MOLECULAR MECHANISM OF ACUTE PHASE RESPONSE: IDENTIFICATION OF SIGNAL TRANSDUCTION PATHWAYS MEDIATING CYTOKINE INHIBITORY EFFECT ON HUMAN PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR ALPHA (PPARα) IN LIVER CELLS

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by

CHEW GUAT SIEW

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My parents, Mr. & Mrs. Chew Seng Leong:

You have enriched my life beyond measure and I cherished you both so much

&

My husband, Eng Leong:

I find more reasons to love you everyday and I am blessed to be your wife

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12-HETE	12-hydroxyeicosatetraenoic acid
13-HODE	13-hydroxyoctadecadienoic acid
15-dPGJ$_2$	15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$
15-HETE	15-hydroxyeicosatetraenoic acid
5'UTR	5' untranslated region
9-HODE	9-hydroxyoctadecadienoic acid
9 cis-RA	9-cis retinoic acid
AD	Activation domain
AF-1	Activation function 1
AF-2	Activation function 2
AP1	Activating protein 1
Apo A-I	Apolipoprotein A-I
Apo A-II	Apolipoprotein A-II
Apo CIII	Apolipoprotein CIII
apoE	Apolipoprotein E
APS	Ammonium persulphate
APP	Acute phase protein
APR	Acute phase response
ARF6	Adipocyte differentiation-dependent regulatory factor
ATCC	American Type Culture Collection
BADGE	Bisphenol diglycidyl ether
BCP	1-Bromo-3-Chloropropane
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
CO_2	Carbon dioxide
CoA	Coactivators

COUP	Chicken ovalbumin upstream promoter-transcription factor
CoR	Corepressor
COX	Cycloxygenase
CPT-I	Muscle-type carnitine palmitoyltransferase type 1
CRE	Cyclic AMP response element
CREB	cAMP-response element binding protein
CRP	C-reactive protein
DAG	Diacylglycerol
dATP	Deoxyadenosine triphosphate
DBD	DNA-binding domain
DEPC	Dietylpyrocarbonate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
DR-1	Direct repeat-1 base spacer
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
EC	Endothelial cell
EDTA	Ethylene diaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
EPA	Eicosapentaenoic acid
ET-1	Endothelin-1
FAT/CD36	Fatty acid translocase
FATP	Fatty acid transport protein
FBS	Fetal bovine serum
FXR	Farnesoid-X-receptor
Gab-1	Grb2-associated binder 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATE	IFNγ-activated transcriptional element
gp80/130	Glycoprotein 80/130

Grb2	Growth-factor-receptor-bound protein 2
H_2O	Water
HAT	Histone acetyltransferases
HDAC	Histone deacetylase
HDL	High-density lipoprotein
HMG-CoA	Mitochondrial 3-hydroxy-3-methyglutaryl-CoA
HNF4	Hepatocyte nuclear factor-4
HODE	Hydroxyoctadecadienoic acids
hPPARα	Human peroxisome proliferator activated receptor alpha
HRP	Horseradish Peroxidase
ICAM-1	Intracellular adhesion molecule-1
IFNγ	Interferon gamma
IL-1α	Interleukin-1-alpha
IL-1β	Interleukin-1-beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
INOS	Inducible nitric oxide synthase
IP-10	IFNγ-inducible protein of 10 kDa
IPTG	Isopropyl-β-D-thiogalactopyranoside
ISGF-RE	Interferon stimulated gene factor response element
I-TAC	IFN-inducible T-cell a-chemoattractant
JAK	Janus kinase
JNK	JUN-amino-terminal kinases
kb	kilobase pairs
LAP	Liver-enriched activated protein
LARII	Luciferase Assay Buffer II
LB	Luria-Bertani
LBD	Ligand-binding domain
LDL	Low density lipoprotein
LDL-R	Low density lipoprotein receptor
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinases
MCP-1	Monocyte-chemoattractant protein-1
MEM/EBSS	Eagle's Minimum essential medium with Earle's BSS medium
$MgCl_2$	Magnesium chloride
Mig	Monokine induced by IFNγ
M-MLV RT	Molony murine leukemia virus reverse transcriptase
MMP-9	Metalloproteinase
MOPS	3-[N-Mopholino]propanesulphonic acid
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor-κB
NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical density
OxLDL	Oxidized low density lipoprotein
PBP	PPAR binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC-1	PPAR gamma coactivator-1
PIAS	Protein inhibitor of activated STAT
PI3K	Phosphoinositide 3-Kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLB	Passive lysis buffer
PLTP	Phospholipid transfer protein
PMSF	Phenylmethylsufonyl fluoride
poly(dI-dC)	Polydeoxyinosinic-deoxycytidylic acid
PPAR	Peroxisome proliferator activated receptor
PPARα	Peroxisome proliferators activated receptor alpha
PPARγ	Peroxisome proliferators activated receptor gamma
PPARs	Peroxisome Proliferator activated receptors

PPRE	Peroxisome proliferator response element
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Reverse transcription
RXR	Retinoic X receptor
SAA	Serum amyloid A
SDS	Sodium dodecyl sulphate
SH2	Src homology 2
SHP2	SH2-domain-containing tyrosine phosphatase
SMC	Smooth muscle cells
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
SOCS	Suppressor of cytokine signalling
SR-A	Scavenger receptor A
SRC-1	Steroid receptor coactivator 1
STAT	Signal transducers and activators of transcription
SUMO	Small ubiquitin related modifier
TAE	Tris-acetate-EDTA
TATA	Binding elelment for TATA-binding protein (TBP)
TB	Terrific broth
TBP	TATA box-binding protein
TBE	Tris-borate-EDTA
TdT	Terminal Deoxynucleotidyl Transferase
TE	Tris-EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
TNFα	Tumour necrosis factor α
TRE	TPA-response element
TZDs	Thiazolidinediones
UV	Ultraviolet
v/v	Volume per volume
VCAM-1	Vascular cell adhesion molecule-1
VSMC	Vascular smooth muscle cells
w/v	Weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

MEKANISME MOLEKUL RANSANGAN FASA AKUT:

PENGENALPASTIAN LALU ISYARAT TRANSDUKSI YANG MEMBATAS KESAN PERENCATAN SITOKINA TERHADAP RESEPTOR AKTIVASI PEMBIAKAN PEROKSISOM ALPHA (PPARα)

