Transcriptional regulation of the LSDP5 gene.

Master Thesis by Hanna Ræder

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Hanna Ræder

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List of Abbreviations

AT	Adipose Tissue
ATP	Adenosine Tri Phosphate
ADRP	Adipose Differentiation-Related Protein
BAT	Brown Adipose Tissue
BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
cAMP	cyclic Adenosine 3`, 5`-Monophosphate
CE	Cholesterol Esters
CMC	CarboxyMethyl Cellulose
DBD	DNA Binding Domain
DMEM	Dulbecco`s Modified Eagle`s Medium
DMSO	Dimethyl Sufolxide
DNA	DeoxyriboNucleic Acid
DR	Direct Repeat
DTT	Dithiothreitol
E. Coli	Escherchia Coli
EMSA	Electro Phoretic Mobility Shift Assay
ER	Everted Repeat
ERR	Estrogen-Related Receptor
FA	Fatty Acid
FCS	Fetal Calf Serum
FFA	Free Fatty Acids
FOXO	ForkheadboxclassO
FXR	Farnesoid X Receptor
GR	Glucocortocoid Receptor
HAT	Histone Acetyl Transferase
HNF	Hepatic Nuclear Receptor
HSL	Hormon Sensitive Lipase
LB Medium	Luria Bertani Medium
IR	Inverted Repeat
LBD	Ligand Binding Domain
LSD	Lipid Storage Droplet
LSDP	Lipid Storage Droplet Protein
Luc	Luciferase
LXR	Liver X Receptor
MCAD	Medium-Chain acyl coenzyme A Dehydrogenase
M-gast	Musculus Gastrocnemius
mLSDP5	mouse-LSDP5
mRNA	messenger RiboNucleic Acid
M-solus	Musculus Soleus
OA	Oleic Acid
NR	Nuclear Receptor
PAT	Perilipin ADRP/Adipofilin TIP47
PEPCK	Phospho Enol Pyruvate Carboxy Kinase
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PGC-1	PPARγ coactivator-1α
PKA	Protein Kinase A
PPAR	Peroxisome Proliferator Activated Receptor
PPRE	PPAR Responsive Element

PRC	PGC- Related Coactivator
PUFAs	Poly Unsaturated Fatty Acids
RE	Response Element
RXR	Retinoic X Receptor
SD	Standard Deviation
TAG	Triacylglycerol
TIP47	Tail-interacting Protein of 47 kDa
TZD	Thiazolidinedione
WAT	White Adipose Tissue
WT	Wild Type
WY	Wy 14.643
YFP	Yellow Fluorescence Protein
9-cis-RA	9-cis-Retinoic Acid

Summary

Lipid droplet storage proteins (LSDPs) associate with lipid droplets and are involved in the molecular processes of deposition and catabolism of stored lipids in the cells. These proteins differ in tissue distribution, binding affinity to lipid storage droplets, protein stability and transcriptional regulation. Due to these differences, they are believed to have evolved to fine tune fatty acid metabolism according to the particular needs of various tissues. Recent knowledge reveals that these proteins are transcriptionally regulated by Peroxisome Proliferator-Activated Receptors (PPAR).

In this thesis I have focused on the newly discovered protein, lipid storage droplet protein 5 (LSDP5), and the transcriptional regulation of this protein. Nebbs group has previously demonstrated in animal studies that LSDP5 is transcriptionally regulated by PPARα in liver. However, it has been difficult to prove through transfection studies that this regulation is mediated through a PPARα responsive DR1 element (PPRE). The main objective for this thesis was thus to establish whether there is a functional PPRE located in the LSDP5 promoter. Furthermore, the aims were to examine whether the transcriptional regulation of LSDP5 is affected by additional regulatory factors; the transcription factor estrogen-related receptor α (ERRα) and the coactivator PPARγ coactivator-1α (PGC-1α).

In this study it was confirmed that LSDP5 contains a functional PPRE, located in the -2077base pairs (bp) to -2064bp upstream region in the LSDP5 gene. Furthermore, it was demonstrated that the PPARα mediated transcriptional regulation of LSDP5 is dependent on PGC-1α. PGC-1α enhanced the transcriptional activity in the LSDP5 gene remarkably. ERRα repressed the expression of LSDP5. We observed that the presence of ERRα reduced the enhancing effect of PGC-1α on LSDP5 induction. Taken together, my data and the previously published data in our group confirm that LSDP5 is a PPARα target gene. Furthermore, our findings provide new aspects in the regulation of LSDP5, regarding the role PGC-1α and ERRα.

Sammendrag

Lipiddråpe-assosierte proteiner (LDAP) er bundet til lipiddråper og involvert i molekylære prosesser som lagring og nedbrytning av lipider som er lagret i celler. Disse proteinene er ulike med hensyn til vevsekspresjon, bindingsaffinitet, proteinstabilitet og transkripsjonell regulering. Man antar at disse ulikhetene skyldes at proteinenes regulering av fettsyremetabolismen er spesifikt tilpasset ulike vev. Nyere kunnskap har vist at LDAP`ene er transkripsjonelt regulert av Peroksisomal-Proliferator-Aktiverte Reseptorer (PPAR).

I dette arbeidet har jeg fokusert på "lipid storage droplet protein" 5 (LSDP5), et nyoppdaget LDAP, og hvordan dette proteinet er transkripsjonelt regulert. Det har tidligere blitt vist i Nebbs gruppe at LSDP5 er transkripsjonelt regulert av PPARα i lever. Det har imidlertid vært vanskelig å bevise gjennom transfeksjonsstudier at denne reguleringen skjer via et PPAR responselement (PPRE). Hovedproblemstillingen i denne oppgaven var derfor å undersøke om det er et funksjonelt PPRE i promoteren til LSDP5. Videre ønsket vi å undersøke om reguleringen av LSDP5 skjer i samspill med andre faktorer, nærmere bestemt transkripsjonsfaktoren estrogen-relatert reseptor α (ERRα) og koaktivatoren PPARγ koaktivator-1α (PGC-1α).

Resultatene fra dette arbeidet bekrefter at LSDP5 inneholder et funksjonelt PPRE, lokalisert -2077basepar (bp) til -2064bp oppstrøms for transkripsjonsstart i LSDP5 genet. Videre viser resultatene at den PPARα-medierte reguleringen av LSDP5 er avhengig av PGC-1α. Denne koaktivatoren økte den transkripsjonelle aktiviteten i LSDP5 genet betydelig. Når det gjelder ERRα, ble det observert at denne transkripsjonsfaktoren hemmet ekspresjonen av LSDP5. Det ser ut til at effekten av PGC-1α på ekspresjonen av LSDP5 blir betydelig redusert når ERRα er til stede. Disse resultatene gir et viktig bidrag til kunnskap om hvordan LSDP5 er transkripsjonelt regulert. Sammen med de tidligere publiserte resultatene fra dyreforsøk, bekrefter disse transfeksjonsstudiene at LSDP5 er et PPARα målgen.

Resultatene i oppgaven bidrar også med nye aspekter i reguleringen av LSDP5, hvor vi nå har sett at PGC-1 og ERRα spiller en viktig rolle i kontroll av den transkripsjonelle reguleringen av LSDP5 genet.

1. Introduction

The storage of energy excess when energy intake exceeds energy cost, and the release of energy in situations where energy access is limited characterize normal regulation of energy balance (1). Increased energy intake might result in an imbalance between fat synthesis and degradation, leading to an increase in circulating fatty acids (FAs) and accumulation of lipids in white adipose tissue (WAT). Whereas most tissues store excess FAs in the form of triacylglycerol (TAG), WAT has a unique capacity to store most of the body's TAG reservoir in lipid droplets (2). The incorporation of FAs in TAGs is very important, as increased level of circulating FAs might cause harmful effects. Elevated plasma concentrations of FAs have been implicated in the etiology of insulin resistance and diabetes (3). However, increased accumulation of lipids in WAT often causes diseases and disorders. In the state of hyperlipidemi, accumulation of lipids exceeds the storage capacity of the WAT and lipids are distributed in non-adipose tissues such as skeletal muscle, cardiac myocytes and pancreatic β-cells, leading to cell dysfunction and possible cell death, a phenomenon called lipotoxicity (4). Whereas lipid accumulation in skeletal muscle is associated with the development of insulin resistance (5), lipid overload in β-cells leads to dysregulated insulin secretion (6). Lipotoxicity in heart might cause heart failure (7). This illustrates important aspects in lipid metabolism, and clarifies the consequences of a dysregulation in lipid metabolism. The molecular processes of deposition or catabolism of the stored lipid components is regulated by a complex system of hormones, transcription factors and other factors that associate with the lipids and mediate their function within the cell.

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1.1 Lipid droplet associated proteins (LDAPs)

The ability to store large amounts of lipids is essential to ensure that energy is available in situations where energy access is limited, for instance during prolonged fasting. Fasting is a powerful stimulator of WAT lipolysis, which gives rise to a profound increase in plasma free FA (FFA) content. These FFAs are readily taken up by the liver or oxidized, converted to ketone bodies, or incorporated into lipid storage droplets (LSDs) (8). Most mammalian cells are able to store triacylglycerols (TAG), cholesterol esters or other lipids in these LSDs. The size of the droplets varies. While most cells store the lipids in relative small (< 1 μm diameter) storage droplets for use as energy source or in membrane biogenesis, the adipocytes contain large TAG-rich droplets that can exceed 50 μm in diameter. Thus, WAT has the capacity of highly efficient packaging of lipids (9). There is lack of knowledge about the mechanisms that control the flux of neutral lipids into and out of LSDs in any type of cell. LSDs are not just lipid depots, but also functional subcellular organelles. They are involved in multiple intracellular processes including lipid metabolism, vesicle traffic, and signalling through interactions with other organelles. This indicates that LSDs are important in lipid homeostasis (10-12). Different proteins are found on the surface of LSDs of basal and lipolytically stimulated 3T3-L1 cells (13) , some are ubiquitously expressed as stomatin; a raft associated protein (14), others are tissue specific expressed as α-synuclein in neurons (15) and stanniocalcin in ovary (16). Caveolins which also are proteins targeted to LSDs, are assumed to play important roles in signal transduction, cholesterol transport and endocytosis (17).

The lipid droplets consist of a core of neutral lipids, surrounded by a monolayer of phosholipids, into which proteins are embedded (18;19)

Among proteins that are found to co-localize on the LDSs surface are proteins involved in LSD coating. These proteins play an important role in the molecular processes that govern either the deposition or catabolism of the stored lipid components in the LSDs (20). The first protein to be experimentally identified to associate with the LSD surface was perilipin (21) Soon thereafter, ADRP (Adipose Differentiation-Related Protein) /adipohilin (22), TIP47 (Tail-interacting protein of 47 kDa)/placental tissue protein 17 (pp17) (23), S3-12 (24) and LSDP5 (25) were identified.

1.1.1 Structure

Perilipin, ADRP and TIP47 exhibit high sequence identity within an amino-terminal PAT-1 domain and a weaker homology in the central and carboxyl-terminal PAT-2 domain (26) (Figure 1.1). Due to their structural similarities, these proteins have been named the PAT-family (**P**erilipin-**A**DRP/adipophilin/**T**IP47). Adipohilin is the human ortholog of ADRP (27). S3-12 shares significant sequence homology to ADRP and TIP-47 in the carboxyl-terminus, but limited identity with the PAT-1 domain. It is therefore considered as a peripheral member of the PAT-family. LSDP5 is a recently identified member of the PAT-family. Structurally, it shares highest homology with TIP47 and ADRP. It is related to perilipin with sequence similarity within the amino-terminal PAT-1 domain region and the 11-mer repeated region. The homology to S3-12 is restricted to a sequence after the PAT-1 domain (28). Common for all the PAT-proteins is a segment of 11-mer helical repeats in the central sequence. This 11-mer repeat is responsible for the binding to the LSD surface, by forming an α11/3 amphipatic helix conformation enabling the hydrophobic and non-hydrophobic amino acids to be positioned on opposite half-sites of the alpha helix and thereby facilitate lipid association (29) (Figure 1.2).

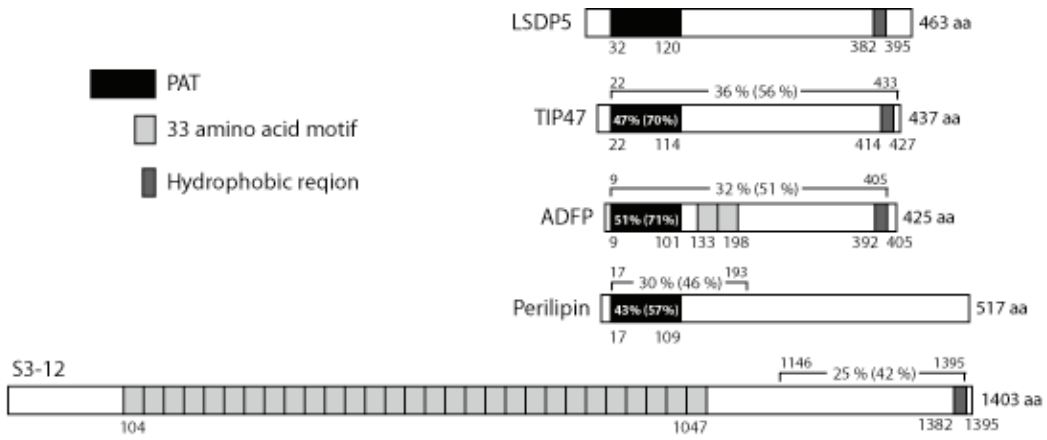

Figure 1.1 A schematic drawing of sequence identity (and similarity) of mouse LSDP5 against the other PAT members. Overall, LSDP5 is highly identical in sequence to TIP47 and ADRP (from aa 33 to 401) with 36 and 32 percent sequence identity (70 and 71 percent similarity), respectively. All PAT members have unique extreme amino- and carboxy- termini of varying sizes. LSDP5 has considerably longer unique N- and C-termini compared to the more related PAT members TIP47 and ADRP (Adapted from (30)).

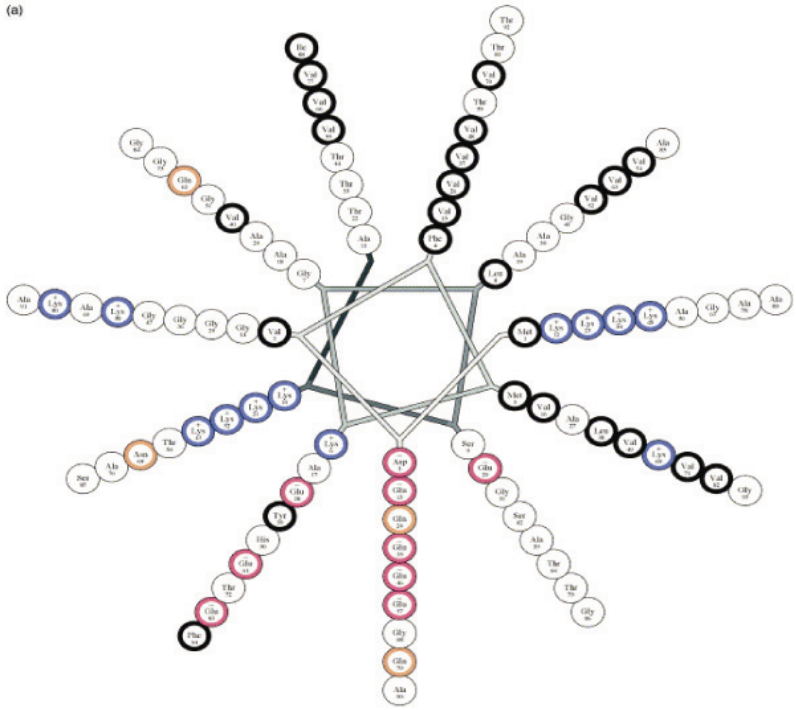

Figure 1.2 Helical pinwheel plot. The 11-mer helix contains hydrophobic amino acids shown on the upper side of this pinwheel plot and polar amino acids on the other side. This structure might give LSDP5 both lipid binding and hydrophilic properties. Hydrophobic residues are in black, charged residues in red or blue, and polar residues in yellow. 11-mer repeat: HxxHxxxHxxx, where H is hydrophobic and x are non-hydrophobic residues. Adapted from (31).

