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STUDY OF THE MOLECULAR MECHANISM UNDERLYING PPAR ACTIVATION BY LT175, A NOVEL DUAL PPARα/γ LIGAND THAT AMELIORATES THE METABOLIC PROFILE AND INSULIN SENSITIVITY IN A MOUSE MODEL OF INSULIN RESISTANCE

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1. INTRODUCTION

1.1 The nuclear receptor family

Cells are constantly exposed to a large variety of lipids and other metabolic intermediates. Traditionally, lipids were thought to serve as simple energy storing molecules. More recently it has been realized that they can also initiate and regulate signaling events that will decisively influence development, cellular differentiation, metabolism and related functions through the regulation of gene expression. Multicellular organisms dedicate a large family of nuclear receptors to these tasks. These proteins combine the defining features of both transcription factors and receptor molecules, and therefore have the unique ability to bind signaling molecules and transduce the appropriate signals derived from lipid environment to the level of gene expression. There are several ways to group and categorize the divergent members of the superfamily. Originally those nuclear receptors whose ligands were identified were called "classic" nuclear receptors. These are tipically endocrine receptors that bind their ligands with high affinity (such as thyroid hormone receptors for the thyroid hormones or the estrogen hormone receptors for estrogens). Other nuclear receptors, whose ligands remained unknown, were classified as orphan nuclear receptors. To compound the naming system, the cognate ligands of some orphan nuclear receptors (including PPARs) were originally unknown but were later identified. Upon the discovery of their cognate ligands this later group of orphan nuclear receptors became "adopted" and was often referred to as adopted nuclear receptors (Kliewer et al., 1999).

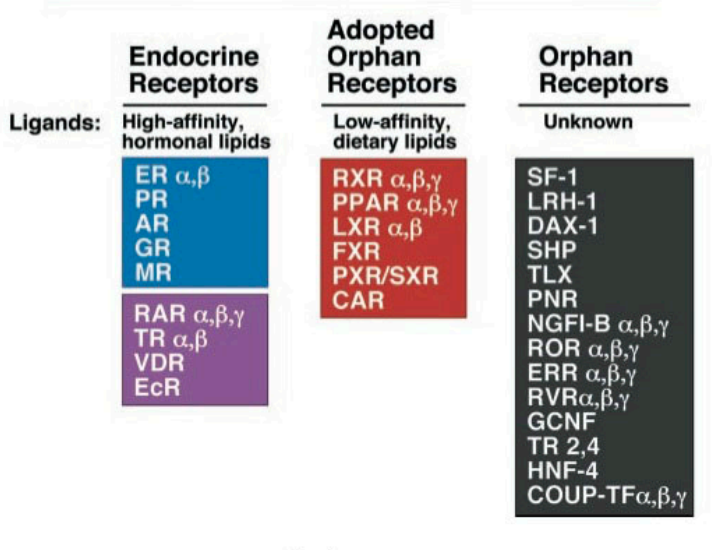


Fig. 1 *nuclear receptors subdivided into three groups, depending on the source and type of their ligand (Chawla et al., 2001)*

This classical grouping system of nuclear receptors has several weaknesses. Maybe the biggest source of inconsistency in this classification derives from the fact that those nuclear receptors are categorized as orphans whose ligand has not been found yet. Since novel ligands are found and characterized all the time, this classification is, by its very nature, always temporary. Reflecting on the weaknesses of the above classification principle based on the presence of endogenous ligands, phylogenetic classification model was introduced (Cell, 1999) in which the main organizing principle was sequence similarity.

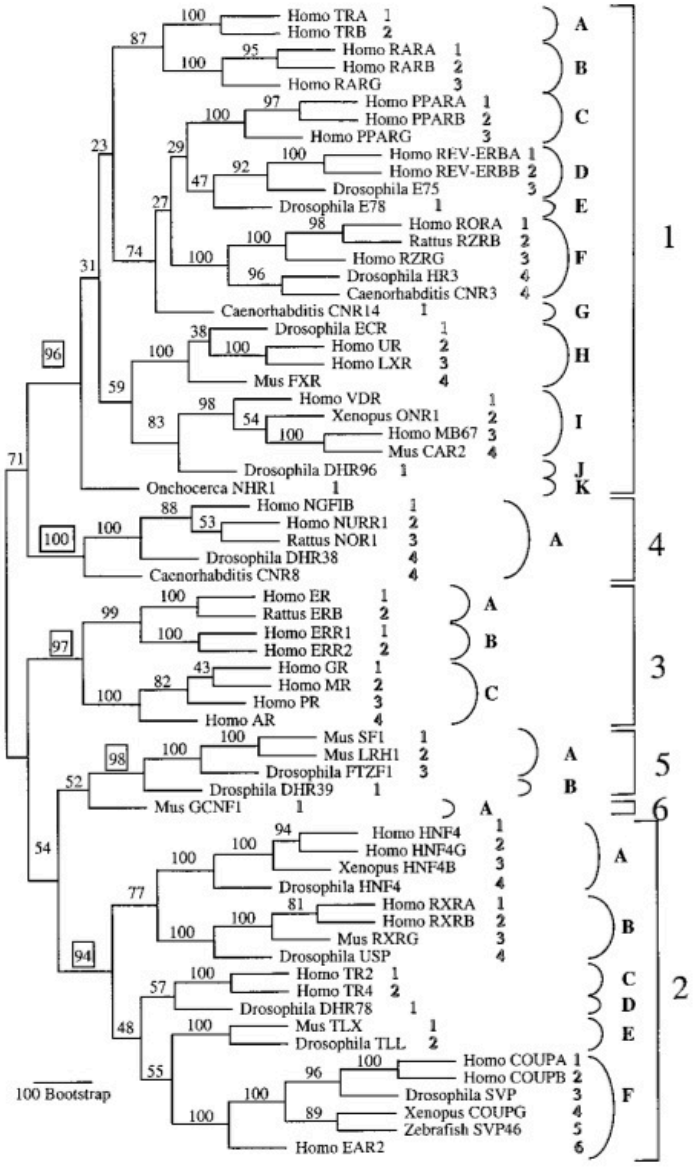


Fig. 2 *phylogenetic tree connecting the 65 known nuclear receptor genes in vertebrates, arthropods, and nematodes (Cell, 1999)*

1.2 Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors are ligand-dependent transcription factors which belong to the nuclear hormone receptor superfamily (Desvergne et al., 1999; Dreyer et al., 1992; Issemann et al., 1990) and are involved in translating the effects of fatty acids and various drugs into the gene expression level. The cellular and systemic roles that have been attributed to PPARs extend far beyond the control of hepatic peroxisome proliferation in rodents after which these receptors were initially named (Michalik et al., 2004).

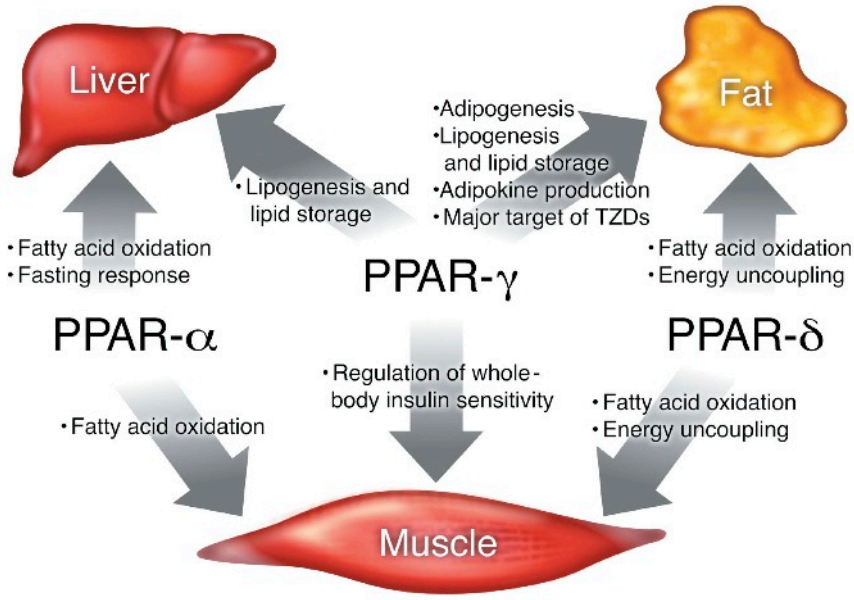


Fig. 3 *metabolic integration by PPARs (Evans et al., 2004)*

PPARs are involved in lipid and glucose metabolism, as they transcriptionally regulate the enzymes and other proteins involved in energy homeostasis. They are also expressed in epithelial cells, which have an essential function in the mucosal immune response, and in dendritic cells, macrophages, B and T lymphocytes, suggesting a role in immunity. The increased severity of inflammatory diseases in PPAR-deficient mice suggests an anti-inflammatory role for either unliganded PPAR or PPAR activated by endogenous ligands (Shah et al., 2007; Adachi et al., 2006). In fact, PPAR ligands repress the expression of cell adhesion molecules on endothelial cells and the secretion of chemokines by epithelial and other cells, decreasing the recruitment of leukocytes to the site of inflammation (Straus et al., 2007).

Three PPAR isotypes have been identified in lower vertebrates and mammals: PPARα or NR1C1, PPARβ/δ or NR1C2, also called NUC-1 or FAAR, and PPARγ or NR1C3 (Nuclear Receptors Nomenclature Committee, 1999).

1.2.1 Structure

PPARs possess the classic domain structure of nuclear receptors (Feige et al., 2006) (figure 4). Specifically, their N-terminal region displays a ligand-independent, weak trans-activation domain called activation function 1 (AF-1). This is followed by a two zinc finger motif comprising DNA-binding domain. The C-terminal region contains the ligand-binding pocket and a ligand-dependent, major trans-activation domain, termed AF-2. The C-terminus is also responsible for dimerisation with the retinoid X receptor (RXR) and for the docking of regulatory co-factor proteins.

Fig. 4 *schematic structure of a typical nuclear receptor (Chawla et al., 2001)*

Mechanistically, they form heterodimers with RXR and activate transcription by binding to a specific DNA element, termed the peroxisome proliferator response element (PPRE), in the regulatory region of a variety of genes encoding proteins that are involved in lipid metabolism and energy balance (Kota et al., 2005; Berger et al., 2005; Michalik et al., 2006). Although the majority of PPREs identified so far consist of a direct repeat of the canonical AGGTCA sequence spaced by one nucleotide direct hexa-nucleotide repeats (DR-1) (Schoonjans et al., 1996), DR-2 elements may also function as PPRE (Gervois et al., 1999). There are some additional features of PPAR target sites, such as the presence of an extended 5-half site, the presence of adenine as the separator single nucleotide or a slightly imperfect hexameric motif (Ijpenberg et al., 1997). All three PPAR subtypes are believed to bind to canonical DR1 elements. Because certain cell types express more than one PPAR subtype, the question arises on what determines PPAR binding to a certain DR-1 element in these cells. A focused study showed that the 5' flanking nucleotides of the core DR-1 elements played an important role in determining the PPAR subtype specificity of PPREs (Nielsen et al., 2006).

1.2.2 Tissue distribution and endogenous ligands

PPARs exhibit different tissue distribution and functions and different ligand specificities (Wahli, 2002). PPARα is highly expressed in the liver, brown adipose tissue, heart, skeletal muscle, kidney and in other organs at lower levels. PPARγ is highly expressed in adipose tissues and is also present in colon and lymphoid organs. PPARβ/δ is expressed ubiquitously, but its levels may vary to a large extent (Desvergne et al., 1999).

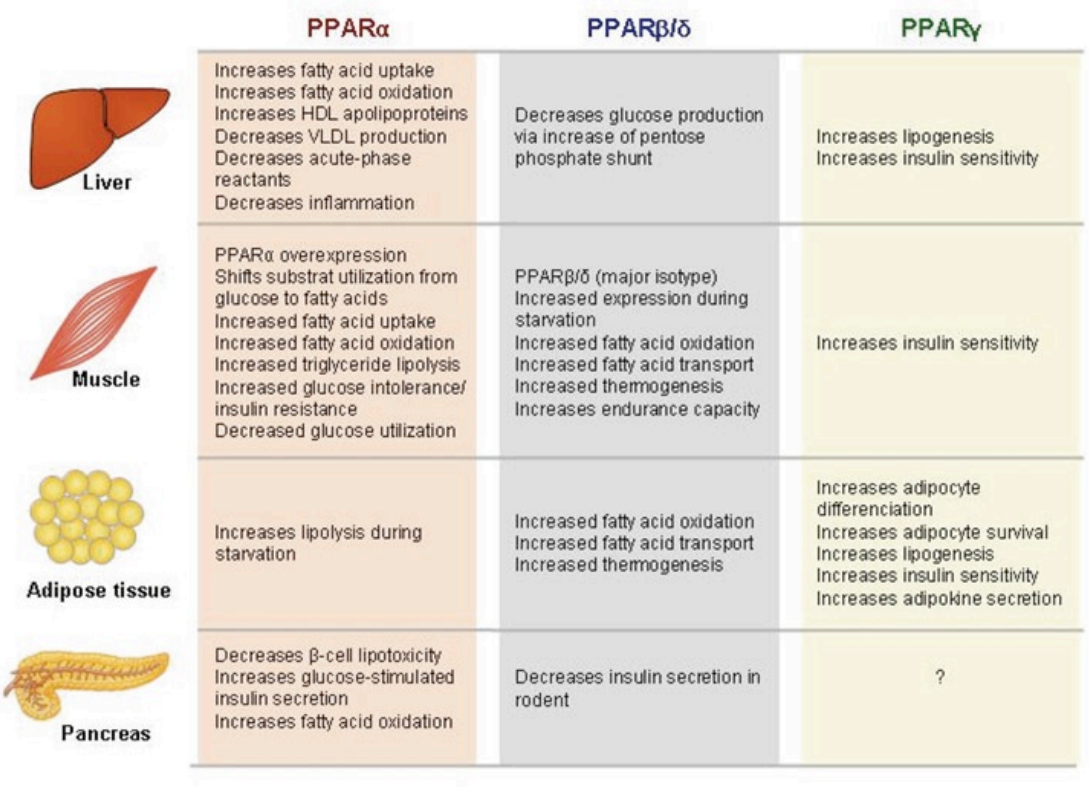


Fig. 5 *PPARs action in different tissues (Yessoufou et al., 2010)*

There is a variety of potential endogenous ligands for the PPARs, such as fatty acids, in particular unsaturated fatty acids, several eicosanoids and metabolites of linoleic acid, which bind to the PPARs with varying affinities, resulting in transcriptional activation of target genes, thus conferring to these receptors the function of lipid sensor which when activated can markedly redirect metabolism. Molecules that were found to bind physically to PPARs include polyunsaturated fatty acids (PUFAs) such as certain ω3-polyunsaturated fatty acids, and certain ω6-polyunsaturated fatty acids. Certain saturated fatty acids were also found to bind PPARα. Another group of PPAR

ligands comprises the conversion products of essential fatty acids (mainly arachidonic acid) by lipoxygenases or cyclooxygenases. The best examples for eicosanoids that are possibly PPARα ligands are hydroxyeicosatetraenoic acids and leukotriene B4. Recent findings led to the hypothesis whereby PPARs are not specific receptors for one particular fatty acid molecule but are sensor molecules that sample the intracellular mixture of available fatty acids species. In line of this idea, it has been shown that PPARγ covalently bind a subset of fatty acids and that it can bind two ligand molecules at the same time (Itoh et al., 2008).

1.2.3 Mechanisms of action

Binding of agonist within the ligand-binding site of PPARs causes a conformational change promoting the interaction with transcriptional coactivators, which in turn affects transcription of genes via direct activation of gene expression, ligand-dependent or –independent repression or transrepression (Glass et al., 2000). It was reported that this exchange of corepressors and coactivators is mediated by TBL1 (transducin β-like 1) and by the related protein TBLR1 (Perissi et al., 2004). Conversely, binding of antagonists results in a conformation that favours the binding of corepressors (Zoete et al., 2007; Yu et al., 2007). Physiologically, PPAR-RXR heterodimers may bind to PPREs in the absence of a ligand. Although the transcriptional activation depends on the ligand-bound PPAR-RXR, the presence of unliganded PPAR-RXR at a PPRE has effects that vary depending on the promoter context and cell type. In fact, for some PPARγ target genes in adipocytes, unliganded PPARγ-RXR heterodimers recruit corepressor complexes, resulting in active repression, whereas for other genes, corepressors are not recruited by unliganded PPARγ-RXR (Guan et al., 2005).

Coregulators allow the DNA-bound transcription factors to transmit their activation or repression properties to the transcriptional machinery. These cofactors are characterized by their ability to interact with a wide variety of transcription factors and their ability to assemble a protein complex that will be the transcription effector. Coactivators are defined as molecules recruited by ligand bound activated nuclear receptors and enhance transcription, and corepressors as molecules that interact with receptors that are not bound to ligand and repress transcription. Current evidences

indicate that this definition can be modified by gene, cell, and signaling context for any one coregulator (Desvergne et al., 2006).

According to their mechanism of action, coactivators can be divided in two large families: the former includes Steroid Receptor Coactivator (SRC) and CBP/p300, that act in part as molecular scaffolds, and in part by acetylating diverse substrates. The latter, including peroxisome proliferator-activated receptor 1α (PGC-1α), does not act by remodeling chromatin, however it is involved in multiple biological responses related to energy homeostasis (Lonard et al., 2007).

On the other hand, corepressors interact directly with the unliganded receptor, leading to enhancement of basal transcription repression and they also have an autonomous repression domain. Among the corepressor family, the first proteins that have been described are the silencing mediator of retinoid and thyroid hormone receptor (SMRT) and the nuclear hormone receptor-corepressor (NCoR) (Lonard et al., 2007).

1.3 PPARα

1.3.1 Functions

PPARα controls lipid catabolism promoting fatty acid oxidation in the liver and is the target for the fibrate class of hypolipidaemic agents such as fenofibrate, clofibrate and gemfibrozil, which are used for treating hypertriglyceridaemia (Kota et al., 2005; Berger et al., 2005; Michalik et al., 2006). It regulates the expression of genes encoding enzymes implicated in the peroxisomal β-oxidation pathway such as acyl-CoA oxidase, enoyl-CoA hydratase/dehydrogenase multi-functional enzyme and 3 ketoacyl-CoA thiolase. PPARα also modulates genes involved in FA uptake, activation to acyl-CoA esters, mitochondrial β-oxidation, by induction of carnitine palmitoyl transferase-1 (CPT1) and modulation of acyl-CoA dehydrogenase (MCAD), and ketone body synthesis controlling the mitochondrial hydroxymethylglutaryl-CoA synthase (mHMG-CoAS) (Schoonjans et al., 1996). It is also involved in the metabolism of apolipoprotein apoA-I and apoA-II, in the expression of genes involved in gluconeogenesis, in the inhibition of transamination and deamination of amino acids, as well as in the blocking of urea synthesis (Zoete et al., 2007). PPARα activation also inhibits the expression of apo C-III, which works as a suppressor of the LPL activity (Zandbergen et al., 2007).

Intracellular FA concentrations are controlled, in part, by the activity of the FA transport protein (FATP), which regulates the entry of FA through the cell membrane, and by acyl-CoA synthetase (ACS), the enzyme trapping FA inside the cells by converting them to ester derivatives. PPARα activation mediates the induction of FATP expression in liver and intestine and the up-regulation of ACS expression in liver and kidney (Martin et al., 1997). PPARα has been demonstrated to affect FA import into mitochondria by up-regulating the expression of muscle and liver type α-carnitine palmitoyltransferase I genes (Mascaro et al., 1998; Kersten et al., 1999). Through their effect on the expression of FA transporter and FA oxidation genes, PPARα activators direct the FA flux to the β-oxidation pathway and therefore diminish the FA pool to be incorporated to triglyceride-rich lipoproteins. Consequently, PPARα maintains lipid homeostasis by controlling the FA flux from peripheral tissues, such as adipose tissue, to the liver. The role of PPARα in hepatic fatty acid metabolism is especially prominent during fasting. In fact, during an overnight or prolonged fast, fatty acids are released from adipose tissue and transported into the liver, where PPARα is robustly induced (Kersten et al., 1999). Moreover, it accumulates according to a daily rhythm with highest levels at the beginning of feeding time (Lemberger et al., 1996). The resulting activation of PPARα by fatty acids promotes hepatic fatty acid oxidation to generate ketone bodies, providing an energy source for peripheral tissues.

In fasted PPARα-null mice, its absence is associated with pronounced hepatic steatosis, decreased levels of plasma glucose and ketone bodies, elevated plasma free fatty acid levels and hypothermia (Kersten et al., 1999; Leone et al., 1999; Hashimoto et al., 2000). These severe metabolic disturbances are the result of the decreased expression of a large number of genes involved in hepatic lipid metabolism, many of which have been identified as direct PPARα target genes (Martin et al., 1997; Sato et al., 2002). In addition, PPARα is a target of hypothalamic hormone signaling as it plays an important role in the anti-inflammatory action of glucocorticoids (Crisafulli et al., 2009). In fact, during fasting, as well as in situations of physical and physiological stress, the hypothalamic ACTH induces the release of glucocorticoids by the adrenal glands, which stimulates the hepatic expression of PPARα.

