Flavanones are present in high concentrations in citrus fruits. The main aglycones in citrus are naringenin, hesperetin, and eriodictyol.

Flavones are much less common than flavonols in fruits and vegetables. The prominent flavones in food are luteolin and apigenin. Parsley and celery are the primary food sources. Isoflavones are flavonoids with structural similarities to estrogens. Soy and soybean-derived products are the main sources of isoflavones in the diet. The 3 soybean isoflavones are genistein, daidzein, and glycitein. Typically, more genistein exists in soybeans and soyfoods than daidzein, and glycitein comprises <10% of the total isoflavone content. Isoflavones are naturally present in the soybean primarily in their ß-glycoside form (genistein, daidzein, and glycitein). Soy protein has gained considerable attention for its potential role in improving risk factors for cardiovascular disease and the FDA recommends that an intake of at least 25g of soy protein per day can lower total and LDL-c (Sacks et al. 2006). However, it remains controversial whether the favorable effects of soy protein are related to protein or its isoflavone content (Bhathena and Velasquez 2002).

1.7.3 Isoflavones

1.7.3.1 Consumption, absorption and metabolism

In Western countries, isoflavone intake does not usually exceed 1-3 mg per day although it is somewhat higher in vegetarians consuming soy milk or yogurt and in women treated with isoflavone supplements (Erdman et al. 2007). The mean daily isoflavone intake among Japanese adults ranges from 25 to 50 mg (expressed as aglycone equivalents). Intake in Hong Kong and Singapore is lower than in Japan, although intake in Shanghai appears to be similar to that in Japan (Messina et al. 2006).

Isoflavones exist primarily in plants in the inactive form as glycosides (Bhathena and Velasquez 2002). Once ingested, isoflavone glycosides (genistin and daidzin) are hydrolyzed in the intestines by bacterial ß-glucosidases and are converted to corresponding bioactive aglycones (genistein and daidzein). The aglycones are then absorbed from the intestinal tract and conjugated mainly in the liver to glucuronides, which are either re-excreted through the bile and re-absorbed by enterohepatic recycling or excreted unchanged in the urine. Daidzein may be further metabolized to equol or dihydrodaidzein in the colon. Daidzein, genistein, equol are the major isoflavones that have been detected in the blood and urine of humans. Dihydrodaidzein have also been detected in human plasma (Erdman et al. 2007).

Concentrations of isoflavones and their metabolites in plasma and urine have been reported in several studies of humans and animals. In healthy humans consuming diets without soy, plasma concentrations of isoflavones are usually in the nanomolar range but are increase markedly in the micromolar range after ingestion of enriched isoflavones products (King and Bursill 1998). Plasma isoflavone concentrations of 1–4 μM have been

reported in various population groups consuming foods rich in isoflavones (Adlercreutz et al. 1993; Xu et al. 2000).

1.7.3.2 Effects on lipid and glucose metabolism

Obesity and diabetes mellitus are association with major cardiovascular risk factors (dyslipidemia, atherosclerosis, and coronary artery disease) responsible for excess morbidity and mortality (Bhathena and Velasquez 2002). Insulin resistance is a common feature of obesity and diabetes and is affected by the nature of dietary fat (Yamashita et al. 1998). Thus, diet interventions to control insulin resistance may be one of the means for the management of obesity and diabetes.

Although it remains unclear whether beneficial effects can be attributed to soy protein or isoflavones, a study on postmenopausal women, showed that the consumption of isoflavones, genistein, and daidzein was associated with lower body mass indexes, fasting insulin concentrations and higher HDL-c (Goodman-Gruen and Kritz-Silverstein 2001). Genistein and daidzein also lowered the insulin response to an oral glucose load. Nonetheless, little information is available on the effects of soy in individuals with type 2 diabetes, who are at higher risk due to hyperlipidemia, lower HDL levels, and abnormalities in LDL/lipoprotein composition (Hermansen et al. 2001; Jayagopal et al. 2002). Indeed, in a recent study, soy isoflavones alone do not confer significant cardiovascular protection or positive effects on glycemic control in this group of patients (Gonzalez et al. 2007).

Assessment of the effects on using either soy proteins differing in their isoflavone contents or with isoflavone extracted from red clover on lipidemia in several studies

(Manach et al. 2005) showed that only a small number of trials displayed an overall increase in HDL-c and a decrease LDL-c (Wangen et al. 2001; Sanders et al. 2002; Nestel et al. 2004). In agreement, a recent scientific advisory by the American Heart Association based on the meta-analysis of 19 studies on the lipidemic effects of soy isoflavones reported that the average effect on LDL-c and other lipid risk factors was nil (Sacks et al. 2006). Hence, they attributed the beneficial effects of soy on cardiovascular and overall health was due to their high content of PUFA, fiber, vitamins and minerals and low content of saturated fat. More studies with isolated isoflavones are needed to establish the hypolipemic effects of isoflavone.

1.7.3.3 Molecular mechanism of isoflavones

The mechanisms by which isoflavones exert their beneficial effects on diabetes and obesity are unclear (Bhathena and Velasquez 2002). As a result of their structural similarities to endogenous estrogens, isoflavones, especially genistein, act as weak estrogens and compete with estradiol for binding to ER to modulate gene transcription. Recently, biochanin A was shown to upregulate hepatic apoA-I mRNA expression through an ER dependent pathway (Chan et al. 2007). Isoflavones may also exert their biological effects via non-estrogen receptor-mediated mechanisms by inhibiting the activity of several enzymes, including protein tyrosine kinases, DNA topoisomerase I and DNA topoisomerase II, and ribosomal S6 kinase. Indeed, some of the cellular and metabolic effects of isoflavones may be through both ER and non-ER mediated mechanisms.

Several lines of evidence suggest that isoflavones may favorably affect glucose homeostasis, insulin secretion, and lipid metabolism (Erdman et al. 2007). A large part of these studies was carried out with genistein. *In vitro* studies suggest that genistein and daidzein inhibits glucose absorption in the intestines and also protects against glucose-induced oxidation of LDL (Vedavanam et al. 1999). Genistein has also been shown to have a positive effect on lipid metabolism in the liver and adipose tissue which was accompanied by a decrease in triacylglycerol content, especially in hepatocytes (Nogowski et al. 1998). Genistein was also reported to decrease the number of high-affinity insulin receptors in the livers of ovariectomized rats (Mackowiak et al. 1999). Thus, genistein appears to have direct effects on lipid metabolism in liver and adipose tissue by affecting both lipogenesis and lipolysis. In skeletal muscle cells, genistein was shown to inhibit glucose uptake stimulated by uncoupling protein 3 (Huppertz et al. 2001). Collectively, isoflavones, in particular genistein, may appear to have favorable biological actions on glucose and lipid metabolism that may explain their potential to benefit obesity and diabetes.

1.7.4 Traditional Chinese medicine, a source of flavonoids

Herbal formulations comprising more than one plant extract have been used in traditional Chinese medicine (TCM) for about three thousand years. Interest on their value and therapeutic efficacy has directed the increasing popularity of these natural remedies in recent years (Qiu 2007). TCM is rich in flavonoids and other phenolic compounds (Jung et al. 2006). While there are indications on the therapeutic potential of flavonoids, the rich source of flavonoids in TCM have yet to be fully exploited.

According to its clinical manifestations, diabetes mellitus is categorized as *xiaokezheng* or *xiaodanzheng*, both of which mean diabetes, in TCM. TCM has its own unique understanding in the etiology, diagnosis and therapeutics of a disease condition. However, some components of TCM have particular relevance to Western medicine. *Xiaokezheng* in TCM correlates closely with the western definition of diabetes mellitus in most but not all cases. Indeed, various symptoms associated with diabetes mellitus such as polyuria (excessive urination), polydipsia (excessive thirst), polyphagia (excessive hunger), weight loss are also seen in *xiaokezheng*.

There are about 33 types of herbs most frequently used in Chinese traditional prescriptions for the clinical treatment of diabetes and its complications according to the Chinese pharmacopoeia (Li et al. 2004). However, regardless of the efficacy of their supposed anti-diabetic properties, the mechanisms of action of these herbs are rarely understood. Among these anti-diabetic herbs, the root of *Astragalus membranaceus* and its subspecies are present in the highest number of anti-diabetic herbal formulation. Another species of herbs purported to be anti-diabetic is the *Pueraria spp.* which belong to the same subfamily of Papilionaceae as *Astragalus.*

1.7.4.1 The anti-diabetic herb, *Pueraria Thomsonii*

The *Pureraria* species commonly associated with anti-diabetic properties are *Pueraria lobata* and *Pueraria thomsonii*. They are usually collected in the autumn or winter and are cut into cubes or sliced cross-sectionally. The antihyperglycemic actions of *Pueraria* extracts have been investigated in diabetic rats (Hsu et al. 2003). In addition some of the studies involved its use in conditions affecting menopausal women, such as osteoporosis,

coronary heart disease, and some hormone-dependent cancers (Wang et al. 2003a; Woo et al. 2003). The molecular basis for these may be due to the estrogenic affects of isoflavones isolated from the plant extract such as genistein, daidzein, formononetin and biochanin A (Ososki and Kennelly 2003). One *Pueraria* flavonoid, kakonein, was reported to be effective in lowering blood glucose level of alloxan or adrenalin-induced diabetic mice while another *Pueraria* flavonoid was useful in the treatment of diabetes complications and hyperlipidemia (Li et al. 2004). Nonetheless, detailed mechanistic studies of its bioactive compounds are few. Indeed, despite recommendations by the World Health Organization (WHO), few traditional antidiabetic plants have received scientific or medical scrutiny although this should be undertaken to ensure the safety and efficacy of its applications (Jung et al. 2006).

1.8 Objectives

Drugs activating PPARα and PPARγ such as fibric acid derivatives and thiazolidinediones respectively, have beneficial effects on diabetes mellitus, dyslipidaemia and the associated metabolic syndrome. The search for compounds with dual PPARα and PPARγ activities is an area of intense pharmaceutical activity. Traditional Chinese herbal decoctions and formulations are extremely popular nutritional products consumed for their anti-diabetic properties, largely without any understanding of their mechanisms of action. We hypothesize that small phenolic molecules present in some 'anti-diabetic' botanical foods may activate the PPAR signaling system. Discovery and characterization of putative PPAR-activating compounds would be an important first step towards their possible application in the management of the metabolic syndrome.

Recently, natural variants of PPAR have been shown to be functionally significant and to be important determinants of cardiovascular and metabolic health. V227A affecting the PPARα hinge region was associated with perturbations in plasma lipid levels in a relatively high proportion of selected Asian populations. However, the impact of this variant on the function of PPARα is not known. Furthermore, while the physiological relevance of V227A is slowly emerging, detailed molecular mechanism of action pertaining to its functionality, if any, remains elusive. Indeed, use of V227A as a molecular tool to functionally characterize the significance of PPARα and its interaction with corresponding proteins, such as coregulators, will aid in further understanding of PPARα physiology.

To address the above issues, the objectives of this study were:

1) To identify, isolate and structurally characterize PPAR-active compounds from an anti-diabetic botanical, *Pueraria Thomsonii* (PT), and to characterize their functional effects in relevant cell models.

2) To examine the effects of the V227A variant on PPARα function and to elucidate the molecular mechanisms for any observed effects.

To address our first objective, we isolate the bioactive compound(s) which are PPARα/PPARγ active from purported anti-diabetic herb, *Pueraria Thomsonii* (PT), using a specific chimeric PPAR screening assay. PT, a herb used in the traditional treatment of diabetes and its complications, was identified as the candidate herb after consultation from the Chinese pharmacopoeia. Following the identification of bioactive compounds from this botanical, we will characterize these PPARα/PPARγ-activating compound(s) together with closely related compounds and compared them with currently available reference drugs. A systematic characterization of their transcriptional properties, function in adipocyte differentiation, and the ability to induce endogenous PPAR regulated gene expressions will assess their potential use in managing metabolic diseases.

To address our second objective, we first examine the functionality of V227A variant using various synthetic and endogenous PPARα ligands together with isoflavones characterized from the herb PT on the transactivation of PPARα regulated genes in lipid metabolism. Other than the nature of the ligand, PPARα activity is regulated through the action of AF-1 and/or AF-2, RXR dimerization, protein regulation and coregulator interaction. Thus, a methodical analysis of V227A PPARα activity, in regards to these mechanistic factors, will elucidate the major mechanism behind any observable effects.

Following identification of the major underlying mechanism, we will functionally characterize, in detail, the PPARα and protein interaction involved and the relevance of V227A on PPARα in such context. Finally, we will investigate whether the molecular mechanism elucidated is physiologically relevant at the chromatin level.

Together, the detailed mechanistic study of PPARα V227A with the isolation and characterization of PPAR active compounds isolated from PT will both serve to further our understanding of PPAR physiology.

CHAPTER 2: MATERIALS AND METHODS

2.1 DNA manipulation

2.1.1 Full length PPAR expression plasmids

Full length human PPARα (pSG5-FL-PPARα) and PPARγ (pPSG5-FL-PPARγ) expression vectors used for Sections 3.1 and 3.2 were obtained from Dr W Wahli (Universite de Lausanne, Switzerland).

From Sections 3.3 to 3.5, full length PPARα expression was attained with pcDNA3.1-WT. These plasmids were obtained from Dr Tai E Shyong (Singapore General Hospital, Singapore). In brief, the full length human PPARα cDNA was cloned by RT-PCR from HepG2 cell RNA using primers with BamHI and ApaI sites into pcDNA3.1/ myc-His A (Invitrogen) to generate pcDNA3.1-PPARαWT. The valine at position 227 was mutated into alanine by PCR site-directed mutagenesis using Quikchange kit (Stratagene) according to the manufacturer's instructions to generate pcDNA3.1-PPARαV227A.

2.1.2 Gal-PPAR-LBD expression plasmids

pGal-PPARαWT-LBD (residues 171-468) was constructed by excising pSG5FL-PPARα with BstUI and BamHI and ligating proximally to the DBD of Gal4 of the pM vector (Clontech). pGal-PPARαV227A-LBD was generated through the Quikchange kit using pGal-PPARαWT-LBD as a template. pGal-PPARγ-LBD was constructed by excising pSG5FL-PPARγ2 with RsaI and blunt-end ligating to Hind III site of pM.

2.1.3 UASG-Luc reporter plasmid

The upstream activating sequence (UASG)-luciferase (Luc) reporter gene was constructed by cloning 5 copies of the upstream activating sequence of Gal4 in tandem to a luciferase gene in a pGL3-basic vector containing the basal thymidine kinase promoter..

2.1.4 CYP4A6-PPRE-Luc reporter plasmid

The pCYP4A6-PPRE-Luc was a gift of Dr W.Wahli, University of Lausanne, Switzerland (Krey et al. 1993).

2.1.5 Full length RXRα expression plasmid

pCMX-hRXRα was generated after excision of VP16 and re-ligation of the HindIII site of pCMX-VP16 hRXRα, a gift of Dr Ronald M Evans, The Salk Institute, San Diego, CA (Forman et al. 1995).

2.1.6 HMGCS2-Luc reporter plasmid

The promoter of mitochondria HMG-CoA synthase (-1081 to + 22) was cloned by RT-PCR of HepG2 cell genomic DNA using primers with NheI and HindIII sites into pGL3 basic vector (Promega) to generate pHMGCS2-Luc.

2.1.7 NCoR C-terminal expression plasmid

pSG424-C-terminal NCoR (amino acid 1575- 2453) was a gift of Dr Tetsuya Tamagi, National Hospital Organization, Japan (Tagami et al. 1998).

2.1.8 Full length NCoR expression plasmid

pSG5-NCoR (full length) was a gift of Dr RN Cohen, University of Chicago, Chicago, IL (Yu et al. 2005).

2.1.9 VP16-PPARα expression plasmid

The hinge and LBD (residues 167-468) of PPARα WT or V227A was cloned into the BamHI and HindIII site of pVP16 (Clontech) to give pVP16-WT and pVP16-V227A. The VP16 control plasmid, pVP16-ΔH1-H2, of helices 3 to 12 of PPARαLBD (residues 245-468) was also cloned using the above strategy.

2.1.10 Gal-p300 expression plasmid

Residues 1-117 of E1A binding protein p300 from pCMVb-p300 HA (Addgene) were cloned into the EcoRI of pSG424 to generate pGal-p300.

2.1.11 Gal-SRC-1 expression plasmid

Residues 213-1061 of pCR3.1-hSRC-1A (Jenster et al. 1997) (a gift from Dr BW O'Malley, Baylor College of Medicine, Houston, TX) was cloned into the Sal1 and XbaI of pM vector to generate pGal-SRC-1

2.1.12 Gal-SMRT expression plasmid

pCMX-Gal-C-SMRT, encoding residues 2004-2517, was also gift of Dr RM Evans (Chen and Evans 1995).

2.1.13 Gal-PRIP expression plasmid

The plasmid encoding PRIP (residues 819-1096) was pSG5-Gal4-hRAP250-del4, a gift of Dr JA Gustafsson, Karolinska Institutet, Sweden (Caira et al. 2000).

2.1.14 Gal-TIF2 expression plasmid

Residues 622-869 of TIF2 (Loy et al. 2003) were cloned into the BamHI and MluI site of pM vector.

2.1.15 Gal-PGC1α expression plasmid

Residues 120-284 of pSV- PGC1α (Addgene) was cloned into the BamHI and MluI site of pM vector.

2.1.16 Gal-NCoR truncation expression plasmids

Gal-NCoR truncated mutants in Fig. 3.5.4 were generated by cloning respective fragments into the EcoRI and SalI sites of the pM vector. GST-NCoR mutants (GST-G1 to GST-G3 in Fig. 3.5.5) was generated by subcloning each NCoR truncated mutant into pGEX-4T-1 (Invitrogen).

2.1.17 VP16-PPARα truncation expression plasmids

VP16-PPARα truncated mutants in Fig. 3.5.6 were generated by cloning respective fragments into the BamHI and HindIII sites of the pVP16 vector.

2.2 Materials and reagents

WY14,643 was purchased from Tocris. [^{3}H]-WY14,643 was purchased from American Radiolabeled Chemicals, Inc. Pioglitazone was a gift from Takeda Chemical Industries.

Biochanin A, genistein, formononetin, calycosin, daidzein, α-linolenic acid, linoleic acid, fenofibrate and trichostatin A (TSA) from Sigma. Flavonoids were kind gifts from Dr. Andrew M. Jenner (Department Biochemistry, National University of Singapore). Additional diosmetin was purchased from Chromadex. Voucher specimens of *Pueraria thomsonii* (SBG-PT-040720) were deposited in the Singapore Herbarium, Botanical Gardens, National Parks Board.

2.3 Cell culture

Preadipocyte 3T3- L1 cells, HepG2 and HeLa cells were obtained from American Type Culture Collection. 3T3-L1 cells were cultured in DMEM. HepG2 and HeLa cells were cultured in minimum essential medium, Eagle modified (Sigma) with 2mM L-glutamine, 1.5g/L sodium bicarbonate, 0.1mM non-essential amino acids, 1mM sodium pyruvate and charcoal-treated fetal bovine serum, 10%.

2.4 Transient transfection and reporter gene assay

Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) or GenePORTER 2 (GTS) according to manufacturer's instructions. Briefly, cells were cultivated in charcoal treated medium for 3 days before seeding at 40,000 cells/well in 24 well microtiter plates and incubated for 24h before transfection. Transfected cells were then exposed to test samples in charcoal treated medium. In reporter gene bioassays, cells were lysed post treatment with 100µl of M-Per (Pierce) per well for 5 min. Luciferase activity was then measured using Glomax 96 Microplate Luminometer (Promega). Experiment results

obtained from reporter gene assays were re-confirmed with measurements of either renilla luciferase (Promega) or β-Gal (Promega) to ensure similar transfection efficiency.

2.5 Ligand binding assay

2.5.1 PPARγ competitor binding assay

A PPARγ competitor assay (PolarScreen, Invitrogen) was applied to evaluate the binding affinity of individual isoflavones to PPARγ -LBD. The assay was performed according to the manufacturer's instructions. Briefly, recombinant PPARγ-LBD was added to a fluorescent PPARγ ligand (fluormone PPAR-Green) to form a PPARγ-LBD/fluormone complex with a high polarization index. This complex was added to individual test samples in 96-well plates, incubated at room temperature for 2 h and polarization values measured with Tecan Ultra 384 fluorescence polarization plate reader. PPARγ-specific ligands in test samples displaced the fluorescent fluormone from the PPARγ-LBD fluormone complex, resulting in lower polarization values. Assays were conducted in triplicate and data presented as means ± SEM. Curve fitting was performed using GraphPad Prism Software.

2.5.2 Radiolabelled competitive binding assay

Competitive PPARα ligand binding assay was performed as previously described (Lim et al. 2000). Briefly, HeLa cells in 24 well microtiter plates were transfected with pcDNA3.1-PPARαWT or PPARαV227A and incubated in 3 nM of [^{3}H]-WY14,643 with

increasing doses of unlabeled WY14,643 at 37°C. The amount of radiolabelled WY14,643 specifically bound was measured after 24h incubation.

2.6 Adipocyte differentiation assay

Murine fibroblast or preadipocyte 3T3-L1 cells were seeded at a density of 40,000 cells/well in 24-well plates and cultured to confluency for 2 days in DMEM. Post-confluent preadipocytes were exposed to induction medium containing 10% charcoal dextran-stripped serum, insulin (5 mg/l), dexamethasone (1 μM), and 3-isobutyl-1-methylxanthine (0.5 μM). Induction medium was removed after 2 days; cells were washed 3 times, and exposed to increasing doses of test compounds for 8 days. Medium was replenished with appropriate ligands every 2 days. After treatment, cells were washed with PBS, fixed with 4% paraformaldehyde, and lipid droplets were stained with 0.5% oil-red O in 60% isopropanol. Stained cells were examined under phase-contrast photomicrography, and a minimum of 3 experiments were performed in duplicate.

2.7 Western analysis

Immunoblotting of samples obtained from co-immunoprecipitation assays or total cell lysates were performed according to standard western blotting protocol, using anti-PPARα rabbit polyclonal antibody (Santa Cruz), anti-PPARγ mouse monoclonal antibody (Santa Cruz), anti-NCoR rabbit polyclonal antibody (Affinity Bioreagents) and anti-β-actin mouse monoclonal antibody (Sigma).

2.8 Reverse transcription polymerase chain reaction (RT-PCR)

2.8.1 Real time RT-PCR

Post-confluent 3T3-L1 cells or HepG2 cells were exposed to ligands before total RNA were isolated with Qiagen's RNAeasy Kit. Reverse transcription of RNA was carried out with SuperScript III Reverse Transcriptase (Invitrogen) following manufacturer's protocol with random primers. Quantitative PCR amplification and detection were performed with TaqMan Universal PCR MasterMix according to the manufacturer's protocol on ABI Prism 7000 Sequence Detection System (Applied Biosystems) The gene expression assay kits for individual genes were as follow: PPARα (Hs00231882_m1), CPT1A (Hs001577079_m1), HMGCS1 (Hs00266810_m1), HMGCS2 (Hs00266810_m1), PPARγ (Mm00440945_m1), aP2 (Mm00445880_m1), adiponectin (Mm00456425_m1) and 18S as internal control. Results are presented as the fold increase compared to vehicle. Values are the mean ± S.E. of two independent experiments.

2.8.2 Duplex semi-quantitative RT-PCR

HepG2 cells were transfected with pcDNA3.1-PPARαWT or –PPARαV227A and treated in the absence or presence of WY14,643. RNA was extracted from treated cells using RNeasy Mini Kit (Qiagen) post treatment. Two μl of cDNA was subjected to PCR with specific primer pairs that match bp 80-99 (PPARα forward, 5'-TCCTGCAAGAAATGGGAAAC-3') and bp 474-493 (PPARα reverse, 5'-CGACAGAAAGGCACTTGTGA-3') of the human PPARα sequence or; bp 143-162 (HMGCS2 forward, 5'-ATACTTGGCCAAAGGACGTG-3') and bp 604-623 (HMGCS2

reverse, 5'-GTGGGACGAGCATTACCACT-3') of the human HMGCS2 sequence using the QuantumRNA^{TM} 18S internal standard kit (Ambion). The amplicons were resolved on 2% agarose electrophoresis and image was captured by Alpha DigiDoc. Bands were quantified using Scion Image and the expression levels of HMGCS2 gene transcript expressed as ratios against 18S rRNA internal control.

2.9 Immunoflourescence

HepG2 cells seeded on cover-slips in 24 well microtiter plates were transfected with pcDNA3.1-PPARαWT or V227A and treated in the absence or presence of WY14,643. At 24 h post treatment, the cells were fixed with cold absolute methanol for 10 min, followed by the incubation with anti-PPARα rabbit polyclonal antibody (Santa Cruz) (1:400) in a humidity chamber for 1 h at 37^{o}C. After washing, the cell monolayer on the cover-slips was incubated with secondary antibodies conjugated with FITC (Amersham Pharmacia Biotech) (1:600). Optical immuno-fluorescence microscope (Olympus IX-81) was used to view the specimens at 100X magnification, excitation wavelengths of 480 nm, for FITC using oil immersion objectives. The nucleus was stained with DAPI.

2.10 siRNA knockdown

BLOCK-iT™ Lentiviral RNAi Expression System (Invitrogen) was used according to the manufacturer's instructions to produce lentiviruses for gene silencing of NCoR in HepG2 cells. Double-stranded oligonucleotide encoding the shRNA of NCoR (siNCoR) (Top strand: 5' CACCGCTGAAGAGGGTTCTGTTTGTCGAAACAAACAGAACCCTCTTC

AGC 3'; Bottom strand: 5' AAAAGCTGAAGAGGGTTCTGTTTGTTTCGACAAACA GAACCCTCTTCAGC 3') were synthesized and oligo-duplexes were cloned into a lentivirus expression vector (pLenti6/BLOCK-iT^{TM}-DEST). The expression plasmid and other virus packaging plasmids were cotransfected into 293FT cells to generate replication-defective lentivirus. The resulting lentivirus was then used to infect HepG2 for gene silencing of NCoR. Successful knockdown of NCoR was assessed using western blotting. A randomized sequence (siScram) (Forward: 5' CACCAGGATGCAACACGTGGAATAGCGAACTATTCCACGTGTTGCATCCT 3'; Reverse: 5' AAAAAGGATGCAACACGTGGAATAGTTCGCTATTCCACGTGTTGC ATCCT 3') not targeting any known human transcript sequences was used as a control.

2.11 Glutathione-S-transferase (GST) pull down

GST fusion proteins were expressed in BL21-DE3 *E. coli* by induction with 0.4 mM isopropylthio-β-d-galactosidase at 37 °C. Proteins were isolated after disruption through sonication and affinity purified on glutathione-Sepharose 4B beads (Amersham Pharmacia) according to manufacturer's protocol. WT and VA PPARα were *in-vitro* translated in reticulocyte lysate (Promega) using T7 polymerase, according to manufacturer's protocol. 25µl of *in vitro* translated proteins was incubated with equal amounts of GST fusion proteins overnight at 4°C. Following this, GST fusion protein complexes were affinity purified with 50µl of glutathione-Sepharose 4B beads for 2h at 4°C. After extensive washing, bound proteins were eluted by boiling in 2X loading buffer and analyzed by Western blotting for PPARα .

2.12 Immunoprecipitation (IP)

HepG2 cells grown on 100 mm plates were transfected with pcDNA3.1-PPARαWT or V227A and treated in the absence or presence of WY14,643. At 24h post treatment, the cells were washed with cold PBS and lysed using RIPA buffer. Following centrifugation at 1500g for 15min, the supernatants were precleared with Protein A/G PLUS agarose beads (Santa Cruz) for 30min and incubated with 2μg/ml anti-PPARα rabbit polyclonal antibody at 4°C overnight. Following this, the antibody-protein complex was pulled down using A/G agarose beads and washed 5 times with RIPA buffer before elution of bound proteins by boiling in 2X loading buffer and analyzed by Western blotting for NCoR or PPARα .

2.13 Chromatin immunoprecipitation (ChIP)

HepG2 cells grown on 100 mm plates were transfected with pcDNA3.1-PPARαWT or V227A and treated in the absence or presence of WY14,643. At 24h post treatment, the cells were treated with formaldehyde to cross-link proteins to DNA and ChIP performed using the ChIP Assay Kit (Upstate) according to manufacturer's instructions. ChIP was performed using the following antibodies at 2μg/ml: normal rabbit IgG, PPARα, SRC-1, NCoR and p300 (Santa Cruz). Primers used to amplify the PPRE region (-198 to -34) of the mitochondria HMG-CoA synthase promoter are: Forward 5' CAGCCATTCCCAC ACATGCTCA 3'; Reverse 5' CAGACTTTATAAAGCCCCAAGACT 3'). In ChIP-re-ChIP experiments, PPARα ChIP complexes were eluted by incubation for 30 min at 37 C in 50 μl 10mM dithiothreitol. After centrifugation, the supernatant was diluted 20 times

with re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) and subjected again to the ChIP procedure using NCoR antibody.

2.14 Isolation and structural characterization of bioactive compounds

2.14.1 Solid-liquid chromatography separation

Ten grams of the ethyl acetate *Pueraria thomsonii* (PT) extract was dry packed with 15g of silica gel (LiChroprep Si 60 40~63 μm, Merck) and applied onto the top of a Medium Pressure Liquid Chromatography (MPLC) (Buchi Chromatography B-680) glass column packed under pressure with 350g silica gel (LiChroprep Si 60 15~25 μm, Merck). The column was successively eluted using mixtures with increasing polarity as follows: 99:1, 98:2, 97:3,...,90:10, 80:20,…,10:90, 0:100, and in the end washed with methanol.

2.14.2 High Pressure Liquid Chromatography (HPLC), Mass Spectrometry (MS), Nuclear Magnetic Resonance (NMR) spectroscopy

The HPLC chromatogram of compounds was by performed using a reverse-phase gradient run in Shimadzu LC2010A HPLC system. The column used was Waters Nova-Pak C18 (3.9 x 150mm). The UV wavelength detector was set at 254nm. The MS and NMR spectroscopy was performed by the Chemical and Molecular Analysis Centre, Department of Chemistry, NUS.

2.15 Statistical analysis

For Sections 3.1 to 3.2, all luciferase experiments were performed at least 3 times on separate occasions. Statistical analyses were conducted using SPSS 13.0 (SPSS Inc.). One-way ANOVA was used to determine dose-dependent activities of PPAR ligands, active herbal extracts, and differential effects of isoflavones on PPARα/PPARγ. For multiple comparisons with the vehicle group, Dunnett's post-hoc test was used. For multiple pair-wise group comparisons, Bonferroni adjustments were applied. Repeated measurement analysis over concentration levels between AM and PT was also performed with Bonferroni correction.

For Sections 3.3 to 3.5, all luciferase experiments were done in duplicates or triplicates at least three times on separate occasions. The difference between WT and V227A at each treatment was analyzed by 2 sample t-test. Adjustment for type 1 error due to multiple comparisons was done by Bonferroni procedure on groups with a significant difference.

CHAPTER 3: RESULTS

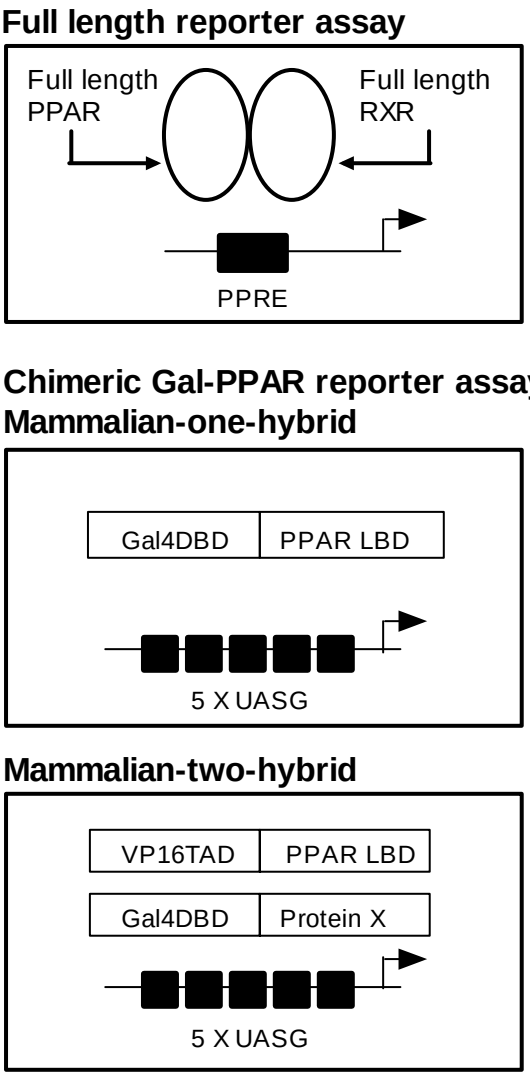
3.1 Discovery of PPAR bioactive flavonoids from the anti-diabetic herb, *Pueraria thomsonii* (PT)

3.1.1 Evaluation of a chimera Gal-PPAR reporter gene bioassay

To isolate PPAR active compounds from the anti-diabetic herb, *Pueraria thomsonii* (PT), a specific and sensitive screening assay was needed. One possible screening method is through cotransfection of the full length PPAR receptor with a PPRE driven reporter gene into human cell lines (Fig 3.1.1A, top panel). An agonist will induce the reporter gene signal in such an assay. However, transcription induced by PPAR and its obligate partner, RXR, on PPAR target genes can be due to the response of either PPAR or RXR agonists alone or in combination (Kliewer et al. 1992; Gearing et al. 1993; Keller et al. 1993). Thus, a full length PPAR reporter assay is not a specific tool for PPAR ligand screening.