1.1.2 Tissue expression

The LDAPs are expressed in distinct tissues, suggesting that they have evolved to fine tune lipid metabolism according to the particular needs of these tissues. Perilipin is found only on the TAG-rich LDs in adipocytes and the cholesterol ester-rich droplets in steroidogenic cells (32). S3-12 is highly expressed in adipose tissue, skeletal muscle and heart (33;34). Originally, ADRP was thought to be mainly expressed in adipocytes, but later it is found that ADRP is ubiquitously expressed, with specifically high expression in metabolic organs and tissues (35). ADRP associates with smaller neutral LDs located within most tissues, but rarely in adipose cells that express perilipin. TIP-47 is ubiquitously expressed. However, a higher expression in skeletal muscle and placenta has been observed (36) According to the newly published data by Dalen et al, LSDP5 is found to be mainly expressed in fatty oxidising tissues and organs such as heart, liver and red muscle (37). These findings are confirmed in another study, performed by Yamaguchi et al (38). The high expression of LSDP5 in muscle is confined to red muscle (M-soleus) (39) (Figure 1.3).

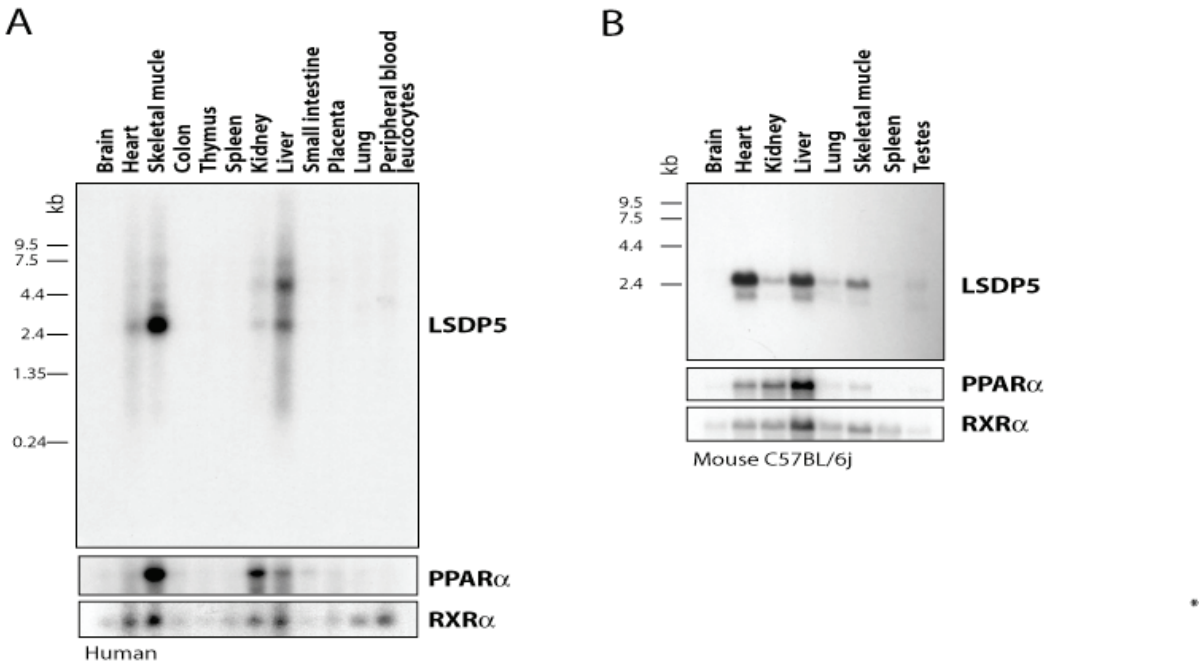

Figure 1.3 Tissue expression of LSDP5 in human and mouse tissues. (A) Expression of LSDP5 mRNA in human tissues. Expression of PPARα and RXRα is shown for comparison. **(B)** Expression of LSDP5 mRNA in mouse tissues (C57/BL strain). Expression of PPARα and RXRα is shown for comparison. For both blots, the most abundant expressed transcript (2.5 kb) is in agreement with the predicted mRNA transcript size. Additional uncharacterized transcripts are observed in human liver (~5 kb) and in specific mice tissues (~2 kb). Adapted from (40).

1.1.3 Function

Earlier the LSDs were regarded as simple lipid reservoirs (41). Recent knowledge demonstrates that they are active intracellular compartments involved in lipid metabolism, by affecting the lipogenetic and lipolytic processes in the body (42) . To date, the function of perilipin is best established.

The main function of perilipin is to protect the LSDs from lipolysis, by preventing access of hormone-sensitive lipase (HSL) to the lipid droplet. Perilipin is a major substrate of cAMP-dependent protein kinase in adipocytes. When perilipin is phosphorylated as a result of hormonal stimulation, HSL gets access to the lipid droplet and initiates lipolysis. Studies with perilipin knockout mice show that these mice have a constitutive lipolysis, normal bodyweight despite an increase in food consumption, smaller fat depots and they are resistant to diet-induced and genetic obesity (43).

Originally, ADRP was thought to be mainly expressed in adipocytes, based on observations of increased ADRP mRNA expression during adipocyte differentiation. Recent studies however show that ADRP has a specialized role in the liver where it associates with TAG-rich LSDs. ADRP ensures that FFAs, released from peripheral tissues during fasting, efficiently are incorporated in the LSDs. This is crucial to prevent high levels of FFA in the liver (44).

The knowledge upon the function of TIP47 is limited. So far, we know that it associates with small LSDs (45). A more specific biological role remains to be clarified.

S3-12 coats nascent LSDs in adipocytes during TAG synthesis, and might play a role in FA uptake or *de novo* TAG synthesis (46).

Dalen et al proved that LSDP5 binds to the surface of LSDs, in the similar manner as the other PAT-proteins (47) (Figure 1.4) . With broadest expression in tissues with high fatty oxidising capacity such as heart, liver and skeletal muscle, it is suggested that LSDP5 plays an important role in oxidation of fatty acids (48).

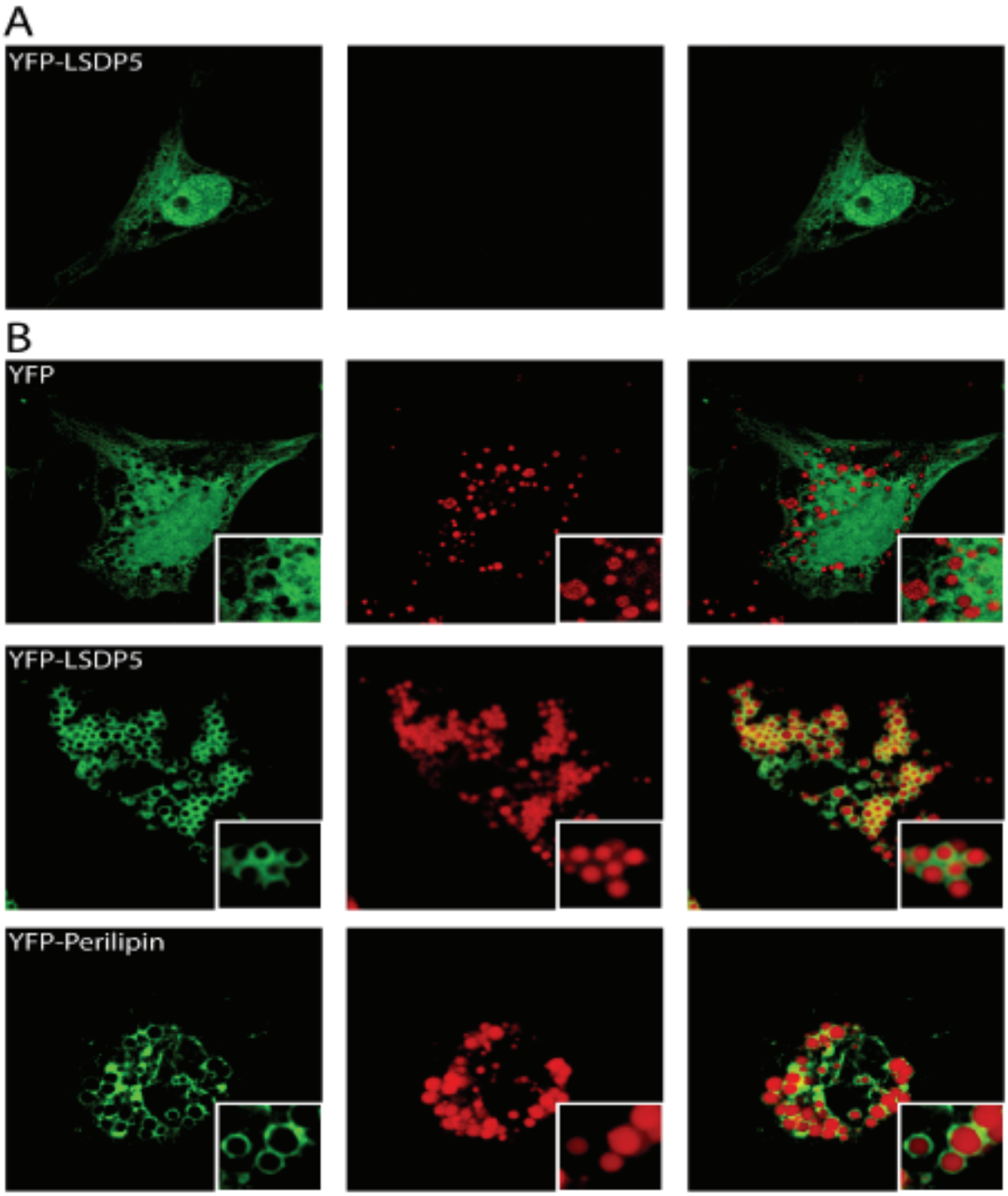

Figure 1.4 LSDP5 selectively associates with the lipid storage droplet surface. Cos-1 cells were transfected with pEYFP-C1-vector or YFP-fused to cDNA coding for LSDP5 or perilipin and incubated with BSA or BSA-bound oleic acid (OA) (100µM) for 24 hours. The cells were fixed with paraformaldehyde/glutaraldehyde prior to staining of lipids with Oil Red O. Cellular localization of PAT proteins was determined with immunofluorescence confocal laser microscopy. Left panels show immunofluorescent signal (green), middle panels show Oil red O staining (red), and right panels show merged images.(A) Expression of YFP-LSDP5 in cells incubated with BSA.(B)Expression of YFP, YFP-LSDP5 and YFP-perilipin in cells incubated with OA-BSA. Adapted from (49).

Recent data indicates that ADRP (50) TIP-47 (51) and LSDP5 (52) in addition to perilipin, play an important role in protecting the stored lipids from degradation by coating of LSDs. Thus, coating of LSDs to prevent lipolysis, seems to be a common feature for the PAT-proteins. This role has not been found for S3-12.

1.2 Transcriptional regulation of the LDAPs

In the organism there are numerous factors involved in transcription of genes controlling metabolism. These transcription factors (TFs) constitute about 3000 of all 30000 protein coding genes in the human genome which indicates a complex and tight regulation of metabolic control (53). The expression of genes is dependent on TF binding to specific DNA sites, response elements (RE), in the regulatory area (promoter) of target genes (54). The promoter is localized in front of the coding sequence of a gene which consist of a transcription initiation site and often a TATA box (thymidine and adenine rich motif) and/ or an initiator element that facilitate the assembly of the general transcription factors (GTFs) required for specific promoter binding by RNA polymerase II (54-56).

Several of the PAT genes are transcriptionally regulated by members of the PPAR family such as S3-12, perilipin, ADRP and LSDP5. This will be described underneath.

1.2.1 PPARs

PPARα, δ and γ are members of the nuclear receptor (NR) superfamily of ligand-dependent transcription factors (Figure 1.5) (57).

1.2.1.1 Nuclear receptors

Nuclear receptors (NRs) regulate the expression of target genes to affect processes as diverse as reproduction, development, and general metabolism. These proteins were

first recognized as the mediators of steroid hormone signalling and provided an important link between transcriptional regulation and physiology. Today, the human genome is reported to contain 48 members of this superfamily (58). The family includes not only the classic endocrine receptors that mediate the actions of steroid hormones, thyroid hormones, and the fat-soluble vitamins A and D, but a large number of so-called orphan nuclear receptors, whose ligands, target genes, and physiological functions were initially unknown. Today this last group is divided into two; the adopted orphan receptors, where the physiological ligand is now known, and the orphan receptors where the physiological ligand is still unknown. Members of the adopted orphan receptor group, also named "sensor receptors"(59) , include e.g. receptors for fatty acids; PPARs, oxysterols; liver X receptor (LXRs), bile acids; farnesoid X receptor (FXR) and vitamin A; retinoid X receptor (RXR) (60) (Figure 1.5).

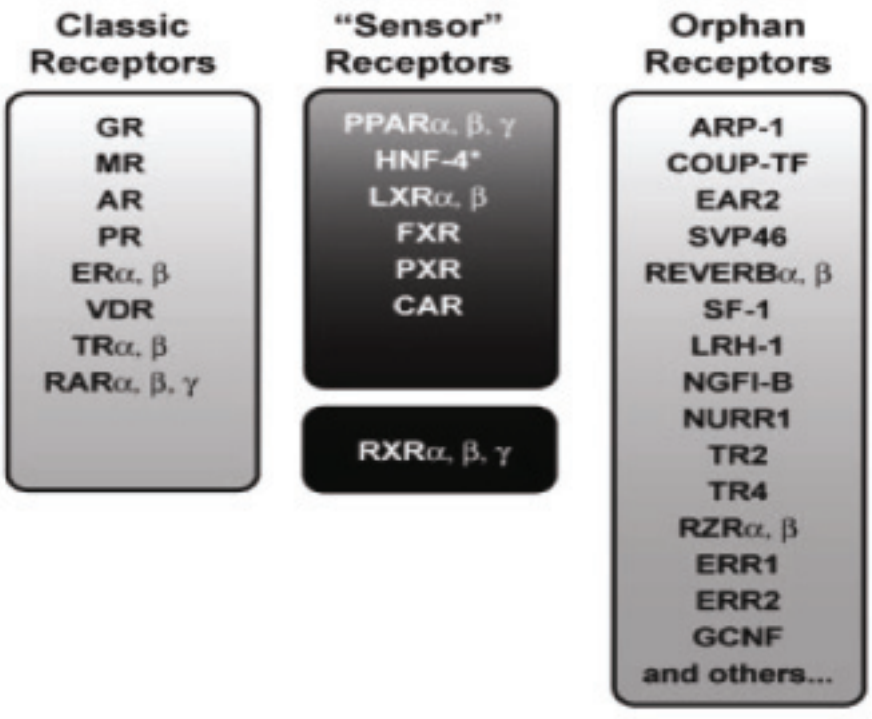

Figure 1.5. The nuclear receptor super family. The classic hormone receptors bind molecules with a high affinity. As key factors of endocrine homeostasis, their activation is associated with many metabolic adjustments. The "sensor" receptors are sensors of the metabolic status, respond to both incoming dietary signals and metabolites generated in the organism, and are responsible for the metabolic adaptation at the cell, organ, and whole organism level. The orphan receptors possess the structural characteristics of nuclear receptors including a sequence consistent with the presence of a ligand binding domain. No ligand has thus far been identified for these receptors. Adapted from (61)

The protein structure of the NRs consists of several domains. At the NH_2- terminal region there is a ligand-independent transcription activation function domain (AF-1 or A/B domain). In the core, a DNA-binding domain (DBD or C domain) is located which contains two highly conserved zinc finger motifs that target the receptor to specific DNA Response elements (RE). A hinge region (D domain) permits protein flexibility to allow for simultaneous receptor dimerization and DNA binding. Adjacent to the hinge region there is a large ligand-binding domain (LBD or E/F domain) that mediates ligand binding, dimerization, interaction with heat shock proteins, nuclear localization and transactivation. At the COOH-terminal region, the AF-2 domain binds co-repressor or co-activator (Figure 1.6A) (62). NRs bind to specific REs at their respective target genes that consist of one or two consensus core half site sequences in the promoter or enhancer region. Binding of the NR occurs either as a monomer, homodimer or a heterodimeric complex with another NR; often with the receptor for 9-*cis*-retinoic acid known as RXR to the consensus sequence, AGGTCA. The consensus sequences are arranged as direct-(DR), inverted- (IR) or everted (ER) repeats separated by 1-6 nucleotides (n) (Figure 1.6B). When a ligand binds a specific NR, the receptor undergoes a conformational change (Figure 1.6C); co-repressors dissociate which further lead to recruitment of co-activators that enable transcriptional activation (Figure 1.6 D).

