Academy of Sciences of the Czech Republic
Institute of Physiology

Charles University in Prague
1st Faculty of Medicine

Control of white adipose tissue metabolism:

role of AMP-activated protein kinase

and

modulation by omega-3 polyunsaturated fatty acids

Mgr. Olga Horáková

Ph. D. Thesis

Supervisor: Jan Kopecký, MD, DSc.

Praha 2006

ACKNOWLEDGEMENTS

Presented thesis was elaborated at the Department of Adipose Tissue Biology of the Institute of Physiology, Academy of Sciences of the Czech Republic. It is based on four primary papers, attached copies of which constitute the key part of the text.

I would like to express my sincere gratitude to all of you who have been involved in this work in one way or another. Special thanks are due to:

- Jan Kopecký, MD, DSc, my supervisor for all his support and for providing me with a lot of scientific freedom and the possibility to participate in the obesity research.
- Collaborators and coautors of our publications
- My friends and colleagues from the Department of Adipose Tissue Biology for their advice, help and encouragement.

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

A. **Matějková O**, Mustard KJ, Šponarová J, Flachs P, Rossmeisl M, Mikšík I, Thomason-Hughes M, Grahame Hardie D, Kopecký 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.

B. Šponarová J, Mustard KJ, **Horáková O**, Flachs P, Rossmeisl M, Brauner P, Bardová K, Thomason-Hughes M, Braunerová R, Janovská P, Grahame Hardie D, Kopecký J. (2005). Involvement of AMP-activated protein kinase in fat depot-specific metabolic changes during starvation. *FEBS Lett.* 579: 6105-6110.

C. Flachs P, **Horáková O**, Brauner P, Rossmeisl M, Pecina P, Franssen-van Hal N., Ružicková J, Šponarová J, Drahota Z, Vlček C, Keijer J, Houštek J, Kopecký J. (2005). Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce β-oxidation in white fat. *Diabetologia* 48: 2365-2375.

D. Flachs P, Mohamed-Ali V, **Horáková O**, Rossmeisl M, Hosseinzadeh-Attar MJ, Hensler M, Ružičková J, Kopecký J. (2006) Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed a high-fat diet. *Diabetologia.* 49 :1-4

CONTENTS

1. LIST OF ABBREVIATIONS

α1 AMPK	α1 isoform of α subunit of AMPK
ACC	acetyl-CoA carboxylase
ACS	acyl-CoA synthetase
ADD-1/SREBP-1	adipocyte determination and differentiation factor-1/sterol regulatory element-binding protein-1c
ADP	adenosine diphosphate
ALA	α-linolenic acid
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AOX	acyl-CoA oxidase 1
aP2	fatty acid binding protein 4
ATP	adenosine triphosphate
BAT	brown adipose tissue
BMI	body mass index
cAMP	cyclic AMP
C/EBP	CCAAT/enhancer-binding protein
COX	cytochrome-c-oxidase
COX IV	subunit IV of mitochondrial cytochrome oxidase
CPT-1	carnitine-palmitoyl transferase I
CR	caloric restriction
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FAS	fatty acid synthase
FABP	fatty acid binding protein
FAT	fatty acid transporter
FAT/CD36	fatty acid translocase/CD36
FATP	fatty acid transport protein
G3P	glycerol-3-phosphate
GPAT	glycerolphosphate acyltransferase
HF	high fat
HSL	hormone-sensitive lipase

LPA	lysophosphatidic acid
LPL	lipoprotein lipase
LXR	liver X receptor
mGPAT	microsomal GPAT
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mtGPAT	mitochondrial GPAT
mtTFA	mitochondrial transcription factor A
nDNA	nuclear DNA
NE	norepinephrine
NEFA	non-esterified fatty acids
NRF	nuclear respiratory factor
OXPHOS	oxidative phosphorylation
PEPCK	phosphoenolpyruvate carboxykinase
PEPCK-C	cytosolic PEPCK
PGC-1	PPAR γ coactivator 1
PKA	protein kinase A
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
SCD	stearoyl-CoA desaturase
TAG	triacylglycerol(s)
TNF-α	tumor necrosis factor α
TZD	thiazolidinediones
UCP	uncoupling protein
WAT	white adipose tissue

2. INTRODUCTION

Regulating energy levels is fundamental process in every living organism. The endogenous systems regulating energy balance have developed during the course of evolution, when the conservation of energy reserves and the efficient replenishment of exhausted energy stores were essential for survival and reproduction [1].

According to 'energy balance equation theory' any increase in energy intake or decrease in energy expenditure, which imbalance the equation, will result in accumulation of fat in the body [2]. Obesity, an excessive accumulation of adipose tissue, will develop if individuals fail to match their energy intake to their energy needs. Enviromental factors, such as the increased avaibility of high caloric food or the decrease of physical activity, contribute to its developement and their influence is amplified by genetic predisposition [3]. Obesity is associated with the risk of developing type 2 diabetes mellitus, coronary heart disease, hypertension, sleep apnoea, asthma, certain form of cancer and osteoarthritis of small and large joints [4].

Mechanisms regulating stable levels of energy stores in the body involve the central control of behavior and thermogenesis mediated by neuroendocrine system, control of metabolite fluxes among various organs, and, finally, energy metabolism within individual tissues. Due to the complex nature of this process, any treatment strategy for obesity is difficult.

Many pieces of evidence suggest that body fat content is controlled, at least partially, by the metabolism of adipose tissue itself. First, many candidate genes for obesity have important roles in adipocytes [5]. Second, mice that are prone or resistant to obesity were created by transgenic modification of adipose tissue (reviewed in [6]). In these transgenic models, metabolic changes in white adipose tissue are mostly responsible for the altered accretion of body fat and associated metabolic disorders. And third, trought its secretory function, white adipose tissue lies at the heart of a complex network of factors capable of either improving (leptin, adiponectin) or reducing (TNF-α, IL-6, resistin) insulin action in relevant tissues, including skeletal muscles and liver. This raises the possibility that the developement of drugs targeting adipose function represents a new terapeutic approach to senzitize peripheral tissues to insulin. This could be of particular

therapeutic benefit in pathology associated with white adipose tissue mass dysregulation, such as lipodystrophy and obesity.

2.1. *WHITE ADIPOSE TISSUE*

In adult mammals adipose tissue, in addition to adipocytes, contains stromal-vascular cells including fibroblastic connective tissue cells, leukocytes, macrophages, and preadipocytes (not yet filled with lipid), which contribute to structural integrity.

White adipose tissue (WAT) amount to 20-30% of normal body weight (,but may vary from a few % up to 70%). WAT was for a long time regarded as a relatively passive site of energy storage and also as mechanical and thermic insulator. The energy is accumulated in the form of triacylglycerols (TAG) during periods of excess food consumption and mobilized when calorie intake is inadequate. WAT is an important site of interconversion of steroid hormones [7], and an important site of oestrogen production in post-menopausal women, probably explaining the protection of obese women from osteoporosis [8]. During past decade, evience has been provided that WAT secretes a number of bioactive molecules, collectively termed 'adipokines'. This newly discovered secretory function has shifted the view on WAT, which is no longer considered only as an energy-storage tissue but also as an endocrine organ influencing whole body homeostasis.

The adipose organ is localised in several depots including white as well as brown adipose tissues. The largest depots are found subcutaneously and in the abdominal region. Visceral and subcutaneous adipose tissues depots display different metabolic properties, manifested by differences in the expression level of genes involved in fat cell metabolism, and in the secretion of adipose factors that could be involved in some pathologies in both rodents and humans [9;10]. The overall effect of sex and regional locations of the adipose tissue on some of the mechanisms affecting the lipolytic capacity was investigated in rats, such as adrenergic receptor balance and several cascade steps at the postreceptor level, such as adenylyl cyclase, protein kinase A (PKA) and hormone-sensitive lipase (HSL) [11].

The lipid droplets in adipocytes can be unilocular and/or multilocular. Unilocular cells contain a single large lipid droplet which pushes the cell nucleus against the plasma membrane, giving the cell a signet-ring shape *(Figure 1)*. Unilocular cells, characteristic of WAT, range in size from 25 to 200 microns. Mitochondria are found predominately in the thicker portion of the cytoplasmic rim near the nucleus. The large lipid droplet does not appear to contain any intracellular organelles.

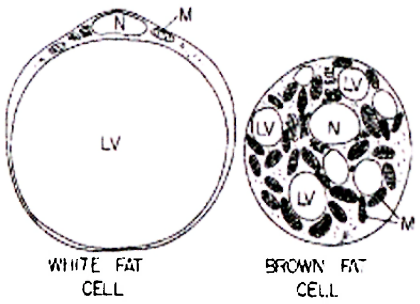

***Figure 1**. White fat cell and brown fat cell. Note the single large lipid vacuole in the white fat cell and the numerous smaller lipid vacuoles in the brown fat cell. LV: lipid vacuole; M: mitochondria; N: nucleus*

Approximately 60 to 85% of the weight of WAT is lipid, with 90-99% being TAG. Small amounts of free fatty acids, diglyceride, cholesterol, phospholipid and minute quantities of cholesterol ester and monoglyceride are also present. In this lipid mixture, six fatty acids make up approximately 90% of the total, and these are myristic, palmitic, palmitoleic, stearic, oleic, and linoleic. Varying the composition of the diet can vary the fatty acid profile in adipose tissue. The remaining weight of WAT is composed of water (5 to 30%) and protein (2 to 3%).

Each adipocyte in WAT 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. Blood flow to adipose tissue varies depending upon body weight and nutritional state, with blood flow increasing during fasting.

The size of adipose tissue mass is a function of both adipocyte number and size. An increase in adipose tissue mass can occur by hyperplastic growth, which is an increase in the number of adipocytes. This increase in number occurs primarily by mitotic activity in precursor cells. Adipose tissue mass can also increase by

hypertrophic growth, which is an increase in the size of adipocytes. This increase in size occurs primarily by lipid accumulation within the cell.

Pluripotent stem cell precursor gives rise to a mesenchymal precursor cell, which has the potential to differentiate along mesodermal lineages of myoblast, chondroblast, osteoblast and adipocyte. Given appropriate stimuli the preadipocyte undergoes clonal expansion and subsequent terminal differentiation into a mature adipocyte. Adipogenesis is driven by transcription factors CCAAT/enhancer-binding protein (C/EBP) α, β and δ, peroxisome proliferator-activated receptor (PPAR) γ and adipocyte determination and differentiation factor-1/sterol regulatory element-binding protein-1c (ADD-1/SREBP-1), whose activation leads leads to the expression of many adipocyte specific proteins involved in glucose and lipid metabolism *(Figure 2;* [12]).

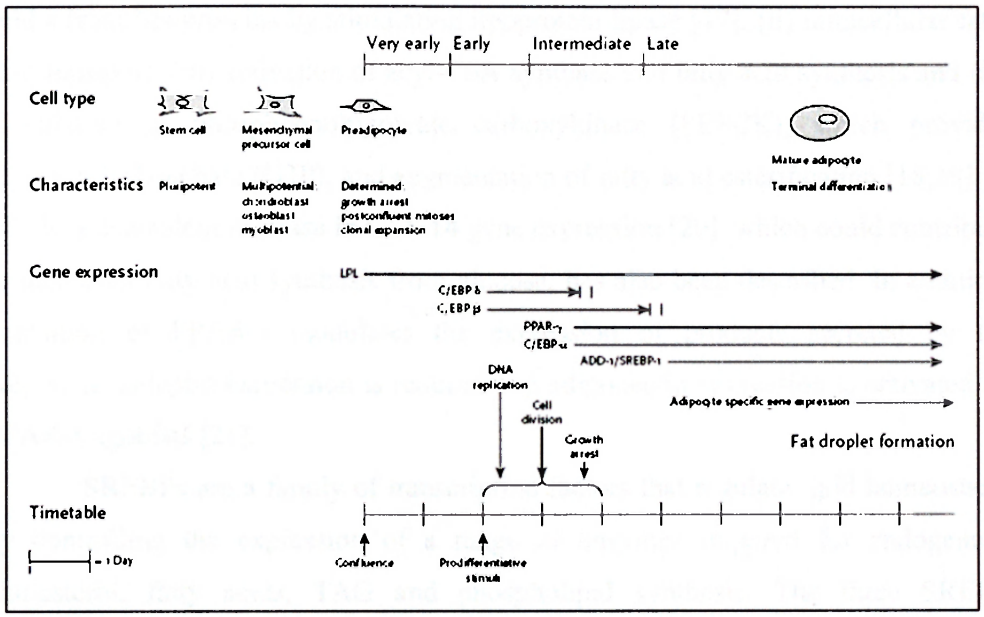

Figure 2. *Addition of mitogens and hormonal stimuli to 3T3-L1 cells leads to a cascade of transcriptional events that account for the expression of most proteins - mediating adipocyte function.*

C/EBPs are a family of transcription factors that all contain a highly conserved, basic-leucine zipper domain at the C-terminus that is involved in dimerization and DNA binding. The pivotal roles of the family in a number of processes, including differentiation, the inflammatory response, liver regeneration, metabolism and numerous other cellular responses.

Both C/EBPβ and C/EBPδ mRNA are induced during early phase of adipocyte differentiation in response to cAMP-elevating agents and glucocorticoid respectively [13]. The pre-adipocytes then exit the cell cycle and begin to express C/EBPα, which is then followed by the induction of adipocyte-specific markers - fatty acid binding protein (aP2), insulin-responsive glucose transporter (GLUT 4), leptin, PPAR-γ, steroyl-CoA desaturase 1 (SCD-1). Individual C/EBP members play the precise role of in the differentiation of white versus brown adipose tissue [14;15].

Another important transcription factor, which critically regulates adipocyte function, is the nuclear hormone receptor PPAR-γ. In addition to its stimulatory effects on pre-adipocyte differentiation, activation of PPAR-γ promotes the storage of fatty acid s in mature adipocytes by acting at several steps [16]: (i) release of fatty acid s from lipoproteins by stimulating lipoprotein lipase [17], (ii) intracellular fatty acid transport, (iii) activation of acyl-CoA synthase and fatty acid synthesis and (iv) stimulation of phosphoenolpyruvate carboxykinase (PEPCK), which provides glycerol-3-phosphate (G3P), and augmentation of fatty acid esterification [18;19]. A PPAR-γ-dependent increase in GLUT4 gene expression [20], which could contribute to increased fatty acid synthesis from glucose, has also been described. In addition, activation of PPAR-γ modulates the expression of products secreted by the adipocyte as leptin expression is reduced and adiponectin expression is activated by PPAR-γ agonists [21].

SREBPs are a family of transcription factors that regulate lipid homeostasis by controlling the expression of a range of enzymes required for endogenous cholesterol, fatty acids, TAG and phospholipid synthesis. The three SREBP isoforms, SREBP-1a, SREBP-1c and SREBP-2, have different roles in lipid synthesis. SREBP-1c is the predominant isoform expressed in most of the tissues, with especially high levels in the liver, WAT, skeletal muscle, adrenal gland and brain [22]. Mice overexpressing hepatic SREBP-1c demonstrate a selective induction of lipogenic genes (for example acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and SCD-1), with no effect on genes of cholesterol synthesis [23]. This isoform seems to be mainly regulated at the transcriptional level by insulin. In a typical counterregulatory fashion, the effects of insulin on SREBP-1c transcription are opposed by glucagon via cAMP [24].

2.1.1. GLUCOSE METABOLISM

WAT expresses the insulin-regulated glucose transporter GLUT4 as well as the non-insulin-regulated transporter GLUTl, which may provide its basal glucose requirements [25]. Glucose uptake from plasma by WAT appears to be regulated more by the prevailing glucose concentration (perhaps reflecting GLUTl activity) than by the insulin concentration [26].

Despite the ability of WAT to take up and oxidize glucose, it is generally accepted that this plays little part in whole-body glucose homeostasis. Although adipose tissue accounts for only a small fraction of insulin-dependent glucose disposal [27], experimental animals with fat-selective knock-out of the GLUT4 gene show impaired glucose tolerance, suggesting that the functional integrity of WAT is crucial in regulating intermediate metabolism [28].

WAT is one of the major sites for conversion of carbohydrate to fat in mammals. During formation of acetyl CoA, which is substrate of lipogenic enzymes, from glucose is form also more than enough reducing equivalents for the synthesis fatty acid *de novo* [29].

2.1.2. LIPID METABOLISM

In the control of fat content in WAT are involved following mechanisms: fatty acid uptake, fatty acid synthesis *de novo*, TAG synthesis, fatty acid oxidation, lipolysis and reesterification *(Figure 3)*.

Fatty acids are either provided by the diet in an esterified structure with glycerol, namely TAG, present in chylomicrons or lipoproteins, or can be synthesized *de novo* from glucose in lipogenic tissues. They are then stored in WAT after esterification with G3P to form TAG droplets. G3P arises from glucose through glycolysis, a pathway occurring at a high rate in postprandrial situations. In contrast, during fasting, fatty acids are released from WAT into the blood via lipolysis of the TAG stores present in that tissue and used for fuel by other tissues in which they are transported and oxidized for energy production. Such a scheme implies that lipolysis occurs in WAT during fasting whereas esterification is activated in postprandial situations. At the same time when adipocytes hydrolyse stored TAG to produce fatty acids they reesterify part of these fatty acids back to TAG, leading to a recycling process.

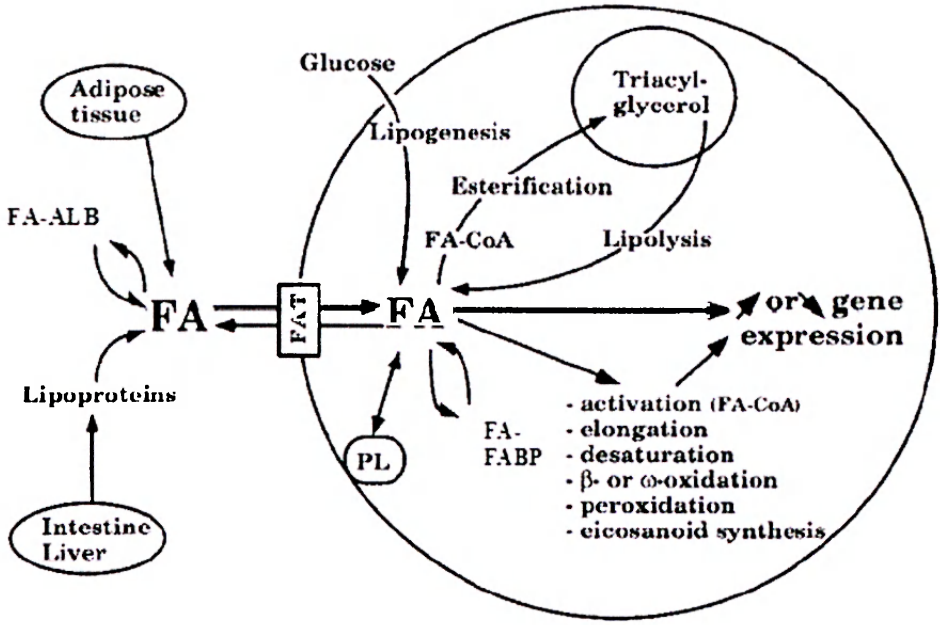

Figure 3. Major pathways of fatty acid production, transport, and metabolism.

2.1.2.1. FATTY ACID UPTAKE

Fatty acids are utilized for diverse cellular processes including mitochondria oxidation, membrane synthesis and energy storage. All of these intracellular processes are dependent upon fatty acids traversing the plasma membrane to get into the cell.

2.1.2.1.1. Lipoprotein lipase

In mammals, lipoprotein lipase (LPL) is synthesized in parenchymal cells of adipose and several other tissues (excluding liver), released from the cells and transferred to the vascular endothelium [30]. Here the lipase acts on TAG in plasma lipoproteins to release fatty acids for uptake by adipose tissue for storage or by other organs such as muscle for oxidation [31]. As LPL is a short-lived enzyme [32], the enzyme activity appears to be rapidly regulated by changes in mRNA levels. Completely reciprocal regulation of the mRNA levels has been found in the two tissues under starvation - down-regulation in adipose tissue and up-regulation in

muscle. It hat been suggested that adipose tissue play a main role in TAG clearance and hydrolysis in the postprandial period when its LPL is activated by insulin [33].

2.1.2.1.2. Membrane transport

Fatty acid uptake into mammalian cells occurr via a protein-mediated system that is highly regulated by physiologic stimuli such as contractile activity and insulin. Fatty acid transfer is govern by the molar ratio of fatty acid to albumin and the concentration of free fatty acid in the circulation. v uptake in adipocytes is facilitated by a membrane transport protein, the fatty acid transporter (FAT), for which six potential candidates, the fatty acid translocase (FAT/CD36), the fatty acid transport protein (FATP), the mitochondrial aspartate aminotransferase, caveolin, the adipose differentiation related protein and the fatty acid binding protein (FABP; a cytosolic protein which can bind to membranes) have been cloned and characterised [34, 35].

These proteins can be induced to translocate from intracellular depots to the the plasma membrane within minutes to increase fatty acid uptake. Fatty acid transfer in adipocyte was stimulated by norepinephrine. FAT/CD36 is increased in diabetes, obesity and by high fat diet. FATP and FABP are regulated by fasting [34].

Once fatty acids have reached the cytosol of the target cells, they need to be activated into acyl-CoA thioesters for further metabolism, e.g. elongation, desaturation, esterification and/or oxidation. The activation process is catalysed by some of five isoformes of acyl-CoA synthetases (ACS) that differ according to their chain length specificities [36].

2.1.2.2. FATTY ACID SYNTHESIS

The synthesis of malonyl-CoA is the first committed step of fatty acid synthesis catalyzed by ACC. Malonyl-CoA is a major precursor for fatty acid synthesis, but also a crucial regulator of mitochondrial fatty acid β-oxidation through its inhibition of carnitine palmitoyltransferase 1 [37].

The synthesis of fatty acids from acetyl-CoA and malonyl-CoA is carried out by the multiple enzymatic activities of FAS. The active enzyme is a dimer of identical subunits. The primary fatty acid synthesized by FAS is palmitate. Palmitate

is then released from the enzyme and can then undergo separate elongation and/or unsaturation to yield other fatty acid molecules

ACC is the major site of regulation of fatty acid synthesis. Its two isoforms, ACC-1 and ACC-2, have distinct cellular (cytoplasma, resp. mitochondrial membrane) and tissue distribution and appear to have distinct functions in the control of fatty acid synthesis and fatty acid oxidation, respectively. In lipogenic tissues, such as WAT and lactating mammary gland, ACC-1 is the major form of ACC expressed. In oxidative tissues, such as heart and skeletal muscle, ACC-2 predominates [38].

The rate of fatty acid 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 fatty acids. There is evidence for protomer into polymer transitions *in vivo*, where the effects of insulin treatment on adipocyte ACC, and of insulin injection or refeeding starved rats on hepatic ACC [39].

ACC is also controlled through hormone mediated phosphorylation. Phosphorylation of ACC by PKA or AMP-activated protein kinase (AMPK) both result in decreased sensitivity of ACC to citrate [40]. There is a diurnal rhythm [41]; ACC is also phosphorylated and inactivated in response to prolonged food absence in liver [42], feeding rats a high-fat diet [41], glucagon administration to whole rats [43] or adrenaline treatment of adipocytes. Most recently, leptin has been shown to activate AMPK in skeletal muscle, resulting in phosphorylation and inactivation of ACC-2 and stimulation of fatty acid oxidation [44].

Stearoyl-CoA desaturase (SCD) is an endoplasmic reticulum enzyme that catalyses the critical committed step in the biosynthesis of monounsaturated fatty acids from saturated fatty acids. SCD has recently been shown to be an important control point in lipid homeostasis [45] and body weight regulation [46]. Four SCD genes have been cloned in mice. SCD-1 which is widely expressed and best characterized is regulated by a number of nutritional and hormonal factors including insulin, cholesterol and polyunsaturated fatty acids (PUFA) [45].

2.1.2.3. TRIACYLGLYCEROL SYNTHESIS

After acylation to the acyl-CoA derivative by the ACS, activated fatty acids are esterified in TAG by the sequential action of the glycerolphosphate acyltransferase (GPAT). Esterification requires G3P formed from dihydroxyacetone phosphate by the action of G3P dehydrogenase. GPAT catalyzes the acylation of G3P to form lysophosphatidic acid (LPA) as the initial and committed step in the *de novo* synthesis of TAG. LPA can converted via phosphatidate to phospholipides or diacylglycerol. The formation of TAG can be catalyzed by several different activities. In adipose tissue is majority diacylglycerol transferase.

The regulation of TAG formation and hydrolysis is likely to be complex, involving transcriptional and post-transcriptional controls that respond to specific hormones, to metabolites derived from individual meals and long-term diet exposures, to fasting and refeeding, and to exercise mediated energy expenditure. Important roles are played by SREBP-1c, PPARγ, and LXR, by their ligands, and by hormonal and nutritional regulators including insulin, carbohydrate, and fatty acids.

Two isoenzymes of GPAT exist, the majority microsomal (mGPAT) and minority mitochondrial (mtGPAT). Their activities have different sensitivities to various reagents and have different fatty acid specificities [47].

Adipose tissue tyrosine kinase reversibly inactivates the mGPAT [48]. Insulin can activate the phosphorylated mGPAT via phosphatase. For mtGPAT, regulation has been shown to occur at the level of transcription in response to changes in differentiation state, feeding status, the hormones insulin and glucagon, and the transcription factor SREBP-1 [49, 50]. AMPK inactivate liver GPAT in isolated mitochondria in a time and ATP-dependent manner [51].

2.1.2.4. FATTY ACID OXIDATION

Mitochondrial β-oxidation of fatty acids is initiated by the sequential action of carnitine-palmitoyl transferase 1 (CPT-1), which is located in the outer membrane, and carnitine-palmitoyl transferase 2 (CPT-2), which is located in the inner membrane together with a carnitine-acylcarnitine translocase. The former enzyme is tought to exert the important regulatory influence on rates of fatty acid oxidation [52]. Proteins highly similar to CPT-1 have been identified in the membrane of peroxisomes [53].

Once fatty acids have gained access to the mitochondrial matrix via the carnitine acyltransferase system, their β-oxidation can proceed. β-oxidation of fatty acids involves the repetitive processing of two carbon units from the fatty acyl chain. The cycle involves four reactions. Each round of β-oxidation produces one NADH, one $FADH_2$, and one acetyl-CoA.

While the metabolic intermediates formed during β-oxidation are chemically identical in both the peroxisomes and mitochondria, different enzymes are involved in the two organelles. Three enzymes are involved in the peroxisomal β-oxidation pathway. In contrast with mitochondria, peroxisomes are capable of oxidizing a wide variety of fatty acids, e.g. very long-chain fatty acids, prostanoids, pristanic acid, dicarboxylic fatty acids, certain xenobiotics and bile acid intermediates. Because they have different affinities for the various fatty acids, peroxisomes and mitochondria act complementarily. The main function of peroxisomal β-oxidation may be chain-shortening and/or preparation of an initially poor substrate for subsequent β-oxidation by mitochondria [54].

β-oxidation may be mainly regulated by the entry of long-chain fatty acids into the mitochondria. Inactivation of ACC in cell leads to decrease in the concentration of the product of ACC, i.e., malonyl-CoA, which is a potent inhibitor of CPT-1 [37]. Regulation of the activity of the overall β-oxidation pathway is not well understood, but recent evidence suggests that PPARα may play a pivotal role [55]. As in mitochondria, PPARs play a pivotal role in the management of peroxisomal β-oxidation rate.

Several factors can also chronically influence CPT-1 activity. First, possibly by changing CPT-1 spatial conformation, membrane fluidity modulates the sensitivity of CPT-1 to inhibitors. Second, expression of CPT-1 (in the liver and probably in the heart) is regulated by hormones and by the abundance of long-chain fatty acids, which are known to activate the nuclear PPAR. While insulin inhibits the transcription of CPT-1, thyroid hormone, cAMP and PPARα stimulate its transcription. Third, disruption of cytoskeleton and inhibition of Ca21/calmodulindependent protein kinase II, which controls the integrity of the cytoskeleton, have been shown to enhance CPT-1 activity in cultured hepatocytes [56].

Mitochondria in WAT have a remarkable capacity for palmitoyl-carnitine oxidation but the physiological relevance of this relatively high capacity for β-

oxidation still remains unclear especially since adipocyte mitochondria contain very low CPT-1 activity.

2.1.2.5. LIPOLYSIS

In case of undernutrition and higher energy demand TAG are rapidly mobilized. Fatty acids are liberated from adipose tissue into the circulation and ultimately for use as fuel. The action of HSL on TAG yields two moles of unesterified fatty acids and one mole of monoglyceride. Hydrolysis of this remaining monoglyceride to one glycerol and one fatty acid moiety occurs readily through the action of monoglycerol lipase, which is presumably not under direct hormonal control *in vivo*.

The rate-limiting step is the activation of HSL, via a cascade of cellular signals. ß-adrenergic agents lead to an increase in the intracellular concentration of the cAMP that activates PKA, which in turn phosphorylates and stimulates HSL. Phosphorylated HSL moves from the cytosol of the adipocyte to the surface of the lipid droplet within the cell [58]. Catecholamines and insulin are the major plasma hormones that regulate lipolysis.

The phosphorylation of perilipins, a family of proteins located on the surface of the lipid droplet, is also required before HSL can catalyze the hydrolysis of the TAG inside the lipid droplet. Unphosphorylated perilipins create a barrier between HSL and cellular lipids, and prevent lipolysis. Phosphorylation of perilipin by PKA enables HSL to gain access to intracellular TAG, possibly by modifying the surface of the lipid droplet [59,60].

Adipose tissue lipolysis is very sensitive to changes in plasma insulin concentration [61]. Primary rat adipocytes were shown an increase of rates of lipolysis in the presence of both high glucose and insulin, associated with an increase in the levels of HSL [62]. Leptin increases glycerol release from adipocytes [63], but, unlike the response most lipolytic stimuli, this is not accompanied by the release of significant amounts of non-esterified fatty acids (NEFA) [64]. The most likely explanation for this is that leptin also increases the expression of several enzymes of fatty acid oxidation, allowing the fatty acids to be oxidized *in situ* within the adipocytes.

Perhaps most surprising is that mice lacking perilipin are more crippled in regard to regulation of fatty acid mobilization than those deficient in HSL [65].

2.1.2.6. REESTERIFICATION

About 30% of fatty acid reesterification in TAG have been shown to occur in WAT during fasting, i.e. at a time when lipolysis is activated, both in rats and humans. Esterification of fatty acids requires G3P formation, which under lipolytic situations does not arise from glycolysis since glucose utilization is strongly reduced under such circumstances. After complete hydrolysis of TAG, leading to three fatty acids and one glycerol, the latter is rephosphorylated into G3P by glycerol kinase. However, glycerol kinase is present at very low concentration in WAT when compared with liver [66]. In WAT is more common an abbreviated version of gluconeogenesis that would use pyruvate to making G3P required for re-esterification of fatty acids. This pathway was named glyceroneogenesis [67]. Glyceroneogenesis can be defined as the synthesis of G3P from non-carbohydrate precursors like pyruvate, lactate and certain amino acids.

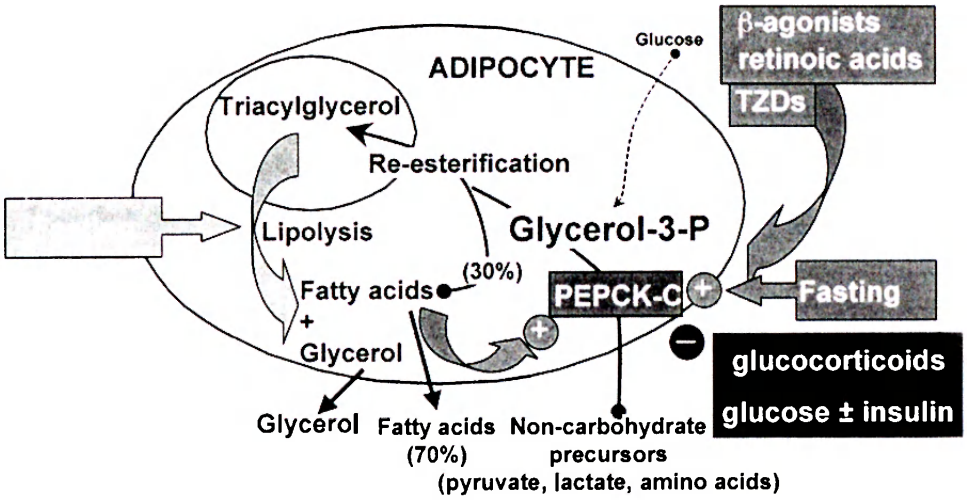

Figure 4. *Simplified representation of adipocyte glyceroneogenesis and of the regulation of its key enzyme, PEPCK-C.*

The key glyceroneogenic enzyme was determined to be cytosolic PEPCK (PEPCK-C) *(Figure 4.,* [68]*)*. PEPCK-C has unique and different tissue specific

functions - gluconeogenesis in liver, ammoniagenesis in the kidney, and glyceroneogenesis in adipose tissue.

The activity of PEPCK-C is increased by fasting, β-adrenergic agonists [69], cis- and trans-retinoic acids, unsaturated fatty acids [70] and thiazolidinediones (TZDs), while glucocorticoids decrease it. Glucose and maybe insulin are also inhibitory. All of these regulatory actions are exerted, at least in part, at the level of the transcription rate of the PEPCK-C gene. Glucocorticoid regulation presents a special interest since these hormones induce PEPCK-C expression in liver while inhibiting it in WAT [71]. *In vitro* differentiated adipocytes from the 3T3-F442A murine preadipocyte cell line express PEPCK-C only in the differentiated state [72].

2.1.3. ENERGY METABOLISM

Like in other tissues, mitochondria represent the main source of ATP even in white fat. Mitochondria are membrane-bounded organelles that produce ATP - the universal energy fuel molecule. The energy derived from the oxidation of substrates is converted into an electrochemical proton gradient. About 90% of standard mammalian oxygen consumption is mitochondrial, of which 80% is coupled to ATP synthesis by F_0F_1 ATP synthase and 20% is uncoupled by the mitochondrial proton leak via uncoupling proteins (UCP), etc. [73].

Mitochondria are usually depicted as elongated cylinders with a diameter of 0.5 - 1 μm. Each mitochondrion is bounded by two highly specialised membranes. Together they create two separate mitochondrial compartments: the intenal matrix space and much narrower intermembrane space. The outer membrane contains a protein called porin, which forms large aqueous channels, permeable to all molecules with molecular mass less than 10 kDa. The inner membrane is folded into numerous cristae, which greatly increases its total surface area. This membrane contains proteins with three types of functions: (i) the enzymes of the respiratory chain, (ii) ATP synthase complexes, and (iii) specific transport proteins that regulate the passage of metabolites across the inner membrane. Since the electrochemical proton gradient is generated across this membrane, it is important that the membrane be impermeable to most small ions. A high proportion of the phospholipid cardiolipin found in the membrane may contribute to this impermeability. The matrix contains a highly concentrated mixture of hundreds of enzymes including

those required for the oxidation of fatty acids and pyruvate and for the citric acid cycle. An important group of enzymes, which metabolise ATP (eg. myokinase, creatine kinase), are trapped in the intermembrane space.

Oxidative phosphorylation (OXPHOS) varies greatly depending on cellular activities, and is regulated by both gene expression and the electrochemical potential difference of H^+. OXPHOS is tightly regulated, not only by allosteric and covalent regulation of catalytic properties of several OXPHOS complexes, mediated by ATP/ADP ratio, but also by numerous fine controls [74]. The phosphocreatine shuttle can influence the ability of adenylates to mediate changes in OXPHOS [57].

Mirochondrial proteins are coded by genes of both nuclear (nDNA) and maternal mitochondrial (mtDNA) DNA. Many conditions that lead to changes in bioenergetics result in mitochondrial proliferation. Expression of both mtDNA and nDNA in response to cellular energy consumption requires several transcription factors. The transcription and replication of mtDNA are regulated by a factor called mitochondrial transcription factor A (mtTFA) [75], in addition to polymerases, ribosomal proteins and other molecules. Nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) are examples of transcription factors for nDNA encoding OXPHOS enzymes. NRF-1 and -2 bind to the promoter region of a broad range of mitochondrial genes encoded in the cell nucleus, including β-ATP synthase, cytchrome-c, cytochrome-c-oxidase subunit IV (COX IV), and mtTFA. It is of particular interest that the NRFs turn on mtTFA, a key transcriptional activator that translocates to the mitochondria and activates mitochondrial DNA replication and transcription. An increase in NRF-1 in response to contractile activity in skeletal muscle have shown [76]. Respiratory uncoupling of HeLa cells, through expression of UCP1, triggers expression of NRF-1 and NRF-1-sensitive genes [77]. An activation of AMPK led to an increased NRF-1 binding to DNA with the subsequent increases of mitochondrial proteins and mitochondrial volume [78].