MANUSIA DALAM SEL HATI

ABSTRAK

Sitokina seperti IL-6 memainkan peranan penting dalam merangsang tindakbalas fasa akut (APR) dalam badan semasa kecederaan dan inflamasi. Dinamik ekspresi dan interaksi oleh komponen laluan isyarat dalam pengawalaturan pembiakan peroksisom alpha (PPARα) semasa APR masih belum dikenalpasti. Dalam kajian ini, kita telah mengenalpasti tiga potensi laluan isyarat, JAK-STAT, PI3K dan MAPK (p38 dan ERK1/2) yang berasas daripada komponen JAK dan SHP2 di bahagian hulu laluan IL-6, terlibat dalam merangsang perencatan pengekspresan gen PPARα. Penggunaan perencat-perencat spesifik terhadap laluan isyarat transduksi JAK, PI3K dan MAPK menunjukkan IL-6 merencat paras mRNA PPARα, melalui ransangan pengikatan STAT1 dan STAT3 ke elemen STAT pada promoter PPARα. Tambahan lagi, ekspresi lampau STAT1 dan STAT3 di dalam sel hati merencat aktiviti promoter PPARα, menunjukkan bahawa peningkatan dalam pengikatan aktiviti DNA dengan STAT merencatkan pengekspresan gen PPARα. Di samping itu, didapati bahawa rawatan dengan perencat-perencat specifik AG490, Rapamycin, SB203580 dan U0126 merencat kesan IL-6 dalam pengikatan aktiviti DNA STAT1 dan STAT3, menyarankan laluan simpang antara laluan-lalaun isyarat tersebut. Sebagai rumusan, di dalam kehadiran perencat-perencat spesifik, kesan IL-6 ke atas pengekspresan protein dan aktiviti pengikatan DNA dengan STAT1 dan STAT3 direncat secara keseluruhan atau

sebahagian. Secara keseluruhannya, kajian ini telah berjaya menemukan laluan baru yang merangsangkan perencatan pengekspresan gen PPARα oleh IL-6 yang melibatkan modulasi laluan JAK-STAT, JAK-PI3K-Akt-mTOR-STAT dan SHP-MAPK (p38-STAT dan ERK1/2-STAT). Kajian ini juga menumpukan perhatian signifikan terhadap JAK-STAT sebagai laluan dominan disebabkan laluan simpang di antara JAK-STAT dengan PI3K, dan, MAPK melalui aktivasi SHP, dan, transkripsi aktivator STAT di dalam pengawalaturan pengekspresan PPARα mRNA. Oleh itu, penentuan laluan yang mengawalatur pengekspresan PPARα di dalam sel hati yang dirawat oleh IL-6 mencadangkan keupayaan peranan fisiologi oleh PPARα memodulasi APR dan berkeupayaan mempunyai implikasi terapeutik di dalam pembentukan APR.

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ABSTRACT

Cytokines, like IL-6, play an important role in triggering the acute phase response (APR) of the body to injury and inflammation. The dynamics of expression and interaction of the IL-6 signalling pathway components in the regulation of peroxisome proliferator-activated receptor alpha (PPARα) during APR remain to be properly identified. In this study, we determined that three possible potential signaling pathways, JAK-STAT, PI3K and MAPK (p38 and ERK1/2) which derived from the upstream JAK and SHP2 components of the IL-6 signalling were involved in IL-6-inhibitory effect on PPARα gene expression. Pre-treatment of cells with the pharmacological inhibitors of JAK2, PI3K and MAPK, demonstrated that IL-6 inhibited the mRNA levels of PPARα via activating the binding of STAT1 and STAT3 to STAT binding site in the PPARα promoter. Moreover, over expression of the STAT1 and STAT3 in the liver cells decreased PPARα promoter activity, indicating that an increase in DNA binding activity of STAT1 and STAT3 inhibited the PPARα gene expression. It was also found that AG490, Rapamycin, SB203580 and U0126 inhibitors attenuated the action of IL-6 on the DNA binding of STAT1 and STAT3, suggesting a cross-talk between the signaling pathways. In short, in the presence of all the inhibitors, the effect of IL-6 on protein expression and DNA binding of STAT1 and STAT3 were either completely or partially inhibited. Taken together, this study has successfully unraveled

novel pathways by which IL-6 inhibited PPARα gene transcription, involving the modulation of JAK-STAT, JAK-associated PI3K-Akt-mTOR-STAT and SHP-mediated MAPK (p38-STAT and ERK1/2-STAT) pathways. The present study also underlines the significance of JAK-STAT as a dominant pathway due to cross-talks between JAK-STAT with PI3K, and, MAPK pathways via SHP activation, and, STAT transcription factors in down regulating the PPARα mRNA expression. Thus, the determination of the regulatory pathways of PPARα in IL-6-treated liver cells strongly suggests the potential physiological role for PPARα in modulating APR and may have immediate therapeutic implications in the development of APR.

CHAPTER 1

INTRODUCTION

1.1 Historical Background

The concept of peroxisome proliferation was first initiated when Hess and his colleagues discovered that the administration of hypolipidemic drug resulted in enlarged liver or hepatomagaly which was caused by an increase in the number and size of intracellular peroxisomes (Hess *et al*., 1965). Subsequently, a group of structurally diverse agents was found to promote and increase the number of hepatic peroxisome in rodents. Therefore, these agents are collectively named "peroxisome proliferators" (Reddy and Krishnakantha, 1975).

By 1980, Reddy and co-workers discovered that chronic, long-term administration of these chemicals to rats also resulted in liver cancer (Reddy *et al*., 1980). These classic observations laid a foundation for the focus of future research to elucidate the mechanisms underlying the effect of peroxisome proliferators. The discovery of nuclear receptors aided this pursuit and it was soon hypothesized that the effects induced by peroxisome proliferators were the result of a receptor-mediated mechanism (Reddy and Lalwai, 1983). However, it was not until 1990 that a receptor was cloned and shown to be activated by this class of chemicals (Issemann and Green, 1990) and thus termed the peroxisome proliferator-activated receptor (PPAR).

A family of transcription factors, known as PPARs which consists of PPARα, PPARβ/δ, PPARγ, has moved from the status of orphan receptor to one of the best characterised nuclear receptors. Their functional characterisation provides unique insights into the role of fat in health and diseases. Recently, this nuclear receptor has also been shown to fulfil critical unique roles in general transcriptional control of numerous cellular processes, with implications in inflammation, atherosclerosis, cancer development and epidermal wound healing (Pineda-Torra *et al*., 2002).

1.2 Peroxisome proliferator-activated receptors (PPARs)

PPARs are members of nuclear hormone receptor (NHR) superfamily, the largest family of transcription factors. There are three distinct PPAR subtypes; PPARα (also referred to as NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3), each encoded by separate genes and with specific tissue distribution pattern and metabolic functions (Issemann and Green, 1990, Dreyer *et al*., 1992, Kliewer *et al*., 1994, Braissant *et al*., 1996, Pineda-Torra *et al*., 2007).

