DIFFERENTIAL REGULATION OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA (PPARγ) BY CYTOKINES IN MURINE MACROPHAGE J774.2 CELL LINE: ELUCIDATION OF SIGNAL TRANSDUCTION PATHWAYS OF TUMOUR NECROSIS FACTOR ALPHA (TNFα) IN REGULATING MACROPHAGE PPARγ GENE EXPRESSION

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2007

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

May 2007

ACKNOWLEDGEMENT

This thesis is truly a collection of hard work and efforts of many people for the past five years. First and foremost, I would like to express my deepest gratitude to my supervisor, Associate Professor Dr. Tengku Sifzizul Tengku Muhammad for his constant support, guidance, encouragement and most of all his patience throughout both the experimental works and the writing of this thesis. I am truly honored to have such a talented, outstanding and generous supervisor.

Many thanks to my co-supervisor, Professor Dr. Nazalan Najimudin for igniting my passion for research during my undergraduates studies. My view of molecular genetics and love of science would not have fully developed without his help. Thanks are also due to Dr. Tan Mei Lan for her guidance in cell culture techniques.

I would like to take this opportunity to express my sincere gratitude to Dr. Akira Sugawara of Tohoku University Graduate School of Medicine, Japan for their generosity in providing the mPPARγ1 promoter constructs and most of all, sharing of their research findings.

Thanks without measure to my mentor, Boon Yin for her technical advice and help. My heartfelt thanks to my wonderful lab members for their kindness and good team work. To Dr. Chew Choy Hoong, Kak Wina, Danley, Eng Keat, Chee Keat, Guat Siew, Leong, Ida, Aya, Azad, Sham, Amir, I will cherish the moments we shared together.

I would also like to take this opportunity to thank the Ministry of Science, Technology and Environment (MOSTE) for their financial support under the National Science Fellowship (NSF) Scheme and acknowledge the Malaysian

Toray Science Foundation (MTSF) for the grant awarded. Thanks are also due to the staffs in Institute of Postgraduate Studies (IPS) and School of Biological Sciences USM for the assistance and facilities provided.

I would like to express my eternal gratitude to my late father and my mother for their never-ending moral support and unconditional love. I could never have done my Phd. project without their support. I owe so much to both of you. Pa, you will always in my heart, and Mum, thanks for everything. My heartfelt thanks to my brothers, sister, sister-in-law and future brother-in-law for the joys and tears we shared together. To Zhi Ann and Zhi Qiang, the boys next door, thanks for brighten up my life during my short stay in hometown after my father's funeral.

I would also like to express my gratitude to my father-, mother-, brothers-in-law for their support and encouragement. Last but not least, this thesis is dedicated to my beloved husband, Kelvin Cheah. Thank you for giving me a happy family, a lovely boy, Benjamin and most of all, walks through with me all the up and down in my life.

Lim Chui Hun

May 2007

This thesis is dedicated to

my father, forever in loving memories;

my husband, Kelvin Cheah

&

my son, Benjamin.

Thank you for being my source of inspiration.

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LIST OF ABBREVIATIONS

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
AF-1	Activation function 1
AF-2	Activation function 2
AP1	Activating protein 1
apoE	Apolipoprotein E
APS	Ammonium persulphate
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
CDDO	Triterpenoid 2-cyano-3, 12-dioxooleana-1,9-dien-28-oic acid
cDNA	Complementary DNA
CO_2	Carbon dioxide
CoA	Coactivators
CoR	Corepressor
COX	Cycloxygenase
CRE	Cyclic AMP response element
CREB	cAMP-response element binding protein
dATP	Deoxyadenosine triphosphate
DBD	DNA-binding domain
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
EPA	Eicosapentaenoic acid
ET-1	Endothelin-1
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H_2O	Water
HAT	Histone acetyltransferases
HDAC	Histone deacetylase
HDL	High-density lipoprotein
HODE	Hydroxyoctadecadienoic acids
HRP	Horseradish Peroxidase
ICAM-1	Intracellular adhesion molecule-1
IFNγ	Interferon gamma
IL-1α	Interleukin 1α
IL-1β	Interleukin 1β
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
kb	kilobase pairs

LB	Luria-Bertani
LBD	Ligand-binding domain
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAP kinase	Mitogen-activated protein kinase
MCP-1	Monocyte-chemoattractant protein-1
$MgCl_2$	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
NaCl	Sodium choride
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
PMSF	Phenylmethylsufonyl fluoride
poly(dI-dC)	Polydeoxyinosinic-deoxycytidylic acid
PPAR	Peroxisome Proliferator Activated-Receptor
PPARγ	peroxisome proliferators activated receptor gamma
PPRE	Peroxisome proliferator response element
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Reverse transcription
RXR	Retinoic X receptor
SDS	Sodium dodecyl sulphate

SMC	Smooth muscle cells
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
SR-A	Scavenger receptor A
SRC-1	Steroid receptor coactivator 1
STAT	Signal transducers and activators of transcription
TAE	Tris-acetate-EDTA
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

PENGAWALATURAN PEMBEZAAN RESEPTOR AKTIVASI PEMBIAKAN PEROKSISOM GAMMA (PPARγ) OLEH SITOKINA DI DALAM SEL TURUNAN MAKROFAJ MURIN J774.2: PENGENALPASTIAN LALUAN ISYARAT TRANSDUKSI FAKTOR NEKROSIS TUMOR ALFA (TNFα) DI DALAM PENGAWALATURAN PENGEKSPRESAN GEN PPARγ

