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Effect of a peroxisome proliferator activator receptor (PPARalpha and PPARgamma) dual agonist in Ob/Ob, LDLR-/-Mice

Delattre, Anne-Isabelle

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EFFECT OF A PEROXISOME PROLIFERATOR ACTIVATOR RECEPTOR (PPARalpha AND PPARgamma) DUAL AGONIST IN Ob/Ob, LDLR-/- MICE.

Mémoire présenté pour l'obtention du grade de

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Anne-Isabelle DELATTRE
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Effect of a Peroxisome Proliferator Activator Receptor (PPARalpha and PPARgamma) dual agonist in Ob/Ob, LDLR-/- mice.

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Summary

Cardiovascular diseases (CVD) remain the leading cause of mortality in the Western societies. Several risk factors predispose to CVD including the metabolic syndrome components diabetes, obesity, dyslipidemia and hypertension. It has been demonstrated that nuclear peroxisome proliferator activated receptors (PPAR) down regulation (mainly obesity-related), is a key feature of metabolic syndrome initiation. The identification of PPARγ and PPARα as being the primary targets for the insulin-sensitizing thiazolidinediones and the lipid lowering fibrates, respectively, has provided opportunities for the identification of novel compounds for the treatment of the metabolic syndrome.

In mice with combined leptin and LDL-receptor deficiency (DKO mice) the metabolic syndrome components are associated with increased oxidative stress, accelerated atherosclerosis and impaired cardiovascular function. Compared to lean mice, PPARα and PPARγ expression was downregulated in these DKO mice.

In this thesis, we further investigated the role of PPARs in metabolism, atherosclerosis, and cardiovascular function in this DKO mouse model, by analyzing the effect of a dual PPARα/γ agonist, [(S)-3-(4-(2-carbazol-9-yl-ethoxy) phenyl-2-ethoxy propionic acid)] developed by Novo Nordisk. This work is a part from the PhD project research of Wim Verreth. Physiological parameters (glucose, cholesterol, lipid, insulin, and adiponectin plasma concentration) were measured at different times during the treatment with the PPAR dual agonist and compared to control mice (without treatment) and placebo mice (treated with a solution without agonist). After 12 weeks of treatment, echocardiographies were performed to analyze heart function. Finally, all mice were sacrificed and gene expression was undertaken in adipose tissue to explain the observed physiological changes. The PPARα/γ dual agonist improved insulin sensitivity and thus restored glucose tolerance in one week. After 12 weeks of treatment, the heart function of PPAR-treated mice was better than the heart function of placebo mice, although no significant decrease in triglycerides, cholesterol or atherosclerosis could be evidenced.

Effet d'un double agoniste de Peroxisome Proliferator Activator Receptor (PPARalpha and PPARgamma) sur des souris Ob/Ob, LDLR-/-.

DELATTRE Anne-Isabelle

Résumé

Les maladies cardiovasculaires sont l'une des principales causes de mortalité dans les sociétés occidentales. De nombreux facteurs prédisposent à ces maladies. Le syndrome métabolique associe le diabète de type 2, l'obésité, la dyslipidémie et l'hypertension. Des études ont démontré que la répression des récepteurs nucléaires de type PPAR, souvent présente en cas d'obésité, est l'une des phases clés dans l'initiation du syndrome métabolique. L'identification de PPARγ comme cible primaire pour les thiazolidinediones, qui améliorent la sensibilité à l'insuline, et de PPARα pour les fibrates, qui réduisent les concentrations sanguines en lipides, a mis en évidence de nouvelles possibilités de traitement pour le syndrome métabolique. Les souris présentant à la fois une déficience combinée en leptine et en récepteur aux LDL (souris DKO), souffrent du syndrome métabolique, associé à une augmentation du stress oxydatif, de l'athérosclérose et ont une fonction cardiaque déficiente. PPARα et PPARγ sont réprimés chez ces souris.
Dans ce mémoire, nous approfondirons le rôle des PPARs dans le métabolisme, le développement de l'athérosclérose ainsi que les fonctions cardiovasculaires dans ce modèle de souris DKO. Pour cela, nous analyserons sur celles-ci l'effet d'un double agoniste de PPARα/γ, [(S)-3-(4-(2-carbazol-9-yl-ethoxy) phenyl-2-ethoxy propionic acid)], développé par Novo Nordisk. Ce travail s'inscrit dans les recherches menées par Wim Verreth pour son doctorat. Nous avons mesuré les paramètres physiologiques (concentrations plasmatiques en glucose, cholestérol, triglycérides, insuline et adiponectine) chez des souris traitées avec le double agoniste, mais également de souris placébos et de souris contrôle. Après 12 semaines de traitement, nous avons réalisé sur chaque souris des échocardiographies, des coupes du cœur pour mesurer l'étendue des plaques d'athérosclérose et nous avons analysé l'expression génique dans le tissu adipeux pour expliquer les changements physiologiques. Le double agoniste améliore la sensibilité à l'insuline et restaure la tolérance au glucose en une semaine. Après 12 semaines, les fonctions cardiaques des souris traitées avec l'agoniste étaient meilleures que celles des autres souris (placébos et contrôles), sans qu'il n'y ait de diminution de cholestérol, de triglycérides ou des lésions atherosclérotiques.

Abbreviation list

ABC	ATP Binding Cassette
ACC	Acetyl-CoA Carboxylase
ACS	AcylCoA Synthetase
ADD1/SREBP1	Adipocyte Differenciation and Determination Factor 1
AF-2	Ligand dependant Activation Function 2
AP-1	Activator Protein 1
Apo CI	Apolipoprotein C 1
apoB	Apolipoprotein B
ATP	Adenosine Triphosphate
BMI	Body Mass Index
BSA	Bovine Serum Albumin
C/EBP CAAT	enhancer binding protein
CAAT	Part of a conserved sequence located about 75-bp upstream of the start point of eukaryotic transcription units which may be involved in RNA polymerase binding; consensus=GG(C|T)CAATCT.
cDNA	complementary DNA
CEHA	Centrum voor Experimentele Heelkunde en Anesthesiologie
CPT-I	Carnityl-Palmitoyl-Transferase I
CPT-II	Carnityl-Palmitoyl-Transferase II
Ct	Threshold Cycle
CVD	CardioVascular Disease
DBD	DNA Binding Domain
DKO	Double Knock-Out
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra-Acetate
EF	Ejection Fraction
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Responsive Kinase
ERK	Extracellular signal Related Kinase
FA	Fatty Acid
Fabp-4	Fatty Acid Binding Protein 4
FAO	Fatty Acid Oxidation
FAS	Fatty Acid Synthase
FAT	Fatty Acid Transporter
FATP	Fatty Acid Transport Protein
FC	Free Cholesterol
FFA	Free Fatty Acid
GLUT-4	Glucose Transporter 4
GSK-3	Glycogen Synthase Kinase-3
HDL	High Density Lipoprotein
HOMA	Homeostasis Model Assessment
HSL	Hormone Sensitive Lipase
Igfbp4	Insulin-like Growth Factor Binding Protein-4
IL-1	Interleukin 1
IL-6	Interleukin 6
IR	Insulin Receptor
IRS-1	Insulin Receptor Substrate 1
IVSTh	InterVentricular Septum Thickness

IRS-1	Insulin Receptor Substrate 1
IVSTh	InterVentricular Septum Thickness
LA	Alpha-Lipoic Acid
LBD	Ligand Binding Domain
LDL	Low Density Lipoprotein
LDL-R	Low-Density-Lipoprotein Receptor
Lpl	Lipoprotein Lipase
LV	Left Ventricle
LVDA	Left Ventricle Diastolic Area
LVEDD	Left Ventricle End Diastolic Area
LVESD	Left Ventricle End Systolic Area
LVmass	Left Ventricle mass
LVSA	Left Ventricle Systolic Area
LXR	Liver X Receptor
MAPK	Mitogen Activated Protein Kinase (MAP kinase)
MCP-1	Monocyte Chemoattractant Protein 1
mRNA	Messenger RNA
NEFA	Non-Esterified Fatty Acids
NFκB	Nuclear Factor kappa B
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDK4	Pyruvate Dehydrogenase Kinase 4
PFA	Polyunsaturated Fatty Acids
PI3-kinase	Phosphatidylinositol 3 Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
Pltp	Phospholipid Transfer Protein
PPAR	Peroxisome Proliferator Activated Receptor
PPRE	Peroxisome Proliferator activated receptor Response Element
PTP-1B	Protein Tyrosine Phosphatase-1B
PWTh	Posterior Wall Thickness
RNA	Ribonucleic Acid
RT-PCR	Real Time, Reverse Transcription, Polymerase Chain Reaction
RXR	9-cis-Retinoic Acid Receptor (retinoic X receptor)
SCD	Stearoyl-CoA Desaturase
SF	Shortening Fraction
SMC	Smooth Muscle Cells
SRBI	Scavenger Receptor Class B member 1
SREBP	Sterol Regulatory Element Binding Protein
TG	Triglyceride
TNF-α	Tumor Necrosis Factor alpha
TZDs	Thiazolidinediones
UCP1	Uncoupling Protein-1
VCAM-1	Vascular Cell adhesion Molecule-1
VLDL	Very Low Density Lipoprotein

Content table

1. Introduction

Figure 1.1: Cardiovascular risk factors
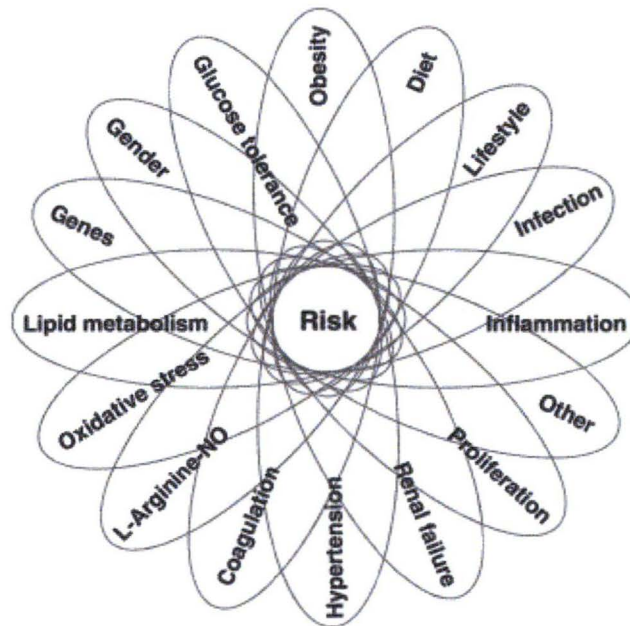
Components contributing to cardiovascular risk show large overlap, making it difficult to assess the separate contribution of these components to overall cardiovascular risk.

Source: Maas et al 2003.

1. Introduction

Ageing, the Western Diet and lack of physical activity contribute to major health problems: cardiovascular diseases, cancer ... The rate of obesity, diabetes, hypertension, and cardiovascular diseases increases particularly in the societies that are following the western life style (few sport, fat and sweet food) and these diseases occur at younger age. Obesity, diabetes and hypertension are components of the "metabolic syndrome" that is related to high disease risk.

In the following paragraphs, we will describe the cardiovascular diseases, their causes and their phenotypes. We will then explain what are the metabolic syndrome and its main mechanisms. We will at last discuss the role of the Proliferator Activated Receptors (PPARs) in the regulation of lipid and glucose metabolism, and thereby cardiovascular function. Finally, we discuss the results of the project. The aim of this study was to assess the effects of a new dual PPARα,γ agonist on atherosclerosis in mice.

1.1. Atherosclerosis and cardiovascular diseases

1.1.1. Cardiovascular diseases

1.1.1.1. Description

Cardiovascular disease (CVD) is a leading cause of mortality and is responsible for one-third of all global deaths [WHO, 2002]. It regroups many different vascular and cardiac diseases including atherosclerosis, peripheral vascular disease, vascular rupture, arrhythmic death and stroke. For review, see [Scott, 2004]

1.1.1.2. Risk factors

The causes of cardiovascular diseases are multiple. Figure 1.1 shows the main factors predisposing to cardiovascular problems, including obesity, hypertriglyceridemia, low high density lipoprotein cholesterol (HDL)-cholesterol levels, hypertension and insulin resistance, which are metabolic syndrome components (see part B). Cardiovascular problems increase with age, because of the accumulation of several risk factors. The life style also influences the development of CDV: smoking, fat food and lacks of sport are important risk factors [Maas and Boger, 2003]. Gender and race play also an important role. In North America, the prevalence of CVD in Korean Americans is higher than in white Americans. One other high-risk group is African American women; high prevalence of obesity and sedentary lifestyle are related to high CVD morbidity and mortality [Allen and Szanton, 2005]. Premenopausal women, compared to men, are relatively spared from coronary heart disease.

1.1.2. Atherosclerosis

1.1.2.1. Description

Atherosclerosis is a common vascular disease. It consists of an accumulation of lipid and fibrous connective tissue in the endothelial wall of arteries, combined with a local inflammatory process. [Lusis, 2000]

These modifications lead to a reduction of the luminal side making it difficult for blood to pass through and may result in a thrombus formation. Complete blockage in the heart and the

Figure 1.2: Structure of a normal vessel wall
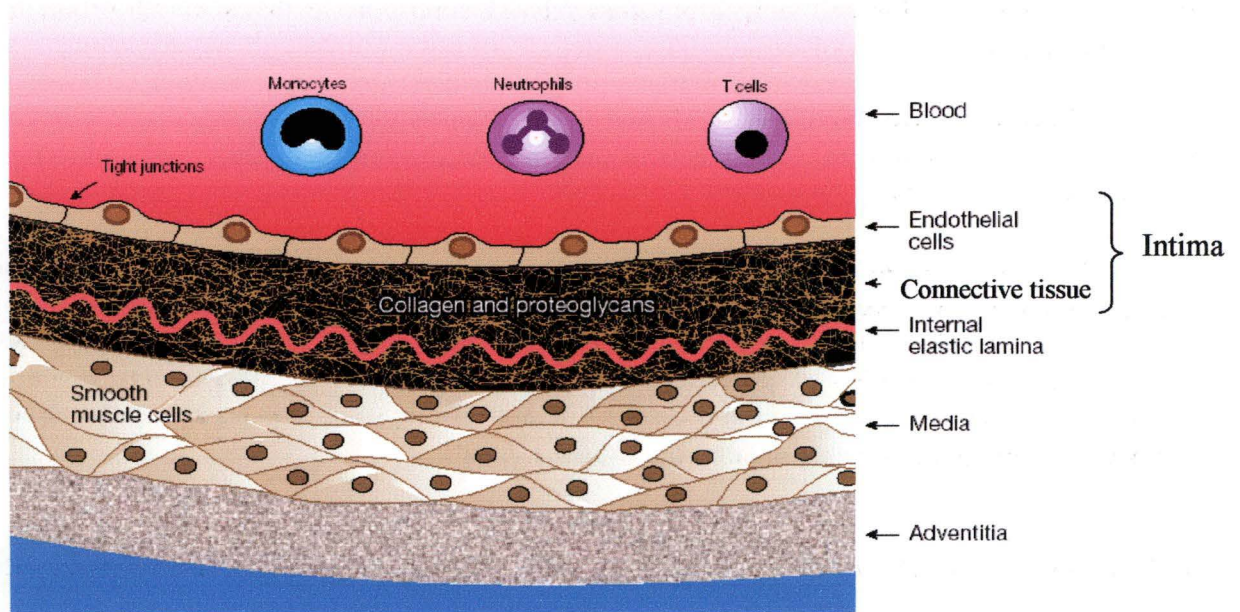
Figure 1.3: Development of atherosclerosis in the vessel wall
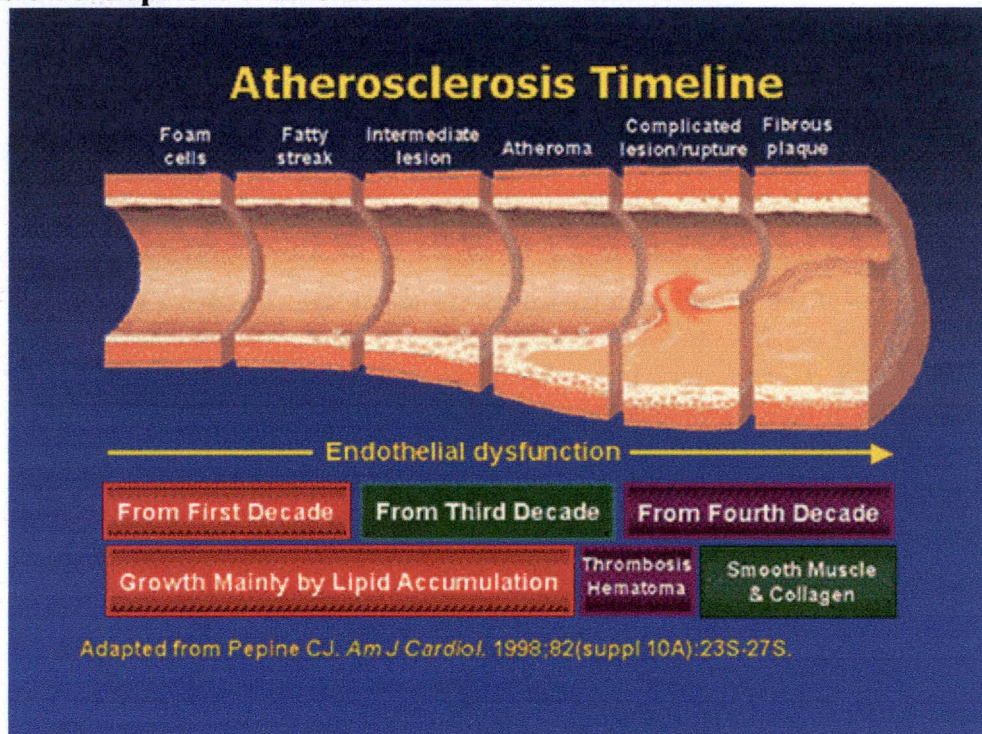
Atherosclerosis is a pathologic modification of the arterial wall, which leads to a reduction of the diameter of the vessel.

brain can lead to a heart attack or stroke, respectively. Development of atherosclerosis occurs preferentially in the carotid bifurcation, coronaries, and other large and medium-sized arteries, where there is shear stress. Inflammation, accumulation of lipid-loaded macrophages (foam cells), and a deregulation of the proliferation of smooth muscular cells (SMC) are the main known mechanisms of atherosclerosis.

1.1.2.2. Normal vessel structure

The arterial wall is composed of three tunicae, from the luminal side (figure 1.2): the tunica intima, the tunica media and the tunica adventitia [Lusis, 2000].

The **tunica intima** is in contact with the blood. The endothelium, a unicellular layer of endothelial cells connected by tight junctions, is a selective barrier for the blood elements and it has secretor functions. This layer is very thin: only 1 µm (thickness of the endothelial cells and of a limited connective layer). The integrity of the endothelium is essential to prevent disorders, e.g. low density lipoprotein (LDL)-cholesterol infiltration. It is separated from the tunica media by the internal elastic lamina, a thin plate of elastic tissue.

SMC and extracellular components (elastic fibres, collagen bundles, proteoglycans and other proteins) form the thick **tunica media**. Its role is to adapt the caliber of the artery to keep the lumen open: the smooth muscle cells are able to contract, and the elastic fibres allow the expansion. The SMC produce components of the extracellular matrix and they are able to migrate and to proliferate, to repair damaged vessels. The external elastic lamina separates the tunica media from the tunica adventitia.

The tunica adventitia is composed of fibroblasts, collagen and elastic bundles, nerves, lymphatic vessels and vasa vasorum (blood vessels). Less dense than the other tunica, it connects vessels to surrounding tissues. The vasa vasorum supply the media in oxygen en nutrients, and the nerves control the SMC.

1.1.2.3. Growth of the lesion

Atherosclerotic plaques are classified in fatty streaks, gelatinous plaques, fibrous plaques, and, finally, complex atheromatous plaques. The morphology and the evolution of atherosclerosclerotic plaques are illustrated in figure 1.3. The cellular mechanisms of the lesion formation, described in the following paragraphs, are illustrated in figure 1.4. For reviews, see [Rivard, 2000; Shah, 2005; Demuth, 1995; Reape, 1999; Geng, 2002; Witztum, 1991]

Fatty streaks
The first type of lesion, the fatty streak, is not pathologic and is reversible. There is an infiltration of LDL from the blood in the vessel wall, which causes a small accumulation of lipid and cholesterol in the endothelium. This lesion can already start to develop at young age.

Gelatinous plaques
This gelatinous plaques does not cause any clinical disease yet. LDL are oxidised, which lead to an inflammatory response, because oxidised LDL (oxLDL) attracts macrophages. There is an accumulation of macrophages in the intima and SMC migrate from the media towards the intima, leading to the neointima, which becomes thicker. Macrophages phagocyte the lipid and the cholesterol (oxLDL) and become foam cells. Foam cells secrete factors, such as

Figure 1.4
Cellular mechanisms of the growth of the atherosclerotic plaque
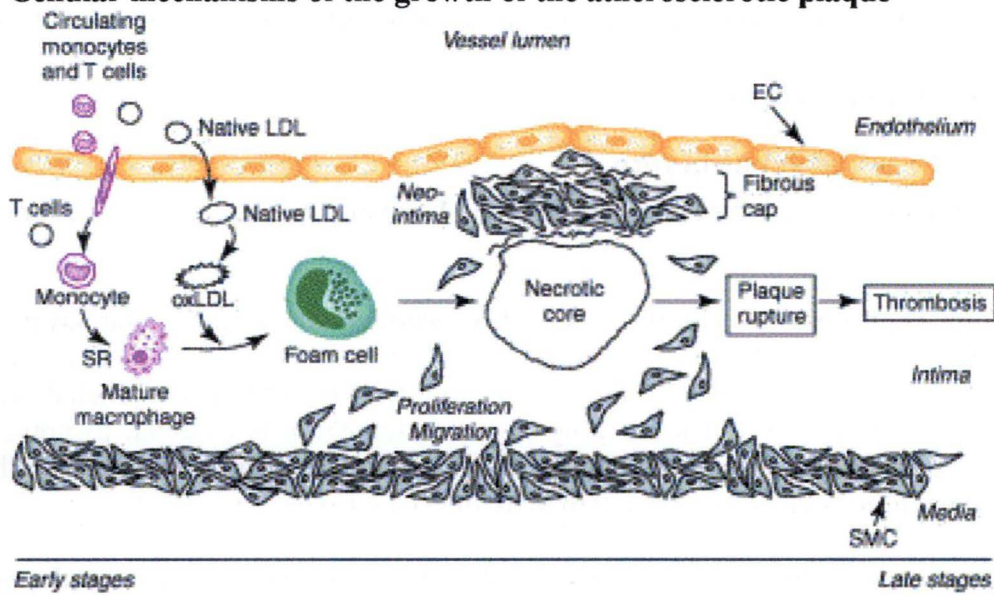
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Atherosclerosis is a complex vascular disease initiated by accumulation and oxidation of plasma low-density lipoprotein (LDL) in the sub-endothelial space of the vessels, followed by endothelial cell (EC) activation resulting in the recruitment of circulating monocytes and T cells. Trapped monocytes differentiate into macrophages that take up oxLDL thanks to their 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.

Source: Duval et al., 2002

Table 1.1
Values for human: risks factors for metabolic syndrome

Risk factors	Limit values for humans
Abdominal obesity (Waist circumference)	Men >120 cm Women > 88 cm
Hypertriglyceridemia	≥ 150 mg/dL
Low HDL-Cholesterol values	Men: < 40 mg/dL Women: < 50 mg/dL
Hypertension: high blood pressure	Systole: > 130 mm Hg Diastole: > 85 mm Hg
High fasting glucose	≥ 110 mg/dL

Source: National Institute of Health, 2001.

chemokines, that result in higher inflammation. The SMC have also the capacity to take up lipids, but less than the macrophages.

