MOLECULAR CLONING AND CHARACTERISATION OF 5'-UNTRANSLATED AND PROMOTER REGIONS OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR ALPHA (PPARα) FROM HUMAN

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Choy Hoong
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CONTENTS

LIST OF FIGURES

LISTS OF TABLES

ABBREVIATIONS

5'-RACE	Rapid amplification of cDNA 5' ends
5'-UTR	5'-untranslated region
6-keto-$PGF_{1\text{-alpha}}$	6-keto prostaglandin F1-alpha
8S-HETE	8-S-hydroxyeicosatetraenoic acid
9 *cis*-RA	9-*cis* retinoic acid
ABC-1	Adenosine triphosphate-binding cassette transporter-1
ACS	Acyl-CoA synthetase
AD	Activation domain
AF-1	Activation function 1
AF-2	Activation function 2
AP-1	Activated protein-1
AP-2	Activating protein-2
Apo A-I	Apolipoprotein A-I
Apo A-II	Apolipoprotein A-II
Apo CIII	Apolipoprotein CIII
BCP	1-Bromo-3-Chloropropane
BFE	Peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
bp	base pair
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
$CaCl_2$	Calcium chloride
CBF	CCAAT-binding factor
CBP/p300	CREB binding protein
cDNA	Complementary DNA
CLA-I	CD-36 and LIMPII analogous 1
CoA	Coactivator
CoR	Corepressor
COX	Cyclooxygenase
CPT-I	Muscle-type carnitine palmitoyltransferase type I
CRE	cAMP response element
CREB	cAMP-responsive binding protein
CRP	C-reactive protein
CTF	CCAAT-binding transcription factor
DBD	DNA-binding domain
DEHA	Diethylhexyladipate
DEHP	Diethylhexylphthalate
DEPC	Diethylpyrocarbonate
DMEM	Dublecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
DR-1	Direct repeat response elements 1
EC	Vascular endothelial cells
EDTA	Ethylene diaminetetraacetic acid
ET-1	Endothelin-1

FAT/CD36	Fatty acid translocase
FATP	Fatty acid transport protein
FBS	Fetal bovine serum
HAT	Histone acetyl transferases
HDAC	Histone deacetylase activity
HMG-CoA	Mitochondrial 3-hydroxy-3-methyglutaryl-CoA
HNF-3β	Hepatocyte nuclear factor 3
HNF-4	Hepatocyte nuclear factor-4
hPPARα	Human Peroxisome proliferators activated receptor alpha
IFNγ	Interferon-gamma
IKK	IκB kinase
IL	Interleukin
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
ITPG	Isopropyl-β-D-thiogalactopyranoside
JAK2-STAT5b	Janus kinase 2-signal transducer and activator of transcription 5b
kb	kilobase
LARII	Luciferase Assay Buffer II
LB	Luria-Bertani
LBD	Ligand-binding domain
LPL	Lipoprotein lipase
LTB4	Leukotriene B4
MAP-K	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MEM/EBSS	Eagle's Minimum essential medium with Earle's BSS medium
$MgCl_2$	Magnesium chloride
MMP-9	Matrix metalloproteinase-9
MOPS	3-[N-Mopholino]propanesulphonic acid
mRNA	Messenger RNA
NCoR	Nuclear receptor corepressor
NF-κB	Nuclear factor-κB
NF-I	Nuclear Factor I
NF-Y	Nuclear Factor Y
NRE	Negative regulatory element
NSAIDs	Non-steroidal anti-inflammatory drugs
Oct-1	Octamer-1
PAI-1	Plasminogen activator inhibitor type 1
PBP	PPAR-binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PKA	Protein kinase A
PLB	Passive lysis buffer
PPARγ	Peroxisome proliferators activated receptor gamma
PPARα	Peroxisome proliferators activated receptor alpha
PPARδ(β)	Peroxisome proliferators activated receptor delta/beta
PPARs	Peroxisome proliferators activated receptors
PPRE	Peroxisome proliferators response elements
PRIP	PPAR-interacting protein

RLM-RACE	RNA ligase-mediated and oligo-capping rapid amplification of cDNA ends
RNA	Ribonucleic acid
RORα1	Retinoid acid receptor-related orphan receptor alpha 1
RXR	Retinoic X receptor
SMC	Vascular smooth muscle cells
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
Sp1	Specificity protein 1
SRA	Scavenger receptor A
SR-BI	Scavenger receptor B type I
SRC-1	Steroid receptor coactivator 1
STATs	Signal transducers and activators of transcription
TAE	Tris-acetate-EDTA
TAFs	TBP-associated factors
TB	Terrific Broth
TBE	Tris-borate-EDTA
TBP	TATA box-binding protein
TCFII	Transcriptional factor II
TE	Tris-EDTA
TF	Tissue factor
THCOX	Peroxisomal trihydroxycoprostanoyl-CoA oxidase
TNF-α	Tumor necrosis factor alpha
tPA	Human tissue-type plasminogen activator
TSR	Template Suppression Reagent
UV	Ultraviolet
v/v	Volume/volume
VCAM-1	Vascular cell adhesion molecule-1
w/v	Weight/volume
Wy-14643	4-Chloro-6-(2, 3-xylidino)-2-pyrimidinlythioacetic acid
X-Gal	5-bromo-4-chloro-3 indolyl-β-D-galactopyranoside

ABSTRACT

Peroxisome proliferator-activated receptor alpha (PPARα) is a ligand-activated transcription factor which belongs to the nuclear receptor superfamily and regulates gene transcription by heterodimerizing with retinoic X receptor (RXR). PPARα has attracted considerable attention since it was demonstrated to be pivotal regulators of lipoprotein metabolism, vascular inflammation, atherosclerosis and carcinogenesis. To date, studies addressing the regulation of human PPARα gene expression remain largely unexplored. In order to understand the structure and molecular mechanisms governing PPARα regulation, it is vital to identify and characterise the 5'-untranslated region (UTR) and promoter region of the human PPARα gene. In this study, six alternatively spliced variants and two new novel exons at the 5'-UTR of human PPARα gene were identified. The putative transcriptional start site of each variant was identified, leading to the discovery of four promoters in the human PPARα gene which are responsible for transcribing these alternatively spliced variants. Three of these four promoters, named promoter B, promoter C and promoter D, were successfully cloned and sequenced. Sequence analysis revealed potential binding sites for transcriptional factors AP-1, AP-2, Sp1, STAT, C/EBP, GATA, CREB and Oct-1. In addition, TATA boxes were found in promoters C and D. However, no TATA boxes but several GC boxes were found within the first 100 nucleotides upstream of the transcriptional start site of promoter B. Finally, transient transfections using luciferase reporter constructs into HepG2 and Hep3B cells showed that both promoter B and promoter C are functional promoters, with promoter B being the more potent and stronger promoter. Deletion analysis of the human PPARα promoters identified a possible negative regulatory element located between the regions -765 to -1147 of promoter B and -413 to -967 of promoter C.