ABSTRAK

Aterosklerosis merupakan punca kematian utama di negara-negara maju. Peranan PPARγ dalam makrofaj yang diaktifkan oleh sitokina adalah penting di dalam patogenesis aterosklerosis. Namun, mekanisme molekul yang tepat yang mana sitokina mengawalatur pengekspresan gen PPARγ masih kurang difahami. Di dalam kajian ini, kami mengkaji kesan empat sitokina iaitu TNFα, IFNγ, IL-1α dan IL-1β ke atas pengekspresan mRNA, protein dan aktiviti pengikatan DNA PPARγ di dalam sel turunan makrofaj murin J774.2, model yang paling lazim digunakan untuk aterosklerosis. TNFα dan IFNγ didapati merencat pengekspresan mRNA dan protein PPARγ serta aktiviti pengikatan DNA. Sebaliknya, IL-1β merangsangkan peningkatan pengekspresan PPARγ pada peringkat mRNA, protein dan aktiviti pengikatan DNA. IL-1α pula tidak mempunyai kesan ke atas pengekspresan PPARγ dan aktiviti pengikatan DNA. Memandangkan perubahan dalam kandungan protein dan aktiviti pengikatan DNA di dalam makrofaj yang dirawat dengan sitokina selaras dengan perubahan dalam mRNA PPARγ, keputusan ini mencadangkan dengan kukuh bahawa pengekspresan PPARγ dan aktiviti pengikatan DNA dikawalatur pada peringkat metabolisme mRNA. Di antara empat sitokina yang digunakan, TNFα didapati paling berkesan di dalam merencat pengekspresan mRNA PPARγ. Ujian aktinomisin D menunjukkan bahawa paras ekspresi mRNA PPARγ dikawalatur pada peringkat kadar transkripsi gen, dan bukannya pada peringkat

kestabilan mRNA dalam sel J774.2 yang dirawat dengan TNFα. Penggunaan perencat-perencat spesifik terhadap laluan isyarat transduksi MAP kinas (PD98095, U0126, SB202190 dan SP600125) menunjukkan TNFα merencat paras mRNA PPARγ melalui laluan p42 ERK dan p46/54 JNK, yang kemudian mengaktifkan dan merangsang pengikatan c-Jun dan ATF2 ke elemen rangsangan cAMP (CRE) pada promoter mPPARγ1. Oleh itu, kajian ini menyediakan pandangan baru untuk laluan berpotensi yang mungkin terlibat di dalam pengawalaturan pengekspresan PPARγ oleh TNFα di dalam sel turunan makrofaj J774.2, dan mencadangkan satu sasaran berpotensi untuk halangan terapeutik terhadap aterosklerosis.

DIFFERENTIAL REGULATION OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA (PPARγ) BY CYTOKINES IN MURINE MACROPHAGE J774.2 CELL LINE: ELUCIDATION OF SIGNAL TRANSDUCTION PATHWAYS OF TUMOUR NECROSIS FACTOR ALPHA (TNFα) IN REGULATING MACROPHAGE PPARγ GENE EXPRESSION

ABSTRACT

Atherosclerosis is the leading cause of death in developed countries. The role of the PPARγ in cytokine-activated macrophages is of crucial importance in the pathogenesis of atherosclerosis. However, the precise molecular mechanisms by which cytokines regulate PPARγ gene expression are poorly understood. In the present study, we evaluated the effects of four cytokines i.e. TNFα, IFNγ, IL-1α and IL-1β on the expression of PPARγ mRNA, protein and DNA binding activity in the murine macrophage J774.2 cell line, the widely used model for atherosclerosis. It was demonstrated that TNFα and IFNγ inhibited the PPARγ mRNA and protein expressions as well as DNA binding activity. By contrast, IL-1β induced a marginal increase at the levels of PPARγ mRNA, protein content and DNA binding activity. IL-1α, however, had no significant effects on the PPARγ gene expression and DNA binding activity. Since the changes observed in the PPARγ protein content and DNA binding activity in cytokine-treated macrophages followed closely the corresponding changes in PPARγ mRNA expression, the results strongly suggest that the PPARγ expression and binding activity were mainly regulated at the levels of mRNA metabolism. Amongst four cytokines used, TNFα was found to produce the most significant inhibition of PPARγ mRNA expression. Actinomycin D experiment showed that the level of PPARγ mRNA expression was mainly regulated at the level of rate of gene transcription and not at the level of mRNA

stability in TNFα-treated J774.2 cells. The use of specific inhibitors against MAP kinase signal transduction pathways (PD98095, U0126, SB202190 and SP600125) demonstrated that TNFα inhibited the mRNA levels of PPARγ via p42 ERK and p46/54 JNKs pathways, which in turn, activated and induced the binding of c-Jun and ATF2 to cAMP-responsive elements (CRE) in mPPARγ1 promoter. Thus, this study provides novel insights into the potential pathways that may be responsible for the molecular regulation of macrophage PPARγ gene expression by TNFα in macrophage J774.2 cell line, and suggests a potential target for therapeutic intervention against atherosclerosis.

CHAPTER 1

INTRODUCTION

1.1 Background

Atherosclerosis is the leading cause of death in the United States and the cause of more than half of all mortality in the developed countries. It is a long-term chronic disease characterized by the accumulation of lipids and fibrous connective tissue in the large arteries, accompanied by a local inflammatory response (Lusis, 2000). As the cholesterol plaque, or lesions, build up in the arteries over time, the risk for disease increases. Atherosclerotic coronary heart disease is the underlying cause for most heart attacks, and one of the most common causes for congestive heart failure, cardiac arrhythmias and sudden death (Lusis, 2000).

Epidemiological studies have revealed several genetic and environmental risk factors predisposing to atherosclerosis. Smoking, metabolic disorders clustering with insulin resistance, such as dyslipidemia, hypertension, diabetes, high cholesterol, and family history of heart disease, are particularly important risk factors. Predisposing symptoms of the disease include high blood pressure and elevated cholesterol, especially elevated LDL-cholesterol.