Fibrous plaques
The fibrous plaque represents the third phase. As the fatty streak progresses, SMC migrate from the media to the subendothelial space where they proliferate and produce connective tissue to form a fibrous cap. A necrotic core, under the fibrous cap, contains cholesterol, lipids and cell debris. The endothelium rises and the lumen becomes reduced.

Complex atheromatous plaques
The final lesion can become very complex, with calcification, ulceration at the luminal surface, and proliferation of the vasa vasora into the lesion.

1.1.2.4. Health problems

The atherosclerotic plaque can provoke major health problems. Advanced lesions reduce the diameter of the vessel and disturb the blood flow. As a result, the risk of infarct/stroke is higher. Moreover, if the fibrous plaque is calcified, it breaks down more easily resulting in thrombus formation. The third problem is caused by the vasa vasora: the vessels, which are growing into the plaque, can provoke haemorrhage when there is erosion of the fibrous caps.

1.1.2.5. Risk factors

Atherosclerosis is characterized by the same risk factors than the other cardiovascular diseases. The most important risk factors belong to the metabolic syndrome, described in the following paragraph. The main factors which predispose to atherosclerosis are physiological (hypertension, hyperhomocysteinemia, dyslipidemia, including hypercholesterolemia, diabetes), behavioural (smoking, sedentary life), and pathological (local inflammation, infection).

1.2. Metabolic syndrome

1.2.1. Characteristics

There are different risk factors predisposing to CVD. Five of these risk factors are clustered in the metabolic syndrome: obesity, hypertriglyceridemia, low HDL-cholesterol levels, and hypertension and insulin resistance. The cut-off values for these risk factors in humans (dependent on gender) are given in Table 1. The term metabolic syndrome is used when a person has at least three of these five risk factors. Many other factors, such as reduced physical exercise, bad nutrition, low socio-economic status, age and genetic predispositions also contribute to the metabolic syndrome [Forest et al, 2005]. Inflammation links the metabolic syndrome to CVD.

The metabolic syndrome is common in the Western societies (in more than 20 % of the total population of the USA [Park et al, 2003]). Extrapolation from the present epidemiological studies predicts that up to 40 % of the population could be affected by the metabolic syndrome in the year 2020 [Forest et al, 2005]. Population studies also strongly suggest the existence of a relationship between the metabolic abnormalities and the development of diabetes and cardiovascular disease.

Figure 1.5: Cardiovascular disease mortality increase in the metabolic syndrome
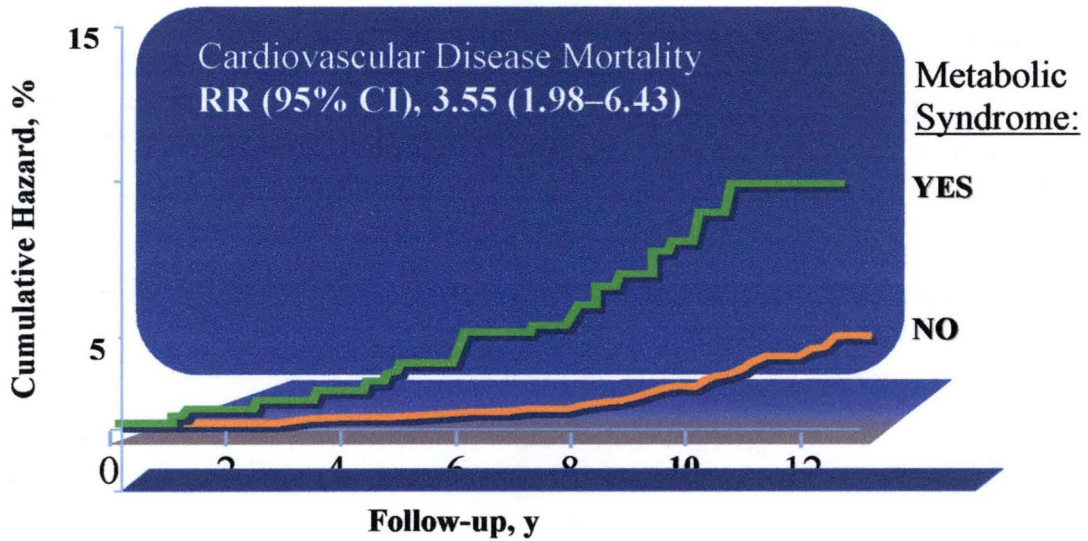
With metabolic syndrome, the mortality due to a cardiovascular problem is multiplied by about 3.5.

Source: Lakka et al. 2002(modified).

Figure 1.6: A working model illustrating how accumulated fat contributes to metabolic syndrome
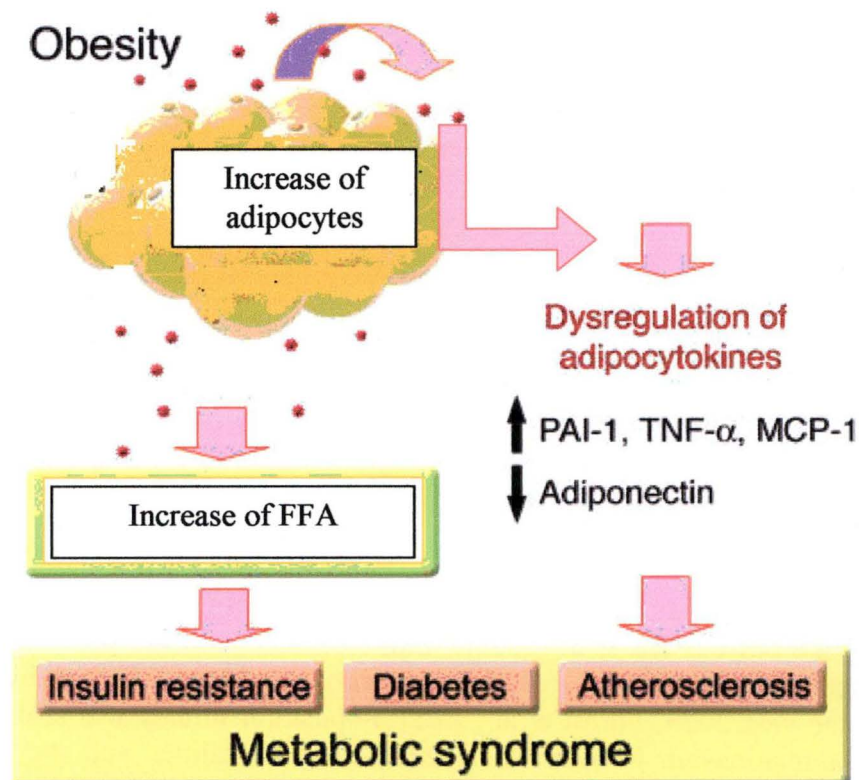
Source: Furukawa et al, 2004

Cardiovascular complications are the main cause of morbidity and mortality in patients with type 2 diabetes (about 75 %) [Gray et al, 1997]. In non-diabetic persons, the mortality as a result of CVD has decreased in the last decennia, but these numbers have increased in patients with type II diabetes [Greenberg, 2003].

Because the metabolic syndrome is often accompanied by type 2 diabetes that develops from insulin resistance, it is important to lower the risk factors, particularly the bad nutrition, in these patients. The metabolic syndrome increases cardiovascular mortality about 3.5-fold (figure 1.5).

It appears that lifestyle modifications can contribute to the prevention of progression to diabetes and the reduction of individual CVD risk factors. Whether use of insulin sensitising and lipid lowering drugs can significantly delay or prevent the progression to diabetes is under investigation. Because of its contribution to the growing prevalence of type 2 diabetes and the associated increased CVD risk, the recognition of the metabolic syndrome and its consequences are critical for morbidity and mortality risk assessment.

1.2.2. Metabolic syndrome components

1.2.2.1. Obesity

Obesity is an excessive accumulation of adipose tissue, which leads to overweight. Body mass index (BMI) is used to assess the weight status. BMI is defined as the weight in kilograms divided by the square of the height in metres (kg/m^2). A BMI over 25 kg/m^2 is defined as overweight and a BMI over 30 kg/m^2 as obesity. Globally, there are more than 1 billion overweight adults, and at least 300 million of them are obese [Haffner and Ashraf, 2000]. Human obesity in developed countries is spreading and appears in children, due to many factors, including bad nutrition and lack of sport. The genetic component of obesity is complex: multiple genes are involved and small differences in expression can increase or reduce the risk of obesity and environmental factors influence the development of obesity very much [Lazar, 2005]. A better paradigm for obesity is the waist-to-hip ratio because it takes into account the fat (visceral) distribution.

Obesity, particularly visceral adiposity, contributes to the clustering of the other metabolic syndrome components, such as insulin resistance, type 2 diabetes, dyslipidemia and hypertension. The metabolic abnormalities induced by visceral adiposity have still to be fully elucidated [Abate et al, 1995]. Figure 1.6 illustrates how fat accumulation can play an important role in the development of metabolic syndrome.

The adipose tissue is not only an energetic reserve; it also has endocrine functions mediated by adipokines [Miner, 2004]. Several adipokines, regulate some of the processes that contribute to the development of atherosclerosis and CVD, including dyslipidemia, arterial hypertension, endothelial dysfunction, insulin resistance, and vascular remodelling. Adipokines are preferentially expressed in intra-abdominal adipose tissue, and the secretion of pro-inflammatory adipokines is elevated with increasing adiposity. TNFα, leptin, resistin and adiponectin are four major adipokines.

Adiponectin is an adipokine secreted exclusively by adipose tissue and it is abundantly present in human plasma. It is lower in obese and type 2 diabetes patients and increases with

Figure 1.7: Glucose homeostasis and role of insulin
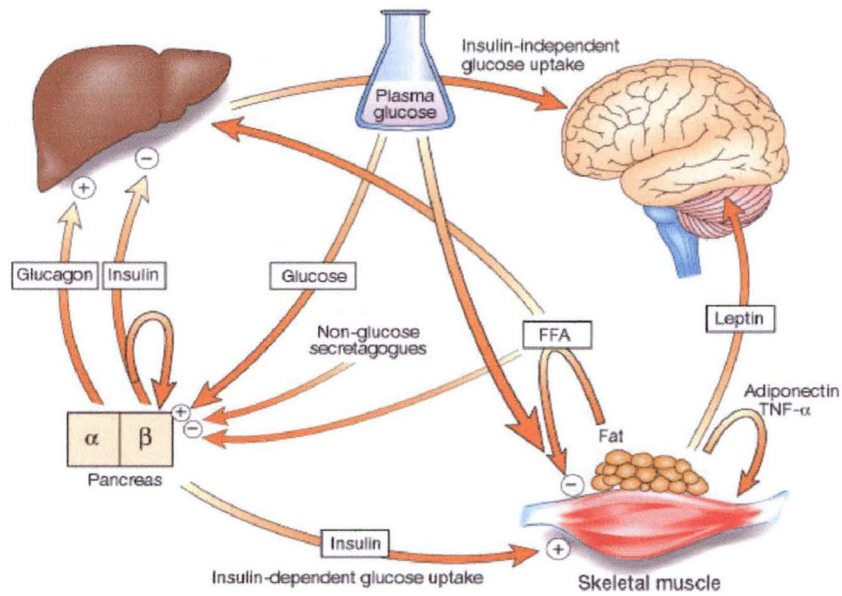
Insulin is secreted from the β-cells of the endocrine pancreas in response to elevations in plasma glucose. The hormone decreases glucose production from the liver, and increases glucose uptake, utilization and storage in fat and muscle. The fat cell is important in metabolic regulation, releasing FFAs that reduce glucose uptake in muscle, insulin secretion from the β-cell, and increase glucose production from the liver. The fat cell can also secrete adipokines such as leptin, adiponectin and TNFα, which regulate food intake, energy expenditure and insulin sensitivity.

Source: Alan., 2001

weight reduction. A negative correlation between circulating adiponectin levels and body mass index and insulin resistance has been demonstrated [Gil-Campos, 2004; Bouskil, 2005].

Leptin is secreted by the fat cells and act on the hypothalamic leptin receptors. Leptin signals the nutritional status to the brain and to peripheral organs. The effects of leptin are multiple: regulation of food intake, activation of immune and reproductive systems [Sader et al, 2003].

Resistin has been shown to antagonize insulin action. It has three postulated physiological roles: a mediator of regulation of metabolism, a mediator of adipogenesis and a relationship to inflammation [Steppan, 2004]. Resistin gene expression is markedly downregulated by treatment with anti-diabetic drugs, e.g. the thiazolidinediones (see part C) [Steppan, 2001].

Tumour necrosis factor-α (TNFα) is a cytokine with multiple biological functions that, in mammals, has been shown to modulate muscle and adipose tissue metabolism. TNF-α down regulates the LPL activity of adipose tissue [Albalat et al, 2005].

Approaches that stimulate adipocyte differentiation or influence gene expression in the adipose tissue could positively alter adipokine levels, thereby reducing the severity of their resulting pathologies.

With an increased prevalence of obesity in developed countries, associated chronic diseases rise in a parallel way. It causes secondary morbidity, as diverse as type 2 diabetes, dyslipidemia, hypertension and cardio-vascular diseases.

1.2.2.2. Insulin resistance

a) Insulin

[For review, see Knutson, 1991]. Insulin is a peptide hormone, produced by pancreatic β-cells as the primary biosynthetic product, preproinsulin, containing 109 amino acid residues. This peptide is rapidly converted to proinsulin (86 amino acid residues) by cleavage of the amino terminal 23 amino acid "pre"sequence. Within the β-cell secretory granules, proinsulin is converted by proteolytic cleavage to insulin (51 amino acids) and C peptide (31 amino acids). The regulation of insulin release is extremely complex, being affected by glucose, amino acids, intestinal hormones, glucagon, neural influences, and other factors. However, glucose is the most important stimulus and, via a feedback effect, a major inhibitor of insulin secretion.

Insulin exerts a dominant effect on the regulation of glucose homeostasis. These glucoregulatory effects are predominantly exerted in three tissues: liver, muscle and adipose tissue. In the liver, insulin inhibits the production of glucose by inhibiting gluconeogenesis and glycogenolysis and promotes glycogen storage. In muscle, insulin stimulates the uptake, storage, and use of glucose. In adipose tissue, insulin facilitates the uptake of glucose and its conversion to fatty acids, and it inhibits the catabolism of fatty acids. Figure 1.7 summarizes the actions of insulin in the body and the mechanisms of glucose homeostasis.

Insulin diverse actions are initiated by interaction with a specific transmembrane protein receptor. The insulin receptor is composed of two subunits, α and β. The extracellular α subunit confers high-affinity insulin binding. The transmembrane component of the β subunit is responsible for transducing the signal of insulin binding to the interior of the cell. After insulin binding, specific tyrosine residues of the intracellular region of the β subunit are

Figure 1.8: Signal transduction in insulin action
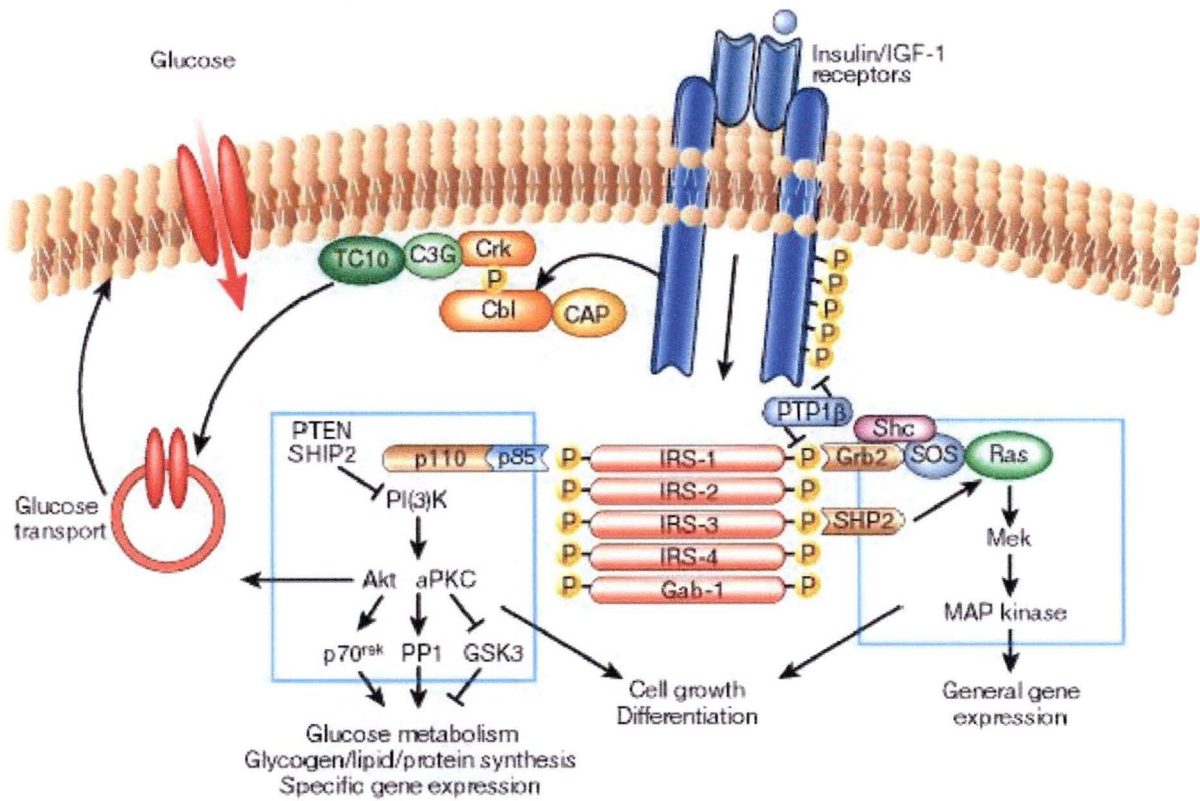
The insulin receptor is a tyrosine kinase that undergoes autophosphorylation, and catalyses the phosphorylation of cellular proteins such as members of the IRS family. Upon tyrosine phosphorylation, these proteins interact with signalling molecules through their SH2 domains, resulting in a diverse series of signalling pathways. These pathways result in the regulation of glucose, lipid and protein metabolism.

Source: Internet site of the Medical College of Wisconsin

Figure 1.9: Model linking obesity to the pathogenesis of insulin resistance and type 2 diabetes
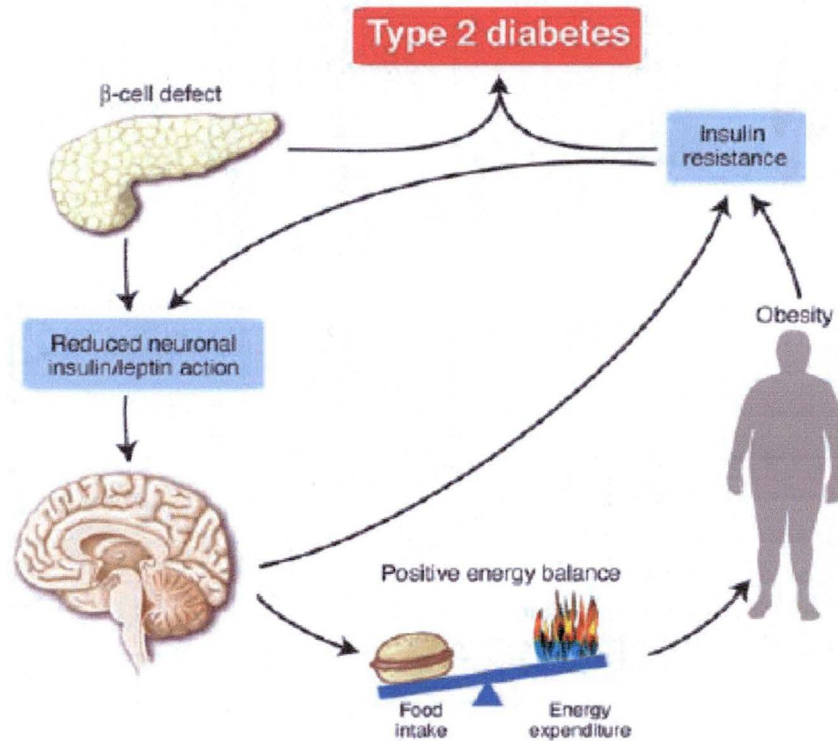
Reduced neuronal input from adiposity or nutrient-related signals favours both positive energy balance and hepatic insulin resistance. As weight gain progresses to obesity, worsening insulin resistance increases the demand for insulin secretion. When combined with a β-cell defect (which reduces insulin action in the brain and periphery), type 2 diabetes ensues.

Source: Schwartz, 2005.

rapidly phosphorylated, and the tyrosine kinase intrinsic to this region is activated. The main pathways activated by the insulin fixation are described in the figure 1.8.

b) Insulin resistance

Insulin resistance is defined as a condition in which the body fails to respond normally to insulin. Himsworth, who observed that elderly or obese diabetic subjects were relatively insensitive to the hypoglycaemic effect of insulin, first described the phenomenon of insulin resistance in 1936. He proposed that patients with diabetes should be divided into two categories, insulin-sensitive and insulin-insensitive. Later, the concept of insulin resistance was connected to the reduced response of insulin in the regulation of glucose homeostasis in muscle, adipose tissue and liver. Studies in the 1960's and 1970's showed the resistance to insulin-stimulated glucose uptake to be a characteristic feature in patients with non-insulin-dependent diabetes mellitus and impaired glucose tolerance. Deterioration of glucose tolerance can only be prevented if the pancreatic β -cell is able to increase its insulin secretory response and maintain a state of chronic hyperinsulinemia. If hyperinsulinemia cannot be maintained, the plasma free fatty acid (FFA) concentration will not be reduced normally, and the resulting increase in plasma FFA production will lead to increased hepatic glucose production. In insulin-resistant individuals, even small increases in hepatic glucose production are likely to lead to significant fasting hyperglycemia under these conditions.

In insulin resistance, the defect of the insulin signalling pathway may occur at the level of the receptor, or of the intracellular signalling pathway. The receptor could have a normal binding affinity for insulin without the autophosphorylation needed for the signal transmission in the cell. Restoring insulin levels do not lead to the normal signalling in the cell [Le Roith and Zick, 2001].

Increase in FFA plays an important role in insulin resistance by inhibiting muscle glucose transport and oxidation via effects on serine/threonine phosphorylation of IRS-1 (insulin receptor substrate-1) [Zick, 2004].

c) Consequences of insulin resistance

I) Insulin resistance and type 2 diabetes

Type 2 diabetes starts from insulin resistance coupled with the inability to produce enough insulin to overcome this resistant state. The pancreatic β-cells increase their insulin production but this production is too high and this may lead to the death of the cells.

The ethiology of type 2 diabetes mellitus is multifactorial and probably genetically based, but it also has strong behavioural components: sport and healthy food prevent the development of diabetes. A familial predisposition to diabetes, excess weight or ingestion of diabetogenic substances, the morphology of skeletal muscle and disturbances in sex steroid hormones have been proposed to be involved in the development of insulin resistance. [O'Rahilly et al, 2005]. Age and dyslipidemia are also factors, which increase the risk of diabetes. It appears often with the age or with obesity. It is the most common form of diabetes. [Lazar, 2005]. Figure 1.9 illustrates links between β-cell defect, obesity, insulin resistance and type 2 diabetes. .

