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STUDIES OF LXR AND CIDEA FUNCTION IN HUMAN ADIPOCYTES

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ABSTRACT

Obesity, defined as a body mass index of 30 or above, is the result of an imbalance of energy intake and energy expenditure. In the last decade, the obesity prevalence has truly reached epidemic proportions with major effects on public health. Obesity is closely associated with insulin resistance, type 2 diabetes, hyperlipidemia and atherosclerosis. On the other end of the spectra, cancer cachexia is a poor diagnostic factor in cancer patients. It is characterized by a state of unintentional weight loss, primarily of body fat but also of lean body mass. Although the mechanisms behind the loss of adipose tissue are not completely understood, lipolysis seems to be a major factor.

Adipose tissue is an important metabolic and endocrine organ. One of the most important functions of the adipocyte is lipolysis, the hydrolysis of triglycerides to free fatty acids (FFA) and glycerol. This is a tightly regulated process of great importance to the whole-body metabolism. The FFAs can either be released into the circulation, to be used as energy substrate by other organs and tissues, or utilized within the adipocyte for re-esterification or lipid oxidation. The process of lipid oxidation in adipocytes is controlled in part by the pyruvate dehydrogenase complex (PDC), an important regulator of substrate oxidation in adipocytes. This complex is inactivated when phosphorylated by pyruvate dehydrogenase kinases (PDKs), which promotes lipid oxidation rather than glucose oxidation.

The aim of this thesis was to investigate how two factors, the liver x receptor (LXR) and cell death-inducing DNA fragmentation factor-α-like effector A (CIDEA) affect adipose tissue metabolism. LXR is a nuclear receptor and a known regulator of cholesterol, lipid and carbohydrate metabolism. CIDEA is almost exclusively expressed in white adipocytes in humans and can affect critical metabolic functions such as lipolysis.

In **paper I**, we investigated the role of CIDEA in cancer cachexia. We measured levels of CIDEA in subcutaneous adipose tissue from subjects suffering from cancer cachexia and compared these to weight-stable cancer patients and noncancer patients. Levels of CIDEA mRNA were increased in cancer cachexia and correlated with elevated levels of FFAs and weight loss. Over-expression of CIDEA increased fatty acid oxidation in human adipocytes in culture and decreased glucose oxidation. Furthermore, augmented levels of CIDEA enhanced the expression of PDK1 and PDK4, and the phosphorylation of PDC. In accordance with this, mRNA levels of PDK1 and PDK4 in the clinical material correlated with CIDEA expression. In conclusion, CIDEA is involved in loss of adipose tissue in cancer cachexia at least in part due to its ability to inactivate PDC and thereby switch substrate oxidation in human adipocytes from glucose to lipids.
In **papers II and III**, the role of LXR in human adipocyte function was studied, with focus on substrate oxidation (paper II) and lipolysis (paper III). In paper II, we treated human adipocytes with the LXR agonist GW3965 and observed an increased fatty acid and decreased glucose oxidation. We showed that LXR activation can increase the mRNA level of PDK4 and thereby the phosphorylation of PDC. We also showed a

decreased activity of PDC, which was found to be dependent on PDK4. Furthermore, we could establish that the effect of GW3965 on lipid oxidation was specific for LXR, since it was abolished upon knockdown of LXR. In conclusion, we suggest that LXR has an important role in the regulation of substrate oxidation in human adipocytes, at least in part by influencing the phosphorylation status of PDC.
In paper III, LXR activation was shown to up-regulate glycerol release from human adipocytes. Based on microarray analysis we found a strong impact of LXR activation on known lipolysis-regulating genes. We showed differences in expression and localization of perilipin 1 (PLIN1) and hormone sensitive lipase (HSL). When PLIN1 is depleted, the effect of LXR is abolished. Furthermore, we showed binding of LXR and its heterodimerizing partner Retinoid X Receptor to the promoters of HSL and PLIN1 upon LXR activation. We also demonstrated that LXRα is the predominant isoform involved in regulation of adipocyte lipolysis within this context. In conclusion, we proposed that LXR activation up-regulates adipocyte lipolysis, at least in part through LXR binding to the promoter of PLIN1 and down-regulation of PLIN1 expression.

In conclusion, we suggest that CIDEA and LXR can affect central functions of adipocyte metabolism, namely lipolysis and substrate oxidation. We show that CIDEA is involved in the loss of adipose tissue in cancer cachexia and that this is at least in part due to a shift in substrate oxidation. Activation of LXR in human adipocytes increases fatty acid oxidation and lipolysis, through effects on PDC and PLIN1. The findings in this thesis are of importance for conditions of dysregulated adipose tissue metabolism, such as obesity and cachexia.

LIST OF PUBLICATIONS

I. Laurencikiene J, **Stenson BM**, Nordström EA, Agustsson T, Langin D, Isaksson B, Permert J, Rydén M, Arner P.
Evidence of an Important Role of CIDEA in Human Cancer Cachexia
Cancer Research 2008 68(22)9247-9254

II. **Stenson BM**, Rydén M, Steffensen KR, Wåhlén K, Pettersson AT, Jocken JW, Arner P, Laurencikiene J.
Activation of Liver X Receptor Regulates Substrate Oxidation in White Adipocytes
Endocrinology 2009 150(9)4104-4113

III. **Stenson BM**, Rydén M, Venteclef N, Dahlman I, Pettersson AML, Mairal A, Åström G, Blomqvist G, Wang V, Jocken JW, Clément K, Langin D, Arner P, Laurencikiene J.
Liver X Receptor (LXR) Regulates Human Adipocyte Lipolysis
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CONTENTS

LIST OF ABBREVIATIONS

5'AMP	5'adenosine monophosphate
AC	Adenylyl Cyclase
AMPK	5'AMP-activated protein kinase
ApoE	Apolipoprotein E
AR	Adrenergic Receptor
AT	Adipose Tissue
ATGL	Adipose Triglyceride Lipase
BAT	Brown Adipose Tissue
BMI	Body Mass Index
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanylate Monophosphate
ChIP	Chromatin Immunoprecipitation
CIDEA	Cell Death-Inducing DNA Fragmentation Factor-α-like Effector A
CIDEC	Cell Death-Inducing DNA Fragmentation Factor-α-like Effector C
Cpt	Carnitine palmitoyl-CoA transferase
C/EBPα	CCAT/enhancer-binding protein α
DEXA	Dual Energy X-ray Absorptiometry
DG	Diglycerol/Diacylglycerol
DMEM	Dulbecco's modified Eagle's Medium
FAS	Fatty Acid Synthase
FFA	Free Fatty Acids
FXR	Farnesoid X Receptor
GLUT	Glucose Transporter
GTP	Guanosine 5'-triphosphate
HADHA	3-hydroxtacyl-CoA-dehydrogenase
HSL	Hormone-Sensitive Lipase
IR	Insulin Receptor
IRS	Insulin Receptor Substrates
LPL	Lipoprotein Lipase
LXR	Liver X Receptor
LXRE	Liver X Receptor Response Element
MCAD	Medium-Chain Acyl-CoA Dehydrogenase
MG	Monoglycerol/Monoacylglycerol
MGL	Monoacylglycerol Lipase
NADH	Nicotinamide Adenine Dinucleotide
NCoR	Nuclear Receptor Co-Repressors
NF-κB	Nuclear Factor kappa-B
NPRA	Natriuretic Peptide Receptor A
NR	Nuclear Receptor
PDC	Pyruvate Dehydrogenase Complex
PDE3B	Phosphodiesterase 3B
PDK	Pyruvate Dehydrogenase Kinase
PI3K	Phosphatidyl Inositol 3-Kinase
PKA	Protein Kinase A

PKB	Protein Kinase B
PKG	Protein Kinase G
PLIER	Probe Logarithmic Intensity Error
PLIN1	Perilipin 1
Pol II	Polymerase II
PPAR	Peroxisome Proliferator-activated Receptor
PPRE	Peroxisome Proliferator-activated Receptor Response Element
PXR	Pregnane X Receptor
RIP140	Receptor-Interacting Protein 140
RNAi	RNA interference
RT-qPCR	Real-Time Quantitive PCR
RQ	Respiratory Quotient
RXR	Retinoid X Receptor
SAM	Significance Analysis of Microarray
SC	Subcutaneous
SCD1	Steoryl-CoA Desaturase 1
SGA	Subjective Global Assessment
SGBS	Simpson-Golabi-Behmel syndrome
SHP	Small Heterodimer Partner
SMRT	Silent Mediator of Retinoic Acid Receptor and Thyroid receptors
SNP	Single Nucelotide Polymorphism
siRNA	Small Interfering RNA
SVF	Stroma-Vascular Fraction
TCA	Tricarboxylic Acid
TG	Triglyceride/Triacylglycerole
TNFα	Tumor Necrosis Factor-α
UCP1	Uncoupling protein 1
VISC	Visceral
VLCD	Very Low Calorie Diet
WAT	White Adipose Tissue
WHO	World Health Organization
ZAG	Zinc-α2-glycoprotein

1 INTRODUCTION

The aim of this thesis is to investigate the role of two distinct factors – liver x receptor (LXR) and cell death-inducing DNA fragmentation factor-α-like effector A (CIDEA) in human adipocytes. Section 1 is a review of literature relevant for the studies and discussions in this thesis. I have summarized the most important concepts in studies of adipocytes and adipose tissue in the context of obesity and cancer cachexia. I have also reviewed published data on CIDEA and LXR in relation to adipocytes and adipocyte biology, focusing on human studies.

1.1 OBESITY AND CACHEXIA

Obesity is an excess of body fat as a result of an imbalance between energy intake and energy expenditure. In the last decade the incident of obesity has truly reached epidemic proportions. According to the world health organization (WHO), 1.5 billion adults were overweight in 2008, and of these around 500 million were obese. In 2010, nearly 43 million children under the age of five were overweight. Obesity is a multifactorial disease, influenced by both genetic and environmental factors. It is associated with adipocyte and adipose tissue dysfunction and, in turn, with severe health problems such as insulin resistance, type 2 diabetes, fatty liver, hypertension, dyslipidemia, atherosclerosis and cancer [1].

On the other end of the spectra, cachexia is a syndrome manifested by involuntary weight loss. Cachexia is associated with cancer and non-malignant conditions such as tuberculosis, HIV, heart and renal failure and chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease [2].

1.1.1 ASSESSMENT OF BODY COMPOSITION

Body mass index (BMI, weight in kg/(height in meters)^2) is a measure used for classification of under- (<18.5 kg/m^2), normal (18.6-24.9 kg/m^2) and overweight (25 kg/m^2), see table 1. Individuals with a BMI over 30 kg/m^2 are considered obese, and this definition is divided into three classes. The cut-off points for obesity and type 2 diabetes are based on epidemiological data relating to risks of weight-related health problems, see table 1.

TABLE 1. BMI CLASSES, DEFINED BY WHO

Classification	**BMI (kg/m^2)**
Underweight	<18.5
Normal weight	18.5-24.9
Overweight	≥25
Pre-obese	25-29.9
Obese	≥30
Obese Class I	30-34.9
Obese Class II	35-39.9
Obese Class III	≥40

BMI is the most used measurement of body composition. It is a simple and easily applicable method since it does not require specialized equipment, but also has some drawbacks. The BMI classification is inappropriate to use in certain groups of patients including children, elderly people and highly trained athletes. Other fairly simple methods that have won ground in the last few years and might be more appropriate are waist-to-hip ratio and waist circumference. More advanced methods, appropriate for clinical examinations but perhaps not larger population studies include dual energy X-ray absorptiometry (DEXA) and bioelectrical impedance. Bioelectrial impedance can give an assessment of fat mass and fat free mass while DEXA provides data on fat mass, muscle mass and lean body mass.