All three PPAR isoforms possess similar structural and functional features. Principally, four functional domains have been identified which are called A/B, C, D and E/F (Figure 1.1). The N-terminal A/B domain contains a ligand-independent activation function 1 (AF-1) (Werman *et al*., 1997) responsible for the phosphorylation of PPAR. The DNA binding domain (DBD) or C domain promotes the binding of PPAR to the peroxisome proliferator response element (PPRE) in the promoter region of target genes (Kliewer *et al*., 1992). The D site is a docking domain for cofactors. The E/F domain or ligand-binding domain (LBD) is responsible for ligand specificity and activation of PPAR binding to the PPRE, which increases the expression of the target genes.

Prior to transcriptional activation, PPAR heterodimerises with RXR to form a complex (Kliewer *et al*., 1992). RXRs are also members of the NHR superfamily that are activated following binding with 9 cis-RA (9-cis retinoic acid) (Desvergne and Wahli, 1999). PPAR/RXR heterodimer recognises sequences situated within the DR1 motif, whereby PPAR interacts with the upstream extended core hexamer of the DR1, whereas RXR occupies the downstream motif (Palmer *et al*., 1995; Ijpenberg *et al*., 1997).

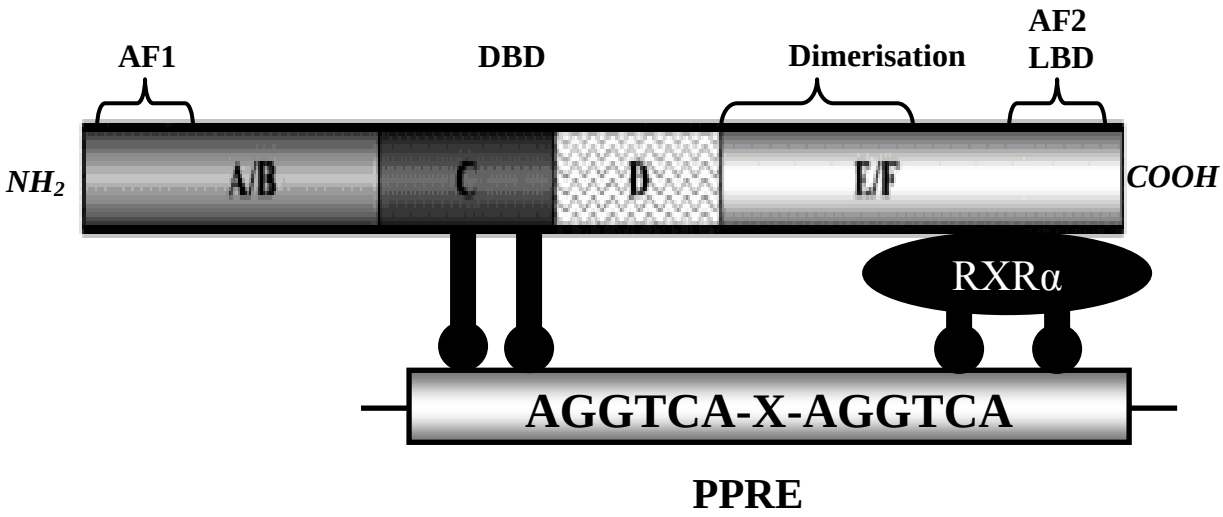

Figure 1.1 Schematic representation of the structural domains of PPAR. PPAR consists of four distinct functional domains. The A/B domain is located at the N-terminal with AF-1 is responsible for phosphorylation, the domain C is implicated in DNA binding, domain D is the docking region for cofactors and domain E/F is the ligand binding domain, containing AF-2, which promotes the recruitment of cofactors required for gene transcription. AF-1, activating function-1; DBD, DNA binding domain; AF-2, activating function-2; LBD, ligand-binding domain; RXR, retinoid-X-receptor; PPRE, peroxisome proliferators response element.

1.3 Peroxisome proliferator-activated receptor α (PPARα)

PPARα was the first PPAR to be identified (Issemann and Green, 1990). PPARα was isolated by screening a mouse liver cDNA library using a probe based on the consensus sequence of the DNA binding domain of several nuclear receptors. Full-length PPARα cDNA was found to encode a 468 amino acid protein with predicted molecular weight of 52 kDa. Analysis of the amino acid sequence demonstrated that PPARα belonged to the steroid hormone receptor superfamily since it had all the typical characteristics of steroid receptor (Evans *et al.*, 1988). The PPARα amino acid sequence displayed high homology to the DNA binding region of nuclear steroid hormone receptors such as the glucocorticoid receptor, estrogen receptor, retinoid X receptor, vitamin D receptor, thyroid receptor and retinoic acid receptor.

PPARα was also cloned from frog, rat, guinea pig and human (Wilson *et al*., 2002). In humans and rodents, high level of PPARα mRNA is found in liver, heart, kidney and muscle. However, the mRNA level of PPARα in human liver appears lower than in rodent liver (Palmer *et al*., 2002). In addition, it is expressed in steroidogenic tissue such as adrenals (Hierlihy *et al*., 2006). Furthermore, human PPARα is also expressed in vascular cells including endothelial cells (Inoue *et al*., 2001), smooth muscle cells (Staels *et al*., 1998a; Diep *et al*., 2006) and monocytes/macrophages (Staels *et al*., 1998a; Chinetti *et al*., 1998; Neve *et al*., 2003).

In mouse and rat, PPARα appears late in the development (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). Therefore, it plays an important role in the regulation of intermediary metabolism, which has been very well studied in liver. Regardless of species, the expression of PPARα correlates with high mitochondrial and peroxisomal β-oxidation activities.

1.4 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 (endogenous) and synthetic (exogenous) substances serve as PPARα ligands, including fatty acids and fatty acid-derived products as well as pharmacological molecules such as plasticizers and herbicides (Isseman and Green, 1990; Forman *et al*., 1997; Krey *et al*., 1997; Ward *et al*., 1998; Lin *et al*., 1999; Kota *et al*., 2005) (Table 1.1). The ability of PPARα to bind multiple natural and synthetic ligands is due to the 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 helixes (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 binding pocket closes and forms a 'mouse trap model' (Wahli, 2002). Xu *et al*. (2006) 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 1.1 The PPARα natural (endogenous) and synthetic (exogenous) ligands. PPARα can be activated by a structurally diverse group of ligands, which bind to PPARα and increase the transcriptional activity.

Natural (Endogenous) Ligands	Synthetic (Exogenous) Ligands
Palmitic acid	Wy-14, 643
Stearic acid	Clofibrate
Oleic acid	Gemfibrozil
Linoleic acid	Nafenopin
Arachhidonic acid	Bezafibrate
Eicosapentaenoic acid	Fenofibrate
Leukotriene B4	Fenoprofen

A range of saturated and unsaturated fatty acids could activate PPARα (Gottlicher *et al*., 1992; Burkart *et al*., 2007). Palimitic acid, oleic acid, linoleic acid and arachidonic acid are examples of saturated acid that can activate PPARα (Banner *et al*., 2007). Notably, PPARα is the only PPAR subtype that binds with high affinity to a wide range of saturated acids. This may be because 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*., 2005). The most notable synthetic PPARα ligand is the hypolipidemic fibrates drugs. 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 (Isseman and Green, 1990).