II) Insulin resistance and lipid metabolism

The link between insulin resistance and lipid metabolism is the focus of an abundant literature over the last years. We will try to summurize these data. For reviews, see [Tomkin et al, 2002; Taskinen et al, 1995; Felbert et al, 1993; Bjorntorp, 1994; Despres and Marette, 1994]

Figure 1.10: Mechanisms relating insulin resistance and dyslipidemia
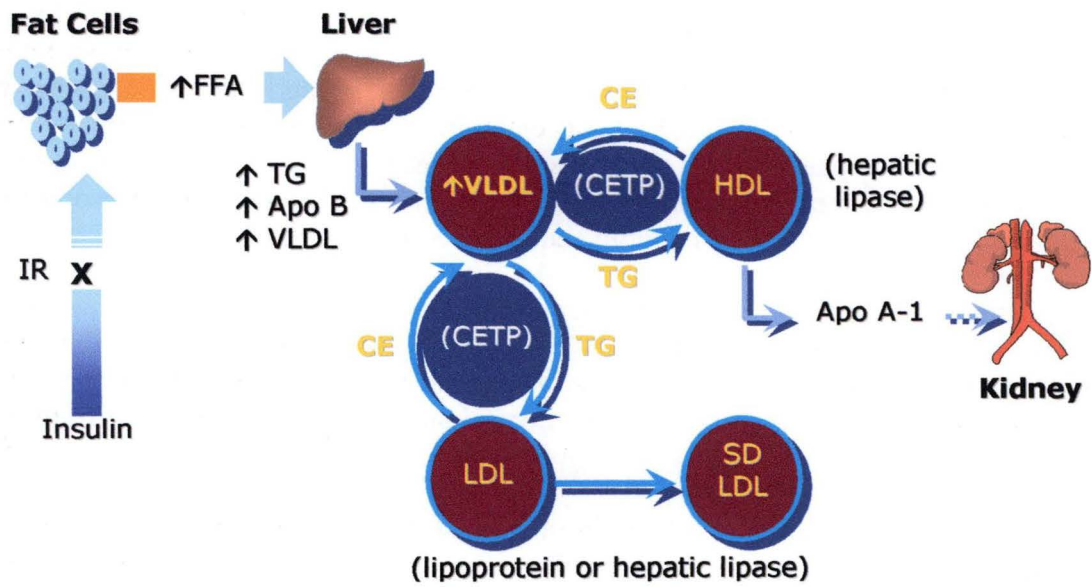
Source: www.lipidonline.orgLipid

The basic components of dyslipidemia in insulin resistance include elevation of triglycerides and lowering of HDL cholesterol [Figure 1.10]. Additional elements have been associated with dyslipidemia, including small, dense LDL, postprandial lipidemia and abnormalities of the fibrinolytic system. All these metabolic disturbances are potentially atherogenic and, because of the mixed interplay and close correlations between the components, this complex network may help to explain the controversy surrounding hypertriglyceridemia as an independent CVD risk factor.

Insulin may play a multifactorial role in the regulation of postprandial lipid metabolism. Normally, it rapidly suppresses the release of nonesterified fatty acids (NEFA) from adipose depots, both by inhibition of hormone-sensitive lipase (HSL) and by stimulation of re-esterification. The reduced delivery of NEFA to the liver, coupled with the direct suppressive effect of insulin, leads to postprandial diminution of hepatic very low density lipoprotein (VLDL)-triglycerides (TG) secretion. At the same time, insulin activates or stimulates the activity of peripheral (particularly adipose tissue) lipoprotein lipase (LPL), aiding the removal of TG-rich lipoproteins from the circulation. The action of LPL on TG-rich lipoproteins is coupled with the transfer of excess surface material, including cholesterol, to HDL. These actions of insulin all have their own characteristic time courses and dose-response relationships.

Among the factors believed to play a central role in causing insulin resistance, elevated free fatty acids (FFA) levels have received considerable attention. It is unlikely that insulin resistance precedes elevated FFA levels, as normal adipose tissue would be sensitive to the antilipolytic effect of insulin. In insulin-resistant conditions, such as abdominal obesity, the inhibiting effect of insulin on the mobilization of FFA from adipose tissue is impaired. Possible mechanisms include the reduced effect of insulin on the inhibition of intracellular hormone-sensitive lipase and the reduced effect on the increase in the re-esterification of fatty acids. An enlarged but yet lipolytically active abdominal, visceral fat depot increases the portal FFA flux and reduces hepatic insulin binding and extraction, thereby causing systemic hyperinsulinemia. The increased delivery of FFA to the liver, coupled with the impaired suppressive effect of insulin, leads to a postprandial increase of hepatic VLDL-TG secretion. In insulin resistance, the elevations in TG concentrations reflect mainly the increased number of the larger, more TG-rich VLDL particles. Decreased LPL activity leads to delayed hydrolysis of VLDL particles, which in turn leads to an increase in VLDL remnants.

The overall effect is likely to be an impairment of the removal of TG-rich lipoproteins and hence an increase in the residence of these lipoproteins in the plasma, with an opportunity for neutral lipid exchange with HDL. Neutral lipid exchange between large, TG-rich VLDL and LDL may also occur, leading ultimately to the formation of small, dense LDL particles.
High levels of portal FFA have been suggested to enhance hepatic glucose production through elevated gluconeogenesis, which significantly contributes to the establishment of hyperglycaemia. At the level of skeletal muscle, high FFA concentrations can reduce insulin-stimulated glucose utilization through substrate competition (the glucose-fatty acid cycle). Both glucose oxidation and storage are inhibited by FFA.

III) Insulin resistance and hypertension

Hypertension is the most prevalent CVD, affecting at least 600 million people and is an important contributor to cardiovascular mortality and morbidity. Elevated heart rate is associated with a greater risk of developing hypertension, atherosclerosis, and cardiovascular events [Palatini and Julius, 1999].

Insulin resistance and hyperinsulinemia may lead to an increase in blood pressure by several mechanisms. First, increases in plasma catecholamine concentrations are associated with increases in plasma insulin concentrations, independent of any change in the plasma glucose. Insulin stimulates the sympathetic nervous system, and high doses of insulin increase cardiac output and heart rate. Numerous studies indicate that feeding promotes insulin production and stimulates sympathetic activity. Insulin causes a dose-related increase in plasma catecholamine concentrations in humans, closely related to increases in pulse rate and blood pressure in insulin clamp test. Second, there is evidence that insulin promotes renal tubular sodium resorption and water reabsorption. Euglycemic insulin clamp tests have shown that increases in circulating insulin inhibit natriuresis, which may lead to volume expansion and elevated blood pressure. Third, insulin stimulates the sodium/hydrogen pump in vascular smooth muscles, resulting in intracellular accumulation of sodium, which has been linked to the enhancement of the presser effect of noradrenaline and angiotensin II. Fourth, insulin is a vascular growth factor by interaction with either its own receptors or those of insulin-like growth factor 1.

Denker and Pollock (1992)[Denker and Pollock, 1992] performed a meta-analysis to determine the relationship between fasting insulin levels and blood pressure in normoglycemic subjects. They found a significant correlation between the fasting insulin concentration and both systolic and diastolic blood pressure, supporting the role of hyperinsulinemia in the pathogenesis of essential hypertension.

IV) Insulin resistance/diabetes and cardiovascular diseases

Insulin resistance is now receiving increasing attention not only as a precursor of type 2 diabetes but also as a predictor of increased risk of CVD. [Haffner and Ashraf, 2000]. Fat distributed in the abdominal region is a risk factor for type 2 diabetes and CVD and is associated closely with insulin resistance [Abate et al, 1995]. Although hyperinsulinemia may prevent direct decompensation of glucose homeostasis, high plasma insulin levels predict the future development of coronary heart disease risk factors, such as type 2 diabetes, hypertension, increased plasma triglyceride concentration and decreased HDL concentration. [Haffner and Ashraf, 2000]. Furthermore, high plasma insulin levels have been associated with an increased risk of ischemic heart disease in several prospective population studies [Casassus et al, 1992; Folsom et al, 1997; Pyorala et al, 1985; Welborn and Wearne, 1979]. With the insulin resistance, the vasculature is exposed to high concentrations of circulating free fatty acids, glucose, and insulin. This leads to chronic vascular inflammation (demonstrated by increased concentration of TNFα, interleukin 1 (IL-1) and IL-6) [Plutzky, 2001] that induces the atherosclerotic process through various mechanisms.

It has been shown that thiazolidinediones, PPARγ agonists, improve insulin sensitivity. It is thus an interesting way to decrease also the other risk factors, linked to the insulin resistance. In the following paragraphes, we will describe these peroxisome proliferators activator receptors, and their pharmacological activities.

8

Table 1.2: Repartition of the PPAR isoforms in an adult rat (simplified table):

	PPARα	PPARβ,δ	PPARγ
Central nervous system	+/-	+ +	-
Kidney	+ + + (proximal tubules)	+ + + (in all the kidney	-
Liver	+ + + +	+ +	-
Pancreas	+	+ +	+
Heart	++	+ +	-
White adipose tissue	+	+	+ + +
Duodenum	+ + + +	+ + +	+ +

- = absent/barely detectable
+ => + + + + = waxing quantity

Figure 1.11: General structure of PPARs

a) Gene

Ligand-independent activation domain (AF-1)

Ligand-dependent activation domain (AF-2)

DBD

LBD-Dim.

DNA Binding Domain (2 zinc fingers)

Ligand Binding and Dimerization Domains

b) Protein

Transcription activation (AF-1)

Dimerization
Ligand binding
Ligand-dependent transcription activation (AF-2)

E/F

A/B

D Dimerization

C

DNA binding
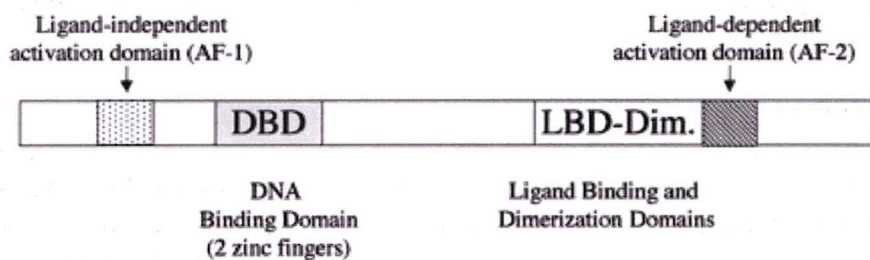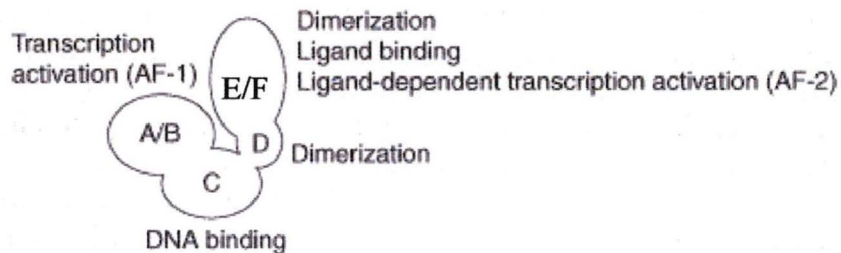
1.3. PPARs in the metabolic syndrome

1.3.1. Introduction

It has been demonstrated that nuclear peroxisome proliferator activated receptors (PPAR) deactivation (mainly obesity-related) is a key phase of metabolic syndrome initiation [Evans et al, 2004; Ferre, 2004; Tenenbaum et al, 2003]. PPARs regulate a number of processes that contribute to the development of atherosclerosis and CVD, including dyslipidemia, arterial hypertension, endothelial dysfunction, insulin resistance, and vascular remodelling. The identification of the nuclear receptors peroxisome proliferator activated receptor-gamma (PPARγ) and PPARalpha (PPARα) as being the primary targets for the insulin-sensitising thiazolidinediones and the lipid lowering fibrates, respectively, has given us a better inside in how PPARs function.

1.3.2. General characteristics

1.3.2.1. Discovery

A synthetic molecule, which belongs to the fibrate family, was originally found to induce the proliferation of the cell peroxisomes in rats. Peroxisomes are organelles that contain oxidative enzymes and a crystalline structure inside a sac, which contains amorphous grey material. The function of peroxisomes is to detoxify or to sequester toxic substances like hydrogen peroxide and other metabolites. Further work showed that the genes activated by the synthetic molecule were involved in fatty acid (FA) oxidation, and that the proliferation of the peroxisomes did not occur in all species. The name "peroxisome proliferator activated receptors" (PPAR) however, was kept.

1.3.2.2. Structure

Peroxisome Proliferator Activated Receptors are transcriptional factors. They belong to the nuclear receptor super-family. PPARs are ubiquitous in the body, and they exist in three isoforms: α, β/δ, γ, which are differentially expressed in different tissues (see table 1.2). The PPAR proteins are composed of five different domains, including four functional domains: a phosphorylation domain, a DNA binding domain, a docking domain for cofactors and a ligand-binding and transcription activation domain. (Figures 1.11, a and b)

The A/B domain is in the N-terminal region. It contains a ligand-independent activation subdomain (AF-1), responsible for the phosphorylation of PPAR. The DNA binding domain, called C or DBD, promotes the binding of PPAR to the peroxisome proliferator response element (PPRE) in the promoter region of target genes. It is composed of two zinc fingers, essential for binding. [Temple et al, 2005]. The D domain is the docking site for the cofactors, which regulate the activity of the PPAR. The E domain, or LBD (ligand binding domain), is the site of ligand fixation. The F domain contains the ligand-dependent activation function 2 (AF-2). The position of the AF2 helix is the primary determinant of whether a nuclear receptor is in the active or inactive conformation. [Stanley et al, 2003]. The E/F domains help to recruit PPAR cofactors. [Blanquart et al, 2003; Kota et al, 2005].

Table 1.3: Natural and synthetic ligands of PPARs

Ligands	PPARα	PPARβ,δ	PPARγ
Synthetic	Fibrates (chlofibrate, ciprofibrate, benzafibrate…)	ETYA Benzafibrate	Thiazolidinedione (pioglitazone, rosiglitazone…) CDDO (partial agonist)
Natural	Linoleic acid arachidonic acid oleic, eicosapentaenoic, stearic acids; **In general, mono-unsaturated acids.** LTB4	Linoleic acid (Fatty acid)	Linoleic acid arachidonic acid **In general polyunsaturated acids.** PGJ2 metabolites

ETYA: 5, 8, 11, 14-eicosatertraynoic acid (synthetic arachidonic acid analog)
LTB4: leukotriene B4
PGJ2: prostaglandine J2
CDDO: triterpenoid 2-cyano-3, 12-dioxooleana-1,9-dien-28-oic acid.

Sources: Krey and Al, 1997; Ferry and al, 2001; Kota and al, 2005.

Figure 1.12: Binding to the consensus sequence of the heterodimer PPAR/RXR
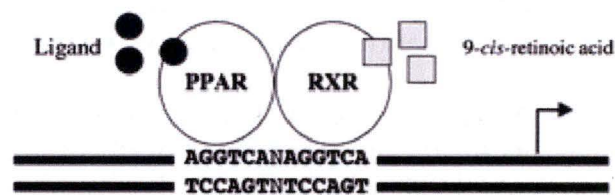
Source: P. Ferre, 2004, (modified)

1.3.2.3. Mechanism

a) Transactivation: DNA binding dependent process

The activation of PPARs occurs in five steps: the ligand fixation helps the dimerisation with the RXR. This dimer then binds to the DNA and attracts cofactors (coactivators or corepressors) resulting in the recruitment of the transcription machinery elements.

I) Ligand fixation

Ligand dependent activation is common to the entire PPAR family. In absence of a ligand, the ligand-binding domain does not have a well-defined structure. The ligand acts as a skeleton, enabling the PPAR ligand-binding domain to interact with other molecular partners. [Cronet et al, 2001]. Correct positioning of the AF2 helix at the C-terminal end of the PPAR LBD by agonists is essential for nuclear receptor activation by enhancing the affinity for coactivator proteins. [Stanley et al, 2003]. However, PPAR have basal activity without ligand [Molnàr et al, 2005].

Natural ligands (table 3)

The endogenous generation of ligands increases the activity of the PPARs. The natural ligands of PPARs are FFA, and their derived products. 9-cis-retinoic acid receptor (RXR) ligand, the 9-cis-retinoic acid, is required for the activity of the PPAR/RXR dimer. Key residues in the ligand-binding pocket are responsible for ligand selectivity among the three isoforms of PPARs. PPARs have substantially larger ligand-binding pockets than other nuclear receptors, which allow PPARs to bind a wide variety of ligands. The PPARα ligands are preferentially mono-unsaturated acids. Many polyunsaturated acids and prostaglandin-derived products are PPARγ ligands. The natural PPARβ/δ ligands are less known. In general, they are fatty acids.

Synthetic ligands (table 3)

The synthetic ligands of PPARα belong to the fibrate family. PPARγ is activated by the thiazolidinediones. The synthetic compounds that activate PPARβ,δ are less developed, but PPARδ can bind some PPARα synthetic ligands. The use of synthetic agonists has been pivotal for unravelling their functions.

II) Heterodimerisation with RXR

The heterodimerization of the PPAR with the 9-cis-retinoic acid receptor (RXR) is facilitated by the E domain (or LBD: ligand-binding domain), because the ligand forces the PPAR to adopt a specific structure, which is more rigid (see figure 1.12). After ligand binding, PPARs form heterodimers with another nuclear receptor, the Retinoic X Receptor (RXR). This dimerization is necessary for the transcriptional activity of the PPAR.

III) Binding to DNA

Once the dimer forms, it binds to a consensus sequence of 13 bases in direct repeat, called the PPAR Responsive Element (PPRE). The consensus sequence is AGGTCA-N-AGGTCA. N is a one or two nucleotide spacer (see figure 1.12). [Blanquart et al, 2003]. The recognition of the sequence by the PPAR is not very stringent: if the zinc-finger structure is maintained, an intact DBD is not required for PPAR binding on PPRE. On the contrary, the RXR recognition is more stringent. [Temple et al, 2005]. The subtypes of both PPAR and RXR participate in the modulation of binding affinity and specificity. [Juge-Aubry et al, 1997].

Figure 1.13: Levels of control of PPAR activity
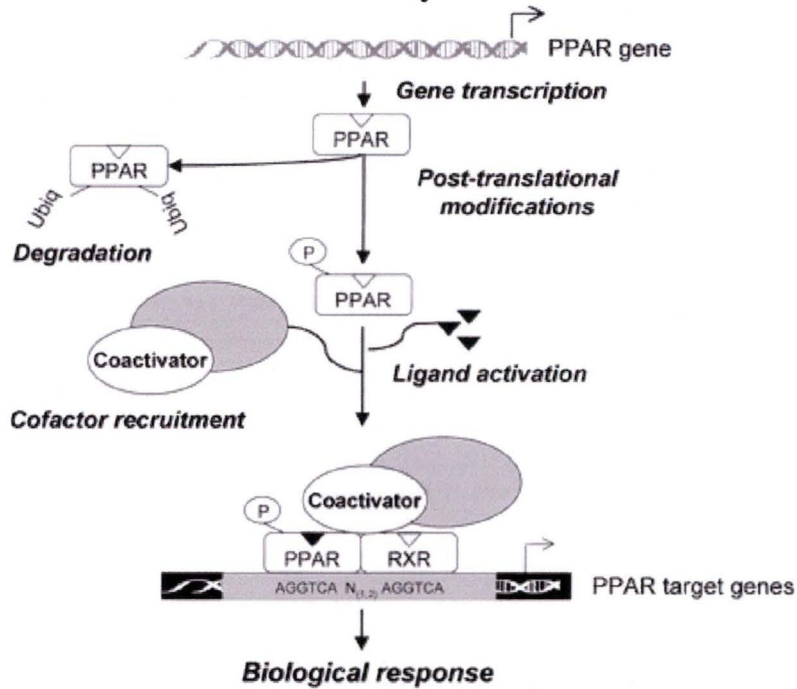
PPAR activity is controlled at the level of gene expression, protein translation and stability, posttranslational modifications affecting receptor stability and potency, availability, receptor specificity and cofactor selectivity of ligands, and consequent differential cofactor recruitment

Source: Barbier and al., 2003

Figure 1.14: Possible mechanisms of PPAR degradation by the ubiquitin–proteasome system
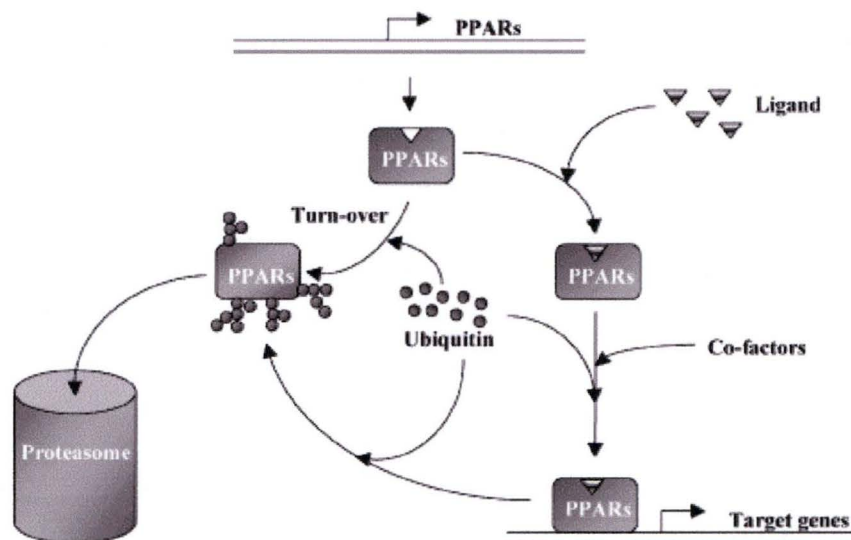
The ubiquitin–proteasome pathway degrades PPAR proteins. This system controls the PPAR proteins level in cells and then the intensity of the response to the ligand. The ligand stabilizes the PPAR proteins by decreasing its ubiquitination. The degradation of the PPAR proteins is a consequence of the cofactor recruitment, in order to stop the response.

Source: Blanquart et al, 2003,

IV) Cofactors and activation of the transcription

Several proteins act as coactivators or corepressors that mediate the ability of PPARs to initiate or stop the transcription process. Coactivators may act in two different ways. They can form a simple bridge between the PPAR and the transcription machinery, but they also can have enzymatic activities and act in modifying chromatin and DNA structure. In general, unactivated PPARs are complexed with corepressors, which extinguish their transcriptional activity by the recruitment of histone deacetylases. When the PPAR is activated by its ligand, its conformation changes and it is dissociated from the corepressor. Coactivators, which have histone acetyl transferase activity, then bind the PPARs and facilitate by this way the target gene transcription. [Auwerx, 1999].

b) Transrepression: DNA binding independent process

PPARs can also repress gene transcription in a DNA-binding-independent manner via protein-protein interactions and cofactor competition, preventing the transcriptional activity of other transcription factors. They interfere thus with other signalling pathways, such as the Activator Protein-1 (AP1), the nuclear factor-kappa B (NFκB), the CAAT box/enhancer binding protein (C/EBP) and the nuclear factor of activated T cells.

1.3.2.4. Regulations

a) General

PPARs are strongly regulated by several mechanisms, on different levels (figure 1.13), because it is necessary to adjust lipid and glucose metabolism to fluctuating needs in energy. An increase in the level of FFA is associated with increased expression of various PPAR responsive genes. The expression of the three isoforms changes according to the tissue type, the physiological and pathological conditions.