1.1.2 CANCER CACHEXIA

Cancer cachexia, or unintentional weight loss concomitant with malignant cancer, is associated with a poor survival rate and an impaired response to chemotherapy [2, 3]. It is commonly defined as cancer with unintentional weight loss of >5% in the last 3 months or >10% in the last 6 months. The weight loss affects both fat mass and lean body mass, however, the loss of fat mass often precedes and is more rapid than that of lean body mass [2, 3]. The mechanisms behind the adipose tissue (AT) loss are not completely clear, although increased lipid mobilization due to augmented adipocyte lipolysis seems to be a major contributing factor [3, 4]. Cancer patients with cachexia have a higher lipolytic rate, i.e. higher ratio of fatty acids or glycerol over fat mass, than cancer patients not suffering from cachexia [5, 6]. They also have higher levels of the important enzyme hormone sensitive lipase (HSL, described in detail in section 1.6.4) in their AT, indicative of an increased lipolysis. Furthermore, isolated subcutaneous (SC) adipocytes from cachexic patients display a higher catecholamine and natriuretic peptide-stimulated lipolysis *in vitro* compared to weight stabile cancer patients and noncancer patients. This effect could be counteracted by HSL-inhibition [5]. Also, the total lipase activity, the activity of both HSL and adipose triglyceride lipase (ATGL, described in detail in section 1.6.4), is higher in visceral (VISC) AT from patients suffering from cancer cachexia compared to weight-stable cancer patients [7].

The size of adipocytes is also affected by cachexia as both human and murine cachexic adipose tissue is characterized by smaller adipocytes and also increased collagen-fiber content in the tissue matrix [8, 9]. Recent studies have shown that the decrease in AT mass in cachexia is due to a reduction of adipocyte volume and not adipocyte number [4, 10]. Global gene expression profiling of the molecular pathways associated with SC AT in cachexia indicates an up-regulation of pathways regulating energy turnover and a down-regulation of pathways concerning cell and tissue structure [10].

1.2 THE ADIPOSE TISSUE

The white adipose tissue (WAT) is a vital organ for mechanical cushioning, thermal insulation and energy storage and in the past it was believed that this was the only function of WAT. Recently it has become appreciated that WAT can act not only as metabolic organ but is also as an important endocrine organ.

The WAT is the largest energy reservoir in the body and a major source of metabolic fuel [11]. Fat is stored in forms of triglycerides (TG) in the adipocytes and the WAT

acts as a buffer by increasing clearance of TGs in plasma and suppressing the release of free fatty acids (FFA) into the circulation [11]. WAT is a loose connective tissue consisting of adipocytes and a stroma-vascular fraction (SVF), in turn containing preadipocytes, macrophages, lymph cells, mast cell, fibroblasts and endothelial cells. The mature adipocyte consists of a large lipid droplet occupying the major part of the cell. A layer of cytoplasm surrounds the lipid droplet and the nucleus is flattened and positioned to the periphery of the cell. In humans, there are two major fat depots: SC and VISC fat. The SC fat is less metabolically active compared to the VISC fat, and the differences in the regulation of the two depots will be discussed in section 1.6.1. In the recent years, another type of adipose tissue, the brown adipose tissue (BAT), has raised some interest in the obesity field after the discovery that BAT is present and active in adult humans and not only in rodents and infants, as previously believed [12-14]. The studies in this thesis, however, have only been performed in cells from and/or in WAT.

The main function of the WAT is to store energy for periods of food deprivation and act as a mechanical shield and insulate the body, as mentioned above. The WAT is also an important endocrine organ secreting different factors, termed adipokines. The adipokines act in an auto and/or paracrine fashion and can thus regulate WAT and other organs such as muscle, liver and the central nervous system. According to recent data, there are over 250 proteins secreted from adipose tissue [15]. Two of the most well-characterized adipokines are tumor necrosis factor-α (TNFα) [16] and leptin [17]. TNFα is a cytokine regulating a series of cellular and biological processes. In the context of WAT and obesity, TNFα is known to stimulate inflammation [16, 18] and at a cellular level, TNFα has been shown to stimulate human adipocyte lipolysis through reduction of phosphodiesterase 3B (PDE3B) [19, 20]. This pathway is described in section 1.6.3. TNFα can also reduce expression of genes important for insulin signaling [21] and fatty acid oxidation and uptake [22]. Leptin is an adipostat, which acts by signaling to the hypothalamus in the brain. Leptin is almost exclusively produced by adipocytes [17, 23] and promotes increased energy consumption while it reduces appetite and stimulates satiety [23, 24]. Leptin signals through the leptin receptor, LEP-R, and mice lacking either the leptin gene (ob/ob) or the leptin receptor (db/db) are severely obese [17]. These mice are important model systems for the study of obesity and type 2 diabetes and humans with a dysfunction in the corresponding human genes display severe obesity [25]. Administration of recombinant leptin to humans or mice deficient in leptin restores body weight to normal levels by increasing metabolic rate and decreasing food intake [23, 26]. However, data from several studies indicate that there might be a general resistance to leptin in obesity. Circulating levels of leptin are directly correlated with adipose tissue mass; hence obese individuals have high levels of leptin in their circulation. Therefore, leptin treatment is not an effective way of treating common obesity.

1.3 INSULIN AND INSULIN RESISTANCE

Insulin is a protein synthesized in the β-cells of the islet of Langerhans in the pancreas and released into the circulation when blood glucose levels rise, e.g. in the postprandial state. When insulin binds to the insulin receptor (IR) on the surface of its target cells, several protein activation cascades are initiated, for example insulin receptor trans-activation results in translocation of glucose transporter (GLUT) 4 to the plasma

membrane, leading to influx of glucose in the cell, glycolysis and glycogen synthesis. For a thorough review of insulin's role in whole-body energy regulation, see biochemistry or physiology text books such as [27] or [28]. Insulin regulates WAT function on several levels: from uptake of FFAs into the adipocyte via lipoprotein lipase (LPL) to re-esterification, lipogenesis and lipolysis as well as GLUT4 translocation. The role of insulin in regulation of lipolysis will be discussed in detail in section 1.6.3.

Insulin resistance is a state when peripheral tissues become less sensitive to insulin and insulin actions. This leads to increased plasma glucose levels, which in turn leads to augmented production of insulin by the pancreas. When resistance persists, the insulin production becomes insufficient, resulting in hyperglycemia and type 2 diabetes [29]. The mechanisms behind the development of insulin-desensitization of the peripheral tissues are not completely understood, but it is known that circulating levels of FFAs are elevated in obesity due to an increased lipolysis, and the distribution of body fat is of importance. Increased levels of FFA in the circulation have been showed to have deleterious effects on peripheral tissues such as liver, muscle and pancreas and can affect insulin action, cell survival and glucose metabolism. A clinical study demonstrated that effects on insulin signaling, related to type 2 diabetes, occur as soon as four weeks after initiation of a high-calorie diet [30]. There is a strong connection between insulin resistance and the obesity-associated development of low-grade inflammation of the WAT, leading to increased FFAs and changes in adipokine release [31, 32].

1.4 ADIPOSE TISSUE INFLAMMATION

Obesity is associated with a chronic low-grade inflammation. This inflammation is tightly coupled to a dysregulation of AT, and the associated alterations change the release of adipokines and FFAs from the WAT, increasing the risk of obesity-associated complications such as insulin resistance [24, 31-35]. Macrophages in the WAT are important regulators of the obesity-associated inflammation. Macrophages are a normal component of the cell population in the WAT, and in the non-obese state these are most probably scavenger cells involved in normal cellular turnover by clearing the tissue from apoptotic cells and lipids. These macrophages are termed M2-macrophages and have anti-inflammatory properties. However, in the obese state, macrophage precursor cells are recruited from the circulation and these macrophages undergo polarization into a class of pro-inflammatory macrophages termed M1. The mechanism behind this polarization is unknown and heavily debated. The M1 macrophages release high levels of pro-inflammatory cytokines such as TNFα and, in turn, stimulate lipolysis and inhibit insulin signaling [36-38].

1.5 FATTY ACID OXIDATION

When the body lacks energy in the fasted state, FFAs produced by lipolysis can be oxidized via mitochondrial β-oxidation. This process generates acetyl-CoA, the entry molecule for the tricarboxylic acid (TCA) cycle which, in turn, is a series of chemical reactions used by all aerobic living organisms to generate energy from carbohydrates, fats and proteins. The major site for β-oxidation is the skeletal muscle, although substrate oxidation in WAT is significant [39] and energy expenditure in human fat

cells is dramatically increased after body weight reduction [40]. Also, the capacity of human WAT to oxidize FFAs may change when the oxidative machinery is altered [41].

Mitochondrial β-oxidation is regulated on several levels. Both the cellular uptake of FFA and the entry of acyl groups into the mitochondria are highly controlled. The latter is mainly regulated by the shuttle enzyme carnitine palmitoyl-CoA transferase (Cpt) I [42]. Also, the activity of the mitochondrial matrix enzymes, for example medium-chain acyl-CoA dehydrogenase (MCAD) and 3-hydroxtacyl-CoA-dehydrogenase (HADHA) is of importance for the rate of mitochondrial β-oxidation [42].

A key component in regulating the switch between glucose and fatty acids as fuel is the pyruvate dehydrogenase complex (PDC) [43]. The PDC catalyzes the oxidative decarboxylation of pyruvate, linking glycolysis (the breakdown of glucose) to acetyl-CoA and nicotinamide adenine dinucleotide (NADH), which can then enter into the TCA cycle. The PDC is inhibited by a group of enzymes termed pyruvate dehydrogenase kinases (PDKs) via phosphorylation of the E1 component of the PDC (PDH-E1). Inhibition of the PDC results in a reduction of glucose oxidation and increase in fatty acid oxidation, since long chain fatty acids are favored over glucose as substrate. Four PDKs (termed PDK1-PDK4) have been identified in mammalian tissues. The regulation of the PDKs is complicated and multifaceted. Pyruvate inactivates PDK activity acutely while products of both the PDC reaction and β-oxidation such as Acetyl-CoA and NADH are important for PDC inactivation in order to spare glucose during prolonged fasting. The different PDK-isoforms phosphorylates the PDC on different sites, leading to a dynamic regulation of fuel usage. PDK4, the most well studied isoform, has been shown to regulate oxidation in cardiac and skeletal muscle, kidney, liver and adipose tissue [44, 45] and its expression is regulated by the nuclear receptor peroxisome proliferator-activated receptor (PPAR)α.

1.6 LIPOLYSIS AND LIPOLYSIS-REGULATION

Lipolysis is the tightly regulated step-wise breakdown of TG to FFA and glycerol [46]. The first step (from TG to diglyceride, (DG)) is catalyzed by ATGL, the next step (from DG to monoacylglycerol, (MG)) by HSL and the final step is catalyzed by monoacylglycerol lipase (MGL) [47], see figure 1. HSL is capable of hydrolyzing all three steps, however it has the highest affinity for DAG. The efflux of FFA and glycerol from the adipocyte is followed by transport of these metabolites to other tissues, for FFAs mainly skeletal muscle, liver and heart and for glycerol, the liver. Some of the FFAs can remain within the adipocyte and be re-esterified to TGs. In non-overweight humans, the mean turnover rate of TG in the fat mass is around 100-300 g/day. There is a constant low level of lipolysis in adipocytes, termed basal or spontaneous lipolysis. Hormones can have a strong influence on the rate of lipolysis and inter-individual variations in AT lipolysis are of importance for the rate of weight loss [46]. The most important regulators of lipolysis-rate are catecholamines, natriuretic peptides (only in humans [48]) and insulin [46, 49].

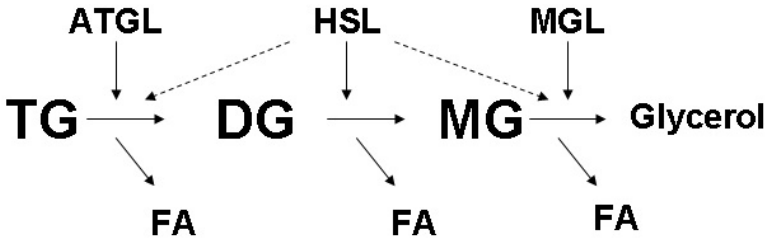

Fig 1. A simplified schematic picture of the lipases controlling the hydrolysis of TG to FA and glycerol. ATGL; adipose triglyceride lipase, DG; diacylglycerol, FA; fatty acid, HSL; hormone sensitive lipase, MG; monoacylglycerol, MGL; MG lipase, TG; triacylglycerol

1.6.1 CATECHOLAMINES

Catecholamines are important regulators of the rate of lipolysis in human adipocytes by activation of adrenergic receptors (AR). In the cell membrane, an enzyme termed adenylyl cyclase (AC) resides, coupled to ARs via trimeric guanosine 5'-triphosphate (GTP)-binding proteins (G-proteins). In human adipocytes, three β-AR (β_1, β_2, β_3) and one α-AR (α2A) have been identified [49]. Functional studies have shown that, among the β-receptors, β_1 and β_2 are most active while β_3 seems to be more important in rodent fat cells. The β-receptors are coupled to G-stimulatory proteins activating AC, thereby increasing the production of the important cellular messenger cyclic adenosine monophosphate (cAMP). This activates the protein kinase A (PKA) complex, leading to phosphorylation and thereby activation of HSL and the lipid droplet coating protein perilipin 1 (PLIN1) [11, 49]. The α-receptors, however, are coupled to the G-inhibitory proteins leading to inactivation of AC. Catecholamines activate all four ARs in human adipocyte, and the balance between activation of α and β determines whether lipolysis is stimulated or inhibited. The stimulatory actions of β-ARs is however usually dominant [49]. A detailed schematic picture of lipolysis regulation is presented in Figure 2A.

