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DIETARY EFFECTS ON GENE REGULATION AND FUNCTION IN HUMAN ADIPOSE TISSUE IN OBESITY

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ABSTRACT

Obesity is an excess of body fat that develops from both genetic and environmental factors, leading to a disturbed balance between energy intake and energy expenditure. It is closely associated with insulin resistance, type 2 diabetes, hyperlipidemia and atherosclerosis. Its increasing prevalence has reached epidemic proportions worldwide.

Adipose tissue produces and secretes a number of important factors that are involved in the regulation of adipose tissue metabolism. These factors could also be of importance in the development of insulin resistance. Previous research has shown that a number of genes seem to be involved in both the development of obesity and different functions of adipose tissue. It is not clear if, and how, weight loss or the energy content and the macronutrient composition of the diet affect the expression of these genes. More knowledge about intracellular pathways and mechanisms underlying the metabolic regulation in adipose tissue is essential for a better understanding of the pathogenic importance of this tissue.

The aims of this thesis were to define dietary effects on the secretory function of, and gene expression in, human adipose tissue, to identify and characterize novel nutrient-sensitive candidate genes in obesity and to study the functional role of one of these novel genes *in vitro*.

In **Papers I** and **II**, we investigated the influence of weight loss and macronutrients on protein secretion and gene regulation in white adipose tissue (WAT). Forty women were randomized to two different ten-week hypoenergetic diets. In **Paper I**, the changes in secretion were compared with the changes in circulating levels and adipose tissue mRNA expression. The effect of energy restriction on different secreted proteins varies. There were no differences between the diet groups and we therefore conclude that the energy supply *per se* and not the macronutrient composition is of importance for the regulation of the protein secretory function and gene expression in human adipose tissue, at least during energy restriction. Furthermore, there was no difference in weight loss between the diet groups.

In **Paper II**, we used microarrays to study the changes in gene expression induced by the two hypoenergetic diets. Key results were confirmed using real-time quantitative PCR. There were no differences between the diet groups, although in both groups, marked effects were obtained on genes regulating the production of polyunsaturated fatty acids and genes regulating obesity in experimental models. Cell death-inducing DFFA-like effector A (CIDE-A) was the most up-regulated gene after weight loss.

In **Paper III**, we investigated the function(s) of CIDE-A in human adipose tissue. We found that CIDE-A is expressed in human, as opposed to mouse, WAT. The gene appears to be protective against obesity and several features of the metabolic syndrome and an important regulator of basal lipolysis in human WAT, most likely through interactions with tumour necrosis factor α (TNF-α) signalling and/or perilipin.

In **Paper IV**, we tried to elucidate the possible mechanism(s) by which CIDE-A regulates basal lipolysis in white adipocytes. We studied effects of over-expression of CIDE-A and interaction between CIDE-A and different nuclear receptors. We found that CIDE-A interacts with the liver X receptors (LXRs) in human adipocytes. We also found that activation of the LXRs stimulates lipolysis, possibly via down-regulation of perilipin. We suggest that CIDE-A mediates its antilipolytic effect via modulation of LXR-regulated gene expression.

LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals (I-IV):

I. **Elisabet Arvidsson**, Nathalie Viguerie, Ingalena Andersson, Camilla Verdich, Dominique Langin and Peter Arner. Effects of Different Hypocaloric Diets on Protein Secretion from Adipose Tissue of Obese Women.
Diabetes 2004;53(8):1966-71

II. Ingrid Dahlman*, Kristina Linder*, **Elisabet Arvidsson Nordström**, Ingalena Andersson, Johan Lidén, Camilla Verdich, Thorkild IA Sørensen and Peter Arner. Changes in Adipose Tissue Gene Expression with Energy-Restricted Diets in Obese Women.
American Journal of Clinical Nutrition 2005;81(6):1275-85
**These authors contributed equally.*

III. **Elisabet Arvidsson Nordström**, Mikael Rydén, Emma C Backlund, Ingrid Dahlman, Maria Kaaman, Lennart Blomqvist, Barbara Cannon, Jan Nedergaard and Peter Arner. A Human-Specific Role of Cell Death-Inducing DFFA (DNA Fragmentation Factor-α)-Like Effector A (CIDEA) in Adipocyte Lipolysis and Obesity.
Diabetes 2005;54(6):1726-34

IV. Jurga Laurenčikienė, **Elisabet Arvidsson Nordström**, Amanda Pettersson, Per Antonson, Michel Tujague, Knut R Steffensen, Susanne Akterin, Mikael Wirén, Jan-Åke Gustafsson, Bart Staels, Mikael Rydén and Peter Arner. Interactions Between CIDEA and LXR in Human Fat Cells of Possible Importance for Lipolysis.
Submitted to FASEB Journal

CONTENTS

LIST OF ABBREVIATIONS

Abhd5	Abhydrolase domain containing 5
AC	Adenylyl cyclase
ANP	Atrial natriuretic peptide
AR	Adrenergic receptor
ATGL	Adipose triglyceride lipase
ATP	Adenosine 5'-triphosphate
BAT	Brown adipose tissue
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BMR	Basal metabolic rate
β2MG	Beta 2-microglobulin
cAMP	Adenosine 3',5'-cyclic monophosphate
cDNA	Complementary DNA
CIDE	Cell death-inducing DFFA-like effector
CNS	Central nervous system
C_t	Cycle threshold
CVD	Cardiovascular disease
DAG	Diacylglycerol
DFF	DNA fragmentation factor
E%	Energy percent
FA	Fatty acid
FFA	Free fatty acid
FSP27	Fat-specific protein 27
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Glycemic index
GL	Glycemic load
GLUT	Glucose transporter
G-protein	GTP-binding protein
GST	Glutathione S-transferase
GTP	Guanosine 5'-triphosphate
$G\alpha_i$	GTP-binding protein, inhibitory α-subunit
$G\alpha_s$	GTP-binding protein, stimulatory α-subunit
HDL	High-density lipoprotein
hMSC	Human mesenchymal stem cell
HMW	High molecular weight
HOMA	Homeostasis model assessment
HSL	Hormone-sensitive lipase
IL	Interleukin
IR	Insulin receptor
JNK	c-jun amino terminal kinase
LXR	Liver X receptor
LXRE	LXR response element
MAG	Monoacylglycerol
MAPK	Mitogen-activated protein kinase

MCP-1	Monocyte chemoattractant protein 1
mRNA	Messenger RNA
NF-κB	Nuclear factor-κB
NR	Nuclear receptor
PAI-1	Plasminogen activator inhibitor type 1
PAL	Physical activity level
PCOS	Polycystic ovary syndrome
PDE	Phosphodiesterase
PKA	cAMP-dependent protein kinase A
PLA	Processed lipoaspirate
PLIN	Perilipin
PPAR	Peroxisome proliferator-activated receptor
REE	Resting energy expenditure
RIP140	Receptor interacting protein 140
RNA	Ribonucleic acid
RNAi	RNA interference
RT-qPCR	Real-time quantitative polymerase chain reaction
RXR	Retinoid X receptor
siRNA	Small interfering RNA
SNS	Sympathetic nervous system
SREBP	Sterol regulatory element-binding protein
TAG	Triacylglycerol
TDEE	Total daily energy expenditure
TGF	Transforming growth factor
TNF-α	Tumour necrosis factor α
UCP-1	Uncoupling protein 1
WAT	White adipose tissue
WHO	World Health Organization
wt	Wildtype
Y2H	Yeast 2-hybrid

1 INTRODUCTION

This thesis aims to contribute to the field of obesity research and more specifically to investigate weight loss-induced effects on adipose tissue gene regulation and function. The prevalence of obesity has reached epidemic proportions worldwide and is constantly increasing. Obesity increases the risk for several severe disorders, such as type 2 diabetes and atherosclerosis, and it is therefore important to gain more knowledge about how it can be prevented and treated. Section 1 is a review of literature relevant for the studies in the individual papers and the discussion in the thesis. Some terms will be briefly mentioned, some described in more detail.

1.1 OBESITY

Obesity is an excess of body fat, often defined as a body mass index (BMI, weight in kg/(height in metre)^2) equal to or greater than 30 kg/m^2. BMI is a height-independent, although not ethnicity-independent, measure of weight that gives an indirect estimation of body adiposity (1). Extremely old, extremely tall or very muscular individuals are exceptions to the reliability of BMI (1). Furthermore, the BMI cutoffs used in the World Health Organization (WHO) classification of overweight and obesity (summarized in ***Table 1***) are not well suited for assessment of obesity in children and adolescents and instead several different age- and gender-specific definitions are currently in use (2).

Table 1. *WHO classification of overweight and obesity*

Classification	**BMI**
Underweight	<18.5
Normal-weight	18.5-24.9
Overweight	≥25
Pre-obese	25.0-29.9
Obese class I	30.0-34.9
Obese class II	35.0-39.9
Obese class III	≥40.0

1.1.1 Prevalence

In spite of more information and better awareness about obesity and its consequences, the prevalence of obesity continues to rise in both developed and developing countries. In 2002, it was estimated that at least 315 million people world-wide were obese and another 750 million people were overweight based on the WHO classification (3). The same year, 65 % of American adults were overweight, of which 30 % were obese (4). The prevalence of obesity in Sweden is low by international comparison, but is increasing in both children, adolescents and adults (5). A recent report from Statistics Sweden (SCB) showed that the prevalence of obesity in Sweden has doubled in 25 years and that 50 % of Swedish men and more than one third of Swedish women are now overweight or obese (6). In many developing countries undergoing rapid economic development, increased prevalence of obesity often

coexists with chronic undernutrition in the same population (e.g., in Brazil (7)). The increasing industrialization, urbanization and use of labour-saving devices and cars occurring in most countries around the world is associated with 'obesogenic' (8), weight gain-promoting, changes in diet and behaviour, in particular, an increased intake of energy-rich foods and a more sedentary lifestyle (e.g., in Egypt (9)). The global obesity epidemic (or 'pandemic' (8)) affects all age groups, not the least children and adolescents (10).

1.1.2 Aetiology of Obesity

Obesity develops from a disturbed balance between energy intake and energy expenditure. Differences in resting energy expenditure (REE) between individuals have a minor effect on body adiposity or the development of obesity (11). The main influences on equilibrium levels of body fat can be divided into biological, behavioural and environmental factors (8). *Biological influences* are age, gender, hormonal factors and genetics, whereas *behavioural influences* are e.g., willpower, habits, emotions, attitudes, beliefs and knowledge. *Environmental influences* are factors that determine the amount and type of food eaten and the amount and type of physical activity taken, e.g., selection and prices of foods in grocery stores and canteens, school attitude to sports, labour-saving devices and availability of local gyms. From studies of identical and fraternal twins, raised together or apart, and adopted children and their biological and adoptive parents, it has been concluded that a large part of the variance in body size and body composition can be attributed to genetic factors (12-14). Several studies have shown associations between polymorphisms in genes encoding adipose tissue-produced molecules or adiposity signals and obesity phenotypes (reviewed in (15)). However, household and physical environment play important roles in establishing food and exercise habits (16). Populations who migrate from a traditional to a westernized environment often adopt westernized behaviours and habits and increase their prevalence of obesity, type 2 diabetes and cardiovascular disease (CVD) (17,18).

1.1.3 Obesity-Related Complications

Obesity, in particular abdominal obesity, is associated with a vast number of different complications, summarized in ***Table 2*** and reviewed in (19). This has personal consequences for obese individuals and financial consequences for the health and medical service. Disability pension is more common among obese and the obese spend more days on sickness absence (20,21) and are at an increased risk of premature mortality compared with normal-weight individuals (22,23). Obesity is associated with impairments in several aspects of quality of life, e.g., lack of sexual desire (24). Some obese people even have worse mental well-being than chronically ill or injured patients, such as cancer survivors (25). Obesity is considered a chronic disease and is treated with behavioural therapy, pharmaceuticals, bariatric surgery, diet, exercise or a combination of different approaches (reviewed in (26)).

Table 2. *Obesity-related complications*	
Cardiovascular	Hypertension
	Atherosclerosis
	Stroke
Metabolic	Type 2 diabetes
	Dyslipidemia
	Glucose intolerance
	Insulin resistance
	Goitre
Respiratory	Sleep apnea
	Pickwick syndrome
Gastrointestinal	Gallbladder disease
	Fatty liver
	Gastroesophageal reflux
Urogenital	Urine incontinency
	Infertility
	Prostate hyperplasia
	Menstrual disturbance
	Polycystic ovary syndrome (PCOS)
	Pregnancy toxicosis
	Complications during delivery
Other	Arthrosis
	Back pain
	Complications in relation to surgery
	Cancer
	Excessive sweating
	Depression

1.1.4 White and Brown Adipose Tissue

Adipose tissue is a heterogeneous tissue that consists of several different cell types, predominantly adipocytes (fat cells), held together by loose connective tissue that is vascularized and innervated. There are two main types of adipose tissue in mammals, termed brown adipose tissue (BAT) and white adipose tissue (WAT). There are several differences between WAT and BAT, e.g., the structure of the fat cells and the function, regulation and anatomical distribution of the tissues (for a thorough review of BAT, see (27)): The adipocytes in WAT are optimized for energy storage and mature adipocytes consist of a large lipid droplet, a small nucleus displaced to the side and few mitochondria. WAT is 95 % triacylglycerol (TAG). BAT, but not WAT, expresses the mitochondrial protein uncoupling protein 1 (UCP-1) through which it is able to generate heat in response to cold, e.g., in hibernating animals, rodents and newborns (including humans). Brown adipocytes have large numbers of mitochondria, a central nucleus and small lipid droplets. BAT can be found around the kidneys and aorta, between the scapulae, near the sternum and around the neck,

whereas subcutaneous and visceral fat are the largest WAT depots. WAT is the predominant type in humans, whereas adult humans have very little BAT (28,29). BAT is therefore considered to be of little clinical importance in humans.