ABSTRAK

Reseptor Aktivasi Pembiakan Peroksisom Alpha (PPARα) merupakan faktor transcripsi yang diaktifkan oleh ligan yang tergolong dalam superfamili reseptor nuklear dan mengawalatur transcripsi gen dengan bergabung dengan reseptor retinoik X (RXR). PPARα didapati berfungsi sebagai pengawaltur penting di dalam metabolisme lipid, inflamasi vaskular, aterosklerosis dan kanser. Sehingga kini, tidak banyak penyelidikan mengenai kawalatur gen PPARα telah dilakukan. Untuk memahami mekanisme molekul yang mengawalatur PPARα, adalah penting untuk mengenalpasti and mencirikan kawasan 5'-tak tertranslasi (UTR) dan kawasan promoter gen PPARα manusia. Daripada kajian ini, enam varian terbahagi alternatif dan dua exon baru pada bahagian 5'-UTR gen PPARα telah dikenalpasti. Tapak permulaan transkripsi putatif untuk setiap varian telah dikenalpasti dan seterusnya menghasilkan penemuan empat promoter PPARα yang bertanggungjawab mentranskripsikan enam varian terbahagi alternatif ini. Tiga daripada empat promoter PPARα, iaitu promoter B, promoter C dan promoter D telah diklonkan dan dijujukkan. Analisis jujukan menemui tapak pengikatan untuk faktor transkripsi AP-1, AP-2, Sp1, STAT, C/EBP, GATA, CREB dan Oct-1. Kotak TATA dijumpai pada promoter C dan D, tetapi tidak pada promoter B, yang sebaliknya mempunyai kotak GC pada lingkungan 100 nucleotida pertama daripada tapak permulaan transkripsi. Transfeksi (transien) dengan menggunakan konstruk pelapor lusiferase ke dalam sel HepG2 dan Hep3B membuktikan kedua-dua promoter B dan C adalah promoter berfungsi. Promoter B merupakan promoter yang lebih kuat dan berpotensi daripada promoter C. Analisis pemotongan kedua-dua promoter PPARα manusia mendapati kehadiran elemen pengawalatur negatif di antara bahagian -765 ke -1147 promoter B dan -713 ke -967 promoter C.

INTRODUCTION

1.1 Background

Coronary heart disease is a major health problem and the leading cause of death in Malaysia. The link between cardiovascular disease and lipids has been appreciated for a long time. Cardiovascular disease is most often as a result of the damage occurring to the inside of the blood vessels through the formation of atherosclerosis. The build up of plaques begins as lipid underlays the endothelium of arteries, which in time, causes the narrowing of the vessels and severely limiting the blood flow in advanced cases. Rapture of plaque or thrombosis may lead to myocardial infarction or stroke (Berliner *et al*., 1995; Glass and Witztum, 2001; Vosper *et al*., 2002).

A family of transcription factors known as the Peroxisome Proliferator-Activated Receptors (PPARs) plays a central role in regulating the lipid homeostasis and carbohydrate metabolism. The discovery of PPARs was highly significant and since then, the receptor has moved from the status of orphan receptor to one of the best characterised nuclear receptors. Their functional characterisation is providing unique insight into the role of fat in health and diseases. Recently, this nuclear receptor has also been shown to fulfil critical and unique roles in general transcriptional control of numerous cellular processes, with implications in inflammation control, atherosclerosis, cancer development and epidermal wound healing.

1.2 Peroxisome Proliferator-Activated Receptors (PPARs)

The peroxisome proliferator-activated receptors (PPARs) were discovered in the early 1990s by Issemann and Green (1990). PPARs are members of the superfamily of nuclear hormone receptors. There are three distinct PPAR subtypes - PPARα (also known as NR1C1), PPARβ (NR1C2) and PPARγ (NR1C3), each encoded by a separate

gene and each with a distinct tissue distribution pattern and metabolic functions (Issemann and Green, 1990; Braissant *et al*., 1996). PPARα is expressed preferentially in tissues exhibiting high catabolic rates of fatty acids, such as liver, kidney, heart and muscle (Auboeuf *et al*., 1997) and in cells of the arterial wall (Staels *et al*., 1998a). PPARβ is more general in terms of tissue distribution, with varying levels in different organs (Wahli, 2002), while PPARγ is expressed predominantly in adipose tissue and in the immune system (Fajas *et al*., 1997) (See Table 1.1).

The PPARs possess a domain structure common to other members of the nuclear receptor gene family (Figure 1.1). Sequence comparison of their DNA-binding domains (DBD) shows that they are highly conserved, while lower level of conservation is seen in the ligand-binding domains (LBD) across the subtypes (Willson *et al*., 2000). There is a significant sequence variation in the residues that line the ligand-binding pocket (Nolte *et al*., 1998; Uppenberg *et al*., 1998). The variation is responsible for determining the ligand selectivity and binding specificity of the PPAR subtypes (Xu *et al*., 2001). The NH_3-terminal region of the receptor shows low sequence identity across the subtypes and is responsible for differences in the biological function of the subtypes (Castillo *et al.*, 1999).

For years, PPARγ has been the most widely studied subtype. Recently, more interest has been generated into the other subtype named PPARα. PPARα has been proven to play pivotal role in fatty acid metabolism. Furthermore, PPARα has also been shown to mediate pleiotropic effects with direct implications on multiple fundamental pathways in the cell.

Table 1.1 Summary of PPAR subtypes and their tissue distribution

Subtype	Tissue distribution
PPARα	Liver, kidney, heart
PPARβ (or δ)	Ubiquitous
PPARγ	Adipose, immune cells

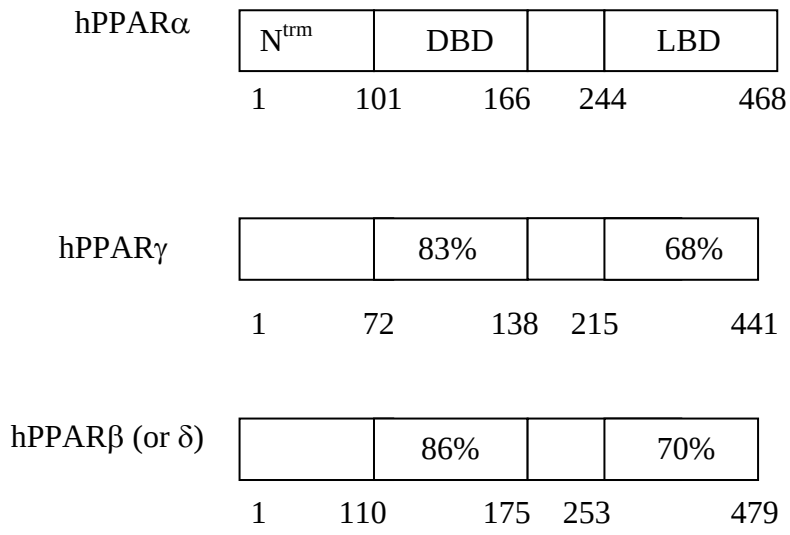


Figure 1.1 Functional domains of the PPARs. N^{trm} represents NH_3-terminal; DBD represents DNA-binding domain; LBD represents ligand-binding domain. Numbers in bars represent the percent (%) amino acid identity between the human subtypes relative to PPARα. Numbers below each bar indicate the position of the last amino acid of each domain.

