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Vliv ektopické syntézy mitochondriálního odpřahujícího proteinu 1 v bílé tukové tkáni na celotělový metabolizmus u myší

Effect of ectopic synthesis of mitochondrial uncoupling protein 1 in white adipose tissue on whole-body metabolism in mice

Dizertační práce

Vedoucí závěrečné práce/školitel:

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Abstrakt (teze):

Prevence a léčba obezity patří mezi nejzávažnější problémy zdravotní péče. Mezi terapeutické cíle patří i metabolizmus tukové tkáně, jejíž hromadění je podstatou vzniku obezity. Pokusy na transgenních myších s ektopickou expresí hnědotukového odpřahujícího proteinu (UCP1) v bílé tukové tkáni prokázaly, že zvýšením výdeje energie v bílé tukové tkáni lze zabránit rozvoji obezity. Jedním z hlavních cílů dizertační práce byla detailní charakterizace fenotypu tohoto unikátního modelu myší rezistentních k obezitě. Ukázali jsme, že odpřažení mitochondriální oxidační fosforylace v bílé tukové tkáni vedlo ke zvýšení oxidace mastných kyselin, snížení lipogeneze a indukci mitochondriální biogeneze v této tkáni. Dále jsme se zaměřili na modulaci náchylnosti k rozvoji obezity zvýšením oxidace mastných kyselin v bílém tuku v reakci na fyziologický podnět, kombinovanou intervenci založenou na zvýšeném příjmu *n*-3 polynenasycených mastných kyselin (PUFA) a mírné kalorické restrikce, u myší krmených dietou s vysokým obsahem tuku. Synergická indukce mitochondriální oxidační kapacity a lipidového katabolizmu v epididymálním bílém tuku byla spojena s potlačením zánětlivé reakce této tkáně, která doprovází obezitu. Zlepšení lipidové (a glukózové) homeostázy bylo nezávislé na chladem indukované termogenezi (a UCP1) a souvisí s indukcí tzv. jalového cyklu, lipolýzou mastných kyselin triacylglycerolů a re-esterifikací mastných kyselin v adipocytech. Zvýšený energetický výdej v bílé tukové tkáni může bránit rozvoji obezity a zvyšovat flexibilitu metabolizmu. Závěr: Indukce katabolizmu lipidů v bílé tukové tkáni jak vlivem transgenní modifikace tak v odpovědi na fyziologickou stimulaci, může brzdit rozvoj obezity a snižovat její nepříznivé dopady. Tyto výsledky by mohly nalézt uplatnění při léčbě obezity a doprovodných onemocnění.

Klíčová slova: obezita, aP2-*Ucp1* transgenní myš, metabolizmus bílé tukové tkáně, *n*-3 PUFA**,** kalorická restrikce

Abstract (thesis):

The prevention and treatment of obesity is a major problem of health care systems in affluent societies. Metabolism of adipose tissue belongs to the therapeutical targets, since accumulation of adipose tissue is the basis of obesity development. Experiments using transgenic mice with ectopic expression of brown-fat uncoupling protein 1 *(*UCP1) in white adipose tissue (WAT), verified a concept that obesity could be ameliorated by increasing energy expenditure in WAT. The goal of the experiments of this PhD Thesis was to characterize in detail the phenotype of this unique animal model of obesity resistance. We have shown that mitochondrial uncoupling in WAT resulted in increased oxidation of fatty acids (FA), in face of decreased lipogenesis and induced mitochondrial biogenesis in this tissue. In further studies, we aimed to modulate propensity to obesity be increasing FA oxidation in WAT in response to physiological stimuli. This could be accomplished in response to the combination treatment using *n*-3 polyunsaturated fatty acids (*n*-3 PUFA) and mild calorie restriction in mice fed high-fat diet. Synergistic induction of mitochondrial oxidative capacity and lipid catabolism in epididymal WAT was associated with suppression of low-grade inflammation of WAT, which is typical for obesity. The improvement of lipid (and glucose) homeostasis were independent of cold-induced thermogenesis (and UCP1) and could be explained by activation of a futile substrate cycle of FA lipolysis of triacylglyceroles and FA re-esterification in adipocytes. Such induction of energy expenditure in WAT may exert anti-obesity effect and improve metabolic flexibility. In summary, increase in lipid catabolism in white fat in response to either transgenic modification or physiological stimuli could counteract obesity and ameliorate its harmful consequences. These results could be used in treatment of obesity and the associated disorders.

Keywords: obesity, aP2-*Ucp1* transgenic mice, white adipose tissue metabolism, *n*-3 PUFA, calorie restriction

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Special thanks to my parents, my family and friends, who gave me the great support.

This thesis is based on the following publications, referred to by their capital letters in the text, as indicated here. For the complete list published articles of the author of the PhD thesis, see 7 LIST OF ALL PUBLICATIONS OF THE CANDIDATE.

A. Kopecký J, Rossmeisl M, Hodný Z, Syrový I, Horáková M, **Kolárová P.** Reduction of dietary obesity in aP2-Ucp transgenic mice: mechanism and adipose tissue morphology. *Am J Physiol.* 1996 May; 270 (5 Pt 1):E776-86, (IF = 4,75).

B. Rossmeisl M, Syrový I, Baumruk F, Flachs P, **Janovská P**, Kopecký J. Decreased fatty acid synthesis due to mitochondrial uncoupling in adipose tissue. *FASEB J.* 2000 Sep;14(12):1793-800, (IF = 6,79).

C. Rossmeisl M, Barbatelli G, Flachs P, Brauner P, Zingaretti MC, Marelli M, **Janovská P**, Horáková M, Syrový I, Cinti S, Kopecký J. Expression of the uncoupling protein 1 from the aP2 gene promoter stimulates mitochondrial biogenesis in unilocular adipocytes in vivo. *Eur J Biochem.* 2002 Jan;269(1):19-28, (IF = 2.85).

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E. **Janovská P**, Flachs P, Kazdová L, Kopecký J. Anti-obesity effect of *n*-3 polyunsaturated fatty acids in mice fed high-fat diet is independent of cold-induced thermogenesis. *Physiol Res.* 2013 Apr 16;62(2):153-61, (IF = 1.55).

LIST OF ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ADIPOR1	adiponectin receptor
ADIPOR2a	diponectin receptor
ALA	α-linolenic acid
AMPK	AMP-activated protein kinase
aP2-*Ucp1* mice	transgenic mice with expression of mitochondrial uncoupling protein 1 from aP2 gene promoter
ATGL	adipose triglyceride lipase
ATP	adenosin triphosphate
BAT	brown adipose tissue
cAMP	cyclic adenosin monophosphate
cHF	corn oil based high-fat diet
cHF+CR	cHF diet with 10% of calorie restriction
cHF+F	corn oil based high-fat diet supplemented with fish oil
cHF+F+CR	cHF+F diet with 10% of calorie restriction
COX	cytochrome c oxidase
CR	calorie restriction
Cyox3	gene for cyclooxygenase
DAG	diacylglycerol
DHA	docosahexaenoic acid
DNS	*de novo* synthesis
DNP	2, 4-dinitrophenol
EC_{50}	50^{th} percentile values
EPA	eicosapentaenoic acid
EPID-WF	epiddidymal white fat
FA	fatty acids
G-3-P	glycerol-3-phosphate
GLUT4	glucose transporter 4

HF	high fat
HMW	high-molecular weight
HSL	hormone-sensitive lipase
IL	interleukin
INCA	indicect calorimetry
LMW	low molecular weight form of adiponectin
LPL	lipoprotein lipase
MAG	monoacylglycerol
MMW	medium molecular weight form of adiponectin
NE	norepinephrine
NEFA	non-esterified fatty acids
Nrf	gene for nuclear respiratory factor
OGTT	oral glucose tolerance test
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1α	peroxisome-proliferator activated receptor γ co-activator 1α
Pgc-1α	gene for peroxisome-proliferator activated receptor γ co-activator 1
PKA	protein kinase A
PPAR	peroxisome proliferator-activated receptor
PRCF	percent relative cumulative frequency
PUFA	polyunsaturated fatty acid
RER	respiratory exchange ratio
RQ	respiratory quotient
Sc-WF	subcutaneous white fat
ST	standard diet
T2D	type 2 diabetes
TAG	triacylglycerols
TAG/FA	futile substrate cycle based on lipolysis of intracellular triacylglycerols and re-esterification of fatty acids
TNFα	tumor necrosis factor-alpha
UCP1	uncoupling protein 1

Ucp1	gene for uncoupling protein1
VCO_2	carbon dioxide production
Vlcad	gene for very long-chain acyl-CoA dehydrogenase
VO_2	oxygen consumption
WAT	white adipose tissue

CONTENT

1 INTRODUCTION

Prevalence of obesity, the excessive accumulation of adipose tissue, is expanding worldwide dramatically during recent decades. Energy imbalance, sedentary lifestyle and over-nutrition, cause obesity and development other metabolic diseases (1). Differences in propensity to obesity among individuals depend on the involved of both genetic and environmental factors. To improve the efficiency of the treatment of obesity, and namely of the diseases associated with obesity, like type 2 diabetes (**T2D**), coronary heart disease, atherosclerosis and hypertension, metabolism of adipose tissue itself needs to be better characterized (2).

1.1 Energy balance

Energy balance of the whole organism is governed by the First Law of Thermo dynamics, and it is often expressed as follows:

Energy intake = energy expenditure + energy stored

In the state of energy balance energy expenditure is equal to energy intake (Fig. 1). Energy expenditure includes all forms of physical activity, basal metabolism, and adaptive thermogenesis. Physical activity represents all voluntary movement as well as muscle shivering. Basal metabolism reflects the multiple biochemical processes necessary to sustain life. Basal metabolic rate is defined as the energy expenditure of a subject relaxed and at rest, at thermoneutrality, 8–12 hr after the last food ingestion. Actual metabolic rate of mammals will obviously differ depending upon environmental conditions to which the organism is exposed. Adaptive thermogenesis reflects energy dissipated in the form of heat in response to environmental changes, such as exposure to cold and/or different energy rich diet and can be divided in two types of processes:

shivering thermogenesis and non-shivering thermogenesis (3, 4). Shivering thermogenesis belongs to mechanisms protecting the organism from acute cold exposure but it is not an acclimatization mechanism. In response to acute cold exposure, organism starts up the activation of the primary motor centre for shivering of the posterior hypothalamus and muscle fibres are starting to contract involuntarily and heat is produced instead of work (4). After a prolonged period in the cold, animals stop to shiver but retain an equally high metabolic rate. This reflects cold-induced increase in the capacity for non-shivering thermogenesis, occurring namely in brown adipose tissue (**BAT**). In BAT heat production occurres in mitochondria, as a consequence of a uncoupling of oxidatice phosphorylation mediated by mitochondrial uncoupling protein 1 (**UCP1**) (5).

Negative energy balance results in a degradation of energy stores, while the positive energy balance leads to increase in energy stores. Increase in lipid storage in adipose tissue thus reflects excess in energy consumption relative to energy expenditure. Energy balance is responsive to various factors including hormones and neural inputs, in addition to psychological and cultural factors.

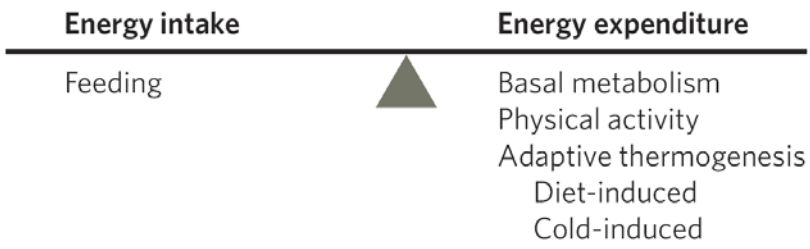

Fig. 1 Energy homeostasis depends upon the balance between caloric intake and energy expenditure. Energy expenditure includes physical activity, basal metabolism, and adaptive thermogenesis. [adapted from (6)].

1.2 Adipose tissue

Two types of adipose tissue, white adipose tissue (**WAT**) and BAT with different functions exist in mammals. White adipose tissue is the primary site of energy storage, which occurs in lipid droplets in adipocytes. The droplets are composed of triglycerides (**TAG**) and cholesteryl esters, with the surface formulated by a phospholipid monolayer, namely by perilipin, which plays an important role in regulation of lipid storage (7, 8). At the same time, WAT represents a major endocrine organ, which affects metabolism in other tissues through secretion of adipokines, which include hormones, cytokines and other proteins with specific biological functions (9).

Until recently, it was thought, that cells of both types of adipose tissue originate from the same progenitor cells but recent studies have shown that classical brown fat (10) as well as myocytes derived from a myf5 expressing progenitor cellular lineage unlike WAT cells, which are derived from a myf5 negative lineage (Fig. 2) (11). However, brown adipose cells can be derived from myf5 negative lineage and they are named „brite cells“ or also beige cells. Brite cells are interspersed in some depots of WAT and exhibit inducibility of thermogenesis by UCP1. The creation or differentiation of brite cells can be induce by cold, sympathetic stimulation, some ligands of peroxisome proliferator-activated receptor-γ (**PPARγ**) or several hormons (2, 11). The key transcriptional regulator in inducing of complete programm of BAT differentiation is protein PRDM16, which coactivates PPARγ coactivator 1 α (**PGC-1α**), as well as PPARγ and suppresses expression of white fat cell markers. Both muscle cells and brown fat cells have numerous mitochondria that burn fuel to release large amounts of energy. Muscle cells exhaust energy generating by oxidative phosphorylation for muscle contraction, while brown fat cells release metabolic energy as heat respiratory uncoupling mediated by UCP1 (12).

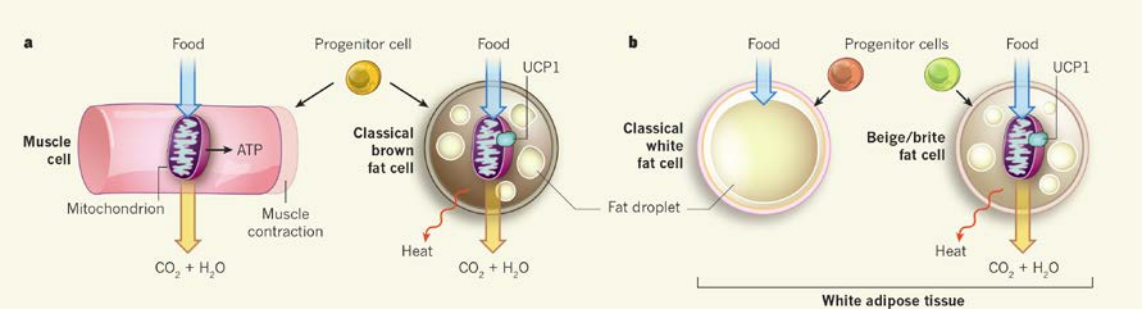

Fig.2 Classical brown adipose cells and muscle cells are derived from the same progenitor cells, unlike the white adipose cells. In WAT were identified two types of progenitor cells and they can differentiate into typical white adipose cells, which store energy in the form of lipid droplets, and/or in „brite cells“, which express UCP1 and generate heat by uncoupling respiration. [adapted from (12)].

When animals are chronically exposed to cold, the ability of the typical brown fat to produce heat is increased in accord with increasing in the total amount of UCP1. In addition certain white adipose depots can undergo a shift in colour from white to brownish (see above) (Fig. 3a). This “browning” process is connected with large increase in expression of the UCP1 gene. It is now known that namely subcutaneous WAT in rodents includes both progenitors of original white fat cells and subpopulation of progenitors of a different type of adipocyte that could express UCP1 (11, 12). However, the contribution of “browning” to total non-shivering thermogenesis is relatively small.