PPARα affects carbohydrate metabolism by means of compensatory insulin secretion by the Islets of Langerhans, influencing glucose and lipid regulation. Other recently

discovered functions are the mediation of female-specific gene repression and the protection of the liver from oestrogen induced toxicity. PPARα regulates the processes that are essential for the maintenance of pregnancy and embryonic development; although PPARα-null mice are viable, they present an increased risk of maternal abortion and neonatal mortality (Yessoufou et al., 2006). Finally, it favours skin healing during the inflammatory phase that follows injury.

Interestingly, PPARα-null diabetic mice are hypoinsulinaemic, with a reduced ability to use glucose and lipids as energy sources (Yessoufou et al., 2006). In contrast, clofibrate treatment was shown to lead to browning of retroperitoneal WAT as evidenced by enhanced uncoupling protein 1 (UCP1) expression (Xue et al., 2005). PPARα-null mice also display normal BAT development. Similarly to PPARγ, it is possible that PPARα activity also modulates obesity-associated inflammation either through its metabolic activity or anti-inflammatory effects. Induction of obesity with high-fat diet in PPARα knockout or wild type mice suggested that PPARα protected against obesity-induced chronic inflammation in the liver. Plasma markers of liver injury and inflammation, including serum amyloid A and alanine aminotransferase activity, were increased in high-fat diet fed PPARα$^{-/-}$, but not in wild-type animals (Stienstra et al., 2007).

1.3.2 Regulation

Several posttranslational modifications were described for PPARα. Insulin mediates the phosphorylation of Ser12 and Ser21, which enhance the transactivation capacity of PPARα (Shalev et al., 1996). Other phosphorylation mechanisms, by the p38 MAPK or protein kinase C (PKC) pathways, were also described. Interestingly, inhibition of PKC had a dual effect on PPARα. PKC inhibition decreased transactivation capacity of PPARα, but enhanced its transrepression activity (Blanquart et al., 2004). The concentration of PPARα mRNA and the receptor itself are affected by the circadian rhythm of circulating glucocorticoids. Stressful situations that elevate these hormones also contribute to increase PPARα in hepatocytes.

1.4 PPARβ/δ

1.4.1 Functions

This isotype is ubiquitously expressed, with the highest level of expression in colon, small intestine, liver and keratinocytes, and is implicated in different processes. The expression of PPARβ/δ is induced during the early phase of white pre-adipocyte differentiation in murine clonal cell lines (Grimaldi et al., 2001). Activation of PPARβ/δ increases lipid catabolism in skeletal muscle, heart and adipose tissue, stimulating the β-oxidation of fatty acids in muscle (Schuler et al., 2006), reverse cholesterol transport (Barish et al., 2006) and influencing lipoprotein homeostasis (Oliver et al., 2001); this leads to an improvement of the serum lipid profile and insulin sensitivity in animal models. It improves hyperglycemia by increasing carbohydrate hepatic catabolism and suppressing glucose capture in the liver. PPARβ/δ is implicated in osteoclastic bone resorption (Mano et al., 2000) moreover, concerning skin wound repair, it enhances keratinocyte survival and migration. PPARβ/δ deletion affects placenta morphogenesis resulting in partial embryonic lethality (Nadra et al., 2006). Surviving PPARβ/δ null mice are healthy and fertile and exhibit a striking reduction in adiposity relative to wild-type animals (Barak et al., 2002). This effect however, is not recapitulated in mice harbouring an adipose tissue-specific deletion of PPARβ/δ, suggesting that the phenotype is fat-non-autonomous and may evolve from perturbations in systemic lipid metabolism (Barak et al., 2002). PPARβ/δ null mice also expend less energy and consequently display increased susceptibility to weight gain coupled with blunted BAT UCP1 expression on a high-fat diet (Wang et al., 2003). In contrast, targeted activation of PPARβ/δ in adipose tissue leads to reduced adiposity and improved lipid profiles. In addition to being lean, these animals are also resistant both to high-fat diet-induced and genetic (*db/db*) obesity, hyper-lipidaemia, and hepatic steatosis (Wang et al., 2003). These genetic models collectively suggest that activation of PPARβ/δ protects against obesity by stimulating thermogenesis in adipose tissues.

In the liver, PPARβ/δ down-regulates the expression of pro-inflammatory genes (Shan et al., 2008). In muscle, after selective deletion of PPARβ/δ, mice exhibit switch in muscle fiber types toward a lower oxidative capacity that precedes the development of obesity and diabetes (Schuler et al., 2008). In this tissue, PPARβ/δ

upregulates fatty oxidation and energy expenditure to a far greater extent than does the lesser-expressed PPARα.

Recently, another anti-inflammatory role for PPARβ/δ was revealed, as it was found to be necessary for the timely clearance of apoptotic cells. PPARβ/δ deficiency caused a delay in the uptake, while PPARβ/δ ligand treatment in wild-type mice caused an enhanced uptake of apoptotic cells. C1q, a component of the classical complement activation pathway was found to be a direct target gene of PPARβ/δ. As a result of the abnormal sensing of apoptotic cells in PPARβ/$\delta^{-/-}$ animals, these mice developed a lupus erythematosus-like autoimmune disease (Mukundan et al., 2009).

1.4.2 Regulation

PPARβ/δ enhances BAT metabolism by mediating, at least in part, the function of PGC-1α which constitutes the central regulator of BAT thermogenesis. Interestingly, TWIST1, a helix-loop-helix containing transcriptional regulator selectively expressed in adipose tissue was recently demonstrated to act as a negative-feedback regulator of PGC-1α/ PPARβ/δ-mediated brown fat metabolism (Pan et al., 2009).

1.5 PPARγ

1.5.1 Structure

The structure of PPARγ consists of an amino terminal activation domain (AF-1), a DNA binding domain (DBD), a ligand binding domain (LBD) and a second carboxy terminal activation domain (AF-2). Like other PPARs, PPARγ has a large binding pocket allowing it to accommodate a wide range of ligands (Gampe et al., 2000).

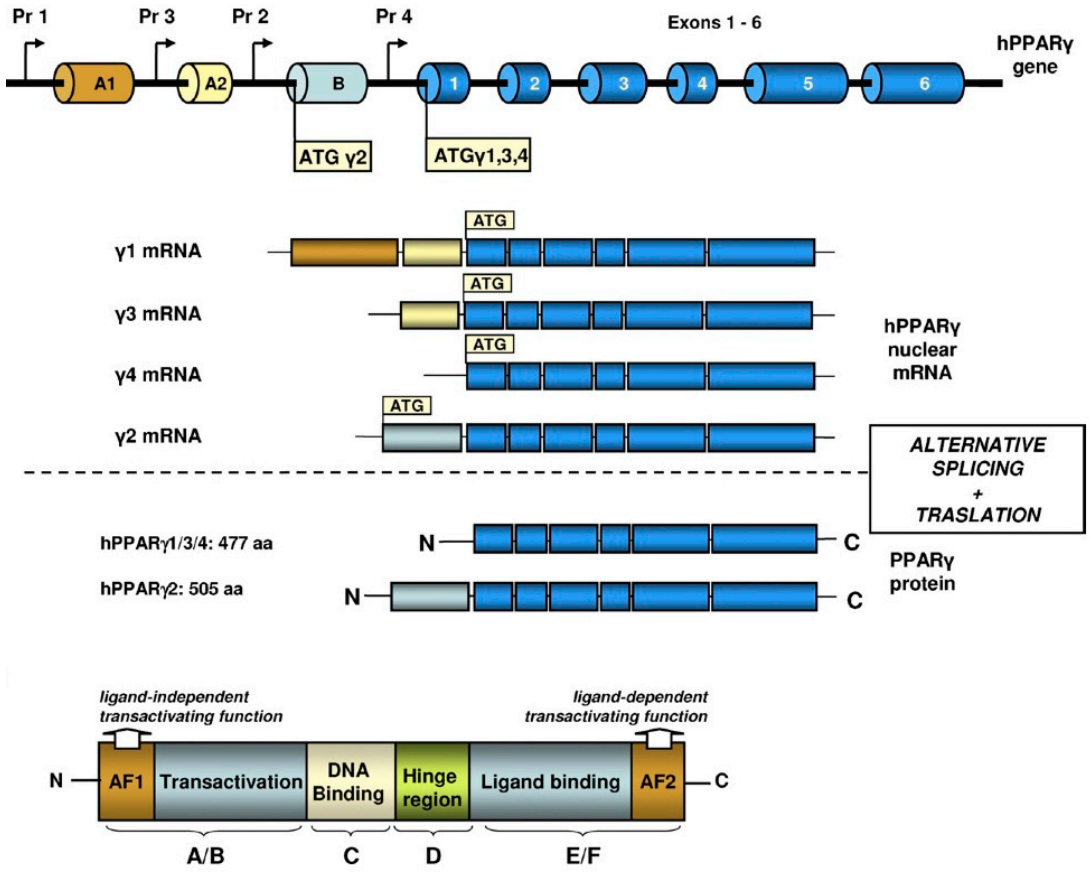


Fig. 6 *PPARγ organization at gene, mRNA and protein levels (Luconi et al., 2010)*

1.5.2 Functions

PPARγ plays a pivotal part in cell-fate determination, lipid biosynthesis, inflammation and insulin sensitivity (Tontonoz et al., 2008; Rosen et al., 2006). In humans, the same gene, consisting of 9 exons of more than 100kb, mapping in ch3p25 is transcribed into 4 mRNAs. Differential promoter usage and alternative splicing produce four PPARγ variants, including two major forms of the protein, PPARγ1 and PPARγ2. PPARγ1 is expressed in a wide range of tissues, including the liver, skeletal

muscle, adipose tissue and bone. PPARγ2, which contains 28 or 30 additional amino acids (in mouse and human respectively) in its N-terminus compared with PPARγ1, is expressed mainly in adipogenic cells. These variants are driven by distinct promoters, but have similar transcriptional activities (Mueller et al., 2002).

PPARγ regulates lipid storage in adipose tissues; it is the target for the insulin sensitizing thiazolidinediones, a class of drugs widely used to treat type 2 diabetes (Forman et al., 1995). By promoting the expression of the lipid droplet associated proteins fat-specific protein (FSP27), cell death inducing DFFA-like effector A (CIDEA) and perilipin, PPARγ promotes efficient storage of triglycerides in unilocular lipid droplets (Puri et al., 2008).

PPARγ is abundantly expressed in adipocytes and plays a pivotal role in adipocyte differentiation (Tontonoz et al., 1994), being the only known factor that is necessary and sufficient for induction of adipocyte differentiation (Rosen et al., 2000). In fact, PPARγ is induced during adipocyte differentiation, and forced expression of PPARγ in nonadipogenic cells effectively converts them into mature adipocytes (Tontonoz et al., 1994). In addition, PPARγ knockout mice fail to develop adipose tissue (Rosen et al., 1999).

However, the functions of PPARγ extend far beyond fat storage and adipocyte differentiation. While PPARγ is highly expressed in adipocytes and adipose tissue, it is present in numerous other cell types and tissues including macrophages, osteocytes, endothelial cells and placenta. Despite its relatively low expression levels in healthy liver, PPARγ is critical for the development of hepatic steatosis (Patsouris et al., 2006; Yu et al., 2003). The role of PPARγ in promoting osteoclast differentiation and bone resorption when activated by rosiglitazone has been recently recognized (Wahli et al., 2008). Its deletion in mouse osteoclast precursors leads to increased bone mass and density and extramedullary haematopoiesis. Conversely, PPARγ down-regulates osteogenesis. In fact, it influences the competition between adipogenic and osteoblastic differentiation of bone marrow progenitors in favour of adipogenesis (Akune et al., 2004; Cock et al., 2004).

All PPARγ-null embryos die at an early developmental stage because of placental defects including impaired vascularisation (Nadra et al., 2006). Using conditional knockout strategies, PPARγ has been selectively deleted in liver, fat and muscle. Mice harboring liver-specific knockouts of PPARγ develop hyperlipidemia, elevated

plasma glucose and insulin levels, and increased adiposity, all of which may be caused by a pronounced delay of hepatic triglyceride uptake (Gavrilova et al., 2003). Knock out of PPARγ after complete adipose tissue development results in a progressive loss of fat, hyperlipidemia, fatty liver, and hepatic insulin resistance, but as long as some fat is present mice can maintain normal whole-body glucose homeostasis and normal muscle insulin sensitivity (He et al., 2003). Studies of muscle knockout mice reveal that loss of muscle PPARγ causes secondary insulin resistance in liver and adipose tissue, although these tissues continue to respond to TZDs (Norris et al., 2003). Macrophage-restricted deletion of PPARγ also results in systemic insulin resistance and glucose intolerance (Odegaard et al., 2007). PPARγ in macrophages is implicated in anti-inflammation, uptake and reverse transport of cholesterol, and subtype specification (Olefsky et al., 2010). The receptor was shown to drive macrophage differentiation into the alternatively activated, anti-inflammatory population (M2), rather than the classically activated, proinflammatory population (M1) (Odegaard et al., 2007).

1.5.3 The role of PPARγ in adipogenesis

Adipogenesis consists of integrated cascades that involve several transcription factors. The initial step of adipogenesis is the lineage commitment of mesenchimal stem cells (MSCs) followed by the expansion of preadipocytes. PPARγ is a critical component in adipogenesis, as indicated by the fact that loss of PPARγ expression in murine embryonic fibroblasts leads to a complete absence of adipogenic capacity (Kubota et al., 1999). The PPARγ2 isoform is induced earlier and more strongly than PPARγ1 during adipogenesis. The receptor is also required for maintaining the proper functions of differentiated adipocytes (Sugii et al., 2009).

While PPARγ is critical in the development of both white (WAT) and brown adipose tissue (BAT) in vivo (Imai et al., 2004), many of its target genes are distinct at least in part due to the presence of different sets of interacting coregulators. Before full adipogenesis takes place in white adipocytes, corepressors such as NCoR and SMRT are associated through their receptor-interacting domains on PPARγ2 and other specific target gene promoters and maintain the repressive states of adipogenic genes (Nofsinger et al., 2008), in a repressive complex containing the retinoblastoma protein (pRb) and histone deacetylase HDAC3 (Guenther et al., 2001). Other corepressor

proteins that are reported to interact with PPARγ and have repressive roles in adipocytes are RIP140, SIRT1, TRB3 and TAZ (Koppen et al., 2010).

During differentiation into white adipocytes, phosphorylation of pRb dislodges the pRb-HDAC3 repressive complex from the receptor (Yu et al., 2005). This may allow coactivator proteins such as p300/CBP to associate with PPARγ and assist with its adipogenic activity. In addition to PPARγ, the CCAAT/enhancer binding protein (C/EBP) family of transcription factors play an important role in adipogenesis. At the initial phase of differentiation, C/EBPβ and C/EBPδ are induced and stimulate expression of C/EBPα and PPARγ. C/EBPα and PPARγ then act reciprocally to induce a number of adipogenic target genes (aP2, CD36, PEPCK, LPL, GLUT4 and ACRP30) and sustain their own expression by a positive feedback loop, resulting in terminal differentiation (Tontonoz et al., 2008). However, PPARγ seems to be the principal driver of the adipogenic program. Thus, while overexpression of PPARγ can induce adipogenesis in C/EBPα-deficient mouse embryonic fibroblasts (MEFs), exogenous C/EBPα expression is unable to do so in PPARγ-deficient MEFs (Tontonoz et al., 2008).

During differentiation into brown adipocytes, PPARγ binds to and cooperates with PGC-1α and PRDM16 to modulate brown adipocyte function (Seale et al., 2009). Very recently, it was found that at the initial stage of adipogenesis another nuclear receptor, glucocorticoid receptor (GR), is transiently recruited along with C/EBPβ to a complex consisting of PBP/MED1/TRAP220 and p300 to enhancer regions of PPARγ2 isoform (Steger et al., 2010). In response to glucocorticoids, this results in a transient increase in H3K9 acetylation and enhances the induction of PPARγ2, which becomes the principal driver of adipogenesis.

1.5.4 Regulation

The gene encoding the murine PPARγ protein exhibits a remarkable circadian expression pattern in liver and adipose tissue, and this rhythmic response is magnified by consumption of a high-fat diet (Yang et al., 2006; Green et al., 2007).

A number of transcription factors are involved in the regulation of PPARγ expression and function. Transcription factors of the CCAAT/enhancer-binding protein (C/EBP) family, C/EBPs α, β and δ, stimulate PPARγ transcription by directly binding to the promoter region (Darlington et al., 1998). PPARγ expression and function is also

positively regulated by other transcription factors during adipogenesis, including sterol regulatory element-binding protein 1c (SREBP1c), Krueppel-like factor (KLF) 5, KLF15, Zinc finger protein 423 (ZFP423) and transcription factor COE1 (also known as early B cell factor; EBF1). KLF2 and GATA-binding proteins 2 and 3, on the other hand, negatively regulate PPARγ expression (Mori et al., 2005; Oishi et al., 2005; Gupta et al., 2010; Banerjee et al., 2003; Tong et al., 2005).

Another level of regulation of PPARγ transcriptional activity is its ability to modify histones. In the absence of ligand binding, PPARγ forms a protein complex with corepressors, such as NCoR and SMRT, as well as histone deacetylases (HDACs), as a result of which PPARγ is transcriptionally silent (Guan et al., 2005). Upon ligand binding, HDACs are dissociated from this protein complex and coactivators, such as CREB-binding protein (CBP) and histone acetyltransferases, are recruited. In fact, PPARγ undergoes conformational changes which provide a contact site for LXXLL motifs in p300/CBP and SRC-1 and other coactivators, through charge clamp in its ligand binding domain (Nolte et al., 1998). In addition to ligand induced binding, p300/CBP proteins can interact with the N-terminal AF-1 domain of PPARγ in a ligand-independent fashion (Gelman et al., 1999). Histone methylation also plays a critical part in the regulation of PPARγ activation as evidenced by the fact that noncanonical Wnt pathways, activated by Wnt5a, suppress PPARγ transcriptional activation via the histone-lysine N-methyltransferase SETDB1 (Takada et al., 2007).

Post-translational modifications of PPARγ are also important to regulate its activity. Phosphorylation of PPARγ2 at Ser 112 within the AF-1 domain by secretory factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), results in impaired PPARγ2 transcriptional activity (Hosooka et al., 2008). Recent studies demonstrated that PPARγ is a direct substrate of Cdk5 (Choi et al., 2010). PPARγ is phosphorylated by Cdk5 at Ser 273, which is situated in a loop within the LBD. Importantly, this phosphorylation alters the expression of only a subset of PPARγ responsive genes including those encoding the fatty acid transporter CD36 and adiponectin. Chromatin binding was unaffected by phosphorylation leading to the suggestion that phosphorylation may serve to alter the recruitment of coregulators.

In addition to phosphorylation, sumoylation at lysine 107 in the AF-1 and lysine 395 in the AF-2 region (Van Beekum et al., 2009) and ubiquitination confer a different level of post-transcriptional PPARγ regulation (Yamashita et al., 2004). The

sumoylated receptor is not able to bind its regular heterodimerization partner RXR. In particular, sumoylation of the AF-1 domain represses PPARγ transcriptional activity; in contrast, lysine 395 sumoylation is not involved in the regulation of direct PPARγ target genes but rather in the trans-repression of inflammatory genes by PPARγ in macrophages (Pascual et al., 2005). The ubiquitination is positively linked to PPARγ transcriptional activity (Van Beekum et al., 2009). Finally, PPARγ has been demonstrated to be nitrated following inflammatory activation of macrophages and such a post-translational modification blocks ligand induced nuclear traslocation of the receptor, resulting in inhibition of anti-PPARγ inflammatory activity (Shibuya et al., 2002).

A further level of regulation of PPARγ activity occurs on intracellular spatial compartmentalization of the receptor. Extracellular shuttling of PPARγ may inhibit its transcriptional activities but it allows the receptor interaction with putative cytosolic and plasma membrane substrates, resulting in a putative positive modulation of nongenomic functions of PPARγ. MEK1 can be one of the PPARγ shuttles (Burgermeister et al., 2007).