Research conducted during the past decade has led to an understanding of a relationship between the role of nuclear receptor peroxisome proliferator activated receptor γ (PPARγ) in macrophage and the biological basis for arthrosclerosis (Tontonoz *et al.*, 1998; Marx, 1998b; Chinetti, 1998; Ricote, 1999). For instance, PPARγ, upon activation, has been demonstrated to promote monocyte differentiation to macrophage and increase the uptake of oxidized LDL by macrophages to be transformed into foam cells (Tontonoz *et al.*, 1998). It has also been shown to be highly

expressed in macrophage-derived foam cells and atherosclerotic plaque (Marx, 1998b). By contrast, PPARγ has also been demonstrated to have an anti-atherogenic effect. For example, it was reported that PPARγ is a potent negative regulator in the development of atherosclerosis (Ricote, 1999) and has the ability to induce apoptosis of human monocyte-derived macrophages (Chinetti, 1998).

1.2 Peroxisome proliferators activated receptors (PPARs)

Peroxisome proliferators activated receptors (PPARs) are a family of transcription factors that belong to the superfamily of nuclear receptors. The PPAR family consists of three distinct subtypes, termed α (NR1C1), β/δ (NR1C2) and γ (NR1C3), all of which display tissue-specific expression patterns reflecting their biological functions (Pineda-Torra *et al.*, 2001).

All three PPAR isoforms possess similar structural and functional features. Principally, four functional domains have been identified, 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 targeted genes.

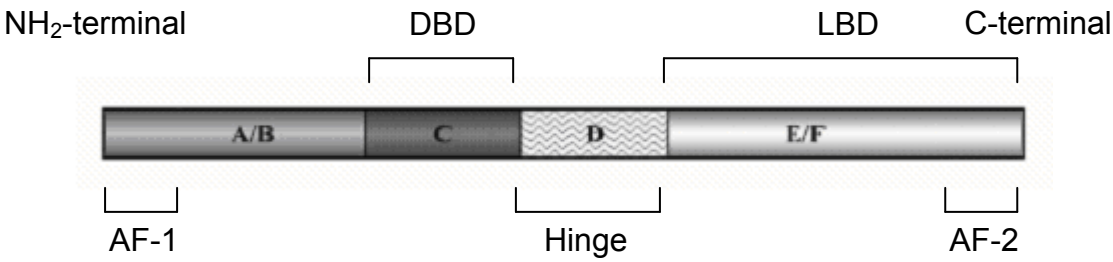

Figure 1.1 Schematic representation of the structural domains of PPAR. PPAR consists of four distinct functional domains. The A/B domain locates at the N-terminal with AF-1 is responsible for phosphorylation, the domain C is implicated in DNA binding, domain D is the docking region for cofactors and domain E/F is the ligand binding domain, containing AF-2, which promotes the recruitment of cofactors required for gene transcription.

Recruitment of PPAR co-factors to assist the gene transcription processes is carried out by the ligand-dependent activation function 2 (AF-2), which is located in the E/F domain (Berger & Moller, 2002).

Like other members of the nuclear receptor gene family, the PPAR subtypes possess a common domain structure which contains DNA-binding domains (DBD) and ligand-binding domains (LBD). Amino acid sequence comparison of DBD amongst PPAR subtypes shows they are highly conserved indicating that they share similar DNA binding site presence on the promoter region of the target genes, while the LBD have a slightly lower level of conservation across the subtypes (Figure 1.2) suggesting that they are ligand-specific. The NH_2-terminal domain of the subtypes shows low sequence identity which is responsible for differences in the biological function of the subtypes (Castillo *et al*., 1999).

1.3 Peroxisome proliferator-activated receptor γ (PPARγ)

PPARγ was first identified as a component of an adipocyte differentiation-dependent regulatory factor (ARF6) that binds to the well-characterized, fat cell-specific enhancer of the adipocyte fatty acid-binding protein (aP2) gene (Tontonoz *et al*., 1994a; Tontonoz *et al*., 1994b).

PPARγ, like the other PPARs, is an obligate heterodimer with another member of the nuclear receptor subfamily, the retinoic X receptors (RXR), the receptor for 9-*cis*-retinoic acid. Upon heterodimerization with RXR, PPARγ binds to peroxisome proliferator response element (PPRE) which in turn regulates downstream target genes (Figure 1.3) (Kliewer *et al*., 1992).

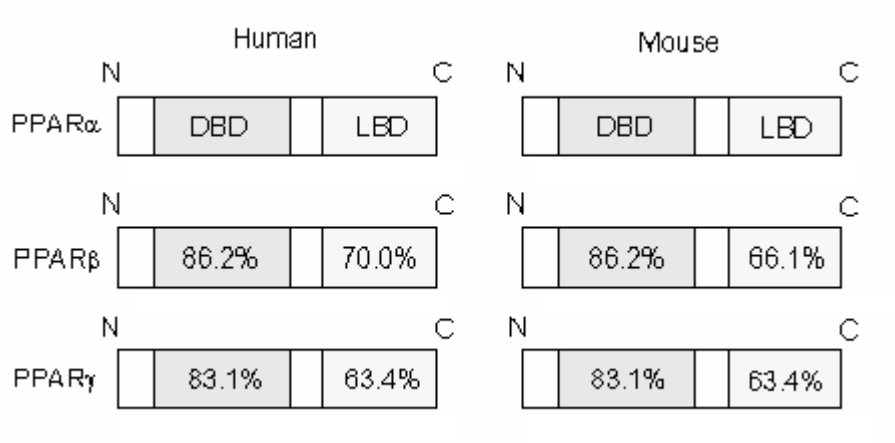

Figure 1.2 Comparison of amino acid identities of the DBD and LBD of human and mouse PPAR isoforms. Amino acid sequences are represented by open bars and numbers in the bars show the percentage of amino acid identity between human and mouse isoforms relative to PPARα. N, N-terminus; DBD, DNA-binding domain; LBD, ligand-bindind domain and C, C-terminus (Murphy & Holder, 2000).

