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Modulation of body fat content by targeting the lipid metabolism of adipose tissue

Ph. D. Thesis

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This thesis is based on the following articles, referred to in the text by their Roman numerals as indicated here:

I. Matejkova O, Mustard KJ, **Sponarova J**, Flachs P, Rossmeisl M, Miksik I, Thomason-Hughes M, Grahame Hardie D, Kopecky J. (2004). Possible involvement of AMP-activated protein kinase in obesity resistance induced by respiratory uncoupling in white fat. *FEBS Lett.* 569(1-3):245-8.

II. Ruzickova J, Rossmeisl M, Prazak T, Flachs P, **Sponarova J**, Veck M, Tvrzicka E, Bryhn M, Kopecky J. (2004). Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. *Lipids* 39(12):1177-85. Erratum in: *Lipids* 2005 40(1):115.

III. Flachs P, Horakova O, Brauner P, Rossmeisl M, Pecina P, Franssen-van Hal N., Ruzickova J, **Sponarova J**, Drahota Z, Vlcek C, Keijer J, Houstek J, Kopecky J. (2005). Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce β-oxidation in white fat. *Diabetologia* 48: 2365-2375.

IV. **Sponarova J,** Mustard KJ, Horakova O, Flachs P, Rossmeisl M, Brauner P, Bardova K, Thomason-Hughes M, Braunerova R, Janovska P, Grahame Hardie D, Kopecky J. (2005). Involvement of AMP-activated protein kinase in fat depot-specific metabolic changes during starvation. *FEBS Lett.* 579: 6105-6110.

The above papers are included in full in this PhD Thesis. For a list of all my published articles, see page 41 and for a list of my poster presentations, see page 43.

CONTENTS

1. LIST OF ABBREVIATIONS

ACC 1, 2	acetyl-CoA carboxylase; isoform 1 and 2
ADP	adenosine diphosphate
ALA	α-linolenic acid
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AOX–1	acyl-coA oxidase 1
aP2	fatty acid binding protein aP2
aP2-*Ucp1* mouse	transgenic mouse with the expression of UCP1 from the fat-specific aP2 gene promoter
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
C/EBP α, β, γ	CCAAT/enhancer binding proteins
cHF	composite high fat diet
cHF-F1 and cHF-F2	composite high fat diet with partial replacement of lipids with EPA/DHA concentrate
CPT-1	carnitine palmitoyltransferase 1
DHA	docosahexaenoic acid
EF1-α	elongation factor 1 α
EPA	eicosapentaenoic acid
FA	fatty acid(s)
FAS	fatty acid synthase
FFA	free fatty acids
GLUT 1, 2, 4	glucose transporter 1, 2, 4
HF	high fat
HSL	hormone-sensitive lipase
LA	linoleic acid
LPL	lipoprotein lipase
MCD	malonyl-coenzyme A desaturase
n-3 PUFA	polyunsaturated fatty acids of n-3 (omega–3) series

NRF-1	nuclear respiratory factor 1
n-3 VLCD	very low calorie diet with addition of EPA/DHA
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1	PPAR gamma coactivator 1
PPAR (α,δ,γ)	peroxisome proliferator-activated receptor - α, δ, γ
PUFA	polyunsaturated fatty acids
qRT-PCR	quantitative real-time PCR
ROS	reactive oxygen species
sHF*f*	semisynthetic high fat diet based on flax-seed oil
sHF*f*-F1 and sHF*f*-F2	semisynthetic high fat diet based on flax-seed oil with partial replacement of lipids with EPA/DHA concentrate
SREBP-1,1c, 2	sterol regulatory element binding protein –1,1c, 2
TG	triacylglyceroles
TNFα	tumor necrosis factor α
TZD	thiazolidinediones
UCP 1, 2, 3	uncoupling protein 1, 2, 3
VLCD	very low calorie diet
VLDL	very low density lipoproteins

2. INTRODUCTION

White adipose tissue is specialized connective tissue that functions as the major storage site for fat in the form of triacylglyceroles (TG). As the major form of energy storage, adipose tissue provides a buffer for energy imbalances when energy intake is not equal to energy output. The excessive accumulation of adipose tissue – obesity (defined by a body mass index, weight divided by the square of height, of 30 kg m^{-2} or greater) – implies a health risk. In particular, it is associated with the development of type 2 diabetes mellitus, dyslipidemia, cardiovascular diseases (together designated as a metabolic syndrome) and certain forms of cancer. These changes are, to a certain extent, dependent on the regional distribution of adipose tissue. Abdominal and, in particular, visceral adiposity is strongly linked to metabolic syndrome diseases, whereas such association is much less consistent with respect to subcutaneous fat mass (1).

However white adipose tissue is more than just a passive depository for excess energy. In the last few years, it became evident that adipocytes secrete a large number of proteins called adipokines, such as leptin, adiponectin, tumor necrosis factor α (TNF-α), interleukin-6 and resistin. These adipokines act both locally and at more distant sites, in order to modulate whole body metabolism, particularly sensitivity of other tissues to insulin (2). Indeed, many candidate genes relating to obesity have important function in adipose tissue, and numerous models of transgenic mice have been created that are prone or resistant to obesity due to the modification of the metabolism in fat tissues (3), suggesting that metabolism of adipose tissue itself contributes to the control of body fat content.

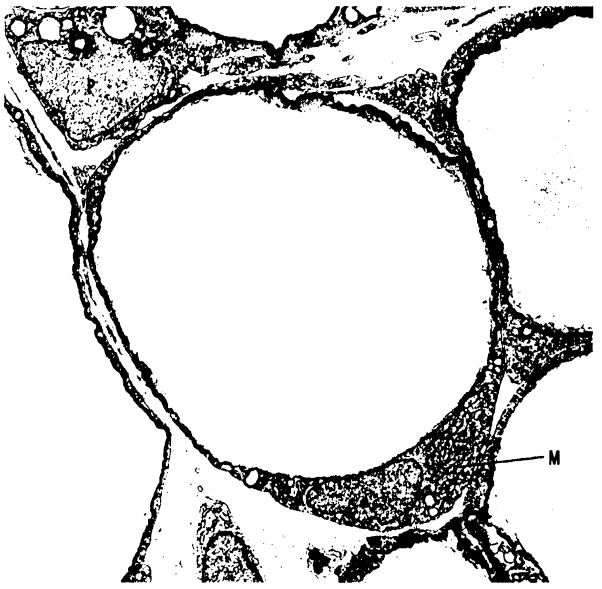

Figure 1. *Electron microscopy of typical white adipocyte. Reproduced from "The adipose organ" (Cinti, S. (1999); Editrice Kurtis, Milano, Italy)*

2.1. Lipid metabolism in white adipose tissue

2.1.1. White adipose tissue

The quantity of body fat stores varies widely in mammals, ranging from 2% - 50% body mass, typically from 10-20% in mice and humans (4). The huge variation in fat mass is unlike that of any other organ in the body. Approximately 60 - 85% of the weight of adipose tissue is lipid, with 90-99% being TG. In contrast to brown adipocytes which contain many small lipid droplets, so-called multilocular cells and rich vascularization and densely packed mitochondria give them its colour; white adipocytes are unilocular cells containing a single large lipid droplet, which pushes the cell nucleus against the plasma membrane. White adipocytes vary enormously in size (20 – 200 μm in diameter), and are able to change their diameter 10-fold and their volumes by several hundred-fold. White adipose tissue is not as richly vascularized as brown adipose tissue, but each adipocyte in white adipose tissue is in contact with at least one capillary. This blood supply provides sufficient support for the active metabolism, which occurs in the thin rim of cytoplasm surrounding the lipid droplet. However, specific metabolic rate and oxidative capacity of white fat are relatively low. The contribution of white fat to the resting metabolic rate of a lean human subject is close to 5 %, while thermogenesis in brown fat in rodents may account for more than 50 % of total metabolic rate (5). The blood flow to adipose tissue varies depending upon body weight and nutritional state, with blood flow increasing during fasting.

The main metabolic pathways of adipose tissue cover lipolysis, i.e. extraction of lipids from circulation mediated by lipoprotein lipase (LPL), in situ fatty acid (FA) synthesis from glucose and the breakdown of TG, which is initiated by hormone-sensitive lipase (HSL). The released FA may be re-esterified or oxidized in adipocytes, or they are exported to other tissues. In normal fed rats, only 0.2 % of endogenous FA in adipocyte are oxidized. In fed rats, approximately 50 % of FA are released, while 49 % are re-esterified (6). Also in human adipocytes, uptake and re-esterification of free fatty acids (FFA) derived from plasma lipoproteins has major importance in fed state (7).

2.1.2. Main metabolic pathways in white adipose tissue

2.1.2.1. Lipolysis

LPL mediated lipolysis

The uptake of FA by white adipose tissue, mediated by LPL, is the main mechanism of plasma TG clearance during the postprandial period – LPL is activated by insulin (8). LPL is expressed by the parenchymal cells of several extrahepatic tissues, including adipocytes. After transport to the endothelial surface, LPL catalyse the hydrolysis of the TG components of chylomicrons and very low density lipoproteins (VLDL), thereby providing FFA and 2-monoacylglycerols for tissue utilization. The activity of LPL in adipose tissue is known to be regulated by nutritional and hormonal factors. Under catabolic condition, e.g. fasting, LPL activity is decreased (9). Other reports showed that catecholamines decrease LPL synthesis and secretion, while insulin supplementation decreases FA levels and restore the LPL activity of adipose tissue (8). Thus, there is an inverse correlation between blood FA levels and LPL activity. Different fat depots vary in their responsiveness to the hormones that regulate lipolysis and this also varies according to fat distribution. In both men and women, the lipolytic response to norepinephrine is more marked in abdominal, rather in gluteal or femoral adipose tissue (10).

HSL mediated lipolysis

The activation of HSL is the final rate-limiting step for the breakdown of TG and diacyglyceroles in adipocytes. The enzyme is also responsible for the hydrolysis of cholesteryl esters and retinyl esters. HSL is activated by catecholamines through cAMP-dependent phosphorylation, whereas insulin prevents this phosphorylation (11). Apart from adipose tissue, the enzyme is also functionally expressed in skeletal muscle and testis. An important step in lipolysis activation *in vivo* is the translocation of HSL from a cytosolic compartment to the surface of the lipid droplet. Lipid droplets are coated with perilipins, structural proteins that stabilize the droplets and prevent the hydrolysis of TG in the basal state. Upon lipolytic stimulation, HSL is translocated from the cytosol to the fat droplets, while, perilipins are moved from the fat droplets to the cytosol. Thus, the physiological

activation of lipolysis is a highly coordinated reaction between phospho-HSL and phospho-perilipins (12).