Alternatively, the chimeric receptor consisting of the PPAR LBD and the yeast Gal4 DBD will allow for specific screening of PPAR ligands only. This mammalian-one-hybrid assay (Fig 3.1.1A, middle panel) is a variation of the mammalian-two-hybrid assay (Fig 3.1.1A, bottom panel). While the mammalian-two-hybrid assay characterizes protein-protein interactions, the mammalian-one-hybrid assay is used to characterize DNA-protein interactions. However, both hybrid assays work on the same principle that in most eukaryotic transcription factors, the activation domain and DBD are modular and can function in close proximity to each other without direct binding (Verschure et al. 2006). The DBD is responsible for binding to DNA and the activating domain is responsible for activation of transcription. In this study, the yeast Gal4 DBD was chosen because it binds strongly to its response element, the upstream activating sequence of galactosidase (UASG), and thereby giving rise to a sensitive assay. AF-2 on helix 12 of

Figure 3.1.1A Schematic representation of PPAR reporter gene bioassays



PPAR (Nolte et al. 1998) provides the activation module in this mammalian-one-hybrid assay. Since activity of AF-2 is dependent on ligand binding, it allows transcription activation of the chimera only in the presence of a ligand, and thereby conferring low background and added sensitivity to the assay. The resulting chimera protein when activated by a PPAR ligand, will bind to UASG and activate the transcription of the luciferase reporter gene to indicate the presence of a specific PPAR ligand. Therefore, to differentiate the ligands of PPAR from that of its heterodimeric partner RXR, we employed the specific and sensitive chimera Gal-PPAR reporter gene bioassay.

To determine the specificity and sensitivity of the chimera Gal-PPAR reporter gene bioassay, we cotransfected chimeric Gal-PPARα-LBD or Gal-PPARγ-LBD together with the UASG-Luc reporter gene in a human cell line expressing low levels of PPAR, HeLa cells. The PPARα specific ligand, WY14,643, dose-dependently activated the chimeric Gal-PPARα construct, with a 50% effective concentration (EC50) of 5μM and a maximal activity 14-fold of the vehicle (Fig. 3.1.1B). Similarly, the PPARγ specific ligand, pioglitazone, increased the activity of the Gal-PPARγ system at a maximum of 40 fold of the vehicle with an EC50 of 3μM (Fig. 3.1.1C). There was minimal cross-reaction between pioglitazone and WY14,643 with PPARα and PPARγ, respectively, in this chimera Gal-PPAR reporter gene bioassay, indicating that this chimera bioassay could be used as a tool to detect PPAR activity of unknown compounds from botanicals.

3.1.2 PPAR activity of PT extract

Ethanolic extracts of botanicals with purported anti-diabetic properties were screened for PPAR activity using the evaluated chimeric Gal-PPAR reporter-gene bioassay in our

laboratory. PT, together with *Astragalus membranaceus* (AM) significantly stimulated both PPARα and PPARγ. PT was also more selective for PPARα. Thus, this project was focused on PT[1].

A dose response curve to quantify the PPAR activity of PT extract was carried out using the Gal-PPAR reporter gene bioassays. PPARα activity of PT was first observed at a concentration of 10μg/ml, rising to a peak at 300μg/ml (Fig 3.1.2). The PPARα activity of PT at its peak was 100% of the maximal activity of WY14,643, a PPARα agonist. The EC50 for PT was ~100μg/ml. PT extract also activated PPARγ. PPARγ activity of PT was first observed at a concentration of 30μg/ml, rising to a peak at 300μg/ml. However, the PPARγ activity of PT at its peak was only 30% of the maximal activity of 15dPGJ2, a natural PPARγ agonist.

Therefore, PT extract from concentrations 30 to 300μg/ml was a dual PPARα/PPARγ activator and also a strong activator of PPARα.

[1] PPAR activity of AM was characterized by other laboratory members.

Figure 3.1.1(B,C) Dose response curves of chimera Gal-PPAR reporter gene bioassays

Using the mammalian-one-hybrid system (inset), HeLa cells were cotransfected with (B) Gal-PPARα or (C) Gal-PPARγ (25ng) and the $(UASG)_5$-Luc reporter gene (250ng) and exposed to WY14,643 or Pioglitazone for 48hr as indicated. Data points (mean±SEM, n=3) represented fold increase in luciferase activity over vehicle. Means without a common letter differ, $p<0.05$.

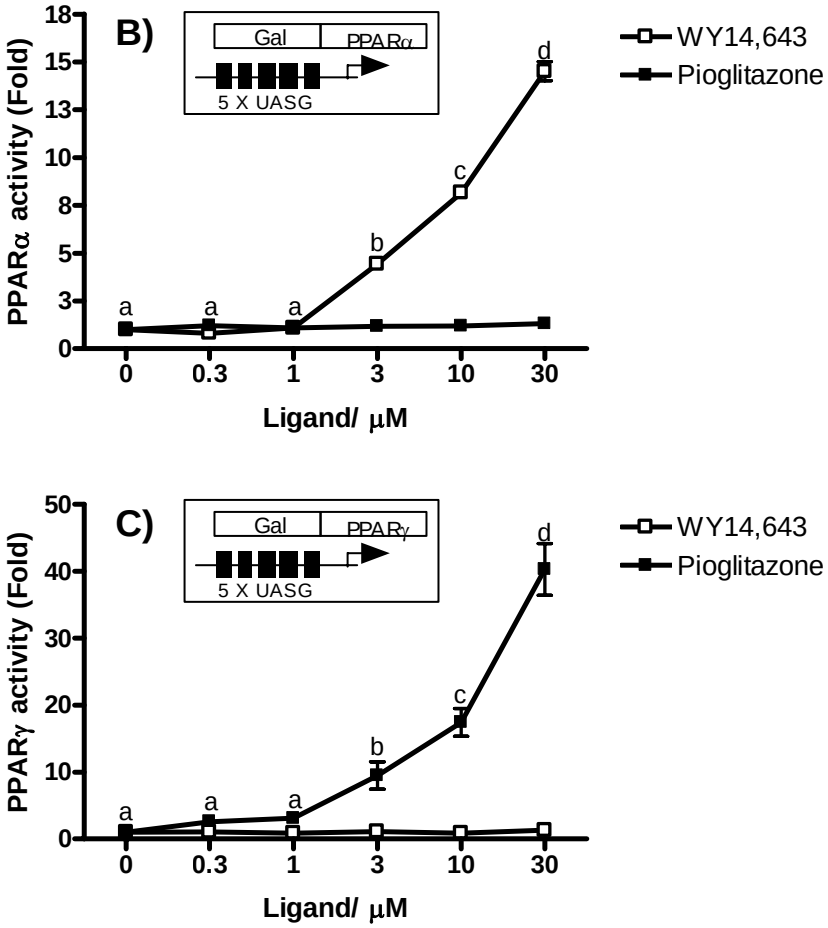
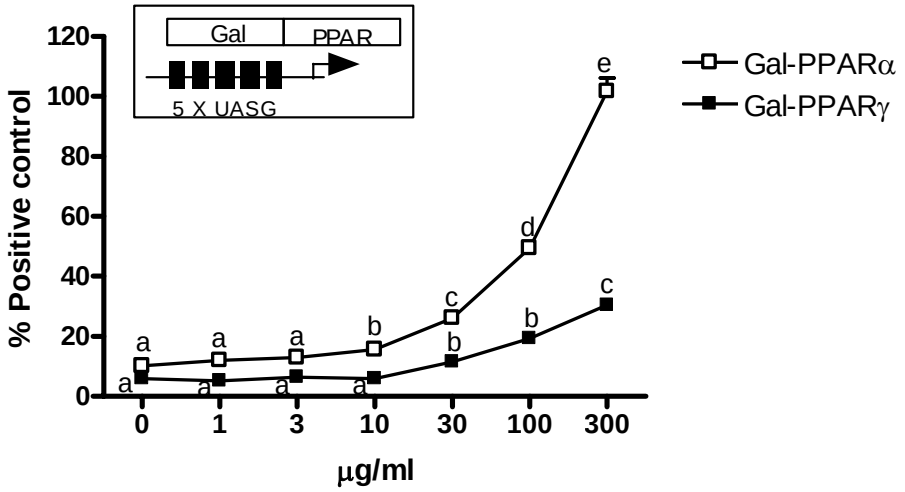


Figure 3.1.2 Effects of *Pueraria thomsonii* (PT) extract on chimera Gal-PPAR reporter gene bioassays

HeLa cells cotransfected with Gal-PPARα or Gal-PPARγ (25ng) and the $(UASG)_5$-Luc reporter gene (250ng) were exposed to PT for 48hr as indicated. Data points (mean±SEM, n=3) represented percentage of the positive controls for PPARα (WY14,643, 30μM) and PPARγ (15dPGJ2, 3μM). Means without a common letter differ, $p<0.05$.

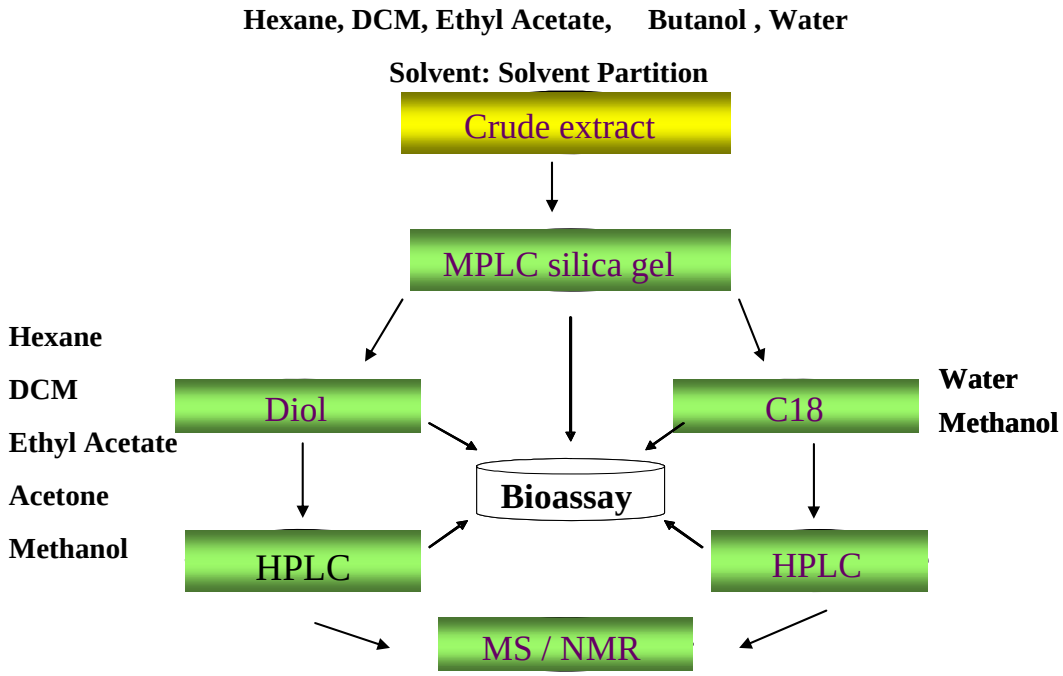


3.1.3 Herbal extraction and bioassay guided fractionation

To understand the molecular basis of the PPAR activity of PT, we performed bioassay-guided fractionation of the crude extracts to isolate the PPAR active components.

A strategy in which the crude extract was sequentially separated with organic solvents of decreasing polarity (Ethyl acetate < Hexane < DCM < Butanol) through solvent-solvent partition was employed (Figure 3.1.3). Solvent layers were assayed for PPARα and PPARγ activity. Active layers identified were then loaded onto a medium pressure liquid chromatography (MPLC) glass column packed with a silica gel matrix. The column was eluted using mixtures of non-polar (hexane) and polar (acetone) solvents. Fractions were collected and assayed for PPARα and PPARγ activity. After which, active fractions were combined and further separated using high pressure liquid chromatography (HPLC) with either Diol or C18 columns with solvents of varying polarity. Active fractions were identified through bioassays, and crystallized. Finally, the identities of the active compounds were confirmed through mass spectrometry (MS) and nuclear magnetic resonance (NMR).

Figure 3.1.3 Schematic representation of strategy for bioactive compounds identification through bioassay guided fractionation



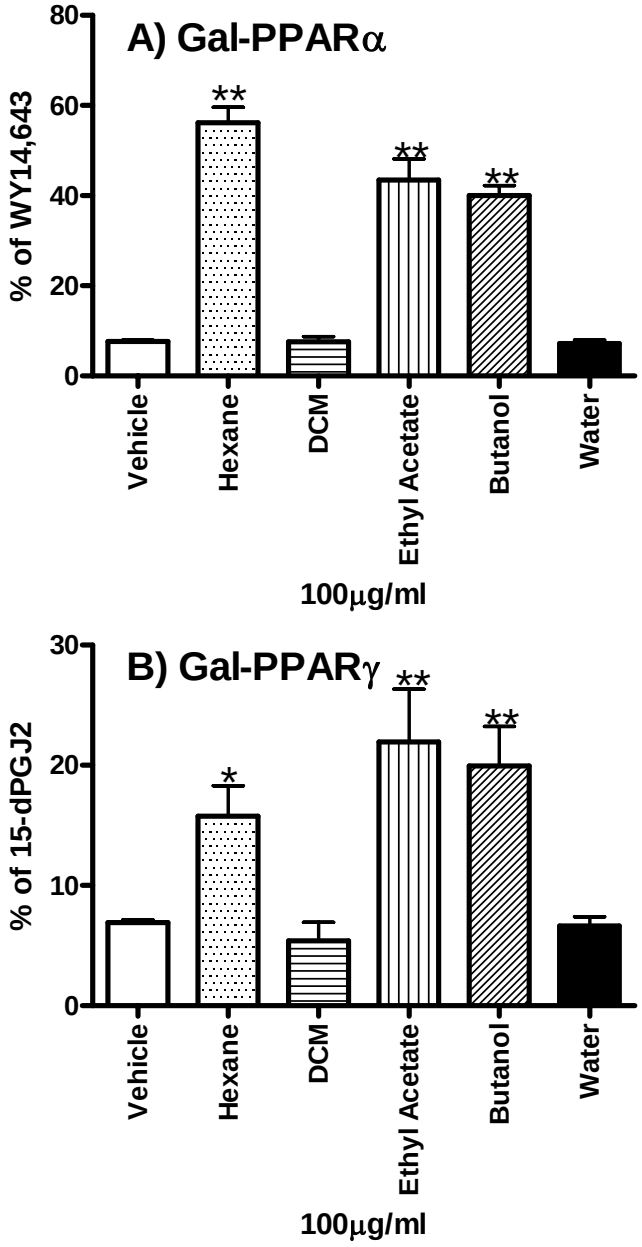
3.1.4 Solvent selection for PT extraction

Using the above bioassay guided strategy, we first established the optimal solvent for extraction of PPAR active compounds from PT. Combinations of hexane/water, DCM/water, ethyl acetate/ water, butanol/water and water alone, were used. These 5 different solvent extracted layers of PT were tested for PPAR activity using the chimeric Gal-PPAR reporter-gene bioassay. Extraction with 100% hexane, 100% ethyl acetate and 100% butanol significantly stimulated both PPARα (Fig. 3.1.4A) and PPARγ (Fig. 3.1.4B) at a dose of 100μg/ml. In contrast, extraction with 100% DCM and water alone exhibited minimal PPAR activity. For all three active solvent layers (hexane, ethyl acetate and butanol), PPARα activity was ~50-60% and PPARγ activity was ~20%. However, cell toxicity was observed to be ~80% and ~20% for the hexane and butanol layers respectively. Minimal cell toxicity was observed only in the ethyl acetate layer.

Since extraction with 100% ethyl acetate resulted in one of the highest PPARα and PPARγ activity and was at the same time, not toxic to cells, subsequent studies were focused on the ethyl acetate layer of PT.

Figure 3.1.4 PPAR activity of solvent extracted fractions of PT

HeLa cells cotransfected with (A) Gal-PPARα or (B) Gal-PPARγ (25ng) and the $(UASG)_5$-Luc reporter gene (250ng) were exposed to each solvent fraction at a final concentration of 100μg/ml for 48hr. Data points (mean±SEM, n=3) represented percentage of the positive controls for PPARα (WY14,643, 30μM) and PPARγ (15-dPGJ2, 3μM). Compared to vehicle; *, $p<0.05$; **, $p<0.01$.



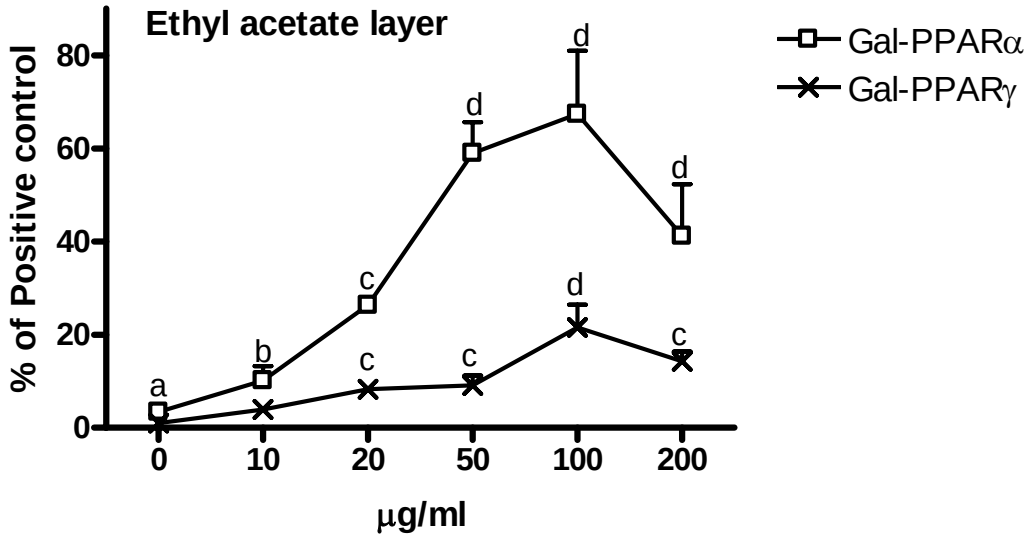
3.1.5 PPAR activity of PT ethyl acetate layer

PT was identified as a PPAR active herb from screening experiments performed previously. A dose response curve to validate its activity after 100% ethyl acetate extraction was carried out using the Gal-PPAR reporter gene bioassays. PPARα activity of PT was first observed at a concentration of 10μg/ml, rising to a peak at 100μg/ml (Fig 3.1.5). The PPARα activity of PT at its peak was 60% of the maximal activity of WY14,643. The EC50 for PT was ~50μg/ml. Ethyl acetate PT layer also activated PPARγ. PPARγ activity of PT was first observed at a concentration of 10μg/ml, rising to a peak at 100μg/ml. However, the PPARγ activity of PT at its peak was only 20% of the maximal activity of pioglitazone.

Therefore, from concentrations 10 to 100μg/ml, the ethyl acetate extracted layer of PT was a dual PPARα and PPARγ activator and was a strong activator of PPARα.

Figure 3.1.5 Dose response of ethyl acetate PT layer on chimera Gal-PPAR reporter gene bioassays

HeLa cells cotransfected with Gal-PPARα or Gal-PPARγ (25ng) and the $(UASG)_5$-Luc reporter gene (250ng) were exposed to PT ethyl acetate fraction for 48hr as indicated. Data points (mean±SEM, n=3) represented percentage of the positive controls for PPARα (WY14,643, 30μM) and PPARγ (Pioglitazone, 30μM). Means without a common letter differ, $p<0.05$.



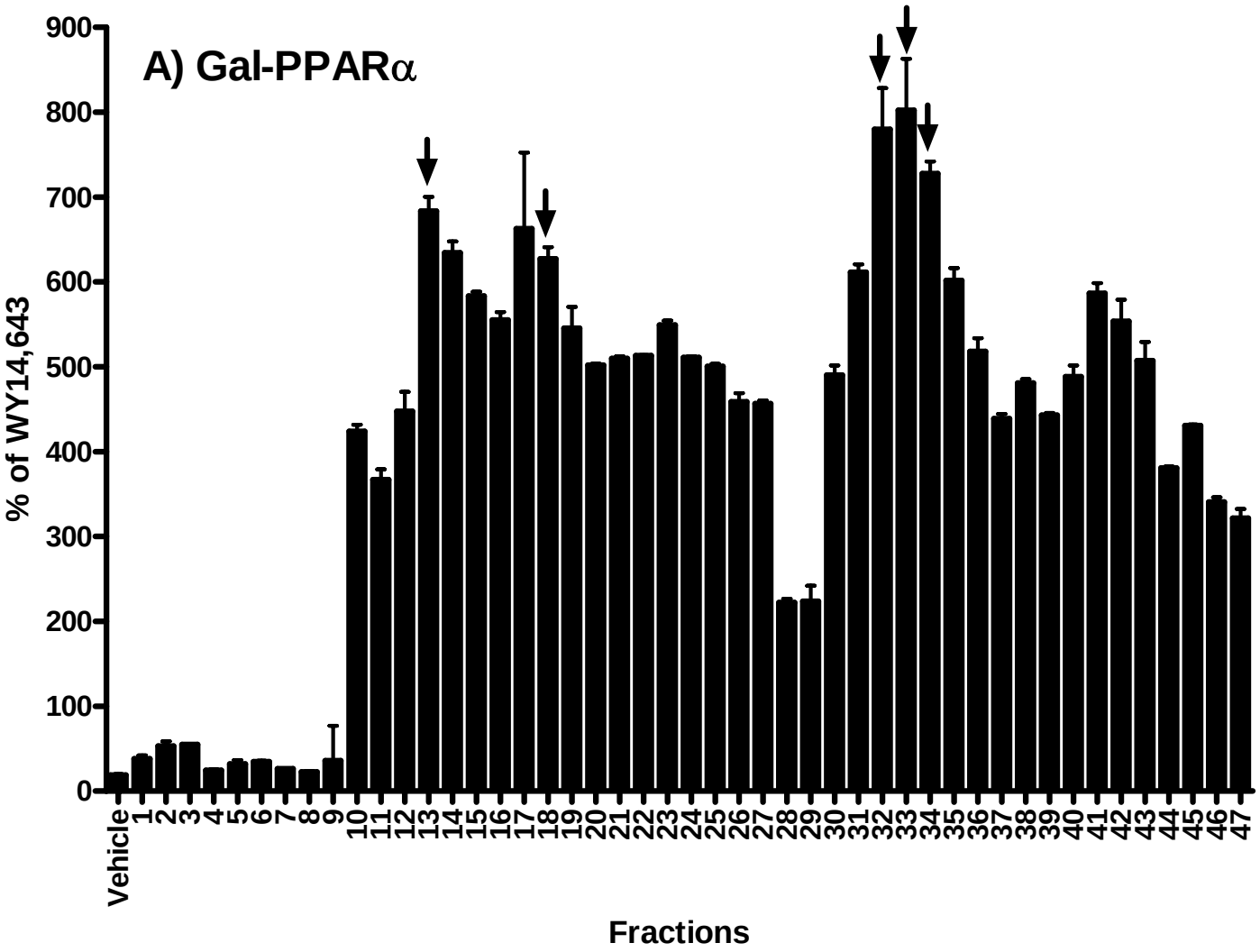
3.1.6 PPAR activity of MPLC separated fractions from PT

To identify the bioactive compound responsible for PPAR activation in the ethyl acetate layer of PT, bioassay guided fractionation was employed. Preliminary fractionation was first performed on 10g of the ethyl acetate PT layer which was dry packed with 15g of silica gel and loaded onto the MPLC. The column was eluted using mixtures of hexane and acetone with increasing polarity and in the end washed with methanol. 47 fractions were collected and assayed for PPARα activity. Majority of the PPARα active compounds were eluted in Fractions 10-27 and 30-47 (Fig 3.1.6A). Among these fractions, major peaks of PPARα activity were observed in Fractions 13, 18 and between Fractions 32 to 34. A majority of PPARγ bioactive compounds were also eluted in Fractions 10-27 and 30-47 (Fig 3.1.6B). Since the final aim was to identify PPARα/PPARγ dual agonists, Fractions 1 to 10 were not tested for PPARγ bioactivity as they showed minimal PPARα activity previously (Fig 3.1.6A). Among these fractions, major peaks of PPARγ activity were observed in Fractions 17, 23, 41 and, again, between Fractions 32 to 34.

To identify PPAR active compounds from PT which are also dual PPARα/PPARγ agonists in nature, active Fraction 32 was chosen for further separation using HPLC.

Figure 3.1.6 MPLC separation of PT ethyl acetate extract using silica as the solid phase matrix

Ten grams of PT ethyl acetate extract was dry packed onto a MPLC glass column containing silica gel. The column was successively eluted using mixtures of Hexane and Acetone. Fractions collected were dried, reconstituted to 20mg/mL and tested for (A) PPARα and (B) PPARγ activity using the chimera Gal-PPAR reporter gene bioassay system at a final concentration of 20μg/mL for 48hr. Data points (means±SEM, n=3) represented percentage of the positive controls for PPARα (WY14,643, 30μM) and PPARγ (Pioglitazone, 30μM). Black arrows indicate major peak of PPAR activity.


A) Gal-PPARα
% of WY14,643
900
800
700
600
500
400
300
200
100
0
Vehicle
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
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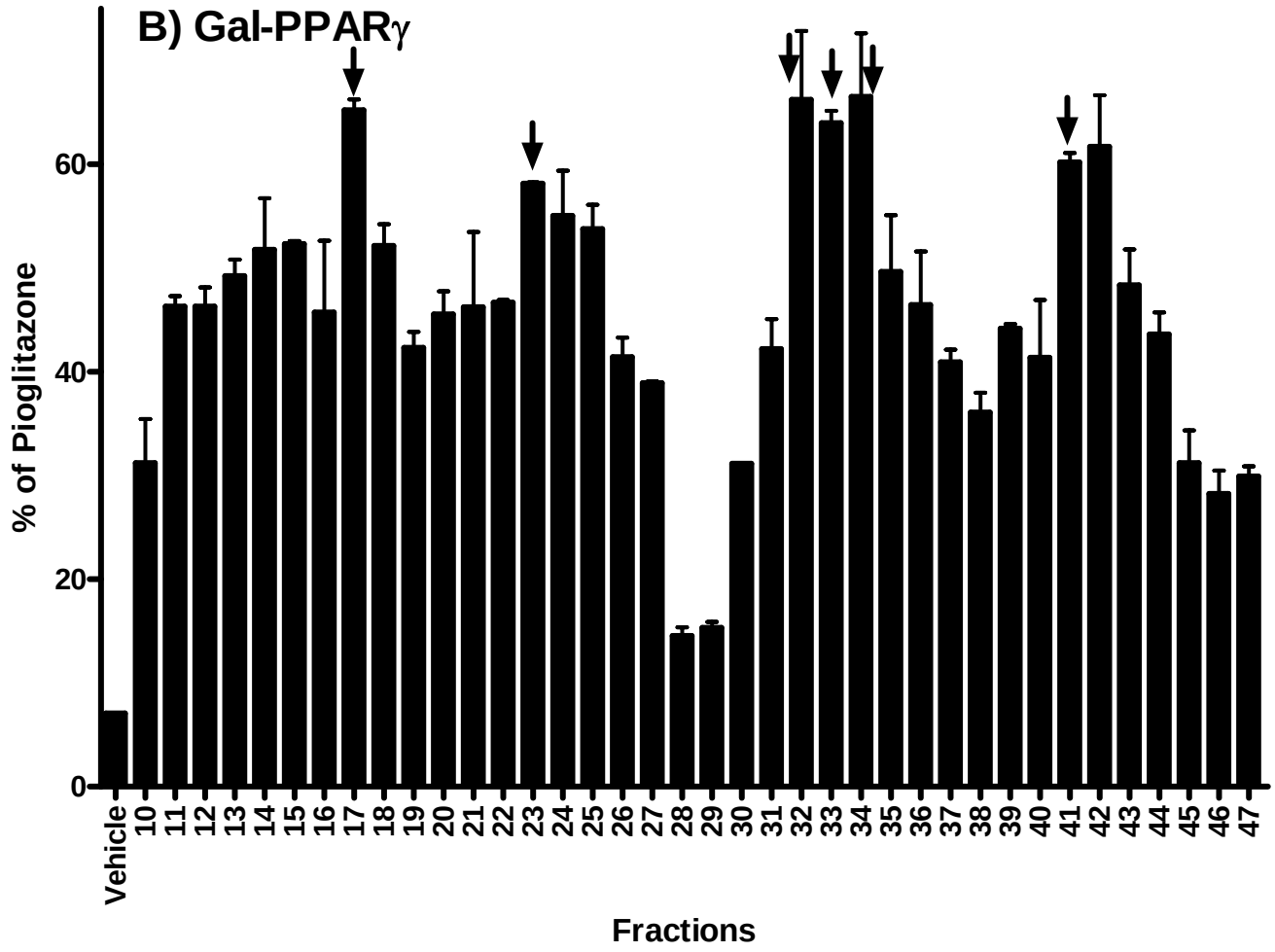
38
39
40
41
42
43
44
45
46
47
Fractions


B) Gal-PPARγ
% of Pioglitazone
0
20
40
60
Vehicle
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
Fractions

3.1.7 PPAR activity of fractions further separated by HPLC

The dual PPARα/PPARγ active, MPLC separated Fraction 32 of PT was further fractionated by HPLC, using a normal phase Diol column, with DCM (100%-50%) and ethyl acetate (0%-50) as the mobile phase. Fractions A to S were collected, dried, and reconstituted to 20mg/mL. Among these fractions, Fraction Q exhibited strong dual PPARα/PPARγ and was present in sufficient quantities to be isolated at 97% purity. Structural characterization with mass spectrometry and NMR indicated that it is daidzin.

3.1.8 Bioactive compounds from PT

Besides daidzin, the dual PPARα/PPARγ active Fraction 32 was also fractionated by another HPLC method, using a reverse phase C18 column, with methanol (100%-50%) and water (0%-50) as the mobile phase (Table 3.1). Of the 11 fractions obtained, sufficient quantities were present in the third fraction to be isolated at 96% purity. Structural characterization with mass spectrometry and NMR indicated that it was genistin.

Two types of HPLC columns were utilized because C18 is able to separate compounds of higher polarity, while diol separates compounds of mid range polarity. Together, both C18 and diol can reversibly bind to a broad range of organic compounds which elute with a range of solvents according to their polarity. Such separation enables selective removal and concentration of organic compounds from complex mixtures. By controlling the elution sequence of the compounds retained on a matrix, biologically active components can be isolated from the fractions collected. From Fraction 32, daidzin

and genistin were isolated from PT through such strategy. Furthermore, because daidzin and genistin was isolated form diol and C18 respectively, this suggests that genistin is more polar than daidzin.

Similar strategies were employed for Fraction 13, 18 and 34 and daidzein, 2'hydroxy-daidzein and puerarin were identified from these fractions respectively.

Figure 3.1.7 PPAR activity of fractions from HPLC separation of PPARα active Fraction 32 with a Diol column

PPAR active fraction 32 of MPLC separated ethyl acetate extracts of *Pueraria thomsonii* (PT) were combined and further separated by HPLC using DCM and ethyl acetate as the mobile phase. Fractions A to S were collected, dried, reconstituted to 20mg/mL and tested for (A) PPARα and (B) PPARγ activity using the chimera Gal-PPAR reporter gene bioassay system at a final concentration of 20μg/mL for 48hr. Data points (means±SEM, n=3) represented percentage of the positive controls for PPARα (WY14,643, 30μM) and PPARγ (Pioglitazone, 30μM). Grey arrows indicate fraction chosen for further characterization.

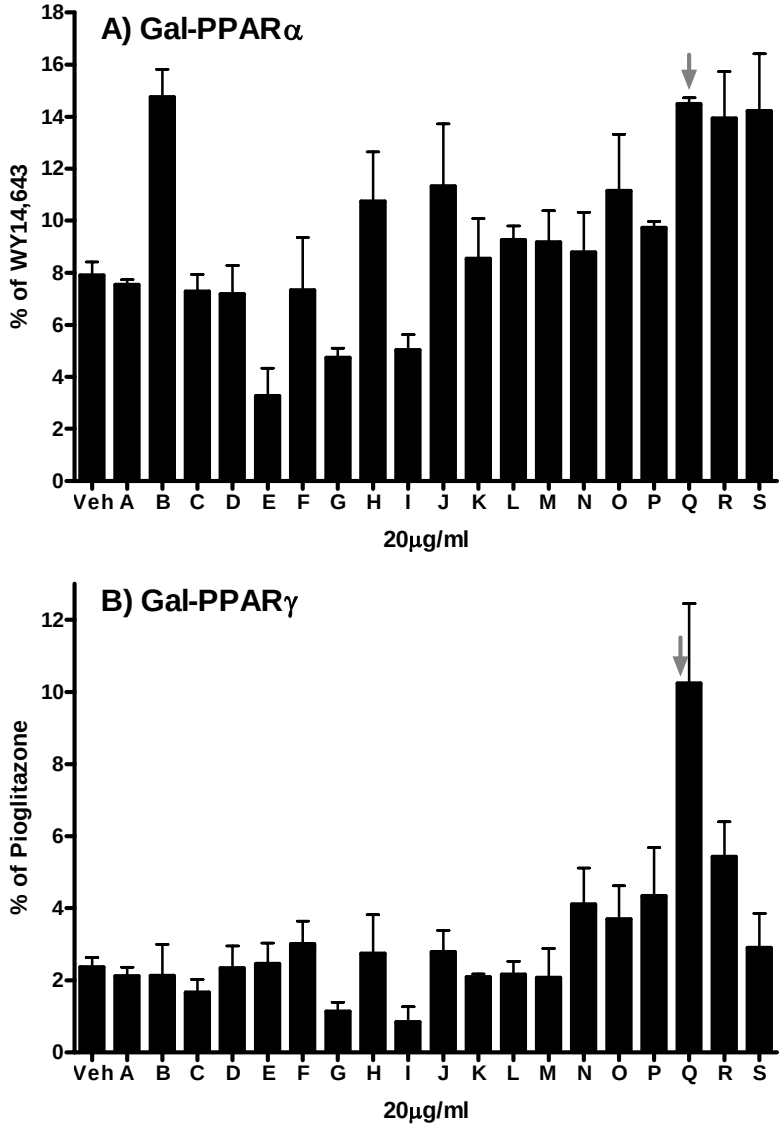


Table 3.1 Compounds isolated from various fractions of MPLC separation of PT ethyl acetate extract

Fractions	Column	Solvent conditions	Compound
32	Diol	DCM: Ethyl Acetate	Daidzin
32	C18	Methanol: Water	Genistin
34	C18	Methanol: Water	Puerarin
13	Diol	Hexane: Acetone	Daidzein
18	C18	Methanol: Water	2'hydroxy-daidzein

3.2 Characterization of flavonoids on PPARα and PPARγ activity

3.2.1 Effects of isoflavones on PPAR using the chimera Gal-PPAR reporter gene bioassay

Among the compounds isolated from the PPAR active herb, PT, daidzein is an isoflavone. From another PPAR active herb, AM, others in our laboratory have also identified the isoflavones, formononetin and calycosin, to be PPAR activating. Together with the commonly studied isoflavones, genistein and its precursor biochanin A, we compared the PPAR actions of these 5 isoflavones concurrently to evaluate their potential as dual PPARα/PPARγ activators.