The expression of both mtDNA (by mtTFA) and nDNA for OXPHOS and UCP (by NRFs, etc.) is coordinated by a factor called PPAR γ coactivator 1 (PGC-1) [79]. PGC-1 and its functions provide a plausible molecular basis for the connection between environmental/hormonal stimuli and mitochondrial biogenesis and respiration when the organism has altered energy or thermogenic requirements. PGC-1 is involved in multiple biological responses related to energy homeostasis, thermal regulation, and glucose metabolism. PGC-1 has been shown to stimulate

genes of fatty acid oxidation associated with an increase in fatty acid oxidation in adipocytes and in heart [80]. This coactivator also induces gene expression for the insulin-sensitive glucose transporter GLUT-4 and increases glucose uptake in muscle [81]. PGC-1 expression in liver is dramatically increased by fasting [82]. PGC-1 stimulates the synthesis of both UCP1 and UCP2 [75].

2.1.4. SECRETORY FUNCTION

Besides free fatty acids WAT release many proteins *(see Table 1)*. The secretory proteins of WAT, collectively referred to as 'adipocytokines'. Proteins secreted by adipose tissue play an important autocrine role in WAT physiology. Adipokines have many physiological effects on different organs including the brain, bone, reproductive organs, liver, skeletal muscles, immune cells and blood vessels [83]. And are involved in obesity-associated complications, such as insulin resistance, endothelial dysfunction, arterial hypertension and atherosclerosis [84].

Table 1. *The most important proteins secreted by adipocytes*

MOLECULE	EFFECT
Leptin	Feedback effect on hypothalamic energy regulation; maturation of reproductive function
Resistin	Appears to impair insulin sensitivity
Adiponectin	Improves insulin sensitivity if administered to rodent models of insulin resistance; improves fatty acid transport and utilisation
Adipsin	Required for the synthesis of ASP, possible link between activation of the complement pathway and adipose tissue metabolism
ASP	Activates diacylglycerol acyltransferase, inhibits hormone sensitive lipase, stimulates GLUT-4 translocation to the cell surface
Tumor necrosis factor α (TNF-α)	Mediator of the acute phase response. Inhibits lipogenesis, stimulates lipolysis and impairs insulin-induced glucose uptake, thus leading to insulin resistance and weight loss
IL-6	Increases hepatic glucose production and TAG synthesis, role in insulin resistance unclear
PAI-1	Potent inhibitor of the fibrinolytic system
Tissue factor	Initiator of the coagulation cascade

Angiotensinogen	Regulator of blood pressure and electrolyte homeostasis
PGI2 and PGF2α	Implicated in inflammation and blood clotting, ovulation and menstruation, acid secretion
TGF-β	Regulates growth and differentiation of numerous cell types
IGF-1	Stimulates cell proliferation and mediates many of the effects of growth hormone
MIF	Involved in proinflammatory processes and immunoregulation

2.1.4.1. LEPTIN

Leptin is a cytokine-like molecule with a molecular mass of 16 kDa synthetised mainly, but not exclusively, in WAT. Its synthesis occurs in proportion to adipose cell size and number, but also in response to acute stimuli. It is released into the blood and reaches its receptors in the brain via facilitated passage across the blood-brain barrier. Activation of these receptors results in a reduction of food intake, which is mediated through various downstream mechanisms including neuropeptide Y and α-melanocyte stimulating hormone [85, 86].

Numerous other actions of leptin have been described, such as effects on endocrine system and on reproduction.

Expression of the *ob* gene (gene coded leptin) is elevated by feeding, acute insulin, or glucocorticoid administration, expression is reduced by fasting [87], adrenergic β agonists [88], and PPAR γ agonists, such as TZDs [89]. The *ob* gene is downregulated by intracellular free fatty acid accumulation, thereby raising the possibility that its product, leptin, is regulated in response to lipolysis [90].

In both normal rodents and rodents with obesity and insulin resistance leptin therapy improves hyperinsulinaemia and hyperglycaemia [91]. The fact that leptin exerts a glucose- and insulin-lowering effect and improves insulin sensitivity *in vivo*, suggests involvement of centrally acting mechanisms. The peripheral mechanism by which leptin exerts its glucose- and insulin-lowering effect might be via promoting fatty acid oxidation and TAG synthesis by activation of AMPK in skeletal muscle. The resulting intramyocellular lipid depletion will enhance insulin sensitivity [44]. Apart from insulin-sensitising effects, leptin diminishes hyperinsulinaemia, probably via inhibition of insulin secretion. Functional leptin receptors have been

demonstrated on insulin-secreting β-cells of the pancreas [92]. High leptin levels *in vivo* cause AMPK activation in the adipocytes [93].

Increases of plasma leptin are much more effective in the low than in high concentration range. This is also consistent with the finding that the body is better protected against weight loss than against weight gain [1]. Since plasma leptin levels are positively correlated with BMI, obesity seems to reflect a leptin-resistant state. The limited efficacy of leptin in obese pacients could be due to the development of leptin resistance after prolonged exposure to increased plasma leptin concentrations [94].

2.1.4.2. ADIPONECTIN

Adiponectin is a recently identified adipocyte-specific secretory protein of about 30 kD that exhibits several actions on skeletal muscle, liver and vessels and appears to be involved in the regulation of energy balance and insulin action and also seems to have anti-inflammatory and anti-atherogenic properties [95, 96]. Adiponectin is the product of the adipose tissue most abundant gene transcript-1 (apM1), which is exclusively expressed in WAT. Adiponectin is specifically expressed during adipocyte differentiation and is not detectable in fibroblasts.

The expression of adiponectin is stimulated by insulin, IGF-1 and the TZDs [95, 97]. Corticosteroids, TNF-α and β-adrenergic stimulation inhibit adiponectin gene expression in 3T3-L1 adipocytes [97, 98, 99].

Serum adiponectin levels and are decreased in humans with obesity and type 2 diabetes as well as in obese and insulin-resistant rodents [100, 101, 102]. In addition, adiponectin gene transcription is decreased in adipocytes from obese and diabetic humans and rodents [96, 102]. Low plasma adiponectin concentrations predicted a decrease in insulin sensitivity and an increase of type 2 diabetes [103].

Administration of adiponectin led to an increase in glucose utilisation and fatty acid oxidation in cultured myocytes and in soleus muscle of mice *in vivo*. In hepatocytes AMPK was activated as well, leading to a reduction in gluconeogenesis [104].

Thus, adiponectin might exert its insulin-sensitising effect via the following mechanisms: i) increased fatty acid oxidation leading to a lower muscle TAG content and lower plasma concentrations of free fatty acids which will both improve

insulin signalling; ii) direct improvement of insulin signalling; iii) inhibition of gluconeogenesis, partly via reduced substrate delivery and partly via reduction of molecules involved in gluconeogenesis by activation of AMPK.

2.2. *AMP-ACTIVATED PROTEIN KINASE*

AMPK is the central component of a protein kinase cascade that plays a key role in regulation of carbohydrate and fat metabolism. The enzyme, highly conservated throughout evolution, serving as a metabolic master switch in response to alterations in cellular energy charge. AMPK phosphorylates numerous target proteins at serine residues in the context of a characteristic sequence recognition motif. Phosphorylation may result in increases or decreases in the rate of the metabolic pathway in which the protein target plays a regulatory role [105]. Recent evidence suggests that AMPK can also influence metabolism by regulating gene expression [106].

AMPK is a heterotrimer of three subunits, i.e., α, β, and γ [107] and each subunit exists in several isoforms [108]. While AMPK is regarded as a ubiquitous enzyme, the expression pattern of individual α, β and γ subunits occurs in a tissue-specific manner.

The 62 kDa α-subunit contains the kinase domain and also contributes to the AMP-binding site [109]. The kinase is activated, in response to the AMP-to-ATP ratio (AMP/ATP), in physiological situations when cellular production of ATP is decreased - exercise, heat shock, glucose or oxygen deprivation. Once activated, it switches on catabolic and switches off anabolic pathways (*Figure 5.*, [110])

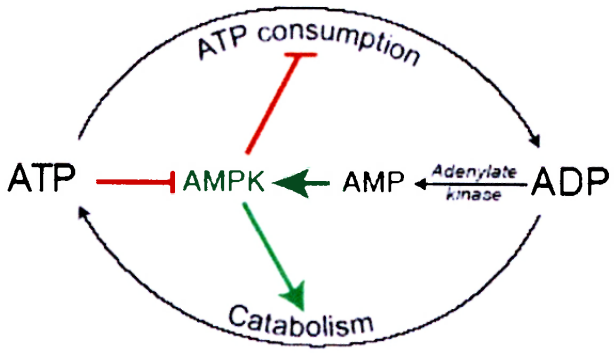

Figure 5. *Regulation of AMPK.*

Two of the classical targets for AMPK in liver are ACC and 3-hydroxy-3-methylglutaryl-CoA reductase, catalyzing the key regulatory steps in fatty acid and sterol synthesis, respectively [109].

Its known effects among others include: (i) inhibition of fatty acid synthesis and lipolysis in adipocytes due to phosphorylation of ACC-α and hormone-sensitive lipase [111, 112, 113]; (ii) stimulation of glucose uptake into adipocytes [114] and myocytes [115]; (iii) activation of fatty acid oxidation in muscle due to phosphorylation of ACC-β [115]; (iv) induction of mitochondrial biogenesis possibly through activation of NRF-1 [78]; and (v) downregulation of lipogenic genes mediated by transcription factor SREBP-1 in liver [116], as well as in 3T3-L1 adipocytes, in the latter case by downregulating PPARγ [117].

AICAR, an specific activator of AMPK, stimulated adipose tissue AMPK α1 activity and adiponectin gene expression, while attenuating the release of TNF-α and IL-6 [118].

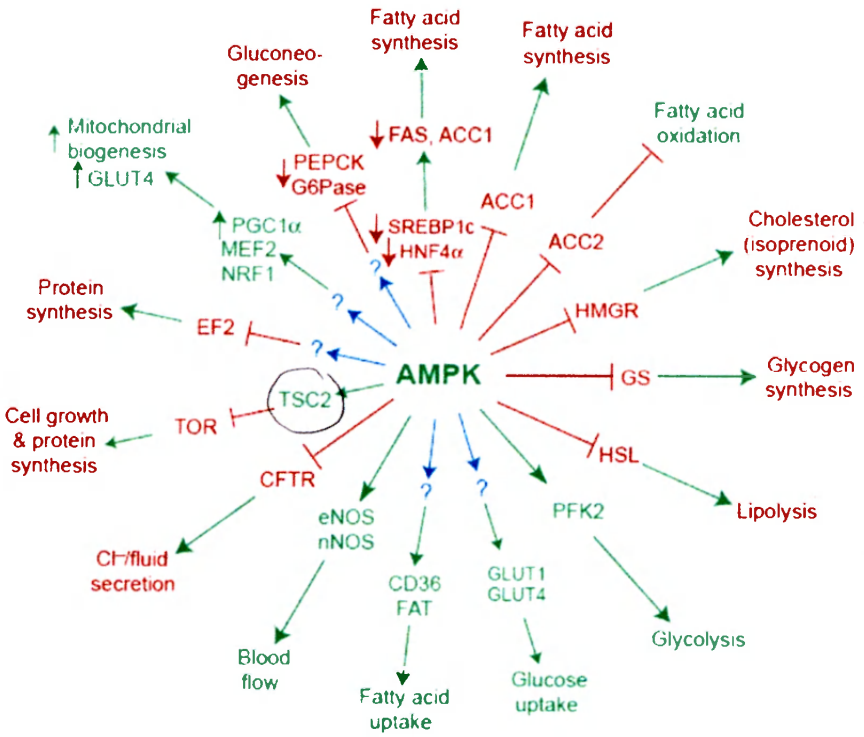

Figure 6. *Targets for AMPK.*
Target proteins and processes activated by AMPK activation are shown in green, and those inhibited by AMPK activation are shown in red. Where the effect is caused by a change in gene expression, an upward-pointing green arrow next to the protein indicates an increase, whereas a downward-pointing red arrow indicates a decrease in expression. Abbreviations: CFTR, cystic fibrosis transmembrane regulator; EF2, elongation factor-2; eNOS/nNOS, endothelial/neuronal isoforms of nitric oxide synthase; G6Pase, glucose-6-phosphatase; GS, glycogen synthase; HMGR, 3- hydroxy-3-methyl-CoA reductase; MEF2, myocyte-specific enhancer factor-2; TOR, mammalian target of rapamycin.

2.3. *UNCOUPLING PROTEINS*

The UCPs regulate discharge the proton gradient that is generated by the respiratory chain across the inner mitochondrial membrane. This energy-dissipatory mechanism can serve functions such as thermogenesis, maintenance of the redox balance, or reduction in the production of reactive oxygen species. The UCPs are integral membrane proteins that have molecular masses between 31 kDa and 34 kDa. The functional carrier unit is a homodimer.

The most well-known member of the family is UCP1, which is uniquely expressed in brown adipose tissue (BAT) and plays an important role in cold- and diet-induced thermogenesis. UCP2 and UCP3 were first cloned and identified in humans in 1997 and subsequently in rodents [119]. They share about 56 % amino acid identity with UCP1 and are themselves about 72 % identical. UCP2 and UCP3 do not appear to be solely involved in thermogenesis, and have proposed roles in modulating generation of reactive oxygen species and in lipid handling, although their physiological roles have not yet been definitively determined. UCP2 mRNA is found in many tissues and at high levels in white adipose tissue, skeletal muscle, spleen and pancreatic β-cells, whereas UCP3 is predominantly expressed in skeletal muscle, heart, and to a lesser extent adipose tissue. This expression pattern is consistent with their proposed roles since adipose tissue and skeletal muscle are major contributors to overall energy metabolism. Variations in activity or regulation of UCP2 and UCP3 in these tissues could contribute to obesity and associated diseases. A number of physiological and pathological states lead to increased expression of UCP2 and UCP3 mRNAs. These include fasting, high fat (HF) diets, suckling of newborn pups, sepsis, acute endurance exercise, and hyperthyroidism, as well as experimental manipulations such as lipid infusion and streptozotocin-induced diabetes [120].

2.3.1. aP2-*UCP1* TRANSGENIC MICE

Transgenic aP2-*Ucp1* mice, 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 excellent model to study the effects of respiratory uncoupling on fat accumulation *in vivo*. These animals are partially resistant to obesity induced by age, genetic background, and HF diet *(Figure 7)*. Their resistance to obesity reflects

lower accumulation of TAG in all fat depots except for gonadal fat, which becomes relatively large [121, 122, 123]. Importantly, obesity resistance results exclusively from a transgenic modification of white fat [124], since brown fat in these mice is greatly atrophied [125].

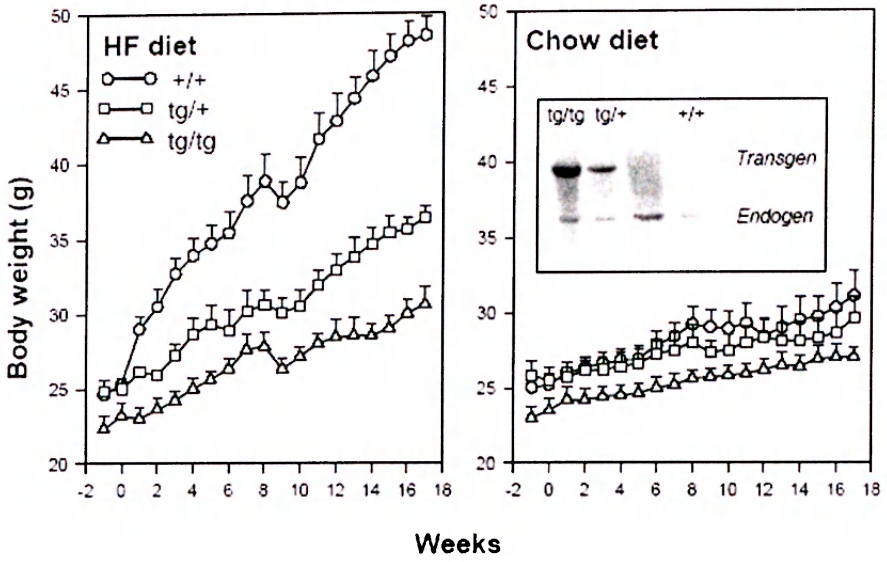

Figure 7. *Resistance against dietary obesity in aP2-Ucp1 mice.*
Body weights of mice fed the Chow (standard) diet or the high fat (HF) diet since 3 months of age. Insert: Southern blot with Ucp1 probe. Symbols: +/+ control mice, tg/+ transgenic mice.

The resistance to obesity reflects lower accumulation of TAG in all fat depots, except for gonadal fat, which becomes relatively large [122, 123]. The strong mitigation of obesity in aP2-*Ucp1* mice, and the fact that UCP1 acts locally to reduce adiposity, indicate that the reduction results not only from increased energy expenditure but also from a differential modification of lipid metabolism in various fat depots.

The transgenic UCP1 expressed in mitochondria of white adipocytes of aP2-*Ucp1* mice modulate mitochondrial membrane potential and rendered the potential sensitive to natural regulatory ligands of UCP1 purine nucleotides and fatty acids [126].

Only unilocular cells were present in white fat of both homo- and heterozygous transgenic mice. Under no circumstances, could ectopic UCP1 induce conversion of unilocular into multilocular adipocytes. The morphometric study of subcutaneous white fat of the adult transgenic animals demonstrated that the

unilocular cells had a larger cytoplasmic area and contained more numerous and larger mitochondria with a relatively high cristae density, compared to control mice. The results of the morphometric analysis indicated induction of mitochondrial biogenesis by ectopic UCP1 in the unilocular adipocytes. Also was elevated the transcripts for UCP2 and COX IV and increased content of mitochondrial cytochromes [127].

Expression of the genes for ACC and FAS, cytoplasmic enzymes engaged in the *de novo* fatty acid synthesis, was significantly depressed by the transgene in both subcutaneous and epididymal white fat depot. Fatty acid synthesis was reduced up to fourfold by ectopic UCP1 in the white fat of transgenic aP2-*Ucp1* mice, reflecting the magnitude of UCP1 expression in different fat depots [128].

The transgenic UCP1 affects norepinephrine-induced lipolysis in adipocytes. The lipolytic effect of noradrenaline was lowered by ectopic UCP1 in white adipocytes of aP2-*Ucp1* transgenic mice, reflecting the magnitude of UCP1 expression and the reduction of the ATP/ADP ratio in different fat depots. Transgenic UCP1 also down-regulated the expression of HSL and lowered its activity. To examine the basis for the mitigation of the lipolytic responsiveness to noradrenaline, the content of cAMP and G-protein levels were analyzed. Incubation of these cells in the presence of noradrenaline resulted in an increase of cAMP levels. But this induction was significantly less in transgenic than in control mice. The adipose tissue content of stimulatory G-protein α subunit was increased while that of inhibitory G-protein α subunits decreased in response to UCP1 expression [129].

UCPs and other protonophores that decrease adiposity by increasing energy expenditure and by mitigating lipogenesis could also depress the catecholamine responsiveness of lipolysis due to lowering mitochondrial ATP synthesis in adipocytes.

2.4. *FASTING*

The early post-absorptive state begins as the nutrient flow from the intestine diminishes. As blood glucose and insulin levels fall back to normal, glucagon release triggered. Glucagon acts to prevent hypoglycemia by promoting glycogenlysis and gluconeogenesis in liver. Decreased insulin also promotes lipolysis and release of

amino acids as alanine and glutamine from muscle. Several tissues use fatty acids in preference to glucose. Glycerol and alanine are substrate for gluconeogenesis and glutamine is used as an energy source by enterocytes.

If a fast becomes prolonged, mobilisation of fatty acids is increased by stimulation of adipose tissue by norepinehrine. Glucose is spared for brain.

Under conditions extraordinarily prolonged fasting (starvation) ketone bodies are mobilized from liver.

In the post-absorptive state the net flow of fatty acids is outwards across the adipose tissue capillary wall, into the circulation. This state is brought about by activation HSL which acts on the stored TAG to release NEFA and glycerol. The major factor may be the progressive removal of suppression by insulin, as insulin concentrations fall with fasting.

Glucagon also stimulates lipolysis *in vitro* in mammals [130]. Studies in humans by microdialysis of subcutaneous WAT imply that the net adrenergic effect is inhibitory rather than stimulatory after overnight fast [131].

Of the fatty acids released in lipolysis, some are reesterified. Larger values are observed *in vitro* than *in vivo* [132]. It has been suggested that these fatty acids take an extracellular route [133]. The fatty acids released probably leave the adipocyte by a carrier-mediated process [134] and diffuse. Because of reesterification glyceroneogenesis is induced by fasting. The expression and activity of PEPCK-C is increased. During fasting one can assume that the obvious glyceroneogenic precursors are lactate and certain amino acids like alanine [135].

Serum insulin levels fall and the uptake of glucose and lipids by the adipocyte diminishes. This leads to a decreased expression of the *ob* gene, which is responsible for leptin formation and hence the plasma leptin concentration falls [136].

Although there is usually no significant dietary TAG present in the circulation in the form of chylomicron TAG after overnight fast, the LPL in WAT is not completely inactive.

The expression of lipogenic enzyme genes (ACC, FAS and citrate cleavage enzyme) is markedly reduced in adipocytes but not in stromal-vascular fraction cells isolated from subcutaneous depot of 48-h starved male rats [137]. During fasting increase also β-oxidation in adipocytes [138].

When animals are refed after fasting, these effects are especially prominent. Under such conditions, the plasma levels of glucose and insulin are high. Fatty acid synthesis is high, whereas fatty acid oxidation and the expression of PPARα and PPARγ are low. Fatty acids are secreted from the liver into circulation and taken up by adipose tissue. These effects are even more pronounced when the diets are enriched in carbohydrates. Under such conditions, the expression of PPARα is low and the effect of PPARα activators are blunted.

Refeeding fasted rats with a high-carbohydrate diet produces a reduction in PEPCK-C activity and mRNA in adipose tissue, just as it does in liver [70].

2.5. *POLYUNSATURATED FATTY ACIDS*

Every organism contains broad range of fatty acids. They are not only the main structural component of every cell, bounded in phospholipids, or an important source of energy, stored as TAGs. But they also play an active role in the cell metabolism. The activities of fatty acids may be dependent on their having a higher or lower ability to interact with enzymes or receptors, as compared with other fatty acids.

Oleic acid (18:1, ω-9) is the most common monoenoic fatty acid in plants and animals. It is a product of *de novo* fatty acid synthesis.

PUFA, which have their first double bond between the third and the fourth carbon atom from the ω carbon, are called omega-3 fatty acids. If the first double bond is between the sixth and seventh carbon atom, then they are called omega-6 fatty acids. Omega-3 and omega-6 fatty acids cannot be interconverted, and both are essential nutrients. Linoleic acid (LA; 18:2, ω-6) is a major fatty acid in plant lipids. In animals it is derived only from dietary vegetables, plants and marine oils. Arachidonic acid (AA; 20:4, ω-6) is a major component of animal phospholipids. It is also a major component of marine algae and some terrestrial species, but very little is found in the diet. α-Linolenic acid (ALA; 18:3, ω-3) is found in higher plants and algae. Eicosapentaenoic acid (EPA; 20:5, ω-3) and docosahexaenoic acid (DHA; 22:6, ω-3) are major fatty acids of marine algae, fish and fish oils. These fatty acids are found in animals, especially in phospholipids in the brain, retina and testes.

The consumption of omega-3 PUFA leads to a substantial decrease in plasma TAG concentrations, but has little effect on blood cholesterol in most circumstances.

In addition to effects on plasma lipids, dietary fatty acids can prevent the developement of insulin resistance [139]. Oxidized omega-3 fatty acids may be an important component of the observed anti-inflammatory effects of fish oil.

Rodents fed fish oil have reduced adipose tissue mass, without a significant change in energy intake. Although these effects may be gender and strain specific [140, 141]. Dietary PUFAs downregulate the expression of SCD-1 in adipose tissue of rats and differentiated 3T3-L1 cells. The downregulation of SCD-1 expression might therefore contribute to the ability of PUFAs to reduce adipose tissue growth [142].

PUFAs enhance uncoupling of OXPHOS. Adding phospholipids containing DHA to mitochondria both *in vitro* and *in vivo* reduces the respiratory control index and in reases the proton permeability of mitochondrial membrane [143]. PUFAs are also able to increase UCP2 mRNA in 3T3-L1 preadipocytes [144]. Rats fed with DHA and EPA have increased cytochrome c oxidase activity in brown adipose tissue and diet induced thermogenesis [145]. This is due to the stimulation of mitochondrial and peroxisomal fatty acid β-oxidation by omega-3 PUFA [146]. Stimulation of fatty acid oxidation was also observed in muscle and liver [147].

Dietary fatty acids and their metabolites are able to modulate protein expression by several mechanisms. They may affect gene transcription, mRNA processing and modulate posttranslational modifications of proteins [148, 149]. PUFAs are known to suppress lipogenic gene transcription by downregulating the expression of the SREBPs [150, 151] and they may function as antagonists of liver X receptors (LXR) [152] and as activators/ligands for the PPARs [153]. Fatty acids or their metabolites are not only reported to be natural ligands for the different PPARs *(reviewed in* [205]*)*, but they can also control the expression of these receptors, as demonstrated for PPARα [154].

Knowledge regarding fatty acids as kinase cascade activators/inhibitors has also accumulated during the last decades [155,156].

There is a high incorporation of EPA and DHA in membrane phospholipids. An increased amount of omega-3 PUFA may alter the physical characteristics of the membranes. Altered fluidity may lead to changes of membrane protein functions and cell signalling. Enzymes in the eicosanoid pathway (cyclooxygenases, lipoxygenases, and P450 epoxygenases) normally use the major omega-6 fatty acid, arachidonic acid, liberated from phospholipids by phospholipases, as substrates. A

number of pathologic states such as inflammation, asthma, hypertension and certain types of cancer are associated with the dysregulation of the eicosanoid pathway [157]. The structurally similar omega-3 PUFAs may replace arachidonic acid in phospholipids. Some omega-3 PUFAs are converted into products with properties distinct from those generated from arachidonic acid, while others, in particular DHA, are inhibitors of cyclooxygenases (and possibly lipoxygenases). The consumption of a diet enriched in omega-3 PUFA (specifically EPA and DHA) may thereby affect eicosanoid biosynthesis [158]. In general, the eicosanoids produced from omega-3 fatty acids, particularly EPA and DHA, are less inflammatory, cause vasodilation, and inhibit platelet aggregation, compared with those produced from omega-6 fatty acids.

3. SPECIFIC AIMS OF WORK

General goal of this work was to learn whether changes in energy charge and metabolism of WAT may affect content of body fat and improve of obesity-associated disorders. Also site-specific response of subcutaneous and epididymal fat pads were studied. The specific aims of this thesis were:

A. to verify the hypothesis that respiratory uncoupling modulates activity of AMPK in white adipose tissue;

B. to investigate whether AMPK could be involved in the different responses of subcutaneous and epididymal fat depots to starvation;

C. to evaluate the effect of diets rich in omega-6 and omega-3 fatty acids on adipose tissue energy metabolism; and

D. to test the hypothesis that whole body effects of EPA and DHA involve induction of adiponectin.

4. RESULTS AND DISCUSSION

4.1. Possible involvement of AMP-activated protein kinase in obesity resistance induced by respiratory uncoupling in white fat (PUBLICATION A)

The specific aim of this study was to verify the hypothesis that respiratory uncoupling modulates activity of AMPK in white adipose tissue.

The UCPs enhance proton leak and through respiratory uncoupling decrease ATP synthesis. Since UCP1-mediated respiratory uncoupling in brown fat is involved in thermogenesis and in the control of energy balance [159], it may be hypothesised that uncoupling of OXPHOS in WAT would also increase energy expenditure and thermogenesis. It could be expect an effect of respiratory uncoupling on lipid metabolism in adipocytes and therefore reduction of body fat content. Consequences of the expression of transgenic UCP1 in white fat in our aP2-*Ucp1* transgenic mice, which induces the obesity resistance, has been studied in great detail *(for details see chapter 1.3.1)*. Reduction of fatty acid synthesis [128] could be explain by a drop in level of ATP induced by respiratory uncoupling. But decrease in ATP level was not a unifying explanation for all of these metabolic changes like - reduction of the lipolytic action of catecholamines [129], as well as increases in mitochondrial biogenesis [127] and in endogenous oxygen consumption [122] in WAT.

Since level of ATP was lower and the ADP/ATP ratio was higher in white fat with ectopic expression of UCP1, we first examined the possibility that transgenic UCP1 expression induce an activation of AMPK via increase of the AMP/ATP ratio. Differences between aP2-*Ucp1* and control in the ADP/ATP ratio in WAT mice were more pronounced in subcutaneous than in epididymal fat, in accordance with a higher content of transgenic UCP1 in the former fat depot [129]. We expected that the AMP/ATP ratio should be also affected due to the adenylate kinase reaction [160].

It was observed that the AMP/ATP ratio in both fat depots of transgenic animals was higher than in control mice *(see Figure 1A in Publication A)*. Because of it the activity of AMPK was determinated in WAT of both genotypes by using

kinase assay. The presence of transgenic UCP1 resulted in a significant, 2-fold increase in the activity of the α1 isoform of AMPK (α1 AMPK) in subcutaneous fat *(see Figure 1B in Publication A)*. The activity of the α2 AMPK was negligible in adipose tissue. Our results are supported by finding that norepinephrine (NE) increased the activity of AMPK in BAT of wild type but not UCP1-KO mice [161]. Also adenovirus-mediated expression of UCP1 in liver of mice resulted in decrease of body fat content, weight of adipose tissue and activation of hepatic AMPK [162].

Not only higher activity but also a significantly higher content of α1 AMPK subunit in transgenic than in control mice in both epididymal and subcutaneous fat was found *(see Figure 2 in Publication A)*. Increased expression of α1 has been observed in other tissues in response to treatments that would be expected to persistently activate AMPK, e.g., during pressure overload hypertrophy in rat heart [163], or endurance training in human skeletal muscle [164].

ACC, rate limiting enzyme of *de novo* fatty acid synthesis *(see chapter 1.1.2.2.)*, is one of known target protein of AMPK. Quantification of total ACC and pACC content in adipose tissue lysates also showed a modest but significant 1.3-fold increase of the pACC/ACC ratio in subcutaneous fat due to the transgenic modification.

Increased phosphorylation of ACC could, in addition to decreasing of *de novo* fatty acid synthesis, raise a rate of fatty acid oxidation. Measurement of oleate oxidation in adipose tissue fragments *(see Figure 3 in Publication A)* showed 2.7-fold increase in the oleate oxidation in subcutaneous fat, but not epididymal fat of transgenic mice.

In order to further characterize the complex changes in lipid metabolism in the transgenic mice, expression of the PPARγ and aP2, its target gene was analyzed. A significant diminution of PPARγ and aP2 mRNA level was found in subcutaneous but not in epididymal fat of aP2-*Ucp1* mice *(see Table 2 in Publication A)*.

In adipocytes, AMPK is known to inhibit both lipolysis and lipogenesis by regulating directly the enzymes engaged in lipid metabolism [111], as well as by downregulating PPARγ expression [117]. All the effects of transgenic UCP1 on the biochemical properties of white fat in the aP2-*Ucp1* mice seem to be in agreement with the activation of AMPK. Indeed, it was observed that UCP1-induced increase in the ATP/ADP ratio in the subcutaneous white fat of transgenic mice [129] was also associated with a reduced ATP/AMP ratio, increased activity of AMPK, and

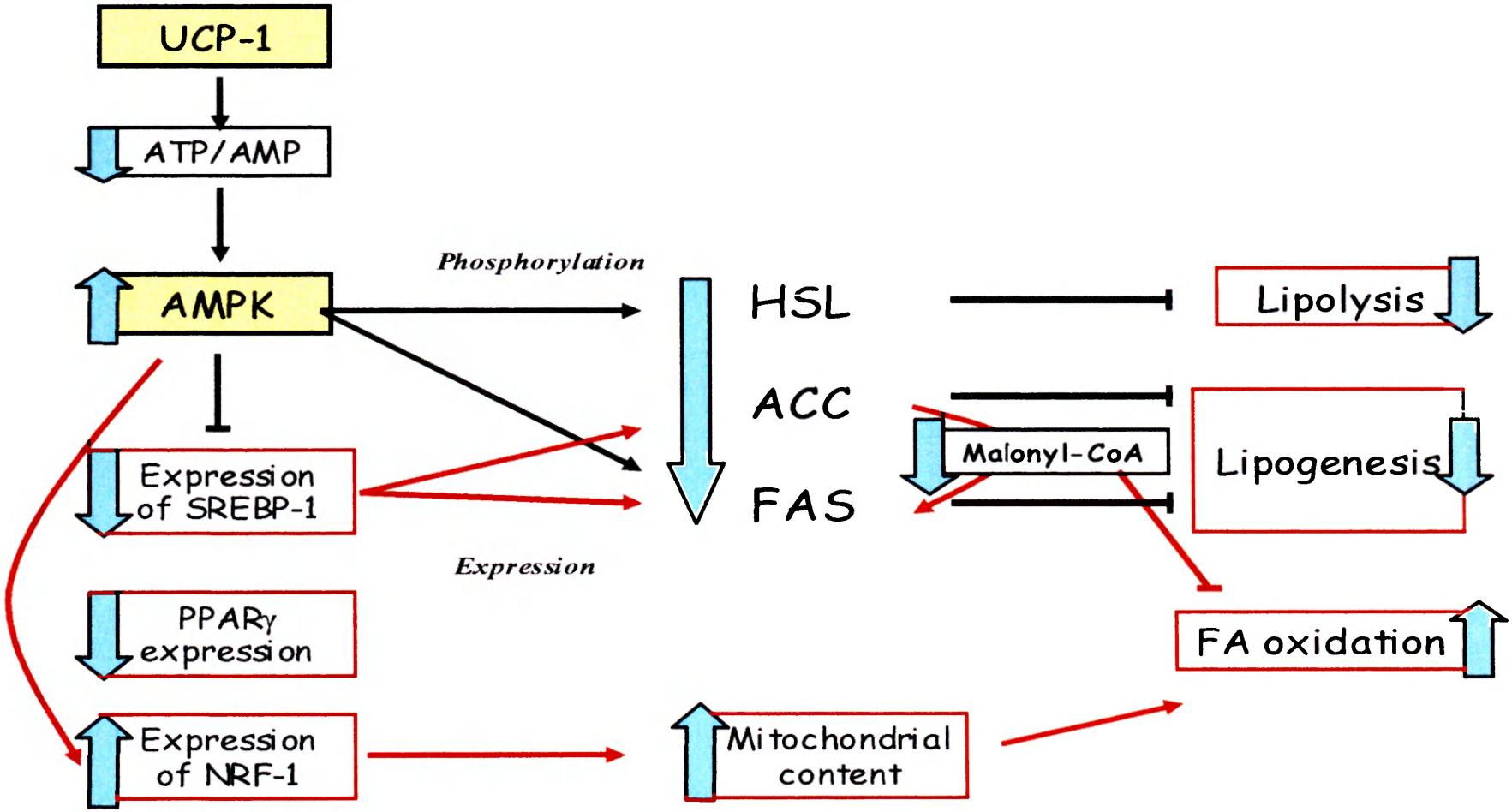

Figure 8. *Scheme of modulation of WAT metabolism by ectopic Ucp1.*

enhanced oxidation of fatty acid. Less dramatic changes were also observed in the gonadal fat of these mice. Thus, activation of AMPK by decreased intracellular energy charge in the white fat of aP2-*Ucp1* mice explains the complexity of changes in adipose tissue metabolism observed in this obesity-resistant transgenic model *(Figure 8.)*.

These results suggest a new link between mitochondrial UCPs and regulation of body weight, showing that lower efficiency of mitochondrial energy conversion in fat cells may decrease adiposity in part by depression of *in situ* lipogenesis. The measurement of fatty acid synthesis is a new way for detecting the activity of UCPs in adipose tissue. Because the oxidative capacity of white fat is relatively low, the link between mitochondrial energetics and fatty acid synthesis may be a superior target for treatment strategies for obesity.
My main contributions to this work were measurement of fatty acid oxidation and collaboration on AMPK and ACC analysis.

4.2. Involvement of AMP-activated protein kinase in fat depot-specific metabolic changes during starvation (PUBLICATION B)

The specific aim of this study was to investigate whether AMPK could be involved in the different responses of subcutaneous and epididymal fat depots to starvation.

The biological significance of the link between energy status and metabolism in adipocytes, which has been shown in aP2-*Ucp1*, and the involvement of AMPK in this link, is supported by experiments in mice deprived of food. This experiments also could show differences of physiological function between white fat depots. Depending upon its anatomical location, white adipose tissue exhibits strong heterogeneity in tends of physiologial and biochemical properties [165]. Different characteristics between intra-abdominal and subcutaneous fat depots have been described, including differences in gene expression, rate of lipolysis and lipogenesis and response to catecholamines [11, 166, 167]..

The time- and depot-dependent effects of starvation were studied during 24 hours food deprivation. Mice (initially weighting 27.1 g) lost an average of 1.2 g after 6 hours, 2.7 g after 12 hours and 3.7 g after 24 hours of fasting. It was observed

decrease in weight in both studying fat depots, epidydimal and subcutaneous. Significant change was detected only in weight of subcutaneous fat after 12 and 24 hours of fasting compared with fed mice. The reduction of weight was accompanied by a decrease in the content of tissue lipids in subcutaneous but not in epididymal fat after 24 h *(see Table 2 in Publication B)*. Starvation increased plasma levels of NEFA. The rise of concentration of NEFA in plasma was significant after 6 hours and it reached a peak after 12 hours of fasting *(see Table 2 in Publication B)*.