1.2.1 Brown adipose tissue and uncoupling protein 1

Brown adipose tissue, a thermogenic organ, is present throughout the whole life in rodents and in most mammals its mass declines after birth. In human, it is present and active in newborns, where it is essential for a successful defense of body temperature right after birth (13). It was thought for a long time that BAT disappears during early human childhood, but recent studies describe metabolically active BAT in adult man in several anatomical locations, namely in the depots of adipose tissue in supraclavicular

and the neck regions with some additional paravertebral, mediastinal, para-aortic, and suprarenal localizations (14, 15) (Fig. 3b).

a) b)

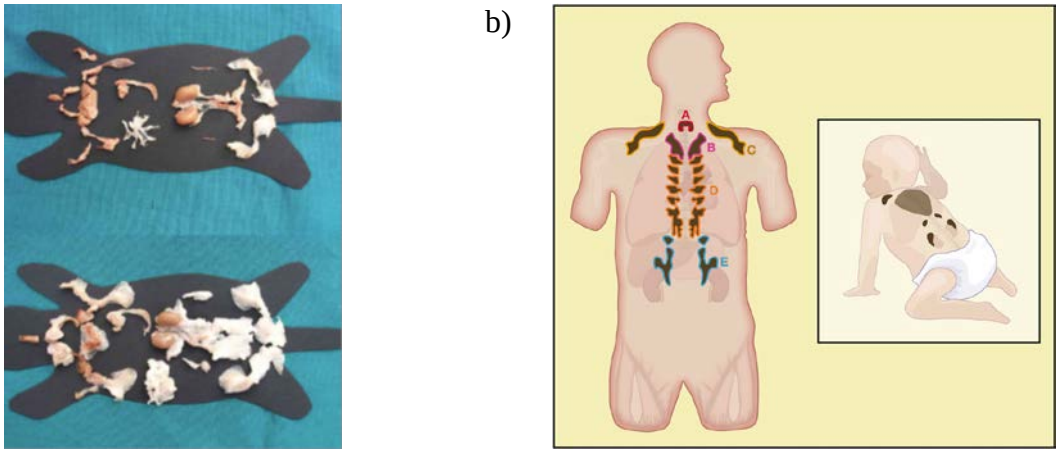

Fig. 3 a) Multidepot adipose organ of adult Sv129 female mice kept at 28 °C (bottom) or at 6 °C (upper) for 10 days. [adapted from (16)]. b) The major human BAT depots in adult man and in newborns: (A) thyroid/tracheal, (B) mediastinal, (C) paracervical/supraclavicular, (D) parathoracical, (E) supra and perirenal. In infants also a thin kite-shaped sheet of BAT between the shoulder blades has been described (in light brown) [adapted from (14)].

The role of BAT in energy metabolism is determined by the amount of UCP1 in the mitochondrial inner membrane. This protein is typically present only in brown fat but recently it has also been identified in thymocytes (17). It converts the electrochemical potential of protons across the inner mitochondrial membrane into heat. Thus regulated uncoupling of oxidative phosphorylation to form heat is the main function of BAT. This tissue accumulates lipids as a source of fatty acids (**FA**) to be oxidized in mitochondria when thermogenesis is activated. Acute regulation of thermogenesis in BAT is mediated by the sympathetic nervous system, where increase food consumption or cold exposure serve as signals for the releasing of neurotransmitter, noradrenaline (**NE**), and initiation of TAG breakdown in the brown adipocytes. This process is primarily mediated via β3-adrenergic receptors. The signal is transmitted via cAMP and protein kinase A (**PKA**) and released FA (13). Fatty acids have a dual function: i) they serve as a fuel for thermogenesis; ii) they activate proton conductance

in UCP1. Noradrenergic stimulation of β3-adrenergic receptors triggers cAMP-responsive pathways (see above) to 1) initiate PKA-dependent lipolysis to release FA that acutely activate UCP1; 2) enhance transcription of UCP1 gene (***Ucp1***) (18). Both mRNA and protein levels of UCP1 are dramatically upregulated on cold-acclimation or noradrenaline treatment. Loss of BAT function is linked to obesity and metabolic disease (19). The UCP1 activity is inhibited by purine nucleoside di- and triphosphates but the role of this mechanism in BAT thermogenesis is still under the discussion. Three competing models are generally described. In the first model FA are obligatory cofactors, which by embedding their carboxyl side-chain into the UCP1 provide a critical proton-buffering site to transport protons across the inner membrane (Fig. 4A). The second model is a "flip-flop" model, where the protonated FA cross the inner membrane and deprotonate in the mitochondrial matrix as governed by the pH gradient. The dissociated FA anion is afterward transported across the inner mitochondrial membrane by UCP1, driven by the membrane potential (Fig. 4B). In the third model, the FA are not necessary for proton transport, but they induce an allosteric change, and overcome the inhibition of nucleotide remaining active UCP1. Simple competitive kinetics can describe the functional interaction (Fig. 4C). Proponents of all the models present favourable pieces of evidence in support of "their" model, thus knowledge of how UCP1 is acutely regulated remains unknown (13, 18).

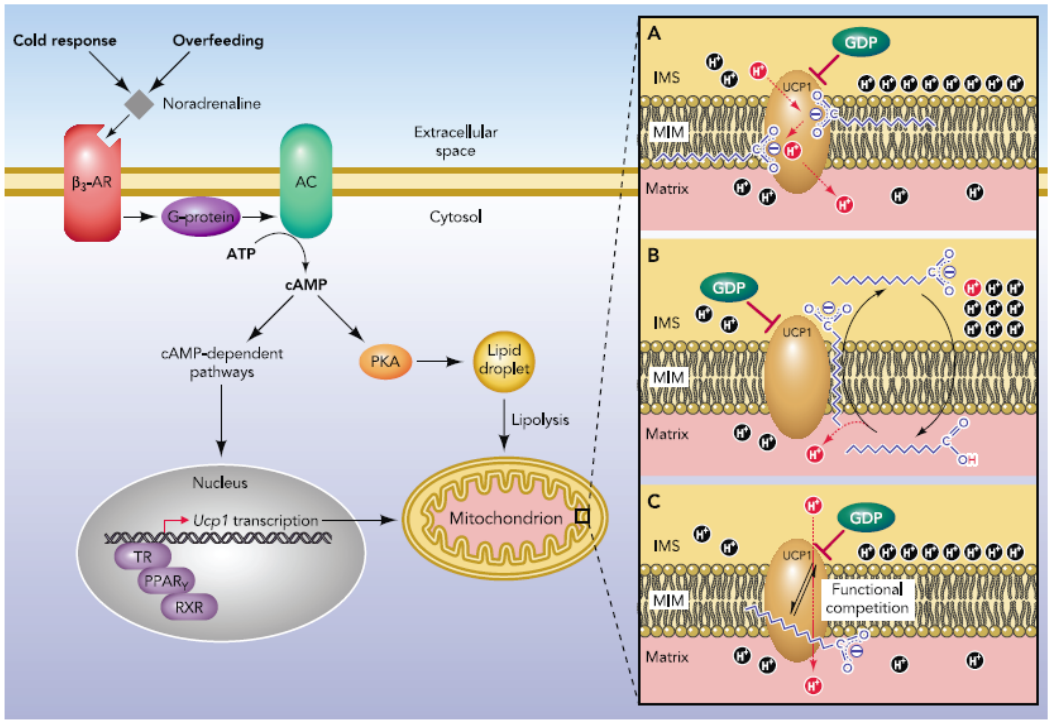

Fig. 4 Activation of thermogenesis in BAT. NE, cold response or overfeeding stimulate β_3-adrenergic receptors, which triggers cAMP pathways to lead in increasing of *Ucp1* gene expression and/or via PKA activation and lipolysis to FA release for UCP1 activation. Three commonly models of UCP1 mechanism in BAT are used: A) FA are as co-factors, their carboxyl groups are embedded into UCP1 protein; B) FA anions are protonated in the intermembrane space and as a neutral molecule enters into the mitochondria; C) FA induce allosteric change for activation of UCP1. [adapted from (17)].

Prolonged cold acclimatization leads to BAT recruitment, the total amount of UCP1 increases about 10-fold or more, but also an increase in total cellularity, total amount of mitochondria, mitochondrial enzymes, FA oxidizing enzymes, etc., contribute to the vastly enhanced oxidative capacity. UCP1-mediated thermogenesis in rodents is inducible not by cold but also by excessive food intake. Thus a cafeteria diet feeding in rats results in hyperphagia, which is accompanied by increase in BAT mass, noradrenaline turnover, oxygen consumption, respiratory enzyme activity and UCP1 protein content (18, 19). Thus, that it is now widely accepted that diet induced thermogenesis, at least in rodents, is only mediated by BAT. However, in contrast with this dogmatic view, it was also found that at ambient temperatures of either 24^{o}C or

28°C, rats fed a cafeteria diet, although showing an increase in whole-body resting oxygen consumption attributable to diet-induced thermogenesis, had the same levels of oxygen consumption by interscapular BAT in comparison with rats fed the control (20). This surprising observation is in fact in accordance with the results of the group of Kozak showing that both control and UCP1-ablated mice had similar oxygen consumption before and after a switching from the chow to high/sucrose diet (21). UCP1 ablation led to development of obesity at thermoneutral temperature, but surprisingly, the UCP1-ablated mice were resistant to high-fat diet induced obesity at low ambient temperature (2).

The resistance to obesity under the above conditions was associated with increased oxidative capacity in WAT (2, 22). These results supported the notion, that regulatable non-shivering thermogenesis independent of UCP1 in BAT, which could take place in other tissues, could counteract obesity (19, 23).

1.2.2 White adipose tissue

For a long time, WAT was considered to serve just a "passive" energy-storing tissue. However, present view is different. It is now known that WAT not only serves as an organ, which stores TAG during excessive energy intake and releases FA into circulation when energy is scarce, but that WAT can regulate the metabolism of other organ and tissues (brain, liver, muscle or pancreas) through various signals, namely adipokines.

1.2.2.1 Lipid metabolism in white adipocytes

Fat accumulation is determinated by the balance between lipogenesis and fat breakdown (lipolysis/FA oxidation), while contribution of FA oxidation within adipocytes is

relatively small (24). Most energy reserves in the animals and human body are stored in adipocytes as TAG, which may arise from either *de novo* lipogenesis or uptake of FA from plasma (25). Between meals, during the periods of food shortage or calorie deficit, such as starvation, fasting or long-term exercise, the energy for other organs is obtained from hydrolysis TAG into FA and glycerol (lipolysis), which are released from adipocytes to plasma circulation. Free FA are bound to plasma albumin and can be transported and utilized by other organs (26).

Lipolysis in adipocytes reflects the hydrolysis of TAG, to release FA and glycerol for use by other organs. Thus FA can enter circulation and be taken up by other organs for β-oxidation and ATP generation (mainly muscle and liver). In addition, FA and glycerol can also serve as substrate for ketogenesis and gluconeogenesis in the liver. Free FA can be again utilized in adipocytes to re-esterification, which always accompanies lipolysis and requires energy ((2); see below). Lipolysis occurs at the surface of lipid droplets of the adipocytes and it is regulated by hormones. In the fasted state, glucocorticoids are elevated and adipose triglyceride lipase (**ATGL**), the major enzyme of lipolysis, is upregulated. ATGL hydrolyzes TAG to diacylglycerol (**DAG**) (27, 28). Furthermore, catecholamines bind to the $G\alpha_s$-coupled β-adrenergic receptors and activate a signaling cascade, which increases cAMP levels and so activates PKA. PKA phosphorylates enzyme hormon-senzitive lipase (**HSL**), which is the major enzyme in hydrolyzing DAG to monoglycerol (**MAG**). PKA also phosphorylates perilipin, the protein covering the lipid droplets. Phosphorylated perilipin triggers a major remodelling of lipid droplets to provide lipases greater access to the lipid droplets (25, 29). MAG can be hydrolyzed by MAG lipase to glycerol and FA. Lipolysis is inhibited in the fed state by insulin, which binds to its receptors, and a by signaling cascade, which decreases cAMP (25, 30).

Under normal conditions, the adipocytes are capable to maintain the balance between lipolysis (TAG breakdown) and lipogenesis (TAG synthesis) in response to physiological requirement. For the maintaining of energy homeostasis and the prevention an abnormal increase in plasma non-esterified (free) FA that is lipotoxic, storage of TAG in adipocytes must be well controlled (31).

De novo synthesis (**DNS**) is the synthesis FA from non-lipid substrates, mainly carbohydrates (glucose), and it is present in liver, WAT and in the mammary glands. DNS is regulated by hormones (insulin, glucagon) and by nutritional conditions (carbohydrates and/or polyunsaturated fatty acids (**PUFA**)). In adipose tissue and muscle, insulin controls TAG synthesis by increasing glucose uptake and conversion of glucose to glycerol-3-phosphate (**G-3-P**) via glycolysis. It seems that G-3-P from this pathway is not the major source for TAG synthesis (26, 32). Glyceroneogenesis, depending on the production of G-3-P from gluconeogenic substrates such as pyruvate, is mainly regulated at the level of cytosolic phosphoenolpyruvate carboxykinase (**PEPCK**). PEPCK catalyzes the conversion of oxalacetate, a citric acid cycle intermediate, to phosphoenolpyruvate, which is converted via several steps to G-3-P for TAG synthesis (32, 33).

Part of FA released by lipolysis is immediately transported back into adipocytes by FA translocase/CD36 (**FAT/CD36**; futile **TAG/FA cycle**) (34-36). TAG/FA cycle is probably essential for buffering of plasma FA levels (32) and so it has an import role in maintaining of metabolic flexibility of adipocytes, as well as the whole organism. In transgenic mice the overproduction of PEPCK, the key enzyme of glyceroneogenesis, in WAT increased glyceroneogenesis, reesterification of free FA, body weight and fat mass, decreased and preserved glucose tolerance and whole-body insulin sensitivity (37). It indicates the key role of PEPCK in the control of FA re-esterification in adipose tissue and, thus, the contribution of glyceroneogenesis to fat accumulation.

Lipolysis in adipocytes can be associated with oxidative stress and energy depletion and also with activation of AMP-activated protein kinase (**AMPK**) (38). AMPK is activated during starvation (39) or during physical activity (40), when also increased activity of mitochondrial enzymes and expression of PEPCK were found. AMPK modulates lipolysis in WAT while affecting PKA, HSL and ATGL (30, 41-43). Activation of AMPK increases acute hydrolysis of TAG, while in long-term, activation of AMPK supports resynthesis of TAG (42). Thus, AMPK may participate in control of TAG/FA cycle and could modulate balance between lipolysis and FA re-esterification.

1.2.2.2 Adipose tissue as an endocrine organ

The secretary function is an important characteristic of WAT, which secretes a large variety of bioactive molecules, known as adipokines (Fig. 5). These molecules exert endo-, auto-, or paracrine signal functions (44). Obesity induces changes in adipokine secretion and increases secretion of pro-inflammatory cytokines such as tumor necrosis factor alpha (**TNFα**), supporting development of insulin resistance (45). Expansion of WAT in obesity leads to increased macrophage infiltration and inflammation underlying enhanced production of pro-inflammatory cytokines. Obesity is also associated with increased release of free FA and dysregulation secretion of anti-inflammatory cytokines, such as leptin, adiponectin and resistin (46).