With the chimeric Gal-PPARα assay, biochanin A, formononetin, and genistein were the most potent activators of PPARα with EC50 of 1.3μM, 1.0μM, and 16μM, respectively, comparing favorably with WY14,643 (EC50 of 5μM) (Fig 3.2.1A, Table 3.2). In terms of maximal PPARα activity, biochanin A and genistein were the most efficacious, exhibiting up to 65–71% of that observed with WY14,643. However, peak activity of genistein for PPARα was observed only at high doses ~30μM, unlike biochanin A, whose peak was 10-fold lower at 3μM (Fig 3.2.1A). For PPARγ, biochanin A, formononetin, and genistein were the most potent activators (EC50 of 3.7μM, 2.6μM, and 23μM vs. pioglitazone; 3 μM) (Fig 3.2.1B, Table 3.2). The 5 isoflavones did not differ in their maximal PPARγ activities, which ranged from 20–35% of pioglitazone (Fig 3.2.1B).

Thus, with the chimera Gal-PPAR reporter gene bioassay, the isoflavones, genistein, biochanin A and formononetin, were identified as strong activators of PPARα and PPARγ. However, biochanin A was most potent as maximal PPARα/PPARγ activity occurred at a physiologically relevant level of 3μM.

3.2.2 Evaluation of a full length PPAR reporter gene bioassay

While the chimera Gal-PPAR reporter gene assay was an efficient tool for PPAR ligand screening, the chimera consisted of the yeast Gal4 DBD and only the LBD of PPAR. This artificial construct provides limited insights to identified PPAR ligands in a physiologically relevant context because full transcription of PPAR in the presence of ligands involves an interplay with its heterodimeric partner, RXR, and the other domains of the receptor.

Thus, to determine whether dual PPAR bioactivity of these isoflavones could be observed in a more natural context, a full length reporter gene bioassay was first evaluated. We cotransfected full length PPARα or PPARγ together with a reporter gene CYP4A6-PPRE-Luc containing two copies of the consensus PPRE driven by the CYP4A6 promoter in a functionally relevant hepatic cell line expressing endogenous RXR (HepG2). The PPARα specific ligand, WY14,643, dose-dependently activated PPARα with a maximal activity 2 fold of the vehicle at 90μM (Fig. 3.2.2A). Similarly, the PPARγ specific ligand, pioglitazone, increased the activity of the PPARγ at a maximum of 5 fold of the vehicle between 5-30μM (Fig. 3.2.2B).

HepG2 cells were also transfected with CYP4A6-PPRE-Luc alone to investigate the effects of endogenous PPAR on the reporter gene. In the presence of WY14,643, there was minimal activation by endogenous PPARα. However, in the presence of pioglitazone, endogenous PPARγ activated the reporter gene up to 3 folds between 30-90μM.

In comparison to the chimera Gal-PPAR bioassay, this bioassay exhibited a lower specificity activity perhaps, reflecting the actions of endogenous receptors and effects of the heterodimeric partner RXR.

3.2.3 Effects of isoflavones on PPAR using the full length reporter gene bioassay

Using the evaluated full length PPAR assay, biochanin A, formononetin and genistein exhibited the highest PPARα (EC50 of 1μM, 3.7μM, and 9.5μM, respectively) and PPARγ (EC50 of 1μM, 4.3μM, 12μM) stimulating activities (Fig 3.2.3, Table 3.2). In particular, biochanin A was an order of magnitude more potent than calycosin and daidzein and comparable to values observed for WY14,643 and pioglitazone for both PPARα and PPARγ. Maximal activities for biochanin A reached 188% and 102% of reference drugs for PPARα and PPARγ, respectively.

Consistent with the chimera Gal-PPAR reporter gene assay, formononetin, genistein and in particular, biochanin A were strong PPARα/PPARγ activators in the full length reporter gene bioassay. Furthermore, in both assays, biochanin A, formononetin and genistein were, in general, more PPAR active than calycosin and daidzein.

Figure 3.2.1 Effect of isoflavones on chimera Gal-PPAR reporter gene bioassays

HeLa cells cotransfected with (A) Gal-PPARα or (B) Gal-PPARγ (25ng) and the $(UASG)_5$-Luc reporter gene (250ng) were exposed to increasing amounts of isoflavones. genistein (Gen), formononetin (For), biochanin A (Bio), calycosin (Cal), and daidzein (Dai) for 48hr. Doses used were 0, 1, 3, 10, 30, 60 and 100μM for all isoflavones, except genistein and biochanin A, where the highest dose was 90μM. Data points (means±SEM, n=3) represented percentage of the positive controls for PPARα (WY14,643, 30μM) and PPARγ (Pioglitazone, 30μM) respectively. Values without a common letter differ, $p<0.05$.

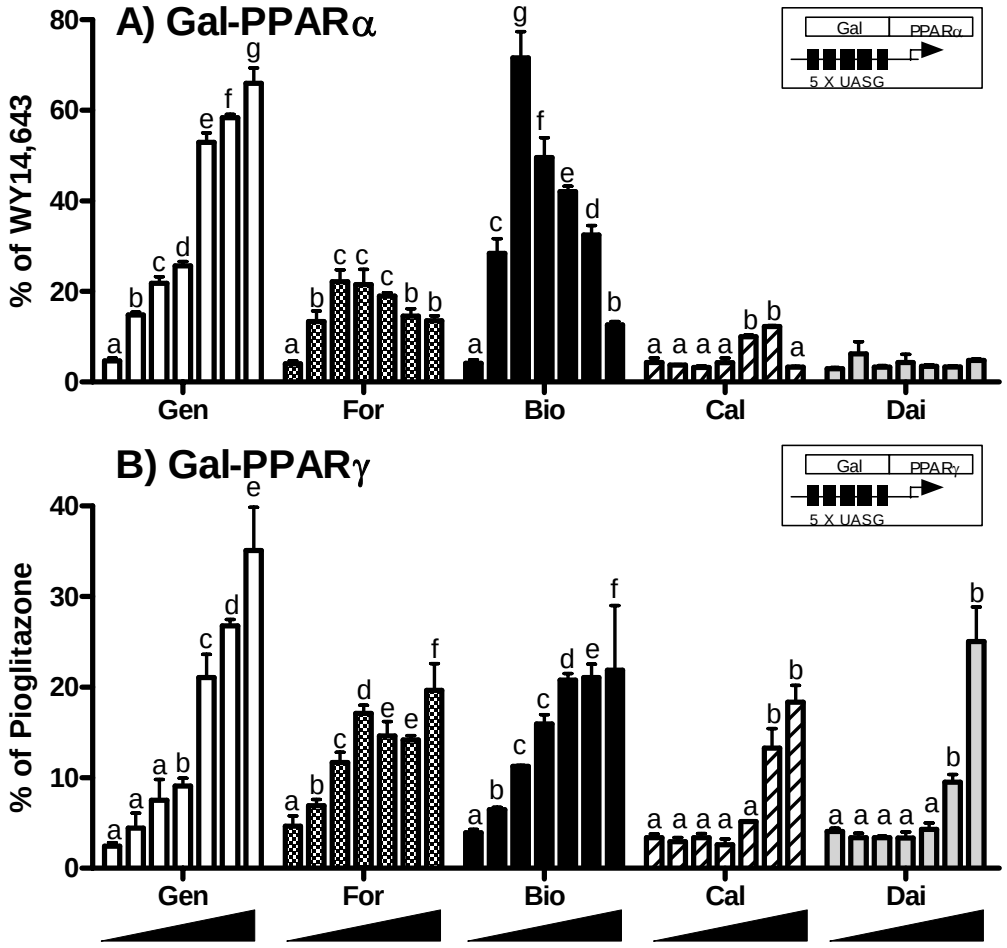


Table 3.2 Comparative PPAR activity of commonly consumed isoflavones

	Gal-PPAR in HeLa				PPAR in HepG2			
	α		γ		α		γ	
	EC_{50} μM	Cmax[1] %	EC_{50} μM	Cmax[2] %	EC_{50} μM	Cmax[1] %	EC_{50} μM	Cmax[2] %
Calycosin	22	12	45	18	22	157	36	93
Formononetin[3]	<1	22	2.6	17	3.7	246	4.3	96
Daidzein	NSA[4]	NSA	73	25	NSA	NSA	NSA	NSA
Genistein	16	65	23	35	9.5	266	12	104
Biochanin A[3]	1.3	71	3.7	20	<1	188	<1	102
WY14,643	5.9	100	NSA[5]	NSA[5]	2.2	100	NSA	NSA
Pioglitazone	NSA	NSA	3.6	100	NSA	NSA	0.5	100

[1] Maximal activity expressed as percentage of a saturating dose (30μM) of the PPARα selective ligand, WY14,643

[2] Maximal activity expressed as percentage of a saturating dose (30μM) of the PPARγ selective ligand, Pioglitazone

[3] Most potent PPARα and PPARγ activators

[4] NSA, no significant activity

[5] No significant activity up to 30μM

3.2.2 Dose response curves of full length PPAR reporter gene bioassays

HepG2 cells cotransfected with full length (A) PPARα or (B) PPARγ (50ng) and the CYP4A6-PPRE-Luc reporter gene (100ng) (inset) were exposed to WY14,643 or Pioglitazone as indicated for 48hr. Cells transfected with CYP4A6-Luc reporter vector only reflects endogenous PPAR activity. Data points (means±SEM, n=3) represented fold increase in luciferase activity over vehicle. Means without a common letter differ, $p<0.05$.

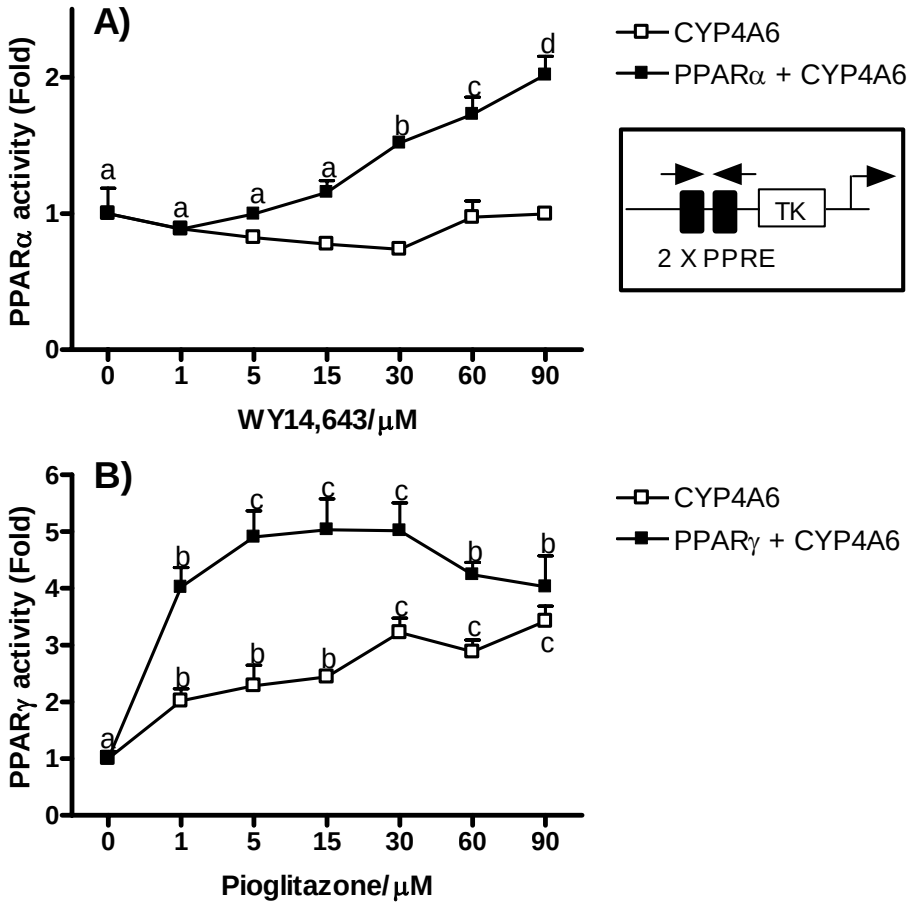
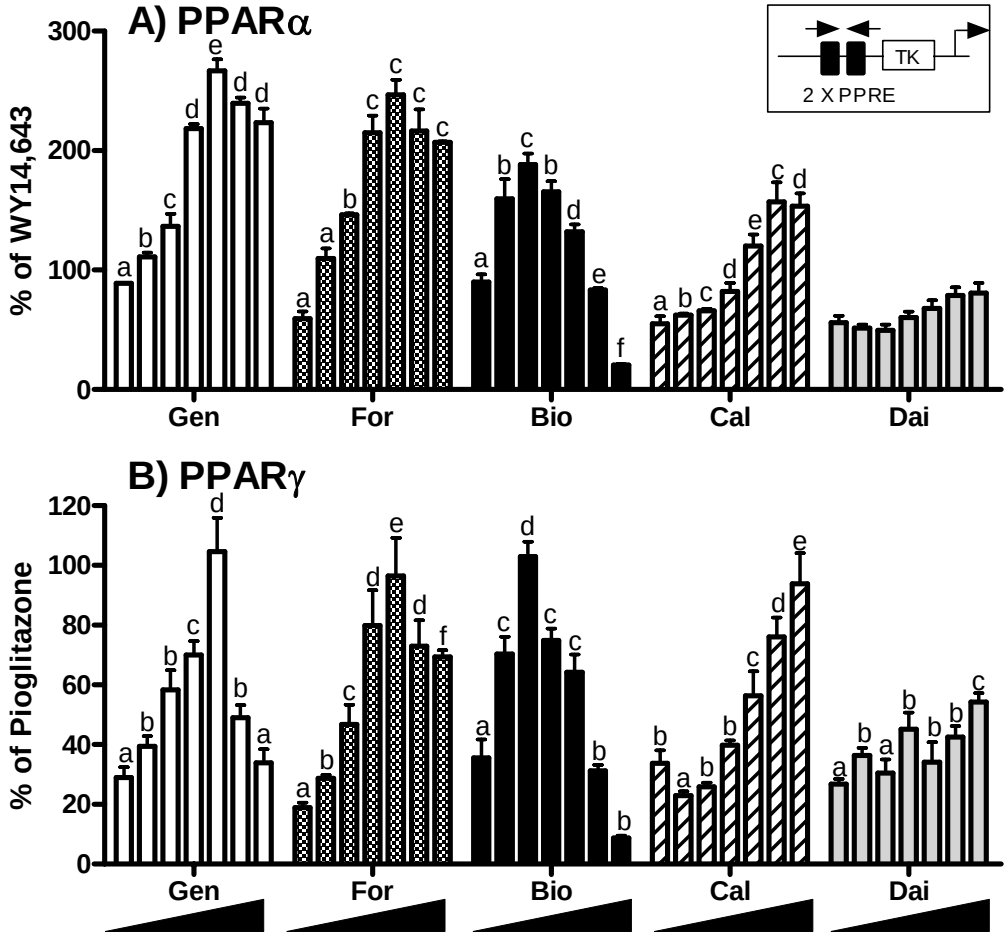


Figure 3.2.3 Effects of isoflavones on full length PPAR activity

HepG2 cells cotransfected with full length (A) PPARα or (B) PPARγ (50ng) and the CYP4A6-PPRE-Luc reporter gene (100ng) were exposed to increasing amounts of isoflavones for 48hr. Genistein (Gen), formononetin (For), biochanin A (Bio), calycosin (Cal), and daidzein (Dai). Doses used were 0, 1, 3, 10, 30, 60 and 100μM for all isoflavones, except genistein and biochanin A, where the highest dose was 90μM. Data points (means±SEM, n=3) represented percentage of the positive controls for PPARα (WY14,643, 30μM) and PPARγ (Pioglitazone, 30μM) respectively. Values without a common letter differ, $p<0.05$.



3.2.4 Effects of isoflavones on selected PPARα regulated genes

To further evaluate the effects of isoflavones on PPARα regulated endogenous genes, full length PPARα was overexpressed in HepG2 cells and treated with isoflavones. PPARα regulated expression of selected genes (PPARα (Pineda Torra et al. 2002), CPT1A (Mascaro et al. 1998) and HMGCS2 (Rodriguez et al. 1994)) were measured by real time RT-PCR. CPT1A is the rate limiting enzyme that controls fatty acid import in mitochondria while HMGCS2 is the key enzyme in ketone bodies conversion during fasting. The HMGCS1 gene, lacking a PPRE on its promoter (Hegardt 1999), was used as a negative control.

As expected, an increase in gene expression for CPT1A (Fig 3.2.4B) and HMGCS2 (Fig 3.2.4D) was observed with WY14,643. However, upregulation of PPARα gene expression was not observed (Fig 3.2.4A). The effects of isoflavones on the expression profile of these three genes were, in general, similar to that of WY14,643. In particular, biochanin A induced the highest increase (2-fold vs. vehicle) in HMGCS2 gene expression among the isoflavones and this increase was comparable to the effects of WY14,643 (Fig 3.2.4D). Gene expression of the negative control, cytosol HMGCS1, was not affected by WY14,643 or isoflavones (Fig 3.2.4C).

Thus, consistent with the chimera Gal-PPAR and full length reporter gene assays, biochanin A, and other isoflavones, upregulates PPARα endogenous genes. Furthermore, the upregulation of PPARα controlled genes, like the HMGCS2 gene, was not due to indirect activation from an upregulation of PPARα itself as treatments with isoflavones did not affect the expression of PPARα.

3.2.5 PPARγ binding properties of isoflavones

Since activation of PPARα activity by isoflavones was not due to indirect mechanism such as PPARα gene upregulation, we investigated whether the different transactivation activity were caused by differential binding affinities. Due the availability of a non-radioactive PPARγ polarization competitor assay, the ability of isoflavones to displace PPARγ-LBD fluorescent-ligand complexes was measured. As expected, doses of pioglitazone (at least 1μM) significantly displaced the fluormone, whereas estradiol did not (Fig 3.2.5). All 5 isoflavones significantly displaced fluormone at doses of at least 1μM, indicating that they bind PPARγ at its LBD pocket. In this assay, calycosin and formononetin exhibited poor solubility and precipitated at doses ~30μM.

Excluding formononetin and calycosin, the 50% inhibitory concentration (IC50) of the other 3 compounds were in the order biochanin A, genistein, daidzein, corresponding to trends in EC50 observed with the Gal-PPARγ and full length PPARγ transactivation assays.

Figure 3.2.4 Effects of isoflavones on selected PPARα regulated genes in HepG2

HepG2 cells cotransfected with full length PPARα (50ng) and exposed to 3μM of isoflavones. Genistein (Gen), formononetin (For), biochanin A (Bio), calycosin (Cal), and daidzein (Dai). At 24h post ligand treatment, total RNA was extracted, and RNA expression for PPARα (A), CPT1A (B), HMGCS1 (C) and HMGCS2 (D) or 18s was evaluated by real-time RT-PCR. Data represents means±SEM of 2 separate experiments done in duplicates. Positive control was WY14,643 at 30μM.

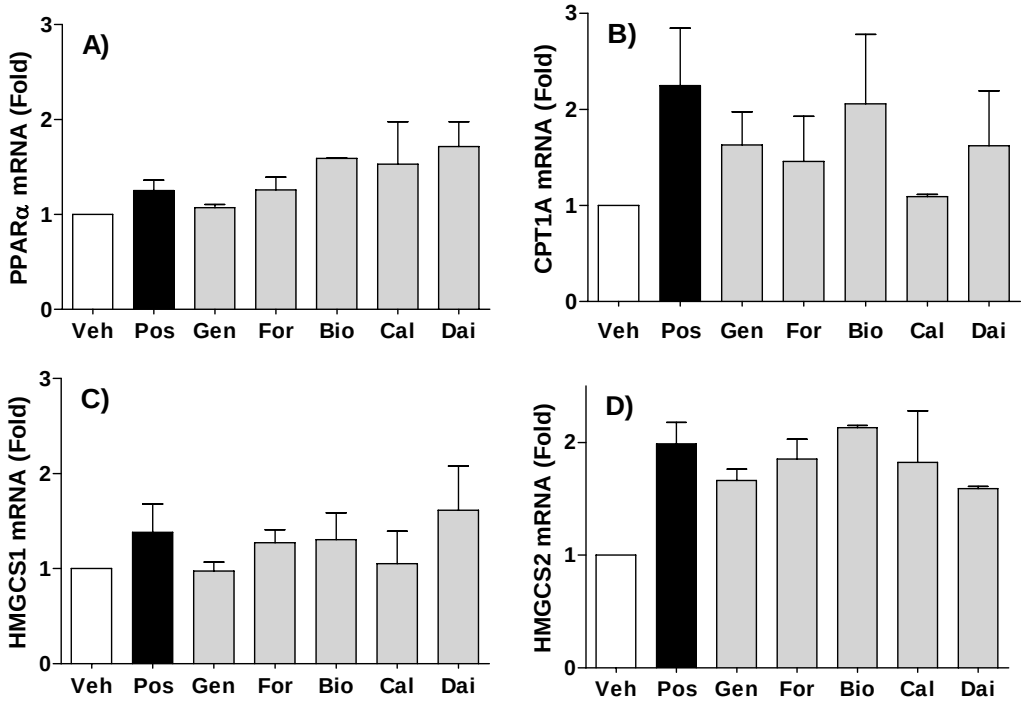
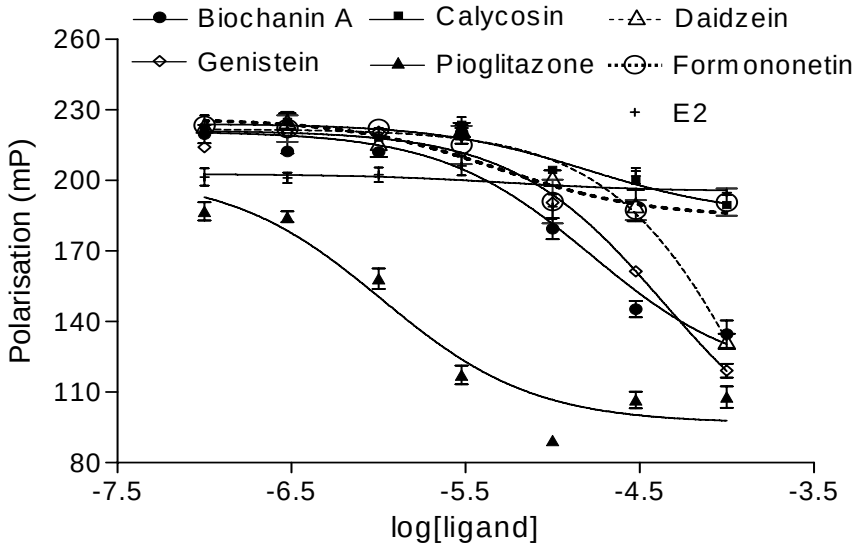


Figure 3.2.5 PPARγ competitive ligand binding assay

Increasing doses (0.1, 0.3, 1, 3, 10, 30 and 100μM) of isoflavones were added to a liganded PPARγ fluorome complex. Changes in polarization values (mP) caused by displacement of fluorescent PPARγ ligand (fluorome) by isoflavones were measured. Values are mean ± SEM, n=3.



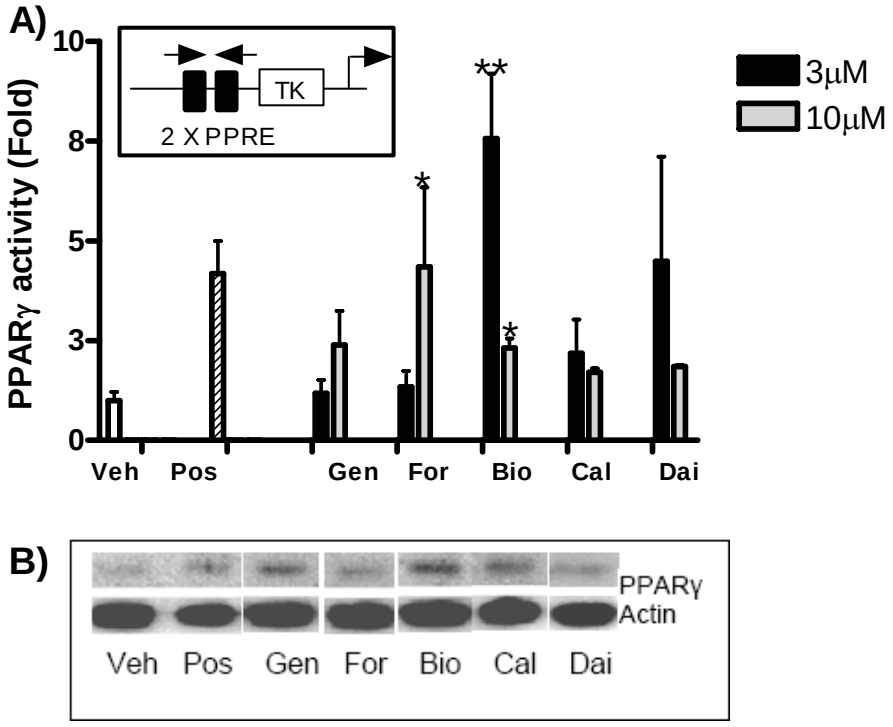
3.2.6 Effects of isoflavones on endogenous PPARγ function

Activation of PPARγ by isoflavones had been demonstrated through the overexpression of PPARγ in the full length reporter assay using various cell lines previously. To investigate the relevance of isoflavones on lipid metabolism in a process of which PPARγ plays a major role, the PPAR-driven reporter-gene CYP4A6-PPRE-Luc was transfected into differentiated 3T3-L1 preadipocytes, to measure endogenous PPARγ-driven responses. 3T3-L1 cells endogenously express high levels of PPARγ after adipocyte differentiation (Chawla et al. 1994). As expected, pioglitazone induced a significant rise in PPARγ activity (4-fold vs. vehicle) (Fig 3.2.6A). At a low dose of 3μM, only biochanin A increased activity of the CYP4A6-PPRE-Luc reporter (7.6-fold vs. vehicle). At a higher dose of 10μM, formononetin was also active (5.5-fold vs. vehicle). Being more potent than the other isoflavones, biochanin A displayed a reversed dose response compared with the other isoflavones. It is likely that the biphasic response of isoflavones may be due to the limitations in coregulator content or differences in protein expression levels at different isoflavone concentrations (eg. 10μM). Western blot analysis with specific PPARγ antibody indicated that these 3T3-L1 adipocytes endogenously expressed PPARγ (Fig 3.2.6B).

These results suggested that both biochanin A and formononetin at low doses could activate endogenous PPARγ in differentiated preadipocytes.

Figure 3.2.6 Effects of isoflavones on endogenous PPARγ function in adipocytes

Differentiated 3T3-L1 cells (A) were transfected with CYP4A6-PPRE-Luc reporter gene only and exposed to genistein (Gen), formononetin (For), biochanin A (Bio), calycosin (Cal), and daidzein (Dai) at 3 and 10μM for 48hr. Positive control (Pos) was Pioglitazone (30 μM). Data (mean ± SEM, n=3) are fold increases in luciferase activity over vehicle. **p*<0.05; ***p*<0.01, compared to vehicle. (B) PPARγ protein expression of differentiated adipocytes. 3T3-L1 cells were exposed to isoflavones (3 μM) and PPARγ protein detected with an anti-PPARγ mouse monoclonal antibody. Protein loading control, actin, was detected with an anti-actin mouse monoclonal antibody.



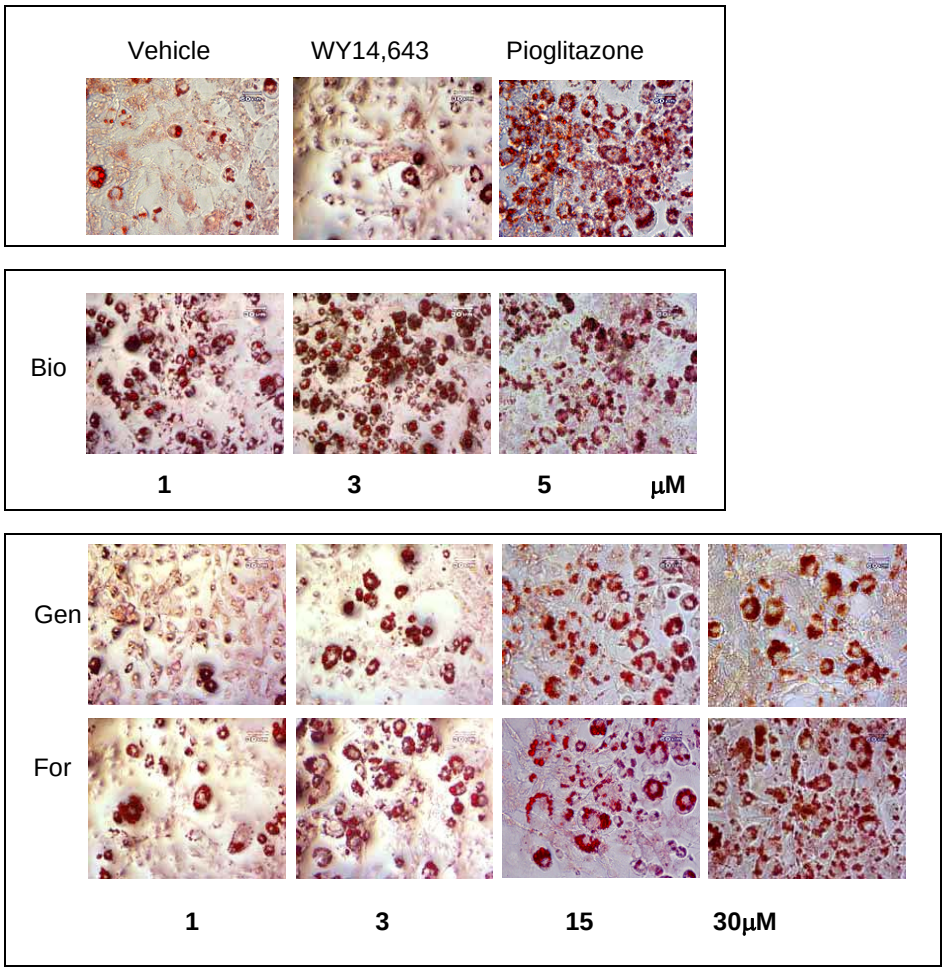
3.2.7 Effects of isoflavones on 3T3-L1 cells differentiation

To determine if activation of PPARγ by biochanin A, genistein, and formononetin was associated with adipose differentiation, we visualized accumulation of lipid droplets with oil-red O staining. Adipocyte differentiation is used as an indication of the functional consequences of PPARγ ligand activation since PPARγ is the master regulator of adipocyte differentiation (Forman et al. 1995). Pioglitazone, but not the vehicle or WY14,643, strongly stimulated preadipocyte differentiation, indicating the PPARγ specificity of the assay (Fig 3.2.7). At a dose of 1μM, only biochanin A stimulated lipid droplet accumulation in preadipocytes above vehicle. Formononetin and genistein stimulated lipid accumulation at higher doses of at least 3μM and 15μM, respectively.

Thus, among the isoflavones tested, biochanin A was the most potent stimulator of PPARγ activity and could lead to adipocyte differentiation a dose of 1μM. Furthermore, together with the characterization of isoflavone's PPARα activity performed previously, we established that isoflavones were dual PPARα/PPARγ active and certain members, in particular, biochanin A, was more potent than the others.

Figure 3.2.7 Effects of isoflavones on 3T3-L1 preadipocyte differentiation

3T3-L1 cells were exposed to induction media and exposed to genistein (Gen), formononetin (For), biochanin A (Bio) at the doses indicated. After 8 days of exposure to isoflavones, differentiated cells were stained with Oil-Red O. Photomicrographs were at 400X magnification. WY14,643 and Pioglitazone used were at 1μM



3.2.8 Screening of various flavonoid groups for PPAR activity

Since isoflavones displayed dual PPARα/PPARγ activity, we investigated whether other structurally similar flavonoids have potential dual PPARα/PPARγ activity using the chimeric Gal-PPAR assays.