1.2 INSULIN RESISTANCE AND THE METABOLIC SYNDROME

Insulin regulates several important metabolic processes in the body, e.g., glucose uptake, lipolysis (the breakdown of TAG to free fatty acids (FFAs) and glycerol) and hepatic gluconeogenesis (i.e., glucose synthesis). Reduced sensitivity to insulin, i.e., insulin resistance, results in lowered activity of these processes and/or compensatory hyperinsulinaemia. Insulin resistance is present in type 2 diabetes, as well as in conditions with increased risk for the development of this disease, such as obesity, familial combined hyperlipidaemia and polycystic ovary syndrome (PCOS). Insulin resistance precedes overt diabetes by several years.

The term 'Syndrome X' was first described by Reaven in 1988 as a cluster of insulin resistance, hyperinsulinaemia, glucose intolerance, triglyceridaemia and decreased levels of high-density lipoprotein (HDL) cholesterol, increasing the risk of CVD (30). In 1999, the WHO defined the 'metabolic syndrome' as glucose intolerance, impaired glucose tolerance or diabetes and/or insulin resistance together with ≥2 of the following criteria: hypertension, triglyceridaemia, low HDL cholesterol, abdominal obesity or microalbuminuria (31). Two years later, the Adult Treatment Panel III (ATP III) of the National Cholesterol Education Program (NCEP) selected five criteria for the diagnosis of the metabolic syndrome: abdominal obesity, elevated fasting glucose, hypertension, low HDL cholesterol and triglyceridaemia, where the metabolic syndrome was considered to be present if ≥3 criteria were met (32). These two definitions have been compared and discussed (33). An extended version, the 'insulin resistance syndrome', including more metabolic abnormalities that increase the risk of clinical manifestations associated with insulin resistance, has recently been described (34). The use of several different definitions has caused confusion and made it difficult to compare prevalence numbers in different studies. An internationally accepted definition is therefore necessary (35) and the International Diabetes Federation (IDF) has recently produced a set of criteria suggested to be used as a new world-wide definition (33).

1.3 LIPOLYSIS

Lipolysis (reviewed in (36-39)) is the hydrolysis of TAG to FFAs and glycerol, controlled by hormones, the sympathetic nervous system (SNS), nutritional status, genetic variability and disease. Lipolysis can be either basal, i.e., spontaneous, or hormone-stimulated. The enzyme adenylyl cyclase (AC) resides in the cell membrane of the adipocyte and is coupled to adrenergic receptors (ARs) via trimeric guanosine 5'-triphosphate (GTP)-binding proteins (G-proteins). Three β-ARs (β_1, β_2 and β_3) and one α-AR (α2A) have been identified in human adipocytes. The β-ARs are associated with the stimulatory form of the G-protein, G_s. Ligands (catecholamines, e.g., adrenaline and noradrenaline) that bind to the β-ARs in the adipocyte cell membrane activate AC via

translocation of the α_s-subunit from the $\beta\gamma$-subunit of the G-proteins. This causes the transformation of adenosine 5'-triphosphate (ATP) to adenosine 3',5'-cyclic monophosphate (cAMP), a process that is inhibited by insulin via activation of phosphodiesterase 3B (PDE3B). The increased intracellular concentration of cAMP stimulates cAMP-dependent protein kinase A (PKA), that phosphorylates and activates hormone-sensitive lipase (HSL) and perilipin (PLIN), leading to the hydrolysis of TAG. β_2-AR is the most important β-AR in humans. In addition, human, but not murine, fat cells express the anti-lipolytic α_2A-AR. This receptor is coupled to $G\alpha_i$ and inhibits AC. This mechanism allows catecholamines to further fine-tune lipolysis. Monoacylglycerol lipase (MGL), which is not hormonally regulated, completes the hydrolysis by acting on monoacylglycerol (MAG).

The FFAs from the lipolysis diffuse into the blood via fatty acid transport protein (FATP), where they bind to the carrier protein serum albumin. The FFAs are transported to tissues where they are used as fuel. Most tissues can oxidize fatty acids (FAs) and only the brain uses glucose as its main fuel. Lipolysis in adipose tissue regulates the release of FFAs into the plasma and therefore also the body's supply of energy from fat. FFAs are not only used for oxidation to yield energy, but also in lipoprotein synthesis. Glycerol is a substrate for gluconeogenesis in the liver and cannot be re-esterified in the adipocytes, since fat cells lack the enzyme glycerol kinase and therefore the ability to synthesize glycerol-3-phosphate from glycerol. Instead, glycerol is transported to the liver via an adipose-specific glycerol channel aquaporin isoform (AQPap7). The rate of lipolysis increases when FAs are for some reason (e.g., exercise, fasting) required as a metabolic substrate.

During the last few years, the picture of lipolysis has been extended by new discoveries, e.g., the identification of a new lipolysis pathway regulated by natriuretic peptides (40) and that of the enzyme adipose triglyceride lipase (ATGL) (41-43). Lipolysis is summarized in ***Figure 1*** and some of its components are described more in detail in the following text.

1.3.1 Hormone-Sensitive Lipase

HSL (44) is a well characterized enzyme that hydrolyzes both TAG and diacylglycerol (DAG) with an enzyme activity that is ten-fold more specific for DAG than TAG (45). In human fat cells, HSL participates in the regulation of basal lipolysis, although is most important for lipolysis stimulated by catecholamines or natriuretic peptide (46,47). Stimulated lipolysis requires the translocation of HSL from the cytosol to the lipid droplet (48). HSL is phosphorylated by PKA and murine studies suggest that HSL could possibly be activated also via other pathways (49). The human HSL promoter was characterized in 1997 (50). HSL knockout mice are sterile and have enlarged fat cells in both BAT and WAT, although are not obese (51,52). HSL was for a long time the only enzyme known to hydrolyze TAG in mammalian adipose tissue, but the finding that HSL knockout mice retain some adipocyte lipolysis and accumulate DAG suggested that additional enzymes are involved in the breakdown of TAG (51-53).

1.3.2 Adipose Triglyceride Lipase

ATGL (also known as desnutrin (42) and calcium-independent phospholipase A2-ζ (43)) was recently identified (41). ATGL primarily hydrolyzes the first ester bond of TAG (41). It has been shown that HSL and ATGL together are responsible for more than 95 % of the TAG hydrolase activity in murine WAT (54). In murine adipocytes, ATGL is up-regulated by fasting and down-regulated in the fed state, i.e., by insulin, and by the cytokine tumour necrosis factor α (TNF-α) (55). The murine ATGL promoter region has been characterized and the ATGL gene was found to be transcriptionally regulated by peroxisome proliferator-activated receptor γ (PPAR-γ)-mediated signals (55). ATGL knockout mice are heavier and have more body fat than wildtype (wt) (56). They accumulate ectopic fat in the heart and several other tissues, leading to cardiac dysfunction and death, although have improved glucose tolerance and insulin sensitivity (56). In humans, different ATGL gene polymorphisms are associated with FFA concentrations, TAG concentrations, glucose levels and risk for type 2 diabetes, suggesting that ATGL may be involved in the metabolic syndrome (57). However, in contrast to HSL, ATGL mRNA expression is not regulated by obesity in humans (58). Furthermore, ATGL has recently been shown to primarily regulate basal lipolysis in humans (47).

1.3.3 Perilipin

The human PLIN gene was isolated in 1998 (59). Several years before that, murine PLIN was first identified as the major substrate for PKA (60) and later shown to be adipocyte-specific and localized to the surface of the lipid droplets of differentiated 3T3-L1 adipocytes (61,62). Differential splicing results in two isoforms in adipocytes, PLIN A and B, respectively (63), where PLIN A is the predominant isoform. Less is known about the functions of PLIN B, although it has been shown to be regulated by insulin and catecholamines (64). PLIN is an important regulator of lipolysis in human adipocytes (65). It inhibits basal and promotes PKA-mediated lipolysis (66). The PLIN-interacting protein Abhd5 (abhydrolase domain containing 5), also known as CGI-58, is an important activator of ATGL and it was recently shown that PLIN regulates ATGL (67,68).

PLIN knockout mice are lean, resistant to diet-induced obesity and have elevated basal lipolysis (69,70), whereas over-expression of PLIN A results in decreased lipolysis and therefore increased storage of TAG (71). Based on these results, it has been suggested that PLIN acts as a barrier to hydrolysis by HSL. However, recent studies in 3T3-L1 cells suggest that PLIN functions more as a lipid droplet scaffold than a physical barrier and regulates the trafficking of different lipolytic effectors involved in PKA-mediated lipolysis (68,72). PLIN is expressed on the same peripheral lipid droplets in the adipocytes where activated HSL is present (72). In the basal state, PLIN interacts with Abhd5 and thereby regulates the activity of ATGL (68). Upon phosphorylation, PLIN and Abhd5 dissociate and HSL translocates to PLIN-containing lipid droplets (68). The PKA-induced translocation of HSL is partially dependent on PLIN phosphorylation whereas the lipolytic action of HSL requires phosphorylation of PLIN (68,73).

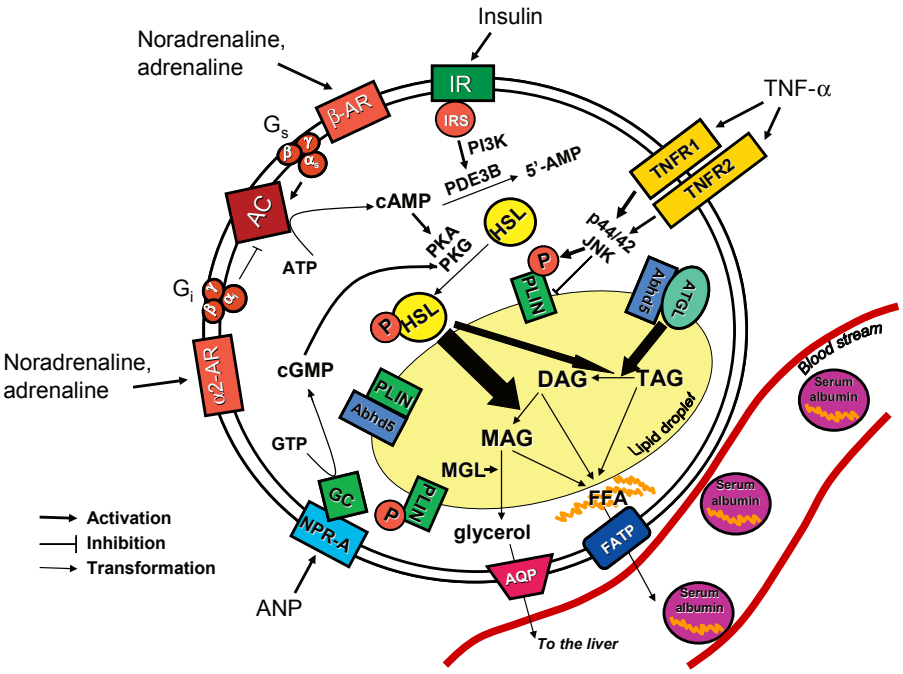

Figure 1. *Lipolysis in human adipocytes. α2-AR, α2-adrenergic receptor; G_i, inhibitory G-protein; AC, adenylyl cyclase; G_s, stimulatory G-protein; β-AR, β-adrenergic receptor; IR, insulin receptor; IRS, insulin receptor substrate; TNF-α, tumour necrosis factor α; TNFR, TNF receptor; JNK, c-jun amino terminal kinase; FATP, fatty acid transport protein; AQP, aquaporin adipose; ANP, atrial natriuretic peptide; NPR-A, natriuretic peptide receptor A; GC, guanylyl cyclase; GTP, guanosine 5'-triphosphate; cGMP, guanosine 3',5'-cyclic monophosphate; ATP, adenosine 5'-triphosphate; cAMP, adenosine 3',5'-cyclic monophosphate; PI3K, phosphatidyl inositol-3 kinase; PDE3B, phosphodiesterase3B; 5'-AMP, adenosine monophosphate; PKA, cAMP-dependent protein kinase A; PKG, cGMP-dependent protein kinase I; HSL, hormone-sensitive lipase; PLIN, perilipin; ATGL, adipose triglyceride lipase; Abhd5, abhydrolase domain containing 5; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; MGL, monoacylglycerol lipase; FFA, free fatty acids.*

1.3.4 Lipolysis Regulation

Lipolysis is regulated by several factors, reviewed in (74) and shown in ***Figure 1***. The most important are catecholamines, natriuretic peptides and insulin. The catecholamines adrenaline and noradrenaline are hormones secreted by the adrenal medulla and reach the adipose tissue via the general circulation. Noradrenaline is also a neurotransmitter that is released from the nerve endings of the SNS, innervating the adipose tissue. The catecholamines can have lipolytic or antilipolytic effects in human adipocytes, depending on which AR subtype they interact with (i.e., α_2A- or β-ARs), as previously described. The activation and distribution of the different types of receptors therefore determine the intracellular cAMP levels. Natriuretic peptides, especially atrial natriuretic peptide (ANP), have recently been shown to regulate human adipose tissue lipolysis via a pathway that does not involve insulin, PDE3B or changes in cAMP levels (40). Instead, ANP stimulates lipolysis via a guanosine 3',5'-cyclic

monophosphate (cGMP)-dependent pathway that induces phosphorylation of HSL and PLIN. This is mediated via activation of the ANP receptor (NPR-A), that has intrinsic guanylyl cyclase (GC) activity, and the cGMP-dependent protein kinase I (cGKI or PKG) (75).