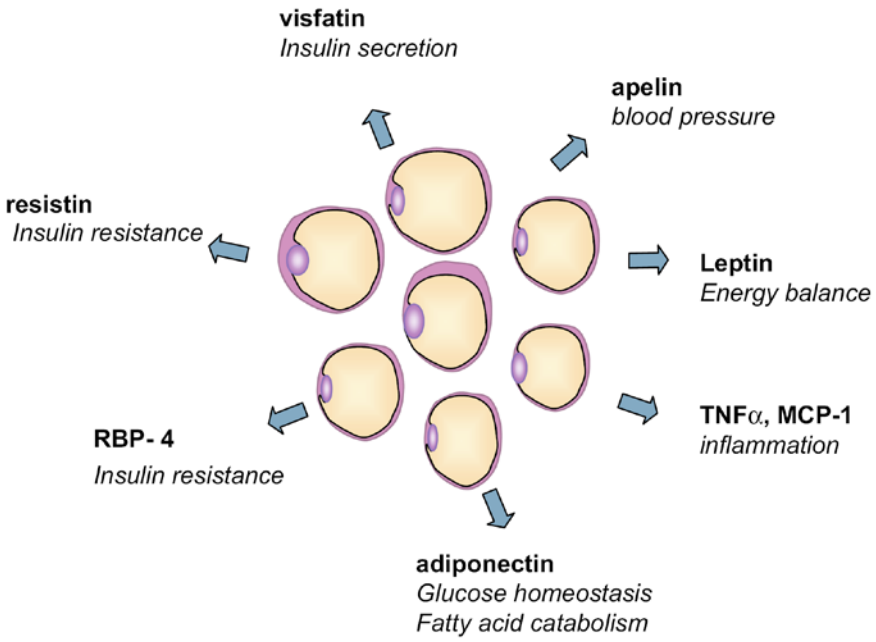

Fig. 5 White adipose tissue regulates whole body homeostasis by secretion several proteins with endo-, auto-, or paracrine functions. Illustration of the major adipokines secreted from adipokines with their metabolic and physiological effects. [adapted from (9)].

Leptin The name of this cytokine comes from greek *leptos*, and means lean. This protein has a molecular mass of 16kDa and regulates energy metabolism, increasing energy expenditure and decreasing energy consumption (9, 47). Plasma leptin levels directly correlate with WAT mass. Leptin is secreted mainly by WAT but also by other organs such as the stomach, placenta, and brain. Leptin controls WAT growth through its action at the central nervous system. Hypothalamus leptin receptors control satiety and energy balance. Activation of the sympathetic nervous system by leptin leads to a decrease of its own secretion and it is a part of a negative feedback loop controlling body weight (48). Except for hypothalamus, leptin receptors are present in various organs such as liver, skeletal muscle, heart, kidney, pancreas, and others. Leptin mRNA is present mainly in WAT, but it was found also in ovary, intestine and in liver. White adipocytes also express leptin receptors, suggesting an autocrine and paracrine role of hormone. Leptin selectively activates the α2 catalytic subunit of AMPK in skeletal muscle, which stimulates FA oxidation (49), reduces ectopic fat accumulation in non-adipose tissues and increases insulin sensitivity. Hyperleptinemia is typical for obese subjects and is defined as decreased sensitivity to the effects of leptin, associated with impaired leptin signalling cascade. Description of these changes led to the concept of leptin resistance (47).

Adiponectin This adipokine is specifically and abundantly expressed in WAT and is an abundant plasma protein as well (50, 51). Adiponectin exists in a wide range of multimer complexes in plasma and it creates 3 major oligomeric forms: a low–molecular weight (**LMW**) trimer, a middle–molecular weight (**MMW**) hexamer, and high–molecular weight (**HMW**) 12- to 18-mer adiponectin (52). There is a strong negative correlation between plasma adiponectin concentrations and fat mass, except some cases of undernutrition and in newborns (50, 53). Adiponectin acts mainly via two receptors, ADIPOR1, which is found mainly in skeletal muscle and WAT and ADIPOR2 that exists mainly in the liver. In skeletal muscle, adiponectin increases glucose uptake (via GLUT4 translocation) and nonoxidative glycolysis, while at the same time it promotes FA oxidation (54). In comparison with mice, human studies do not demonstrate altered ADIPOR1/ADIPOR2 mRNA levels associated with insulin-resistant states. *In vitro*

study on the myotubes of obese patients with or without T2D showed impairment of adiponectin-stimulated AMPK phosphorylation and fatty FA oxidation (55). In the liver adiponectin has several effects. The main role is in the lowering systemic glucose levels by suppression of hepatic glucose output. Adiponectin suppresses the expression and activity of key regulators in gluconeogenesis, such as PEPCK and glucose-6-phosphatase (54, 56). Adiponectin also influences FA metabolism in the liver, with secondary effects on circulating levels of TAG and free FA. Adiponectin may play an important role as a starvation factor, since it inhibits energy expenditure, promotes food intake centrally, and stimulates FA utilization in peripheral tissues (57).

Resistin The peptide hormone resistin is expressed within adipocytes of rodents and macrophages of humans. Its production is increased with feeding and obesity and decreased by PPARγ ligands such thiazonolinediones (53). In humans resistin gene is expressesed mainly in WAT and to lesser extent also in BAT, and in mammary gland. This hormone acts in skeletal muscle and adipose WAT but the mechanism and role of resistin are in these tissues still unknown. In mice resistin plays important role in glucose homeostasis and insulin resistance probably through decreased activity of AMPK and increased expression of gluconeogenic enzymes in liver. Strong expression of resistin gene was found in perigonadal fat of female mice and the association of obesity and high serum concentration of this hormone was demonstated (44, 58).

TNFα A pro-inflammatory cytokine, which was described as the first molecular link between inflammation and obesity. This inflammatory cytokine is overexpressed in the WAT of rodent models of obesity and in the WAT as well as muscle of obese humans (46). The primary sources of TNFα are macrophages, which infiltrate into WAT due to obesity (59). TNF-α inhibits lipogenesis and stimulates lipolysis (53) as well as the production of other cytokines, such as IL-1 and IL-6.

1.3 aP2-*Ucp1* transgenic mice

To learn whether energy expenditure in WAT could be induced to the extent, which compromises accumulation of body fat and obesity, a unique transgenic mice was developed, which harboured ectopic *Ucp1* expression in WAT (**aP2-*Ucp1*** transgenic mice). To this end, the transgene was constructed by using the promoter of the adipose lipid binding - protein (**aP2**) gene, and the whole genomic sequence of *Ucp1*, and mice of the C57BL/6J genetic background (60). Overexpression of *Ucp1* from the transgene was observed in both BAT and WAT, but overexpression of *Ucp1* in BAT caused the atrophy of this tissue, apparently due to a collapse of energy metabolism of brown adipocytes. In mice fed chow diet with ectopic expression of *Ucp1,* no differences in body weight during the first two months were observed. At 3 months of age, the reduction of the size of the subcutaneous WAT tissue in transgenic mice was observed. Lower body weight gain was observed in transgenic mice in comparison with their nontransgenic littermate in resposne to high-fat (**HF**) diet-feeding, starting from 3 months of age. Gonadal fat was enlarged and subcutaneous fat depot was decreased in aP2-*Ucp1* mice as compared with the control mice. Transgenic mice also showed improved glucose intolerance and lower insulin level on HF diet as compared with control mice (61).

Later studies showed stronger expression of *Ucp1* in subcutaneous than in epididymal WAT, and decline in this expression during ageing. Ectopic UCP1 decreased mitochondrial membrane potential in adipocytes of transgenic mice, and it was regulated by FA and adenine nucleotides as expected, indicating that UCP1 was properly inserted in mitochondrial membrane and that is was functional (62). In WAT of transgenic mice, increased lipoprotein lipase (**LPL**) activity was detected, especially in epididymal WAT (63). Moreover, ectopic expression of *Ucp1* resulted in decreased FA synthesis (64) and down-regulation of FA re-esterification (63), inhibition of NE-stimulated lipolysis and reduced HSL activity in WAT (65). It was also found that transgenesis was associated with up-regulation of glycolysis and mitochondrial biogenesis and increasing lactate production in WAT (66). Expression of *Ucp1* in WAT resulted in decrease of expression

PPARγ and aP2 genes (67). Therefore, for the first time, experiments in aP2-*Ucp1* mice verified the concept that increased energy expenditure in WAT may counteract obesity.

1.4 *n*-3 polyunsaturated fatty acids and calorie restriction in prevention of obesity

The dietary FA can affect the modulation of metabolic processes in the body, including those in adipose tissue (68). Ratio of *n*-6 and *n*-3 PUFA, which was changed during the ages due to changes in dietary habits, may affect the risk of obesity and metabolic syndrome (69). *n*-3 PUFA, especially α-linolenic acid (**ALA**; 18:3*n*−3), eicosapentaenoic acid (**EPA**; 20:5*n*-3), and docosahexaenoic acid (**DHA**; 22:6*n*-3), have cardioprotective effect, act anti-inflammatory, as hypolipidemics, and these lipids may reduce obesity in rodents (see below).

Humans and animals can synthesize saturated and monosaturated FA *de novo*, but both lack the enzyme responsible for insert a *cis* double bond at the *n*-3 or the *n*-6 position of a FA to synthetize ALA or linoleic acid, respectively (69). ALA is the essential FA and can be metabolized into EPA and DHA, which have hypolipidaemic effect, regulate glucose transport in muscle and in adipocytes and protect against insulin resistance and obesity in rodents fed HF diets (70). *n*-3 PUFA suppress new FA synthesis, induce FA oxidation in liver, skeletal muscle and in WAT (71, 72), regulate gene expression acting through the PPARα and sterol regulatory element-binding protein pathways (73, 74). In human *n*-3 PUFA exert beneficial effects in improvements in lipid metabolism and decreasing the rate of fatal coronary heart disease in diabetic patients who had a myocardial infarction (75). *n*-3 PUFA have also anti-inflammatory effect in diseases such as rheumatoid arthritis and Crohn's disease (76).

Calorie restriction (**CR**), reduction of daily calorie intake, affects adipocyte function and reduces body weight (77). CR provides health benefits and extends life span in diverse organisms, including mammals and modulates adipose physiology, such as fat cell size, augments lipolytic rates within subcutaneous fat and increases circulating

adiponectin. CR in mice modulates also gene expression, upregulates genes for FA oxidation and downregulates genes for FA synthesis. Calorie restriction increases mitochondrial respiration as well as mitochondrial number in WAT (78).

It has been demonstrated at our laboratory that dietary intake of *n*-3 PUFA resulted in upregulation of mitochondrial biogenesis in WAT in mice fed HF diet, as well as in increased lipid catabolism in WAT (71), and in the induction of adiponectin (79). Moreover, we have shown that supplementation of HF diet using *n*-3 PUFA resulted in decreased level of endocannabinoids in WAT (80). This could be a part of the mechanism, by which *n*-3 PUFA could induce mitochondrial biogenesis (81).

2 HYPOTHESIS AND SPECIFIC AIMS

General goal of this PhD thesis was to verify the hypothesis that accumulation of body fat and obesity could be counteracted by increasing energy expenditure in WAT, independent of UCP1.

The first part of the work was focused on the detailed characterization of the phenotype of the aP2-*Ucp1* transgenic mice, especially on the metabolic changes induced by ectopic UCP1 in WAT. In the second part of this work, I have contributed to the studies aimed to learn whether lower accumulation of body fat could be induced in response to a physiological intervention increasing energy expenditure in WAT, namely the combination treatment with calorie restriction and *n*-3 PUFA in mice fed obesogenic HF diet.

The specific aims of the PhD thesis were:

1. characterization of the biochemical and morphological changes in white and BAT of aP2-*Ucp1* mice fed standard chow or high-fat diet (*see Publication A - 4.1*);

2. characterization of the link between efficiency of energy conversion and the rate of FA synthesis in adipocytes and verification of the hypothesis that respiratory uncoupling in WAT could modulate adiposity via decreasing *de novo* lipogenesis (*see Publication B - 4.2*);

3. characterization of the effect of respiratory uncoupling in WAT on mitochondrial biogenesis (*see Publication C - 4.3*);

4. characterization of the role of WAT metabolism in the effect of the combination treatment based on calorie restriction and *n*-3 PUFA, with a major focus on the induction of energy expenditure in WAT, the underlying mechanism and metabolic flexibility (*see Publication D - 4.4*); and

5. verification of the idea that the the induction of FA oxidation in WAT, and the anti-obesity effect of *n*-3 PUFA, are independent of cold-induced UCP1-mediated thermogenesis (*see Publication E - 4.5*).

3 METHODS

All the methods and techniques, including characterization of animals and treatments used, are described in the particular publications (see Section 9). In the following text, only methods performed by the author of the PhD thesis, which were used the publications underlying this thesis, are described.

3.1 Evaluatioin of tissue respiration using Warburg apparatus

Fragments of BAT, epididymal fat, or liver (40-60, 200-300, and 40-60 mg, respectively) were incubated in Warburg vessels with 3 ml of the incubation medium [90mM NaCl, 4.7mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 3.6 mM $NaHCO_3$, 25 mM glycylglycine (pH 7.4) and 10mM glucose]. Respiration of tissue fragments was measured at 30^oC under the atmosphere 95% O_2 - 5% CO_2, monitored by manometer and recorded every 10 min during 150 min of measurement. The data are means ± SE and they were evaluated as µl of oxygen consumption per mg of DNA (82, 83).

1.3 DNA measurements

The method is based on the enhancement of fluorescence seen when bisbenzimidazole (Hoechst 33258) binds to DNA. DNA was estimated in 10 to 20 µl aliquots of 10% BAT and 20% WAT homogenates, prepared in TE medium (10 mM Tris.Cl and 2 mM EDTA, pH 7.4). The all-glass homogenizer was used (83, 84).

3.3 Differentiation of 3T3-L1 adipocytes in cell cultures

3T3-L1 cells were grown in Dulbecco's modified Eagle's medium at 37^{o}C in 10% CO_2 in air. After cell confluence (~4-5 days after plating) the differentiation medium with 2 µM dexamethasone (for the first 48 h) and 100 nM 5-(4-[2-(N-methyl-N-(2-pyridyl) amino) ethoxy] benzyl) thiazolidine-2, 4-dione maleic acid salt (BRL 49653; in methyl sulfoxide) was used. For experiments cell cultures were used, which contained 50-60% of differentiated adipocytes (12-14 days after confluence). Before each measurement, a complete change of medium was performed. For the induction of uncoupling oxidative phosphorylation in mitochondrial membrane of cells, 2, 4-dinitrophenol (**DNP**) of different concentrations was used (66).

3.4 Measurement of FA oxidation in 3T3-L1 adipocytes

FA oxidation was assessed according Zhou et al., with some modification (85). Adipocytes in cell culture dish were incubated for 4 h at 37 ^{o}C in medium with 0.4% BSA and 5µCi (9, 10[n-^{3}H] oleic acid (10.3 mCi/nmol; Amersham). After incubation, medium was collected and excess of [^{3}H]oleic acid was removed by repeated precipitation with an equal volume of 10% trichloroacetic acid with 2% BSA. Supernatants were transferred to a 1.5 ml microcentrifuge tube placed into scintillation vial containing 0.5 ml sterile unlabeled water and incubated 18 hr at 50^{o}C. As the standard, 1.6µCi/ml $^{3}H_2O$ was used. After 18 hr, the microcentrifuge tube was removed and radioactivity was measured by scintillation counting (66, 85).

3.5 Measurement of FA synthesis in 3T3-L1 adipocytes

FA synthesis was measured by the incorporation of ^{14}C from acetate into saponifiable FA. The adipocytes were incubated for 4 hr at 37^{o}C in the presence of 1 µCi/ml [2-^{14}C] acetic acid. After 4 hr incubation, the medium was discarded and cells were washed twice by phosphate buffered saline (pH 7.4) and solubilised by proteinase K solution for 15 min at 20^{o}C. Aliquots were used for DNA measurement (see 3.2) and assessment of incorporation of radioactivity into FA. The latter aliquot was incubated in the presence of KOH (final concentration 22.5%) for 15 min at 70^{o}C and after incubation 96% ethanol (final concentration 41%) was added. The mixture was again incubated for 120 min at 70^{o}C. After adjunction of 450µl of 5.4 M H_2SO_4 and 3 ml petroleum ether with vigorous mixing and separation and evaporation of petroleum ether, scintillation coctail was added and radioactivity was measured (66, 86).