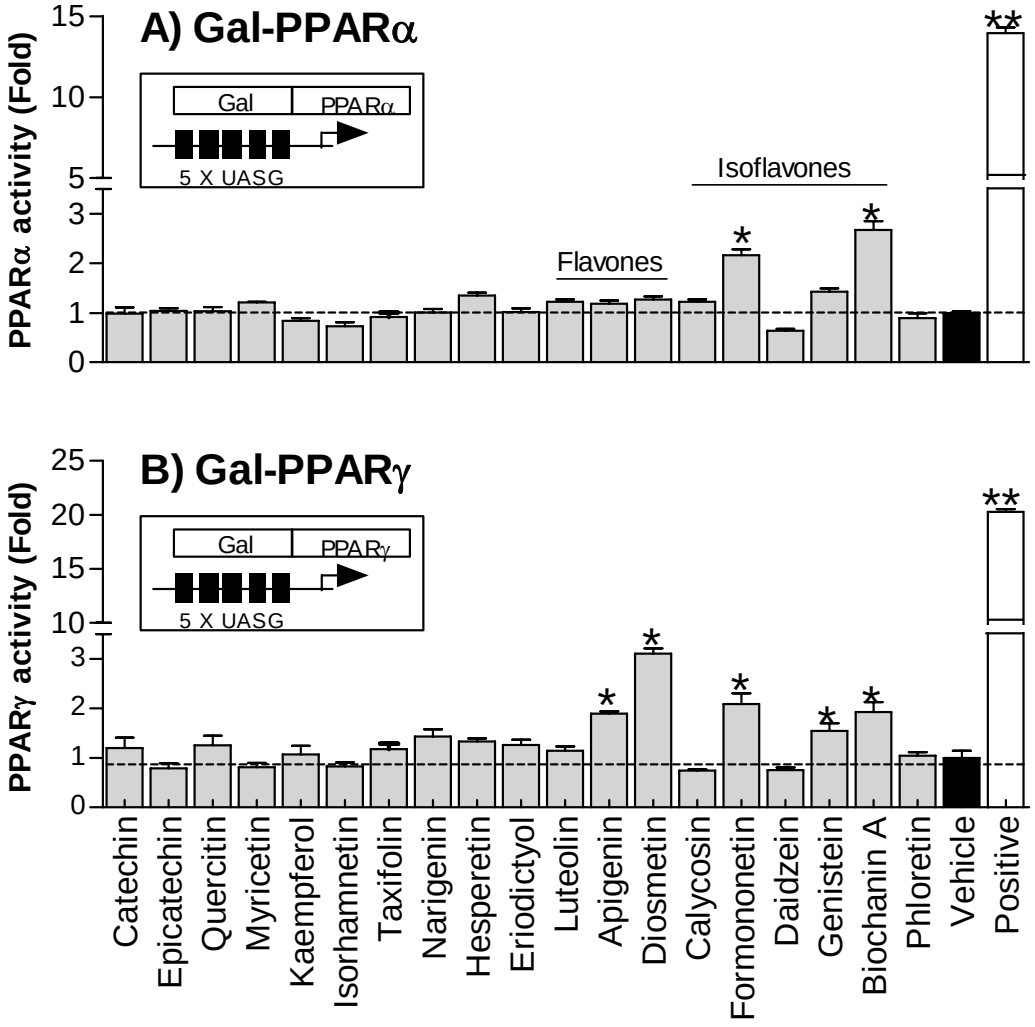
The 19 flavonoids[2] screened were grouped according to anthocyanins (catechin and epicatechin), flavonols (quercitin, myricetin, kaempferol, isorhamnetin and taxifolin), flavanones (naringenin, hesperetin and eriodictyol), flavones (luteolin, apigenin and diosmetin), isoflavones (daidzein, genistein, biochanin A, formononetin and calycosin), and chalcone (phloretin). As shown previously, isoflavones were PPAR active (Fig 3.2.8). Surprisingly, the flavone group was the only other flavonoid group that was PPAR active. One of these flavones, diosmetin, showed PPARγ selective activity. At a dose of 3 μM, diosmetin increased PPARγ activity up to 3 fold vs. vehicle, and had the highest PPARγ activity among the flavonoids tested (Fig 3.2.8B). In contrast, its PPARα activation was relatively weak (1.3 fold vs. vehicle) (Fig 3.2.8A). In the same flavone group, apigenin also showed moderate PPARγ activity (1.9 fold vs. vehicle).

This suggested that other than isoflavones, which were dual PPARα/PPARγ agonists, flavones in the flavonoids group were also PPAR active. In particular, diosmetin was PPARγ selective.

[2] Structures of flavonoids in Fig 1.8

Figure 3.2.8 Screening of different groups of flavonoids for PPAR activity

HeLa cells cotransfected with (A) Gal-PPARα or (B) Gal-PPARγ (25ng) and the $(UASG)_5$-Luc reporter gene (250ng) were exposed to 3μM of selected flavonoids for 48hr. Data points (means±SEM, n=3) are fold increases in luciferase activity over vehicle. Positive controls were WY14,643 (30μM) and Pioglitazone (30μM) for PPARα and PPARγ respectively. **p*<0.05; ***p*<0.01, compared to vehicle.



3.2.9 Effects of diosmetin, from the flavone group of flavonoids, on PPAR

To characterize the unexpected PPARγ selective activation of diosmetin, cells were co-transfected with full length PPARα, PPARβ or PPARγ expression vector and the reporter vector CYP4A6-PPRE-Luc, and exposed to diosmetin. Diosmetin activated full length PPARγ in a dose dependant manner, and the maximal activity (2.7 fold) at 10μM was ~50% of the positive control (pioglitazone, 5.7 fold induction vs. vehicle) (Fig 3.2.9A). In contrast, diosmetin did not activate PPARα or PPARβ.

In PPARγ competitor binding assays, diosmetin replaced fluormone at 1μM and displayed an IC50 of 8.4μM, indicating that it transactivates PPARγ by binding to its ligand-binding pocket (Fig 3.2.9B). In 3T3-L1 differentiated cells, diosmetin induced transcriptional activity dose-dependently (up to 5.1 fold) and was comparable to pioglitazone even at a low dose of 10μM (Fig 3.2.9C). Similarly, diosmetin moderately stimulate preadipocyte differentiation (Fig 3.2.9D).

In all, diosmetin showed selective PPARγ activation, through binding of its ligand binding pocket, in PPAR reporter gene and PPARγ functional studies.

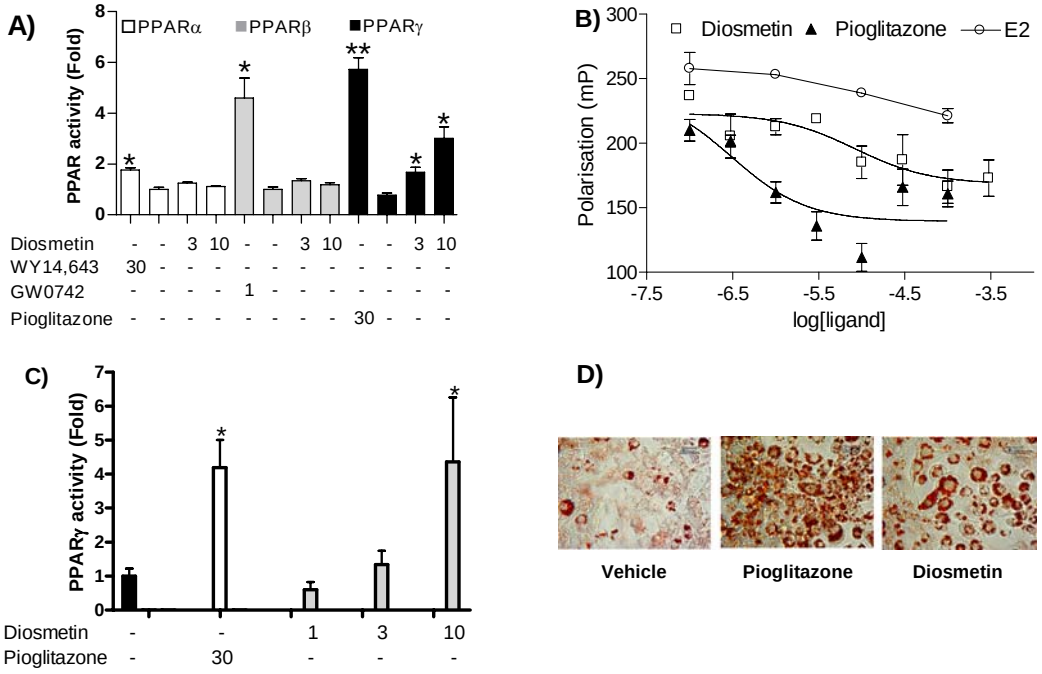
Figure 3.2.9 Identification of the non-isoflavonoid, Diosmetin, as a PPARγ selective agonist

(A) HeLa cells cotransfected with full length PPARα, PPARγ or PPARβ (50ng) and the CYP4A6-PPRE-Luc reporter gene (100ng) were exposed to increasing amounts of diosmetin (μM) as indicated for 48hr. Positive controls were WY14,643, Pioglitazone and GW0742, in μM, for PPARα, PPARγ and PPARβ respectively. Data points (means±SEM, n=3) are fold increases in luciferase activity over vehicle. **p*<0.05; ***p*<0.01, compared to vehicle.

(B) Increasing doses (0.1, 0.3, 1, 3, 10, 30 and 100μM) of diosmetin were added to a liganded PPARγ fluorome complex. Changes in polarization values (mP) caused by displacement of fluorescent PPARγ ligand (fluorome) by isoflavones were measured. Values are mean ± SEM, n=3.

(C) Differentiated 3T3-L1 cells were transfected with CYP4A6-PPRE-Luc reporter gene only and exposed to Diosmetin as indicated for 48hr. Positive control (Pos) was Pioglitazone (30 μM). Data (mean ± SEM, n=3) are fold increases in luciferase activity over vehicle. **p*<0.05; compared to vehicle.

(D) 3T3-L1 cells were exposed to induction media and exposed to diosmetin (1μM) or Pioglitazone (1μM). After 8 days of exposure to ligands, differentiated cells were stained with Oil-Red O. Photomicrographs at 400X magnification.



3.2.10 Effects of isoflavones and diosmetin on selected PPARγ regulated genes

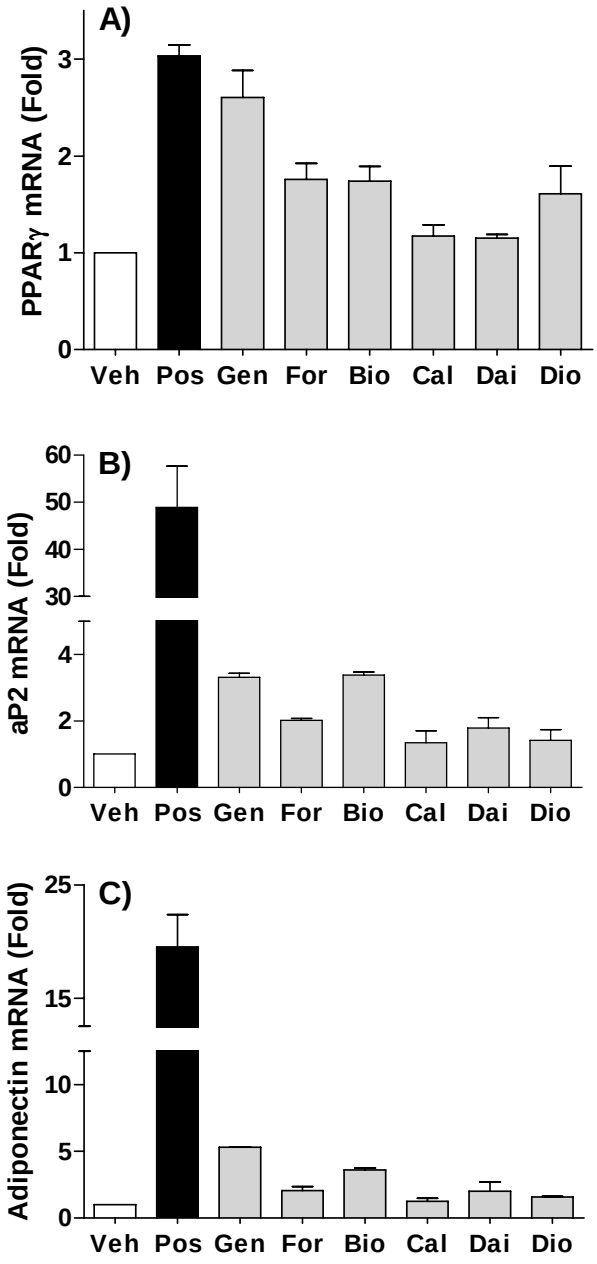
Together with diosmetin and isoflavones, we studied the effects of these flavonoids on PPARγ regulated endogenous genes. 3T3-L1 cells were exposed to these flavonoids and the effects on selected PPARγ regulated genes (aP2 (Tontonoz et al. 1994) and adiponectin (Iwaki et al. 2003)) measured by real-time RT-PCR. aP2 is involved in the regulation of lipid and glucose metabolism while adiponectin, an adipokine, is an insulin sensitizer. PPARγ has not been reported to be self-regulatory but was measured to exclude the possibility that activation of PPARγ regulated genes by flavonoids is due to an upregulation of PPARγ gene expression through a PPARγ independent pathway.

As expected, pioglitazone increased the gene expression of aP2 and adiponectin up to 48 and 18 folds respectively (Fig. 3.2.10B and C). Surprisingly, pioglitazone also increased the gene expression of PPARγ up to 3 folds. Diosmetin showed relatively weak upregulation of adiponectin (1.6 fold vs. vehicle) and had no effect on the aP2 gene. For isoflavones, only genistein and biochanin A increased the gene expression of both aP2 (~ 3 fold) and adiponectin (~4 fold). However, these increases were relatively weak compared to pioglitazone. In addition, increase in PPARγ gene expression with genistein was comparable to that of pioglitazone.

Altogether, we show previously and herein that isoflavones, especially genistein and biochanin A, are dual PPARα/PPARγ agonists which affected PPAR reporter gene, PPARγ function and PPAR endogenous genes. Furthermore, our work also suggests that parent botanicals of isoflavones, such as PT, are potential anti-diabetic agents useful for lipid profile management.

Figure 3.2.10 Effects of flavonoids on selected PPAR regulated genes in differentiated 3T3-L1 cells

3T3-L1 cells were exposed to induction media and exposed to 3μM of isoflavones for 8 days. Genistein (Gen), formononetin (For), biochanin A (Bio), calycosin (Cal), and daidzein (Dai). Total RNA was extracted, and RNA expression for PPARγ (A), aP2 (B) and adiponectin (C) or 18s was evaluated by real-time RT-PCR. Data represents means±SEM, n=2. Positive control was Pioglitazone at 30μM.



3.3 Characterization of flavonoids and PPARα ligands on a natural PPARα V227A variant

Meta-analysis of soy isoflavones intake in human subjects indicates their beneficial role on lipid profiles in the body (Zhan and Ho 2005). However, the overall efficacy of soy isoflavones still remains debatable (Erdman et al. 2007). Adding to this debate is a recent study which reported no overall improvements in lipid plasma concentration except in a subset of the participants who are carriers of an estrogen receptor β polymorphism. Carriers of this polymorphism, ERβ(cx), experienced a significant increase in HDL-c in response to isoflavone intake (Hall et al. 2006). Isoflavones are also reported ligands of the NRs, ERβ and ERα.

Similar gene-diet interactions have also been reported for PPARα, an important NR involved in the regulation of lipid metabolism (Lefebvre et al. 2006). In women carriers of the PPARα V227A polymorphism, an increase in dietary PUFA intake was associated with lower HDL-c concentration (Chan et al. 2006). Similar to isoflavones, PUFA are ligands of PPARα. While the gene-diet interaction between PPARα and PUFA was reported, the dietary effect of isoflavones on lipid profiles in relation to PPARα polymorphisms is still unknown.

Unlike ERβ(cx) which is a splice variant (Ogawa et al. 1998), V227A is a point mutation which occurs on helix 2 of the PPARα LBD. This mutation lies within the region where point mutations A234T, R243Q, and R243W on the thyroid receptor β (T3Rβ) has been reported and functionally characterized in thyroid hormone resistant patients (Behr and Loos 1992; Onigata et al. 1995; Yagi et al. 1997; Safer et al. 1998)

(Fig 3.3.1A). Another reported point mutation on the PPARα is the L162V polymorphism (Vohl et al. 2000). While L162V occurs on the DNA binding domain of PPARα and is functionally active *in vitro* (Flavell et al. 2000; Sapone et al. 2000), function of V227A at the LBD is unknown. Furthermore, effects of isoflavones on V227A activity are also unclear.

3.3.1 Transcriptional activity of V227A with genistein and biochanin A on the CYP4A6 PPRE

To investigate the transcription function of the variant in the presence of isoflavones (genistein and biochanin A) characterized previously, HepG2 cells were co-transfected with plasmids encoding either full length wild type (WT) or V227A PPARα, and a reporter gene CYP4A6-PPRE-Luc containing two copies of the consensus PPRE derived from the CYP4A6 promoter (Krey et al. 1993). In the presence of genistein (Fig 3.3.1B) or biochanin A (Fig 3.3.1C), both WT and V227A displayed similar transactivation activity from 1 to 10μM. Only at a high dose of 30μM genistein was V227A 27% weaker.

Thus, in the presence of physiologically relevant levels of the isoflavones, there was no difference in PPARα activity between WT and V227A.

Figure 3.3.1(A) Amino acid sequence comparison of Helices 1 and Helix 2 of T3Rβ and PPARα

The V227A PPARα variants and T3Rβ (A234, R243) mutants associated with resistance to thyroid hormones are marked with asterisks.

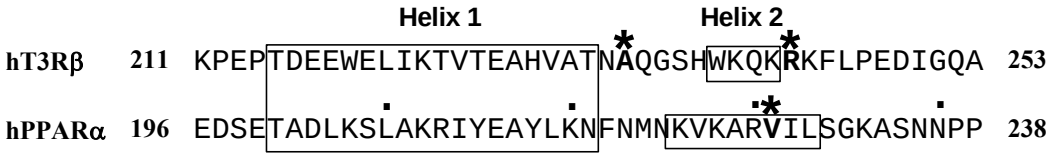
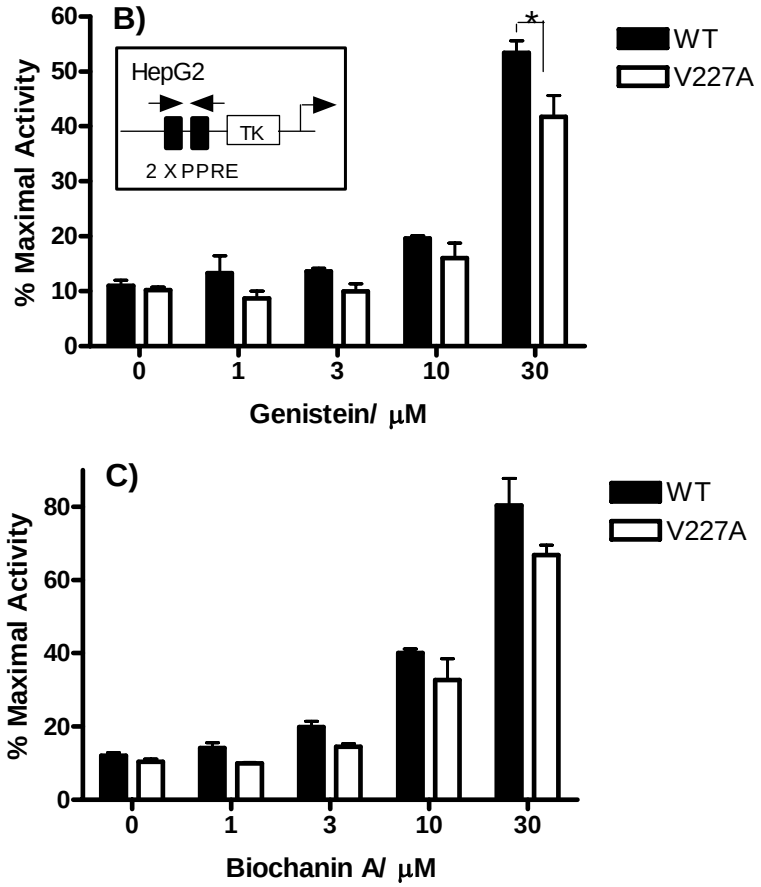


Figure 3.3.1(B,C) The PPARα V227A variant exhibits similar transactivation activity on the consensus CYP4A6-PPRE with genistein and biochanin A

HepG2 cells were co-transfected with reporter vector CYP4A6-PPRE-Luc (100ng) and full length WT or V227A PPARα (50ng) before treatment with genistein (B) or biochanin A (C) for 48 h. Values are mean ± SE of three replicates, and expressed as percentage of maximal WT activity (WY14,643, 100μM). * $p<0.05$.



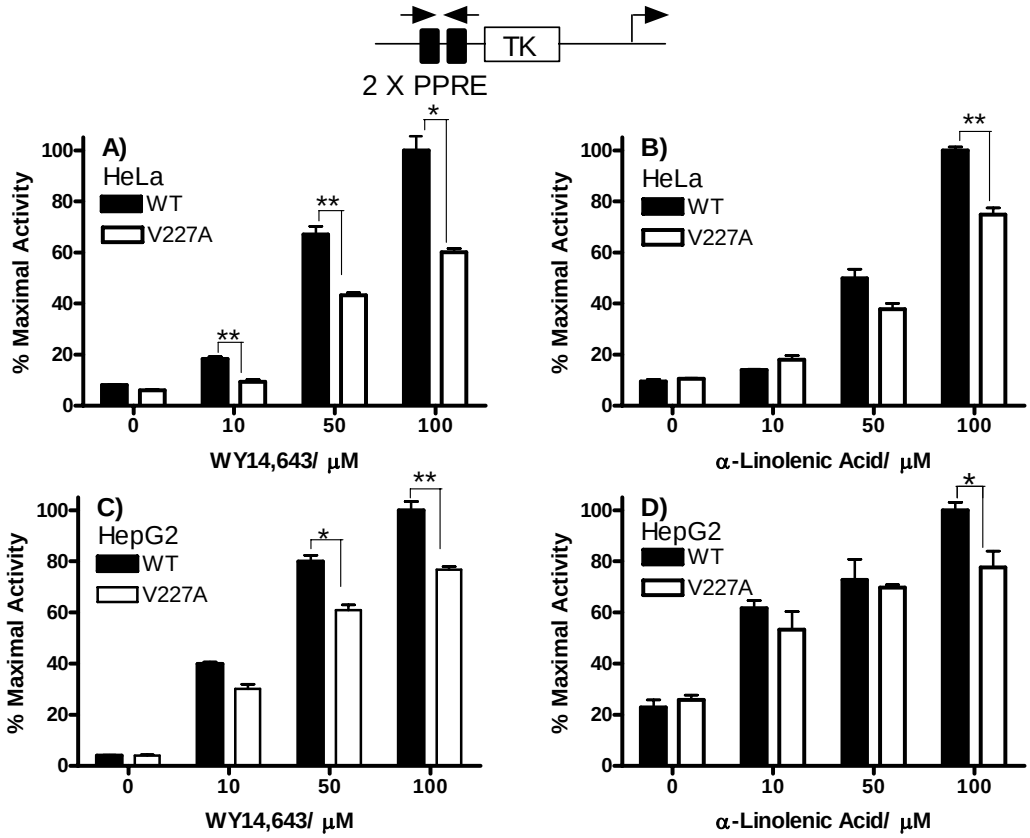
3.3.2 Transcriptional activity of V227A with WY14,643 and α-linolenic acid on the CYP4A6 PPRE

Although there was no difference in transcription activity between WT and V227A with isoflavones, we investigated the transcription function of the variant in the presence of the potent PPARα synthetic ligand, WY14,643 and the endogenous PPARα ligand, α-linolenic acid. HeLa and HepG2 cells were co-transfected with plasmids encoding either WT or V227A PPARα, and the reporter gene CYP4A6-PPRE-Luc. In HeLa cells, V227A was 35%-49% weaker than WT on exposure to the potent fibrate WY14,643 (Fig. 3.3.2A); and about ~25% weaker than WT at high doses of the less potent, α-linolenic acid (Fig. 3.3.2B). In HepG2 cells, V227A was ~23% weaker with WY14,643 (Fig 3.3.2C); and in the presence of the α-linolenic acid, V227A was 22% weaker than WT at the highest dose (Fig 3.3.2D). Lesser differences between WT and V227A observed in HepG2 cells may be due to interference from endogenously expressed WT PPARα.

Thus with the CYP4A6 PPRE, we demonstrate that V227A is a functional PPARα variant in the presence of isoflavones, the fibrate drug WY14,643 and the endogenous ligand α-linolenic acid. Interestingly, V227A exhibited subtle, yet significantly weaker transactivation activity compared to WT with WY14,643 and α-linolenic acid.

Figure 3.3.2 The PPARα V227A variant exhibits lower transactivation activity on the CYP4A6-PPRE in the presence of WY14,643 and α-linolenic acid

HeLa (A, B) and HepG2 (C, D) cells were co-transfected with reporter vector CYP4A6-PPRE-Luc (100ng) and full length WT or V227A PPARα (50ng) before treatment with WY14,643 or α-Linolenic acid for 48 h. Values are mean ± SE of three replicates, and expressed as percentage of maximal WT activity. * $p<0.05$; ** $p<0.01$.



3.3.3 Transcriptional activity of V227A with fenofibrate and linoleic acid on the CYP4A6 PPRE

Fenofibrate is a PPARα agonist and belongs to the same class of lipid lowering drug as WY14,643. Linoleic acid, an ω-6 fatty acid, and α-linolenic acid, a ω-3 fatty acid, are PUFA commonly found in the diet. Since V227A exhibited lower transactivation activity with WY14,643 and α-linolenic acid, we also investigated the effects of related ligands, fenofibrate and linoleic acid, from each respective group on the transactivation of V227A. In contrast to WY14,643 and α-linolenic acid, transactivation activity of V227A in the presence of fenofibrate or linoleic acid was similar to WT at all doses of ligands tested (Fig 3.3.3).

3.3.4 Transcriptional activity of V227A with GW9662 on the CYP4A6 PPRE

Besides the fibrates and PUFA groups of PPARα agonists, we also investigated V227A transcriptional activity in the presence of GW9662, a PPARγ antagonist but also a partial PPARα agonist (Leesnitzer et al. 2002; Seimandi et al. 2005). In HeLa cells, activation of WT by GW9662 was highest at 50μM. However, maximal activation of WT by GW9662 was only ~40% of the positive control (WY14,643) and was consistent with its partial agonist activity. In comparison, V227A was 30% and 38% weaker on exposure to GW9662 at 20 and 50μM respectively (Fig 3.3.4).

Among the PPARα ligands tested, we demonstrate that weaker transcriptional activity of V227A was only observed on selected ligands and was most marked with

WY14,643. Therefore, to study the molecular mechanism behind the lower transactivation of V227A in subsequent work, WY14,643 was the primary ligand used.

3.3.5 Lower transactivation of V227A is independent of PPARα amount transfected

Before the molecular mechanism behind the weaker activity of V227A was elucidated, we investigated whether lower transcription function of V227A with WY14,643 was due to varying amounts of V227A transfected. HeLa cells were co-transfected with plasmids encoding either WT or V227A PPARα, at varying amounts, and the reporter gene CYP4A6-PPRE-Luc. Varying the amounts of V227A (25-75ng) transfected, V227A transactivation activity remained lower (~76%-44%) than WT in the presence of WY14,643 (Fig. 3.3.5A). Conversely, at varying amounts of WT (25-75ng), WT transactivation activity remained higher than V227A with WY14,643 (Fig. 3.3.5B). These data suggest that the lower transactivation activity observed for V227A was not due to unequal transfection efficiency.

In addition, to improve the expression efficiency of PPARα variant in HepG2 cells, an adenovirus-mediated expression system was employed to allow for a consistently high expression of mutant and WT PPARα (see Appendix, Pg 244). In this setting, the V227A variant consistently induced about half the transactivation activity observed with the WT PPARα. Hence, this suggests that the overexpression experiments are valid methods to explore the physiological relevance of gene variants and allows for further pathogenic mechanisms to be deduced.

Figure 3.3.3 The PPARα V227A variant exhibits similar transactivation activity on the CYP4A6-PPRE with linoleic acid and fenofibrate

HepG2 cells were co-transfected with reporter vector CYP4A6-PPRE-Luc (100ng) and full length WT or V227A PPARα (50ng) before treatment with linoleic acid or fenofibrate for 48 h. Values are mean ± SE of three replicates, and expressed as percentage of maximal WT activity with linoleic acid.

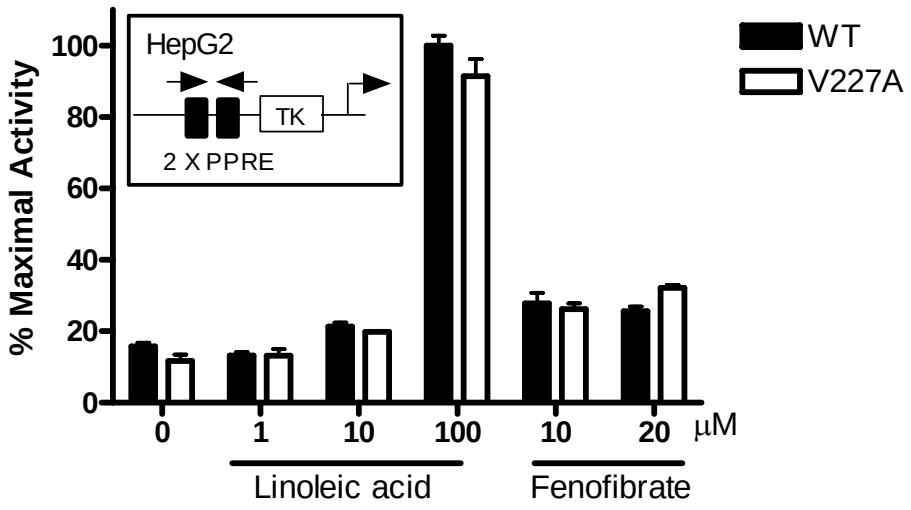


Figure 3.3.4 The PPARα V227A variant exhibits lower transactivation activity on the CYP4A6-PPRE with GW9662

HeLa cells were co-transfected with reporter vector CYP4A6-PPRE-Luc (100ng) and full length WT or V227A PPARα (50ng) before treatment with GW9662 for 48 h. Values are mean ± SE of three replicates, and expressed as percentage of maximal WT activity (+; WY14,643, 100μM). * $p<0.05$; ** $p<0.01$.

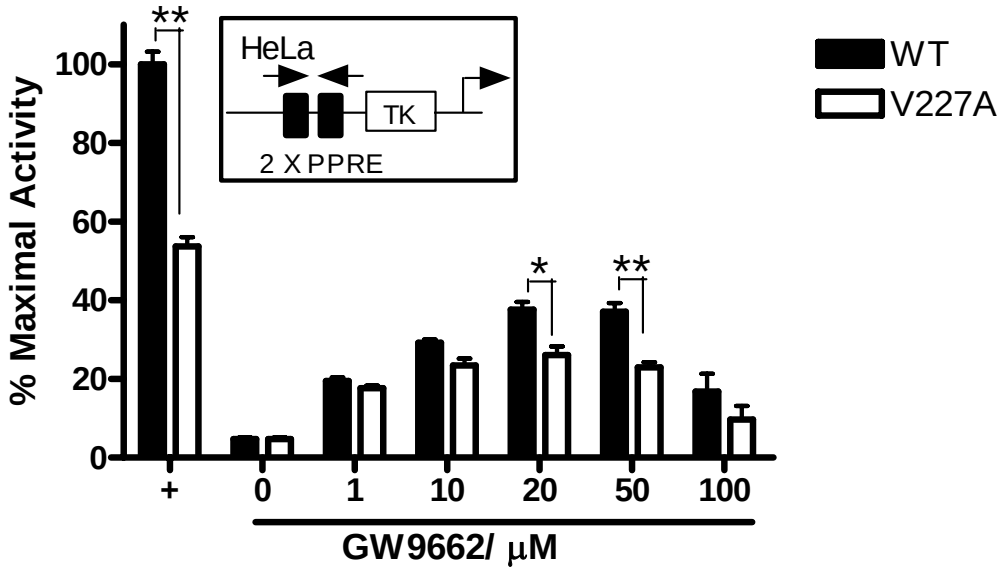
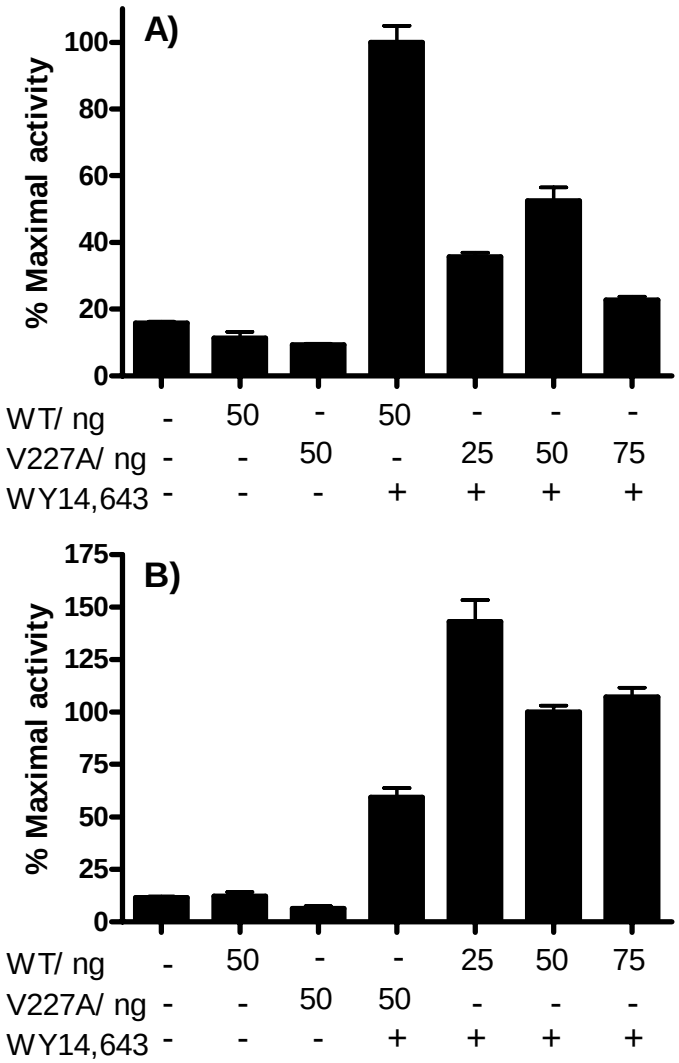


Figure 3.3.5 Lower PPARα V227A variant transactivation activity on the consensus CYP4A6-PPRE in the presence of WY14,643 is independent of PPARα amount

HeLa cells were co-transfected with reporter vector CYP4A6-PPRE-Luc (100ng) and varying amounts of full length V227A (A) or WT (B) PPARα before treatment with WY14,643 (μM) for 48 h. Values are mean ± SE of three replicates, and expressed as percentage of maximal WT activity (WY14,643 at 100μM, 50ng).