1.3 Peroxisome Proliferator-Activated Receptor Alpha (PPARα)

PPARα was the first PPAR to be identified (Issemann and Green, 1990). PPARα was originally cloned from a mouse liver cDNA library and has since been cloned from frogs, rats, guinea pigs and humans (Willson *et al*., 2000).

PPARα tissue distribution in rodents and humans revealed high levels of mRNA in tissues, such as liver, heart, kidney and muscle (Table 1.1). In summary, regardless of species, the expression of PPARα correlates with high mitochondrial and peroxisomal *β*-oxidation activities.

In mouse and rat, PPARα appears relatively late in development in tissues where it will be expressed in adulthood (Braissant and Wahli, 1998; Desvergne and Wahli, 1999). In adult rat, relatively high levels of PPARα mRNA are detected in brown fat, liver, kidney, heart and the mucosa of stomach and duodenum. In addition, significant amounts of PPARα mRNA are also expressed in the retina, adrenal gland, skeletal muscle and pancreatic islets (Braissant and Wahli, 1998).

Like rodents, human PPARα is highly expressed in the liver, heart, kidney, skeletal muscle and large intestine. However, the mRNA levels of PPARα in human liver appear lower than in the rodent liver (Palmer *et al*., 1998). In addition, it is expressed in steroidogenic tissues such as adrenals (Zhou and Waxman, 1998). Furthermore, human PPARα is also expressed in vascular cells including endothelial and smooth muscle cells and macrophages/foam cells (Staels *et al*., 1998a; Chinetti *et al*., 2000a; Neve *et al*., 2000).

1.4 PPARα: Mechanism of action

Prior to transcriptional activation, PPARα heterodimerize with the retinoic X receptor (RXR) to form a complex (Kliewer *et al*., 1992). RXRs are also members of the nuclear hormone receptor superfamily that are activated following binding with 9 *cis*-RA (9-*cis* retinoic acid) (Desvergne and Wahli, 1999). The PPARα/RXR complex then binds to a specific sequence in the 5' end of the target genes, known as the peroxisome proliferator response element (PPRE).

The PPRE was first characterised by using synthetic oligonucleotides (Kliewer *et al*., 1992) and consists of a direct repeat of two copies of a hexameric nucleotide sequence AGGTCA separated by one nucleotide, known as the DR-1 response elements (Tugwood *et al.,* 1992). A PPARα-selective PPRE is further characterized by a 5'-flanking C(C/G)(A/G)A(A/T)(C/T) consensus sequence. Conservation of this 5'-flanking region is particularly essential for PPARα binding and therefore is an important determinant of the PPARα/RXR specificity (Issemann *et al*., 1993; Juge-Aubry *et al*., 1997).

PPARα/RXR heterodimer recognizes sequences situated within the DR-1 motif, whereby PPARα interacts with the upstream extended core hexamer of the DR-1, whereas RXR occupies the downstream motif (Palmer *et al*., 1995; IJpenberg *et al*., 1997; DiRenzo *et al.,* 1997) (Figure 1.2).

Both the formation of PPARα/RXR heterodimer and the subsequent transcriptional activation of the target gene are ligand dependent. Ligand binding may evoke conformational changes within the DNA-binding domain, thereby altering the potential to stimulate transcription of target genes. Interaction of PPARα with RXR results in a permissive heterodimer, in which binding of 9-*cis* retinoic acid to RXR can alter activation as well (DiRenzo *et al*., 1997).

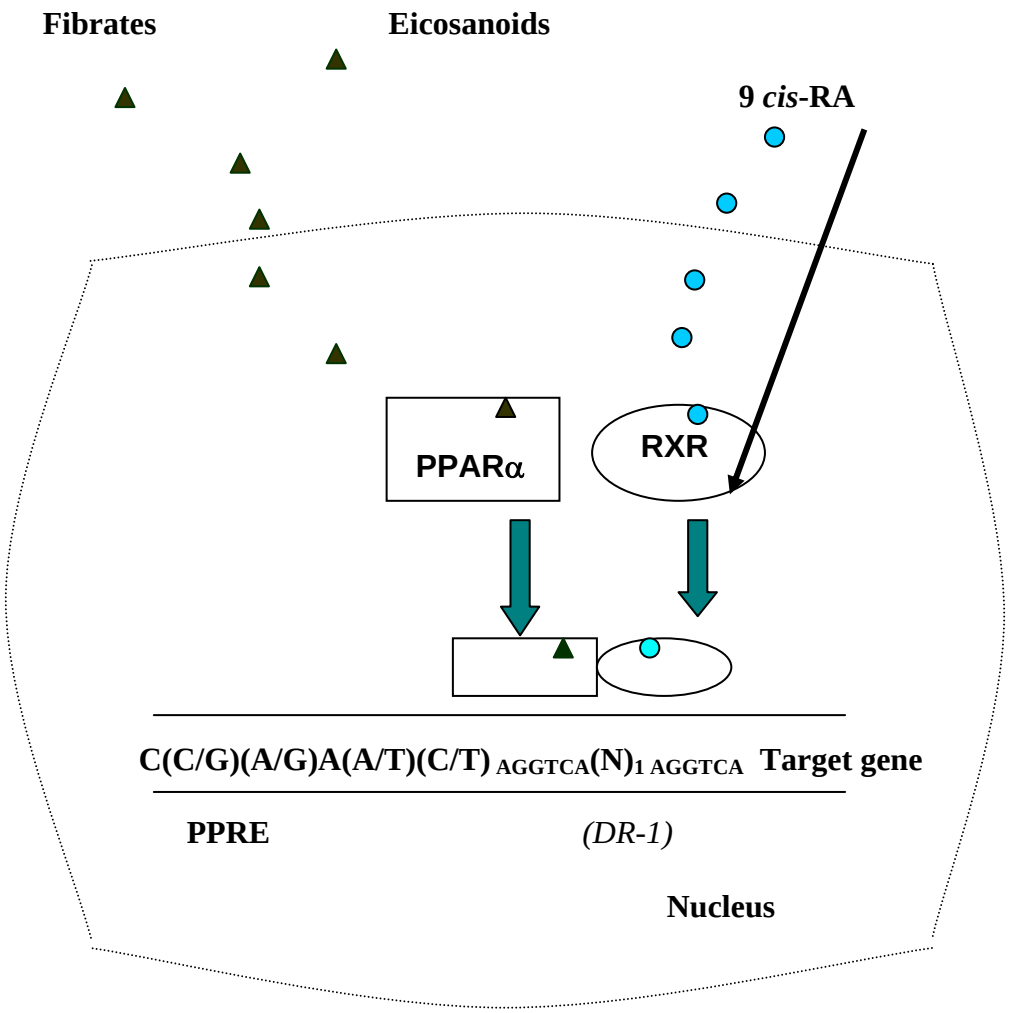


Figure 1.2 Mechanism of action of PPARα. Alteration of target gene transcription via binding of peroxisome proliferator response elements (PPRE) to the activated peroxisome proliferator-activated receptor alpha (PPARα) and the retinoic X receptor (RXR). 9 *cis*-RA represents 9 *cis*-retinoic acid; DR-1 represents the direct repeat of two copies of a hexameric nucleotide sequence AGGTCA separated by one nucleotide.