3.6 Measurement of lactate production in 3T3-L1 adipocytes

Lactate released by cells differentiated in cell culture in to medium was measured using a coupled enzyme assay (kit 139 084 from Boehringer Mannheim, Germany) after 24 hr of culture in the presence of DNP. L-lactic acid (**L-lactate**) is oxidized by nicotinamide-adeninedinucleotide (**NAD**) in the presence of L-lactate dehydrogenase to pyruvate. The trapping of pyruvate by subsequent reaction catalysed by the enzyme glutamate-pyruvate transaminase in the presence of L-glutamate can display the reaction in favour of pyruvate and NADH. The amount of NADH is stochiometric to the amount of L-lactic acid and the increase in NADH was determinated by means of its light absorbance at 340 nm (66).

3.7 RNA isolation for analysis of gene expression

Total RNA was isolated from adipocytes in cell culture using guanidinium thiocyanate as described by Chomczynski and analysed by Northern blot analysis. The cells were washed twice by PBS and dissolved in denaturating solution [4M guanidinium thyocyanate, 25mM sodium citrate, pH 7.0, 0.5% (wt/vol) *N*-laurosylsarcosine (Sarkosyl) and 0.1M 2-mercaptoethanol], collected to the 2ml tubes and kept on ice. Sodium citrate, phenol, water, and mix of chloroform and isoamylalcohol (24:1) were gradually added during shaking. After centrifugation the supernatant with DNA and RNA was collected. Isopropylalcohol was added, mixted and after precipitation in -20^{o}C and centrifugation was supernatant immediately removed and pellet with RNA was washed twice with ethanol and dried in vacuum. RNA was dissolved in formamide during night and RNA concentration and purity was determinated by spetrophotometrically (64, 87, 88).

3.8 Animal experiments and dietary interventions

Male mice C57BL/6J were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred at the animal facility of Institute of Physiology for several generations. From the weaning were mice maintained onto standard laboratory chow (**Chow**; extruded R/MH diet; Sniff Spezialdiäten, Soest, Germany). Two month old mice were singly caged and habituated to a corn-oil based high-fat diet (**cHF**; lipid content 35%, wt/wt) for two weeks and then randomly divided for 5 weeks or 15 weeks to various dietary treatments: (1) cHF, ad libitum; (2) cHF supplemented with *n*-3 PUFA concentrate (46% wt/wt, DHA, 14%, wt/wt EPA; product EPAX 1050 TG; EPAX, Alesund, Norway), which replaced 15% of lipids of dietary lipids, specifically the corn oil part, (**cHF+F**), ad libitum; (3) cHF with restriction of energy intake, the ratio was reduced by 10% wt/wt in comparison with mice fed ad libitum with the same type of diet (**CR; cHF+CR**); and (4) **cHF+F+CR**. The body weight and food intake were

monitored weakly. During the fourth week of the experiment indirect calorimetry measurement was performed (70, 89).

3.9 Indirect calorimetry and metabolic flexibility

The indirect calorimetry was performed using teh system INCA (Somedic, Horby, Sweden), this technique has been introduced during 2004-6 to our laboratory by the author of this PhD thesis. The INCA system is designed for determination of energy expenditure and metabolic flexibility in rodents (Fig. 6). The animal is placed into the measuring chamber with a constant airflow and controlled temperature, where the oxygen consumption ($\boldsymbol{VO_2}$) as well as carbon dioxide production ($\boldsymbol{VCO_2}$) of the animal is measured every 2 min. The respiratory quotient (**RQ**) is calculated from these two measurements (VCO_2/VO_2). After implantation of the transponder (Mini-Mitter ER4000) into the abdomibal cavity of animal, simultaneous recordings of body core temperature, total locomotor activity and heart rate can also be measured. Data from this transponder is collected in parallel with the metabolic data and are storaged in the computer (90).

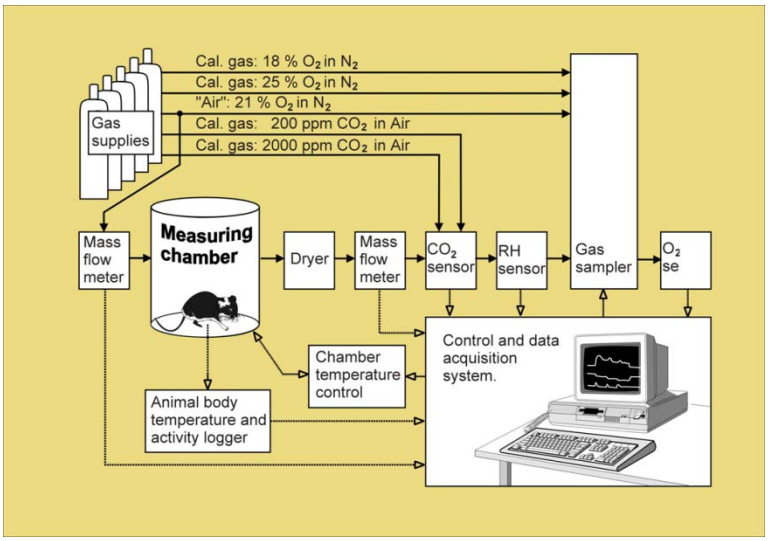

Fig. 6 INCA system. The system is calibrated by calibration gas (18% and 20% O_2 in N_2) before the start of the actual measurement. The airflow is regulated by mass flow meter on entering into measuring chamber with animal. At the output is the air dried and amount of CO_2, O_2 and humidity (RH sensor) is measured every 2 min. Composition of the incoming air is also check every 2 min. System is under computer control, where the data are stored [adapted from (90)].

For assessment of energy expenditure and metabolic flexibility, we used two experiments protocols at 30^{o}C ambient temperature (close to the thermoneutral temperature). In the experiment aimed to characterize the effectiveness of the combination treatment with *n*-3 PUFA and calorie restriction, we assessed maximal induction in RQ in response to a glucose load (0.45 ml of 50% [wt/vol.] D-glucose] administrated by intragastic gavage to overnight (12 h) fasted animals. The induction of RQ (**ΔRQ**) was calculated as a difference of averaged RQ values from 60 min intervals before and after the gavage (70).

In the experiment focused on the effects of *n*-3 PUFA, we evaluated RQ in 6-month-old C57BL/6J male mice fed standard low-fat (**ST**), or cHF diet, or cHF diet supplemented with EPA and DHA (cHF+F). During the INCA assessment, mice were fed the experimental diets for 22h, starting at 3:00 p.m. and the level of substrate partition was assessed by calculating respiratory exchange ratio (**RER**; VCO_2/VO_2 ratio). Percent relative cumulative frequency (**PRCF**) curve pooled from the all animals within a given dietary groups (6-8 animals per group) were constructed, based on the RER values. Both $\mathbf{EC_{50}}$ and Hillslope values were evaluated (91-93).

4 RESULTS OF SELECTED PUBLICATIONS

4.1 Publication A: White and brown adipose tissue, aP2-*Ucp1* transgenic mice, diet-induced obesity

Summary of results of the article "**Reduction of dietary obesity in aP2-*Ucp* transgenic mice: mechanism and adipose tissue morphology"**, by Kopecký J., Rossmeisl M., Hodný Z., Syrový I., Horáková M., **Kolářová P**., published in American J. Physiol., 1996.

The aim of this study was to describe the biochemical and morphological changes in WAT and BAT of aP2-Ucp1 mice fed standard chow or high-fat diet.

Experiment was accomplished mostly using 3-mo-old nontransgenic and aP2-*Ucp1* animals (transgenic mice) fed the standard Chow or HF diet during 6 months period. Chow diet contained 25, 9, and 66% calories as protein, fat, and carbohydrate, respectively. The HF diet, which was used because of its obesogenic character, contained 13, 60, and 27% calories protein, fat, and carbohydrate. Interscapular BAT, subcutaneous (dorsolumbal) and visceral (epididymal) WAT were used for the respiratory measurement and DNA, RNA, proteins, enzymes and histology analysis.

In the aP2-*Ucp1* mice, expression of *Ucp1* was detected only in white and brown fat but not in other tissues and it was associated with mitochondrial membranes. UCP1 concentration in mitochondria from epididymal WAT of transgenic mice was ~14-fold lower in comparison with BAT mitochondria of nontransgenic mice maintained at 20^{o}C. While there were no differences in the amount of UCP1 protein in BAT between the transgenic and nontransgenic mice fed Chow diet, the amount of this protein was significantly increased in nontransgenic mice fed HF diet. This increase was absent in transgenic mice. In epididymal WAT, antigen UCP1 was detected only in transgenic

mice and its content was significantly increased in mice fed HF diet in comparison with mice fed Chow diet.

For the evaluation of mitochondrial oxidative capacity, activity and mRNA level of cytochrome c oxidase (**COX**) was determined. In nontransgenic mice, COX activity in WAT was ~10-fold lower than in BAT, while HF diet increased mitochondrial oxidative capacity in both type of adipose tissues. The increase in the subcutaneous WAT was 9.1-fold as compared with the Chow diet mice. The COX activity significantly declined in BAT in transgenic mice in comparison with nontransgenic mice, especially on HF diet, indicating a lower mitochondrial oxidative capacity.

The *Ucp1* mRNA level in BAT of transgenic mice was raised compare to nontransgenic mice, reflecting a contribution of both aP2-*Ucp1* transgene and endogenous *Ucp1*. In WAT depots, *Ucp1* mRNA was detected only in transgenic but not in nontransgenic mice and dietary condition has no significant effect on the expression in any fat depot.

Reflecting the earlier indirect results on the induction of energy dissipation in WAT of transgenic mice, the Warburg apparatus was used for the characterization of tissue respiration measurement. The rate of endogenous oxygen consumption in the epididymal fat was ~1.5-fold higher in the transgenic as compared with the nontransgenic mice. Surprisingly, an inverse effect (a 2-fold difference) of the transgen was found in BAT (Fig. 7).

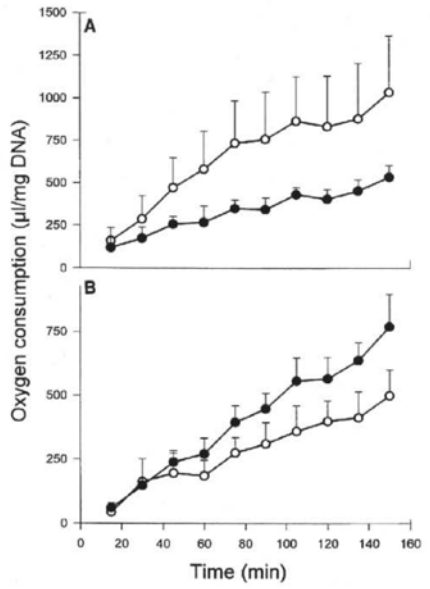

Fig. 7 Endogenous oxygen consumption in brown (A) and epididymal (B) adipose tissue from 8-to 10-mo-old transgenic (○) and nontransgenic (●) mice fed Chow diet. Data are expressed relative to tissue DNA content. Data are means ± SE. The difference between transgenic and nontransgenic mice were significant (P<0.05), (83).

For determination of possible differences between subcutaneous and abdominal WAT in lipid metabolism in the response to aP2-*Ucp1* transgene, the activity of LPL, the main enzyme of regulation of triglyceride uptake, was assessed. The LPL activity was significantly increased on HF diet without any major effect of the transgene in epididymal fat. In subcutaneous fat was no difference in LPL activity in mice fed Chow diet between both genotypes was detected, but HF diet induced LPL activity much more in transgenic mice then in nontransgenic mice.

The DNA concentration was increased in BAT in comparison to WAT, where in epididymal fat it was much lower than in subcutaneous fat in both genotypes. Surprisingly, in spite of the lower weight of the epididymal fat in transgenic mice, the total DNA content was significantly higher than in nontransgenic mice fed both Chow (~1.5-fold) and HF (~1.4-fold) diet. DNA concentration in epididymal fat tended to be higher in Chow diet but in HF diet was DNA increased ~3.5-fold in transgenic in comparison to nontransgenic mice. These results reflected the proliferation of novel small cells in the epididymal fat by the aP2-*Ucp1* expression and were confirmed by

histological analysis. These small cells did not accumulate significant amounts of lipids and they were much more abundant in the transgenic HF diet-fed than in the Chow diet-fed mice.

In conclusion, endogenous *Ucp1* expressed in WAT of aP2-*Ucp1* transgenic mice has thermogenic activity, whereas its expression in BAT suppressed this activity. Altered mitochondrial energy metabolism in transgenic mice modulated adiposity and energy expenditure and pointed out on different roles of white adipose depots in lipid metabolism.

My contribution to this work was the determination of endogenous oxygen consumption in adipose tissue using Warburg apparatus and the help with the biochemical measurements.

4.2 Publication B: Lipogenesis, aP2-*Ucp1* transgenic mice, 3T3-L1 cells, obesity

Summary of results of the article "**Decreased fatty acid synthesis due to mitochondrial uncoupling in adipose tissue"**, by Rossmeisl M., Syrovy I., Baumruk F., Flachs P., **Janovska P.**, Kopecky J., published in The FASEB Journal 2000.

Based on the published data showing that transgenic UCP1 in WAT affects body weight, and the preliminary data concerning the increase in FA oxidation and decreased lipogenesis in in WAT of aP2-Ucp1 transgenic mice, we focused on the analysis on the link between Ucp1 expression and FA metabolism in WAT.

To characterize the effect of mitochondrial uncoupling on the metabolism of adipocytes, the first experiment on differentiated 3T3-L1 incubated or not with chemical uncoupler DNP was performed. With increasing concentration of DNP, FA oxidation increased and lactate production and FA synthesis became inhibited. Parallel measurement of FA synthesis with estimation of the mitochondrial membrane potential ($\Delta\psi_m$) in whole cells confirmed previous effect of DNP and they showed a strong correlation between decreased FA synthesis and decreased $\Delta\psi_m$ in adipocytes (Fig. 8).

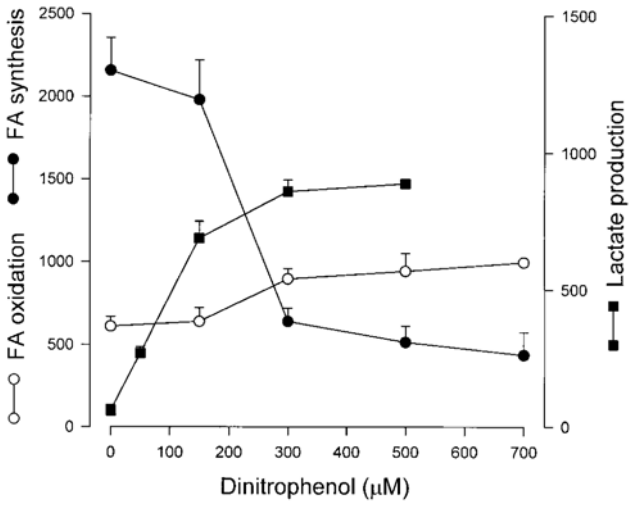

Fig. 8 The FA oxidation, FA synthesis and lactate production were measured in presence of different concentration of DNP in 3T3-L1 differentiated cells (adipocytes). Values are means ± SE of three independent experiments (66).

For *in vivo* experiment, aP2-*Ucp1* mice and their nontransgenic littermates as a model of mitochondrial uncoupling induced by expression of ectopic *Ucp1* in WAT were used. Expression of the genes for ACC and FAS, the lipogenic enzymes, was significantly declined by the *Ucp1* transgene in both subcutaneous and epididymal WAT. The transgene decreased also FA synthesis in both WAT depots in experiment *in vivo*, as well as in experiment *in vitro* (Fig. 9), this suppression was detected only in adipose tissue but not in other tissues, reflecting the specific ectopic expression of *Ucp*1 in transgenic mice.