1.5 Structural organisation of PPARα

The human PPARα has been mapped to chromosome 22q12-q13.1 by somatic cell hybridisation and linkage analysis (Sher *et al*., 1993). The human PPARα gene is composed of twelve exons which spanned approximately 85kb, with 5'-untranslated region coded by exons A, 1a, 2a, 2b and part of exon 3 (Vohl *et al*., 2000; Chew *et al*., 2003). The coding region of PPARα comprises the remainder of exon 3 and exon 4-8, with the 3'-untranslated region consisting of the last 232 bp of exon 8. The introns length vary between 0.6 kb to 20 kb as indicated in Figure 1.2.

Recently, four promoters (A, B, C, D) which are responsible in transcribing six alternatively spliced variants in the 5'-untranslated region (UTR) of human PPARα were identified (Chew *et al*., 2003) (Figure 1.2). Promoters A and B are responsible in transcribing two variants each, while promoters C and D transcribe one variant each (Chew *et al*., 2003; Chew *et al*., 2007).

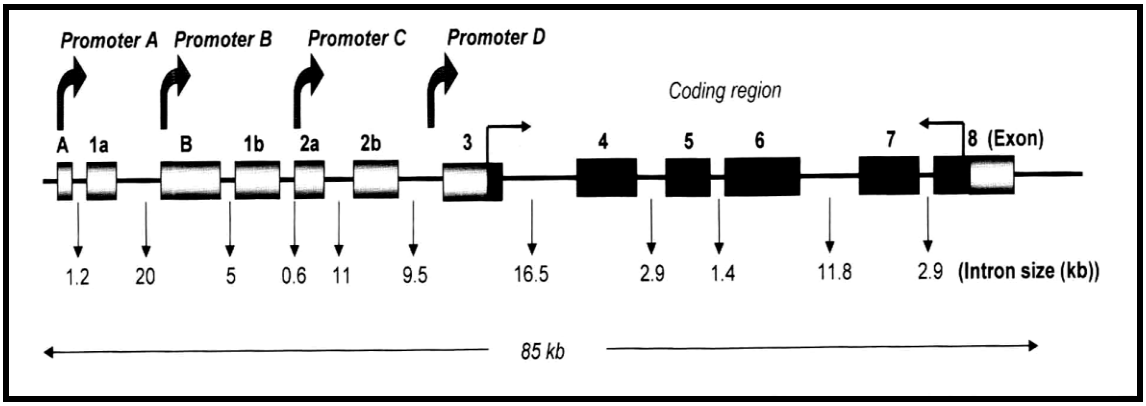

Figure 1.2 Schematic representation of the structural organisation of human PPARα gene. The gene spans 85 kb of genomic DNA. The spatial localisation of exons within the gene and the size of introns are indicated in the upper and lower panel respectively. Untranslated region □ coding regions ■ are also indicated. Block arrows indicate the location of the four promoters A, B, C and D.

1.6 Physiological roles of PPARα

1.6.1 PPARα and fatty metabolism

PPARα serves a fundamental role in mammals by acting as a central modulator of signalling molecules that mediate changes in gene expression to maintain lipid homeostasis. PPARα is highly expressed in tissues with elevated rates of fatty acid catabolism, whereby through the interaction with PPRE on the promoter region of several genes, PPARα and its ligands regulate the transcription of the genes of key enzymes and proteins such as fatty acid transport protein (FATP), fatty acid translocase (FAT/CD36) and Acyl-CoA synthetase (ACS) that play crucial roles in the lipid and fatty acid metabolism (Schoonjans *et al*., 1996a; Chinetti *et al*., 2001; Ye *et al*., 2001; van Raalte *et al*., 2004; Israelian-Konaraki and Reaven, 2005).

Intracellular fatty acid concentrations are partly regulated by import and export system that is controlled by FATP, FAT/CD36 and ACS. These proteins facilitate the transport of fatty acids through the cell membrane and their esterification preventing their efflux (Abumrad *et al*., 1993, Schoonjans *et al*,, 1995, Martin *et al*., 1997, Tontonoz and Mangelsdorf, 2001).

PPARα acts as a regulator of intracellular fatty acid uptake controls. Treatment with PPARα agonists (activators) has been shown to induce FATP mRNA levels in rat liver and intestine, and induce ACS mRNA levels in liver and kidney (Schoonjans *et al*., 1995, Martin *et al*., 1997, Motojima *et al*., 1998, Fruchart *et al*., 2004). Recent experiments carried out using LDL receptor-deficient mice also showed similar induction in ACS mRNA (Srivasta *et al*., 2006). 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 mitochondria where its metabolism takes place. Muscle-type carnitine palmitoyl transferase 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*., 2001, Louet *et al*., 2001). Furthermore, activation of PPARα was proven to up-regulate the gene expression of mitochondrial 3-hydroxy-3-methyglutaryl-CoA (HMG-CoA) synthase (Rodriguez *et al*., 1994, Meertens *et al*., 2005). 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 (Rodriguez *et al*., 1994, Meerten *et al*., 2005). 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 the synthesis of ketones (Chinetti *et al*., 2001).

1.6.2 PPARα and triglyceride-rich lipoprotein metabolism

There is increasing evidence that serum triglycerides are strong risk factors in cardiovascular disease. 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 trigyceride-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 PPRE-mediated manner. 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).

Research carried out by Srivastava *et al*. (2006) further supported the above-mentioned mechanisms. A PPARα ligand, fenofibrate, was demonstrated to improve lipid abnormalities, such as lowering serum triglycerides and cholesterol, improving insulin sensitivity, and preventing accumulation of lipids in the aorta. In addition, this research showed that fenofibrate mediated its effect partly via inhibition of triglyceride production and partly via clearance of triglyceride-rich apolipoprotein B (Apo B) particles by elevating LPL and reducing apolipoprotein CIII (Apo CIII).

Apo CIII plays a key role in delaying the catabolim 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*., 2007). PPARα activators were also proven to decrease Apo 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 might supress 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 hepatocytes 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-1, Ear3/COUP-TF or Rev-erb-alpha (Vu-Dac *et al*., 1998, van Raalte *et al*., 2004, Becker *et al*., 2006).