1.6 Biochemical action of PPAR ligands

1.6.1 PPARα ligands

In a variety of mouse models, PPARα agonists lower plasma triglycerides, reduce adiposity and improve hepatic and muscle steatosis, consequently improving insulin sensitivity (Chou et al., 2002; Kim et al., 2003). In particular, studies carried out both in rodents and humans led to the establishment of four major metabolic pathways affected by fibrates and explaining their effects on lipoprotein metabolism. First, the induction of lipoprotein lypolisis as a result of either an increase in intrinsic lipoprotein lipase activity or an increased accessibility of TG-rich lipoprotein particles for lipolysis due to reduced TG-rich lipoprotein apo C-III content (Malmendier et al., 1989). Second, limitation of hepatic TG synthesis and VLDL production due to increased FA catabolism and reduced FA synthesis (Martin et al., 1997). Third, increase in LDL particle removal as a result of changes in plasma LDL composition and subsequent increase in LDL affinity for its receptor leading to enhanced LDL catabolism (Caslake et al., 1993). Fourth, increase in HDL production and stimulation of reverse cholesterol transport. Moreover, fibrates increase the production of apolipoprotein A-I and A-II in human liver (Berthou et al., 1996), which leads to increased plasma HDL concentrations and enhanced reverse cholesterol transport. By contrast, these genes are repressed in rodents (Berthou et al., 1996).

PPARα agonists have already been suggested against cardiovascular inflammatory responses. The PPARα agonist fenofibrate is a potent anti-inflammatory drug used in the treatment of patients with hypertriglyceridemia and rheumatoid arthritis (Okamoto et al., 2005). When used as a lipid-lowering agent in patients with atherosclerosis, fenofibrate induces a decrease in circulating TNF-α, IL-1β and IFN-γ (Madej et al., 1998).

1.6.2 PPARβ/δ ligands

PPARβ/δ ligands prevent weight gain and suppress macrophage derived inflammation (Coll et al., 2009). The PPARβ/δ agonist GW501516 was shown to lower plasma triglyceride levels in obese monkeys while raising high-density lipoprotein levels, prompting the initiation of clinical trials to assess its efficacy in hyperlipidemic patients (Oliver et al., 2001). In mice, this ligand significantly retards weight gain, but does not affect food consumption in animals fed a high fat diet. Treatment with a

PPARβ/δ agonist also improves insulin resistance induced by a high fat diet, probably as a consequence of increased fat burning by muscle and overall improvement in systemic lipid metabolism.

1.6.3 PPARγ ligands

Thiazolidinediones, a class of synthetic compounds, bind to PPARγ resulting in improvement in insulin resistance in rodents and humans, and have widely been used to treat type 2 diabetes mellitus (Nolan et al., 1994). The mechanisms by which thiazolidinediones improve insulin resistance involve multiple pathways. Thiazolidinediones increase PPARγ expression and stimulate PPARγ transcriptional activity in adipose tissue, resulting in the up-regulation of genes involved in lipid metabolism, such as CD36, FABP4, GLUT4 and LPL (Kawai et al., 2010). These changes in gene expression enhance the incorporation of free fatty acids (FFA) into adipose tissue, which eventually decreases serum FFA levels and reduces lipotoxicity in liver and skeletal muscle, thereby improving insulin sensitivity (Yamauchi et al., 2001). Second, PPARγ specific drugs alter the release of signaling molecules from fat, including leptin (Spiegelman et al., 2006), TNF-α, resistin and adiponectin, which by virtue of serum transport have far-reaching metabolic effects in other tissues. For example, PPARγ agonists inhibit the expression of TNF-α and resistin, which both promote insulin resistance (Peraldi et al., 1997; Rajala et al., 2003). On the other hand, PPARγ agonists stimulate the production of adiponectin, in particular the more metabolically active high molecular weight form HMW adiponectin (Lara-Castro et al., 2006), which promotes fatty acid oxidation and insulin sensitivity in muscle and liver. As a result, hepatic glucose production is reduced and muscle glucose use is increased (Yamauchi et al., 2001).

Lipid redistribution from visceral to subcutaneous adipose tissue depots by thiazolidinediones also has an important role for the improvement of insulin sensitivity, because visceral adipose tissue, rather than subcutaneous adipose tissue, is associated with the development of metabolic complications related to obesity (Shadid et al., 2003).

The treatment with TZDs in lipodystrophic mice improves insulin sensitivity, indicating also a beneficial activity outside of adipose tissue (Kim et al., 2003).

Moreover, TZDs reduce glucose levels, improve HDL-cholesterol levels and decrease thickening of the intima-media thickness (DeFronzo et al., 2009).

In the muscle, the treatment with PPARγ agonists decreased the expression of the protein PDK4, which inhibits the glucose oxidative metabolism and the expression of genes involved in the transport and oxidation of fatty acids (Way et al., 2001). In macrophages, PPARγ ligands have anti-inflammatory effects by repressing the expression of a subset of Toll-like receptor (TLR) target genes by a molecular mechanism termed ligand-dependent transrepression (Shah et al., 2007), in which PPARγ action is not mediated by its binding to canonical PPRE target DNA sequences. PPARγ was shown to bind directly other transcription factors, such as NF-kB or activator protein 1 (AP-1), interfering with the DNA binding capacity of these transactivators (Chung et al., 2000).

Finally, the PPARγ agonists thiazolidinediones efficiently normalize skin homeostasis when orally administrated to patients suffering from psoriasis, suggesting that their beneficial effects are most likely due to systemic anti-inflammatory functions of PPARγ (Michalik et al., 2008).

Despite improvement in glucose and lipid metabolism and variable effects on the cardiovascular system, a growing body of evidence demonstrates unexpected adverse effects of these drugs: weight gain, due to increased adipose tissue mass, fluid retention, and effects on skeletal metabolism (DeFronzo et al., 2009; Home et al., 2009). For example, activation of PPARγ by thiazolidinediones suppresses several key osteogenic transcription factors in both mice and humans (Shockley et al., 2009), such as homeobox protein Dlx5, Runt-related transcription factor 2 (Runx2) and Osterix, and these changes contribute to a decrease in bone mass. In addition, thiazolidinediones can stimulate bone resorption, further uncoupling the bone remodeling unit.

1.6.4 SPPARMs

Recent studies have demonstrated that full and partial activation, as well as antagonism, of PPARγ can all improve insulin sensitivity. Coupled with additional evidence, from mouse models and humans with polymorphisms in PPARγ, it appears that moderate activation of PPARγ might be better suited to uncouple the insulin-sensitizing effects from the adverse side-effects (Doshi et al., 2010). Investigation of

partial or selective PPARγ modulators (SPPARMs) have proved promising and suggests that they represent an attractive alternative approach to avoid the unwanted effects of PPARγ ligands (Doshi et al., 2010; Feldman et al., 2008). Unlike full PPARγ agonists, in vitro and in vivo studies demonstrate that the SPPARMs exhibit limited effects on adipogenesis and fluid retention but still increase adiponectin profiles and insulin sensitivity in a manner comparable to, or even superior to, the TZDs (Doshi et al., 2010).

At molecular level, the interaction of SPPARMs with PPARγ is different to that of full agonists which results in distinct changes in receptor conformation, leading to alternate co-factor recruitment, thereby eliciting discrete cellular responses (Zhang et al., 2007). Typically, binding of full agonists results in stabilization of the AF2 helix in the active conformation causing dissociation of corepressors such as NCoR and allowing binding of coactivators, which leads to increased transcription of genes responsible for the pleiotropic effects of PPARγ. In contrast, the structurally diverse SPPARMs appear to promote activation of PPARγ by stabilizing an alternate region of PPARγ, involving the β-sheet and helix 3 regions (Bruning et al., 2007; Pochetti et al., 2007), and it is thought that this results in limited recruitment of coactivators underpinning the SPPARMs restricted and more favourable effects (Doshi et al., 2010; Zhang et al., 2007; Montanari et al., 2008; Pochetti et al., 2010; Porcelli et al., 2011).

1.7 PPARs and the metabolic syndrome

The three PPAR subtypes are strategically positioned in the regulation of metabolic reactions and inflammation in different tissues and organs. For this reason they have been exploited as suitable targets in the correction of metabolic derangements typically occurring in obesity, type 2 diabetes and metabolic syndrome.

Metabolic syndrome is defined as the combination of multiple metabolic disorders, including obesity, dyslipidaemia, glucose intolerance, inflammation and hypertension. The pathophysiology determining this syndrome involves an interaction of key factors, but two of these, insulin resistance and obesity, play major roles. These chronic disorders are related to alterations in lipid and glucose metabolism.

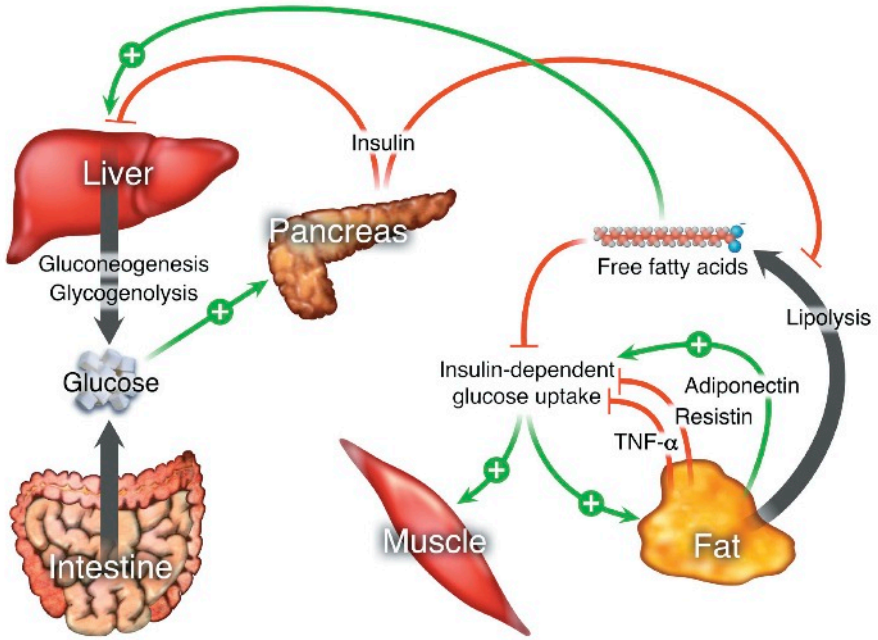


Fig. 7 *tissue-tissue crosstalk in glucose and lipid homeostasis (Evans et al., 2004)*

Currently two main competing theories are proposed that could explain the emergence of impaired glucose tolerance. First, obesity is now recognized as a state of chronic, low grade inflammation (Wellen et al., 2003; Hotamisligil et al., 2006). Current models suggest that constant excess nutrient supply results in hypertrophy of adipocytes and adipose tissue. This leads to a series of changes that include an increase in adipose tissue hypoxia and an increase in oxidative and ER stress within the adipocyte. These changes lead to alterations in gene expression, cellular

metabolism and a profound change in the adipocyte secretome. Adipocytes secrete a large number of factors, collectively termed adipokines, including the hormones leptin and adiponectin as well as the pro-inflammatory cytokines TNFα, IL-6 and MCP-1. Under hypertrophic conditions production of these pro-inflammatory cytokines is increased whilst production of the anti-inflammatory hormone adiponectin is decreased. These pro-inflammatory signals serve to recruit circulating monocytes and activate resident macrophages within the inflamed adipose tissue, which leads to further increase in the local concentration of pro-inflammatory molecules. Increased activation of intracellular stress pathways, including JNK and NF-κB, which inhibit insulin signaling in key organs and tissues such as liver, skeletal muscle and adipose tissue itself, gives rise to a state of insulin resistance. Dysregulated adipokine secretion from WAT of obese individuals also contributes to the development of systemic insulin resistance and metabolic disease (Rosen et al., 2006). Second, spillover of excess fatty acids and their metabolites into other tissues, so-called ectopic fat, leads to induction of insulin resistance in tissues including liver and skeletal muscle (Morino et al., 2006).

Fat metabolism and sugar homeostasis are inherently related. Insulin, recognized for its role in promoting glucose uptake, has long been known to regulate triglyceride catabolism through its inhibition of hormone-sensitive lipase. Lipid abnormalities also have profound effects on glucose homeostasis.

Development of systemic insulin resistance represents the first hit on the journey to type 2 diabetes mellitus, the major source of morbidity and mortality in the obese population. It is characterized by insulin resistance in target tissues, namely the failure of insulin to stimulate glucose disposal in muscle and adipose tissue and to inhibit gluconeogenesis in the liver. Additionally, pancreatic beta cells fail to compensate for this resistance by adequately increasing the secretion of insulin. Under these circumstances a defect in β-cell capacity may develop, as a result of lipotoxicity and glucotoxicity, leading to reduced β-cell number and insulin production (DeFronzo et al., 2010). As mentioned above, the three PPAR subtypes play a pivotal role in the regulation of glucose and lipid metabolism in different tissues, and the PPAR ligands are useful tools against type 2 diabetes and metabolic syndrome.

To design ligands modulating the activity of PPARs with improved therapeutical index, it is necessary to decipher the structural features and molecular mechanisms

underlying the ligand binding and the consequent activation of these receptors as a function of the chemical structure of ligands that dictates a peculiar transcription program. The investigations performed during the doctorate program was focused on the elucidations of the biochemical properties of a new synthetic PPAR ligand, here used a model to unveil novel structural and biochemical features of PPAR activation.

2. AIM OF THE STUDY

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily, ligand-dependent transcription factors that play a key role in the regulation of lipid, glucose and energy metabolism. As such, these nuclear receptors represent ideal targets for the treatment of pathological conditions characterized by dysregulation of metabolic pathways such as obesity, insulin resistance and T2D. Synthetic molecules have been already on the market but unfortunately they also evidenced unwanted effects that limit their therapeutic potential. Recent investigations aim at developing new PPAR ligands devoid of the side effects of the marketed antidiabetic agents thiazolidinediones and the dual PPAR α/γ agonists glitazars. To design new molecules with an improved profile of action, it is fundamental to understand the structural basis and the molecular mechanism underlying PPAR activation induced by different ligands. Our laboratory, in collaboration with chemists and crystallographers has recently described a new molecule that binds and activates both PPARα and γ subtypes (Montanari et al., 2008). On the basis of these preliminary findings, the aim of the work presented in this doctorate thesis was to study the peculiar molecular mechanism of action of LT175, a novel dual PPAR α/γ ligand, to better understand how the ligand-receptor complex works. To this end, in vitro and in vivo approaches were set, using different cell-free as well as cellular and animal models.

In details, the specific aims of the doctorate thesis were:

1. To study the recruitment of coregulators on the liganded receptors using Fluorescence Resonance Energy Transfer (FRET) approach.
2. To investigate the ability of the ligand to stimulate adipocyte differentiation and to induce lipid accumulation.
3. To evaluate the in vivo bioavailability and transcriptional activity of the compound in PPRE-LUC reporter mice.
4. To test the in vivo influence on metabolic profile induced by the ligand LT175 in C57Bl/6 mice, which were made insulin resistant by feeding them with a high fat diet (DIO).

The overall goal was to decipher the mechanistic basis of PPAR activation that explain the behaviour of LT175, here used as a model molecule to aid the design of novel PPAR scaffold molecules that could be refined to develop novel treatments of obesity and type 2 diabetes.

3. MATERIALS AND METHODS

3.1 Coregulator recruitment assay

Coregulator Interaction Assays were performed using Fluorescence Resonance Energy Transfer (FRET) approach. The ligand binding domain (LBD) of human PPARγ was expressed as N-terminal His-tagged protein using a pET28a vector and purified as following. Freshly transformed Escherichia coli BL21 DE3 were grown in LB medium with 30 μg of kanamycin mL^{-1} at 37 °C to an OD of 0.6. The culture was then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside and further incubated at 18 °C for 20 h. Cells were harvested and resuspended in a 20 mL x L^{-1} culture of buffer A (20 mM Tris, 150 mM NaCl, 10% glycerol, 1 mM Tris 2-carboxyethylphosphine HCl, pH 8) in the presence of protease inhibitors (Complete Mini EDTA-free; Roche Applied Science). Cells were sonicated, and the soluble fraction was isolated by centrifugation (35000g for 45 min). The supernatant was loaded onto a $Ni2^{+}$-nitrilo-triacetic acid column (GE Healthcare) and eluted with a gradient of imidazole 0-300 mM in buffer A with a PU980 HPLC system (Jasco, Lecco, Italy). The fractions containing the protein were collected, quantitated with a Bradford assay, and analyzed on 12% SDS-PAGE. The protein was then dialyzed over buffer C (20 mM Tris, 20 mM NaCl, 10% glycerol, 1 mM Tris 2-carboxyethylphosphine HCl, pH 8) to remove imidazole. The identity of the His-tagged protein was determined on the basis of the molecular weight and of the sequencing of the tryptic peptides obtained by liquid chromatography-electrospray ionization mass spectrometry and tandem mass spectrometry (LTQ; ThermoElectron Co., San Jose, CA), respectively. The protein was then loaded onto a Q-Sepharose HP column (GE Healthcare) and eluted with a gradient of NaCl 0-500 mM in buffer B (20 mM Tris, 10% glycerol, 1 mM Tris 2-carboxyethylphosphine HCl, pH 8). The protein was then dialyzed over buffer B and kept frozen in aliquots at a concentration of 1 mg 3 mL^{-1}.

Fluorescence resonance energy transfer assays to evaluate peptide recruitment were performed in 384-well plates in a final volume of 10 μL. A mix of 8 ng of human PPARγ-LBD, 0.8 ng of europium-labeled anti-His antibody (Perkin-Elmer, Monza, Italia), and 86 ng of allophycocyanin-labeled streptavidin (Perkin-Elmer), in a FRET buffer containing 50 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, and 0.1% free fatty acids BSA was prepared. The tested ligands were added to the mix at the concentration ensuring the saturation of the receptor. The biotinylated peptides

(PRIMM, Milano, Italia) were added in 12-point dose response curves starting at 9 μM as the highest concentration. The reactions were equilibrated for 1 h at room temperature and then measured in an Envision multiplate reader (Perkin-Elmer) using 340 nm as excitation and 615 and 665 nm as emission wavelengths. The ratio between 665 (APC signal) and 615 (europium signal) was used to evaluate the peptide recruitment on the receptor.

The peptide sequences used are Biotin-CPSSHSSLTERH-KILHRLLQEGSPS-COOH (NR box 2) for SRC-1 spanning from amino acid 676 to 700 (reference number NP_671766), Biotin-DGTPPPQEAEEPSLLKKLLLAPANT-COOH (NR box 1) for PGC-1R spanning from amino acid 130 to 154 (reference number NP_037393), Biotin-LERNNIKQAANNSLLLHLLKSQ-TIP-COOH (NR box 4) spanning from amino acid 366 to 390, and Biotin-PVSPQDFSFSKNGLLSRLLRQNQDSYL-COOH (NR box 7) spanning from amino acid 805 to 831 for RIP140 (reference number NP_003480), Biotin-SGNLVPDAASKHKQLSELLRG-GSGS-COOH (NR box 1) spanning from amino acid 56 to 80 and Biotin-SVQPPRSISPSALQDLLRTLKSP-COOH (NR box 4) spanning from amino acid 2055 to 2078 for CBP (reference number NP_004371), Biotin-GSTHGTSLKEKHKILHRLLQDSSSPVD- COOH (NR box 2) for TIF2 spanning from amino acid 676 to 702 (reference number NP_006531), Biotin-SFADPASNLGLEDIIR-KALMGSFDD-COOH (NR box 1) for N-CoR1 spanning from amino acid 2253 to 2277 (reference number NP_006302), and Biotin-APGVKGHQRVVTLAQHISEVITQ-COOH (NR box 1) for SMRT spanning from amino acid 2123 to 2145 (reference number NP_006303).

3.2 Cell cultures

3.2.1 3T3-L1

Differentiation protocol

3T3-L1 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% bovine calf serum. Two days after reaching confluence, differentiation was induced in DMEM supplemented with 10% fetal calf serum and 1 μM dexamethasone, 0.5 mM 3-isobuthyl-1-methylxanthine (IBMX), and 10 μg insulin·mL^{-1}. After 48 h, cells were supplemented with medium containing 10 μg insulin·mL^{-1} for an additional four days. Alternatively, cells were differentiated

with medium containing 5 μg insulin·mL^{-1} and 1 μM rosiglitazone or the other ligands. Medium was replenished with ligands every other day.

Oil red-O staining

Lipid content was determined by the staining with Oil Red O. The medium was removed and cells were incubated for 5 min RT with 10% formalin. The formalin was discarded and an equal volume of fresh formalin was added, incubating for 1 hour. Cells were then washed with 60% isopropanol and Oil Red O was added for 10 min. After washing four times with H_2O pictures were taken with an Axiovert 200 microscope at 20X magnification. The quantitation of the intracellular dye was performed after extraction by isopropanol and by reading the absorbance at 550 nm.

3.2.2 C3H10T1/2

Differentiation protocol

C3H10T1/2 cells were cultured in DMEM supplemented with 10% fetal bovine serum. One day after reaching confluence, differentiation was induced in DMEM supplemented with 10% fetal bovine serum and 1 μM dexamethasone, 0.5 mM 3-isobuthyl-1-methylxanthine (IBMX), 10 μg insulin·mL^{-1} and 5μM rosiglitazone. After 48 h, cells were supplemented with medium containing 10 μg insulin·mL^{-1} and 5μM rosiglitazone for an additional four days. Alternatively, cells were differentiated with medium containing 5 μg insulin·mL^{-1} and 1 μM rosiglitazone or the other ligands. Medium was replenished with ligands every other day.