Obesity is characterized by an increased spontaneous lipolysis, partly due to increased adipocyte size, and a blunted stimulated lipolysis. However, there are some discrepancies between the two major fat depots. In the SC WAT from obese individuals, the expression of α2-AR is increased. At the same time β2-AR and HSL mRNA expression is decreased. This leads to a blunted stimulated lipolysis in obese SC WAT [49, 50]. However, in VISC WAT, β3-AR mRNA expression is increased while the levels of α2-AR are decreased, which results in an increased stimulated lipolysis [51]. FFA from the VISC fat has a stronger negative effect on other tissues, especially the liver. The VISC fat is drained to the liver via the portal vein. Therefore, FFAs from the VISC depot easily reach the liver, which can result in dyslipidemia, hyperinsulinemia, hyperglycemia and hepatic insulin resistance. Also, the release of FFA is stronger in the VISC depot due to a more pronounced effect of catecholamines and lower anti-lipolytic effect of insulin.

1.6.2 NATRIURETIC PEPTIDES

Besides catecholamines, natriuretic peptides can also contribute to an up-regulation of lipolysis. These effects are mediated via the natriuretic peptide receptor A (NPRA) and seem only to be present in humans. The receptor induces guanylyl cyclase, cyclic

guanylate monophosphate (cGMP) production, activation of protein kinase G (PKG) and finally HSL-phosphorylation [11, 49], see figure 2A.

1.6.3 INSULIN

Insulin is the most powerful endogenous inhibitor of lipolysis. Insulin binding to IR on adipocytes, starts a phosphorylation cascade involving the proteins insulin receptor substrates (IRS1 and -2) and the protein complex phosphatidyl inositol 3-kinase (PI3K). The PI3K-complex activates in turn protein kinase B (PKB) and PDE3B, catalyzing the breakdown of cAMP to its inactive form, 5'adenosine monophosphate (5'AMP). This leads to lower levels of cAMP and a decreased phosphorylation of HSL and PLIN1 through PKA, see figure 2A.

1.6.4 LIPASES

As mentioned in the introduction to this section and as indicated in figure 1, there are three major lipases involved in the hydrolysis of FFA; ATGL, HSL and MGL. HSL was considered the key enzyme in adipocyte lipolysis for a long time. However, in 2004, three groups independently reported that an enzyme capable of hydrolyzing TG, named ATGL (or desnutrin or phospholipase A2ξ) had been discovered [52-54].

1.6.4.1 HSL

HSL can hydrolyze both the first (from TG to DG) and second (from DG to MG) step in the lipolysis process (see figure 1), however the affinity of HSL *in vitro* is about 10 times higher for DG, compared to TG [55]. HSL knockout mice are sterile and have enlarged adipocytes, both in BAT and WAT, but are not obese [55, 56]. Administration of an HSL inhibitor reduces plasma level of FFA in mice, rats and dogs, indicating that it has an important role *in vivo* [57]. Phosphorylation of HSL by PKA, at least on most Ser phosphorylation sites, leads to an increased activity of the enzyme. HSL activity is thereby controlled by catecholamines and insulin, since these factors can modulate levels of cAMP and thereby phosphorylation status of HSL. In rat HSL, three phosphorylation sites leading to increased activity have been characterized, Ser^{563}, Ser^{659} and Ser^{660} [58], corresponding to Ser^{552}, Ser^{649} and Ser^{650} in humans, respectively. HSL is also negatively regulated by phosphorylation on Ser^{554} (corresponding to rat Ser^{565}). According to a recent paper, Ser^{552} and Ser^{554} might be the most important sites in relation to lipolysis in human obesity [59].

1.6.4.2 ATGL

In mice, expression of ATGL is up-regulated by fasting and down-regulated in the postprandial state by insulin and TNF-α [60]. ATGL knockout mice have an increased body weight compared to wild type, and have more body fat. They accumulate fat in the heart and other tissues, leading to cardiac dysfunction [61]. However, they are glucose tolerant and insulin sensitive. In humans, several ATGL polymorphisms have been identified. These are associated with increasing circulating concentrations of FFA, TG and glucose and risk for type 2 diabetes, indicating that ATGL might be involved in the metabolic syndrome [62]. ATGL has been shown to be able to regulate basal lipolysis in humans [63]. However, ATGL is not regulated by obesity in humans, unlike HSL [64]. ATGL is also important for stimulated lipolysis. According to a

recent study in human adipocytes, ATGL was translocated from larger to smaller lipid droplets upon catecholamine stimulation. Furthermore, the stimulation increased the colocalization of ATGL and HSL [65]. The activity of ATGL requires the presence of a co-factor termed comparative gene identification 58 (CGI-58) [11]. In the unstimulated state, CGI-58 is bound to PLIN1 and thereby unable to activate ATGL. When the intracellular levels of cAMP rise, PLIN1 is phosphorylated, leading to dissociation of CGI-58 from PLIN1. CGI-58 is released from the lipid droplet, activates ATGL and thereby hydrolysis of TG [66-69]. The dynamic regulation of proteins on the surface of the lipid droplet is illustrated in Figure 2B.

1.6.4.3 MGL

The third lipase involved in TG hydrolysis in human adipocyte, MGL, is not hormonally regulated nor considered rate limiting, due to its abundance. To this end, it has not been the focus of many studies, compared to HSL and ATGL. However, a recent knockdown study indicates that MGL deficiency impairs lipolysis and attenuates diet-induced insulin resistance [70].

1.6.5 LIPID-DROPLET ASSOCIATED PROTEINS

Since FFAs are highly toxic to cells, re-esterification of FFAs into TGs is vital for cell survival. Within cells, TGs are stored in lipid droplets. The lipid-droplet coating proteins protect cells from the deleterious effects of FFA and are also important for the regulation of lipolysis when the fat is needed as energy. There are several lipid-droplet associated proteins and below I will describe two of the most important ones, PLIN1 and cell-death inducing DNA fragmentation factor, α subunit-like effector C (CIDEC).

1.6.5.1 PLIN1

The predominant protein family for lipid droplets is the PLIN family, and in adipocytes PLIN1 is the main factor. PLIN1 is the most highly phosphorylated protein in adipocytes and is essential for lipolysis [11]. Low levels of PLIN1 are associated with increased basal lipolysis and attenuated stimulated lipolysis in both rodents [71, 72] and humans [73]. Genetic ablation of PLIN1 gives a lean phenotype and resistance to diet- or genetically induced obesity in mice [71, 72]. It also affects lipid oxidation and biosynthesis, not only in AT but also other peripheral tissues such as liver, skeletal muscle and kidney [74]. PLIN1 has a dual role in regulation of lipolysis. In the basal state, PLIN1 probably works as a barrier, protecting the TGs from hydrolysis. As described above and in Figure 2B, when PLIN1 is phosphorylated by PKA, CGI-58 is released, activating ATGL and thereby lipolysis. Also, PKA-phosphorylation of PLIN1 causes a conformational change of the protein, leading to recruitment of phosphorylated HSL to the lipid droplet surface and increased lipolysis [75].

1.6.5.2 CIDEC

Another important lipid droplet-associated protein regulating lipolysis is CIDEC [76]. CIDEC is predominantly expressed in adipocytes from the SC depot and is down-regulated in response to caloric restriction [77]. Down-regulation of CIDEC by siRNA has been shown to increase lipolysis and decrease the size of lipid droplets in 3T3-L1 adipocytes [77, 78]. Also, cells lacking CIDEC are less responsive to adrenergic

stimulation of lipolysis [77], further indicating a possible role for CIDEC in lipolysis regulation and lipid droplet biology. CIDEC belongs to the CIDE-proteins, and CIDEA, the major protein studied in paper I, will be discussed in detail below.

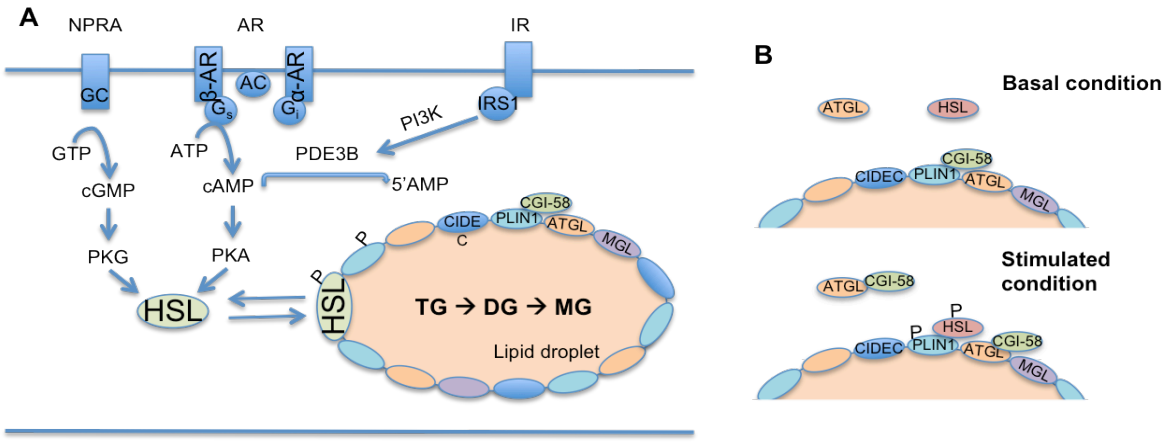

Fig 2 A: Lipolysis regulation in human adipocytes. B: Changes on the lipid droplet surface upon lipolysis activation. AR, adrenergic receptors; AC, adenylyl cyclase; AMP, adenosine monophosphate; ATGL, adipocyte triglyceride lipase; ATP, adenosine 5'-triphosphate; cAMP, cyclic AMP; CGI-58, comparative gene identification-58; cGMP, cyclic guanosine monophosphate; CIDEC, cell death-inducing DNA fragmentation factor-α-like effector C; DG, diacylglycerol; $G_{i/s}$, inhibitory/stimulatory GTP-binding protein; GC, guanylyl cyclase; GTP, guanosine triphosphate; HSL, hormone sensitive lipase; IR, insulin receptor; IRS, IR substrate; MG, monoacylglycerol; MGL, MG lipase; NPRA, natriuretic peptide receptor A; PDE3B, phosphodiesterase 3B; PLIN1, perilipin 1; PKA, protein kinase A, PKG, protein kinase G; TG, triacylglycerol

1.6.6 ZINC-α2-GLYCOPROTEIN (ZAG)

Zinc-α2-glycoprotein (ZAG) has been considered a candidate molecule responsible for the activation of lipolysis in cancer cachexia. ZAG is a protein expressed and secreted by several normal tissues however over-expressed in several types of tumors. It can stimulate lipolysis in both rodent and human fat cells, at least *in vitro*. Furthermore, AT from patients suffering from cancer cachexia has an increased gene expression and secretion of ZAG. This secretion is also proportional to the degree of weight loss [9]. However, a recent paper suggests that ZAG is activated in the AT by the catabolic state when the need for energy-rich lipids is high. The ZAG protein is not secreted but acts locally to up-regulate lipolysis and is an effect of the catabolic state *per se*, rather than the cancer cachexia [79].