3.3.6 Lower transactivation activity of V227A on the natural HMGCS2 promoter and its gene expression

While we had shown previously that V227A was weaker on the PPRE, the effects of this variant was further evaluate in the process whereby PPARα plays a key role, lipid metabolism. The PPRE on the HMGCS2 promoter is the most PPAR responsive among 16 known PPREs compared (Juge-Aubry et al. 1997) and is also a well characterized PPARα responsive gene (Rodriguez et al. 1994). The HMGCS2 enzyme is involved in ketone bodies formation during fasting and has a partial role in cholesterol metabolism (Hegardt 1999) (Fig 1.3). Thus, examination of V227A on such a PPARα regulated endogenous gene would enable a clearer understanding towards the role of V227A in a physiologically relevant context.

Cells were co-transfected with WT and V227A PPARα, and a luciferase-reporter driven by residues -1081 to +22 of HMGCS2 promoter (HMGCS2- Luc) (Rodriguez et al. 1994). In this system, V227A was 22-25% weaker than WT with WY14,643 (Fig. 3.3.6A), and ~27% weaker than WT with high doses of α-linolenic acid in HeLa cells (Fig. 3.3.6B). In HepG2 cells, V227A was 17%-14% weaker than WT with 50μM and 100μM of WY14,643 respectively (Fig 3.3.6C); and in the presence of α-linolenic acid (100μM), V227A was 25% weaker compared to WT (Fig 3.3.6D).

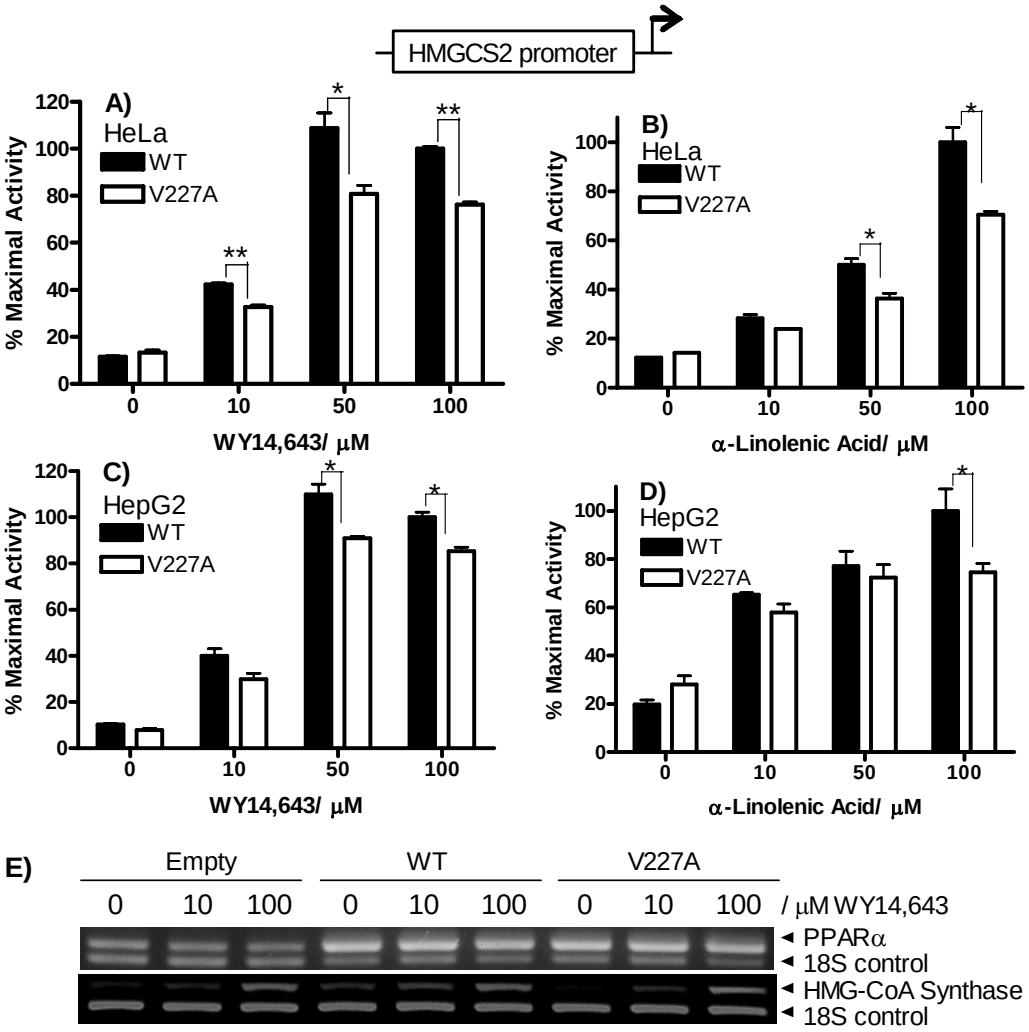
Next, to evaluate the effects of V227A in its functional context, we measured expression of HMGCS2 mRNA by duplex semi-quantitative RT-PCR (Walter et al. 2004). WT or V227A were expressed in HepG2 cells and exposed to WY14,643. Ligand treatment did not affect WT or V227A PPARα gene expression (Fig. 3.3.6E, upper panels). In cells transfected with empty vector, WY14,643 dose-dependently induced

HMGCS2 mRNA expression consistent with endogenous PPARα effect (Fig. 3.3.6E). As expected, transfection of PPARα plasmids increased expression of PPARα mRNA, and no differences were detected in the expression of the WT and V227A variant (Fig. 3.3.6E, upper panels). The presence of ligand dose-dependently increased HMGCS2 mRNA, such that mRNA content was about 75% higher compared to baseline at maximal dose, consistent with HMGCS2 being a PPARα-regulated gene (Fig. 3.3.6E, lower panels). With the V227A plasmid, HMGCS2 mRNA expression was consistently lower compared to WT. Transactivation was ~36% lower in the absence of ligand and was ~20%-10% lower with WY14,643 (10μM, 100μM) in three independent experiments.

In agreement with V227A's transactivation activity on the CYP4A6 PPRE (Fig 3.3.2), V227A induced lower promoter activity in the HMGCS2 gene.

Figure 3.3.6 The PPARα V227A variant exhibits lower transactivation activity on the mitochondria HMG-CoA synthase (HMGCS2) promoter activity

HeLa cells (A,B) and HepG2 cells (C,D), were co-transfected with either full length WT or V227A PPARα (50ng), and a reporter construct driven by residues -1081 to +22 of the HMGCS2 promoter (100ng). Cells were exposed to indicated doses of WY14,643 or α-linolenic acid for 48 h and luciferase activity are expressed as percentage of maximal WT activity (mean ± SE of three replicates). * $p<0.05$; **$p<0.01$. (E) HMGCS2 mRNA expression. HepG2 cells were transfected with full length WT or V227A PPARα (50ng) and treated WY14,643 (10, 100μM) for 24 h. RNA was extracted and subjected to duplex semi-quantitative RT-PCR. Quantification of mRNA encoding PPARα and HMGCS2 were performed using specific primers with 18s rRNA as endogenous standard. Upper and lower panels were amplified with primers specific for PPARα and HMGCS2 respectively. A representative data set from three independent experiments is shown



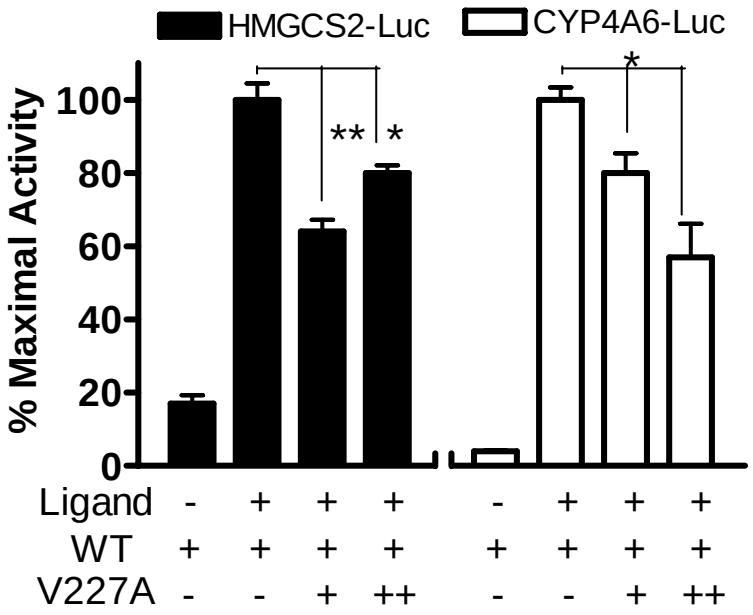
3.3.7 V227A inhibits transactivation by WT PPARα in a dominant negative manner

Lower transactivation activity of a PPARα splice variant (Gervois et al. 1999) and several artificial PPARα mutants (Michalik et al. 2005; Seimandi et al. 2005) have been reported to occur in a dominant negative way. Therefore, to evaluate the effects of mutant on WT activity, WT or V227A PPARα were co-expressed in HepG2 cells (Fig. 3.3.7). WT PPARα transactivity was attenuated by 36% and 20% for the HMGCS2 and CYP4A6-PPRE promoters respectively, in the presence of V227A plasmid (Fig 3.3.7). This dominant negative effect was most marked for the CYP4A6 promoter where a 43% reduction in WT activity was observed with 10-fold excess of V227A.

In all, the V227A variant exhibited weaker transactivation activity in both CYP4A6-PPRE and HMGCS2 promoters in HeLa and HepG2 cells in a dominant-negative manner.

Figure 3.3.7 The PPARα V227A variant exhibits dominant negative activity

HepG2 cells were co-transfected with both full length WT (100ng) and V227A PPARα (100ng or 1000ng); with either the HMGCS2-Luc (200ng) or CYP4A6-PPRE-Luc (200ng) reporter genes, in the absence or presence of WY14,643 (100μM) for 48 h. Values are mean ± SE of three replicates, and expressed as percentage of maximal WT activity. * $p<0.05$; ** $p<0.01$.



3.4 Mechanism(s) elucidation for attenuated PPARα V227A activity

3.4.1 Role of V227A on the LBD

To dissect the molecular basis of reduced transactivation, we systematically studied the effects of the V227A polymorphism on PPARα-mediated signaling pathways. Since the mutation occurs at the ligand binding domain of PPARα, we first investigated the effects of V227A on the properties of the LBD.

Using the mammalian-one-hybrid system as described in Section 3.1.1, chimeric receptors consisting of the PPARα LBD (residues 171-468) of the WT or V227A variants, and the yeast Gal4DBD were cotransfected with the UASG-luc reporter gene into HeLa cells to examine the effects of the V227A polymorphism in the LBD on transcriptional activation. In the presence of WY14,643, V227A LBD exhibited 62%-66% lower transactivation activity compared to the WT (Fig. 3.4.1A). Similarly, Gal-V227A was 28% weaker than Gal-WT with high doses of α-linolenic acid, suggesting that the mechanism for defective transactivation resides in reduced LBD activation function.

To determine if the V227A substitution affected ligand affinity, the ligand binding properties of the mutant receptor was examined in a competition assay. In this assay, [^{3}H]-WY14,643 binding to WT PPARα was largely replaced by unlabeled WY14,643 at doses ≥5nM, whereas the non-PPAR ligand, dihydrotestosterone (DHT), did not affect [^{3}H]-WY14,643 binding (Fig. 3.4.1B). No significant differences in the relative binding affinity of WY14,643 to WT or V227A PPARα were observed, indicating the differences in V227A transactivation function were unlikely to be due to defective ligand binding at ≥5nM.

Here, we demonstrated that while lower transactivation in V227A was a result of reduced LBD function, a difference in ligand binding was not likely to be the cause.

3.4.2 Effects of RXR content on V227A

The mammalian-one-hybrid assay was used as a tool in the identification and characterization of specific PPAR ligands in Section 3.1 and 3.2 previously. This is useful because activation by PPAR ligand in this system is independent of the obligate heterodimerization partner, RXR. Thus, the results in Fig 3.4.1A suggested that the weaker transcriptional activity was independent of RXR.

To confirm this and to examine whether endogenous RXR was limiting due to overexpression of the PPARα receptor, cells were co-transfected with plasmids encoding this obligate heterodimer, PPARα WT or V227A and the CYP4A6 PPRE reporter gene. Differences between WT and V227A were still observed, suggesting that weaker activity of V227A was not stoichiometrically limited by RXR (Fig 3.5.2).

Figure 3.4.1 Effects of V227A on the ligand binding domain

HeLa (A) cells were co-transfected with chimeric Gal-PPARαWT-LBD or Gal-PPARαV227A-LBD (50ng) together with 100ng of $(UASG)_5$-Luc reporter before treatment with ligands for 48 h in the mammalian one-hybrid assay. Values are mean ± SE of three replicates, and expressed as percentage of maximal WT activity. * $p<0.05$; **$p<0.01$. (B) Ligand binding affinity. WT or V227A PPARα in HeLa cells were exposed to 3nM [^{3}H]-WY14,643 alone or with increasing concentrations of unlabeled WY14,643. The amount of [^{3}H]-WY14,643 specifically bound after 24hours incubation was measured and expressed as a percentage of relative to controls not exposed to cold hormone. Data represents means±SEM, n=2.

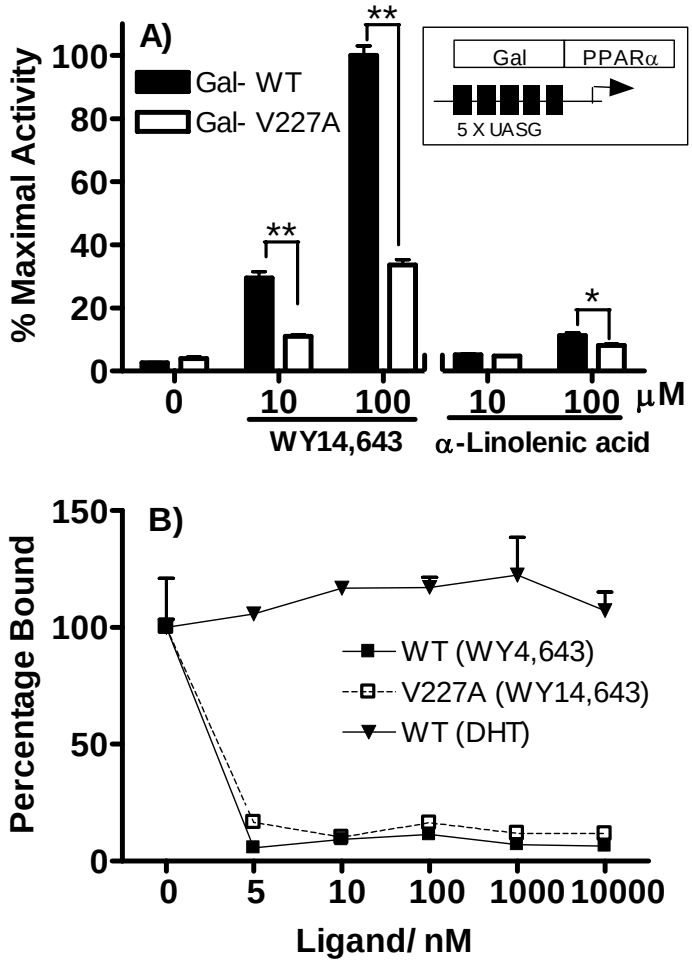
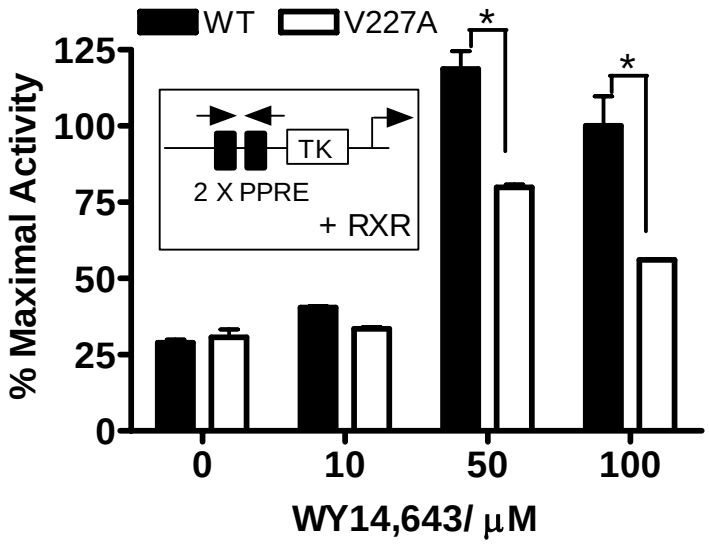


Figure 3.4.2 Weaker transactivation of V227A is independent of RXR content

WT or V227A PPARα (50ng) plasmids were co-transfected in HepG2 cells with 50ng of RXRα and 100ng of CYP4A6-PPRE-Luc reporter plasmids prior to the treatment with WY14,643 in HepG2 for 48 h. Values are mean ± SE of three replicates, and expressed as percentage of maximal WT activity. * $p<0.05$.



3.4.3 Role of V227A on PPARα expression and localization

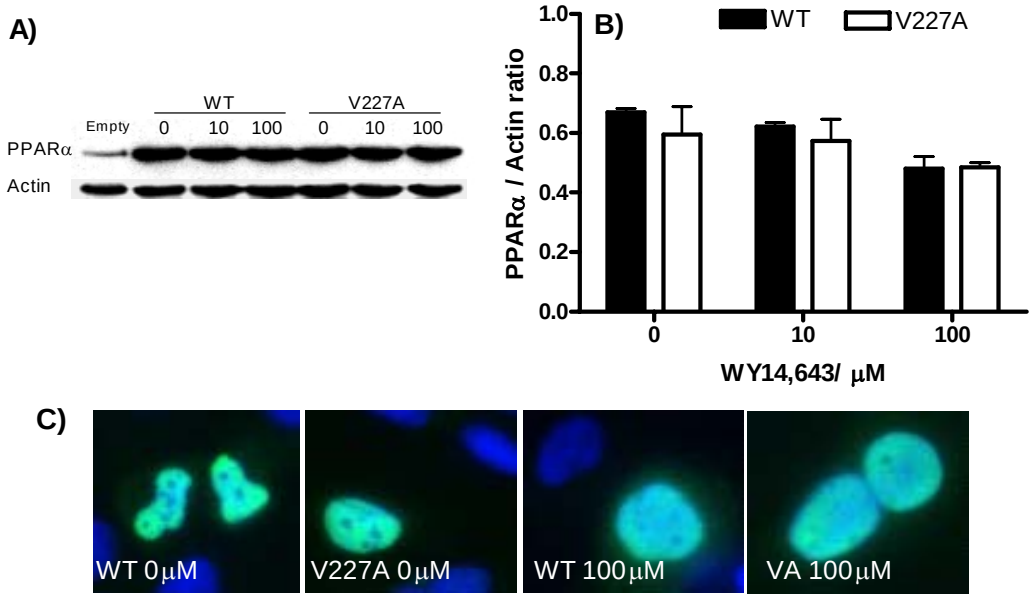
Since lower transactivation of V227A was not dependent on ligand binding and RXR, we next examined whether weaker activity was due to differences in protein expression. Immunoblot studies revealed that WT and V227A protein content in HepG2 cells were comparable in the absence and presence of ligand (Fig. 3.4.3A and B), indicating the substitution did not affect PPARα protein stability.

A natural PPARα splice variant has been reported to affect nuclear location (Gervois et al. 1999), thus, we investigated whether the variant affected sub-cellular localization. Immunofluorescence studies showed that overexpressed WT and V227A PPARα resided in the nucleus, in the presence and absence of ligand, and no differences in sub-cellular localization patterns were observed (Fig. 3.4.3C).

Taken together, weaker V227A activity was not due to differences in protein expression and nuclear localization.

Figure 3.4.3 Weaker transactivation of V227A is independent of protein expression and nuclear localization

HepG2 cells (A) were transfected with 50ng of WT or V227A PPARα and treated with increasing doses of WY14,643 and harvested 48 h post-treatment. PPARα protein was detected with specific antibody and quantified using ScionImage analyzer. (B) Ratio of PPARα and Actin using Scion Image. (C) Sub-cellular localization of PPARα proteins. Full length WT or V227A PPARα (50ng) were transfected into HepG2 before treatment with WY14,643 for 48 h. The resulting cells were stained with PPARα polyclonal antibody and goat anti-rabbit antibody conjugated with FITC. Nucleus was stained with DAPI stain.



3.4.4 Role of V227A on coregulator interaction

To further elucidate the mechanism(s) for the decrease in transcriptional activity of the variant, we compared interactions of WT and V227A with major coregulator proteins known to interact with PPARα (Yu and Reddy 2007). Interactions between PPARα LBD (residues 167-468) linked to VP16 activation domain, and Gal-NR interacting domains of major coregulators (Dowell et al. 1997; Voegel et al. 1998; Dowell et al. 1999; Caira et al. 2000; Vega et al. 2000; Xu et al. 2002) known to interact with PPARα (Table 3.3), in the mammalian-two-hybrid assay, a sensitive method for the measurement of protein-protein interaction.

No obvious differences between WT and V227A were observed in the recruitment of the coactivators SRC-1, TIF2, PGC-1, p300 and PRIP, in the absence or presence of ligand (Table 3.3). Strikingly, marked differences (about 2-fold higher for V227A) were observed in the relative binding of the corepressors, NCoR and SMRT, in the absence of ligand (Table 3.3). Lesser but still significantly increased interactions with V227A were still observed with low dose of ligand, suggesting that increased corepressor recruitment contributed to reduced transactivation function of V227A.

Table 3.3: Interaction of coregulators with PPARα in the Mammalian-Two-Hybrid Assay[1]

	VP16-PPARα (167-468)			
	WT		V227A	
Ligand	-	+	-	+
Gal- Coactivator[2]				
SRC-1 (213-1061)	3.1 ± 0.3	5.5 ± 0.8	4.9 ± 0.5	6.0 ± 0.4
TIF2 (622-869)	1.6 ± 0.0	2.2 ± 0.1	1.8 ± 0.1	2.4 ± 0.1
PGC1α (120-284)	1.1 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	2.3 ± 0.1
p300 (1-117)	26.5 ± 1.0	27.6 ± 3.0	22.7 ± 0.1	24.3 ± 0.1
PRIP (819-1096)	4.8 ± 0.5	7.4 ± 0.6	6.9 ± 1.1	8.2 ± 0.8
Gal-Corepressor[2]				
SMRT (2004-2517)	1.9 ± 0.1	1.9 ± 0.2	4.0 ± 0.3	2.8 ± 0.3
NCoR (1575-2453)	5.3 ± 0.8	4.3 ± 0.7	10.4 ± 0.4	6.7 ± 0.5

[1] HeLa cells were co-transfected with chimeric VP16-WT, VP16-V227A (100ng) and Gal-coregulator (100ng) together with $(UASG)_5$-Luc reporter (500ng). Cells were exposed to the absence or presence of WY14,643 (10μM) for 48 h. Interaction was expressed as fold (mean±SE of three replicates) of VP16 alone.

[2] The coregulator regions, cloned downstream of GAL4DBD, were based on residues reported to interact with PPARα: SRC-1 (residues 579-932) (Dowell et al. 1997); TIF2 (residues 624-869) (Caira et al. 2000); PGC1α (residues 120-284) (Vega et al. 2000), p300 (residues 39-117)(Dowell et al. 1997); PRIP (residues 887-891) (Voegel et al. 1998); SMRT (residues 2124-2149, 2329-2358) (Xu et al. 2002); and NCoR (residues 2110-2453) (Dowell et al. 1999).

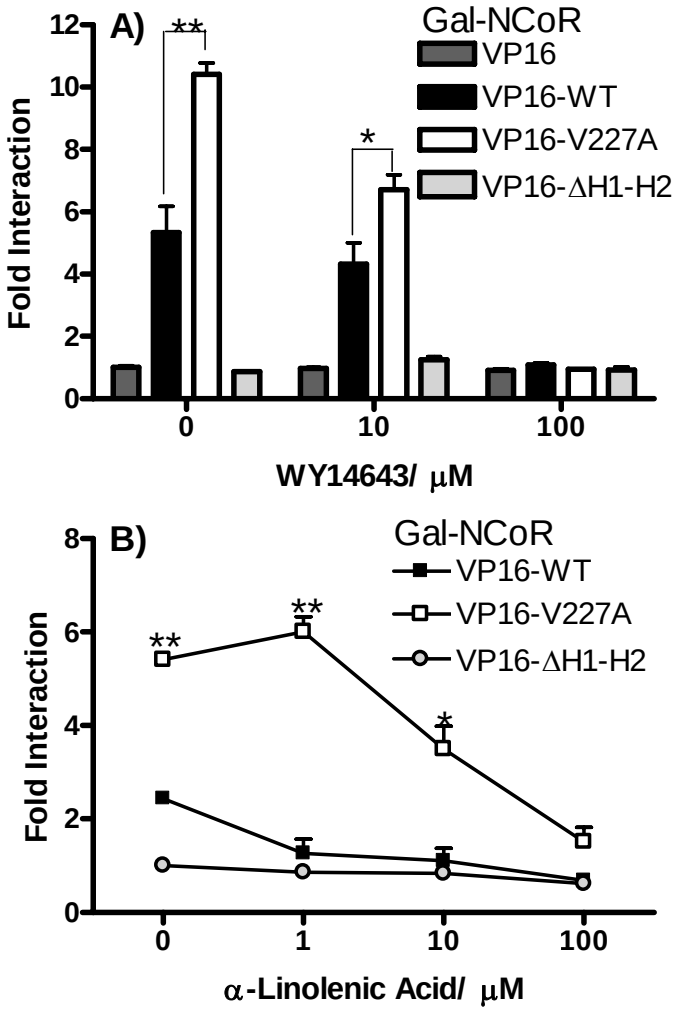
3.4.5 Role of V227A on nuclear corepressor (NCoR) interaction

To confirm that V227A had increase interaction with corepressors and this effect was due to the function of helices 1 and 2 where V227A resides, a LBD mutant (residues 245- 468) (ΔH1-H2) lacking helices 1 and 2 was compared with WT and V227A. In the absence of ligand, WT PPARα bound strongly to NCoR, while ΔH1-H2 exhibited minimal binding (Fig. 3.4.4A). The presence of ligand induced release of NCoR from WT PPAR-LBD in a dose-dependent manner; and at maximal ligand dose, no NCoR binding was observed. Strikingly V227A bound with 100% higher avidity to NCoR compared to WT. The presence of ligand dose-dependently caused release of corepressor, but to a lesser degree compared to WT (Fig. 3.4.4A). Similar differences were observed with α-linolenic acid, wherein V227A bound NCoR with greater avidity versus WT (Fig. 3.4.4B).

Together, the data suggested that increase NCoR recruitment likely contributed to the reduction in transactivation function of V227A. Interestingly, the role of NCoR in PPARα has been controversial (Semple et al. 2005). In order to understand the mechanistic basis of this increased corepressor recruitment and the significance of NCoR in PPARα function, we undertook detailed experiments to examine NCoR/PPARα interactions.

Figure 3.4.4. Interaction of NCoR in the mammalian-two-hybrid assay

HeLa (A) and HepG2 (B) cells were co-transfected with chimeric VP16-WT, VP16-V227A or VP16-ΔH1-H2 (100ng) and Gal-NCoR (100ng) together with $(UASG)_5$-Luc reporter (500ng). Cells were exposed to indicated doses of WY14,643 (A) or α-linolenic acid (B) for 48 h. Interaction was expressed as fold (mean±SE of three replicates) of VP16 alone. * $p<0.05$; ** $p<0.01$.



3.5 Molecular mechanism of attenuated PPARα V227A activity by NCoR

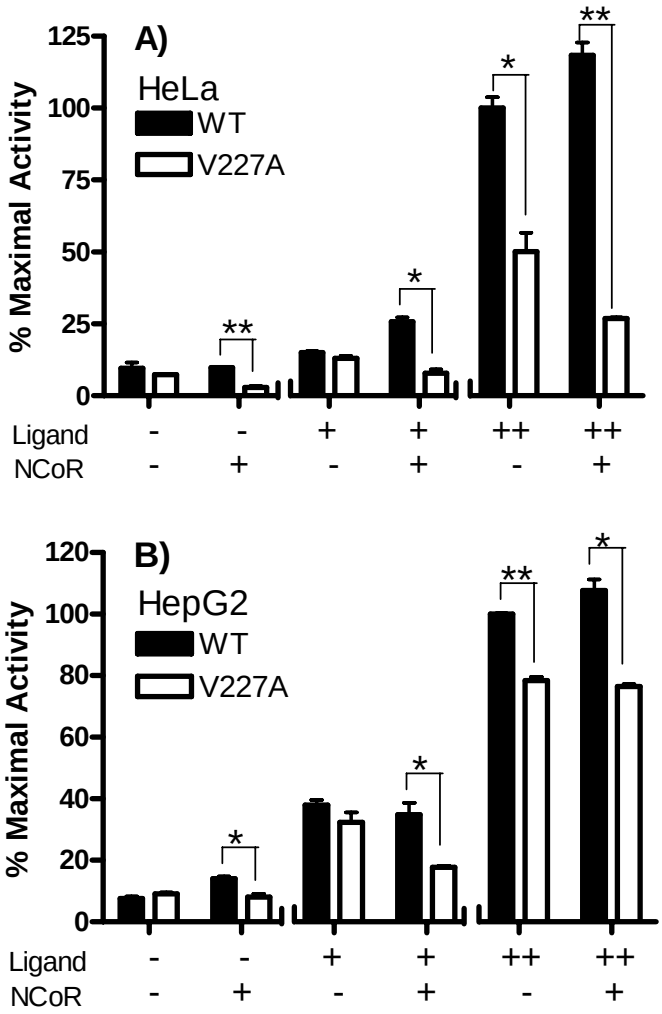
3.5.1 Effects of NCoR overexpression

NCoR is a modular protein that contains N-terminal repression domains and C-terminal nuclear receptor interaction domain (ID) (Horlein et al. 1995; Seol et al. 1996; Zamir et al. 1996; Cohen et al. 1998; Webb et al. 2000). Using the mammalian-two-hybrid system, we previously established an increased in V227A interaction with the C-terminal of NCoR (residues 1575-2453). As the mammalian-two-hybrid system only measures protein-protein interaction, we next evaluated the relevance of this interaction on the PPRE with cotransfected full length NCoR. In the absence of ligand, overexpressing NCoR increased the difference in transcription activity between full-length WT and V227A from 23% to 69% in HeLa cells (Fig 3.5.1A). Increased suppression of V227A versus WT transactivity by NCoR was also observed in the presence of ligand. Similarly, differences between WT and V227A were more evident in the presence of NCoR in HepG2 cells (Fig 3.5.1B). Interestingly, comparing rows 1 and 2 of Fig 3.5.1A, the presence of NCoR did not affect the activity of WT. In contrast, overexpression of NCoR caused a further decrease in V227A activity and was observed with or without ligands in both cell lines tested.

Taken together, the data suggested that the activity of V227A was more sensitive to NCoR content on the PPRE than WT. Consequently, the difference in activity between V227A and WT increased.

Figure 3.5.1. Transcriptional activity of PPARαV227A is sensitive to NCoR overexpression

HeLa (A) or HepG2 cells (B) were co-transfected with either full length WT or V227A PPARα (50ng) and reporter vector CYP4A6-PPRE-Luc (100ng) in the absence or presence of full length NCoR (10ng). Transfected cells were exposed to increasing doses of WY14,643 (10, 100 μM) for 48 h. Values are mean ± SE of three replicates, and expressed as percentage of maximal WT activity. * $p<0.05$; ** $p<0.01$.