3.3 PPRE-Luc

Experiments with the PPRE-Luc reporter mice were conducted in collaboration with Dr. Adriana Maggi (Dipartimento di Scienze Farmacologiche). These mice belong to C57Bl/6xDBA/2 F2 strain and carry the construct pMAR-PPRE5X-tk-Luc-MAR (Ciana et al., 2007). All experiments were carried on with 3- to 5-month-old male PPRE-Luc transgenic mice. Mice were kept under a 12 h light/dark regimen. To maximize the reporter response to drug treatments, metabolic activation of PPARs was minimized by feeding mice only during the night (Ciana et al., 2007) for the 48h preceding the experiment. All the experiments were carried out in the afternoon.

PPAR ligands were administered orally by gavage in 0.5% hypromellose (Sigma Aldrich). Treatments were performed in the morning (9:00 AM), and photon detection was assayed 6 h later (3:00 PM).

3.3.1 In vivo bioluminescence reporter imaging

Mice were visualized with a Night Owl imaging unit (Berthold Technologies, Bad Wildbad, Germany) consisting of a Peltier-cooled charge-coupled device slow-scan camera equipped with a 25-mm, f/0.95 lens. Images were generated by a Night Owl LB981 image processor and transferred via video cable to a peripheral component interconnect frame grabber using WinLight32 software (Berthold Technologies). For the detection of bioluminescence, mice were anesthetized using an s.c. injection of 50 μl of ketamine-xylazine solution composed of 78% ketamine (Ketavet 50; Intervet, Peschiera Borromeo, Italy), 15% xylazine (2% solution, Rompun; Bayer, Leverkusen, Germany), and 7% water. The mice then received an i.p. injection of 25 mg/kg D-luciferin (Promega, Madison, WI) 20 min before bioluminescence quantification, to obtain an uniform biodistribution of the substrate. Mice were placed in the light-tight chamber and a grayscale photo of the animals was first taken with dimmed light. Photon emission was then integrated over a period of 5 min.

3.3.2 Ex vivo luciferase enzymatic assay

For enzymatic assay of luciferase activity, tissues were dissected and immediately frozen on dry ice. Protein extracts were prepared by homogenization in a TissueLyser (QIAGEN GmbH, Hilden, Germany) in 200 μl of 100 mM KPO4 lysis buffer, pH 7.8, containing 1 mM dithiothreitol, 4 mM EGTA, 4 mM EDTA, and 0.7 mM phenylmethylsulfonyl fluoride), three cycles of freezing-thawing, and 30 min of minifuge centrifugation (Eppendorf AG, Hamburg, Germany) at maximum speed. Supernatants containing luciferase were collected, and protein concentrations were determined by Bradford assay. Luciferase enzymatic activity was measured by a commercial kit (Luciferase assay system; Promega, Madison, WI) according to the supplier's instructions. The light intensity was measured with a luminometer (Veritas; Promega, Madison, WI) over a 10 s time period and expressed as relative light units (RLU) per microgram of protein.

3.4 Quantification of LT175 in plasma

C57Bl/6 mice were treated for 15 days with 100 mg LT175/kg/day. Blood samples were collected from retrorbital plexus with a Pasteur glass pipette at days 0,1,2,3,4,5,10 and 15. After centrifugation at 8000 rpm 4 °C for 5 min plasma was collected. One hundred µl aliquots of plasma sample were mixed with 50 µl of internal standard working solution (100 ng/µl of clofibric acid). Formic acid was added to a final concentration of 0.1%. One ml mixture of di-ethyl ether and ethyl acetate (1:1, v/v) was added and vortexed for 30 sec followed by a centrifugation at 3000 rpm for 2 min. Using a Pasteur glass pipette, the organic layer was transferred in an Eppendorf tube and evaporated to dryness by nitrogen flushing. The organic extraction procedure was performed twice for each sample. Pooled residues were reconstituted with 0.2ml of methanol:water:isopropilic alcohol (1:1:1, v/v/v), transferred in autosampler vials and 10 µl were injected in to the LC-MS/MS system.
The chromatography was performed on a Surveyor MS Pump Plus (Thermo-Electron Co, San Josè, CA, USA) with cooling auto-sampler and column oven enabling temperature control of the analytical column. An Inertsil ODS-2 C18 column (150 X 4.6mm, 3 µm; GL Sciences Inc., Tokyo, Japan) was employed. The column temperature maintained at 40°C and chromatographic separation was achieved using an isocratic gradient of mobile phase consisting of methanol–water–isopropilic alcohol (1:1:1, v/v/v) delivered with a flow rate of 0.5ml/min. MS analysis were performed in Negative ion mode on a mass spectrometer equipped with an Electro Spray Ionization (ESI) source.

3.5 Diet Induced Obese mice

3.5.1 Experimental procedure and diet

Six weeks old C57Bl/6J male mice (at least 6 animals/group) (Charles River Laboratories, Calco, Italy) were fed a high fat diet containing 45% fat as the calorie source for 16 weeks. PPAR ligands were administered once a day for 2 weeks by oral gavage in 0.5% hypromellose (Sigma Aldrich). LT175 was administered at 100 mg/kg/day. Fenofibrate (100 mg/kg/day), rosiglitazone (10 mg/kg/day) and AZ12063233 (5 mg/kg/day) were used as reference compounds for PPARα, γ and α/γ activation, respectively. All animal studies were approved by the local ethical committee and followed the Italian and European Community legislation.

3.5.2 In vivo Magnetic Resonance Imaging

MRI analyses were performed in collaboration with Dr. Uliano Guerrini (Dipartimento di Scienze Farmacologiche). At day 12 of treatment with ligands, mice underwent in vivo magnetic resonance imaging (MRI) analysis to monitor total body fat distribution. Mice were anesthetized with 1% isoflurane and were analyzed in a 4.7 T Avance II MRI scanner (Bruker Corporation, Karlsrhue, Germany). After a gradient echo scout, 16 axial 1 mm thick T1 weighted slices were placed in the abdominal region spanning from kidneys to bladder included. The field of view was 30 × 30 mm2 with a matrix of 128 × 128 pixels. Four averages of a spin echo sequence with TE = 10 ms and TR = 400 ms were acquired in 3′25″ to provided a contrast between fat and other tissues. In order to maximize consistency between different animals, the slice immediately frontal with respect to the ilium bone was chosen for visceral fat estimation and was computed as (fat area)/(slice area). Areas were semiautomatically delimited and measured with Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, California).

3.5.3 Plasma analysis

At sacrifice, blood was taken for the determination of biochemical parameters and circulating hormones. After centrifugation at 8000 rpm 4 °C for 5 min plasma was collected. Total plasma cholesterol, triglycerides, NEFA, and glucose were measured with standard commercial kits (for cholesterol, Horiba ABX, Roma, Italy; for triglycerides and glucose, Sentinel Diagnostics, Milano, Italy; for NEFA Wako, Neuss, Germany), following owner's instructions. Insulin, adiponectin and FGF21 levels were determined with ELISA kit (Insulin: Mercodia, Uppsala Sweden; Adiponectin: Genway, San Diego, CA; FGF21: Biovendor, Germany).

3.5.4 Cholesterol and triglycerides distribution in lipoprotein fractions

Fast protein liquid chromatography for lipoprotein separation and quantification of cholesterol and tryglicerides were set as follows: Superose 6 column 300 mm x 10 mm was used. The flow rate of the pump was set at 500µl/min and the reagent flow rate was set at 60 fractions. 100µl of plasma were used, for a total elution volume of 24 ml. The run time was 50 min. After the FPLC, cholesterol and triglycerides were

dosed by colorimetric kit using 50μl of the separated fractions. The UV detector was set at 230nm.

3.5.5 Cholesterol and triglycerides levels in the liver

100mg of tissue were omogenized with TissueLyser (QIAGEN GmbH, Hilden, Germany). Fifty μg of 5α-Colestane were added as internal standard. Twenty ml of a solution of chloroform-methanol (2:1, v/v) were added to the samples, following an O/N incubation. The acqueous phase was removed, and the organic phase was filtered and dried by nitrogen flushing. Samples were dissolved in 1ml exane and divided into two different aliquots. The first aliquot was dried by nitrogen flushing and dissolved in 100μl of a solution 5% Triton X-100 in chloroform, then dried again and dissolved in 100μl of a solution 0.9% NaCl in H_2O. Triglycerides were then measured with commercial kit (Sentinel Diagnostic). The second aliquot followed a saponification for determination of total cholesterol levels, adding 2ml NaOH in 90% EtOH and incubating the samples for 1h at 60°C. After diluting samples with 1ml H_2O, 10ml petroleum ether were added and the solution were shacked vigorously. Samples were then washed passing by sodium sulfate filters.

3.5.6 Oral Glucose Tolerance Test and Insulin Tolerance Test

For the oral glucose tolerance test (OGTT), mice were fasted overnight and were administered 2g glucose·kg^{-1} per os. Blood was taken from the tail at 15 min intervals, and glucose concentration was determined with OneTouch Ultra glucometer (LifeScan, Milano, Italy). For the insulin tolerance test (ITT), mice were fasted six hours and were administered 0.6 IU·kg^{-1} of insulin (Eli Lilly, Firenze, Italy) with an intraperitoneal injection. Blood samples were taken from the tail at 15 min intervals, and glucose concentration was determined with OneTouch Ultra glucometer (LifeScan).

3.6 RNA extraction and gene expression analyses by Real Time qPCR

Cell cultures

Total RNA was extracted from 3T3-L1 adipocytes with Trizol (Sigma, Milano, Italy) followed by purification on Nucleospin RNA II column (Macherey Nagel, Germany) according to the manufacturer's instructions.

Tissues

Mouse tissues were snap frozen in liquid nitrogen for gene expression analysis. Total RNA was extracted with Trizol (Sigma, Milano, Italy) followed by purification on Nucleospin RNA II column (Macherey Nagel, Germany). RNeasy lipid cartridges (Qiagen, Milano, Italy) were used with tissues rich in lipids, according to the manufacturer's instructions.

Purified total RNA samples were quantitated with Nanodrop (Thermo Scientific, Wilmington, DE). One microgram of total RNA was used to quantitate the mRNA levels of the different PPAR target genes by real time qPCR using iScriptTM One Step RT-PCR for Probes (Bio-Rad, Milano, Italia), in a CFX 384 thermal cycler (Bio-Rad). 36B4 was used as house keeping gene for data normalization.

3.6.1 Primers for gene expression analysis

The following set of primers were used for the gene expression analysis:

GENE	SEQUENCE	
m36B4	fwd	AGATGCAGCAGATCCGCAT
	rev	GTTCTTGCCCATCAGCACC
	probe	CGCTCCGAGGGAAGGCCG
mIDH3α	fwd	ACGGAAGGAGAATACAGTGG
	rev	GTACTCGAAGGCAAACTCTG
	probe	ACCCCATCAACGATCACATGCTCA
mCYTC	fwd	GAAAAGGGAGGCAAGCAT
	rev	ACTCCATCAGGGTATCCTCT
	probe	AACAAGAACAAAGGCATCACCTGGG
mGLUT4	fwd	TGTCGCTGGTTTCTCCAACTG
	rev	CCATACGATCCGCAACATACTG
	probe	ACCTGTAACTTCATTGTCGGCATGGGTTT
mSUCLG1	fwd	AATGATCCAGCCACAGAAGG
	rev	AGCAATGAAGGACACTACAGG
	probe	AGCATAACTCAGGTCCAAAGGCCAA
mHADH	fwd	TCTTGACTATGTTGGACTGGATAC
	rev	AAGGACTGGGCTGAAATAAGG
	probe	CTTGGACGGGTGGCATGAAATGG
mUCP1	fwd	GAGCTGGTAACATATGACCTC
	rev	GAGCTGACAGTAAATGGCA
	probe	ACAAAATACTGGCAGATGACGTCCC
mCD36	fwd	GCGACATGATTAATGGCACAG
	rev	GATCCGAACACAGCGTAGATAG
	probe	CAACAAAAGGTGGAAAGGAGGCTGC
mCPT1α	fwd	CCAAGTATCTGGCAGTCGA
	rev	CGCCACAGGACACATAGT
	probe	CATGGCTCAGACAGTACCTCCTTCA
mCPT1β	fwd	GATGCAGTTCCAGAGAATCC
	rev	CTTGTTCTTGCCAGAGCT
	probe	TCTGCCCACTCTACCCTTCCTC
mACRP30	fwd	AGGCATCCCAGGACATC
	rev	CCTGTCATTCCAACATCTCC
	probe	CCTTAGGACCAAGAAGACCTGCATCTC
mFABP4	fwd	GGCGTGGAATTCGATGAA
	rev	GCTTGTCACCATCTCGTT
	probe	TGATGCTCTTCACCTTCCTGTCGT

mDIO2	fwd	CTGTGTCTGGAACAGCTT
	rev	CACTGGAATTGGGAGCAT
	probe	CTAGATGCCTACAAACAGGTTAAACTGGGT
mELOVL3	fwd	TGCTTTGCCATCTACACG
	rev	CAGTGGACAAAGATGAGTGG
	probe	TGAACTGGGAGACACGGCCTT
mENACγ	fwd	AAAGCTCAACAAGACTGACC
	rev	CACCAAAGTTAGACAGGAGC
	probe	AGAGCCCAGCCAACAGTATTGAGA
mMCAD	fwd	ACCCAGATCCTAAAGTACCC
	rev	CGAAAGCAATTCCTCTGGTG
	probe	TGGCCCATGTTTAGTTCCTTTTTTCCAA
mHMGCS2	fwd	CTGGCTCGGTTGATGTT
	rev	GAGCCTTGTCTACATCCTTG
	probe	CTTCCAGCTTTAGACCCCTGAAGGC
mACOX1	fwd	TCACGTTTACCCCGGC
	rev	CAAGTACGACACCATACCAC
	probe	CATCAAGAACCTGGCCGTCTGC
mACADL	fwd	GAAACCAGGAACTACGTGAAG
	rev	GCTGTCCACAAAAGCTCT
	probe	CACACATACAGACGGTGCAGCATA
mPGC-1α	fwd	CATTTGATGCACTGACAGATGGA
	rev	GTCAGGCATGGAGGAAGGAC
	probe	CCGTGACCACTGACA ACGAGGCC
mGK	fwd	GTGTACCAATATCTGGGTGTT
	rev	AGAAAATACACACTTATGGCCC
	probe	CAAAAACACGTATGGAACAGGGTGCT
mTFAM	fwd	CACCCAGATGCAAAACTTTCAG
	rev	CTGCTCTTTATACTTGCTCACAG
	probe	CCACAGGGCTGCAATTTTCCTAACC
mPCK1	fwd	TTGAACTGACAGACTCGCCCT
	rev	TGCCCATCCGAGTCATGA
	probe	CCGCATGCTGGCCACCACA
mHSL	fwd	GCTCCCTTTCCCCGA
	rev	ATGCAGAGATTCCCACCT
	probe	CACTGTGACCTGCTTGGTTCAACT

Primers for real-time PCRs were designed with IDT software available on line and optimized to work in a two-step protocol (40 cycles of amplifications each consisting of a denaturation step at 95° C for 30s and an annealing/extension step at 60° C for 60s) or one-step protocol (10 min at 50°C for reverse transcription, 40 cycles of amplifications each consisting of a denaturation step at 95° C for 10s and an annealing/extension step at 60° C for 30s). The oligonucleotides used for Real-Time PCR were synthesized by Primm (Milano, Italy) or Eurofin MWG Operon (Ebersberg, Germany).

3.7 Statistical analysis

Statistical analyses were performed via one-way ANOVA with post-test analysis for multiple group comparisons, using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA). Differences with *p* values less than 0.05 were considered statistically significant.

4. RESULTS

4.1 LT175 binds and activates PPARα and PPARγ

Two enantiomeric derivatives of clofibric acid, LT175 and EX34 (figure 2), were synthesized by the group of Prof. Loiodice, Dipartimento Farmaco-Chimico, Università degli Studi di Bari "Aldo Moro" (Pinelli et al., 2005; Montanari et al., 2008). The ***S***-enantiomer LT175 binds to and activates PPARα and γ.

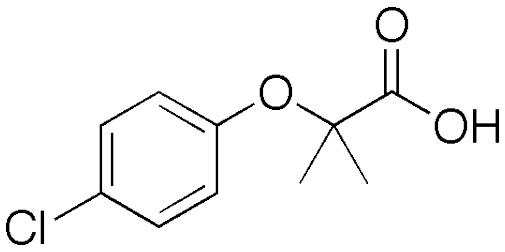


Fig 1*: structure of clofibric acid*

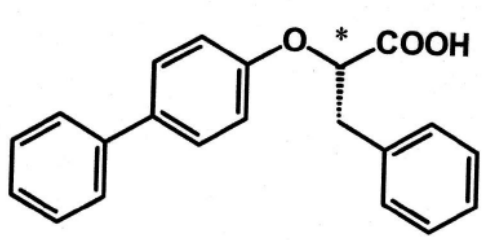


Fig 2*: structure of (S) LT175 and (R) EX34*

To gain insights in the molecular mechanisms governing the activation of PPARα and PPARγ we used fluorescence resonance energy transfer (FRET) assays to study the recruitment of coregulators induced by LT175. FRET assays were set at saturating concentrations of the ligand and increasing amounts of a panel of biotinylated peptides, corresponding to known cofactors of PPARs. LT175 acts as a full PPARα agonist, inducing the recruitment of different coactivators on the receptor and avoiding the recruitment of the corepressor NCoR (figure 3). Differently from rosiglitazone, LT175 does not allow the recruitment of the coactivator CBP on PPARγ. Furthermore NCoR still interacts with PPARγ in the presence of LT175, whereas the full agonist rosiglitazone does not allow the interaction of PPARγ with the corepressor NCoR (figure 4).

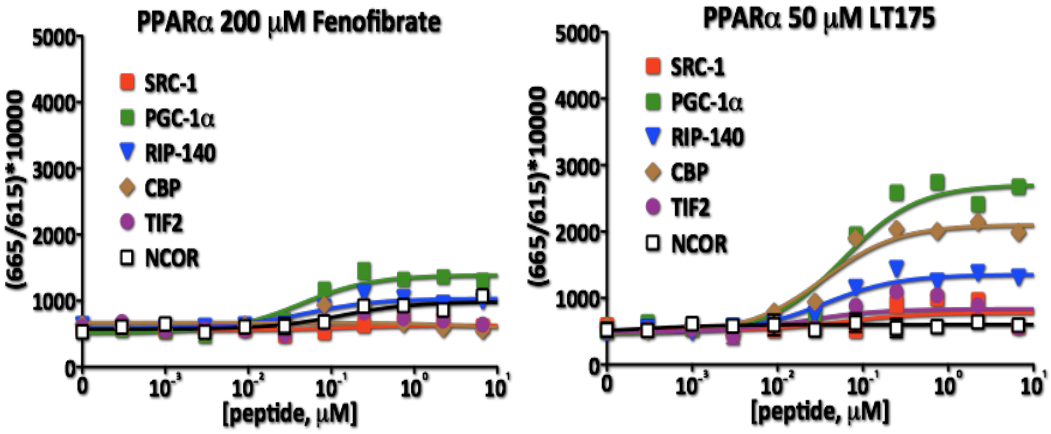


Fig 3: *coregulator recruitment on PPARα by fenofibrate and LT175*

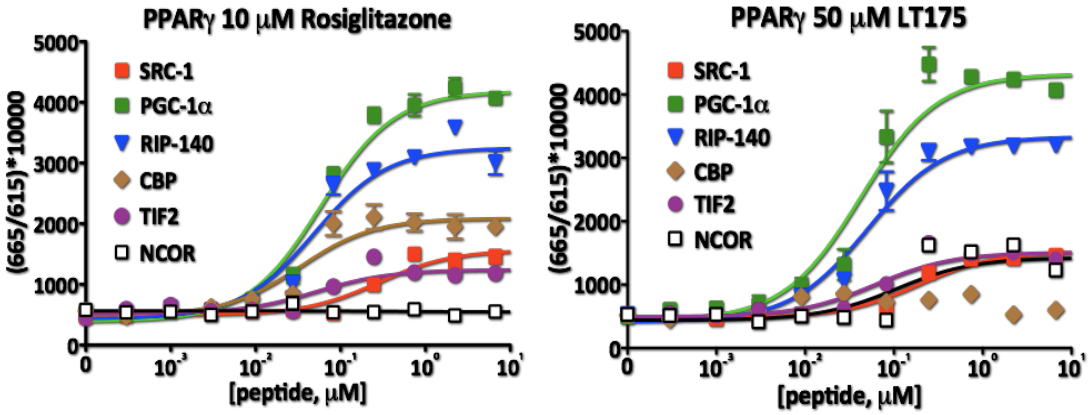


Fig 4*: coregulator recruitment on PPARγ by rosiglitazone and LT175*

4.2 LT175 activates PPARγ and reduces lipid accumulation in adipocytes

The biochemical mechanisms underlying the insulin sensitizer effect of thiazolidinediones depends, at least in part, on their action on adipose tissues (Tontonoz and Spiegelman, 2008). However, because one of the most important side effects induced by their action is lipid accumulation in adipocytes, we analyzed the in vitro biological activity of LT175 by evaluating the effect of LT175 on the differentiation of 3T3-L1 murine fibroblasts to adipocytes. Confluent cultures of 3T3-L1 preadipocytes were incubated with insulin and different PPAR ligands for seven days. Staining with Oil-Red O shows that adipocytes differentiated in the presence of

LT175 accumulate less lipids as compared to those treated with rosiglitazone, suggesting that this molecule may have lower adipogenic activity (figures 5 and 6).