1.7 CIDEA

CIDEA belongs to a family of apoptotic proteins termed the CIDE-proteins [80]. The most important family members in the context of this thesis are, except for CIDEA, CIDEB and CIDEC. These three proteins show high sequence similarity but different tissue expression, CIDEB is expressed in human liver and small intestine and murine

liver while CIDEC is expressed in human heart, small intestine and liver and human and murine WAT.

In our laboratory, we came across CIDEA in a microarray where the mRNA of this gene was down-regulated in WAT of obese subjects, and up-regulated upon weight loss [81, 82]. Also, it was shown that CIDEA could regulate lipolysis [82]. Since then, in work from our group, a single nucleotide polymorphism (SNP) in CIDEA has been associated with obesity [83], the human promoter of CIDEA has been described [84], and in paper I, we propose a role for CIDEA in cancer cachexia.

1.7.1 ROLE OF CIDEA IN ADIPOCYTES

There appears to be a clear discrepancy between the role of CIDEA in mice and humans. CIDEA is highly expressed in murine BAT and is lowly expressed in murine WAT [80, 85-87]. Mice lacking CIDEA are lean and resistant to diet-induced obesity. They show an increased metabolic rate, probably due to increased protein levels and activity of 5'AMP-activated protein kinase (AMPK) in BAT, combined with an increased β-oxidation in brown adipocytes [87, 88]. However, in humans, CIDEA is almost exclusively expressed in WAT. Obese humans have a lower expression of CIDEA in WAT compared to lean [81, 82, 89], and CIDEA was the most up-regulated protein in human SC WAT following diet-induced weight loss [82] in a Swedish cohort. Furthermore, low CIDEA expression is associated with several features of the metabolic syndrome, and RNAi-mediated depletion of CIDEA in human adipocytes increased lipolysis and TNF-α-secretion [82]. There is also a coding SNP in the human CIDEA gene associated with obesity [82, 83]. Taken together, this indicates that CIDEA might be important for the regulation of fat mass in human adipocytes.

1.7.2 LOCALIZATION OF CIDEA IN ADIPOCYTES

There has been controversy about the localization of CIDEA in fat cells. Several reports have shown that both endogenous and ectopically expressed CIDEA can localize to the lipid droplet, affecting lipid storage [90-92]. In brown adipocytes, ectopically expressed CIDEA was detected on the endoplasmatic reticulum [88]. A recent paper from our group indicates that CIDEA is localized to both the cytoplasm and the nucleus of human white fat cells. In the same paper, it is shown that CIDEA can regulate LXR [93]. This will be discussed further in section 5. In paper I, we show that CIDEA is involved in adipose tissue loss in cancer cachexia, at least in part by inactivation of PDC and thereby allowing a switch in substrate oxidation from glucose to fat in human adipocytes.

1.8 LXR

1.8.1 INTRODUCTION TO NUCLEAR RECEPTORS AND LXR

LXRs are transcription factors belonging to the nuclear receptor super family. Nuclear receptors (NR) are a class of ligand-activated transcription factors involved in numerous biological processes. NRs exert their transcriptional regulation through direct binding to DNA and through interaction with multiple coactivator or corepressor complexes. This leads to modifications of chromatin environment and alters transcription of target genes. NRs have a common modular structure and consist of the

following domains: N-terminal regulatory domain, DNA-binding domain, hinge region, ligand-binding domain and C-terminal domain. An important aspect of NRs is their ability to activate or repress gene transcription by binding of a ligand, for example hormones, vitamins, lipid metabolites or drugs. This feature makes the NRs attractive potential drug targets. For a review on nuclear receptors in general, see [94].

LXRs have in the last 15 years been identified as important metabolic regulators in many tissues and cell types. Upon activation, they bind DNA at specific sites termed LXR response elements (LXREs) as heterodimers with the retinoid x receptor (RXR) (for a review on RXR, see [95]) and affect the transcription of their target genes. The most well studied aspects of LXR regulation are their role in cholesterol metabolism and atherosclerosis. For a review on LXR, see [96] and figure 3. This thesis will focus on the role of LXR in adipose tissue and adipocytes.

Both isoforms of LXR, LXRα and LXRβ - coded by the NR1H3 and NR1H2 genes, respectively, are expressed in mature murine and human adipocytes. LXRβ is ubiquitously expressed, while LXRα is predominantly expressed in WAT, liver, small intestine and macrophages [97] and is up-regulated during adipocyte differentiation. The expression of LXRβ is not changed during adipogenesis. A study from our group has showed that obese women have higher levels of LXRα in SC WAT compared to lean. Also, SNPs in both isoforms are associated with obesity [98]. Three different splicing forms of LXRα have been identified, however the role of these is still not clear [99]. LXR was first discovered as an orphan receptor, a receptor without an identified ligand, but was deorphanized with the discovery of the oxysterols as the natural ligand. LXR is important for several aspects of adipocyte metabolism, however the role of LXR in regulation of important adipocyte functions such as adipogenesis and lipogenesis is still a matter of debate.

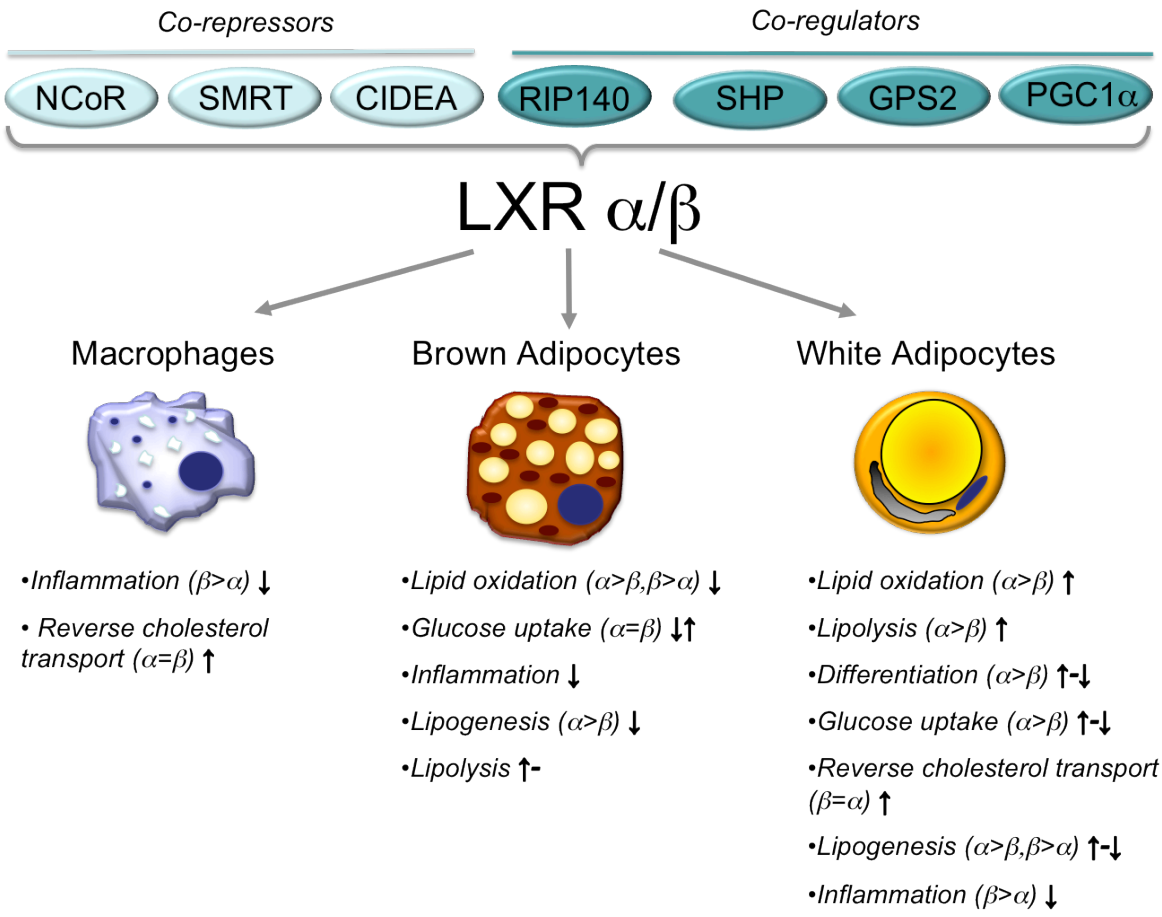

Figure 3 Summary of functions attributed to LXR in different cell types. The proposed roles of LXR in different cellular processes are summarized for macrophages, brown and white adipocytes. Where available, information on the main receptor-isoform (i.e. α or β) mediating the effect is indicated. Positive (↑), negative (↓) or no (-) effects following LXR activation are indicated as reported in the literature. Figure from Laurencikiene and Rydén, submitted.

1.8.2 LXR AND ADIPOGENESIS

The reports concerning LXR and adipogenesis, the process of cell differentiation by which preadipocytes become adipocytes, are inconclusive. A central question is the potential crosstalk between LXR and a master regulator of adipogenesis, PPARγ. Different studies have reported that LXR ligands can suppress, activate or not at all affect adipogenesis. As mentioned above, LXRα is up-regulated during adipocyte differentiation *in vitro*. This induction is mediated by the indispensible adipogenic genes PPARγ and CCAT/enhancer-binding protein α (C/EBPα), at least in human *in vitro* differentiated Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes and murine 3T3-L1 cells [100-102]. Also, several treatments interfering with adipogenesis, such as ultraviolet light A, flavonoids and some polyunsaturated fatty acids, cause a simultaneous down-regulation of LXRα and PPARγ [103, 104]. Pioglitazone (a PPARγ activator) treatment of non-diabetic subjects has been shown to up-regulate LXRα mRNA expression [105]. The potential crosstalk between PPARγ and LXR is even more complex since both PPARγ and some of its target genes, for example the fatty

acid binding protein aP2, are in turn LXRα target genes in adipocytes [106, 107]. Several previous reports have shown that LXR activation during adipogenesis, a process heavily driven by PPARγ, can potentiate fat accumulation in both human and murine cells [100, 107], and siRNA-meditated knockdown of LXRα suppresses adipocyte differentiation, however only in mice expressing PPARγ [107]. Other studies show no or even a negative effect of LXR on adipogenesis [108, 109]. Even though LXR could act as a modulator of adipocyte differentiation, it is not essential for murine adipogenesis since LXRαβ-/- mice have functional, although somewhat reduced AT [110, 111].

1.8.3 LXR AND CHOLESTEROL TRANSPORT IN ADIPOCYTES

One of the most well studied functions of the LXRs is their ability to regulate cholesterol transport and metabolism by up-regulation of important cholesterol transporters in liver, intestine and macrophages. LXR can also up-regulate the expression of the rate-limiting enzyme in bile acid synthesis, Cyp7 (only in mice), and the HDL-associated lipoprotein E (ApoE) [112]. In murine adipocytes, LXR has been shown to induce expression of several genes related to cholesterol metabolism, for example ApoE [113, 114], and LXR activation leads to an increased expression of both the cholesterol transporters ABCG1 and A1 and ApoE in wild type mice, but not mice lacking LXR. In papers II and III, we have used up-regulation of ABCG1 as a control for proper LXR activation.

1.8.4 LXR AND LIPOGENESIS

In the liver, activation of LXR both via endogenous and synthetic agonists induces lipogenesis. This is mediated via up-regulation of SREBP1c and its response genes, for example fatty acid synthase (FAS) and steoryl-CoA desaturase 1 (SCD1) [97]. In adipocytes, the role of LXR is less clear due to conflicting data from different laboratories. However, taken together the data speaks against a major role for LXR in lipogenesis in human and murine adipocytes. Mice lacking LXR have smaller fat depots [111] and an increased expression of FAS in WAT [110, 115], indicating a role for LXR in lipogenesis. Furthermore, treatment with LXR ligand of 3T3-L1 adipocytes during differentiation has been shown to increase the expression of FAS and SREBP1c, and up-regulate lipid accumulation [100, 107]. A different study indicated that the LXR ligand T0901317 up-regulated FAS and SREBP1c in murine AT *in vivo* [107]. Conversely, stimulation of human primary adipocytes with LXR agonist for 7 days in a different study only marginally affected the expression of SREBP1c, FAS and SCD1 [116]. Also, LXR activation has been shown to induce up-regulation of SREBP1c in 3T3-L1 cells, but without SREBP1c recruitment to the FAS promoter or induction of activity of a FAS promoter-driven reporter gene. As a result, there was no effect on FAS after LXR activation, despite up-regulation of SREBP1c [117]. Different cell culture conditions and experimental setup, such as time-point of LXR stimulation, could explain at least a part of these discrepancies. It is also worth mentioning in this context that human adipocytes in general display a very low level of *de novo* lipogenesis.