Insulin is the major antilipolytic factor in humans. The binding of insulin to insulin receptors (IRs) on the cell-surface of adipocytes causes phosphorylation and activation of PDE3B via IR substrates (IRSs) and the phosphatidyl inositol-3 kinase (PI3K) complex. Activated PDE3B catalyzes the breakdown of cAMP to inactive 5'-AMP (adenosine monophosphate). The activation of PDE3B by acute insulin stimulation therefore causes a decrease in intracellular cAMP and PKA activity, quenching stimulatory signals from β-ARs (76). In contrast, prolonged stimulation of the IRs (such as in type 2 diabetes) results in sensitization of β_2-AR $G\alpha_s$ signalling and enhanced cAMP production (77). However, the elevated levels of insulin also inhibit β-ARs from activating PKA (78).

1.3.5 Clinical Importance

The regulation of lipolysis is disturbed in a number of different disorders, for example obesity (reviewed in (37)) and PCOS (79). This is characterized by an increased basal, but blunted catecholamine-stimulated, lipolysis. Elevated circulating FFA levels caused by enhanced basal lipolysis in obesity could cause perturbations such as insulin resistance and type 2 diabetes (reviewed in (80)). It is not fully known why basal lipolysis is increased in obesity, although increased β_3-AR function and a slightly decreased α_2A-AR function in human visceral fat cells could be two factors (81,82). β_3-AR is, however, of minor importance in humans, compared with rodents (37). Catecholamine resistance is present in abdominal obesity due to low β_2-AR density and the response to these hormones is therefore blunted (83). The production and secretion of TNF-α is enhanced in obesity and this cytokine is therefore an important candidate for increasing basal lipolysis (84).

1.3.6 Regional Differences

The distribution of the body fat is an important determinant of obesity-related complications and adipose tissue metabolism (reviewed in (85)). Most adipose tissue, in obese and non-obese individuals alike, is subcutaneous. Visceral fat (around the viscera, i.e., intraabdominal organs) only constitutes about 500-1000 g, however, is more metabolically active than subcutaneous fat. Hormone-stimulated adipocyte lipolysis is enhanced in visceral adipose tissue in obesity and FFAs from the degradation of TAG in this tissue are drained by the portal vein to the liver (86), which is thought to contribute to the development of insulin resistance (reviewed in (87)). In contrast, FFAs from subcutaneous adipose tissue are drained by the peripheral vein system as shown in ***Figure 2***.

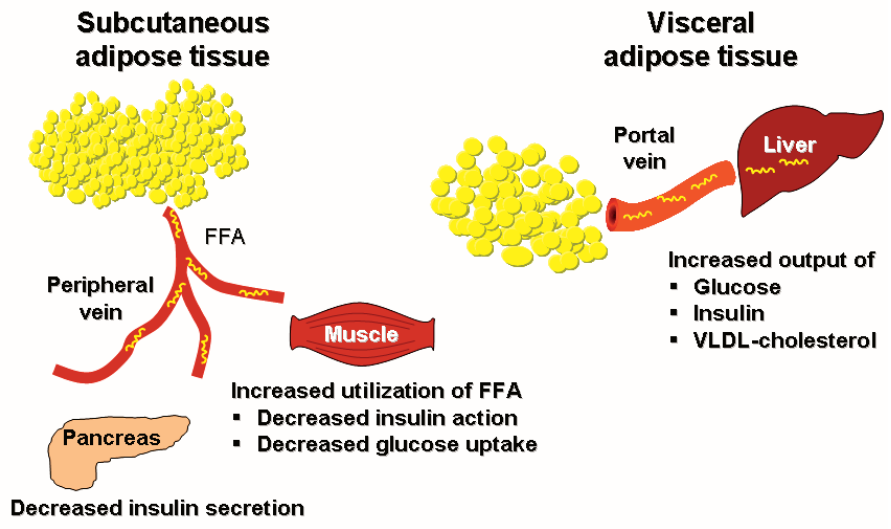

Figure 2. *Regional differences in lipolysis. Free fatty acids (FFA) from lipolysis in subcutaneous and visceral adipose tissue are drained via peripheral veins and the portal vein, respectively. The increased lipolysis in obesity has effects on liver, skeletal muscle and pancreas, as indicated.*

1.4 THE SECRETORY FUNCTION OF ADIPOSE TISSUE

Adipose tissue is an active endocrine organ that produces and secretes a number of important factors with auto-, para- and endocrine action of relevance for e.g., insulin resistance and energy homeostasis. Among these are several cytokines and chemokines (e.g., TNF-α (88), monocyte chemoattractant protein 1 (MCP-1) (89), interleukin (IL)-6 (90) and -8 (91)), hormones (e.g., leptin (92) and adiponectin (93-95)), enzymes (e.g., ACE, angiotensin converting enzyme (96)) and a number of other factors (e.g., plasminogen activator inhibitor type 1 (PAI-1) (97)). The effects of weight loss on protein secretion from adipose tissue were studied in the first paper of this thesis. The six proteins investigated in the paper will be described in this section.

1.4.1 Leptin

Leptin is the gene product of the obese (ob) gene (92) and is primarily produced and secreted by adipose tissue (98). The discovery of leptin opened up a new field in obesity research and leptin was for some time thought to be a potential obesity drug that would solve the obesity problem. Leptin is involved in several important mechanisms in the body, e.g., regulation of appetite, food intake and energy expenditure (99-102). Mutations in the leptin (ob) or leptin receptor (db) gene in rodents result in severe obesity (103,104). In humans, similar single mutations also cause extreme obesity (105,106), which proves that leptin is an important regulator of energy homeostasis also in man. However, such mutations are extremely rare in humans, even in individuals with severe, early-onset obesity (107). Furthermore, the finding that leptin levels are dramatically increased in obese humans suggests that

obesity is a state of leptin resistance (108). Leptin is thought to signal to the brain about the size of the fat depots in the body (109). The leptin mRNA expression and secretion rate are higher in subcutaneous than visceral adipose tissue in both obese and lean individuals, particularly in women (110,111).

1.4.2 Tumour Necrosis Factor α

TNF-α is a cytokine expressed in adipose tissue (88). It is synthesized as a transmembrane protein that can be processed to release a soluble form. The production of the transmembrane form is increased in adipose tissue in obesity (112) and the fact that adipose-derived TNF-α is not released into the systemic circulation (113) suggests that it has mainly auto- or paracrine, rather than endocrine, effects in this tissue.

TNF-α expression and secretion from human and rodent adipose tissue are associated with insulin resistance (88,114). TNF-α is increased in obese rodents (88) and humans (115) and decreased after weight reduction (116). It is thought to alter insulin sensitivity partly by down-regulating adiponectin levels, partly by inhibiting insulin signalling in adipocytes, mediated by changes in insulin-stimulated tyrosine phosphorylation cascades and probably also changes in gene expression (117,118). TNF-α induces apoptosis (programmed cell death) of human adipocytes *in vitro* (119) and influences the expression of a number of genes in adipocytes (120).

TNF-α stimulates basal lipolysis in rodent (121) and human (84,122) adipocytes and the elevated levels of FFAs are thought to be another cause of insulin resistance (reviewed in (80)). TNF-α participates in the regulation of fat mass by increasing energy expenditure and lipolysis and decreasing appetite (reviewed in (123)), indicating that adipose-derived TNF-α might play a role in limiting obesity (124). The lipolysis-stimulating effects of TNF-α in human fat cells are mediated by increased phosphorylation and decreased expression of PLIN via the mitogen-activated protein kinases (MAPKs) p44/42 and c-jun amino terminal kinase (JNK), as shown in ***Figure 1*** (122,125). In murine (126), but not human (125), adipocytes, TNF-α enhances lipolysis via down-regulation of $G\alpha_i$. Both HSL and PLIN are down-regulated by TNF-α in human adipocytes when nuclear factor-κB (NF-κB) is inhibited, which suggests that NF-κB is important for TNF-α-induced lipolysis in these cells (127).

1.4.3 Interleukin-6

The pro-inflammatory cytokine IL-6 is secreted from adipose tissue (90) and the secretion from subcutaneous adipose tissue, measured as plasma IL-6, correlates with BMI (113,114). Plasma IL-6 concentrations are higher in the portal vein than in the radial artery in extremely obese subjects, indicating that visceral fat is an important source of IL-6 secretion (128). IL-6 levels in adipose tissue from lean control subjects are very low (129). The expression of IL-6 is increased in adipocytes from insulin-resistant individuals and IL-6 treatment of rodent adipocytes leads to decreased glucose uptake in these cells, which suggests that IL-6 plays an important role in the insulin resistance seen in obesity and type 2 diabetes (130). However, IL-6 knockout

mice develop several features of the metabolic syndrome, e.g., obesity, insulin resistance and impaired glucose tolerance (131). IL-6 receptors are expressed in pituitary cells (132) and IL-6 is released systemically (113), which suggests that this cytokine plays a role in the central nervous system (CNS). TNF-α up-regulates IL-6 expression and secretion in human adipocytes (120). Insulin has anti-inflammatory effects in adipocytes by impairing IL-6 signalling at several levels (133,134).

1.4.4 Interleukin-8

IL-8 is a member of the CXC (cysteine, any amino acid, cysteine) chemokine superfamily. Both mature isolated fat cells and cultured adipose tissue produce and secrete IL-8 (91). TNF-α treatment up-regulates IL-8 in mature adipocytes (135). The plasma concentrations of the pro-inflammatory cytokines and chemokines TNF-α, IL-6 and IL-8 are higher in non-obese women with a high percentage of body fat than in non-obese women with a different body composition but similar body weight, indicating a stage of early inflammation (136). Macrophages, rather than mature adipocytes, have been shown to be the main source of IL-8 and other pro-inflammatory chemokines in adipose tissue (133,137). Insulin stimulates and the glycosphingolipid sulfatide decreases IL-8 production by adipose tissue, which might be of clinical importance since insulin levels are increased and sulfatide levels are decreased in type 2 diabetes (133,138).

1.4.5 Plasminogen Activator Inhibitor Type 1

PAI-1 inhibits fibrinolysis and promotes coagulation (reviewed in (139)). It is involved in the development of atherothrombosis (140). The protein is expressed in adipose tissue (97) and plasma PAI-1 levels have been shown to correlate with BMI and the intra-abdominal visceral fat depot in humans and rats (140,141). The mRNA expression and secretion of PAI-1 are higher in visceral than subcutaneous adipose tissue from obese individuals (142). PAI-1 production in adipose tissue is mainly derived from stromal cells and not adipocytes (142). PAI-1 mRNA levels increase in visceral fat during the development of obesity, which suggests that PAI-1 may be involved in the increased risk of vascular disease seen in obesity (141). A common polymorphism in the human PAI-1 gene is strongly linked to obesity (143). TNF-α up-regulates PAI-1 expression in human adipose tissue (144).

1.4.6 Adiponectin

Adiponectin (also known as apM1, Acrp30 and adipoQ) is an adipose-specific secretory protein and the gene product of apM1, adipose most abundant gene transcript 1 (93-95). Adiponectin forms homo-trimers that interact with each other and generate homo-oligomeric structures (93). Four species of adiponectin multimers have been identified in human plasma: trimer, albumin-binding trimer, hexamer and high molecular weight (HMW) adiponectin (145). Trimeric, hexameric and HMW adiponectin activate different signal transduction pathways and bind to different

receptors, which suggests that oligomerization is important for at least some of the biological activities of adiponectin (146). Diabetes-associated mutations in the human apM1 gene cause impaired oligomerization (147).

Although the plasma levels of most adipose-derived proteins increase with an increasing fat mass, the plasma concentrations of adiponectin are lower in obese than in lean subjects (148) and the mRNA expression of adiponectin is reduced in adipose tissue from both obese mice and humans (95). It is not known what mechanisms control the concentration of plasma adiponectin, although the production of adiponectin is influenced by nutritional status (149). Weight loss increases the concentration of circulating adiponectin in both nondiabetic and diabetic humans (150). Since adiponectin is produced by adipose tissue only, this suggests the presence of an inhibitory feedback regulation of adiponectin production in obesity (151).

Adiponectin protects against the development of atherosclerosis (152) and the higher plasma levels in women than men (148) could be part of the explanation for why atherosclerosis is more common in men than women. Adiponectin may also play a role in FA and energy homeostasis, since treatment of mice with adiponectin causes weight reduction, decreased plasma FFA levels and increased FA oxidation (153). Adiponectin knockout mice have delayed clearance of FFA in plasma and high levels of TNF-α in adipose tissue and plasma (154). Adiponectin increases insulin sensitivity in insulin-resistant mouse models of obesity (155) and is thought to be involved in the development of the metabolic syndrome (156). In agreement with these data, adiponectin knockout mice develop severe diet-induced insulin resistance (154).