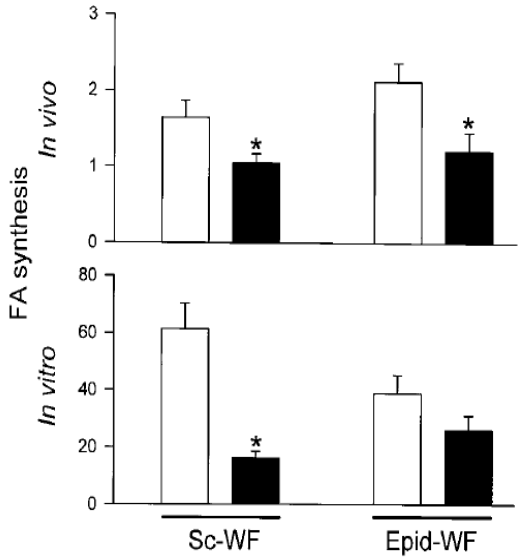

Fig. 9 The FA synthesis in subcutaneous (Sc-WF) and epididymal (Epid-WF) white fat was measured in experiment *in vivo* by incorporation of injected $^{3}H_2O$ into saponifiable FA and in experiment *in vitro*, as the incorporation of ^{14}C into FA extracted from tissue fragments incubated in the presence of D-[U-^{14}C] glucose. Values are means ± SE. Asterisks indicate statistically significant differences between genotypes (control mice – empty bars, transgenic mice – solid bars). The difference between Sc-WF and Epid-WF of control animals was significant in experiment *in vitro* (66).

In summary, these results showed that uncoupling of oxidative phosphorylation in mitochondria in WAT depressed FA synthesis in adipocytes, which reflected not only decreased reduction of adiposity but also increased energy expenditure.

I contributed to this work by performing the cells culture experiments, and I have measured FA oxidation, lactate production, and FA synthesis in 3T3-L1 adipocytes.

4.3 Publication C: Uncoupling protein 1, adipocytes, mitochondria

Summary of results of the article "**Expression of the uncoupling protein 1 from the *aP2* gene promoter stimulates mitochondrial biogenesis in unilocular adipocytes *in vivo*"**, by Rossmeisl M., Barbatelli G., Flachs P., Brauner P., Zingaretti MC., Marelli M.**,** **Janovska P.**, Horakova M., Syrovy I., Cinti S., Kopecky J., published in Eur. J. Biochem. 2002.

The aim of this study was to characterize the mechanism of the uncoupling respiration in white adipose tissue during ageing of aP2-Ucp1 transgenic mice in relation to mitochondrial biogenesis and to tissue morphology.

The experiments were established on the differentiated 3T3-L1 cells or adipocytes isolated from the WAT of aP2-*Ucp1* heterozygous transgenic mice or on their littermates. *Ucp1* expression was detected by the immunohistological determination in multilocular cells of subcutaneous fat of young mice regardless of the genotype. The higher *Ucp1* expression was found in BAT of transgenic mice in comparison to their control nontransgenic littermates, which was with agreement with expression of both *Ucp1* endogene and aP2-*Ucp1* transgene in transgenic mice. In adult mice only in transgenic mice, unilocular adipocytes of both type of white adipose tissue contained UCP1. The levels of *Ucp1* mRNA and UCP1 protein were decreased during ageing in both type of adipose tissue of transgenic mice. The levels of *Ucp1* mRNA of control mice were by one order of magnitude lower in comparison with the transgenic mice, whereas the amount of UCP1 was lower only about 2-fold. In epididymal fat were UCP1 transcript and protein detected only in transgenic mice. These results also indicated a higher levels of transgenic UCP1 in subcutaneous than in epididymal adipose tissue, both mRNA and protein content (Fig. 10a).

For the characterization of the relationship between UCP1 and mitochondrial biogenesis, the transcript level of COX IV (a nuclear gene for one of the subunits of

mitochondrial COX) was assessed. The main effect of the genotype was observed on the level of COX IV mRNA in both types of WAT with a higher effect in the transgenic mice during ageing. The levels of transcript didn't change during the ageing except for decreasing of COX IV mRNA in subcutaneous fat between the first and second month of age, independently of the genotype (Fig. 10b).

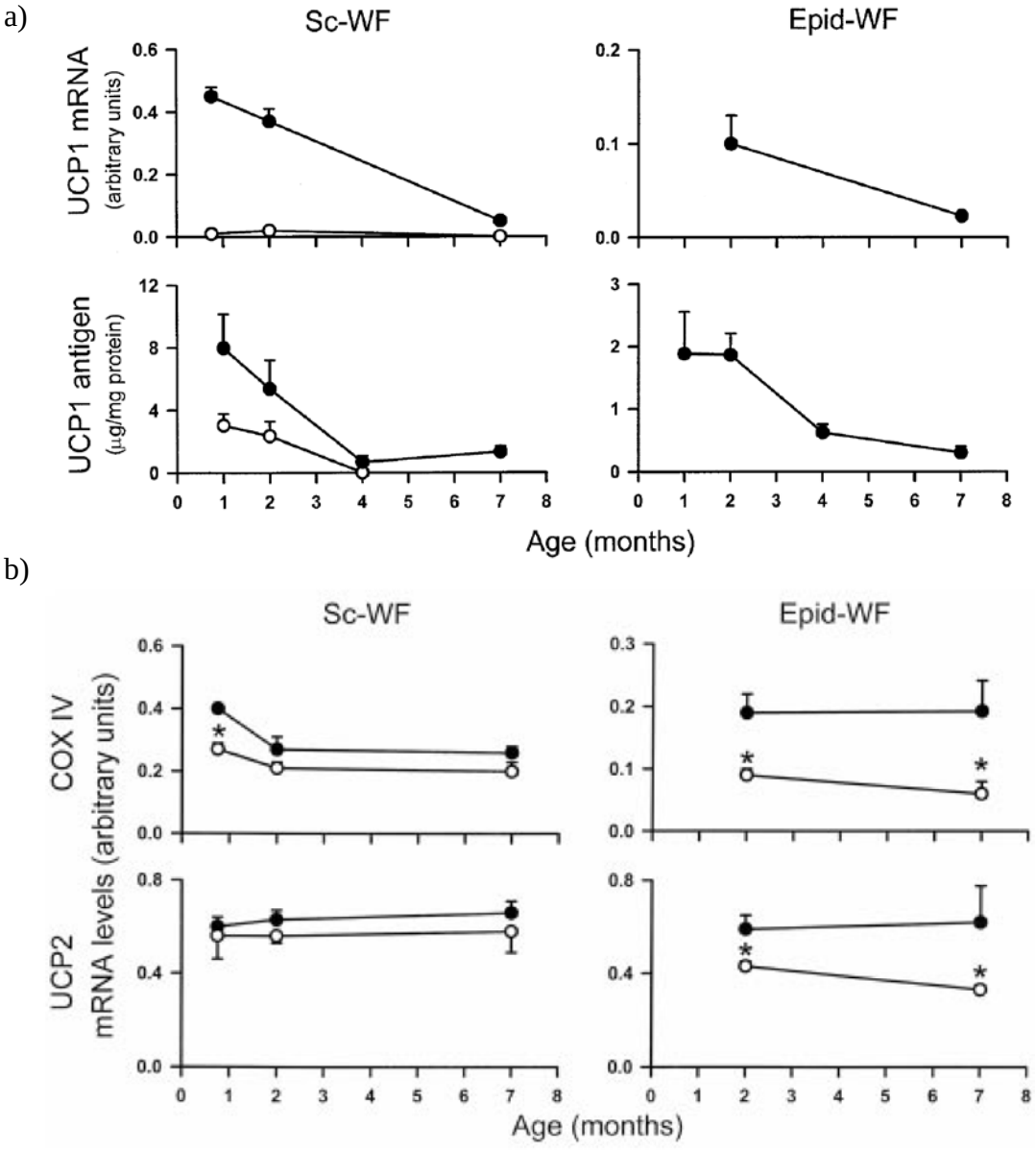

Fig. 10 Quantification of mRNA and protein for UCP1 (a) and mRNA for mitochondrial markers in white adipose tissue depots during ageing (b). Analyses were performed in subcutaneous (Sc-WF) and

epididymal fat (Epid-WF) of control (open symbols) and transgenic (full symbol) mice of indicated ages. Values are means ± SE. All differences in quantification of UCP1 between genotypes were significant. Asterisks indicate significant differences between genotypes within the same age group (64).

The content of cytochromes *b*, and *a + a3*, respectively, the subcutaneous fat was compared between young (5weeks old) and adult (7-9 month old) mice of both genotypes. When the content of the cytochromes was expressed relative to the mass of tissue, there was a main effect of age on cytochrome *b* content. Transgenic young mice had 1.7-fold higher cytochrome *b* content in comparison to the control mice (Fig. 11).

The results of the connection between respiratory uncoupling in adipocytes and stimulation of mitochondrial biogenesis were supported the determination of the levels of gene expression in the experiment with 3T3-L1 cells differentiated in cell culture.

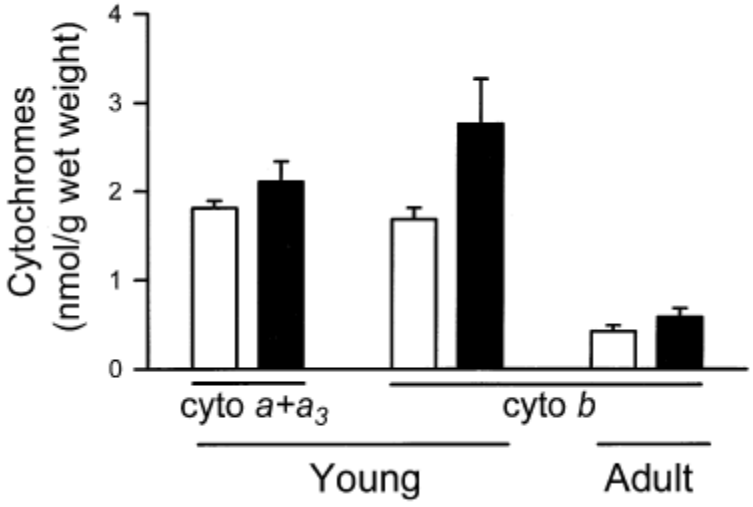

Fig. 11 Conntent of mitochondrial cytochromes *a + a3* and cytochrome *b* in young and adult mice in subcutaneous white fat. There were compared control (open bars) and transgenic (solid bars) mice of indicated age. Values are means ± SE. The cytochromes *a + a3* content of adult animals were under the limit of detection (64).

In conclusion, these results indicated that respiratory uncoupling *per se* is capable of inducing mitochondrial biogenesis in WAT *in vivo.*

Within this study, I have conducted all the cells culture experiments and RNA isolation from the cell cultures and from adipose tissue depots of the mice.

4.4 Publication D: *n*-3 PUFA, metabolic syndrome, white adipose tissue

Summary of results of the article "**Synergistic induction of lipid catabolism and anti-inflammatory lipids in white fat of dietary obese mice in response to calorie restriction and *n*-3 fatty acids"**, by Flachs P., Rühl R., Hensler M., **Janovska P.**, Zouhar P., Kus V., Jilkova ZM., Papp E., Kuda O., Svobodova M., Rossmeisl M., Tsenov G., Mohamed-Ali V., Kopecky J., published in Diabetologia 2011.

The goal of this study was to prove additive effects of CR combined with n-3 PUFA intake on lipid catabolism in WAT and assess the potential of the combined intervention in the treatment of obesity and metabolic syndrome.

Two months-old male mice C57BL/6J were fed for 2 weeks corn-oil-based high fat diet (cHF; lipid content 35%) and then, after random division into four groups mice were subjected to various treatments for 5 or 15 weeks: (1) cHF, ad libitum; (2) cHF supplemented with *n*-3 PUFA concentrate (46% wt/wt DHA, 14% wt/wt EPA; product EPAX 1050 TG; EPAX, Alesund, Norway) replacing 15% wt/wt of dietary lipids (cHF+F), ad libitum; (3) cHF with restriction of energy intake (10% wt/wt in comparison with mice fed ad libitum with the same type of diet, CR; cHF+CR); and (4) combination supplementary diet and calorie restriction (cHF+F+CR). Mice fed cHF increased their body weight during intervention in comparison with mice treated with cHF+F or cHF+CR, which were partially protected against the dietary obesity. Mice treated cHF+F+CR had full protection against body weight gain. The combination treatment significantly reduced both adiposity and fat cell size in all fat depots (epididymal, mesenteric and subcutaneous fat) and all the treatments decreased TAG accumulation in the liver after 5 weeks of treatment, when the effect of cHF+F+CR was stronger than at 15 weeks of the treatment. At this time plasma TAG levels at ad libitum fed state were strongly reduced, plasma glucose levels were similar in all groups and insulin levels were decreased by CR, and namely in the combination treatment.

Metabolic flexibility was analyzed as a metabolic response to the FASTED/RE-FED transition at 4 weeks of the experiment and only in plasma metabolites of FASTED state differences was found among the groups. The combination treatment showed the highest NEFA and the lowest glucose levels and displayed the largest differences in both plasma metabolites levels between FASTED and RE-FED conditions in comparison to the cHF mice (Fig. 12a, b). To characterize whole body metabolic flexibility and capacity to use carbohydrate and lipids fuels, the response to the intragastric gavage of glucose applied after overnight was evaluated using fasting by INCA was performed during the 4 week of the treatment. The maximal induction of ΔRQ by the combination treatment revealed that this treatment preserved metabolic flexibility better than any of the treatments applied separately (Fig. 12c).

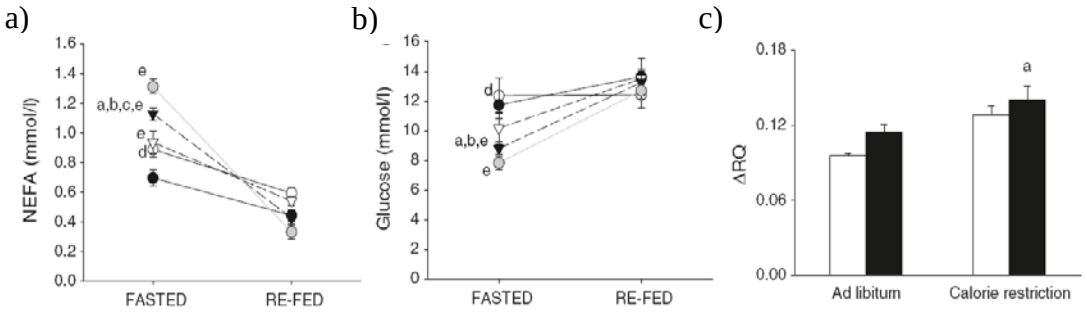

Fig. 12 Metabolic flexibility determinated in FASTED and RE-FED state. Plasma levels of NEFA (a) and glucose (b) after 4 weeks of the treatment. White circles, cHF; black circles, cHF+F; white triangles, cHF+CR; black triangles, cHF+F+CR; grey circles, Chow. During the 4 weeks of the treatment metabolic flexibility by indirect calorimetry was measured. Maximal induction in RQ values was assessed in overnight fasted mice after intragastic gavage of glucose. White bars, cHF or cHF+CR diet; black bars, cHF+F or cHF+F+CR. Data are means ± SEM. [a,b,c]Significant difference (ANOVA) compared with cHF, cHF+F, and cHF+CR, respectively; [d]significant difference (t test) compared with Chow-fed mice [e]significant difference (repeated-measures ANOVA) between the FASTED and RE-FED states (70).