Given that PPARs form heterodimers with RXR, regulations on RXR also have an effect on the transcriptional activity of PPARs. It has indeed been shown that the activity of the PPARγ/RXRα heterodimer is maximal when both receptors are bound to their respective ligands [Tontonoz P et al, 1997]. There are also two important post-translational regulations, the ubiquitination and the phosphorylation.

b) Ubiquitination

Ubiquitination is a modification that leads to the degradation of the proteins by the proteasome. It consists in adding 8kDa polypeptide, called ubiquitin, on the lysine residues of the proteins that have to be degraded. The poly-ubiquitinated proteins are rapidly degraded by the 26S proteasome. (Figure 1.14)

Factors that initiate the ubiquitination are different according to the isoform. PPARα is less degraded by the proteasome when it has a ligand bound to it, whereas PPARγ has a co-repressor that protects it from degradation [Blanquart et al, 2003]. In general, it seems that the recruitment of cofactors, which modify PPARα activity, could interfere with the PPARα protein stability [Blanquart et al, 2004].

c) Phosphorylation

A protein kinase is an enzyme that transfers a phosphate group from a donor molecule (usually ATP) to an amino acid residue of a protein. The protein kinase functions in signal

Figure 1.15

a) Representation of kinase pathways implicated in the phosphorylation of PPARs and in the regulation of their transcriptional activity.
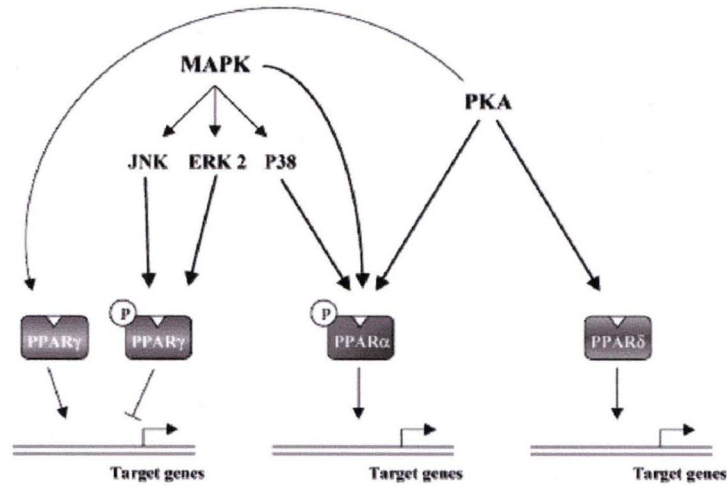
The PPARs are targets for kinases. The function of the phosphorylation of PPARs appears to be specific of the kinase implicated and of the PPAR isotype phosphorylated. Indeed, MAPkinase phosphorylation increases the activity of PPARα and decreases the activity of PPARγ. However, PKA induces an increase of the transcriptional activity of the three PPAR isotypes. Since the kinases are activated by numerous extracellular signals and since the kinases modulate the PPAR activities, it appears that PPARs are regulated by physiological changes leading to the production of kinase activators. Ⓟ: described phosphorylation by kinases.

Source: Blanquart et al, 2003

b) Phosphorylation sites of PPARα and PPARγ.
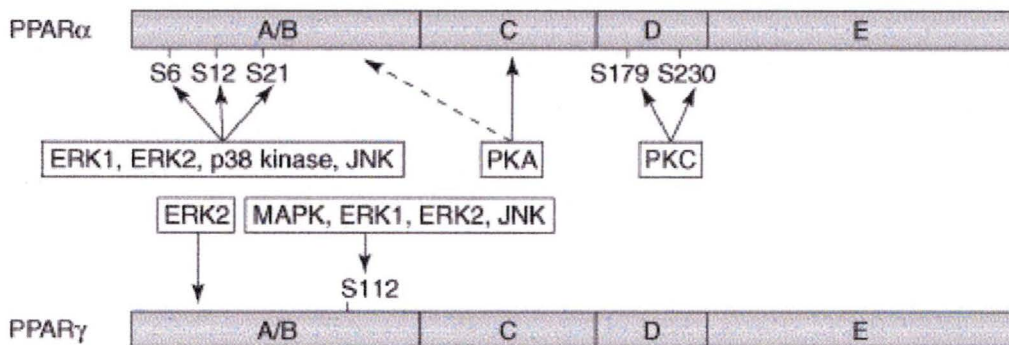
Source: Gelman, 2005, (modified).

transduction for the regulation of enzymes: phosphorylation can activate (or inhibit) the activity of an enzyme. PPARs are phosphoproteins and they can be regulated by phosphorylation. (Figure 1.15). The phosphorylation is a fine tuning mechanism of the PPAR activity that allows the integration of signals coming from the cell membrane. Three kinase families (mitogen activated protein kinase (MAPK), protein kinase C (PKC), protein kinase A (PKA)) are known to be involved in PPAR phosphorylation. The effects of the phosphorylation on PPAR activity depend (Figure 1.15 b) on the PPAR isotype, the phosphorylated residue, the kinase, the stimuli, and the cell type. The regulations by phosphorylation are complex. For example, PKC decreases the transactivation enhanced by PPARα, but PKA increases it. [Blanquart et al, 2004; Gelman et al, 2005].

1.3.3. PPARα

3.1. Localisation

PPARα is expressed mainly in metabolically active tissues; it is involved in the regulation of genes important in FA oxidation. It is the predominant PPAR isotype in the liver, the kidney, the muscles and the heart. It is present, although less expressed, in adipose tissue, cells from the vascular wall (endothelial cells, monocytes/ macrophages, vascular smooth muscular cells) and enterocytes.

3.2. Functions of PPARα

Genes activated by PPARα are mostly genes involved in lipid metabolism.

a) PPARα in the liver

In the liver (and partially in adipose tissue), PPARα stimulates the uptake of FFA in the cells, by increasing the expression of transporters, such as the fatty acid transporter (FAT) and the fatty acid transport protein (FATP) [Motojima et al, 1998]. In addition to regulating the polyunsaturated fatty acids (PFA) uptake, PPARα also increases the cellular retention by increasing the expression of the fatty acid binding protein (FABP) and acylCoA synthetase (ACS) resulting in the esterification of FA preparing it for catabolism. PPARα favourizes mitochondrial uptake of the PFA by increasing carnityl-palmitoyl-transferase I and II (CPT-I/II) and upregulates their oxidation by increasing the expression of β-oxidation enzymes (figure 1.16)[Leone et al, 1999; Schoonjans et al, 1996; Wahli et al, 1995]. PPARα also influences lipid metabolism. It reduces the synthesis of triglycerides by increasing the expression of enzymes that catalyze the degradation of FA, such as the lipoprotein lipase (LPL) or enzymes involved in the β-hydroxylation [Kota et al, 2005]. It also decreases ApoCIII (an inhibitor of LPL activity) expression. PPARα also influences hepatic glucose production during fasting: the glycerol is metabolised to glucose when PPARα levels are high. [Patsouris. et al. 2004].

b) PPARα and reverse transport cholesterol

The cholesterol efflux is the first step of the reverse cholesterol transport pathway that brings cholesterol from the peripheral cells back to the liver. Macrophage cholesterol efflux to HDL occurs via active efflux mediated by the ATP-binding cassette (ABC) proteins, such as ABCA-1. ABCA-1 allows the removal of intracellular free cholesterol (FC) from peripheral cells towards HDL rich in apolipoproteins (Apo) ApoAI and ApoAII, and HDL are then

Figure 1.16: PPARα and the control of lipid metabolism
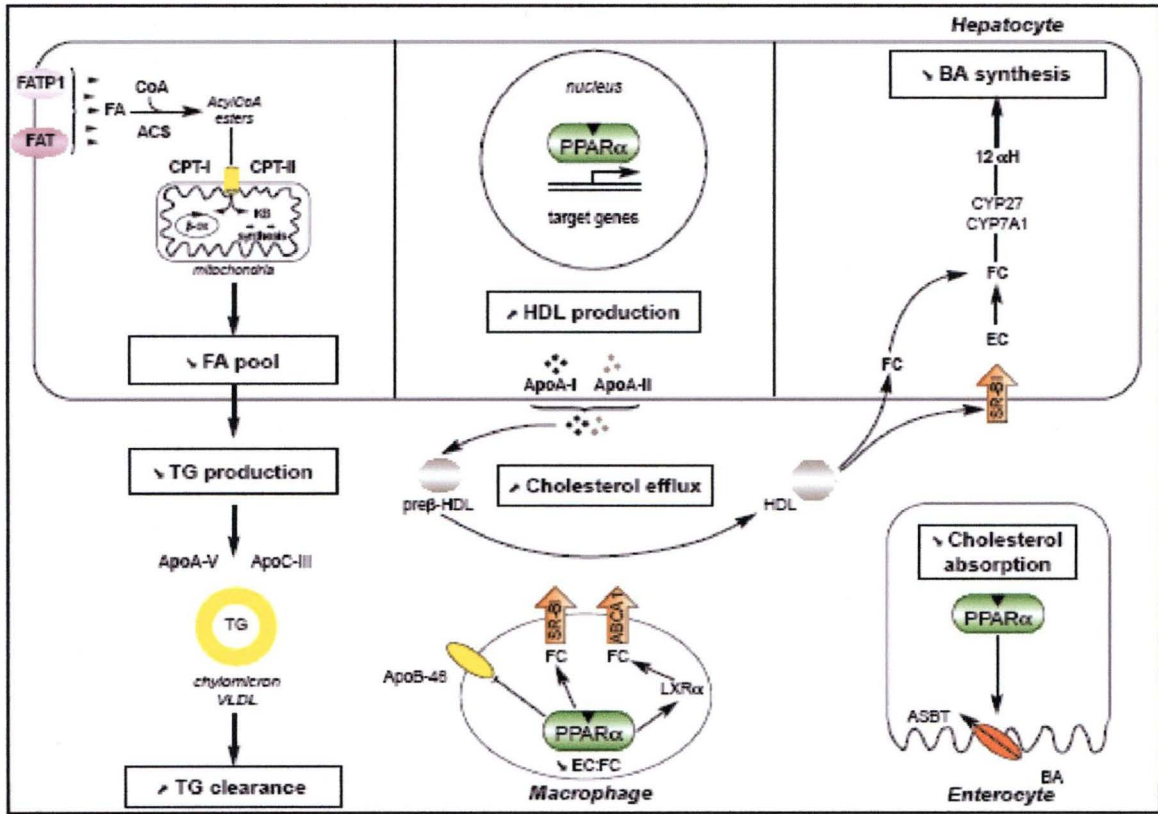
PPARα increases the entry of fatty acids (FA) into the hepatocyte and favours their activation, intracellular transport and catabolism via the β-oxidation cycle (β-ox) thus diminishing FA pool and triglyceride (TG) production. PPARα also increases ketone body (KB) synthesis and TG clearance by modulating the expression of genes implicated in TG lipolysis. In addition, PPARα influences high-density lipoprotein (HDL) production in the hepatocyte by upregulating apolipoprotein (Apo) A-I and A-II gene expression. PPARα further controls the reverse cholesterol transport by increasing cholesterol efflux from macrophages and HDL capture by the liver. The net effect on bile acid (BA) synthesis by the liver is less clear. Finally, PPARα also acts on cholesterol absorption in the intestine. Regulation of gene expression: bold – upregulated; thin – downregulated. EC: esterified cholesterol; FC: free cholesterol; CoA: coenzyme A; 12α-H: 12α -hydroxylase.

Source: Duval C., 2004

captured by the liver through the scavenger receptor B1 (SRBI) receptor, belonging to the family of scavenger receptors. PPARα plays an important role in reverse cholesterol metabolism by increasing the expression of ABCA-1 and of SRBI. This results in an enhanced availability of free cholesterol for efflux through the ABCA-1 pathway.

c) PPARα in muscle

In the muscle (skeletal muscle but also heart), PPARα increases FA oxidation. It also induces the economy of glucose, e.g. in increasing the expression of the pyruvate dehydrogenase kinase 4 (PDK4). These regulations allow cells to spare energy.

d) PPARα and inflammation

The role of PPARα in the control of the inflammation came from the observation that the inflammatory response is prolonged in PPARα-/- mice. [Blanquart, 2001]. Overall, PPARα interferes with different steps of the inflammatory response by modulating the expression of cytokines, cytokine receptors, adhesion molecules and acute phase protein. It decreases the expression of fibrinogen and of cytokines (e.g. TNFα) involved in the inflammatory response, resulting in the inhibition of inflammatory pathways. In addition, PPARα represses the cytokine-induced expression of vascular cell-adhesion molecule-1 (VCAM-1) and thrombin-induced endothelin-1 expression in endothelial cells. The diapedesis of the lymphocytes is decreased in this way.

1.3.4. PPARγ

1.3.4.1. Localisation

PPARγ is predominant in the adipose tissue and in skeletal muscles. It is also expressed in the liver and, at a low level, in the heart and other cell types, as the endothelial cells.

PPARγ exists in human in three isoforms, PPARγ1, PPARγ2 and PPARγ3, due to three different promoters in the PPARγ gene. [Blanquart and al, 2001]. PPARγ1 and PPARγ3 give the same protein, called PPARγ1. PPARγ1 protein has the broadest repartition, and it is found in the liver, the adipose tissue, the endothelial cells, the smooth muscle cells and in the macrophages. PPARγ2 is restricted to the adipose tissue and it plays an important role in adipocyte differentiation.

1.3.4.2. Functions of PPARγ

PPARγ induces the transcription of genes involved in adipocyte differentiation, insulin sensitivity, lipid metabolism and inflammation. To study PPARγ function, PPARγ agonists were used. These agonists also have effects independent of PPARγ. Actually, there are new studies to separate the PPARγ from PPARγ independent effects, but some functions are not totally clear. The functions off PPARγ described thereafter may thus be not correct.

a) PPARγ in adipose tissue

PPARγ is mainly expressed in adipose tissue where its activation stimulates adipogenesis and the expression of genes involved in fatty acid uptake and storage and in TG metabolism. It is essential for adipocyte proliferation and differentiation. PPARγ plays a central role in maintaining a functional and differentiated adipose tissue (figure 1.17); [Hammarstedt, 2003].

Figure 1.17 : Role of PPARγ in adipose tissue
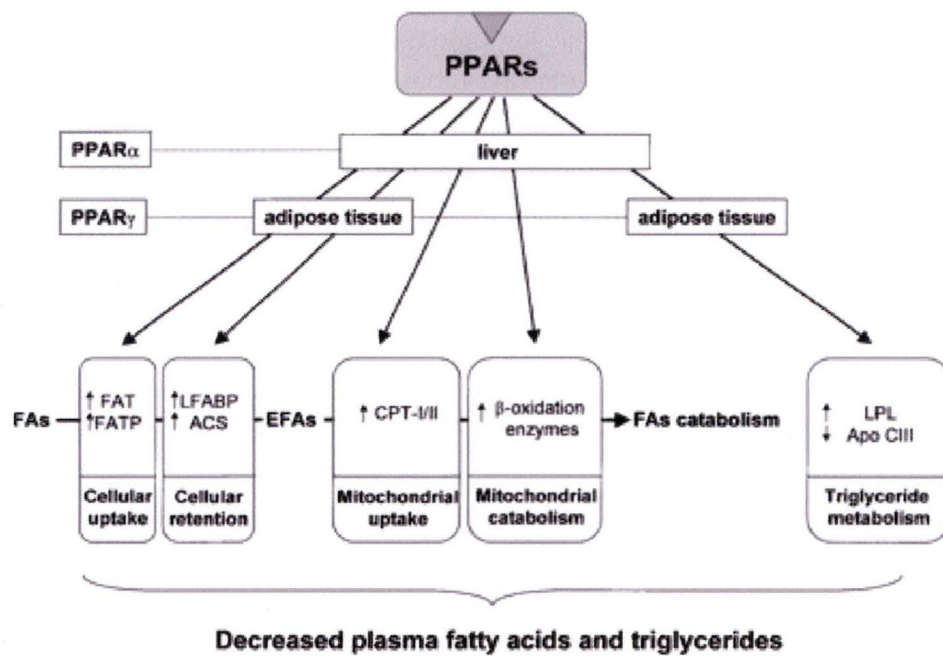
Source: Ferre, 2004

b) PPARγ in muscles

The muscle-specific disruption of PPARγ resulted in a state of severe insulin resistance. Therefore, PPARγ has a direct effect on the insulin sensitivity in the muscle. PPARγ pathway seems also to be involved in the molecular response to hypertrophic stimuli in the heart, trough the inhibition of augmented protein synthesis, preventing increases in protein content and cell size, and the activation of NFκB [Mehrabi et al, 2003].

c) PPARγ in endothelial cells and inflammation

The role of PPARγ in the inflammatory process is contested. The observed anti-inflammatory effects often vary according to the agonists used and are not always consistent with their capacity for receptor binding. Nevertheless, in the endothelial cells, PPARγ enhances the iNOS activity and inhibits leucocyte/endothelial cell interactions. These two actions could inhibit inflammation. It is thus possible that PPARγ has, per se, a potent anti-inflammatory role [Wang, 2002].

d) PPARγ in atherosclerotic lesions

PPARγ is expressed in atherosclerotic lesions. It inhibits growth-factor induced proliferation and migration of smooth muscle cells. It induces the scavenger receptor CD36 in macrophages, which increase the foam cells in the atherosclerotic lesion: it appears to coordinate a complex response to oxLDL that results in both increased scavenging and increased lipid efflux from macrophages. The result of its action in macrophages may be the removal of modified lipoproteins from the artery wall [Castrillo and Tontonoz, 2004]. .

1.3.5. PPARβ/δ

1.3.5.1. Localisation

PPARβ/δ is the product of the PPARβ/δ gene, also called Nuc-1 or FAAR (Fatty Acid Activated Receptor): FAAR is the mouse homologue of human Nuc-1 [Amri et al, 1995]. It is expressed at relatively high levels in the adipose tissue, the skin and the brain.
It is also present in smooth muscle cells and its expression is higher during the formation of the vascular lesion, in the neointima [Barbier et al, 2003].

1.3.5.2. Function of PPARβ/δ

In adipocytes, PPARβ/γ upregulates FA oxidation and energy expenditure: it decreases adiposity. PPARβ/δ deficient mice have reduced energy uncoupling and develop obesity more easily [Wang et al, 2003].

PPARβ/δ is a regulator of oxidised lipids. It favours the cholesterol accumulation in the macrophages by increasing the expression of genes involved in the accumulation of cholesterol (e.g. CD36) and by decreasing the activity of genes involved in the metabolism and efflux of cholesterol (e.g. ApoE and ABCA1). PPARβ/δ plays a central role in regulating fatty acid catabolism in muscles. In PPARα-/- mice, PPARβ/δ compensates partially the lack of PPARα.

Figure 1.18: Metabolic and vascular actions of PPARα and PPARγ agonists
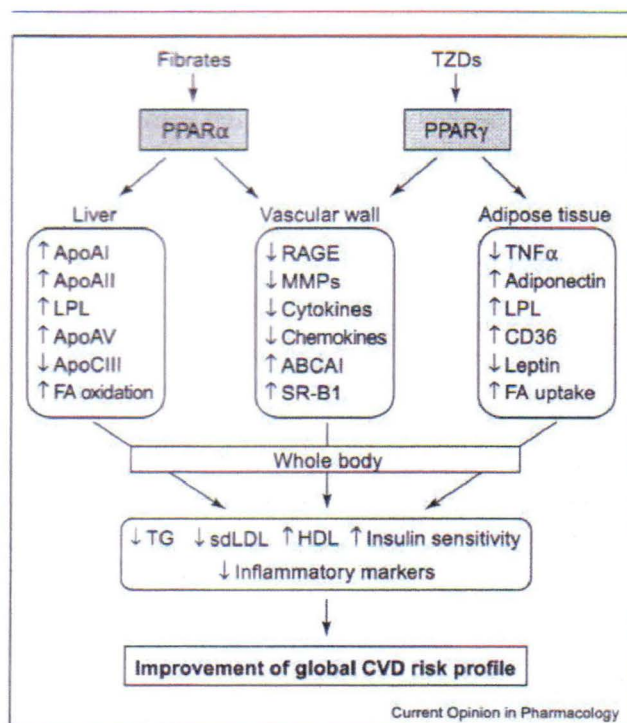
In the liver, fibrates, through PPARα activation, regulate the expression of genes involved in FA oxidation, TG synthesis and HDL metabolism. TZDs, PPARγ activators, exert their action primarily in adipose tissue where they interfere with insulin signalling, FA metabolism and cytokine production. Both drug classes reduce plasma concentrations of several inflammatory biomarkers. ABCA1: ATP binding cassette A1. SRBI: scavenger receptor BI

Source: Chinetti-Gbaguidi and al, 2005

The role of PPARβ/δ in atherogenesis is still unclear. PPARβ/δ could contribute to lesion development, due to its role in modulating the proliferation of smooth cells. However, it could also normalize the lipid profile, which is beneficial for the vessel wall [Marx et al, 2004].

1.3.6. PPAR agonists: pharmacological interest

Synthetic PPAR agonists (see table 1.3) are used as drugs. The fibrate are PPARα agonists, and the thiazolidinediones (TZDs) are PPARγ agonists (Figure 1.18).

1.3.6.1. Specific effects of PPAR agonists

a) Thiazolidinedione and diabetes

The antidiabetic activity of the thiazolidinediones (TZDs), well described PPARγ agonists, is now well known. PPARγ enhances insulin signalling, thereby improving insulin sensitivity (see function of PPARγ, 1.3.4.2). Rosiglitazone and pioglitazone are two synthetic PPARγ ligands, used as pharmaceutical insulin sensitizers.

b) Fibrate and dyslipidemia

Fibrates, PPARα agonists, have beneficial effect on lipid levels: they are used for cholesterol reduction, often in association with other molecules, such as statins or TZDs. There are many different fibrates used as lipid lowering drugs, including clorofibrate (first discovered), fenofibrates, and ciprofibrate. Beside their beneficial effects on lipids, PPAR agonists also exert pleiotropic effect. For instance, fenofibrate prevents the development of diabetes by reducing adiposity, improving peripheral action, and exerting beneficial effects on pancreatic β-cells [Koh et al, 2003].

1.3.6.2. Other effect of synthetic PPAR agonists

a) On atherosclerosis

I) Indirect effects on atherosclerosis

The indirect effects of PPARs are relevant for treating atherosclerosis, because of the decrease of the circulating lipids and the improvement of the insulin sensitivity.

II) Directs effects on vessel wall

The PPARs have positive and negative direct effects on the atherosclerosis development in the vessel wall.
One of the negative effects is that PPARs intervene in the first state of atherogenesis, the chemoattraction and the recruitment of the leucocytes [Duval et al, 2002]. For instance, the PPARα agonist stimulates the expression of monocyte chemoattractant protein 1 (MCP-1). As a positive effect, PPARs stimulate the efflux of cholesterol from foam cells. The thiazolidinediones normalize endothelial function and correct structural vascular abnormalities. PPARs induce ABCA1, which stimulates cholesterol efflux. Furthermore, they are inhibitors of inflammation (Figure 1.19). The TZDs generally cause a shift toward larger LDL particles, which are less prone to oxidative modifications and are therefore thought to be less atherogenic [van Wijk et al, 2004]. In summary, as illustrated in figure 1.18, fibrates and

Figure 1.19

Atherosclerotic blood vessel

Macrophage

Atherogenic inflammation

PPARγ   PPARα   PPARβ/δ

LXRα   ?