1.5 The structural organization of human PPARα gene

The human PPARα has been mapped to chromosome 22q12-q13.1 by somatic cell hybridization and linkage analysis (Sher *et al*., 1993). As in the mouse, the human PPARα gene is composed of eight exons which spanned 83.7 kb, with a 5'-untranslated region encoded by exons 1, 2 and part of exon 3 (Figure 1.2) (Vohl *et al*., 2000). The coding region of PPARα comprises the remainder of exon 3 and exons 4-8, with the 3'-untranslated region consisting of the last 232 bp of exon 8. The intron lengths vary between 1.4 kb to 24.8 kb as indicated in Figure 1.3 (Vohl *et al*., 2000).

Recently, the number of exons which forms the PPARα gene was extended to ten exons due to the discovery of two new exons within the 5'-untranslated region of the PPARα gene (Pineda-Torra *et al*., 2002; GenBank Accession No. BC000052). These exons were named as Exons 1b and 2b, respectively, while the previously known Exon 1 and 2 were named Exons 1a and 2a, respectively (Figure 1.4).

Little research has been performed on the mRNA variants (isoforms) of PPARα, unlike the other PPAR subtype, the PPARγ, whose mRNA variants were determined in both human and mouse (Zhu *et al*., 1995, Fajas *et al*., 1998). In the human PPARγ, four mRNA variants were determined while two mRNA variants were identified in the mouse PPARγ, due to different promoter usage and alternative splicing (Zhu *et al*., 1995, Fajas *et al*., 1998).

In human PPARα, a splice mRNA variant which lacked the entire exon 6 was identified (Gervois *et al*., 1999). This deletion, due to alternative splicing, formed a truncated PPARα protein with dominant negative activity which interferes with the activity of the functional full-length PPARα protein (Gervois *et al*., 1999).

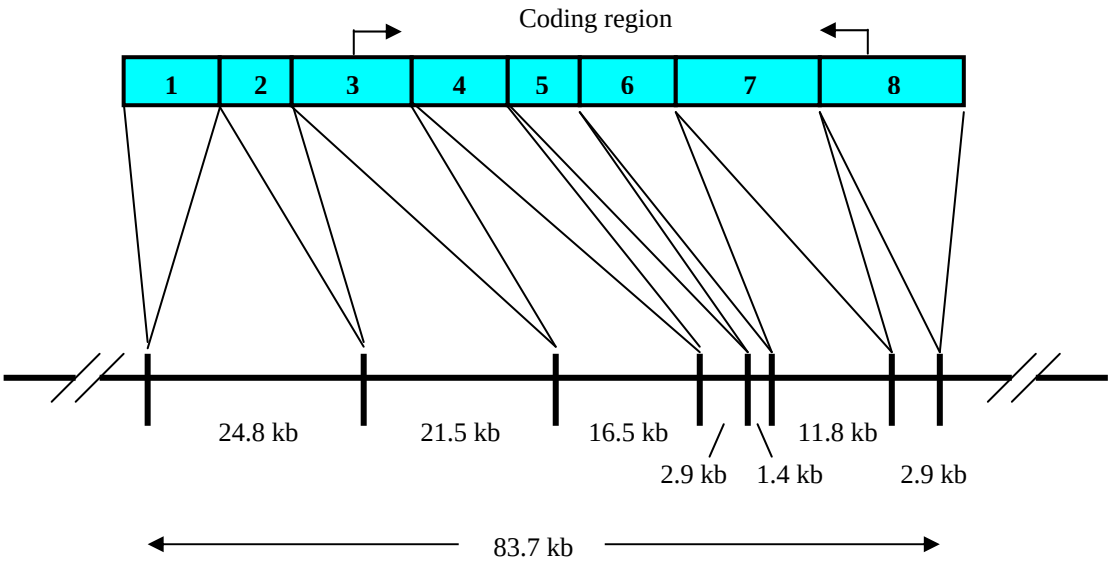


Figure 1.3 Schematic representation of the human PPARα gene. The gene spans 83.7 kb of genomic DNA. Upper panel represents the human PPARα messenger RNA. Arrows indicate translation start (methionine) and stop codons. The spatial localization of exons within the gene is shown in the lower panel. Exon numbers and the size of intron are also indicated.

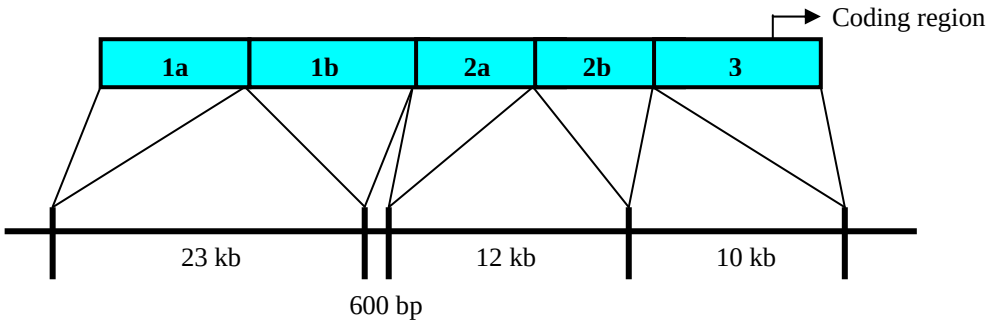


Figure 1.4 Schematic representation of the newly reported 5' flanking region human PPARα gene. The upper panel represents the 5'-flanking region human mRNA. Arrow indicates the start codon (methionine). The lower panel represents the spatial localization of exons of the 5' flanking region. The size of introns are indicated below the lower panel.

As with most members of the nuclear hormone superfamily, PPARα protein shares a common structure of five functional domains named A/B, C, D and E (Figure 1.5). However, the F domain is absent from all PPARs compared with other members of the nuclear superfamily (Lazennec *et al*., 2000). The A/B domain (or the NH_3-terminal) contains the ligand-independent transcription activation function 1 (AF-1) (Hi *et al*., 1999). The C domain (or the DNA-binding domain) has a characteristic helix-loop-helix structure stabilized by two zinc atoms and is responsible for the binding to peroxisome proliferator response elements (PPREs) in the promoter region of target genes. The D domain is the hinge region that can modulate the DNA binding ability of the receptor and also involved in corepressor binding (Lazennec *et al*., 2000). The E domain (or COOH-terminal region) encompasses a ligand binding function and exhibits a strong ligand-dependent activation function (AF-2). The ligand binding domain facilitates the heterodimerization of PPARα with the retinoic X receptor (RXR). In its active state, this heterodimer is able to bind to a PPRE and modulate the expression of its target genes (Krey *et al*., 1997; Lazennec *et al*., 2000).