In response to nutritional status we expected changes in lipogenesis. The expression of SREBP-1, a transcription factor controlling lipogenesis, declined only transiently in subcutaneous fat, while the expression was progressively abolished during 24h of starvation in epididymal fat *(see Figure 1A in Publication B)*. A gradual inhibition of the expression of FAS was observed in both depots, with a stronger effect in the epididymal fat *(see Figure 1B in Publication B)*. In parallel, the activity of fatty acid synthesis also decreased to different extents in the two fat depots *(see Figure 1C in Publication B)*.

Since decrease in the rate of lipogenesis was observed due to starvation in WAT, also cellular level of malonyl-CoA, a potent inhibitor of fatty acid oxidation, could be affected. Previously, stimulation of fatty acid oxidation in adipocytes isolated from epididymal and perirenal fat of starved rats has been shown [168]. Our measurement of oleate oxidation in WAT fragments from fed mice and mice starved for 12 h confirmed these findings and showed significant stimulation of fatty acid oxidation by starvation in epididymal fat *(see Figure 9.)*. Fatty acid oxidation also tended to be increased during starvation in subcutaneous fat, but this effect was not significant *(see Figure 9.)*.

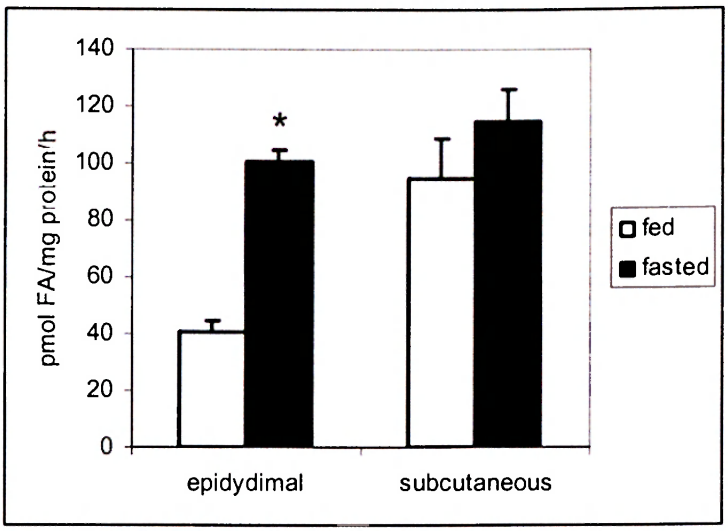

Figure 9. *Increase of fatty acid oxidation in WAT after 12h of food deprivation*

Previously was shown that activation of AMPK in liver do the following: (i) to phosphorylate and inactivate ACC [105]; (ii) to suppress the transcriptional regulator SREBP-1, an effect that decreases the expression of ACC, FAS [116], and the first cominitted enzyme in the pathway of glycerolipid synthesis GPAT [51]; and (iii) to increase the activity [169] and expression [170] of CPT-1.

The separate regulation of lipid metabolism in epididymal and subcutaneous fats in response to starvation was in agreement with a differential activation of the AMPK. Since AMPK complexes contain one of the two catalytic subunits (α1 or α2), which respond differently to various stimuli [44, 171], we determined activity of α1 and α2 subunit separately. AMPK complexes were immunoprecipitated from lysates, using antibodies against α1 or α2 subunits. In response to starvation α1 AMPK activity was stimulated in epididymal fat. The activity peaked after 12h of starvation *(see Figure 3A in Publication B).* In contrast, no changes of α1 AMPK activity could be detected in subcutaneous fat *(see Figure 3A in Publication B).* The α2 AMPK activity was very low and did not change significantly in either fat depot.

By using immunobloting we detected total content of ACC and the phosphorylation state of Ser79 of ACC, the specific site of inhibitory phosphorylation by AMPK. The phosphorylation state was raised in accord with the differential activation of α1 AMPK activity *(see Figure 3B in Publication B).* Total ACC content declined during 24 h of starvation, especially in subcutaneous fat *(see Figure 3C in Publication B).* This findings are in accord with established changes in

the phosphorylation status of AMPK of fasted and refed rats published recently. AMPK activity was 2-fold higher in the epidydimal fat pad of fasted compared to refed rats [172]. AMPK activity was also accompanied with change in the phosphorylation of Ser79 ACC.

Mice normally eat most of their daily intake during the dark phase, and in response to prolonged starvation they exhibit torpor, i.e. a lowering of their body temperature and energy expenditure [173]. AMP-activated protein kinase activity in adipocytes is represented mainly by the α1 isoform. Whether this has a functional significance is presently unclear although it can be emphasized that AMPK complexes containing this isoform are much less sensitive to AMP [174]. AMPK in adipocytes is induced by all stimuli which increase cAMP in adipocytes, e.g fasting or cold [172, 175].

In order to separate any response to cold from that to starvation, was the effect on the activity of the AMPK studied following a new experimental protocol *(see Protocol B in Materials and methods of Publication B)*. Mice were deprived of food just before entering the dark phase and the analysis was performed 6 hours later, in both starved mice and ad libitum fed controls sacrified at the same time like starved animals. This experiment was performed in animals maintained at two different temperatures (20 and 30°C). Also in this experimental protocol was observed the rise in phosphorylation state of AMPK and ACC in epididymal fat after 6 h of starvation in both temperatures. Yet the pACC/ACC ratio decreased in subcutaneous fat in this experimental model *(see Table 3 in Publication B and Figure 10)*.

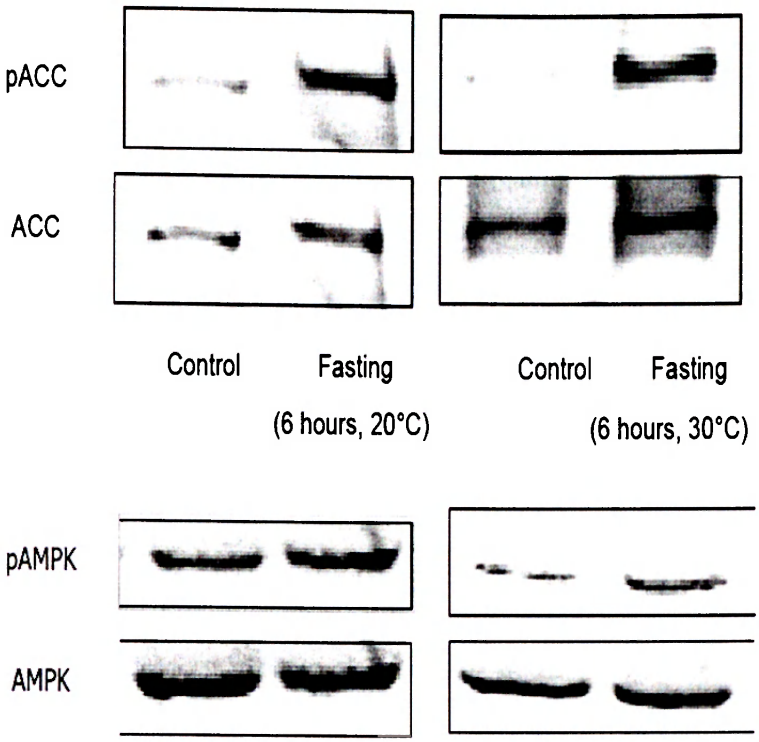

Figure 10. *Effect of 6-hour starvation on phosphorylation of ACC and AMPK in epididymal WAT in mice maintained at 20° or 30° C.*

Moreover, in the animals at 20°C, core body temperature as well as whole body oxygen consumption were followed for more than 16 h, both in starved mice and controls with free access to food. These measurements were performed using a modern system for indirect calorimetry combined with telemetry of body temperature *(see Figure 11.)*. Core body temperature was slightly lower (about 1°C difference) in the animals starved for 2 h and 6 h, respectively, than in at the same time in ad libitum fed mice, and the temperature started to drop progressively compared with the controls only after 6 h of starvation. These results indicate that mice starved for 6 h have not entered torpor, and that torpor appeared only after 6 h of starvation. Therefore, the activation of AMPK, observed under the conditions of the new experiment was not induced by lowering of body temperature. This conclusion was further supported by the fact that the activation of AMPK as observed in mice maintained at both 20 °C and the temperatute of 30°C, i.e. close to thermoneutrality for mice. It could be argued that the activation of both AMPK and ACC in the epididymal fat by starvation, as revealed by the quantification of the

phosphorylation of the enzymes, was lower at 30°C than at 20°C *(see Table 3)*, and therefore, the activation of the AMPK depended in part on body temperature and not only on the starvation. Lower ambient temperature may really augment the effect of starvation on the AMPK in specific WAT depots.

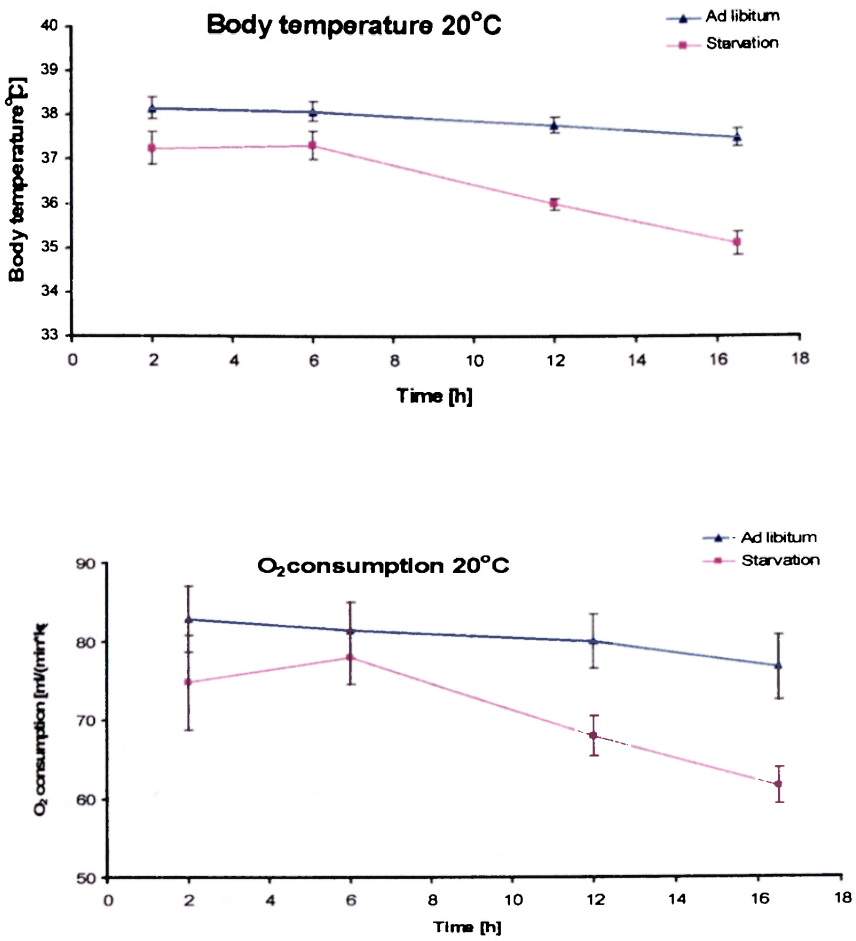

Figure 11. *Whole body oxygen consumption and deep body temperature in mice at 20°C.*

In order to understand the mechanism of the differential effect of fasting on lipid metabolism and activation of AMPK in subcutaneous and epididymal fat, we examined whether either PEPCK or UCP2 could be involved. Expression of both PEPCK [67] and UCP2 [176] genes in adipose tissue is known to be stimulated by

food deprivation. In both cases, the stimulation may result in depression of the energy charge in adipocytes, because PEPCK govens ATP-consuming fatty acid re-esterification in the adipocyte during fasting [67], while UCP2, which is abundant in white fat [177], may decrease the rate of ATP formation due to respiratory uncoupling in mitochondria [178, 179].

In fed mice, the mRNA levels of PEPCK in epididymal fat were 2-fold higher than in subcutaneous fat, and these levels increased substantially during 24 h of fasting *(see Figure 2B in Publication B).* In contrast, a smaller and transient increase of PEPCK mRNA levels was observed under similar conditions in subcutaneous fat, resulting in about 6-fold higher activity in epididymal than in subcutaneous fat in animals fasted for 24 h *(see Figure 2B in Publication B).* Levels of UCP2 mRNA were similar in both depots of fed mice, and the expression increased during fasting *(see Figure 2 in Publication A).* However, the increase was much higher in epididymal than in subcutaneous fat, with a maximal expression reached 12 h after food deprivation, resulting in a 4-fold difference between epididymal and subcutaneous fat.

In different response of both fad pads to starvation could be involved fatty acid reesterification driven by PEPCK. PEPCK is strictly transcriptionally regulated [180] and is essential for the synthesis of G3P from non-glucose precursors in adipocytes during fasting. Up to 30% of the fatty acid released by lipolysis in epididymal fat of the fasted rat are reesterified back to TAG [19]. This futile cycle could decrease intracellular ATP levels. A strong induction of PEPCK in epididymal fat during fasting correlates with the activation of AMPK, similar to the situation in fasted liver [67]. By analogy with the induction of fat accumulation by transgenic overexpression of PEPCK in adipocytes [181], a stronger stimulation of PEPCK-dependent fatty acid reesterification in epididymal fat may explain the slower decline in weight and lipid content of this fat depot during food deprivation.

The induction of UCP2 expression observed during fasting, which was higher in epididymal than subcutaneous fat, might increase the proton leak in mitochondria [178, 179], inhibit synthesis of ATP during oxidative phosphorylation and contribute to the differential activation of AMPK cascade in fat depots. In support of this, respiratory uncoupling in muscle cells *in vitro* [182] and in white adipose tissue in vivo in aP2-*Ucp1* mice *(see Publication A)*, both stimulated AMPK. These results suggest that UCP2 could act upstream of AMPK. On the other hand,

expression of the UCP2 gene could be increased by AMPK, as already demonstrated for both UCP2 and UCP3 in myocytes [183]. Induction of UCP2 could be a protection against damage by reactive oxygen species.

The metabolism of WAT is under a complex neurohormonal control, exerted primarily by insulin and catecholamines [184]. The notion that AMPK drive the changes in lipid metabolism during the starved-fed transition is compatible with earlier studies that linked these changes to increases in plasma insulin and decreases in glucagon [42]. Thus insulin has been reported to decrease AMPK activity in isolated hepatocytes and glucagon to activate hepatic AMPK [42].

The α1 AMPK exhibits activation by stimuli which increased cAMP, for example β-adrenergic agonist [172]. It was observed that glycerol released in adipocytes isolated from epididymal adipose tissue in response to NE was higher than that of adipocytes isolated from the subcutaneous depot of adult rats [185]. In adipocytes from fed animals the α_2-adrenoceptor number was greater in adipocyte membranes from subcutaneous (inguinal and popliteal) than from internal (perirenal and epididymal) adipose tissues. On the other hand was shown that starvation induced a decrease in α_2-adrenoceptor number and an increase in β-adrenergic sensitivity. The effect was greater in adipocytes from subcutaneous than from epidydimal fad pad in hamster [186]. Several studies have proposed another explanations for the site-specific differences in lipolytic response: (i) differences in nerve control [187], (ii) differences in the balance in α_2- and β-adrenoceptors [131], (iii) differences in the sensitivity of adipocytes to adenosine-mediated suppression and (iv) differences in the adenylate-cyclase activity [188].

There is evidence that the anti-lipolytic effect is a natural role of the adipose predominant α subunit isoform of AMPK - α1. In mice lacking α1 AMPK was observed (i) that the size of adipocytes is considerably reduced (ii) that basal and isoproterenol-stimulated lipolysis in these cells is higher than that of control adipocytes and (iii) that AICAR has no inhibitory effect on lipolysis [172]. Also in liver of starvated rats was found decrease in activity of AMPK after refeeding, with the α1-isoform being most responsive [189].

The differencial response to starvation exhibited by epidydimal and subcutaneous fat show a different biological role played by these tissues in organism. The control of lipid and glucose metabolism in WAT by AMPK may

represent a basic biological mechanism that contributes to regional differences in the metabolic properties of adipose tissue depots.

My main contributions to this work were measurement of fatty acid oxidation and AMPK and ACC analysis.

4.3. Polyunsaturated fatty acids of marine origin up-regulate mitochondrial biogenesis and induce β -oxidation in white fat (PUBLICATION C)

The specific aim of this study was to evaluate the effect of diets rich in omega-6 and omega-3 fatty acids on adipose tissue energy metabolism.

Omega-3 PUFA of marine origin reduce adiposity in animals fed high-fat diet. Our aim was to learn how EPA and DHA limit development of obesity and reduce cellularity of adipose tissue and whether is effect on adipose tissue direct or not. The effects of EPA/DHA concentrate (6 % EPA, 51 % DHA) admixed to form two types of high-fat diet were studied in male C57BL/6J mice.

At 3 to 4 months of age, the animals were randomly assigned to experimental HF diets: 1) obesity-promoting cHF diet, derived from the standard chow (*Surwit 1988),* containing 35,2 % lipids consisting of 95 % rapeseed oil and 5 % sunflower oil rich in oleic acid (18:3, ω-9) or 2) sHFf diet containing 20 % lipids consisting of flax-seed oil rich in ALA (18:3 ω-3). When indicated, 15 % (cHF-F1 diet) or 44 % (sHFf-F2 diet) (wt/wt) of a fat component in the HF diets was replaced by the concentrate of omega-3 PUFA of marine origin rich in DHA (EPAX 1050 TG; Pronova Biocare a.s., Lysaker, Norway). EPA and DHA collectively form 45 – 64% (wt/wt) of the concentrates and they are present as TAG.

After 4 weeks of feeding sHFf-F2 diet the body weight of mice decreased of 11±2 % and weight of epididymal fat was 30±3 % lower compared with control mice feeding sHFf diet. The weight of dorsolumbar fat did not change significantly *(see Table 1 in Publication C).* Changes in gene expression in epididymal and subcutaneous fat from mice fed either sHFf or sHFf-F2 was analyzed by using three methods - cDNA PCR-subtraction, oligonucleotide microarray analysis and qRT-PCR *(for results see Table 2 in Publication C).* The most of detected upregulated

genes in both depots belong to mitochondrial coded gene for components of the mitochondrial oxidative phosphorylation system (complex I , COX and subunit 6 of ATPase). Also gene for adipsin, fatty acid transport protein, was upregulated by EPA/DHA dietary uptake. On the other side was observed downregulation in expression gene for SCD-1 in both epididymal and subcutaneous fat and acyl-CoA:diacylglycerol acyltransferase in epididymal depot only.

To verify if the upregulated gene expression of subunits of the mitochondrial oxidative phosphorylation system result in induction of protein levels were they detected using immunoblots. In epididymal fat, levels of SDH_{70}, COX1, COX6 and α-subunit F_1-ATPase were elevated approximately 2-fold by the sHFf-F2 diet compared with the sHFf diet. In contrast to mRNA levels in the subcutaneous fat, no effect of the diet on any of the analysed antigens was observed. These resultes lead to the hypothesis about different posttranscription regulation in both fat depots.

An increase of the mitochondrial proteins levels indicate induction of mitochondrial biogenesis in epididymal WAT caused by dietary intake of EPA/DHA concentrate. Therefore we analysed potential changes in expression of two transcription factors, coordinating mitochondrial biogenesis, PGC-1 and NRF-1 *(see Chapter 1.1.3.)*. qRT-PCR analysis revealed approximately 3.4-fold induction of both PGC-1 and NRF-1 transcripts in epididymal fat by the sHFf-F2 diet compared with the sHFf diet *(see Figure 2a and b in Publication C)*. Also another PGC-1 downstream target CPT-1 was upregulated in epididymal depot of mice fed sHFf-F2 diet *(see Figure 2c in Publication C)*.

WAT mitochondria have a remarkable capacity for palmitoyl-carnitine oxidation but the physiological relevance of this relatively high capacity for β-oxidation still remains unclear especially since adipocyte mitochondria contain very low CPT-1 activity. The upregulation of the gene for CPT-1 suggests stimulation of fatty acid oxidation in the epididymal fat of the sHFf-F2 mice. Indeed, oxidation of oleate was 1.5- to 1.8-fold higher in epididymal fat of the sHFf-F2 compared with the sHFf mice while fatty acid synthesis was decreased. In subcutaneous fat, similar rates were observed in both diet groups *(see Figure 12)*.

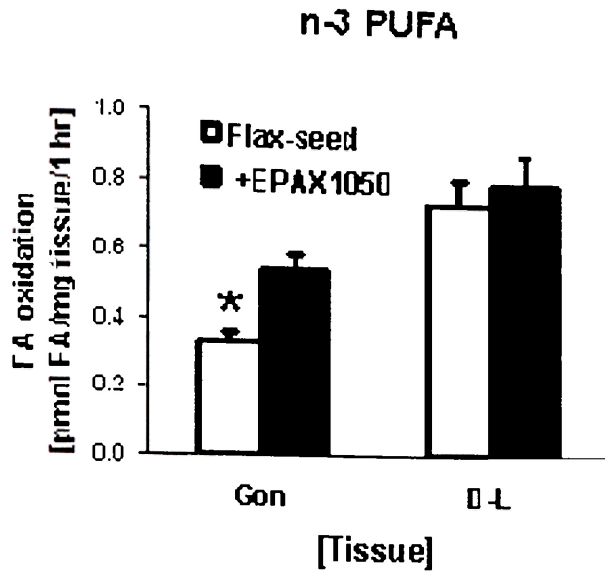

Figure 12. *Fatty acid oxidation in epididymal (Gon) and subcutaneous (D-L) WAT of mice fed sHFf diet (whie bars) or sHFf-F2 diet (black bars). * significant differences between diets.*

The effect of EPA/DHA on body fat reduction, induction of mitochondrial biogenesis and β-oxidation was verify under obesity-induced conditions in mice fed cHF-F1 compared with cHF.

In contrast to different impact of EPA/DHA to CTP I mRNA levels, the expression of marker of peroxisomal fatty acid oxidation acyl-CoA oxidase 1 (AOX) was increased in both fad pads in mice fed sHFf-F2 compared with the sHFf. PUFA may be the ligands for PPARα, which would activate PPARα to upregulate the expression of AOX *(Reddy & Hashimoto 2001)*. Others also found that dietary fish oil increased the expression of AOX in rodent livers and muscles and suggested that dietary PUFA could increase the fatty acid oxidation in these tissues [147]. These results all suggest that PUFA increase fatty acid oxidation through increase of gene expression of enzymes involved in fatty acid oxidation. Our results indicate that omega-3 PUFA of marine origin (EPA and DHA) are more potent then omega-3 PUFA of plant (ALA). And they prove specific effect in WAT. In contrast to white adipose tissue, especially the epididymal fat, no significant changes in the expression of PGC-1, NRF-1 and CPT-1 genes were observed in liver (not shown). Only the

levels of AOX mRNA were significantly higher in liver of mice fed the sHFf-F2 than in those fed the sHFf diet.

In rodents, numerous studies have shown that PUFA from fish oils suppressed the expression of lipogenic genes in the liver and also upregulated the expression of genes involved in lipid oxidation in the liver and muscle tissues [147, 191]. The PUFA, particularly omega-3 series, may play a role in partitioning intracellular fuel to reduce lipid accumulation [192]. Transcriptional control of gene expression is a common mechanism by which lipids as well as other nutrients affect metabolism. While the exact mechanisms by which PUFAs exert their effects are not fully understood, PUFAs have been shown to modulate gene transcription by interacting with at least three nuclear receptors- LXRα and β, HNF-4α, and PPAR α, β, and γ - and by regulating the transcription factor SREBP-1 and 2 [193].

The most recent additions to the host of transcription factors regulated by PUFAs are the LXRs α and β [152]. LXRs upregulate transcription of the lipogenic transcription factor SREBP-1c by binding to an LXRE in the promoter of the SREBP-1c gene. Some evidence suggests that binding of PUFAs to LXRs results in the inability of LXR to induce transcription of SREBP-1c, causing a consequent decrease in lipogenesis [194]. PPARs, which are activated by PUFAs, have also been shown to directly bind LXRs and antagonize their lipogenic effects, thus providing another mechanism whereby PUFAs interact with LXRs to regulate lipogenesis [195].

Other mechanisms of action for PUFA regulation of gene expression have also been characterized. PUFAs such as arachidonic acid repress the SCD-1 gene in 3T3-L1 adipocytes by decreasing SCD-1 mRNA stability [196]. SCD-1mRNA levels in adipocytes are differentially decreased by other PUFA such as linoleic and linolenic acids and EPA, indicating that the repression of SCD-1 mRNA expression is a selective response to PUFA.

The anti-adipogenic effect of omega-3 PUFA could be direct or mediated by action of PUFA on other organs e.g liver or muscle. Suppression of lipogenesis in liver and lower VLDL secretion together with increase of lipid oxidation in liver and muscle mediated by dietary fish oil PUFAs lead to reduction of plasma TAG levels and lipid supply to adipose tissue. Also prostaglandins, reactive intermediates of PUFA, could be involved. Prostaglandins play a critical role in the adipocyte differentiation process [197]. Omega-3 PUFA exert an anti-adipogenic effect in

adipose tissue in a site-specific manner by down-regulating series 2 prostaglandin synthesis. They prevent the development of visceral adipose tissue by down-regulating adipocyte differentiation [198].

In order to further address the molecular basis by which PUFA might direct influence adipose tissue gene expression were quantified PGC-1 and NRF-1 mRNA levels in 3T3-L1 adipocytes incubated for 24 h with various fatty acids: oleic acid alone, oleic acid plus ALA, or oleic acid plus DHA *(see Figure 4 in Publication C)*. Compared with oleate, both ALA and DHA significantly increased the levels of both transcripts. The stimulation by DHA seemed to be more pronounced than that by ALA, but the difference was not statistically significant.

This finding indicates that PUFA-mediated regulation of adipose tissue gene expression would not necessarily need extra-adipose factors. And the activation of PGC-1 transcription factor is acute and direct effect of omega-3 PUFA on WAT. All isoforms of PPAR physically interact with gene for PGC-1α [199]. A recent study indicates that treatment of *ob/ob* mice with rosiglitazone, a PPARγ ligand of the TZD family and a widely used antidiabetic drug, increases mitochondrial mass, palmitate oxidation and the expression of genes encoding PGC-1α , CPT-1 and UCP1 in epididymal fat [200]. Except for the up-regulation of the gene for UCP1, all these effects are similar to those elicited in WAT by dietary intake of EPA/DHA, suggesting that both omega-3 PUFA and rosiglitazone induce similar metabolic changes in white adipose tissue cells, while increasing mitochondrial oxidative capacity and stimulating glucose uptake into adipocytes. However, in contrast to EPA/DHA, TZDs also support an adipogenic programme. Surprisingly, rosiglitazone induces the accumulation of omega-3 PUFA in white adipose tissue of diabetic mice [201]. Whether the mechanism of the effect of rosiglitazone depends on the accumulation of omega-3 PUFA in adipocytes remains to be clarified.

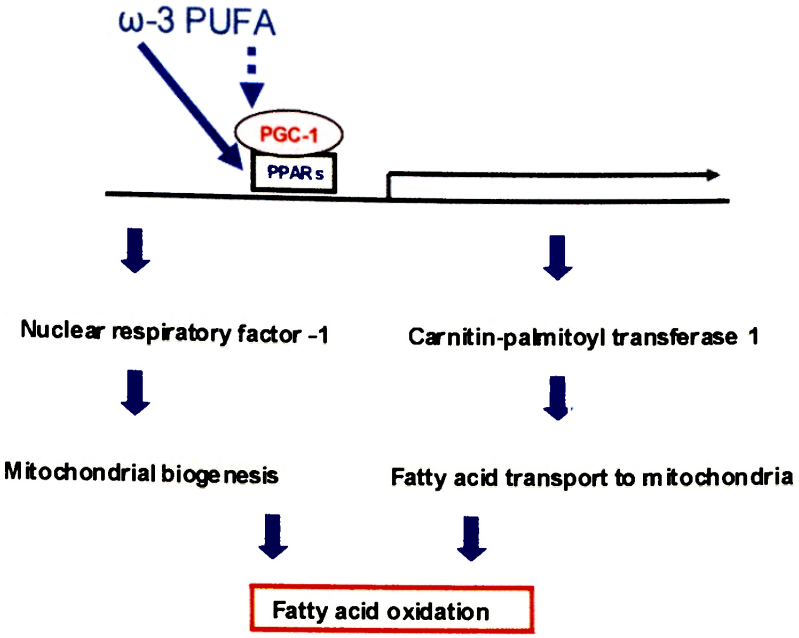

Figure 13. *The mechanism EPA/DHA mediated metabolic switch from lipid storage to lipid oxidation.*

PUFAs act as potent hypolipidemic agents due to their ability to increase expression of genes encoding enzymes of lipid oxidation while concurrently decreasing those encoding lipogenic enzymes. EPA/DHA seems to be more potential then other PUFA. The mechanism of EPA/DHA mediated metabolic switch from lipid storage to lipid oxidation includes (i) attenuation of the malonyl-CoA inhibitory effect on CPT-1, (ii) induction of CPT-1 gene expression and (iii) stimulation of mitochondrial biogenesis via up-regulation of NRF-1. This metabolic switch in adipocytes is induced independently of ALA intake and could reduce the development of obesity. Relatively minor changes in the composition of lipids in an obesity-promoting diet exert surprisingly pronounced effects.

My main contributions to this work were measurement of fatty acid oxidation and cell culture experiments.

4.4. Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed high-fat diet (PUBLICATION D)

The specific aim of this study was to test the hypothesis that whole body effects of EPA and DHA involve induction of adiponectin.

In previous study was shown effect of dietary intake of fish oil omega-3 PUFA on hyperinsulinemia caused by HF diet. It also protected against down-regulation of GLUT4, an insulin-sensitive glucose transporter, in WAT by the high-fat diet. The expression of glucose transporter GLUT4 partially restored in adipose tissue of mice treated with EPA/DHA suggests that improved insulin sensitivity of adipose tissue could be responsible, at least partly, for the enhancement in whole body insulin sensitivity. We hypothesized that one of the adipose tissue secreted factors could be involved. Both leptin and adiponectin are responsible for increased fatty acid oxidation in response to the activation of AMPK in muscle [44, 104].

Adult male mice fed cHF or cHF-F1 diets *ad libitum* or 70% *ad libitum* (caloric restriction (CR)) for 5 weeks were compared *(for characterization of diets see Materials and methods in Publication D).* Only 9 % of dietary fat was substituted by EPA/DHA in cHF-F1 diet. This treatment, corresponding to about 2.6 mg EPA/DHA per g body weight/day and 5.3 % oftotal energy intate formed by EPA/DHA [202]. Body weight gain was about 2.7 g lower in mice fed a cHF-F1 diet enriched with EPA/DHA compared with cHF diet in ad libitum fed mice. Weight loss of about 10 g was observed in food restricted animals fed the control cHF diet. However, a combination of CR and the cHF-F1 diet had no further effect on body weight compared with CR alone *(see Tab. 1 in Publication D).* The intake of EPA/DHA did not affect food consumption of the animals. In accordance with the previous studies our results showed limited accumulation of epididymal fat by omega-3 PUFA of marine origin admixed to HF diet. Subcutaneous fat was less affected.

First, effect of the partial replacement of dietary lipids by EPA/DHA concentrate (with EPA/DHA ratio 1:8,5) on plasma markers of lipid and glucose metabolism, and plasma levels of selected adipokines was studied. Decrease in plasma levels of NEFA was detected only in mice fed cHF-F1 diet *ad libitum.* TAG levels were lower in both groups animals with CR and was also affected by cHF-F1

diet. Levels of glucose and leptin declined due to caloric restriction only, where changes in leptin levels were dramatical. On the other side levels of insulin and adiponectin were affected by dietary omega-3 PUFA only. Changes were independent of CR. Feeding of cHF-F1 diet leads to 10-fold fall of insulin levels in plasma of both animals fed *ad libitum* and these with CR. Levels of adiponectin were increased by cHF-F1 diet to a similar extent in both *ad libitum* fed and in the CR mice (by 34 and 22 %, respectively). *(For details see Table 1 in Publication D).*

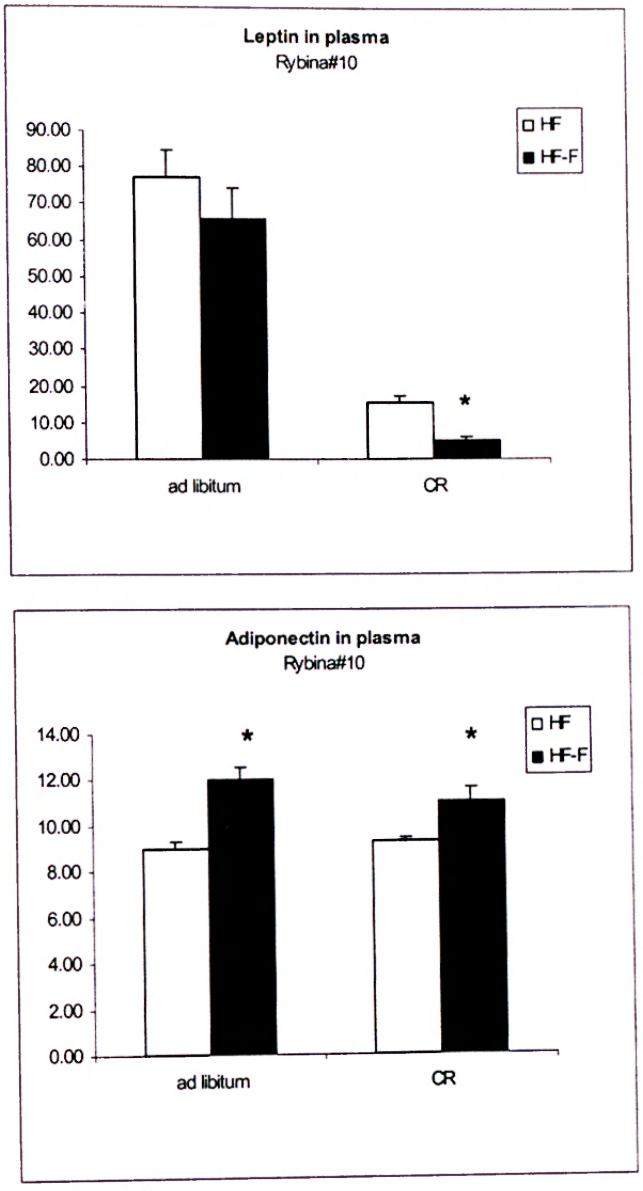

Figure 14. *Plasma levels of leptin and adiponectin in mice fed cHF (white bars) or cHF-F1 (black bars) diet ad libitum (left) or 70% ad libitum (right). *significant differences.*

Second, the effect of EPA/DHA on the expression and production of adiponectin and leptin was investigated in WAT of *ad libitum* fed mice. Production of the adipokines was measured in tissue explants. Significant effect of the diet neither on expression nor secretion of leptin was detected. In adipocytes isolated from both depots expression of adiponectin was stimulated by EPA/DHA although changes in expression were not found in whole tissues. The stimulative effect of intake of cHF-F1 diet was stronger in adipocytes from epididymal fat. Levels of secreted adiponectin were increased by EPA/DHA in epididymal fat only *(for details see Table 2 in Publication D).*

Although both caloric restriction and dietary intake of EPA/DHA resulted in decrease in body weight and reduction of adipose tissue mass, effect on lipid and glucose metabolism markers and secretion of adipokines is different. By dietary EPA and DHA were regulated predominantly NEFA, insulin and adiponectin. Interestingly, EPA/DHA significantly reduced circulating insulin levels in a dose-dependent manner, suggesting improved whole-body insulin sensitivity and/or direct effect on pancreatic β-cells [202, 203].

In contrast to adiponectin, leptin plasma levels were markedly decreased by CR, but neither its levels or leptin expression were affected by EPA/DHA. Plasma leptin concentrations as well as leptin gene expression in epididymal fat positively correlated with adiposity under different dietary regimens, suggesting that leptin secretion from adipose cells followed the changes in adiposity and glucose metabolism in adipocytes [204]. That EPA/DHA induces adiponectin but not leptin indicates that mechanisms controlling the expression of genes for these two adipokines are different.

The mRNA expression of adiponectin and its plasma level are significantly reduced in obese and diabetic mice and humans [96, 101]. Although strong negative correlations between plasma adiponectin levels and BMI have been shown both in humans and in animals, the induction of adiponectin by EPA/DHA could not result from reduction of adiposity, since the strong decrease of fat content due to CR did not influence adiponectin levels.

As with the TZD, EPA and DHA may upregulate adiponectin expression by acting as ligands of PPAR-γ, the transcriptional regulator interacting directly with adiponectin promoter [206]. The potential importance of adiponectin as a target for

the insulin-sensitizing action of TZD is underscored by the substantial increase in plasma adiponectin levels in response to treatment with TZD [207]. Because PPARγ is predominantly expressed in adipose tissue and adipose tissue appears to be essential for TZD induced improvement in insulin sensitivity, this upregulation of adiponectin by TZD suggests that adiponectin plays an important role in mediating the anti-diabetic effect of TZD. Interestingly, an association between circulating adiponectin and plasma omega-3 PUFA, and DHA in particular, was found recently in healthy humans [208]. Both obese and diabetic objects are characterized by elevated concentrations of TNF-α, a cytokine that was found to lower adiponectin expression in adipocytes [98]. Both dietary fish oil and treatment with TZDs lower the expression of TNFαin WAT [209, 210].