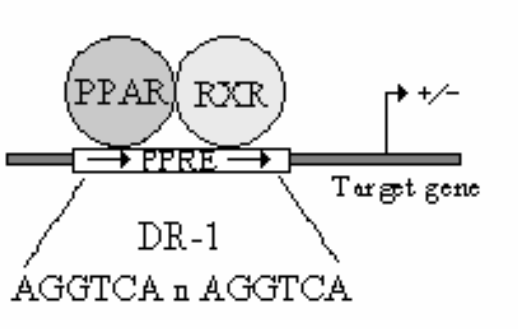

Figure 1.3 Gene transcription mechanisms of PPARγ. PPAR/RXR heterodimer binds to a PPRE in the regulatory regions of target genes, thereby governing the expression of the downstream target genes.

Structurally, PPRE consists of direct repeat of the nuclear receptor hexameric DNA core recognition motif AGGTCA separated by one nucleotide, known as DR-1 response elements (Lemberger *et al*., 1996; Juge-Aubry *et al*., 1997).

1.3.1 The structural organization of PPARγ gene

PPARγ has been cloned from a number of species, including mouse (Zhu *et al*., 1993; Kliewer *et al*., 1994), hamster (Aperlo *et al*., 1995), cattle (Sundvold *et al*., 1997), pig (Houseknecht *et al*., 1998) and human (Greene *et al*., 1995; Elbrecht *et al*., 1996).

The PPARγ gene, which has 9 exons (Figure 1.4) and extends over more than 100kb of genomic DNA for human (Fajas *et al*., 1997) and 105kb for mouse (Zhu *et al*., 1995), is mapped to chromosome 6 E3-F1 by *in situ* hybridization (Zhu *et al*., 1995).

In contrast to human, in which four PPARγ mRNA isoforms have been identified so far, i.e., PPARγ1, γ2 (Fajas *et al*., 1997), γ3 (Fajas *et al*., 1998) and γ4 (Sunvold & Lien, 2001), in mouse, only two PPARγ mRNA isoforms have been detected, termed PPARγ1 and γ2 (Zhu *et al*., 1995). The two mRNA isoforms of PPARγ arise as products of different promoter usage and alternative splicing from a single PPARγ gene, which differ only at their 5' ends (Figure 1.4).

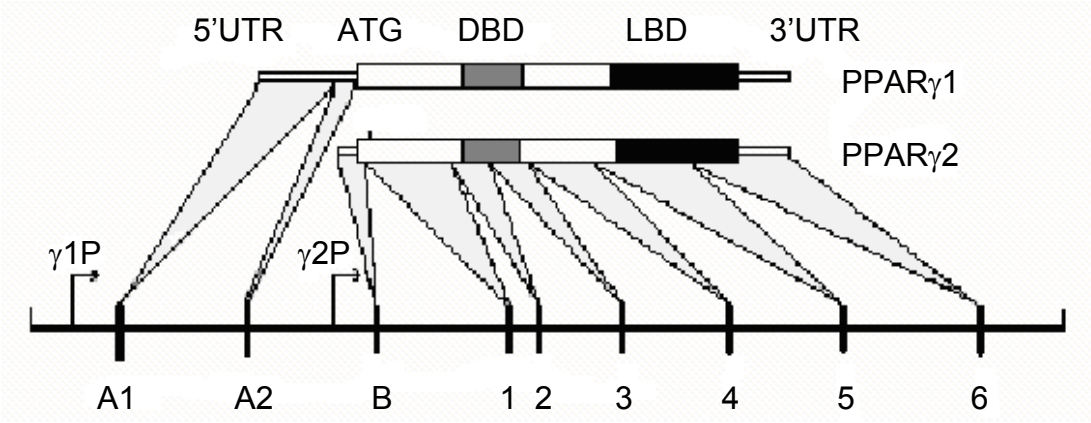

Figure 1.4 Structural organization of mPPARγ gene. The eight exons (A1, A2, and 1-6) encoding the mPPARγ1 and the seven exons (B and 1-6) encoding the mPPARγ2 are shown in the genomic DNA. γ1P and γ2P represent the promoter of mPPARγ1 and mPPARγ2, respectively.

The PPARγ1 is encoded by 8 exons whereas PPARγ2 is encoded by 7 exons (Figure 1.4). Consistent with the production of two PPARγ mRNAs, there are two PPARγ promoters, each with a specific and distinctive expression pattern (Zhu *et al*., 1995). The two PPARγ transcripts differ in their 5'end. PPARγ1 mRNA codes for one protein, while PPARγ2 codes for a different protein containing 28 additional amino acids at the N-terminus to the start codon of PPARγ1 for human (Sundvold *et al*., 1997) and 30 additional amino acids for mouse (Zhu *et al*., 1995).

In PPARγ1, the two most upstream exons A1 and A2 comprise the 5' untranslated region (UTR) and are spliced to the six most 3' proximal exons (Kliewer *et al*., 1992) which encompass the common coding region shared by the two isoforms. The 5' untranslated region (UTR) of PPARγ2 plus the additional 30 N-terminal amino acids specific to PPARγ2 are encoded by exon B, located between exon A2 and exon 1 (Zhu *et al*., 1995).

Thus, exons A1 and A2 are spliced with exon 1 to 6 to give rise to PPARγ1 mRNA. PPARγ2 mRNA is generated by splicing of exon B to exon 1 to 6. Each of the two zinc fingers of the DNA-binding domains of mPPARγ is encoded by a separate exon (exon 2 and 3, respectively). The ligand-binding domain is encoded by two exons which are exons 5 and 6.

1.3.2 Tissue distribution and expression patterns of PPARγ

PPARγ mRNA is expressed in a tissue-specific manner. A comparison of the tissue-distribution of PPARγ transcripts among different species shows PPARγ mRNAs are specifically expressed at high levels in mammalian adipose tissue, large intestine and hematopoietic cells (Tontonoz *et al*., 1994b) and variable, but generally at lower levels in other tissues like kidney, liver and small intestine (Aperlo *et al*., 1995). Interestingly, PPARγ is barely detectable in muscle (Fajas *et al*., 1997; Auboeuf *et al*., 1997).