1.8.5 LXR AND LIPOLYSIS

LXR has been shown to increase the degree of basal lipolysis in several studies. The basal release of FFA was increased in murine 3T3-L1 cells with over-expressed and activated LXRα, and administration of the LXR agonist T0901317 to mice led to increased serum FFA and glycerol [108], indicating an increased lipolysis. Furthermore, a similar treatment with another LXR agonist, GW3965, resulted in smaller adipocytes [118], also indicating increased lipolysis. In paper III, we show that stimulation with GW3965 increased basal lipolysis in both human and murine adipocytes in culture. We also show that this is at least in part mediated by binding of LXR to the promoter of PLIN1, leading to decreased levels of PLIN1.

The role of LXR in hormone-stimulated lipolysis is puzzling. In 3T3-L1 cells with over-expressed LXRα, treatment with LXR agonist did not change the maximal rate of FFA or glycerol release upon stimulation with an agonist for the β3-adrenoreceptor [108]. However, isolated adipocytes from LXRαβ-/- mice have been shown to display a three times higher maximal response to noradrenalin stimulation (acting as a non-selective α- and β-adrenoreceptor activator), compared to adipocytes from wild-type littermates [110]. Also, the microarray data presented in paper III indicate that several genes known to affect stimulated lipolysis are influenced by LXR activation in human adipocytes. However, only LXRs effect on basal lipolysis is studied in paper III.

1.8.6 LXR AND LIPID OXIDATION

Activation of LXR has been shown to up-regulate skeletal palmitate oxidation [119] and cardiac expression of PDK4 [120]. Furthermore, administration of an LXR agonist to mice caused an increase in hepatic peroxisomal β-oxidation and up-regulated MCAD and HADHA [121]. Also, LXR-KO mice show increased FFA synthesis and energy consumption in WAT, indicating that LXR might be important for energy homeostasis [115]. Interestingly, LXR has been implicated in the negative regulation of oxidation and energy expenditure in brown adipocytes [122, 123]. In paper II, we show that activation of LXR in white human and murine adipocytes up-regulates fatty acid oxidation and down-regulates glucose oxidation. This is, at least in part, mediated by PDK4-dependent phosphorylation of the PDC, shifting the fuel usage from glucose to fatty acids.

1.8.7 LXR-KNOCKOUT MICE

Even though there is still no data on adipose tissue-specific LXR-knockout mice, a lot can be retrieved in this area from the double and isoform-specific LXR knockouts that have been described. LXRαβ-/- mice are glucose tolerant and insulin sensitive. They have 15-20% smaller AT depots compared to their wild type littermates, a discrepancy that increases with age [100, 115]. LXRαβ-/- mice are resistant to diet induced obesity, probably due to an increased metabolic rate, and have smaller adipocytes compared to wild type [111, 115, 124]. WAT from knockout mice have higher expression levels of both PLIN1 and HSL, compared to wild type littermates [110]. There appears to be a difference with regards to the energy-expenditure regulation, two studies show increased expression of uncoupling protein 1 (UCP1) in muscle and AT from LXRαβ-

/- mice [115, 122] while in another study, UCP1 is increased in BAT [123] but not WAT [124].

1.8.8 LXR ISOFORMS

As mentioned above, the LXR isoforms display different tissue distribution patterns. Gene expression profiling in gonadal WAT from isoform-specific knockouts, compared to wild type, shows that a similar number of genes, around 400, are positively or negatively affected by knockdown of LXRα. However, only 155 genes are up-regulated by LXRβ knockdown, compared to 328 down-regulated genes [125]. Analysis of gene ontology indicates that while genes related to carbohydrate metabolism are more LXRα-specific, genes coding for cytokines was higher regulated by LXRβ. The cholesterol transporters are equally regulated by both isoforms [114, 126]. Mice lacking LXRα were similar to wild type concerning fat accumulation on high fat-diet, however they remained insulin sensitive and had an increased expression of UCP1 [111, 122, 124]. Mice lacking LXRβ, on the other hand, were resistant to diet-induced obesity, had smaller fat cells and impaired fat storage in adipocytes, similar to the LXRαβ-/- mice [111, 124]. This indicates that the LXR isoforms might have diverse roles in the regulation of metabolism. In paper III, we show that up-regulation of lipolysis by LXR activation is an effect mainly dependent on LXRα and not LXRβ.

1.8.9 LXR CO-FACTORS

Since the LXRs are nuclear receptors ligands, NR dimerization partners and transcriptional co-regulators regulate their transcriptional activity. LXRs associate with NR Co-repressors (NCoR), Silent Mediator of Retinoic acid receptor and thyroid receptors (SMRT) and small heterodimer partner (SHP) [127-129], but direct interaction with either of these proteins and LXR has not been shown in adipocytes. However, both LXRα and β have been shown to interact with receptor-interacting protein 140 (RIP140) [130, 131]. Furthermore, a recent paper from our group show a direct interaction between LXR and CIDEA [93].

2 AIMS

The aim of this thesis was to investigate how LXR and CIDEA can affect important aspects of adipocyte metabolism. Further knowledge on the regulation of adipocyte metabolism and its regulators could be of great importance in future treatments of states of AT dysfunction, such as obesity and cachexia.

I. The aim of paper one was to study the role of CIDEA in relation to fat loss in cancer cachexia
II. The aim of the second paper was to investigate the effect of LXR on substrate oxidation in human adipocytes
III. The studies in the third paper aimed at examining the role of LXR in relation to basal lipolysis in human adipocytes

3 COMMENTS ON MATERIALS AND METHODS

The experimental methods used in the papers included in the thesis have been described in detail in the respective papers. In this section I will briefly describe some of the techniques and their advantages and/or disadvantages compared to alternative methods.

3.1 SUBJECTS AND ADIPOSE TISSUE SAMPLE COLLECTION

All studies were conducted in accordance with the guidelines of the Declaration of Helsinki and approved by regional ethical committees at Karolinska University Hospital. Written consent was obtained from all participants. For paper I, all patients scheduled for gastrointestinal cancer operations between January 2004 and December 2005 were evaluated, selections were made and subjects were divided into groups based on diagnosis after surgery. Details on exclusion and inclusion are described in detail in the paper. Around 450 patients were evaluated. Patients who were fit in spite of their cancer, had not received prior anti-cancer treatment and were willing to participate were included, n = 48. The patients were then divided into three groups based on the diagnosis after surgery:

Cancer cachexia (n = 23): gastrointestinal cancer with unintentional weight loss of >5% in the last 3 months or >10% in the last 6 months.
Weight-stable cancer (n= 17): patients with gastrointestinal cancer who reported no important weight change during the last year.
Control group (n = 8): patients with pre-diagnosed gastrointestinal cancer but who did not have a malignancy according to final histological evaluations.

It should be noted that all of the cancer patients included in the study suffered from gastrointestinal cancer, which should be taken into consideration when evaluating and interpreting the results from the study. Also, a large proportion (75%) of the patients is male and of fairly high age. However, since gastrointestinal cancer is more common in men and in older patients, this factor is difficult to circumvent. All studies have been performed in SUC WAT since we did not have access to VISC WAT from these patients.

For the molecular studies in all three papers, SC adipose tissue was obtained from healthy subjects undergoing cosmetic lipoaspiration. They were not selected for age, sex or body weight.

3.2 CELL CULTURE

The use of different in vitro systems such as cell lines and primary cultures are essential to the type of research performed in this thesis. For the molecular studies we have used primary cultures of human in vitro differentiated adipocytes whenever possible, and used the murine cell line 3T3-L1 as a complimentary system to see potential species differences, or when technical problems made it difficult to use primary human cells.

At present, there is no readily available, well-working cell line of human adipocytes. Therefore, either primary human adipocytes or the murine cell line 3T3-L1 is often used. Unfortunately, this approach has several drawbacks. Cell lines in general have changes in the cell cycle machinery, making them easy to work with since they can be frozen and thawed whenever needed. Also, every passage is identical to the previous ones, giving high reproducibility. However, the changes in cell cycle machinery gives an altered phenotype of the cells and makes them more prone to spontaneous mutations, compared to primary cell cultures. 3T3-L1 cells are of murine origin, raising questions about species differences. However, the primary cell cultures also have drawbacks. There might be higher individual variation, depending on the individual donors, the supply is unpredictable and they seem to be sensitive to infections. Also, isolating and culturing the primary adipocytes is a time-consuming task.

In the papers in this thesis, 3T3-L1 cells were cultured and differentiated to adipocytes using a standard protocol [132]. The in vitro differentiated human adipocytes were isolated, cultured and differentiated as previously described [133, 134]. In brief, WAT was cut into small pieces, digested with collagenase and centrifuged. The stroma-vascular fraction was resuspended and filtered after which the cells were plated in inoculation media. After 24 hours the medium was changed to differentiation media, containing Dulbecco's modified eagle's medium (DMEM)/F12 with fetal bovine serum, penicillin-streptomycin, amphotericin B, human insulin, triiodo-L-thyroine, human transferring, biotin, panthotenate, cortisol and rosiglitazone. The rosiglitazone, a PPARγ agonist, was present for the first 3-6 days and then removed. After differentiation, around 70% of the preadipocytes displayed a round shape with a completely filled cytoplasm. Plates with less than 70% differentiation density were discarded.

3.3 OVER EXPRESSION OF CIDEA

In paper I, human CIDEA (hCIDEA) was over-expressed in murine 3T3-L1 cells and *in vitro* differentiated human adipocytes, since these cells express very low endogenous levels of CIDEA. The CIDEA expression vector was obtained by insertion of hCIDEA into a plasmid. Constructs were verified by sequencing and protein expression was confirmed using *in vitro* translation. For the transfections, Lipofectamine and Plus Reagent were used after careful optimization of transfection conditions, using green fluorescent protein-containing plasmids. The efficiency of transfection was around 40%.

3.4 FATTY ACID OXIDATION

In papers I and II, palmitate oxidation was assessed in *in vitro* differentiated human adipocytes and 3T3-L1 cells. As described in the introduction, β-oxidation supplies the cells with fuel for the TCA cycle. In this method, cells are fed ^{14}C-labeled palmitate and the amount of $^{14}CO_2$ and ^{14}C-labeled metabolites, side products of the TCA cycle, are measured. The rate-limiting factor carnitine was supplied in excess and a powerful stimulator of β-oxidation was used as a positive control.

When assessing substrate oxidation in vivo, calculation of respiratory quotient (RQ) is a common method. It is calculated from removal of carbon dioxide and consumption of

oxygen and results in a unit less number reveling the major energy source. An RQ of 1, for example, represents pure carbohydrate oxidation and a number around 0.7 indicates that fat is the major substrate for oxidation in the patient. This method is used in paper I.

3.5 GLUCOSE OXIDATION

In papers I and II, glucose oxidation was also assessed. The method is similar to the fatty acid oxidation method described above; however labeled glucose is added to the cells instead of palmitate. Radioactive CO_2 was collected and measured by scintillation counting.

3.6 ACTIVITY AND PHOSPHORYLATION OF PDC

An important part of both papers I and II was the experiments showing differences in phosphorylation status and/or activity of the PDC. As described above, PDC acts as a cellular fuel switch, regulating if glucose or FFAs are the primary energy source for the TCA cycle. When PDC is inactivated by phosphorylation, glucose oxidation is inhibited in favor of lipid oxidation. In papers I and II we used a PDC pull-down kit based on the immunoprecipitation technique. Protein lysates from 3T3-L1 cells and/or human *in vitro* differentiated adipocytes were immunoprecipitated using an anti-PDH-E1 antibody coupled to Sepharose beads. The E1 subunit is the subunit phosphorylated when PDC is inactivated. After washing and elution, the proteins were separated by SDS-PAGE and transferred to a membrane. An anti-phosphoserine antibody was used for detection. In the studies, two different controls for equal loading were used for technical reasons. In the murine experiment, and also in paper I, we used the anti-phosphoserine antibody to detect levels of input material as a loading control, a standard way of assessing equal loading when performing IPs. However, a better control is measurement of total amount of PDC-E1, and then relating this to the amount of phosphorylated PDC-E1. This was used in the experiments performed in the later part of the study, namely the human adipocytes.