2.1.2.2. Lipogenesis

De novo FA synthesis in adipocytes is an important mechanism involved in the control of body fat content. The lipogenic capacity of human adipose tissue is believed to be much smaller than that of liver. However, even in humans, adipose tissue may account for up to 40 % of the whole-body lipogenesis and may contribute to the development of obesity (13). The biosynthesis of FA requires cooperation between mitochondrial and cytoplasmatic enzymes and involves fluxes of metabolites across mitochondrial membranes. The synthesis of malonyl-CoA is the first committed step of FA synthesis and the enzyme that catalyzes this reaction, acetyl-CoA carboxylase (ACC), is the major site of regulation of FA synthesis. In animals, including humans, two carboxylase isoforms (ACC-1 and ACC-2) having distinct tissue distribution are encoded by separate genes. ACC-1 is the predominant isoform in lipogenic tissues such as liver, adipose tissue, and lactating mammary gland. ACC-2 is highly expressed in the skeletal muscle, heart, and, to a considerable degree, in the liver. In lipogenic tissues, malonyl-CoA is the source of C_2 moiety used for the synthesis of FA by fatty acid synthase (FAS). The rate of FA synthesis is controlled by the equilibrium between monomeric ACC and polymeric ACC. The activity of ACC requires polymerization. This conformational change is enhanced by citrate and inhibited by long-chain FA. Insulin is known to activate ACC and FAS, whereas glucagon and epinephrine inactivate the enzymes (14;15). Both ACCs and FAS are modulated by AMP-activated kinase (AMPK) (16). Another major player in the regulation of fatty acid metabolism is malonyl-CoA decarboxylase, which degrades malonyl-CoA (17).

2.1.2.3. β–oxidation

Adipocytes have active mitochondrial machinery for FA oxidation, although the rate of oxidation is generally lower than in liver or muscle cells. Only 0.2 % of endogenous FA are oxidized in white adipose tissue, however a slight shift in this pathway could have a significant impact on the fuel economy of the whole body (6). Carnitine

palmitoyl transferase-1 (CPT-1), the enzyme of the inner mitochondrial membrane that controls the transfer of cytosolic long chain acyl-CoA into mitochondria acts as a gatekeeper for acyl-CoA to enter the β-oxidation pathway. However, a major factor that regulates intracellular FA oxidation in a variety of tissues, including adipocytes, is malonyl-CoA, an allosteric inhibitor of CPT-1. Interestingly, its concentration changes in response to alterations in the cellular fuel availability and energy expenditure. Therefore, malonyl-CoA is both an intermediate in the *de novo* synthesis of long-chain FA and an inhibitor of CPT-1, and represents an important check-point in metabolism of FA (17).

2.1.2.4. Fatty acid re-esterification

Re-esterification of FA within adipocytes plays an important role in intracellular fuel partitioning and regulation of adipose tissue mass. Under normal conditions, the glycerol released from the hydrolysis of TG in adipocytes cannot be re-utilized for the esterification of FA because of the very low activity of glycerol kinase in white fat (18). The "lack" of glycerol kinase normally prevents adipocytes from recycling the glycerol in a "futile" cycle of TG hydrolysis and re-synthesis. The 3-glycerol phosphate required for TG synthesis must be generated from either glucose via glycolysis or, alternatively, via a truncated form of gluconeogenesis termed glycerogenesis (19). The key enzyme in this glycerogenesis is the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK) that catalyzes the conversion of oxaloacetate to phosphoenolpyruvate and could be therefore considered as the rate-limiting enzyme for FA re-esterification under normal conditions. However, it has been recently shown that glycerol kinase could be substantially activated with thiazolidinediones (TZD), a class of insulin-sensitizing agents and widely used anti-diabetic drugs, and that treatment under these conditions could considerably participate in the overall rate of re-esterification (18;20). Increased expression of PEPCK in adipose tissue of mice has been associated with obesity (21). Its down-regulation, however, may occure as a result of mutation in the promoter binding site for its regulatory factor, the peroxisome proliferator-activated receptor γ (PPARγ), resulting in reduced adipose tissue size (22). During fasting, the low level of insulin and increased intracellular cAMP leads to decreased glucose utilization and increased PEPCK gene expression (19).

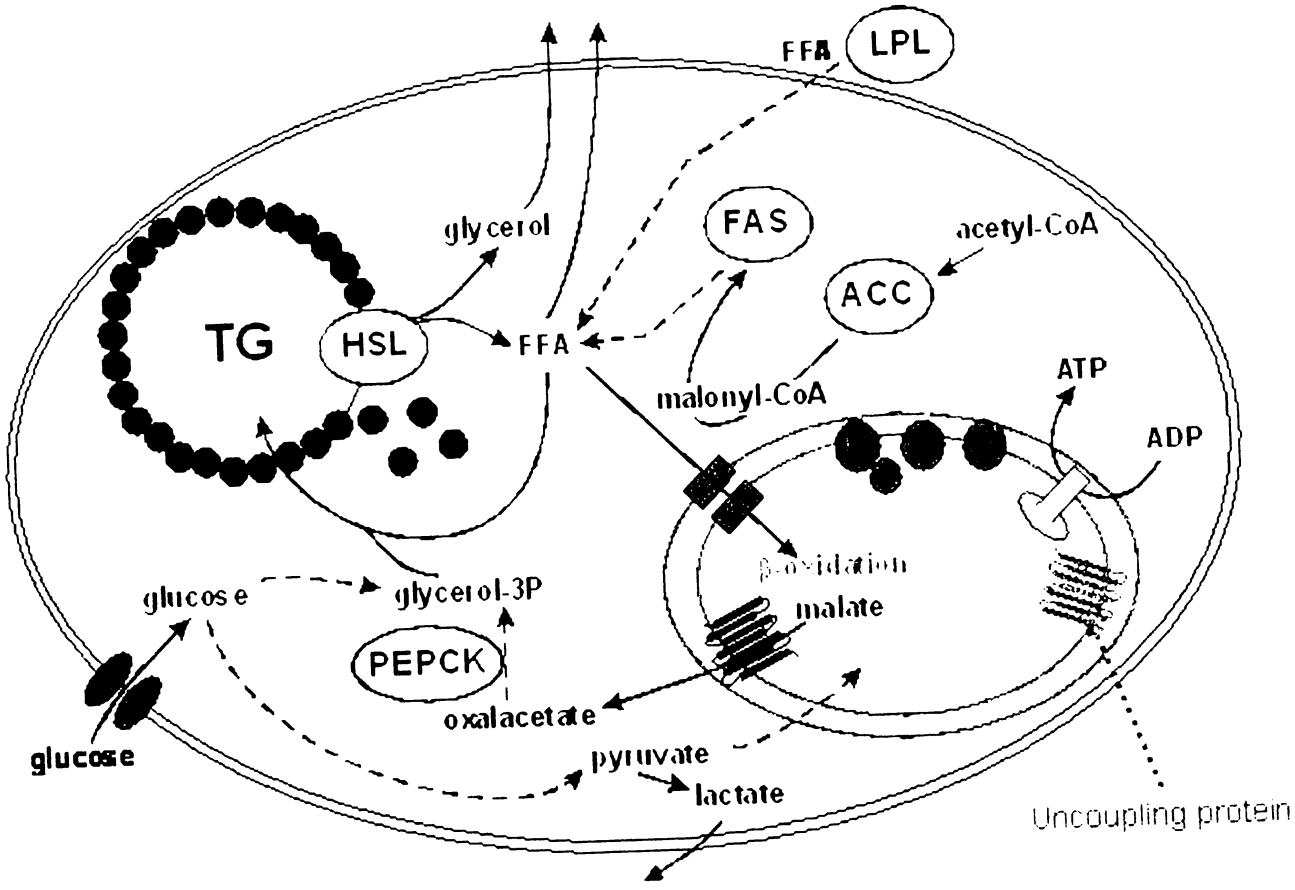

Figure 2. *Main metabolic pathways in white adipose tissue*

2.1.3. Regulation of gene expression in white adipocytes

Studies of transcription biding sites in the regulatory regions of genes expressed specifically in mature adipocytes resulted in the identification of three main families of transcription factors which play critical roles in the regulation of lipid metabolism as well as in the process of adipocyte differentiation.

2.1.3.1. PPARs

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptors family involved in cellular differentiation and metabolic homeostasis. Their transcriptional activities are modulated by ligand - receptor interaction with retinoid X receptor (23). To date, three PPAR isoforms (α,γ,δ) with tissue selective expression and ligand specific activation have been identified. Many PPAR-regulated genes encode proteins that regulate FA oxidation and storage (e.g. LPL, adipose-specific lipid binding protein (aP2), PEPCK, FAS, glucose transporter GLUT-2 and uncoupling protein 1 (UCP1)) (24). PPARα is highly expressed in the liver and skeletal muscle, and plays an important role in regulating expression of enzymes in the β-oxidation pathway. PPAR-γ is preferentially expressed in adipocytes, and modifies FA synthesis and storage in adipose tissue. Expression of PPARγ is induced and necessary for normal adipocyte differentiation (25), and ligand-activated PPARγ is sufficient to induce adipose conversion of fibroblasts (26). PPARδ is expressed in most cell types with relative abundance and so far, little is known about its role, but recent data suggest that PPARδ is also involved in adipocyte differentiation (27) and alteration of lipid metabolism (28). Long-chain FA, whether saturated or not, and certain prostaglandines (e.g. prostaglandin J2 derivatives) were shown to be naturally occurring activators for all three PPARs. Polyunsaturated fatty acids (PUFA) were shown to be most potent (29). Other PPARγ activators –TZD regulate glucose metabolism and insulin sensitivity, by reducing systemic FA availibility (24).

2.1.3.2. SREBPs

Sterol regulatory element binding proteins (SREBPs) are known to modulate transcription of numerous genes which function in both cholesterol and FA metabolism

(30). SREBPs are unique transcription factors in as much as they contain two transmembrane domains which anchor the protein to the endoplasmatic reticulum. The N-terminal fragment can be cleaved and translocated to the nucleus. This cleavage step is regulated by a putative sterol-sensing molecule, SREBP-activating protein that forms a complex with SREBP and traffics between the rough endoplasmic reticulum and the Golgi apparatus. Animal experiments involving transgenic and knockout mice suggest that three isoforms, SREBP-1a, -1c, and -2, have different roles in lipid synthesis. In differentiated tissues and organs, SREBP-1c is involved in FA synthesis, whereas SREBP-2 plays a major role in the regulation of cholesterol synthesis (30).

SREBP-1c, which is predominantly expressed in adipose tissue, has been identified as a nutritionally regulated form of SREBP-1. This isoform regulates the expression of many of the genes involved in *de novo* FA and TG synthesis, including ACC and FAS. Many groups have established that insulin upregulates SREBP1c mRNA and mature SREBP protein and is accompanied by increases in FA biosynthetic gene expression. As the product of the ACC-catalysed reaction, there is also an increase in the levels of malonyl-CoA, leading to the inhibition of CPT1 and mitochondrial FA oxidation (31).