Analysis of the tissue distribution of the two PPARγ isoforms revealed that PPARγ1 shows rather ubiquitous distribution, whereas PPARγ2 had a more restricted distribution. PPARγ2 is much less abundant in all tissues analyzed compared to PPARγ1, the predominant PPARγ isoform. The only tissue expressing significant amounts of PPARγ2 is adipose tissue, where its mRNA makes up about 20% of total PPARγ mRNA (Fajas *et al*., 1997; Auboeuf *et al*., 1997).

Previous research showed that the expression of PPARγ2 mRNA is markedly increased very early during adipocyte differentiation (Chawla *et al*., 1994; Tontonoz *et al*., 1994b; Tontonoz *et al*., 1994c). Early induction of PPARγ2 expression during adipocyte differentiation and its adipose tissue selectivity suggesting its pivotal role in the regulation of adipocyte differentiation.

In addition to the role in adipocyte differentiation, PPARγ has also been shown to play a pivotal role in monocytes differentiation. It was reported that PPARγ is expressed in cells of the monocyte/macrophage lineage (Tontonoz *et al*., 1998; Greene *et al*., 1995; Ricote *et al*., 1998b; Jiang *et al*., 1998; Chinetti *et al*., 1998; Marx *et al*., 1998b) suggesting that PPARγ is involved in the development of monocyte along the macrophage lineage, in particular in the conversion of monocytes to foam cell in the development of atherosclerosis (Tontonoz *et al*., 1998).

PPARγ is also found expressed in several carcinomas, suggesting a role in the differentiation of cancer cell lines and in cell cycle regulation (Tontonoz *et al*., 1997; Altiok *et al*., 1997; Kubota *et al*., 1998; Mueller *et al*., 1998; DuBois *et al*., 1998).

1.3.3 Natural and synthetic ligands of PPARγ

PPARγ is a ligand-activated transcription factor. The binding of ligands to the receptor greatly increases its transcriptional activity. The ligand binding domain (LBD) of PPARγ consist of 13 α helices and a small four-stranded β sheet forming a large Y-shaped hydrophobic pocket (Figure 1.5). This pocket represents the ligand binding cavity and has a volume of approximately 1300 $Å^3$, which is about twice that of the other nuclear receptors (Wagner *et al*., 1995).

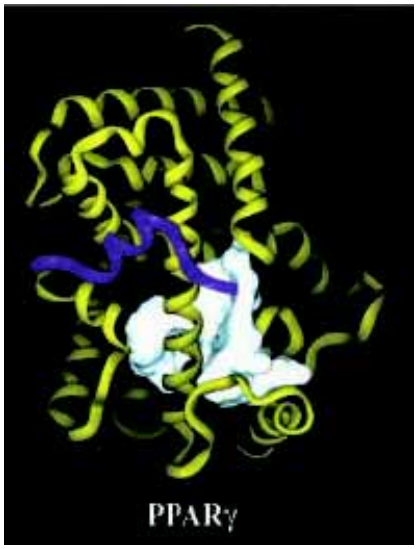

Figure 1.5 Three-dimensional structure of ligand binding domains of PPARγ. An X-ray crystal structure of PPARγ (yellow ribbon) is shown. PPARγ is shown associated to LXXLL peptides (blue ribbons), the signature motif of the receptor coactivators. The solvent-accessible ligand binding pocket is displayed as an off-white surface (from Xu *et al*., 2001).

The PPARγ ligands occupy ~30 –40% of the pocket, in contrast to the thyroid hormone receptor, where the ligand fills around 90% of the pocket (Wagner *et al*., 1995). Besides its large size, another characteristic feature of the PPARγ ligand binding pocket is that its bottom portion is sealed by helix 2', which is absent in other nuclear receptors. This particular helix may increase the size of the pocket, and possibly participates in an entry channel for the ligand.

The structural alignment of the ligand binding cavities of PPARγ showed that the ligand selectivity depends on the identity of a single amino acid histidine in helix 5. This selectivity seems to be conserved between different ligand classes and corresponds to an intrinsic property of the receptors (Xu *et al*., 2001). The characteristics of the PPARγ LBD give insight into the propensity of PPARγ to interact with a variety of natural and synthetic compounds (Xu *et al*., 1999; Nolte *et al*., 1998).

A broad spectrum of synthetic and naturally occurring substances can serve as PPARγ ligands, including pharmacological molecules, as well as fatty acids and fatty acid-derived products. PPARγ is bound and activated by naturally occurring arachidonic acid metabolites derived from cycloxygenase and lipoxygenase pathways, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-dPGJ_2), 12-hydroxyeicosatetraenoic acid (12-HETE) and 15-hydroxyeicosa-tetraenoic acid (15-HETE) (Forman *et al*., 1995; Kliewer *et al*., 1995; Nagy *et al*., 1998; Huang *et al*., 1999) (Figure 1.6).