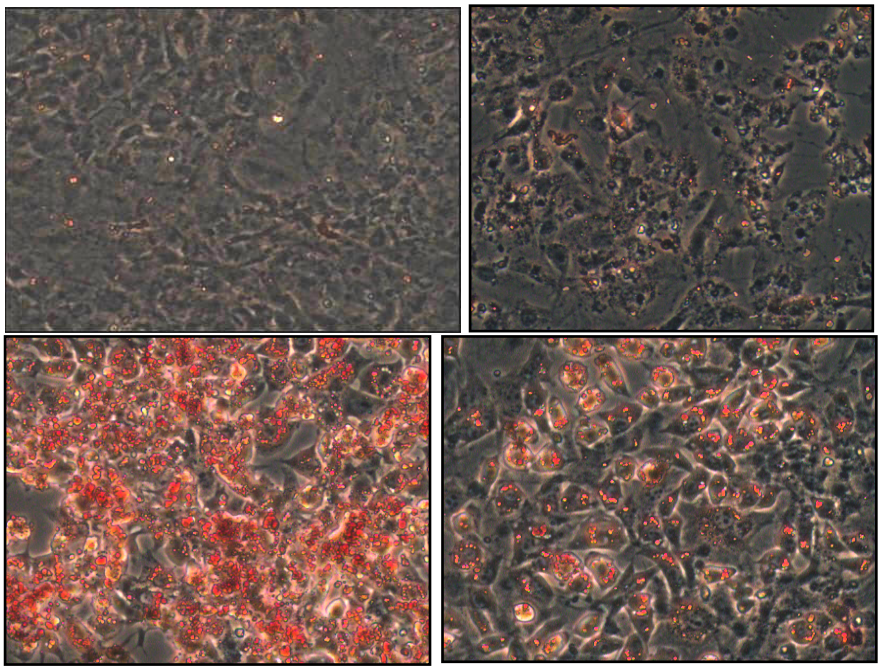


***Fig 5**: 3T3-L1 lipid accumulation by Oil-Red O staining; upper left panel: ctrl; upper right: insulin; lower left: ins + 1 μM rosiglitazone; lower right: ins + 5 μM LT175*

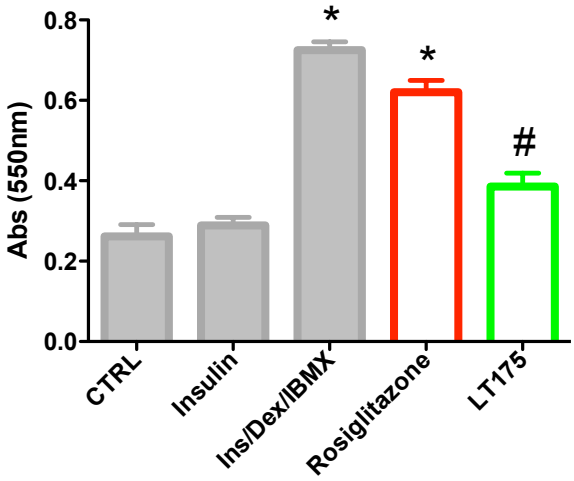


***Fig 6**: Oil Red O staining quantification; * P<0.05 vs Insulin, # P<0.05 vs rosiglitazone*

The analysis of the gene expression profile indicates that LT175, like rosiglitazone, is able to activate the PPARγ-dependent program of differentiation in adipocyte cultures by increasing the mRNA levels of typical PPARγ target genes like the insulin-dependent glucose transporter Glut4, adiponectin (Acrp30), a peptide hormone involved in insulin sensitivity, and of fatty acid binding protein 4 (Fabp4) also known as aP2, a factor involved in the accumulation of triglycerides. Interestingly, LT175 also increases the expression of hormone sensitive lipase (Hsl), contributing to free cholesterol and fatty acids (figure 7).

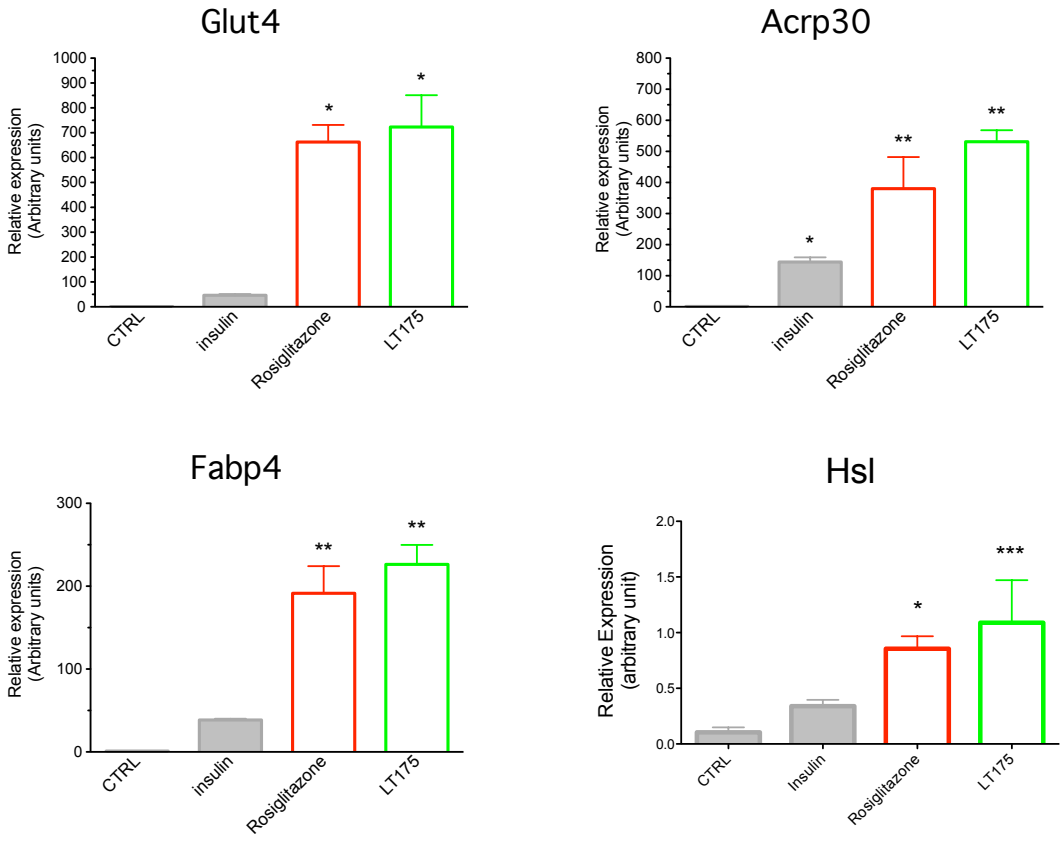


***Fig** 7: gene expression profile of 3T3-L1 preadipocytes differentiated in presence of rosiglitazone or LT175; * P<0.05 vs Insulin, ** P<0.01 vs Insulin, *** P<0.001 vs Insulin*

To corroborate these results, similar experiments were performed in another cell line, the murine fibroblast C3H/10T1/2, which can also be differentiated to adipocytes using a protocol similar to the one used for 3T3-L1 fibroblasts. As shown in figure 8,

LT175 is able to activate the PPARγ-dependent program of differentiation also in this cellular model, enhancing the expression of Glut4, Acrp30 and Fabp4.

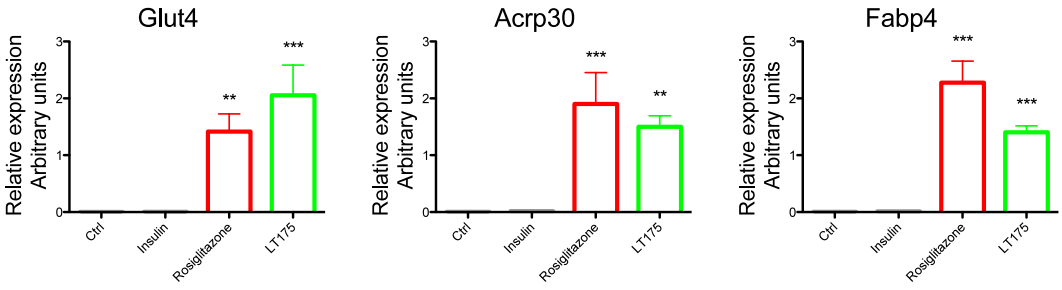


Fig 8: *gene expression profile of C3H10T1/2 differentiated in presence of rosiglitazone or LT175; ** P<0.01 vs Insulin, *** P<0.001 vs Insulin*

4.3 LT175 increases PPAR-mediated transcription in PPRE-Luc mice

Having assessed that LT175 binds to and activates both PPARα and γ subtypes, we wanted to evaluate the biological activity of this ligand in vivo. First, the bioavailability and the in vivo transcriptional activity of LT175 was evaluated by in vivo imaging in the chest and ex vivo by enzymatic assay in different tissues and organs of the reporter mouse model PPRE-Luc. LT175 was orally administered to PPRE-Luc mice for three days at the dose of 100 mg/kg body weight/day. The results show that LT175 induces PPRE-driven transcription of the luciferase reporter in the liver, WAT, BAT, indicating that the compound reaches these target tissues where it activates the PPAR-dependent transcription program (figure 9). Ten mg/kg body weight/day rosiglitazone and 100 mg/kg body weight/day fenofibrate were used as reference PPARγ and PPARα ligands, respectively. AZ12063233 was kindly provided by AstraZeneca as dual PPARα/γ reference agonist and was administered at 5 mg/kg body weight/day. As expected, rosiglitazone increases luciferase in white adipose tissue (WAT) but not in the liver, whereas the PPARα reference ligand fenofibrate was only effective in the liver. The dual PPARα/γ ligand AZ12063233 enhances luciferase activity in WAT and in the liver, although the latter value did not reach statistical significance. LT175 increases luciferase activity in the liver, white and brown adipose tissue.

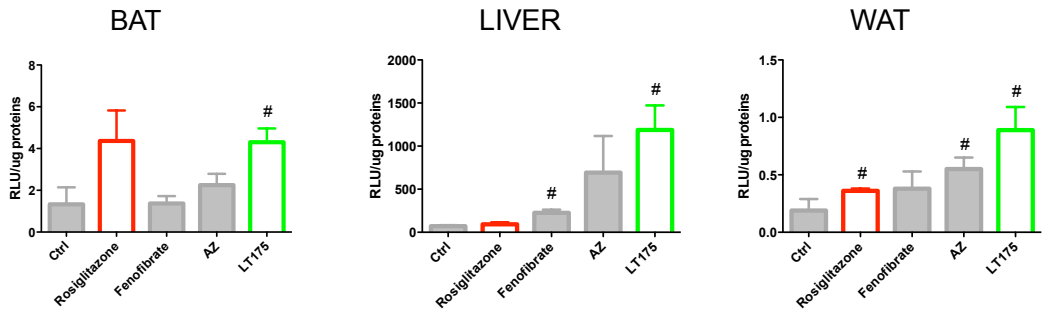


***Fig 9**: relative luciferase activity in the liver, white and brown adipose tissue of PPRE-LUC mice; # P<0.05 vs Ctrl*

We next sought to determine the kinetics of PPAR-mediated transcription when mice are exposed to a longer treatment with LT175. The experiment was run in parallel in PPRE-Luc mice administered with the PPARα agonist Wy14,643 used as a reference for comparison. To this end, PPRE-Luc reporter mice were treated for 19 days with different PPAR ligands, and the magnitude and distribution of luminescence induced by PPAR activation was evaluated by in vivo imaging every day in chest and abdomen areas. The results show a different spatial PPAR activation induced by LT175 during the days of treatment, compared to the reference PPARα agonist Wy14,643 (figures 10, 11, 12, 13 and 14). As shown in figure 10, mice treated with vehicle show a very small PPAR activation during the 19 days of treatment. In contrast, mice treated with Wy14,643 show a greater PPAR activation in the chest area during the 19 days of treatment (figure 11). In the case of LT175, mice show a more consistent PPAR activation also in the abdomen, especially during the last days of treatment (figure 12).

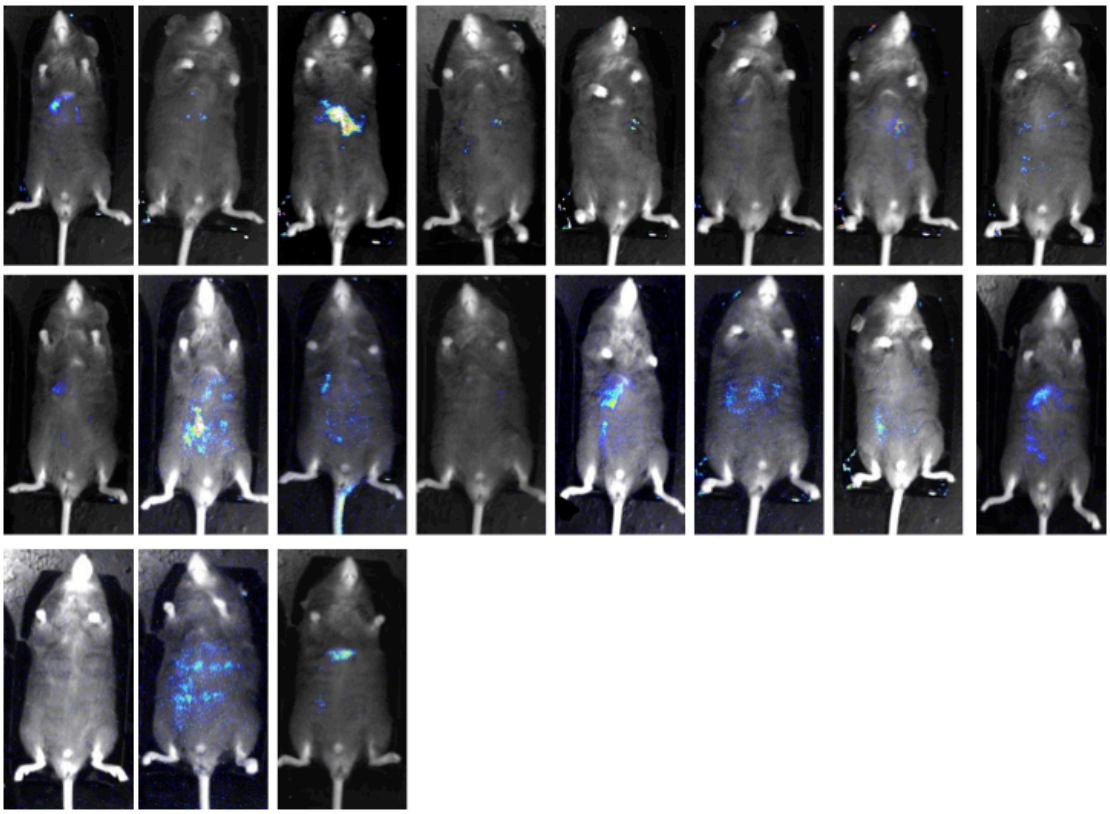


Fig 10: *luciferase activity in PPRE-LUC mouse treated for 19 days with the vehicle*

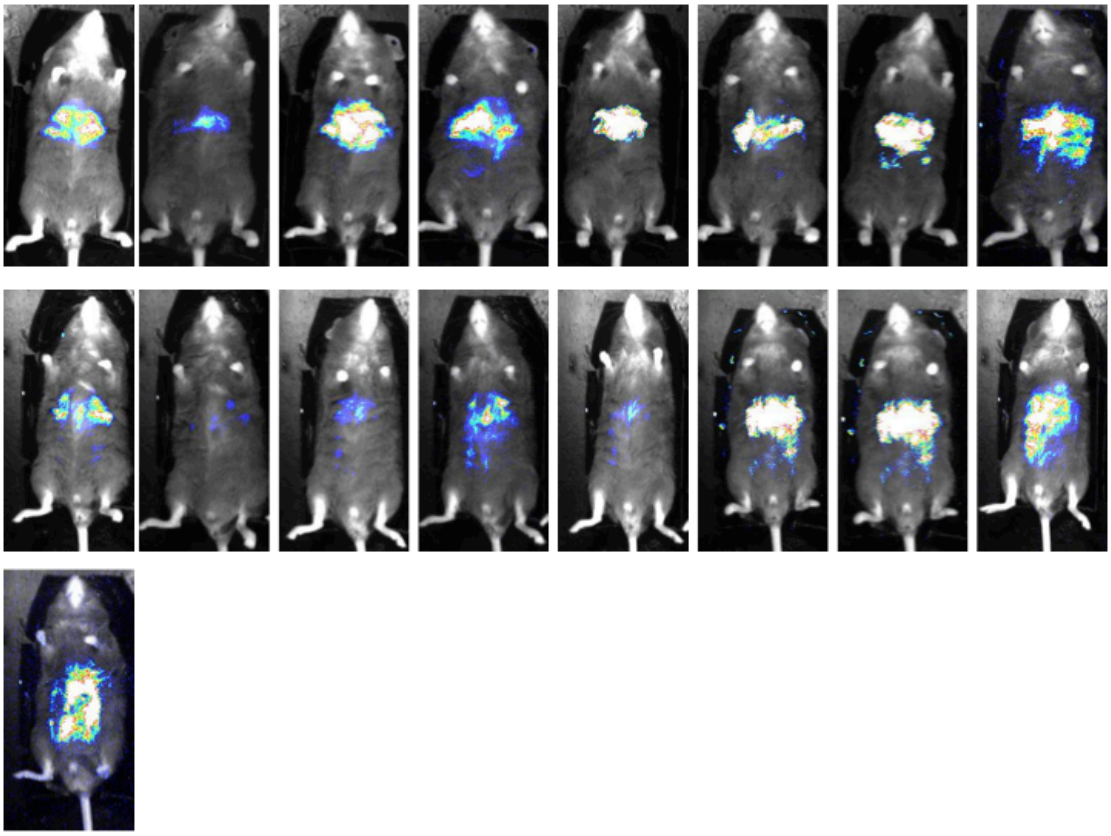


Fig 11: *luciferase activity in PPRE-LUC mouse treated for 17 days with Wy14,643*

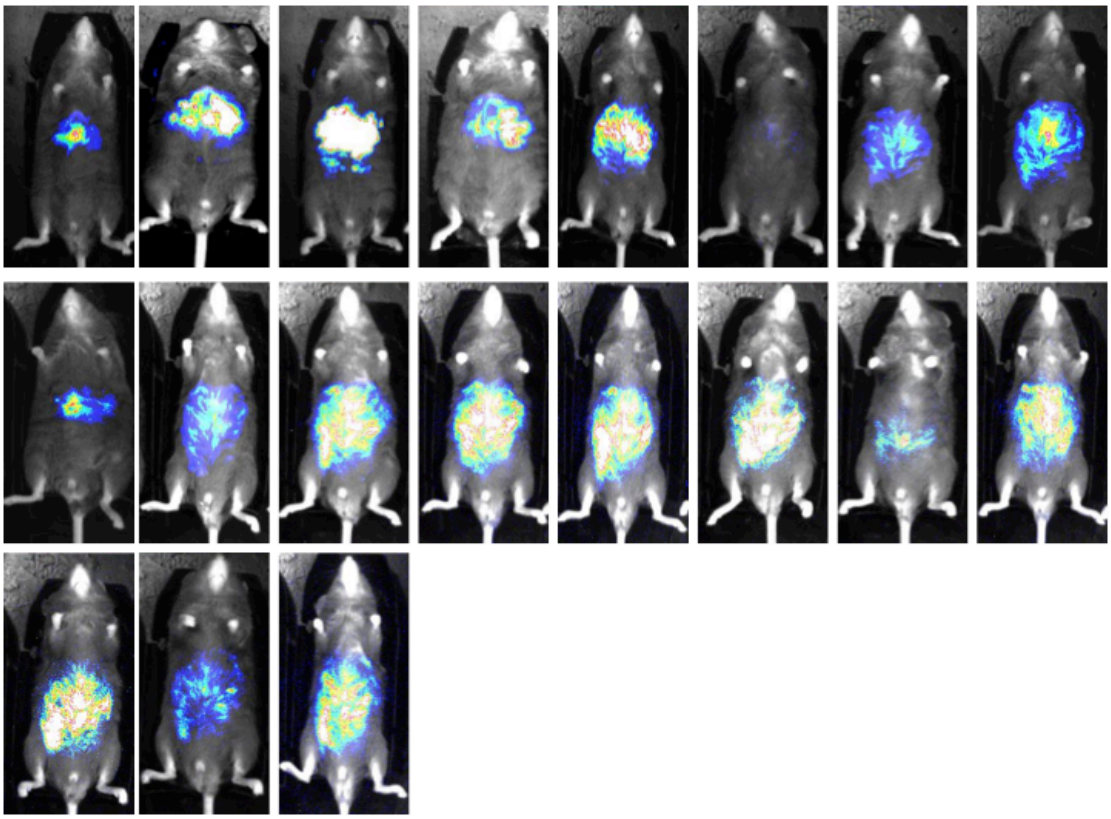


***Fig 12**: luciferase activity in PPRE-LUC mouse treated for 19 days with LT175*

Quantification of bioluminescence shown in figure 13 (chest area, mostly referred to the liver), and in figure 14 (abdomen area) indicates that LT175 induces different receptor activation than Wy14,643 throughout the treatment. Interestingly, after seven days, animals treated with LT175 show loss in the receptor activation, while luciferase activity oscillates to greater values in mice on Wy14,643, suggesting that these two ligands may regulate hepatic PPARα activity with different modalities.