2.1.3.3. C/EBPs

The CCAAT/enhancer-binding proteins (C/EBPs) belong to a large family of leucine zipper transcription factors that can form homo/heterodimers (23). Three of these family members C/EBPα, C/EBPβ, and C/EBPδ, are expressed in both white and brown adipose tissue and have been recognized as key activators of adipocyte differentiation process (32). C/EBPs can modulate the transcription of a large variety of genes for metabolic enzymes including aP2, GLUT-4, leptin, PPARγ and UCP-1. In mature adipocytes, phosphorylation plays a key role in the modulation of C/EBPs function (23). For example, protein kinase C can phosphorylate C/EBPα at several sites, leading to an attenuation of its DNA-binding activity, while insulin can activate a dephosphorylation-mediated degradation of C/EBPα.

2.2. Role of UCPs in white adipose tissue and respiratory uncoupling

As in other tissues, mitochondria represent the main source of ATP even in white fat. The efficiency of ATP synthesis during oxidative phosphorylation and hence the rate of ATP synthesis depend on the proton leak of the inner mitochondrial membrane. Brand and colleagues (1999) suggest a general occurrence of proton leak in mitochondria *in vivo*. Several candidate genes encoding for proteins that could enable the regulated proton leak are expressed in adipocytes, namely the genes for various uncoupling proteins (UCPs). UCPs are mitochondrial transporters present in the inner membrane of mitochondria. They belong to the family of anion mitochondrial carriers and are found in all mammals and plants. UCP1 is unique to brown adipocytes and is essential to nonshivering thermogenesis and in order to control body temperature (33). Brown fat is present in all mammalian neonates. A rapid respiration not coupled with ATP synthesis represents a powerful thermogenic process and the UCP1 content reflects the thermogenic activity of brown adipose deposits.

More recently, homologues of the brown fat UCP has been isolated and named UCP2 and UCP3. UCP2 and UCP3 share 72% and 57% amino acid identity, respectively, toward UCP1 (34). UCP2 is present in many organs and cell types (35), with the highest levels found in white adipose tissue, where UCP2 expression negatively correlates with obesity (36). Therefore a role in the regulation of lipid metabolism in adipocytes was suggested. The third uncoupling protein to be described to date, UCP3, is abundantly and preferentially expressed in muscle in humans and also in brown adipose tissue and muscle in rodents. In the white fat, only UCP2 and brain mitochondrial carrier (BMCP1, referred to as UCP5) genes are normally active. However, even in adult humans, relatively low levels of the UCP1 transcript can be detected in various fat depots (37). Similarly to UCP1, UCP2 and UCP3 may also enhance mitochondrial proton leak, induce respiratory uncoupling, and decrease ATP synthesis (38). In vitro studies demonstrated that a decrease in mitochondrial ATP production resulted in the inhibition of both FA synthesis (39) and the lipolytic action of catecholamines (40). In turn, lipolytic hormones, such as insulin, have been shown to decrease ATP levels in adipocytes (41).

Numerous reports have described significant changes in expression of UCP2 and/or UCP3 by dietary manipulation. Starvation was shown to increase the mRNA levels for muscle UCP2 and mainly UCP3 in rodents (42) or humans, while refeeding led to the down regulation of UCP3 gene expression. Mice over-expressing UCP3 in skeletal muscle are lean and resist diet-induced obesity and diabetes (43). Starvation and LPS treatment increase mRNA UCP2 level in skeletal muscle and liver mitochondria (35). High-blood levels of leptin resulted in a dramatic increase of the UCP2 mRNA expression in the white adipose tissue (44). It has been observed that TZD stimulates UCP2 mRNA expression in cell lines representing white adipose tissue (45) and increased mRNA UCP2 in white adipose tissue was also seen in mice fed with polyunsaturated fatty acids of n-3 (omega-3) series (n-3 PUFA) in the diet compared to a control group (46).

2.2.1. aP2-*Ucp1* transgenic mice

Transgenic aP2-*Ucp1* mice with C57BL/6J background, in which the UCP1 gene is driven by the fat specific aP2 promoter to achieve enhanced expression in both brown and white fat, represent an established model to study the effects of respiratory uncoupling in adipose tissue (47). These animals are partially resistant to obesity induced by age, genetic background (47) and high-fat (HF) diet (48). Their resistance to obesity reflects lower accumulation of TG in all fat depots except for epididymal fat, which becomes relatively large. (47;48). Interestingly, reduction in total body weight becomes apparent only under obesity-promoting conditions (such as feeding HF diet (48)) and importantly, resistance to obesity results exclusively from a transgenic modification of white fat, since brown fat in these mice is greatly atrophied (49). Ectopic expression of transgenic UCP1 in white fat and its consequences have been studied in great detail (47;48;50-54). Transgenic UCP1 is contained in all unilocular adipocytes. Expression of the transgene differs in various fat depots with greatest expression in subcutaneous fat and relatively low expression in epididymal fat (47). However, even in epididymal fat transgenic UCP1 is capable of decreasing mitochondrial membrane potential in adipocytes (52). In adult mice, the total content of transgenic UCP1 in white fat does not exceed 2% of the total UCP1 found in interscapular brown fat. Apparently, only minute amounts of

ectopic UCP1 in unilocular adipocytes of white fat can uncouple oxidative phosphorylation (52) and reduce the accumulation of fat.

The phenotype of aP2-*Ucp1* transgenic mice exhibited a reduction of FA synthesis (54) and in the lipolytic action of catecholamines (53), as well as increases in mitochondrial biogenesis (51) and in endogenous oxygen consumption (50) in white adipose tissue. A drop in ATP/ADP ratio in transgenic animals when compared to control group also was shown (53). All these effects were more pronounced in subcutaneous fat compare to epididymal fat depot, most likely reflecting the magnitude of UCP1 expression (47). The phenotype of aP2-*Ucp1* mice suggest that respiratory uncoupling is involved in the modulation of white adipose tissue metabolism.

2.3. AMP-activated protein kinase – an energy checkpoint

AMP-activated protein kinase (AMPK) is the central component of protein kinase cascade that plays a key role in the regulation of energy control. AMPK is activated in response to ATP depletion, and vice versa the activation is antagonized by high concentrations of ATP. The AMP/ATP ratio varies as the square of the ADP/ATP ratio. It is therefore a much more sensitive indicator of cellular energy status and is the parameter that is, in a highly sensitive manner monitored by the AMPK system (55). On the contrary, high glycogen content in skeletal muscle appears to repress AMPK activation both in animals and in humans. In cell-free assays, AMPK is also allosterically inhibited by physiologically relevant concentrations of phosphocreatine. Thus, the AMPK system may be able to monitor not only the immediate availability of energy in the form of intracellular adenine nucleotids, but also the medium- and long-term reserves of cellular energy in the form of phosphocreatine and glycogen respectively. AMPK occurs as heterotrimeric complexes comprising a catalytic α subunit and regulatory β and γ subunits. In humans, each subunit is encoded by two or three genes ($\alpha1$, $\alpha2$; $\beta1$,$\beta2$; $\gamma1$,$\gamma2$,$\gamma3$). The different $\alpha\beta\gamma$ combinations exhibit differences in their response to AMP and their subcellular localization (56). In white adipose tissue, the activity of $\alpha1$-AMPK complexes is predominant and accounts for more than 90% of total AMPK activity (57).

The AMPK cascade could be stimulated by any stress that interferes with ATP production such as metabolic poisoning, oxidative stress, hypoxia, nutrient deprivation or physiological stress, e.g. exercise or starvation. Once activated, AMPK phosphorylates a number of downstream substrates, the overall effect of which is to switch-off ATP-consuming pathways and switch-on ATP-generating pathways. In addition to the acute effect of AMPK, activation of AMPK has long-term effects, altering both gene expression and protein expression (reviewed in (55;58;59)). A full discussion of the downstream targets and processes regulated by AMPK can be found elsewhere. Activation of AMPK in skeletal muscle increases glucose uptake both acutely, via increased translocation of GLUT-4 to the plasma membrane (60) and chronically, by increased transcription of the GLUT-4 gene (61). AMPK also up-regulates the nuclear-respiratory factor 1 (NRF-1) and the PPARγ-coactivator-1 (PGC-1), transcription factor that were shown to induce mitochondrial biogenesis (62). AMPK activation in liver also represses the transcription of the genes encoding enzymes of gluconeogenesis (PEPCK and glucose-6-phosphatase) (63). Thus, AMPK activation stimulates glucose utilization by muscle and inhibits glucose production by liver. The system also acts as "master switch" for lipid metabolism. AMPK activation switched off FA and cholesterol synthesis in liver acutely by increasing phosphorylation of ACC and hydroxymethyl-glutaryl-CoA reductase (64) and also chronically, by decreasing transcription of the genes encoding ACC and FAS (65). This activation is mediated by suppression of the transcription factor SREBP-1 (66), which up-regulates genes engaged in lipogenesis (see above). In muscle, it stimulates FA oxidation via phosphorylation of ACC-2 isoform leading to a decrease in malonyl-CoA and a consequent increase in FA transport into mitochondria due to the relief of malonyl-CoA inhibition of CPT-1 (17). In adipocytes, AMPK is known to inhibit both lipolysis and lipogenesis by phosphorylation of HSL and ACC-1 (57;67), as well as by downregulating PPARγ expression. Moreover, *in vitro* studies suggest an inhibitory role for AMPK in adipocyte differentiation (68). Recent studies indicate the involvement of AMPK in the effects of physical excercise (17), adiponectin (69), TZD (70), leptin (71;72), and the most recently also in the effect of PUFA (73). Nevertheless the role of AMPK in adipose tissue remains relatively unexplored.