ABCG1   ABCA1

Cholesterol efflux to HDL acceptor   apoE, PLTP   Cholesterol efflux to apoAI acceptor
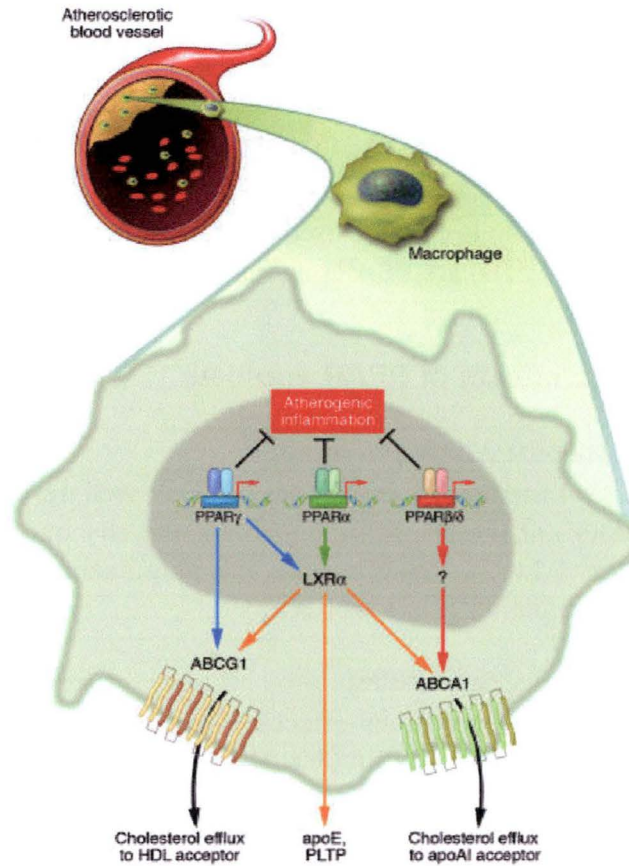
PPAR signalling pathways influence macrophage gene expression and foam-cell formation. Ligand activation of PPARα and PPARγ, but not PPARβ/δ, inhibits the development of atherosclerosis in LDLR-/-mice. Both systemic and local mechanisms might contribute to these beneficial effects. PPARα and PPARγ increase LXRα expression in macrophages and promote expression of ABCA1, which mediates cholesterol efflux to apoAI. PPARγ may also inhibit cholesterol accumulation in macrophages through direct regulation of ABCG1, which has been implicated in cholesterol efflux to HDL. Activation of each of the PPARs with selective agonists also inhibits the expression of inflammatory markers in the artery wall. PTLP, phospholipid transfer protein.

Source: Castrillo and Tontonoz, 2004

Table 1.4: Phenotypes of the different murine models

	C57BL6J	Ob/Ob	LDLR(-/-)	LDLR(-/-);Ob/Ob
Obesity	-	++	-	++
Insulin resistance Diabetes	-	++	-	++
Dyslipidemia	-	-	Chol↑	Chol↑, TG↑
Hypertension	-	-	+	++
Impaired heart function	-	-	-	++

TZDs are generally considered to improve the global cardiovascular risk profile [Chinetti-Gbaguidi et al, 2005].

b)On cells proliferation

The TZDs have also totally other effects: they have anti-proliferative effects on tumoral cells [Theocharisa, 2003]. These effects seems to be PPARγ independent.

1.3.6.3. Dual agonists

As seen before, the dyslipidemia and the diabetes are two main factors of the cardiovascular disease. PPARα is mainly involved in lipid metabolism and PPARγ acts on adipogenesis and glucose homeostasis. Moreover, the PPARs have some ligands in common. It is thus interesting to develop dual PPARα and PPARγ activators. These dual activators will be useful for the treatment of dyslipidemia associated with type 2 diabetes. The dual agonists may thus reduce the risk of CVD, especially atherosclerosis, because of the control of inflammation by the PPARs.

Different dual agonists have already been produced. They have, indeed, an antidiabetic and hypolipidemic potential. [Chakrabarti et al, 2004]. Anti-tumor effects are described for the dual agonist, as for TZDs, but this mechanism seems to be PPAR independent and we will not describe these effects [Liu et al, 2004].

Figure 1.20: comparison between DKO and normal mice.

Left: DKO mice; right, C57BL6J mice.
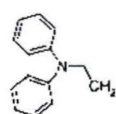
1.4. DKO mice (LDLR(-/-);Ob/Ob) and metabolic syndrome

To study the mechanisms of the metabolic syndrome and to test the effects of new molecules on the development of this disease, it is necessary to find a representative animal model. Leptin deficient mice (Ob/Ob) are an excellent model for obesity and insulin resistance. [Barouch et al, 2003]. However, high concentrations of HDL protect these mice against the development of atherosclerosis, even on a background of C57BL6 mice, which are genetically sensitive to the development of atherosclerosis. LDL-receptor deficient mice [LDLR(-/-)] only develop atherosclerosis on a high fat diet, and obesity and diabetes on a diabetogenic diet.[Mertens et al, 2003; Verreth et al, 2004a] In our laboratory (CEHA) Ob/Ob mice were crossed with LDLR(-/-) mice (both on a C57BL6J background).

The resulting LDLR(-/-);Ob/Ob mice (or double knock out mice (DKO)) are obese (figure 1.20), insulin-resistant, dyslipidemic and hypertensive. Increased oxidative stress in these mice is associated with accelerated atherosclerosis and impaired cardiac function (table 1.4). [Van Eck et al, 2003].

Compared to lean mice, PPARα and PPARγ expression was downregulated in DKO mice. However, when these DKO mice were diet-restricted, they showed a significant weight-loss and increased expression of PPARα and PPARγ in the adipose tissue, the aortic arch, and the heart. These changes in expression were associated with increased insulin-sensitivity, decreased hypertriglyceridemia, reduced mean 24-hour blood pressure and heart rate, restored circadian variations of blood pressure and heart rate, better heart function, and reduced atherosclerosis. [Verreth et al, 2004].

Figure 1.20 A: EC50 obtained for the dual agonist in an *in vitro* transactivation test in HEK 293 cells.

Compound number	Structure	hPPARα		hPPARγ		hPPARβ/δ	
		EC_{50} +/- SD μM	% max +/- SD	EC_{50} +/- SD μM	% max +/- SD	EC_{50} +/- SD μM	% max +/- SD
3q		0.36 +/- 0.16	140.4 +/- 11.6	0.17 +/- 0.081	108.7 +/- 17.6		3 +/- 0

EC_{50}: Effective concentration; the dosage at which the desired response is present for 50 percent of the population.

% max: fold activation relative to maximum activation obtained with WY14643 for PPARα and with rosiglitazone for PPARγ.

Source: Sauerberg and al, 2001

Figure 1.20 B: Crystal structure of the PPARγ receptor binding domain complexed with 3q.
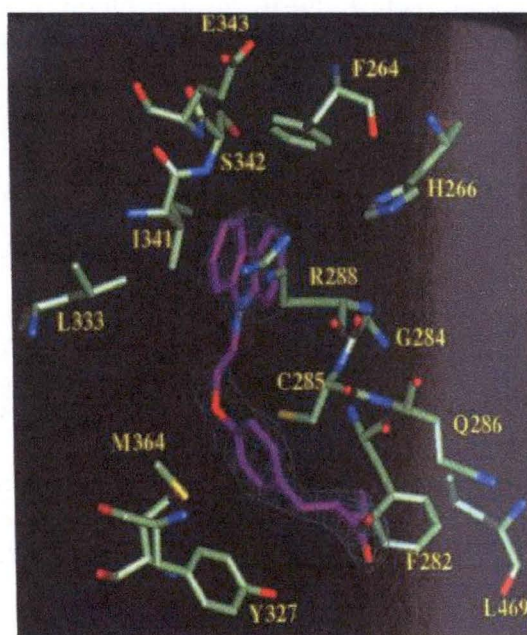
The ligand carbon atoms are shown in magenta. Amino acids of the PPARγ receptor neighboring the ligand are shown with green-colored carbon atoms and with their residue type and sequence number written in yellow.

1.5. Goal of the thesis

As previously described in the introduction, the metabolic syndrome (cluster of obesity, hypertension, diabetes and other metabolic disorders) becomes a common disease. The CEHA laboratory (KUL) has developed a murine model for this syndrome, the DKO mice, deficient for leptin and for the LDL-R. The laboratory is presently involved in several intervening studies in this model: mice are treated either with a statine (Crestor) that decreases cholesterol, either diet restricted or treated with PPAR agonists. The analyses are focused on lipid and glucose metabolism, as well as on gene expression.

In this thesis we further investigated the role of PPARs in metabolism, atherosclerosis, and cardiovascular function in this mouse model by analyzing the effect of a dual PPARα/γ agonist [(S)-3-(4-(2-carbazol-9-yl-ethoxy) phenyl-2-ethoxy propionic acid)] developed by Novo Nordisk. The structure and the chemical characteristics of this agonist are given in figure 1.21. Previously, this agonist was shown to improve insulin sensitivity in diabetic db/db mice more than the PPARγ agonists, pioglitazone and rosiglitazone. Moreover, the agonist lowered plasma triglycerides and cholesterol in high cholesterol fed rats after 4 days treatment, indicating *in vivo* PPARα activity [Sauerberg et al, 2002]. Per Sauerberg provided the CEHA with this agonist. Wim Verreth, in his PhD thesis, already started experiments with this agonist on the LDL-/-, Ob/Ob mice, but it was necessary to confirm the results and to increase the number of treated-mice, in order to allow a reliable statistical analysis of the data.

The practical work in this thesis can be subdivided in the following parts.
- The first part was the daily treatment of the mice with the PPAR agonist.
- The second part was the collection of blood samples every week, in order to analyze the effects of the agonist on several plasma parameters. For each sample, glucose, insulin, lipid (triglycerides and cholesterol), as well as adiponectin concentrations were determined, to get a better insight of the possible beneficial effect of the agonist on glucose and lipid metabolism.
- In the third part, the agonist was evaluated on heart function using an echocardiographic approach and on atherosclerotic lesions in the aortic root, by immunostaining tissue sections for oxidized LDL, macrophages and smooth muscle cells.
- Finally, gene expression analysis was undertaken in adipose tissue to compare agonist-treated and placebo mice, using quantitative RT-PCR. We measured the abundance of mRNA for the genes encoding the three PPAR isoforms, as well as for the gene encoding *Glut-4*, a glucose carrier involved in glucose extraction from the blood.

In the DKO mice model used in this work, we found that the dual agonist improved insulin sensitivity, but we observed no reduction, neither in triglycerides nor in cholesterol concentration. There was also no reduction of the atherosclerotic lesions volume. The gene expression analysis confirmed that the effect of this agonist was tissue specific and that the dual agonist had no effect on the expression of PPARδ.

2. Materials and Methods

Figure 2.1: Genotyping of the mice using diagnostic PCR with Ob primer:
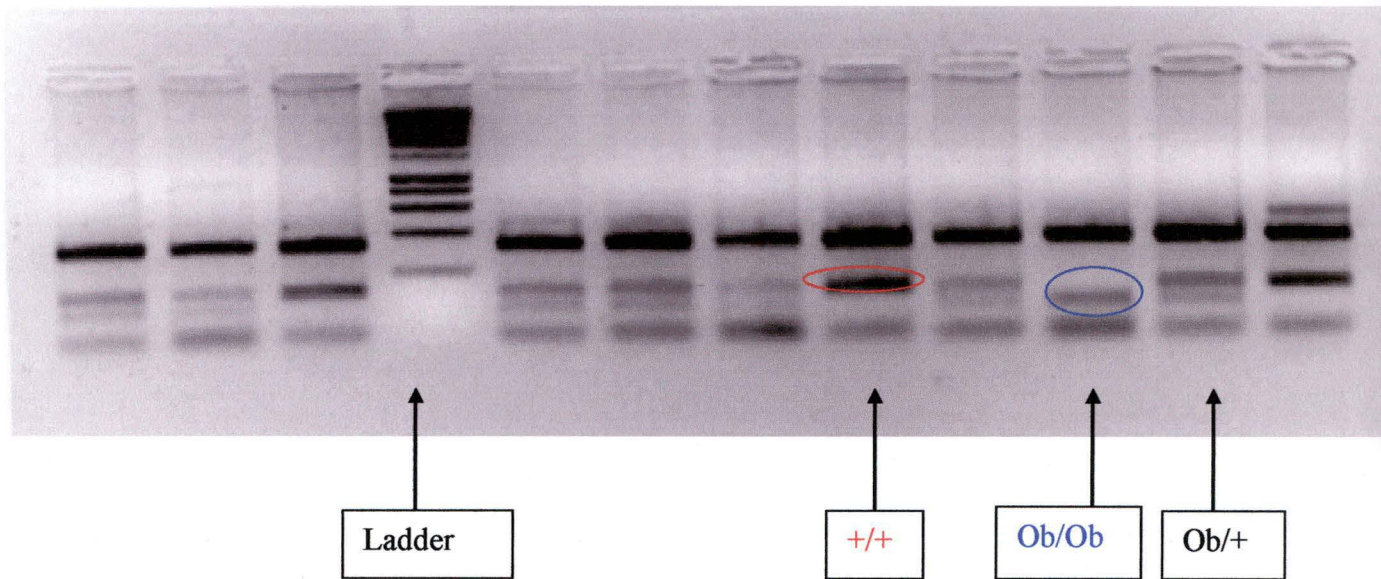
2. Materials and Methods

2.1. DKO mice: breeding and characterization

2.1.1. Breeding of the mice

Homozygous LDL-receptor knockout mice (LDLR(-/-)) and heterozygous Ob/+, and C57BL6J mice were purchased from Jackson Laboratory, Bar Harbor, ME. LDLR(-/-) mice were backcrossed into a C57BL6J background to the 10th generation. To Obtain leptin deficiency (Ob/Ob) on a background of LDLR deficiency, LDLR(-/-) and Ob/+ mice were crossed, and the F1 progeny of this mating (LDLR(-/+);Ob/+) were then crossed to obtain mice that had either zero, one, or both normal LDLR alleles and were leptin-deficient (LDLR(-/-);Ob/Ob, LDLR(+/-);Ob/Ob, and LDLR(+/+);Ob/Ob, respectively) as well as control LDLR(-/-), LDLR(+/-), and wild type mice, as previously described [Mertens et al, 2003]. All offspring mice were genotyped by PCR techniques (description in the following paragraph). All mice were housed at 22°C on a fixed 14/10-h light/dark cycle. Experimental procedures in animals were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

2.1.2. Genotyping of the mice

2.1.2.1. DNA extraction

The extremity of the tail of the 3-4 weeks old mice was cut (approximately 1cm). The tip was incubated overnight at 55°C, in 700 µl lysis buffer (100 mM Tris-HCL; pH 8.5; 5 mM EDTA; 0.2 % SDS and 200 mM NaCl) with proteinase K (700 µl for 20 ml of lysis buffer). The solution was shaken for 1 min and then centrifuged for 10 min at 14 000 rpm (rotation per minute).

The supernatants were transferred into 600 µl isopropanol, where the DNA becomes visible. The DNA is then dissolved in 200 µl TE (10 mM Tris-HCl; pH 7.0; 1 mM EDTA), and incubated for one hour at 56°C. 200 µl fenol/chloroform were then added and, after 1 minute of agitation, the solution is centrifuged at 14 000 rpm. The supernatants were transferred into 600 µl isopropanol. The DNA is transferred again in 200 µl TE. The DNA is stored at 4°C.

2.1.2.2. DNA analysis

The Ob/Ob mice do not produce functional leptin because of a point mutation in the Ob gene that results in a shorter leptin mRNA. With this mutation appears a new site of restriction for the restriction enzyme DdeI. After PCR (polymerase chain reaction) using the Ob forward (F) and reverse (R) primer (for sequence, see next paragraph), DdeI (Roche) was added and incubated at 37°C overnight. The DNA is put on an agarose gel to identify whether the mice are Ob/Ob, Ob/+ or +/+ (figure 2.1).

In LDLR(-/-) mice the gene for the LDL receptor is replaced by the neomycine gene (neo gene). Using primers for both genes (functional LDL Receptor gene and neo gene) in two

Figure 2.2: Detection of LDLR -/- mice by diagnostic PCR

A) neo-gene
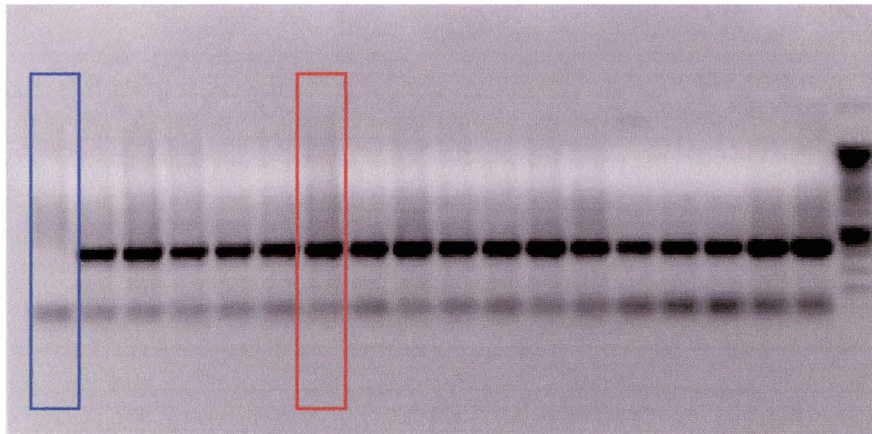
Mouse LDLR -/-

MouseLDLR +/+

B) LDLR gene
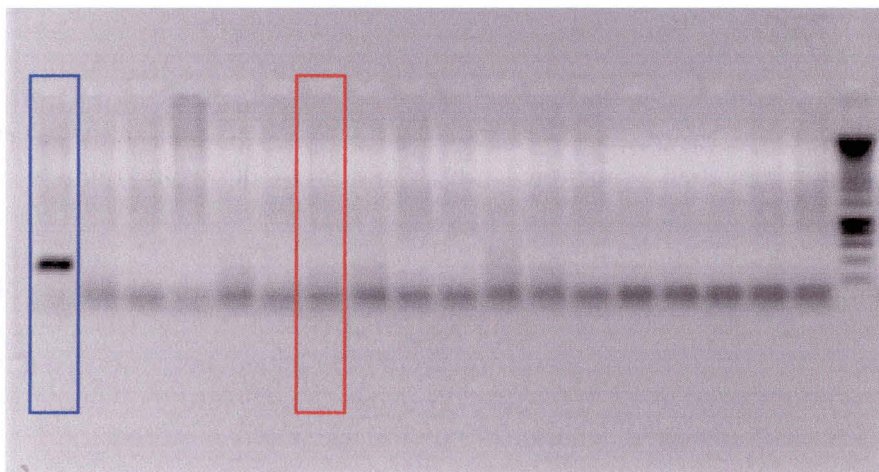
different PCR enables us to identify whether the mice are homozygous -/-, heterozygous -/+ or wild type +/+ (figure 2.2).

<div style="border:1px solid black; padding:10px;">

Primers used for DNA analyses:

➢ **Ob gene**
  F: CTGGTTCTTCACGGATATCATTG
  R: AGGGAGCAGCTCTTGGAGAA

➢ **LDLR gene**
  • **LDLR gene**
  F: 5' ACCCCAAGACGTGCTCCCAGGATG 3'
  R: 5' CGCAGTGCTCCTCATTCGACTTGT 3'

  • **Neo gene**
  F: 5' CAAGATGGATTGCACGCAGGTTCTCC 3'
  R: 5' CACGAGGAAGCGGTCAGCCCATTCG 3'

</div>

2.1.2.3. Treatment of the mice with the dual PPARα,γ agonist

The PPARα,γ agonist [(S)-3-(4-(2-carbazol-9-yl-ethoxy)phenyl-2-ethoxy propionic acid)] was a kind gift from Per Sauerberg, Novo Nordisk A/S. Previously, this agonist was shown to improve insulin sensitivity in diabetic db/db mice more than the PPARγ agonists pioglitazone and rosiglitazone. Moreover, the agonist lowered plasma triglycerides and cholesterol in high cholesterol fed rats after 4 days treatment, indicating in vivo PPARα activity [Sauerberg et al, 2002].

The agonist was prepared as suspension in 0.2 % CMC + 0.4 % Tween-80 in saline. Fresh suspensions were made for 7 days and kept at + 4°C. The agonist was administered orally (10 mL/kg; 3 mg/kg/day) for 12 weeks starting at the age of 12 weeks (n=9). Placebo mice (n=7) received saline suspension without the agonist. Control mice (n=3) received no medication.

Figure 2.3: Echocardiography

A) M-mode
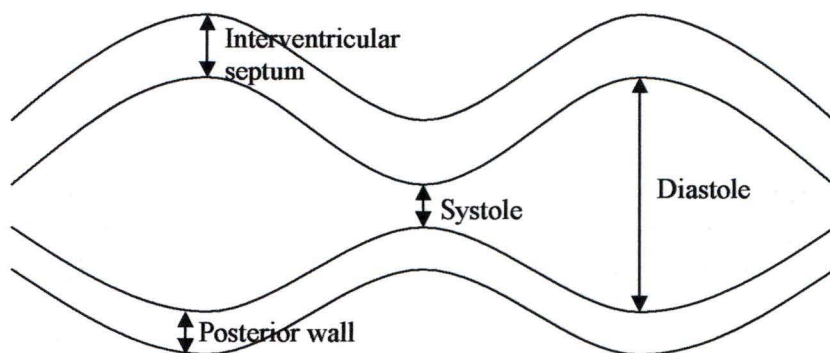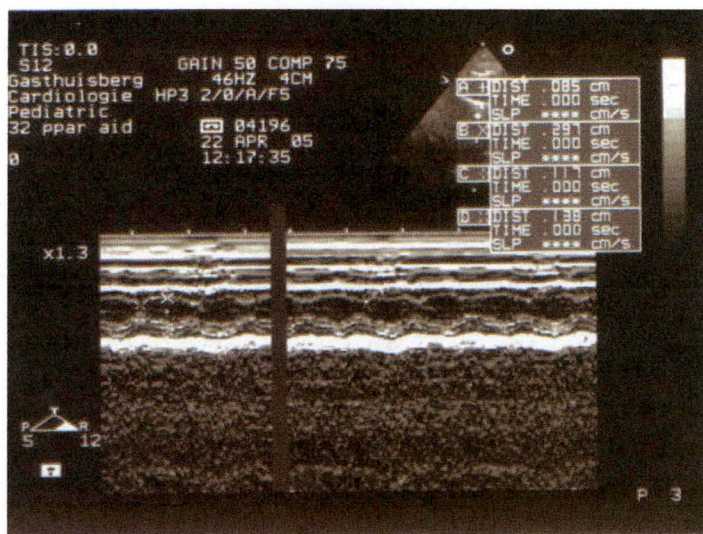
B) 2D-mode
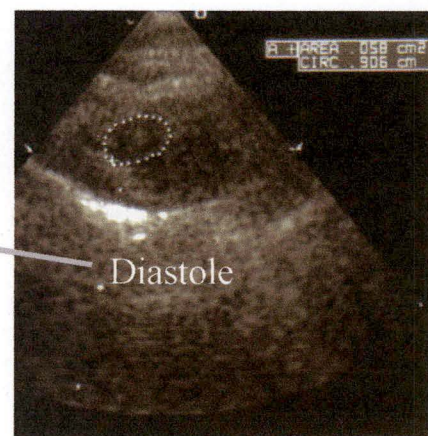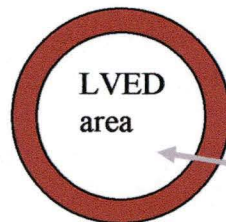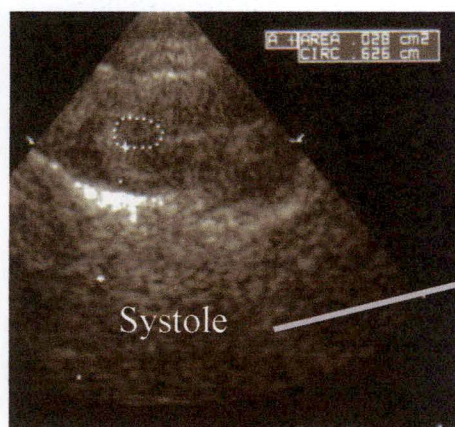
2.2. Physiological parameters

2.2.1. Biochemical analyses

Blood of conscious mice was collected by tail bleeding into tubes with 30 µL EDTA after an overnight fast. Plasma was obtained by centrifugation, 10 minutes at 3000 rpm. Glucose was measured with a glucometer (Menarini Diagnostics) and plasma insulin with mouse insulin Enzyme-Linked Immunosorbent Assay (ELISA), Mercodia. Insulin resistance was calculated by a homeostasis model assessment (HOMA):

HOMA = fasting serum insulin (mU/L) x fasting blood glucose (mmol/L)/22.5.