For further characterization of glucose homeostasis, oral glucose tolerance test (**OGTT**) was performed and fasted plasma insulin levels was measured during 4-5 weeks of the treatment. Only the combination treatment significantly decreased fasted insulinemia, HOMA index and significantly improved glucose homeostasis.

Gene expression analysis revealed a synergistic induction of genes of mitochondrial biogenesis and lipid catabolism (*Pgc-1α, PPARα, Nrf, Cyox3* and *Vlcad*, *Lcad*). No induction of *Ucp1* by any of the treatments either in epididymal fat or in interscapular BAT was detected. The synergistic induction of mitochondrial FA oxidation was confirmed ex vivo by [1-^{14}C]palmitate oxidation in fragments of epididymal WAT after 5 weeks of the dietary treatment, where only the combination treatment significantly stimulated the oxidation. The higher activity of COX and mitochondrial oxidative capacity in adipocytes were also confirmed using respirometry in the cHF+F+CR mice in comparison with the cHF mice. Results suggested induction of futile cycling of FA between lipolysis and FA re-esterification in adipocytes.

Immunohistochemical analysis of epididymal WAT displayed suppression of low-grade inflammation which was in accordance with changes in tissue levels of anti-inflammatory lipid mediators, namely 15-deoxy-Δ12,15-prostaglandin J2 and protectin D1.

In conclusion, dietary *n*-3 PUFAs augment the anti-obesity effects of CR in mice while improving glucose homeostasis and increasing lipid catabolism in WAT. These effects probably reflect in large synergistic induction of mitochondrial FA oxidation in WAT, in the absence of mitochondrial uncoupling, reflecting the induction of futile cycling of FA between lipolysis and FA re-esterification in adipocytes.

Within this study, I was involved in conducting the animal feeding experiment. I have performed INCA measurements and isolation of adipocytes for evaluation of mitochondrial respiratory capacity and oxidative phosphorylation.

4.5 Publication E: *n*-3 PUFA, metabolic syndrome, lipogenesis, white adipose tissue

Summary of results of the article "**Anti-obesity Effect of *n*-3 Polyunsaturated Fatty Acids in Mice Fed High-Fat Diet Is Independent of Cold-Induced Thermogenesis"**, by **Janovská P.**, Flachs P., Kazdová L., Kopecký J., published in Physiol. Research 2013.

The main goal of this study was to confirm that induction of lipid catabolism in WAT by n-3 PUFA is independent of UCP1-mediated thermogenesis.

The C57BL/6J male mice born and maintained at 20°C were weaned at 4 weeks of age to either the standard (ST; 21, 3, and 56% calories as protein, fat, and carbohydrate, respectively) or HF diet (cHF; 15, 59, and 26% calories as protein, fat, and carbohydrate, respectively) or cHF diet was supplemented with EPA and DHA (cHF+F), added as a concentrate of *n*-3 PUFA (46% DHA, 14% EPA; EPAX 1050 TG, EPAX a.s., Lysaker, Norway), which replaced 15% of dietary lipids (specifically, 5.25 g of corn oil/100 g cHF diet) and the ambient temperature was increased to 30°C for the whole diet experiment (i.e., from the time of weaning to 8 months of age).

At the end of experiment mice fed cHF were heavier as compared with the ST diet-fed mice, while the cHF+F diet tended to counteract the obesity. The weight of epididymal fat depot was significantly lower and the weight of dorsolumbar fat depot tended to be lower in the cHF+F group, as compared with the cHF diet fed mice. While plasma levels of TAG tended to be elevated and levels of NEFA were significantly increased in response to the cHF diet-feeding, the supplementation of the cHF diet with *n*-3 PUFA exerted a protective, anti-hyperlipidaemic effect.

Glucose homeostasis was tested 2 weeks before termination of experiment by intraperitoneal glucose tolerance test (**IP GTT**). cHF diet feeding increased fasting glucose levels and deteriorated glucose tolerance but supplemented diet normalized blood glucose levels and improved glucose tolerance.

For the evaluation of the possible changes in energy expenditure, indirect calorimetry at 22h at 30°C, INCA was performed and RER and PRCF curves were evaluated. The PRCF curves shifted to the left in response to both cHF-based diets, and a trend for a difference between the EC_{50} value of the cHF and cHF+F curves was observed, supporting a shift from lipid to carbohydrate oxidation in response to the *n*-3 PUFA supplementation. The cHF curve was significantly steeper than both the cHF+F and the ST curves suggests (i) a relatively homogeneous distribution of RER values in the cHF diet fed mice (92), reflecting a strong drive for oxidation of abundantly supplied dietary lipids, and (ii) that the supplementation of the cHF diet with *n*-3 PUFA could unmask an inherent heterogenity of the mice with respect to the preservation of glucose homeostasis by the *n*-3 PUFA supplementation (Fig. 13).

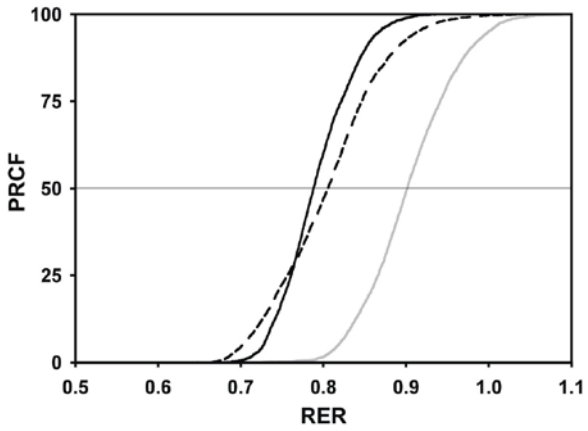

Fig. 13 Evaluation of energy expenditure by indirect calorimetry. Percent relative cumulative frequency (PRCF) curves were constructed based on RER values pooled from all the animals within a given dietary group (6-8 animals per group) during the whole measurement period (92). Mice had free access to ST diet (gray line), or cHF diet (black line), or cHF+F diet (black dash line) and water (91, 93).

Ex vivo metabolism analysis of epididymal fat confirmed beneficial effect of *n*-3 PUFA on the prevention of obesity also at thermoneutral temperature, where the activity of UCP1 is suppressed. Incorporation of ^{14}Cglucose into total lipids (Fig. 14A), as well as into the acyl groups (Fig. 14B) in epididymal WAT, were significantly decreased in association with the cHF diet-feeding and this decrease was partially prevented by the

n-3 PUFA supplementation, namely under the insulin-stimulated conditions (Fig. 14B). Moreover, as suggested by the changes in incorporation of radiolabeled glucose into glycerol residues (Fig. 14C), i.e., the marker of *de novo* glycerol synthesis and FA re-esterification cHF-feeding depresses FA re-esterification in WAT, while *n*-3 PUFA could preserve this activity, namely under the basal conditions, in the absence of the insulin stimulation.

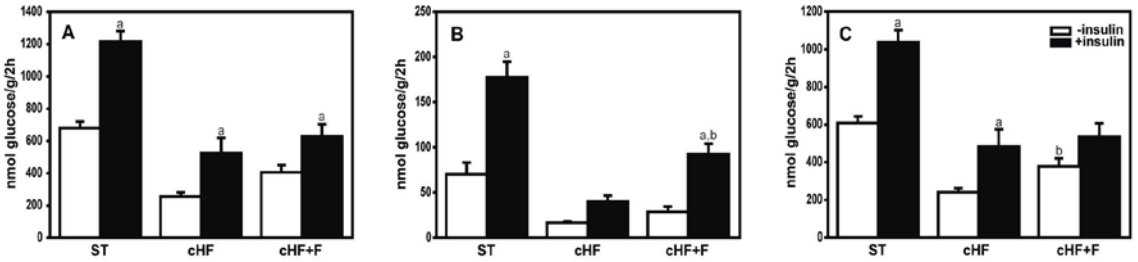

Fig. 14 Ex vivo lipid metabolism in epididymal fat at the end of experiment: Incorporation of ^{14}Cglucose into neutral lipids (TG synthesis); (A), and incorporation of ^{14}C-glucose into acyl groups (*de novo* FA synthesis); (B), Incorporation of ^{14}C- glucose into glycerol residue (FA re-esterification); (C) was calculated based on the data in A and B. [a] $p<0.05$ for the effect of insulin, [b] $p<0.05$ for the effect of cHF+F compared to cHF diet (93) .

In summary, these results suggested that the anti-obesity effect of *n-3* PUFA in rodents depends on the activation of the UCP1-independent thermogenesis in WAT, using mechanisms distinct from those mediating classical adaptive cold-induced thermogenesis. Futile cycling of FA between lipolysis and FA-reesterification in adipocytes was probably involved (TAG/FA substrate cycle).

Within this study, I was responsible for conducting all the animal experiments including INCA measurement, and for the collaborative arrangements with the researches in IKEM.

5 SUMMARY

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

1. We confirmed the thermogenic effect of the ectopic UCP1 in WAT of aP2-*Ucp1* transgenic mice by respirometry using Warburg and adipose tissue fragments. The rate of oxygen consumption in epididymal WAT was ~1.5-fold higher in transgenic mice in comparison with the non-transgenic mice. The inverse effect was observed in BAT, which could be attributed to energy collapse in adipocytes of the ectopic UCP1. Ectopic UCP1 in WAT is thermogenically active.

2. Mitochondrial uncoupling in 3T3-L1 adipocytes differentiated in cell culture resulted in increased FA oxidation and lactate production, in accordance with the inhibition of FA synthesis. Depression of FA synthesis was also documented in WAT with ectopic expression of *Ucp1*, where the reduction of the expression of the genes for lipogenic enzymes was detected.

3. Ectopic UCP1 in unilocular adipocytes of WAT induced mitochondrial biogenesis.

4. Metabolism in WAT was altered in response to combination treatment using CR and *n*-3 PUFA in mice fed cHF diet. The improvement of metabolic flexibility was observed both in whole body fuel partitioning and on plasma metabolites levels. Synergistic induction of mitochondrial oxidative capacity and lipid catabolism in epididymal fat was associated with suppression of low-grade inflammation of WAT and induction of specific anti-inflammatory lipid mediators, namely 15-deoxy-Δ12,15-prostaglandin J2 and protectin D1. The induction of lipid catabolism in WAT in response to the combined intervention using *n*-3 PUFA and CR could be explained by activation of energy demanding futile TAG/FA substrate cycle in adipocytes.

5. The effect of *n-3* PUFA on body fat accumulation, improvement of lipid and glucose homeostasis and induction of FA re-esterification was confirmed in mice maintained at the thermoneutral temperature, thus independent of cold-inducted thermogenesis (and UCP1) in WAT. Induction of energy expenditure by activation of the futile TAG/FA substrate cycle in adipocytes in WAT may exert anti-obesity effect and improve metabolic health.

6 DISCUSSION AND CONCLUSIONS

The main general conclusion of this PhD thesis is that in spite of the relatively low contribution of WAT to basal metabolic rate (5-10%; (94)), modulation of WAT metabolism could contribute to reduction of adiposity and prevent the development of obesity (95). Obesity belongs to the main factors in the development of metabolic syndrome, cluster of the risk factor of cardiovascular disease and T2D like hyperglycaemia, insulin resistance, hypertension and hyperlipidemia (69, 96). WAT contributes to the control of body fat content and is important in development of insulin resistance, which results from increasing accumulation of lipids in peripheral tissues due to hypertrophy fat cells and excessive releasing of FA from fat (97).

The main goal of pharmacological treatments in prevention and treatment of obesity is to decrease energy intake and/or increase energy expenditure (98). BAT is able to dissipate the energy into heat and so reduce fat storing by UCP1-mediated mechanisms, unique for this tissue. For study of modulation of total energy balance and adiposity, the transgenic mice with ectopic *Ucp1* expression in adipose tissue was constructed (99). The three studies in this thesis describe the mechanism of respiratory uncoupling in WAT, which reduces amount of fat in aP2-*Ucp1* transgenic mice. The first study (83) was focus on the confirmation of the UCP1 thermogenic activity and mitochondrial capacity in white fat. UCP1 in white fat was detected both in unilocular and multilocular cells and amount of UCP1 was ~14-fold lower than in mitochondria from BAT of nontransgenic mice. This minor amount of ectopic UCP1 is able to uncouple oxidative phosphorylation and increase the endogenous respiration of epididymal WAT ~1.5-fold. The BAT of transgenic mice points out atrophy, probably toxic effect of excessive amounts of UCP1, which was confirmed by lower (2~fold) endogenous respiration in comparison with nontransgenic mice. The effect of respiratory uncoupling on triglyceride uptake, regulated by LPL, was characterized in both epididymal and subcutaneous WAT. HF diet induced LPL activity, but only in subcutaneous fat was this increasing suppressed by transgene. These findings are in agreement with reduction of body weight, fat content and regional fat redistribution in transgenic mice fed HF diet (61).

Due to evidence of the ability to modulate the mitochondrial membrane potential in white adipocytes from aP2-*Ucp1* transgenic mice (62), the second study of this thesis was aimed for FA metabolism in this tissue. The reduction of FA synthesis was found in subcutaneous, but not in epididymal fat of transgenic mice in experiment *in vitro*. The suppression of FA synthesis by ectopic U*cp*1 expresion in WAT was supported by *in vitro* experiment, where chemical uncoupler DNP strong reduced FA synthesis and increased lactate production in 3T3-L1 adipocytes. These results were testified on the gene expression level. ACC and FAS mRNA levels were down-regulated, which probably reflect the depression of lipogenic pathways (66).

The main focus of the third study was the identification of the cell type involved in expression of ectopic *Ucp1*. Multilocular cells were detected only in subcutaneous white fat of young mice. Therefore, this result documents that prevention of obesity in adult transgenic mice reflects respiratory uncoupling in unilocular white adipocytes. The transgen affected the mitochondrial content, which was performed by the increased in the level of COX IV transcript, gene in controlling oxidative capacity. The morphometric study validated the mitochondrial biogenesis by detection of unilocular cells with more amount and larger mitochondria in adipocytes of transgenic subcutaneous WAT (64).

Transgenic expression of *Ucp1* in WAT is associated with depression of cellular energy charge, activation of AMPK, downregulation of adipogenic genes, and increase in lipid oxidation (67). Activation of AMPK is associated with up-regulation of mitochondrial biogenesis (100). Furthermore, acute activation of AMPK in WAT adipocytes inhibits both lipogenesis and lipolysis and increases both FA oxidation and glucose transport (98, 101, 102). Because the main role of AMPK in WAT is the control and restraining of energy depletion, the reduction of lipolysis by AMPK could be feedback mechanism limiting the cellular energy release (42). Thus, AMPK is activated in response to mitochondrial uncoupling and it could explain depression of lipogenesis (66) and lipolysis (65) as well as increase in FA oxidation (67) and mitochondrial content (64) in WAT of aP2-*Ucp1* transgenic mice.

Epidemiological research documented that *n*-3 PUFA have beneficial effects including improve insulin resistance and modulation lipid metabolism. Calorie

restriction and weight loss are responsible for a modification of gene expression in WAT (103) and can improve metabolic flexibility (104). Combination treatment *n*-3 PUFA and CR, presented in the fourth study, potentiated beneficial effects described above. The combination treatment demonstrated improvement of metabolic flexibility both the whole body level and plasma markers. This improvement of metabolic flexibility correlated with changes in body weight, adiposity and glucose homeostasis. Combination treatment causes induction of mitochondrial FA oxidation in WAT and inhibits of formation pro-inflammatory lipid mediators and promotes the anti-inflammatory molecules, namely 15-deoxy-Δ12,15-prostaglandin J2 and protectin D1.