Adiponectin influences glucose and fatty acid metabolism through an insulin-sensitizing effect that appears to be mediated through activation of AMPK in liver, muscle and adipocytes [104, 114]. In parallel with its activation of AMPK, adiponectin stimulates phosphorylation of ACC, fatty acid oxidation, glucose uptake and lactate production in myocytes, phosphorylation of ACC and reduction of molecules involved in gluconeogenesis in the liver, and reduction of glucose levels *in vivo* [104]. Moreover, insulin-stimulated tyrosine phosphorylation of signaling molecules, including insulin receptor and insulin receptor substrate 1 in skeletal muscle, was also enhanced by adiponectin [102].

Peripheral insulin resistance and increased insulin secretion arise simultaneously during high-saturated-fat feeding in rats through a common action on skeletal muscle and pancreatic β-cells. This phenomenon is countered by small amounts of dietary long-chain omega-3 PUFA. Our results suggest that the protection against HF diet-induced insulin resistance is at least partially mediated by adiponectin, but not leptin.

We show for the first time that EPA and DHA stimulate adiponectin expression and increase the levels of circulating adiponectin, relatively independent of food intake and body fat mass. It is clear that adiponectin has potential as new factor in the treatment of obesity-related diseases. Induction of adiponectin production by omega-3 PUFA originate from fish oil could be an alternative approach both for pharmacological intervention in obesity.

My main contributions to this work were tissue explants experiments.

5. CONCLUSIONS

A. The activation of AMPK can explain the complexity of metabolic changes in adipose tissue of aP2-*Ucp1* mice and suggests a new link between mitochondrial energy conversion and regulation of body weight.

B. The activation of AMPK is involved in differential response of epidydimal and subcutaneous fat to starvation. The control of lipid and glucose metabolism in WAT by AMPK may represent a basic biological mechanism that contributes to regional differences in metabolic properties of adipose tissue depots.

C. Dietary intake of EPA/DHA induces a metabolic switch from lipid storage to lipid oxidation in WAT by increasing expression and activity of CPT-1 and inducing of mitochondrial biogenesis via up-regulation of PGC-1 and NRF-1.

D. The intake of EPA/DHA stimulates adiponectin expression and increases the levels of circulating adiponectin, relatively independent of food intake and body fat mass.

6. REFERENCES

1. Lev-Ran, A. (1999) Thrifty fenotype: how applicable is it to obesity and type 2 diabetes? Diabetes Rev. 7, 1-22

2. Spiegelman, B.M., and Flier, J.S. (2001) Obesity and the regulation of energy balance. Cell 104(4), 531-43.

3. Hill, J.O., and Peters, J.C. (1998) Enviromental contributtions to the obesity epidemic. Science 280, 1371-1374

4. Bray, G.A. (2000) Overweight, mortality and morbidity. In: Bouchard C. ed. Physical activity and obesity. Champain, IL: Human Kinetics, 31-53

5. Arner, P. (2000) Obesity is genetic disease of adipose tissue? Br. J. Nut.r 83 (Suppl 1), S9–S16

6. Kopecky, J., Rossmeisl, M., Flachs, P., Bardova, K., and Brauner P. (2001) Mitochondrial uncoupling and lipid metabolism in adipocytes. Biochem. Soc. Trans. 29, 791–79.

7. Boulton KL., Hudson DU., Coppack SW., Frayn KN. (1992) Steroid hormone interconversions in human adipose tissue in vivo. Metabolism 41, 556-559

8. Dawson-Hughes, B., Shipp, C., Sadowski, L., and Dallal, G. (1987) Bone density of the radius, spine, and hip in relation to percent of ideal body weight in postmenopausal women. Calcif. Tissue Int. 40, 310-314

9. Arner, P. (2001) Regional differences in protein production by human adipose tissue. Biochem. Soc. Trans. 29, 72–75

10. Wajchenberg, B.L. (2000) Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr. Rev. 21, 697–738

11. Pujol, E., Rodriguez-Cuenca, S., Frontera, M., Justo, R., Llado, I., Kraemer, F.B., Gianotti, M., and Roca, P. (2003) Gender- and site-related effects on lipolytic capacity of rat white adipose tissue. Cell. Mol. Life. Sci. 60, 1982–1989

12. Gregoire, F.M., Smas, C.M., Sul, H.S. (1998) Understanding adipocyte differentiation. Physiol Rev 78(3),783-809.

13. Cao, Z., Umek, R.M., and McKnight, S.L. (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes. Dev. 5, 1538-1552

14. Darlington, G.J., Ross, S.E., and MacDougald, O.A. (1998) The role of C/EBP genes in adipocyte differentiation. J. Biol. Chem. 273, 30057-30060

15. Rosen ED., Walkey, C.J., Puigserver, P. and Spiegelman, B.M. (2000) Transcriptional regulation of adipogenesis. Genes Dev. 14, 1293-1307

16. Rosen, E.D., and Spiegelman, B.M. (2001) PPARgamma: a nuclear regulator of metabolism, differentiation, and cell growth. J. Biol. Chem. 276, 37731–37734

17. Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A. (1996) PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. EMBO J. 15, 5336–5348

18. Tontonoz, P., Hu, E., Devine, J., Beale, E. & Spiegelman B. (1995) PPAR-c2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. Mol Cell Biol 15, 351–357.

19. Olswang, Y., Cohen, H., Papo, O. (2002) A mutation in the peroxisome proliferator-activated receptor gammabinding site in the gene for the cytosolic form of phosphoenolpyruvate carboxykinase reduces adipose tissue size and fat content in mice. Proc. Natl. Acad. Sci. USA 99, 625–630

20. Wu, Z., Xie, Y., Morrison, R.F., Bucher, N.L.R., and Farmer, S.R. (1998) PPARgamma induces the insulin-dependent glucose transporter GLUT4 in the absence of C/EBPalpha during the conversion of 3T3 fibroblasts into adipocytes. J. Clin. Invest. 101, 22–32.

21. Walczak, R., and Tontonoz, P. (2002) PPARadigms and PPARadoxes: expanding roles for PPARgamma in the control of lipid metabolism. J. Lipid. Res. 43, 177–186

22. Shimomura, I., Shimano, H., Horton, J.D., Goldstein, J.L., and Brown, M.S. (1997) Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. J. Clin. Invest. 99, 838–845

23. Shimano, H.,. Horton, J.D, Shimomura, I., Hammer, R.E., Brown, M.S., and Goldstein, J.L. (1997) Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. J. Clin. Invest. 99, 846–854

24. Foretz, M., Guichard, C., Ferre, P., and Foufelle, F. (1999) Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes, Proc. Natl. Acad. Sci. USA 96, 12737–12742

25. Pedersen, O., Kahn, C.R., and Kahn, B.B. (1992) Divergent regulation of the Glut 1 and Glut 4 glucose transporters in isolated adipocytes from Zucker rats. J Clin Invest 89, 1964-1973

26. Coppack, S.W., Frayn, K.N., Whyte, P.L., and Humphreys, S.M. (1989) Carbohydrate metabolism in human adipose tissue in vivo. Biochem Soc Trans 17, 145-146

27. Klip, A., and Paquet, M.R. (1990) Glucose transport and glucose transporters in muscle and their metabolic regulation. Diabetes Care 13, 228–243.

28. Abel, E.D., Peroni, O., Kim, J.K. (2001) Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. Nature 409, 729–733.

29. Flatt, J.P. (1970) Conversion of carbohydrate to fat in adipose tissue: an energy-yielding and, therefore, self-limiting process. J Lipid Res. 11, 131-43

30. Camps, L., Reina, M., Llobera, M., Vilaro, S., and Olivecrona, T. (1990) Lipoprotein lipase: cellular origin and functional distribution. Am J Physiol 258, C673-C681

31. Nilsson-Ehle, P., Garfinkel, A., and Schotz, M.C. (1980) Lipolytic enzymes and plasma lipoprotein metabolism. Annu Rev Biochem 49, 667-693.

32. Vannier, C., Ez-Zoubir, A., Etienne, J., Negrel, R., and Ailhaud, G. (1985) Maturation and secretion of lipoprotein lipase in cultured adipose cells, I. Intracellular activation of the enzyme. J Biol Chem 26, 4424-443

33. Frayn, K.N, and Summers, L.K.M. (1998) Substrate fluxes in skeletal muscle and white adipose tissue and their importance in the development of obesity. In Clinical Obesity. Kopelman, P.G.and Stock, M.J. Blackwell Science Ltd, Oxford. 129-157

34. Abumrad N., Harmon C., Ibrahimi A. (1998) Membrane transport of long-chain fatty acids: evidence for a facilitated process. J Lipid Res 39, 2309-2318

35. Gao, J., and Serrero, G. (1999) Adipose differentiation related protein (ADRP) expressed in transfected COS-7 cells selectively stimulates long chain fatty acid uptake. J Biol Chem 274,16825-16830

36. Coleman, R.A, Lewin, T.M, Van Horn, C.G, and Gonzalez-Baro, M.R. (2002) Do long-chain acyl-CoA synthetases regulate fatty acid entry into synthetic versus degradative pathways? J Nutr 132, 2123-6

37. McGarry, J. D. and Brown, N. F. (1997) Mouse white adipocytes and 3T3-L1 cells display an anomalous pattern of carnitine palmitoyltransferase (CPT) I isoform expression during differentiation. Inter-tissue and inter-species expression of CPT I and CPT II enzymes. Eur J Biochem. 244, 1-14

38. Trumble, G. E., Smith, M. A. and Winder, W.W. (1995) Purification and characterization of rat skeletal muscle acetyl-CoA carboxylase. Eur J Biochem. 231, 192-198

39. Borthwick, A. C., Edgell, N. J. and Denton, R. M. (1987) Use of rapid gel-permeation chromatography to explore the inter-relationships between polymerization, phosphorylation and activity of acetyl-CoA carboxylase. Effects of insulin and phosphorylation by cyclic AMP-dependent protein kinase. Biochem J 241, 773-778

40. Munday, M. R., Campbell, D. G., Carling, D. and Hardie, D. G. (1988) Identification by amino acid sequencing of three major regulatory phosphorylation sites on rat acetyl-CoA carboxylase. Eur J Biochem. 175, 331-338

41. Davies, S. P., Carling, D., Munday, M. R. and Hardie, D. G.(1992) Diurnal rhythm of phosphorylation of rat liver acetyl-CoA carboxylase by the AMP-activated protein kinase, demonstrated using freeze-clamping. Effects of high fat diets. Eur J Biochem 203, 615-623

42. Munday, M.R, Milic, M.R, Takhar, S., Holness, M.J, and Sugden, M.C. (1991) The short-term regulation of hepatic acetyl-CoA carboxylase during starvation and re-feeding in the rat. Biochem J 280, 733-737

43. Mabrouk, G. M., Helmy, I. M., Thampy, K. G. and Wakil, S. J. (1990) Acute hormonal control of acetyl-CoA carboxylase. The roles of insulin, glucagon, and epinephrine. J Biol Chem 265, 6330-6338

44. Minokoshi, Y., Kim, Y. B., Peroni, O. D., Fryer, L. G., Muller, C., Carling, D., and Kahn, B.B. (2002) Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature 415, 339-343

45. Ntambi, J.M, and Miyazaki, M. (2003) Recent insights into stearoyl-CoA desaturase-1. Curr Opin Lipidol 14, 255–261.

46. Dobrzyn, A., and Ntambi, J.M. (2004) The role of stearoyl-CoA desaturase in body weight regulation. Trends Cardiovasc Med 14, 77–81.

47. Coleman, R. A., Lewin, T. M., and Muoio, D. M. (2000) Physiological and nutritional regulation of enzymes of triacylglycerol synthesis. Annu Rev Nutr 20, 77– 103.

48. Lau, T.E., and Rodriguez, M.A. (1996) A protein tyrosine kinase associated with the ATP-dependent inactivation of adipose diacylglycerol acyltransferase. Lipids 31, 277-83.

49. Soncini, M., Yet, S.F., Moon, Y. K., and Sul, H. S. (1995) Hormonal and nutritional control of the fatty acid synthase promoter in transgenic mice. Biochemistry 34, 7303–7310

50. Horton, J. D., and Shimomura, I. (1999) Sterol regulatory element-binding proteins: activators of cholesterol and fatty acid biosynthesis. Curr Opin Lipidol 10, 143–150

51. Muoio, D.M, Seefeld, K., Witters, L.A., and Coleman, R.A. (1999) AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target. Biochem J 338, 783-791

52. Sugden, M.C, and Holness, M.J. (1994) Interactive regulation of the pyruvate dehydrogenase complex and the carnitine palmitoyltransferase system. FASEB J 8, 54-61

53. Zammit, V.A. (1999) Carnitine acyltransferases: functional significance of subcellular distribution and membrane topology. Prog Lipid Res 38, 199–224

54. van Veldhoven, P.P., and Mannaerts, G.P. (1999) Role and organization of peroxisomal beta-oxidation. Adv Exp Medicine and Biology 466, 261–272

55. Aoyama, T., Peters, J.M., Iritani, N. (1998) Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor alpha (PPARalpha). J Biol Chem 273, 5678–5684

56. Cook, G.A., and Park, E.A. (1999) Expression and regulation of carnitine palmitoyltransferase-Ia and-Ib genes. Am J Med Sciences 318, 43–48

57. Balaban, R.S. (1990) Regulation of oxidative phosphorylation in the mamalian cell. Am J Physiol 258, 377-89

58. Egan, J.J. (1992) Mechanism of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipase to the lipid storage droplet. Proc Natl Acad Sci 89, 8537–8541

59. Londos, C., Brasaemle, D.L., Gruia-Gray, J., Servetnick, D.A., Schultz, C.J., Levin, D.M., and Kimmel, A.R. (1995) Perilipin: unique proteins associated with intracellular muetral lipid droplets in adipocytes and steroidogenic cells. Biochem Soc Trans 23, 611–615

60. Garcia, A., Sekowski, A., Subramanian, V., and Brasaemle, D.L. (2003) The central domain is required to target and anchor perilipin A to lipid droplets. J Biol Chem 278, 625–635

61. Campbell, P.J., Carlson MG, Hill JO, Nurjhan N. (1992) Regulation of free fatty acid metabolism by insulin in humans: role of lipolysis and reesterification. Am J Physiol 26, E1063–E1069

62. Botion, L. M. and Green, A. (1999) Long-term regulation of lipolysis and hormone sensitive lipase by insulin and glucose. Diabetes 48, 1691–1697

63. Siegrist-Kaiser, C. A., Pauli, V., Juge-Aubry, C. E., Boss, O., Pernin, A., Chin, W. W.,Cusin, I., Rohner-Jeanrenaud, F., Burger, A. G., Zapf, J. and Meier, C. A. (1997) Direkt effects of leptin on brown and white adipose tissue. J Clin Invest 100, 2858–2864

64. Wang, M.Y., Lee, Y., and Unger, R. H. (1999) Novel form of lipolysis induced by leptin. J Biol Chem 274, 17541–17544

65. Haemmerle, G., Zimmermann, R., Hayn, M., Theussl, C., Waeg, G., Wagner, E., Sattler, W., Magin, T.M., Wagner, E.F., and Zechner, R. (2002) Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis. J Biol Chem 277, 4806-15

66. Reshef, L., Olswang, Y., Cassuto, H., Blum, B., Croniger, C.M., Kalhan, S.C., Tilghman, S.M. and Hanson, R.W. (2003) Glyceroneogenesis and the triglyceride/fatty acid cycle. J Biol Chem 278, 30413–30416

67. Gorin, E., Tal-Or, Z. and Shafrir, E. (1969) Glyceroneogenesis in adipose tissue of fasted, diabetic and triamcinolone treated rats. Eur J Biochem 8, 370–375

68. Ballard, F.J., Hanson, R.W. and Leveille, G.A. (1967) Phosphoenolpyruvate carboxykinase and the synthesis of glyceride-glycerol from pyruvate in adipose tissue. J Biol Chem. 242, 2746–2750

69. Franckhauser, S., Antras-Ferry, J., Robin, P., Robin, D., Granner, D.K. and Forest, C. (1995) Expression of the phosphoenolpyruvate carboxykinase gene in 3T3-F442A adipose cells: opposite effects of dexamethasone and isoprenaline on transcription. Biochem J. 305, 65–71

70. Antras-Ferry, J., Robin, P., Robin, D. and Forest, C. (1995) Fatty acids and fibrates are potent inducers of transcription of the phosphenolpyruvate carboxykinase gene in adipocytes. Eur J Biochem. 234, 390–396

71. Reshef, L., Ballard, F.J., and Hanson, R.W. (1969) The role of the adrenals in the regulation of phosphoenolpyruvate carboxykinase of rat adipose tissue. J Biol Chem 244, 5577-81

72. Dani, C., Doglio, A., Grimaldi, P. and Ailhaud, G. (1986) Expression of the phosphoenolpyruvate carboxykinase gene and its insulin regulation during differentiation of preadipose cell lines. Biochem Biophys Res Commun 138, 468–475

73. Rolfe, D. F., and Brown, G. C. (1997) Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol Rev 77, 731- 758.

74. Brown, G.C. (1992) Control of respiration and ATP synthesis in mammalian mitochondria and cells. Biochem J 284, 1-13

75. Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V. Troy, A., Cinti, A., Lowell, B., Scarpulla, R. C., and Spiegelman, B. M. (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98, 115-124.

76. Adhihetty, P.J, In-cher I., Joseph, A.M., Ljubicic, V., and Hood, D.A. (2003) Plasticity of skeletal muscle mitochondria in response to contractile activity. Exp Physiol 88, 99-107

77. Li, B., Holloszy, J.O., and Semenkovich, C.F. (1999) Respiratory uncoupling induces delta-aminolevulinate synthase expression through a nuclear respiratory factor-1-dependent mechanism in HeLa cells. J Biol Chem 274, 17534-40

78. Bergeron, R., Ren, J.M., Cadman, K.S., Moore, I.K., Pen-et, P., Pypaert, M., Young, L.H., Semenkovich, C.F., and Shulman, G.I. (2001) Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. Am J Physiol Endocrinol Metab 281, E1340-6.

79. Wallace, D.C. (1999) Mitochondrial diseases in man and mouse. Science. 283, 1482-8.

80. Vega, R.B., Huss, J.M., and Kelly, D.P. (2000) The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor a in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. Mol Cell Biol 20, 1868-1876

81. Michael, L.F., Wu, Z., Cheatham, R.B., Puigserver, P., Adelmant, G., Lehman, J.J., Kelly, D.P., and Spiegelman, B.M. (2001) Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. Proc Natl Acad Sci U S A. 98, 3820-5.

82. Yoon, J.C., Puigserver, P., Chen, G., Donovan, J., Wu, Z., Rhee, J., Adelmant. G., Stafford. J., Kahn, C.R., Granner, D.K., Newgard, C.B., and Spiegelman, B.M. (2001) Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature 413, 131-8.

83. Rajala, M.W., and Scherer, P.E. (2003) Minireview: the adipocyte—at the crossroads of energy homeostasis, inflammation, and atherosclerosis. Endocrinology 144, 3765– 3773

84. Trayhurn, P., and Beattie, J.H: (2001) Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. Proc Nutr Soc 60, 329-339

85. Friedman, J.M., and Halaas, J.L. (1998) Leptin and the regulation of body weight in mammals. Nature 395, 763-770

86. Ahima, R.S., and Flier, J.S. (2001) Leptin. Annu Rev Physiol 62, 413-437

87. Saladin, R., De, V.P., Guerre, M.M., Leturque, A., Girard, J., Staels, B., and Auwerx, J. (1995) Transient increase in obese gene expression after food intake or insulin administration. Nature 377, 527–529

88. Trayhurn, P., Duncan, J.S., and Rayner, D.V. (1995) Acute cold-induced suppression of ob (obese) gene expression in white adipose tissue of mice: mediation by the sympathetic system. Biochem. J 311, 729–733.

89. Vos, P.D., Lefebvre, A., Briggs, M.R., and Auwerx, J. (1996) Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator-activated receptor c. J Clin Invest 98, 1004–1009

90. Arai, T, Kawakami, Y, Matsushima, T, Okuda, Y, and Yamashita, K. (2002) Intracellular fatty acid downregulates ob gene expression in 3T3-L1 adipocytes. Biochem Biophys Res Commun 297, 1291-6.

91. Yaspelkis, B. III., Davis, J.R., Saberi M., et al. (2001) Leptin administration improves skeletal muscle insulin responsiveness in diet-induced insulin-resistant rats. Am J Physiol Endocrinol Metab 280, E130-42.

92. Fehmann, H.C., Peiser, C., Bode, H.P., et al. (1997) Leptin: a potent inhibitor of insulin secretion. Peptides 18, 1267-73.

93. Orci, L., Cook, W.S., Ravazzola, M., Wang, M.Y., Park, B.H., Montesano, R., and Unger, R.H. (2004) Rapid transformation of white adipocytes into fat-oxidizing machines. Proc Natl Acad Sci U S A 101, 2058-63.

94. Schwartz, M.W., Woods, S.C., Porte, D., Seeley, R.J., and Baskin, D.G. (2000) Central nervous system control of food intake. Nature 404, 661-671

95. Scherer, P.E., Williams, S., Fogliano, M., Baldini, G., and Lodish, H.F. (1995) A novel serum protein similar to C1q, produced exclusively in adipocytes. J Biol Chem 270, 26746-9.

96. Hu, E., Liang, P., and Spiegelman, B.M. (1996) AdipoQ is a novel adipose-specific gene dysregulated in obesity. J Biol Chem 271, 10697-703

97. Halleux, C.M., Takahashi, M., Delporte, M.L., et al. (2001) Secretion of adiponectin and regulation of apM1 gene expression in human visceral adipose tissue. Biochem Biophys Res Commun 288, 1102-7.

98. Kappes, A., and Loffler, G. (2000) Influences of ionomycin, dibutyryl-cycloAMP and tumour necrosis factor-alpha on intracellular amount and secretion of apM1 in differentiating primary human preadipocytes. Horm Metab Res 32, 548-54.

99. Fassnauer, M., Klein, J., Neumann, S., Eszlinger, M., and Paschke, R. (2001) Adiponectin gene expression is inhibited by beta-adrenergic stimulation via proteinkinase A in 3T3-L1 adipocytes. FEBS Lett 507, 142-6.

100. Weyer, C., Funahashi, T., Tanaka, S., et al. (2001) Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. J Clin Endocrinol Metab 86, 1930-5.

101. Arita, Y., Kihara, S., Ouchi, N., et al. (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun 257, 79-83.

102. Yamauchi, T., Kamon, J., Waki, H., et al. (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat Med 7, 941-6.

103. Stefan, N., Vozarova, B., Funahashi, T., et al. (2002) Plasma adiponectin concentration is associated with skeletal muscle insulin receptor tyrosine phosphorylation, and low plasma concentration precedes a decrease in whole-body insulin sensitivity in humans. Diabetes 51, 1884-8.

104. Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., and Kadowaki, T. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 8, 1288-95.

105. Hardie, D. G., and Carling, D. (1997) The AMP-activated protein kinase. Fuel gauge of the mammalian cell? Eur. J. Biochem. 246, 259–273

106. Leclerc I, Kahn A, Doiron B. (1998) The 5'-AMP-activated protein kinase inhibits the transcriptional stimulation by glucose in liver cells, acting through the glucose response complex. FEBS Lett 431, 180-4

107. Hardie, DG, Carling, D, and Carlson, M. (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem. 67, 821-55.

108. Stapleton, D., Mitchelhill, K.I., Gao, G., Widmer, J., Michell, B.J., Teh, T. , House, C.M., Fernandez, C.S., Cox, T., Witters, L.A., Kemp, B.E. (1996) Mammalian AMP-activated protein kinase subfamily, J.Biol. Chem. 271, 611–614.

109. Carling, D, Clarke, PR, Zammit, VA, Hardie, DG. (1989) Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. Eur J Biochem 186, 129-36.

110. Hardie, DG. (2004) The AMP-activated protein kinase pathway--new players upstream and downstream. J Cell Sci 117, 5479-87.

111. Sullivan, JE, Brocklehurst, KJ, Marley, AE, Carey, F, Carling, D, Beri, RK. (1994) Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMPactivated protein kinase. FEBS Lett 353, 33–36.

112. Garton, A.J., Campbell, D.G., Carling, D., Hardie, D.G., Colbran R.J., and Yeaman, S.J. (1989) Phosphorylation of bovine hormone-sensitive lipase by the AMP-

activated protein kinase. A possible antilipolytic mechanism. Eur. J Biochem 179, 249–254

113. Corton, J.M., Gillespie, J.G., Hawley, S.A., and Hardie, D.G. (1995) 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur. J. Biochem 229, 558–565.

114. Wu, X.D., Motoshima, H., Mahadev, K., Stalker, T.J., Scalia, R., Goldstein, B.J. (2003) Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. Diabetes 52, 1355–1363.

115. Merrill, G.F., Kurth, E.J., Hardie, D.G., and Winder, W.W. (1997) AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am. J. Physiol 36, E1107-E1112.

116. Zhou, G, Myers, R, Li, Y, Chen, Y, Shen, X, Fenyk-Melody, J, Wu, M, Ventre, J, Doebber, T, Fujii, N, Musí, N, Hirshman, MF, Goodyear, LJ, and Moller, DE. (2001) Role of AMP-activated protein kinase in mechanism of metfomiin action. J Clin Invest 108, 1167-1174

117. Habinowski, SA, Witters, LA. (2001) The effects of AICAR on adipocyte differentiation of 3T3-L1 cells. Biochem Biophys Res Commun 286, 852–856.

118. Lihn, AS, Jessen, N, Pedersen, SB, Lund, S, Richelsen, B. (2004) AICAR stimulates adiponectin and inhibits cytokines in adipose tissue. Biochem Biophys Res Commun 316, 853-8.

119. Boss, O., Hagen, T. and Lowell, B.B. (2000) Uncoupling proteins 2 and 3: potential regulators of mitochondrial energy metabolism. Diabetes 49, 143–156.

120. Thompson, MP, Kim, D. (2004) Links between fatty acids and expression of UCP2 and UCP3 mRNAs. FEBS Lett 568, 4-9.

121. Kopecky, J, Clarke, G, Enerback, S, Spiegelman, B, Kozak, LP. (1995) Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. J Clin Invest 96, 2914-23.

122. Kopecky, J., Rossmeisl, M., Hodny, Z., Syrovy, I., Horakova, M., and Kolarova, P. (1996) Reduction of dietary obesity in the aP2-Ucp transgenic mice: mechanism and adipose tissue morphology. Am.J.Physiol 270, E776-E786.

123. Kopecky,J., Hodny,Z., Rossmeisl,M., Syrovy,I., and Kozak,L.P. (1996) Reduction of dietary obesity in the aP2-Ucp transgenic mice: physiology and adipose tissue distribution. Am.J.Physiol. 270, E768-E775.

124. Kopecky, J, Rossmeisl, M, Flachs, P, Bardova, K, Brauner, P. (2001) Mitochondrial uncoupling and lipid metabolism in adipocytes. Biochem Soc Trans 29, 791–797.

125. Stefl, B, Janovska, A, Hodny, Z, Rossmeisl, M, Horakova, M, Syrovy, I, Bemova, J, Bendlova, B, Kopecky, J. (1998) Brown fat is essential for cold-induced thermogenesis but not for obesity resistance in aP2-Ucp mice. Am J Physiol 274, E527-33.

126. Baumruk, F., Flachs, P., Horáková, M., Floryk, D., and Kopecký J. (1999) Transgenic UCP1 inwhite adipocytes modulates mitochondrial membrane potential, FEBS Letters 444, 206-210

127. Rossmeisl, M., Barbatelli, G., Flachs, P., Brauner, P., Zingaretti, M.C., Marelli, M., Janovská, P., Horaková, M., Syrový, I., Cinti, S., and Kopecký, J. (2002) Expression of the uncoupling protein 1 from the aP2 gene promoter stimulates mitochondrial biogenesis in unilocular adipocytes in vivo. Eur J Biochem 269, 19-28

128. Rossmeisl, M., Syrový, I., Baumruk, F., Flachs, P., Janovská, P., and Kopecký, J. (2000) Decreased fatty acid synthesis due to respiratory uncoupling in adipose tissue. FASEB J 14, 1793-1799

129. Flachs, P., Novotný, J., Baumruk, F., Bardová, K., Bouřová, L., Mikšík, I. , Šponarová, J., Svoboda, P., and Kopecký, J. (2002) Impaired noradrenaline-induced lipolysis in white fat of aP2-Ucp1 transgenic mice is associated with changes in G-protein levels. Biochem J 364, 396-376

130. Lefebvre, P. (1975) Glucagon and adipose tissue. Biochem Pharmacol 24, 1261-1266,

131. Arner, P, Hellstrom, L, Wahrenberg, H, Bronnegard, M. (1990) Beta-adrenoceptor expression in human fat cells from different regions. J Clin Invest 86, 1595-600.

132. Hammond, VA, Johnston, DG. (1987) Substrate cycling between triglyceride and fatty acid in human adipocytes. Metabolism 36, 308-13.

133. Edens, NK, Leibel, RL, Hirsch, J. (1990) Mechanism of free fatty acid re-esterification in human adipocytes *in vitro*. J Lipid Res 31, 1423-31.

134. Harmon, CM, Luce, P, Abumrad, NA. (1992) Labelling of an 88 kDa adipocyte membrane protein by sulpho-N-succinimidyl long-chain fatty acids: inhibition of fatty acid transport. Biochem Soc Trans 20, 811-3.

135. Forest, C., Franckhauser, S., Glorian, M., Antras-Ferry, J., Robin, D. and Robin, P. (1997) Regulation of gene transcription by fatty acids, fibrates and prostaglandins: the phosphoenolpyruvate carboxykinase gene as a model.Prostaglandins Leukotrienes Essential Fatty Acids 57, 47–56

136. Guerre-Millo, M. (1997) Regulation of ob gene and overexpression in obesity. Biomed Pharmacother 51, 318-23.

137. Thumelin, S, Kohl, C, Girard, J, Pegorier, JP. (1999) Atypical expression of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in subcutaneous adipose tissue of male rats. J Lipid Res 40, 1071-7

138. Commerford, SR, Pagliassotti, MJ, Melby, CL, Wei, Y, Gayles, EC, Hill, JO. (2000) Fat oxidation, lipolysis, and free fatty acid cycling in obesity-prone and obesity-resistant rats. Am J Physiol Endocrinol Metab 279, E875-85

139. Storlien, LH, Kraegen, EW, Chisholm, DJ, Ford, GL, Bruce, DG, Pascoe, WS. (1987) Fish oil prevents insulin resistance induced by high-fat feeding in rats. Science 237, 885-8.

140. Belzung, F, Raclot, T, Groscolas, R. (1993) Fish oil n-3 fatty acids selectively limit the hypertrophy of abdominal fat depots in growing rats fed high-fat diets. Am J Physiol 264, R1111-8.

141. Fickova, M, Hubert, P, Cremel, G, Leray, C. (1998) Dietary (n-3) and (n-6) polyunsaturated fatty acids rapidly modify fatty acid composition and insulin effects in rat adipocytes. J Nutr 128, 512-9.

142. Jones, B., Maher, M., Banz, W., Zemel, M., Whelan, J., Smith, P., Moustaid, N., (1996) Adipose tissue stearoyl-CoA desaturase mRNA is increased by obesity and decreased by polyunsaturated fatty acids, Am. J. Physiol 271, E44–E99.

143. Stillwell, W., Jenski, L. J., Crump, F. T., and Ehringer, W. (1997) Effect of docosahexaenoic acid on mouse mitochondrial membrane properties. Lipids 32, 497-506

144. Reilly, J.M., and Thompson, M.P. (2000) Dietary fatty acids up-regulate the expression of UCP2 in 3T3-L1 preadipocytes, Biochem Biophys Res Commun 277, 541–545.

145. Oudart, H., Groscolas, R., Calgaari, C., Nibbelink, M., Leray, C., Le Maho, Y., and Malan, A. (1997) Brown fat thermogenesis in rats fed high-fat diets enriched with n-3 polyunsaturated fatty acids. Int J Obesity 21, 955- 962

146. Ide, T., Murata, M., and Sugano, M. (1996) Stimulation of the activities of hepatic fatty acid oxidation enzymes by dietary fat rich in alpha-linolenic acid in rats. J. Lipid Res 37, 148-463.

147. Baillie, RA, Takada, R, Nakamura, M, Clarke, SD. (1999) Coordinate induction of peroxisomal acyl-CoA oxidase and UCP-3 by dietary fish oil: a mechanism for decreased body fat deposition. Prostaglandins Leukot Essent Fatty Acids. 60, 351-6.

148. Clarke, S.D. (2004) The multi-dimensional regulation of gene expression by fatty acids: polyunsaturated fats as nutrient sensors. Curr Opin Lipidol 15, 13–18

149. Wahle, K., Rotondo, D., and Heys, SD. (2003) Polyunsaturated fatty acids and gene expression in mammalian systems. Proc Nutr Soc 62, 349–360.

150. Apfel, R, Benbrook, D, Lernhardt, E, Ortiz, MA, Salbert, G, Pfahl, M. (1994) A novel orphan receptor specific for a subset of thyroid hormone-responsive elements and its interaction with the retinoid/thyroid hormone receptor subfamily. Mol Cell Biol 14, 7025-7035.

151. Schultz, JR, Tu, H, Luk, A, et al. (2000) Role of LXRs in control of lipogenesis. Genes Dev 14, 2831-2838.

152. Ou, J, Tu ,H, Shan, B, et al. (2001) Unsaturated fatty acids inhibit transcription of sterol regulatory element binding protein 1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. Proc Natl Acad Sci U S A 98, 6027-6032.

153. Forman, B.M., Chen, J., and. Evans, R.M (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta, Proc Natl Acad Sci 94, 4312–4317.

154. Steineger, HH, Sørensen, HN, Tugwood, JD, Skrede, S, Spydevold, Ø, Gautvik, KM. (1994) Dexamethasone and insulin demonstrate marked and opposite regulation of the steady-state mRNA level of the peroxisomal proliferator-activated receptor (PPAR) in hepatic cells. Hormonal modulation of fatty-acid- induced transcription. Eur J Biochem 225, 967–974

155. Jiang, Y.H., Lupton, J.R., Chang, W.C., Jolly, C.A., Aukema, H.M. and Chapkin, R.S. (1996) Dietary fat and fiber differentially alter intracellular second messengers during tumor development in rat colon. Carcinogenesis 17, 1227–1233

156. Kawaguchi, T., Osatomi, K., Yamashita, H., Kabashima, T. and Uyeda, K. (2002) Mechanism for fatty acid sparing effect on glucose-induced transcription. Regulation of carbohydrate-responsive element-binding protein by amp-activated protein kinase. J. Biol. Chem. 277, 3829–3835

157. Wallance, J.M. (2002) Nutritional and botanical modulation of the inflammatory cascade — eicosanoids, cyclooxygenases, and lipoxygenases — as an adjunct in cancer therapy. Integr. Cancer Ther 1, 7–37.

158. Grimm, H., Mayer, K., Mayser, P. and Eigenbrodt, E. (2002) Regulatory potential of n-3 fatty acids in immunological and inflammatory processes. Br. J. Nutr 87, S59–S67

159. Ricquier D., Boullaud F. (2000) The uncoupling protein homologes. Biochem J 345, 161-79

160. Hardie, D.G., and Hawley, S.A. (2001) AMP-activated protein kinase: the energy charge hypothesis revisited. Bioessays 23, 1112–1119.

161. Inokuma, K, Ogura-Okamatsu, Y., Toda, Ch., Kimura, K., Yamashita, H. and Saito, M. (2005) Uncoupling Protein 1 Is Necessary for Norepinephrine-Induced Glucose Utilization in Brown Adipose Tissue. Diabetes 54, 1385–1391

162. Ishigaki Y, Katagiri H, Yamada T, Ogihara T, Imai J, Uno K, Hasegawa Y, Gao J, Ishihara H, Shimosegawa T, Sakoda H, Asano T, Oka Y. (2005) Dissipating excess energy stored in the liver is a potential treatment strategy for diabetes associated with obesity. Diabetes 54, 322-32.

163. Tian, R., Musi, N., D'Agostino, J., Hirshman, M.F. and Goodyear, L.J. (2001) Increased adenosine monophosphate-activated protein kinase activity in rat hearts with pressure-overload hypertrophy. Circulation 104, 1664–1669.

164. Frosig, C., Jorgensen, S.B., Hardie, D.G., Richter, E.A. and Wojtaszewski, J.F. (2004) 5'-AMP-activated protein kinase activity and protein expression are regulated by endurance training in human skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 286, E411–E417.

165. Pond, CM, Mattacks, CA. (1991) The effects of noradrenaline and insulin on lipolysis in adipocytes isolated from nine different adipose depots of guinea-pigs. Int J Obes 15, 609-18.

166. Bertile, F, Criscuolo, F, Oudart, H, Le Maho, Y, Raclot, T. (2003) Differences in the expression of lipolytic-related genes in rat white adipose tissues. Biochem Biophys Res Commun 307, 540-6.