1.6.3 PPARα in inflammation, atherosclerosis and thrombosis

Atherosclerosis is a long term process which involves recruitment and activation of different cell types, leading to inflammatory response. It is a multifactorial disease in which the occurrence of lesions may result in ischemia of the heart, brain, resulting in infarction (Zaman *et al*., 2000).

Specifically, the process of formation of atherosclerotic plaque involves recruitment of circulating monocytes, which must first adhere to the endothelium before their invasion into vessel intima, where they subsequently develop into tissue macrophages. These macrophages accumulate intracellular lipid to become foam cells which in turn, produce cytokines and other pro-inflammatory signals, which further stimulate monocyte recruitment to the plaque. These signals also induce vascular smooth cell proliferation and invasion. Developments in the plaque will induce apoptosis of cells in the centre as well as possible plaque destabilization and rupture with subsequent thrombus formation (Vosper *et al*., 2002). PPARα has been widely shown to play an important role in the development of atherosclerosis (Zanbergen and Plutzky, 2007; Izzo *et al*., 2009). PPARα is abundantly present in the atherosclerotic lesions and in primary cultures of endothelial cells, smooth muscle cells and macrophage foam cells (Chinetti *et al*., 1998; Staels *et al*., 1998a). In addition, PPARα is present in isolated human monocyte and its expression increases upon differentiation into macrophages (Chinetti *et al*., 1998). Clinical studies performed showed that PPARα activators (fibrates) lower the progression of atherosclerosis in both human and animals (Hahmann *et al*., 1991; Fruchart *et al*., 1999; Fruchart, 2001; Fruchart, 2009).

Adhesion of circulating monocytes is a critical early step in atherogenesis. Marx *et al*. (1999) reported that PPARα inhibits cytokine-induced (TNF-α) vascular cell adhesion molecule-1 (VCAM-1), an adhesion molecule critical for monocytes recruitment to atherosclerotic lesions (Marx *et al*., 1999; Chinetti *et al*., 2001). This strongly suggests that PPARα may reduce the recruitment and adherance of monocyte to the endothelium. In addition, PPARα also reduces VCAM-1 expression in endothelial cells (Chinetti *et al*., 1998; Neve *et al*., 2000).

In human vascular endothelial cells, PPARα activation represses thrombin-induced expression of endothelin-1 (ET-1), a potent vasoconstrictor peptide and inducer of smooth muscle cell proliferation (Delerive *et al*., 1999b). PPARα activators repress the ET-1 production by interfering with AP-1 signalling pathway, which mediates thrombin activation of endothelin-1 gene transcription (Delerive *et al*., 1999b) (Figure 1.3).

PPARα has also been implicated to interfere with transcription of several inflammatory response genes. For example, PPARα activators inhibit the expression of inducible cyclo-oxygenase-2 (COX-2) through negative interference with NFκB activation (Staels *et al*., 1998a; Meade *et al*., 1999). COX-2 is a catalyser of the production of prostanoids, which are major effectors of inflammation response (Paik *et al*., 2000). In human aortic smooth muscle cells, PPARα activators inhibit interleukin-1 (IL-1)-induced secretion of interleukin-6 (IL-6) and keto prostaglandin F1-alpha (6-keto-$PGF_{1\text{-alpha}}$). IL-6 controls macrophages and T-cell activation, as well as vascular smooth muscle cell proliferation and migration. It also plays a major regulatory role in the acute phase response and is considered an accurate marker of vascular inflammation. IL-6 also induces the potent monocyte chemoattractant protein-1 (MCP-1) in peripheral blood mononuclear cells, which the inhibition of IL-6

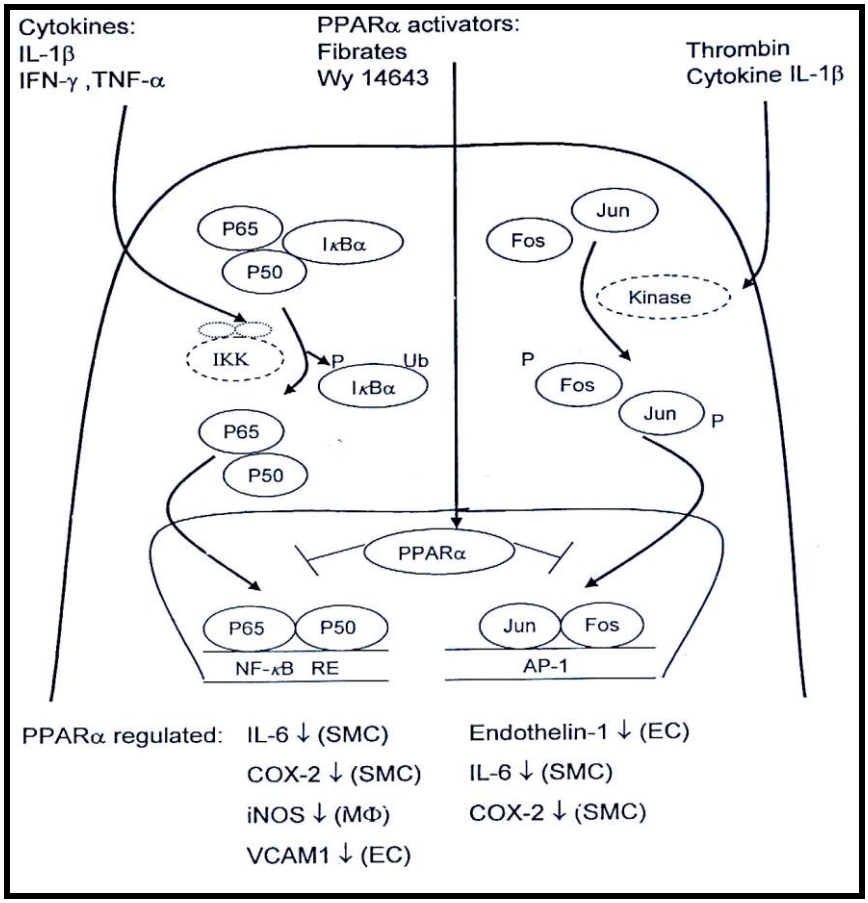

Figure 1.3 Peroxisome proliferator-activated receptor alpha activators inhibit vascular inflammation, induce apoptosis and decrease endothelin-1 secretion by endothelial cells. NFκB, Nuclear factor -κB; AP-1, activated protein-1; IFN-γ, interferon-gamma; IKK, IκB kinase; IL, interleukin; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; TNFα, tumour necrosis factor alpha; EC, vascular endothelial cells; SMC, vascular smooth muscle cells; Mϕ, macrophages.

will slow down recruitment of monocytes to developing plaques. PPARα activators repress the expression of both IL-6 and 6-keto-$PGF_{1\text{-alpha}}$ through negative cross-talk of activated PPARα with transcriptional factors NFκB and AP-1 signalling pathways (Staels *et al*., 1998a; Delerive *et al*., 1999a; Delerive *et al*., 2001).