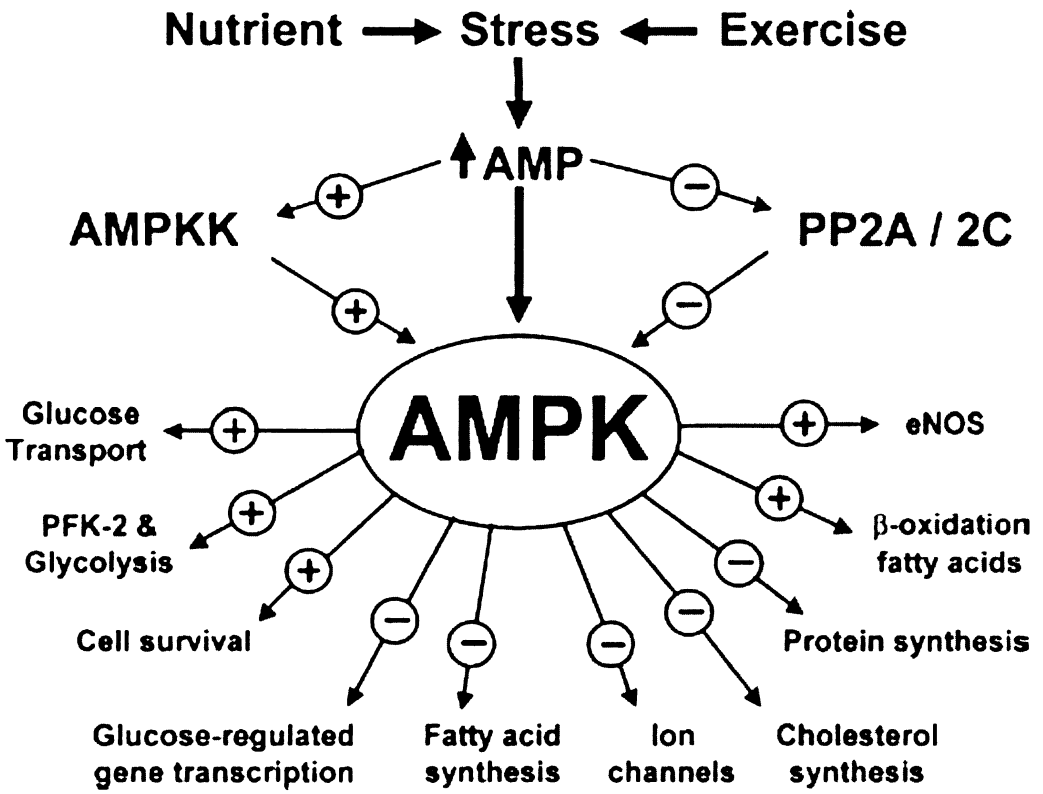

Figure 3. AMPK regulation and function (B.E.Kemp et. all; 2001)

2.4. n-3 polyunsaturated fatty acids

TG from adipose tissue contain a wide spectrum of fatty acids, ranging in chain length from C_{12} to C_{24} with zero to six double bonds, which depend mainly on the FA composition of the diet (74). In several cases for both, rodents (75;76) and humans (77) it has been reported that PUFA, especially omega-3 polyunsaturated FA (n-3 PUFA) eicosapentaenoic acid (EPA, $20:5^{\Delta5,8,11,14,17}$) and docosahexaenoic acid (DHA, $22:6^{\Delta7,10,13,16,19}$) are less effective in promoting the accumulation of adipose tissue than saturated FA. The n-3 PUFA family has their first double bond three carbons from the methyl end. The α-linolenic acid (ALA,$18:3^{\Delta9,12,15}$) is the parent acid for n-3 PUFA and it is an essential FA for mammals because they do not possess the ability to insert double bonds into 18-carbon PUFA between the methyl end and the middle of the molecule, therefore must be obtained from the diet. ALA is found in plants, zooplankton, phytoplankton and marine species. In mammals, ALA is a precursor of EPA and DHA, but is rapidly oxidized in the organism and its conversion to EPA and DHA is only about 15% (78). In contrast, feeding on fish oil leads to a high dose dependent increase in tissue levels of the long, highly unsaturated FA (79). EPA and DHA account for 15-20% of the FA in most fish oils.

The n-3 PUFA, in particular EPA and DHA act as hypolipidaemics, exert prophylactic effects on cardiovascular diseases and may improve insulin sensitivity (80). They are highly incorporated into membrane phospholipides (81;82) and also act as mediators of gene expression (83;84). The effect of n-3 PUFA on fat deposition does not result from the reduction in food intake (46;76;85), but rather reflects the metabolic changes in several tissues (83;86;87). PUFA were shown to be the most naturally occurring activators of PPARs, the transcription factors family that is involved in regulation of the whole body lipid metabolism (see above). Genes encoding both glycolytic and lipogenic enzymes, likewise key enzymes involved in FA oxidation are regulated by dietary n-3 PUFA. In liver, EPA and DHA have been reported to stimulate FA mitochondrial and peroxisomal oxidation (partially by activation of PPARα), suppress FA synthesis and reduce activity of esterifying enzymes, partially by suppression of transcription factor SREBP-1 (86). Stimulation of FA oxidation was also observed in muscle (88). At the level

of adipocytes, a decrease in lipogenesis and in lipogenic genes (87) as well as in basal lipolysis (89) has been shown. Studies in rodents indicate complex modulation of gene expression in white adipose tissue (83;84). In humans, the whole body lipid oxidation was shown to be increased by dietary fish oil (77). n-3 PUFA are also potent stimulators of diet-induced thermogenesis (90) and enhance UCP1 content in brown adipose tissue. Most recently, it was reported that n-3 PUFA stimulated AMPK activity in rat liver (73).

2.5. Effect of starvation on white adipose tissue

Calorie restriction is one of the main conditions leading to a reduction of body fat mass and is also known to be effective for reducing the complications of obesity. Even though the tissues preferentially use carbohydrate for energy over both fat and protein, the quantity of carbohydrate stores in the body is only a few hundred grams (mainly glycogen in the liver and muscles). Therefore, except for the first few hours (in humans) of starvation, the major source of energy is proteins and, in particular adipose tissue. Transgenic mice lacking white adipose tissue are unable to survive starvation which confirms the important role of white adipose tissue in supplying energy for utilization under this condition (4).

During starvation (and in the presence of higher demand for energy, e.g. exercise), FA are predominantly generated within the adipocytes by the action of HSL on stored TG. The FA are than mobilized out of the adipose tissue for delivery to other tissues, such as skeletal muscle, heart and liver. Furthermore, lipolysis *via* LPL as well as its gene expression in white adipose tissue is decreased, while is activated in skeletal muscle (9). This leads to the supplementation of FFA to the liver, muscle and heart for use as a main energy fuel, instead of glucose. However, the FA flux initiated by adipose tissue HSL-mediated lipolysis during starvation is partially futile and is characterized by ~ 50% re-esterification of lipolysed FA back into adipose TG (91). PEPCK, the enzyme that catalyzes the first step of the glyceroneogenesis pathway is up-regulated in adipocytes during starvation, providing glycerol-3-phosphate for TG re-esterification, when the diet cannot (19). In adipose tissue, as in other tissues in which FA serve as the main fuel of

energy, β-oxidation pathway of FA is also increased. Since starvation generally down-regulates the enzymes of TG synthesis (92), and at the same time total TG hydrolysis via HSL is increased and the rate of re-esterification stays on (6), more acyl-CoA become available to the mitochondria. Indeed, starvation down-regulates the expression and activity of ACC, therefore the production of malonyl-CoA, the allosteric inhibitor of CPT-1, is reduced (17) and can promote FA oxidation. Additionally, as a consequence of low insulin levels, glucose uptake in starved adipocytes is diminished (93), and the cells are shifted to increase FA oxidation for energy production. However, starvation causes profound alternations in the physiological environment and additional mechanisms responsible for the regulation of FA oxidation are likely to be involved. It has been shown that adipocytes in different locations of the body differ in their response to calorie restriction (94). Starvation also leads to a selective activation of sympathetic innervations of white adipose tissue (unlike the other tissues) (95) and may stimulate AMPK in this tissue (57).

3. SPECIFIC AIMS OF THE WORK

The general aim of this thesis is to verify a hypothesis that obesity may be limited by modulation of metabolism in white adipose tissue, and that intracellular energy charge is involved in this control. The aP2-*Ucp1* transgenic mice and starvation were used as model situations where intracellular energy charge is altered, with a special focus on the role of AMPK in the control of fat metabolism. Last but not least, the effect of omega-3 PUFA has been also studied. The following specific aims were addressed in experiments on mice

1. whether AMPK-cascade is involved in the induction of obesity resistance by respiratory uncoupling induced by transgenic UCP1 in white adipose tissue;

2. whether AMPK could be involved in the differential response of various depots of white adipose tissue to starvation;

3. whether dietary intake of EPA and DHA could limit obesity by modulation of gene expression and metabolism in white adipose tissue.

The last aim was also verified in a clinical study.

4. COMMENTS AND DISCUSSIONS TO SELECTED PUBLICATIONS

4.1. Involvement of AMP-activated protein kinase in resistance to obesity induced by respiratory uncoupling in white fat (Paper I).

The ectopic expression of UCP1 in the white fat of aP2-*Ucp1* transgenic mice mitigates obesity induced by genetic or dietary factors (47;50). The phenotype was partly described before (see introduction). Among others, it involves the reduction of FA synthesis and the lipolytic action of catecholamines, as well as the increases in mitochondrial biogenesis and endogenous oxygen consumption in white adipose tissue. Indeed, both ATP content and ATP/ADP ratio were previously shown to be depressed in white fat of transgenic mice (53). All these effects are in accordance with the activation of AMPK (see introduction). Based on these findings, the main aim of this study was to verify possible involvement of AMPK cascade and further characterize the metabolic changes behind the phenotype of aP2-*Ucp1* transgenic mice. The AMP/ATP ratio (one of possible activating stimuli), the activity and the total content of α1 catalytic subunit of AMPK and the phosphorylated form of AMPK in two different adipose depots from aP2-*Ucp1* transgenic mice compare to control mice were examined.

As we expected in consequence of ectopic expression of UCP1 in white adipose tissue, HPLC analysis of ATP and AMP levels in both subcutaneous and epididymal fat depots revealed elevation of AMP/ATP ratio in transgenic animals compare to controls. This suggested that AMPK activity would be also affected. Indeed, 2-fold increase in the activity of α1 isoform AMPK in subcutaneous fat was shown. In the case of epididymal fat, the enzyme activity also tended to be higher in the transgenic than in the control mice, but the difference was not statistically significant (Fig.4). Furthermore, significantly higher content of α1 AMPK and its phosphorylation, in both fat depots, were shown due to transgenic modification. The increased expression of α1 subunit has been also observed in other tissues in response to treatment that would be expected to persistently activate AMPK e.g. endurance training in human skeletal muscle (96). To test whether the induction of AMPK activity promoted phosphorylation of its downstream

targets in subcutaneous fat, phosphorylation of ACC-1 was assed using Western blots. A modest induction of the ACC-1 phosphorylation was observed in the transgenic mice.