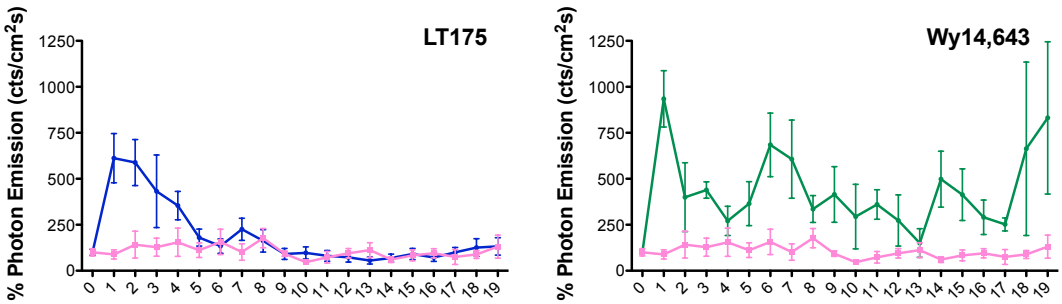


***Fig 13**: chest area, % of photon emission of animals treated with LT175 (blue) and Wy 14,643 (green) compared to the vehicle (pink). Data are expressed as percentage relative to values at day 0*

In the abdomen, LT175 stimulates the transcription from the first day of treatment and this stimulation remains at these levels until the end of the experiment. Wy14,643 induces luciferase activity only after ten days.

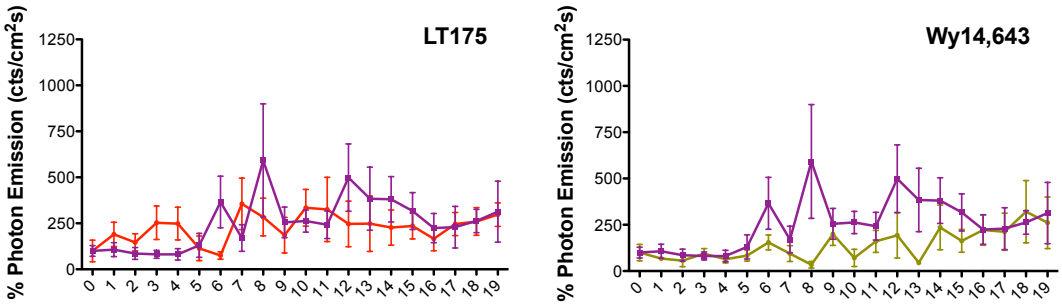


***Fig 14**: abdomen area, % of photon emission of animals treated with LT175 (red) and Wy 14,643 (brown) compared to the vehicle (purple)*

We wanted to investigate further the loss of receptor activation noticed with LT175. This effect may be caused either by desensitization of the receptor, or by increased rate of degradation of the ligand during the days of treatment due to induction of some cytochrome P450 metabolizing the ligand. Therefore, C57Bl/6 mice were treated for 15 days with 100 mg LT175/kg/day. Blood samples were collected from retrorbital plexus at days 0,1,2,3,4,5,10 and 15 and plasma was obtained and analyzed by LC-MS/MS system. As shown in figure 15, plasma concentration of LT175 increases during the days of treatment, reaching a plateu after 7 days. This result clearly indicates that the gradual loss of PPRE-driven transcription observed in PPRE-Luc mice cannot be explained with increased metabolism of the ligand, as LT175 is well detectable in the blood of mice up to 15 days after the beginning of the administration.

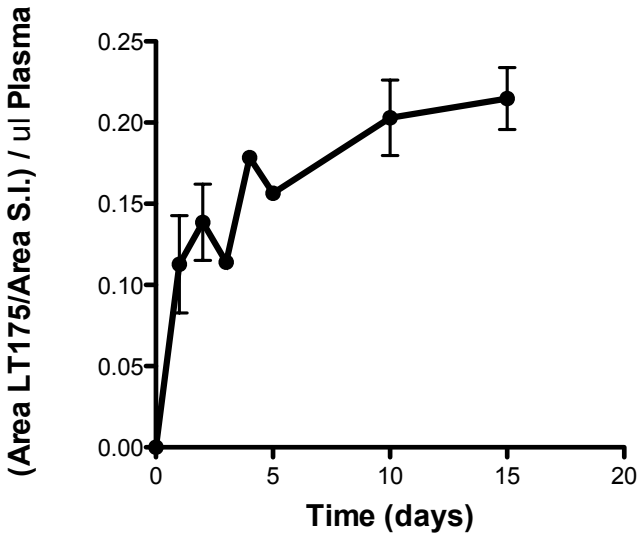
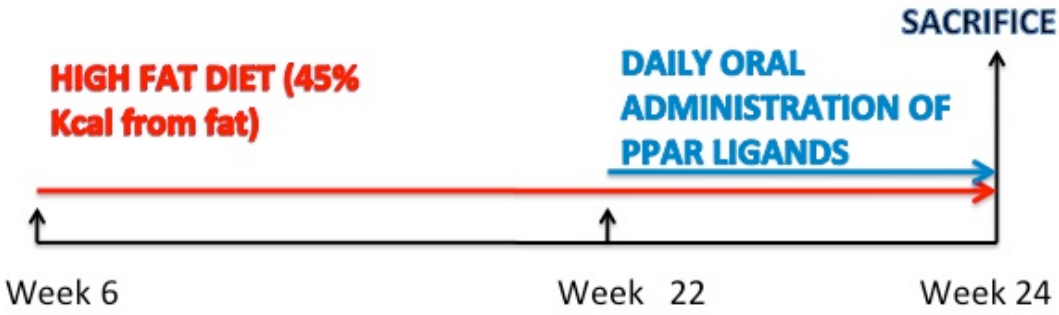


Fig 15: *quantification of LT175 in murine plasma during 15 days treatment*

4.4 LT175 improves the diabetic phenotype of insulin resistant mice

To test the in vivo influence on metabolic profile induced by LT175, the compound was orally administered (100 mg/kg/day) for two weeks to C57Bl/6 mice, which were made insulin resistant by feeding them with a high fat diet (Diet Induced Obese mice, DIO) for 16 weeks. The experimental protocol is shown in the following scheme:



4.4.1 LT175 decreases total body weight and visceral fat

10 mg/kg/day rosiglitazone, 100 mg/kg/day fenofibrate and 5 mg/kg/day AZ12063233 were used for comparison as reference PPARγ or PPARα or PPARα/γ dual ligands, respectively. After two weeks of treatment with PPAR ligands, the groups administered with AZ12063233 and LT175 show a strong decrease of body weight while the other PPAR ligands did not change this parameter (figure 16).

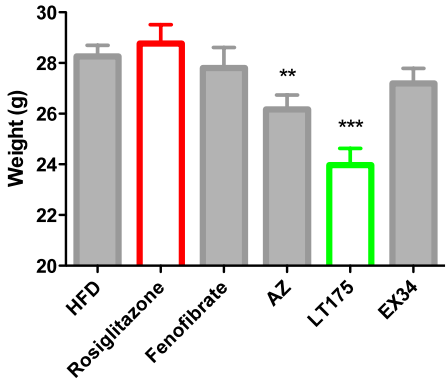


***Fig 16**: total body weight quantification in DIO mice; ** P<0.01 vs HFD, *** P<0.001 vs HFD*

To explain the total body weight reduction observed in these two animal groups we performed magnetic resonance imaging (MRI) analysis and found that visceral adipose tissue decreases in mice treated with ligands with PPARα activity, however the most dramatic effect on visceral fat was observed with LT175 (figures 17 and 18). No significant differences of subcutaneous fat were observed with the tested ligands.

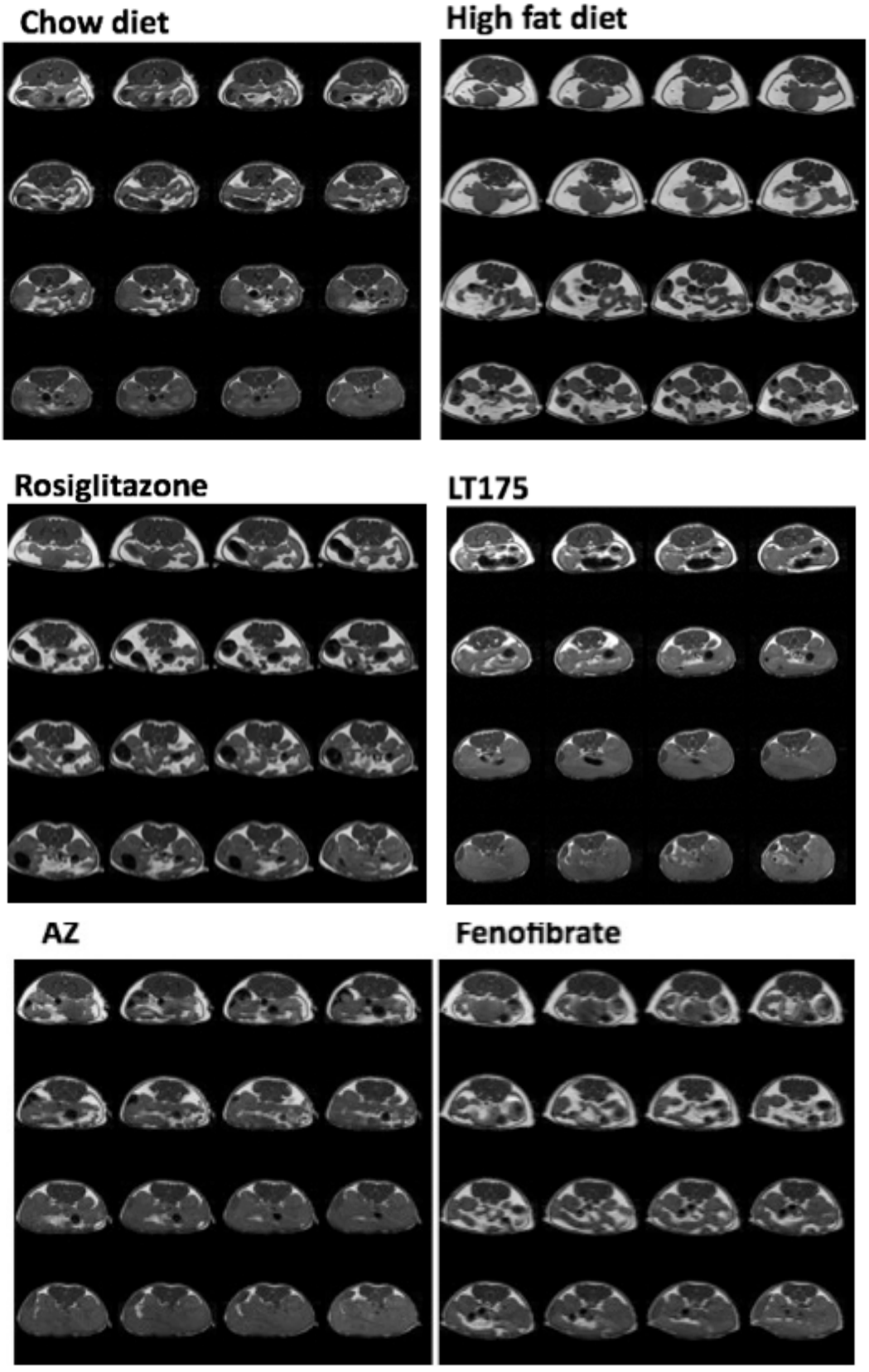


***Fig 17**: MRI analyses of DIO mice. 16 axial 1mm thick T1 weighted slices placed in the abdominal region spanning from kidneys to bladder included; fat area in white*

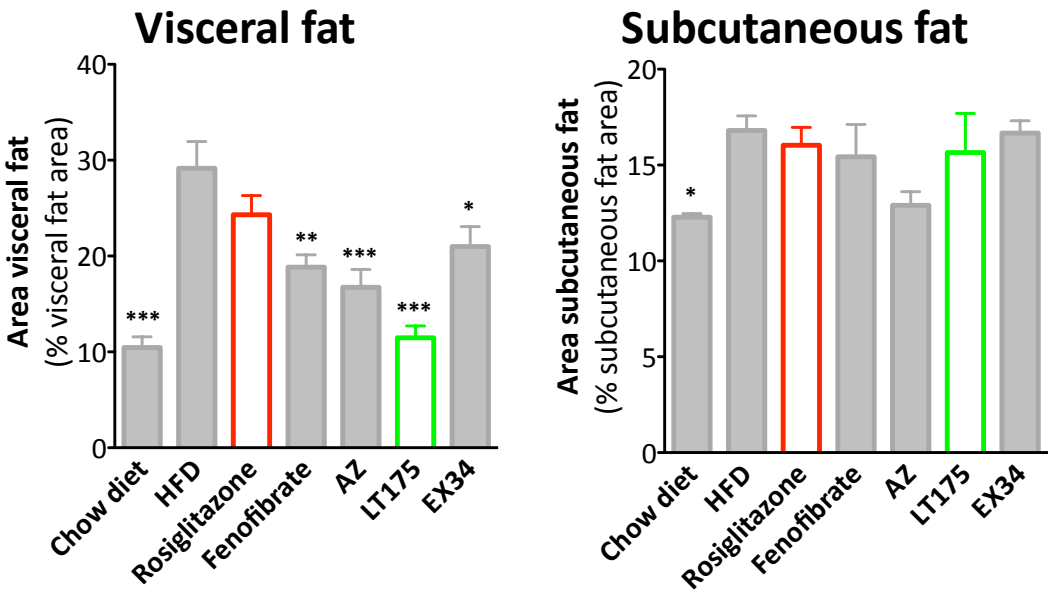


***Fig 18**: visceral and subcutaneous fat area in DIO mice; * P<0.05 vs HFD, ** P<0.01 vs HFD, *** P<0.001 vs HFD*

Surprisingly, treatment with LT175 induces significantly the mass of brown adipose tissue (BAT), whereas the other ligands do not affect this parameter (figure 19). Altogether, the analysis of fat content by MRI and the expanded BAT mass suggest that the reduction of body weight observed in mice treated with LT175 could be, at least in part, explained with a remodeling of adipose tissues induced by this ligand.

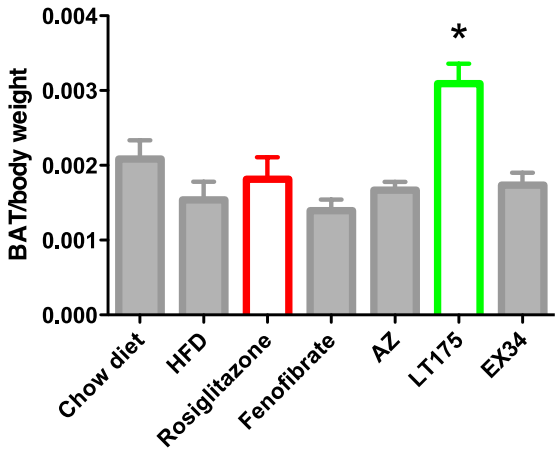


***Fig 19**: Brown adipose tissue mass in DIO mice; * P<0.05 vs HFD*

4.4.2 LT175 improves metabolic parameters in obese mice

The treatment with LT175 decreases plasma glucose, insulin, not-esterified fatty acids (NEFA), triglycerides and cholesterol (figure 20). Notably, LT175 also increases the circulating levels of the insulin sensitizing adipose-derived hormone adiponectin (figure 20) and the member of the fibroblast growth factor (FGF) family FGF21 (figure 21), a peptide hormone inducing several beneficial metabolic effects (Kharitonenkov et al., 2009).

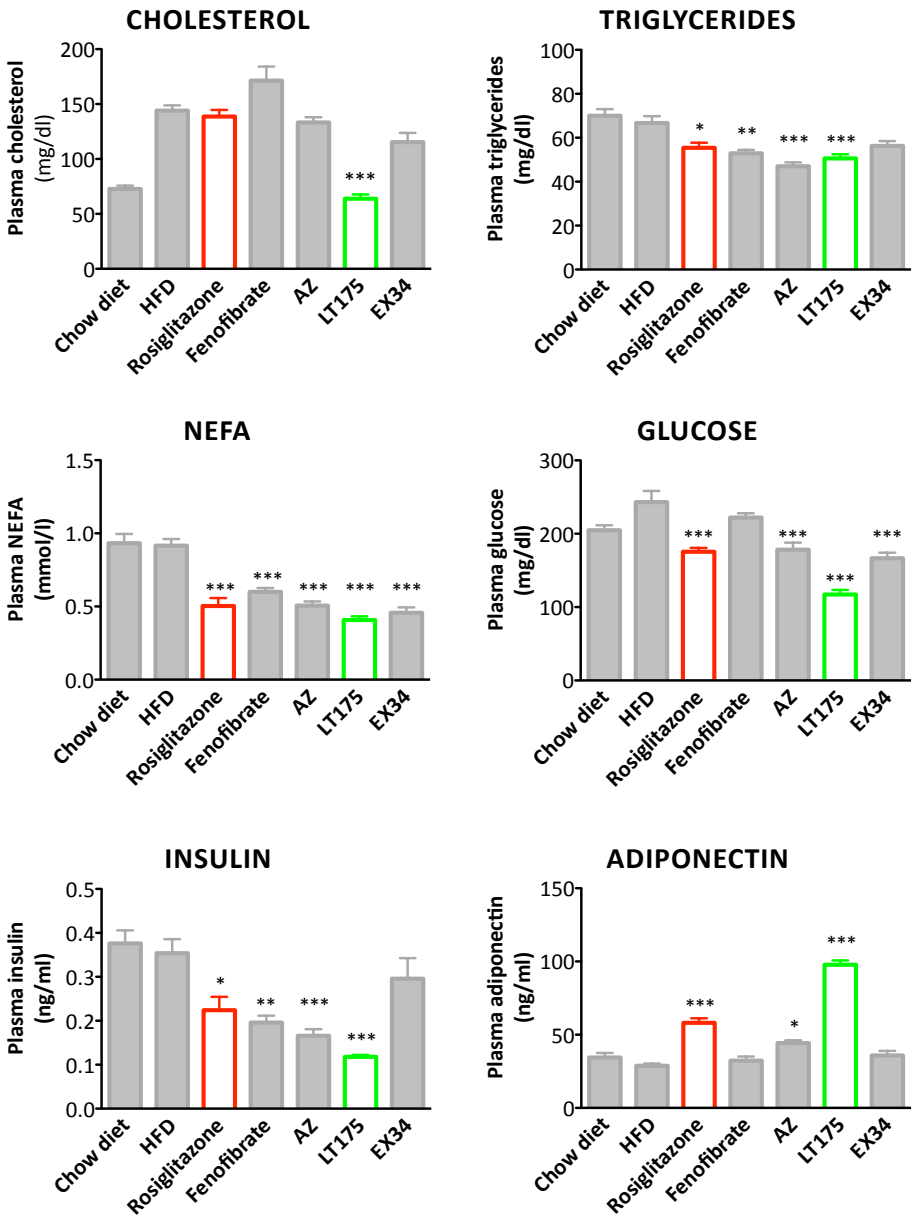


***Fig 20**: plasma levels of cholesterol, triglycerides, not-esterified fatty acids, glucose, insulin and adiponectin after treatment with the different PPAR ligands; * P<0.05 vs HFD, ** P<0.01 vs HFD, *** P<0.001 vs HFD*

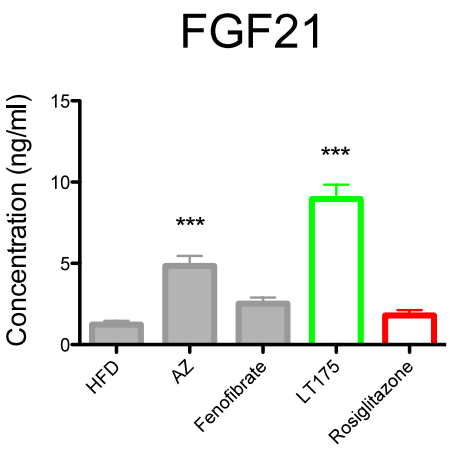


***Fig 21**: circulating levels of FGF21 after treatment with the different PPAR ligands; *** P<0.001 vs HFD*

The interesting results obtained with the analysis of plasma parameters led us to investigate further the levels of cholesterol and triglycerides in the liver and their distribution in lipoprotein fractions. As expected, LT175 decreased cholesterol in the HDL fraction (figure 22), most likely as a consequence of the repressive effects of PPARα activation in rodents (Vu-Dac et al., 1998), and lowered VLDL triglycerides, possibly by increasing triglyceride catabolism (figure 23).