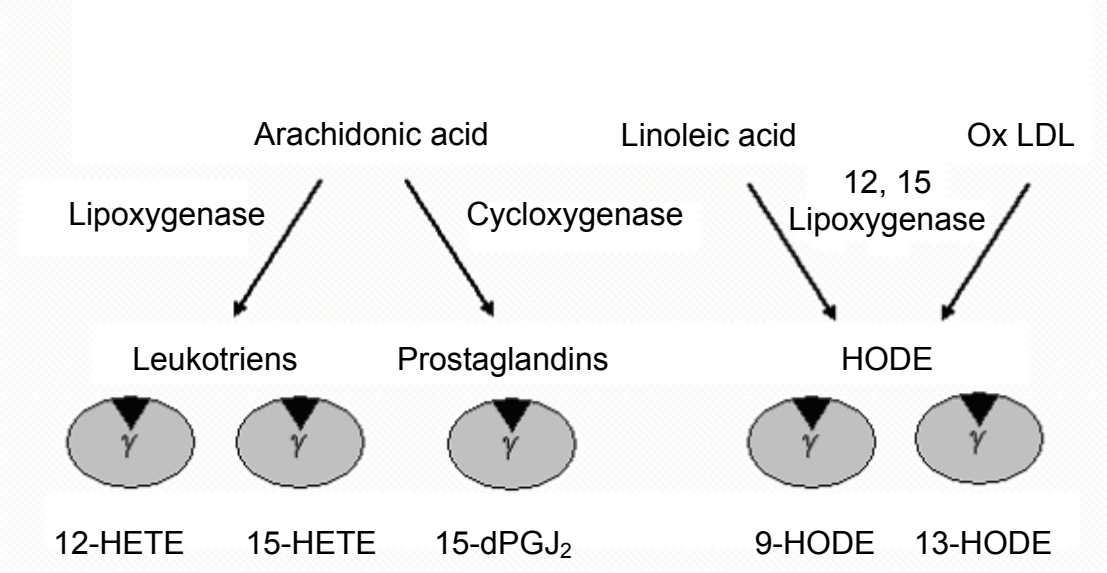

Figure 1.6 Natural ligands of PPARγ. PPARγ is activated by natural activators derived from fatty acids through the cycloxygenase and lipoxygenase pathways such as 15-dPGJ$_2$, 12-HETE, 15-HETE, 9-HODE and 13-HODE.

In addition, other eicosanoids and unsaturated fatty acids are also reported to bind and activated PPARγ. This has been shown for the ω-3 polyunsaturated fatty acids, α-linolenic acid, eicosapentaenoic acid and docohexanoic acid (Krey *et al*., 1997; Kliewer *et al*., 1997). It was also shown that two eicosanoids present in oxidized low density lipoproteins (oxLDL) i.e. 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) are potent endogenous PPARγ ligands (Nagy *et al*., 1998) (Figures 1.6 and 1.7).

The synthetic compounds, thiazolidinediones (TZDs) or "glitazones" which include troglitazone, pioglitazone and rosiglitazone (Figure 1.8) are the first compounds reported as high-affinity PPARγ agonists (Lehmann *et al*., 1995). TZDs are currently being used for the treatment of insulin resistance and type II diabetes mellitus. TZD treatment results in a concomitant fall in glucose and insulin levels, through its insulin-enhancing action (Schwartz *et al*., 1998).

Non-TZDs such as isoxazolidinedione JTT-501 (Shibata *et al*., 1999) and the tyrosine-based GW-7845 (Figure 1.8) have PPARγ activation properties with significant anti-diabetic and anti-carcinogenic activities in rodents (Cobb *et al*., 1998; Suh *et al*., 1999).

Certain non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin and ibuprofen, had been shown to bind and activate PPARγ at high micromolar concentrations (Lehmann *et al*., 1997). Several other NSAIDs, including fenoprofen and flufenamic acid, were also shown to be weak PPARγ agonists (Lehmann *et al*., 1997).

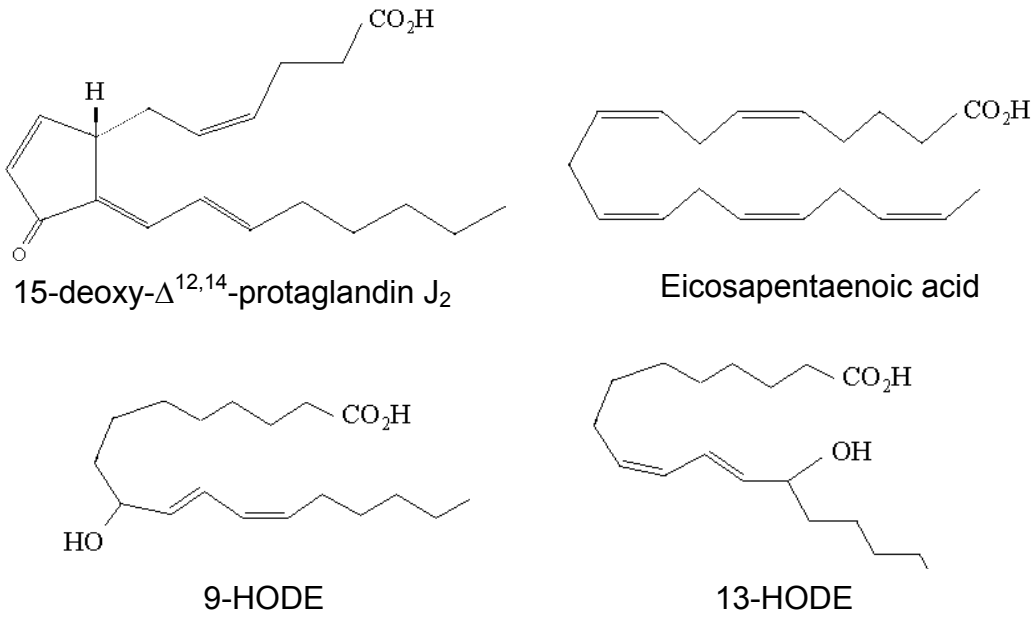

Figure 1.7 Structure of natural ligands of PPARγ. 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-dPGJ_2), eicosapentaenoic acid (EPA), 9-hydroxy-octadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) are potent PPARγ ligands.