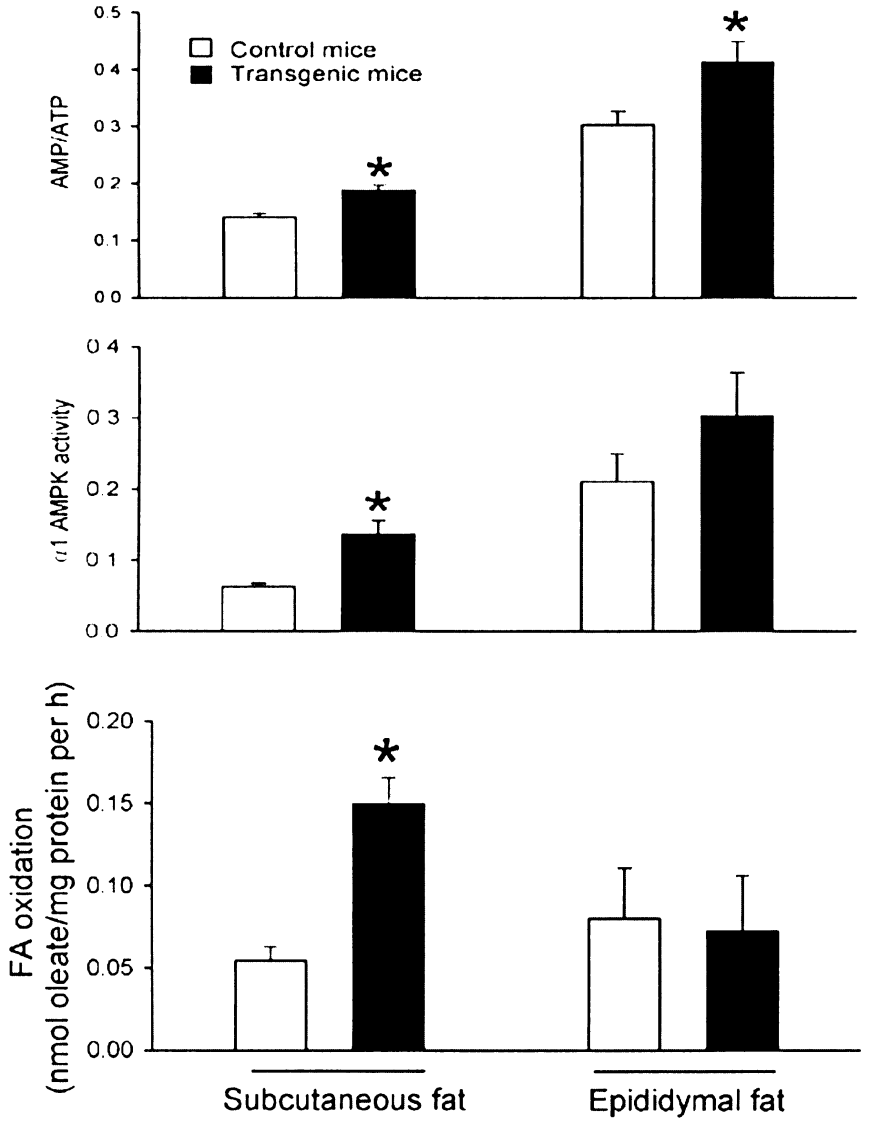

Figure 4. AMP/ATP ratio, AMPK activity and FA oxidation in white fat depots.
Values are means± SE (AMPK activity in units, n=15, 6, 6; respectively).A statistically significant difference between the genotypes is indicated by an asterisk.

In order to further characterize the complex changes in lipid metabolism in the transgenic mice, expression of the PPARγ gene was analyzed. PPARγ plays a crucial regulatory role in adipogenesis (see introduction) and its expression is inhibited by AMPK activation during differentiation of 3T3-L1 adipocytes in culture (68). A significant diminution of PPARγ mRNA level was found in subcutaneous but not in epididymal fat of aP2-*Ucp1* mice. Moreover the expression of a PPARγ target gene - aP2 was also down-regulated in subcutaneous fat. Finally, measurement of oleate oxidation in adipose tissue fragments revealed increase in the subcutaneous, but not in epididymal fat depots (Fig.4) from transgenic animals, which is also consistent with the known effects of AMPK.

The experiments with aP2-*Ucp1* mice suggest that the main function of mitochondrial protonophores in white fat is the modulation of lipid metabolism through the effect on ATP/ADP ratio in adipocytes. Induction of AMPK activity in adipocytes of transgenic mice, mediated most likely by the increased AMP/ATP ratio, represents a mechanism by which respiratory uncoupling could directly modulate fluxes through metabolic pathways of lipogenesis, FA oxidation and lipolysis, and stimulate mitochondrial biogenesis. The differential effect of the transgene on the AMPK activity in various fat depots was in accordance with higher expression of the UCP1 in subcutaneous than epididymal fat, and with the preferential effect of the transgene to reduce an accumulation of lipids in the subcutaneous fat. Thus, activation of AMPK in white fat of aP2-*Ucp1* mice can explain the complexity of the changes in adipose tissue metabolism in this transgenic model and might represent an important mechanism by which body fat stores are regulated. *My main contributions to this work were HPLC analysis and gene expression analysis.*

4.2. Differential response of various white adipose tissue depots to starvation - involvement of AMP activated protein kinase cascade (Paper IV).

Starvation is a common physiological condition leading to a decrease of body fat content and modulation of adipose tissue metabolism itself contributes to the overall effect. Diverse adipose tissue depots have different response to the starvation (94;97); however the mechanism is largely unknown. Our main interest was to see if AMPK in white adipose tissue is involved in metabolic changes during starvation, as well as we have previously seen in the obesity resistant aP2-*Ucp1* transgenic mice (Paper I.). Stimulation of AMPK in liver during starvation has also been reported (98). Meanwhile finalisation of this work Daval et al. (57) published that starvation stimulates the AMPK in epididymal white adipose tissue of rodents, which is involved in inhibition of FA synthesis and basal lipolysis during starvation. Therefore we focused on the differential responses of subcutaneous and epididymal fat depots to starvation and on verification of possible involvement of AMPK in this phenomenon. Male C57BL/6J mice were used for all experiments. The effect of 6, 12, and 24 h of starvation was studied.

Starvation resulted in a progressive loss of body weight over 24 h. Significantly greater suppression of lipogenic genes in epididymal compared to subcutaneous fat was observed. The expression of SREBP-1, a transcription factor controlling lipogenesis in response to nutritional status, was progressively abolished in epididymal fat while in subcutaneous fat went down only transiently (Fig. 5A). We also observed a gradual inhibition of the expression of FAS, a downstream target of SREBP-1, in both fat depots, with a stronger effect in the epididymal fat (Fig. 5B). In parallel, the activity of FA synthesis also decreased to a different extent in the two fat depots (Fig. 5C). Finally, measurement of oleate oxidation in white adipose tissue fragments from fed mice and mice starved for 12 h showed significant >2-fold stimulation of FA oxidation by starvation in epididymal fat while only transient increase in subcutaneous fat. Thus, the adipocytes from starved mice seemed to be switched from lipogenic into lipid burning cells and this phenomenon was more pronounced in epididymal fat.

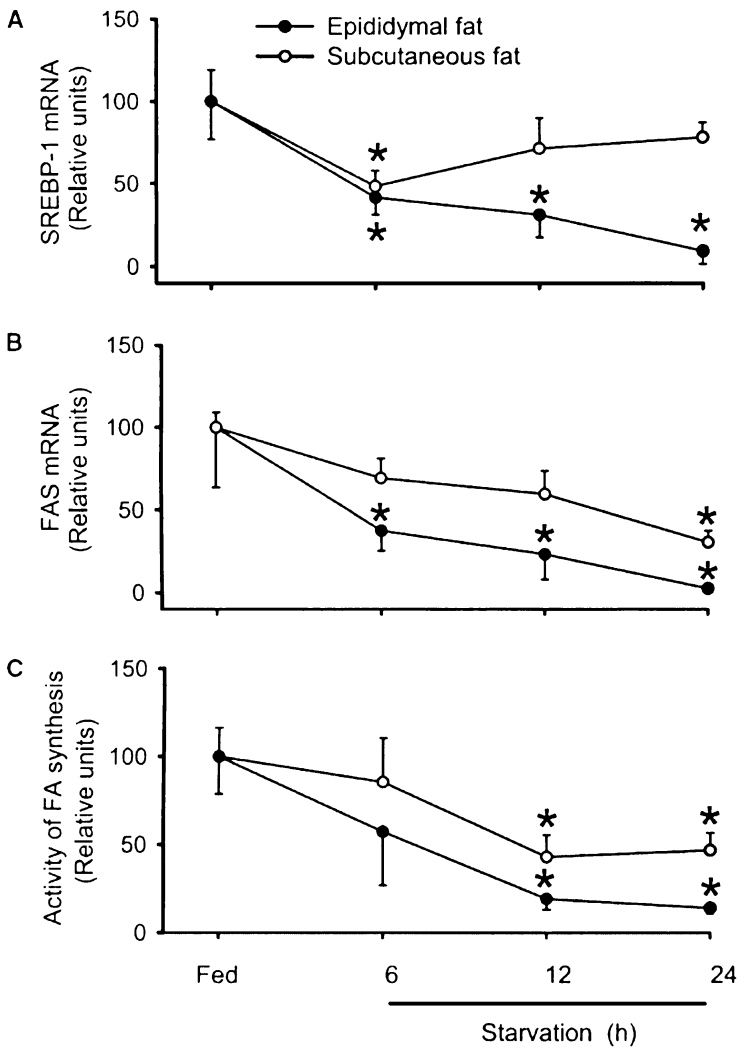

Figure 5. ***Effect of starvation on gene expression and FA synthesis in adipose tissue.*** *All data are expressed in % relative to fed mice and they are means ± S.E.; n = 6–8. (A) SREBP-1 and (B) FAS mRNA level (C) Activities of FA synthesis measured in tissue fragments. Asterisks indicate significant effect of starvation.*

All these effects of the starvation on biochemical characteristics of white fat are in accordance with the activation of AMPK cascade and suggest different impact on subcutaneous and epididymal fat. Assays of the α1 AMPK activity in immunoprecipitates revealed a significant activation after 12 h and no further changes during the subsequent 12 h of starvation in epididymal fat. In contrast, no changes of α1 AMPK activity could be detected in subcutaneous fat (Fig. 6). To confirm the differential activation of α1 AMPK activity in the two fat depots, the phosphorylation status of its downstream target, ACC-1, was evaluated. Starvation significantly increased the amount of pACC, expressed relative to total ACC, in epididymal but not in subcutaneous fat (Fig. 6) with a similar time course to α1 AMPK activity. These observations are in full agreement with our previous study

with aP2-*Ucp1* transgenic mice where we also demonstrated different activation of AMPK in different fat depots which reflected other biochemical characteristic.