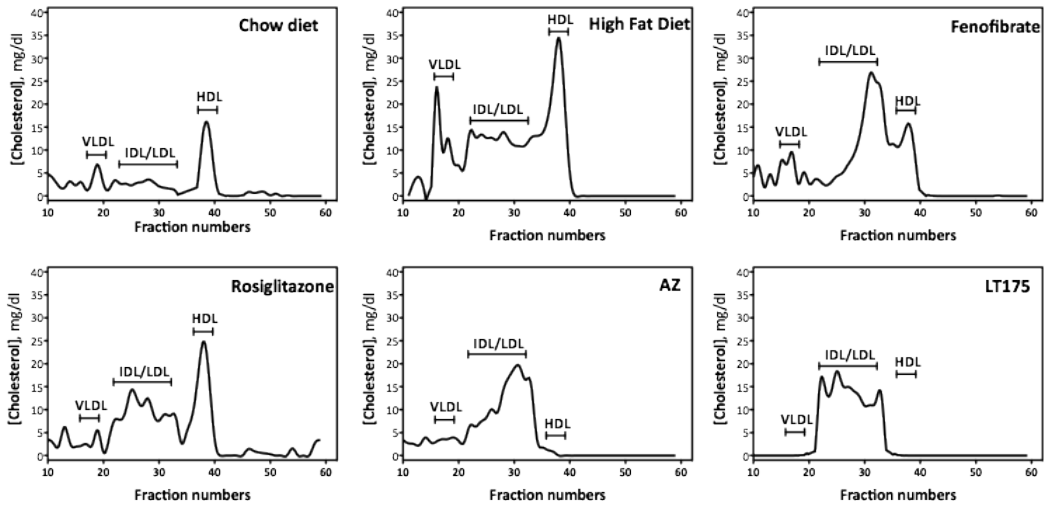


***Fig 22**: cholesterol distribution in lipoprotein fractions*

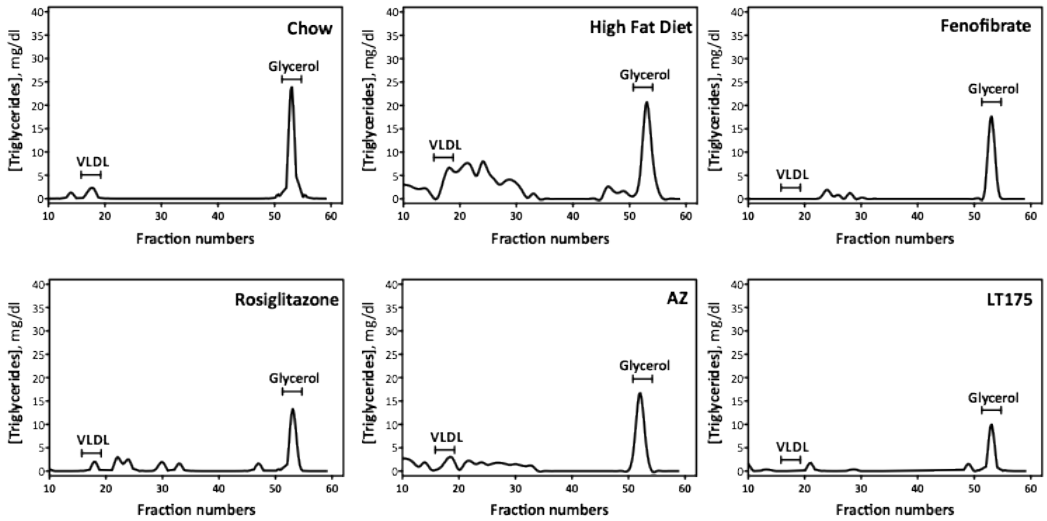


***Fig 23**: triglycerides distribution in lipoprotein fractions*

Hepatic cholesterol content does not change with any of the tested ligands except AZ12063233 (figure 24). Liver triglyceride levels increase with fenofibrate, AZ12063233 and LT175, however the levels of hepatic triglycerides in mice treated with LT175 are significantly lower than those with fenofibrate and AZ12063233 (figure 24).

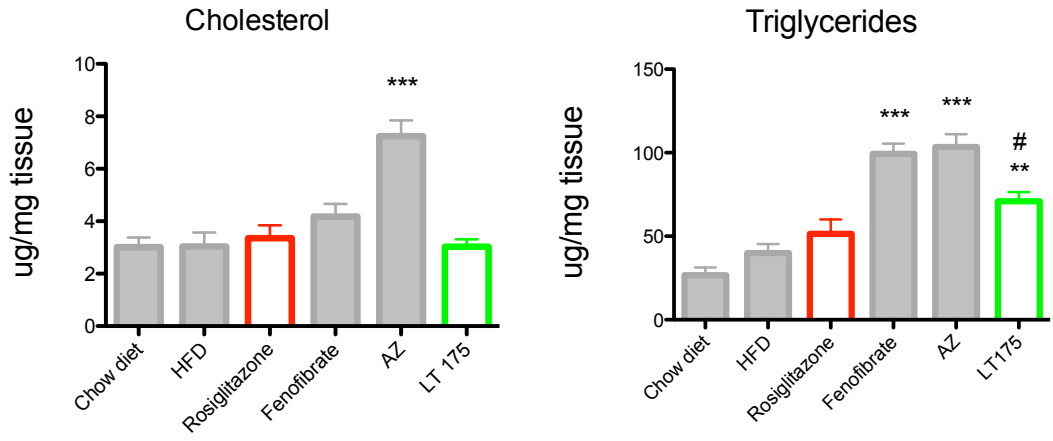


***Fig 24**: spectrophotometric assay on liver cholesterol and triglycerides levels; *** P<0.001 vs HFD, # P<0.05 vs Fenofibrate and AZ*

4.4.3 LT175 is an insulin sensitizer agent

The decreased body weight and visceral fat content and the improvement of plasma and hepatic metabolites and hormones suggested that LT175 may have insulin sensitizing and antidiabetic properties.

To test this hypothesis the antidiabetic potential of LT175 was determined by the oral glucose tolerance test and by the insulin tolerance test, which show that LT175, as well as the other compounds used, improves glucose clearance and insulin sensitivity (figures 25 and 26).

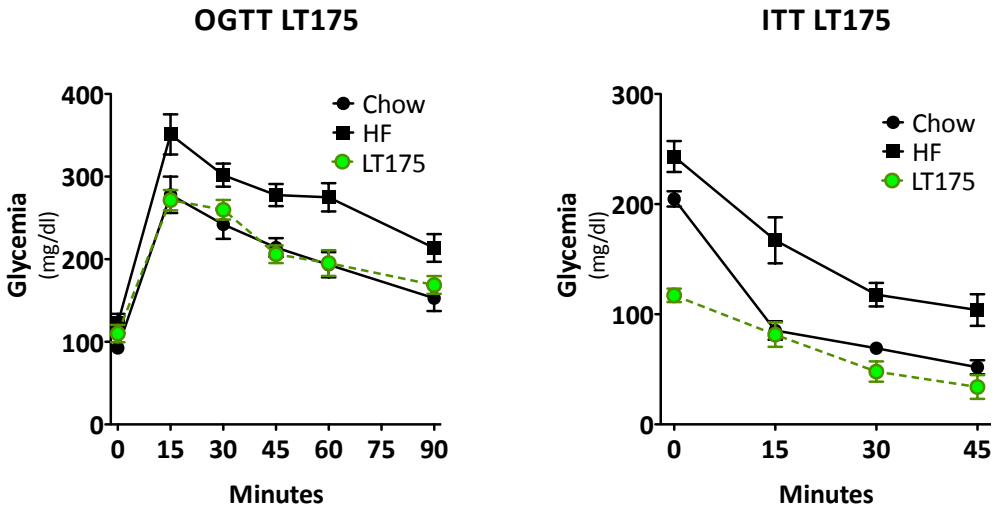


***Fig 25**: glycemic curves for the OGTT and ITT of mice following the chow diet, the high fat diet, or high fat diet + treatment with LT175*

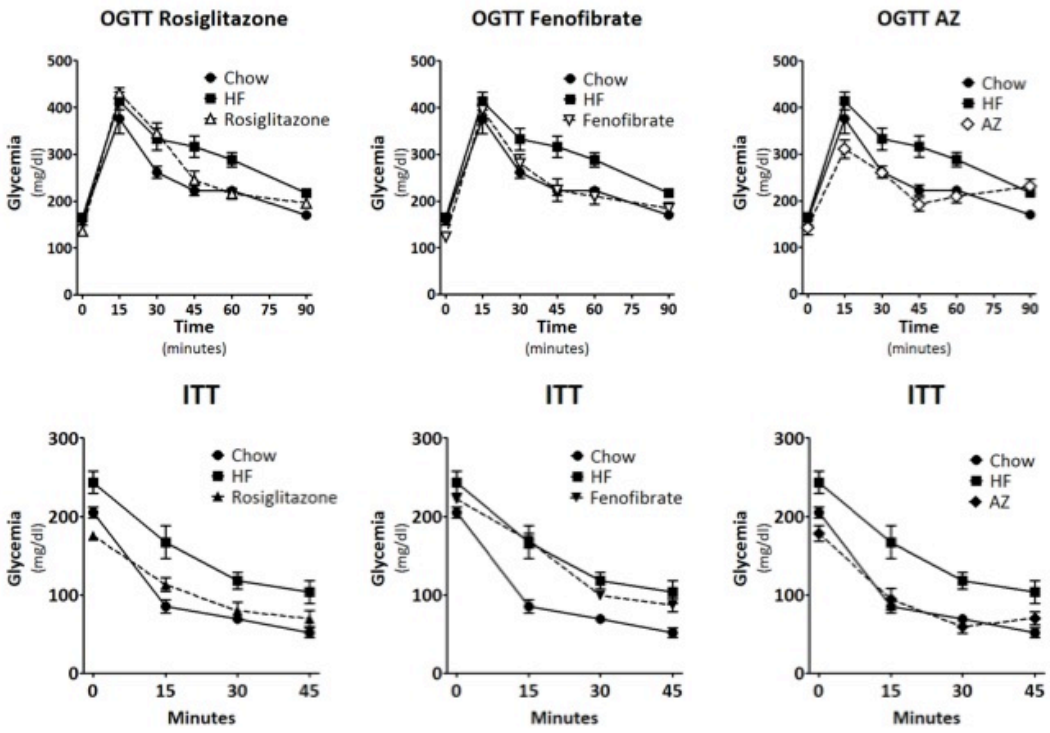


***Fig 26**: glycemic curves for the OGTT and ITT of mice treated with rosiglitazone, Fenofibrate and AZ12063233*

Collectively, the results shown so far demonstrate that LT175 improves metabolic parameters in vivo leading to insulin sensitizing effects in a mouse model of diet induced obesity and insulin resistance (DIO mice).

4.5 Molecular analysis of insulin sensitizing effect of LT175 via PPAR dependent transcriptional program

To assess whether LT175 induces the PPAR dependent transcriptional program, we measured gene expression in the liver, white and brown adipose tissue of different PPAR target genes in C57Bl/6 mice treated for three days with the PPAR ligands. In particular, we focused our attention on genes of fatty β-oxidation (Acyl-CoA dehydrogenase, long chain Acadl - Acyl-CoA dehydrogenase, medium chain Mcad – Hydroxyacyl-CoA dehydrogenase Hadh - Acyl-CoA oxidase 1 Acox1 - Carnitine palmitoyltransferase 1α Cpt1α - Cpt1β), mitochondrial biogenesis (Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha Pgc1α - mitochondrial Transcription factor A, Tfam), TCA cycle and electron transport chain (Isocitrate dehydrogenase 3 (NAD+) alpha IDH3α - CytC - Succinate-CoA ligase, alpha subunit

SUCLG1), ketone bodies synthesis (3-oxoacid CoA transferase 1 Oxct1 - HMG-CoA synthase 2 Hmgcs2) and triglycerides biosynthesis (Phosphoenolpyruvate carboxykinase 1 Pck1 - Glycerol kinase Gk). The results are shown in figures 27, 28, 29 (liver), 30 (WAT) and 31 (BAT). In the liver, the genes of the β-oxidation pathway, as expected, increase their expression with the compounds that show PPARα agonism. Referring to the mitochondrial related genes, Pgc-1α follow a particular trend: it decreases with every compound used, except for rosiglitazone. In the triglycerides biosynthesis pathway, Gk follows the expected trend, while Pck1 does not change.

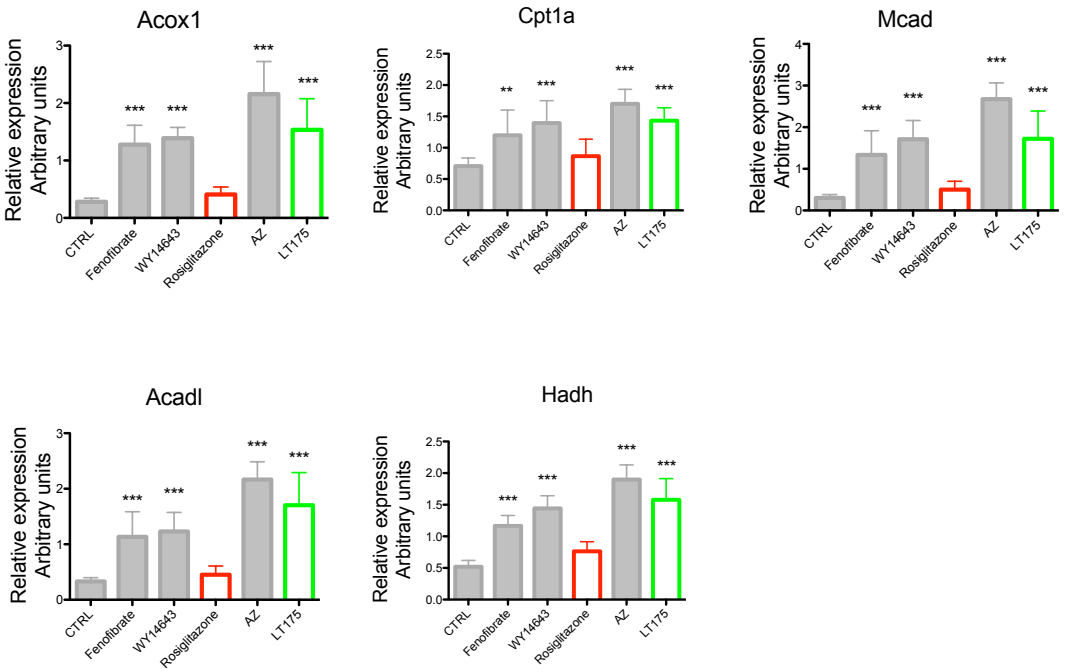


Fig 27 *expression of liver genes involved in fatty acid β-oxidation; ** P<0.01 vs Ctrl, *** P<0.001 vs Ctrl*

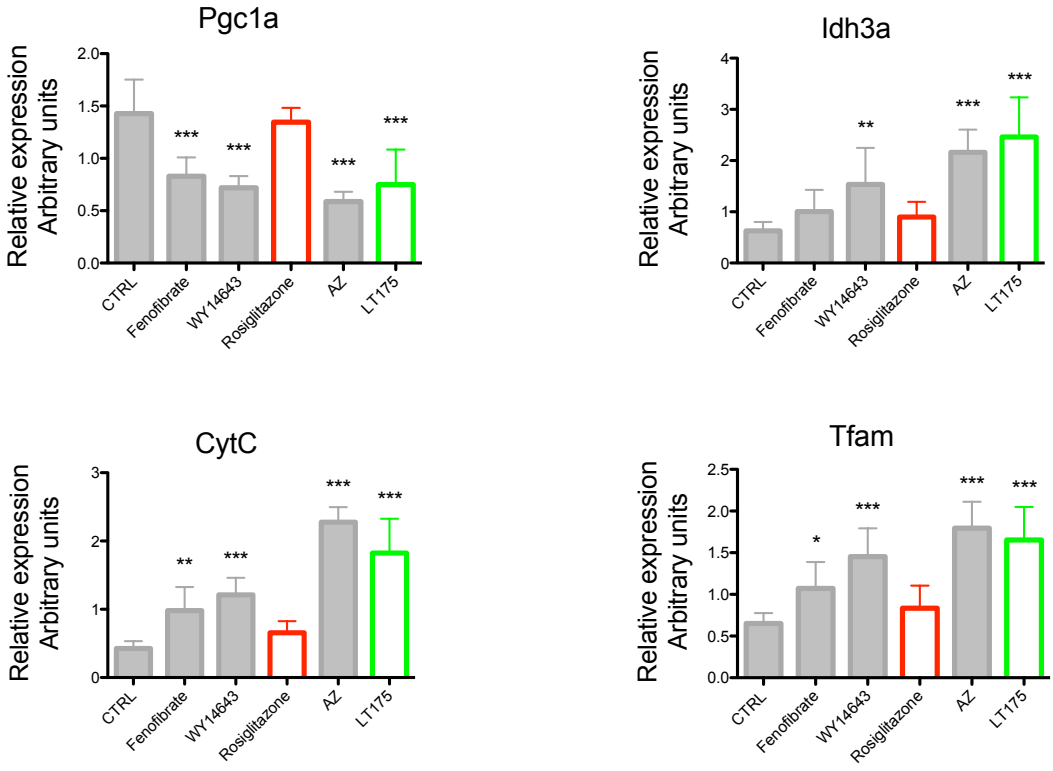


***Fig 28**: expression of liver genes involved in mitochondrial biogenesis; * P<0.05 vs Ctrl, ** P<0.01 vs Ctrl, *** P<0.001 vs Ctrl*

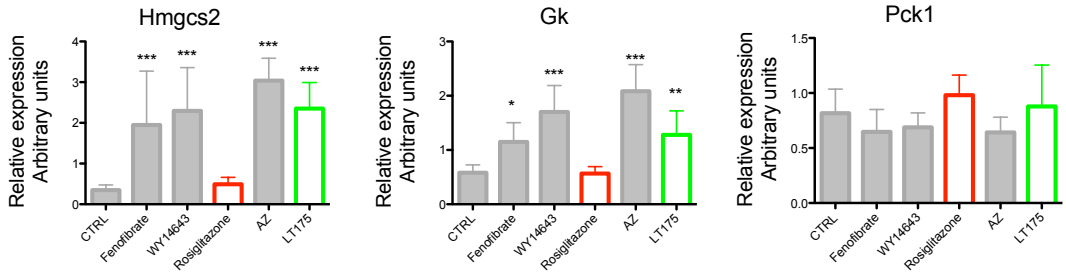


***Fig 29**: expression of liver genes involved in ketone bodies synthesis and triglycerides biosinthesis; * P<0.05 vs Ctrl, ** P<0.01 vs Ctrl, *** P<0.001 vs Ctrl*

In white adipose tissue, the genes analysed were all stimulated by the compounds that present PPARγ agonism (figure 30).

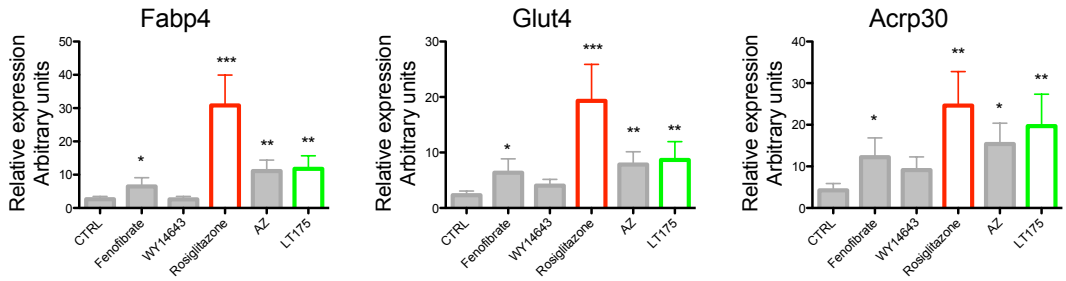


***Fig 30**: gene expression of PPAR target genes in white adipose tissue*

In brown adipose tissue, LT175 induces an increase in Dio2 and Cpt1β expression, while there is a difference in Fabp4, Elovl3 and Ucp1 expression induced either by LT175 or rosiglitazone (figure 31).