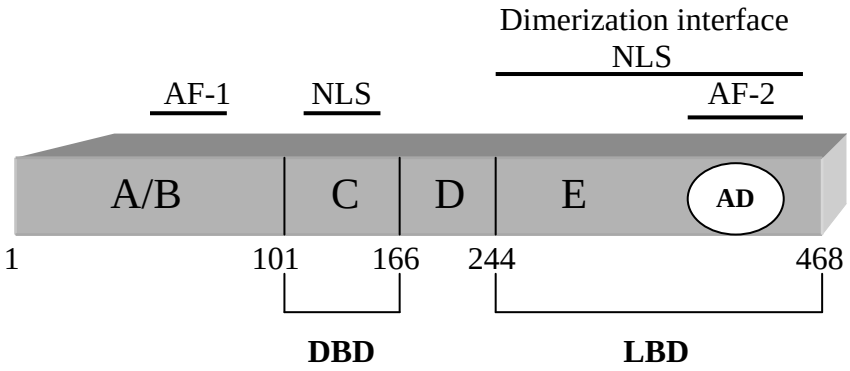


Figure 1.5 Structural and functional organization of the nuclear receptor PPARα protein. AD, activation domain; AF, activation function; DBD, DNA-binding domain; LBD, ligand-binding domain; NLS, nuclear localization signal. Numbers below each bar indicate the position of the last amino acid of each domain.

1.6 Ligands of PPARα

PPARα is a ligand-activated transcription factor. The binding of ligands to the receptor greatly increases its transcriptional activity. A diverse range of compounds which include natural and synthetic substances serve as PPARα ligands, including fatty acids and fatty acid-derived products as well as pharmacological molecules such as plasticizers and herbicides (Issemann and Green, 1990; Krey *at al*., 1997; Forman *et al*., 1997; Ward *et al*., 1998; Lin *et al*., 1999; Roberts-Thomson, 2000) (Table 1.2).

The ability of PPARα to bind such a diverse variety of natural and synthetic compounds has been puzzling. Partial explanation for this ability to bind to multiple ligands came with the description of the crystal structure of the ligand binding domain of PPARα. The ligand binding domain is made out of a three-dimensional fold, which consists of an antiparallel α-helical sandwich of 12 helices (Helix 1 to Helix 12) organised in three layers with a central ligand binding hydrophobic pocket (Bourguet *et al*., 1995; Xu *et al*., 2001; Wahli, 2002). Upon ligand binding, the ligand pocket closes and forms a "mouse trap model" (Wahli, 2002). The ligand binding pocket of PPARα is much larger than that of other nuclear receptors with a volume of approximately 1300$Å^3$, of which the ligand occupies only 30-40% (Xu *et al*., 2001; Wahli, 2002). Interestingly, Xu *et al.* (2001) identified the major determinant of selectivity of ligands in PPARα is the amino acid residue Tyr-314. This amino acid plays an important part in the transcriptional activation of PPARα receptor by ligands.

Table 1.2 The peroxisome proliferator-activated receptor α (PPARα) activating ligands. PPARα is a ligand-activated transcription factor. The binding of ligands to the receptor greatly increases its transcriptional activity.

Class of compound	Name
Fatty acids	Linoleic acid Linolenic acid Oleic acid Arachidonic acid
Eicosanoids	8(S)-Hydroxyeicosatetraenoic Acid Leukotriene B_4
Fibrates	Clofibric acids Ciprofibrate Gemfibrozil 4-Chloro-6-(2, 3-xylidino)-2-pyrimidinlythioacetic acid (Wy-14643)
Plasticizers	Diethylhexylphthalate (DEHP) Diethylhexyladipate (DEHA)
Herbicide	Lactofen 2, 4-Dichlorophenoxyacetic acid

1.6.1 Natural ligands of PPARα

A range of saturated and unsaturated fatty acids could activate PPARα (Gottlicher *et al*., 1992; Willson *et al*., 2000). Palmitic acid, oleic acid, linoleic acid and arachidonic acid are examples of saturated acid that can activate PPARα (Banner *et al*., 1993). Notably, PPARα is the only subtype that binds with high affinity to a wide range of saturated acids (Xu *et al*., 1999b). This may be due to the fact that the PPARα pocket is more lipophilic and less solvent compared to the other PPAR subtypes, explaining its selectivity for more lipophilic saturated fatty acids (Xu *et al*., 2001).

Eicosanoids, a class of fatty acids mainly derived from arachidonic acid, are activators of PPARα. 8-S-hydroxyeicosatetraenoic acid (8S-HETE), a compound associated with inflammation, and leukotriene B4 (LTB4), a chemotactic inflammation mediator, are examples of eicosanoids that can activate PPARα (Yu *et al*., 1995; Devchand *et al*., 1996; Forman *et al*., 1997) (Figure 1.6).

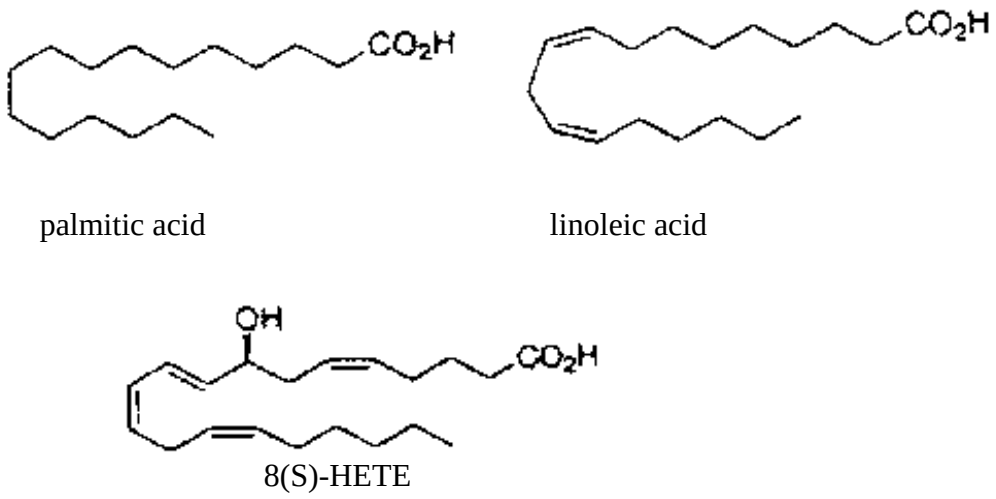
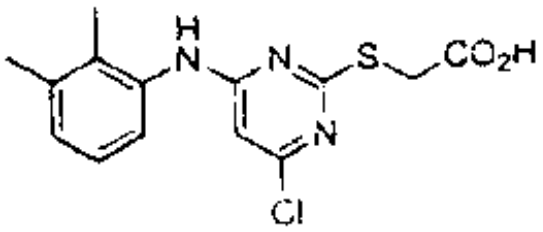


Figure 1.6 Chemical structures of natural ligands of PPARα. Palmitic acid, linoleic acid and 8-S-hydroxyeicosatetraenoic acid (8S-HETE) are potent PPARα ligands.

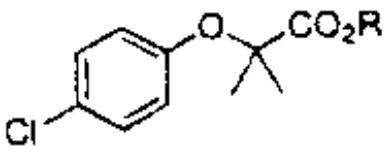
1.6.2 Synthetic ligands of PPARα

The hypolipidemic fibrates drugs are important class of PPARα ligands (Issemann and Green, 1990). Wy-14643, clofibric acid, ciprofibrate, fenofibrate and gemfibrozil are examples of fibrates that can activate PPARα. Clofibric acid and fenofibric acid are dual activators of PPARα and PPARγ, with 10-fold selectivity for PPARα. These fibrates are widely used in the treatment of hypertriglyceridemia and combined hyperlipidemia (Forman *et al*., 1997). Another compound known as ureidofibrates, such as GW 2331 and GW 9578, have been reported as potent and subtype-selective PPARα agonists with lipid lowering activity (Brown *et al.*, 1999).