1.4.7 Adipose Tissue and Inflammation

Recent studies have shown that adipose tissue is infiltrated by macrophages in obesity and that these cells are responsible for most of the TNF-α and IL-6 expression in adipose tissue (157,158). It is not known what triggers this infiltration although chemoattractant proteins such as MCP-1 are thought to play an important role (159). The increased number of macrophages in visceral adipose tissue from obese individuals might also be responsible for the enhanced production of other pro-inflammatory substances (e.g., chemokines) present in obesity (137). Cytokines, among them TNF-α, are involved in the regulation of adipose tissue metabolism (reviewed in (160)). Signalling molecules from the gut, liver and adipose tissue are integrated by the CNS and play important roles in both the regulation of energy homeostasis and insulin action in the body (reviewed in (161)). Furthermore, adipose tissue-derived hormones and cytokines affect food intake and lipid metabolism (149). The release of cyto- and chemokines from adipose tissue is proportional to the adipocyte size (162) and influences the circulating cytokine levels (129). The term ‘adipokines’ (or ‘adipocytokines’) originally referred to substances produced by adipocytes, such as leptin and adiponectin, but is often used as a collective name for cyto- and chemokines produced by adipose tissue. At present, most adipose-derived substances have no clinical applications and are only measured in research settings.

1.5 MACRONUTRIENTS AND WEIGHT LOSS

The effects of energy restriction and dietary macronutrient composition on adipose tissue protein secretion and gene expression were investigated in the first two papers of this thesis. Macronutrients are the energy-rich compounds protein, fat and carbohydrate. The different macronutrients are briefly described in sections 1.5.1-3 and weight loss diets are discussed in section 1.5.4. The Swedish nutrition recommendations for adults and children over the age of two years are summarized in ***Table 3*** (163).

1.5.1 Protein

Dietary protein supplies the body with nitrogen and amino acids that are used in the synthesis of other amino acids, nitrogenous compounds and body proteins, i.e., molecules with very diverse functions in the body: enzymes, receptors, transport proteins, hormones, structural proteins, proteins in the immune system and more. Amino acids can also be used as a source of energy. There is a continuous turnover of proteins in the body, where proteins are degraded and replaced. Twenty amino acids are essential and must be supplied by the diet. Meat, fish, eggs, beans and lentils are examples of foods rich in protein.

1.5.2 Fat

Dietary fat, or lipid, mainly consists of TAG that is metabolized to FFAs and MAG by different enzymes, lipases, and absorbed in the intestine. FAs consist of a charged carboxyl group ($-COO^-$) and a carbon chain of various length and degree of saturation, i.e., with no (termed saturated FA), one (monounsaturated FA) or several (polyunsaturated FA) double bonds. A high content of unsaturated FAs makes the fat soft or liquid, as in oils. FAs primarily supply the body with energy, but are also used in the synthesis of cholesterol, steroid hormones, phospholipids and lipoproteins. Two unsaturated FAs, linoleic acid and linolenic acid, are precursors in the synthesis of certain very-long-chain FAs and must be supplied by the diet. TAG and FFAs can be transported in the blood as complexes with the protein serum albumin or as components of lipoprotein particles. Dietary fat is very energy dense: one gram of fat provides more than twice as much energy as one gram of protein or carbohydrate. Thus, an obvious method for reducing total energy intake is to reduce dietary fat, obtained from e.g., margarine, ice cream, deep-fried foods or chocolate.

1.5.3 Carbohydrate

Dietary carbohydrates are digestible (e.g., sugar, starch) or indigestible (e.g., cellulose, 'fibre') molecules that consist of monosaccharides (e.g., glucose and fructose). They can be divided into mono-, di-, oligo- or polysaccharides depending on the number of monosaccharide units. The body has a small carbohydrate energy

reserve in the form of hepatic glycogen that guarantees an adequate glucose supply to the brain during a short-term fast. The glycogen stores are depleted overnight if not refilled by food intake, causing muscle protein degradation so that amino acids can be used as carbon skeletons in gluconeogenesis. The brain has an absolute requirement for a constant supply of glucose. However, during starvation or a prolonged fast the brain can use ketone bodies, synthesized in the liver from FAs, as fuel. Foods high in fibre (e.g., whole grain bread, fruit and vegetables) are important in weight regulation since they give a feeling of fullness and satiety, have low energy density and take time to eat (chew), so that satiety hormones have the time to act. Products rich in starch are e.g., potatoes, pasta and rice.

Table 3. *Swedish nutrition recommendations (E%, percent of total energy intake)*

Dietary fat, total	25-35 E%
Saturated and *trans* fatty acids	Max 10 E%
Monounsaturated fatty acids	10-15 E%
Polyunsaturated fatty acids	5-10 E%, of which 1 E% Ω-3*
Carbohydrate, total	50-60 E%
Dietary fibre	25-35 g/day
Refined sugars	Max 10 E%
Protein, total	10-20 E%

*Ω-3 fatty acids are primarily obtained from fish

1.5.4 Diets and Weight Loss

Why some people in our 'obesogenic' environment are susceptible to weight gain and others are not is not fully known, although it has been suggested that the weight-gainers share certain characteristics, e.g., a weak satiety response to high-fat meals and a preference for palatable foods, i.e., foods high in fat and sugar (164). Palatable food offsets normal appetite regulation by increasing the expression of hunger and satiety signals. Furthermore, it blunts the response to satiety signals and activates the reward system in the brain (reviewed in (165)). The optimal macronutrient composition of weight reducing diets—and the existence of such a diet—has been and still is widely debated. Furthermore, causes and consequences of weight maintenance or weight reducing diets high or low in certain macronutrients are also discussed.

Glycemic index (GI) is a measure of postprandial blood glucose response per gram of carbohydrate and glycemic load (GL) is GI x dietary carbohydrate content (166). Low-carbohydrate diets can be classified as ketogenic, e.g., the so called 'Atkins diet' (high-fat), or nonketogenic (low-fat) (167). Ketogenic diets rely on inducing a state of ketosis, i.e., when the liver produces ketone bodies from fat for the body to use as fuel. Ketogenic diets have been associated with adverse effects in some studies (167-169), whereas other studies have not found any association with increased risks (170). The reduction in body weight was shown to be independent of the macronutrient composition and GI in several studies (167,171). A recent study (further discussed in the 'concluding remarks' section) compared four different weight loss diets with various macronutrient compositions and found that the subjects on Atkins diet lost

significantly more weight without any more negative secondary outcomes than the other groups (172). Hypocaloric diets high in protein have been associated with more satisfaction, less hunger and reduced loss of lean body mass (173). However, when protein- and carbohydrate-rich meals (meat and bread, respectively) ingested *ad libitum* by obese subjects were compared, there was no difference in satiating effect (174). Carbohydrates in the form of sugar in drinks supply a lot of energy but give little feeling of satiety, which suggests that a high consumption of sweetened beverages could contribute to the development of obesity through an unintentional intake of excess energy (175).

High carbohydrate intake has been associated with higher fasting TAG and high sugar intake with low HDL cholesterol (176). A low-carbohydrate hypoenergetic diet caused reduced TAG and glucose levels and increased HDL cholesterol levels (177). These studies suggest that low-carbohydrate diets would have favourable effects, however, there was no difference in effects on insulin sensitivity or glycemic profiles between eucaloric, weight-maintenance diets with high or low sucrose content and no association between a high carbohydrate intake, high GI or high GL and insulin resistance in other populations (178,179). Both diets high in carbohydrate with low GI and diets low in fat have been shown to cause reduced low-density lipoprotein (LDL) cholesterol levels (171,177).

Metabolic and/or behavioural changes, e.g., increased appetite, decreased physical activity or decreased metabolic rate, are compensatory mechanisms that affect the outcome of hypoenergetic diets (180,181). Physiological adjustments like changes in appetite or metabolic rate seem to be more pronounced in response to weight loss, especially rapid weight reduction, than weight gain (8). Therefore it is important to aim for a slow, or long-term, loss of body fat. The main problem with weight loss is that most people regain the lost weight within a rather short time period. Eating habits that include a low energy density are associated with long-term weight maintenance (182) and a decreased risk of developing obesity, hyperinsulinaemia and the metabolic syndrome (183). Restricting the variety in different food groups can also be a means to maintain long-term weight loss (184). Large portions are associated with excess energy intake (185) and reductions in portion size and energy density reduced energy intake without increasing hunger or decreasing fullness in a short-term study (186). A high intake of dietary fibre and a low intake of dietary fat were associated not only with sustained weight reduction, but also with a decreased risk of developing type 2 diabetes in a Finnish population (187). In contrast, energy-restricted diets with high or low GL did not differentially influence diabetes risk factors above that associated with the weight loss *per se* (188), which suggests that energy restriction is more important for beneficial effects of weight loss than the dietary macronutrient composition.

1.6 CELL DEATH-INDUCING DFFA-LIKE EFFECTOR A (CIDE-A)

Cell death-inducing DFFA-like effector A (CIDE-A) was the most up-regulated gene after weight loss in Paper II and Papers III and IV therefore concern CIDE-A, its regulation and function(s). The two mammalian genes CIDE-A and CIDE-B were identified in 1998 in a database search for genes with homology to the DNA fragmentation factor (DFF) subunit DFF45 (also called DFFA (189)) (190). CIDE-A

and CIDE-B are highly homologous to fat-specific protein 27 (FSP27), which is regulated by TNF-α (191). The homology between these three genes and the subunits of DFF, DFF45 and DFF40, is restricted to an N-terminal region called the CIDE-N domain (190).

1.6.1 CIDE-A and CIDE-B

There are five CIDE-N domain containing proteins in the transcriptomes of humans and mice; DFF40, DFF45, FSP27, CIDE-A and CIDE-B, collectively called the CIDE family (192). The CIDE-N domain of CIDE-B can interact with the CIDE-N domains of DFF45 and DFF40 (193), whereas a sequence in the C-terminal region of CIDE-B is required for homo- and heterodimerization between CIDE-B and CIDE-A (194). CIDE-A and CIDE-B are present as both monomers and dimers (194,195). Few papers have been published concerning the functions of CIDE-A and CIDE-B, although the genes have occurred as up- or down-regulated in a number of gene expression studies (e.g., (196-198)).

Ectopic expression of CIDE-A and CIDE-B induces apoptosis in mammalian cells, probably via their C-terminal region (190) and possibly by localizing to mitochondria (194). The cell death is regulated by the CIDE-N domain and inhibited by DFF45 (190,199). However, there was no difference in cell death between BAT from wt and CIDE-A knockout mice, respectively (189). CIDE-A is a glycoprotein and glycosylated CIDE-A is predominantly localized to the nucleus (195). Transforming growth factor-β1 (TGFβ1) promotes translocation of CIDE-A from the nucleus to the cytoplasm by inhibiting its glycosylation (195).

CIDE-A transcripts are present at varying levels in human heart, skeletal muscle, brain, lymph node, thymus, appendix, bone marrow, placenta, kidney, heart, brain and lung, whereas CIDE-B transcripts are expressed primarily in adult and fetal liver and at lower levels in spleen, lymphocytes and bone marrow (190). The regulation of the transcription of the CIDE-A gene has not yet been studied but two promoters for the CIDE-B gene have been identified (200).

1.6.2 CIDE-A, Obesity and Fatty Acid Oxidation

In mice, CIDE-A mRNA is expressed at low levels in various tissues (e.g., heart, brain and skeletal muscle). However, CIDE-A protein is only expressed at high levels in brown, but not white, adipose tissue (189). CIDE-A knockout mice are resistant to high-fat diet-induced obesity and diabetes and have much less WAT and lower leptin levels than wt, suggesting that CIDE-A is important for the regulation of adiposity (189). Furthermore, the knockout mice have higher core body temperature, higher rate of lipid metabolism and enhanced basal and stimulated lipolysis from BAT than wt mice, indicating that CIDE-A may be important for the regulation of energy expenditure (189).

Green fluorescent protein (GFP)-labelled CIDE-A and CIDE-B localize to mitochondria in BAT and COS1 cells, respectively (189,194). The mitochondria

localization signal in CIDE-B resides in the same C-terminal region of the protein as the dimerization interface (194). CIDE-A interacts with, and possibly inhibits, UCP-1 in BAT and CIDE-A depletion might therefore cause an enhanced uncoupling activity and increased lipolysis, increased FA oxidation and less fat accumulation (201). However, since human adults have little or no BAT (28,202), it is difficult to predict the role of CIDE-A in human obesity (203). Adenovirus-mediated expression of the PPAR-γ coactivator PGC-1α in human white adipocytes induced the expression of mitochondrial proteins and brown adipocyte markers, e.g., UCP-1, and increased the FA oxidation in the adipocytes (204). The role of CIDE-A in these brown adipocyte-like cells has not yet been investigated. However, a polymorphism encoding a V115F amino acid substitution in exon 4 of the CIDE-A gene is associated with obesity in Swedish men and women (205), suggesting a role for CIDE-A in human obesity.

The co-repressor nuclear-receptor-interacting protein 1 (Nrip1 or RIP140) knockout mice are smaller than wt and heterozygous littermates, with a 20 % reduction in body weight and a 70 % reduction in total body fat content compared with wt (206,207). CIDE-A is repressed by RIP140 and is up-regulated in cells from WAT of RIP140 knockout mice (208). The white adipocytes from RIP140 knockout mice were differentiated in the presence of rosiglitazone and had 1.5-fold higher total FA oxidation than wt (208). Rosiglitazone treatment of ob/ob mice was previously shown to result in the induced expression of a number of genes that would be expected to influence energy metabolism and FA oxidation and that are not normally expressed in WAT but only in BAT, e.g., UCP-1 and CIDE-A (209).

1.7 LIVER X RECEPTOR (LXR)

In the last paper of this thesis, we found that CIDE-A interacts with a number of nuclear hormone receptors (NRs) *in vitro* and with the liver X receptors (LXRs) *in vivo*. The activation, endogenous ligands and tissue distribution of the LXRs are described in section 1.7.1. The known functions of the receptors in cholesterol, FA and glucose metabolism, and the associations between the LXRs and obesity and atherosclerosis, respectively, are reviewed in sections 1.7.2-4.