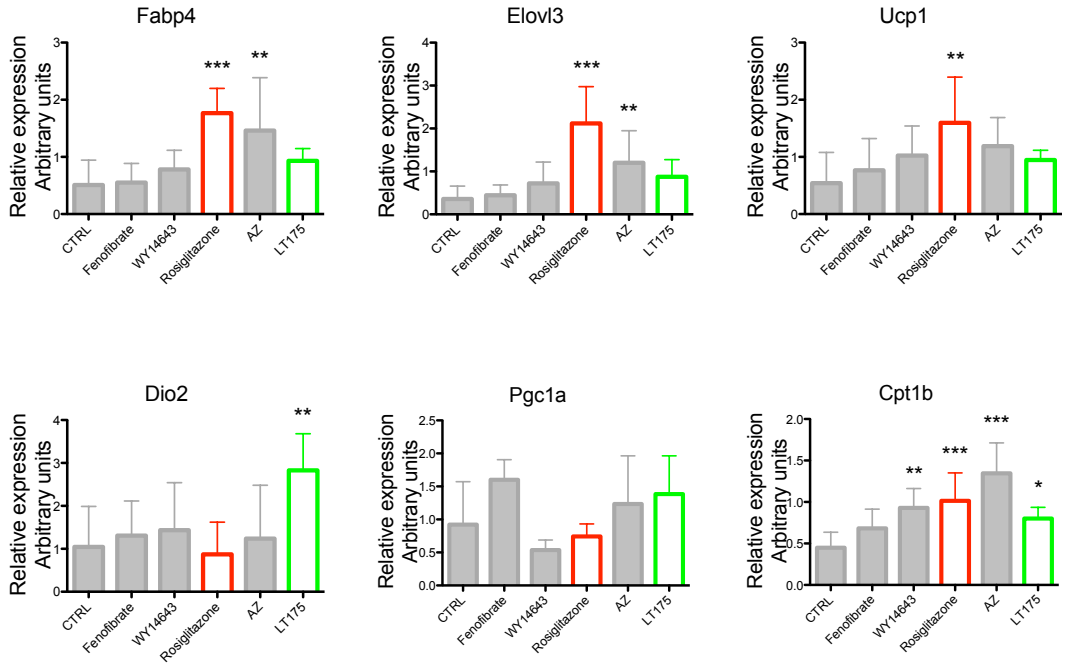


***Fig 31**: gene expression of PPAR target genes in brown adipose tissue*

4.6 LT175 does not alter the expression of ENaCγ

It has been reported that PPARγ full agonists enhance the expression of ENaCγ, the gene encoding for renal sodium transporter; the increased expression of this gene can be involved in fluid retention. Our data show that the PPARγ full agonist rosiglitazone increases the expression of the ENaCγ subunit, whereas LT175 does not elicit any effect (figure 32).

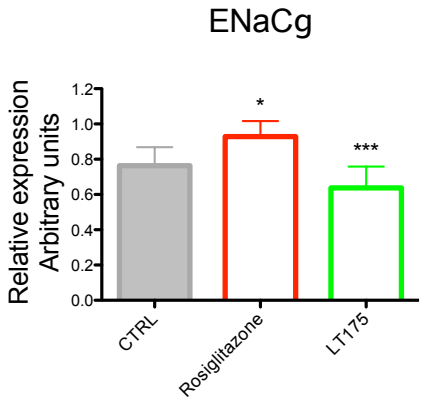


***Fig 32**: ENaCγ gene expression on mice kidneys; * P<0.05 vs Ctrl, *** P<0.001 vs rosiglitazone*

4.7 LT175 causes a decreased lipid accumulation in vitro and in vivo inducing no changes in genes involved in lipid uptake and storage

To further elucidate the biochemical basis of the metabolic effects elicited by LT175, we focused on the mechanisms involved in the decreased lipid accumulation observed in vitro and in vivo. These effects could be due to differential induction of PPARγ targets by the ligands. Two possible mechanisms can be involved: an increase in the expression of mitochondrial related genes, that can leads to an enhanced lipid catabolism by oxidative metabolism, or alternatively a reduced induction of the genes involved in lipid uptake and storage in adipocytes. The ability of the ligands to stimulate the 3T3-L1 preadipocytes differentiation to adipocytes was investigated, and no differences between the treatment with LT175 or with rosiglitazone on differentiation was observed; in fact, 3T3-L1 cells treated with both rosiglitazone and LT175 differentiate into adipocytes and express the typical adipocyte markers (figures 5 and 7). Moreover, the expression of genes involved in lipid catabolism and mitochondrial oxidative metabolism was investigated by measuring mRNA levels of Acadl, CytC, Suclg1, Idh3α, showing no influences on these parameters induced by LT175 (figure 33). In addition, LT175 did not change mitochondrial DNA content, a parameter reflecting mitochondrial biogenesis. These results rule out the hypothesis

whereby increased mitochondrial oxidative metabolism underlies the decreased lipid content in adipocytes.

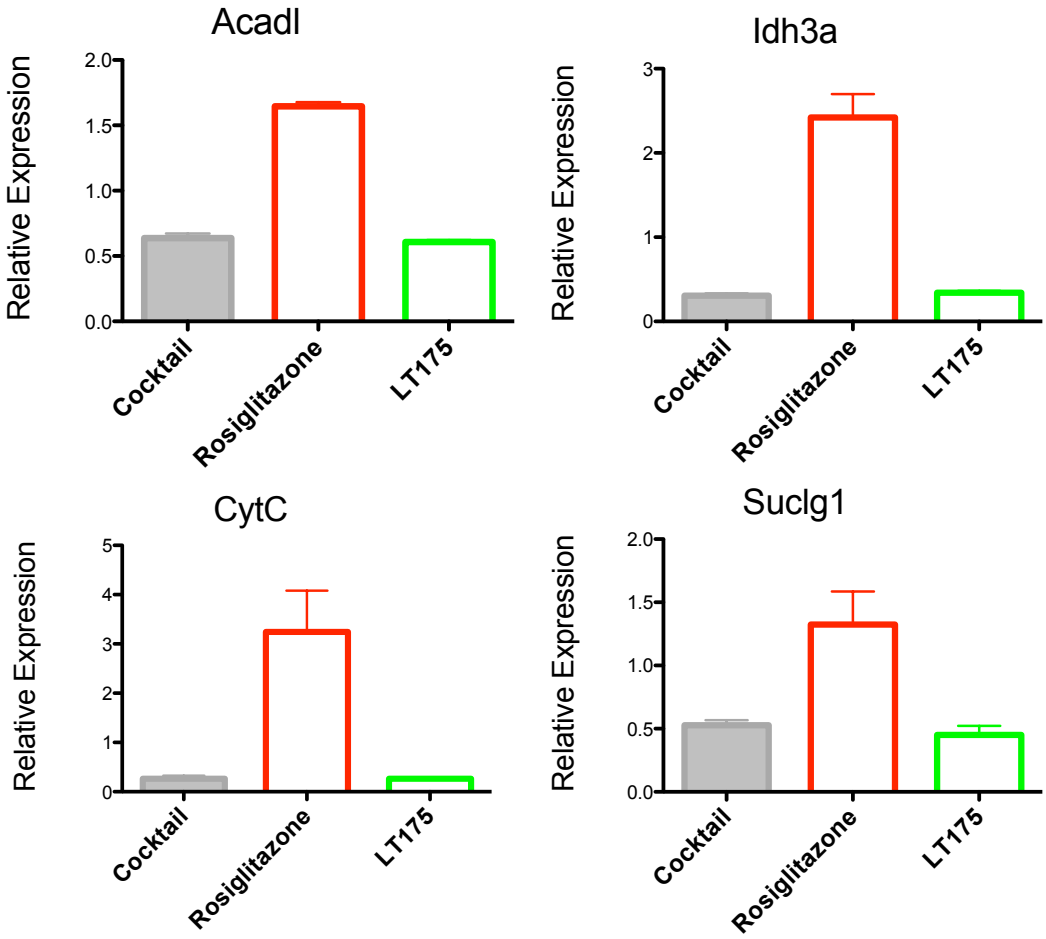


***Fig 33**: gene expression of mitochondrial related genes in 3T3-L1 cells treated with rosiglitazone or LT175; *** P<0.001 vs Cocktail*

Therefore, to explain the decreased lipid accumulation in adipocytes treated with LT175, the expression of genes involved in lipid uptake and storage was tested. Rosiglitazone exerts its action by enhancing the expression of genes for fatty acid uptake (Cd36) and glycerol 3-phosphate formation (Pck1 and Gk), while LT175 does not increase the expression of these genes (figure 34). These results suggest that decreased expression of genes for glycerol 3-phosphate production, required for fatty acid esterification and storage in adipose tissue, may explain the lower lipid accumulation in adipocytes in vitro.

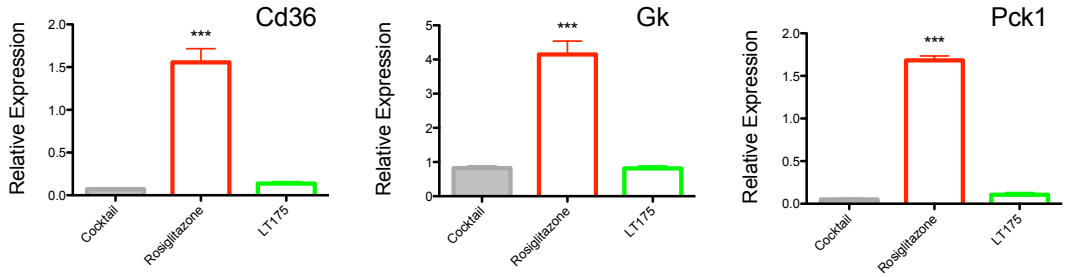


***Fig 34**: gene expression of genes involved in lipid uptake and storage in 3T3-L1 treated with rosiglitazone or LT175; *** P<0.001 vs Cocktail*

5. DISCUSSION

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors which belong to the nuclear hormone receptor superfamily and are involved in translating the effects of lipid soluble factors into the gene expression level (Desvergne et al., 1999; Dreyer et al., 1992; Issemann et al., 1990). They are involved in lipid and glucose metabolism, as they transcriptionally regulate the enzymes and other proteins involved in energy homeostasis (Nolan et al., 1994). Due to their implication at different levels in energy metabolism, they are target of different classes of compounds. In particular the thiazolidinediones, a synthetic class of compounds, bind to PPARγ resulting in improvement in insulin resistance in rodents and humans, and have widely been used to treat type 2 diabetes. Despite improvement in glucose and lipid metabolism and variable effects on the cardiovascular system, a growing body of evidence demonstrates unexpected adverse effects of these molecules: weight gain, due to increased adipose tissue mass, fluid retention, and effects on skeletal metabolism (DeFronzo et al., 2009; Home et al., 2009). PPARα/γ agonists have been postulated as a strategy to combine positive effects due to PPARα and PPARγ activation, in fact the propensity of PPARγ activation to induce adipogenic effects may be offset by the ability of PPARα activation to stimulate lipid catabolism (Chaput et al., 2000). Moreover, pharmacological studies have demonstrated that full and partial activation, as well as antagonism, of PPARγ can all improve insulin sensitivity. The moderate activation of PPARγ might be better suited to uncouple the insulin-sensitizing effects from the adverse side-effects (Doshi et al., 2010). Investigation of partial or selective PPARγ modulators (SPPARMs) have proved promising and suggests that they represent an attractive therapeutic approach (Doshi et al., 2010; Feldman et al., 2008).

Our laboratory has recently described a new molecule that binds and activates both PPARα and γ subtypes (Montanari et al., 2008).

The experiments performed in 3T3-L1 preadipocytes indicate that LT175 is able to activate the PPARγ-dependent program of differentiation in adipocyte cultures (and the results obtained in C3H10T1/2 corroborate this hypothesis), in fact there are no differences in adipocyte differentiation of cells treated with rosiglitazone or LT175; however, adipocytes differentiated in the presence of LT175 accumulate less lipids as compared to those treated with rosiglitazone. Two possible mechanisms can be involved to explain the peculiar behaviour of this ligand: an increase in the expression

of mitochondrial related genes, that leads to enhanced lipid catabolism via fatty acid β-oxidation, or reduced induction of the genes involved in lipid uptake and storage in adipocytes. The gene expression profile of 3T3-L1 treated with different PPAR ligands shows that the mechanism involved in the decreased lipid accumulation observed in vitro and in vivo depends on the genes involved in lipid uptake and storage. In fact, rosiglitazone exerts its action by enhancing the expression of genes for fatty acid uptake (Cd36) and glycerol 3-phosphate formation (Pck1 and Gk), while LT175 does not increase the expression of these genes. These results suggest that decreased expression of genes for glycerol 3-phosphate production, required for fatty acid esterification and storage in adipose tissue, may explain the lower lipid accumulation in adipocytes in vitro.

Going further in understanding the biochemical mechanism of LT175 action, we decided to use PPRE-Luc mice as a useful experimental model to quantify the magnitude and the spatial distribution of PPAR activation in the mouse body. In this particular animal model, the luciferase is under the control of PPAR responsive element, and the receptor activation induced by the ligand results in luminescence. Mice show a cyclic PPAR activation state during the 19 days of the experiment. Importantly, LT175 induces a peculiar PPAR activation, with the most prominent peaks from day 1 to 4, that ends starting from the day 7 of treatment. In contrast, PPAR activation by Wy14,643 results in cyclic response to the ligand. Referring to the abdomen area, LT175 seems to induce a stronger photon emission than Wy14,643, suggesting a different spatial PPAR activation induced by the ligand. Also, while LT175 was able to increase the transcription as early as one day after the beginning of the treatment, Wy14,643 increased luciferase activity only after ten days. A possible explanation could be that the dual PPARα/γ activity of LT175 may allow the early increase of luciferase activity via PPARγ, which is highly expressed in visceral fat and large intestine, while Wy14,643, being a PPARα agonist, increases luciferase activity in the abdominal area by activating PPARα in the intestine where this receptor is expressed at low levels. Alternatively, the bioavailability, absorption and distribution of LT175 and Wy14,643 in the abdominal area may differ and this would explain the different temporal activation of PPAR-dependent transcription observed in this area with the two ligands. Further experiments will be necessaries to determine the different behavior of these two ligands. The enzymatic assay demonstrates also

that, in a treatment of three days, LT175 does not activate the PPAR-mediated transcription in tissues other than liver, WAT and BAT. The quantification of LT175 in the murine plasma during 15 days of treatment allowed us to hypothesize that the loss of PPAR-mediated transcription in the liver noticed in PPRE-Luc mice after 7 days of treatment can derive from the desensitization of the receptor. These findings will be useful in the future also to investigate possible different administration regimens to induce constant receptor activation and to study the bioavailability of the compound in long term treatments.

Despite genetic models of insulin resistance, like *db/db* mice, are widely used for the characterization of the in vivo influence on metabolic profile induced by PPAR ligands, we choose to use a model of Diet Induced Obese mice (DIO), that can be more predictive of the patophysiology of type 2 diabetes. Looking at the plasma parameters, every ligand used provoked a decrease in triglycerides, NEFA, glucose, insulin and an increase in adiponectin levels, but LT175 showed the stronger variation in all these parameters. LT175 is also able to increase FGF21 levels. The importance of this result depends on the fact that FGF21 prevents lipid accumulation by stimulating hepatic lipolysis and ketogenesis, decreases circulating levels of glucose by stimulating its uptake in white adipose tissue, and reduces circulating insulin and triglycerides. Of note, LT175 is the only tested ligand that induced a decrease in circulating cholesterol levels. The analysis of cholesterol distribution in lipoprotein fraction indicates that LT175 decreased HDL cholesterol in mice. This is expected, because ligand-activated PPARα downregulates ApoA-I, that is major component of HDL, in mice, whereas human ApoA-I gene is upregulated by PPARα (Vu-Dac et al., 1998). We also determined the distribution of triglycerides in lipoprotein fractions and as expected LT175, being a PPARα activator, decreased the VLDL triglycerides. However, while mice administered with the other two molecules with PPARα activity, fenofibrate and AZ12063233, showed high levels of triglycerides in the liver, in the group of mice treated with LT175 the hepatic levels of triglycerides were significantly lower. These results correlate with the reduced body weight and fat accumulation observed in mice treated with LT175. Presently, we do not have an explanation of this behavior, however since LT175 expanded brown fat mass it is conceivable to hypothesize that the increased fatty acid oxidation in this tissue can be a major driver contributing to reduce lipid accumulation in several tissues including the liver.

As assessed by in vivo Magnetic Resonance Imaging (MRI), LT175 strongly decreases visceral fat. This effect together with the increased brown fat mass is most likely responsible of the reduction of total body weight. It is positive that the decrease in fat area derives from a reduction of visceral fat, which is implicated in the low-grade inflammation state affecting negatively systemic insulin sensitivity and ultimately leading to the onset of type 2 diabetes. The expansion of brown adipose tissue mass observed with LT175 is likely to contribute to the decreased body weight and visceral fat depot via fatty acid β-oxidation and thermogenesis resulting from uncoupling electron transport chain from oxidative phosphorylation. However, further studies need to be performed to confirm the functionality of BAT in mice treated with LT175. Globally, the analysis of the gene expression in the liver, WAT and BAT shows an induction of PPAR target genes in these tissues, due to the activation of the PPAR dependent transcription machinery, leading to the biological effects seen in this animal model.

Concerning fluid retention, another major side effect of thiazolidinediones reported in several patients, while rosiglitazone enhances the expression of ENaCγ, the gene encoding for renal sodium transporter and in part responsible of this side effect, LT175 did not affect renal ENaCγ expression in our animal model. This result suggests that LT175 may influences fluid retention less than thiazolidinediones, however further studies are necessaries to asses this hypothesis.

Collectively, these results show that LT175 behaves differently as compared to the PPARγ full agonist rosiglitazone in these experimental models, mainly concerning weight gain, the most important side effect induced by the thiazolidinediones.

Finally, to explain at the molecular level the different behaviour of the PPARγ partial agonist LT175 as opposed to the full agonist rosiglitazone, we focused on the different interaction of the ligand with the receptor. In previous work published by our laboratory (Montanari et al., 2008), the crystal structure of LT175 complexed with the PPARγ-LBD was solved and a molecular explanation for its different behaviour was proposed. The dynamic behaviour of helix 12 (H12) of nuclear receptors plays a key role in coactivator recruitment. Binding to the receptor of ligands with different chemical structure modulates the stabilization of H12 in different ways, determining a different biological cellular response (Love et al., 2002) either acting as a LBD global stabilizer (Cronet et al., 2001) or influencing the H12 stability through additional

structural changes such as repositioning of H11 or different conformations of the loop 11/12. Full agonists shift the equilibrium between active and inactive conformation of helix 12 towards the active state, leading to coactivator recruitment. Partial agonists are incapable of shifting this equilibrium in favor of the active conformation to the same extent as full agonists; they are not able to stabilize the H12 in the proper position, due to the lack of a few key interactions and even a slight unpositioning of H12 may result in an attenuated transcriptional response (Steinmetz et al., 2001). LT175, showing a partial agonism behaviour, induces only a lower stabilization of H11 and H12 and increases the stabilization of H3 (Montanari et al, 2008). The mechanisms of full or partial agonism behavior of PPAR ligands rely on the differential structural conformation induced by the ligand, which then allows the selective recruitment of a different set of coregulators and ultimately dictates the selectivity of the response to the ligand. The analysis of coregulator recruitment by FRET experiments showed that in presence of LT175 the corepressor NCoR still interacts with PPARγ. These results support the hypothesis that the compound is a full PPARα agonist and a partial PPARγ agonist that could therefore have the beneficial effects induced by the thiazolidinediones, avoiding or at least reducing their known side effects. The biochemical analysis of in vitro recruitment of PPAR coregulators can predict the behavior of different PPAR ligands depending on the absolute content and the ratio of coactivators/corepressors in different tissues, and can be useful to better understand the mechanisms underlying the effects elicited by different ligands.

All together, the results obtained during the doctorate program allowed elucidating the mechanisms underlying PPAR activation by different ligands. LT175 has been characterized as a new dual PPARα/γ ligand with improved therapeutic profile in diabetic mice. This integrated experimental approach will be exploited to design scaffold molecules that could be refined to develop novel treatments of obesity and type 2 diabetes.

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