3.5.2 Effects of NCoR silencing

While overexpression of NCoR further augmented the difference between WT and V227A, we also investigated the effects of silencing endogenous NCoR expression on transcriptional activity of V227A. HepG2 cells were transiently infected with lentiviral constructs encoding short hairpin sequences complementary to NCoR (siNCoR) or a randomized sequence (siScram). Cell extracts immunoblotted with anti-NCoR confirmed that expression of the corepressor protein was reduced in cells expressing siNCoR compared to siScram (Fig. 3.5.2). Reduction of NCoR was associated with a relative increase in V227A activity, such that differences between WT and variant PPARα became less evident (36% versus 23% attenuation) (Fig. 3.5.2).

Thus, while additional amounts of NCoR increased the difference between WT and VA, silencing of NCoR decreased this difference.

3.5.3 Effects of histone deacetylase (HDAC) inhibition

As repression of transactivation activity by NCoR was dependent on its HDAC properties, we investigated the effects of the HDAC inhibitor, TSA. The presence of TSA dose-dependently restored V227A activity (Fig 3.5.3). Functional differences between WT and V227A decreased from 46.4% in the absence of TSA to 17.6% and 7.6% with the addition of 100 and 200 nM of TSA respectively.

In all, we demonstrated that transcriptional activity of V227A was further decreased with NCoR overexpression and the weaker transcriptional activity of V227A can be partially recovered after NCoR knockdown and in the presence of HDAC inhibitor. These results suggested that NCoR is a *bona-fide* repressor of PPARα activity and that

reduced transactivation activity of the V227A variant can be explained, at least partially, by its increased affinity for this corepressor.

3.5.4 Identification of receptor-interacting domain (ID) on NCoR for PPARα interaction

While the nuclear receptor interaction domains on the C-terminal of NCoR have been described for TR and RAR, RXR and PPARγ (Horlein et al. 1995; Seol et al. 1996; Hu and Lazar 1999; Cohen et al. 2000; Webb et al. 2000; Cohen et al. 2001; Hu et al. 2001; Makowski et al. 2003), it remains unclear whether any, or specific combinations, of the three identified domains interacts with PPARα.

In order to characterize domains mediating NCoR/PPARα interactions, we constructed Gal-NCoR expression plasmids containing one, two, or all three receptor interaction domains (ID) (Fig. 3.5.4A). The ID contained a core consensus nonapeptide motif (LXXI/HIXXXI/L) termed CoRNR box which mediates corepressor assembly on nuclear receptors (Hu and Lazar 1999; Hu et al. 2001). Chimeric Gal-NCoR proteins were co-expressed with VP16-PPARα-LBD, in the mammalian two-hybrid interaction assay. The absence of interactions with NCoR fragments G5 and G6 (containing ID2 and ID3), and G7 (containing ID3), indicated that ID2 and ID3 did not interact with PPARα (Fig 3.5.4B). On the other hand PPARα exhibited strongest interactions with the N-terminal NCoR truncation fragments G3 and G4 (containing ID1), both in the absence and presence of ligand, suggesting that ID1 was the most critical interaction domain (Fig. 3.5.4B). Fragment G2 (containing the CoRNR box and C-terminal flanking sequences of ID1) exhibited >9-fold stronger binding compared to G1 (without an intact CoRNR box), indicating the critical important of the CoRNR box to PPARα interactions. Fragment G3

(containing the CoRNR box and both C- and N-terminal flanking sequences of ID1) induced 2.7-fold higher interactions than Fragment G2, indicating a role of the first 24 amino acids (2251-2274) immediately N-terminal of the ID1 CoRNR box for PPARα/NCoR interactions. Intriguingly Fragment G4 (containing ID1 and ID2) exhibited stronger interactions compared to Fragment G8 (containing all 3 ID and N-terminal sequences) suggesting a potential suppressive function in NCoR residues 1575-2039.

The aggregate data indicate that ID 1, but not ID2 and ID3, was the PPARα ID. In particular, NCoR residues (residues 2251 to 2280) were necessary and sufficient for optimal interactions with PPARα. Compared to WT, the V227A variant showed stronger interaction with NCoR fragments, both in the absence and presence of ligand (Fig.3.5.4B), consistent with previous experiments and its lower transactivation activity.

Figure 3.5.2 NCoR silencing decreases the transcriptional difference between PPARα WT and V227A

HepG2 cells were transiently infected by pLenti6-GW/U6-scramble^{shRNA} (siScram) or pLenti6-GW/U6-NCoR^{shRNA} (siNCoR) and co-transfected with either full length WT or V227A PPARα (50ng) and reporter vector CYP4A6-PPRE-Luc (100ng) before exposure to WY14,643 (100μM) for 48 h. Values are mean ± SE of two replicates, and expressed as percentage of maximal WT activity of siNCoR. * $p<0.05$. Cell lysates were immunoblotted with anti-NCoR to evaluate effects of NCoR knockdown. Cell lysates immunoblotted with anti-PPARα indicated that PPARα expression was affected.

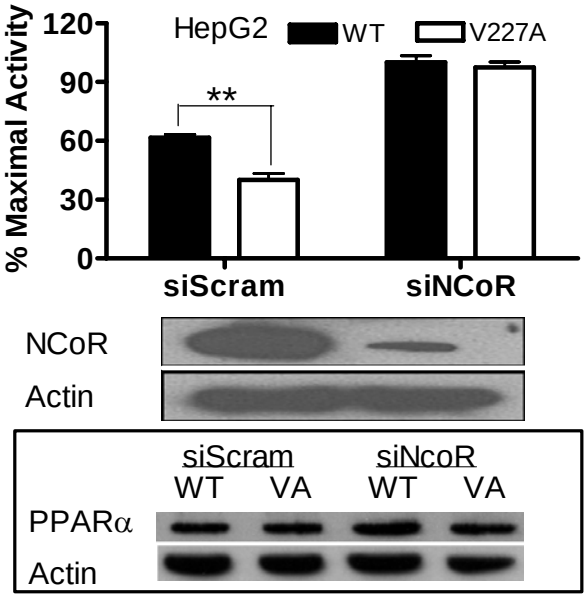


Figure 3.5.3 Transcriptional activity of PPARαV227A is sensitive to HDAC inhibitor

HeLa cells overexpressing WT or V227A PPARα (50ng) together with CYP4A6-PPRE-Luc (100ng) were exposed to an increasing dose of trichostatin A (TSA) (100nM, 200nM) in the absence or presence of WY14,643 (100 μM) for 48 h. Values are mean ± SE of three replicates, and expressed as percentage of maximal WT activity (WY14,643, 100μM) **p<0.01. Cell lysates immunoblotted with anti-PPARα indicated that PPARα expression was affected.

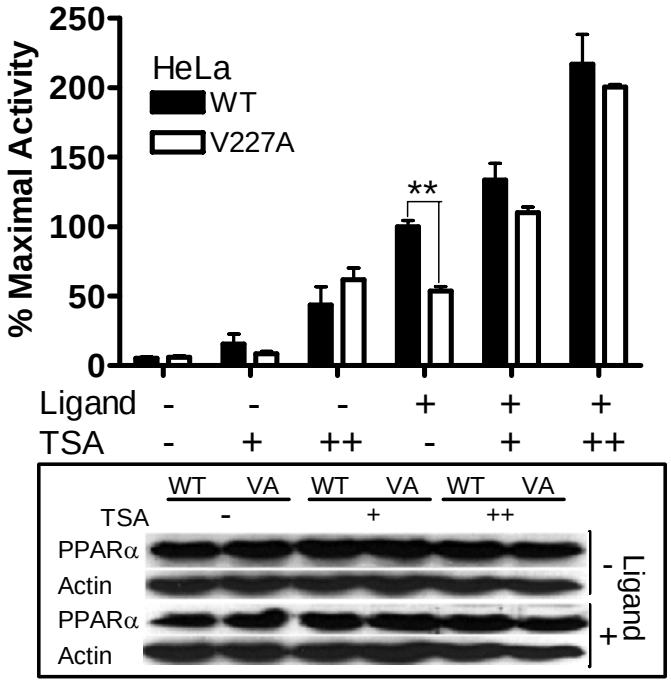
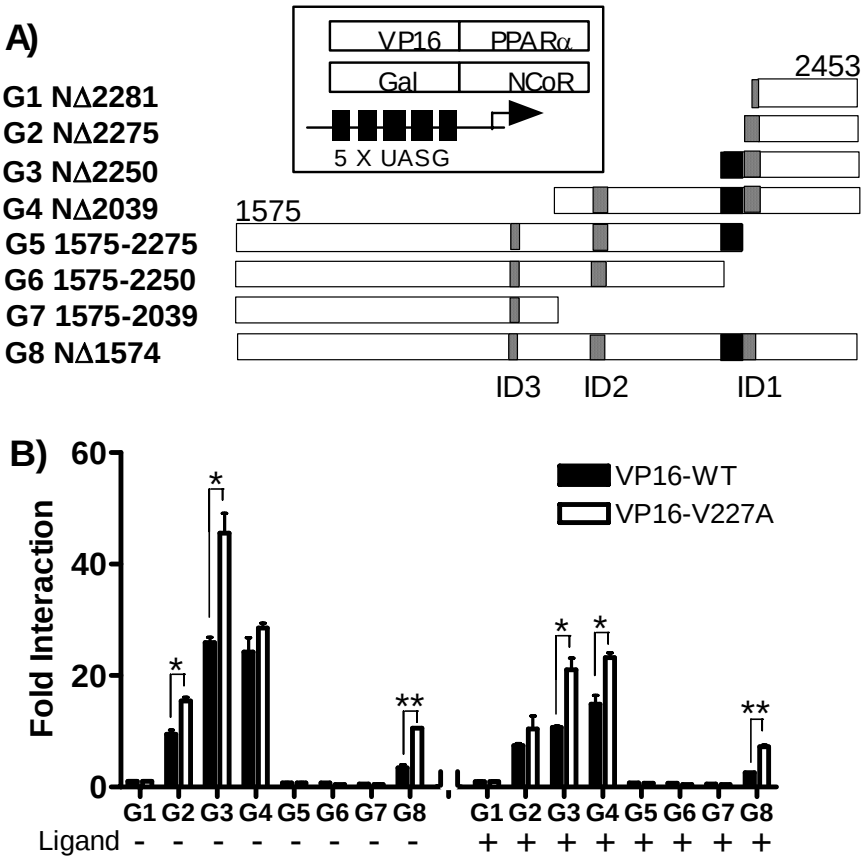


Figure 3.5.4. Interactions between PPARα with receptor interaction domains of NCoR

(A) Schematic diagram of Gal-NCoR truncation fragments and the mammalian-two-hybrid system. Grey boxes represent unique CoRNR box sequences within each receptor interaction domain (ID), residues 2277-2285, 2073-2081, 1949-1953 for ID1, ID2 and ID3 respectively. Black boxes represents the first 24 amino acids immediately N-terminal of the ID1 CoRNR box. (B) HeLa cells were co-transfected with 100ng of PPARα LBD chimeras, VP16-WT- or VP-V227A (100ng), and indicated C-terminal Gal-NCoR truncated fragments (100ng) with the $(UASG)_5$-Luc reporter gene (500ng), in the absence or presence of WY14,643 (10μM) for 48 h. Fold interaction (mean ± SE of three replicates) was expressed as folds of G1 at 0μM. * $p<0.05$; ** $p<0.01$.



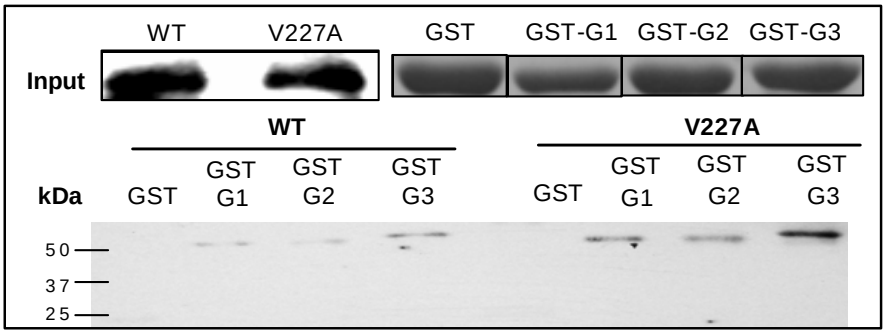
3.5.5 Role of V227A on NCoR-PPARα interaction *in-vitro*

To further confirm residues 2251 to 2280 of ID1 is the PPARα ID, we constructed GST-NCoR fragments in the GST-pull down assay. Chimeric NCoR fragments were incubated with *in vitro* translated full length PPARα WT or V227A and bound proteins were evaluated by western blot analysis using anti-PPARα rabbit polyclonal antibody. Consistent with previous results, interaction of the NCoR truncated mutants with WT and V227A was strongest with NCoR fragment G3, containing the entire ID1. Strikingly, interactions of PPARα V227A with G3 were stronger than WT, confirming the critical importance of this residue to PPAR/corepressor binding (Fig. 3.5.5).

Our results showed for the first time that PPARα/NCoR interactions are mediated predominantly by ID1 on the NCoR, and that the V227A substitution significantly increases this interaction.

Figure 3.5.5 Interactions of PPARα and NCoR using GST pull down assay

GST-NCoR truncated fragments (G1: 2282-2453; G2: 2276-2453; G3: 2251-2453) were incubated with equal amounts of *in-vitro* translated full length PPARα WT or V227A and GST pulldown performed using Sepharose 4B beads. Bound proteins were identified using anti-PPARα rabbit polyclonal antibody. Input shows the immunoblot for *in-vitro* translated PPARα and the coomassie blue staining of GST proteins expressed.



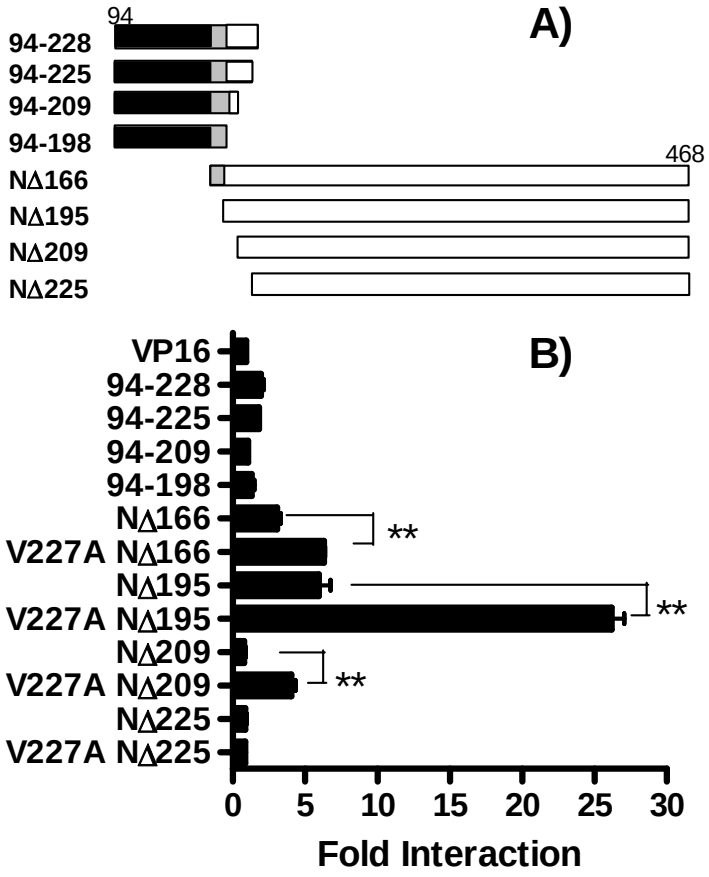
3.5.6 Role of residue 227A on NCoR ID1 interaction

To further define the role of residue 227 for NCoR interactions, several VP16-PPARα truncation fragments, centered on the hinge region (residues 167-244) (Desvergne and Wahli 1999) that begins at the termination of DBD and extending to helix 2 were constructed for the mammalian two-hybrid assay (Fig. 3.5.6A).

The Gal4-NCoR truncated fragment G3 (NΔ2250) was used for the interaction study since this fragment exhibited highest binding to PPARα (Fig. 3.5.4). Fragments containing PPARα-DBD and the pre-helix 1 region (residues 94-198) by themselves did not interact with NCoR (Fig. 3.5.6B). Interactions were strongest with the PPARα fragment containing the entire helices 1-12 (residues 196-468) but excluding the pre-helix 1 region (Fig. 3.5.6B). Unexpectedly inclusion of the pre-helix 1 region 167-198 to the LBD, reduced interaction with NCoR. Mutant LBD fragments missing portions of helix 1 or helix 2 resulted in drastic reduction of interactions, suggesting a critical role for these 2 helices in PPARα/NCoR interactions. Most strikingly, the V227ANΔ195 variant bound NCoR with >4-fold stronger avidity than the corresponding WTNΔ195 fragment (Fig. 3.5.6B), indicating that the V227A substitution introduces a residue that greatly stabilizes PPARα-NCoR ID1 interactions.

Figure 3.5.6 Interactions of NCoR with deletion fragments of PPARα hinge

(A) Schematic diagram of VP16-PPARα truncation fragments. Black boxes represent DBD (residues 94-166), gray boxes represent residues 167-195 before helix 1. White boxes represent residues 196-468 of helix 1 to helix 12. (B) WT or V227A PPARα VP16 truncated fragments (100ng) were co-expressed with G3 (NΔ2250) (Fig. 3.5.4) (100ng) and the $(UASG)_5$-Luc reporter gene (500ng) in the mammalian two-hybrid assay. Fold interaction (mean ± SE of three replicates) was expressed as folds of VP16 empty vector. **p<0.01



3.5.7 Role of V227A on ternary interactions with coregulators

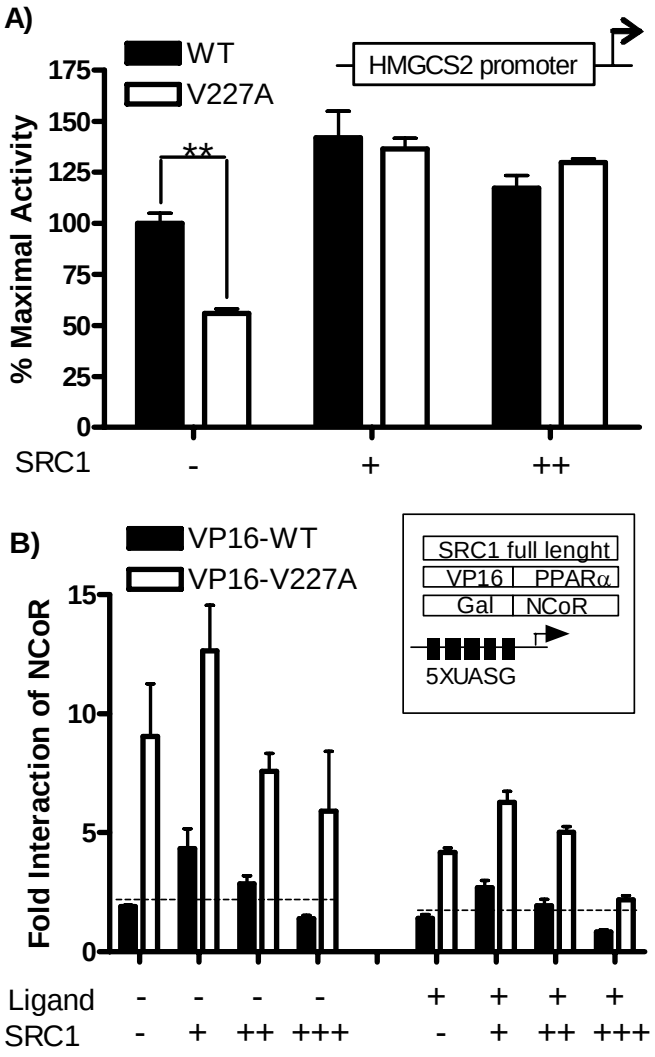
Our previous data have established that V227A provides additional contacts for PPARα-NCoR interaction. Since NCoR and SRC-1 have been shown to compete for the same binding site on PPARα (Xu et al. 2002), we next investigated the dynamics of corepressor and coactivator interactions with PPAR and the role that V227A played in such context.

We first observed the effects of SRC-1 on PPARα-regulated transactivation on the HMGCS2 promoter (Fig. 3.5.7A). Interestingly, exogenous SRC-1 could dose-dependently restore transactivation defect of V227A, indicating a competitive role between NCoR and SRC-1.

In order to measure the effects of SRC-1 on NCoR/PPARα interactions, we performed the mammalian-two-hybrid assay in the presence of increasing doses of full length SRC-1. Low doses of exogenous coactivator increased transactivity of the Gal-NCoR/VP16-PPARα complex as expected (Fig. 3.5.7B). However increasing doses of SRC-1 reduced WT PPARα/NCoR interactions to below baseline, consistent with a model in which NCoR and SRC-1 competed for the same PPARα binding site. The pattern of binding of NCoR to V227A was similar to WT except that it was higher at all doses of SRC-1, indicating that the substitution interfered with the dynamics of corepressor/coactivator exchange.

Figure 3.5.7 Ternary interactions between SRC-1, NCoR and PPARα

(A) SRC-1 overexpression. HeLa cells were co-transfected with either full length WT or V227A PPARα (50ng), a reporter construct driven by residues -1081 to +22 of the HMGCS2 promoter (100ng) and increasing amounts of full length SRC-1 (50, 100ng) with 100 μM WY 14,643 for 48 h. Values (mean ± SE of three replicates) were expressed as percentage of maximal WT activity (WY14,643, 100 μM). (B) Effects of SRC-1 on NCoR/PPARα interactions. HeLa cells were co-transfected with chimeric VP16-WT, VP16-V227A (100ng) and Gal-NCoR (100ng) together with the $(UASG)_5$-Luc reporter (500ng). Cells were exposed to WY14,643 (10 μM) for 48 h at increasing doses (50, 100, 200ng) of full length SRC-1. Fold interaction (mean ± SE of three replicates) was expressed as folds of VP16 empty vector. * $p<0.05$; ** $p<0.01$



3.5.8 Role of V227A on NCoR-PPARα interaction *in-vivo*

To compare corepressor binding between WT and V227A *in vivo*, immunoprecipitation of PPARα with NCoR was performed in HepG2 cells. Cell lysates were precipitated with PPARα antibody, and bound corepressor quantified by immunoblotting with anti-NCoR. In the absence of ligand, both WT and V227A PPARα bound NCoR (Fig. 3.5.8). The addition of 100 μM WY14,643 resulted in almost complete dissociation of NCoR from WT, whereas V227A retained observable NCoR, suggesting that ligand-dependent release of NCoR from V227A was reduced (Fig. 3.5.8, lowest panel). These data were in agreement to our observations previously.

3.5.9 Role of V227A on recruitment of NCoR by PPARα on chromatin

Combinatorial roles of multiple coregulator complexes are required for mediating transcription regulation (Perissi and Rosenfeld 2005). While we have established the role of NCoR in PPARα transcription and the interplay with SRC-1 in such context, we have yet to show the relevance of such dynamics on the chromatin *in-vivo*.

To evaluate PPARα V227A-NCoR complex formation on the HMGCS2 promoter, we performed chromatin immunoprecipitation assays. HepG2 cells overexpressing WT or V227A were cultured in the presence or absence of ligand. Transcription factors interacting with genomic DNA were cross-linked with formaldehyde, and chromatin immunoprecipitated (ChIP) with specific antibodies for PPARα, p300, SRC-1 and NCoR. Genomic DNA fragments recovered after ChIP served as template for PCR reaction with primers for the PPRE of the HMGCS2 promoter. With anti-PPAR, the amount of PPRE

pulled down by WT or V227A PPARα was similar, indicating that receptor-DNA binding did not contribute to weaker transactivation activity of the V227A (Fig. 3.5.9).

Binding of coactivator p300 to PPRE was not affected by the substitution. Interestingly both SRC-1 and NCoR were bound to the WT PPARα/PPRE complex without added ligand, suggesting partial activation by endogenous ligand in HepG2 cells. In comparison, V227A preferentially bound NCoR and much less SRC-1 (Fig. 3.5.9). The presence of ligand induced complete release of NCoR from the WT PPARα/PPRE transcription complex and its replacement by SRC-1, consistent with full transactivation function. In contrast, V227A recruited corepressor strongly to PPRE without added ligand, and exhibited defective NCoR release with exogenous ligand. Competition between NCoR and SRC-1 was evident in that complete activation of the WT PPARα/PPRE transcription complex by ligand was associated with complete release of NCoR and its replacement by SRC-1. In contrast, defective release of NCoR resulted in both NCoR and SRC-1 being present simultaneously in the V227A PPARα/PPRE transcription complex.

This increased binding of corepressor in the presence, and absence, of hormone indicated that mechanism of reduced transactivation was due to increased binding of corepressor to chromatin as a result of the substitution.

Figure 3.5.8 Effect of V227A on PPARα interaction with NCoR

HepG2 cells transfected with full length PPARα-WT or V227A (24μg) were incubated with anti-PPARα in the absence or presence of 100μM WY14,643 for 24 h. Precipitates were probed with anti-NCoR antibody. Inputs were 5% and 10% cell lysate western blotted for PPARα and NCoR expression respectively.

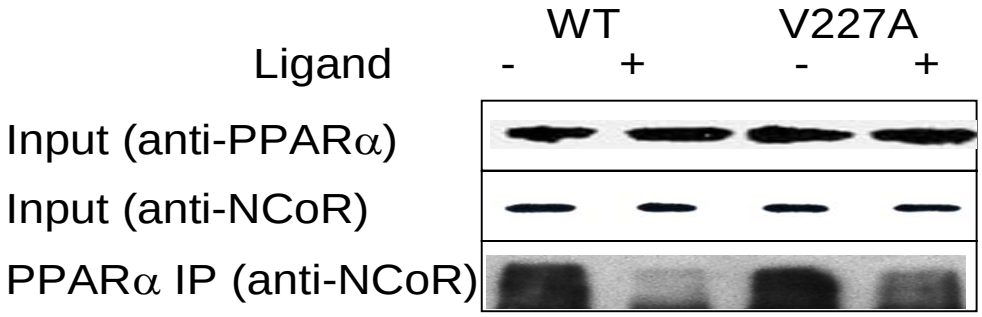
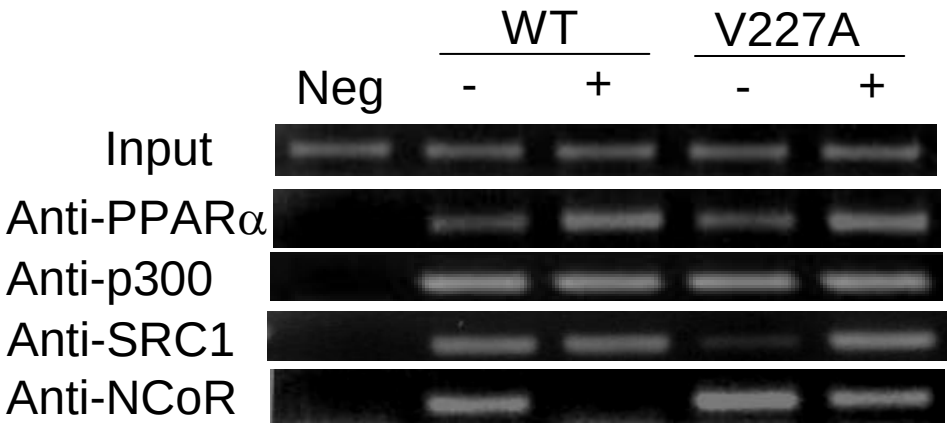


Figure 3.5.9 Effects of V227A in the recruitment of coregulators to the HMGCS2 promoter

HepG2 cells were transfected with 24μg of full length PPARα-WT or V227A, with or without WY14,643 (100μM) for 24 h. ChIP analysis was carried out using antibodies against PPARα, p300, SRC-1, NCoR or rabbit IgG (Neg- negative control). PCR for the PPRE of the HMGCS2 promoter were performed up to 33 cycles. The input was a representative of soluble chromatin (1%) that was reverse cross-linked and amplified under the same PCR conditions.



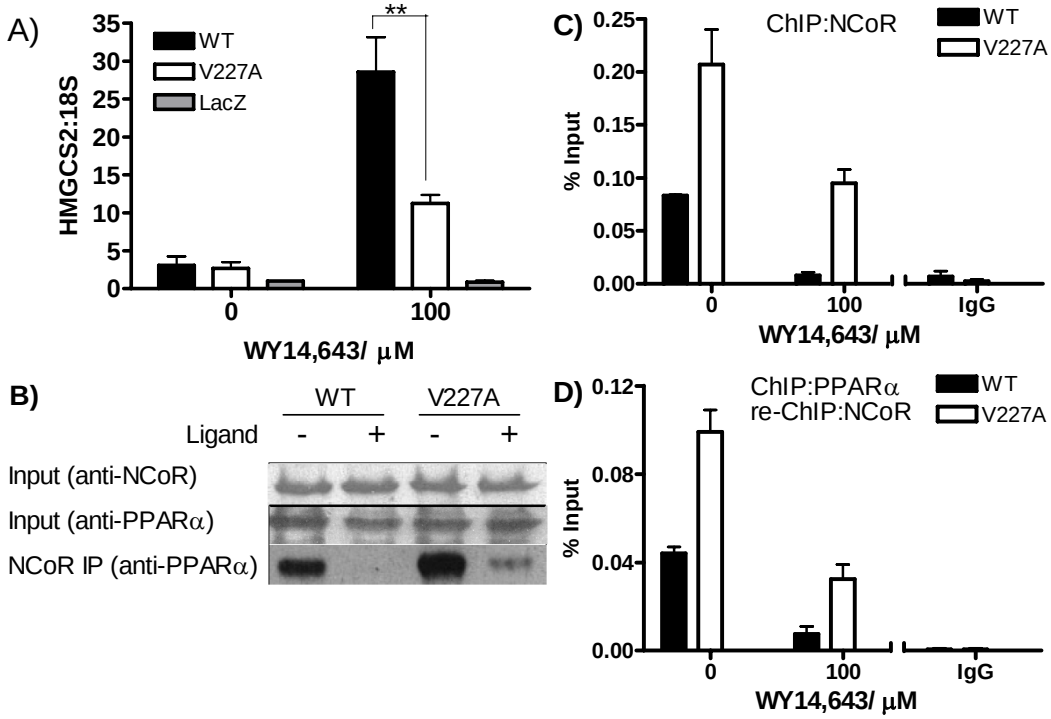
3.5.10 Correlation of V227A gene expression with recruitment of NCoR to the PPRE of the HMGCS2 promoter

To correlate PPARα-regulated gene expression with recruitment of NCoR to the PPRE of the HMGCS2 promoter, HepG2 cells were infected with adenovirus expressing PPARα WT, V227A or LacZ, and HMGCS2mRNA expression measured with quantitative real time RT-PCR. PPARα V227A regulated HMGCS2 mRNA expression was 60% lower compared to WT in the presence of WY14,643 (Fig 3.5.10A). Co-immunoprecipitation experiments performed on replicates indicated that decreased HMGCS2 mRNA expression in the presence of ligand was associated with defective release of NCoR from V227A (Fig. 3.5.10B). Simultaneous chromatin immunoprecipitation with anti-NCoR showed that decreased mRNA expression was associated with increased recruitment of NCoR to the PPRE in the HMGCS2 promoter (Fig. 3.5.10C). Increased binding of the V227A/NCoR complex to PPRE in the presence, and absence, of hormone was also evident in ChiP-re-ChiP experiments, wherein chromatin complexes were sequentially immunoprecipitated with antibodies to PPARα, and then NCoR (Fig. 3.5.10D).

In aggregate, our experiments indicate that the mechanism of reduced transactivation of the substitution was due to increased binding of NCoR to V227A/PPRE chromatin complex.

Figure 3.5.10. Correlation of PPARα-regulated gene expression with recruitment of NCoR to PPRE in the HMGCS2 promoter

HepG2 cells were infected with adenovirus expressing WT PPARα, V227A or empty LacZ vector and treated with, or without, WY14,643 for 24h. (A) HMGCS2 mRNA levels were measured by quantitative RT-PCR and normalized to 18S rRNA. Values are mean ± SD of three replicates, and expressed in relative quantities to LacZ in the absence of ligand. (B) Immunoprecipitation: Cells from representative replicates in (A) were incubated with anti-NCoR rabbit polyclonal antibody overnight, and precipitates probed with anti-PPARα mouse monoclonal antibody. The input was a representative of soluble chromatin (1%) that was reverse cross-linked and amplified under the same PCR conditions. (C) Recruitment of NCoR to HMGCS2 promoter. ChIP analyses were performed on parallel replicates depicted in (A), using antibodies against NCoR or rabbit IgG. Real time PCR was used to quantify amount of PPRE of the HMGCS2 promoter bound to NCoR. D) ChiP-re-ChIP experiments. ChIP experiments were carried out as in (C) and PPARα ChIP complexes were eluted and subjected again to the ChIP procedure using NCoR antibody. Values are mean ± SD of three replicates.



3.6 Summary of results

Two aspects on transcriptional regulation of PPARα were addressed in this work. Dietary flavonoids and their parent botanicals as ligand modulators of PPARα function were first investigated. Extending from this aim, benefits of these flavonoids as dual PPARα/PPARγ agonists were evaluated. In the second part, the role of NCoR in PPARα function was revealed through the study of V227A, a natural PPARα polymorphism.

Our work demonstrated that the purported anti-diabetic effects of the herb, PT, were in part due to its PPAR activation ability. Bioassay-guided fractionation resulted in the identity of daidzin, genistin, puerarin, daidzein and 2'hydroxy-daidzein as bioactive compounds from PT. Because of the clinical benefits of a dual PPARα/PPARγ agonist, we characterized the PPAR effects of isoflavones formononetin and calycosin isolated from another anti-diabetic herb, AM, together with daidzein from PT and 2 common isoflavones, genistein and biochanin A, using chimeric and full-length PPAR constructs *in vitro*.