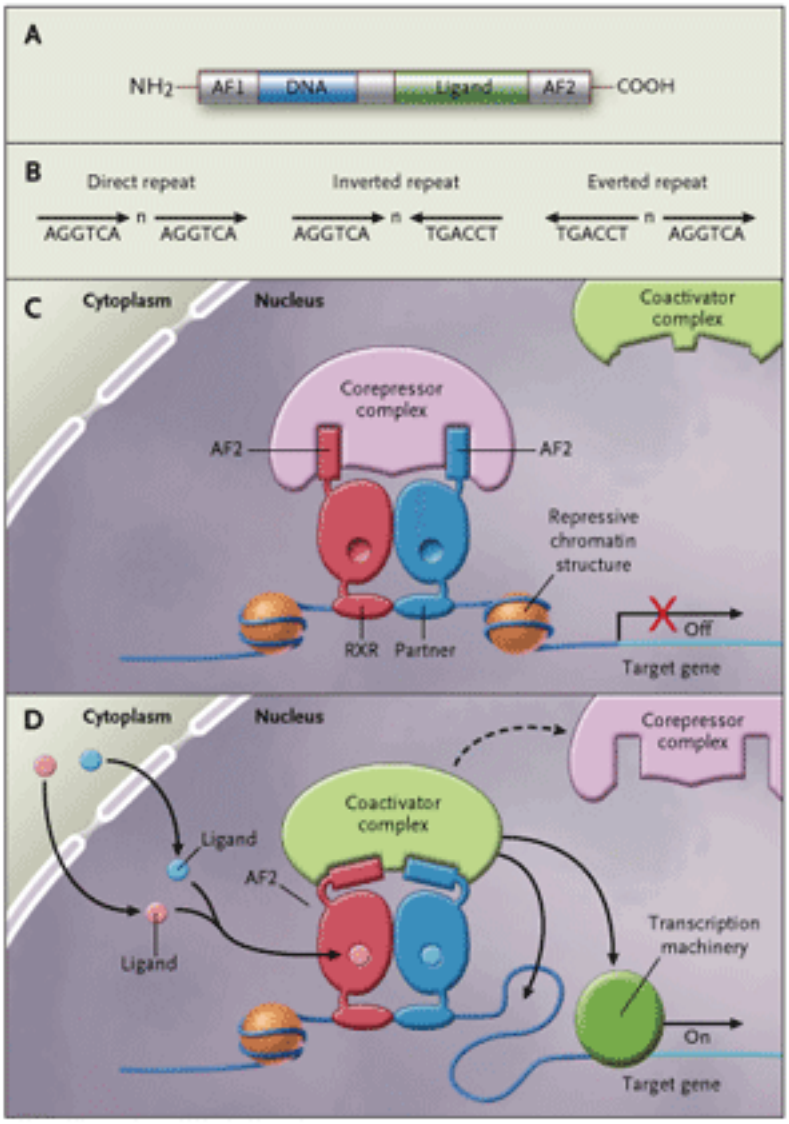

Figure 1.6 Nuclear Receptors as Ligand-Dependent Transcription Factors. Panel A shows the canonical structure of the nuclear receptor, which includes N-terminal activation function 1 (AF1), DNA binding, ligand binding, and C-terminal AF2 domains. Responsive elements in the nuclear receptor can be configured as either direct, inverted, or everted repeats of the hexad core sequence AGGTCA (Panel B). The number of nucleotides between the two core elements (n) confers additional specificity. As shown in Panels C and D, RXR heterodimers constitutively bind to response elements in the promoter regions of target genes. In Panel C, in the absence of agonist ligand, the AF2 domain conformation promotes receptor interaction with co-repressors. The multiple-subunit co-repressor complex stabilizes repressive local chromatin structure and blocks access of the transcription machinery (red X) to the promoter. As shown in Panel D, ligands diffuse across the nuclear membrane and bind to receptor ligand-binding domains. Ligand binding triggers a conformational change in the AF2 domain, which destabilizes co-repressor interaction and promotes co-activator binding. Multiple-subunit co-activator complexes activate local chromatin structure and recruit the transcription machinery to the promoter, where target-gene transcription commences. Adapted from (63).

The PPARs transcriptional regulates their specific target genes by binding to a PPRE located in the regulatory area of the gene (figure 1.7). This DNA element is a DR1 (for some genes a DR2 element) which means that the RE consists of a direct repeat of the consensus sequence, AGGTCA half site spaced by one (or two) nucleotides (64).

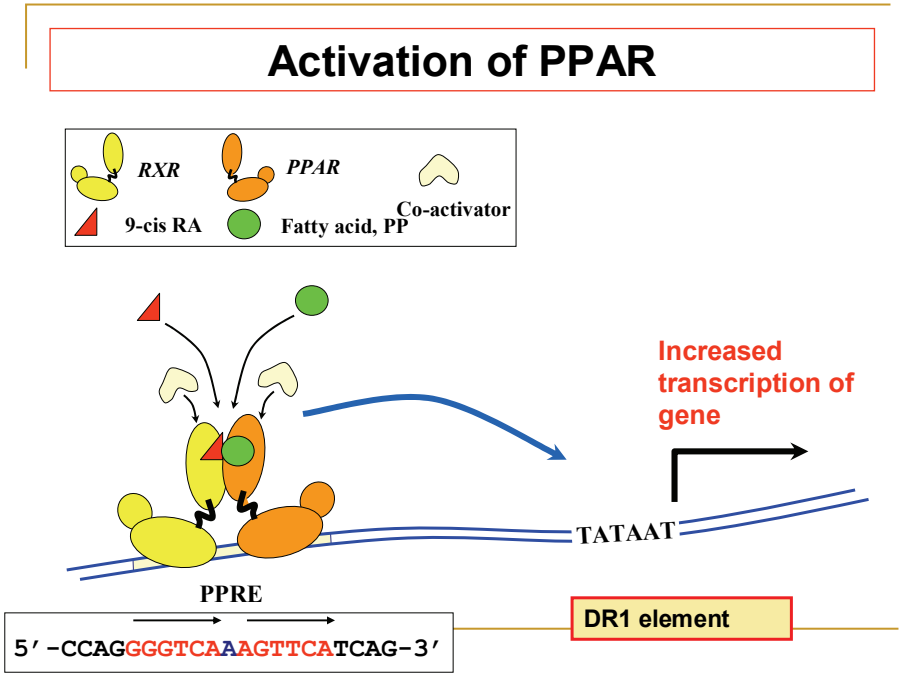

Figure 1.7: When PPAR is activated by ligands, PPAR heterodimerizes with RXR and regulate transcription by binding to a specific PPAR response element (PPRE) in the promoter in the target gene.

A common feature among a few of the NR members is that they transcriptional regulate their target genes as heterodimers with retinoid X receptors (RXRs). The NRs are activated by specific ligands, where they bind to their ligand binding domain. PPARs are activated by fatty acids, naturally occurring fatty acid-derived molecules and synthetic ligands (65). The PPARs are important pharmacological targets for treatment of obesity, diabetes and lipid disorders.

The PPARs include three different isoforms designated PPARα, PPAR β/δ and PPAR γ. Although all three isoforms share similar protein sequence and structure, they differ in tissue distribution, ligand selectivity and biological actions. Figure 1.8 gives and overview over the signalling pathways the PPARs regulate and how their metabolic pathways integrate.

PPARα is expressed in tissues with high β-oxidation activity corresponding to its role in regulating the oxidation of FAs (66), such as liver, brown adipose tissue, muscle, heart and kidney. Thus, the main role of PPARα is to transcriptional regulate genes that are involved in lipid and lipoprotein metabolism (67). Furthermore, PPARα is irreplaceable for a proper response to fasting in liver (68). PPARα ligands are polyunsaturated fatty acids, including DHA and EPA, oxidised phospholipids, lipoprotein lipolytic products and fibrates (69).

PPARγ is highly expressed in WAT and macrophages. PPARγ ligands are naturally occurring FA derivatives, prostaglandin derivatives and synthetic compounds such as the antidiabetic thiazolidinediones (TZDs). By acting as direct agonists for PPARγ, these drugs reduce peripheral insulin resistance and thus lower blood glucose levels in patients with type 2 diabetes. The activation of PPARγ results in a reduced release of FFAs and insulin resistance-mediating adiopocytokines, such as tumor necrosis factor α (TNFα), leptin or resistin, and an increased production of the antidiabetic adiponectin. The consequence of this is improved insulin sensitivity in liver and skeletal muscle (70). The high expression of PPARγ in WAT is linked to its role as a crucial regulator of the adiopogenesis, the process of preadipocytes maturing into adipocytes (71).

PPAR β/δ is ubiquitously expressed. It is activated by polyunsaturated FAs (PUFAs), prostaglandins and synthetic compounds. Similar to PPARα, PPAR β/δ plays an important role in the regulation of glucose and lipid levels in plasma, by stimulating FA oxidation in heart and skeletal muscle. Treatment with PPAR β/δ agonists in obese phenotype db/db mice, normalized the lipid profile and reversed diet-induced obesity and insulin resistance in mice (72).

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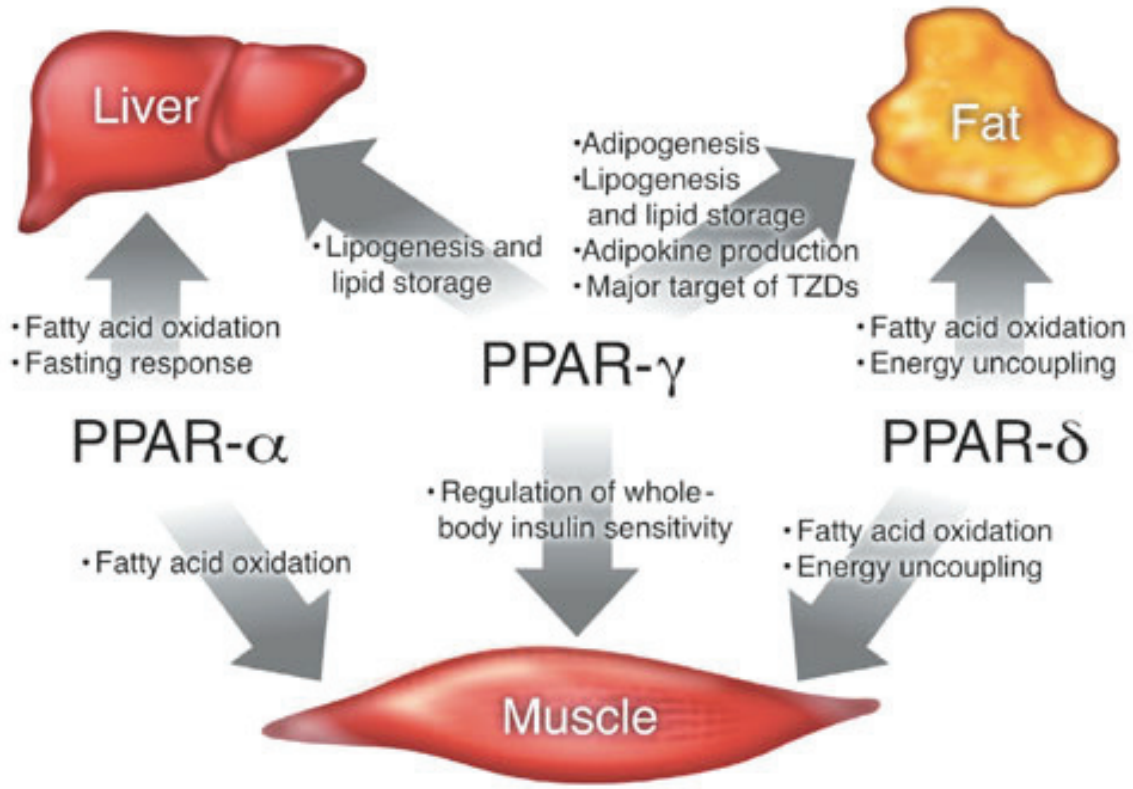

Figure 1.8 Metabolic integration by PPARs. The three PPAR isoforms regulate lipid and glucose homeostasis through coordinated activities in liver, muscle and adipose tissue. Adopted from (73)

1.2.2 PPARs regulate the transcription of LDAPs

The tissue expression of PPARs is closely related to the expression of the LDAPs, and recently published studies confirm that the distinct members of the PAT-family are transcriptionally regulated by the PPARs (74).

S3-12 and perilipin have been demonstrated to be PPARγ target genes in WAT (75). In a study performed by Dalen et al (2), it was shown that these promoters of the genes contain an evolutionary conserved PPRE. They also found that S3-12 and perilipin are induced during adipocyte differentiation, matching the timing of PPARγ

induction, and induced by PPARγ activation (76). The function of S3-12 and perilipin in WAT are believed to be their involvement in storage of lipids in LSDs by stimulating uptake of FAs and *de novo* TAG-synthesis (S3-12) and by preventing the release of FAs from the lipid droplets (perilipin).

TIP47 has not yet been demonstrated to be regulated by PPARs.

While S3-12 and perilipin are regulated by PPARγ in WAT, it has recently been demonstrated that ADRP is regulated by PPARα in liver (77). This regulation is mediated through a highly conserved DR-1 element. Both *in vitro* and *in vivo* studies have shown that PPARα activation by the synthetic ligand WY-14643 induces the expression of ADRP (78). Along this line, a functional PPRE was identified both in the human adipophilin and mouse ADRP promoters. Recent findings by Dalen et al (79) furthermore demonstrated that the expression of ADRP is stimulated by fasting in the liver *in vivo*. Based upon the knowledge that the expression of PPARα is also induced during fasting and its target genes, it was therefore reasonable to believe that the upregulation of ADRP during fasting is due to PPARα activation. However, surprisingly, studies with PPARα knockout mice demonstrated that the expression of ADRP during fasting is independent of PPARα (80), suggesting that ADRP is regulated by other mechanisms during fasting. This is opposite from ADRP gene regulation by PPARα agonists as discussed above. The accumulation of ADRP and the formation of LSDs are highly correlated (81): fasting and the presence of FAs facilitate formation of LSDs and increased formation of LSDs results in a higher cellular content of ADRP. When ADRP is not bound to LSDs, it is rapidly degraded by proteasomes (82). The main function of ADRP in liver is to ensure that FAs released from peripheral tissues during fasting, are incorporated in the TAG-containing LSDs.

The knowledge upon regulation of the newly identified LSDP5 is still limited, but so far studies indicate that it is regulated by PPARα and that the regulation of the

LSDP5 gene is, at least in part, similar to the regulation of the ADRP gene (83). Like ADRP, LSDP5 is induced during fasting, independently on a functional PPARα. However, the basal expression of LSDP5 is dependent upon PPARα. This was demonstrated in PPARα wild-type mice and PPARα knockout mice (84).