1.7.1 LXR-α and LXR-β

Two members of the LXR family have been identified and isolated: LXR-α (also known as RLD-1) (210,211) and LXR-β (also called NER, UR, OR-1 and RIP15) (212-215). LXR-α and LXR-β belong to the superfamily of NRs and are ligand-activated transcription factors. The LXRs bind to LXR response elements (LXREs) that consist of two hexanucleotide repeats (AGGTCA) separated by four nucleotides (direct repeat 4, DR4) (210,213,216). They become transcriptionally active by forming heterodimers with the retinoid X receptor (RXR) (217). The heterodimer is then further activated by binding the RXR ligand (9-*cis* retinoic acid), or one of the LXR ligands, or both ligands concurrently (218). The endogenous ligands for LXRs are oxidized cholesterol derivatives, oxysterols (219). In addition, D-glucose and D-glucose-6-phosphate were recently shown to be endogenous ligands of the receptors

(220). LXR-β is ubiquitously expressed while LXR-α is expressed in liver, kidney, small and large intestine, pituitary gland, spleen, fat tissue, lung, skin and adrenals (210,213,221). Both LXR-α and LXR-β translocate from the nucleus to the cytosol under certain conditions (222,223).

1.7.2 LXR, Cholesterol and Fatty Acid Metabolism

LXR activation leads to the up-regulation of several FA metabolism-associated genes, among others sterol regulatory element-binding protein 1c (SREBP-1c), fatty acid synthase (FAS) and acyl carrier protein (ACP) (224). LXR-αβ double knockout mice are unable to induce SREBP-1c-dependent lipogenesis (225). Although LXR activation by the synthetic agonist T0901317 causes a transient increase in plasma TAG in rodents (224,226), the agonist GW3965 (227) has potent antiatherogenic effects (228). LXRs have been identified as endogenous inhibitors of atherosclerosis and important determinants of macrophage lipid accumulation and foam cell formation (229). LXR-α knockout mice lack the ability to respond normally to dietary cholesterol and have an abnormal expression of genes involved in bile acid metabolism, sterol and FA synthesis, which suggests that the receptor functions as an essential regulatory component of cholesterol homeostasis (230). LXR-α is up-regulated by PPAR-α activators, e.g., FAs, which suggests that LXR-α could mediate cross-talk between FA and cholesterol metabolism (231). Furthermore, LXR-α is up-regulated by insulin and deletion of both LXRs suppresses insulin-mediated induction of enzymes involved in FA and cholesterol metabolism (232). Ectopic expression of LXR-α in murine adipocytes in combination with LXR activation by T0901317 was shown to increase the rate of FFA release (233). Although glycerol release did not increase in these cells, both FFA and glycerol levels in serum increased when mice were injected with T0901317 and the authors thus suggest that activation of the LXRs increases basal lipolysis (233).

1.7.3 LXR and Glucose Metabolism

The LXR agonists T0901317 and GW3965 have been shown to lower plasma glucose and improve insulin sensitivity in insulin-resistant rodents, at least in part by regulating key enzymes in glucose metabolism (234,235). The agonists have also been shown to improve TNF-α-induced insulin resistance in BAT (236). LXR-β knockout mice are glucose intolerant but not insulin resistant due to impaired insulin secretion (237). In skeletal muscle and adipose tissue, LXR activation increases uptake and oxidation of both fat and glucose, the latter by inducing expression of the insulin-responsive glucose transporter GLUT4 in adipocytes (235,238,239). LXR-α seems to be particularly important for basal and insulin-regulated expression of GLUT4 in WAT (238).

1.7.4 LXR and Obesity

LXR-α mRNA levels and polymorphisms in the LXR-α and LXR-β alleles are associated with obesity in humans (240). LXR-αβ double knockout mice have a significantly decreased amount of both WAT and BAT compared with wt mice, indicating that LXRs play a role in fat accumulation (241). The decreased amount of adipose tissue is due to a decrease in adipocyte size, possibly caused by reduced delivery of hepatic TAG to adipocytes (237). The double knockouts are resistant to diet-induced obesity, although only when dietary cholesterol is present (225). LXR-β knockout mice are also resistant to diet-induced obesity (237). Selective activation of LXR-β is thought to have several favourable effects, e.g., inhibit atherosclerosis without increasing hepatic lipogenesis. However, no LXR-β-specific agonist is yet commercially available (242,243).

2 AIMS

The specific aims of this thesis were:

1) to define dietary effects regulating the secretion of different polypeptides from human adipose tissue. (Paper I)
2) to define dietary effects regulating differential gene expression in human adipose tissue. (Paper II)
3) to identify and characterize novel nutrient-sensitive candidate genes in obesity. (Paper III)
4) to study the functional role of a novel candidate gene *in vitro*. (Papers III and IV)

3 COMMENTS ON MATERIAL AND METHODS

The studies in Papers I-IV were approved by the committee on ethics at Huddinge University Hospital and explained in detail to each of the subjects, from whom informed consent was obtained. The animal experimentation in Paper III was performed in accordance with institutional guidelines. Ethical permission was obtained from the Northern Stockholm animal ethics committee. All details about the methods used can be found in the individual papers. In this section I will discuss some of the techniques, their advantages and disadvantages compared with alternative techniques and recent developments.

3.1 SUBJECTS

All subjects except three included in the different studies were healthy (apart from obesity) and free of regular medication. In Papers I and II, one woman was treated with thyroid hormone for goitre. In Paper III, one woman was treated for goitre and one for mild hypertension. In Papers I and II, all subjects except one were of Scandinavian origin. All subjects in Papers III and IV were Caucasians. Five women in Papers I and II were postmenopausal. Although cytokine levels change during the menopausal transition (244), the results in these two studies were not significantly altered if the postmenopausal women were excluded from the analyses. The obese subjects in Papers I and II were participants in a European multi-centre study investigating effects of hypoenergetic diets with different macronutrient compositions. They underwent adipose tissue needle biopsies before and after a ten-week diet. The obese subjects in Papers III and IV were either undergoing bariatric surgery to treat their obesity or scheduled for either elective cosmetic liposuction or non-therapeutic needle biopsy. The non-obese subjects in Papers III and IV were either scheduled for elective liposuction or needle biopsy, or undergoing surgery for non-malignant disease, such as gallstone. The characteristics of the subjects in Papers I-IV are summarized in ***Table 4***. Note that there is an overlap between the different groups in Papers I and II.

Table 4. *Characteristics of the subjects in Papers I-IV. (hMSC, human mesenchymal stem cells)*

Paper	F/M	Obese/ non-obese	BMI range	Age range	Comment
I & II	40/0	40/0	31-48	21-49	5 post-menopausal, 1 goitre, 1 not Scandinavian
II	23/0	23/0	31-48	21-49	Microarray
	21/0	21/0	32-48	21-49	Microarray + real-time quantitative PCR
	15/0	15/0	32-44	23-48	Real-time quantitative PCR
III	146/40	143/43	18-50	18-63	1 goitre, 1 mild hypertension
	9/3	12/0	35-50	24-50	Bariatric surgery
	14/3	9/8	21-46	24-57	Isolated adipocytes + adipose tissue
IV	2/0	1/1	23-31	29-39	Preadipocytes for immunocytochemistry
	2/0	0/2	23-26	19-31	Liposuction for hMSC (245)
	3/0	0/3	23-27	27-50	Preadipocytes for lipolysis experiments
	3/0	2/1	23-35	27-55	Preadipocytes for mRNA measurements

The needle biopsies of abdominal subcutaneous adipose tissue were obtained under local anaesthesia and immediately rinsed in saline before being brought to the laboratory. Although local anaesthesia inhibits glucose transport and lipolysis in adipocytes and adipocyte growth in culture, the effects only last until the anaesthetic is removed by the washing of the biopsy (246). Catecholamine-induced lipolysis is not influenced by local as compared with general anaesthesia (247).

To understand obesity-related complications and to study risks and benefits from weight loss, it is important to achieve correct estimations of body composition, i.e., fat mass and fat free mass, and possibly also the body fat distribution, i.e., subcutaneous or visceral. Among the many available methods (reviewed in (248)), the choice depends on several different factors, e.g., the purpose of the study and the number of subjects. In Papers I and II, body composition was estimated using leg-to-leg bioelectrical impedance analysis (BIA). The current used in bioimpedance is only led through the fat free mass, where the water and electrolytes are, and therefore gives a measure of total body water that can be converted to fat free mass. The method is non-invasive, non-expensive and quick, and therefore well suited for studies with large numbers of subjects, such as the multi-centre study in Papers I and II. The disadvantages of the method are that it cannot be applied on patients who are hypersensitive to electricity or those with pacemaker, and that the result depends on changes in body water and its distribution, e.g., during different stages of the menstrual cycle or oedema.

Dual X-ray absorptiometry (DXA) has recently been shown to be a more valid method for the estimation of body composition of weight loss in very obese individuals (BMI >37) than air displacement plethysmography (ADP, also called BodPod) and BIA, since the latter two methods rely on a fixed hydration of fat free mass that is affected by obesity, diabetes and weight loss (249). In normal- or overweight individuals, however, ADP and BIA are sensitive enough to detect rather small changes in body composition (250,251). Waist circumference or waist-to-hip ratio gives an approximation of abdominal obesity. Although magnetic resonance imaging (MRI) and computed tomography (CT) give very accurate estimations of fat distribution and amount (252), these methods are expensive, time-consuming and invasive, and therefore not suitable for large studies or most clinical work. In Paper III, we used waist circumference as a measure of abdominal obesity.

Total daily energy expenditure (TDEE) in humans is the sum of basal metabolic rate (BMR) or REE, diet-induced thermogenesis and energy expenditure of physical activity (253,254). BMR is measured in the morning after sleep and an overnight fast, in the supine position, at physical and mental rest and pleasant temperature. REE is very similar to BMR, with the exception that it is measured at total rest although not just after sleep (253). REE is quantified by indirect calorimetry, measured using e.g., a mask, hood or chamber. Deltatrac, the ventilated hood indirect calorimeter used in Papers I and II, is considered the most accurate equipment and has been validated in several different laboratory and clinical situations (254). TDEE and REE depend on several different factors, e.g., body size, body composition, body surface (heat loss), gender and age (assessed in children in (255)). Diet-induced thermogenesis is the increase in energy expenditure accompanying the intake and digestion of food. The extent of energy expenditure from physical activity depends on both exercise and movements in daily life. The factor physical activity level (PAL) is TDEE divided by

BMR. PAL 1.6 corresponds to sedentary work and limited physical activity in the spare time, whereas sedentary work and regular physical activity (e.g., a brisk 60 min-walk daily) gives a PAL of approximately 1.8 (163). In Papers I and II, TDEE was estimated by multiplying REE with PAL 1.3, which corresponds to sedentary work and very little or no exercise, which can be expected from very obese people such as the participants in the two studies.

In Papers I-III, the glucose and insulin levels in plasma were determined and used for the calculation of homeostasis model assessment (HOMA) index, an indirect measure of *in vivo* insulin sensitivity, using the formula ([plasma glucose (mmol/l) x plasma insulin (mU/l)]/22.5) (256). The purpose of the study and the number of subjects must be taken into account when chosing a method for estimating insulin resistance. In Papers I-III, HOMA was chosen since it is an appropriate method for large studies (recall that the subjects in Papers I and II were participants in a multi-centre study), whereas the 'gold standard' test, hyperinsulinemic euglycemic clamp (257), is feasible only if the number of subjects is small. Moreover, HOMA gives an estimate of basal insulin resistance, whereas most other methods measure stimulated insulin resistance (reviewed in (258)).

In Paper III, one cohort of obese individuals underwent gastric banding to treat their obesity. These subjects were weight-stable and had decreased their BMI by on average 15 kg/m^2 in 2-4 years. Bariatric surgery is the only obesity treatment so far shown to result in substantial weight loss 10 years after intervention, with gastric bypass being one of the most efficient although also most complicated methods (259).

A total of 18 male mice of three different strains (NMRI, C57BL/6J and 129Sv/Pas) were included in Paper III. C57BL/6J and 129Sv/Pas are inbred, i.e., brothers and sisters have been mated for at least 20 generations, resulting in a genetic variability comparable to twins. In contrast, NMRI (Naval Medical Research Institute) mice are outbred, i.e., have normal genetic variability. Inbreeding increases the risk of disease and C57BL/6J mice are glucose intolerant and have impaired insulin secretion, recently shown to be caused by a deletion in a gene involved in β-cell mitochondrial metabolism (260).

3.2 DIETS

In Papers I and II, the dietary targets for fat content in the two hypoenergetic diets were 20–25 energy percent (E%) for the low-fat diet and 40–45 E% for the moderate-fat diet. Dietary targets for protein and carbohydrates were 15–20 E% from protein in both diets and 60–65 E% and 40–45 E% in the low-fat and moderate-fat diet, respectively, from carbohydrates. These targets are not very different from the Swedish nutrition recommendations (summarized in ***Table 3***). Long-term effects of more extreme diets, like those in the study by Suljkovicova *et al.* (261) (15 E% and 65 E% from fat in the two diets, respectively), would be interesting to study, although compliance would probably be a major problem. Hence, the study by Suljkovicova *et al.* was small (9 subjects), short-term (5+5 days) and all food was provided by the investigators.