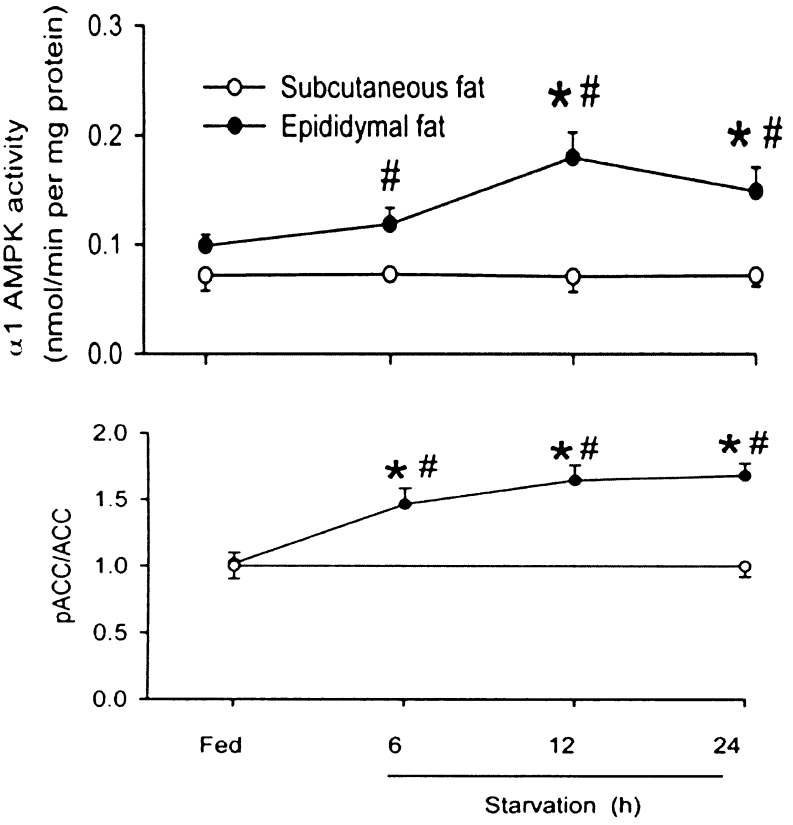

Figure 6. Effect of starvation on α1 AMPK activity and phosphorylation of ACC-1, expresed relative to total ACC. *α1 AMPK activity is expressed in nmol phosphate incorporated/min per mg protein of tissue extract used for immunoprecipitation. Total ACC and pACC were quantified and the results are expressed as a ratio, relative to the ratio obtained at time zero. All data are means ± S.E. (n = 5–6). Asterisks indicate significant effect of starvation and hatched crosses significant differences between fat depots.*

Because AMPK could stimulate glucose uptake in white adipose tissue by mechanism independent of insulin (99), it could also induce glycerogenesis and recycling of FA into TG during starvation (91). Recycling of FA consumes ATP, therefore may decrease intracellular energy charge and, in turn, activate AMPK. In epididymal fat of fed mice the mRNA levels of PEPCK, an enzyme that is strictly transcriptionally regulated and is required for glyceroneogenesis during starvation (19), were 2-fold higher than in subcutaneous fat and increased substantially during 24 h of starvation (Fig. 7). In contrast, only a smaller and transient increase of PEPCK mRNA was observed in subcutaneous fat. In our study both subcutaneous and epididymal fat tended to decrease in weight, although this was only significant for subcutaneous fat at 12 h and 24 h after food deprivation and accompanied by a decrease in the content of tissue lipids in subcutaneous but not in

epididymal fat after 24 h. Stronger stimulation of PEPCK-dependent FA re-esterification in epididymal fat could be one of possible explanations. Furthermore, expression of UCP2 gene was previously shown to be stimulated in white adipose tissue by food deprivation (100). UCP2 might increase the proton leak in mitochondria and thus reduce synthesis of ATP (101) and activate AMPK, similarly to transgenic mice over-expressing UCP3 in skeletal muscle(102) or UCP1 in white adipose tissue (103). On the other hand, increased expression of the UCP2 gene might be a consequence of AMPK activation, since AMPK can induce both UCP2 and UCP3 in rat skeletal muscle (104). In our study, levels of UCP2 mRNA were similar in both depots of fed mice, and increased during starvation (Fig. 7). This increase was much greater in epididymal fat, peaking at 12 h.

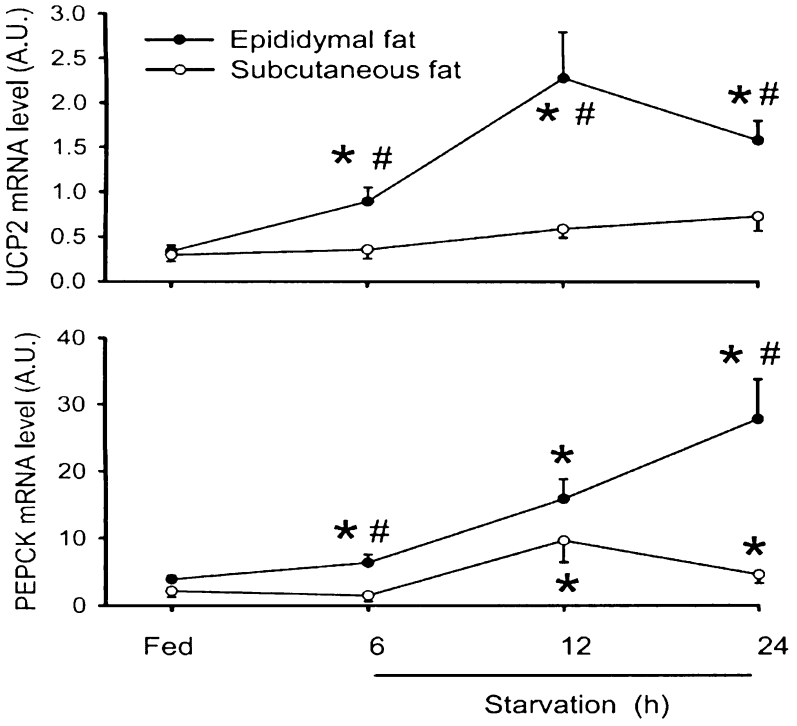

Figure 7. Effect of starvation on UCP2 and PEPCK gene expression levels. *All data are means ± S.E. (n = 6–8). Asterisks indicate significant effect of starvation and hatched crosses significant differences between fat depots.*

The control of lipid and glucose metabolism in white adipose tissue by AMPK may represent a basic biological mechanism that contributes to regional differences in the metabolic properties of adipose tissue depots.

The results described in the section above represent the outcome of my main project. Colleagues of mine contribute with the AMPK, ACC analysis and FA oxidation measurements.

4.3. The modulation of metabolism and gene expression of white adipose tissue by dietary intake of EPA and DHA (Paper II, III).

4.3.1. Anti-obesity effect of EPA/DHA.

The n-3 PUFA of marine origin have been shown to play an important role in the modulation of the whole body metabolism (see introduction). Especially EPA and DHA are less effective in promoting accumulation of adipose tissue than saturated fats (76;85;105), most likely by modulating fuel partitioning by down-regulating lipogenesis and stimulating lipid oxidation (84;106). However the mechanism for the reduction of body fat stores is still unclear. The former study (Paper II) was focused on whether dietary EPA and DHA could limit the development of obesity and proliferation of adipose tissue cells induced by HF diet, and whether other dietary FA, namely FA of plant origin – ALA and linoleic acid (LA), could influence the effect of EPA/DHA. Male C57BL/6J mice were used for all experiments. Large scale of diets with different macronutrient composition and energy density as well as different ratios of EPA/DHA has been tested. For full information about diets see the article itself. The main points of these extensive studies are summarized below.

During the development of obesity induced by composite HF diet (cHF; 35% of fat in a diet (wt/wt), accumulation of epididymal fat was significantly reduced (1.4-fold and 3.9-fold) when 15% (cHF-F1) or 44% (cHF-F2) of dietary fat, respectively, was replaced by EPA/DHA. 30% caloric restriction resulted in equal depression of epididymal fat tissue as 44% (wt/wt) fat replacement by EPA/DHA. The combination of caloric restriction and 15% (wt/wt) fat replacement by EPA/DHA had no further effect. On the other hand, in mice fed with semisynthetic HF diet (sHF*f*; rich in ALA, the precursor of EPA/DHA; only 20% of fat in a diet; wt/wt), which did not promote obesity, only the higher dose, i.e. 44% dietary fat replacement by EPA/DHA (sHF*f*-F2) was effective in reduction of adiposity. Interestingly, subcutaneous fat was less affected in all the cases. It was the low ratio of EPA to DHA that promoted the effect, which is in agreement with previous observations (75;85).

Since each cell contains constant amount of DNA, tissue DNA concentration and its amount could be used as markers of mean cell size and tissue cellularity, respectively. Quantification of adipose tissue DNA revealed that in case of the cHF diet with EPA/DHA added, the observed reduction of epididymal fat was associated with 34-50% depression of cellularity, similar to the 30% caloric restriction. DNA content of adipose tissue also decreases under other circumstances leading to depression of adiposity such as the induction of energy dissipation by ectopic UCP1 in adipocytes (47;50), or due to calorie restriction (107).

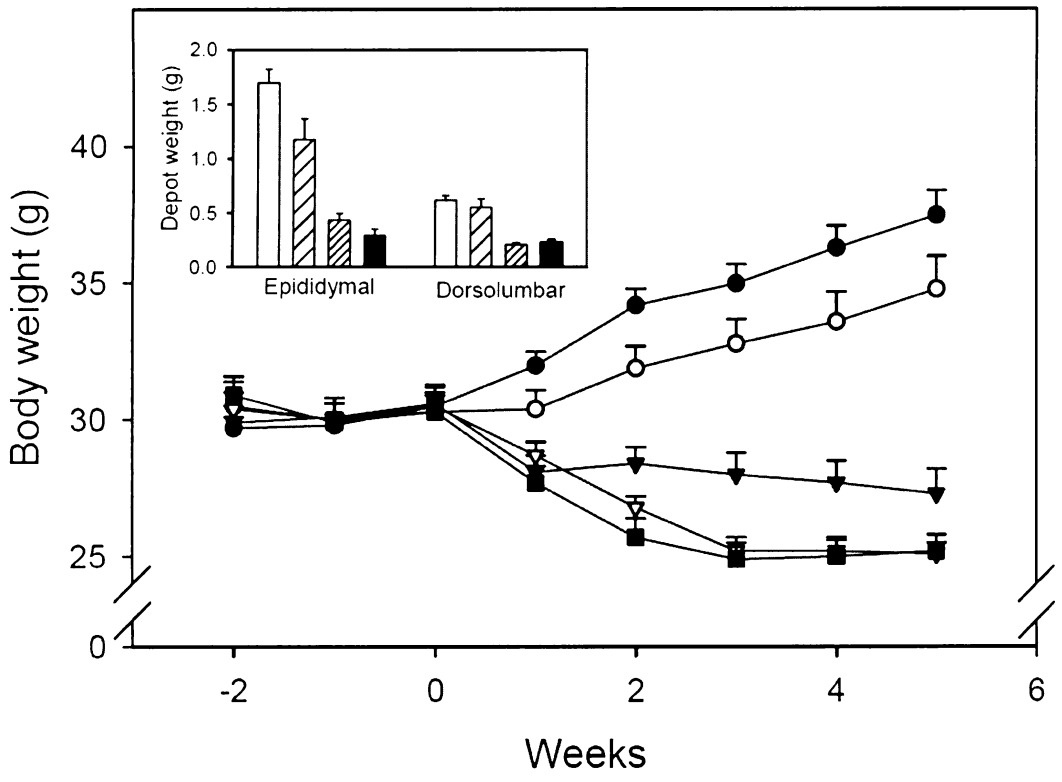

Figure 8. Effects of long chain n-3 PUFA and calorie restriction on body weight and fat accumulation in mice fed high fat diet. *At 4 months of age (week -2), chow diet was replaced by high fat. Two weeks later (week 0), animals were divided into 5 subgroups and fed ad libitum, either with no further modification (full circles) of diet, or with replacement of 15% (wt/wt; empty circles) or 44% (wt/wt; full triangles) of its fat content by EPA/DHA concentrate. Mice on the calorie restriction regime were fed either the high fat diet alone (empty triangles) or the diet, in which 15% (wt/wt) of lipids was formed by EPA/DHA concentrate (full squares). After 5 weeks, the animals were sacrificed. Inset: weights of fat depots in animals fed ad libitum high fat diet (empty bars), diet in which 15% (coarse hatched bars) or 44% (fine hatched bars) of its fat content was formed by EPA/DHA concentrate, and the calorie restriction group fed high fat diet alone (full bars). Data are means ± S.E. (n = 10).*

As in previous studies (85;108), the reduced accumulation of body fat in the EPA/DHA treated animals did not result from lower food consumption. The effect may be secondary to the stimulation of mitochondrial and peroxisomal FA oxidation in liver and muscle, and inhibition of hepatic lipogenesis and VLDL formation (83-85). All these actions contribute to the hypolipidemic effect of EPA/DHA, thus limiting the supply of FA to adipocytes. In accordance with this idea, we observed that even low concentration of EPA/DHA admixed into a sHF diet (sHF*f*-F1), which did not affect adiposity, was sufficient to induce depression of plasma TG.