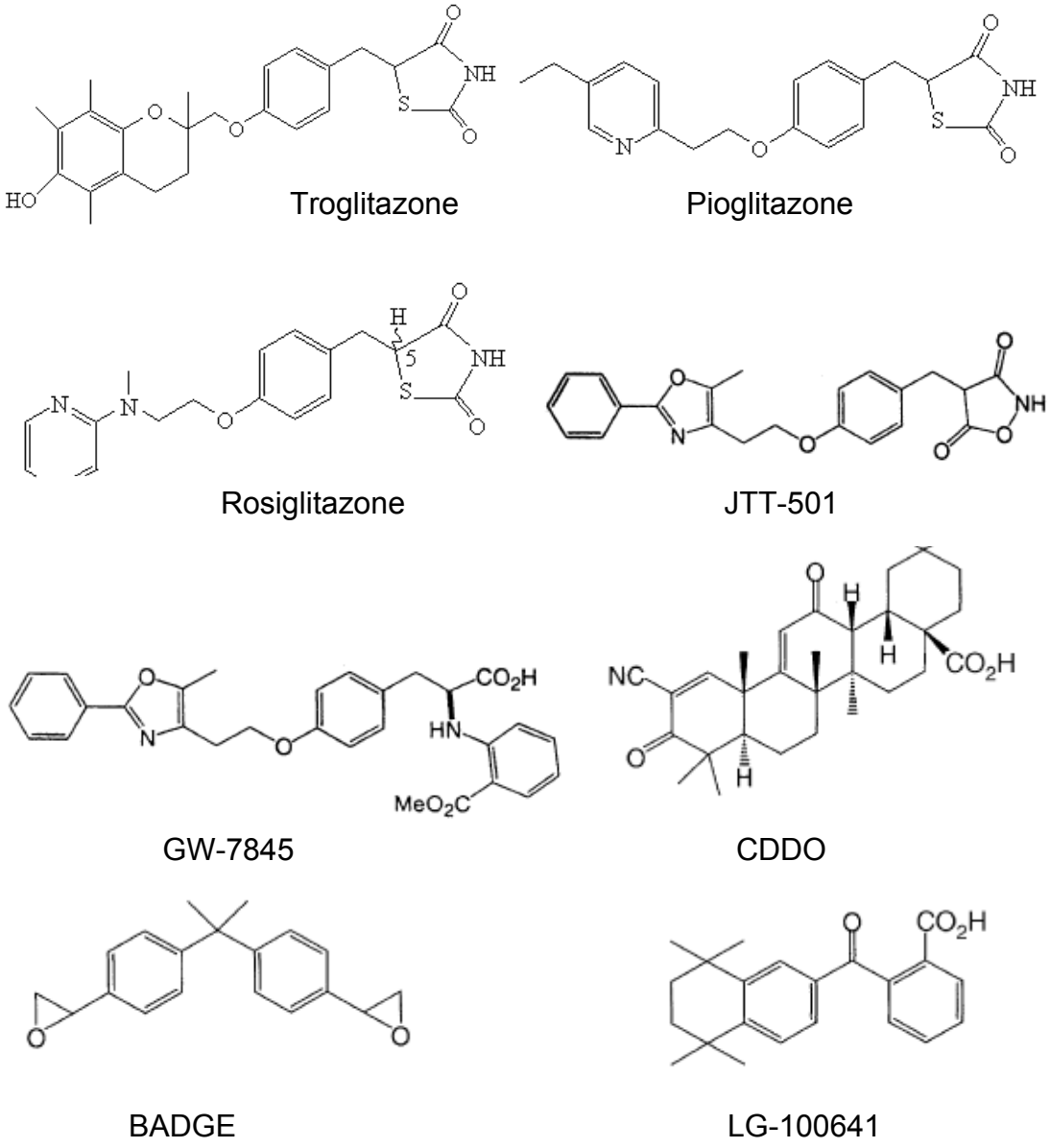

Figure 1.8 Structure of synthetic agonists and antagonists of PPARγ. Troglitazone, pioglitazone, rosiglitazone, JTT-501, GW-7845 and CDDO are agonists of PPARγ; BADGE and LG-100641 are antagonists of PPARγ.

Novel PPARγ partial agonists and antagonists have been recently identified. Triterpenoid 2-cyano-3, 12-dioxooleana-1,9-dien-28-oic acid (CDDO) (Figure 1.8) is a partial agonist with anti-inflammatory properties (Wang *et al*., 2000). Bisphenol diglycidyl ether (BADGE) and LG-100641 (Figure 1.8) are recently identified PPARγ antagonists (Wright *et al*., 2000; Mukherjee *et al*., 2000). Although these compounds have less clinical significance, they may be useful in understanding PPARγ physiology and the identification of new ligands.

In addition to synthetic chemical methods, research in natural products has also yielded potent PPARγ agonists from several medicinal plants. Saurufuran A from *Saururus chinensis* (*Saururaceae*) (Hwang *et al*., 2002), flavonoids such as chrysin, apigenin and kampferol (Liang *et al*., 2001) and phenolic compounds from *Glycyrrhiza uralensis* (*Fabaceae)* (Kuroda *et al*., 2003) are recently identified PPARγ agonists.

1.3.4 Cofactors for the PPARγ

Cofactors have been shown to play an important part in the transcriptional control of PPARγ. They act as coactivators or corepressors that mediate the ability of PPARγ to initiate or suppress the transcription process. They interact with nuclear receptors in a ligand-dependent manner (Lemberger et al., 1996).

Initially, it was thought that the cofactors simply bridge PPARγ with the basic transcriptional machinery. However, it has become clear that these cofactors also carried several enzymatic activities, suggesting that they could control gene expression by specifically modifying chromatin and DNA structure (Glass *et al*., 1997; Pazin & Kadonaga, 1997; Moras & Gronemeyer, 1998). It is suggested that in the absence of any ligand, PPARγ may bind to corepressors which extinguish its transcriptional activity by the recruitment of histone deacetylases. Histone hypoacetylation is associated with condensed nucleosomes and thereby transcriptionally silent (Glass *et al*., 1997; Pazin & Kadonaga, 1997; Moras & Gronemeyer, 1998).

Ligand binding induces a conformational change in the receptor that results in the dissociation of corepressors and removal of histone deacetylases from DNA with subsequent recruitment of coactivator complexes that contain proteins with histone acetyltransferase activity. Acetylation is associated with changes of nucleosome conformation which modulates accessibility of promoter regions and facilitates transcription, thereby increases the transcription of target gene (Glass *et al*., 1997; Pazin & Kadonaga, 1997; Moras & Gronemeyer, 1998) (Figure 1.9).