The subjects in Papers I and II were prescribed a daily energy intake 600 kcal lower than their TDEE, based on REE x PAL 1.3. According to the Harris-Benedict equation for calculation of BMR already published in 1918 (262), the average woman in these studies (age 35 y, weight 102 kg, BMI 37 kg/m^2; see Table 1 in Paper I) would have a BMR of approximately 1800 kcal/day, compared with 1400 kcal/day for a 63 kg-reference woman (163). Considering the probable difference in activity level, the energy requirement of the average subject and the normal-weight reference woman might not differ (approximately 2300 kcal/day and 2200-2500 kcal/day, respectively, with PAL 1.3 for the subject and PAL 1.6-1.8 for the reference woman). An energy intake of 600 kcal (equal to 2.5 MJ) corresponds to two McDonald's cheese burgers and the energy reduction in the studies was thus quite modest.

Dietary intake can be assessed by e.g., food frequency questionnaires, weighed diet records and dietary recall(s). There are advantages and disadvantages with all these methods (reviewed in (263)). In Papers I and II, the subjects kept food diaries and recorded everything they ate or drank during the study. The dietician reinforced the dietary instructions and assessed the compliance of the subjects weekly. Furthermore, a three-day weighed food record was performed before the study and during the last week of intervention and one-day weighed food records were completed three times during the study. Under-reporting of energy intake or intake of certain foods is a common problem in assessments of dietary intake, especially among overweight or obese subjects, and can be established e.g., by concurrent measurement of energy expenditure by doubly labelled water or estimation of protein intake by 24-hour urinary nitrogen output (264-266). However, these methods are expensive and time-consuming and thus not feasible in large studies.

3.3 CELL SYSTEMS

In many research areas, human cell lines are commercially available and can be used for repeated experiments, purchased, frozen and thawed whenever needed. However, cell lines do not fully reflect the characteristics of primary cells. In studies of adipocytes, murine cell lines such as 3T3-L1 cells are often used, since there are no commercially available human fat cell lines. There are many differences between murine and human adipocytes and we have therefore chosen to work mainly with human primary fat cells, isolated from adipose tissue obtained from biopsies or liposuction (Papers III and IV). The drawbacks of this are the unpredictable and irregular availability of tissue, the limited amounts of tissue and the limited lifespan of the cells. Once the differentiated preadipocytes mature, they round up and loosen from the surface of the cell culture plate. Mature adipocytes float and are difficult to keep in culture, compared with preadipocytes. Furthermore, adherent cells such as preadipocytes are easy to treat with different substances, such as TNF-α and MAPK inhibitors (Paper III) or the LXR agonists T0901317 or GW3965 (Paper IV).

Human mesenchymal stem cells (hMSC) can be differentiated into adipocytes with similar characteristics as adipose-derived adipocytes (267). We have recently isolated hMSC from adipose tissue, differentiated them into adipocytes and managed to keep these 'cell line-like' cells in culture with retained differentiation capacity for up to fifteen passages or more (245). These cells, termed PLA (processed lipoaspirate),

were used for some of the experiments in Paper IV. However, although much work has been done, these cells need to be further characterized. Also, although much longer than primary cells, the lifespan of the PLA cells is still limited. Furthermore, different PLA 'cell lines' are derived from different individuals with different characteristics and therefore do not have identical properties. The lack of human fat cell lines also means that it is not possible to achieve stably transfected fat cells similar to the CIDE-A-expressing MCF-7 cells in the paper by Iwahana *et al*. (195). It is, however, possible to use adenoviral infection of primary differentiated preadipocytes for studies of protein over-expression (204) and to transiently transfect PLA cells using cationic lipid such as Lipofectamine (as in Paper IV). Furthermore, we have managed to transiently transfect preadipocytes with small interfering RNA (siRNA), although with different degrees of knockdown, probably due to individual differences between the tissue donors (Paper III). These and other methods for transfection of mammalian cells are reviewed in (268).

3.4 PROTEIN DETECTION AND INTERACTION

For studies of protein secretion (leptin, IL-6 and -8, TNF-α, PAI-1, adiponectin and MCP-1) and protein expression (PLIN) in Papers I and III, we used standard methods such as radioimmunoassays (RIAs) (269), enzyme-linked immunosorbent assays (ELISAs) (270) and Western blot (271).

In Paper I, we used fresh adipose tissue cut into small pieces and short-time incubation (2 h) since collagenase isolation and prolonged incubation or culture of tissue explants cause increased TNF-α secretion and altered adipocyte expression of inflammatory mediators such as TNF-α and IL-6 (272,273). Long-term incubation (6 h or more) of isolated adipocytes or differentiated preadipocytes with bovine serum albumin (BSA) also affects cytokine release and gene expression (274). However, in Papers I and III, adipose tissue was only exposed to BSA for 2 h in secretion and lipolysis experiments. For preadipocytes (used in Papers III and IV), BSA was only used during the isolation of the fat cells (275). Thereafter, fetal calf serum was used during the first 24 h of cell culture and after that, only serum-free medium was used.

Besides using Western blot for the determination of PLIN protein expression in Paper III, we also ran a number of Western blots for the detection of CIDE-A protein in adipose tissue and adipocytes, although did not manage to detect bands of the appropriate size with any of a number of different commercial antibodies. Other groups have also tried to detect endogenous CIDE-A protein by Western blotting and failed due to lack of high affinity antibodies (195). Furthermore, CIDE-A is a glycoprotein that forms dimers (195) and interacts with other proteins (Paper IV). Low protein expression, post-translational modifications such as glycosylation and dimerization, or complex formation with other proteins may prevent efficient detection of endogenous protein using Western blot.

Modifications such as glycosylation or complex formation could also be part of the explanation to why we did not get any positive results from our yeast 2-hybrid (Y2H) (276) screenings, in spite of the use of two different cDNA libraries (Paper IV). It is possible that other proteins are required for the interaction between CIDE-A and

proteins like LXR. Besides, the Y2H system that we used only detects nuclear interaction, whereas the interactions we found using other methods in Paper IV were predominantly cytosolic. Surprisingly, the same antibodies that did not work for Western blotting worked perfectly for immunohistochemistry (Paper IV), as evidenced by incubations with specific blocking peptides. It is possible that the endogenous levels of CIDE-A in human adipocytes are too low to be detected by Western blot or that the protein is too sensitive to be run on denaturing gels. However, it is also possible that CIDE-A changes conformation upon binding to other proteins, which would make it difficult to detect endogenous CIDE-A even on non-denaturing gels, although this remains to be tried. Until we or others find a solution to these problems, one way of studying effects of CIDE-A protein in adipocytes would be to transfect PLA cells with FLAG-tagged (277) CIDE-A (as in the immunofluorescence experiments with 3T3-L1 cells in Paper IV), since PLA cells hardly express any CIDE-A (Nordström, EA, unpublished observations) and untransfected PLA cells therefore can be used as controls.

Y2H, mammalian 2-hybrid and the transactivation assay used in Paper IV (described in ***Figure 3***) are based on essentially the same principle, namely that one protein (called 'bait' in Y2H) is fused to a binding domain (BD) that binds to a certain response element and activates a reporter gene (e.g., a gene encoding an essential amino acid or luciferase) under specific conditions. In the 2-hybrid systems, interaction with another protein (termed 'prey' in Y2H) fused to an activation domain (AD), with or without ligand/agonist, is required for activation of the reporter gene. In the transactivation assay, the activation induced by the binding of a Gal4-fusion protein (LXR) to a response element (Gal4 binding site) and of ligand/agonist (GW3965) to this protein is modified by co-activators or co-repressors (CIDE-A). This results in increased or repressed transcription of the reporter gene (measured as luminescence).

At least in our hands, glutathione S-transferase (GST) pull-down (278) does not seem to be sensitive enough to distinguish between interactions with different NRs. GST pull-down is an *in vitro* method and it is possible that other factors, such as co-activators or co-repressors that are present in cells but not in artificial systems, are needed to make interactions with different NRs more specific. For the GST pull-down experiments we used CIDE-A protein that was *in vitro* translated in the presence of [^{35}S]methionine using the rabbit reticulocyte system that was also used for the initial *in vitro* translation of the CIDE-A construct (Paper IV). This system can be used either with biotinylated lysine (non-radioactive) or with radioactively labelled amino acids.

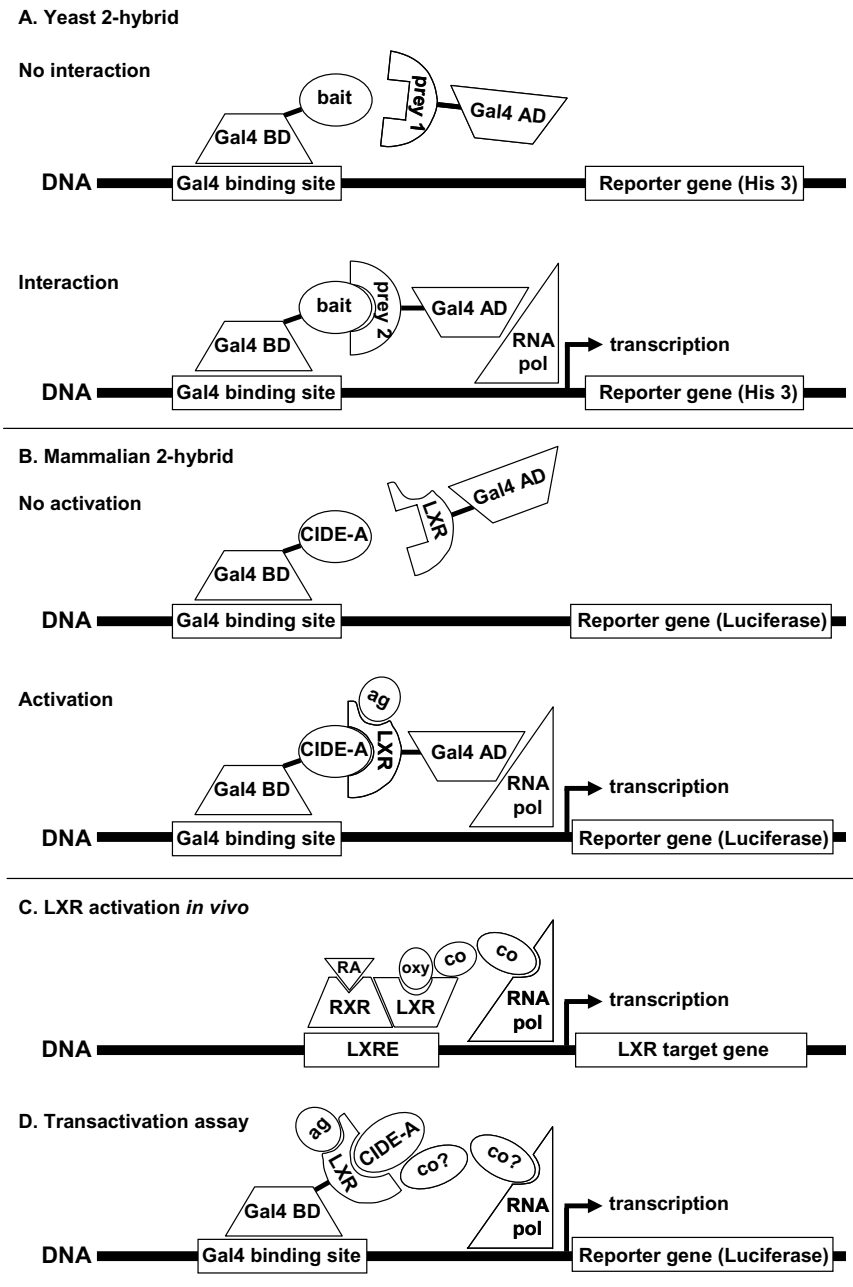

Figure 3. *Principles of yeast 2-hybrid (A), mammalian 2-hybrid (B), LXR activation in vivo (C) and the transactivation assay used in Paper IV (D). BD, binding domain; AD, activation domain; His 3, gene encoding the amino acid histidine; RNA pol, RNA polymerase; CIDE-A, cell death-inducing DFFA-like effector A; LXR, liver X receptor; RXR, retinoid X receptor; ag, agonist; RA, 9-cis retinoic acid; oxy, oxysterols; co, co-activator or co-repressor.*

3.5 LIPOLYSIS

At the same time as TAGs are broken down to glycerol and FFAs, new TAGs are formed in the cell. FFAs are re-utilized in this process. As mentioned previously, glycerol cannot be re-esterified in the adipocyte due to lack of the enzyme glycerol kinase that converts glycerol to glycerol-3-phosphate. It is therefore preferable to measure glycerol, rather than FFA, release as an index of lipolysis. The rate of basal lipolysis in adipose tissue is rather low and a very sensitive method is therefore needed, particularly if the amount of adipose tissue available for the experiment is small (279).

In Papers III and IV, we used an ultrasensitive *in vitro* method based on luciferase, luciferin, glycerol kinase and ATP (279,280). Luciferase produces light in the presence of luciferin, ATP and a number of co-factors. Glycerol kinase produces glycerol-1-phosphate and adenosine 5'-diphosphate (ADP) when glycerol and ATP are present (280). This causes a reduction in light intensity. The emitted light can be measured in a luminometer and the amount of glycerol calculated.