Despite these findings indicate that LSDP5 is transcriptionally regulated by PPARα, it has so far not been confirmed that the LSDP5 gene contains a functional conserved DR-1 element.

1.2.3 New aspects in PPARα regulation of LDAPs

Recently, transcriptional regulation of PPARα target genes has been related to two other proteins that are demonstrated to have an important influence on the regulation of PPARα target genes. Estrogen-related receptor α (ERRα) and PPARγ coactivator-1α (PGC-1α) are both involved in regulation of genes involved in energy production pathways, including cellular FA uptake, FA oxidation and mitochondrial electron transport/oxidative phosphorylation.

ERRs are orphan NRs, and consist of three isoforms, ERRα, ERRβ and ERRγ (85). ERRα and ERRγ are predominantly expressed in highly active metabolic tissues such as BAT, heart and skeletal muscle (86). These tissues use primary FAs as energy substrate. After birth, the expression of ERRα in heart increases dramatically, coincident with the postnatal switch to FAs as energy substrate (87). This demonstrates ERRα`s function in FA oxidation in heart. It is found that ERRα regulates the expression of medium-chain acyl coenzyme A dehydrogenase (MCAD), which is a key enzyme in the FA β-oxidation pathway in the mitochondria (88). However, the ERRα mediated gene regulation of MCAD, seems to be dependent on the coactivator, PGC-1α. Early attempts to prove ERRα`s role in transcription of MCAD failed without the presence of this coactivator (89). PGC-1α belongs to the PGC-1 family along with the two other isotypes, PGC-1β and PGC-related coactivator (PRC) (90). The expression of PGC-1α is most prominent in tissues with high energy demands (91), similar to the expression pattern of ERRα. PGC-1α is a

key regulator in cellular energy metabolic pathways, where it primary regulates FA oxidation and increases the number of mitochondria (92).

Originally, PGC-1α was identified as a PPARγ-interacting protein in BAT, from which the term PPARγ coactivator-1α is adopted (93). To date, it is known that it interacts with many different transcription factors, including PPARα and ERRα (94).

Coactivators play an important role in gene regulation. PGC-1 coactivators have powerful transcriptional activity when linked to a DNA binding domain or when they dock on a transcription factor (95) (Figure 1.6 D). They bind a protein complex with histone acetyl transferase (HAT)-activity at the amino-terminal region. The HAT-containing proteins acetylate histones and remodel chromatin structure to allow transcriptional activity. When PGC-1α is bound to this activation complex, repressor proteins such as histone deacetylase and small heterodimer partner (SHP) is replaced and this results in increased transcription (96).

The importance of this coactivator in oxidative metabolism pathways is demonstrated in animal studies. In PGC-1α knockout mice it is demonstrated reduced mRNA expression of genes involved in mitochondrial oxidative metabolism in tissues such as heart, liver, BAT and skeletal muscle. PGC-1α is required for the normal expression of these genes.

Both ERRα and PGC-1α are involved in the same metabolic pathways as PPARα. It is also shown that the ERRα mediated regulation of the FA utilization genes occurs through direct activation of PPARα gene transcription (97). Several of the genes involved in FA catabolism that ERRα and PGC-1α regulates, are target genes of PPARα. It is therefore interesting to consider whether the PPARα mediated regulation of LDAPs might be influenced by ERRα and PGC-1α.

2. Objectives

The main objective in this project was to study the transcriptional regulation of LSDP5. Recent studies indicate that LSDP5 is a PPARα target gene. However, it has been difficult to show through transfection studies that the LSDP5 promoter contains a functional PPRE. In this thesis I wanted to examine whether the LSDP5 gene contains a PPRE in addition to study whether the PPARα mediated regulation of LSDP5 is influenced by additional regulatory proteins. I have focused on the NR ERRα and the coactivator PGC-1α.

Following *sub-objectives* were addressed;

- Does the LSDP5 gene contain a conserved PPAR response element?
- Does PGC-1α enhance the transcriptional activity of the LSDP5 gene?
- Is the PPARα mediated regulation of LSDP5 affected by ERRα?

3. Materials and methods

3.1 Materials

Bacteria	Manufacturer
Competent E.coli	Invitrogen

Cell lines	Manufacturer
Monkey kidney COS-1 cells	ATCC

Cell medium	Manufacturer
Dulbecco`s Modified Eagle`s Medium (DMEM)	Sigma
Fetal Calf Serum	Sigma

Chemicals	Manufacturer
Ampicillin	Sigma
Bacto-Agar	Merck
Bacto-peptone	Merck
Bacto-yeast	Merck
CaCl2	Merck
Coenzyme A	Sigma
Dextrose	Sigma
DTT (DL-Dithionthreitol)	Sigma
EDTA (ethylenediaminetetraacetic acid)	Sigma
Ethanol	Merck
Glucose	Sigma
Glycerol	Fluka
HCl	Chemi-teknikk AS
Hepes (Hydroxyethylpiperazineethanesulfonic acid)	Sigma
Isopropanol	Arcus
KCl	Merck
KH2PO4	Merck
L-glutamine	Merck
Luciferin	Sigma
Lysis buffer x 5	Promega
MgSO4 x 7H2O	Sigma
MgSO4	Sigma
Na2HPO4x2H20	Merck
NaCl	Sigma
NaH2PO4	Merck
NaOH	Chemi-teknikk AS
PBS (Phophat buffered saline)	Gibco

Pencillin/Streptomycin	Sigma
Trypsin	BioWhittaker
WY-14.643	Sigma
9-cis-retinoic acid	Sigma

Equipment	Manufacturer
Cell flasks and plates	Corning Incorporated
Falcon tubes	Falcon
Microtubes	Sarsted
Parafilm	American National Can
Pipettes	Biohit
Pipette tips	Integra bioscenses
96-well plates	Sigma
Pipeteboy Comfort	Integra bioscenses

Instrument	Manufacturer
Biofuge fresco (Centrifuge)	Heraeus instruments
Cell counter model Z1	Coulter electronics
Freezer, -20 C	Forma Scientific
Incubator	Forma Scientific
Luminometer TD-20/20	Turner design
MilliQ-synthesis	Millipore

Kits	Manufacturer
Jet Star Maxiprep Kit	Genomed
BC Assay Protein quantitation kit	Uptima

Plasmids

pGL3-mLSDP5-luc
pCMX-hERRα
pcDNA3-mPGC-1α
pSG5-mPPARα
pSG5-mRXRα

Software and internet resources	Manufacturer
Adobe Illustrator	Adobe Incorporated
Microsoft Office 2003	Microsoft Incorporated
Pubmed	National Library of Medicine
Reference Manager 10	ISI Research Soft
SPSS 14.0	SPSS Incorporated

3.2 Methods

In order to study if PPARα regulates the transcriptional activity of the LSDP5 gene, the LSDP5 promoter was characterized with respect to whether PPARα and its heterodimer, RXR as well as ERRα and PGC-1α are able to induce the transcriptional activity of the LSDP5 gene. To study this, an LSDP5 reporter construct (pGL3-(m)LSDP5-Luc [-2324 to +244]) was cotransfected with following expression plasmids ERRα, PPARα, RXRα and PGC-1α (alone or together) into Cos-1 cells. After this transient transfection the cells were harvested and transcriptional activity was measured by the Luciferase assay.

3.2.1 Culturing and seeding cells

Monkey kidney Cos-1 cells (ATTC # CRL-1650) were cultured in high glucose (4,5 g/L) containing Dulbecco`s modified Eagle`s medium (DMEM, Sigma; #6546) supplemented with 10 % heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37 C in 5 % CO_2. Cells were kept subconfluent prior to experiments.

The cell number was quantified using the Coulter Counter.

The day before transfection, the cells were seeded at 90 % confluency in six-well plates, 200 000 cells per well.

3.2.2 Transformation of plasmids into bacteria

Before transfecting the cells with reporter plasmids and expression plasmids we had to produce a large amount of each plasmid by using bacteria that are able to copy the DNA plasmids very fast (Figure 3.1). The plasmids behave as accessory genetic units that replicate and are inherited independently of the bacterial chromosome.

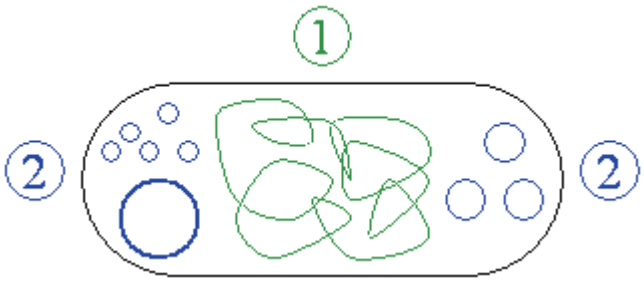

Figure 3.1A: Schematic drawing of a bacterium with plasmids enclosed. 1: Chromosomal DNA. 2: Plasmids. (Adapted from Wikipedia).

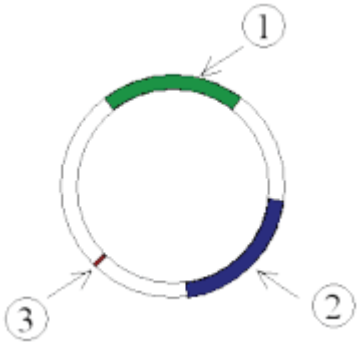

Figure 3.1B: Schematic drawing of a plasmid with antibiotic resistances. 1 & 2 Genes that code for resistance. 3 Origin of replication, or Ori (a starting point for DNA replication). (Adapted from Wikipedia).

Transformation is an artificial process where plasmid DNA is introduced into bacteria. By growing the bacteria, the inserted plasmid vector will replicate, and it is possible to obtain high concentrations of the plasmid DNA. Plasmid vectors contain genetic markers, and these markers provide specific resistance to antibiotics such as the kanamycins, ampicillin and the tetracyclines. Selection of transformed bacteria is therefore possible by growing the bacteria in medium with specific antibiotics (figure 3.2) (98).

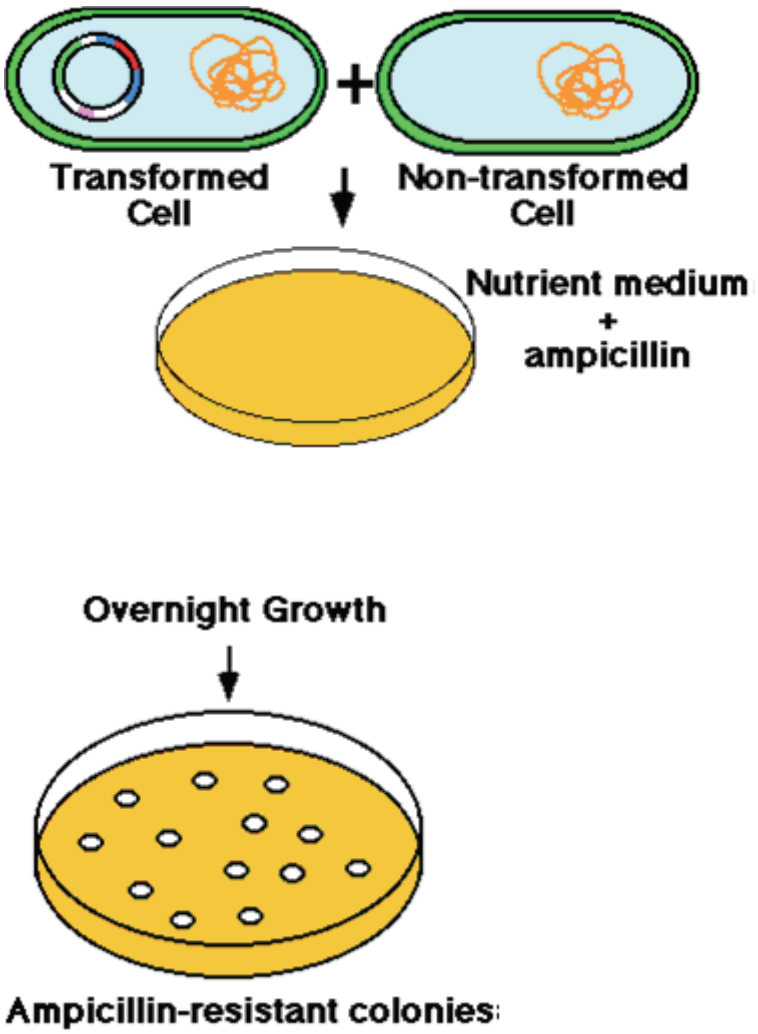

Figure 3.2: The transformed bacteria, containing the ampicillin-resistance gene, are grown overnight, and this results in ampicillin-resistant colonies in the agar plates.

3.2.3 Plasmids

The plasmids used in this project were available in the laboratory prior to initiation of the transfections; The pCMX-mERRα (gift from Prof. Vincent Giguere, Molecular Oncology Group, McGill University Health Centre, Canada), pcDNA3-mPGC-1α (gift from Prof. Vincent Giguere, Molecular Oncology Group, McGill University

Health Centre, Canada), pSG5-mRXRα and pSG5-mPPARα expression vectors (provided by Jan-Åke Gustafsson (Department of Bioscience and Medical Nutrition, Novum, Huddinge, Sweden)) have been described previously. The pGL3-(m)LSDP5-Luc reporter vectors were generated as described below.

Identification, cloning and mutagenesis of the LSDP5 promoter were made as follow by Post.Doc Knut Tomas Dalen: The full-length mouse LSDP5 cDNA sequence was used to search nonredundant and high-throughput genomic sequence databases by the basic local alignment search tool (BLAST) to identify the LSDP5 promoter sequence. The sequence spanning the transcription start site (-4000 to +2000) was scanned with a consensus PPRE (RGGBSAAAGGTCA) with the use of the gcg program package.

The full-length mouse LSDP5 promoter (pGL3-(m)LSDP5-Luc [-2324 to +244]) was amplified with PfuTurbo (Stratagene) with mouse genomic DNA (Clontech, #6650-1) as template using PCR-settings as described (99). Primers used:

5-m-LSDP5-promoter (HindIII): 5'-
TAAAGCTTGCCAGGAATGCTATTCTCGGACT-3',

3-m-LSDP5-promoter (HindIII): 5'-
TAAAGCTTTCAGGGCTCATGCCCTATGTATC-3',

The full-length mouse promoter (pGL3-(m)LSDP5-Luc [-2324 to +244]) was next inserted into a pGL3-Basic-Vector (Promega # E1751) as described (Appendix, Protocol 1).

The deletion construct was generated by restriction digestion with SacI followed by religation of the vector to generate the pGL3-(m)LSDP5-Luc [-1329 to +244] vector. Site-directed mutagenesis of the DR-1 element to generate the pGL3-(m)LSDP5-Luc [-2324 to +244-DR-1 mut] was performed with site-directed mutagenesis as described (25). Oligos used: m-LSDP5-PPRE-s-mut: 5'-GAGCCTGTGGACGTCAGACGGCTCCTTGCAGGA-3' and m-LSDP5-PPRE-a-mut: 5'-TCCTGCAAGGAGCCGTCTGACGTCCACAGGCTC-3'.

The plasmids were transformed into competent DH5α-E.coli bacteria according to the protocol (Protocol 2).

Protocol 2

Solutions

LB-medium is a growth medium with optimized growth conditions. LA-medium is LB-medium supplemented with bacteria agar, allowing the growth medium to be solid in dishes.

LB medium

To 2 L of distilled water, add: 20 g peptone, 10 g yeast extract, 20 g NaCl. Adjust the pH to 7.0. The solution is autoclaved to be sterilized.

LA medium

Add 1.5 % Bact agar to the LB medium. The solution is autoclaved to be sterilized.

Agar plates

After autoclave, allow the LA medium to cool to 50°C. Add 500 µl ampicillin (50 mg/ml) to 500 ml LA-medium. Distribute the LA medium on plates.