167. Budd, TJ, Salter, AM, Buttery, PJ. (1993) Depot specific effects of insulin and isoproterenol on porcine adipose tissue metabolism. Biochem Soc Trans 21, 149S.

168. Wang, T, Zang, Y, Ling, W, Corkey, BE, Guo, W. (2003) Metabolic partitioning of endogenous fatty acid in adipocytes. Obes Res 11, 880-7.

169. Velasco, G, Gomez del Pulgar, T, Carling, D, and Guzman, M. (1998) Evidence that the AMP-activated protein kinase stimulates rat liver carnitine palmitoyltransferase I by phosphorylating cytoskeletal components. FEBS Lett 439, 317-320

170. Suchankova, G, Tekle, M, Saha, AK, Ruderman, NB, Clarke, SD, and Gettys, TW. (2005) Dietary polyunsaturated fatty acids enhance hepatic AMP- activated protein kinase activity in rats. Biochem Biophys Res Commun 326, 851-858

171. Minokoshi, Y, Alquier, T, Furukawa, N, Kim, YB, Lee, A, Xue, B, Mu, J, Foufelle, F, Ferre, P, Birnbaum, MJ, Stuck, BJ, Kahn, BB. (2004) AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 428, 569-74.

172. Daval, M, Diot-Dupuy, F, Bazin, R, Hainault, I, Viollet, B, Vaulont, S, Hajduch, E, Ferre, P, Foufelle, F. (2005) Anti-lipolytic action of AMP-activated protein kinase in rodent adipocytes. J Biol Chem 280, 25250-7.

173. Overton, J.M. and Williams, T.D. (2004). Behavioral and physiologic responses to caloric restriction in mice. Physiol. Behav. 81, 749-754.

174. Salt, I., Celler, J. W., Hawley, S. A., Prescott, A., Woods, A., Carling, D., and Hardie, D. G. (1998) AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform. Biochem J 7334, 177-187

175. Yin, W., Mu, J., and Birnbaum, M. J. (2003) Role of AMP-activated protein kinase in cyclic AMP-dependent lipolysis In 3T3-L1 adipocytes. J Biol Chem 278, 43074-43080

176. Millet, L, Vidal, H, Andreelli, F, Larrouy, D, Riou, JP, Ricquier, D, Laville, M, Langin, D. (1997) Increased uncoupling protein-2 and -3 mRNA expression during fasting in obese and lean humans. J Clin Invest 100, 2665-70.

177. Pecqueur, C, Alves-Guerra, MC, Gelly, C, Levi-Meyrueis, C, Couplan, E, Collins, S, Ricquier, D, Bouillaud, F, Miroux, B. (2001) Uncoupling protein 2, in vivo distribution, induction upon oxidative stress, and evidence for translational regulation. J Biol Chem 276, 8705-12.

178. Echtay, KS, Winkler, E, Frischmuth, K, Klingenberg, M. (2001) Uncoupling proteins 2 and 3 are highly active H(+) transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone). Proc Natl Acad Sci U S A. 98, 1416-21.

179. Fink, BD, Hong, YS, Mathahs, MM, Scholz, TD, Dillon, JS, Sivitz, WI. (2002) UCP2-dependent proton leak in isolated mammalian mitochondria. J Biol Chem 277, 3918-25.

180. Hanson, RW, Reshef, L. (1997) Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annu Rev Biochem 66, 581-611.

181. Franckhauser, S., Munoz, S., Pujol, A., Casellas, A., Riu, E., Otaegui, P., Su, B., and Bosch, F. (2002). Increased fatty acid re-esterification by PEPCK overexpression in adipose tissue leads to obesity without insulin resistance. Diabetes 51, 624-630.

182. Fryer, L. G., Parbu-Patel, A., and Carling, D. (2002) Protein kinase inhibitors block the stimulation of the AMP-activated protein kinase by 5-amino-4-imidazolecarboxamide riboside. J Biol Chem 277, 25226-25232

183. Pedersen, SB, Lund, S, Buhl, ES, Richelsen, B. (2001) Insulin and contraction directly stimulate UCP2 and UCP3 mRNA expression in rat skeletal muscle *in vitro*. Biochem Biophys Res Commun 283, 19-25.

184. Kopelman, P.G. (1998). Effects of obesity on fat topography: Metabolic and endocrine determinants. In *Clinical obesity* (Kopelman, P.G. & Stock, M.J., eds), pp. 158-175. Blackwell Science Ltd, Oxford.

185. Hartman, AD, and Christ, DW. (1978) Effect of cell size, age and anatomical location on the lipolytic response of adipocytes. Life Sci. 22, 1087-96.

186. Carpene, C., Galitzky, J., Saulnier-Blache, J.S., Lafontan, M. (1990) Selective reduction of $\alpha 2$-adrenergic responsiveness in hamster adipose tissue during prolonged starvation. Am J Physiol 259, E80-8.

187. Bray, GA, Inoue, S, and Nishizawa, Y. (1981) Hypothalamic obesity. The autonomic hypothesis and the lateral hypothalamus. Diabetologia 20 Suppl, 366-77.

188. Dieudonne, MN, Pecquery, R, and Giudicelli, Y. (1992) Characteristics of the alpha 2/beta-adrenoceptor-coupled adenylate cyclase system and their relationship with adrenergic responsiveness in hamster fat cells from different anatomical sites. Eur J Biochem 205, 867-73.

189. Assifi, M.M., Suchankova, G., Constant, S., Prentki, M., Saha A.K., and Ruderman, N.B (2005) AMP-activated protein kinase and coordination of hepatic fatty acid metabolism of starved/carbohydrate-refed rats , AJP - Endo 289, 794-800

190. Reddy, JK, and Hashimoto, T. (2001) Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. Annu Rev Nutr 21:193-230.

191. Yahagi, N, Shimano, H, Hasty, AH, Amemiya-Kudo, M, Okazaki, H, Tamura, Y, Iizuka, Y, Shionoiri, F, Ohashi, K, Osuga, J, Harada, K, Gotoda, T, Nagai, R, Ishibashi, S, and Yamada, N. (1999) A crucial role of sterol regulatory element-binding protein-1 in the regulation of lipogenic gene expression by polyunsaturated fatty acids. J Biol Chem 274, 35840-4.

192. Clarke, SD. (2001) Polyunsaturated fatty acid regulation of gene transcription: a molecular mechanism to improve the metabolic syndrome. J Nutr 131, 1129-32.

193. Jump, DB. (2002) Dietary polyunsaturated fatty acids and regulation of gene transcription. Curr Opin Lipidol 13, 155-164.

194. Repa, JJ, and Mangelsdorf, DJ. (2000) The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. Annu Rev Cell Dev Biol 16, 459-481.

195. Miyata, KS, McCaw, SE, Patel, HV, Rachubinski, RA, and Capone, JP. (1996) The orphan nuclear hormone receptor LXR alpha interacts with the peroxisome proliferator-activated receptor and inhibits peroxisome proliferator signaling. J Biol Chem 271, 9189-9192.

196. Sessler, AM, Kaur, N, Palta, JP, and Ntambi, JM. (1996) Regulation of stearoyl-CoA desaturase 1 mRNA stability by polyunsaturated fatty acids in 3T3-L1 adipocytes. J Biol Chem 271, 29854-29858.

197. Forman, BM, Tontonoz, P, Chen, J, Brun, RP, Spiegelman, BM, and Evans, RM. (1995) 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. Cell 83, 803-812.

198. Okuno, M, Kajiwara, K, Imai, S, Kobayashi, T, Honma, N, Maki, T, Sumga, K, Goda, T, Takase, S, Muto, Y, and Moriwaki, H. (1997) Perilla oil prevents the excessive growth of visceral adipose tissue in rats by down-regulating adipocyte differentiation. J Nutrition 127, 1752-1757.

199. Puigserver, P, and Spiegelman, BM. (2003) Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. Endocr Rev 24, 78-90

200. Wilson-Fritch, L, Nicoloro, S, Chouinard, M, et al (2004) Mitochondrial remodeling in adipose tissue associated with obesity and treatment with rosiglitazone. J Clin Invest 114, 1281-1289

201. Watkins, SM, Reifsnyder, PR, Pan, HJ, German, JB, and Leiter, EH. (2002) Lipid metabolome- wide effects of the PPAR gamma agonist rosiglitazone. J LipidRes 43, 1809-1817

202. Ruzickova, J, Rossmeisl, M, Prazak, T, Flachs, P, Sponarova, J, Vecka, 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, 1177-85.

203. Ikemoto, S, Thompson, KS, Itakura, H, Lane, MD, and Ezaki, O. (1995) Expression of an insulin-responsive glucose transporter (GLUT4) minigene in transgenic mice: effect of exercise and role in glucose homeostasis. Proc Natl Acad Sci U S A 92, 865-9.

204. Hun, CS, Hasegawa, K, Kawabata, T, Kato, M, Shimokawa, T, and Kagawa, Y. (1999) Increased uncoupling protein2 mRNA in white adipose tissue, and decrease in leptin, visceral fat, blood glucose, and cholesterol in KK-Ay mice fed with eicosapentaenoic and docosahexaenoic acids in addition to linolenic acid. Biochem Biophys Res Commun 259, 85-90.

205. Brun, RP, and Spiegelman, BM. (1997) PPARgand the molecular control of adipogenesis. J Endocrinol 155, 217–218

206. Iwaki, M, Matsuda, M, Maeda, N, et al. (2003). Induction of adiponectin, a fat derived antidiabetic and antiatherogenic factor, by nuclear receptors. Diabetes 52, 1655-1663

207. Maeda, N, Takahashi, M, Funahashi, T, Kihara, S, Nishizawa, H, Kishida, K, Nagaretani, H, Matsuda, M, Komuro, R, Ouchi, N, Kuriyama, H, Hotta, K,

Nakamura, T, Shimomura, I, and Matsuzawa, Y. (2001) PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. Diabetes 50, 2094–2099.

208. Fernandez-Real, JM, Vendrell, J, and Ricart, W (2005) Circulating adiponectin and plasma fatty acid profile. Clin Chem 51, 603-609.

209. Endres, S., Ghorbani, R., Kelley, V. E., Georgilis, K., Lonnemann, G., van der Meer, J. W., Cannon, J. G., Rogers, T. S., Klempner, M. S. and Weber, P. C. (1989) The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med 320, 265-271

210. Hofmann, C, Lorenz, K, Braithwaite, SS, Colca, JR, Palazuk, BJ, Hotamisligil, GS, and Spiegelman, BM. (1994) Altered gene expression for tumor necrosis factor-α and its receptors during drug and dietary modulation of insulin resistance. Endocrinology 134, 264–270

PUBLICATION A.

Possible involvement of AMP-activated protein kinase in obesity resistance induced by respiratory uncoupling in white fat.

Matějková O, Mustard KJ, Šponarová J, Flachs P, Rossmeisl M, Mikšík I, Thomason-Hughes M, Grahame Hardie D, Kopecký J

FEBS Lett. 569(1-3):245-8 (2004)

FEBS 28541

FEBS Letters 569 (2004) 245–248

Possible involvement of AMP-activated protein kinase in obesity resistance induced by respiratory uncoupling in white fat

Olga Matejkova[a], Kirsty J. Mustard[b], Jana Sponarova[a], Pavel Flachs[a], Martin Rossmeisl[a], Ivan Miksik[c], Michaela Thomason-Hughes[b], D. Grahame Hardie[b], Jan Kopecky[a,*]

[a]*Department of Adipose Tissue Biology and Center for Integrated Genomics, Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 14220 Prague, Czech Republic*
[b]*Division of Molecular Physiology, School of Life Sciences, Wellcome Trust Biocentre, University of Dundee, Dow Street, Dundee DD1 5EH, UK*
[c]*Department of Biologically Active Compounds, Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 14220 Prague, Czech Republic*

Received 21 April 2004; revised 14 May 2004; accepted 3 June 2004

Available online 15 June 2004

Edited by Vladimir Skulachev

Abstract The AMP-activated protein kinase (AMPK) cascade is a sensor of cellular energy charge that promotes catabolic and inhibits anabolic pathways. However, the role of AMPK in adipocytes is poorly understood. We show that transgenic expression of mitochondrial uncoupling protein 1 in white fat, which induces obesity resistance in mice, is associated with depression of cellular energy charge, activation of AMPK, downregulation of adipogenic genes, and increase in lipid oxidation. Activation of AMPK may explain the complex metabolic changes in adipose tissue of these animals and our results support a role for adipocyte AMPK in the regulation of storage of body fat.

Keywords: ATP; Lipid metabolism; Mitochondrion; Adipocyte; Transgenic mouse

1. Introduction

It is becoming evident that metabolism of adipose tissue is important for the control of body fat content [1,2] and that intracellular energy charge is involved in the control of lipid metabolism in adipocytes. In vitro studies demonstrated that a decrease of mitochondrial ATP production resulted in the inhibition of both fatty acid (FA) synthesis [3] and the lipolytic action of catecholamines [4]. In turn, lipolytic hormones decreased ATP levels in adipocytes [5,6]. The intracellular energy charge is also low [7,8] in transgenic mice [9] rendered resistant to obesity by ectopic expression of mitochondrial uncoupling protein 1 (UCP1) in white fat (aP2-*Ucp1* mice). These animals exhibited a reduction of FA synthesis [10] and the lipolytic action of catecholamines [7], as well as increases in mitochondrial biogenesis [11] and endogenous oxygen consumption [12] in white adipose tissue. At that time, a unifying explanation for all of these metabolic changes was not apparent.

The AMP-activated protein kinase (AMPK) is a sensor of cellular energy charge that, once activated by an increase in the cellular AMP/ATP ratio, acts as a metabolic master switch [13,14]. Its known effects include: (a) inhibition of FA synthesis and lipolysis in adipocytes due to phosphorylation of acetyl-CoA carboxylase-1 (ACC-1) and hormone-sensitive lipase [15–17]; (b) stimulation of glucose uptake into adipocytes [18] and myocytes [19]; (c) activation of FA oxidation in muscle due to phosphorylation of ACC-2 [19,20]; (d) induction of mitochondrial biogenesis possibly through activation of nuclear respiratory factor-1 [21]; and (e) downregulation of lipogenic genes in liver [22], as well as in 3T3-L1 adipocytes, in the latter case by downregulating peroxisome proliferator-activated receptor γ (PPARγ; [23]). The role of AMPK in adipose tissue remains relatively unexplored, although recent studies indicate the involvement of AMPK in the effects of physical exercise [24], adiponectin [18], thiazolidinediones [25], and leptin [26,27].

The aim of this study was to verify whether changes in the energy charge of adipocytes in vivo, due to respiratory uncoupling in aP2-*Ucp1* mice, could activate AMPK in adipocytes, and to clarify further the role of AMPK in white adipose tissue.

2. Materials and methods

2.1. Animals

Male C57BL/6J control mice and their transgenic littermates, hemizygous for the aP2-*Ucp1* transgene [9,12,28], were kept in a controlled environment (20 °C; 12 h light/dark cycle) with free access to water and standard chow diet. At 6 months of age, animals were sacrificed by cervical dislocation under diethylether anesthesia. Epididymal and subcutaneous dorsolumbar white fat depots [12] were dissected, flash frozen and stored in liquid nitrogen for biochemical and RNA analysis.

2.2. RNA analysis

Gene expression was analyzed by reverse transcription followed by the real time quantitative PCR (LightCycler Instrument, Roche, Germany) with primers specific for PPARγ, adipocyte lipid binding protein 2 (aP2) and β-actin (Table 1). Levels of β-actin were used to correct for inter-sample variations. Detailed protocol was described before [7].

*Corresponding author. Fax: +420-241-062-599.
E-mail address: kopecky@biomed.cas.cz (J. Kopecky).

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; aP2, adipocyte lipid binding protein 2; aP2-*Ucp1* mice, transgenic mice expressing UCP1 gene from aP2 gene promoter; FA, fatty acids; pACC, phosphorylated form of ACC; pAMPK, phosphorylated form of AMPK; PPARγ, peroxisome proliferator-activated receptor γ; TBS, 10 mM Tris–HCl, pH 7.4, 0.5 M NaCl; UCP, uncoupling protein

0014-5793/$22.00
doi:10.1016/j.febslet.2004.06.002

Table 1
Sequences of PCR primers

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	GenBank Accession No. for cDNA
PPARγ[a]	GGCGAGGGCGATCTTGACAGG	GGGCTTCCGCAGGTTTTTGAGG	U09138
β-Actin	GAACCCTAAGGCCAACCGTGAAAAGAT	ACCGCTCGTTGCCAATAGTGATG	XO3765
aP2	AACACCGAGATTTCCTTCAA	AGTCACGCCTTTCATAACACA	M13385

[a] Both isoforms γ1 and γ2 are amplified.

2.3. Determination of ATP and AMP

Flash-frozen tissues dissected immediately after sacrifice were stored in liquid nitrogen. Tissues were homogenized in 6% (w/v) perchloric acid. After centrifugation, supernatants were neutralized and adenine nucleotides determined using HPLC [7].

2.4. Activity of α1 isoform of AMPK

AMPK was immunoprecipitated from tissue extracts prepared by homogenization in buffer A and the activity was assayed using a peptide substrate [29]. Protein concentration was estimated using bicinchoninic acid procedure and BSA as a standard [7]. AMPK activity is expressed in Units (nmol phosphate/min) per mg of protein in the adipocyte extract used for immunoprecipitation.

2.5. Oxidation of FA

Oxidation of oleic acid was measured using a modified protocol of Wang et al. [30]. Adipose tissue (~35 mg) sliced up into 5–10 fragments was pre-labeled with 40 μCi/mL [9,10(n)-^{3}H]oleic acid in 300 μl of 2% FA-free BSA-KRB buffer containing 5 mM glucose for 75 min at 37 °C and under 5% CO_2 (gentle shaking). Fragments were washed three times and re-suspended in 500 μl of the buffer and incubated for additional 4 h at 37 °C. Oxidation was assessed by measuring the amount of ^3H_2O released into the medium. The content of free FA in adipose tissue fragments was measured in 5% homogenate (w/v in H_2O) using a NEFA C kit from Wako Chemicals (Richmond, VA) and results were calculated according to a published formula [30].

2.6. Quantification of AMPK and ACC

The total content of the α1 catalytic subunit of AMPK (α1 AMPK) and the phosphorylated form of AMPK (pAMPK) was determined in tissue lysates by Western blotting [29,31] using antibodies against total α1 AMPK [31] and phosphospecific antibodies against Thr-172 [32]. For the quantification of total ACC and the phosphorylated form of ACC (pACC), tissue lysates (10 μg protein) were subjected to SDS-PAGE using pre-cast 3–8% Tris-acetate gels (Invitrogen). Protein was transferred to nitrocellulose membranes (BioRad, Hercules, CA) and the membranes incubated in Odyssey Blocking buffer (Li-Cor Biosciences, Lincoln, NE). Phosphospecific sheep antibodies against the Ser-221 site on ACC-2 [29], that also recognize the homologous site (Ser-79) on ACC-1 (1.46 μg/ml in blocking buffer containing 0.2% Tween-20), were used to quantify pACC (incubation for 1 h). The membranes were washed 6 × 5 min with TBS (10 mM Tris–HCl, pH 7.4, 0.5 M NaCl) containing Tween-20 (0.2%) and immersed in blocking buffer containing 0.2% Tween-20 and 1 μg/ml anti-sheep IgG conjugated to IR dye 680 (Molecular Probes, Leiden, The Netherlands) and 1 μg/ml streptavidin conjugated to IR Dye 800 (Rockland Inc., Philadelphia, PA) and left shaking for 1 h, protected from light. The membranes were then washed 6 × 5 min using TBS-Tween (0.2%) and 1 × 5 min in PBS and scanned using the Odyssey IR Imager (Li-Cor Biosciences). The results were quantified using Odyssey software. A linear response was obtained between 0.3 and 20 μg lysate protein analyzed (not shown).

2.7. Statistics

Statistical significance was evaluated using unpaired *t*-tests. Differences were judged to be significant at $P < 0.05$.

3. Results

3.1. Increased cellular AMP/ATP ratio

We observed previously [7] that in white fat of the aP2-*Ucp1* mice ATP content was lower and ADP/ATP ratio was higher than in control mice. These differences were more pronounced in subcutaneous than in epididymal fat, in accordance with a higher content of transgenic UCP1 in the former fat depot [7]. Due to the adenylate kinase reaction [13], we expected that the AMP/ATP ratio should be also affected by ectopic expression of UCP1 in white fat. It was observed that the AMP/ATP ratio in both fat depots of transgenic animals was higher than in control mice (Fig. 1A). No statistically significant differences between the two genotypes could be detected at the level of tissue contents of individual nucleotides (not shown).

3.2. Enhancement of AMPK activity

The increase of the AMP/ATP ratio produced by ectopic expression of UCP1 in white fat suggested that AMPK activity in adipose tissue would be also affected. Indeed, the presence of transgenic UCP1 resulted in a significant, 2-fold increase in the activity of the α1 isoform (α1 AMPK) in subcutaneous fat (Fig. 1B). The activity also tended to be higher in the epididymal fat, although this was not statistically significant (Fig. 1B). The activity of the α2 isoform was negligible in adipose tissue (not shown). Quantification of the expression of α1 AMPK and phosphorylation of Thr-172 using Western blots (Fig. 2A) indicated a significantly higher content of the α1 subunit in transgenic than in control mice in both epididymal and subcutaneous fat (Fig. 2B) and, after correction for α1 content, a significant increase in α1 AMPK phosphoryla-

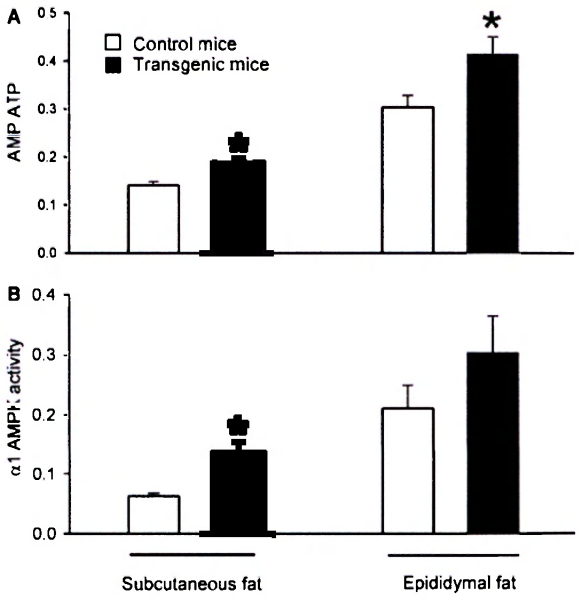

Fig. 1. AMP/ATP ratio and AMPK activity in adipose tissue. (A) Concentrations of ATP and AMP were estimated by HPLC in tissue extracts ($n = 15$). (B) Activity of α1 AMPK (in units; $n = 6$). Values are means ± S.E. Asterisks indicate statistically significant differences between genotypes.

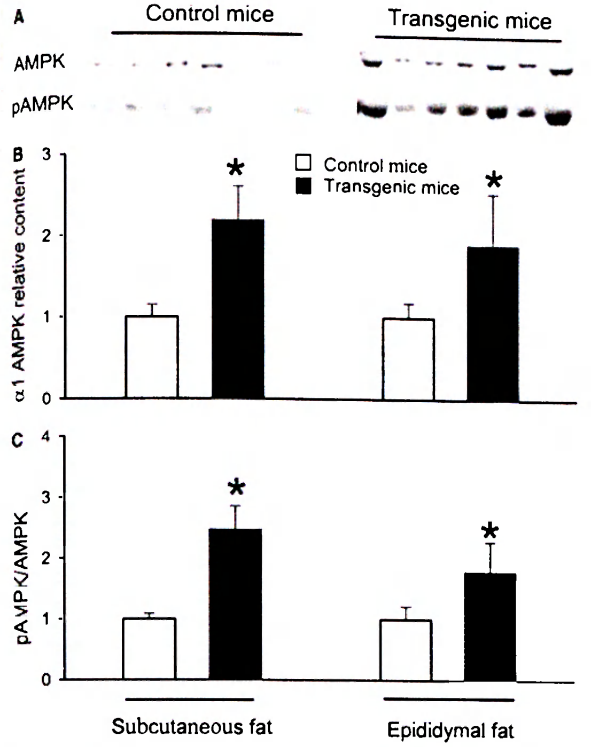

Fig. 2. Content and phosphorylation of AMPK in adipose tissue. (A) Total α1 AMPK and phosphorylated form of α1 AMPK (pAMPK) were quantified using Western blots, as illustrated for the analysis in subcutaneous fat. (B) Total content of α1 AMPK. (C) Phosphorylation of AMPK. Values are means ± S.E. ($n = 6$). Asterisks indicate statistically significant differences between genotypes.

tion in both fat depots due to the transgenic modification (Fig. 2C). Quantification of total ACC and pACC content in adipose tissue lysates (see Section 2) also showed a modest but significant increase of the pACC/ACC ratio (1.3-fold; $P < 0.05$; $n = 6$) in subcutaneous fat due to the transgenic modification. Increased phosphorylation of both α1 AMPK and ACC in the transgenic mice further supports the activation of AMPK by the ectopic UCP1 in white fat.

3.3. Decreased expression of PPARγ and aP2 genes

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 and its expression is inhibited by AMPK activation during differentiation of 3T3-L1 adipocytes in culture [23]. A significant diminution of PPARγ mRNA level was found in subcutaneous but not in epididymal fat of aP2-*Ucp1* mice (Table 2). Moreover, the expression of a PPARγ target gene, i.e., aP2, was also downregulated in subcutaneous fat (Table 2).

3.4. Enhancement of FA oxidation

Previous experiments showed a higher rate of oxidation of endogenous substrates in white fat of transgenic compared with control mice [12]. Measurement of oleate oxidation in adipose tissue fragments (Fig. 3) revealed a similar rate of oxidation in the subcutaneous and epididymal fat of control mice. However, the presence of transgenic UCP1 resulted in a significant, 2.7-fold increase in the oleate oxidation in subcutaneous fat, but not epididymal fat.

Table 2
Downregulation of PPARγ and aP2 gene expression in adipose tissue of transgenic mice

Sample	mRNA level (relative units %)	
	PPARγ	aP2
Subcutaneous fat		
+/+	100.0 ± 15.6	100 ± 17.6
tg/+	22.9 ± 6.2*	32.8 ± 6.4*
Epididymal fat		
+/+	100.0 ± 11.6	100 ± 12.5
tg/+	68.1 ± 26.1	80.7 ± 12.6

Transcripts were quantified in adipose tissue of control (+/+) and transgenic mice (tg/+). Data are means ± S.E. ($n = 5$ to 6). Asterisks indicate statistically significant differences between genotypes. Results were verified using Northern blot analysis (not shown).

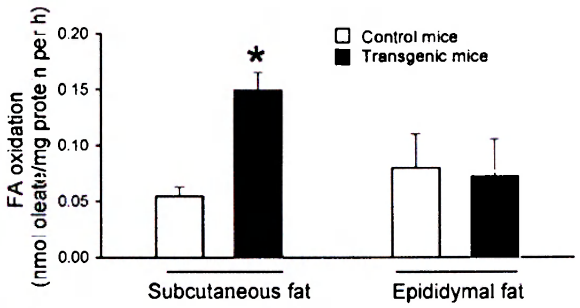

Fig. 3. Oxidation of oleate in adipose tissue. Values are means ± S.E. ($n = 6$). Asterisk indicates statistically significant differences between genotypes.

4. Discussion

Our results demonstrate that persistent depression of energy charge in adipocytes of the transgenic mice results in both an increase of α1 AMPK expression, and an increase in its phosphorylation and activity. Increased expression of α1 has been observed in other tissues in response to treatments that would be expected to persistently activate AMPK, e.g., during pressure overload hypertrophy in rat heart [33], or endurance training in human skeletal muscle [34]. The differential effect of the transgene on AMPK activity in the subcutaneous versus the epididymal fat depots was in accordance with the 2- to 3-fold higher expression of the transgene in the former, detected at both the mRNA and protein levels [10,11], and also with the preferential effect of the transgene on lipogenesis [10], lipolysis [7], and FA oxidation (this report, Fig. 3) in the subcutaneous fat. The strong induction of FA oxidation, the increase in mitochondrial content [11], and the depression of lipogenesis [10] in subcutaneous fat of the transgenic mice are consistent with the known effects of AMPK in white fat [15,16] and other tissues [13,14,20,21] and explain the preferential reduction of subcutaneous fat in the transgenic mice [9,28]. Moreover, the strong downregulation of PPARγ and aP2 genes in the subcutaneous fat of the transgenic mice, most probably due to the activation of AMPK [23], may suppress the adipogenic potential of the tissue. Thus, activation of AMPK in white fat of aP2-*Ucp1* mice, by depression of intracellular energy charge, helps to explain the complexity of the changes in adipose tissue metabolism and obesity resistance in this transgenic model.

Besides the effect of transgenic UCP1 on adipose tissue, several other studies support a link between energy charge and metabolism in adipocytes, and the involvement of AMPK: (a) hyperleptinemia [26] and bezafibrate treatment [35] deplete body fat in rats, whereas expression of UCP1 and UCP2 in white adipose tissue is up-regulated, FA oxidation is increased, and expression of lipogenic genes is profoundly suppressed; (b) in isolated adipocytes leptin inhibits lipid synthesis while up-regulating UCP2 [36]; (c) antidiabetic drugs such as thiazolidinediones stimulate AMPK in adipose tissue [25], while inducing glycerol kinases [37] and phosphoenolpyruvate carboxykinase [38], leading presumably to futile cycling of FA re-esterification in adipocytes. Thiazolidinediones also stimulate AMPK in muscle cells while increasing the intracellular AMP/ATP ratio [39]. In fact, the induction of UCPs by leptin in adipose tissue, leading to a drop in the intracellular energy charge, may partly explain the activation of AMPK in adipose tissue under these conditions [27].

AMPK appears to represent a switch that converts adipocytes to lipid burning cells while suppressing in situ lipogenesis. This mechanism, which reduces the fat content of adipocytes, exists not only in aP2-*Ucp1* mice, but also under other circumstances such as hyperleptinemia [27]. Interestingly, FA oxidation and synthesis in adipose tissue are also reciprocally regulated during fasting [30]. Finally, the control of adipose tissue metabolism by intracellular energy charge and AMPK may represent a basic biological mechanism that contributes to the regional differences in the metabolic properties of adipose tissue depots. This mechanism could be affected by physiological stimuli, as well as by pharmacological agents, and it represents a promising target for the development of strategies for the treatment of both obesity and insulin resistance.

Acknowledgements: We thank J. Bemova, S. Hornova, and D. Salkova for technical assistance, and Prof. B.D. Nelson for valuable discussions. This work was supported by the Grant Agency of the Czech Republic (303/02/1220) and the research project AVOZ 5011922. M.T.H. and D.G.H. were supported by a Project Grant from Diabetes UK. K.J.M. was supported by a Research Studentship jointly funded by the Biotechnology and Biological Sciences Research Council (UK) and by Novo-Nordisk.

References

[1] Arner, P. (2000) Br. J. Nutr. 83 (Suppl. 1), S9–16.
[2] Kopecky, J., Rossmeisl, M., Flachs, P., Bardova, K. and Brauner, P. (2001) Biochem. Soc. Trans. 29, 791–797.
[3] Rognstad, R. and Katz, J. (1969) Biochem. J. 111, 431–444.
[4] Huber, C.T., Duckworth, W.C. and Solomon, S.S. (1981) Biochim. Biophys. Acta 666, 462–467.
[5] Ho, R.J., England, R. and Meng, H.C. (1970) Life Sci. 9, 137–150.
[6] Stein, J.M. (1975) Biochem. Pharmacol. 24, 1659–1662.
[7] Flachs, P., Novotny, J., Baumruk, F., Bardova, K., Bourova, L., Miksik, I., Sponarova, J., Svoboda, P. and Kopecky, J. (2002) Biochem. J. 364, 369–376.
[8] Baumruk, F., Flachs, P., Horakova, M., Floryk, D. and Kopecky, J. (1999) FEBS Lett. 444, 206–210.
[9] Kopecky, J., Clarke, G., Enerback, S., Spiegelman, B. and Kozak, L.P. (1995) J. Clin. Invest. 96, 2914–2923.
[10] Rossmeisl, M., Syrovy, I., Baumruk, F., Flachs, P., Janovska, P. and Kopecky, J. (2000) FASEB J. 14, 1793–1800.
[11] Rossmeisl, M., Barbatelli, G., Flachs, P., Brauner, P., Zingaretti, M.C., Marelli, M., Janovska, P., Horakova, M., Syrovy, I., Cinti, S. and Kopecky, J. (2002) Eur. J. Biochem. 269, 1–10.
[12] Kopecky, J., Rossmeisl, M., Hodny, Z., Syrovy, I., Horakova, M. and Kolarova, P. (1996) Am. J. Physiol. 270, E776–E786.
[13] Hardie, D.G. and Hawley, S.A. (2001) Bioessays 23, 1112–1119.
[14] Yeh, L.A., Lee, K.H. and Kim, K.H. (1980) J. Biol. Chem. 255, 2308–2314.
[15] Sullivan, J.E., Brocklehurst, K.J., Marley, A.E., Carey, F., Carling, D. and Beri, R.K. (1994) FEBS Lett. 353, 33–36.
[16] Garton, A.J., Campbell, D.G., Carling, D., Hardie, D.G., Colbran, R.J. and Yeaman, S.J. (1989) Eur. J Biochem 179, 249–254.
[17] Corton, J.M., Gillespie, J.G., Hawley, S.A. and Hardie, D.G. (1995) Eur. J. Biochem. 229, 558–565.
[18] Wu, X., Motoshima, H., Mahadev, K., Stalker, T.J., Scalia, R. and Goldstein, B.J. (2003) Diabetes 52, 1355–1363.
[19] Merrill, G.F., Kurth, E.J., Hardie, D.G. and Winder, W.W. (1997) Am. J. Physiol. 36, E1107–E1112.
[20] Winder, W.W., Wilson, H.A., Hardie, D.G., Rasmussen, B.B., Hutber, C.A., Call, G.B., Clayton, R.D., Conley, L.M., Yoon, S. and Zhou, B. (1997) A. J. Appl. Physiol. 82, 219–225.
[21] Bergeron, R., Ren, J.M., Cadman, K.S., Moore, I.K., Perret, P., Pypaert, M., Young, L.H., Semenkovich, C.F. and Shulman, G.I. (2001) Am. J. Physiol. Endocrinol. Metab. 281, E1340–E1346.
[22] Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J. and Moller, D.E. (2001) J. Clin. Invest. 108, 1167–1174.
[23] Habinowski, S.A. and Witters, L.A. (2001) Biochem. Biophys. Res. Commun. 286, 852–856.
[24] Ruderman, N.B., Park, H., Kaushik, V.K., Dean, D., Constant, S., Prentki, M. and Saha, A.K. (2003) Acta Physiol. Scand. 178, 435–442.
[25] Saha, A.K., Avilucea, P.R., Ye, J.M., Assifi, M.M., Kraegen, E.W. and Ruderman, N.B. (2004) Biochem. Biophys. Res. Commun. 314, 580–585.
[26] Zhou, Y.-T., Wang, Z.-W., Higa, M., Newgard, C.B. and Unger, R.H. (1999) Proc. Natl. Acad. Sci. USA 96, 2391–2395.
[27] Orci, L., Cook, W.S., Ravazzola, M., Wang, M.Y., Park, B.H., Montesano, R. and Unger, R.H. (2004) Proc. Natl. Acad. Sci. USA 101, 2058–2063.
[28] Kopecky, J., Hodny, Z., Rossmeisl, M., Syrovy, I. and Kozak, L.P. (1996) Am. J. Physiol. 270, E768–E775.
[29] Hawley, S.A., Gadalla, A.E., Olsen, G.S. and Hardie, D.G. (2002) Diabetes 51, 2420–2425.
[30] Wang, T., Zang, Y., Ling, W., Corkey, B.E. and Guo, W. (2003) Obes. Res. 11, 880–887.
[31] Woods, A., Salt, I., Scott, J., Hardie, D.G. and Carling, D. (1996) FEBS Lett. 397, 347–351.
[32] Sugden, C., Crawford, R.M., Halford, N.G. and Hardie, D.G. (1999) Plant J. 19, 1–7.
[33] Tian, R., Musi, N., D'Agostino, J., Hirshman, M.F. and Goodyear, L.J. (2001) Circulation 104, 1664–1669.
[34] Frosig, C., Jorgensen, S.B., Hardie, D.G., Richter, E.A. and Wojtaszewski, J.F. (2004) Am. J. Physiol. Endocrinol. Metab. 286, E411–E417.
[35] Cabrero, A., Alegret, M., Sanchez, R.M., Adzet, T., Laguna, J.C. and Vazquez, M. (2001) Diabetes 50, 1883–1890.
[36] Ceddia, R.B., William, W.N., Lima, F.B., Flandin, P., Curi, R. and Giacobino, J.P. (2000) Eur. J. Biochem. 267, 5952–5958.
[37] Guan, H.P., Li, Y., Jensen, M.V., Newgard, C.B., Steppan, C.M. and Lazar, M.A. (2002) Nat. Med. 8, 1122–1128.
[38] Tordjman, J., Chauvet, G., Quette, J., Beale, E.G., Forest, C. and Antoine, B. (2003) J. Biol. Chem. 278, 18785–18790.
[39] Fryer, L.G.D., Parbu-Patel, A. and Carling, D. (2002) J. Biol. Chem. 277, 25226–25232.