There was a subtle hierarchy of PPARα/PPARγ activities, indicating that biochanin A, formononetin, and genistein were more potent than calycosin and daidzein in chimeric as well as full-length receptor assays. Respective PPARα and PPARγ regulated gene expression levels reflected a similar trend. At low doses, only biochanin A and formononetin, but not genistein, calycosin, or daidzein, activated PPARγ-driven reporter-gene activity and induced differentiation of 3T3-L1 preadipocytes. Our data suggested the potential value of isoflavones, especially biochanin A and their parent botanicals, as anti-diabetic agents and for use in regulating lipid metabolism. Screening of

other structurally related compounds showed that flavones were the only other flavonoid group with PPAR activity. In particular, diosmetin was PPARγ selective.

The functional significance of the V227A substitution on PPARα was also addressed. The polymorphism significantly attenuated PPARα-mediated transactivation of the CYP4A6 and HMGCS2 genes, with polyunsaturated fatty acids and the fibrate, WY14,643, in a dominant-negative manner. Lower transactivation of V227A was ligand dependent as it was only evident with WY14,643 and α-linolenic acid among the panel of PPARα ligands examined. This lower activity was localized to the ligand binding domain, was independent of its obligate partner, RXR, content, and was not due to a change in receptor ligand-binding properties, protein expression or sub-cellular localization.

Screening of a panel of PPARα coregulators revealed that V227A enhanced recruitment of the nuclear corepressor, NCoR. Weaker transactivation activity of V227A could be restored by silencing NCoR, or by inhibition of HDAC activity. Deletion studies indicated that PPARα interacted with NCoR ID1, but not ID2 or ID3. This interaction was dependent on the intact consensus nonapeptide nuclear receptor interaction motif in NCoR ID1, and was enhanced by the adjacent 24 N-terminal residues. Novel corepressor interaction determinants involving PPARα helices 1 and 2 were identified. V227A interact stronger with NCoR *in vivo*. The V227A substitution stabilized PPARα/NCoR interactions in the unliganded state, and caused defective corepressor/coactivator exchange in the presence of ligands, on the HMGCS2 promoter in hepatic cells. These results provided the first indication that defective function of a natural PPARα variant was due to increased corepressor binding. Our data suggest that the PPARα/NCoR

interaction is physiologically relevant, and can produce a discernable phenotype when the magnitude of the interaction is altered by a naturally occurring variation.

CHAPTER 4: DISCUSSION

4.1 Botanicals as a rich source of PPAR active ligands

Approximately one-third of the top-selling drugs in the world are natural products or their derivatives (Kartal 2007). Despite major scientific and technological progress in combinatorial chemistry, the cost of developing a synthetic drug still averages between USD 80 million to several billion and can take several years before final approval for use. Indeed, although over 50 Investigational New Drug applications have been filed for PPAR ligands with the FDA in the last 7 years, none has successfully reached the market (Shearer and Billin 2007). Natural products, on the other hand, offer unsurpassed chemical diversity, novelty, affinity characteristics and evolutionary selection for biological activity than synthetic drugs, and at a fraction of the cost (Chapman 2004). Consequently, attention is now returning to the wealth of compounds derived from natural products.

The criteria for approval of herbal mixtures as medicines are starting to relax (Qiu 2007). In June 2004, the FDA issued new guidelines that permit the approval of herbal mixtures if they can be shown to be safe and effective, even if the active constituents are not known. Indeed, the world market for such medicine has reached US$60 billion with annual growth rates between 5% and 15% (Kartal 2007). Such commercial potential has lead to a boom in herbal research which is mirrored by an exponential increase in related patents at the US Patent and Trademark Office.

Many of the herbal extracts exhibited multiple pharmacological actions. However, there is a general lack of scientific rigor on how these mixtures work in terms of efficacy and mechanism of action. Of these extracts, many contain phytochemicals with structural similarities that mimic endogenous molecules involved in nuclear receptor signaling. A

prototype of such structural similarity is the soy isoflavone, genistein and the ER hormone, estradiol.

In search of novel activators for PPAR, we hypothesize that botanicals traditionally used in the treatment of anti-diabetic related diseases contain compounds which are PPAR active. Isolation and characterization of these PPAR active constituents may provide a means to assess the biological efficacy of herbal extracts purported to be anti-diabetic.

We identified the roots of *Pueraria thomsonii* (PT) as a PPAR stimulator. At PT concentrations of > 30μg/ml, it mediated the dual activation of PPARα and PPARγ with a higher preference for PPARα (Fig 3.1.2). In a recent screening of 52 herbal extracts, others showed that nearly half of the screened herbs significantly activated PPARγ (Rau et al. 2006). Of these, 14 were also activators of PPARα and 3 of them activated all three isoforms. Together with our findings, the collective data suggest that botanicals are a natural source of PPAR activators.

The roots of the *Pueraria* species is a popular herb for use in traditional Chinese medicine in the management of diabetes (Chen et al. 2007). The roots of *Pueraria lobata* and PT have been officially recorded in all editions of The Chinese Pharmacopoeia under the same monograph 'Gegen' (*Radix Puerariae)*. However, in its 2005 edition, the two species were separated into individual monographs, namely 'Gegen' (*Radix Puerariae Lobatae*) and 'Fenge' (*Radix Puerariae Thomsonii*), respectively (Chinese Pharmacopoeia Committee 2005), due to their different chemical profiles (Chen et al. 2006). While the roots of *P. lobata* are often used for clinical prescriptions with other supporting herbs (Sun et al. 2007), PT is used as a soup resource in southern China (Jiang

et al. 2005) and as dietary supplements in North America (Prasain et al. 2003). The wider application of PT is perhaps a reflection of lesser potential side effects since it can be taken regularly in food and as a single herb.

Identification of PT as a PPAR stimulator had led to the isolation of several isoflavonoids with PPAR activity. Seven major isoflavonoids from the roots of the *Pueraria* species have been reported (Chen et al. 2007). They are 3′-hydroxypuerarin, puerarin, 3′-methoxypuerarin, daidzin, genistin, formononetin-7-glucoside and daidzein. Through the Gal-PPAR chimera reporter gene bioassay, we identified daidzin, genistin, puerarin, daidzein and 2'hydroxy-daidzein as PPAR active. Thus, of the 7 major isoflavonoids of the *Pueraria* species, we showed that at least 4 of them in PT are PPAR activators. This also provided mechanistic insights and a possible rationale for the traditional use of *Pueraria* species for their anti-diabetic properties.

Among the PPAR active isoflavonoids identified in our study, daidzin, genistin and puerarin are glycosides of the aglycones daidzein, genistein and puerarein respectively. Most flavonoids occur in plants as glycosides and reach the stomach and small intestine intact before being absorbed after the removal of the sugar molecule (Erdman et al. 2007). Among the flavonoids, isoflavones are particularly well absorbed in humans and it is likely that the aglycones forms, of which the findings will be discussed in detail below, or the methylated, glucuronidated and sulfated metabolites are the molecules responsible for PPAR activity in the body. The total isoflavone content of the roots of *Pueraria* species had been reported to be approximately 30 times higher than that extracted from soybeans (Haluzik and Haluzik 2006). While soybeans has been the most prominent source of isoflavones in the diet (Erdman et al. 2007), botanicals such as PT

may provide an alternative supply of isoflavones. In this work, we showed that isoflavones in PT and another anti-diabetic herb, AM, are PPAR active and supports the hypothesis that botanicals are a rich source for PPAR active ligands.

4.2 Isoflavones in anti-diabetic botanicals are PPARα/PPARγ dual agonists

Isoflavonoids can be found in large abundance in the plant family of Leguminosae. Of the 13,000 species of legumes from this family, only about 20 are commonly consumed by humans (Mazur 1998). Studies on isoflavone levels of legume and non-legume based foods have been done. Table 4.1 lists common foods or botanicals containing significant amounts of the selected isoflavones examined in this work and their ranges (Mazur 1998; Ma et al. 2002; USDA 2002; Wu et al. 2003; Delmonte and Rader 2006). Focus has been typically on isoflavones in soy and its products. Although genistein and daidzein can be found in large quantities in soy, daidzein is also present in high amounts in the roots of the *Pueraria* species. Biochanin A and formononetin can be found mainly in legumes such as clover, pea and sprouts. An isoflavone less commonly found in food, calycosin, is found in clover and at high amounts in AM. While isoflavones are well characterized for their estrogenic properties, we demonstrated that isoflavones also have dual PPARα/PPARγ activity.

Our study documents that the "anti-diabetic" herbs AM and PT are dual PPARα/γ activators due to their isoflavone constituents. Furthermore closely-related isoflavones exhibited significant differences in transcriptional potencies and abilities to regulate adipocyte differentiation. In the chimeric PPARα assay, formononetin and biochanin A had potencies (EC50 of <1.0 μM and 1.3 μM respectively) (Table 3.2) that were

Table 4.1. Common botanicals/foods rich in selected isoflavones[1]

Isoflavone	*Botanical name* Common name	Quantity (mg/100g)	Reference
Calycosin	*Trifolium pratense* Red Clover	21- 184	Wu et al. 2003
	Astragalus membranaceus Astragalus	7400	Ma et al. 2002
Formononetin	*Trifolium spp.* Clover Sprouts	2.28	USDA 2002
	Pueraria lobata Kudzu Root	7.09	Mazur et al. 1998
	Medicago Sativa Alfalfa Sprouts	Trace- 261	USDA 2002
	Trifolium pratense Red Clover	22.3- 1322	Mazur et al. 1998; USDA 2002; Wu et al. 2003
Daidzein	*Glycine max* Soybean	1- 56[2]	
	- Tofu	1.15- 25.8[2]	USDA 2002
	- Tempeh	4.67- 27.3[2]	
	- Miso	7.1- 36.64[2]	
	Trifolium pratense Red Clover	4- 99	Mazur et al. 1998; Wu et al. 2003
	Pueraria lobata Kudzu Root	48.1- 185	Mazur et al. 1998
Genistein	*Glycine max* Soybean	4.6- 84.1	Mazur et al. 1998
	- Tofu	2.89- 37.7[2]	
	- Tempeh	1.11- 39.77[2]	USDA 2002
	- Miso	11.7- 52.39[2]	
	Trifolium pratense Red Clover	6- 580	Mazur et al. 1998; Wu et al. 2003
	Pueraria lobata Kudzu Root	12.6- 56.1	Mazur et al. 1998
Biochanin A	*Trifolium pratense* Red Clover	20.4- 833	Mazur et al. 1998; USDA 2002
	Pisum sativum Chinese peas	9.31	USDA 2002
	Pueraria lobata Kudzu Root	1.4	Mazur et al. 1998
	Cicer arietinum Chickpea	3.1- 0.8	Mazur et al. 1998

[1] For meaningful comparisons, only recent (since 1998) and comprehensive studies are included. Figures expressed as a percentage of the dry weight of the food, unless otherwise stated.
[2] mg/100g of edible portion

comparable to that reported for synthesized PPARα/γ agonists such as ragaglitazar (3 μM) (Lohray et al. 2001), tesaglitazar (3 μM) (Davis 2002) and muraglitazar (0.3 μM) (Devasthale et al. 2005). With respect to PPARγ, formononetin and biochanin A (EC50=2.6 μM and 3.7 μM respectively) (Table 3.2) were less potent compared to ragaglitazar (0.093 μM) (Lohray et al. 2001), tesaglitazar (0.149 μM) (Davis 2002) and muraglitazar (0.11 μM) (Devasthale et al. 2005). Nevertheless, these isoflavones exhibited balanced ratios of PPARα/PPARγ activity ratios of 1:3 in chimeric and almost 1:1 in full-length PPARα/γ assays (Table 3.2). This balanced activity PPARα/PPARγ profile may potentially enhance the attractiveness of biochanin and formononetin, and foods containing them, in the management of the metabolic syndrome (Eckel et al. 2005).

Synthetic dual PPARα/γ drugs exhibit considerable side effects including edema, carcinogenicity in rodent toxicity models and increased cardiovascular risks (Lohray et al. 2001; Davis 2002; Saad et al. 2004; Barlocco 2005; Devasthale et al. 2005; Rubenstrunk et al. 2007). Although dual PPAR agonists like muraglitazar and tesaglitazar have elicited high hopes and were evaluated in large scale phase III clinical trials of type 2 diabetic patients, they have recently been discontinued for development (Nissen et al. 2005; Rubenstrunk et al. 2007).

It is noteworthy that of the failed PPAR agonists, none were PPARα-preferential dual agonists (Rubenstrunk et al. 2007). For most, a higher concentration of dual agonist is required for PPARα activity to be maximal than for PPARγ. For example, PPARα/PPARγ activity ratio for ragaglitazar was 32:1 (Table 4.2). In contrast, PPARα/PPARγ activity ratio for the more balanced dual agonist, formononetin, was around 1:1. Indeed, most safety issues that led to development discontinuations were

Table 4.2 Comparisons of activity ratios between natural and synthetic dual PPARα/PPARγ dual agonists

Ligand	EC50-PPARα (μM)	EC50-PPARγ (μM)	Activity Ratio (α:γ)
Natural dual agonists[1]			
Calycosin	22	36	0.6:1
Formononetin	3.7	4.3	0.9:1
Genistein	9.5	12	0.8:1
Biochanin A	<1	<1	~ 1:1
Synthetic dual agonists			
Ragaglitazar[2]	3	0.093	32:1
Testaglitazar[3]	3	0.149	20:1
Muraglitazar[4]	0.3	0.11	3:1

[1] From Table 3.2, full length PPAR reporter assay
[2] Loray et al. 2001, chimera Gal-PPAR reporter assay
[3] Davis 2004, full length PPAR reporter assay
[4] Devasthale et al. 2005, full length PPAR reporter assay

associated to over-activation of PPARγ rather than to the action of PPARα. This implies a potential role of PPARα preferential dual agonist or one with a more balanced activity ratio for a more efficient approach to PPAR activation with limited side effects.

In this work, we identified isoflavonoids, and the parent botanical PT, to possess such favourable activity ratios. Indeed, the discovery such herbs and isoflavones might lead to PPAR agonists with improved risk-benefit profiles. Furthermore these isoflavones and their parent foods are available immediately for clinical evaluation.

4.3 Flavonoid structure and PPAR activity

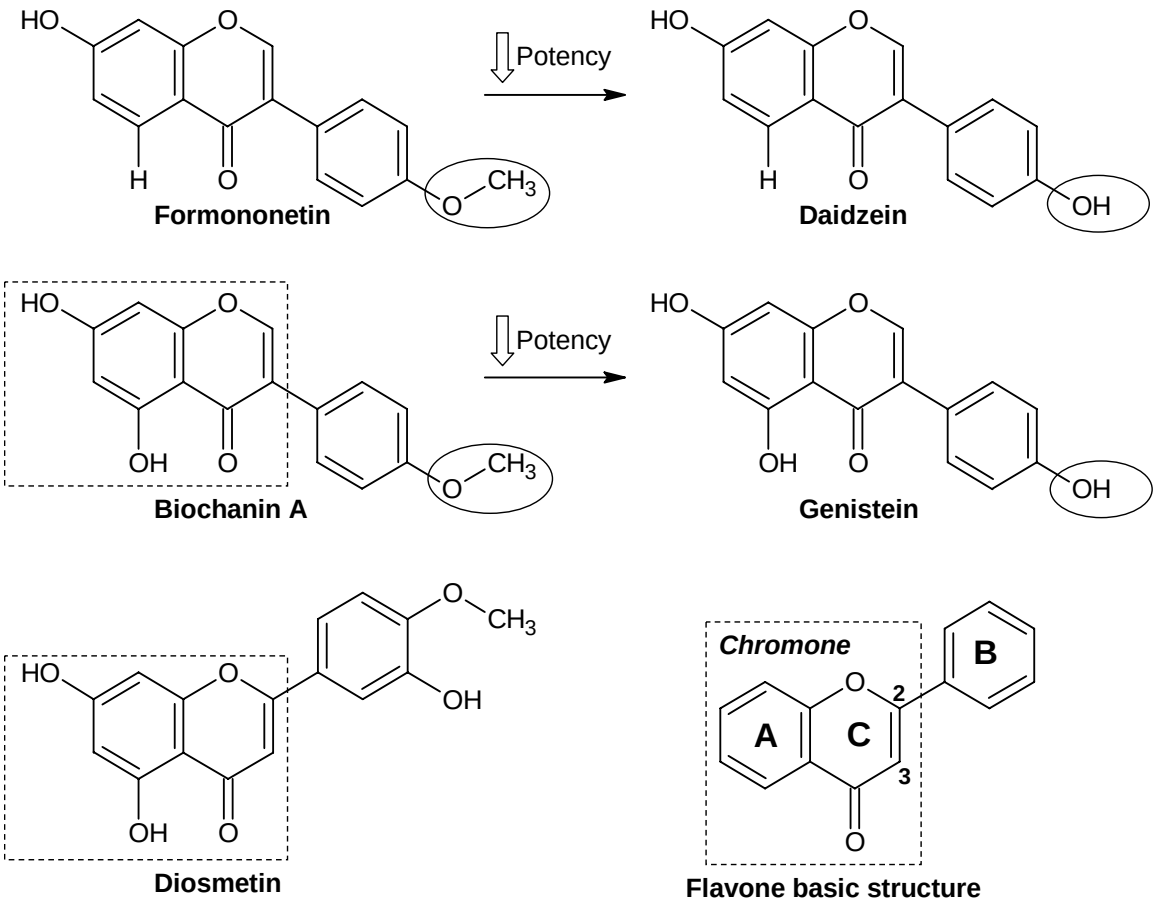
Our data add to the increasing evidence (Dang et al. 2003; Kim et al. 2004; Ricketts et al. 2005) that isoflavones are dual PPARα/γ activators. Besides being phytoestrogens, isoflavones exhibit antioxidant effects and perturb the action of DNA topoisomerase II (Ricketts et al. 2005). Our study indicates that closely-related isoflavones exhibit significant differences in bioactivities. Biochanin A differs from genistein by only an additional methyl moiety in the phenyl B ring but the former is several-fold more potent than the latter (Table 3.2 and Fig 4.1). Similarly formononetin, with an additional methyl moiety, was at least an order of magnitude more potent compared to its metabolite daidzein. The differences in transactivation potencies were consistently observed across several cells lines and on PPAR-regulated adipocyte differentiation, suggesting that they reflect *bona fide* functional differences. Differences in transactivation may be partly due to differences in binding affinity as clear differences in the ability of isoflavone to displace bound PPARγ-fluormone were observed. Biochanin A and genistein displayed the strongest binding affinity corresponding to their strong PPARγ transactivity.

Flavonoids comprised of two aromatic rings (A and B) linked by an oxygenated heterocyclic ring (C) (Fig 4.1). Functional groups and oxidation state of the C ring differentiates flavonoids into different subclasses (Erdman et al. 2007). Although isoflavones are PPAR activators, not all closely-related flavonoids with the basic flavonoid structure are PPAR active. Together with diosmetin of the flavone group, we showed that among the flavonoids, only selected members of the flavone and isoflavone groups activate PPAR.

Both flavones and isoflavones share the same basic structure consisting of two benzene rings (A and B) linked by a heterocyclic pyrone ring (C). In correlation to the structures of biochanin A, genistein and diosmetin with their respective PPAR activity, the similarities suggested that the chromone structure (comprising of the benzene ring A and the heterocyclic pyrone ring C) may favour PPAR activation. Hydrophobicity of the chromone may also influence the strength of PPAR activity. The most marked example being genistein and daidzein which differs only by the additional hydroxyl group on the A ring of the chromone but the former is several-fold more potent than the latter (Table 3.2 and Fig 4.1). In addition, isoflavones differ from flavones by having the B ring attached at position 3 on the C ring instead of position 2 like the latter. Comparing structures of biochanin A and diosmetin, differences of the position of the B ring and the type of chemical moieties attached to it may play a role in PPAR selectivity.

Nonetheless, further molecular and structural studies are necessary to understand the mechanistic basis for these differences, whether they are related to different abilities to recruit co-activators or co-repressors (Guan et al. 2005), and/or cross-activation of other steroid receptors such as ER or RXR (Dang and Lowik 2004).

Figure 4.1 Flavonoid structure and PPAR activity



4.4 Potential application of diosmetin as a selective PPARγ ligand

The anti-inflammatory drug, Daflon, containing 90% diosmin and 10% hesperidin, has been used in the treatment of non-diabetic related diseases for many years. When diosmin was administered orally to healthy volunteers, only its aglycone, diosmetin, was detected in plasma (Struckmann and Nicolaides 1994). In this study, we identified diosmetin as a PPARγ selective ligand (Fig. 3.2.9)

Although the drug Daflon is known to be anti-oxidative and inhibits prostaglandins biosynthesis, detailed molecular mechanism of Daflon's efficacy remains poorly understood (Manthey 2000; Bergan et al. 2001). Our work extends on the biological properties of diosmetin and provides new evidences at the molecular level for new applications of the drug, Daflon.

The current disappointing clinical outcomes in the development of PPAR dual agonist have hastened the quest for new selective PPAR modulating (SPPARM) drugs that can deliver improved therapeutic benefits (Shearer and Billin 2007). The principle of this application is that ligands which modulate the activity of PPAR receptors in a tissue and pharmacological specific manner would allow regulation of desired pathways while limiting undesirable side effect. SPPARMs are similar in concept to selective estrogen receptor modulators such as tamoxifen which displays tissue specific affects. One such SPPARM, Metaglidasen, is currently in Phase III clinical trial after demonstrating significant improvements in metabolic parameters without the side effects of edema or weight gain (Rubenstrunk et al. 2007). Although we only showed moderate activation by diosmetin on the adiponectin gene (Fig 3.2.10), we demonstrated that diosmetin activates PPARγ through ligand binding on a consensus PPRE (Fig 3.2.9). These results may

suggest that diosmetin, and the drug Daflon, may potentially function as a SPPARM on certain PPAR regulated genes yet to be examined.

While more work is required to explore the detailed mechanism of diosmetin induced PPARγ activation, our study suggests the potential new applications of a safe drug already available on the market. Although in comparison with other potent PPARγ agonists, diosmetin is only a mild PPARγ activator, less-potent ligands may be more beneficial for long term application especially in chronic diseases such as the metabolic syndrome.

4.5 Potential application of flavonoids and their parent botanicals as PPAR activators

Isoflavones from soy (Kirk et al. 1998; Mezei et al. 2003) and licorice (Mae et al. 2003) exert anti-diabetic and hypo-lipidemic effects in animal models. There is evidence that soy extracts have anti-lipidemic properties in humans (Bhathena and Velasquez 2002; Zhan and Ho 2005; Taku et al. 2007) and evidence is emerging that they play a beneficial role in obesity and diabetes (Li et al. 2005). The FDA recommends the consumption of at least 25g of soy protein daily for cardiovascular health. Soy-based diets can result in beneficial changes to measures of glycemic control in type II diabetics (Jayagopal et al. 2002; Li et al. 2005). Nonetheless isoflavones are relatively poorly absorbed and serum levels seldom exceed 10μM (Bloedon et al. 2002; Takimoto et al. 2003). Our data indicate that genistein, although exerting strong maximal activity *in vitro* may not have much *in vivo* activity due to its high EC_{50} values (3.0-23 μM). Consumption of a soy beverage (90mg) high in genistein resulted in mean genistein levels of only 0.6μM (Van

Patten et al. 2002). Hence, it is not surprising that effects on dyslipidemia following administration of isoflavones based on soy products have been mixed (Hall et al. 2006; Sacks et al. 2006; Taku et al. 2007). Indeed, due to the conflicting reports, the American Heart Association has issued a summary statement not recommending isoflavone supplements in food or pills due to its nil effects on lipid risk factors (Sacks et al. 2006).

Nonetheless, our data may help explain differences following administration of preparations enriched for biochanin A compared to formononetin on lipid levels (Nestel et al. 2004), based on the higher potency of the former. Although stimulation by biochanin A was weaker than that observed for pioglitazone, its effect on adipocyte differentiation was observed at a low dose of 1 μM, a property not observed for other isoflavones. The marked differences in potency between different isoflavones make it important that studies should be done with foods containing precisely defined amounts of isoflavones in an order that nutritional effects mediated through the PPAR pathway may be evident.

While PPAR agonists are used for the treatment of risk factors associated with the metabolic syndrome and type 2 diabetes, they display side effects which limit their clinical development and therapeutic use. New concepts of specific and selective pharmacological modulation of PPAR activity have emerged (Gervois et al. 2007). Current strategies aimed at reducing side effects involved SPPARMs identification, partial PPAR agonists and the optimization of activity ratios between different PPAR isotype (Rubenstrunk et al. 2007). It is clear that the development of modulators which attain efficient therapeutic activity without PPAR related side effects will be important to fulfill unmet clinical needs in the treatment of metabolic disorders. Our study has

provided additional candidates for PPAR modulation that is in line with the current concepts in PPAR drug design. Not only are these isoflavones and their parent botanicals dual agonists, they also possess favourable activity ratios. Further work using micorarray analysis can determine the potential exploitation of flavonoids, especially diosmetin, as a SPPARM.

4.6 Gene-environment interactions

How genetic variations interact with the environment, and specifically dietary intake, to influence overall cardiovascular heart disease (CVD) risk is an area in which understanding is under rapid expansion (Ordovas 2006a). Gene–environment interaction refers to the differential phenotypic effects of different environments on individuals with the same genotype, or to the differential effects of the same environment on individuals with different genotypes (Ordovas 2006b). Dyslipidemia is one of the risk factors of CVD (Smith 2007). An important group of drugs used in the treatment of dyslipidemia are the fibrates, an agonist of PPARα (Duval et al. 2007).

Recently, it has been suggested that gene–drug and gene–diet interactions may modulate the impact of the PPARα L162V polymorphism. For example, L162V may alter the response of HDL-c to gemfibrozil (Bosse et al. 2002) as well as to fenofibrate (Brisson et al. 2002). Furthermore, it has been suggested that the effect of the L162V on plasma TG and apoC-III concentrations is dependent on the dietary intake of PUFA (Tai et al. 2005). On the other hand, the PPARα V227A polymorphism was associated with perturbations in plasma lipid levels and modulated the association between dietary PUFA and HDL-c (Chan et al. 2006).

Although several natural PPARα variants are known (Naito et al. 2006), mechanistic studies have not been performed on any of them. This is the first demonstration that a natural PPARα variant attenuates transcription because of increased interaction with the corepressors. The V227A polymorphism induced significantly weaker transcriptional effects on a promoter containing the consensus CYP4A6-PPRE in presence of a fibrate drug (WY14,643) and a natural PUFA ligand (α-linolenic acid) in physiologically relevant cells (Fig 3.3.2). The transactivation defect was about 25%-49% weaker compared to the WT and was cell-type dependent. Furthermore, the V227A mutant exhibited significantly weaker activity on the HMGCS2 proximal promoter in reporter gene assays and lower mRNA expression in hepatic cells (Fig 3.3.6).

While attenuation of PPARα V227A activity was consistently observed with WY14,643 and α-linolenic acid, such a trend was not evident with PPARα ligands like the isoflavones biochanin A and genistein (Fig 3.3.1), the fibrate fenofibrate and the PUFA linoleic acid (Fig 3.3.3). Indeed, different ligands make distinct contacts with PPARα, resulting in conformational alterations which facilitate interaction of the receptor with coregulators and/or the general transcription machinery that underlie the molecular basis of ligand-dependent transcriptional activation (Dowell et al. 1997b). In hyperlipidemic patients and human apolipoprotein AI (apoA-I) transgenic mice, while both fenofibrate and gemfibrozil increased HDL-c, the levels of PPARα regulated main constituent of HDL, apoA-I, was only increased by fenofibrate (Duez et al. 2005). This was due to more efficient coactivator recruitment by fenofibrate induction on the apoA-I promoter. Thus, our data suggest that with gene-drug interactions, it is unlikely that differences in response to fenofibrate will be observed between non-carriers and carriers

of the variant. Since WY14,643 was never employed clinically because of hepatocarcinogenesis (Zahradka 2007), ongoing studies comparing gemfibrozil and fenofibrate will shed light on whether carriers of the variant may respond differently to gemfibrozil.

Although Asians consume a high amount of isoflavones compared to their western counterparts (Erdman et al. 2007), our data (Fig 3.3.1) suggest that with gene-diet interactions, it is less likely that differences in PPARα regulated response to isoflavones will play a significant role in any distinction observed between non-carriers and carriers of the variant from their diet.

While there was no distinction in PPARα transactivity between WT and V227A with linoleic acid (Fig 3.3.3), V227A was weaker than WT in the presence of α-linolenic acid (Fig 3.3.2). α-linolenic acid is a ω-3 PUFA while linoleic acid is a ω-6 PUFA. Gene-diet interaction between ω-3 and ω-6 with another better studied PPARα polymorphism, L162V, has been described (Tai et al. 2005). Increased ω-6 intake in carriers of the L162 variant was associated with a marked reduction in TG whereas this association was not observed in non-carriers. However, both experienced beneficial decrease in TG when ω-3 intake was considered. In women who carried the V227A polymorphism, increasing dietary PUFA intake was associated with lower HDL cholesterol (Chan et al. 2006). Our transactivity data with one of the ω-3 PUFA found commonly in the diet were in agreement with Chan *et al.*'s observation as V227A activity was lower than WT in the presence of α-linolenic acid (Fig 3.3.2). While distinction between the type of PUFA intake was not made in Chan *et al* 's association study and α-linolenic acid forms only a part of the total dietary PUFA intake, our data suggested that carriers with the V227A

polymorphism may experience less beneficial effects related to PPARα regulated lipid factors associated with increased ω-3 intake, since V227A activity was lower with α-linolenic acid. In contrast, no such distinction may be observed in association with ω-6 intake.

While others have shown that PPAR activity of L162V was higher than WT, we show here that another PPARα variant, V227A, had reduced PPAR activity. Interestingly, the impact of the polymorphisms on plasma lipid/lipoprotein levels is similar on certain aspects (eg. association with lower TG levels) despite the contrast in PPARα activity. As the regulation of lipid metabolism is a complex process, further studies are needed to compare the differences in the regulation of PPARα specific genes (eg. apoA-I, ACO) by the two polymorphisms to explain such discrepancy.

A major limitation of this work is that the study was dependent on the overexpression system. Furthermore, overexpression was largely carried out using HepG2 cells with a relatively high WT background. While no comparable cell line expressing the V227A variant is currently available, further understanding of V227A activity could be achieved through comparisons between the blood cell populations of carriers of this variant and the wild type. Indeed, differences of the two groups of blood cell populations in response to PPARα ligands and the effects on relevant PPARα regulated genes will provide a clearer characterization of V227A function.

The importance of making a distinction between functional single nucleotide polymorphism (SNP) and nonfunctional SNPs to avoid the use of nonfunctional genetic variants in association studies has been strongly recommended (Humphries et al. 2004; Corella and Ordovas 2005). Here we provide the first example that V227A, a variant

which is present in relatively high frequency in populations of Asia, is functional. This paves the way for further studies to better understand the phenotypes associated with this polymorphism in health and diseases.

4.7 Mechanism(s) for attenuated PPARα V227A activity

In order to understand the mechanisms involved in PPARα V227A transcriptional attenuation, several aspects previously reported to affect transcription of PPARα and other nuclear receptors where first investigated. Although PPARα turnover by the proteasome system through post-translational ubiquitin modification has been shown to affect the ligand-induced expression level of its target genes (Blanquart et al. 2002; Blanquart et al. 2004a), we showed that lower V227A activity was not due to a decrease in its protein levels (Fig 3.4.3A and B).

However, we did not investigate the effects of another post-translational modification, phosphorylation, on PPARα V227A activity. While MAPK and PKA phosphorylation occurs mainly at the N-terminal, PKC phosphorylation occurs at the hinge and LBD of PPARα (Blanquart et al. 2004b). In particular, inhibition of serine residue 230 phosphorylation impaired ligand activated PPARα transcription. However, the detailed mechanism for this repression is unclear. For the estrogen receptor, the mechanistic consequence of phosphorylation at its hinge altered receptor function through ligand, DNA, and coactivator binding (Likhite et al. 2006). DNA binding, through ChIP pulldown with antibodies against PPARα (Fig 3.5.9, second panel), and ligand binding, through radiolabelled competitive binding assays (Fig 3.4.1B), was not affected by V227A since there were minimal differences in comparison to WT. Thus,

while V227A's effect on S230 phosphorylation status was not examined, our results suggest that V227A had minimal effect on the phosphorylation status of S230 even though it is in close proximity.

It has been reported that a natural PPARα splice variant lacking the entire LBD resides in the cytoplasm and exhibits dominant negative activity when localized to the nucleus (Soderstrom et al. 1997). Interaction with Hsp90, a cytoplasmic protein, at the hinge and LBD of PPARα also represses PPARα activity (Sumanasekera et al. 2003a; Sumanasekera et al. 2003b). Activity and subcellular compartmentalization of PPARα was also altered by the centrosome-associated protein CAP350 although specific interaction domains were not investigated (Patel et al. 2005). Our data showed that the lower activity of V227A was not due to a difference in subcellular localization as V227A did not display distinction in co-localization like the PPARα splice variant or from a possible consequence of differential association with other PPARα interacting cytoplasmic proteins in the presence or absence of WY14,643 (Fig 3.4.3C).