Experimental procedure

Bacteria are stored at -70°C. Thaw the cells on ice. Use a sterile pipette tip to transfer 100 µl of the competent cells to polypropylene tubes. Store the tubes on ice. (Glass tubes should not be used as they lower the efficiency of transformation by 10-fold). Add 2 µl plasmid diluted to a concentration of 10 ng/µl to the bacteria. Swirl the

tubes gently several times to mix their contents. Store the tubes on ice for 60 minutes. Transfer the tubes into a preheated 42°C circulating water bath for 60 seconds. This step is called “heat shock” and is necessary for the pores in the bacteria membrane to open. Now it is possible for the DNA to be inserted into the bacteria. Then, rapidly transfer the tubes back to ice and allow the cells to cool for at least 2 minutes. This step is crucial to ensure that the pores close and that the DNA is kept inside the bacteria. Transfer 30 µl of the transformed cells onto agar plates. Sterilize a bent glass rod by dipping it into ethanol and then in flame of a Bunsen burner. When the rod has cooled to room temperature, spread the transformed cells gently over the surface of the agar plate, containing 50 µg ampicillin/ml. Incubate the plates over night at 37°C. Include a negative control: Add cells without inserted DNA onto an agar plate with ampicillin. No bacterial colonies should grow on this plate ((98), modified procedure).

3.2.4 Bacterial growth

After plasmids were transformed and bacteria were incubated overnight at 37°C, bacteria colonies were grown in the plates. Then one of these colonies was picked and transferred to a flask with LB-medium according to the protocol (Protocol 3). Next day, E.coli cells were grown to log phase.

Protocol 3

Experimental procedure

Pick a single colony (2-3 mm in diameter) from the agar plate, and transfer the colony into a bottle with 100 ml LB-medium, supplemented with 50 µg ampicillin/ml. Incubate overnight at 37°C in an orbital incubator. Include a negative control: prepare

a small tube with only medium and ampicillin (2 µl ampicillin and 2 ml medium). No bacteria culture is expected in this tube.

3.2.5 Isolation of plasmids

After transformation of plasmids into bacteria and growing them overnight, the next step is to isolate the plasmids from the bacteria. This is obtained by centrifugation of the bacteria and then purifying the plasmids from the bacteria pellet. Maxi preparation of DNA is used for isolation of large amounts of highly purified plasmid DNA. The technique is based on the principle that the bacteria are lysated and neutralized. Then the lysate is applied onto a column and the DNA is bound to the anion exchange resin. Washing the resin removes RNA and all other impurities. Finally the purified plasmid DNA is eluted from the column and concentrated by an alcohol precipitation (Jet Star Protocol). In this project, plasmid isolation was performed according to the Jet Star Maxi protocol (Appendix, Protocol 4).

Solutions

Jet Star Maxi prep kit (GENOMED GmbH, # 220020)

Isopropanol

Ethanol, 70%

Experimental procedure

E.coli cells are pelleted by centrifugation. Transfer the bacteria to centrifugation tubes. Centrifuge at 5000 rpm for 10 minutes at 4 °C, and remove the supernatant. All traces of medium should be removed carefully. Then follow the steps in the Jet

Star Maxi Prep protocol (Appendix, protocol 4). Dissolve the precipitated plasmids in 300 µl MilliQ H_2O, and quantify DNA concentration on a photometer prior to transfection of plasmids into Cos-1 cells.

3.2.6 Transfection, harvesting and lysis

Transfection is introduction of cloned DNA into cultured eukaryotic cells. There are different strategies to deliver genes into eukaryotic cells, and the choice of method depends on several factors, such as the ability of cell line to survive the stress of transfection and the efficiency required of the system. There are three categories of transfection techniques: transfection by biochemical methods, transfection by physical methods, and virus-mediated transduction.

The calcium-phosphate-mediated method is one of the biochemical methods and has been used for more than 30 years. It was originally presented by Graham and Van der Eb in 1973 (100), and is based on the principle that the uptake of DNA by cells in culture is markedly enhanced when the nucleic acid is presented as a coprecipitate of calcium phosphate and DNA. The calcium phosphate DNA-coprecipitate attaches to the cell surface and is absorbed by endocytosis. After entering the cells by endocytosis, some of the coprecipitate escapes from endosomes or lysosomes and enters cytoplasm, from where it is transferred to the nucleus. This results in expression of the transfected genes in up to 50 % of the cells (98).

In this project, a reporter plasmid, the pGL3-LSDP5(-2324/+244)luc or the 5'-deletion constructs of the LSDP5 promoter described above, was cotransfected with the expression plasmids for either pCMX-hERRα, pcDNA3-mPGC-1α, pSG5-mRXRα and pSG5-mPPARα expression vectors, by the calcium-phosphate-mediated method (Protocol 5). The expression plasmids were added to investigate both the effect of them individually, and in combination with the other expression plasmids. An empty vector, pGL3-Basic-Vector (without LSDP5 inserted), was added to ensure equal concentrations of DNA in all wells. Cells were treated with a PPARα and a RXRα agonist, WY 14.463 (Sigma, # C7081) and 9-cis retinoic acid (9-cis-RA)

(Sigma, # R4643), respectively. After 72 hours incubation, the cells were washed and harvested in the reporter lysis buffer, and luciferase and protein measurements were performed, according to the protocol (Protocol 6).

Protocol 5

Solutions

2 x Hepes-buffer-saline (2 x HBS)

1.6 g NaCl, 0.074 g KCl, 0.024 g $Na_2HPO_4x\ 2H_2O$, 0.2 g dextrose, 1 g hepes. Dissolve in ddH20 to a total volume of 100 ml and adjust to pH 7.05. Sterilize by filtration (0.2μm in diameter).

$CaCl_2$ (250 mM)

3, 676 g $CaCl_2$ x $2H_20$. Dissolve in ddH_20 in a total volume of 100 ml. Sterilize by filtration (0.2μm in diameter).

10 x PBS

1 tablet (Gibco, # 18912-014)) in 10 ml H_20. Sterilize by filtration (0.2μm in diameter).

2xHBS/PBS

10 μl 10xPBS/ml 2xHBS

Ligands

Ligands, 10 μM Wy 14.463 and 1 μM 9-cis-RA dissolved in DMSO, were added to growth medium.

Reporter Lysis 5 x buffer (Promega, # E397A)

1x PBS

Experimental procedure

Prepare the plasmids

Mix the plasmids in Falcon tubes (15 ml). Total amount of DNA to each well should be 10 µg: 5 µg of the reporter plasmid (pGL3-LSDP5(-2324/+244)luc), 1 ug of each of the expression plasmids (pSG5-hERRα, pSG5-mPPARα, pSG5mRXRα or pSG5m-PGC-1α). Regulate with empty vector to obtain the correct amount DNA (Table 1).

Stimulation with ligands

Seed the cells in six-well plates, and allow them to grow overnight. Remove medium from the cells. Add control medium or medium with ligands (10 µM PPARα and 1 µM 9-cis-RA), 3 ml/well, prior to the transfections.

Transfection

Mix 2 x HBS and 10 x PBS in a Falcon tube. Transfer (250 µl) of this mix to the tubes with plasmids. Vortex in at least 20 seconds. During vortexing, add (250 µl) calcium chloride in droplets. This will result in formation of an insoluble calcium phosphate coprecipitate with DNA. Add (500 µl) of the transfection mix to each well (Table 1). The coprecipitate will now attach to the cells and uptake by endocytosis will take place. Incubate for 72 hours, 37°C.

Reporter plasmid (LSDP5-luc)	5 μg
Expression plasmids (PPARα, RXRα, ERRα or PGC-1α)	1-4 μg
Empty vector	to 10μg
Tot	*10 μg*
2xHBS/10xPBS	250 μl
CaCl2	250 μl
Tot (mix)	*500 μl*

Table 1: Plasmids and transfection mix given to each well in a six-well plate. Each well was added 3 ml medium +/- ligand prior to the transfection mix and plasmids.

Harvesting and lysis

Remove growth medium from the cells. Wash the cells with 1x PBS twice. Add 200 ul 1 x lysis buffer to each well. Incubate for 15 minutes at room temperature. Scrape attached cells from the dish, and transfer them to tubes on ice. Store the tubes in the freezer at -20°C, or continue with luciferase and protein measurements.

3.2.7 Luciferase activity- and protein measurements

The Luciferase Assay

The Luciferase assay is commonly used in many laboratories. The purpose of this reporter assay is to measure the regulatory potential of an unknown DNA-sequence. This can be done by linking a promoter sequence to an easily detectable reporter gene. Luciferase serves as an excellent reporter gene in promoter analysis studies. Most luciferase marker genes currently in use are derived from the luciferase gene of the the firefly (Photinus pyralis). Luciferase is an enzyme, which acts as a reporter by

emitting light during oxidation of their chemical substrate, luciferin. The firefly luciferase catalyzes the bioluminescent oxidation of the luciferin in the presence of ATP, magnesium and oxygen (Figure 3.3). The light generated during this process is captured, amplified and measured in a luminometer. The assay provides accurate measurements of the level of gene expression (98). Thus, measuring luciferase activity is a good strategy to study transcriptional activity in the promoter of a gene.

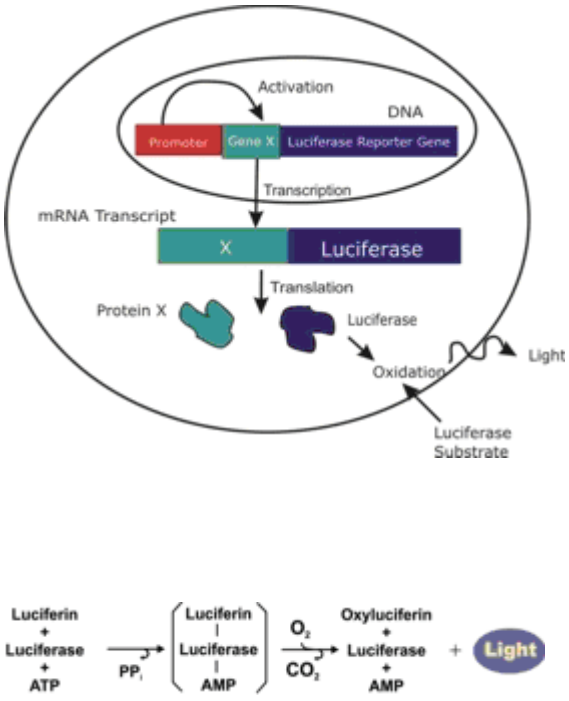

Figure 3.3: The principle of Luciferase Assay

Luciferase activity measurement

After cells were harvested, luciferase activity was measured according to the protocol (Protocol 6). Prior to the measurements, cells were mixed by vortexing and then centrifuged. The supernatant was used to the luciferase measurements.

Protein concentrations measurement

Luciferase activity was related to the protein concentrations in the cells. Protein concentrations were measured prior to luciferase activity, by using the BC Assay. This is a colorimetric protein assay based on a reaction where Cu^{2+} is reduced to Cu^{+} when bound to the peptidic bounds of proteins, leading to the formation of a water soluble purple coloured complex. The reaction is measured by the high optical absorbance of the final Cu+ complex at 562 nm. Absorbance is directly proportional to the protein concentration, which can be calculated with a reference curve obtained for a standard protein (Appendix, Protocol 7).

Protocol 6

Solutions

Luciferase assay reagent: 470 uM luciferin, 530 μM ATP, 270 μM coenzyme A, 20 nM tricine, 3.74 mM $MgSO_4$ x $7H_2O$, 0.1 mM EDTA, 33.3 mM DTT. Add 100 ml H_2O.

BC Assay Protein quantitation kit: reagent A, reagent B, Albumin standard

Experimental procedure

Protein measurement

Vortex the cells in at least 20 seconds to mix the content carefully. Measure protein concentrations with the BC Assay method according to the manufacturers protocol (Appendix, Protocol 7).

Luciferase activity

After protein concentrations are measured, centrifuge the samples at 4°C, 13 000 rpm for 2 minutes. Transfer 100 µl of the luciferase assay reagent into each luminometer tube. Then add 10 µl of each sample with cell lysate to a luminometer tube. Vortex before inserting the tube into the luminometer. Initate reading.

4. Statistical analysis

The results from transfection studies are presented as means + SD, and are representative for three (four) individual experiments performed in triplicates. Due to the small sample size, a non-parametric method was used to examine statistical significance between independent samples. Statistically significance was set at 5 % ($P<0.05$).

5. Results

The main objective in this project was to study the transcriptional regulation of LSDP5. Dalen et al.(101) demonstrated that LSDP5 is transcriptionally stimulated by activation of PPARα in mouse liver and heart. To study this, wild type and PPARα knockout mice were fed with vehicle (CMC) or a potent synthetic PPARα activator, WY 14.643, 10 mg/kg daily for one week. mRNA analysis from liver and heart tissues were performed, and a six-fold induction of the LSDP5 mRNA was found in the liver of wild type mice, compared to no induction in the PPARα knockout mice. The transcriptional regulation of LSDP5 by WY-14.643 feeding was much weaker in heart, with only a 1.3 fold induction of the LSDP5 mRNA. Furthermore, it was shown that PPARα is important for the basal expression of the LSDP5 gene both in liver and heart. In PPARα knockout mice, the basal expression of the LSDP5 gene was approximately 90 % lower in liver and 60 % lower in heart tissue, compared to wild type mice (102). These findings suggest that transcriptional regulation of LSDP5 is dependent on a functional PPARα.

So far, it has been difficult to show through transfection studies that the LSDP5 gene contains an evolutionarily conserved PPARα responsive DR-1 element. It has thus been necessary with more transfection studies to prove that PPARα regulates the transcription of the LSDP5 gene through a PPRE. In this project, the identification a possible PPRE and testing of its functionality was studied by cotransfecting a full-length LSDP5 reporter construct with PPARα and RXRα expression vectors into Cos-1 cells. To study this, transfected cells were treated with WY 14.643 and 9-cis-retinoic acid to activate PPARα and RXRα, respectively. Since it previously in preliminary studies has been difficult to observe any transcriptional regulation of the LSDP5 gene by cotransfection with PPARα/RXRα alone, additional factors were included in this thesis to examine whether the regulation of this gene by PPARα/RXRα is dependent on other regulatory proteins.

Sub-objectives in this project were therefore to examine if the PPARα/RXRα mediated transcriptional regulation of LSDP5 is dependent on the NR ERRα and/or the coactivator PGC-1α. The reason why these proteins were included in the transfection assay is that both ERRα and PGC-1α are highly expressed in metabolic tissues, and they are known to be involved in the regulation of FA oxidation pathways. It is therefore likely that they cross-talk with PPARα/RXRα in the transcriptional regulation of the LSDP5 gene based upon what is known about the function of LSDP5. It is demonstrated that ERRα upregulates genes that are important regulators of FA oxidation in liver and most of them are known PPARα target genes (103). It is also shown that PPARα itself is a direct ERRα target gene (104). Furthermore, several studies indicate that PGC-1α is a potent coactivator of both ERRα (105-107) and PPARα (108;109) in transcriptional regulation of their target genes. It is thus likely that the PPARα mediated regulation of the LSDP5 gene might be affected by ERRα, and that PGC-1α might play an important role as a coactivator that enhances the expression of the LSDP5 gene.

In order to study this, transfection studies were performed to investigate the effects of PPARα/RXRα, ERRα and PGC-1α on LSDP5 expression, both individually and in combination. Both full-length LSPD5 promoter (pGL3-(m)LSDP5-Luc [-2324 to +244]) construct, a deletion construct (pGL3-(m)LSDP5-Luc [-1329 to +244]) and a mutation construct (pGL3-(m)LSDP5-Luc [-2324 to +244-DR-1 mut]) were used to map the most PPARα -responsive region in the promoter of the LSDP5 gene.