In paper II, we confirmed the increased phosphorylation of the PDC by showing decreased activity of the complex. This was performed using a then novel ELISA-based technique utilizing the lateral flow concept. Antibodies are captured onto nitrocellulose membranes on the dipstic, and a wicking pad draws the protein lysate through the antibody band. The intensity of the bands represents the activity of the complex, since the PDH activity reaction produces NADH. NADH, in turn, is coupled to the reduction and precipitation of a colored dye, showing on the dipstick as a band.

3.7 LIPOLYSIS

In paper III, lipolysis was assessed as release of glycerol from in vitro differentiated human adipocytes and murine 3T3-L1 cells. Lipolysis is, as described above, a process where TG are hydrolyzed to three FFA and one molecule of glycerol. Since FFA can be utilized within the adipocyte in a process termed re-esterification, lipolysis is preferentially measured as release of glycerol, and not of FFA, into the cell culture media. The method used to assess the levels of glycerol in paper III is a bioluminescence method based on luciferase, luciferine, glycerol kinase and ATP [135-

137]. Two competing reactions, one catalyzed by glycerol kinase and one by luciferase compete for the available ATP. A higher concentration of glycerol in the sample will decrease the amount of light emitted. This light is measured in a luminometer and glycerol levels are calculated, based on a standard curve with known glycerol concentrations.

3.8 GENE EXPRESSION

In all three papers, quantitative real-time polymerase chain reaction (RT-qPCR) is used to assess mRNA levels of specific genes. The mRNA levels of a gene can often reflect protein levels of the respective gene in the cells. In the PCR reaction, certain sequences are amplified using specific primers and enzymes and the reaction is monitored in real-time using a PCR machine. This instrument measures the amount of PCR product, based on fluorescence, in the reaction. Fluorescence is generated in each PCR cycle and this amount is directly proportional to the amount of PCR product in the reaction. In the papers in this thesis, two different detection methods have been used: SYBR green and Taqman.

SYBR green is a non-specific dye that fluoresces only when bound to double-stranded DNA. SYBR green is a less specific, however simpler and cheaper method of detecting amount of PCR product in the samples. There is a risk for false-positive results since the dye detects not only specific products but also primer-dimers and unspecific products. However, this problem can be avoided with careful primer design and the use of dissociation curves and agarose gel electrophoresis, making sure that a single product is obtained.

The Taqman assays, purchased from the manufacturer, contain fluorescent-labeled probes complementary to a sequence in the target gene. The probes are labeled with a flourophore and a quencher, absorbing the fluorescence when the probe is unbound. The target-bound probe is degraded by the Taq polymerase during elongation, releasing the flourophore from the quencher. This emits fluorescence. Taqman is a more sensitive detection method than SYBR green but the method has its drawbacks. It is more expensive, requires more material (in our setup it requires twice the amount of cDNA as SYBR green) and is dependent on that a probe for your gene of interest is available.

The results of the PCRs, independent of detection method, are C_t-values. This value represents the point in the reaction when the fluorescence reaches above the threshold value, due to the amplification. There is a linear relationship between the log of the starting amount in the sample and their corresponding C_t. We have used the $2^{-\Delta\Delta C}$T comparative method to determine the mRNA levels of the different genes [138].

When running RT-qPCR it is essential to use reliable reference genes or “house-keeping” genes, genes not affected by the experimental conditions. These genes are amplified in parallel reactions. We have used several different reference genes and compared the results between them, making sure that we get similar relative results independent of reference gene. Two of the reference genes used are 18S rRNA and

LRP10, the latter one a gene previously shown to be a dependable reference gene for human WAT [139].

3.9 RNAI

In papers II and III, RNA interference (RNAi) was utilized to silence expression of different genes using small interfering RNA (siRNA). In this method, small double stranded RNA molecules are transfected into primary cell cultures, interfering with the expression of a specific target gene. For technical reasons, siRNA from two different companies, Qiagen and Dharmacon, where purchased and used in paper II. In paper III, only siRNA from Dharmacon was used since this company provides a pool of four different siRNAs targeting different sequences in the same mRNA, resulting in a better knockdown. As a negative control, we used non-targeting siRNA from the respective company. The non-targeting siRNA have no known similarities to human sequences. In the supplemental data of paper II, we performed extensive methodological experiments indicating that the control oligonucelotides do not cause any important off-target effects in our system. We also show that siRNA treatment against a housekeeping gene does not influence lipolysis.

3.10 MICROARRAY

The RNA used for the microarray was carefully quality-checked and the array was run according to standard protocols by the core facility at Karolinska Institutet, Stockholm, Sweden. For the analysis of differences in expression of individual genes on the microarray between LXR activated and vehicle-treated samples, the data set was normalized using probe logarithmic intensity error (PLIER). The normalized data set was then analyzed using significance analysis of microarrays (SAM) with a false discovery rate of 1%. For table I in paper III, a list of genes that could be of importance and that had an absolute call over 150 and genes known to regulate lipolysis in adipocytes (even genes or genes with a lower absolute call) was compiled and it was indicated whether these were significant according to SAM or not. All genes that we studied further were confirmed using qRT-PCR.

3.11 PROTEIN MEASUREMENTS

In all three papers, Western Blot was used to quantify amount of protein in samples of different treatments. Western Blot is a standard technique of verifying the identity of protein using antibody recognition and size, and the migration of denatured proteins compared to a known standard scale is assessed. The detection method used in the present papers is chemiluminiscence and the intensity of the bands was quantified using the Quantity-One software. The selection of specific and efficient antibodies is vital to obtain good results when using Western Blot, and all antibodies were carefully tested. In the case of HSL in paper III, we used two different antibodies, both giving similar results.

3.12 CHROMATIN IMMUNOPRECIPITATION (CHIP)

ChIP is an assay based on the interaction between chromatin and transcription factors and was used in paper III to show direct binding of LXR to the promoters of PLIN1 and HSL upon activation of LXR. The DNA-protein-complexes are crosslinked, isolated

and sonicated to smaller fragments. The fragments are then immunoprecipitated using an antibody, in this case against LXR, after which the DNA was extracted and analyzed using qRT-PCR with specific primers against sequences in the promoters of PLIN1, HSL and, as a control, the known LXR target gene ABCG1. ChIP is a specific method and is very useful in the study of transcription factors such as LXR. The biggest caveats are the necessity of a specific antibody and that large quantities of cells are needed. Also, the assay is dependent on the correct timing of the crosslinking since protein-DNA interactions are dynamic. As a negative control, we have used an IgG antibody.

3.13 IMMUNOSTAINING, MICROSCOPY AND IMAGE ANALYSIS

In paper III, we studied the localization and colocalization of PLIN1 and HSL. To this end, we performed sequential double immunocytochemical stainings with different secondary fluorescing antibodies. We compared the protein localization around the rim of the lipid droplet of both proteins in cells that had been treated with the LXR agonist GW3965 to vehicle-treated cells. We studied this using at least three z-stacks after deconvolution from three different lipid droplets in three different cells per patient. We calculated the relative amount of PLIN1 and also the ratio of PLIN1 over HSL and compared the results from the vehicle-treated and LXR-agonist treated cells.

3.14 STATISTICS

For statistical analyses in general, it is often recommended that the number of subjects and objects should not be lower than 30 per group in order to ensure that the data will be normally distributed. This is however often not possible when it comes to experimental research, due to technical limitations. It would take very long time; the material might be precious and the reagents very expensive. In our studies, primary cells from 3-6 individuals are used and non-parametric tests are applied.

With regards to the clinical data in paper I, a power calculation based on the previously known distribution of CIDEA mRNA expression was performed before subjects were recruited. The patients were recruited in a ratio of 1:2:3 with regards to control patients, weight stable cancer patients and cancer cachexia patients. This ratio suggested, based on the assumption of a 10% experimental failure rate and mean and SD of CIDEA mRNA, that 1:16:24 patients had to be recruited to detect a 2-fold difference between cachexia and the two other groups at <0.05 (using ANOVA) with 80% power using sample power (SPSS, Inc). In the analysis of data, results were compared using ANOVA and appropriate post hoc test, chi-squared test, unpaired or paired t-test and linear/multiple regression analysis. When comparing SGA and tumor scores, Kruskal-Wallis and Mann-Whitney tests were used.

For the experimental part of paper I and in papers II and III, the statistical analysis was performed using Student's t-test or Mann-Whitney test, using standard statistical software.

4 RESULTS AND DISCUSSION

In this section, I will summarize and comment the results from the individual papers, focusing on the aims previously outlined in section 2. In section 5, I will summarize the results from the different papers and provide some future perspectives.

4.1 PAPER I

The aim of paper I was to study a novel concept in the role of fat loss in cancer cachexia, namely the function of CIDEA. Also, a number of metabolism-related clinical parameters were assessed and compared between the three study groups: cancer cachexia, weight-stable cancer patients and noncancer patients.

The clinical data is shown in table 1 in paper I. Even though the subjects with cancer cachexia had a lower BMI, body fat percent and total amount of body fat compared to the other groups, lean body mass was similar in both cancer groups. The cancer cachexia group reported a more pronounced weight loss, and had a higher subjective global assessment score (SGA). SGA is a nutritional assessment method where a high score is indicative of malnourishment. The patients with cancer cachexia also had lower levels of markers of anabolism, such as plasma TG and serum albumin, compared to the other two groups. Lipid mobilization, measured as plasma FFA or plasma glycerol concentrations, corrected for total body fat, was increased in the cancer cachexia group. Finally, the RQ was significantly decreased in the cancer cachexia group, compared to the noncancer and weight-stable cancer group, indicating that this group had a high level of lipid oxidation, rather than carbohydrate oxidation.

CIDEA is an interesting gene since it is almost exclusively expressed in white adipocytes in humans and brown adipocytes in mice. It has also been shown to be up-regulated in human WAT after weight loss [82]. Since earlier studies indicated that CIDEA might be important for adipocyte metabolism, we decided to investigate if it could play a role in cancer cachexia. We measured the mRNA levels of CIDEA in WAT from patients suffering from cancer cachexia and could show that the expression of CIDEA was higher in this group, compared to the weight stable cancer patients and the noncancer patients. BMI and adipose mass correlated strongly and negatively with CIDEA levels. There was no correlation between CIDEA levels and lean body mass, indicating that CIDEA expression in adipocytes might be important for total fat mass. It is difficult to elucidate whether the strong correlation between reported weight loss and CIDEA mRNA expression in WAT is due to a loss of body weight *per se* or to the catabolic state of the cancer cachexia patients.

It has previously been shown that changes in the metabolism can influence the FFA oxidation in WAT. Patients with cancer cachexia have an increased lipolysis and an elevated FFA mobilization. In this study, it was evident that the cancer cachectic patients had a lower RQ compared to the other groups, and that CIDEA expression in all subjects was strongly correlated with RQ. A low RQ, indicating a high FFA-oxidation, was associated with high CIDEA mRNA levels, and as much as 25% of the variation in RQ could be explained by CIDEA, according to calculations of adjusted r^2. Also, reported weight loss was negatively correlated with RQ. Furthermore, multiple regression analysis showed that only CIDEA was a significant

regressor for RQ when either BMI or body fat was used together with CIDEA as an independent regressor and RQ as the dependent variable.

Next, we wanted to elucidate the potential impact of high CIDEA levels on metabolism in white adipocytes. Human CIDEA was over-expressed in two different cell systems: murine 3T3-L1 and human preadipocytes, differentiated *in vitro*. In both these cell systems, CIDEA is expressed at very low levels. Cells with increased levels of CIDEA showed a higher fatty acid oxidation, compared to control. This is in line with the clinical data showing that high mRNA levels of CIDEA correlates with low RQ, indicating a high fatty acid oxidation. Also in line with these findings, over-expression of CIDEA decreased glucose oxidation in 3T3-L1 cells. Since CIDEA belongs to a proapoptotic family, it is important to note that CIDEA did not induce necrosis or apoptosis when over-expressed in this setting.