Adiponectin was measured with a mice adiponectin ELISA (mouse adiponectin ELISA cat nummer EA2500-1-A5, from the firm Biocat.

To determine glucose tolerance, glucose was measured in samples obtained by tail bleeding before and 15, 30, 60, 120 and 240 minutes after intra-peritoneal glucose administration (20 % glucose solution; 2 g/kg). Total cholesterol and triglycerides were measured using standard enzymatic colorimetric assays (Boehringer Mannheim).

2.2.2. Echocardiography

Transthoracic echocardiography of 24-week C57BL6, placebo and PPAR agonist-treated DKO mice, was performed with the use of a Philips SONOS 5500 with a 5-12 MHz S12 neonatal ultra band cardiac phased probe [Yang et al, 1999] . Mice were anesthetized with pentobarbital and put on a heating pad to maintain body temperature. Heart rate was measured in M-mode. The fractional area change, a measure for the ejection fraction (EF), was calculated from the left ventricle (LV) cross-sectional area (2-D short axis) using the equation: EF (%) = ((LVDA – LVSA)/LVDA) x 100, wherein LVDA is the LV diastolic area and LVSA is the LV systolic area.

Shortening fraction (SF), a measure of LV systolic function, was calculated from the M-mode LV dimensions using the equation: SF (%) = ((LVEDD- LVESD)/LVEDD) x 100, wherein LVEDD is the LV end diastolic diameter, and LVESD is the LV end systolic diameter.

The left-ventricular mass (LVmass) was calculated from LVEDD, the inter ventricular septum thickness (IVSTh) and the posterior wall thickness (PWTh) all measured in M-mode using the equation:

LVmass = (1.055[(IVSTh + LVEDD + PWTh) ^{3} - (LVEDD) ^{3}].

In this formula, 1.055 is the specific gravity of the mouse myocardium.

Representative images of echocardiography are given in figure 2.3.

2.2.3. Histological analyses

2.2.3.1. Slice preparation

After dissection, the hearts were put in physiological saline. They were put in Tissue-Tek (Einbettmedium, of Jung [Leica microsystems]). A syringe was used to inject Tissue-Tek in the heart. Hearts were put in plastic cups of 5 mL with Tissue-Tek and these plastic cups were then placed in 2-methylbutan and frozen in liquid nitrogen. They were stored at −20°C. They were cut in sections (thickness: 7 μm). The slices were stored at -80°C.

2.2.3.2. Histological staining

Three stainings were used on different slices: one classic staining, to measure atherosclerosis, and three immune-stainings, to detect the ox-LDL, the macrophages and the SMC.

a) Oil red O

This staining visualizes the lipids in the arterial wall. We measured the area of the atherosclerosis plaque on these slices.

The slices were rinsed with distilled water, and they were incubated 18 min in oil-red-O. After 3-times washing with distilled water, they were counterstained in Harris Haematoxyline (the slices are incubated 30 sec). Harris Haematoxyline colors the nucleus and the basophilic tissues in dark blue. The slices were washed with distilled water and glycerol/gelatin was added to fix the cover glass.

b) ox LDL

This staining visualizes ox-LDL.

The slices were incubated 5 min in TBS-buffer, then 45 min in 10 % goat serum, diluted in TBS with 2 % bovine serum albumin (BSA), for the saturation of the non-specific binding sites. The slices were incubated overnight with the primary antibody (monoclonal antibody), against mice ox-LDL (Ox4E6, 1 μg/ml). After 3-fold washing for 5 min with TBS-buffer, slices were incubated one hour with the secondary antibody, against mice antibodies (goat antibodies, marked with alkaline phosphatase, GAM/AP, 1 μg/ml). Slices were washed 4 times (3-times with TBS, 1 with Tris) and then fuchsin substrate was added during 12 min. Levamisol (Dako) was added in the fuchsine substrate in order to inhibited the endogenous alkaline phosphatase, according to the recommendation provided in the Dako kit protocol, Fuchsin Substrate-Chromogen System for Immunohistochemistry. The conterstaining is a Harris Haematoxylin and the coverslip was fixed with glycerol/gelatin.

c) Macrophages

It is almost the same process than for ox-LDL staining, but the serum used was rabbit serum, but here a step more was done with a biotinylated secondary antibody, to amplify the signal. The primary antibody was Mac3 (Pharmingen, 10 μg/ml), a rat monoclonal antibody, incubated overnight, and the secondary antibody: RaRa/B (Dako, 5 μg/ml), anti-rat rabbit antibody biotined. After 3 rinsings, streptavidine labeled with alkaline phosphatase (Str/AP), dissolved in TNB (0.1 M Tris-HCl, 0.15 M NaCl; 0.5 % blocking reagents from TSA Biotin System Kit from PerkinElmer Life Sciences, Inc. pH 7.5) was added in TNT (0.1 M Tris-HCl, 0.15 M NaCl; pH 7.5; 0.05 % Tween(R)20). After one washing with TBS buffer, 2

Figure 2.4: RNA profile with the Agilent bioanalyzer

Z925 Adipose tissue

Corrected RNA Area: 197.95   rRNA Ratio [28S / 18S]:   0.00
RNA Concentration: 110.86 ng/ul
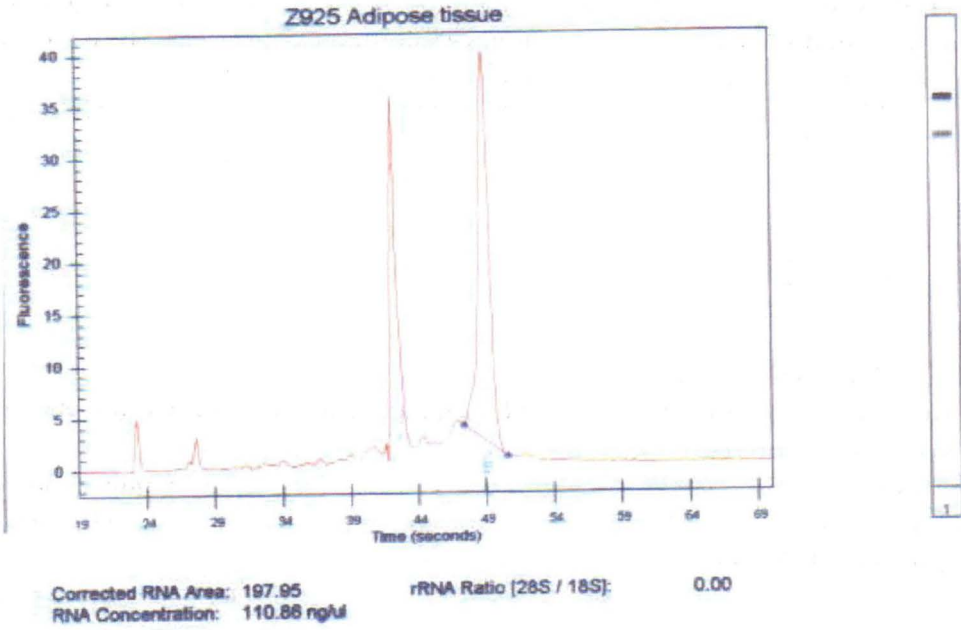
#	Name	Start Time (s)	End Time (s)	Corr.Area	% of total Area
1	18S	47.35	50.55	72.75	38.75

washings with Tris, the slices were coloured with fuchsine substrate and then with Harris Haematoxylin.

d) SMC

The same protocole than for ox-LDL staining was applied, but the primary antibody Klon 1A4 (70 µg/ml, dilution 1/300), a mice monoclonal antibody, recognized SMC-α-actin. The secondary antibody is GAM-APHRP goat (0.2 µg/ml), peroxidase conjugated, and the substrate is DAB (3'-diaminobenzidine-tetrahydrochloride-dihydrate: Sigma powder, 25 mg powder in 200 ml Tris buffer).

2.2.3.3. Measurement of atherosclerosis

The extent of atherosclerosis was determined by analysis of cross-sections from the aortic root between the beginning and the end of the valves. Approximately 10 7-µm frozen sections per animal were used for morphometric and immunohistochemical analysis. Blinded analysis of positive immunostained sections was performed with the Quantimet600 image analyzer (Leica) [Mertens et al, 2003]. The same process was used to measure the SMC, the macrophages and the ox-LDL.

2.3. Real Time RT-PCR

2.3.1. RNA extraction

Total RNA was extracted from each tissue using the Trizol reagent (Invitrogen) and purified on an RNeasy kit column (Qiagen). First strand cDNA was generated from total RNA by reverse transcription using random primers from Takara and Superscript III reverse transcriptase (Invitrogen).

2.3.2. Control of RNA quality

The Agilent bioanalyzer was used to control RNA quality. The protocol is described in the agilent site (http://www.chem.agilent.com). A graph obtained with good quality RNA is given in figure 2.4.

2.3.3. RT-PCR

Quantitative real time (RT) PCR was performed using Sybr®Green master mix according to the supplier protocols (Applied Biosystems). Oligonucleotides (Invitrogen) used as forward primer (F) and reverse primer (R) are described in the following paragraph. The mRNA abundance for PPARα, PPARγ and PPARδ in different tissues from C57BL6, control and PPAR agonist-treated DKO mice at 24 weeks was estimated by quantitative real time RT-PCR.

The abundance of mRNA for PPARα, PPARγ and PPARδ was calculated using the threshold cycle (Ct) value, i.e. the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold. β-actin was choosen as housekeeping gene. For each sample, both the Ct for the gene of interest and for the housekeeping gene β-actin were determined to calculate ΔCt, thus normalizing the data and correcting for differences in amount and/or quality between the different RNA samples.

ΔCt(sample) = Ct(target gene) – Ct(housekeeping gene)
The expression levels were related to an external calibrator consisting of intra-abdominal adipose tissue from C57BL6 control mice. Subsequently, ΔΔCt was determined:
ΔΔCt = ΔCt(sample) – ΔCt(calibrator)

The relative expression levels were calculated from 2–ΔΔCt, according to the manufacturer's instructions (Applied Biosystems). The mRNA expression abundances are indicated as arbitrary units ± SD [Verreth et al, 2004].

Primer used: For RT-PCR:

➢ - **For mouse PPARα:**
  F: 5'- TCAGGGTACCACTACGGAGTTCA -3';
  R: 5'- CCGAATAGTTCGCCGAAAGA -3';

➢ - **For mouse PPARγ:**
  F: 5'- GCAGCTACTGCATGTGATCAAGA -3';
  R: 5'- GTCAGCGGGTGGGACTTTC -3';

➢ - **For mouse PPARδ:**
  F: 5'- CCCCGGTGTAGCCATGAC -3';
  R: 5'- CTCACAGATCACCAGTCCTAAGAACA -3';

➢ - **For mouse β-actin:**
  F: 5'- ACGGCCAGGTCATCACTATTG -3';
  R: 5'- CACAGGATTCCATACCCAAGAAG -3'.

2.4. Statistical analysis

Groups were compared using the Kruskal-Wallis test (Graph Pad Prism version 3.02) followed by Dunn's Multiple Comparisons test. Correlations were calculated using the non-parametrical Spearman correlation coefficient. A P-value of less than 0.05 was considered statistically significant.

3. Results

Figure 3.1: Chronology of the experiments
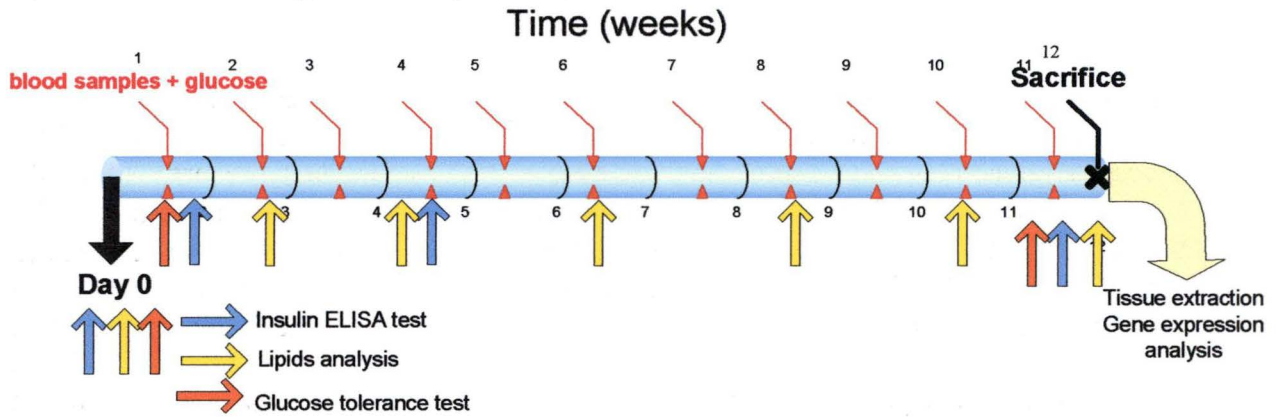
Figure 3.2: Weight change as a function of time in PPAR-treated mice and in placebo mice.
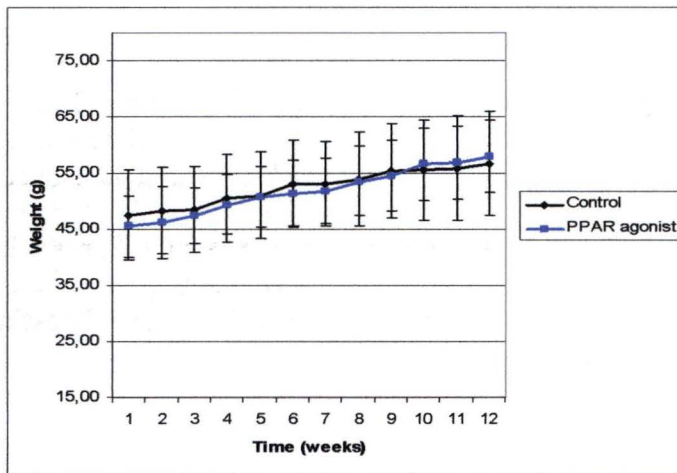
n treated = 9
n control (3) + n placebo (7) = 10 controls
mean is given +/- standard deviation

Figure 3.3: Blood glucose concentration as function of time in PPAR-treated mice and in placebo mice.
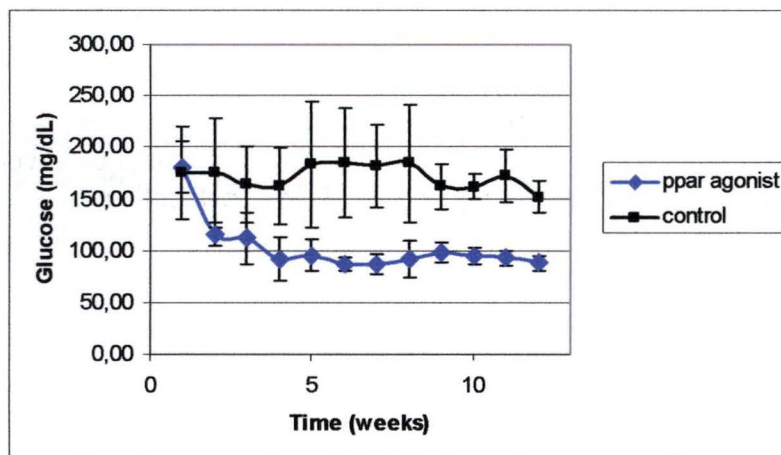
n treated = 9
n control (3) + n placebo (7)
= 10 controls
mean is given +/- standard deviation

The glucose concentration is measured with a glucometer (Menarini Diagnostics).

3. Results

The CEHA laboratory is involved in several intervening studies in LDL-/-, Ob/Ob mice (DKO mice). In these DKO mice, diet restriction leads to weight loss, and decrease of blood glucose, triglycerides, cholesterol and atherosclerotic lesions. Gene expression analysis showed an overexpression of PPARα and PPARγ. We thus treated these mice with a PPARα/γ dual agonist. Several physiological parameters were measured: blood parameters (concentrations of glucose, insulin, adiponectin, triglycerides and cholesterol) and cardiovascular parameters (echocardiography for monitoring the heart function and histological analysis for estimating the atherosclerotic lesion extension). Gene expression was also studied, to analyze the tissue-specific action of the dual agonist and to link the physological data with molecular data. Our study was focused on adipose tissue, while other tissues were investigated by Wim Verreth (unpublished results).

3.1. Chronology of the experiments

The mice were treated with the PPAR α/γ dual agonist or placebo each day during 12 weeks starting at the age of 12 weeks, as described in the paragraphs 2.1.1, of the Materials and Methods. To study the metabolic effects of the drug in the mice, blood was taken once a week. Different analyses were done on the collected plasma, at different intervals. A plan of the experimental setup is given in figure 3.1. The tissues used for gene expression studies were taken the last day (sacrifice). Two PPAR-agonist treated mice died during the 4th week of experiment. The cause of the death is unknown, but they seemed to have intestinal problems: the large intestine was open before dissection.

3.2. Systemic analysis

3.2.1. Weight

Mice treated with the PPAR dual agonist and control (placebo included) mice show similar weight profiles as a function of time (figure 3.2). The treatment had no effect on weight in DKO mice.

3.2.2. Blood parameters

To have a better insight of the possible effects of the PPAR agonist, we investigated several blood parameters as a function of time, in relation with glucose and lipid metabolism.

Figure 3.4 Glucose tolerance test (GTT).
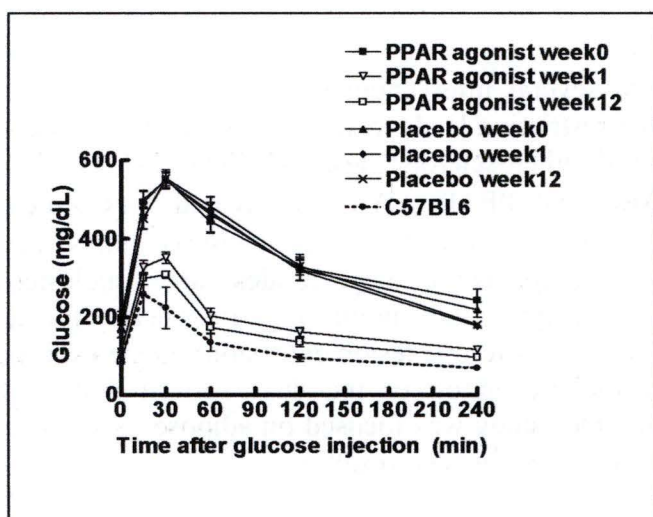
n treated = 9
n placebo = 10
n C57BL6 = 10
mean is given +/- standard deviation.

The glucose concentration is measured with a glucometer (Menarini Diagnostics) at times (min): 0; 15; 30; 60; 120; 240.
Time 0: After measuring fasting glucose concentration, intra-peritoneal glucose administration (20 % glucose solution, 2 g/kg).

Figure 3.5: Insulin in PPAR agonist-treated DKO mice at times 0, 1, 4, and 12 weeks.
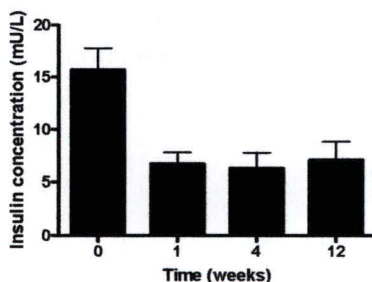
n = 9
mean +/- standard deviation

Insulin concentration was measured with an insulin ELISA (Mercodia test).

Figure 3.6: HOMA in PPAR agonist-treated DKO mice at times 0, 1, 4 and 12 weeks.
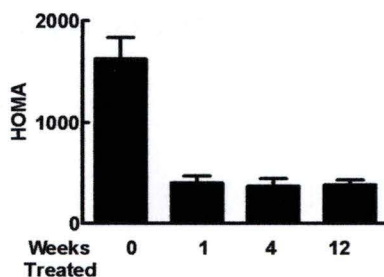
n = 9
mean +/- standard deviation

HOMA = fasting serum insulin (mU/L) * fasting blood glucose (mmol/L) / 22.5.

3.2.2.1. Glucose metabolism

a) Glucose concentration

At 12 weeks, DKO mice had high blood glucose (153 +/- 24 mmol/L). The glucose concentration decreased with 45 % in the first week of the treatment. During the rest of the treatment, the blood concentration stayed at this low level (84 +/- 16 mg/dL) (see figure 3.3). The glucose tolerance test (GTT) shows the ability of the body to cope with a sudden increase of blood glucose. The DKO mice have a low capacity to extract the glucose from the blood. The glucose concentration stays high (322 +/-86 mg/dL), even at 120 min after the injection (see figure 3.4). The rate of glucose decrease was comparable between non-treated DKO mice, at time 0 (12 weeks old mice) and at time 12 (24 weeks old mice). The treatment restored glucose tolerance: after 12 weeks of treatment, glucose tolerance in PPAR-agonist treated DKO mice was improved. It is similar to that in normal mice (C57BL6).

b) Insulin concentration

The decrease of insulin also occurred after one week (from 15 +/- 6.8 µg/mL to. 6.9 +/- 3.7 µg/mL). Thereafter, this parameter did not change (figure 3.5).

c) HOMA

The HOMA (homeostasis model assessment) is a function of the concentration of glucose and insulin and is an indicator of insulin resistance. A decrease of the HOMA shows that the insulin sensitivity is increased. The HOMA decreased with 77 % in the PPAR-agonist treated mice after one week of treatment and remained stable until the end of the treatment (figure 3.6). These data confirm the results obtained with the glucose tolerance test (figure 3.4).

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Figure 3.7: Effect of the PPAR-agonist on lipid metabolism.