4.3.2. Metabolic switch in white adipose tissue.

More pronounced effect of EPA/DHA on adiposity, as compared with their precursor ALA may be also mediated by changes in gene expression in adipocytes (83;84;106). In order to identify the specifically regulated genes (Paper III), cDNA PCR-substraction and oligonucleotide microarray analysis were performed with RNA samples isolated from both epididymal and subcutaneous fat from mice maintained on sHF diet with/without 44% EPA/DHA fat replacement (sHF*f* x sHF*f*-F2). Expressions of selected genes were verified using quantitative real-time PCR method (qRT-PCR). This analysis revealed that the majority of the most up-regulated genes in both fat depots belong to mitochondrial genome and code for components of the oxidative phosphorylation system. Conversely, the most down-regulated genes were those supporting lipogenesis. The subunits of mitochondrial oxidative phosphorylation complexes were also quantified on a protein level using immunoblots. In epididymal fat, the analyzed antigens were generally elevated by the EPA/DHA rich diet, while in the subcutaneous fat no effect of the diet was observed. These results suggest distinct post-transcriptional control of the expression of the genes for mitochondrial proteins in different fat depots.

PGC-1α and its downstream target NRF-1 are known to be nuclear transcriptional regulators implicated in mitochondrial biogenesis. Importantly, their expression is responsive to physiological signals mediating e.g. termogenesis or gluconeogenesis (109). We hypothesized that induction of PGC-1α and NRF-1 could orchestrate the induction of mitochondrial biogenesis we observed. qRT-PCR analysis

revealed induction of both PGC-1α and NRF-1 transcript levels in epididymal fat by the sHF/-F2 (Figure 9A,B). Both genes tended to be up-regulated also in subcutaneous fat, but the effects were much smaller. CPT-1, which is necessary for the translocation of FA into mitochondria, is another down-stream target for PGC-1α. Indeed, also CPT-1 gene expression was also up-regulated by the sHF/-F2 (Figure 9C) in epididymal but not in subcutaneous fat. Positive correlations between the expressions of PGC-1α and CPT-1 within identical tissues and diets were observed (Figure 9D). The up-regulation of the markers of mitochondrial biogenesis and β-oxidation and supression of lipogenic genes were verified in experiments when induction of obesity by cHF diet was reduced by only 15% replacement of dietary lipids by EPA/DHA (cHF-F1), as well as in cell culture, using 3T3-L1 adipocyte cell line treated with various FA for 24 hours (see the article itself).

The up-regulation of the CPT-1 gene suggests stimulation of FA oxidation. Indeed, oxidation of oleate was 1.5 to 1.8-fold higher in epididymal fat from sHF/-F2 compared to sHF/ mice while FA synthesis was lower. For further characterization we also analyzed gene expression of acyl-CoA oxidase 1 (AOX-1), the marker of peroxisomal FA oxidation. The mRNA levels of AOX-1 were significantly higher in mice fed sHF/-F2 than sHF/ diet, in both epididymal and subcutaneous adipose tissue. Thus, in contrast to CPT-1, similar stimulation of AOX-1 gene transcription was observed in both fat depots of sHF/-F2 mice. These results, as well as the preferential stimulation of PGC1α and NRF-1 gene expression in epidydimal fat suggest the involvement of mitochondria rather than peroxisomes in the stimulatory effect of EPA and DHA on the FA oxidation in epididymal fat.

n-3 PUFA and their metabolites, eicosanoids (83), belong to ligands of various isoforms of PPARs. All PPARs physically interact with PGC-1α (110) which is up-regulated by EPA/DHA in adipocytes. A recent study indicates that treatment of ob/ob mice (model of genetically induced obesity) with rosiglitazone, PPARγ ligand from the TZD family and a widely used antidiabetic drug, increases mitochondrial mass, palmitate oxidation, as well PGC-1α and CPT-1 (111). All these effects are similar to those elicited in white adipose tissue by EPA/DHA suggesting that both n-3 PUFA and rosiglitazone induce similar metabolic changes in adipocytes, while increasing mitochondrial oxidative capacity

and stimulating glucose uptake into adipocytes. However, in contrast to EPA/DHA, TZD also support an adipogenic program (111).

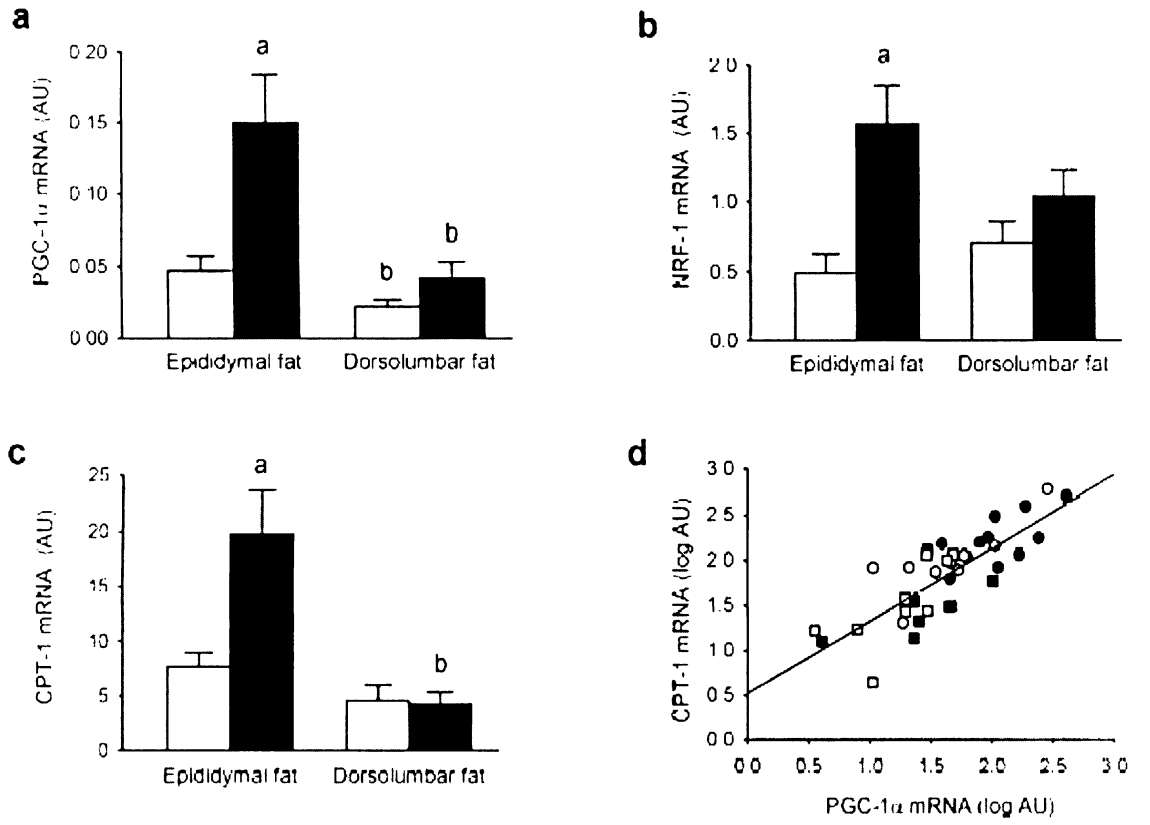

Figure 9. Gene expression in adipose tissue. *(A-C) transcript levels were evaluated in total RNA isolated from adipose tissue depots of mice fed* sHF*f (white bars) or* sHF*f*-F2 *(black bars) using qRT-PCR; *significant differences between diets; †significant differences between fat depots; data are means ± SE (n = 9 - 15). (D) correlation between PGC-1α and CPT-1 mRNA levels in epididymal fat of the* sHF*f (white circles) and* sHF*f*-F2 *(black circles) fed mice, and in subcutaneous fat of the* sHF*f (white squares) and* sHF*f*-F2 *(black squares) fed mice. Spearman correlation coefficient for each set of data was 0.72 - 0.83 and for pooled data the coefficient was 0.79. All the correlations were statistically significant (P < 0.01).*

4.3.3. The influence of EPA/DHA on adipose tissue gene expression in obese women during very low calorie diet (*unpublished data*)

Encouraged by the results from studies on mice, we were interested in seeing the impact of dietary n-3 PUFA on gene expression in white adipose tissue in humans. Twenty severely obese women were randomly assigned for a three week period to a very low calorie diet (VLCD; 2200 kJ/ day) with addition of EPA/DHA concentrate (2.8 g /day, ratio 2/1; n-3 VLCD group) or with placebo (saline solution; VLCD group). Higher weight and hip circumference losses as well as significantly higher increase in β-hydroxybutyrate, suggesting higher FA oxidation, were found in the n-3 VLCD group compare to controls. This suggested that EPA/DHA addition in the diet could potentiate the effect of VLCD itself. For more details about the study design and results from clinical part see *Kunesova et al.* (112).

To verify whether changes in body fat content and whole lipid metabolism are accompanied by changes in adipose tissue gene expression, and to see the possible impact of EPA/DHA on it, the total RNA was isolated from biopsies of subcutaneous abdominal fat (~ 100mg), taken under local anesthesia, in the day 0 and 21. The RNA was isolated and purified using RNaeasy Lipid Tissue Mini Kit (Qiagene) and cluster of transcripts (Table 1.) was analyzed by qRT-PCR method using GeneAmp 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Levels of all transcripts were correlated with that of the gene for elongation factor 1α (EF1α) since quantity of this transcript is not affected by the dietary treatment (data not shown). Lasergene software (DNAstar, Madison, WI, USA) was used to design oligonucleotide primers (Table 1.).