Other methods for the determination of lipolysis are e.g., microdialysis, tracer techniques (radio-labelled isotopes) and the arteriovenous technique. Microdialysis can be used for *in situ* lipolysis investigations, e.g., of the extracellular space in subcutaneous adipose tissue or skeletal muscle (281,282). This gives an estimate of the true interstitial concentration of glycerol. The concentration of glycerol in the dialysate can be measured with the bioluminescence assay described above. The method is time-consuming, rather expensive, invasive and not suitable for studies with large numbers of subjects, such as that in Paper III (n=186). However, it allows the introduction of lipolytic stimuli concurrent with lipolysis measurements. Glycerol or FAs that are radioactively labelled can be used for *in vivo* studies of whole body lipolysis (reviewed in (283)), although are expensive and not well suited for studies of regional differences in lipolysis. The arteriovenous technique (284) only allows the study of one depot, and measures total glycerol release from lipolysis of TAG from adipose tissue and chylomicrons (i.e., dietary TAG) concurrently (285).

3.6 MEASUREMENTS OF GENE EXPRESSION AND REGULATION

Real-time quantitative polymerase chain reaction (RT-qPCR) (reviewed in (286)) is a highly sensitive standard method for the determination of mRNA levels. As in PCR (287), certain sequences are amplified by the use of specific primers and enzymes, although this can be studied 'online' or 'in real time' using RT-qPCR. The mRNA levels can be detected in a number of ways, with intercalating dyes such as SYBR Green being the simplest although also the least sensitive method, since the dye binds to all double-stranded DNA. Hence, there is a risk for false-positive results from primer-dimers and unspecific products. However, this can be avoided by careful primer design and the use of dissociation curves and agarose gel electrophoresis, showing that a single product is amplified. Furthermore, DNase treatment during the RNA isolation and RNA quality controls done with e.g., Agilent Bioanalyzer, decreases the risk for DNA contamination to a minimum. Different types of probe-based chemistry, e.g., TaqMan, are much more specific than SYBR Green and

eliminate the need for dissociation curves etc. Both SYBR Green- and TaqMan-based chemistry were used in Papers I-IV.

To ensure that equal amounts of cDNA in different samples are compared, the determination of the mRNA levels of one or more reference genes or 'house-keeping' genes, i.e., genes that are not affected by the conditions studied, is essential. The gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as reference gene in Papers II-IV (mGAPDH in Paper IV), is very commonly used. However, it has been found to be regulated by a number of different conditions (e.g., by hypoxia (288)). In Paper II, we found that GAPDH was regulated by weight loss, although the increase in C_t (cycle threshold; the higher the C_t value, the lower the mRNA expression) value after diet was minor, <1 %, and the coefficient of variation (CV) was 5-6 %. Thus, we considered GAPDH a valid reference gene in Paper II. In Paper III, we predominantly used β2-microglobulin (β2MG), a reference gene not affected by obesity or weight loss, although also used GAPDH for some experiments, since we found that β2MG at least in our hands is regulated by TNF-α. The reference gene used in Papers I and IV, 18S rRNA, was not affected by the conditions studied in these papers, although was ranked 6 out of 10 in an evaluation of reference genes for studies of gene expression in human adipose tissue (289). In future studies, the top-ranked reference gene, LRP10 (low density lipoprotein receptor-related protein 10; a membrane protein), should be used alone or, preferably, in combination with other reference genes.

To our knowledge, we were the first to successfully use RNA interference (RNAi) (290,291) on human primary differentiated preadipocytes (Paper III). Much has happened in this field during the last few years and among other things, the concentrations of siRNA used in the cell culture medium have decreased dramatically (to 1-5 nM) to try to avoid off-target effects (292). Furthermore, 'scrambled' siRNA, i.e., siRNA without homology to any known sequence in the human genome, are now in standard use and effects from knockdown of several reference genes are often measured in parallel with the gene of interest. We used siRNA directed against mouse HSL as a control in the experiments in Paper III.

In Paper II, a prerequisite for total RNA samples to be included in the microarray was a total yield of ≥8 μg RNA per sample and a nondegraded pattern in the Agilent Bioanalyzer analysis of RNA quality. Thus, microarray analyses were only performed for 23 out of the 40 subjects, primarily due to lack of RNA but in some cases due to RNA of insufficient quality. Today, similar experiments require 100 ng-1μg of RNA, the latter ensuring enough RNA for quality analysis. In addition, the quality and purity of the RNA obtained by the use of modern kits are very high. To increase the RNA concentration in some of the samples in Paper II, we precipitated the RNA using ethanol. Today we would most probably use a column-based 'clean-up' kit that increases concentration and improves quality (i.e., removes contaminations) concurrently.

3.7 DATA ANALYSIS

In microarray analyses with thousands of gene transcripts there is a statistical risk of false-positive and false-negative results caused by multiple comparisons. The

significance analysis of microarrays method (SAM) (293), used in Paper II, adjusts for these multiple comparisons by using permutations, i.e., rearrangements of data into unique orderings. It is often more interesting to study coordinate changes in whole pathways than changed expression of individual genes, and additional information can be gained if genes that demonstrate minor, nonsignificant changes are included in the pathway analysis (294). This can be done using e.g., MAPPFinder (295) together with GenMAPP (296) and Gene Ontology (297). Microarray suite (MAS) checks the quality of the data from the microarrays by controlling the hybridization and specificity of the different probes in each probe set representing a gene transcript on the array. Each transcript is represented by both 'perfect match' probes and mismatch probes.

For statistical analyses, it is often recommended that the number of subjects or objects studied should be at least 30 in each group to ensure normally distributed data. In experimental research, this is most often not possible due to e.g., lack of tissue or cost of reagents. In addition, although there are sometimes large differences between primary cells from different individuals, 3-6 experiments are usually enough to provide statistically significant results. Data can be analyzed for normal distribution using e.g., the Kolmogorov-Smirnov and Shapiro-Wilk tests (as in Paper II) and log transformed if not normally distributed (as in Paper I). Alternatively, non-parametric tests can be used (as in Paper III).

The number of subjects needed for each diet group in Paper I was determined by power calculations during the planning of the study. In brief, based on the known variation in leptin secretion in obese subjects (111,298) and with a desired *P*-value of 0.025 after a Bon-Ferroni correction to adjust for multiple analyses, we found that 20 subjects in each group would ensure a power of at least 80 %.

4 COMMENTS ON RESULTS

In this section I will summarize and comment on the results from the individual papers, taking the specific aims of the thesis (see section 2) as a starting point for the discussion. All details about the individual results, *P*-values, graphs and tables can be found in Papers I-IV. I will then try to tie the different papers and results together in section 5 and draw conclusions from the thesis as a whole, before ending with future perspectives in section 6.

4.1 DIETARY EFFECTS ON PROTEIN SECRETION (PAPER I)

The aim of Paper I was to define dietary effects on the regulation of the secretion of different polypeptides from adipose tissue. Forty women were randomly allocated to either a low-fat, high-carbohydrate diet or a moderate-fat, moderate-carbohydrate diet for ten weeks. The fat and carbohydrate contents of the two diets were significantly different, however, were not extreme and not very different from the Swedish nutrition recommendations. Both diets were hypoenergetic, although the energy restriction was mild (600 kcal/day). We did not expect any dramatic changes in the different parameters studied, since the energy restriction was moderate, the diet period rather long and the differences in composition between the diets rather small.

During the dietary intervention, the women in the moderate-fat group were more satisfied and happy than the women in the low-fat group, who complained about headache and being cold. The total amount of calories from fat during the intervention was within the targeted range in the moderate-fat diet group. However, the fat intake in the low-fat diet group was slightly higher than the target. There was no difference in compliance between the groups, although it seems that more effort was needed from the women in the low-fat group to stay within the recommendations. The weekly 'pep talks' from the dietician were probably needed, especially in the low-fat group, and resulted in 100 % of the subjects completing the study.

There was a slight, although significant, difference in protein intake between the two groups and a significant difference in intake of dietary fibre, with the highest intake of both protein and fibre in the low-fat group. Protein and fibre give a feeling of satiety and fullness, which possibly contributed to the compliance in the low-fat group. However, the habitual fat intake in the low-fat group was higher than that of the hypoenergetic diet, and apparently the low fat intake was the main problem for the subjects in this group. Furthermore, since the intake of both protein and fat in the low-fat group was slightly higher than the targeted maximum levels, the carbohydrate intake in the low-fat group was quite far from the targeted 60-65 E% (52.4 E%). Thus, the diet should not be termed 'high-carbohydrate' and the differences between the diet groups were smaller than planned.

We found no significant differences between the diet groups in weight reduction, protein secretion, circulating levels or gene expression, although weight-loss resulted in decreased levels of pro-inflammatory cytokines and improved insulin sensitivity. The secreted proteins could be divided into three groups depending on the sensitivity of their regulation to energy restriction, with leptin as the most sensitive, TNF-α, IL-6 and

IL-8 as an intermediate group and adiponectin and PAI-1 as the least sensitive. Only leptin and IL-6 mRNA expression changed significantly, which suggests that they are transcriptionally regulated, whereas TNF-α and IL-8 are mainly regulated post-translationally. The circulating levels of leptin and PAI-1 changed dramatically and plasma levels of IL-6 showed a less marked, although significant change. Plasma glucose, plasma insulin, HOMA index and several other parameters changed significantly only when the two groups were analyzed together. This was probably due to the rather small number of subjects in the two groups and it is possible that a larger study with more rapid and more extensive weight loss and/or more extreme diets would result in more dramatic changes.

From the studies in Paper I, we conclude that the energy supply *per se* and not the macronutrient composition is of importance for the regulation of the protein secretory function and gene expression in human adipose tissue, at least during energy restriction. The sensitivity of different secreted proteins to energy restriction varies and subcutaneous adipose tissue does not seem to contribute to a major extent to the plasma levels of PAI-1, since plasma PAI-1 levels but not PAI-1 secretion changed significantly.

4.2 DIETARY EFFECTS ON GENE EXPRESSION (PAPER II)

Paper II aimed to define dietary effects on the regulation of adipose tissue gene expression. The subjects and diets were the same as in Paper I. Using microarrays with approximately 8500 of the best described human genes we searched for genes regulated by weight loss and/or dietary macronutrient composition. As discussed in section 3, for technical reasons it was only possible to perform microarray assays for 23 out of the 40 women in the study. However, in total, key results were confirmed by RT-qPCR in 36 out of the 40 women, with no significant differences between those involved in the microarrays and those not involved. Furthermore, there were no significant differences in clinical parameters such as weight loss between the subjects involved in the microarray assays and the rest, and we therefore concluded that the women participating in the arrays were representative of the whole group. There was no overlap between these women and those in the paper by Viguerie *et al.* (299), although they were all recruited to the same multi-centre study**.**

A number of different statistical analyses showed that 52 genes were up-regulated and 44 genes were down-regulated, with almost identical patterns in the two dietary groups. In particular, the quantitatively most up- or down-regulated genes were essentially the same after both diets. All data from the 23 subjects participating in the arrays were therefore analyzed together, just as in Paper I, and the RT-qPCR results were also calculated for the two dietary groups pooled together.

Out of the seven genes that changed the most due to the energy restriction, four fit into the same pathway leading from acetyl-CoA or malonyl-CoA to polyunsaturated FAs. The physiological importance of this is unclear. However, since these FAs have various functions in adipocytes there are several different feasible explanations, as discussed in Paper II. Another finding of this study was that the expression of several genes known to regulate obesity in experimental models, e.g., knockout mice, was affected by the

energy restriction, highlighting the importance of such models in the search for candidate genes in obesity. However, there are numerous differences in many different aspects between mice and humans (e.g., concerning adipokines (300)) and it was therefore particularly interesting that CIDE-A was the most up-regulated gene after weight loss, since CIDE-A knockout mice are lean and resistant to diet-induced obesity. Furthermore, in rodents, CIDE-A is only expressed in BAT, as opposed to WAT (189). It is notable that CIDE-A was not regulated by energy restriction in the paper by Viguerie *et al.* (299), although the women in that study were of a different genetic background than the women in our study.

As discussed in section 1, recent research has shown that obesity is characterized by a low-grade inflammation and macrophage infiltration (157,158). Surprisingly and in contrast to another microarray study (301), we did not find any important changes in the expression of inflammatory genes. However, the study by Clément *et al*. was based on a very low calorie diet, inducing rapid weight loss in only 28 days, completely different from our study design.

In agreement with the results from Paper I, we conclude that the macronutrient composition, at least the fat and carbohydrate content, is of little or no importance for changes in gene expression in human adipose tissue of obese women following energy restriction. Marked effects were obtained on genes regulating the production of polyunsaturated FAs and genes regulating obesity in experimental models, with CIDE-A being the most up-regulated gene after weight loss.

4.3 CIDE-A, A NOVEL CANDIDATE GENE IN OBESITY (PAPER III)

The apparent species differences between our results concerning CIDE-A in Paper II and those of Zhou *et al.* (189) made us investigate CIDE-A further, aiming to characterize this novel nutrient-sensitive candidate gene in obesity. A first characterization was done in Paper III and continued in Paper IV. In this section I will discuss some of the results from Paper III, to be continued and expanded by the addition of results from Paper IV in section 4.4.