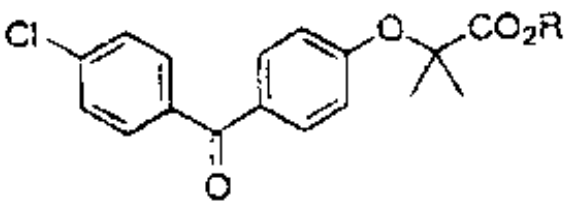
Interestingly, certain non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin, fenoprofen, flufenamic and ibuprofen, have been shown to bind and activate PPARα at high micromolar concentrations, with fenoprofen activating the receptor to a degree comparable to that obtained with Wy-14643 (Lehmann *et al.*, 1997) (Figure 1.7).



Wy-14643



clofibrate, R=Et
clofibric acid, R=H



Fenofibrate

Figure 1.7 Chemical structures of synthetic ligands of PPARα.

1.6.3 PPARα cofactors

PPARα is modulated by cofactors that either enhance (coactivators) or silence (corepressor) its transcriptional activity (Xu *et al*., 1999a; Glass and Rosenfeld, 2000). Initially, it was thought that the cofactors simply bridge PPARα with basic transcriptional machinery. Recently, it has become clear that these cofactors carried out several enzymatic activities, suggesting the fact that they can control gene expression by specifically modifying chromatin and DNA structure (Dowell *et al*., 1997a, Glass *et al.,* 1997; Pazin and Kadonaga, 1997; Moras and Gronemeyer, 1998; Latchman, 1998).

Ligand binding induces a conformational change in the receptor that results in dissociation of corepressors and removal of histone deacetylases from DNA. Subsequently, coactivators' complexes that contain proteins with histone acetyltransferase activity are recruited. Acetylation is associated with alteration of nucleosome structure, producing a more open chromatin structure to modulate accessibility of promoter regions, thereby increasing the transcription of target gene (Dowell *et al*., 1997a; Glass *et al.,* 1997; Pazin and Kadonaga, 1997; Moras and Gronemeyer, 1998) (Figure 1.8).

Some of these cofactors identified include CREB binding protein (CBP/p300) and steroid receptor coactivator (SRC)-1, as well as PPAR-binding protein (PBP), PPAR-interacting protein (PRIP), peroxisomal trihydroxycoprostanoyl-CoA oxidase (THCOX), silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (NCoR).

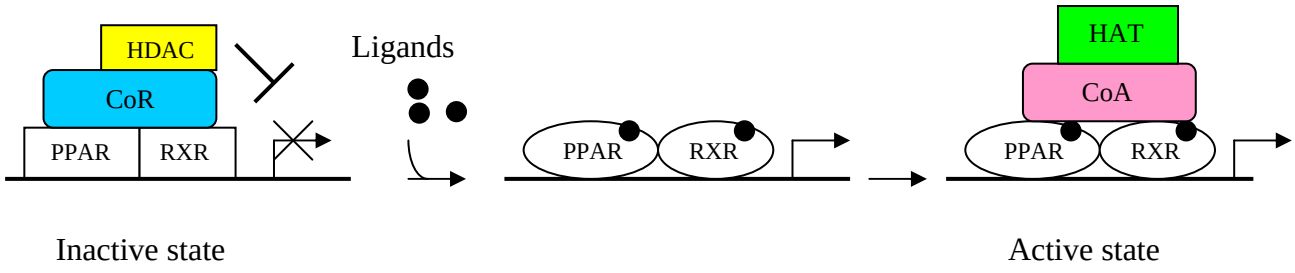


Figure 1.8 Transcriptional activation of PPARα by cofactors. Transcriptional activity, in general, is activated with the release of corepressor (CoR) complexes, which contain histone deacetylase activity (HDAC), and the recruitment of coactivators (CoA), which target histone acetyl transferases (HAT) to the promoter. The differential docking of cofactors is facilitated by structural changes brought about by ligand-binding coreceptor phosphorylation.

CBP/p300 is widely expressed (Chrivia *et al*., 1993; Janknecht and Hunter, 1996; Misiti *et al*., 1998) and coactivates numerous transcription factors including several nuclear receptors (Chakravarti *et al*., 1996; Hanstein *et al*., 1996; Kamei *et al*., 1996; Smith *et al*., 1996; Dowell *et al*., 1997b; Kraus and Kadonaga, 1998). CBP/p300 are coactivators that exhibit histone acetyltransferase activity and also interact with another histone acetyltransferase known as P/CAF (Dowell *et al*., 1997b).

Dowell *et al*. (1997b) demonstrated that 79 amino acids of p300-(39-117) and 115 amino acids of CBP-(1-115) are sufficient for interaction with PPARα (Dowell *et al*., 1997b; Gelman *et al*., 1999). A L*XX*LL nuclear interaction motif, which is a signature motif for the binding of cofactor to nuclear receptors, has been identified within these regions (amino acids 81-85 of p300 and 69-73 of CBP) and this single motif is sufficient for interaction with PPARα (Dowell *et al*., 1997b). p300 interacts with PPARα through the amino acids within the COOH-terminal portion (amino acids 448-468) (AF-2) as well as the residues within the hinge region known as the T-box of

the PPARα receptor (amino acids 166-179) (Dowell *et al*., 1997b). The constitutive presence of CBP/p300 could enhance the basal ligand independent transcriptional activity of PPARα *in vivo* and could thereby explain the high level of basal activity of PPARα.

The steroid receptor coactivator (SRC)-1 is the first described nuclear receptor coactivator (Onate *et al*., 1995; Smith *et al.,* 1996). SRC-1 has intrinsic histone acetyl transferase activity (Spencer *et al*., 1997) and is ubiquitously expressed (Onate *et al*., 1995; Misiti *et al.*, 1998). Two L*XX*LL motifs have been mapped in SRC-1. Its interaction with PPARα appears to be ligand-dependent. The AF-2 of the PPARα LBD serves as an adaptor surface for the interaction with SRC-1 (Nolte *et al*., 1998). The two L*XX*LL motifs of a single SRC-1 molecule interact separately with the AF-2 helix of each receptor molecule of a dimer, thus forming a stable ternary complex of two PPARα LBDs and one SRC-1 molecule (Nolte *et al*., 1998).

The PPAR-binding protein (PBP) and the PPAR-interaction protein (PRIP) were isolated on the basis of its interaction with the LBD of PPARα in the yeast two-hybrid system (Zhu *et al.*, 1997; Zhu *et al.*, 2000). PBP and PRIP both contain two copies of the sequence motif L*XX*LL and their respective interaction with PPARα, coupled with the presence of a ligand, increase the transcriptional activity of the nuclear receptor. These two coactivators, which are devoid of histone acetyltransferase activities, appear to be involved in the second step of coactivation, which is to serve as facilitators linking the receptor complex to the basal transcriptional machinery (Zhu *et al.*, 2000).