PUBLICATION B.

Involvement of AMP-activated protein kinase in fat depot-specific metabolic changes during starvation

Šponarová J, Mustard KJ, **Horáková O**, Flachs P, Rossmeisl M, Brauner P, Bardová K, Thomason-Hughes M, Braunerová R, Janovská P, Grahame Hardie D, Kopecký J.

FEBS Lett. 579: 6105-6110 (2005)

:EBS 30068

FEBS Letters 579 (2005) 6105–6110

Involvement of AMP-activated protein kinase in fat depot-specific metabolic changes during starvation

Jana Sponarova[a], Kirsty J. Mustard[b], Olga Horakova[a], Pavel Flachs[a], Martin Rossmeisl[a], Petr Brauner[a], Kristina Bardova[a], Michaela Thomason-Hughes[b], Radka Braunerova[a], Petra Janovska[a], D. Grahame Hardie[b], Jan Kopecky[a,*]

[a] *Department of Adipose Tissue Biology, Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 14220 Prague, Czech Republic*

[b] *Division of Molecular Physiology, School of Life Sciences, Wellcome Trust Biocentre, Dundee University, Dow Street, DD1 5EH, Dundee, Scotland, UK*

Received 23 August 2005; accepted 22 September 2005

Available online 7 October 2005

Edited by Vladimir Skulachev

Abstract The mechanisms controlling fat depot-specific metabolism are poorly understood. During starvation of mice, downregulation of lipogenic genes, suppression of fatty acid synthesis, and increases in lipid oxidation were all more pronounced in epididymal than in subcutaneous fat. In epididymal fat, relatively strong upregulation of uncoupling protein 2 and phosphoenolpyruvate carboxykinase genes was found. In mice maintained both at 20 and 30 °C, AMP-activated protein kinase was activated in epididymal but did not change in subcutaneous fat. Our results suggest that AMPK may have a role in the different response of various fat depots to starvation.

Keywords: Lipid metabolism; Adipocyte; AMPK; Lipogenesis; Acetyl-CoA carboxylase

1. Introduction

The metabolic properties of white adipose tissue (WAT) depend on its anatomical localization in the body and the health consequences of obesity are also affected by the site of fat accumulation. However, the mechanisms that control fat depot-specific differences in WAT metabolism are largely unknown, including the differential metabolic responses of fat depots to starvation, a common physiological condition leading to a decrease of body fat content [1–3]. In both epididymal WAT [4] and liver [5] of rodents, starvation stimulated the AMP-activated protein kinase (AMPK). This enzyme is a sensor of cellular energy stress that, once phosphorylated due to an increase in the cellular AMP/ATP ratio or other stimulus, activates ATP-producing processes while switching off ATP-consuming metabolic pathways [6]. Activation of AMPK in WAT results in inhibition of fatty acid (FA) synthesis and basal lipolysis due to phosphorylation of acetyl-CoA carboxylase-1 (ACC-1) and hormone-sensitive lipase [4,7–9], and also has effects on glucose uptake into adipocytes [10]. Our previous studies demonstrated that induction of resistance to obesity in mice by transgenic expression of mitochondrial uncoupling protein 1 (UCP1) in WAT [11] was associated with depression of cellular energy charge, activation of AMPK [12], downregulation of adipogenic genes, increases in lipid oxidation [12,13], impaired lipolytic action of catecholamines [14], and increased mitochondrial biogenesis [15]. Other recent studies suggest the involvement of AMPK in effects of exercise [16], adiponectin [17], thiazolidinediones [18], and leptin [19] in WAT.

The aim of this study was to investigate whether AMPK could be involved in the different responses of subcutaneous and epididymal fat depots to starvation.

*Corresponding author. Fax: +420 241 062 599.
E-mail address: kopecky@biomed.cas.cz (J. Kopecky).

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; α1 (α2) AMPK, α1 and α2 isoforms of the catalytic subunit of AMPK; FA, fatty acids; FAS, fatty acid synthase; NEFA, non-esterified fatty acids; PEPCK, phosphoenolpyruvate carboxykinase; pACC, the phosphorylated form of ACC; pAMPK, phosphorylated form of AMPK; SREBP-1, sterol regulatory element-binding protein-1; UCP, uncoupling protein; WAT, white adipose tissue

2. Materials and methods

2.1. Animals

Three- to four-month-old male C57BL/6J mice were used for all experiments. Animals were housed in a controlled environment (20 °C; 12-h light–dark cycle; light from 6:00 a.m.; five mice/cage) with free access to water and standard chow diet [11]. Two experimental protocols were used: A, all mice were caged singly for one week at 20 °C and than sacrificed between 9:00 and 10:00 a.m., while some of these mice were denied access to food for 6, 12, and 24 h before the sacrifice, respectively, and controls were allowed free access to food. B, mice were caged singly for two weeks either at 20 or at 30 °C and at 5:30 p.m. of the day of the experiment, mice were denied food or not and both groups were sacrificed 6 h later under dimmed red light. In both protocols, mice were sacrificed by cervical dislocation and epididymal and subcutaneous dorsolumbar WAT depots [14] were dissected.

2.2. Measurements of plasma non-esterified FA

Blood was collected by tail bleeds just before sacrifice and plasma was prepared using EDTA. Concentrations of non-esterified fatty acids (NEFA) were evaluated enzymatically (kit NEFA C from Wako Chemicals, Richmond, CA).

2.3. FA synthesis and oxidation in adipose tissue

Activity of FA synthesis was measured in fragments of adipose tissue incubated in the presence of glucose and insulin, and incorporation of radioactivity into saponifiable FA was estimated as described

0014-5793/$30.00
doi:10.1016/j.febslet.2005.09.078

previously [13], except that incubation medium also contained 1 mM pyruvate and ^3H_2O (0.5 mCi/ml), which was used as a radioactive tracer instead of glucose. Oxidation of oleate in the tissue fragments was measured as described previously [12].

2.4. RNA analysis

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was treated with RNase-free DNase. Gene expression was analyzed by reverse transcription followed by real time quantitative PCR (LightCycler Instrument, F. Hoffman-La Roche Ltd., Basel, Switzerland) with primers specific for sterol regulatory element-binding protein-1 (SREBP-1), fatty acid synthase (FAS), phosphoenolpyruvate carboxykinase (PEPCK), and UCP2 (Table 1). Levels of β-actin mRNA were used to correct for inter-sample variations [14].

2.5. Other analytical procedures

Quantification of tissue lipids [20], and evaluation of activity of the α1 isoform of AMPK (α1 AMPK) using immunoprecipitation of AMPK from tissue extracts and specific peptide assay was performed as described previously [12,21]. Western blotting was used for quantification of the total content of α1 AMPK and phosphorylated form of AMPK (pAMPK) and of the total content of acetyl-CoA carboxylase (ACC), and the phosphorylated form of ACC (pACC; see [12] for the detailed protocol).

2.6. Statistics

Statistical significance was evaluated using unpaired *t*-tests or two-way ANOVA. Differences were judged to be significant at $P < 0.05$.

3. Results

3.1. Changes in body weight, adiposity and NEFA in plasma

In initial experiments, effects of 6, 12, and 24 h of starvation were studied in mice maintained at 20 °C, while sacrificing all animals during the early light phase of the day (Protocol A in Section 2). Starvation resulted in a progressive loss of body weight over 24 h (Table 2). Both subcutaneous and epididymal fat tended to decrease in weight, although this was only significant for subcutaneous fat at 12 and 24 h after food deprivation. The reduction of adipose tissue weight during starvation was accompanied by a decrease in the content of tissue lipids in subcutaneous but not in epididymal fat after 24 h (Table 2). Starvation is known to be associated with the induction of lipolysis in WAT, and this was reflected by a rise in the plasma concentration of NEFA that was significant by 6 h and peaked at 12 h. All these data indicate that major changes of WAT metabolism had occurred in mice denied access to food for 6–12 h.

3.2. Fat depot-specific modulation of lipogenesis and FA oxidation

Suppression of the lipogenic genes in WAT due to starvation was observed. It was significantly greater in epididymal compared with subcutaneous fat. Thus, expression of SREBP-1, a transcription factor controlling lipogenesis in response to nutritional status [22], declined only transiently in subcutaneous fat, while the expression was progressively abolished during 24 h of starvation in epididymal fat (Fig. 1A). A gradual inhibition of the expression of FAS was observed in both depots, with a stronger effect in the epididymal fat (Fig. 1B). In parallel, the activity of FA synthesis also decreased to different extents in the two fat depots (Fig. 1C).

Previous studies have shown stimulation of FA oxidation in adipocytes isolated from epididymal and perirenal fat of starved rats [23]. Our measurement of oleate oxidation in

Table 1
Sequences of PCR primers

Gene	Sense primer (5′ → 3′)	Antisense primer (5′ → 3′)	GenBank Accession No. for cDNA
SREBP-1[a]	GCTTCCGGCCTGCTATGA	CAGGGCCTCGGTGTGCTC	AB017337, AF374266
FAS	GGCTGCCTCCGTGGACCTTATC	GTCTAGCCCTCCCGTACACTCACTCGT	X13135
PEPCK	CCAGCCAGTGCCCCATTATTGAC	TTTGCCGAAGTTGTAGCCGAAGAA	NM 011044
UCP2	GCCCGGGCTGGTGGTGGTC	CCCCGAAGGCAGAAGTGAAGTGG	U94593
β-Actin	GAACCCTAAGGCCAACCGTGAAAAGAT	ACCGCTCGTTGCCAATAGTGATG	X03765

[a]The primers detect the following splicing variants: SREBP-1A, SREBP-1A-W42, SREBP-1C and SREBP-1C-W42.

Table 2
Effects of starvation on body weight, fat pads weight, adipose tissue lipid content and plasma NEFA levels

	Fed mice	Starved mice			
		6 h	12 h	24 h	*n*
Loss of body weight (g)[a]	NA	1.2 ± 0.2	2.7 ± 0.1	3.7 ± 0.2	14–16
Weight of fat depots (mg)					
Subcutaneous fat	228 ± 20	197 ± 7	165 ± 9[b]	168 ± 15[b]	14–16
Epididymal fat	332 ± 24	313 ± 14	290 ± 18	297 ± 24	14–16
Tissue lipids (mg/depot)					
Subcutaneous fat	47.3 ± 7.7	52.9 ± 9.8	34.6 ± 7.7	25.8 ± 5.1[b]	4
Epididymal fat	163 ± 25	130 ± 37	124 ± 20	146 ± 40	4
NEFA (mM)	0.67 ± 0.05	1.17 ± 0.06[b]	1.85 ± 0.06[b,c]	1.51 ± 0.07[b,d]	14–16

Fed mice, and mice starved for various periods of time (Protocol A, see Section 2) were analyzed. The initial body weights in all groups were simila (27.1 ± 0.7 to 27.6 ± 0.8 g).
[a]The difference between the initial and final body weight is indicated; the corresponding values for four animals used for the estimation of tissue lipid were 1.9 ± 0.1, 3.2 ± 0.5, and 4.4 ± 0.1 g, respectively. Data are means ± S.E.
[b]Significant difference between fed and starved mice.
[c]Significant difference between 6 and 12 h of starvation.
[d]Significant difference between 12 and 24 h of starvation.

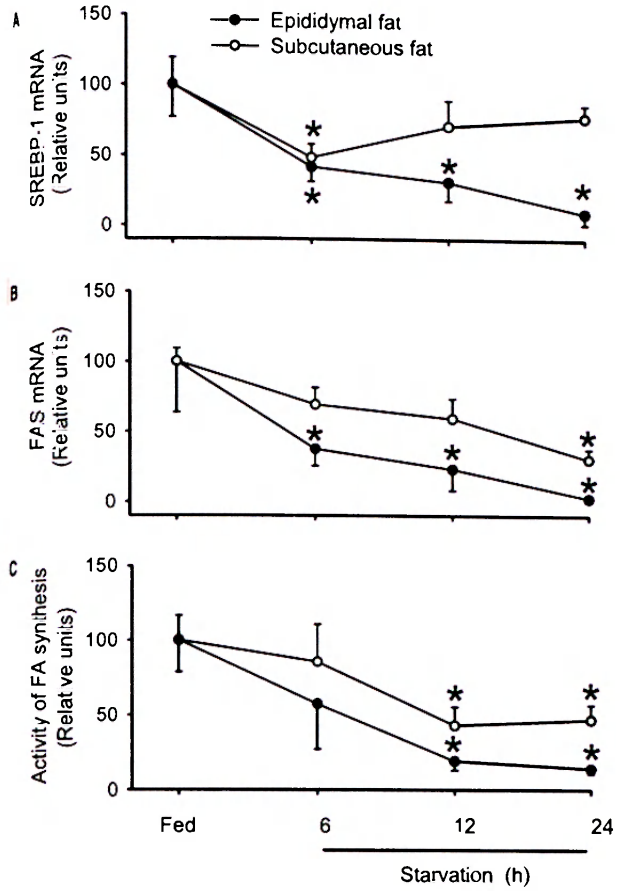

Fig. 1. Effect of starvation (Protocol A) on gene expression and FA synthesis in adipose tissue. All data are expressed in % relative to fed mice and they are means ± S.E.; $n = 6–8$. (A) SREBP-1 and (B) FAS mRNA level. In subcutaneous fat of fed mice, the levels of SREBP-1 transcript were similar, and that of FAS were significantly (2.1-fold) higher, compared with epididymal fat. (C) Activities of FA synthesis measured in tissue fragments. In subcutaneous adipose tissue of fed mice, the activities were significantly (2.3-fold) higher than in epididymal fat. Similar results were also obtained in measurements of FA synthesis in vivo (data not shown). Asterisks indicate significant effect of starvation.

WAT fragments from fed mice and mice starved for 12 h ($n = 7–9$) confirmed these findings and showed significant >2-fold stimulation of FA oxidation by starvation in epididymal fat (41 ± 4 vs. 101 ± 4 pmol FA/mg protein/h). In contrast, no change of FA oxidation could be detected in subcutaneous fat (95 ± 14 vs. 115 ± 11 pmol FA/mg protein/h).

3.3. Differential induction of PEPCK and UCP2 genes in epididymal and subcutaneous fat

Expression of both UCP2 [24] and PEPCK [25] genes in WAT is stimulated by food deprivation and either effect might be linked to the activation of AMPK (see Section 4). Levels of UCP2 mRNA were similar in both depots of fed mice, and increased during starvation (Fig. 2A). However, this increase was much greater in epididymal fat, peaking at 12 h after which there was a 4-fold difference between epididymal and subcutaneous fat. In epididymal fat of fed mice the mRNA levels of PEPCK were 2-fold higher than in subcutaneous fat and increased substantially during 24 h of starvation (Fig. 2B). By

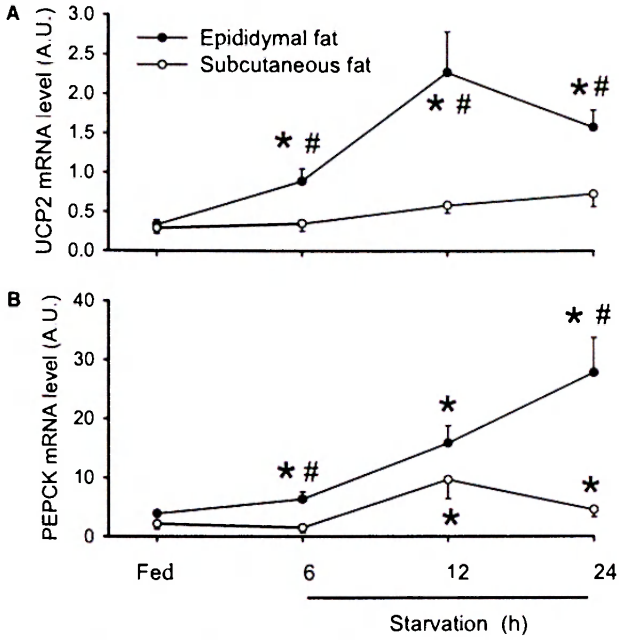

Fig. 2. Effect of starvation (Protocol A) on gene expression in adipose tissue. (A) UCP2 and (B) PEPCK mRNA level. All data are means ± S.E. ($n = 6–8$). Similar results were obtained when total RNA isolated from collagenase-released adipocytes was used for the analysis (data not shown). Asterisks indicate significant effect of starvation and hatched crosses significant differences between fat depots.

contrast, only a smaller and transient increase of PEPCK mRNA was observed in subcutaneous fat, so that after 24 h starvation the levels were about 6-fold lower than in epididymal fat (Fig. 2B).

3.4. Activation of the AMPK cascade

The above effects of starvation were all consistent with a differential activation of the AMPK cascade in epididymal and subcutaneous fat. Indeed, assays of the α1 AMPK activity in immunoprecipitates revealed a significant activation (1.9-fold) after 12 h, and no further changes during the subsequent 12 h of starvation in epididymal fat (Fig. 3A). In contrast, no changes of α1 AMPK activity could be detected in subcutaneous fat (Fig. 3A). The α2 AMPK activity was very low and did not change significantly in either fat depot (not shown). 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 (Fig. 3B). Total ACC content declined during 24 h of starvation, especially in subcutaneous fat (Fig. 3C). However, starvation significantly increased (1.6-fold) the amount of pACC, expressed relative to total ACC, in epididymal but not in subcutaneous fat (Fig. 3D) with a similar time course to α1 AMPK activity (Fig. 3A).

Mice normally eat most of their daily intake during the dark phase, and in response to prolonged starvation they exhibit torpor, i.e., a lowering of their body temperature and energy expenditure [26]. In order to separate any response to cold from that to starvation, activity of AMPK cascade in WAT was analyzed in animals maintained either at 20 or 30 °C (Table 3). In this experiment, animals were deprived of food just before entering the dark phase and sacrificed 6 h later,

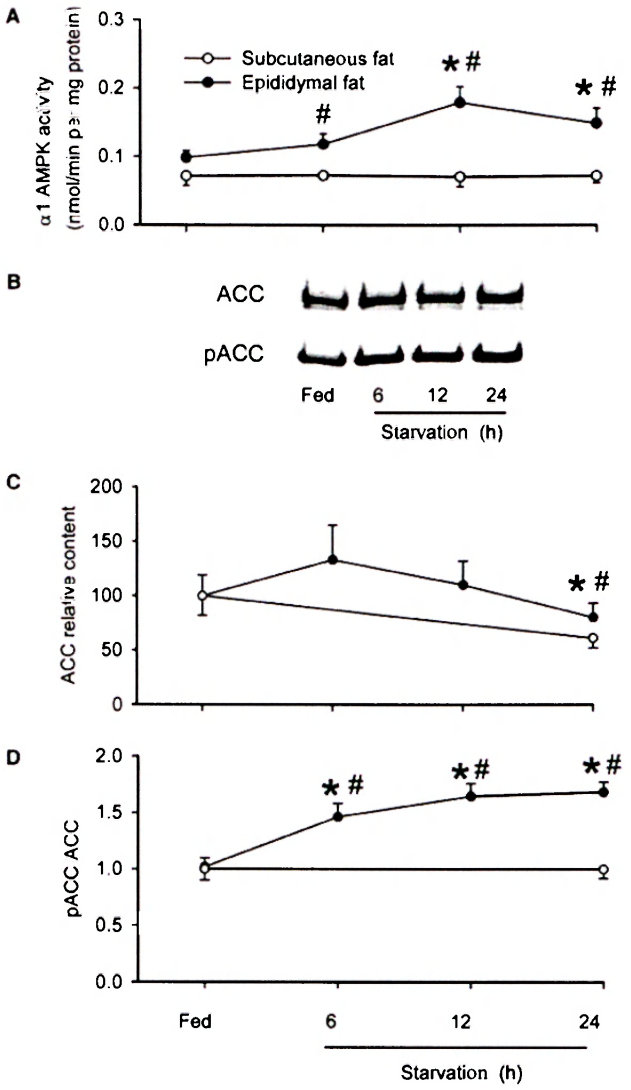

Fig. 3. Effects of starvation (Protocol A) on AMPK and ACC in adipose tissue. (A) α1 AMPK activity is expressed in nmol phosphate incorporated/min per mg protein of tissue extract used for immunoprecipitation. (B) Total content of ACC and pACC were quantified using Western blots, as illustrated for the analysis of epididymal fat. (C) Total content of ACC, relative to the content at time zero. (D) ACC phosphorylation; 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). For other details, see legend to Fig. 2.

together with the controls (Protocol B in Section 2). Even at 20 °C, only marginal depression (by about 0.8 °C) of core body temperature occurred after 6 h of starvation and whole body oxygen consumption was not affected until 12 h of starvation (data not shown). Estimation of AMPK phosphorylation using Western blots revealed activation of the enzyme due to starvation in epididymal but not in subcutaneous fat. In epididymal fat, starvation stimulated phosphorylation of ACC while an opposite effect was observed in subcutaneous fat. All these effects were similar at 20 and at 30 °C. In both temperatures, the total content of AMPK or ACC did not change in response to the starvation lasting for 6 h (Table 3).

4. Discussion

The principal finding of this report is that there is a fat depot-specific activation of the AMPK cascade in WAT during starvation. This activation, found in epididymal and not in subcutaneous fat, was convincingly documented using different and complementary experimental protocols in mice and it is in agreement with a similar activation of the AMPK found in the epididymal fat of starved rats [4]. The activation was not evoked by a decrease of body temperature occurring during starvation in mice although it could be augmented at lower ambient temperature.

Activation of the AMPK signalling pathway during starvation would contribute to a large depression of lipogenesis in epididymal fat. It may also account for stimulation of FA oxidation [23] selectively in epididymal fat, accompanied by a shift of glucose metabolism to a non-oxidative pattern [2]. In adipose tissue of starved rats, lactate becomes a major metabolite of glucose and the increase in lactate production with starvation is much higher in epididymal than in subcutaneous fat [2]. Because AMPK could stimulate glucose uptake in WAT by a mechanism independent of insulin [10], it could also induce glyceroneogenesis and recycling of FA into triglyceride in epididymal fat during starvation [27]. Recycling of FA consumes ATP and may decrease intracellular energy charge, and, in turn, activate AMPK. The recycling would be favoured by the much greater induction of PEPCK gene transcription in epididymal rather than subcutaneous fat that we report here. PEPCK is strictly transcriptionally regulated [28] and is required for glyceroneogenesis during starvation [29].

By analogy with the induction of fat accumulation by transgenic overexpression of PEPCK in adipocytes [30], a stronger stimulation of PEPCK-dependent FA re-esterification in epididymal fat may explain the lack of a decline in weight and lipid content of this fat depot during 24 h of food deprivation (see Table 2). In addition, the inhibition of basal lipolysis by AMPK in epididymal fat may result in a slower release of FA from adipocytes [31]. The changes in both FA re-esterification and release may balance the effects of increased FA oxidation and depressed lipogenesis on lipid content and tissue weight in epididymal fat and may be essential to preserve the energy pool in this tissue during the initial phases of food deprivation. A high rate of futile cycling of FA inside adipocytes would favor a fast activation of FA release during prolonged starvation [27]. Indeed, increased sensitivity of epididymal fat to the lipolytic action of epinephrine was noticed in rats starved for 72 h [32].

We report that the upregulation of UCP2 during starvation was greater in epididymal than in subcutaneous fat. UCP2 might increase the proton leak in mitochondria and thus reduce synthesis of ATP [33,34] and activate AMPK, similarly to transgenic mice over-expressing UCP3 in skeletal muscle [35] or UCP1 in WAT [12]. 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 [36]. The role of UCP2 in protection of WAT cells against the damage caused by reactive oxygen species, which would be formed at a higher rate when FA oxidation in mitochondria is increased, should be also considered [34].

The metabolism of WAT is under a complex neurohormonal control, exerted primarily by insulin and catecholamines [1]. Insulin inhibits AMPK in heart [37], while catecholamines acti-

Table 3
Effects of 6 h-starvation on phosphorylation and total contents of AMPK and ACC in white fat of mice maintained at different temperatures

	20 °C		30 °C	
	Fed mice	Starved mice	Fed mice	Starved mice
pAMPK/AMPK				
Subcutaneous fat	1.00 ± 0.02	1.10 ± 0.06	0.99 ± 0.09	1.04 ± 0.10
Epididymal fat	1.00 ± 0.07	1.55 ± 0.13[a]	1.00 ± 0.04	1.21 ± 0.05[a]
AMPK				
Subcutaneous fat	1.00 ± 0.11	0.80 ± 0.07	0.94 ± 0.10	0.86 ± 0.15
Epididymal fat	1.00 ± 0.08	0.80 ± 0.09	0.80 ± 0.07	0.74 ± 0.06
pACC/ACC				
Subcutaneous fat	1.00 ± 0.05	0.56 ± 0.07[a]	0.91 ± 0.11	0.65 ± 0.07[a]
Epididymal fat	1.00 ± 0.38	1.81 ± 0.24[a]	1.00 ± 0.14	1.48 ± 0.14[a]
ACC				
Subcutaneous fat	1.00 ± 0.23	1.14 ± 0.29	1.07 ± 0.04	0.85 ± 0.03[b]
Epididymal fat	1.00 ± 0.11	0.86 ± 0.13	1.06 ± 0.27	1.10 ± 0.18

Mice fed or starved (Protocol B, see Section 2) were analyzed. Content of pAMPK, total AMPK, pACC, and total ACC was quantified using Western blots (see Fig. 2 of this manuscript and Fig. 2 of [12]). Phosphorylation of AMPK and ACC, respectively, is expressed as a ratio between two forms of the enzymes. Results are expressed relative to the values for fed mice at 20 °C. Data are means ± S.E. ($n = 6$).
[a]Significant effect of starvation.
[b]Significant effect of temperature.

vate AMPK in adipocytes [4]. Responsiveness of adipocytes to both insulin [38] and β-adrenergic agonist [39] is higher in epididymal than subcutaneous fat. Therefore, the differential activation of AMPK in the two fat depots during starvation may be related to the effects of insulin and circulating catecholamines. Moreover, activation of sympathetic innervation of WAT in the abdomen was found in rats during prolonged fasting [40] and differential sympathetic (see [41]) as well as parasympathetic [42] innervation of abdominal and subcutaneous WAT exists in rodents. This suggests a possibility for the involvement of autonomic innervation in the responsiveness of various fat depots to starvation and other stimuli like exposure to cold, which also increases sympathetic innervation of WAT [43].

In summary, the control of lipid and glucose metabolism in WAT by AMPK may represent a basic biological mechanism that contributes to regional differences in the metabolic properties of adipose tissue depots. This mechanism may contribute to the development of adverse health consequences of obesity and represents a promising therapeutic target.

Acknowledgments: This work was supported by the Czech Science Foundation (303/05/2580), the Grant Agency of the ASCR (KJB5011303), the Wellcome Trust Collaborative Research Initiative Grant (02760), and the European Commission (LSHM-CT-2004-005272). M.T.H. and D.G.H. were supported by a Project Grant from Diabetes UK. K.J.M. was supported by a Research Studentship jointly funded by the Biotechnology and Biological Sciences Research Council (UK) and by Novo-Nordisk.

References

[1] Kopelman, P.G. (1998) Effects of obesity on fat topography: metabolic and endocrine determinants in: Clinical Obesity (Kopelman, P.G. and Stock, M.J., Eds.), pp. 158–175, Blackwell Science Ltd, Oxford.
[2] Newby, F.D., Sykes, M.N. and DiGirolamo, M. (1988) Regional differences in adipocyte lactate production from glucose. Am. J. Physiol. 255, E716–E722.
[3] Li, Y., Bujo, H., Takahashi, K., Shibasaki, M., Zhu, Y., Yoshida, Y., Otsuka, Y., Hashimoto, N. and Saito, Y. (2003) Visceral fat: higher responsiveness of fat mass and gene expression to calorie restriction than subcutaneous fat. Exp. Biol. Med. (Maywood) 228, 1118–1123.
[4] Daval, M., Diot-Dupuy, F., Bazin, R., Hainault, I., Viollet, B., Vaulont, S., Hajduch, E., Ferre, P. and Foufelle, F. (2005) Anti-lipolytic action of AMP-activated protein kinase in rodent adipocytes. J. Biol. Chem. 280, 25250–25257.
[5] Witters, L.A., Gao, G., Kemp, B.E. and Quistorff, B. (1994) Hepatic 5′-AMP-activated protein kinase: Zonal distribution and relationship to acetyl-CoA carboxylase activity in varying nutritional states. Arch. Biochem. Biophys. 308, 413–419.
[6] Hardie, D.G. (2004) The AMP-activated protein kinase pathway – new players upstream and downstream. J. Cell Sci. 117, 5479–5487.
[7] Sullivan, J.E., Brocklehurst, K.J., Marley, A.E., Carey, F., Carling, D. and Beri, R.K. (1994) Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase. FEBS Lett. 353, 33–36.
[8] Garton, A.J., Campbell, D.G., Carling, D., Hardie, D.G., Colbran, R.J. and Yeaman, S.J. (1989) Phosphorylation of bovine hormone-sensitive lipase by the AMP-activated protein kinase. A possible antilipolytic mechanism. Eur. J. Biochem. 179, 249–254.
[9] Corton, J.M., Gillespie, J.G., Hawley, S.A. and Hardie, D.G. (1995) 5-Aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur. J. Biochem. 229, 558–565.
[10] Salt, I.P., Connell, J.M. and Gould, G.W. (2000) 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) inhibits insulin-stimulated glucose transport in 3T3-L1 adipocytes. Diabetes 49, 1649–1656.
[11] Kopecky, J., Clarke, G., Enerback, S., Spiegelman, B. and Kozak, L.P. (1995) Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. J. Clin. Invest. 96, 2914–2923.
[12] Matejkova, O., Mustard, K.J., Sponarova, J., Flachs, P., Rossmeisl, M., Miksik, I., Thomason-Hughes, M., Hardie, D.G. and Kopecky, J. (2004) Possible involvement of AMP-activated protein kinase in obesity resistance induced by respiratory uncoupling in white fat. FEBS Lett. 569, 245–248.
[13] Rossmeisl, M., Syrový, I., Baumruk, F., Flachs, P., Janovská, P. and Kopecký, J. (2000) Decreased fatty acid synthesis due to mitochondrial uncoupling in adipose tissue. FASEB J. 14, 1793–1800.
[14] Flachs, P., Novotny, J., Baumruk, F., Bardova, K., Bourova, L., Miksik, I., Sponarova, J., Svoboda, P. and Kopecky, J. (2002) Impaired noradrenaline-induced lipolysis in white fat of

aP2-Ucp1 transgenic mice is associated with changes in G-protein levels. Biochem. J. 364, 369–376.
[15] Rossmeisl, M., Barbatelli, G., Flachs, P., Brauner, P., Zingaretti, M.C., Marelli, M., Janovska, P., Horakova, M., Syrovy, I., Cinti, S. and Kopecky, J. (2002) Expression of the uncoupling protein 1 from the aP2 gene promoter stimulates mitochondrial biogenesis in unilocular adipocytes in vivo. Eur. J. Biochem. 269, 1–10.
[16] Ruderman, N.B., Park, H., Kaushik, V.K., Dean, D., Constant, S., Prentki, M. and Saha, A.K. (2003) AMPK as a metabolic switch in rat muscle, liver and adipose tissue after exercise. Acta Physiol. Scand. 178, 435–442.
[17] Wu, X., Motoshima, H., Mahadev, K., Stalker, T.J., Scalia, R. and Goldstein, B.J. (2003) Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. Diabetes 52, 1355–1363.
[18] Saha, A.K., Avilucea, P.R., Ye, J.M., Assifi, M.M., Kraegen, E.W. and Ruderman, N.B. (2004) Pioglitazone treatment activates AMP-activated protein kinase in rat liver and adipose tissue in vivo. Biochem. Biophys. Res. Commun. 314, 580–585.
[19] Orci, L., Cook, W.S., Ravazzola, M., Wang, M.Y., Park, B.H., Montesano, R. and Unger, R.H. (2004) Rapid transformation of white adipocytes into fat-oxidizing machines. Proc. Natl. Acad. Sci. USA 101, 2058–2063.
[20] Dole, V.P. (1956) A relation between non-esterified fatty acid in plasma and the metabolism of glucose. J. Clin. Invest. 35, 150–154.
[21] Hawley, S.A., Gadalla, A.E., Olsen, G.S. and Hardie, D.G. (2002) The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. Diabetes 51, 2420–2425.
[22] Kim, J.B., Sarraf, P., Wright, M., Yao, K.M., Mueller, E., Solanes, G., Lowell, B.B. and Spiegelman, B.M. (1998) Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. J. Clin. Invest. 101, 1–9.
[23] Wang, T., Zang, Y., Ling, W., Corkey, B.E. and Guo, W. (2003) Metabolic partitioning of endogenous fatty acid in adipocytes. Obes. Res. 11, 880–887.
[24] Millet, L., Vidal, H., Andreelli, F., Larrouy, D., Riou, J.P., Ricquier, D., Laville, M. and Langin, D. (1997) Increased uncoupling protein-2 and -3 mRNA expression during fasting in obese and lean humans. J. Clin. Invest. 100, 2665–2670.
[25] Gorin, E., Tal-Or, Z. and Shafrir, E. (1969) Glyceroneogenesis in adipose tissue of fasted, diabetic and triamcinolone treated rats. Eur. J. Biochem. 8, 370–375.
[26] Overton, J.M. and Williams, T.D. (2004) Behavioral and physiologic responses to caloric restriction in mice. Physiol. Behav. 81, 749–754.
[27] Kalderon, B., Mayorek, N., Berry, E., Zevit, N. and Bar-Tana, J. (2000) Fatty acid cycling in the fasting rat. Am. J. Physiol. Endocrinol. Metab. 279, E221–E227.
[28] Hanson, R.W. and Reshef, L. (1997) Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annu. Rev. Biochem. 66, 581–611.
[29] Beale, E.G., Hammer, R.E., Antoine, B. and Forest, C. (2002) Glyceroneogenesis comes of age. FASEB J. 16, 1695–1696.
[30] Franckhauser, S., Munoz, S., Pujol, A., Casellas, A., Riu, E., Otaegui, P., Su, B. and Bosch, F. (2002) Increased fatty acid re-esterification by PEPCK overexpression in adipose tissue leads to obesity without insulin resistance. Diabetes 51, 624–630.
[31] Lyon, I., Ookhtens, M., Montisano, D. and Baker, N. (1988) Fat pad triacylglycerol fatty acid loss and oxidation as indices of total body triacylglycerol fatty acid mobilization and oxidation in starving mice. Biochim. Biophys. Acta 958, 188–198.
[32] Zapf, J., Waldvogel, M. and Froesch, E.R. (1977) Increased sensitivity of rat adipose tissue to the lipolytic action of epinephrine during fasting and its reversal during re-feeding. FEBS Lett. 76, 135–138.
[33] Echtay, K.S., Winkler, E., Frischmuth, K. and Klingenberg, M. (2001) Uncoupling proteins 2 and 3 are highly active H+ transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone). Proc. Natl. Acad. Sci. USA 98, 1416–1421.
[34] Joseph, J.W., Koshkin, V., Saleh, M.C., Sivitz, W.I., Zhang, C.Y., Lowell, B.B., Chan, C.B. and Wheeler, M.B. (2004) Free fatty acid-induced beta-cell defects are dependent on uncoupling protein 2 expression. J. Biol. Chem. 279, 51049–51056.
[35] Schrauwen, P., Hardie, D.G., Roorda, B., Clapham, J.C., Abuin, A., Thomason-Hughes, M., Green, K., Frederik, P.M. and Hesselink, M.K. (2004) Improved glucose homeostasis in mice overexpressing human UCP3: A role for AMP-kinase? Int. J. Obes. Relat. Metab. Disord. 28, 824–828.
[36] Pedersen, S.B., Lund, S., Buhl, E.S. and Richelsen, B. (2001) Insulin and contraction directly stimulate UCP2 and UCP3 mRNA expression in rat skeletal muscle in vitro. Biochem. Biophys. Res. Commun. 283, 19–25.
[37] Beauloye, C., Marsin, A.S., Bertrand, L., Krause, U., Hardie, D.G., Vanoverschelde, J.L. and Hue, L. (2001) Insulin antagonizes AMP-activated protein kinase activation by ischemia or anoxia in rat hearts, without affecting total adenine nucleotides. FEBS Lett. 505, 348–352.
[38] Santos, R.F., Sztalryd, C. and Reaven, G. (1991) Effect of anatomical site on insulin action and insulin receptor phosphorylation in isolated rat adipocytes. Int. J. Obes. 15, 755–762.
[39] Portillo, M.P., Villaro, J.M., Torres, M.I. and Macarulla, M.T. (2000) In vivo lipolysis in adipose tissue from two anatomical locations measured by microdialysis. Life Sci. 67, 437–445.
[40] Migliorini, R.H., Garofalo, M.A.R. and Kettelhut, I.C. (1997) Increased sympathetic activity in rat white adipose tissue during prolonged fasting. Am. J. Physiol. 272, R656–R661.
[41] Bowers, R.R., Festuccia, W.T., Song, C.K., Shi, H., Migliorini, R.H. and Bartness, T.J. (2004) Sympathetic innervation of white adipose tissue and its regulation of fat cell number. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286, R1167–R1175.
[42] Kreier, F., Fliers, E., Voshol, P.J., Van Eden, C.G., Havekes, L.M., Kalsbeek, A., Van Heijningen, C.L., Sluiter, A.A., Mettenleiter, T.C., Romijn, J.A., Sauerwein, H.P. and Buijs, R.M. (2002) Selective parasympathetic innervation of subcutaneous and intra-abdominal fat-functional implications. J. Clin. Invest. 110, 1243–1250.
[43] Garofalo, M.A.R., Kettelhut, C., Roselino, J.E.S. and Migliorini, R.H. (1996) Effect of acute cold exposure on norepinephrine turnover in rat white adipose tissue. J. Auton. Nerv. Syst. 60, 206–208.