In our experiments we demonstrated that *n*-3 PUFA are able to increase mitochondrial oxidative capacity in WAT without up-regulation of UCP1 gene in adipose tissue. The experiments from the last study, performed on mice maintained at thermoneutrality, independent of cold-induced thermogenesis, sustained anti-obesity metabolic effect of *n*-3 PUFA independent of UCP1-mediated thermogenesis. *n*-3 PUFA supplementation modulated WAT metabolism, namely induction of de novo lipogenesis, observed both at 20^{o}C and 30^{o}C.

Our findings suggest that *n*-3 PUFA, namely in combined treatment with CR, could induce activity of TAG/FA cycle, mitochondrial oxidative phosphorylation and FA oxidation in WAT. Results suggest the involvement of AMPK in metabolic and anti-inflammatory effects of the treatments (70).

In summary, not only transgenic modification of WAT, but also a combination treatment based on physiological stimuli, namely calorie restriction and intake of *n*-3 PUFA, could counteract obesity by pushing lipid catabolism in WAT to its limits. These results are important for developing new strategies for treatment of obesity and associated metabolic and immune disorders.

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9 SELECTED PUBLICATIONS

Publication A

Kopecky J, Rossmeisl M, Hodny Z, Syrovy I, Horaková M, and **Kolarova P**.

Reduction of dietary obesity in the aP2- Ucp transgenic mice: mechanism and adipose tissue morphology

Am.J.Physiol. 270:E776-E786, 1996. (IF = 4.75)

Reduction of dietary obesity in aP2-*Ucp* transgenic mice: mechanism and adipose tissue morphology

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Kopecký, Jan, Martin Rossmeisl, Zdeněk Hodný, Ivo Syrový, Milada Horáková, and Petra Kolářová. Reduction of dietary obesity in aP2-*Ucp* transgenic mice: mechanism and adipose tissue morphology. *Am. J. Physiol.* 270 (*Endocrinol. Metab.* 33): E776–E786, 1996.—C57BL6/J mice with the expression of the mitochondrial uncoupling protein (UCP) gene from the fat-specific aP2 gene promoter were used to study the mechanism by which the aP2-*Ucp* transgene affects adiposity and reduces high-fat diet induced obesity. In the transgenic mice, UCP synthesized in white fat was inserted into mitochondria, and oxygen uptake by epididymal fat fragments indicated UCP-induced thermogenesis. The respirometry data, UCP content, cytochrome oxidase activity, and tissue morphology suggested functional involution of brown fat. Despite 25- to 50-fold lower mitochondrial cytochrome oxidase activity in white than in brown fat cells, total oxidative capacity in white and brown adipose tissue is comparable. Appearance of novel small cells in the gonadal fat of the transgenic mice was associated with a higher DNA content than that of the nontransgenic mice. The results prove a potential of transgenically altered mitochondria in white fat to modulate adiposity and energy expenditure and suggest the existence of a yet unidentified site-specific link between energy metabolism in adipocytes and cellularity.

C57BL6/J mouse strain; uncoupling protein; white fat mitochondria; high-fat diet; cellularity

DESPITE EXTENSIVE RESEARCH of lipid metabolism and obesity, control of adiposity is still not well understood. Not only efficiency of energy conversion but also signals for proliferation of preadipocytes (1, 19, 26) and mechanisms underlying regional differences in adipose tissue growth and metabolism (2) require further studies. Recent gene transfer experiments in mice (see Refs. 15, 16) suggested that, at least under specific circumstances, adiposity may be influenced by mitochondria in white adipose tissue.

Mitochondria in white fat are equipped for ATP synthesis, as are mitochondria in other tissues (23). The principal substrates for mitochondrial oxidation in adipocytes are fatty acyl-CoA compounds (22). A relatively lower capacity for carnitine-mediated transport of fatty acyl-CoA compounds in white fat mitochondria directs acyl groups toward esterification and lipogenesis, whereas brown fat mitochondria show higher capacity for fatty acid (FA) oxidation (22). In contrast to white fat, mitochondria in brown fat are clearly involved in nonshivering thermogenesis and control of total energy balance (for review see Ref. 9). These mitochondria contain uncoupling protein (UCP), the key component of the thermogenic mechanism (9), and exhibit a unique stoichiometry of protein complexes in the inner mitochondrial membrane, with a high content of cytochrome oxidase (COX) and low ATP synthetase (12, 22). The content of mitochondria is extremely high in brown adipocytes, whereas it is much lower in white fat cells, where they form clusters in cytoplasm (5).

Expression of the UCP gene (*Ucp*), driven by the fat-specific promoter of the adipocyte lipid-binding protein (aP2) gene in mice, prevented to a large extent development of dietary (16) or genetically (15) induced obesity, as well as obesity due to hypothalamic lesions (unpublished data). In the aP2-*Ucp* transgenic mice, dissipation of energy not only in brown but also in white fat mitochondria may influence adiposity and total energy balance. However, direct proof of the link between ectopic synthesis of UCP in white fat of aP2-*Ucp* transgenic mice and a decrease in the efficiency of energy conversion is still required. It also remains to be explained why the effect of the transgene on adiposity differs among various fat depots (always resulting in reduced subcutaneous and relatively enlarged gonadal fat) and why reduction in total body weight only becomes apparent when the animals become obese.

The primary objective of the experiments described in this paper was to seek morphological and biochemical correlations in white and brown adipose tissue with the effects of the aP2-*Ucp* transgene on adiposity. The model chosen was a normal and an aP2-*Ucp* transgenic C57BL6/J mouse with high-fat (HF) diet-induced obesity, which is the mouse discussed in the accompanying paper (16). The secondary objective was to assess oxidative capacity of white compared with brown adipose tissue in the mouse to characterize the limits of thermogenesis induced by the uncoupling of oxidative phosphorylation in white fat mitochondria.

The results indicate that the changes in adiposity associated with the expression of the aP2-*Ucp* transgene are linked to energy dissipation in white and not in brown adipose tissue and that the oxidative capacity of white fat is adequate for the changes of the total energy balance induced by the transgene. The mechanisms of the regionally dependent changes of adiposity induced by the transgene expression and the escalation of the transgene effect in the obese state are discussed.

MATERIALS AND METHODS

Animals and tissues. For all of the experiments, only male C57BL6/J mice were used, mainly the 9-mo-old nontransgenic and aP2-*Ucp* transgenic animals from the feeding study described in the accompanying paper (16). Mice were fed the standard chow diet (Chow diet) or the high-fat diet (HF diet) for 6 mo before they were killed by cervical dislocation (16). Otherwise, mice fed the Chow diet were used. The following

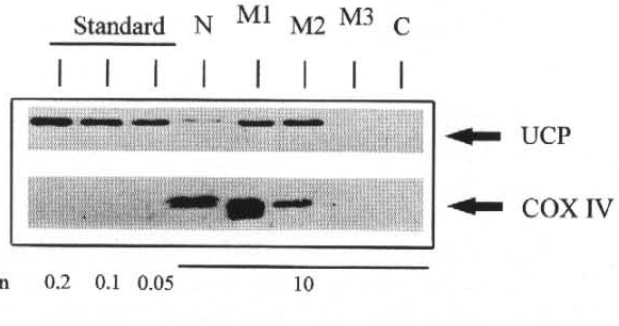

Fig. 1. Subcellular localization of mitochondrial uncoupling protein (UCP) in white adipose tissue of transgenic mice. Epididymal fat dissected from 4-mo-old mice was used for isolation of different subcellular fractions, as described in MATERIALS AND METHODS. The fractions were analyzed by immunoblotting with antibodies against mitochondrial UCP and subunit IV of cytochrome oxidase (COX). Isolated UCP was also used as standard. N, nuclear fraction; M1 and M2, mitochondrial fractions sedimented at 10,000 g_{max} and 30,000 g_{max}, respectively; M3, microsomal fraction; C, cytosol. Immunodecoration was performed using enhanced chemiluminescence.

tissues (16) were analyzed: interscapular portion of brown adipose tissue, white adipose tissue depots located subcutaneously at the lower part of the back (dorsolumbal depot), epididymal white fat, and liver.

Respirometry. Tissue fragments (40–60, 200–300, and 40–60 mg of interscapular brown fat, epididymal fat, and liver, respectively) were incubated in Warburg vessels with a total volume of 18 ml. The incubation medium (3 ml) contained (in mM) 90 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 3.6 $NaHCO_3$, 25 glycylglycine (pH 7.4), and 10 glucose (8). Respiration was measured at 30°C under an atmosphere of 95% O_2-5% CO_2.

Fractionation of adipose tissue and enzymatic measurements. For the characterization of the subcellular localization of UCP in the white fat of transgenic mice (see Fig. 1), homogenate was prepared in 0.25 M sucrose, 10 mM tris(hydroxymethyl)aminomethane (Tris)·Cl, and 0.1 mM EDTA, pH 7.4, from freshly dissected epididymal fat. The homogenate was fractionated by differential centrifugation at 4°C, as described for isolation of mitochondria from brown adipose tissue (22). Cell nuclei sedimented at a maximum of 600 g_{max} and mitochondria (M1) sedimented at 10,000 g_{max} (10 min) were collected. The fraction containing light mitochondria (M2; see Ref. 21) was isolated by centrifugation of the 10,000 g_{max} supernatant at 30,000 g_{max} for 10 min. The 30,000 g_{max} supernatant was centrifuged further at 100,000 g_{max} for 60 min to separate the microsomal fraction (M3) and cell cytosol. All of the sedimented protein fractions were suspended in a medium containing 10 mM Tris·Cl and 2 mM EDTA, pH 7.4 (TE medium) and the following inhibitors of proteases at a concentration of 1 μg/ml: antipain, leupeptin, aprotinin, and pepstatin A.

For estimating the total activity of COX and the content of the UCP and COX subunit IV antigens in fat depots (see below), tissue samples stored at −70°C (16) were homogenized on ice in the TE medium and centrifuged at 400 g_{max} for 5 min. The supernatant solution was centrifuged at 100,000 g_{max} for 30 min. Sedimented proteins (crude cell membranes) were suspended in the TE medium, which also contained the protease inhibitors just mentioned. The activity of COX was estimated spectrophotometrically (32) at 25°C, with 25 μM reduced cytochrome *c* (from horse heart; C 2506, Sigma Chemical) in the whole tissue homogenates (total COX activity) and also in the crude cell mebranes. The yield of the isolation of the crude cell membranes was then evaluated through recovery of the COX activity (between 40 and 60% with respect to the total COX activity in the homogenate) and used to calculate the total content of antigens in the fat depots (see *Immunoblotting*).

Activity of lipoprotein lipase (LPL) was estimated in detergent extracts of fat fragments (40–80 mg) stored at −70°C (16), as previously described (13). Skim milk standard was used as a control for interassay variations (13). Glycerol [1-^{14}C]trioleate was from Amersham (CFA 258).

Immunoblotting. Different subcellular fractions (see Fig. 1), crude cell membranes isolated from interscapular brown fat (2–10 μg protein), and epididymal fat (30 μg protein) were electrophoresed on 10% polyacrylamide gels and analyzed by immunoblotting as before (15), with rabbit anti-hamster UCP and anti-bovine COX subunit IV polyclonal antibodies (dilution 1:2,000). The immunocomplexes were detected using swine anti-rabbit immunoglobulin G (IgG) labeled by peroxidase (dilution 1:2,000; SEVAC, Prague), enhanced chemiluminescence (kit from Amersham), and densitometry. In later experiments (see Figs. 4 and 5), ^{125}I-labeled whole donkey antibody against rabbit IgG (IM 134, Amersham) was used as the second antibody, at a concentration of 0.2 μCi/ml (15), and radioactivity was evaluated using PhosphorImager SF (Molecular Dynamics). All of the values were corrected for the yield of the isolated crude cell membranes. Quantification of UCP and COX was performed using standards of purified UCP [50–200 ng; (20)] and mitochondria (5–10 μg protein) isolated (22) from mouse heart, respectively. Molar quantities of UCP monomer were calculated using a molecular mass of 32 kDa (6). For COX quantification, the content of cytochrome aa_3 in the heart mitochondria standard (0.70 nmol aa_3, i.e., 0.35 nmol COX/mg mitochondrial protein) was estimated spectrophotometrically (30). Concentration of isolated UCP was measured according to Schaffner and Weissmann (24), and protein in all of the other samples was determined by the bicinchoninic acid procedure, using bovine serum albumin as a standard (see Ref. 16).

RNA analysis. Total RNA was isolated from adipose tissue samples (16), and *Ucp* mRNA was analyzed by Northern blot analysis, as before (15), by using the 320-bp *Bgl*II restriction fragment derived from the first exon of the *Ucp*. The radioactivity of the 1,230-bp major *Ucp* mRNA species (17) was evaluated by the PhosphorImager SF. Total RNA isolated from the brown fat of adult nontransgenic C57BL6/J mice reared at 20°C served as a standard. Blots were rehybridized with the 1,100-bp *Pst*I restriction fragment of mouse γ-actin cDNA, and the signal was used to correct for small intersample variations.

DNA measurements. DNA was estimated fluorometrically (18) in 10- to 20-μl aliquots of 10% brown fat and 20% white fat homogenates, prepared in TE medium with an all-glass homogenizer.

Histology. Tissues were placed in Bouin's fixative and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Statistical analysis. The data were analyzed as described in Ref. 16. All comparisons were judged to be significant at $P < 0.05$.

RESULTS

Ectopically synthesized UCP inserted into white fat mitochondria. In the aP2-*Ucp* transgenic mice, UCP could be detected only in white and brown fat but not in other tissues, and it was associated with crude cell

membranes (15). To assess whether UCP was inserted into mitochondria, a homogenate of epididymal fat of the transgenic mice was fractionated to separate the crude nuclear fraction; mitochondrial subpopulations sedimented at 10,000 g_{max} (M1) and 30,000 g_{max} (M2); microsomes (M3); and cytosolic proteins. UCP was detected by immunoblotting (see Fig. 1). The amount of COX, a marker of the inner mitochondrial membrane, was also evaluated using the antiserum specific for subunit IV of the enzyme. As expected, a majority of both, UCP and the COX subunit IV, was associated with the M1 and M2 mitochondria. The nuclear fraction contained less of either antigen than the mitochondrial fractions. Traces of UCP and COX were present in the microsomal fraction in some experiments (not shown), and no antigens could be detected in the cytosol. The data suggest that UCP in white fat of the transgenic mice was properly inserted into mitochondria.

UCP concentration in the M1 mitochondria isolated from epididymal fat of the aP2-*Ucp* transgenic mice was ~14-fold lower than in mitochondria prepared from brown fat of the nontransgenic C57BL6/J mice reared at 20°C (5.6 ± 2.5 and 78 ± 9 μg UCP/mg mitochondrial protein, corresponding to 0.175 ± 0.078 and 2.43 ± 0.30 nmol UCP/mg mitochondrial protein; n = 5–8). The specific activity of COX was threefold lower in the M1 mitochondria isolated from epididymal as opposed to brown fat (103 ± 16 and 306 ± 30 μmol cytochrome c oxidized·min^{-1}·mg protein^{-1}, respectively).

Respirometry. Despite the indirect data indicating induction of energy dissipation in white fat by UCP synthesized in the aP2-*Ucp* transgenic mice, direct functional proof was still required. The rate of endogenous oxygen consumption of brown and epididymal adipose tissue fragments from the nontransgenic and the aP2-*Ucp* transgenic mice was estimated by using a Warburg apparatus (Fig. 2). In all of the tissues analyzed, the rate of oxygen consumption was almost linear during the first 100 min of the incubation. When expressed relative to the DNA of the tissue (see also Table 3), a rate of oxygen consumption ~1.5-fold higher was detected in the epididymal fat from the transgenic compared with the nontransgenic mice. Surprisingly, an inverse effect (a 2-fold difference) of the transgene was found in brown adipose tissue. Therefore, in the nontransgenic mice, the DNA-adjusted oxygen consumption was ~2.5-fold higher in brown than in epididymal fat, whereas in the transgenic mice, the endogenous respiration of epididymal fat was ~1.4-fold higher than that of brown fat. No significant effect of the transgene on the respiration of liver fragments was noticed (not shown).