The next step in mapping the pathway or pathways behind CIDEAs effects on substrate oxidation was to compare mRNA levels of important regulatory genes in the oxidation cascade in cells with over expressed CIDEA against control. However, the increased levels of CIDEA did not affect known regulators such as Cpt-I and HADHA. In contrast, levels of PDK1 and PDK4 were increased in cells with high levels of CIDEA, compared to control. In the clinical material, there was a correlation between expression of PDK1/PDK4 and CIDEA mRNA levels. The correlation was strong, as much as 46% of the variation in PDK1 and 58% of the variation in PDK4 could be explained by variation of CIDEA.

As described in the introduction, the PDKs regulate the activity of the PDC – and thereby the cellular substrate oxidation. The PDKs inhibits the PDC by phosphorylation, which results in a higher degree of lipid oxidation and a lower glucose oxidation. Therefore, we investigated the phosphorylation status of the PDC. In lysates from cells with over-expressed CIDEA the phosphorylation of PDC was stronger compared to control. This is indicative of a decreased activity of the complex and thereby and a shift in the substrate oxidation from glucose to lipids. The up-regulation of FFA oxidation by CIDEA might be a protective mechanism for handling the increased levels of FFA due to the augmented lipolysis connected to the cancer cachexia. It should be mentioned that CIDEA has previously been shown to inhibit lipolysis. However, in the case of cancer cachexia the stimulated lipolysis is increased, most probably due to enhanced expression and function of HSL [5], overriding the inhibitory effects on basal lipolysis caused by high CIDEA.

In this paper, we suggest that CIDEA is involved in the altered lipid metabolism associated with cancer cachexia. In our cohort, the cancer cachexia patients show clear signs of malnutrition, however the lean body mass is unaltered. Furthermore, they display an increased lipolysis, FA mobilization and lipid oxidation, compared to the noncancer and the weight-stable group. We propose that expression of CIDEA is increased, leading to up-regulation of PDK1 and PDK4. The PDKs phosphorylate the PDC, causing a decreased activity and thereby inducing the use FFAs instead of glucose for acetyl-CoA oxidation via the TCA cycle. It is difficult to speculate about the mechanism behind CIDEA up-regulation in cancer cachexia. There have been discussions regarding the possible presence of cachexia factors. The hypothesis suggests that so far unknown factor, or several factors, could up-regulate the expression of HSL, and thereby increase lipolysis in adipocytes. ZAG has been proposed as a potential cachexia factor since it has been shown to up-regulate lipolysis [140, 141]. A recent paper suggests that ZAG acts locally in the adipose

tissue [79]. This paper also suggests that ZAG might be a catabolism factor and not necessarily a cancer cachexia aspect, since ZAG levels in the AT and plasma glycerol and free fatty acids are enhanced in subjects given a very low calorie diet (VLCD). In a different study, CIDEA levels were increased in the AT of patients on VLCD [89]. Taken together, this suggests a possible co-regulation of ZAG and CIDEA. Both proteins seem to be up-regulated by a catabolic state and it is not possible to say if one regulates the other or if an unknown factor regulates both proteins simultaneously. It is difficult to speculate about how CIDEA could regulate the expression levels of PDK1 and 4 since CIDEAs role in gene regulation in general is unknown. However, it could be of great interest to further study the relationship of CIDEA and ZAG to acquire additional insight into the regulation of adipocyte metabolism in cancer cachexia. Since cancer cachexia in many ways is the opposite of obesity, gaining more knowledge about the pathways involved is of interest also with regards to obesity and type 2 diabetes, besides the importance in the cancer field.

4.2 PAPER II

The aim of paper II was to study the effect of LXR activation on substrate oxidation in human adipocytes and try to elucidate the underlying mechanism. As previously described, the oxidation of FFAs might function as a protective mechanism when the circulating levels of FFAs rise. High circulating levels of FFA are well known to promote insulin resistance and, in turn, type 2 diabetes. Even though skeletal muscle is the major site for lipid oxidation in humans, the adipocytes contribute to the total energy expenditure [39], and the capacity of human WAT to oxidize FFAs may change when the oxidative machinery is altered [41].

When we stimulated *in vitro* differentiated human adipocytes with the LXR agonist GW3965 β-oxidation was up-regulated, both in a time- and concentration-dependent manner. To try to find a pathway for the up-regulation of lipid oxidation, we measured the mRNA levels of several important β-oxidation genes in cells treated with GW3965 or vehicle. Similar to the results from paper I, we could not see any effect on known regulators such as Cpt-I and HADHA. Since LXR has previously been shown to up-regulate levels of PDK4 in muscle cells, we measured the mRNA levels of PDK1 and PDK4. We could show that stimulation with a specific LXR agonist increased the levels of PDK4 in human adipocytes, on mRNA and also on protein level. However, PDK1 was not affected. We then investigated the effect of LXR on the phosphorylation of the PDC. Since PDK4 can phosphorylate, and thereby inactivate, PDC and thus turn the cell towards favoring lipids over glucose as substrate for the TCA cycle, this could be how LXR regulates lipid oxidation. When LXR is activated in human adipocytes, the phosphorylation of PDC is increased and the activity, measured by a novel dipstick assay, is decreased. As expected, glucose oxidation is also decreased.

In order to see if the effect of LXR on substrate oxidation is PDK4-specific, we performed knockdown studies. We successfully silenced PDK4 in human adipocytes using siRNA and subsequently measured the effect of GW3965-stimulation on glucose oxidation, lipid oxidation and PDC-phosphorylation. We also measured a set of genes not directly related to β-oxidation, in order to exclude possible non-target effects in our system. We also measured PDK1, which was not affected by the PDK4 knockdown. The knockdown of PDK4 in human adipocytes abolished the effect of GW3965 on

glucose oxidation and diminished the PDC phosphorylation upon treatment with the LXR agonist. The knockdown of PDK4 did not affect lipid oxidation in the adipocytes, indicating that other mechanisms might be important for the LXR-activated up-regulation of β-oxidation. The elevation of FFAs themselves might cause an up-regulation of lipid oxidation, since it has previously been shown in muscle that reduced FFA availability can increase PDH activity through differences in the NADH/NAD+ ratio [142].

We also performed knockdown studies of LXR to investigate whether the effect of the LXR agonist truly were mediated by LXR. We knocked down both isoforms simultaneously and measured lipid oxidation. In cells lacking LXR no GW3965-mediated up-regulation of β-oxidation was present, indicating that the effects of the LXR agonist are specific for LXR activation.

In the last section of paper II we looked into the effect of LXR on substrate oxidation in murine 3T3-L1 adipocytes. The results were highly similar to the data obtained in human adipocytes, and described above. Lipid oxidation was up-regulated, glucose oxidation attenuated, mRNA levels of PDK4 increased and the PDC showed an increased degree of phosphorylation. This strengthens the results from the human adipocytes and suggests no species specificity in this process, at least concerning humans and mice.

In this paper, we demonstrate that LXR activation can increase lipid oxidation and decrease glucose oxidation. This is, at least regarding glucose oxidation, due to an increase in PDK4 and a concomitant phosphorylation of the PDC. We show that the LXR-agonist mediated effects are true LXR effects since they are abrogated by LXR silencing. Up-regulation of PDK4 and decrease in PDC activity in muscle have been associated with development of diabetes [143]. The effect in adipocytes, however, might be different since it might decrease FFA output from adipocytes, diminishing deleterious effects of increased circulating levels of FFA connected to a increased lipolysis. The possible effect of LXR activation in WAT in general will be discussed more in detail in paragraph 5.

4.3 PAPER III

In paper III, we have studied the role of LXR activation on basal human adipocyte lipolysis and described a potential mechanism for LXR action in this pathway. As described above, lipolysis is one of the central events in adipocyte metabolism. The process of lipolysis is tightly regulated in order to ensure a good supply of energy to other tissues in the form of lipids when needed. At the same time, it is important to keep the rate of lipolysis at an appropriate level so that energy stores will not be wasted at incorrect times. This balance is disrupted in obesity, where the basal lipolysis is increased, partly due to increased adipocyte size. There is a clear connection between augmented levels of circulating FFAs and insulin resistance, and thereby type 2 diabetes.

When we stimulated *in vitro* differentiated human adipocytes and murine 3T3-L1 cells with the LXR agonist GW3965, the release of glycerol was increased. Since the end

products of lipolysis are one molecule of glycerol and three FFAs, and glycerol cannot be utilized within the adipocytes, levels of glycerol are an appropriate measurement of lipolysis. We could also show that the LXR-effect of lipolysis is independent of re-esterification, since the relative up-regulation of lipolysis by LXR activation was similar in cells treated with an inhibitor of re-esterification and control cells. Next, we wanted to elucidate the pathway behind the increased lipolysis. We performed microarray analysis on RNA from human adipocytes, differentiated *in vitro* and treated with GW3965 for 24h. We analyzed cells from five different subjects; details on the statistical analysis have been described in section 3.10. The results from the microarray showed that around 6% of all genes represented on the microarray ChIP was affected by the LXR activation and among the genes involved in lipolysis, 25% of the genes were affected. These genes are listed in table 1 in paper III.

To elucidate the pathway behind the LXR-related up-regulation of lipolysis and confirm the results from the microarray, we performed qRT-PCR measurements on samples from adipocytes treated with GW3965 or vehicle for 6, 12 or 24 hours. We could confirm several genes indicated as potential candidates from the microarray analysis and also showed that the effect was rather quick, the mRNA levels of several genes were significantly altered after only 6 or 12 hours of GW3965-stimulation. We saw a dramatic reduction of mRNA levels of HSL, PLIN1, CGI-58 and CIDEC upon treatment with GW3965. However, the expression of PPARγ was not affected by the LXR activation. Of these, we could confirm HSL, PLIN1 and CIDEC on protein level and decided to continue with HSL and PLIN1, two major lipolysis-regulators, potentially regulated by LXR. We studied the localization of both these proteins simultaneously in cells treated with GW3965 or vehicle and could detect a difference in the localization pattern around the lipid droplet. The presence of PLIN1 around the lipid droplet was lower in cells with activated LXR, compared to control. Also, the ratio of HSL/PLIN1 was higher. A higher level of HSL combined with a lower amount of PLIN1 on the rim of the lipid droplet could increase lipolysis. Next, we studied if HSL phosphorylation and activity is affected by the LXR activation.

When HSL is phosphorylated by PKA, lipolysis is affected. We used Western Blot to investigate the phosphorylation status of some HSL-phosphorylation sites known to affect lipase activity. We could conclude that LXR does not affect HSL phosphorylation, at least not on the sites investigated here, since the phosphorylated forms of HSL were down-regulated to the same extent as total HSL protein. Using the specific HSL inhibitor BAY we could show that LXR's effect on lipolysis is dependent on functional HSL, since the up-regulation of glycerol release is abolished when HSL is inhibited in human *in vitro* differentiated adipocytes.

Since the data above indicates that HSL activity is most probably unaffected by LXR activation and the relative down-regulation of PLIN1 protein compared to HSL is more dramatic, we hypothesized that PLIN1 might be the main factor in LXR-mediated lipolysis. We knocked down PLIN1 in human adipocytes using RNAi and measured the levels of glycerol release. As expected, a diminished expression of PLIN1 caused an increased basal lipolysis. However, we could show that the LXR-effect was abolished in cells lacking PLIN1. This indicates that the down regulation of PLIN1 might be the main mechanism for LXR-mediated up regulation of lipolysis.

Since LXR acts as a transcription factor, we hypothesized that LXR could bind to the promoter of PLIN1. In order to investigate this, we performed ChIP assays. We applied a bioinformatical search using the MatInspector software to search for potential LXREs in the PLIN1 promoter, 3.5 kbp upstream of the transcription start site. However, we could not detect any LXREs using the MatInspector software. Instead, we designed nine primer pairs. The amplified sequences overlap potential PPARγ response elements (PPREs), RXR sites or represent the most proximal regions of the promoter. We compared the enrichment of LXR on the different promoter fragments in material from adipocytes treated with GW3965 or vehicle and could show that LXR activation lead to a higher degree of enrichment of LXR binding on the most proximal parts of the PLIN1 promoter. We also performed ChIP using an antibody against polymerase II (Pol II). The results indicate that there is less binding of Pol II to the most proximal parts of the PLIN1 promoter, in line with the diminished transcription of PLIN1 determined by qRT-PCR.