PUBLICATION C.

Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce β-oxidation in white fat

Flachs P, **Horáková O**, Brauner P, Rossmeisl M, Pecina P, Franssen-van Hal N., Ružicková J, Šponarová J, Drahota Z, Vlček C, Keijer J, Houštek J, Kopecký J.

Diabetologia 48: 2365-2375 (2005)

Diabetologia (2005) 48: 2365–2375
DOI 10.1007/s00125-005-1944-7

ARTICLE

P. Flachs · O. Horakova · P. Brauner · M. Rossmeisl · P. Pecina · N. Franssen-van Hal · J. Ruzickova · J. Sponarova · Z. Drahota · C. Vlcek · J. Keijer · J. Houstek · J. Kopecky

Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce β-oxidation in white fat

Received: 10 March 2005 / Accepted: 13 June 2005 / Published online: 5 October 2005

Abstract *Aims/hypothesis:* Intake of *n*-3 polyunsaturated fatty acids reduces adipose tissue mass, preferentially in the abdomen. The more pronounced effect of marine-derived eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids on adiposity, compared with their precursor α-linolenic acid, may be mediated by changes in gene expression and metabolism in white fat. *Methods:* The effects of EPA/DHA concentrate (6% EPA, 51% DHA) admixed to form two types of high-fat diet were studied in C57BL/6J mice. Oligonucleotide microarrays, cDNA PCR subtraction and quantitative real-time RT-PCR were used to characterise gene expression. Mitochondrial proteins were quantified using immunoblots. Fatty acid oxidation and synthesis were measured in adipose tissue fragments. *Results:* Expression screens revealed upregulation of genes for mitochondrial proteins, predominantly in epididymal fat when EPA/DHA concentrate was admixed to a semisynthetic high-fat diet rich in α-linolenic acid. This was associated with a threefold stimulation of the expression of genes encoding regulatory factors for mitochondrial biogenesis and oxidative metabolism (peroxisome proliferator-activated receptor gamma coactivator 1 alpha [*Ppargc1a*, also known as *Pgc1α*] and nuclear respiratory factor-1 [*Nrf1*] respectively). Expression of genes for carnitine palmitoyltransferase 1A and fatty acid oxidation was increased in epididymal but not subcutaneous fat. In the former depot, lipogenesis was depressed. Similar changes in adipose gene expression were detected after replacement of as little as 15% of lipids in the composite high-fat diet with EPA/DHA concentrate, while the development of obesity was reduced. The expression of *Ppargc1a* and *Nrf1* was also stimulated by *n*-3 polyunsaturated fatty acids in 3T3-L1 cells. *Conclusions/interpretation:* The anti-adipogenic effect of EPA/DHA may involve a metabolic switch in adipocytes that includes enhancement of β-oxidation and upregulation of mitochondrial biogenesis.

Keywords Adipose tissue · Carnitine palmitoyltransferase 1 · Fish oil · Flax-seed oil · Metabolism · Mitochondria · *n*-3 Polyunsaturated fatty acids · PGC-1 · PPARGC1A

Abbreviations ACOX1: acyl-Coenzyme A oxidase 1 · ALA: α-linolenic acid · ATP5A1: subunit α of F_1-ATPase · AU: arbitrary units · cHF diet: composite high-fat diet based on chow · cHF-F1 and sHFf-F2: high-fat diets with partial replacement of lipids by fish oil concentrate · COX6: cytochrome c oxidase subunit VI · CPT1A: carnitine palmitoyltransferase 1A · DHA: docosahexaenoic acid · EPA: eicosapentaenoic acid · EPA/DHA concentrate: concentrate of *n*-3 polyunsaturated fatty acids from fish oil (6% EPA and 51% DHA) · ESM: Electronic Supplementary Material · MT-ATP6 and MT-ATP8: subunits 6 and 8 of mitochondrial ATPase · MT-CO1 and MT-CO3: mitochondrial cytochrome *c* oxidase subunits I and III · MT-ND1: subunit 1 of mitochondrial NADH dehydrogenase (complex I) · MT-ND4: subunit 4 of mitochondrial NADH dehydrogenase (complex I) · NRF1: nuclear respiratory factor-1 · PPAR: peroxisome

Electronic Supplementary Material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s00125-005-1944-7

P. Flachs · O. Horakova · P. Brauner · M. Rossmeisl · J. Ruzickova · J. Sponarova · J. Kopecky (✉)
Department of Adipose Tissue Biology, Institute of Physiology, Academy of Sciences of the Czech Republic,
Videnska 1083,
142 20 Prague, Czech Republic
e-mail: kopecky@biomed.cas.cz
Tel.: +420-24106255
Fax: +420-241062599

P. Pecina · Z. Drahota · J. Houstek
Department of Bioenergetics,
Institute of Physiology,
Academy of Sciences,
Prague, Czech Republic

N. Franssen-van Hal · J. Keijer
RIKILT-Institute of Food Safety,
Wageningen, The Netherlands

C. Vlcek
Department of Genomics and Bioinformatics,
Institute of Molecular Genetics, Academy of Sciences,
Prague, Czech Republic

proliferative activated receptor · PPAR GC1A: PPARγ coactivator-1α · PTP4A1: protein tyrosine phosphatase 4a1 · PUFA: polyunsaturated fatty acids · qRT-PCR: quantitative real-time RT-PCR · SCD1: stearoyl-CoA desaturase 1 · SDHA: 70 kDa protein subunit of mitochondrial complex II · sHFf diet: semisynthetic high-fat diet · UCP: uncoupling protein

Introduction

Type 2 diabetes exhibits a much stronger dependence on obesity than other health consequences of excessive accumulation of body fat. One of the major causes of obesity is high intake of lipids [1]. Studies in rodents [2–7] have demonstrated that saturated fats facilitate adipose tissue deposition more effectively than polyunsaturated fatty acids (PUFA). The long-chain *n*-3 PUFA eicosapentaenoic acid (EPA; 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*-3), which are abundant in marine fish oil, are more potent in reducing adiposity than *n*-3 PUFA from plants, such as α-linolenic acid (ALA, 18:3 *n*-3 [4, 8]). The intake of DHA appears to be particularly important [5, 8]. In mammals, ALA is a precursor of EPA and DHA, but is rapidly oxidised in the organism and its conversion to EPA and DHA is quite inefficient [6, 9, 10]. Hence, dietary fish oil supplement is a more effective source of tissue DHA than ALA [6, 10]. Also in humans, dietary fish oil may decrease body fat content [11]. Furthermore, EPA and DHA act as hypolipidaemics, exert prophylactic effects on cardiovascular disease and may improve insulin sensitivity (references, see [4, 9]). Therefore, current dietary recommendations favour a substantial decrease in the *n*-6: *n*-3 PUFA ratio [6, 9].

The effect of *n*-3 PUFA on fat deposition does not result from a reduction in food intake [2–6, 8, 11], but rather reflects metabolic changes in several tissues [6, 12], such as stimulation of lipid oxidation and inhibition of lipogenesis in liver (references, see [6, 12]), stimulation of fatty acid oxidation in muscle [13], as well as adaptive thermogenesis mediated by mitochondrial uncoupling protein 1 (UCP1) in brown fat [2, 14]. In humans, whole-body lipid oxidation was increased by dietary fish oil [11]. The anti-adipogenic effect of *n*-3 PUFA may be also mediated by changes occurring in white adipose tissue itself [5, 15]. Studies in rodents indicate a complex modulation of gene expression in white adipose tissue by *n*-3 PUFA [6, 12], especially in fat depots in the abdominal region, which are preferentially reduced [3, 5, 6, 8, 16]. The changes in gene expression suggest a decrease in lipogenesis and fatty acid release from adipocytes [5] and an enhancement of mitochondrial respiration [3] and glucose uptake [8] in these cells.

The main goals of this study were to investigate the effects of EPA and DHA, compared with ALA, on gene expression in white adipose tissue, and to test the hypothesis that these effects limit the development of obesity. Experiments were conducted in mice fed two types of high-fat diet, either a semisynthetic diet (sHFf) or a composite diet based on chow (cHF; see Materials and methods and [8]). The sHFf diet is well defined and made it possible to study the separate effects of EPA and DHA, and ALA, respectively. Similar purified diets have been used in previous studies in rodents. While these diets do not promote obesity in mice [2–5, 8, 13, 14], the cHF diet does [8, 17]. We show here that replacement of some of the dietary lipids with EPA and DHA induced expression of mitochondrial genes in white adipose tissue, as well as the gene encoding their upstream regulatory factor, PPARγ coactivator-1α (PPARGC1A, also known as PGC1α). This factor links nuclear receptors to the transcriptional program of mitochondrial biogenesis and oxidative metabolism in adipocytes and muscle cells [18–20] and to gluconeogenesis in liver [21]. We also demonstrate that the expression of the gene for carnitine palmitoyltransferase 1A(*Cpt1a*) and oxidation of fatty acids was stimulated in epididymal but not subcutaneous white adipose tissue. Our data suggest that upregulation of mitochondrial biogenesis and induction of β-oxidation, as well as suppression of lipogenesis in adipocytes, are involved in the preferential reduction of lipid accumulation by EPA and DHA in abdominal fat.

Materials and methods

Animals and diet

Male C57BL/6J mice were imported from the Jackson Laboratory (Bar Habor, ME, USA) and bred at the Institute of Physiology for up to 16 generations. Male mice were used for the experiments. Animals were housed in a controlled environment (20°C; 12-h light–12-h dark cycle; light from 06.00 h) with free access to water and standard chow [17]. At 4 months of age, singly caged animals were randomly assigned for 4 weeks to one of the high-fat diets described previously (see Tables 1, 2, 3 of Ref. [8] and the Electronic Supplementary Material [ESM] Tables 1 and 2); the sHFf diet, which contained 20% (wt/wt) flax-seed oil (rich in ALA) as the only lipid; or the sHFf-F2 diet, which had the same composition as the preceding diet except that 44% of lipids were replaced by *n*-3 PUFA concentrate containing 6% EPA and 51% DHA (EPAX 1050TG; Pronova Biocare, Lysaker, Norway; EPA/DHA concentrate). Some 4-month-old animals were also habituated for 2 weeks to the cHF diet, derived from standard chow and containing 35% (wt/wt) lipids of very low *n*-3 PUFA content. These were then assigned for 5 weeks to the cHF diet or to the cHF-F1 diet, which had the same composition as the cHF diet except that 15% (wt/wt) of lipids were replaced with EPAX 1050TG [8]. Individual food intake was determined as before [8]. Mice were killed by cervical dislocation and subcutaneous dorsolumbar and epididymal white adipose tissue depots [8] were dissected. Tissues were either used immediately for measurement of fatty acid metabolism, or stored in liquid nitrogen for RNA analysis and immunoblotting. The experiments were conducted under the guidelines for the use and care of laboratory animals of the Institute of Physiology, Academy of Sciences of the Czech Republic.

Table 1 Effects of EPA/DHA concentrate on body weight, fat depots and food consumption

	Diet			
	sHFf	sHFf-F2	cHF	cHF-F1
Body weight (g)				
Initial[a]	26.4±0.5	26.4±0.4	27.5±0.6	27.8±0.7
Final[a]	26.9±0.7	23.4±0.6[b]	35.4±1.1	33.4±0.7
Change[a]	0.4±0.5	−2.9±0.8[b]	7.75±0.7	6.25±0.6[c]
Epididymal fat weight (mg)	481±38	326±24[b]	2093±97	1741±122[c]
Dorsolumbar fat weight (mg)	215±12	197±16	679±45	640±40
Food consumption[a] (kJ/day per animal)	71.2±6.2	70.8±5.1	65.5±3.1	66.3±4.2
n	12	12	11	12

Data are means±SEM
Mice were fed semisynthetic diets (sHFf or sHFf-F2) and used for the analysis of gene expression in adipose tissue (Table 2, Fig. 2), or fed composite diets (cHF or cHF-F2) and used for the analysis of gene expression in collagenase-liberated adipocytes (Table 4)
[a]With respect to the whole period of dietary treatment
[b]Statistically significant difference compared with sHFf diet
[c]Statistically significant difference compared with cHF diet

Differentiation of 3T3-L1 adipocytes

Eleven days after induction of cell differentiation [22] and 24 h before RNA isolation, a complete change of the medium was performed using serum-free Dulbecco's modified Eagle medium containing 0.5% fatty acid-free BSA, 830 nmol/l insulin and 0.5 mmol/l oleic acid in the form of BSA/fatty acid sodium salt. Medium was further supplemented with ethanolic solution of fatty acid: (1) 0.2 mmol/l oleic acid; (2) 0.2 mmol/l ALA; or (3) 0.2 mmol/l DHA, and incubated for 1 h before use.

RNA isolation

Total RNA was isolated and purified using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol of the supplier with additional phenol/chloroform extraction.

DNA and protein content

DNA was measured fluorometrically in tissue samples digested with proteinase K, and protein concentration was assessed using the bicinchoninic acid procedure and BSA as standard [23].

cDNA PCR subtraction

RNA was isolated from epididymal fat pads pooled from six mice fed the sHFf or the sHFf-F2 diet. cDNA was generated using the SMART PCR cDNA Synthesis Kit (BD Biosciences, San Jose, CA, USA). To detect the genes up-regulated by the sHFf-F2 diet, PCR-based cDNA subtraction was carried out with the PCR-Select cDNA Subtraction Kit (BD Biosciences). Subtracted cDNA libraries were subcloned (T/A cloning) into bacteria and analysed by colony array hybridisation using ^{32}P-labelled cDNA probes (DECAprime II DNA Labeling Kit; Ambion, Austin, TX, USA). Selected cDNAs were sequenced (DNA sequencer CEQ2000XL; Beckman Coulter, Harbor Boulevard, CA, USA) and DNA sequences were analysed using BLAST (GenBank, NIH).

Oligonucleotide microarray

Each RNA sample isolated from dorsolumbar and epididymal fat of sHFf and sHFf-F2 mice (nine animals per group) was indirectly labelled [24] and processed separately. In the reverse transcription step, aminoallyl dUTP was incorporated and then chemically coupled to fluorophores. RNA samples (30 μg) were separately labelled with Cy5 dye. A common reference sample (a pool representing all the investigated samples) was also labelled with Cy3. High-density microarrays, based on commercial oligonucleotide library (Mouse 10k A set; MWG Biotech, Ebersberg bei München, Germany) were printed on epoxy slides (Quantifoil, Jena, Germany) using a MicroGrid II (Genomic Solutions, Ann Arbor, MI, USA), pretreated, hybridised overnight at 42°C in a humid hybridisation chamber, and read using a laser scanner (ScanArray Express; Perkin-Elmer, Boston, MA, USA). To correct for fluctuations in RNA sample size and the amount of DNA spotted, signals were normalised [25]. All Cy3 and Cy5 signals lower than 2.5-fold background value were excluded from the analysis (approximately 60% of spots). Identification of differentially expressed genes was done using discriminant analysis, which is a supervised form of principal components analysis (Genemath software; Applied Maths, Sint-Martens-Lathem, Belgium). Samples were first assigned to their diet intervention group, then separated along the dimension of maximum

Table 2 Differential gene expression in mice fed sHFf and sHFf-F2 diets

Transcript	Accession number	Function	Epididymal fat				Dorsolumbar fat		
			cDNA subtraction[a]	Microarray[b] (fold change)	qRT-PCR[c] (AU)		Microarray[b] (fold change)	qRT-PCR[c] (AU)	
					sHFf diet	sHF-F2 diet		sHFf diet	sHF-F2 diet
Induction by sHFf-F2 diet									
mt-Co1	J01420_3	OXPHOS		1.6[d]	0.41±0.01	1.21±0.02[d]	1.2	0.78±0.02	0.97±0.02
mt-Co3	AB042809_7	OXPHOS	Yes	1.8[d]	0.79±0.02	1.44±0.02[d]	1.7[d]	0.63±0.02	1.19±0.01[d]
mt-Nd1	AB049357_1	OXPHOS		1.8[d]			1.7[d]		
mt-Nd4	AB042809_10	OXPHOS		1.9[d]			1.8[d]		
mt-Atp6	AB042432_6	OXPHOS	Yes	1.7[d]			1.8[d]		
mt-Atp8	AB042432_5	OXPHOS		1.7[d]			1.7[d]		
Adipsin	NM_013459_1	Fatty acid intake		1.7[d]	0.82±0.01	1.88±0.04[d]	1.8[d]	0.64±0.01	1.27±0.02[d]
Septin 6	NM_019942_1	Cell division		2.1[d]			1.4		
mKiaa0195	BC007157_1	Unknown		4.0[d]			1.1		
cPges	AB024935	Prostaglandin metabolism	Yes		0.33±0.01	0.65±0.01[d]			
Ptp4a1	AK078120	Cell differentiation	Yes		0.19±0.01	0.39±0.01[d]			
Arkadia	AK036351	Cytokine signalling	Yes						
Hgrg8	AK031616	High glucose response	Yes						
Depression by sHFf-F2 diet									
Actin beta	NM_007393_1	Cytoskeleton		3.3[d]	0.44±0.01	0.20±0.02[d]	2.1[d]		
Scd1	NM_009127_1	Lipogenesis		2.5[d]	0.29±0.04	0.17±0.05[d]	3.2[d]		
Dgat2	NM_026384_1	Lipogenesis		2.2[d]			1.0		
Resistin like α	NM_020509_1	Insulin sensitivity		2.5[d]			1.8		
Gnas	NM_010309_1	Hormonesignalling		2.5[d]			1.4		
Npc2	NM_023409_1	Lipid transport		2.5[d]			2.5[d]		
P-lysozyme	NM_013590_1	Non-specific immunity		2.5[d]			2.4[d]		
Ubiquitin c	NM_019639_1	Proteolysis		1.2			2.3[d]		
A730008L03Rik	NM_021393	Unknown		0.9			3.1[d]		

[a] The analysis was designed to detect genes upregulated by the sHFf-F2 diet

[b] The most discriminating genes with significantly different levels of expression in at least one of the tissues are listed (see Results and Materials and methods)

[c] Analysis was performed only where indicated; data are means±SEM (n=12)

[d] Significant effect of diet

AU arbitrary units; *mt-Atp6* and *mt-Atp8* subunits 6 and 8 of mitochondrial ATPase; *cPges* cytosolic prostaglandin E_2 synthase; *Gnas*α-subunits of stimulatory G-protein; *Hgrg8* high glucose-regulated protein 8; *mt-Nd1* and *mt-Nd4* subunits 1 and 4 of mitochondrial NADH dehydrogenase (complex I); *OXPHOS* oxidative phosphorylation; *Npc2* Niemann–Pick type C2 protein; *Ptp4a1* protein tyrosine phosphatase 4a1

See Results for other abbreviations. The animals described in Table 1 were used

Table 3 Quantification of the protein components of the oxidative phosphorylation system in sHFf and sHFf-F2 diet-fed mice

Fat depot	Diet	SDHA	MT-CO1	COX6	ATP5A1
Epididymal fat	sHFf	0.25±0.04	0.37±0.07	0.28±0.02	0.30±0.06
	sHFf-F2	0.48±0.06[a]	0.68±0.08[a]	0.67±0.14[a]	0.44±0.03[a]
Dorsolumbar fat	sHFf	0.42±0.04[b]	0.34±0.06	0.43±0.08	0.33±0.07
	sHFf-F2	0.46±0.07	0.32±0.07[b]	0.38±0.07	0.39±0.07

Individual antigens were quantified in crude cell membranes isolated from adipose tissues using immunoblots and their content was expressed in AU using isolated mouse liver mitochondria as a standard (see Fig. 1)
Data are means±SEM (n=9). The effect of diet in fat depots (two-way ANOVA) was different, except for ATP5A1
[a]Significant differences between diets
[b]Significant differences between fat depots

variation in gene expression between the groups and the 0.5% of genes that contributed most to the variation (the discriminating genes) were subsequently selected. Finally, from the selected genes only those genes were considered that exerted significantly different (t-test) levels of expression in at least one of the tissues analysed.

Quantitative real-time RT-PCR

This analysis (qRT-PCR) was performed using a PCR kit (QuantiTect SYBR Green; Qiagen, Hilden, Germany) and LightCycler (Hoffman-La Roche, Basel, Switzerland). All samples were treated with DNAase. Dilution of cDNA samples before PCR quantification of mitochondrially encoded genes was 100-fold higher compared with nuclear-encoded genes. Levels of all transcripts were correlated with that of the gene for cyclophilin-β since quantity of this transcript is not affected by the dietary treatments [8] and is expressed in arbitrary units (AU). Lasergene software (DNAStar, Madison, WI, USA) was used to design oligonucleotide primers (see ESM Table 3).

Quantification of mitochondrial proteins

Frozen tissues were immersed in a buffer containing 0.25 mol/l sucrose, 50 mmol/l Tris and 5 mmol/l EDTA (pH 7.5), and homogenised for 7 min on ice using a Teflon/glass homogeniser. Tissue fragments were removed by centrifugation for 10 min at 4°C and 600 g and crude cell membranes were separated from the supernatant by centrifugation for 35 min at 4°C and 100,000 g. Samples of the crude cell membranes were analysed using SDS-PAGE and immunoblots [26]. Polyclonal rabbit antibody [26] was used (1:5,000) to detect cytochrome c oxidase subunits I and VI (MT-CO1 and COX6). Monoclonal antibody was used to detect the 70 kDa subunit of complex II (SDHA; 1:2,000; Molecular Probes A-11142, Eugene, OR, USA) and subunit α of F_1-ATPase (ATP5A1; 1:200,000, lot 20D6 [27]). Peroxidase-labelled goat anti-mouse IgG (1:1,000, A8924; Sigma, St Louis, MO, USA) or goat anti-rabbit IgG (1:1,000, F0382; Sigma) was used as a secondary antibody. Antigens were visualised by luminescence and detected on a LAS 1000 CCD camera system (Fuji, Tokyo, Japan). Liver mitochondria isolated from chow diet-fed mice were used as standard to quantify the relative content of each antigen.

Oxidation and synthesis of fatty acid in fragments of adipose tissue

Fatty acid oxidation was measured as the amount of radioactivity released into the medium from fragments (50 mg tissue chopped into about ten pieces using a razor blade) prelabelled with [9,10(n)-^{3}H]oleic acid [28]. Synthesis of fatty acids was measured as before [22] except that the medium also contained 1 mM pyruvate and ^3H_2O (18.5 MBq/ml) was used instead of radioactive glucose.

Statistics

The data were evaluated by one-way (diet) or two-way ANOVA with one grouping factor (diet) and one trial factor (depot). Logarithmic or square root transformation was used to stabilise variance in cells when necessary. The Spearman correlation coefficient was used to evaluate the relationship between transcript levels. The level of significance for all tests was set at p=0.05.

Results

Screening for genes regulated by EPA and DHA in white adipose tissue

To identify the genes specifically regulated in white adipose tissue by marine-derived n-3 PUFA, mice were maintained for 4 weeks on the sHFf diet, rich in ALA and free of DHA or EPA, while the other mice were fed a similar diet, except that 44% of dietary lipids were replaced by EPA/DHA concentrate (sHFf-F2 diet). In accordance with previous results [8], animals fed the sHFf diet maintained stable body weight during 4 weeks of the treatment, while in the sHFf-F2 group the mean body weight declined by 11±2%. The weight of epididymal fat was 30±3% lower than in the sHFf group (mean±SEM of four independent experiments, n=7–12), while the weight of dorsolumbar fat did not change significantly. As observed before [8], food consumption was not affected (Table 1).

First, cDNA PCR subtraction was used to identify genes upregulated in the sHFf-F2-fed mice, starting from total RNA prepared from epididymal pads pooled from mice fed either the sHFf or the sHFf-F2 diet. In total, 52 clones were identified corresponding to 19 differentially expressed genes, six of them having known functions (Table 2): genes for cytochrome *c* oxidase subunit III (*mt-Co3*) and subunit 6 of ATPase (*mt-Atp6*), i.e. the key components of the mitochondrial oxidative phosphorylation system, encoded by mitochondrial DNA, and four nuclear genes engaged in regulatory pathways.

Secondly, oligonucleotide microarray analysis was applied to RNA samples isolated from both epididymal and dorsolumbar fat. Tissues from individual animals fed either sHFf or sHFf-F2 diet (Table 1) were processed and analysed separately. Only 0.5% of the genes most discriminating in both directions (see Materials and methods) between the sHFf and sHFf-F2 diets were considered (Table 2). In accordance with the results of cDNA PCR subtraction, the majority of the most upregulated genes in both fat depots of the sHFf-F2-fed mice belong to the mitochondrial genome and code for components of the oxidative phosphorylation system (complex I, cytochrome *c* oxidase and complex V, ATP synthase). Also, the nuclear gene for adipsin, the adipocyte hormone engaged in the control of fatty acid intake [29], was upregulated. Conversely, the most downregulated genes in the sHFf-F2-fed animals were those supporting lipogenesis and encoding stearoyl-CoA desaturase (*Scd1* [30]), in both epididymal and subcutaneous fat depots, and acyl-CoA:diacylglycerol acyltransferase (*Dgat2*), in the former depot only (Table 2).

Thirdly, seven of the 18 differentially expressed genes were verified using qRT-PCR (Table 1). The differences in the expression of the gene for *mt-Co1* were more pronounced in the epididymal than in the dorsolumbar fat depot (Table 2).

Specific induction of mitochondrial proteins in epididymal fat by the sHFf-F2 diet

Subunits of the mitochondrial oxidative phosphorylation complexes were quantified in crude cell membranes prepared from white adipose tissue using immunoblots (Fig. 1, Table 3). In epididymal fat, levels of SDHA, MT-CO1, COX6 and ATP5A1 were elevated approximately 2-fold by the sHFf-F2 diet compared with the sHFf diet, resulting, in the case of MT-CO1, in higher levels in epididymal than in dorsolumbar fat in the sHFf-F2-fed animals. In the dorsolumbar fat, no effect of the diet on any of the analysed antigens was observed. In contrast to the differential effect of the sHF-F2 diet on the quantity of SDHA and ATP5A1 in two fat depots, qRT-PCR analysis (n=7–9) revealed significant induction of transcripts for these genes by sHF-F2 diet in both epididymal fat (*Sdha*, 1.35±0.16 vs 2.71±0.35 AU; *Atp5a1*, 1.82±0.08 vs 3.97±0.75 AU) and subcutaneous fat (*Sdha*, 1.96±0.19 vs 2.75±0.26 AU; *Atp5a1*, 1.44±0.23 vs 3.31±0.54 AU).

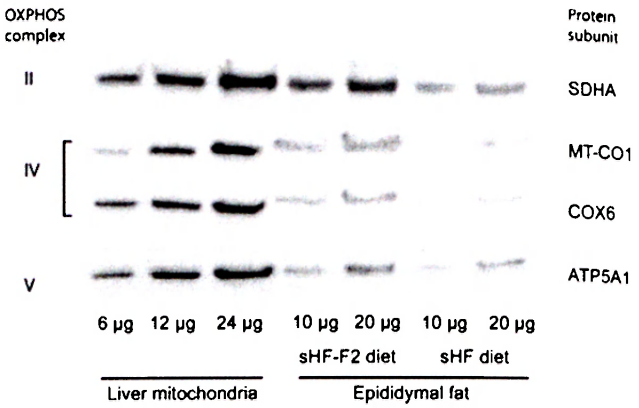

Fig. 1 Immunoblotting of mitochondrial proteins in epididymal fat of the mice fed sHFf and mice fed sHFf-F2 diets. The analysis was performed using crude cell membranes isolated from adipose tissue. The amount of protein in each lane is indicated. Isolated mouse liver mitochondria were used as a standard (see Table 3)

Induction of Ppargc1a, Nrf1, Cpt1a, fatty acid oxidation and depression of fatty acid synthesis in white adipose tissue by the sHFf-F2 diet

We hypothesised that the induction of PPARGC1A, as well as NRF1 [18, 19], which is a downstream target for PPARGC1A and orchestrates mitochondrial biogenesis [19], is involved in the effect of EPA/DHA concentrate on white adipose tissue. qRT-PCR analysis revealed approximately 3.4-fold induction of both *Ppargc1a* and *Nrf1* transcripts in epididymal fat by the sHFf-F2 diet compared with the sHFf diet (Figs. 2a and b). Both genes tended to be upregulated also in dorsolumbar fat, but the effects were much smaller and not statistically significant. Also, CPT1, which is necessary for the translocation of fatty acid into mitochondria, represents a downstream target for PPARG C1A [31]. qRT-PCR analysis revealed that the liver isoform of the gene encoding CPT1 (*Cpt1a*) was upregulated by the sHFf-F2 diet in epididymal but not in dorsolumbar fat (Fig. 2c). Positive correlations between the expressions of genes encoding PPARGC1A and CPT1A within identical tissues and diets were observed, while correlations between the expression of genes encoding PPARGC1A and NRF1 were not statistically significant (Fig. 2d and legend). The upregulation of the gene for CPT1A suggests stimulation of fatty acid oxidation in the epididymal fat of the sHFf-F2 mice. Indeed, oxidation of oleate was 1.5- to 1.8-fold higher in epididymal fat of the sHFf-F2 compared with the sHFf mice, when the rate of oxidation was related either to tissue weight, protein, or DNA (Fig. 3). In dorsolumbar fat, similar rates were observed in both diet groups (Fig. 3). Fatty acid synthesis was lower in epididymal fat of sHFf-F2 than sHFf mice (83±7 vs 114±12 dpm of ^3H_2O incorporated into saponifiable fatty acids per mg of tissue DNA; n=7).

Levels of acyl-CoA oxidase 1 (*Acox1*) mRNA, the marker of peroxisomal fatty acid oxidation, were significantly higher in mice fed the sHFf-F2 diet than in those fed the sHFf diet, in both epididymal (0.27±0.05 vs 0.14±0.02 AU)

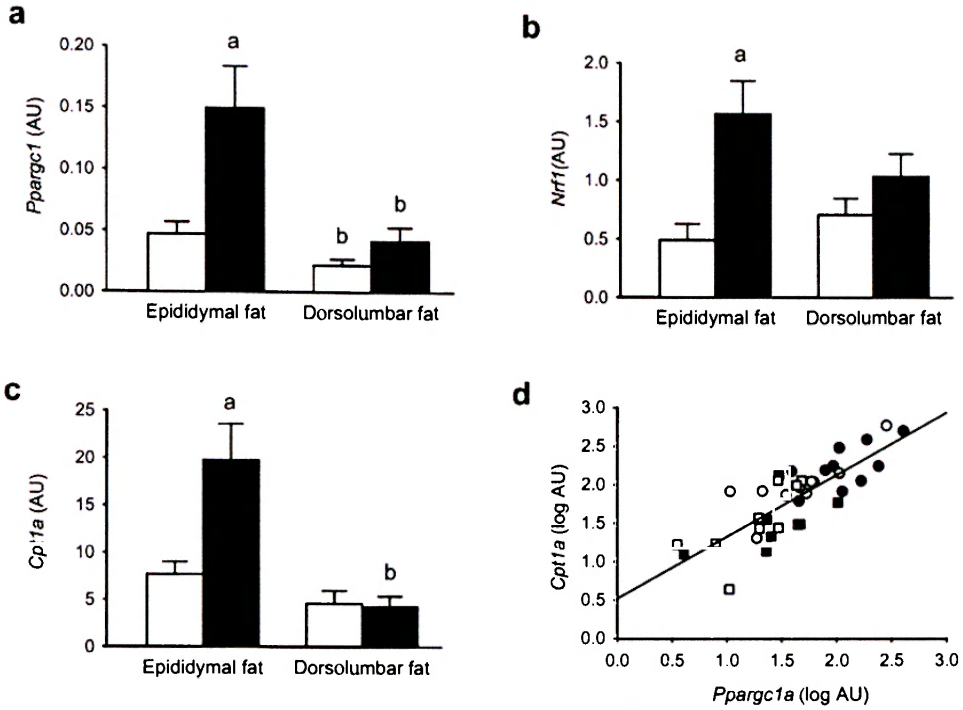

Fig. 2 Gene expression in adipose tissue. **a–c** Using qRT-PCR, transcript levels were evaluated in total RNA isolated from adipose tissue depots of mice fed the sHFf diet (*white bars*) or the sHFf-F2 diet (*black bars*). For all transcripts, the effect of diet in fat depots was different (two-way ANOVA). Significant differences between diets; significant differences between fat depots. Data are means± SEM (n=9–15). **d** Correlation between *Ppargc1a* and *Cpt1a* mRNA levels in epididymal fat of the mice fed sHFf (*white circles*) and sHFf-F2 (*black circles*) and in dorsolumbar fat of the mice fed sHFf (*white squares*) and sHFf-F2 (*black squares*). Spearman correlation coefficients for each set of data were 0.72–0.83. Corresponding values for the correlation between *Ppargc1a* and *Nrf1* transcripts were 0.38–0.62. Only the correlations between *Ppargc1a* and *Cpt1a* transcripts (**d**) were statistically significant (p<0.01)

and dorsolumbar (0.09±0.02 vs 0.04±0.01 AU) adipose tissue. Thus, in contrast to the gene encoding CPT1A, similar stimulation of transcription of the gene for ACOX1 was observed in both fat depots of sHFf-F2 mice (n=13).

In contrast to white adipose tissue, especially the epididymal fat, no significant changes in the expression of *Ppargc1a*, *Nrf1* and *Cpt1a* were observed in liver (not shown). Only the levels of *Acox1* mRNA were significantly higher in mice fed the sHFf-F2 than in those fed the sHFf diet (32±1.5 vs 21±1.3 AU; n=5).

Upregulation of the markers of mitochondrial biogenesis and β-oxidation and suppression of lipogenic genes in adipocytes from mice fed the composite diet cHF-F1

These experiments were designed to verify whether mitochondrial biogenesis and β-oxidation are stimulated when induction of obesity by the cHF diet is reduced by replacement of only 15% of dietary lipids by EPA/DHA concentrate (cHF-F1 diet). In accordance with our previous

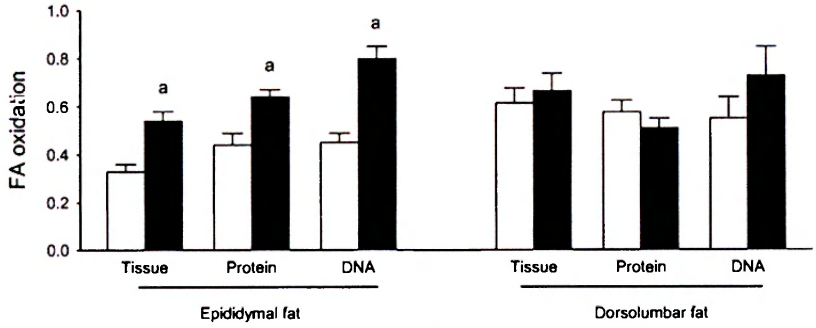

Fig. 3 Oxidation of oleate in fat depots of mice fed sHFf (*white bars*) or sHFf-F2 (*black bars*). Activity is expressed as pmol fatty acids h^{-1}·mg^{-1} tissue (*Tissue*), pmol fatty acids h^{-1}·mg^{-1} protein·10^{-2} (*Protein*), or pmol fatty acids·h^{-1}·μg^{-1} DNA (*DNA*). There was a different effect of diet in fat depots (two-way ANOVA). Data are means±SEM (n=9). The tissue DNA concentration was 0.78±0.08 and 0.76±0.11 μg DNA/mg tissue in epididymal fat of sHFf and sHFf-F2 mice, respectively. Corresponding values for dorsolumbar fat were 1.27±0.16 and 1.05±0.20. Significant difference between diets

Table 4 Gene expression in adipocytes isolated from epididymal fat of mice fed cHF or cHF-F1 diet

Diet	Ppargc1a	mt-Co3	Cpt1a	Scd1	Ucp2	Ucp3
cHF	0.14±0.03	9.25±1.02	1.40±0.32	1.57±0.18	0.87±0.11	0.82±0.02
cHF-F1	0.51±0.09[a]	12.81±0.64[a]	2.32±0.61[a]	0.40±0.08[a]	0.40±0.09[a]	1.01±0.03

Data are means±SEM (n=7)
[a]Significant difference between diets

study [8], a significant 22±7% reduction of body weight gain was observed in the cHF-F1-fed mice compared with the cHF-fed mice after 5 weeks of the treatment. The weight of epididymal fat in the cHF-F1-fed mice was 24±3% lower than in the cHF group (mean±SEM of three independent experiments, n=10–13), while the weight of dorsolumbar fat was not significantly different. As observed before [8], food consumption was not affected (see Table 1 for data from a typical experiment).