By determining the CIDE-A mRNA expression in adipose tissue from a large cohort (n=186) with a wide range of BMI and from very obese subjects before and after substantial surgery-induced weight loss, we could confirm the results from Paper II: CIDE-A is expressed in WAT in humans, down-regulated by obesity and up-regulated by weight loss. Furthermore, we could confirm the results from the study by Zhou *et al*. (189) and show that in rodents, CIDE-A is expressed in brown, but not white, adipose tissue. Human CIDE-A mRNA levels were inversely correlated with different features of the metabolic syndrome, such as abdominal obesity, insulin resistance, enhanced basal lipolysis and fat cell size. Interestingly, C57BL/6J mice, which are glucose intolerant and have impaired insulin secretion, had the lowest CIDE-A mRNA expression, compared with the other two mouse strains in the study ($P<0.01$ and $P=0.06$ for comparison with 129Sv/Pas and NMRI, respectively; unpaired two-tailed Student's *t*-test; unpublished). Another interesting finding was that the body weight increase in 129Sv/Pas mice on normal diet was marginally larger than for the mice on cafeteria diet, even though food consumption was equally increased in all three mouse

strains when on cafeteria diet. A possible explanation for this, at least in part, was that the expression of UCP-1 was significantly higher in BAT of 129Sv/Pas mice than the other two strains ($P \leq 0.01$, unpaired two-tailed Student's *t*-test), and it is therefore feasible that the extra energy from the cafeteria diet dissipated as heat (Nordström, EA, unpublished observations).

In this paper, we found that GAPDH mRNA expression was markedly regulated by obesity, compared with the slight decrease after weight loss in Paper II. Thus, GAPDH is not well suited as a reference gene in studies of obesity, unless thoroughly analyzed on its own as in Paper II. Instead, we used β2MG as reference gene when BMI was one of the investigated parameters. However, this gene is regulated by TNF-α and we therefore used GAPDH as a reference gene in our cell-based experiments. Since FSP27, homologous to CIDE-A, is down-regulated by TNF-α (191), we treated differentiated preadipocytes with TNF-α with or without the addition of specific MAPK inhibitors. We found that this cytokine down-regulates the expression of CIDE-A in human fat cells via the MAPK JNK. To further investigate the function(s) of CIDE-A we used RNAi, discussed in the section below.

4.4 THE FUNCTIONAL ROLE OF CIDE-A (PAPERS III AND IV)

The last aim of this thesis, to study the functional role of a novel candidate gene *in vitro*, was fulfilled by siRNA-mediated knockdown of CIDE-A in Paper III and over-expression of the gene in Paper IV. Moreover, potential interactions between CIDE-A and other proteins were studied in Paper IV.

Down-regulation of CIDE-A mRNA expression by RNAi experiments showed a strong inverse relationship between glycerol release (i.e., basal lipolysis) from human primary differentiated preadipocytes and CIDE-A mRNA levels. We also found that PLIN mRNA is down-regulated in parallel with CIDE-A in siRNA-treated cells and that down-regulation of CIDE-A increases the secretion of TNF-α, which could explain the increase in lipolysis. These effects appeared to be specific for TNF-α, since neither MCP-1, nor adiponectin secretion was affected. However, we could not detect a significant down-regulation of PLIN protein in these samples.

CIDE-A over-expression has been shown to induce apoptosis in mammalian cell lines (190) and taken together with the homology between CIDE-A and DFF, it has been concluded that CIDE-A is a pro-apoptotic gene. However, in Paper IV we show that over-expression of human CIDE-A in 3T3-L1 adipocytes for 48 h does not induce apoptosis or necrosis. Compared with 293T or MCF-7 cells, 3T3-L1 cells appear to be very sensitive to the introduction of foreign DNA (i.e., transfection), since Inohara *et al*. hardly found any apoptosis 24 h after transfection with the empty pcDNA3 vector (190). Apart from this study of CIDE-A over-expression, to our knowledge, no other studies have been performed where CIDE-A has been shown to induce apoptosis. Over-expression of CIDE-A in MCF-7 cells for 24-48 h even inhibited TGFβ1-induced apoptosis, although over-expression for more than 72 h enhanced cell death (195). Such a prolonged over-expression has not been studied in the other papers and the increase in cell death could be mediated via several different mechanisms, not necessarily involving CIDE-A expression *per se*. Furthermore, there was no difference in cell death

between BAT from wt and CIDE-A knockout mice, respectively, indicating that CIDE-A is not directly involved in controlling apoptosis in this tissue (189). Most studies in which CIDE-A has been mentioned have been microarray-based and have only detected CIDE-A as up- or down-regulated by different conditions in various tissues or cell lines (e.g., (196,198,208)). CIDE-B, on the other hand, has been shown to induce morphological signs of apoptosis in mammalian cell lines in at least three studies (190,194,197). Furthermore, the CIDE-A gene is located on chromosome 18p11, associated with diabetes and obesity (302), whereas CIDE-B is located on chromosome 14q11, a locus associated with apoptosis (303). It has been suggested that mitochondria localization and homo- or heterodimerization are required for CIDE-B to induce apoptosis (194). One could hypothesize that CIDE-B, rather than CIDE-A, is cell death-inducing, as heterodimers with CIDE-A or as homodimers. CIDE-B has a rather restricted expression pattern, although to our knowledge, it has not been determined whether CIDE-B is expressed in 3T3-L1 cells or human adipose tissue. A lack of CIDE-B expression in adipocytes could possibly explain why we did not get substantial apoptosis in our experiments. However, it remains to explain why CIDE-A over-expression in cells expressing CIDE-A but not CIDE-B induces apoptosis (190).

In Paper III, we found that low CIDE-A expression is associated with increased basal lipolysis, both *in vitro* (by RNAi) and *in vivo* (in 186 human subjects). In Paper IV, we further proved that CIDE-A plays an important role in the regulation of lipolysis, since CIDE-A over-expression decreased basal lipolysis. By performing Y2H screens and careful analysis of the CIDE-A sequence, we searched for possible interacting proteins. The presence of NR boxes and a CoRNR (Co-Repressor/Nuclear Receptor) box in the sequence made us investigate whether CIDE-A interacts with NRs. By using several different techniques we found that CIDE-A interacts with LXR-α and -β, possibly as a co-repressor. Furthermore, we found that activation of the LXRs using a synthetic agonist enhanced basal lipolysis and down-regulated the lipolysis regulator PLIN in human adipocytes. In agreement with Iwahana *et al.* (195), we found that CIDE-A localizes both to the cytosol and nucleus, although the nuclear staining was very limited. This could at least partly explain why we did not find any nuclear interactions in our Y2H screens. Although localized to the cytosol under certain conditions (222,223), the LXRs mainly reside in the nucleus.

Although these data do not fully explain how CIDE-A regulates basal lipolysis in human adipocytes, we suggest that CIDE-A mediates its antilipolytic effect by regulating (possibly suppressing) LXR action. Further studies are needed to clarify the exact mechanisms of CIDEA-LXR interactions and their effects on basal lipolysis, as discussed in the final section of this thesis.

5 CONCLUDING REMARKS

The four specific aims for the thesis (see section 2) can be merged to two overall aims:

1) to investigate effects of energy restriction and dietary macronutrient composition on the regulation of gene expression and protein secretion in human adipose tissue

2) to search for a novel, nutrient-sensitive candidate gene in obesity and study its regulation and function.

From the studies in Papers I-IV we conclude that

1) the dietary macronutrient composition is of little or no importance for the gene expression and protein secretion in human adipose tissue; it is the energy restriction *per se* that is important (discussed in section 5.1 below)

2) the gene CIDE-A is a novel candidate gene in obesity with antilipolytic properties, possibly exerted by regulating LXR action (discussed in section 5.2).

5.1 ENERGY RESTRICTION AND MACRONUTRIENT COMPOSITION

The effects of energy restriction and weight loss on adipose tissue-derived proteins have been investigated in a number of studies both before and after Paper I was published (e.g., (304-306) and references in Paper I). Furthermore, effects of the macronutrient composition of weight loss diets have also been thoroughly studied, as reviewed in section 1. However, to our knowledge, no other studies have concurrently investigated the effects of weight loss and different hypoenergetic diets on mRNA expression, protein secretion and circulating levels of as many as six different proteins. As for Paper II, it was the first microarray study comparing the effects on adipose tissue gene expression of hypoenergetic diets with different macronutrient composition. We have only investigated the effects of these diets on human subcutaneous adipose tissue from women. It is possible that other tissues or organs would respond differently and that protein secretion from visceral adipose tissue or the gene expression in e.g., skeletal muscle or liver would be affected in different ways. It is also possible that other results would be obtained from similar studies with male subjects.

As already mentioned, the results of the comprehensive studies in Papers I and II suggest that the dietary macronutrient composition is of little or no importance for weight loss and adipose tissue-derived protein secretion and gene expression. Instead, it is the energy restriction *per se* that gives beneficial effects. This is in agreement with several studies demonstrating that there is no 'magic bullet'; the essential prerequisite for weight loss and weight loss maintenance is energy restriction (307,308). In striking contrast to this are the results in the recent paper by Gardner *et al.* (172), showing that the Atkins diet, aiming for an extremely low carbohydrate intake, caused a greater weight loss than three other diets with various fat, protein and carbohydrate contents. Furthermore, conflicting results concerning low-carbohydrate diets and mortality (168-

170) further fuel the debate about pros and cons of different dietary macronutrient compositions.

From a nutritional point of view, extreme weight loss diets such as the Atkins diet are not sustainable in the long run, since the exclusion of certain foods brings a risk of deficiency symptoms resulting from lack of vitamins or minerals. A vast body of evidence shows that a complex and varying diet is essential for good health, although extreme diets can induce short-term weight loss. In agreement with our results and in spite of their positive results from the Atkins diet, Gardner *et al*. conclude that 'long-term success [in losing weight] requires permanent alterations in energy intake and energy expenditure, regardless of macronutrient content' (172).

5.2 CIDE-A AND BASAL LIPOLYSIS

Our results in Paper I show that even a modest weight loss decreases the risk for obesity-related co-morbidities by decreasing the levels of secreted pro-inflammatory cyto- and chemokines, some known to induce insulin resistance, and by improving insulin sensitivity. As previously mentioned, increased basal lipolysis plays an important role in obesity-related complications. The changes in basal lipolysis in the two diet groups studied in Papers I and II have been investigated (309). Basal lipolysis decreased slightly, although not significantly (P=0.09 for the two groups pooled together, paired two-tailed Student's t-test; Löfgren, P *et al.*, unpublished). Weight loss initially causes increased lipolysis, although the establishment of a lower body weight after more extensive weight loss than in Papers I and II is accompanied by decreased basal lipolysis (310).

The weight loss-related improvements mentioned above—decreased levels of cyto- and chemokines, improved insulin sensitivity and decreased basal lipolysis—are mediated at least in part by changes in gene expression, studied in Papers I and II. We believe that CIDE-A is an important player in obesity, since CIDE-A was the most up-regulated gene after diet-induced weight loss in Paper II and since Paper III shows that a low CIDE-A mRNA expression (present in obesity) is associated with high TNF-α secretion, insulin resistance and high basal lipolysis. Based on the results in Paper IV, we believe that CIDE-A exerts anti-lipolytic actions by regulating LXR. CIDE-A over-expression causes decreased basal lipolysis whereas LXR activation causes increased basal lipolysis and decreased mRNA expression of the lipolysis regulator, PLIN. Furthermore, Paper III shows that CIDE-A and PLIN mRNA are down-regulated in parallel. It remains to clarify how exactly CIDE-A and LXR interact, whether more proteins are involved in the complex between them and the mechanisms for how CIDE-A and LXR regulate lipolysis. This is further discussed in the final section of this thesis, 'Future perspectives'.

6 FUTURE PERSPECTIVES

The findings in Paper IV raised new questions concerning the mechanism(s) for how CIDE-A is involved in the regulation of basal lipolysis. As mentioned previously, our hypothesis is that CIDE-A exerts its antilipolytic actions by suppressing LXR-regulated gene expression. Several different approaches are at hand to further clarify the interactions between CIDE-A and LXR:

Firstly, careful bioinformatic analysis of the HSL and PLIN promoters will provide information about whether these sequences hold LXREs or not. If so, expression vectors with fragments of different lengths should be subcloned and used for promoter studies in adipocytes.

Secondly, we do not know as yet which LXR is responsible for the effects on lipolysis (if not both), since the synthetic agonists GW3965 and T0901317 are unspecific and activate both LXR-α and LXR-β. By using siRNA specific for the two isoforms, with and without the addition of agonist, we hope to gain information about the roles of the individual receptors. Furthermore, an LXR-α-specific agonist has been developed (243) and if it becomes commercially available, it will provide further information. However, compensatory effects between LXR-α and LXR-β have been observed in knockout models and could provide difficulties in the interpretation of data (237,311).

Thirdly, preliminary data from our lab indicate that interactions between CIDE-A and LXR might also be involved in the regulation of FA oxidation in human fat cells (Laurenčikienė, J, unpublished observations). This should be further investigated by expression studies of genes involved in FA oxidation.

To better understand the regulation and function(s) of CIDE-A it is also important to gain information about the CIDE-A promoter, which has not been described yet. The sequence upstream of the gene should be investigated in a similar way as suggested for the HSL and PLIN promoters above. It would also be interesting to study the CIDE-A-LXR interactions by investigating the potential complex between the two and possibly also other proteins *in vivo* and to study the cross-talk between CIDE-A and LXR *in vivo*. However, these latter studies would require the use of biochemical techniques that are not established in our group at present, e.g., crystallography, high-performance liquid chromatography (HPLC) or nuclear magnetic resonance (NMR). Simpler methods such as co-immunoprecipitation are not feasible as long as there are no good, commercially available antibodies directed against endogenous CIDE-A, as previously discussed.

Since elevated FFA levels are at the heart of obesity-related complications such as insulin resistance and type 2 diabetes, it is of paramount importance to find ways to decrease the negative effects of these circulating FAs. The work in this thesis suggests that pharmaceutical agents that either up-regulate CIDE-A expression or alter the interactions between the LXRs and CIDE-A could be one approach towards lower FFA levels in obese individuals.

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