We also wanted to study if LXR could bind directly to the promoter of HSL, using a similar approach. We could identify two weak LXREs, represented in primer pair 1 and 2. However, we could not detect LXR binding to these sites. Instead, we could show enrichment of LXR to the most proximal parts of the HSL promoter, were the MatInspector software had detected several potential RXR sites. Also, the recruitment of Pol II to the HSL promoter was decreased in cells treated with GW3965, compared to control. This is similar to the results seen with PLIN1 and is in line with the decreased mRNA expression. LXR binding to the promoter of the well-established LXR gene ABCG1 was also enriched upon LXR activation. Furthermore, we show that LXR's heterodimerization partner, RXR, is also recruited to the LXR binding regions of PLIN1 and HSL upon LXR stimulation in human adipocytes. It should be noted that the relative enrichment of LXR to PLIN1 was stronger than the enrichment of LXR to HSL upon LXR activation.

We show that LXR binds to the promoters of both PLIN1 and HSL and that this binding leads to a negative gene regulation, supported by the Pol II data and mRNA and protein measurements. In general, LXREs have mostly been associated with positive gene regulation; however there are some reports indicating that LXR can suppress gene expression via interactions with cofactors and other transcription factors [96, 144]. We do not know if LXR binds directly to the promoters and/or if a cofactor is involved, however the kinetic mRNA data and ChIP results indicates that LXR regulates gene expression of HSL and PLIN1 through direct recruitment to the respective promoters of the genes.

Both LXRα and LXRβ are expressed in adipocytes. To elucidate which isoform is responsible for the up-regulation of lipolysis in human adipocytes, we performed isoform-specific knockdowns using siRNA and measured expression of PLIN1 and glycerol release. We could show that although cells lacking LXRβ show a similar PLIN1-expression upon GW3965-stimulation, the down-regulatory effect of LXR activation is much weaker in cells lacking LXRα, but not in cells with diminished expression of LXRβ. This indicates that LXRα is the main isoform mediating the effects of LXR activation on lipolysis in human primary adipocytes.

In this paper, we show that activation of LXRα increases basal lipolysis in human and murine adipocytes in culture. Microarray analysis indicated that 25% of lipolysis-related genes are affected by stimulation with the LXR agonist GW3965 and of these, we suggest that the down-regulation of PLIN1 is the most important factor. We propose that LXR binds to the promoter of PLIN1 upon LXR activation, thereby repressing PLIN1 expression. The localization of PLIN1 and HSL around the lipid droplet is also affected by GW3965, and furthermore, knockdown of PLIN1 abolished LXR's up-regulatory effect on glycerol release in human adipocytes. Through isoform-specific knockdown studies, we show that LXRα is the major isoform in LXR-related up-regulation of lipolysis.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

5.1 **PAPER I:** We show that CIDEA is involved in lipid oxidation in adipose tissue and the loss of fat mass associated with cancer cachexia. The ability of CIDEA to inactivate PDC, thereby switching the substrate oxidation in human adipocytes from glucose to lipids contributes to this process.

5.2 **PAPER II:** We propose that LXR is an important regulator for substrate oxidation in both human and murine adipocytes. LXR activation inactivates the PDC, resulting in an increased lipid oxidation and decreased glucose oxidation.

5.3 **PAPER III:** We suggest a new role for LXRα in the regulation of basal lipolysis in human and murine adipocytes. Ligand-mediated activation of LXR increases lipolysis, at least in part through binding to the promoter of PLIN1 and down-regulation of PLIN1 expression.

There is still some controversy on the general role of CIDEA in adipocytes and also regarding the localization of CIDEA in human adipocytes. It could be appealing to study the cellular localization of CIDEA further, and also investigate whether post-translational modifications might affect the localization, and thereby function, of the protein. Further studies of CIDEA are hampered by the lack of a known activator of the protein, which would be very helpful if one postulates that CIDEA can affect transcription of for example PDK1 and PDK4.

The role of CIDEA in adipocyte metabolism in general and also in cancer cachexia needs be evaluated further. There might be a co-regulation of ZAG and CIDEA with effects on catabolism and/or cancer cachexia in human adipose tissue. Furthermore, it could be interesting to investigate the mechanism behind the down-regulation of CIDEA in obesity and the normalization of CIDEA expression upon weight loss.

In paper II and III, the role of LXR in human adipocytes has been investigated. As described in the introduction, the role of LXR in adipocyte metabolism is puzzling due to controversial data from multiple laboratories. We suggest that LXR activation can increase both lipolysis and β-oxidation. This is in line with previous mouse model studies, where LXR agonist administration to mice resulted in smaller adipocytes and higher levels of glycerol in circulation, indicating that both breakdown and utilization of lipids is stimulated by LXR activation. These two processes might be coupled since an up-regulation of β-oxidation within the adipocyte could serve as a protective mechanism against the deleterious effects of increased FFA in circulation. If the adipocytes can metabolize a larger amount of the FFA, this could decrease the negative effects of circulating FFAs in other tissues, such as skeletal muscle and liver. Also, since WAT inflammation is coupled to increased circulating FFAs, an up-regulation of β-oxidation in adipocytes might also be important in the sense of adipocyte inflammation. LXRs role in adipocyte lipogenesis is debated, as described in the introduction. In the studies presented in this thesis, we have not showed measurements

of lipogenesis. However, since LXR is shown to increase both lipolysis and β-oxidation, it seems unlikely that LXR activation would also increase lipogenesis in human adipocytes. The concept of manipulating white adipocytes to be more like brown adipocytes, adaptive thermogenesis, has been debated in the last few years. An increased fatty acid oxidation could be of great interest in this model, since the aim is to create a metabolic shift in white adipocytes from fat storage to lipid utilization. If the fatty acid oxidation could be increased by pharmacological means in the adipocyte itself, the burden of the FFA produced by lipolysis on other organs could be eased.

We show that both LXR and RXR are recruited to two important genes in adipocytes, HSL and PLIN1, thereby repressing gene expression. This is an unusual finding in LXR biology, since LXREs have mostly been implicated in positive gene regulation. Also, we could not identify any LXREs in the regions of the promoters to which LXR and RXR are recruited. This might indicate indirect binding of LXR to the promoters of PLIN1 and HSL, or the presence of unknown LXREs, perhaps more strongly related to negative regulation of transcriptions. However, there are potential binding sites for nuclear factor kappa-B (NF-κB) in the regions where LXR is enriched on the promoters of both HSL and PLIN1. NF-κB has previously been shown to play a role in regulation of lipolysis in human adipocytes [145], and LXR can interfere with NF-κB signalling in macrophages [146]. Further studies on the potential role of the NF-κB site in the PLIN1 promoter will be of great interest in the future.

As mentioned in the introduction, NRs have been regarded as promising targets for development of new pharmaceutical treatment. LXR was considered a very attractive candidate since it promotes reverse cholesterol transport and limit inflammation in macrophages. However, administration of LXR agonist to mice induced lipogenesis in liver and raised triglyceride levels. The data presented in this thesis indicate that further research on this is needed, and that isoform- and tissue specific agonists would be of great interest in this aspect. Previous reports have indicated that LXRα might be the main isoform regulating adipocyte metabolism [108, 114, 126] and in a previous study from our group, expression levels of LXRα was higher in WAT from obese women, compared to lean [98]. On this note, our data in paper III indicates that up-regulation of LXRα could have deleterious effects by increasing lipolysis, a process strongly linked to the development of insulin resistance and type 2 diabetes. It might be more attractive to up-regulate LXRβ, if the positive effects of LXR up-regulation on fatty acid oxidation could be maintained, without the deleterious effects on lipolysis. It should be noted, however, that we have not investigated isoform regulation in the aspect of substrate oxidation, and that mice lacking LXRβ show a different adipocyte phenotype, compared to wild type mice [111]. The generation of isoform- and tissue-specific knockout mice could be of great interest in this concept, since it would open to possibilities of investigating the role of each isoform in the adipose tissue specifically. As LXRβ is ubiquitously expressed, the effects seen in complete knockout mice might also be related to LXRs role in other tissues.

Recently, a genome-wide profiling analysis of LXR, RXR and PPARα in mouse liver was published [147]. In this paper, the authors have performed ChIP sequencing of these three NRs and compared the data sets. This approach would be very interesting to apply in our human adipocyte system for identification of target genes regulated by

LXR. Furthermore, given the differential role for LXR isoforms, performing this analysis with isoform-specific LXR agonists or human cells lacking one of the isoforms would greatly add to our understanding.

It has been proposed that LXRs are constitutively activated in adipocytes since these cells contain large deposits of cholesterols – a potential source of natural ligands. However, others, and we, have shown that LXRs can be activated to a high degree both *in vivo* and *in vitro* by endogenous and exogenous treatments. We have used the artificial LXR agonist GW3965 in our studies. Another alternative would have been TO91317, a readily used agonist. However, this agent is not specific for LXR since it also activates two other nuclear receptors, the farnesoid x receptor (FXR) and the pregnane x receptor (PXR) [148, 149]. On the other hand, we have used TO91317 as a parallel treatment in our systems and have seen similar results. Also, according to our own, unpublished results, neither FXR nor PXR is expressed in human adipocytes. It would be very interesting to find the endogenous activator/activators of LXR in adipocytes, and to study if this potential substance/substances activates both isoforms symmetrically and at the same time, or if there is a dynamic regulation of the LXR isoforms, as seen in adipocyte differentiation, where LXRα is up-regulated while LXRβ is unchanged.

There are three splice variants of LXRα [99], as mentioned in the introduction. Preliminary results from our laboratory indicate that one of the less abundant splice variants might be present in human fat cells. It is not known, to date, what the respective roles of these splice variants are. This would be attractive to explores, since the role of LXR in adipocytes appears to diverge from the role of LXR in other cell types

In a recent paper from our group, CIDEA was shown to interact with LXR [93]. CIDEA binds LXR both *in vitro* and *ex vivo* and can repress LXR-regulated reporter constructs in 3T3-L1 cells. These findings might seem confusing since both LXR and CIDEA can enhance substrate oxidation by up-regulation of PDK4 (paper I and II) and also, both proteins have been shown to up-regulate lipolysis (paper III and [82]). However, the physiological importance of the interaction of CIDEA is not known, and since LXR might affect several aspects of adipocyte metabolism, additional studies are required to see which aspects of LXR function that might be affected by a potential crosstalk between CIDEA and LXR. It would be very interesting to investigate the interaction between CIDEA and LXR further, and include studies in human cells.

The cell studies in this thesis have been performed in *in vitro* differentiated human adipocytes and 3T3-L1 cells. As described in section 3.2, these systems have their drawbacks. It could therefore be an attractive approach to try to confirm the data obtained in this thesis in a different cell system, namely mature human adipocytes. This approach might be more physiological, but is also technically challenging. The mature adipocytes, isolated fresh from human WAT, are fragile and difficult to keep in culture for long periods of time. We have nonetheless recently adapted a technique where we can keep the cells alive in culture for up to 72 hours. These cells respond well to lipolysis stimulation and stimulation with an LXR agonist. According to unpublished data, the mRNA expression of both CIDEA and LXR might be different in these cells.

Also, the effects of LXR activation might differ in these cells, compared to *in vitro* differentiated adipocytes.

In conclusion, we have shown that LXR and CIDEA can regulate important functions of human adipocyte metabolism. This is of importance for states with disturbed AT function, such as obesity and cancer cachexia. The findings presented in this thesis open the field for many more potential research projects, including elucidating the possible co-regulation of CIDEA and ZAG, further in-depth analysis of LXRs role in adipocytes using different techniques such as ChIP-sequencing and isoform- and/or tissue-specific knockout mice and further examinations of protein-protein interactions between LXR and CIDEA. It would also be of interest to examine the role of LXR and CIDEA in human mature adipocytes. Since states affecting the AT, foremost obesity but also cachexia, are major health problems it is of great substance to understand adipocyte biology and how it is affected by states of health and disease.

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