In animals fed the cHF and cHF-F1 diets (Table 1), gene expression was studied using RNA prepared from the adipocytes isolated from epididymal fat (Table 4). Expression of the genes for PPARGC1A, MT-CO3 and CPT1A was enhanced by the cHF-F1 diet compared with the cHF diet, while the gene encoding SCD1 was down-regulated. Since PPARGC1A can induce the expression of the gene for mitochondrial UCP1 [18, 20] and marine-derived n-3 PUFA have been shown to induce expression of this gene in brown fat [14], the *Ucp1* transcript was also measured. However, it was barely detectable (levels were at least four orders of magnitude lower than in interscapular brown fat) and no induction of the *Ucp1* transcript by cHF-F1 diet was observed (not shown). Levels of *Ucp2* transcript were lower in adipocytes from the cHF-F1-fed than the cHF-fed mice, while levels of *Ucp3* transcript were similar in the two groups of mice (Table 4).

Direct effect of n-3 PUFA on adipocytes

3T3-L1 adipocytes differentiated in cell culture were incubated for 24 h with various fatty acids: oleate alone, oleate plus ALA, or oleate plus DHA, and *Ppargc1a* and *Nrf1* transcripts were quantified (Fig. 4). Compared with oleate, both ALA and DHA significantly increased the levels of both transcripts. The stimulation by DHA seemed to be more pronounced than that by ALA, but the difference was not statistically significant. Similar results were obtained in primary cultures of adipocytes (not shown).

Discussion

The results of this study demonstrate the involvement of white adipose tissue, in addition to the liver, brown fat and other organs, in the mechanism whereby marine-derived n-3 PUFA reduce adiposity and body weight. Our finding that EPA/DHA concentrate can specifically stimulate expression of *Ppargc1a* and *Cpt1a*, increase the content of mitochondrial proteins and induce β-oxidation in epididymal but not dorsolumbar fat is consistent with the preferential reduction of adipose tissue accumulation in the abdomen.

The idea that changes induced in white adipocytes by EPA and DHA could contribute to the reduction of obesity is strongly supported by the upregulation of genes encoding PPARGC1A and MT-CO3 and downregulation of the gene encoding SCD1 in adipocytes prepared from epididymal fat of the cHF-F1-fed mice. Under these conditions (mouse fed the cHF-F1 diet), only 15% of dietary fat was replaced by EPA/DHA concentrate, resulting in a substitution of about 10% of the fat for EPA/DHA. This led to a significant reduction in weight gain compared with mice fed the cHF diet.

Increased expression of *Nrf1* is consistent with the upregulation of genes for mitochondrial proteins by EPA/DHA. The majority of these genes (i.e. *mt-Co1*, *mt-Co3*, *mt-Nd1*, *mt-Nd4*, *mt-Atp6* and *mt-Atp8*) are encoded by mitochondrial DNA. However, immunoblotting analysis revealed that the biosynthesis of nuclear-encoded mitochondrial proteins (COX6, SDHA and ATP5A1) is also upregulated. Expression of genes encoding PPARGC1A and NRF1 is more stimulated in epididymal than in dorsolumbar

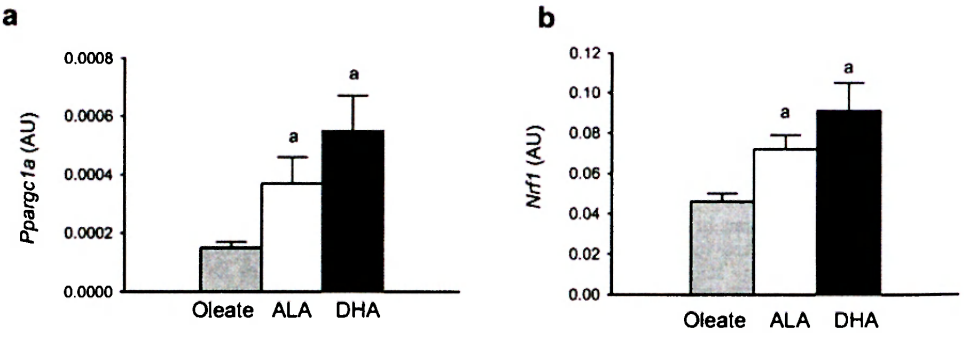

Fig. 4 Effect of n-3 PUFA on gene expression in 3T3-L1 cells. Postconfluent cells were treated for 24 h with oleate, oleate plus ALA, or oleate plus DHA (see Materials and methods), and transcripts were quantified using qRT-PCR. Data are means± SEM (n=12). Significant differences versus oleate

fat, and striking fat depot-specific differences were observed in the effect of EPA/DHA on the expression of the gene encoding CPT1A, fatty acid oxidation activity and the quantity of oxidative phosphorylation proteins. In contrast, EPA/DHA induced transcripts for most of the mitochondrial proteins as well as the marker of peroxisomal fatty acid oxidation, *Acox1*, to a similar extent in both fat depots. These results suggest distinct post-transcriptional control in different fat depots of the expression of the genes for mitochondrial proteins and the involvement of mitochondria rather than peroxisomes in the stimulatory effect of EPA and DHA on fatty acid oxidation in epididymal fat.

Our in vitro experiments suggest that *n*-3 PUFA can modulate gene expression in white adipose tissue by direct interaction with adipocytes. PUFA of the *n*-3 series and their metabolites, eicosanoids [6], are ligands [32] of various isoforms of the peroxisome proliferator-activated receptor (PPAR [33–35]). All PPARs (α, γ and δ) physically interact with the gene encoding PPARGC1A [18, 34, 35], which is upregulated by EPA/DHA in adipocytes. A recent study indicates that treatment of *ob/ob* mice with rosiglitazone, a PPARγ ligand of the thiazolidinedione family and a widely used antidiabetic drug, increases mitochondrial mass, palmitate oxidation and the expression of genes encoding PPARGC1A, CPT1A and UCP1 in epididymal fat [36]. Except for the upregulation of the gene for UCP1, 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 white adipose tissue cells, while increasing mitochondrial oxidative capacity and stimulating glucose uptake into adipocytes. However, in contrast to EPA/DHA, thiazolidinediones also support an adipogenic programme [36]. Surprisingly, rosiglitazone induces the accumulation of *n*-3 PUFA in white adipose tissue of diabetic mice [37]. Whether the mechanism of the effect of rosiglitazone depends on the accumulation of *n*-3 PUFA in adipocytes remains to be clarified.

Forced expression of the gene for PPARGC1A in both 3T3-F442A (references, see [18]) and human adipocytes [20] induces the conversion of adipocytes to energy-dissipating cells, including the recruitment of the gene for UCP1. Low levels of transgenic UCP1 in white adipose tissue may protect mice against obesity [17] while stimulating oxidation of fatty acids [28] and mitochondrial biogenesis [23], and depressing lipogenesis [22] in adipocytes. Also, leptin [38, 39], β_3-adrenergic agonists [40] and bezafibrate [41] have similar effects in rodents [38, 41], including induction of the genes for CPT1A [39, 41] and PPARGC1A [38] in white adipose tissue. Apparently, various stimuli can convert adipocytes into fat-burning cells and depress the accumulation of body fat, even without induction of UCPs in these cells, as documented by the effect of EPA/DHA. Lack of recruitment of UCP1 in the EPA/DHA-treated mice may be explained by the absence of other cofactors or hormones required in addition to PPARGC1A [18, 20]. The absence of UCP1 also suggests that the increase in fatty acid oxidation occurs in white rather than brown fat cells. This idea is supported by the induction of the gene for CPT1A, which is preferentially expressed in white adipose tissue in mice [42]. Because of the low activity of CPT1A, the rate of β-oxidation in white adipose tissue is normally low and fatty acids are directed to esterification [42, 43]. Thus, the enhancement of fatty acid oxidation in white adipose tissue by EPA/DHA concentrate could depend on the increase in the expression of the gene encoding CPT1A. Decreased production of malonyl CoA due to suppression of lipogenesis may result in further augmentation of fatty acid oxidation in mitochondria via the CPT1A-mediated mechanism [44].

It was found recently that expression of the gene encoding PPARGC1A is reduced in the adipose tissue of morbidly obese subjects [45] and inversely correlated with adipose cell mass [46], and that *Ppargc1a* polymorphisms segregate with obesity [47] and type 2 diabetes [48]. Onset of obesity in *ob/ob* mice is associated with downregulation of genes coding for mitochondrial proteins [36], and heat production by white adipose tissue cells from obese humans is lower than in lean individuals [49]. All these findings are compatible with the hypothesis that changes in fuel partitioning and reduced oxidation of lipids in white adipocytes contribute to the development of obesity and type 2 diabetes.

In conclusion, we show for the first time that dietary EPA and DHA preferentially upregulate genes for mitochondrial proteins, including their regulatory genes *Ppargc1a* and *Nrf1*, and increase β-oxidation while depressing lipogenesis in abdominal fat. This metabolic switch is induced independently of ALA intake and could reduce the development of obesity. Relatively minor changes in the composition of lipids in an obesity-promoting diet exert surprisingly pronounced effects. Our findings have important implications for the design of novel dietary and pharmacological strategies for the prevention and treatment of obesity and the metabolic syndrome.

Acknowledgements We thank J. Bemova, S. Hornova, D. Salkova, V. Fialova, C. Vink for technical assistance, J. Vorlicek for statistical analysis, and Drs A. Kotyk and V. Mohamed-Ali for critical reading of the manuscript. This work was supported by the Grant Agency of the ASCR (KJB5011410 and KJB5011303), the Czech Science Foundation (303/05/2580 and 303/03/0749), the MEYS (LL00A079), research projects AVOZ 50110509 and 1M6837805002 (Czech Republic), Pronova Biocare a.s. (Norway) and the Ministry of Agriculture, Nature Management and Food Control (The Netherlands).

References

1. Kopelman PG (2000) Obesity as a medical problem. Nature 404:635–643
2. Oudart H, Groscolas R, Calgari C et al (1997) Brown fat thermogenesis in rats fed high-fat diets enriched with *n*-3 polyunsaturated fatty acids. Int J Obes Relat Metab Disord 21:955–962
3. Hun CS, Hasegawa K, Kawabata T, Kato M, Shimokawa T, Kagawa Y (1999) Increased uncoupling protein 2 mRNA in white adipose tissue, and decrease in leptin, visceral fat, blood glucose, and cholesterol in KK-Ay mice fed with eicosapentaenoic and docosahexaenoic acids in addition to linolenic acid. Biochem Biophys Res Commun 259:85–90

4. Ikemoto S, Takahashi M, Tsunoda N, Maruyama K, Itakura H, Ezaki O (1996) High-fat diet-induced hyperglycemia and obesity in mice: differential effects of dietary oils. Metabolism 45:1539–1546
5. Raclot T, Groscolas R, Langin D, Ferre P (1997) Site-specific regulation of gene expression by *n*-3 polyunsaturated fatty acids in rat white adipose tissues. J Lipid Res 38:1963–1972
6. Azain MJ (2004) Role of fatty acids in adipocyte growth and development. J Anim Sci 82:916–924
7. Hill JO, Peters JC, Lin D, Yakubu F, Greene H, Swift L (1993) Lipid accumulation and body fat distribution is influenced by type of dietary fat fed to rats. Int J Obes Relat Metab Disord 17:223–236
8. Ruzickova J, Rossmeisl M, Prazak T et al (2004) Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. Lipids 39:1177–1185
9. Ruxton CH, Reed SC, Simpson MJ, Millington KJ (2004) The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. J Hum Nutr Diet 17:449–459
10. Sinclair AJ, Attar-Bashi NM, Li D (2002) What is the role of alpha-linolenic acid for mammals? Lipids 37:1113–1123
11. Couet C, Delarue J, Ritz P, Antoine J-M, Lamisse F (1997) Effect of dietary fish oil on body fat mass and basal fat oxidation in healthy adults. Int J Obes Relat Metab Disord 21:637–643
12. Lapillonne A, Clarke SD, Heird WC (2004) Polyunsaturated fatty acids and gene expression. Curr Opin Clin Nutr Metab Care 7:151–156
13. Baillie RA, Takada R, Nakamura M, Clarke SD (1999) Coordinate induction of peroxisomal acyl-CoA oxidase and UCP-3 by dietary fish oil: a mechanism for decreased body fat deposition. Prostaglandins Leukot Essent Fat Acids 60:351–356
14. Takahashi Y, Ide T (2000) Dietary *n*-3 fatty acids affect mRNA level of brown adipose tissue uncoupling protein 1, and white adipose tissue leptin and glucose transporter 4 in the rat. Br J Nutr 84:175–184
15. Raclot T, Oudart H (1999) Selectivity of fatty acids on lipid metabolism and gene expression. Proc Nutr Soc 58:633–646
16. Newman RE, Bryden WL, Fleck E et al (2002) Dietary *n*-3 and *n*-6 fatty acids alter avian metabolism: metabolism and abdominal fat deposition. Br J Nutr 88:11–18
17. Kopecky J, Hodny Z, Rossmeisl M, Syrovy I, Kozak LP (1996) Reduction of dietary obesity in the aP2-Ucp transgenic mice: physiology and adipose tissue distribution. Am J Physiol 270: E768–E775
18. Puigserver P, Spiegelman BM (2003) Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. Endocr Rev 24:78–90
19. Kelly DP, Scarpulla RC (2004) Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev 18:357–368
20. Tiraby C, Tavernier G, Lefort C et al (2003) Acquirement of brown fat cell features by human white adipocytes. J Biol Chem 278:33370–33376
21. Lin J, Wu PH, Tarr PT et al (2004) Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. Cell 119:121–135
22. Rossmeisl M, Syrový I, Baumruk F, Flachs P, Janovská P, Kopecký J (2000) Decreased fatty acid synthesis due to mitochondrial uncoupling in adipose tissue. FASEB J 14:1793–1800
23. Rossmeisl M, Barbatelli G, Flachs P et al (2002) Expression of the uncoupling protein 1 from the *aP2* gene promoter stimulates mitochondrial biogenesis in unilocular adipocytes in vivo. Eur J Biochem 269:1–10
24. Henegariu O, Bray-Ward P, Ward DC (2000) Custom fluorescent-nucleotide synthesis as an alternative method for nucleic acid labeling. Nat Biotechnol 18:345–348
25. Pellis L, Franssen-van Hal NL, Burema J, Keijer J (2003) The intraclass correlation coefficient applied for evaluation of data correction, labeling methods, and rectal biopsy sampling in DNA microarray experiments. Physiol Genomics 16:99–106
26. Antonicka H, Floryk D, Klement P et al (1999) Defective kinetics of cytochrome *c* oxidase and alteration of mitochondrial membrane potential in fibroblasts and cytoplasmic hybrid cells with the mutation for myoclonus epilepsy with ragged-red fibres ('MERRF') at position 8344 nt. Biochem J 342:537–544
27. Moradi-Ameli M, Godinot C (1983) Characterization of monoclonal antibodies against mitochondrial F1-ATPase. Proc Natl Acad Sci U S A 80:6167–6171
28. Matejkova O, Mustard KJ, Sponarova J et al (2004) Possible involvement of AMP-activated protein kinase in obesity resistance induced by respiratory uncoupling in white fat. FEBS Lett 569:245–248
29. Cianflone K, Xia Z, Chen LY (2003) Critical review of acylation-stimulating protein physiology in humans and rodents. Biochim Biophys Acta 1609:127–143
30. Dobrzyn A, Ntambi JM (2004) The role of stearoyl-CoA desaturase in body weight regulation. Trends Cardiovasc Med 14:77–81
31. Song S, Zhang Y, Ma K et al (2004) Peroxisomal proliferator activated receptor gamma coactivator (PGC-1alpha) stimulates carnitine palmitoyltransferase I (CPT-Ialpha) though the first intron. Biochim Biophys Acta 1679:164–173
32. Forman BM, Chen J, Evans RM (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A 94:4312–4317
33. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell 92:829–839
34. Vega RB, Huss JM, Kelly DP (2000) The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. Mol Cell Biol 20:1868–1876
35. Wang YX, Lee CH, Tiep S et al (2003) Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. Cell 113:159–170
36. Wilson-Fritch L, Nicoloro S, Chouinard M et al (2004) Mitochondrial remodeling in adipose tissue associated with obesity and treatment with rosiglitazone. J Clin Invest 114: 1281–1289
37. Watkins SM, Reifsnyder PR, Pan HJ, German JB, Leiter EH (2002) Lipid metabolome-wide effects of the PPARgamma agonist rosiglitazone. J Lipid Res 43:1809–1817
38. Orci L, Cook WS, Ravazzola M et al (2004) Rapid transformation of white adipocytes into fat-oxidizing machines. Proc Natl Acad Sci U S A 101:2058–2063
39. Wang MY, Lee Y, Unger RH (1999) Novel form of lipolysis induced by leptin. J Biol Chem 274:17541–17544
40. Himms-Hagen J, Melnyk A, Zingaretti MC, Ceresi E, Barbatelli G, Cinti S (2000) Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. Am J Physiol Cell Physiol 279:C670–C681
41. Cabrero A, Alegret M, Sanchez RM, Adzet T, Laguna JC, Vazquez M (2001) Bezafibrate reduces mRNA levels of adipocyte markers and increases fatty acid oxidation in primary culture of adipocytes. Diabetes 50:1883–1890
42. Brown NF, Hill JK, Esser V et al (1997) Mouse white adipocytes and 3T3-L1 cells display an anomalous pattern of carnitine palmitoyltransferase (CPT) I isoform expression during differentiation. Inter-tissue and inter-species expression of CPT I and CPT II enzymes. Biochem J 327:225–231
43. Martin BR, Denton RM (1970) The intracellular localization of enzymes in white-adipose-tissue fat-cells and permeability properties of fat-cell mitochondria. Biochem J 117:861–877

44. Ruderman NB, Saha AK, Kraegen EW (2003) Minireview: malonyl CoA, AMP-activated protein kinase, and adiposity. Endocrinology 144:5166–5171
45. Semple RK, Crowley VC, Sewter CP et al (2004) Expression of the thermogenic nuclear hormone receptor coactivator PGC-1alpha is reduced in the adipose tissue of morbidly obese subjects. Int J Obes Relat Metab Disord 28:176–179
46. Yang X, Enerback S, Smith U (2003) Reduced expression of FOXC2 and brown adipogenic genes in human subjects with insulin resistance. Obes Res 11:1182–1191
47. Esterbauer H, Oberkofler H, Linnemayr V et al (2002) Peroxisome proliferator-activated receptor-gamma coactivator-1 gene locus: associations with obesity indices in middle-aged women. Diabetes 51:1281–1286
48. Oberkofler H, Linnemayr V, Weitgasser R et al (2004) Complex haplotypes of the PGC-1alpha gene are associated with carbohydrate metabolism and type 2 diabetes. Diabetes 53: 1385–1393
49. Bottcher H, Furst P (1997) Decreased white fat cell thermogenesis in obese individuals. Int J Obes 21:439–444

PUBLICATION D.

Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed a high-fat diet

Flachs P, Mohamed-Ali V, **Horáková O**, Rossmeisl M, Hosseinzadeh-Attar MJ, Hensler M, Ružičková J, Kopecký J.

Diabetologia. 49 :1-4 (2006)

Diabetologia
DOI 10.1007/s00125-005-0053-y

SHORT COMMUNICATION

P. Flachs · V. Mohamed-Ali · O. Horakova · M. Rossmeisl · M. J. Hosscinzadch-Attar · M. Hensler · J. Ruzickova · J. Kopecky

Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed a high-fat diet

Received: 30 June 2005 / Accepted: 30 August 2005

Abstract *Aims/hypothesis:* Diets rich in *n*-3 polyunsaturated fatty acids, namely eicosapentacnoic acid (EPA) and docosahexaenoic acid (DHA), protect against insulin resistance and obesity in rodents and increase insulin sensitivity in healthy humans. We tested whether the anti-diabetic effects of EPA and DHA involve enhanced production of the endogenous insulin sensitiser, adiponectin. *Methods:* We studied the effects, in an obesity-promoting high-fat diet, of partial replacement of vegetable oils by EPA/DHA concentrate (6% EPA, 51% DHA) over a 5-week period in adult male C57BL/6J mice that either had free access to food or had their food intake restricted by 30%. At the end of the treatment, systemic markers of lipid and glucose metabolism and full-length adiponectin and leptin were measured. Adiponectin (*Adipoq*) and leptin (*Lep*) gene expression in dorsolumbar and epididymal white adipose tissue (WAT) and isolated adipocytes was quantified and adipokine production from WAT explants evaluated. *Results:* In mice with free access to food, plasma triacylglycerols, NEFA, and insulin levels were lower in the presence of EPA/DHA, while glucose and leptin levels were not significantly altered. Food restriction decreased plasma triacylglycerols, glucose, insulin and leptin, but not adiponectin. EPA/DHA increased plasma adiponectin levels, independent of food intake, reflecting the stimulation of *Adipoq* expression in adipocytes and the release of adiponectin from WAT, particularly from epididymal fat. Expression of *Lep* and the release of leptin from WAT, while being extremely sensitive to caloric restriction, was unaltered by EPA/DHA. *Conclusions/interpretation:* Intake of diets rich in EPA and DHA leads to elevated systemic concentrations of adiponectin, largely independent of food intake or adiposity and explain, to some extent, their anti-diabetic effects.

Keywords Adipokines · Adiponectin · Adipose tissue · Fish oil · High-fat diet · *n*-3 PUFA

Abbreviations *Adipoq*: Gene encoding adiponectin · AMPK: AMP-activated protein kinase · cHF diet: Composite high-fat diet based on chow · cHF-F1 diet: Composite high-fat diet with partial replacement of lipids by fish oil concentrate · DHA: Docosahexaenoic acid · EPA: Eicosapentaenoic acid · EPA/DHA: Concentrate of EPA and DHA from sea fish (6% EPA and 51% DHA) · *Lep*: Gene encoding leptin · *Ppib*: Gene encoding peptidylpropyl isomerase β, also called Cyclophilin B · PUFA: Polyunsaturated fatty acids · qRT-PCR: Quantitative real-time RT-PCR · *Slc2a4*: Gene encoding solute carrier family 2 member 4, also called *Glut4* · WAT: White adipose tissue.

Introduction

Intake of polyunsaturated fatty acids (PUFA) of marine origin, namely eicosapentaenoic acid (EPA; 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*-3), has numerous beneficial effects on health. EPA and DHA act as hypolipidaemics, exert prophylactic effects on cardiovascular disease, protect against insulin resistance and obesity in rodents fed high-fat diets, and reduce insulin response to glucose in healthy humans [1–3 and references therein]. We, and others, have shown [1] that in a semi-synthetic high-fat diet rich in α-linolenic acid (18:3 *n*-3) partial replacement of lipids with concentrate of EPA and DHA from sea fish (EPA/DHA) resulted in suppression of insulinaemia. It also protected against down-regulation of solute carrier family 2 member 4 (*Slc2a4*, also called *Glut4*)

P. Flachs · O. Horakova · M. Rossmeisl · M. Hensler · J. Ruzickova · J. Kopecky (✉)
Department of Adipose Tissue Biology,
Institute of Physiology, Academy of Sciences,
Videnska 1083,
142 20, Prague, Czech Republic
e-mail: kopecky@biomed.cas.cz
Tel.: +420-241-062554
Fax: +420-241-062599

V. Mohamed-Ali · M. J. Hosseinzadeh-Attar
Adipokines and Metabolism Research Group,
Department of Medicine, University College London,
London, UK

in white adipose tissue (WAT) by the high-fat diet. The effect on *Slc2a4* was part of a complex modulation of WAT gene expression and metabolism, resulting in stimulation of lipid oxidation and inhibition of lipogenesis, especially in the epididymal fat, which led to reduced accumulation of this tissue while food consumption remained unaffected [1, 4].

The aim of this work was to test the hypothesis that the anti-diabetic effects of EPA and DHA involve induction of adiponectin, an adipocyte-derived hormone, which stimulates glucose utilisation and fatty acid oxidation in muscles and decreases hepatic gluconeogenesis as a result of the activation of AMP-activated protein kinase (AMPK) [5]. We show that partial replacement of dietary lipids with EPA/DHA in a composite high-fat (cHF) diet in rodents stimulates expression of the gene encoding adiponectin (*Adipoq*) and increases adiponectin production in epididymal WAT as well as systemic levels of adiponectin by mechanism(s) relatively independent of food intake or adiposity.

Materials and methods

Animals and diets Experiments were performed on adult male C57BL/6J mice as described previously [1, 4]. Mice were imported from the Jackson Laboratory (Bar Harbor, ME, USA) and bread at the Institute of Physiology for up to 16 generations. The animals studied were habituated for 2 weeks to the cHF diet, which was derived from standard chow and contained 35% (wt/wt) lipids of very low *n*-3 PUFA content, and then assigned for 5 weeks to the cHF diet, or a diet of the same composition, i.e. a composite high-fat diet, but with partial replacement of lipids by fish oil concentrate (cHF-F1 diet) such that 15% (wt/wt) of lipids was replaced by the EPA/DHA concentrate containing 6% EPA, 51% DHA, and 4 mg/g α-tocopherol as antioxidant (EPAX 1050TG; Pronova Biocare, Lysaker, Norway). Detailed analysis of fatty acid composition of the diets was performed previously [1]. When indicated, the ration was reduced by 30% (caloric restriction) compared with mice on the same type of diet but with free access to chow. Mice were killed by cervical dislocation, and subcutaneous dorsolumbar and epididymal WAT depots were dissected. Tissues were used immediately for explant studies or isolation of adipocytes [4], or stored in liquid nitrogen for RNA analysis (see below). EDTA plasma was obtained from truncal blood and stored at −70°C. The experiments were conducted according to the Institute's guidelines for the use and care of laboratory animals.

WAT explants Tissue (100 mg) was incubated in 1.0 ml of serum-free medium (Cellgro; Mediatech, Herndon, VA, USA) for 24 h at 37°C/5% CO_2. At the end of the incubation the culture supernatant was stored at −70°C.

Quantification of metabolites and hormones in plasma and supernatant from white adipose tissue explants Triacylglycerol, NEFA, glucose and insulin were estimated as described before [1]. Leptin and full-length adiponectin were measured by 2-site ELISA (R & D Systems, Minneapolis, MN, USA) with inter- and intra-assay CVs of 5.8 and 8.9 for leptin and 6.3 and 9.4% for adiponectin.

Quantitative real-time RT-PCR Total RNA was isolated as before [4] and quantitative real-time *RT-PCR* (qRT-PCR) was performed using a qPCR kit (DyNAmoCapillary SYBRGreen; Finnzymes, Espoo, Finland) and LightCycler (Hoffman-La Roche, Basel, Switzerland). Levels of transcripts were standardised using the gene encoding peptidylpropyl isomerase B (*Ppib*, also called cyclophilin B) and expressed in arbitrary units [4]. Lasergene software (DNASTAR, Madison, WI, USA) was used to design oligonucleotide primers (forward/reverse) for the following genes: *Adipoq* – TCCGGGACTCTACTACTTCTCTTAC CAC / GTCCCCATCCCCATACACCTG; *Ppib* – ACTAC GGGCCTGGCTGGGTGAG / TGCCGGAGTCGACAA

Table 1 Effects of EPA/DHA and caloric restriction on body weight, markers of lipid and glucose metabolism, and adipokines in plasma

	Diet				
	Free access to food		Caloric restriction		
	cHF	cHF-F1	cHF	cHF-F1	*n*
Body weight (g)[a]	37.5±1.2	34.8±1.2[b]	25.1±0.4[c]	25.2±0.6[c]	10
Plasma levels of:	–	–	–	–	–
NEFA (mmol/l)	0.47±0.02	0.33±0.02[b]	0.44±0.05	0.38±0.05	10
Triacylglycerols (mmol/l)	1.46±0.11	0.79±0.07[b]	0.51±0.06[c]	0.52±0.05[c]	10
Glucose (mmol/l)	10.10±0.22	10.04±0.38	7.55±0.44[c]	7.27±0.33[c]	10
Insulin (ng/ml)	2.77±0.52	0.17±0.04[b]	1.68±0.22[c]	0.16±0.06[b]	7
Adiponectin (μg/ml)	9.0±0.3	12.1±0.5[b]	9.2±1.1	11.2±0.5[b]	10
Leptin (ng/ml)	42.5±7.9	40.1±0.9	2.4±0.4[c]	2.5±0.2[c]	10

Mice had either free access to cHF or cHF-F1 diets or were subject to caloric restriction. Data are means±SEM. [a]Animals were used in our previous study [1]. [b]Significant effect of diet. [c]Significant effect of caloric restriction

Table 2 Gene expression and adipokine production in WAT depots of mice with free access to cHF or cHF-F1 diets

	Dorsolumbar fat		Epididymal fat		
	cHF	cHF-F1	cHF	cHF-F1	*n*
Gene expression	–	–	–	–	–
Tissue	–	–	–	–	–
Adipoq (AU)	0.77±0.08	0.94±0.07	0.82±0.03	0.88±0.06	10
Lep (AU)	0.51±0.05	0.48±0.03	0.61±0.04	0.47±0.07	10
Adipocytes	–	–	–	–	–
Adipoq (AU)	3.21±0.25	6.13±0.87[b]	2.83±0.23	7.18±0.82[b]	7–8
Lep (AU)	2.92±0.83	3.57±0.91	1.70±0.40	3.11±1.06	7–8
Adipokine production[a]	–	–	–	–	–
Adiponectin (μg/ml)	0.31±0.04	0.32±0.03	0.32±0.03	0.47±0.03[b]	9
Leptin (ng/ml)	36.1±3.1	34.7±3.6	33.6±6.4	20.9±3.3	9

Transcript levels were evaluated by qRT-PCR using RNA isolated from whole tissues or collagenase-liberated adipocytes. Tissue explants were used to measure adipokine production. Data are means±SEM. [a]Levels in medium after incubation of WAT explants (see Materials and methods). [b]Significant effect of diet. AU, arbitrary units

TGATGA; and leptin (*Lep*) – CCGCCAAGCAGAGGGT CAC / GCATTCAGGGCTAACATCCAACT.

Statistics The data were evaluated by ANOVA as described before [4]. The level of significance of all tests was set at p=0.05.

Results

Mice that had free access to cHF or cHF-F1 diets or a calorie-restricted diet for 5 weeks were compared (Table 1). In accordance with our previous results in mice with free access to standard chow [1, 4], the cHF diet induced obesity, while the cHF-F1 diet resulted in 2.7 g lower mean body weight and about 30% reduction of epididymal fat mass, with no change in dorsolumbar fat. Under these conditions, food consumption was not affected [4]. Caloric restriction resulted in net loss of body weight and adipose tissue mass [1, 4] (Table 1). Plasma levels (Table 1) of NEFA were lower in cHF-F1 than cHF mice with free access to food, but they were not affected by the diet in animals on the calorie-restricted diet, and no independent effect of caloric restriction on NEFA levels was seen. In mice with free access to food, levels of triacylglycerols were lower in cHF-F1 than in cHF mice, but the effect of the cHF-F1 diet was masked by caloric restriction, which decreased triacylglycerol levels (Table 1). Glucose and leptin levels were not affected by the cHF-F1 diet, but they were reduced by caloric restriction. Insulin levels were substantially lower in cHF-F1 than cHF mice, independently of food intake. Also caloric restriction decreased insulin levels, but to a much lower degree than the decrease induced by cHF-F1 diet. Levels of adiponectin were increased by cHF-F1 diet to a similar extent in mice with free access to food as in the mice on a calorie-restricted diet (34 and 22%, respectively), with this effect being independent of caloric restriction.

The effect of EPA/DHA on the expression of *Adipoq* and *Lep* and production of the adipokines was investigated in WAT of mice with free access to chow (Table 2). At the whole-tissue level, no significant effect of the diet on expression of either *Adipoq* or *Lep* was detected. However, in adipocytes isolated from both depots, transcript levels for both adipokines were much higher than in the whole tissues, and *Adipoq* expression was stimulated by EPA/DHA, while *Lep* expression was unaffected. The stimulation of *Adipoq* expression was more pronounced in epididymal than in dorsolumbar fat (2.5- and 1.9-fold stimulation, respectively). Production of adiponectin was significantly increased by EPA/DHA in explants of epididymal but not dorsolumbar fat, while production of leptin was unchanged in the depots studied.

Discussion

This study demonstrates that caloric restriction and the intake of n-3 PUFA of marine origin both lead to weight loss and a decrease in adipose tissue. The latter treatment, corresponding to about 5.3% of total energy intake derived from EPA and DHA, led to a significant reduction of weight gain, involving preferential reduction of epididymal fat in the abdomen [1]. Of note, however, is the differential effect of these two treatments on glucose and lipid metabolism and systemic adipokines. While changes in circulating glucose and leptin were almost entirely mediated by caloric restriction, NEFA, insulin and adiponectin were regulated predominantly by dietary EPA and DHA, and triacylglycerols levels were decreased by both treatments. Various effects of caloric restriction and EPA/DHA respectively reflect modulation of metabolism in several tissues and all the interactions are difficult to dissect. However, as demonstrated in this report, one of the main effects of EPA/DHA is the stimulation of *Adipoq* expression in mature adipocytes and the production of adiponectin, mainly in epididymal fat, leading to increased plasma adiponectin levels.

Plasma levels of adiponectin decrease in obese humans [6]. In our study, however, the induction of adiponectin by EPA/DHA could not result from reduction of adiposity,

since the strong decrease of fat content due to caloric restriction did not influence adiponectin levels. In contrast to adiponectin, leptin plasma levels were decreased dramatically by caloric restriction, but neither these nor *Lep* expression were affected by EPA/DHA, supporting the idea that circulating leptin correlates with adiposity and glucose metabolism in adipocytes [7]. The induction of adiponectin but not of leptin by EPA/DHA indicates that the mechanisms controlling the expression of genes for these two adipokines are different. As with thiazolidinediones, EPA and DHA may upregulate *Adipoq* by acting as ligands of peroxisome proliferator-activated receptor-γ, the transcriptional regulator interacting directly with *Adipoq* promoter [8]. The stimulation of *Adipoq* may also depend on the activation of AMPK, since AMPK in adipocytes stimulates *Adipoq* [9] and EPA and DHA activate hepatic AMPK [10].

In accordance with other studies in rodents fed high-fat diets and in healthy humans [1, 3], our insulin data indicate that EPA and DHA exert a large improvement in insulin sensitivity/action. Indeed, EPA/DHA prevented a rise in plasma insulin due to the cHF diet, since insulin levels in mice fed a standard chow diet were much lower than in the cHF mice, i.e. 0.59±0.10 ng/ml [1] (compare with Table 1). Our results suggest that protection against insulin resistance induced by a high-fat diet is at least partially mediated by adiponectin, but not leptin. Since EPA and DHA in humans were able to prevent but not reverse insulin resistance [3], it should be established whether the failure of dietary EPA and DHA to reverse type 2 diabetes is due to an inability to sufficiently induce adiponectin. Interestingly, an association between circulating adiponectin and plasma *n*-3 PUFA, and DHA in particular, was recently found in healthy humans [11].

In conclusion, we show for the first time that EPA and DHA stimulate *Adipoq* expression and increase the levels of circulating adiponectin, an effect that is relatively independent of food intake and body fat mass. Our findings are relevant for prevention and treatment of obesity-associated pathologies.

Acknowledgements This work was supported by grants from the Academy of Sciences (KJB5011410), Czech Science Foundation (303/05/2580) and Ministry of Education, Youth and Sports (1M6837805002) of the Czech Republic, as well as from the Wellcome Trust (070821/Z/03/Z), and from the European Commission (LSHM-CT-2004-005272), Research Project AVOZ50110509, and by Pronova Biocare (Norway).

References

1. Ruzickova J, Rossmeisl M, Prazak T et al (2004) Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. Lipids 39:1177–1185
2. Ruxton CH, Reed SC, Simpson MJ, Millington KJ (2004) The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. J Hum Nutr Diet 17:449–459
3. Delarue J, LeFoll C, Corporeau C, Lucas D (2004) N-3 long chain polyunsaturated fatty acids: a nutritional tool to prevent insulin resistance associated to type 2 diabetes and obesity? Reprod Nutr Dev 44:289–299
4. Flachs P, Horakova O, Brauner P et al (2005) Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce beta-oxidation in white fat. Diabetologia 48:2365–2375
5. Yamauchi T, Kamon J, Minokoshi Y et al (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 8:1288–1295
6. Arita Y, Kihara S, Ouchi N et al (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun 257:79–83
7. Wellhoener P, Fruehwald-Schultes B, Kern W et al (2000) Glucose metabolism rather than insulin is a main determinant of leptin secretion in humans. J Clin Endocrinol Metab 85:1267–1271
8. Iwaki M, Matsuda M, Maeda N et al (2003). Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. Diabetes 52:1655–1663
9. Lihn AS, Jessen N, Pedersen SB, Lund S, Richelsen B (2004) AICAR stimulates adiponectin and inhibits cytokines in adipose tissue. Biochem Biophys Res Commun 316:853–858
10. Suchankova G, Tekle M, Saha AK, Ruderman NB, Clarke SD, Gettys TW (2005) Dietary polyunsaturated fatty acids enhance hepatic AMP-activated protein kinase activity in rats. Biochem Biophys Res Commun 326:851–858
11. Fernandez-Real JM, Vendrell J, Ricart W (2005) Circulating adiponectin and plasma fatty acid profile. Clin Chem 51:603–609