5.1 PPARα alone gives no significant induction of the LSDP5 gene

Firstly, the basal activity in the cells was examined; that means the transcriptional activity with only endogenously expressed receptors and ligands present. Further, the activity in these cells will be referred to as “control”. These cells were transfected with full-length LSDP5 promoter (pGL3-(m)LSDP5-Luc [-2324 to +244]) only, to measure the transcriptional activity in the LSDP5 gene, affected by endogenous ligands and transcription factors exclusively. The basal activity was compared to activity in cells which were stimulated with synthetic ligands for PPARα and RXRα, WY-14.643 and 9-cis-RA, respectively. Activation of endogenously expressed PPARα /RXRα by synthetic ligands resulted in a marginal (1.4-fold) induction of LSDP5, compared to control (Figure 6.1). When pGL3-(m)LSDP5-Luc [-2324 to +244] was cotransfected with PPARα and RXRα to examine the effect of exogenously added PPARα/RXRα on transcriptional activity of the LSDP5 gene, I did not observe a consistent reporter gene activity. Whereas treatment with WY-14.643 gave a 3.5-fold reporter gene activity in one experiment (Figure 6.2), PPARα activation resulted in only 0.8-fold induction in another experiment (Figure 6.3). These results indicate that PPARα/RXRα alone is not sufficient to give a significant transcriptional induction of the LSDP5 gene.

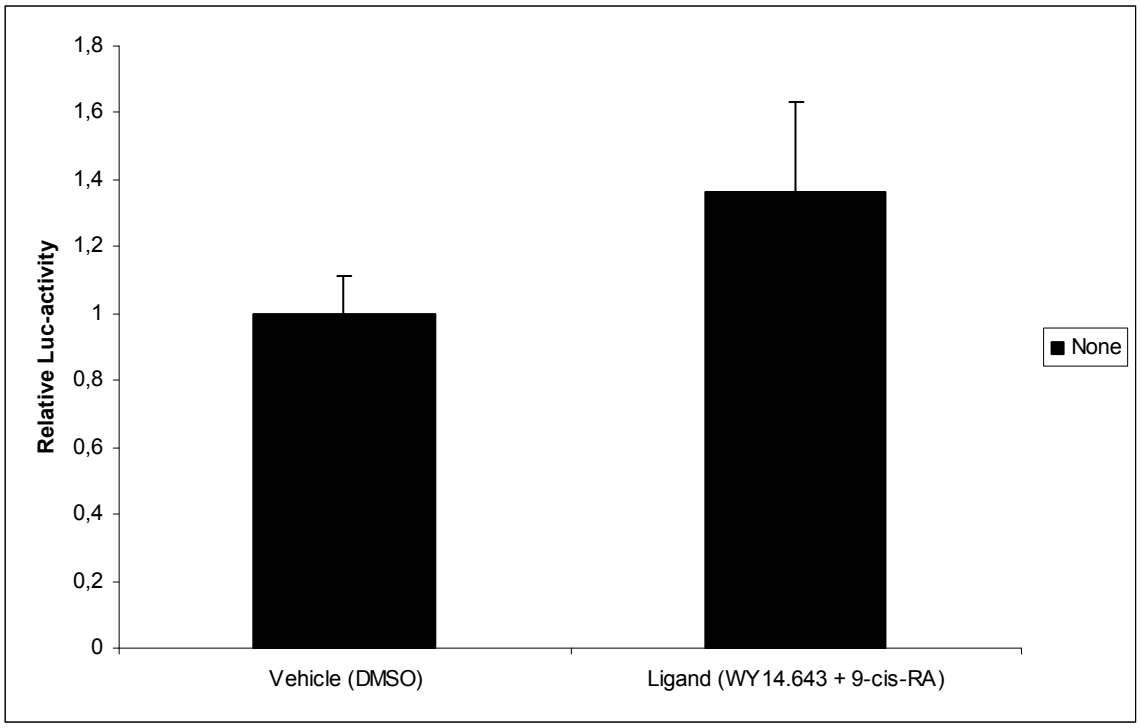

Figure 6.1: Basal activity in Cos-1 cells transfected with pGL3-(m)LSDP5-Luc [-2324 to +244] . Then the cells were treated with vehicle (DMSO) or ligand (10μM WY-14.643 and 1μM 9-cis-RA).

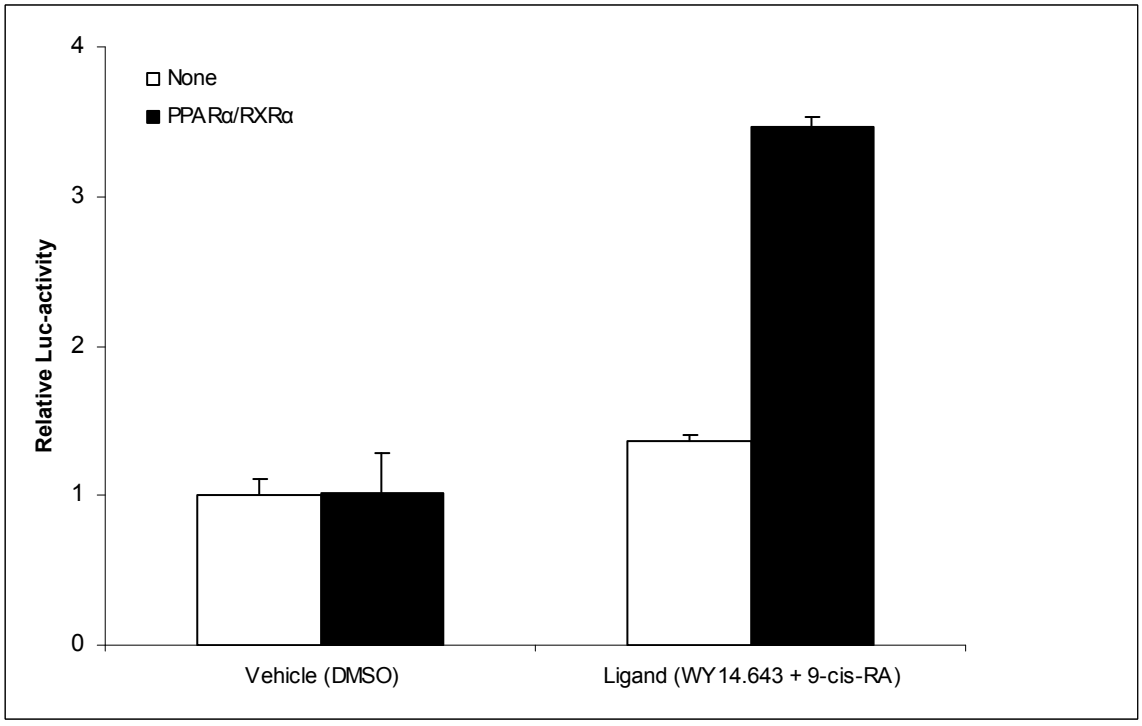

Figure 6.2: LSDP5 reporter vector cotransfected with none or RXRα and PPARα expression vectors. Then the cells were treated with vehicle (DMSO) or ligand (10μM WY-14.643 and 1μM 9-cis-RA).

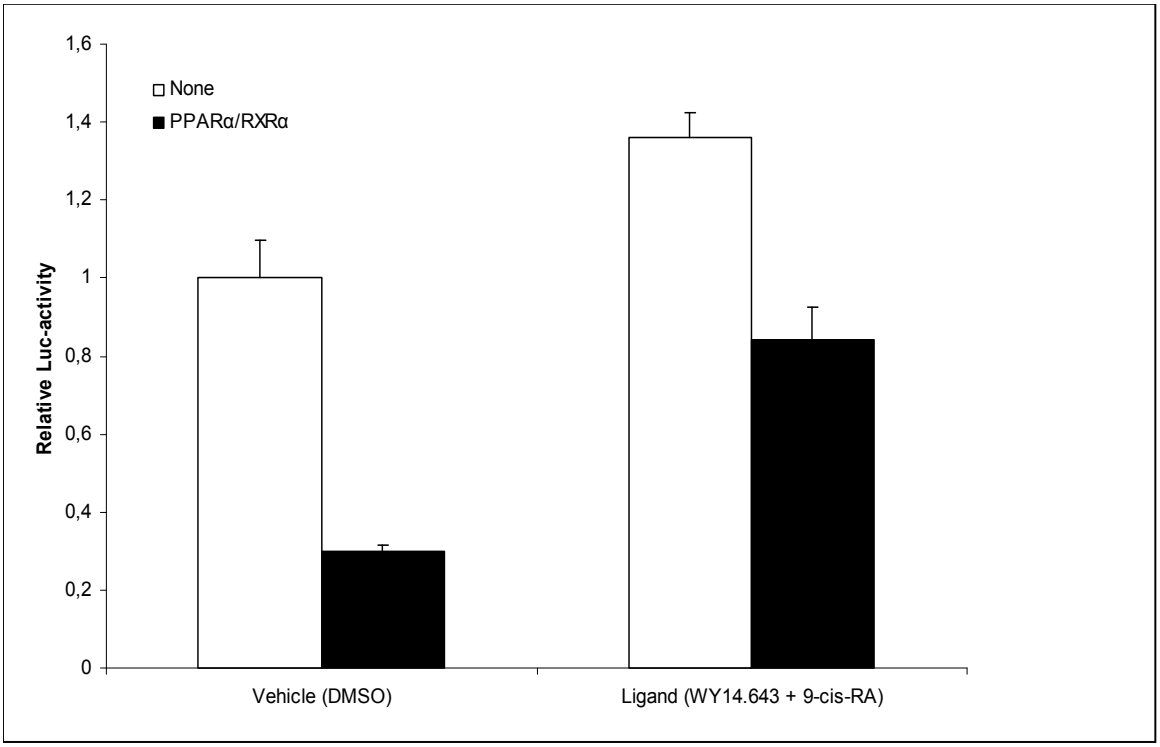

Figure 6.3: LSDP5 reporter vector cotransfected with none or RXRα and PPARα expression vectors. Then the cells were treated with vehicle (DMSO) or ligand (10μM WY-14.643 and 1μM 9-cis-RA).

5.2 PGC-1α enhanced the transcriptional activity of the LSDP5 gene

Coactivator PGC-1α highly induced the transcriptional activity of the LSDP5 gene. The maximal effect was observed when exogenously expressed PPARα and RXRα were present in Cos-1 cells together with PGC-1α.

Firstly, the full-length LSDP5 promoter (pGL3-(m)LSDP5-Luc [-2324 to +244]) was cotransfected with the expression plasmid of PGC-1α, to examine the effect of this co-activator on the transcriptional induction of the LSDP5 gene. Exogenously expressed PGC-1α present in the cells enhanced the reporter activity with 4.8-fold when cells were treated with WY-14.643. Without treatment, the activity was only marginally increased (1.3-fold) (Figure 6.4).

Next, the full-length LSDP5 (pGL3-(m)LSDP5-Luc [-2324 to +244]) luc reporter construct was cotransfected with PPARα, RXRα and PGC-1α expression vectors. Activation by WY-14.643 in these cells resulted in 17-fold induction of the reporter gene (Figure 6.4).

Despite there was a certain level of variation in luciferase values between the four experiments, it was possible to observe a clear trend, indicating the enhanced co-activator effect of PGC-1α, through PPARα/RXRα in the LSDP5 promoter gene, measured by increased reporter gene activity (Figure 6.5). All experiments demonstrated that the presence of PGC-1α along with exogenously expressed PPARα and RXRα resulted in a high transcriptional activity of LSDP5.

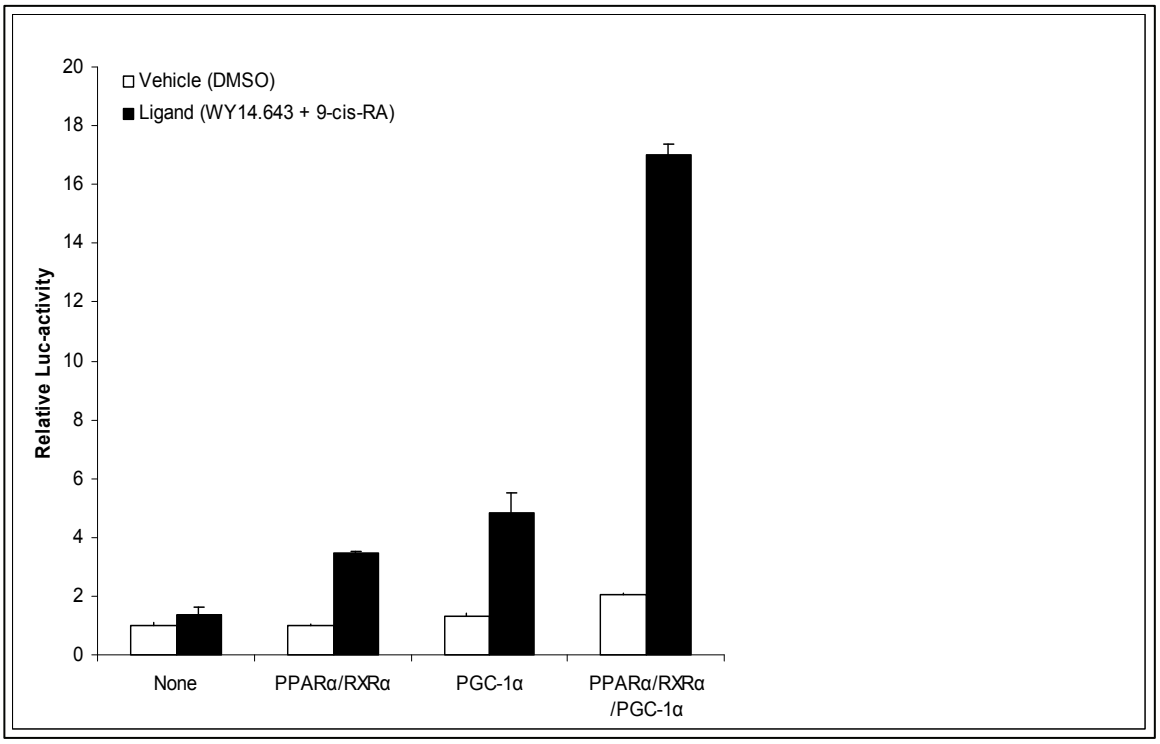

Figure 6.4: LSDP5 reporter vector cotransfected with none, PGC-1α- or RXRα and PPARα expression vectors in Cos-1 cells. Then the cells were treated with vehicle (DMSO) or ligand (10μM WY-14.643 and 1μM 9-cis-RA). The result is representative of four individual experiments performed in triplicates. Results are given as mean + SD. (None, vehicle = 1).