PPAR agonist = PPAR agonist-treated mice
means are given +/- standard deviation

A) Cholesterol concentration as a function of time
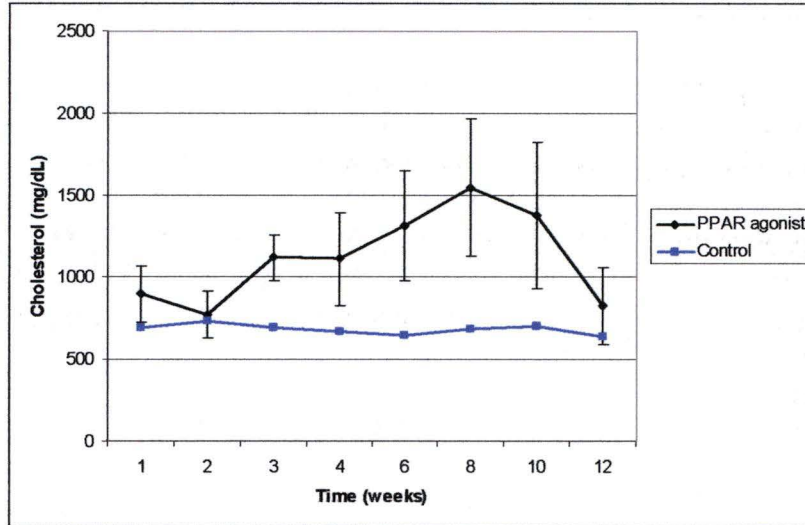
B) Triglycerides concentration as a function of time
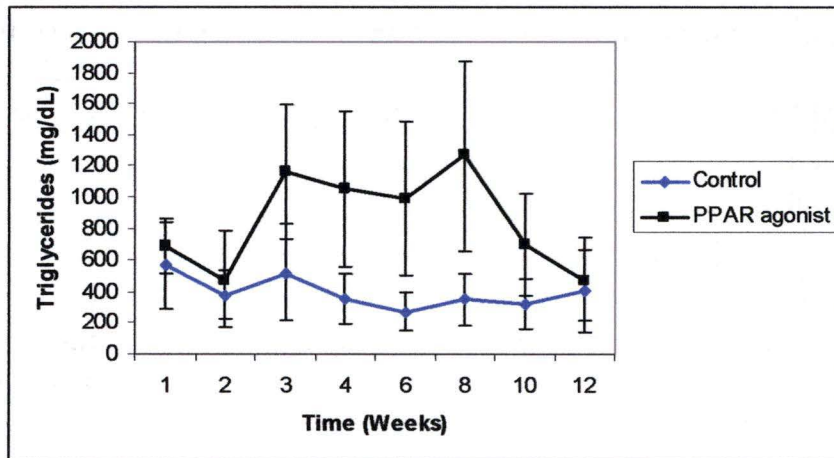
Figure 3.8: Adiponectin blood concentration in placebo and PPAR agonist treated DKO mice.
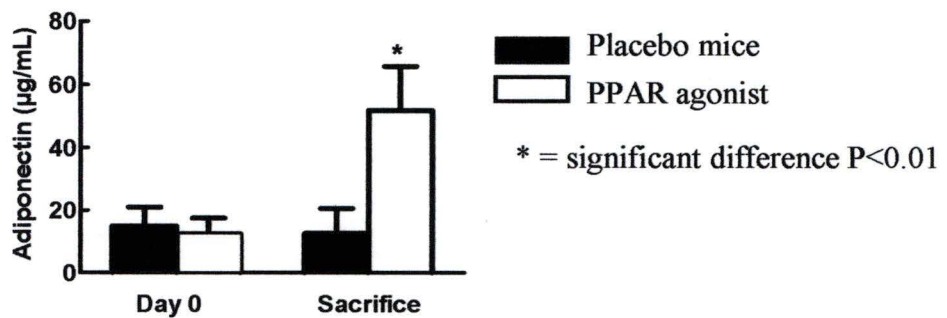
3.2.2.2 Lipids and adiponectin

We further investigated the possible effects of the dual agonist on lipid metabolism. Therefore, triglycerides (TG) and cholesterol were measured in PPAR agonist-treated and in control mice. Adiponectin, an adipokine lower in obese and diabetic patients (as discussed in 1.2.2), was also monitored by ELISA.

a) Lipids

TG and cholesterol are higher in DKO mice, compared to C57BL6 mice (Verreth et al., 2004). However, the agonist did not reduce TG and cholesterol levels even after 12-weeks of treatment: there are no significant differences between the control and the PPAR-agonist treated DKO. (See table 3.1). By contrast, there was even an unexpected increase of the TG and the total cholesterol in the PPAR-agonist treated mice between the weeks 3 to 8. Thereafter, TG and cholesterol levels decrease towards control values (figure 3.7).

Table 3.1: Triglycerides and cholesterol concentrations in PPAR agonist-treated and placebo mices.

	day 0		day 84	
	Cholesterol (mg/dL)	TG (mg/dL)	Cholesterol (mg/dL)	TG (mg/dL)
PPAR treated	895 +/- 174	557 +/- 272	822 +/- 234	477 +/- 266
Placebo	686 +/- 182	566 +/- 272	629 +/- 255	400 +/- 294

b) Adiponectin

The adiponectin is an adipokine present in lower rates in obese and diabetic patients (as discussed in 1.2.2.1). Adiponectin levels increased 4.3-times after a 12-week treatment with the agonist (52 +/- 14 µg/mL vs. 12 +/- 4.8 µg/mL) (see figure 3.8). Adiponectin concentration in placebo mice was the same on day 0 and at sacrifice.

Table 3.2: Effects of the PPAR-agonist on heart characteristics, estimated from images obtained by echocardiography.

Characteristic	C57BL6	DKO placebo	DKO PPAR agonist	ANOVA P
Heart rate (bpm)	406±57	455±83	407±61	NS
Septum thickness (mm)	1.00±0.15	0.99±0.22	1.02±0.14	NS
Posterior wall thickness (mm)	0.99±0.07	1.03±0.28	1.15±0.21	NS
LVED diameter (mm)	2.72±0.49	3.66±0.65 ‡	3.72±0.59 †	<0.0001
LVES diameter (mm)	1.60±0.29	2.66±0.58 ‡	2.18±0.35	<0.0001
LV mass (mg)	93±29	152±58 *	162±33 *	<0.001
LVED area (mm²)	4.16±1.02	4.65±1.11	4.82±1.40	NS
LVES area (mm²)	2.07±0.64	2.86±0.70 †	2.49±0.74	<0.05
EF (%)	50±6	38±6 ‡	48±5 §	<0.0001
SF (%)	40±7	28±7 *	41±3 §	<0.0001

Data are mean±SD. *$P<0.05$, †$P<0.01$ and ‡$P<0.001$ compared to C57BL6; §$P<0.01$ compared to placebo DKO.
(LV= left ventricular; LVED = left ventricular end diastolic; LVES = left ventricular end systolic; EF= ejection fraction; SF= shortening fraction)

3.3 Organ and tissue analysis

In a second part, we wanted to confirm the effects of the PPAR-agonist at the level of organs and tissues that are affected in the metabolic syndrome. That is why heart and adipose tissue were selected in this work. Other tissues were analyzed by Wim Verreth.

3.3.1. Heart: echocardiography

In order to evaluate the cardiac function, we used heart echocardiography, with a particular attention on left ventricular (LV) function (as explained in section 2.2.2 of the Materials and Methods). Representative images obtained by echocardiography are shown in the methods (See figure 2.3). Table 3.2 summarizes the data obtained with this method, with the corresponding statistical analysis.

At 24 weeks, C57BL6 lean mice and DKO placebo mice had comparable LV mass, heart rate, septum and posterior wall thickness. However, SF and EF were significantly lower in DKO mice compared to lean mice indicating that their LV function is impaired.

Compared to placebo DKO, PPAR agonist-treated DKO mice had higher EF and SF, the latter as result of a lower LVES diameter. These data show that the PPAR agonist improved heart function in DKO mice.

The comparison between C57BL6 lean mice and PPAR agonist treated DKO mice showed that the agonist restored LV function. The EF and the FS are comparable in these two groups of mice.

Figure 3.9: Histological analysis of the aortic root from PPAR-agonist treated and control mice

A) Representative section in the aortic root of a DKO mice stained with Oil-Red-O to visualize plaque volume and counterstained with Harris Haematoxilin.
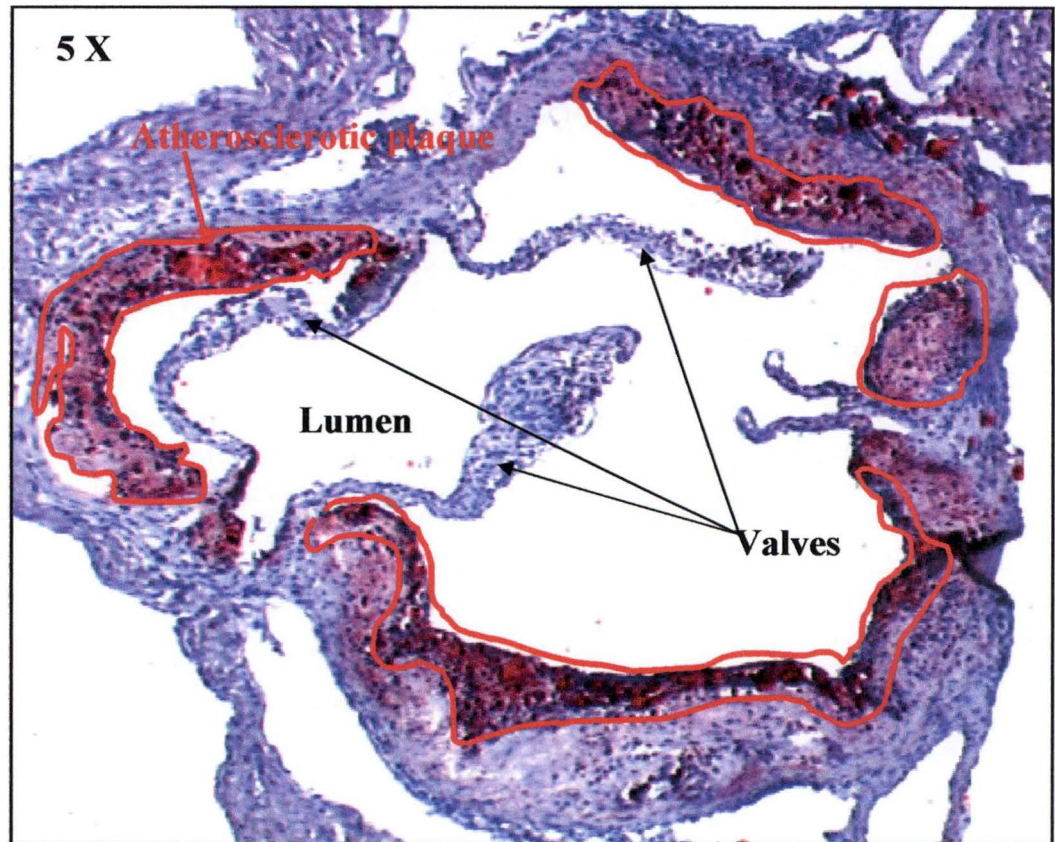
B) Estimation of plaque volume in PPAR agonist-treated and control mice.
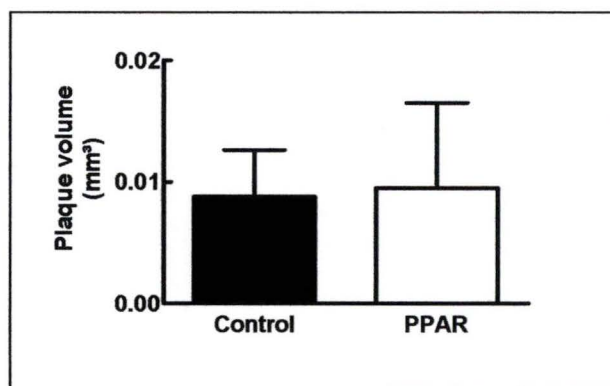
Plaque volume was estimated by measuring the lesion area (circled in red) in serial sections. Data are given +/- standard deviation.

3.3.2. Aortic root: histology and immunostaining

After sacrifice (figure 3.1), hearts were dissected and treated for histological staining and immunostaining, on 7 μm cryo-sections (see paragraph 2.2.3.1 of the Materials and Methods). Representative sections of the aortic root of DKO mice are illustrated in figure 3.9. These sections were analyzed using an image analyzer to estimate the lesion volume (see Section 2.2.3.3 of the Materials and Methods).

There was no significant difference in plaque volume between PPAR-agonist treated mice and control mice (0.082+-0.037 mm³ vs. 0.074+-0.067mm³ respectively, figure 3.7 A). The improvement of the heart function, evidenced by echocardiography, is thus not a consequence of a decrease of atherosclerosis.

We also monitored the abundance of ox-LDL, macrophages and SMC in the lesion. Surprisingly, the levels of ox-LDL (figure 3.10 A) and macrophages (figure 3.10 B) were higher in the PPAR agonist-treated mice, but the SMC content (figure 3.10 C) was similar in both groups. Increase of ox-LDL and macrophages could make the plaque more instable.

Figure 3.10: Comparative immunostaining of the aortic root from PPAR-agonist-treated and control mice.

Sections were immunostained for ox-LDL (A), and macrophage (B), and counterstained with Harris hematoxylin. In (C), section was immunolabelled for smooth muscle cells (SMC), without counterstaining. For more information about the staining, see part II (methods).

Each image is given with the corresponding quantitative data obtained with the image analyzer (mean +/- standard deviation).
PPAR = PPAR agonist-treated mice.
* = significant difference, P<0.05

A)

10 X

%Ox-LDL in plaque surface

Control   PPAR

*

B)

10 X

%Macrophages in plaque surface

Control   PPAR

*

C)

10 X

%SMC in plaque surface

Control   PPAR
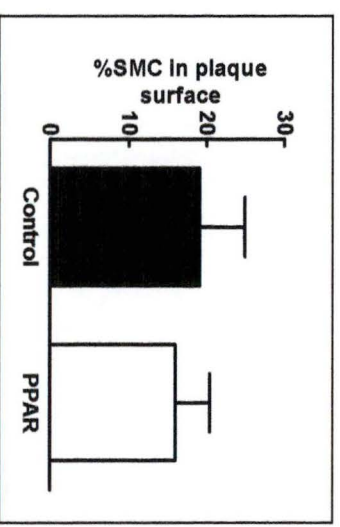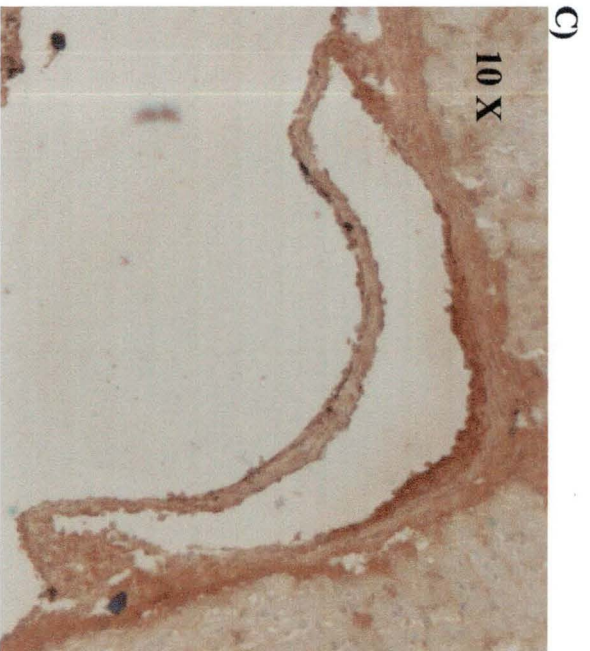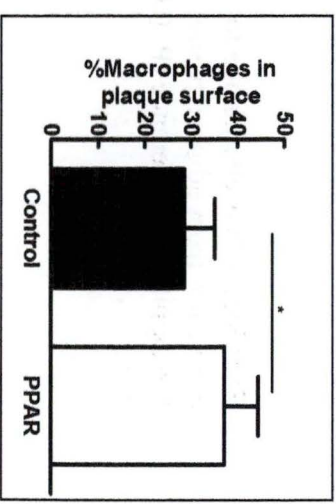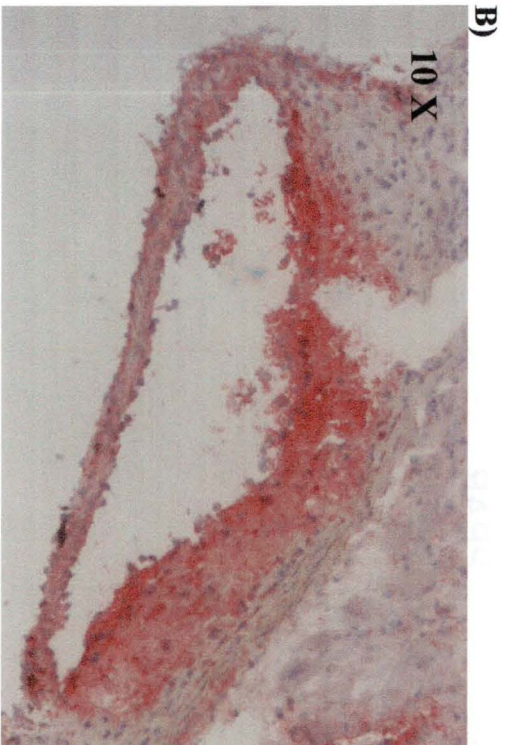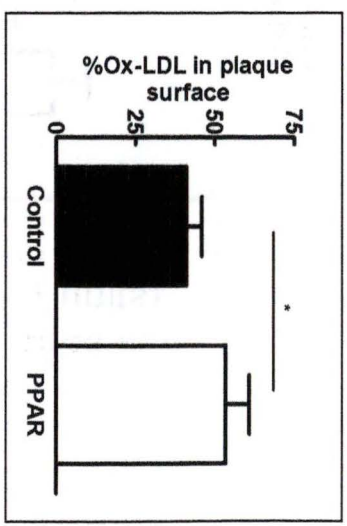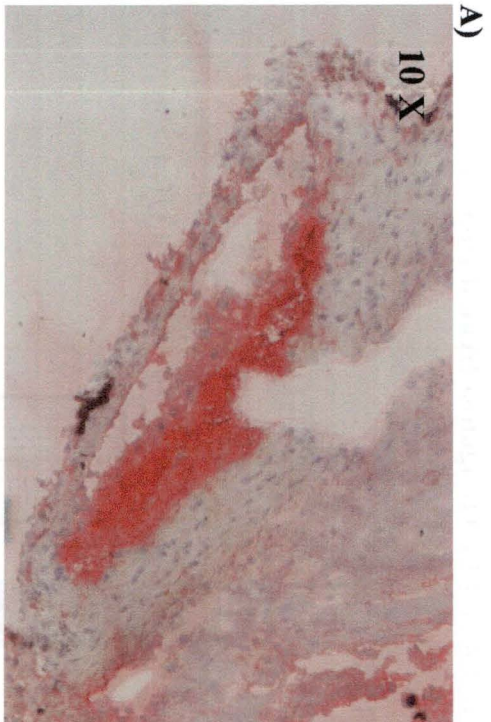
Figure 3.11: Gene expression in placebo and PPAR agonist treated DKO mice.
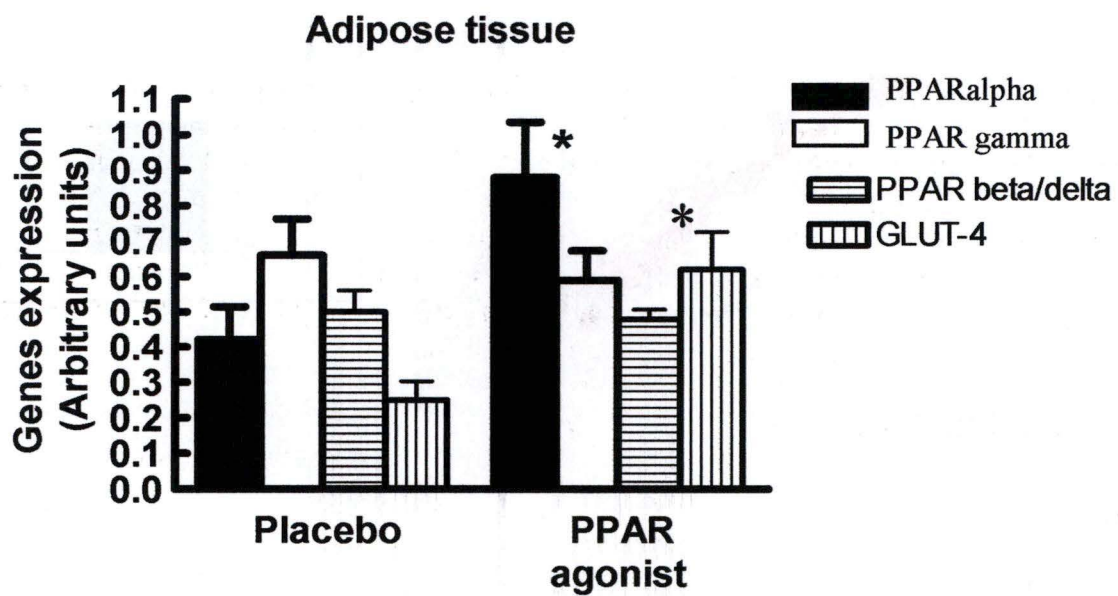
Adipose tissue

Relative mRNA abundance for 4 genes, obtained with real time RT-PCR after RNA extraction from adipose tissue.
* = significant difference (P<0.05)

3.4. Adipose tissue: gene expression study

In the context of this investigation, gene expression analysis has also been undertaken using high density DNA microarrays (Wim Verreth's thesis). To complete the biochemical and physiological data presented here above, we undertook a gene expression study limited to the genes encoding the different PPAR isoforms. PPARβ/δ share some functions and ligands with PPARα and PPARγ. It could compensate partially the lack of one of the other isoforms (see part 1.3.5), and then hiding some effects. Besides this possible compensating effect, dual agonists have been shown to have tissue specific effects [Hegarty et al, 2004]. Glut-4 is also important, given its role in extracting glucose from the blood.

3.4.1. Real time RT-PCR on PPARs

Real time RT-PCR was used to measure relative PPARα, β/δ, γ, mRNA expression (figure 3.9).

The RT-PCR on PPAR was necessary to prove the specificity of the dual agonist in adipose tissue (figure 3.11). PPARα mRNA was twice more abundant in the PPAR-treated mice compared to the placebo mice (ratio mRNA PPARα / mRNA housekeeping gene (β-actin) = 0.88 +/- 0.41 in the PPAR agonist-treated mice, versus. 0.41 +/- 0.25 in the placebo mice). PPARγ expression was not significantly modified (placebo: 0.66+/-0.27;PPAR agonist-treated:0.59+/-0.22. There were no effects on PPARβ/δ expression (placebo: 0.50 +/- 0.16; PPAR-treated: 0.48 +/- 0.07).
Wim Verreth has also analysed the PPAR expression in other tissues (liver, aortic arch, skeletal muscle, heart). The changes of PPARα and PPARγ expression are not the same in all the tissues, but the agonist never had an effect on PPARδ expression. We will discuss these results in the discussion because they give us a better insight in the improvement of the heart function, revealed by echocardiography.

3.4.2. RT-PCR on GLUT-4

GLUT-4 is an insulin responsive glucose carrier: fixation of insulin on its receptor leads to the transport of GLUT4 from an intracellular location to the plasma membrane where GLUT4 facilitates entry of glucose into the cell, according to the glucose gradient. Analysis of the expression of this gene showed a 2.5-fold increase in PPAR-agonist treated mice (0.62 +/- 0.28) compared to placebo mice (0.25 +/-0.14) (figure 3.11). The increase in GLUT-4 expression is one of the mechanisms which could explain the improvement of the insulin sensitivity after agonist treatment.
Other genes were previously studied in micro-array analyses performed in our laboratory. We will mention the main changes in gene expression observed after PPAR-agonist treatment in the discussion (Table 4.2), because they give us a better insight in the changes in glucose tolerance and insulin sensitivity.

Table 3.3: Summary of the metabolic and functional effect of the dual PPAR agonist at 24 weeks.

	C57BL6	Placebo DKO mice	PPAR agonist treated DKO mice
Obesitas	-	+++	+++
Insulin resistance	-	+++	+
Dyslipidemia	-	++	++
Atherosclerosis	-	++	++
Heart function	very good	impaired	good

3.5. Summary of the results

The PPARα/γ dual agonist improved insulin sensitivity and thus restored glucose tolerance in one week. After 12 weeks of treatment, the heart function of PPAR agonist-treated mice was better than the heart function of placebo mice, without a detectable decrease in TG, cholesterol or atherosclerosis. The main changes in physiological parameters are summarized in table 3.3

4. Discussion

Table 4.1: Effect of the dual PPAR agonist on the expression of PPAR isoforms in DKO mice tissues.

	PPARα	PPARγ	PPARβ/δ
Heart	=	+	=
Skeletal muscles	=	+	=
Adipose tissue	+	=	=
Liver	=	+	=
Aorta	+	=	=

= : Expression comparable to placebo-treated DKO mice
+: Higher expression than in placebo-treated DKO mice
- : Lower expression than in placebo-treated DKO mice

4. Discussion

In our laboratory, Verreth et al (2004) have shown that the DKO mice used in this work, when diet restricted, showed a significant weight loss, reduced atherosclerosis and improved cardiovascular function. Diet restriction also caused a significant upregulation of PPARα and PPARγ, as well as of several genes under the transcriptional control of these PPARs. These genes are involved for instance in the regulation of glucose transport, insulin sensitivity, lipid metabolism, oxidative stress, and inflammation.

Given these beneficial effects of the upregulation of the PPARs, an intervening study with a dual agonist of PPARα and PPARγ was started in the laboratory, and this thesis was part of this intervening study. In the following discussion, we will try to integrate our data into the global set of data obtained in the laboratory (unpublished results).