Recently, Neve *et al*. (2001) demonstrated that PPARα agonists inhibit tissue factor (TF) expression in human monocytes and macrophages (Neve *et al*., 2001). Monocytic TF expression contributes to trombogenicity associated with plaque rapture and may propagate thrombus formation at the site of vascular lesions. In addition, TF also mediates adhesion and migration of monocytes (Neve *et al*., 2001). In addition, synthetic PPARα agonist WY-14643 has been shown to reduce inducible nitric oxide synthase (iNOS) which is key inflammatory enzyme in macrophages. The inhibition by PPARα is suggested to be mediated through the modulation of the stress protein, heme oxygenase 1 (Colville-Nash *et al*., 1998; Fruchart *et al*., 1999).

Scavenger receptor A (SR-A) is significant in the generation of atherosclerosis plaque. It mediates the uptake of modified low-density lipoprotein (LDL) which plays key role in the formation of the foam cell (Vosper *et al*., 2002). Matrix metalloproteinase (MMP)-9 (gelatinase B) is produced by macrophages to degrade collagen IV in the basement membrane, facilitating invasion through the vessel wall and into the intima. PPARα agonists are capable of blocking expression of both SR-A and MMP-9, and thus act in an anti-inflammatory and atherosclerotic manner (Fruchart *et al*., 1999; Vosper *et al*., 2002).

Finally, there is evidence that PPARα agonists are able to reduce levels of plasma pro-coagulant factors fibrinogen in human, which is an acute-phase protein whose expression is up-regulated by cytokines during inflammation (Staels *et al*., 1998a). This, in turn, reduces the likelihood of thrombogenesis (Staels *et al*., 1998a;

Kockx *et al*., 1999; Vosper *et al*., 2002). IL-6 is known to up-regulate fibrinogen expression, and it may be through the negative effects of PPARα agonists on IL-6 expression that reduces the fibrinogen levels (Castell *et al*., 1989).

In addition, PPARα agonists also significantly decrease plasma levels of cytokines and acute phase proteins such as C-reactive protein (CRP), which are established risk factors for cardiovascular disease. Thus, these data taken together indicate that PPARα activators exert anti-inflammatory activities in humans (Staels *et al*., 1998b; Chinetti *et al*., 2001).

Therefore, PPARα plays a novel role in atherosclerosis. PPARα may interfere with proatherogenic processes at different levels. Firstly, PPARα exerts beneficial effects on atherosclerosis by changing plasma lipid and lipoprotein profiles toward less atherogenic levels. Secondly, PPARα interferes with the development of atherosclerosis by inhibiting inflammatory response at the level of vascular wall. PPARα may interfere with the early stages of atherosclerotic lesion development by affecting monocyte recruitment by inhibiting TNF-α-induced VCAM-1 expression in endothelial cells. Furthermore, PPARα may also influence the later stages of atherosclerosis by inducing apoptosis of activated human macrophages. In Figure 1.4, the summary of the effects PPARα activation on vascular inflammation, atherosclerosis and thrombosis is shown (Chinetti *et al*., 2001; Fruchart, 2009).

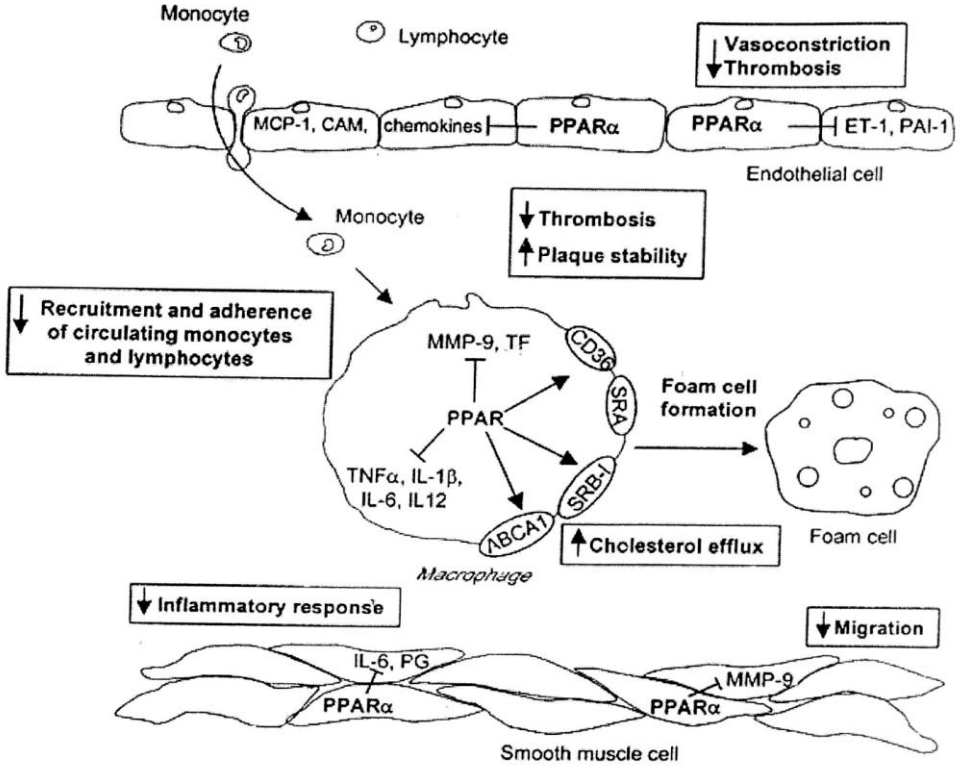

Figure 1.4 PPARα controls vascular inflammation and thrombosis related to atherosclerosis. CAM, cellular adhesion molecule; ET-1, endothelin-1, IL, interleukin; MCP1, monocyte chemoattractant protein; MMP, metaloproteinase; PAI-1, plasminogen activator inhibitor type 1; PG, prostaglandin, TF, tissue factor; TNF, tumour necrosis factor.

1.7 Acute phase response (APR)

The APR is a prominent systemic reaction of the organism to local or systemic disturbances in its homeostasis caused by infection, tissue injury, trauma and surgery, neoplastic growth or immunological disorders (Baumann and Gauldine, 1994; Moshage, 1997; Bengmark, 2004; Lowenstein and Matsushita, 2004; Gruys *et al*., 2005). The APR is beneficial to the injured organism with the aim of restoring the disturbed physiological homeostasis (Moshage, 1997; Lowenstein and Matsushita, 2004; Gruys *et al*., 2005).