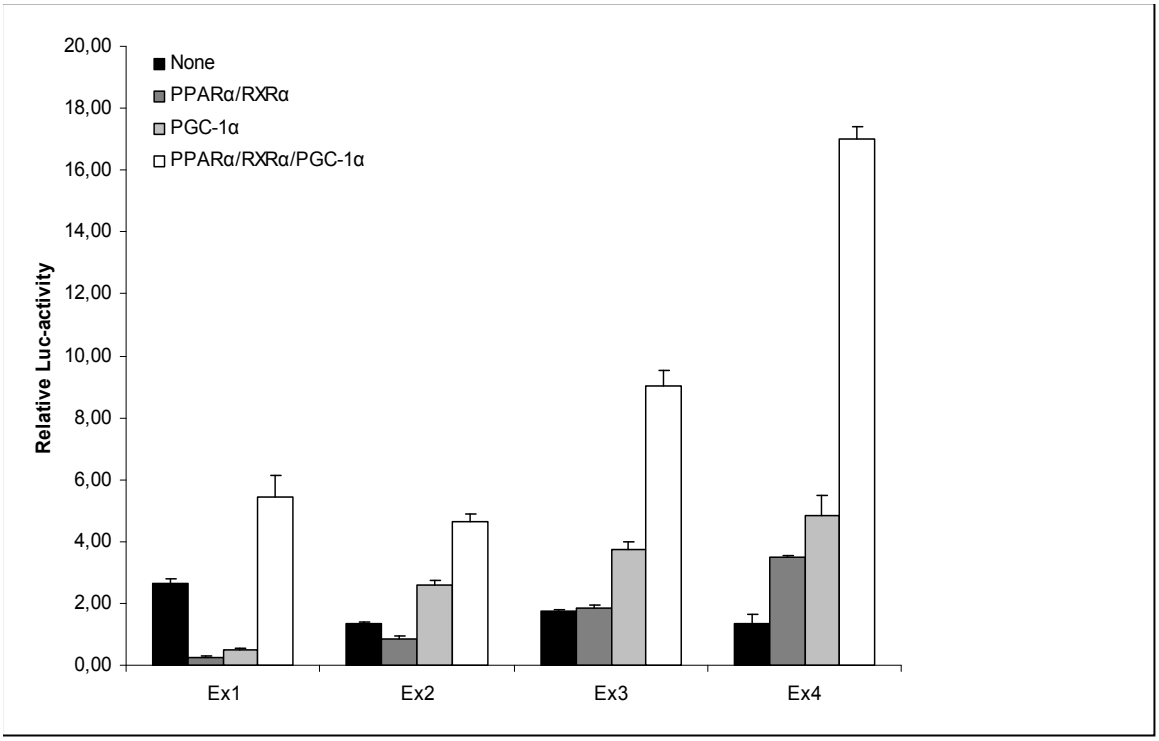

Figure 6.5: Four transfection experiments with LSDP5 reporter vector cotransfected with none, PPARα/RXRα, PGC-1α and PPARα/RXRα/PGC-1α in Cos-1 cells. The figure shows only stimulated cells (10μM WY-14.643 and 1μM 9-cis-RA). Results are given as mean + SD. (None, vehicle = 1).

5.3 ERRα downregulated the expression of LSDP5

Surprisingly, full length LSDP5 pGL3-(m)LSDP5-Luc [-2324 to +244]) luc reporter cotransfected with ERRα resulted in remarkable low induction of LSDP5. There was almost no difference between stimulated (0.5-fold induction) and unstimulated (0.4-fold induction) cells (Figure 6.6).

LSDP5 luc reporter cotransfected with ERRα, PPARα and RXRα expression vectors reduced the induction of LSDP5 compared to control. Treatment with WY-14.643 gave a 0.9-fold reporter gene activity.

When full length LSDP5 luc construct was cotransfected with PGC-1α in addition to ERRα, PPARα and RXRα, and stimulated with WY-14.643, the induction of luc activity increased 7.0-fold. This indicates that PGC-1α strongly upregulates the expression of LSDP5, which is consistent with the previous results. However, it seems like the effect of PGC-1α is reduced (from 17-fold to 7.0 fold) when ERRα is coexpressed in the cells.

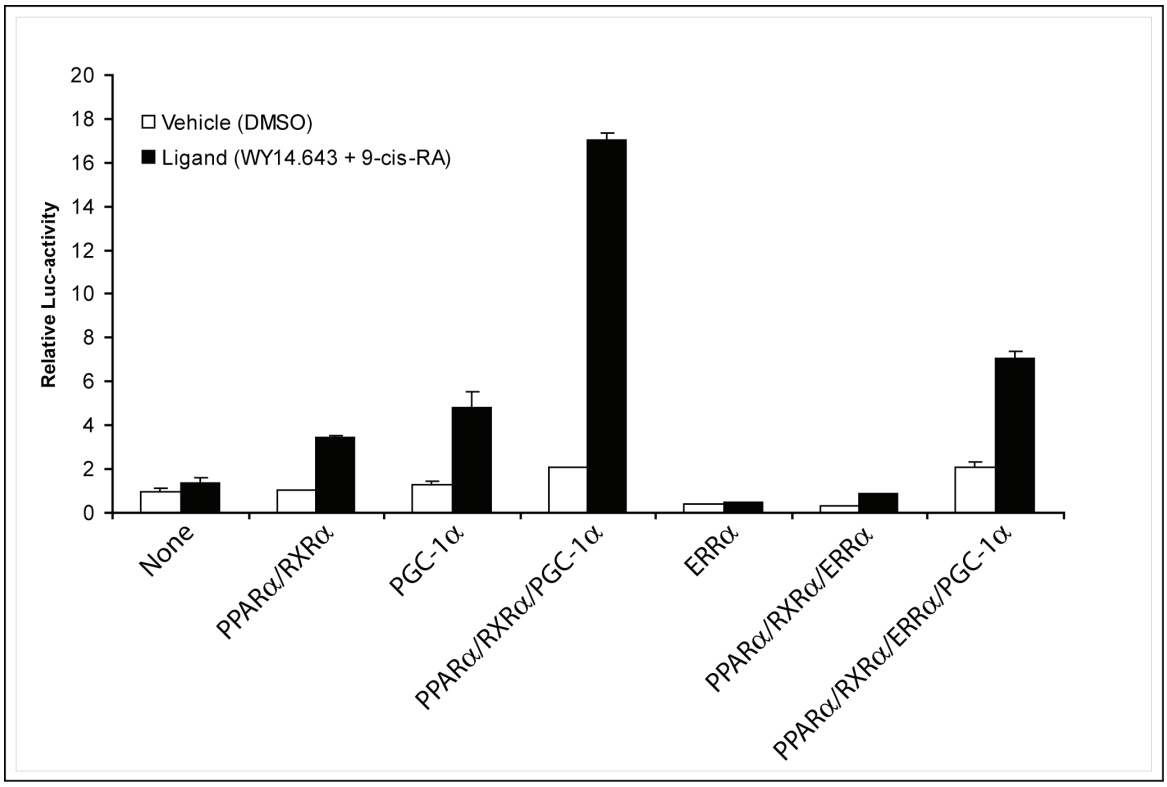

Figure 6.6: LSDP5 reporter vector cotransfected with none-, PPARα/RXRα, PGC-1α, PPARα/RXRα/PGC-1α, ERRα, PPARα/RXRα/ERRα or PPARα/RXRα/ERRα/PGC-1α expression vectors in Cos-1 cells. Then the cells were treated with vehicle (DMSO) or ligand (10μM WY-14.643 and 1μM 9-cis-RA). Results are given as mean + SD. (None, vehicle = 1).

5.4 The LSDP5 promoter contains an evolutionarily conserved PPAR responsive DR-1 element

The transcriptional regulation of LSDP5 was expected to be mediated through a PPRE (DR1). To map the most PPARα-responsive region in the LSDP5 promoter, one 5'- deletion construct made by Post.doc. Knut Tomas Dalen (Figure 6.7) was transiently transfected into COS-1 cells together with the expression plasmids decribed earlier. Since the previous results demonstrated that exogenously expressed PGC-1α in combination with PPARα and RXRα strongly elevated the induced expression of the LSDP5 gene, PPARα, RXRα and PGC-1α expression vectors were cotransfected with the LSDP5 deletion construct (pGL3-(m)LSDP5-Luc [-1329 to +244]). There was no significant reporter activity using this LSDP5 reporter plasmid, compared to a 9-fold induction of reporter activity using the wild type (WT) LSDP5 (pGL3-(m)LSDP5-Luc [-2324 to +244]) promoter (Figure 6.8). These results indicate that the promoter region from -2324 bp to -1329 bp upstream from the transcriptional start site might contain a DR1 element that mediates PPARα transcriptional regulation of the LSDP5 gene.

One 5`- mutation construct (pGL3-(m)LSDP5-Luc [-2324 to +244-DR-1 mut]) (fig.6.4A), also made by Knut Tomas Dalen, was cotransfected into Cos-1 cells. The mutation was performed by "site directed mutagenesis" where one nucleotide in each half site is mutated, resulting in a DR1 element that is not recognizable for the PPARα and thereby leads to no PPARα activation. Cotransfection with the mutated construct did not induce reporter activity (Figure 6.9). This finding confirms that the LSDP5 promoter region from -2324 bp to -2064 bp upstream from transcriptional start site might contain a conserved PPAR response DR-1 element, and that this DR-1 element more specifically is located in the -2077 to -2064 upstream region in the LSDP5 promoter.

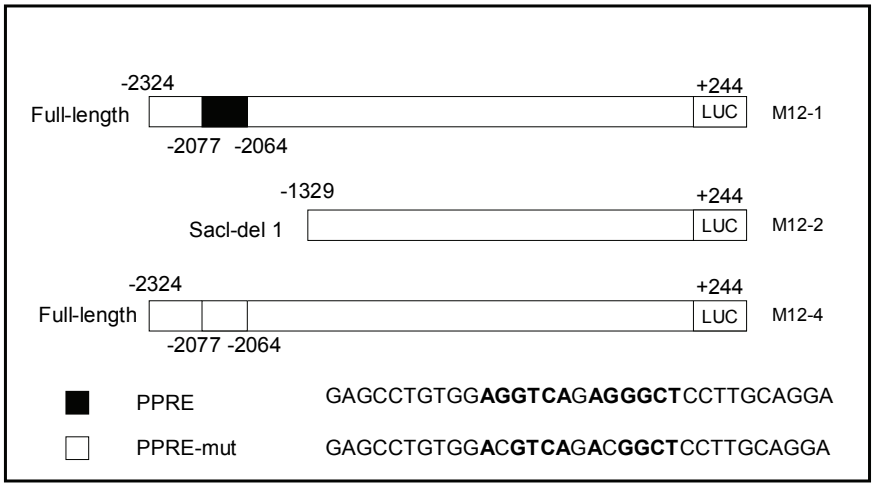

Figure 6.7: The LSDP5 constructs that were transfected into Cos-1 cells: one full-length construct (M12-1), one deletion construct (M12-2) and one mutation construct (M12-4).

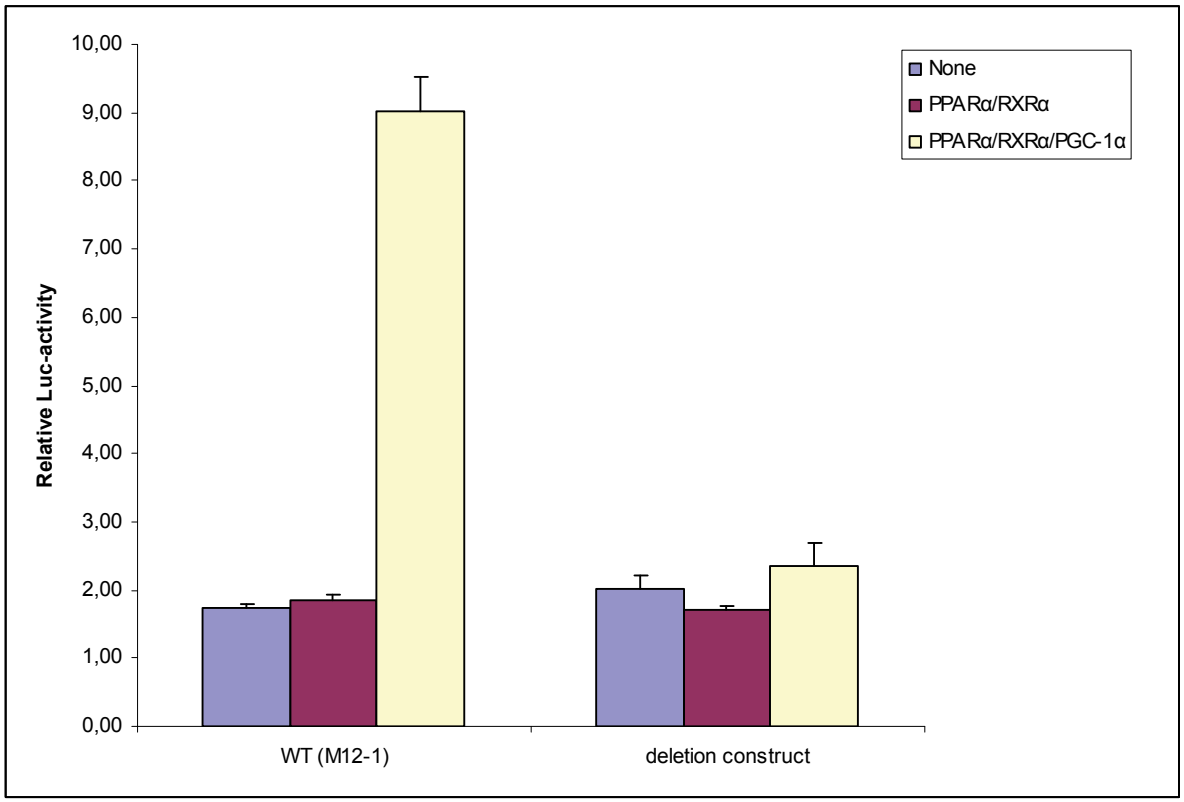

Figure 6.8: Full length LSDP5 (pGL3-(m)LSDP5-Luc [-2324 to +244]) promoter construct (WT) and a LSDP5 deletion construct (pGL3-(m)LSDP5-Luc [-1329 to +244]) cotransfected with none-, PPARα/RXRα, PGC-1α, PPARα/RXRα/PGC-1α, ERRα, PPARα/RXRα/ERRa or PPARα/RXRα/ERRα/PGC-1α expression vectors. The figure shows only stimulated cells (10μM WY-14.643 and 1μM 9-cis-RA). Results are given as mean + SD. (None, vehicle = 1).

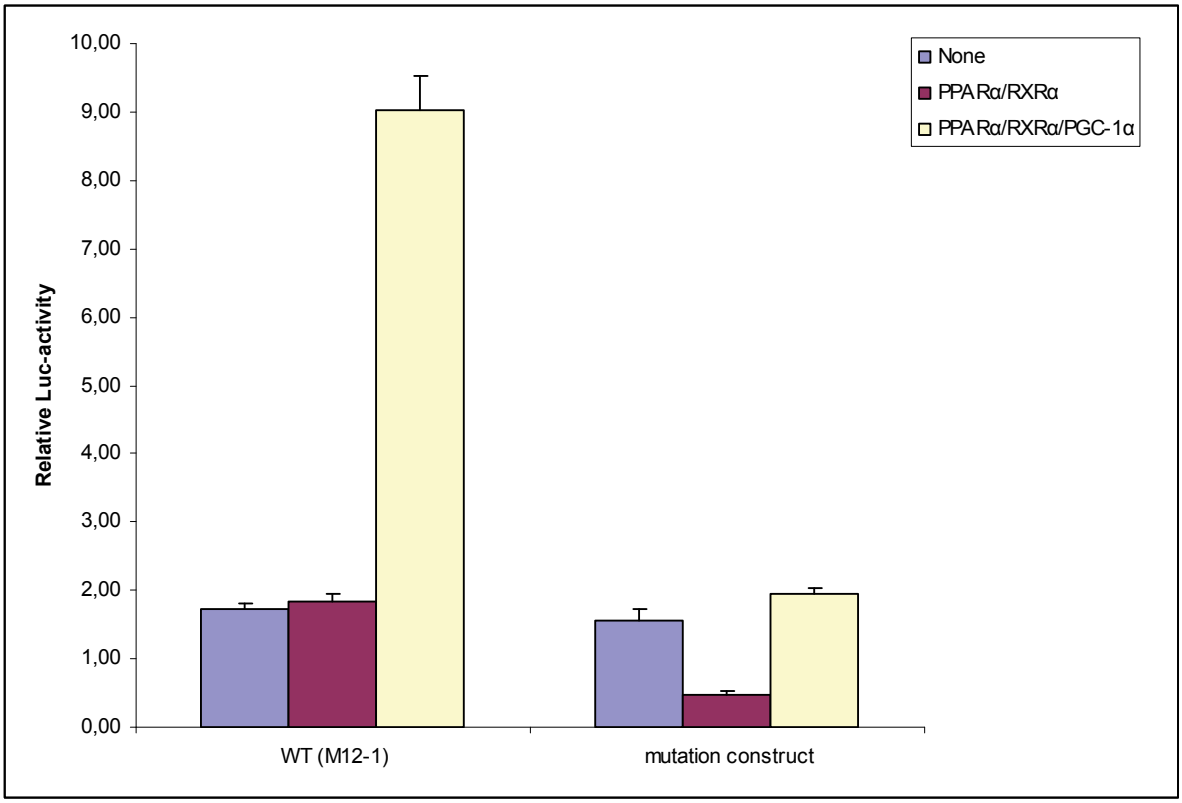

Figure 6.9: Full length LSDP5 (pGL3-(m)LSDP5-Luc [-2324 to +244]) promoter construct (WT) and a mutation construct (pGL3-(m)LSDP5-Luc [-2324 to +244-DR-1 mut]) cotransfected with none-, PPARα/RXRα, PGC-1α, PPARα/RXRα/PGC-1α, ERRα, PPARα/RXRα/ERRa or PPARα/RXRα/ERRα/PGC-1α expression vectors. The figure shows only stimulated cells (10μM WY-14.643 and 1μM 9-cis-RA). Results are given as mean + SD. (None, vehicle = 1).