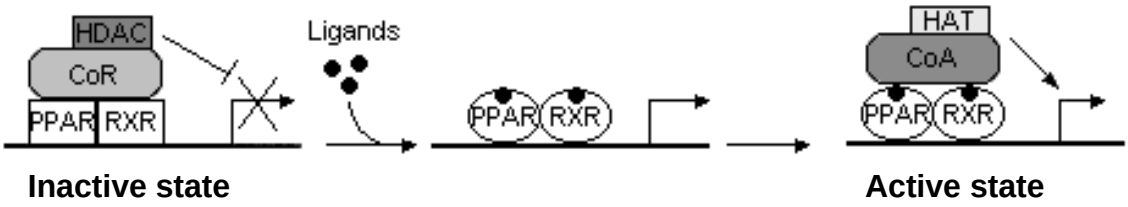

Figure 1.9 Transcriptional activation of nuclear receptors. Transcriptional activation of nuclear receptors requires, in general, the release of corepressor (CoR) complexes, which contain histone deacetylase activity (HDAC), and the recruitment of coactivators (CoA), which target histone acetyl transferases (HAT) to the promoter. The differential docking of cofactors is facilitated by structural changes brought about by ligand-binding or receptor phosphorylation.

Some of these cofactors include members of two families of histone acetylases, i. e. CBP/p300 and steroid receptor coactivator (SRC)-1, as well as PPAR binding protein (PBP), PPAR gamma coactivator (PGC)-1 and silencing mediator for retinoid and thyroid hormone receptors (SMRT).

CBP and p300 were originally identified as CREB (cAMP-responsive binding protein) and E1 A interacting factors (Chrivia *et al*., 1993; Eckner *et al*., 1994; Janknecht & Hunter, 1996a; Janknecht & Hunter, 1996b). CBP/p300 are widely expressed (Misiti *et al*., 1998) and coactivate 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*., 1997; Kraus & Kadonaga, 1998). CBP/p300 interacts with PPARγ through multiple domains in each protein (Gelman *et al*., 1999). Most notably, the NH_2-terminal region of PPARγ can dimerize with CBP/p300 in the absence of ligand and this association enhances its constitutive AF-1 transcriptional activity (Gelman *et al*., 1999). The constitutive presence of CBP/p300 could enhance the basal ligand-independent transcriptional activity of PPARγ *in vivo* and could thereby explain the high level of basal activity of PPARγ.

1.4 Atherosclerosis

Atherosclerosis is a complex vascular disease initiated by accumulation and oxidation of plasma low-density lipoprotein (LDL) in the sub-endothelial space of the vessels. The development of atherosclerosis, however, is a complex long term process which involves recruitment and activation of different cell types, including monocytes/macrophages, endothelial cells, smooth muscle cells and T-lymphocytes in the intima of the arteries, thus leading to a local inflammatory response (Ross, 1999).

The trapped monocytes differentiate into macrophages that take up oxidized low-density lipoproteins (OxLDL) through scavenger receptors (SR), thus forming foam cells. Activated smooth muscle cells (SMC) proliferate and migrate from the media thus leading to neo-intima formation. Activation of these cells leads to the release of pro-inflammatory cytokines, which combined with the secretion of metalloproteases and expression of pro-coagulant factors, results in chronic inflammation and plaque instability. This can further evolve to plaque rupture and acute occlusion by thrombosis, resulting in myocardial infarction and stroke (Figure 1.10) [Ross, 1993; Ross, 1995; Ross, 1999; Lusis, 2000].

PPARγ has been reported to play an important role in the development of atherosclerosis. Interestingly, there are contradicting reports on the role of PPARγ in atherogenesis having demonstrated to produce pro-atherogenic effects in some contexts but anti-atherogenic effects in others.

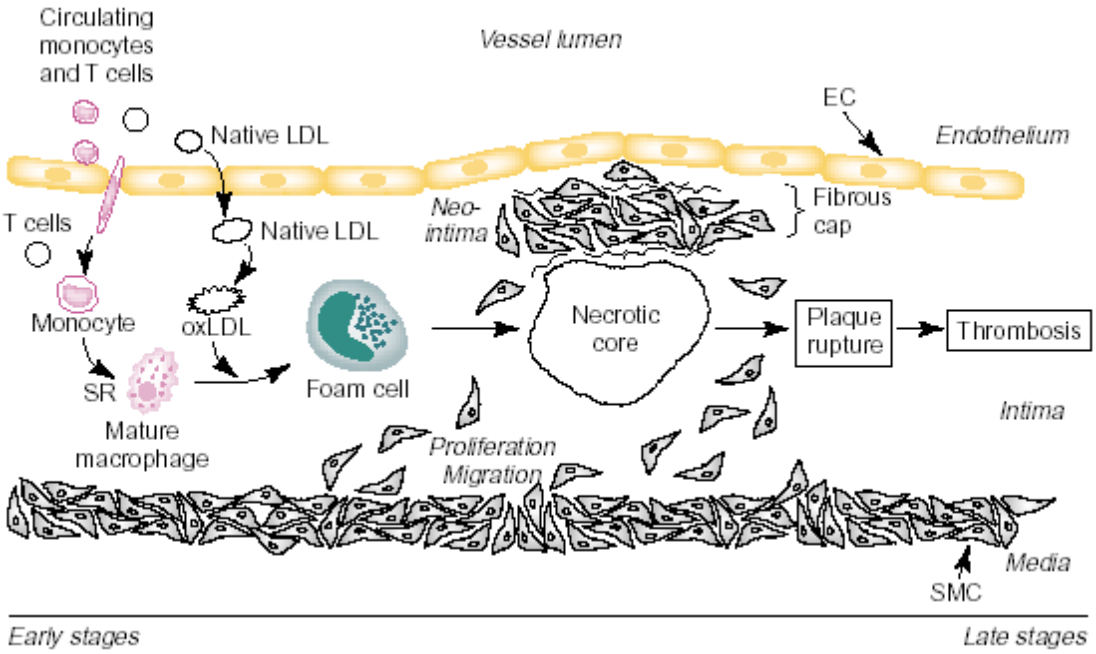

Figure 1.10 The atherosclerosis process. (from Lusis, 2000).