The reason for the existence of a multitude of coactivators remains elusive. One possibility is that different coactivators may preferentially participate in the transcription of specific target genes. It is also possible that PPARα uses only distinct subset of coactivators for optimal transcriptional activity due to the differences in

peroxisome proliferator signalling and other complex cross-talk mechanisms (Zhu *et al.*, 2000).

Most of these coactivators directly interact with the COOH-terminal portion of the ligand-binding domain (LBD) of the PPARα, which contains a ligand-dependent activation function (AF) motif AF-2 (Nolte *et al*., 1998; Glass and Rosenfeld, 2000). However, recently, another coactivator known as peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (bifunctional enzyme, BFE) was identified as a strong interaction partner and activator of the NH_3-terminal domain of PPARα which contains the ligand-independent activation function motif AF-1 (Juge-Aubry *et al*., 2001). The BFE is suggested to activate the basal transcriptional activity of PPARα by modulating the recruitment of proteins such as histone acetylases or other transactivating proteins (Juge-Aubry *et al*., 2001). Alternatively, it may mediate the activity of PPARα by modifying the intracellular import of PPARα into the nucleus (Juge-Aubry *et al*., 2001).

Juge-Aubry *et al*. (2001) also suggested the possibility of BFE forming a ternary complex with PPARα and another peroxisomal enzyme, named peroxisomal trihydroxycoprostanoyl-CoA oxidase (THCOX), and therefore modulates the activity of PPARα through a novel peroxisomal-nuclear feed-forward loop. Interestingly, THCOX, which catalyses the first step of the peroxisomal oxidation of the CoA esters of bile acid intermediates, is also a coactivator of PPARα and interacts with the NH_3-terminal of PPARα (Juge-Aubry *et al*., 2001).

The silencer mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor coreceptor (NCoR) are corepressors identified using yeast two-hybrid system and are suggested to down-modulate PPARα-mediated gene transcription (Lavinsky *et al*., 1998; Dowell *et al.,* 1999). It has been shown that NCoR, which couples with other proteins with histone deacetylase activities, binds to the receptor and

induce a more tightly packed chromatin structure (Latchman, 1998). These corepressors are ligand independent. Hence, the binding of ligand to PPARα induces it to release a corepressor complex with histone deacetylase activity and bind coactivators with histone acetylase activity. These coactivators produce a more open chromatin structure compatible with transcription. There has also been a speculation that the NCoR corepressor protein interacts with the NH_3-terminal of PPARα and partially silent the activity of AF-1 domain, thus explaining the down-regulation of the PPARα gene expression (Juge-Aubry *et al*. 1999).

1.7 Alternative pathways for PPARα transcriptional activation

1.7.1 Activation by phosphorylation

Ligand-dependent transcriptional activation by PPARα is mediated by a large, complex COOH-terminal domain that integrates several critical functions: ligand binding, dimerization, trans-activation and interaction with transcriptional coactivators (Glass *et al*., 1997).

Several nuclear hormone receptors, including PPARs, are regulated by phosphorylation in addition to ligand-dependent activation. PPARα was first shown to be a phosphoprotein in primary rat adipocytes in culture. Treatment of these cells with insulin increases the PPARα phosphorylation (Shalev *et al*., 1996).

Juge-Aubry *et al*. (1999) demonstrated that the 1-92 amino acids (NH_3-terminal) of PPARα contain AF-1 like *trans*-activation domain, which is further activated by insulin through pathways involving the mitogen-activated protein kinases (MAP-K) p42/p44 and the phosphorylation of two serine residues at positions 12 and 21 of PPARα (Juge-Aubry *et al*., 1999). The phosphorylation sites in PPARα are found to be highly conserved across species (Juge-Aubry *et al*., 1999).

The phosphorylation of the NH_3-terminal of PPARα results in the dissociation of corepressor proteins, which may then result in a further transcriptional enhancement of this domain (Juge-Aubry *et al*., 1999).

Lazennec *et al.* (2000) demonstrated that phosphorylation of PPARα by protein kinase A (PKA) pathway is an important modulator of PPARα activity. A combination of PKA activators and PPARα ligands lead to the stabilization of the PPARα DNA binding, which in turn increases the transcriptional activity of PPARα (Lazennec *et al.*, 2000).

However, phosphorylation has also been shown to inhibit the PPARα signalling pathway. For example, transcriptional activity of PPARα is inhibited up to 80% by growth hormone through the Janus kinase 2-signal transducer and activator of transcription 5b (JAK2-STAT5b) pathway (Zhou and Waxman, 1999b). STAT5b inhibits PPARα activity by directly interacting and phosphorylating the NH_3-terminal of PPARα. However, the phosphorylation was shown to be independent from the MAP kinase phosphorylation of serine residues at positions 12 and 21 as in the case with insulin (Zhou and Waxman, 1999b).

Therefore, the effect of phosphorylation on the PPARα signalling pathway, depending upon the nature of triggering signal, is suggested to involve specific pleiotropic actions or ‘cross-talking’ between different kinase pathways (Desvergne and Wahli, 1999).

1.7.2 Activation of PPARα:RXR heterodimer by RXR agonists

Another alternative activation pathway of PPARα:RXR heterodimer occurs through ligand binding to RXR. PPARα forms a permissive heterodimer with RXR, meaning that either partner can regulate the transcriptional activity of the DNA-bound

complex by interacting with its cognate ligand, on its own or when both partners are liganded (Desvergne and Wahli, 1999). Cotransfection studies have shown that both members of the PPARα:RXR complex can mediate a response in the presence of their respective ligand and cotreatment of cells with both ligands results in additive effects (Mangelsdorf *et al.*, 1991; Kliewer *et al*., 1992; Gearing *et al*., 1993; Keller *et al*., 1993; Kersten *et al*., 1995; Desvergne and Wahli, 1999).

RXR forms inactive tetramers in the absence of its ligands and addition of RXR-specific ligands preferentially directs the formation of homodimers rather than heterodimers (Kersten *et al*., 1995). However, the specificity of action behind the formation of PPARα:RXR heterodimers rather than RXR homodimers remains undetermined (Mangelsdorf *et al.*, 1991; Desvergne and Wahli, 1999).

1.8 PPARα and lipoprotein metabolism

1.8.1 PPARα and free fatty acid metabolism

PPARα is highly expressed in tissues with elevated rates of fatty acid catabolism, where it regulates genes involved in fatty acid uptake, activation into acyl-CoA esters, degradation via the peroxisomal and mitochondrial β-oxidation pathways and ketone synthesis (Schoonjans *et al*., 1996a; Chinetti *et al*., 2000a).

PPARα acts as regulator of intracellular fatty acid uptake controls. Intracellular fatty acid concentrations are partly regulated by import and export system that is controlled by proteins such as fatty acid transport protein (FATP), fatty acid translocase (FAT/CD36) and Acyl-CoA synthetase (ACS), which facilitate the transport of fatty acids through the cell membrane and their esterification preventing their efflux (Abumrad *et al*., 1993; Martin *et al.*, 1997; Tontonoz *et al*., 1998).