6. Discussion

6.1 Methodology

The main focus in this thesis was to examine whether PPARα, through a PRRE (DR1) in the LSDP5 promoter, regulates the expression of the LSDP5 gene. Transfection studies are frequently used for this purpose. By transfecting the reporter plasmid (pGL3-(m)LSDP5-Luc [-2324 to +244) into Cos-1 cells, it is possible to analyze the transcriptional activity of this particular gene after cotransfection with different expression plasmids as described earlier. Then expression plasmids might be added one at the time or combined, to examine their effect on the reporter activity. Thus, transfection studies give important information about the transcriptional activity in a particular gene. In this case, a LSDP5 promoter construct cloned into a luciferase reporter vector and PPARα, RXRα, ERRα and PGC-1α expression plasmids, were used to study their influence on the transcriptional activity of the LSDP5 gene.

All data presented in this thesis are based on *in vitro* experiments. The transfection method is an important tool to study the transcriptional activity in a particular gene. However, there are also limitations related to this method. Transfection studies alone give no information of mRNA or protein expression in distinct tissues or how a protein is regulated upon physiological conditions, compared to *in vivo* studies. *In vivo* studies and *in vitro* studies combined are required to obtain a complete picture of how a gene is transcriptionally regulated. Prior to this thesis, *in vivo* experiments with knockout mice had shown that LSDP5 expression in liver and heart was dependent on a functional PPARα. Transfection studies were needed to confirm that the LSDP5 gene is transcriptionally regulated by PPARα through a specific PPARα response element.

The transfections in this thesis were performed by the *calcium phosphate precipitation method*. I will further discuss advantages and limitations of this method.

The calcium phosphate precipitation method is a well established transfection method that has been used for 30 years. The principle behind this method is the formation of a calcium phosphate precipitate that occurs in supersaturated solutions. The forming precipitate binds DNA and the complex of calcium phosphate and DNA is absorbed by the cells. Despite a rapidly growing choice of efficient transfection reagents, this method remains highly attractive. It is a significant goal to achieve high degree of transfection efficiency when performing a transfection. Although a wide range of conditions will lead to precipitates, high transfection efficiencies are only obtained within a narrow range of optimized parameters that assure certain properties of the precipitate (110). These parameters will be discussed in more detail in the following section.

The *calcium concentration* influences the formation of precipitates. Calcium is present in the medium, in which cells are cultured. However, the concentration is kept low due to its poor solubility in the presence of phosphate and carbonate ions. Increased calcium concentration during transfection leads to spontaneously formation of microprecipitates, which is indicated to enhance the transfection efficiency.

The precipitate of calcium and phosphate may undergo changes during transfection. The *solubility of the precipitate* is another parameter, which influences the efficiency of the transfection. Calcium phosphate precipitation is a dynamic and reversible process, and supersaturation of the solution favours the formation of precipitates. The main factors determining the supersaturation are the concentrations of calcium and phosphate. The pH also plays an important role, by influencing the solubility of phosphate, and should be kept at pH=7.05. In addition, temperature, DNA concentration and the degree of purity of the DNA affects the solubility. The fact that transfection efficiency highly depends on the characteristics of the solution, is one of the limitations with this method, since there are many factors that might influence the solution and thus affect the results in a negative manner. There are highly efficient

transfection reagents available today, which remain chemically stable and thus are easier to deal with. However, the calcium phosphate precipitation method works well when paying attention to the factors mentioned above.

The formation of the calcium phosphate precipitate is affected by the *DNA concentrations*. At lower concentrations, DNA`s positive effect does not achieve its full potential, while at high concentrations the DNA can be detrimental to the precipitation. 25 μg DNA/ml is optimal concentration to obtain highest degree of transfection efficiency (111). In my experiments 20 μg DNA/ml was used based on optimalizations prior to the experiments.

It is crucial that the *cells are in exponential growth phase* when used in transfections, thereby increasing the transfection efficiency. The cells will undergo mitosis right after the transfection, and that results in nuclear entry of the DNA in the cells. When adherent cells are kept subconfluent, it allows one more doubling. In my experiments, cells were seeded at 90 % confluency the day before transfection.

The efficiency of transient transfection is determined largely by the *cell line* that is used. Different lines of cultured cells vary by several orders of magnitude in their ability to take up and express exogenously added DNA. It is also important that the cells do not express the expression vectors of interest. In my transfections adherent Cos-1 cells were used. This cell line is known as an especially suited host for transfections due to its low expression of transcription factors that might influence the expression of the gene of interest.

One of the challenges in using the calcium phosphate precipitate method is to avoid *cytotoxicity*. Calcium phosphate precipitates are cytotoxic in a dose- and time-dependent manner. Calcium present in the form of calcium precipitates may cause cell damage and cell death. Toxic effects can be avoided by limiting exposure time to the precipitate to a few hours.

When the parameters mentioned above are optimized, the calcium phosphate precipitate method works well according to my experience. The main advantages are

that it is cheap and easy to perform. Beneficially, it is the only method without traces of synthetic chemical components, since only natural compounds are used. This has a positive influence on the viability of cells and for the quality and purity of the product. Limitations include the time consuming procedure, and the usage of large amounts of DNA.

In addition to the calcium phosphate precipitation method, several other methods have been described to introduce DNA expression vectors into mammalian cells in vitro and in vivo and other transfection reagents can be used for this purpose. The calcium phosphate precipitation method includes the use of chemical reagents. Another chemical reagent that is available to use is DEAE-dextran, a cationic polymer that associates with negatively charged nucleic acids. An excess of positive charge contributed by the polymer in the DNA/polymer complex allows the complex to come closer into association with the negatively charged cell membrane. Uptake of the complex is presumably by endocytosis. This method suits particularly well for transient transfections.

The use of artificial liposomes is a second alternative among chemical reagents, and Lipofectamine is a widely used transfection reagent today. Liposome-mediated nucleic acid delivery is based on the principle that the cationic portion of the lipid molecule associates with the negatively charged nucleic acids, resulting in a compaction of the nucleic acid in a liposome/nucleic acid complex. For cultured cells, an overall net positive charge of the liposome/nucleic acid complex generally results in higher transfer efficiencies, presumably because this allows closer association of the complex with the negatively charged cell membrane. Follwing endocytosis, the complexes appear in the endosomes, and later in the nucleus. Lipofectamine works with many primary cell lines and provides high transfection efficiency.

6.2 General discussion

LSDP5 is a recently identified member of the PAT family. The knowledge upon this protein is still limited. Nebb`s group has previously shown in animal studies that this protein is transcriptionally regulated by PPARα. However, it has been difficult to prove through transfection studies that this regulation is mediated through a PPARα response DR1 element. The main objective for this thesis was thus to establish whether there is a functional PPAR response element located in the LSDP5 promoter. Furthermore, the aims were to examine whether the transcriptional regulation of LSDP5 is affected by additional regulatory factors; the transcription factor ERRα and the coactivator PGC-1α. Our data confirms that LSDP5 contains a functional PPRE. The results also indicate that ERRα and PGC-1a are involved in the PPARα mediated regulation of LSDP5.

Previous experiments have failed to prove that PPARα transcriptionally regulates the LSDP5 gene through a conserved PPRE. Neither this study showed any consistent induction of LSDP5 mediated by PPARα, when we studied the isolated effect of PPARα. Since previous results have shown that the expression of LSDP5 in liver and heart is dependent on a functional PPARα, it is still likely that PPARα regulates the expression of LSDP5, however the full picture of this regulation is not completely understood. Based on these observations, we hypothesized that the regulation of LSDP5 might be affected by ERRα and PGC-1α, since it is well known that they play an important role in controlling the expression of genes that play a major role in FA oxidation pathways in metabolic tissues, like the liver and skeletal muscle.

In our transfection studies we found that PGC-1α strongly enhanced the transcriptional activity of the LSDP5 gene together with PPARα/RXRα after Wy-14.643 stimulation of Cos-1 cells. These results indicate that this co-activator is an important regulator of the expression of LSDP5, and that it mediates its action by an interaction with PPARα through a PPRE in the LSDP5 promoter. These findings are consistent with emerging knowledge of PGC-1α. PGC-1α is known as a potent

activator with a powerful transcriptional activity, which interacts with most members of the NR family, including PPARα (112). The close relationship between PPARα and PGC-1α is illustrated by the fact that they both are co-expressed in tissues with a high FA oxidation activity such as heart, liver and BAT. Furthermore, it is confirmed that there is a direct link between PGC-1α and PPARα. PGC-1α coactivates PPARα in the transcriptional regulation of FA oxidation genes. This was demonstrated in protein-protein interaction studies, performed by Vega and collaborators (113). They demonstrated that PGC-1α physically interacts with PPARα and that the PGC-1α-PPARα interaction involves an LXXLL domain in PGC-1α and an AF3 region in PPARα. Furthermore, it was shown that PGC-1α binds PPARα in a ligand-dependent manner (114). This is consistent with my results, where I observed a maximal transcriptional activation of the LSDP5 gene after stimulation with the PPARα ligand. PGC-1α was originally identified as a PPARγ interacting protein. Interestingly, PGC-1-PPARγ interaction is independent on ligand activation.

Dalen and colleges showed that LSDP5 is highly induced in heart during fasting (115). Both PPARα and PGC-1α play an important role in a fasting response, by regulating the expression of genes involved in FA oxidation. Despite that it is found that induction of the LSDP5 expression is independent on a functional PPARα, it is observed that presence of PPARα facilitates the expression of LSDP5 during fasting. PGC-1α is induced in a tissue-specific manner by signals that represent metabolic needs. One of these signals is starvation, resulting in a marked increase of PGC-1α in liver and heart. The role of PGC-1α in liver is to regulate the metabolic changes that occur in the liver during fasting, such as activation of gluconeogenesis, FA oxidation and synthesis and secretion of ketone bodies. These changes facilitate a shift from the usage of glucose to fats and ketone bodies by peripheral tissues, to ensure that glucose levels are maintained. PGC-1α mediates its regulation by coactivating key hepatic transcription factors such as HNF4α, PPARα, GR, FOXO1, FXR and LXR. Based on my observations, the current knowledge of PGC-1α and the fact that PGC-1α and LSDP5 are coexpressed in liver and heart during fasting, is it reasonable to hypothesize that PGC-1α might regulate the expression of LSDP5 during fasting, by

coactivating PPARα. To our knowledge, no studies have investigated this so far, and further studies are needed to support these findings. It is of significant interest to examine whether the expression of LSDP5 is reduced in mice lacking a functional PGC-1α protein.

When we examined the influence by the transcription factor ERRα, we found that the LSDP5 transcriptional activity in the cotransfection studies described in "Results" to be clearly reduced. This was a bit unexpected, since ERRα is highly coexpressed with PPARα and PGC-1α in tissues with high capacity of FA oxidation. Furthermore, ERRα is induced in liver during fasting, similar to PPARα, PGC-1α and LSDP5 (116). However, ERRα has previously been reported both as an activator, a repressor and a DNA-binding factor with little activity (117). Whereas it is reported that ERRα activates several genes in FA oxidation pathways, it is demonstrated that ERRα represses the induction of other genes such as the gene encoding phosphoenol-pyruvate carboxykinase (PEPCK), one of the rate-determining gluconeogenetic enzymes. These findings demonstrate that ERRα decreases the level of genes involved in gluconeogenesis in a fed state, by antagonizing the stimulatory effects of PGC-1α (118). The same mechanisms may underlie the observations from my experiments; that ERRα represses the induction of LSDP5, by antagonizing the stimulatory effects of PGC-1α. It is not fully understood how ERRα regulates the PEPCK gene expression, but it is possible that ERRα binds to a DNA response element of the PEPCK promoter called gAF3 (119). Bound to this DNA element, ERRα fails to cooperate properly with adjacent transcription factors as well as prevents the binding of another factor, perhaps a NR that promotes the formation of a transcriptionally active and PGC-1α responsive complex. Alternatively, ERRα bound at the gAF3 sequence may actively recruit co-repressors (120). However, according to our data the induction of LSDP5 was lower when PGC-1α was not present in the cells. Thus, it is possible that the repression by ERRα might be independent on PGC-1α, and that it is mediated through other mechanisms. These results indicate that ERRα represses the expression of LSDP5, but future studies are needed to study the mechanisms that underlie this repression.

Taken together, our data confirmed that LSDP5 contains a conserved PPRE. The enhancing effect of PGC-1α on LSDP5 induction was studied in three LSDP5 reporter constructs; one mutation and one deletion construct as well as a full-length promoter construct. Since I had already observed that LSDP5 was highly induced with exogenously expressed PGC-1α in combination with PPARα and RXRα, these three expression constructs were cotransfected with the distinct reporter constructs into the Cos-1 cells. Only the full length promoter construct showed a significant induction of LSDP5, suggesting that this promoter contains a DNA response element to which PPARα might bind and activates LSDP5 transcription. Collectively, my data and the recently published results of LSDP5 as a protein which expression is induced by the PPARα ligand (121;122), suggest that LSDP5 is a PPARα target gene.

6.3 Conclusion

The newly discovered PAT protein LSDP5 is highly expressed in liver, red muscle and heart, and plays an important role during fasting.

In this master thesis I have showed that;

- LSDP5 is transcriptionally regulated by PPARα, through a conserved PPAR DR-1 element, located in the -2077 to -2064 upstream region in the LSDP5 promoter.
- The PPARα mediated regulation of LSDP5 is dependent on PGC-1α. The presence of this coactivator enhanced the induction of LSDP5 remarkably.
- ERRα represses the expression of LSDP5. ERRα present reduces the enhancing effect of PGC-1α on LSDP5 induction. Further studies are needed, to investigate the mechanisms that underlie the inhibitory effect of ERRα.

6.3.1 Future perspectives

It would be of interest to pursue further studies to investigate the influence of PGC-1α and ERRα on the expression of LSDP5. Studies with PGC-1α -/- mice are necessary to study whether the expression of LSDP5 is reduced in mice lacking a functional PGC-1α. Experiments with ERRα knockout mice will contribute with important information of the effect of ERRα on the induction of LSDP5. ERRα and PGC-1α double knock-out studies would be a significant tool to study the possible interplay between ERRα and PGC-1α in the PPARα mediated regulation of LSDP5. An electrophoretic mobility shift assay (EMSA) has to be used to investigate whether nuclear extracted PPARα and RXRα proteins bind to the identified PPRE in the LSDP5 promoter. Search for an ERRα response element located in the LSDP5 promoter will also be of interest to address through transfection and EMSA studies whether ERRα binds directly to the regulatory area of the LSDP5 gene.

Appendix

Protocol 1: pGL3-Basic-Vector

Protocol 4: Jet Star Maxi Prep

Protocol 7: BC Assay

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