4.1. Specificity of the PPARα/γ dual agonist

4.1.1. Expression in different tissues

In previous studies PPAR agonists have been shown to exert tissue-specific actions [Hegarty, 2004]. Therefore, a previous study in the lab determined the effect of the agonist on PPARα and PPARγ expression in different tissues (table 4.1); [unpublished data].
The agonist increased the expression of PPARα in the adipose tissue (confirmed in this work) and the aorta, but not in the heart and the liver. It increased PPARγ expression solely in skeletal muscle and the heart.
These data demonstrate tissue-specific direct actions of the PPAR agonist. The lower PPARγ expression in the liver of agonist-treated mice is most probably an indirect effect of the improved insulin sensitivity.

4.1.2. Compensation by PPARβ/γ

Because PPARβ/δ expression might compensate for variations in PPARα and PPARγ expression [Muoio, 2002], we also looked at the expression of PPARβ/δ. The agonist had no effect on PPARβ/δ expression. Thus, we can exclude any compensating effects of PPARβ/δ overexpression.

Figure 4.1: Correlation between PPARγ expression in the muscle and HOMA.
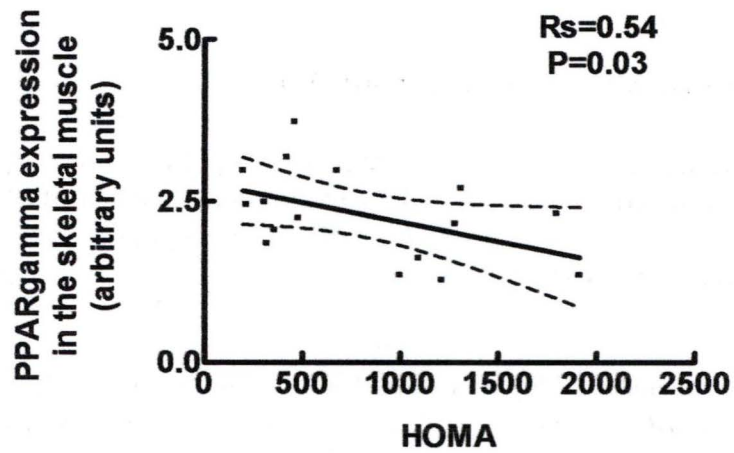
Figure 4.2: Correlation between PPARγ expression in heart and shortening fraction.
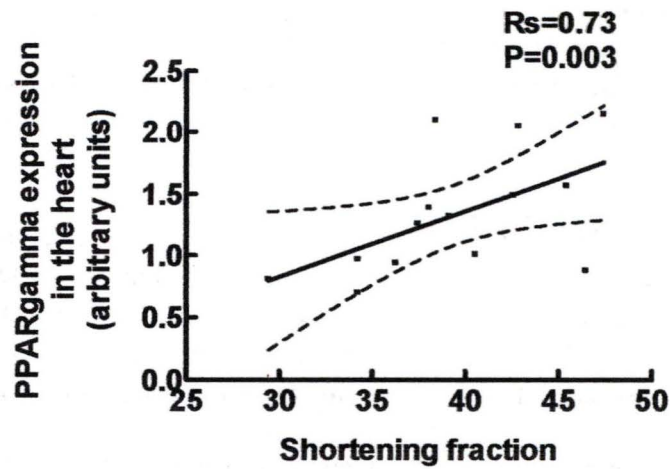
4.2. Insulin sensitivity and glucose metabolism

4.2.1. Insulin sensitivity in the muscle

The muscle is the primary organ for insulin-stimulated glucose disposal, leading to the theory that *in vivo* insulin resistance cannot occur without a defect in muscle. A decrease in the number of insulin receptors, impaired glucose transport and utilization, and decreased basal glucose uptake by skeletal muscle, all play a role in the development of insulin resistance of muscle in Ob/Ob mice.

Disturbances in lipid metabolism, particularly increased availability of circulating lipids and their accumulation within skeletal muscle and heart, are strongly associated with the development of insulin resistance and associated diseases of the metabolic syndrome (obesity, type-2 diabetes, cardiovascular disease) (See part 1.2). The general concept is that the ability of PPAR agonists to up-regulate lipid metabolism in adipose tissue and liver, is central to their insulin-sensitizing effects. In this work, we show that PPAR agonist treatment increased, however, the insulin sensitivity of DKO mice in the absence of an effect on circulating lipids.

Despite relatively low mRNA expression, there are significant protein levels of PPARα and PPARγ in skeletal muscle. The muscle-specific disruption of PPARγ, resulted in a state of severe insulin resistance [Hevener et al, 2003]. Activation of PPARγ by rosiglitazone corrected impaired muscle insulin action [Zierath et al, 1998]. Furthermore, rosiglitazone treatment led to increased muscle insulin-stimulated glucose uptake in Ob/Ob mice, irrespective of muscle TG content. In our laboratory, it was shown that the higher levels of PPARγ expression in the skeletal muscles and in the heart of dual agonist-treated DKO mice, correlated negatively with the HOMA index (figure 4.1).

In the aggregate, our data suggest that the agonist exerts its effect on insulin resistance directly in the skeletal muscle and the heart of DKO mice via upregulation of PPARγ. Furthermore, upregulation of PPARγ in the heart also correlated with the shortening fraction, indicating the importance of this transcription factor in improving cardiac function (figure 4.2).

Next to the effect on insulin sensitivity, PPARγ effects in the heart could also occur through infiltrating macrophages and other blood-borne cells, or could be the result of endocrine actions mediated indirectly by other organs through hormonal effects (See part 1.3.4).

Gilde et al.[2003] showed that PPARα and PPARβ/δ, but not PPARγ, modulate the expression of genes involved in cardiac lipid metabolism. PPARα has a role in the metabolic remodeling of the heart known to occur in several physiological (fasting) and pathophysiological (diabetes, hypertrophy, heart failure and cardiomyopathy) conditions. It is a critical regulator of myocardial fatty acid uptake and utilization. The activation of cardiac PPARα regulatory pathways results in a reciprocal repression of glucose uptake and utilization pathways. (See part 1.3.3). PPARβ/δ plays a role in fat burning in the heart (See part 1.3.5).

Table 4.2: Micro array results. Gene expression in the visceral adipose tissue of PPAR agonist treated compared to placebo-treated DKO mice.

Systematic Name	Gene Name	PPAR agonist-treated/Placebo
NM_008509	Lipoprotein Lipase	0.56
NM_011125	Phospholipid transfer protein	1.6
2310011D22	Fatty acid transporter-4	4.2
NM_007616	Caveolin-1	0.27
NM_010719	Hormone sensitive lipase	3.9
NM_009204	Glucose transporter-4	1.8
BC002148	Fatty acid binding protein-4, adipocyte	2.2
NM_010517	Insulin-like growth factor binding protein-4	2.4

Figure 4.3: Correlation between GLUT-4 expression in adipose tissue and HOMA.

$R_s=-0.65$
$P=0.016$
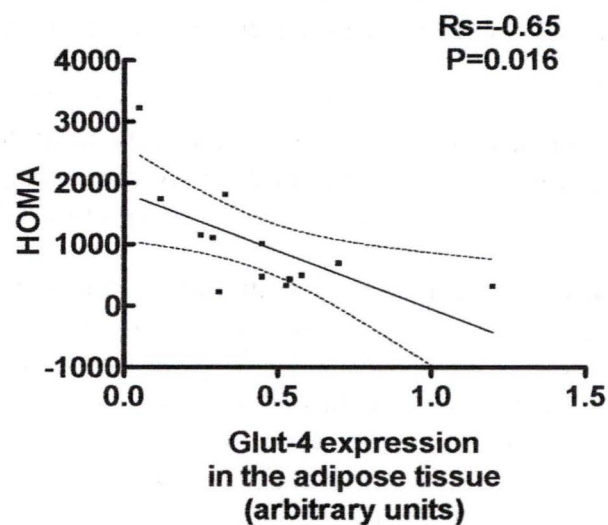
Both PPARα and PPARβ/δ expression in the heart of placebo-treated DKO mice were not different from that in lean C57BL6 mice. The dual agonist had no effect on their expression in the heart of DKO mice, despite the agonist improved cardiac function. This data suggest that PPARα and PPARβ/δ are not important for regulating cardiac function in DKO mice.

4.2.2. Role of the adipose tissue in regulating insulin sensitivity

4.2.2.1 PPARγ

One hypothesis was that PPARγ could directly regulate glucose homeostasis in the adipose tissue. Agonist treatment, however, did not increase PPARγ expression in the adipose tissue of DKO mice. The effect of the dual agonist on insulin sensitivity was thus independent of PPARγ expression in the adipose tissue. As seen before, muscle was the main tissue where PPARγ was acting to reduce insulin resistance.

4.2.2.2. PPARα

Although PPARα has mostly been connected with fatty acid metabolism, it could also affect glucose homeostasis (see part 1.3.3). PPAR agonist treatment resulted in an upregulation of PPARα in the adipose tissue. We observed simultaneously that HOMA and plasma glucose levels were decreasing, indicating an improvement of insulin sensitivity. This demonstrated that PPARα upregulation in the adipose tissue could have an effect on insulin sensitivity.

4.2.2.3. Other genes

Gene expression in the adipose tissue of agonist-treated DKO mice was previously compared with that in placebo-treated mice on high affinity DNA micro arrays, to further identify genes in the adipose tissue that could be involved in regulating insulin sensitivity. (See table 4.1)[unpublished data, Wim Verreth].

a) Genes with higher expression

There are five main genes with higher expression in the fat of the PPAR agonist-treated mice. The comparative values are given in table 4.2. They include genes involved in glucose uptake and lipid metabolism. These genes code for the glucose transporter-4 (*Glut-4*), the adipocyte fatty acid binding protein-4 (*Fabp-4*), the phospholipid transfer protein (*Pltp*) the fatty acid transporter-4 and the insulin-like growth factor binding protein 4 (*Igfbp-4*).

Glut-4 is an insulin responsive glucose transporter: binding of insulin on its receptor leads to the transport of Glut-4 from an intracellular location to the plasma membrane where Glut-4 facilitates entry of glucose into the cell. Increase of *Glut-4* expression correlated with improvement of insulin sensitivity (figure 4.3). *Glut-4* is thus one of the target genes that could explain the decrease of blood glucose. It would be worthwhile to confirm this overexpression at the protein level.

Adipocyte fatty acid binding protein Fabp-4 is a 15 kDa member of the intracellular fatty acid binding protein (Fabp) family, which is known for its ability to bind fatty acids and related compounds (bile acids or retinoids) in an internal cavity. *Fabp-4* is expressed in a differentiation-dependent fashion in adipocytes and is a critical gene in the regulation of the

biological function of these cells. In mice, targeted mutations in *Fabp-4* provide significant protection from hyperinsulinemia and insulin resistance in the context of both dietary and genetic obesity.

A study [Boord et al, 2002] also demonstrated *Fabp* over-expression in macrophages upon differentiation and activation. In these cells, Fabp-4 modulates inflammatory responses and cholesterol ester accumulation, and total or macrophage specific Fabp-4 deficiency confers dramatic protection against atherosclerosis in the apoE-/- mice. These results indicate a central role for Fabp-4 in the development of major components of the metabolic syndrome through its distinct actions in adipocytes and macrophages [Gertow et al, 2004]. However, in our experiment, the increase of *Fabp-4* expression is not associated with a decrease of atherosclerosis, given that there was no significant difference in the plaque volume between control and PPAR agonist-treated mice.

The phospholipid transfer protein (*Pltp*) is elevated in diabetes mellitus (both type 1 and type 2), obesity and insulin resistance. It is involved in remodelling of HDL fractions, resulting in lowering plasma HDL levels by increasing HDL catabolism, and also in VLDL turnover. Elevation of plasma phospholipid transfer protein in transgenic mice, increases VLDL secretion [Lie et al., 2002]. High plasma Pltp is associated with increased coronary artery disease [Schlitt et al., 2003], while *Pltp* deficiency is rather protective [Schlitt et al., 2005]. The elevation of the expression of this gene in PPAR-treated mice is thus consistent with our histological data, since we observe no improvement of the atherosclerotic lesions after PPAR-agonist treatment.

The increased expression of *Igfbp-4* in the adipose tissue of PPAR-agonist treated mice is interesting. It has, indeed been shown that insulin deficiency and chronic hyperinsulinemia in diabetes upregulate the Igf-1 receptor and downregulate Igfbp-4 in the aorta and that these effects may be a major cause of the increased vascular contractility induced by insulin administration and by hyperinsulinemia in established diabetes, resulting in hypertension [Kobayashi et al, 2003]

b)Genes with lower expression

Only two genes appear as having significantly lower expression in agonist treated mice: the genes encoding lipoprotein lipase (*Lpl*) and caveolin-1 (see table 4.2).
Lipoprotein lipase is located on the luminal surface of the capillary endothelium near muscle and adipose tissue. It is the rate-limiting enzyme for the hydrolysis and clearance of circulating triglycerides (TG). It also modulates the binding of TG rich VLDL particles to the VLDL receptor. Because there is a direct relationship between FFA uptake and LPL activity, the relative activity of the enzyme determines FFA availability in muscle and adipose tissue.

With a lower *Lpl* expression, TG levels could increase. However, a mutant mice line with the same genetic background as our mice (C57BL6)) that express lipoprotein lipase in cardiac muscle, but not in skeletal muscle and adipose tissue, shows a transient increase of TG in young mice and then normal plasma TG and HDL in the adults [Levak-Frank, 1999]. This is in agreement with our data, given that we observed also an increase, followed by a decrease of TG levels (figure 3.5).

Caveolin-1 can be redirected from the cell surface to intracellular lipid droplets in various cell types and in particular in the adipocyte and is therefore is a determinant of lipolytic activity

[Cohen et al, 2004]. Indeed, electron microscopy of adipose tissue revealed dramatic perturbations in the architecture of the "lipid droplet cortex" (the interface between the lipid droplet surface and the cytoplasm) in caveolin-1 null adipocytes resulting in attenuated lipolysis and lipid droplet formation. Cav-1 deficient mice are lean, resistant to diet-induced obesity and show hypertriglyceridemia, with severe adipocyte abnormalities. The decreased level of Cav-1 expression we observed is consistent with disturbed lipid metabolism and adipose tissue function [Razani et al, 2002].

In summary, these gene expression data in the adipose tissue, although they should be confirmed at the protein level, are consistent with a disturbed systemic lipid homeostasis. This means that the dual agonist doesn't improve the lipid metabolism, at least during a12 week treatment.

4.3. Indirect Effects of the PPAR Agonist

Previously, it has been proposed that upregulation of PPARγ in the liver of Ob/Ob mice could serve as a compensating mechanism for the impaired insulin resistance in these mice. In our laboratory, Wim Verreth (unpublished data) showed a similar overexpression of PPARγ in the liver of DKO mice. The increased insulin sensitivity in agonist-treated mice was associated with a 1.6-fold lower liver expression of PPARγ. Most likely, the increase of the insulin sensitivity in agonist-treated mice caused the liver to stop compensating for the insulin resistance by decreasing PPARγ expression.

4.4 Lack of effect of the dual agonist on dyslipidemia

PPARs are transcription factors that have important effects on lipid homeostasis via regulation of the expression of genes involved in lipid metabolism (see part 1.3).

4.4.1. PPARα
PPARα is predominantly expressed in the liver where it regulates the transcription of genes involved in hepatic fatty acid uptake and oxidation (see part 1.3.3). PPARα expression in the liver of placebo treated DKO mice was comparable to that in C57BL6 mice (ratio is approximately 1) indicating that obesity, dyslipidemia and insulin resistance in DKO mice was not associated with a change in liver PPARα expression. These data suggest that PPARα expression in the liver is most likely not responsible for the observed dyslipidemia in DKO mice, and that the lack of effect of the agonist on PPARα expression in the liver is thus most likely not the cause of the persistent dyslipidemia in agonist-treated DKO mice.

4.4.2. PPARγ
PPARγ is mainly expressed in adipose tissue where its activation stimulates adipogenesis and the expression of genes involved in fatty acid uptake and storage. PPARα, normally low in adipocytes, can also induce fatty acid uptake and storage in the adipose tissue. Both PPARα

and PPARγ expression in the adipose tissue of placebo treated DKO mice was lower compared to C57BL6 mice indicating that they both could be related to the observed dyslipidemia in DKO mice. In a previous study in the laboratory, it was shown that weight loss induced in these mice by diet-restriction, resulted in an increase of both PPARα and PPARγ expression in the adipose tissue and a decrease in triglyceride levels [Verreth et al, 2004]. In this work, we showed that the dual agonist treatment resulted in an increase in PPARα expression, but not in PPARγ expression in the adipose tissue, and with no change in triglyceride levels. These data suggests that PPARγ is the most important PPAR isoform in regulating lipid metabolism in the adipose tissue of DKO mice and that the lack of effect of the dual agonist on PPARγ expression in the adipose tissue is most probably the cause for its lack of effect on triglyceride levels.

4.4.3. PPARβ/δ

PPARβ/δ has been suggested as a key regulator of fat burning in the adipose tissue, a role that counteracts the fat-storing function of PPARγ (and PPARα). The data in our laboratory show that PPARβ/δ expression is downregulated in the adipose tissue of placebo-treated DKO mice indicating that PPARβ/δ could also play an important role in the observed dyslipidemia in DKO mice [unpublished data]. Agonist treatment had no effect on PPARβ/δ expression, which is in agreement with the lack of effect on dyslipidemia.

4.5. Lack of effect on atherosclerosis

Previously, weight loss in DKO mice resulted in lower plasma triglyceride levels that were associated with lower plaque volume [Verreth and al, 2004]. In the present study, agonist treatment had no effect on triglyceride levels and no effect on plaque volume. In aggregate, the two sets of data suggest that the triglyceride level is the predominant determinant of plaque volume in DKO mice.

Weight loss induced by diet restriction also led to an increase in both PPARα and PPARγ expression in the aortic arch indicating that this increase could be responsible for the inhibition of atherosclerosis. The agonist only induced an increase in PPARα expression in the aortic arch. Thus, the lack of effect of the agonist on PPARγ expression in the aortic arch could explain the lack of effect on atherosclerosis.

The role of PPARβ/δ in the aortic arch is poorly understood. It is upregulated in vascular SMC during vascular lesion formation and it promotes lipid accumulation in human macrophages, suggesting an atherogenic effect. It, however, also attenuates inflammation, suggesting an anti-atherogenic effect. In DKO mice, atherosclerosis was clearly associated with a down-regulation of PPARβ/δ, supporting its anti-atherogenic action. The agonist had no effect on PPARβ/δ expression in the aortic arch.

The increase of the ox-LDL and of the macrophages, that we observed in the atherosclerotic lesions, could make the plaque more instable and increase the risk of thrombus.

4.6 Improvement of cardiovascular functions

4.6.1. Better glucose utilization

DKO mice develop atherosclerosis and hypertension (see part 1.4), increasing afterload, which leads to a dilatation and hypertrophy of the heart at the age of 24 weeks. Increased glucose utilization and decreased fatty acid oxidation (FAO) are some of the possible molecular adaptations when the heart becomes hypertrophic. However, in our model as well as in uncontrolled diabetes, the heart is constrained to switching to glucose oxidation due to insulin resistance, resulting in impaired glucose utilization and an almost exclusive use of FAO to provide the ATP needs of the myocardium.

High circulating levels and uptake of TG and free fatty acids by the diabetic heart, lead to excess intramyocardial lipid accumulation. The subsequent lipotoxicity plays a role in the development of contractile dysfunction and myocyte death observed in the diabetic hypertrophic heart. The lipotoxicity and glucotoxicity could explain the decreased LV functioning in DKO mice at 24 weeks (lower EF and SF) which is due to a systolic dysfunction.

Agonist treatment improved insulin sensitivity after one week of treatment resulting in a prevention of loss of cardiac function in DKO mice. We hypothesize that because of the agonist treatment, the hypertrophic heart of the DKO mice becomes able to switch from FAO to glucose utilization, leading to less accumulation of intramyocardial lipids and glycolytic intermediates.

4.6.2. Central role of adiponectin

Adiponectin levels correlated positively with PPARγ expression in the heart. This indicates that the increase in PPARγ in the heart of agonist-treated DKO mice could also be the result of the positive effect of the agonist on plasma adiponectin levels. Our data are thus in agreement with data in man, monkey and mouse demonstrating that adiponectin is an insulin sensitizing adipocytokine [Hotta et al, 2001; Weyer et al, 2001; Yamauchi et al, 2001; Berg, 2001]. The increase in adiponectin in agonist-treated mice suggests a link between the adipose tissue and insulin sensitivity in the heart, and could therefore have an effect on LV function. The agonist induced PPARα but had no effect on PPARγ in the adipose tissue. Adiponectin correlated positively with PPARα in the adipose tissue that correlated with insulin sensitivity. Our data, thus, indicate that PPARα besides regulating fatty acid metabolism also affects glucose homeostasis.

4.7. Study limitations

We here show that next to PPARα and PPARγ, also PPARβ/δ is downregulated in the adipose tissue and the aortic arch of the DKO mice. Because PPARα/γ agonist treatment had no effect on PPARβ/δ expression we were unable to determine its role in the development of obesity, dyslipidemia, insulin resistance and the associated atherosclerosis and impaired left ventricular function in DKO mice. The lack of effect of the dual agonist on dyslipidemia was surprising. It could also be due to the difference of binding affinity of the 3q dual agonist with the two isoforms (more affinity for PPARγ than for PPARα). It would be also interesting to confirm the variation in gene expression (PPAR, Glut-4, Caveolin-1 ...) at the protein level.

4.8. Conclusion and perspectives

The metabolic syndrome is an actual health problem that is spreading. The finding of new molecules to treat this disease is thus important, but this pathology is complex and in vitro approaches are not sufficient. For example, the PPARα/γ dual agonist 3q used in this work has an action on both PPARα and PPARγ, but it has tissue specific actions in leptin-deficient obese, dyslipidemic insulin resistant mice. The effects are thus mitigated: insulin sensitivity and left ventricle functions are improved, but lipids or atherosclerosis are not reduced. These results can be explained by expressional changes in the adipose tissue of key genes regulating lipid metabolism and insulin sensitivity, most of which are under the transcriptional control of PPARs.

The 'Adipochip' developed in Namur could give some other complementary information on the change in gene expression, especially in adipocyte differentiation. The Adipochip is a low density DNA microarray, bearing 89 capture probes, in order to estimate the expression of genes involved in adipogenesis, lipid and glucose metabolism, stress response, inflammation, ... This tool was developed by Van Koningsloo et al (in preparation) in collaboration with Eppendorf Array technology (EAT, Namur).

Our data also suggest that next to PPARα and PPARγ downregulation, PPARβ/δ deactivation might be a key feature of metabolic syndrome initiation in DKO mice. Our observations point out the critical role of these transcription factors in both the pathogenesis and the treatment of atherosclerosis and cardiac function in the metabolic syndrome.

The effect of PPARα and PPARγ specific agonists separately are now well described. However, we did not know the specific tissue action of each agonist in the DKO murine model. New experiments are now beginning with a PPARγ agonist (rosiglitazone). In a few weeks, another experiment with PPARα agonist will begin. We will thus see if the tissue specific action is the same in the two cases. Dual agonists are in development, and it could be interesting to repeat the experiments with another dual agonist than the 3q, to see if the tissue specific action of 3q is also seen with other dual agonists and if the lipids and the atherosclerosis will remain unchanged as observed with the3q agonsit or decrease in the same time than the insulin resistance. A study with PPARβ/δ agonists could also help to define better the role of this third PPAR isoform in atherosclerosis, insulin resistance and dyslipidemia.

5. Bibliography

5. Bibliography

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