There are three main components of APR, i.e. activators, regulators and effectors, as depicted in Figure 1.5. At the site of invasion by the microorganism and the place of tissue injury, a number of responses of the tissue itself are initiated. Pro-inflammatory cytokines which would act as activators of APR are released, and the vascular system and inflammatory cells are activated. These responses in turn are associated with production of more cytokines and other inflammatory mediators, which diffuse to the extracellular fluid compartment, circulate in the blood and bind to receptors on endothelial cells and hepatocytes, thus amplifying a local response into a systemic inflammatory response. Activators of the APR initiate an intracellular signal transduction cascade that activates the three major transcription factors (STAT3, NFκB, C/EBPβ) which alone or in combination with other proteins regulate transcription of APR effector genes. For example, previous reports have suggested a role of STAT3 in regulation of haptoglobin in liver cells. The STATs are latent transcription factors that are activated by tyrosine phosphorylation. After phosphorylation, the STAT proteins homo- or heterodimerize and translocate to the nucleus to activate the transcription of many target genes, including APPs (Darnell, 1997; Kurash *et al*., 2004).

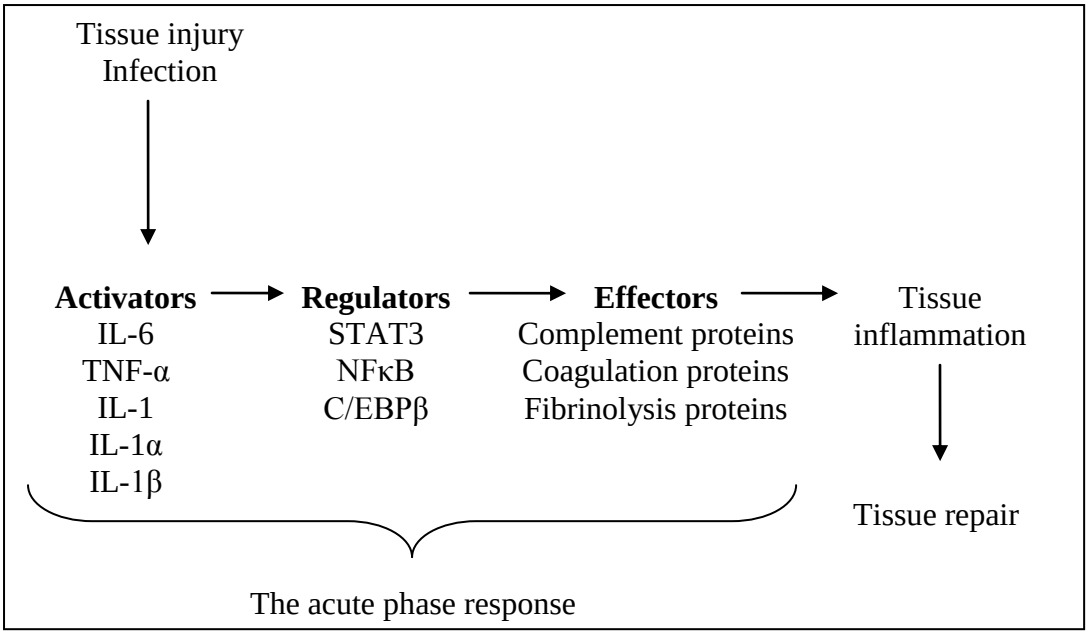

Figure 1.5 Components of the acute phase response. Activated innate immune cells release activators of the APR (IL-6, TNF-α, IL-1). Activators interact with their respective receptors, inducing transcriptional regulators of the APR, including STAT3, NFκB and C/EBPβ. These transcriptional factors direct the synthesis of acute phase effector proteins, which mediate the APR. (Adapted from Lowenstain and Matsushita, 2004)

The APR is accompanied by specific changes in the concentration of plasma proteins, which would act as effectors of the APR. Proteins that increased by at least 25% during the APR are positive APP (e.g. CRP, serum amyloid A (SAA) and fibrinogen), whereas proteins that decreased are negative APPs (e.g. albumin, transferrin, and α-fetoprotein). Changes in APP concentrations are largely attributable to alterations in their rate of synthesis in the liver (Morley and Kushner, 1982; Gabey and Kushner, 1999; Khovidhunkit *et al*., 2004).

In the liver, TNF-α, IL-1 and IL-6 play a key role in APR (Le and Vilcek, 1989; Sehgal *et al*., 1989a; Heinrich *et al*., 1991). These cytokines activate hepatocytic secretion of most of the APP via activating hepatocytic receptors (Le and Vilcek, 1989; Sehgal *et al*., 1989a; Heinrich *et al*., 2003). IL-6 is the major mediator for the hepatocytic secretion of the most of the APP (Le and Vilcek, 1989; Sehgal *et al*., 1989b; Heinrich *et al*., 2003). Table 1.2 shows the major cytokines involved in APR, their cellular sources and biological functions. Furthermore, it has been shown that Kupper cells play an intermediate role. After stimulation by the pro-inflammatory cytokines, the Kupper cells release IL-6 and suppress mononuclear phagocytic production of IL-1 and TNF-α (Schindler *et al*., 1990; Gruys *et al*., 2005), thus mitigating the whole cascade reaction. Down-regulation of the hepatocytic APR is achieved by rapid hepatic removal of circulating cytokines (Heinrich *et al*., 1991; Heinrich *et al*., 2003).

Table 1.2 List of cytokines important in acute phase response, their most important cellular source and biological function

Cytokine	Cellular Source	Biological Activity
IL-1α IL-1β	Monocyte Macrophages, B-cells and dendritic cells	Promote inflammation; activate the coagulation pathway; stimulate the liver to produce APP; catabolism of fat for energy conversion; stimulate the synthesis of collagen and collagenase for scar tissue formation; stimulate the synthesis of adhesion factors on endothelial cells and leukocytes for diapedesis; and activate macrophages
IL-6	Monocytes, macrophages, fibroblasts, Th2 cells, stromal cells and endothelial cells	Stimulates the liver to produce APP; stimulates the proliferation of B-lymphocytes; antibody production and increases neutrophil production; induces myeloma and plasmacytoma growth; nerve cell differentiation.
TNF-α	Monocytes, macrophages, Th1 cells, dendritic cells and NK cells	Works synergistically with IL-1 to enhance inflammation. Functions include acting on endothelial cells to stimulate inflammation and the coagulation pathway; stimulates macrophages to secrete IL-1 for redundancy; activates neutrophils and promoting extracellular killing by neutrophils; stimulates the liver to produce APP, and acts on muscles and fat to stimulate catabolism for energy conversion

1.7.1 PPARα and acute phase response

Several studies have shown that the expression levels of APP are regulated by fibrates, which act via PPARα dependent mechanisms (Staels *et al*., 1998a; Gervois *et al*., 2001; Jonkers *et al*., 2002; Kleeman *et al*., 2003). Several APR markers such as fibrinogen, CRP, SAA, α2-macroglobulin and plasminogen are lowered after fenofibrate treatment in humans, whereas levels of albumin, which is a negative APR protein, is raised.