Overexpression of PPARα obligate partner, RXR, did not affect V227A's transactivation (Fig 3.4.2). This suggests that V227A's weaker activity was not stoichiometrically limited by RXR. The N-terminal of PPAR has been reported to limit receptor activity through interaction with different PPAR domains (Hummasti and Tontonoz 2006). Using the chimeric Gal-PPARα LBD, we show that the reduction in transcriptional activity of V227A was not dependent on interdomain N-C interactions as differences in WT and V227A activity remained in the absence of the N-terminal (Fig 3.4.1A). Hence, this suggested that AF-1 at the N-terminal was not involved in the mediation of V227A's weaker activity. However, the impaired ligand induction of

V227A was not due to a change in ligand binding properties at concentration ≥ 5nm (Fig 3.4.1B). This is surprising because crystallization studies on PPARβ suggested that the region where the mutation lies defined the entrance of the PPARα ligand binding pocket (Fyffe et al. 2006). Collectively, our data suggest that while ligand binding properties for WT and V227A remained the same, protein conformation changes upon ligand binding and the subsequent recruitment of various transcriptional proteins, especially coregulators, may contribute to the contrast in V227A activity.

4.8 Coactivators and PPARα interaction

The combinatorial roles of multiple coregulator complexes are required for the finely tuned modulation of nuclear receptor mediated regulation on a highly specific and diversified set of genes (Perissi and Rosenfeld 2005). PPARα has been reported to interact with various groups of coregulators (Table 4.3) (Dowell et al. 1997a; Zhu et al. 1997; Miyata et al. 1998; Dowell et al. 1999; Vega et al. 2000; Zhu et al. 2000; Xu et al. 2001; Surapureddi et al. 2002; Xu et al. 2002a; Tien et al. 2004) to achieve this intricate control over gene activation and repression.

To evaluate the involvement of coregulators on V227A attenuation, we compared interactions of WT and V227A with major coregulator proteins known to interact with PPARα (Dowell et al. 1997a; Voegel et al. 1998; Dowell et al. 1999; Caira et al. 2000; Vega et al. 2000; Xu et al. 2002a; Yu and Reddy 2007) (Table 3.3).

There was no obvious distinction between WT and V227A recruitment of the coactivators SRC-1, TIF2, PGC-1, p300 and PRIP, in the absence or presence of ligand (Table 3.3). This was the case even for coactivators, p300 and SRC-1 (Dowell et al.

1997a), previously reported to interact near or within the region where the V227A mutation lies. Among the coactivators, interaction with p300 was highest for both WT and V227A. In concordance with observations made by others (Zhou et al. 1998; Mukherjee et al. 2002; Feige et al. 2006), p300/CBP's high affinity highlights the important role it plays in PPARα activation. Consistent with previous report (Dowell et al. 1997a), there was strong interaction between p300 and the hinge-LBD of WT and which was only modestly enhanced by WY14,643. No significant difference in interaction with p300, especially in the presence of ligand, was observed for WT and V227A, thus suggesting that p300 interaction may not be a major factor contributing to the reduction of V227A activity.

In agreement with others (Dowell et al. 1997a), SRC-1 interacted with WT in the absence of ligand. Interaction with SRC-1 was similar between WT and V227A in the presence of ligand (Table 3.3). However, comparing respective SRC-1 interactions in the presence and absence of ligand, the increase in interaction with WY14,643 was higher in WT (~70% increase) than in V227A (~20%). This suggested that the V227A LBD is less sensitive to ligand induced SRC-1 recruitment. This impaired ligand induced recruitment of SRC-1 may contribute in part to the attenuation of V227A transcriptional activity. Crystallographic data indicated that the second interaction motif of SRC-1 contacts with helix 3 and AF2 of PPARα in the presence of ligand (Xu et al. 2001). This model does not support a role of helix 2, where the mutation lies. However, SRC-1 has more than one receptor interaction motif (Ding et al. 1998; McInerney et al. 1998). While the crystallographic study examined the interaction of a single SRC-1 motif on PPARα (Xu et al. 2001), our mammalian-two-hybrid system, with a truncation fragment that

Table 4.3 Summary of coregulator interaction of PPARα

Cofactor	Ligand	Interaction region on PPARα	Effect of interaction on transcription	Reference
p300	Dependent, WY14,643	Hinge, carboxyl terminal	↑	Dowell et al. 1997
SRC-1	Dependent, WY14,643	Hinge, carboxyl terminal	↑	Dowell et al. 1997
	GW409544	SRC-1 motif interacts with helix 3, 3', 4, 5		Xu et al. 2001
PGC-1α	Dependent, Oleic acid, ETYA	Carboxyl terminal	↑	Vega et al. 2000
PBP/ TRAP220	Dependent, WY14,643	Region not defined	↑	Zhu et al. 1997
PRIP	Dependent, WY14,643	Region not defined	↑	Zhu et al. 2000
CITED2	Independent, WY14,643	Hinge	↑	Tien et al. 2004
PRIC285	Dependent, LTB_4, Ciprofibrate	Region not defined	↑	Surapureddi et al. 2002
NCoR	Independent, WY14,643	Hinge	↓	Dowell et al. 1999
SMRT	Dependent, GW6471	SMRT motif interacts with helix 3, 3', 4, 5	↓	Xu et al. 2002
RIP140	Independent, WY14,643	Carboxyl terminal	↓	Miyata et al. 1998

included three of the four reported interaction domains of SRC-1 fused to Gal, provided the summated effect of interaction between this SRC-1 fragment and PPARα. Thus, V227A's effects on the recruitment of SRC-1 may suggest the role of other helices, like helix 2, in SRC-1-PPARα interaction, not revealed by the crystal.

4.9 Corepressors and PPARα interaction

The most marked difference in coregulator interaction between WT and V227A occurred with the corepressors (Table 3.4). Presence of the V227A mutation markedly increased interaction with NCoR in the absence and at low doses of ligand (Fig 3.4.4). There have been limited studies of corepressors recruitment by PPAR (Table 4.4). For PPARα, studies were few and conflicting. Ligand dependent interaction between PPARα and NCoR has been shown in yeast-two-hybrid assay and this NCoR interaction suppressed the reporter activity of a PPRE driven plasmid in HEK 293 cells (Dowell et al. 1999). However, NCoR did not suppress PPARα activity on the PPRE in other cell lines (Lim et al. 2004). On the other hand, a PPARα-Gal fusion protein could not repress constitutive reporter activity in CV-1 cells which indicated the lack of corepressor recruitment of PPARα (Shi et al. 2002). The same group was unable to detect DNA-bound PPARα-RXR-SMRT complexes. PPARα-SMRT interaction has been shown in the presence of an antagonist (Xu et al. 2002a) and partial agonist (Leesnitzer et al. 2002). Adding to these conflicting results is the observation that minimal interaction was detected for C-terminal NCoR and SMRT with PPARα in a mammalian-two-hybrid assay (Semple et al. 2005).

The roles of corepressors in a closely related protein, PPARγ, have also been controversial. Similar to PPARα, others were unable to detect DNA-bound PPARγ-RXR-SMRT complexes and DNA-bound PPARγ-RXR-NCoR complexes (Zamir et al. 1997; Shi et al. 2002). Similar to SMRT interaction with PPARγ (Lavinsky et al. 1998; Agostini et al. 2004; Wang et al. 2004), NCoR-PPARγ interaction has also been shown through GST-pull down assays and mammalian-two-hybrid (DiRenzo et al. 1997; Zamir et al.

1997; Gurnell et al. 2000). The functional relevance of NCoR-PPARγ's interaction in solution, in contrast to the interaction on DNA, was demonstrated with NF-κB regulated inflammatory gene promoters where sumoylated PPARγ prevented the degradation of NCoR complexes on these promoters and target genes were maintained in a repressed state (Pascual et al. 2005). The role of corepressors on the repression of PPARγ mediated transcriptional activity on specific promoters was only recently shown on the glycerol kinase promoter in adipocytes (Guan et al. 2005) and in the ability to inhibit adipocyte differentiation (Yu et al. 2005). However, studies on corepressor-PPARα interaction are conflicting and the function on specific PPARα target genes is lacking.

We have shown in our study that V227A on PPARα enhanced the interaction of PPARα and NCoR as determined by the mammalian-two-hybrid assay. Subsequently, we demonstrated that the recruitment of NCoR on the PPRE of the HMGCS2 promoter by ChIP and the interaction in solution with NCoR by Co-IP was more with PPARα V227A. These results highlight a role of NCoR transcriptional mediation of PPARα and support the need for ongoing studies on the functional significance of PPARα-NCoR interaction in solution, such as PPARα transrepressive role in T cells and B lymphocytes in inflammation (Jones et al. 2002; Jones et al. 2003; Dunn et al. 2007); and on the chromatin, such as PPARα transrepression of AGP (Mouthiers et al. 2005), fibrinogen β (Gervois et al. 2001) , apoC3 (Gervois et al. 1999) and hepatic SCARB1 (Mardones et al. 2003).

Table 4.4 Summary of corepressor interaction with PPAR

Reference	PPAR	Corepressor	Method	Interaction[1]
DiRenzo et al. 1997	γ	NCoR	GST	-
Zamir et al. 1997	γ	NCoR, SMRT	GST, EMSA	+ GST - EMSA
Lavinsky et al. 1998	γ	SMRT	Co-IP	+
Dowell et al. 1999	α	NCoR	GST	+
Gurnell et al. 2000	γ	NCoR, SMRT	Mammalian-two-hybrid, Co-IP	+
Leesnitzer et al. 2002	α, β, γ	NCoR	Time resolved flurometry	+
Shi et al. 2002	α, γ, β	SMRT	EMSA	+ β - α, γ
Xu et al. 2002	α	SMRT	Co-crystallization of PPARα LBD with SMRT peptide in the presence of antagonist.	+
Agostini et al. 2004	γ	SMRT	Mammalian-two-hybrid	+
Wang et al. 2004	γ	SMRT	Co-IP	+
Lim et al. 2004	α, β	SMRT, NCoR	Reporter Gene	+ β - α
Guan et al. 2005	γ	NCoR, SMRT	ChIP	+ GyK - aP2
Yu et al. 2005	γ	NCoR, SMRT	Mammalian-two-hybrid, siRNA	+
Semple et al. 2005	α	NCoR, SMRT	Mammalian-two-hybrid	-

[1] -, no interaction; +, interaction

4.10 NCoR ID and PPARα interaction

NCoR is a modular protein that contains N-terminal repression domains and C-terminal nuclear receptor interaction domains (IDs) (Horlein et al. 1995; Seol et al. 1996; Zamir et al. 1996; Cohen et al. 1998; Webb et al. 2000). The NCoR possess three nuclear receptor IDs (Seol et al. 1996; Webb et al. 2000). Although the IDs show much homology, they mediate interactions with nuclear receptors differently.

There is preliminary evidence that PPARα interacts with ID1 (Dowell et al. 1999) but the specific ID preference pattern for PPARα is still uncertain, as interaction studies comparing all three IDs have not been performed. Here we show conclusively that NCoR ID1, not ID 2 or ID3, was necessary and sufficient for maximal PPARα-NCoR interactions in mammalian two-hybrid (Fig 3.5.4) and GST-pull down assays (Fig 3.5.5). An intact CoRNR box was necessary, because deletion of the terminal 4 residues of the ID1 CoRNR box nonapeptide abolished PPARα-NCoR interactions (Fig 3.5.4). In addition, our deletion mutants indicate the importance of the N-terminal sequences (2251-2274) flanking the CoRNR box, as their deletion leads to weaker PPARα/NCoR interactions. Interaction with PPARα was lower with NCoR fragment G8 (residues 1575-2453) compared to G4 (2039-2453) suggesting a potential suppressive function in NCoR residues 1575-2039. Intriguingly this fragment has been reported to harbor a sin3/HDAC3 recruiting site (Heinzel et al. 1997; Li et al. 2000).

Nuclear receptors have distinct binding preferences for the three IDs of NCoR (Table 4.5). The TR specifically requires the presence of all three ID1, ID2, ID3 all of which act cooperatively to induce maximal TR-NCoR interaction (Horlein et al. 1995; Seol et al. 1996; Hu and Lazar 1999; Cohen et al. 2000; Webb et al. 2000; Cohen et al.

2001; Hu et al. 2001; Makowski et al. 2003). On the other hand, RAR requires ID2 and interacts only minimally with ID1 (Seol et al. 1996; Hu et al. 2001), whilst its heterodimeric partner RXR interacts with ID1 not ID2 (Hu and Lazar 1999; Hu et al. 2001), giving rise to the model that RXR/RAR heterodimer could be bound to the same corepressor molecule via the two adjacent ID1 and ID2. However this model is not applicable to PPARα, since both PPARα and its heterodimeric partner RXR interacts with ID1, and not ID2. Detailed knowledge of the ID preferences of PPARα may lead to the design of peptidomimetics that can block one of the other of NCoR IDs resulting in targeted effects on PPARα function (Mettu et al. 2007).

Table 4.5 Summary of NCoR interaction domain (ID) binding preferences to selected nuclear receptors

Paper	Receptor	ID1	ID2	ID3	Notes
Horlein et al. 1995	TR	+	NS	NS[1]	
Seol et al. 1996	TR	+	++	NS	Both receptor binds ID2 stronger than ID1.
	RAR	+	++		
Dowell et al. 1999	PPARα	+	NS	NS	ID2 fragment studied was incomplete
Hu et al. 1999	TR	+	++	NS	
	RXR	+	-		
Cohen et al. 2000	TR	+	++	NS	TR prefers to bind to NCoR, on ID2. RAR prefers to bind to SMRT in ID2.
Webb et al. 2000	TR	++	+	+++	All three IDs bind TR alone. ID3 strongest, ID2 weakest. But ID3+ID2 bind TR the strongest.
Hu et al. 2001	TR	++	+	NS	For optimized interaction:
	RXR	+	-		TR-extra resides at N terminal
	PPARγ	+	-		RevErb- extra N and C terminal residues within ID1
	RevErb	+	++		
	RAR	-	+		RAR- extra residues at proximal N terminal
Cohen et al. 2001	TR	+	+	+	ID3 is vital for TR binding
	RAR	-	+	-	
Makowski et al. 2003	TR	+	+	+	While ID3 lacked the extended motif, an isoleucine just proximal to this motif is critical for TR binding

[1] NS: Not studied

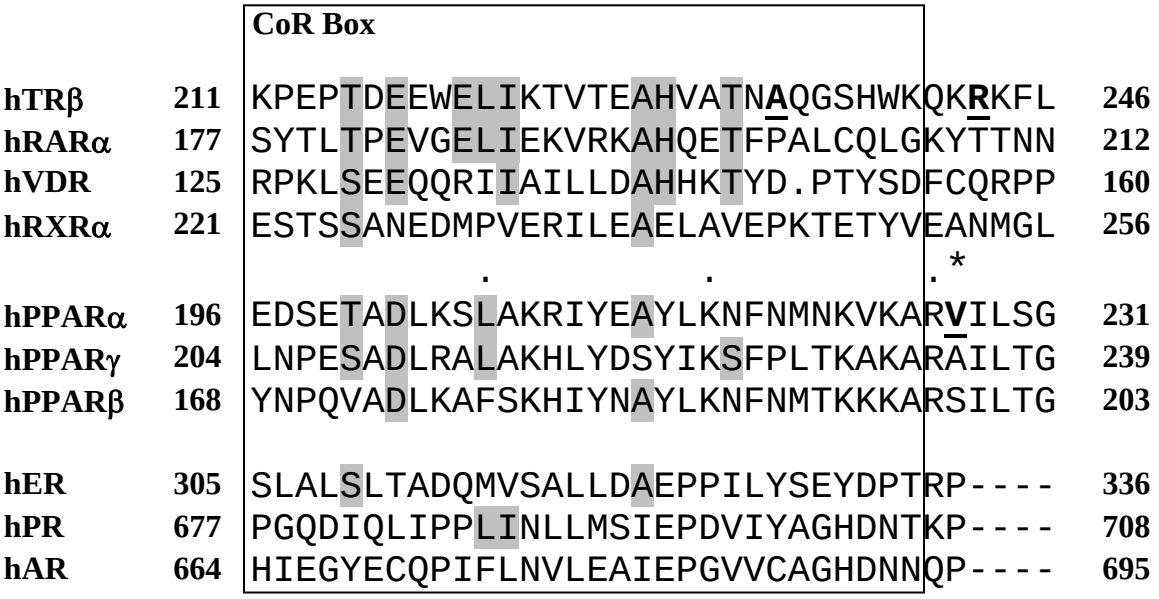
4.11 Function of PPARα hinge in corepressor interaction

The role of the hinge in PPAR is not well studied. Artificial mutations of PPARα in the C-terminal helix 12 are known to increase interactions with NCoR. Deletion of the terminal 13 residues encoding helix 12, results in a truncation mutant (PPARαΔ13) that was able to bind agonists, but did not stimulate transcription (Michalik et al. 2005). This PPARαΔ13 mutant exerted dominant-negative effects, in that it was able to repress the activity of co-expressed WT PPAR. Similarly substitutions in PPARα helix 12 (L459A and G462A), which were created based on PPARγ variants associated with severe insulin resistance, diabetes mellitus, and hypertension (Barroso et al. 1999), resulted in an artificial dominant-negative PPARα mutant that recruited NCoR in a ligand-dissociable manner (Semple et al. 2005). These mutations may block helix 12 from assuming an active conformation resulting in a larger pocket that can accommodate the three-turn corepressor motif, as was observed for antagonist bound PPARα (Xu et al. 2002a).

Besides helix 12, our data indicate that the N-terminal portion of LBD, the short hinge region that begins at the termination of DBD and extending to helix 2 (Huber et al. 2003), plays an important repressor role in PPARα function. Evidence exists from other nuclear receptors that the hinge region plays a key role in receptor/NCoR interactions. Deletion of the AR hinge results in a mutant that is hyperactive, suggesting a repressor function in this domain (Wang et al. 2001). The ERα (Webb et al. 2003) and PR (Jackson et al. 1997) hinge regions recruit NCoR in the presence of partial agonists, and hinge deletion mutants abolished interactions with NCoR. The role of the N-terminal helix in TR and other receptors known to interact with NCoR had been established previously (Horlein et al. 1995). Termed the CoR box (Fig 4.2), this box is well conserved between

Figure 4.2 Sequence alignment of the NCoR box of TR against PPAR and other nuclear receptors

Sequence alignment of TRβ CoR box (residues 211-240) (Horlein et al. 1995) with selected nuclear receptors according to Wurtz et al. 1996. Conserved residues between TRβ and other nuclear receptors highlighted in grey. Natural point mutations on TRβ (A234 and A243) and PPARα in bold underline. V227A PPARα marked with asterisk.



TR and RAR and identifies a regulatory domain in a region which was previously considered to be poorly conserved on the nuclear receptor (Horlein et al. 1995). However, it has been noted by others that a conserved CoR box on PPAR is missing (DiRenzo et al. 1997) (Fig 4.2). This CoR box is also missing on steroid receptors, ER, AR and PR, even though interaction with NCoR had been reported (Jackson et al. 1997; Wang et al. 2001; Webb et al. 2003). Furthermore, NCoR failed to interact with VDR, which is highly conserved within the CoR box, and suggest the possibility of additional requirements for high affinity interaction within the N-terminal portion of the LBD (Horlein et al. 1995), like helix 2 of PPARα.

Intriguingly, sequence alignment indicates that PPARα residue 227 is located between two TRβ hinge residues 234 and 243 that were encountered in patients with resistance to thyroid hormone (Safer et al. 1998). Like the TRβ mutants, our V227A PPARα mutant exhibited dominant negative activity. High resolution crystallography indicates that mutations affecting TRβ hinge residues 234 and 243 modulated the flexibility of the N-terminal region such that higher concentrations of ligand are required for optimal LBD assembly and stability (Huber et al. 2003). Others noted that many of the PPARα residues involved in SMRT interaction of the PPARα-SMRT crystal (Xu et al. 2002a) are not conserved in the LBD of other NRs and suggest that if the structure of the LBD is sufficiently altered, the recruitment of corepressors may be substantially altered as well (Makowski et al. 2003). Indeed, defective release of NCoR by hinge mutants of the TR (Safer et al. 1998) involved residues A234 and R243 which are not well conserved among NR. Furthermore, R243 also lies outside the reported TR NCoR box (Horlein et al. 1995).

It is also interesting to note that the equivalent PPARα 227 residue on PPARγ (residue 235) is already an alanine. The distinction at this single residue between the two PPARs may provide some insights into coregulator (eg. NCoR) affinity and preferences, even though disparity at other residues are likely to be involved as well.

Here we show that PPARα helices 1 and 2, but not the residues preceding helix 1, make important contributions to NCoR interactions, and that the V227A substitution enhances this interaction (Fig 3.5.6). The exact structural basis whereby V227A in helix 2 mediates increased corepressor recruitment, whether by directly modifying contact points with NCoR or as a secondary structural determinant, requires crystallographic analysis.

4.12 Molecular mechanism of attenuated PPARα V227A transcription

The crystal structure of antagonist-bound PPARα revealed that the CoRNR motif fits tightly into a groove formed by PPARα helices 3, 3', 4 and 5 (Xu et al. 2002a). Interestingly, this corepressor groove has also been identified as the critical docking site for coactivators. Although there is overlap, the co-repressor site has a larger interaction interface occupied by the three α-helical turns generated by LXXI/HIXXXI/L of the CoRNR motif, compared to two turns of the SRC-1 coactivator motif, LXXLL (Figure 1.6). It is hypothesized that nuclear receptors distinguish corepressors from coactivators by the length of their interaction motifs. Consistent with this model, our data indicate that NCoR and SRC-1 competed for the same PPARα binding site in solution and in chromatin, and that the V227A substitution favored the binding of the corepressor, resulting in defective recruitment of the coactivator, SRC-1 (Fig 3.5.7).

The dynamic interactions between coactivators and corepressors in PPARα-regulated promoters are controversial. TRβ, RAR, RXR, and PPARγ receptors interact with SMRT and/or NCoR in the absence of hormone. Others, such as the androgen, estrogen and progesterone receptors bind corepressors in the presence of hormone antagonists. Whereas it is clear that PPARα can interact with corepressor in solution (Dowell et al. 1999) or in the presence of antagonists (Xu et al. 2002b), it has not been demonstrated conclusively that NCoR-PPARα binding occurs in PPRE and their presence in chromatin is controversial (Dowell et al. 1999; Semple et al. 2005). It has been suggested that WT PPARα, like PPARγ (Zamir et al. 1997), interacts with SMRT and NCoR in solution, but not on DNA. Our chromatin immunoprecipitation assays show that

WT PPARα interacts with NCoR on the HMGCS2 promoter in the unliganded state, and exogenous ligands induced complete release of NCoR and the simultaneous recruitment of SRC-1 (Fig 3.5.9). The V227A substitution disrupted this process causing incomplete release of NCoR. Defective V227A transactivation function observed with high doses of ligand where NCoR no longer binds to V227A suggest that corepressors other than NCoR may have a role in defective mutant function (Fig 3.4.4). One such corepressor may be SMRT which also binds avidly to V227A (Table 3.4). Ligand dependent corepressors such as RIP140 may play a role as well.

The conventional view that receptors remain stably bound on their DNA response element has been challenged in the past few years (Perissi and Rosenfeld 2005). ChIP analysis of promoter occupancy by nuclear receptors, such as ERα binding on the pS2 promoter (Reid et al. 2003), have shown that binding of the receptor, together with its coregulators, to DNA is characterized by cycles of recruitment and release. Due to the kinetics of coregulator recruitment, the simultaneous occupancy of a chromatin region with corepressors and coactivators is possible, as observed in this work and that of others (Degenhardt et al. 2006). Interestingly, on the recently identified PPARα regulated IGFBP gene (Degenhardt et al. 2006), co-occupancy by corepressor, NCoR, and coactivator, PGC-1α, on the PPRE were observed at various time points in the presence of ligand. On the other hand, since either PPARα or RXR agonist alone is enough to activate transcription of the PPAR/RXR heterodimer (Keller et al. 1993), recruitment of corepressor and coactivator separately by each member of the heterodimer, may account for the co-occupancy of both types of coregulators on the PPRE in the presence or absence of PPARα ligand.

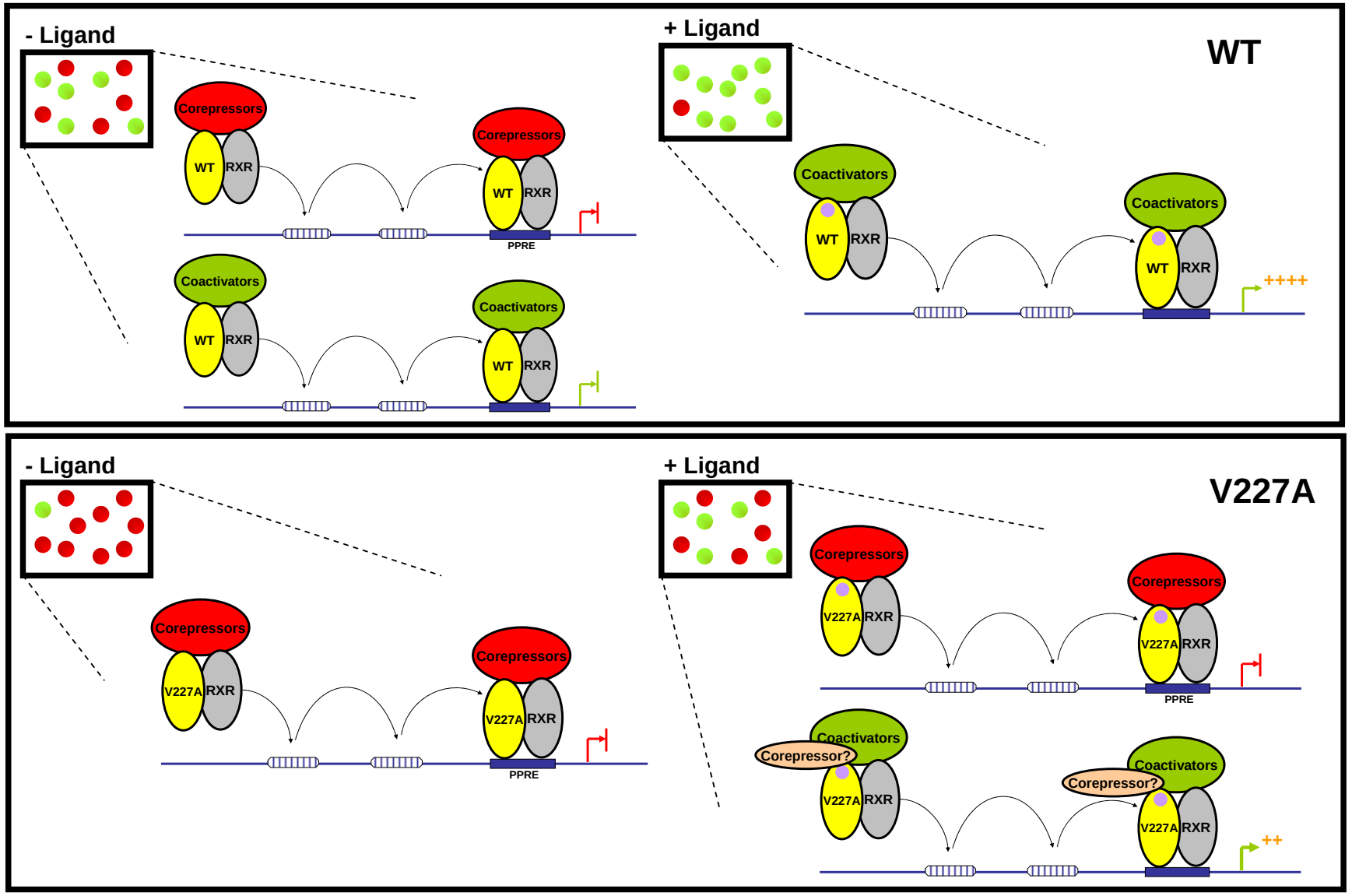
Unliganded ERα were reported to cycle on the promoter and is thought to keep the target gene ready for activation when ligand stimulation occurs (Metivier et al. 2004). Such an observation is compatible to the recently proposed genome scanning model of NR mediated activation that is largely derived from live cell imaging data (Feige et al. 2006). Indeed, in genome scanning, PPAR may transiently interact with the chromatin even in the absence of ligand. Furthermore, mobility of the constitutively associated PPAR/RXR complex indicated the association of large complexes such as corepressor or coactivators complexes during genome scanning (Feige et al. 2005). In addition, these large PPAR coregulator complexes were already pre-assembled before binding onto chromatin and provide a new paradigm in the functional role of coregulator-receptor interaction in solution. Based on this current model of genome scanning and the data derived from our study, we hereby propose a molecular mechanism of action for V227A in Figure 4.3.

While live cell imaging techniques reflect the complexity of a single cell, the ChIP technique reflects overall conditions on a population of cell. Ongoing studies on the nuclear behavior of V227A with coregulators and/or chromatin in living cells will shed light on the mutant's mode of action in the extremely dynamic and multi-compartmentalized nucleus. Indeed, understanding the behavior of NRs in the nucleus of living cells and the underlying impact on transcription will necessitate the combination of microscopy techniques with ChIP experiments. Together this can further facilitate the revelation of potential novel mechanisms of PPARα by the V227A variant.

Figure 4.3 Proposed molecular mechanism of V227A using the genome scanning model

The ChIP technique reflects overall conditions in a population of cells. In our model, total cell population is represented by the black box. Live cell imaging, where the genome scanning model has been largely derived from, reflects a single cell. Here, a single cell is represented by a coloured circle within the black box. The colour of the circle reflects PPARα-coregulator association within each single cell. Thus, PPARα-corepressor complex is red and PPARα-coactivator-complex is green. In the absence of ligand, our ChIP data for WT suggest that a similar portion of the cell population has WT PPARα associated with either corepressors (eg. NCoR) or coactivators (eg. SRC-1). These WT-coregulator complexes roam the nucleus where they interact transiently with chromatin, both on genuine PPREs, as suggested in our ChIP data, and unspecific binding sites. Since there is no ligand, WT-coactivator complexes does not activate transcription on genuine PPREs and is thus silent like WT-corepressor-PPRE complexes. Upon ligand binding, WT-corepressors exchange for the coactivator complexes and a higher proportion of the cell population now has WT-coactivator complexes for association on PPREs, as reflected in our ChIP data. WT-coactivator complexes may transiently bind to "non-specific" sites on chromatin, performing a three dimensional-scanning of the genome, until they encounter a genuine response element in a promoter, at which chromatin remodeling and transcription are initiated. Interestingly, our data suggests a different story for V227A.

Our mammalian-two-hybrid, GST-pull down and Co-IP data all indicate that V227A associates stronger in solution with corepressors (eg. NCoR) in the absence of ligand. Consequently, this may suggest a larger portion of the cell population has primarily V227A PPARα-corepressor complexes. These V227A-corepressor complexes roam the nucleus where they interact transiently with chromatin on genuine PPREs and unspecific binding sites as suggested by our ChIP data. Genuine PPRE remains silent and basal transcription activity of V227A is comparable to that of WT as reflected in our reporter gene assays. In the presence of ligand, V227A-corepressors exchange for the coactivator complexes. However, due to the additional interaction introduced by V227A, release of corepressors is impaired and coactivator recruitment becomes aberrant. Thus, proportion of the cell population that is associated with coactivator is lesser than WT. Lesser proportion of cells will have V227A-coactivator complexes available for association with their genuine PPRE during genome scanning and this results in lower total transcription. In addition, defective V227A activity observed at high doses of ligand where NCoR no longer binds to V227A suggest that corepressors (eg.SMRT) other than NCoR may have a role in defective mutant function.


- Ligand
+ Ligand
WT
Corepressors
WT
RXR
PPRE
Coactivators
++++
- Ligand
+ Ligand
V227A
V227A
RXR
Corepressor?
++

4.13 Conclusion

Our data and that of others extend the number of potential PPAR active compounds since isoflavones are present in many herbs and foods of botanical origins. Traditional PPARγ drugs such as pioglitazone are potent but have serious adverse effects such as obesity and edema. Preliminary data suggest that less potent drugs may still be efficacious while avoiding adverse effects associated with more potent ligands (Liu et al. 2005). The challenge of the future is to determine whether isoflavones with different PPARα/γ potencies and their parent botanicals have any enhanced benefit-risk profiles for management of the epidemic of diabetes, dyslipidemia and the metabolic syndrome.

The physiological consequences of PPAR activity in the absence of ligand are still unclear (Feige et al. 2006) and we have just begun to understand the relevance of abrogated ligand dependent and independent activity in PPARα through V227A. The importance of corepressor interaction for PPARα is currently poorly understood and there is a clear need to study the actions of corepressors on specific PPARα target genes. While we have provided the first available information about the important role of NCoR in the mediation of transcriptional activity of PPARα on the HMGCS2 gene, we have also identified a population of human subjects bearing this aberrant ligand independent activity with further ligand dependent consequences that permits us a chance to further dissect the role of PPARα in various diseases. This study further allows for the design of future human studies to identify other benefits and risks associated with this mutation.

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APPENDIX

Transactivation activity of PPARα V227A variant on a consensus CYP4A6-PPRE and the mitochondria HMGCS2 promoter in the adenovirus expression system

HepG2 cells were infected with adenovirus expressing WT PPARα, V227A or LacZ before transfection with CYP4A6-PPRE-Luc (100ng) (A), or HMGCS2-Luc (100ng) (B) and treatment with WY14,643 as indicated. PPARα and actin protein levels from total protein lysates (20-25μg) of representative replicates were detected with specific antibodies. Values are mean ± SD of three replicates, and expressed as percentage of maximal WT activity. * $p<0.05$; ** $p<0.01$.