The expression levels of individual transcripts are summarized in Table 2. Since we have noticed some disproportion in these levels within the whole group before the start of a reducing period, in particular for UCP2, FAS and ACC-2, the influence of VLCD and n-3 VLCD on gene expression is also expressed as a ratio of the gene expression levels after/before (end/start) dietary treatment for each single patient (Fig. 10); i.e., ratio end/start > 1 shows up-regulation of selected genes and ratio end/start < 1 means down-regulation.

Table 1: Oligonucleotide primers sequences

Gen		*Oligonucleotide*	*Gene bank No.*
genes involved in biogenesis and oxidative capacity of mitochondria:			
NRF-1	5`	GAAGATCAGCAAACGCAAACACAGG	42406291
	3`	CTCCATCAGCCACGGCAGAATAAT	
COX3	5`	TGGCGCGATGTAACACGAGAAAG	17981852
	3`	AGGGGCTAGGCTGGAGTGGTAAAAG	
PGC-1	5`	TGGGGTCAGAGGAAGAGATAAAGT	42406221
	3`	TCATGGAGCAATAAAGCGAAGAGTA	
UCP2	5`	AAGACCATTGCCCGAGAGGAAGG	2772905
	3`	TCTACAGGGGAGGCGATGACAGTGGT	
CPT-1β	5`	GTGCTCTCGGAACCCTGGCGTCTC	23238252
	3`	CAGGGCTTTGCGGATGTGGTTTC	
genes involved in lipogenesis:			
FAS	5`	TGAGCTTTGCGGCCCGGTCCTTCT	1049052
	3`	ACCCGTCTTGGCCCGCAGTAGCATC	
ACC-1	5`	AGGCATGTCTTCAGAGGCAGGGTGGGTTAC	38679959
	3`	TGGGGGAGGAGGCATTACAGGGTTCT	
ACC-2	5`	AGAATTCCCACATCATCCTCACA	4501854
	3`	GTATAAACCCCCTCAAAGTCATCTG	
others:			
EF-1α	5`	GAACCATCCAGGCCAAATAAGC	15421128
	3`	CCACCGCAACTGTCTGTCTCATA	
Leptin	5`	GGGAACCCTGCTTGCACTTTGTA	4557714
	3`	CCTGTTGGCTGTTATGGTCTTATGTATTTT	
GLUT-4	5`	CCGTCGGGCTTCCAACAGATAGG	186552
	3`	ACGGAAAAGATGGCCACGGAGAG	
IL1-Rα	5`	GAGAGTGGGGGTGATGATGA	22001412
	3`	ACGGGGAACTAGGAATGTGTCT	

EF-1α, elongation factor 1α; UCP2, uncoupling protein 2; FAS, fatty acid synthasa; NRF-1, nuclear respiratory receptor 1; COX3, cytochrom-c oxidase 3; ACC-1, acetyl-CoA karboxylase 1; ACC-2, acetyl-CoA karboxylase 2; CPT-1β, carnitine-palmitoyl transferase 1β; GLUT4 – glukose transporter 4; IL1-Rα, interleukin receptor 1α; PGC-1β, PPARγ coactivator 1β .

Table 2: Quantification of gene expression in human white adipose tissue

	VLCD/start	VLCD/end	n-3 VLCD/start	n-3 VLCD/end
COX3	100,0 ± 19,2	59,8 ± 12,6[a]	97,0 ± 10,8	66,6 ± 9,1[b]
NRF-1	100,0 ± 12,3	106,7 ± 9,4	137,2 ± 11,4	119,6 ± 12,8
UCP2	100,0 ± 18,7	104,1 ± 12,6	170,6 ± 17,5[c]	144,7 ± 14,7
ACC-1	100,0 ± 17,8	58,4 ± 10,7[a]	114,0 ± 14,1	55,6 ± 8,8[b]
FAS	100,0 ± 20,6	53,4 ± 24,7[a]	167,3 ± 24,3[c]	67,9 ± 12,8[b]
ACC-2	100,0 ± 12,9	59,3 ± 12,3[a]	156,7 ± 13,0[c]	93,6 ± 17,0[b]
CPT-1β	100,0 ± 11,5	98,5 ± 8,7	98,6 ± 6,0	120,6 ± 6,6[b]
GLUT-4	100,0 ± 25,1	62,7 ± 27,1[a]	106,6 ± 25,5	58,2 ± 16,5[b]
Leptin	100,0 ± 9,6	48,6 ± 17,3[a]	102,5 ± 13,5	54,4 ± 9,0[b]
IL1-Rα	100,0 ± 12,9	75,9 ± 10,1	90,6 ± 12,2	92,6 ± 6,7
PGC-1β	ND	ND	ND	ND

The levels of gene expression are represented in relative units (mean ± SE) as % before and after 3-weeks dietary intervention. VLCD/start, biopsies taken before switch onto VLCD (n=8); VLCD/end, the same group after treatment (n=8); n-3 VLCD/start, biopsies taken before switch onto n-3 VLCD (n=10); n-3 VLCD/end, the same group after treatment (n=10). ND, not detected.
[a]$P<0.05$ (ANOVA), VLCD/start x VLCD/end; [b]$P<0.05$ (ANOVA), n-3 VLCD/start x n-3 VLCD/end ; [c]$P<0.05$ (ANOVA), VLCD/start x n-3 VLCD/start

In summary, we observed that: (i) there was no effect of VLCD or n-3 VLCD on the expression of either NRF-1, the marker of mitochondrial biogenesis, or UCP2, the marker of efficiency of oxidative phosphorylation; (ii) the expression of leptin, which serves as a marker of adiposity (44) was decreased after VLCD with no additional effect in case of n-3 VLCD; (iii) also the expression of lipogenic genes (ACC1, FAS, ACC2) was decreased after VLCD, and this was even more pronounced after n-3 VLCD suggesting a potentiation by EPA/ DHA; (iv) expression of Glut-4 was depressed by VLCD however this could be anatagonized by EPA/DHA, since the decrease in expression was lower in case of n-3 VLCD; (v) the expression of anti-inflammatory cytokine IL1Rα was increased after n3-VLCD compare to VLCD and supports an anti-inflammatory effect of n-3 PUFA (113); (vi) whereas the expression of COX3, the marker of mitochondrial oxidation was depressed after VLCD, the expression of CPT-1, the marker of oxidation of FA in

mitochondria was not influenced by VLCD, indeed, tended to increase after n-3 VLCD. This is in agreement with our observations in mice and also with previous observation in humans (77). Taken together, the results suggest that n-3 PUFA may contribute to reduce body fat content by decreasing lipogenesis and increasing lipid oxidation in human adipocytes. The beneficial effect of n-3 PUFA addition to the diet of obese subjects could be more expressed under conditions of lower energy deficit. The second round of the study, with reduced calorie restriction is now in progress and the results are getting ready to be published. *My main contribution in these presented papers was DNA analysis and qRT-PCR analysis in mice and the gene expression study in humans.*

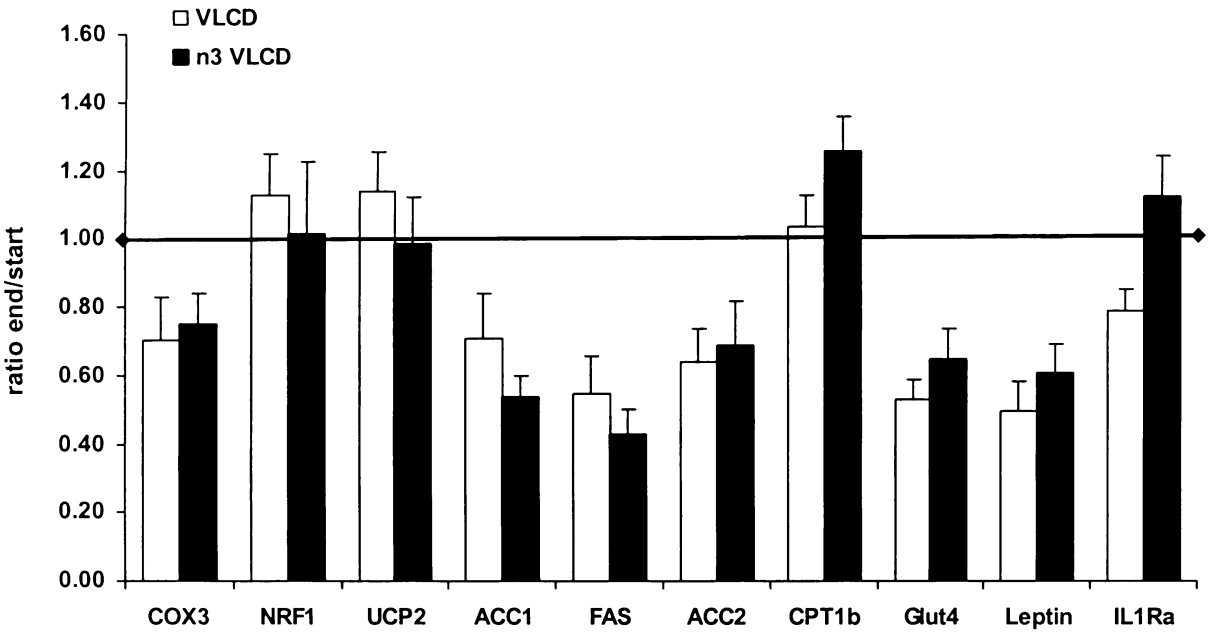

Figure 10. Gene expression in human white adipose tissue. *Transcript levels are expressed as a ratio of the gene expression levels after (end) and before (start) dietary treatment for each single patient. Data are means ± SE (VLCD, n=8; n-3 VLCD, n=10)*

5. CONCLUSIONS

Concerning the specific aims of the thesis, following conclusions may be formulated:

1. Induction of obesity resistance in mice by transgenic expression of UCP1 in white fat can be explained by a metabolic switch in adipocytes, due to depression of cellular energy charge, *in situ* activation of AMPK, down-regulation of adipogenic genes and increase in lipid oxidation.

2. a) AMPK cascade is involved in the fat depot-specific metabolic responses in various fat depots to starvation. Activation of the cascade occurred in epididymal but not in subcutaneous fat in mice.

b) The activation of AMPK in adipocytes might represent an important mechanism by which body fat stores are regulated and may contributes to regional differences in the metabolic properties of adipose tissue depots.

3) a) Dietary EPA and DHA reduce development of obesity induced in mice by high fat diets, in part due to counteracting increase in tissue cellularity, particularly in epididymal fat. Low EPA/DHA ratio potentiates the anti-adipogenic effect.

b) Dietary EPA and DHA induce a metabolic shift in murine white adipose tissue by up-regulating genes for mitochondrial proteins, including their regulatory genes PGC1α and NRF-1, and increase β-oxidation while depressing lipogenesis, preferentially in the epididymal fat in the abdomen.

c) In human severely obese patients, three weeks of very low calorie diet altered gene expression profile in adipose tissue. The expression of lipogenic genes ACC1, FAS, ACC2 was decreased. EPA/DHA tended to augment these changes while the expression of CPT-1, the marker of oxidation of FA, was increased.

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