Interestingly, IL-6 effects on acute phase gene expression are fully suppressed in fenofibrate-treated wild type, but not in PPARα deficient mice (Gervois *et al*., 2004). The global effect of chronic PPARα activation on the expression of positive and negative acute phase genes suggests the existence of an upstream suppression of IL-6 pathway. IL-6 induces acute phase genes via a receptor system, consisting of the IL-6R (gp80) and gp130 proteins, which initiate a signaling cascade leading to downstream activation of transcription factors, such as C/EBP and STAT. In fenofibrate-treated wild-type mice, PPARα down-regulates expression levels of the IL-6R (gp80) and the signal transducer gp130 and reduces levels of phosphorylated STAT3 (Gervois *et al*., 2004), thus contributing to the global suppression of IL-6 induced acute phase gene transcription by PPARα agonists. Moreover, PPARα was also regulated by the expression of several key APP induced by IL-6. Even though the expression of PPARα has been found to be down-regulated by cytokines and LPS during physiological and pathophysiological changes (Beier *et al*., 1997; Beigneux, *et al*., 2000; Fang *et al*., 2004; Feingold *et al*., 2004), limited research has been carried out to look into the action of IL-6 on PPARα gene expression.

Recently, Chew *et al*. (2007) reported the molecular mechanisms by which PPARα was regulated by IL-6 in human HepG2 cells. IL-6-mediated inhibition of

PPARα gene expression was discovered to involve the activation of C/EBP isoforms. Interestingly, LPS and cytokine administration also decreases both protein and mRNA levels of PPARα in the liver of hamster and mice (Beigneux *et al*., 2000; Beigneux *et al*., 2002; Kim *et al*., 2003). In addition, LPS administration significantly reduced the expression of NHR such as PPARγ, PPARβ/δ, RXR and FXR (Beigneux *et al*., 2000; Beigneux *et al*., 2002; Kim *et al*., 2003; Khovidhunkit *et al*., 2004). Therefore, these published reports strongly indicate the implication of roles played by PPARα in inflammation, lipid metabolism and atherosclerosis, which are linked to APR in the mechanisms of coordinating the regulation of multiple genes induced by cytokines.

The action of PPARα on acute phase gene expression is not restricted to the IL-6 signalling pathway. As shown in the case of CRP, chronic activation of PPARα also prevents IL-1 stimulation of acute phase genes such as SAA in vivo (Zambon *et al*., 2006). PPARα activation thus, impairs cytokine-signalling pathways in the liver, acting at different levels, resulting in a potent modulation of APR reaction. PPARα is known to have the ability to sense intracellular lipid levels and orchestrated changes in lipid metabolism, therefore, the receptor has been recognised as liposensor (Chawla *et al*., 2001; Khovidhunkit *et al*., 2004).

Alterations observed in the activity of NHR liposensors are likely to play a pivotal role in the coordinated regulation of fatty acid, cholesterol metabolism and reverse cholesterol transport that occurs during the APR. The fatty acid binding protein (FABP) is well known to be down-regulated in response to infection or inflammation and is also well known target for PPARα (Memon *et al*., 1999; Landrier *et al*., 2004; Bornar *et al*., 2006).

1.8 Cytokines and IL-6

Cytokines are small hormone-like proteins that play a pivotal role in the development and pathology of human disease, including diseases of the immune response. Since their discovery and cloning, it has become abundantly clear that cytokines play critical roles in regulating immune and inflammatory cells (Heinrich *et al*., 1991).They generally act over short distances and short time spans and at very low concentrations. The most remarkable characteristics of cytokines is that they can act on cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). It is common for different cell types to secrete the same cytokine or a single cytokine to act on several different cell types (pletotropy). Cytokines are redundant in their activity, which means that different cytokines can also stimulate a similar function in cells. Cytokines are often produced in cascade, as one cytokine stimulates its target cells to make additional cytokines. Cytokines can interact synergistically or antagonistically with each other (Heinrich *et al*., 2003).

Cytokines have been classified (1) on the basis of their biological responses, (2) according to the receptors used or (3) according to their dimensional structures (Heinrich *et al*., 1998). Based on the classification of their biological responses, three main groups of cytokines can be distinguished: (1) cytokines that primarily act as positive or negative growth factors for a variety of cells (IL-2, IL-3, IL-4, IL-7, IL-10, IL-11, IL-12 and granulocyte-macrophage colony stimulating factor), (2) cytokines with pro-inflammatory properties (TNFα/β, IL-1α/β, IL-6, IFN-α/γ, IL-8, and macrophage inhibitory protein-1), and (3) factors with anti-inflammatory activity (IL-1 receptor antagonists, TNF-α binding protein and IL-1 binding protein).

Hence, IL-6 being a pro-inflammatory cytokine, plays an important role as mediator involved in the regulation of the acute-phase response to injury and infection. Besides their functions in inflammation and the immune response, IL-6 also plays a crucial role in haematopoiesis, liver and neuronal regeneration, embryonal development and fertility (Gervois *et al*., 2004; Chang *et al*., 2005; Song and Kellum, 2005).

IL-6 was first discovered and named interferon-β2 (IFN-β2) in 1980 by Weissenbach and colleagues (Weissenbach *et al*., 1980) during an effort to clone and characterize the interferon-β gene in human fibroblasts. The cytokine was subsequently named B-cell stimulatory factor-2 (Hirano *et al*., 1985), B cell differentiation factor, T cell-replacing factor, 26-kDa protein (Haegeman *et al*., 1986), hybridoma growth factor (Brakenhoff *et al*., 1987; Van Snick *et al*., 1986), interleukin hybridoma plasmacytoma factor 1, plasmacytoma growth factor (Nordan *et al*., 1987), hepatocyte-stimulating factor (Gauldie *et al*., 1987), macrophage granulocyte-inducing factor 2, cytotoxic T cell differentiation factor (Takai *et al*., 1988) and thrombopoietein due to its biological functions. In 1989, when these variously named proteins were found to be identical on the basis of their amino acid and/or nucleotide sequences, the name IL-6 was settled upon (Akira and Kishimoto, 1992; Song and Kellum, 2005).

IL-6 is a member of a cytokine family that consists of leukemia inhibitory factor (LIF), ciliary neurotropic factor (CNTF), IL-11, oncostatin M (OSM) and cardiotrophin 1 (CT-1). Members of this cytokine family contain four antiparallel α-helices termed A, B, C and D that are connected by two long, and one short, loops (Figure 1.6) (Heinrich *et al*., 2003; Song and Kellum, 2005). Although each of the IL-6 type cytokine is recognized by a specific receptor complex, they all share a common