Treatment with PPARα agonists (activators) has been shown to induce fatty acid transport protein (FATP) mRNA levels in rat liver and intestine, and induced acyl-CoA synthetase (ACS) mRNA levels in liver and kidney (Martin *et al.*, 1997; Motojima *et al.*, 1998). The regulation of fatty acid transport protein and acyl-CoA synthetase expression by PPARα activators has been shown to occur at transcriptional level (Motojima *et al*., 1998; Fruchart *et al*., 1999).

All these evidence show that PPARα activators influence fatty acid cellular uptake, which is a crucial regulatory step in lipid metabolism. Once inside the cells, fatty acid must penetrate into the mitochondrial where its metabolism takes place. Muscle-type carnitine palmitoyltransferase type I (CPT-I), a key enzyme in mitochondrial fatty acid catabolism, contains a PPRE in its promoter region and is regulated by PPARα activators (Brandt *et al*., 1998; Mascaro *et al*., 1998; Chinetti *et al.*, 2000a).

Mitochondrial 3-hydroxy-3-methyglutaryl-CoA (HMG-CoA) synthase is a key enzyme in ketogenesis. HMG-CoA catalyses the condensation of acetyl-CoA and generates HMG-CoA, which is eventually converted into ketone bodies (Meertens *et al.*, 1998). A PPRE was identified in the mitochondrial HMG-CoA gene and activation of PPARα was proven to up-regulate the gene expression of HMG-CoA (Meertens *et al*., 1998). These observations, taken together, indicate that PPARα controls fatty acid uptake, activation into acyl-CoA esters and degradation through the peroxisomal and mitochondrial *β*-oxidation pathways, and also the synthesis of ketones (Chinetti *et al.*, 2000a).

1.8.2 PPARα and triglyceride-rich lipoprotein metabolism

There is increasing evidence that serum triglycerides are strong risk factors in cardiovascular diseases. One of the major effects of PPARα activation on lipid metabolism is to reduce triglyceride-rich levels in plasma. PPARα activators alter the synthesis and the catabolism of the triglyceride-rich lipoproteins in a way that decreases plasma triglyceride levels via induction of the lipoprotein lipase (LPL) activity (Schoonjans *et al*., 1996b).

Schoonjans *et al*. (1996b) demonstrated that PPARα mediates the triglyceride-lowering action of PPARα activators by increasing lipoprotein lipase gene expression in a PPRE-mediated manner. A sequence element identified as a PPRE was found to be present in the human lipoprotein lipase promoter which was responsible in stimulating the expression of the gene. Two distinct mechanisms may be involved in PPARα induction of lipoprotein lipase activity: firstly, through the stimulation of the LPL gene expression and secondly, by the induction of hydrolytic activity of enzyme for triglyceride-rich lipoproteins secreted following treatment with PPARα activators (Fruchart *et al*., 1999).

Apolipoprotein CIII (Apo CIII) plays a key role in delaying the catabolism of triglyceride rich particles, by inhibiting their binding to the endothelial surface and lipolysis by LPL (Hertz *et al.*, 1995; Staels *et al.*, 1995; Desvergne and Wahli, 1999; Vosper *et al*., 2002). PPARα activators were also proven to decrease apolipoprotein CIII levels, thus resulting in an enhanced lipolytic activity (Hertz *et al.*, 1995; Staels *et al*., 1995). A few mechanisms may be involved in this negative regulation. Firstly, PPARα activators may suppress Apo CIII by displacing the strong transcriptional activator of the apolipoprotein gene with a lesser active complex, resulting in lower Apo CIII promoter activity (Hertz *et al.,* 1995). Secondly, there is a possibility that PPARα

activators indirectly decrease the expression of a strong transcriptional activator of the Apo CIII gene, i.e. the hepatocyte nuclear factor-4 (HNF-4) (Hertz *et al*., 1995). Alternatively, PPARα activators may induce the expression of repressor proteins of the Apo CIII gene, such as Apolipoprotein A-I regulatory protein-I, Ear3/COUP-TF or Rev-erb-alpha (Vu-Dac *et al.,* 1998).

1.8.3 PPARα and high-density lipoprotein (HDL) metabolism

High-Density Lipoprotein (HDL) metabolism plays important and protective role against cardiovascular diseases. HDL transports cholesterol to the liver for metabolism and excretion, resulting in reduced serum cholesterol availability (Barter and Rye, 1994; Vosper *et al*., 2002). PPARα activation by fibrates influences the expression of genes encoding for proteins involved in HDL metabolism by five key genes. These five key genes are apolipoprotein A-I, apolipoprotein A-II, lipoprotein lipase, scavenger receptor class B type I and adenosine triphosphate-binding cassette transporter-1.

1.8.3.1 Apolipoprotein A-I and apolipoprotein A-II

Recent studies have demonstrated PPARα activation by fibrates increases plasma HDL concentrations through the induction of expression of the human Apolipoprotein A-I (Apo A-I) and Apolipoprotein A-II (Apo A-II) genes (Vu-Dac *et al*., 1994; Vu-Dac *et al.*, 1995; Fruchart, 2001). PPARα directly influences the expression of human Apolipoprotein A-I and Apolipoprotein A-II genes. The transcription rate of the human Apo A-I gene was shown to be induced by PPARα which interacts with a positive PPRE located in the human Apo A-I gene promoter liver specific enhancer (Vu-Dac *et al*., 1994). PPARα was also shown to bind with high

affinity to a DR-I-type PPRE located in the human apolipoprotein A-II promoter, thereby activating apolipoprotein A-II gene transcription (Vu-Dac *et al*., 1995).

1.8.3.2 Lipoprotein lipase

Activation of PPARα by fibrates induces lipoprotein lipase expression via transcriptional level in the liver, resulting in increased lipolysis (Schoonjans *et al*., 1996b). Activated PPARα binds to PPRE in the human lipoprotein lipase gene promoter. An increase in lipolysis leads to an increase in pre-*β*-HDL, which is the key acceptor of cholesterol from peripheral cells during the process of reverse cholesterol transport (Fruchart, 2001).

1.8.3.3 CD-36 and LIMPII analogous 1 (CLA-I)/scavenger receptor B type I (SR-BI)

Scavenger receptor B type I (SR-BI) and its human homologue (CLA-I) are cell surface receptors. The roles played by SR-BI and CLA-I are to bind HDL with high affinity and mediate the selective uptake of cholesteryl esters from HDL in liver and steroidogenic tissues (Chinetti *et al*., 2000b; Fruchart, 2001). SR-BI promotes cholesterol removal from peripheral cells, including macrophages, thus suggesting that CLA-I/SR-BI may play a key role in reverse cholesterol transport pathway (Chinetti *et al*., 2000b; Fruchart, 2001). PPARα activation is seen to induce both CLA-I and SR-BI expressions (Fruchart, 2001; Chinetti *et al*., 2000b).

1.8.3.4 Adenosine triphosphate-binding cassette transporter-1 (ABC-1)

ABC-1 plays pivotal role in HDL metabolism by exporting unesterified cholesterol and phospholipids from cells (Fruchart, 2001). PPARα activation induces