Activity of COX in brown and white adipose tissue depots. Mitochondrial oxidative capacity could influence UCP-induced energy dissipation in the transgenic mice. Therefore the activity of COX, the terminal component of the respiratory chain in mitochondria, was evaluated in interscapular brown fat, subcutaneous white adipose tissue from the dorsolumbal region, and epididymal fat (Table 1).

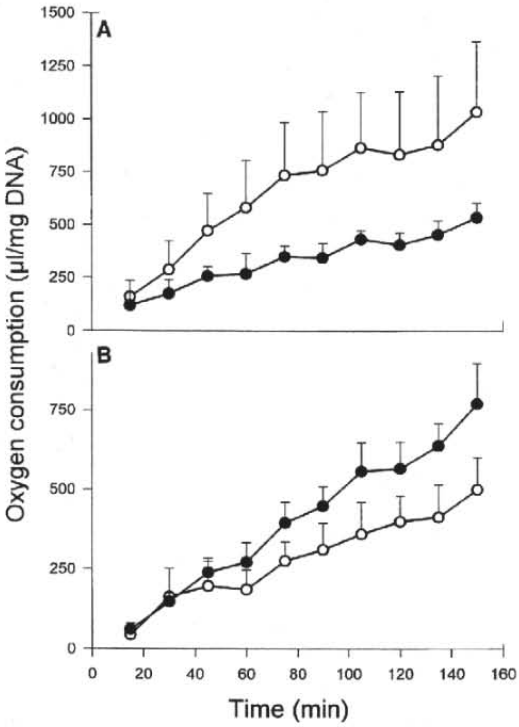

Fig. 2. Endogenous respiration of interscapular brown fat (*A*) and epididymal fat (*B*). Tissues were dissected from the nontransgenic (○) and transgenic (●) 8- to 10-mo-old Chow diet-fed mice (n = 6). Oxygen consumption was measured by a Warburg respirometer and expressed relative to tissue DNA content (see MATERIALS AND METHODS). Data are means ± SE. Beginning from 45 min (brown fat) or 75 min (gonadal fat) of incubation, the differences between transgenic and nontransgenic mice were significant ($P < 0.05$).

In the nontransgenic mice fed the Chow diet, total COX activity was ~10-fold lower in each of the two white fat depots than in interscapular brown fat. In the HF diet-fed nontransgenic mice, the total activity in brown and epididymal depots increased 2.1- and 1.5-fold, respectively, compared with the Chow diet-fed mice, whereas in the subcutaneous white fat depot a much higher increase (9.1-fold) was observed. Under both sets of dietary conditions, the transgene induced a 3-fold decrease of total activity in the subcutaneous white fat depot and a significant 1.4-fold elevation of total COX activity in the epididymal fat. It also significantly decreased the COX activity in the brown fat of the HF diet-fed mice.

In all subgroups of mice, the total COX activity closely matched the differences in cellularity within each type of fat depot, as revealed by the ratio between total COX activity and total DNA content (Table 1), with two exceptions: *1*) the dorsolumbal depot of the Chow diet-fed transgenic mice (a decreased ratio compared with the other experimental situations) and *2*) brown fat [a lower COX-to-DNA ratio (COX/DNA) in the transgenic compared with the nontransgenic mice]. Except for the above deviations, COX/DNA was similar in the dorsolumbal and epididymal fat depots, and it was ~50-fold higher in brown than in white fat. When

Table 1. *Activity of COX in adipose tissue depots of male mice*

		Fat Depot								
		Interscapular			Dorsolumbal			Epididymal		
	n	TA	DNA	WW	TA	DNA	WW	TA	DNA	WW
HF/non-tg	6	$3,505 \pm 554^a$	8.6 ± 3.0^a	14.4 ± 2.6^a	$1,001 \pm 262^a$	0.37 ± 0.04^a	0.38 ± 0.09^a	225 ± 52^a	0.40 ± 0.04	0.10 ± 0.01^a
HF/tg	7	$1,446 \pm 128^b$	7.5 ± 1.0^b	10.0 ± 1.1^b	270 ± 46^b	0.29 ± 0.04^a	0.34 ± 0.08^a	322 ± 57^a	0.40 ± 0.09	0.28 ± 0.07^b
Chow/non-tg	6	$1,646 \pm 204^b$	9.6 ± 1.1^a	16.8 ± 1.1^a	165 ± 41^c	0.33 ± 0.05^a	0.50 ± 0.10^a	149 ± 19^b	0.58 ± 0.07	0.23 ± 0.05^b
Chow/tg	8	$1,397 \pm 164^b$	6.3 ± 0.4^b	14.9 ± 1.6^a	53 ± 8.4^d	0.13 ± 0.02^c	0.19 ± 0.04^b	198 ± 29^b	0.50 ± 0.10	0.24 ± 0.06^b

Values are means ± SE; n, no. of animals/group. Activities of cytochrome oxidase (COX) were estimated in tissue homogenates and are expressed as total activity in the depot (TA; μmol cytochrome c oxidized·min^{-1}·depot^{-1}) related to DNA content (DNA; mmol cytochrome c oxidized·min^{-1}·mg^{-1} DNA) or wet weight of the depot (WW; μmol cytochrome c oxidized·min^{-1}·mg tissue^{-1}). HF, mice fed a high-fat diet; tg and non-tg, transgenic and nontransgenic mice, respectively. For DNA contents see Table 3. For comparison, within each column a different superscript indicates a significant difference between subgroups.

COX activity was adjusted to the weight of the fat depots (Table 1), trends could be observed similar to those when the activity was related to tissue DNA content. Only in the epididymal fat of the HF diet-fed nontransgenic mice was a significantly lower specific COX activity observed in contrast to the other subgroups of mice (Table 1).

UCP mRNA in brown and white adipose tissue depots. Semiquantitative analysis of gene expression was performed at the mRNA level (Fig. 3) with total RNA isolated from interscapular, subcutaneous dorsolumbal, and epididymal fat. In the white fat depots, the UCP messenger could be detected only in the transgenic but not in the nontransgenic mice, and the UCP mRNA levels were about one order of magnitude lower than those in the brown adipose tissue. Importantly, similar *Ucp* mRNA levels were present in both white fat depots, and the dietary conditions had no significant effect on expression in any fat depot. In the brown fat of the transgenic mice significantly higher UCP mRNA levels were detected compared with the nontransgenic animals, reflecting a contribution of both the endogenous *Ucp* gene and the aP2-*Ucp* transgene (15). These data exclude a possibility that site- or diet-dependent changes of the transgene expression determine the transgenic mice phenotype (see DISCUSSION).

Quantification of UCP and COX subunit IV antigens in brown and white fat. The two critical components for thermogenesis in adipose tissue mitochondria of the transgenic mice, UCP and COX, were quantified by immunoblotting (Figs. 4 and 5). Total content of UCP (Fig. 4) in the brown fat of the nontransgenic mice increased significantly (3-fold) in response to the HF diet, indicating increased capacity for nonshivering thermogenesis. This increase in UCP content was completely eliminated by the transgene. In the Chow diet-fed mice, no effect of the transgene on the UCP content was observed, and the content did not differ from the HF diet-fed transgenic mice. In the epididymal fat, the UCP antigen was detected only in the transgenic, but not in the nontransgenic mice, and its total content was significantly (2.5-fold) higher with the HF diet than with the Chow diet. Total content of UCP in the interscapular brown fat was about two orders of magnitude higher than in the epididymal fat of the transgenic mice. UCP content adjusted to DNA content in brown fat tends to be higher in the HF- compared with the Chow diet-fed mice, whereas in epididymal fat no significant effect of the dietary conditions on UCP/DNA was observed. In brown fat, the higher UCP mRNA levels in the transgenic compared with the nontransgenic mice (see Fig. 3) did not induce a significantly higher UCP specific content (Fig. 4). According to UCP/DNA, cellular UCP content in the

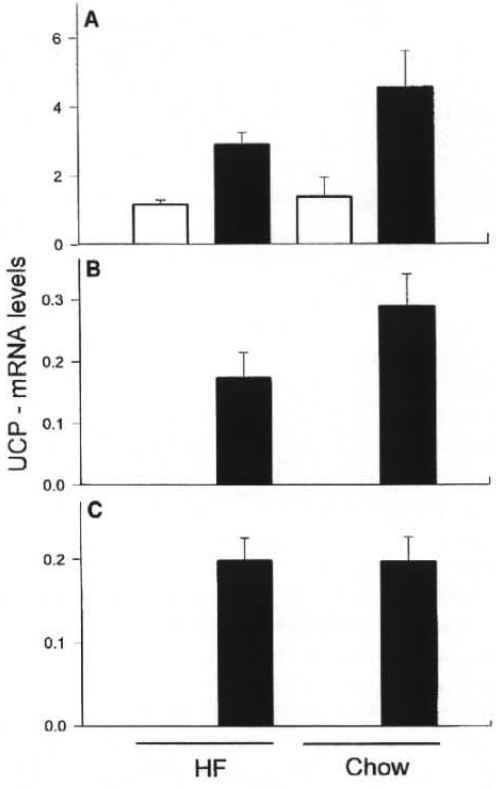

Fig. 3. Northern blot analysis of *Ucp* expression in male adipose tissue depots. Total RNA was isolated from interscapular brown adipose tissue (*A*), dorsolumbal subcutaneous (*B*), and epididymal fat pads (*C*) of nontransgenic (open bars) and transgenic (filled bars) mice fed the high-fat (HF) or Chow diet (n = 6–8); see Table 1 of Ref. 16. The quantity of *Ucp* mRNA was estimated as described in MATERIALS AND METHODS and expressed relative to standard RNA. Values are means ± SE. In brown fat, the transgene significantly increased *Ucp* expression without a major effect of the diet. In dorsolumbal and epididymal fat pads, no significant effect of the diet was detected.

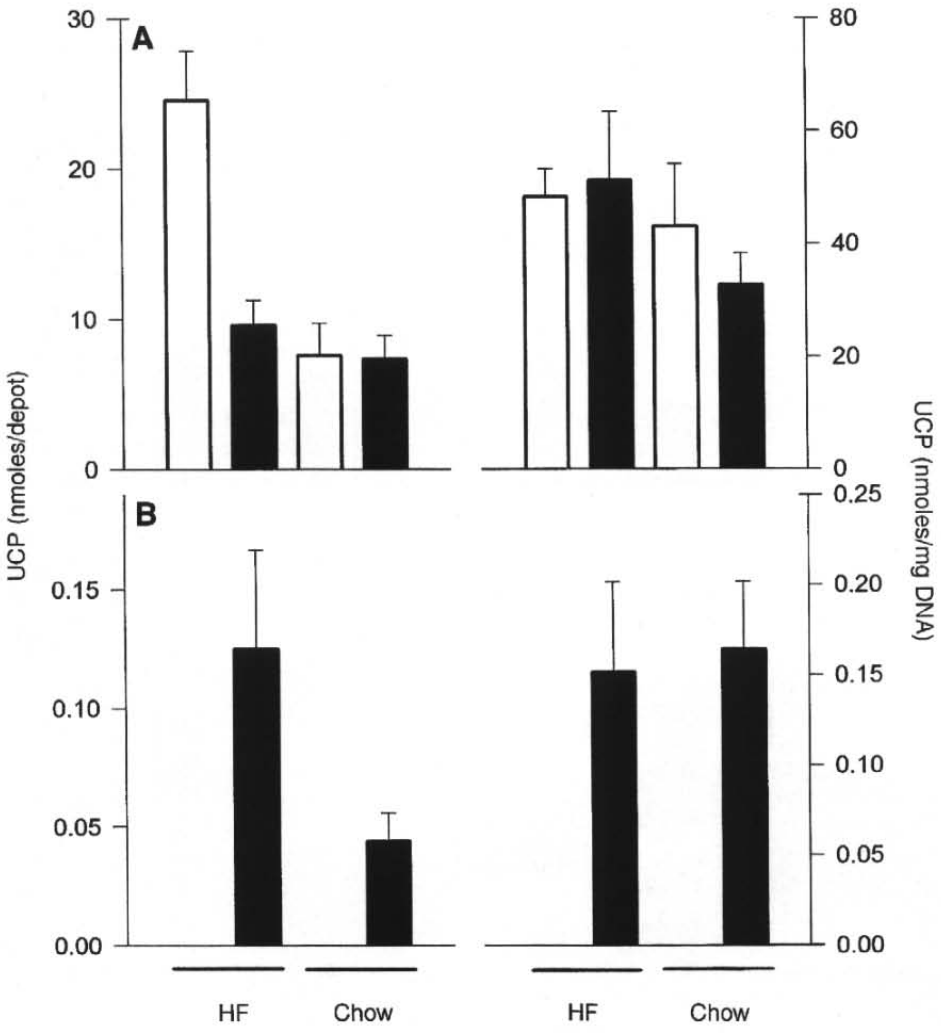

Fig. 4. Quantification of UCP in interscapular brown adipose tissue (*A*) and epididymal fat (*B*) in male mice. Analysis was performed by immunoblotting in crude cell membranes isolated from nontransgenic (open bars) and transgenic (filled bars) mice fed the Chow or HF diet (n = 6–8; see Table 1 of Ref. 16) as described in MATERIALS AND METHODS. UCP content was also related to total DNA in each depot by use of values in Table 3. Values are means ± SE. Significance levels are given in the text.

transgenic mice is ~300-fold higher in brown than in white adipocytes.

Total content of the COX subunit IV antigen in brown fat (Fig. 5) of the HF diet-fed mice was approximately twofold higher than that of the Chow diet-fed animals. A major effect of the transgene was observed, with the transgenic mice showing a lower COX subunit IV content compared with the nontransgenic, mainly the HF diet-fed mice, just as in the case of COX activity measurements (Table 1). The content of the COX subunit IV was 7- to 24-fold higher in brown than in epididymal fat, depending on the subgroup of mice (Fig. 5). A significant increase induced by the HF diet in the epididymal fat was detected (2- to 4-fold in the transgenic and the nontransgenic mice, respectively), but no major effect of the transgene could be observed.

COX/DNA ratio was ~25-fold higher in brown fat than in white fat. In accordance with a similar trend observed in the COX activity measurements (Table 1), transgenic mice showed a significantly lower specific COX subunit IV content in brown adipose tissue than did the nontransgenic mice. However, in contrast to COX activity measurements, the immunoblotting data adjusted to the DNA content suggested that the cellular COX subunit IV content in the epididymal fat was similar in the nontransgenic HF diet-fed and the transgenic Chow diet-fed mice, and higher compared with the other two subgroups of mice, which also showed approximately equal specific COX subunit IV content (see DISCUSSION).

Activity of LPL in white adipose tissue depots. We tested whether intrinsic differences in lipid metabolism between subcutaneous and gonadal fat depots could contribute to responses of the mice toward the aP2-*Ucp* transgene. The activity of LPL, the main enzyme regulator of triglyceride uptake, was estimated in the dorsolumbal and the epididymal fat depots (Table 2). In both white fat depots, total LPL activity differed notably depending on the diet and/or the transgene. Thus, in the subcutaneous fat of the nontransgenic mice fed the HF diet, total enzyme activity was ~20-fold higher than in the Chow diet-fed mice. In transgenic mice, this HF diet-induced increase in LPL activity was largely eliminated, resulting in only fourfold higher activity than that of the nontransgenic mice fed the Chow diet. No significant difference of total LPL activity between the transgenic and nontransgenic Chow diet-fed mice was detected.

In the epididymal fat of the nontransgenic mice, total LPL activity increased about fivefold in response to the

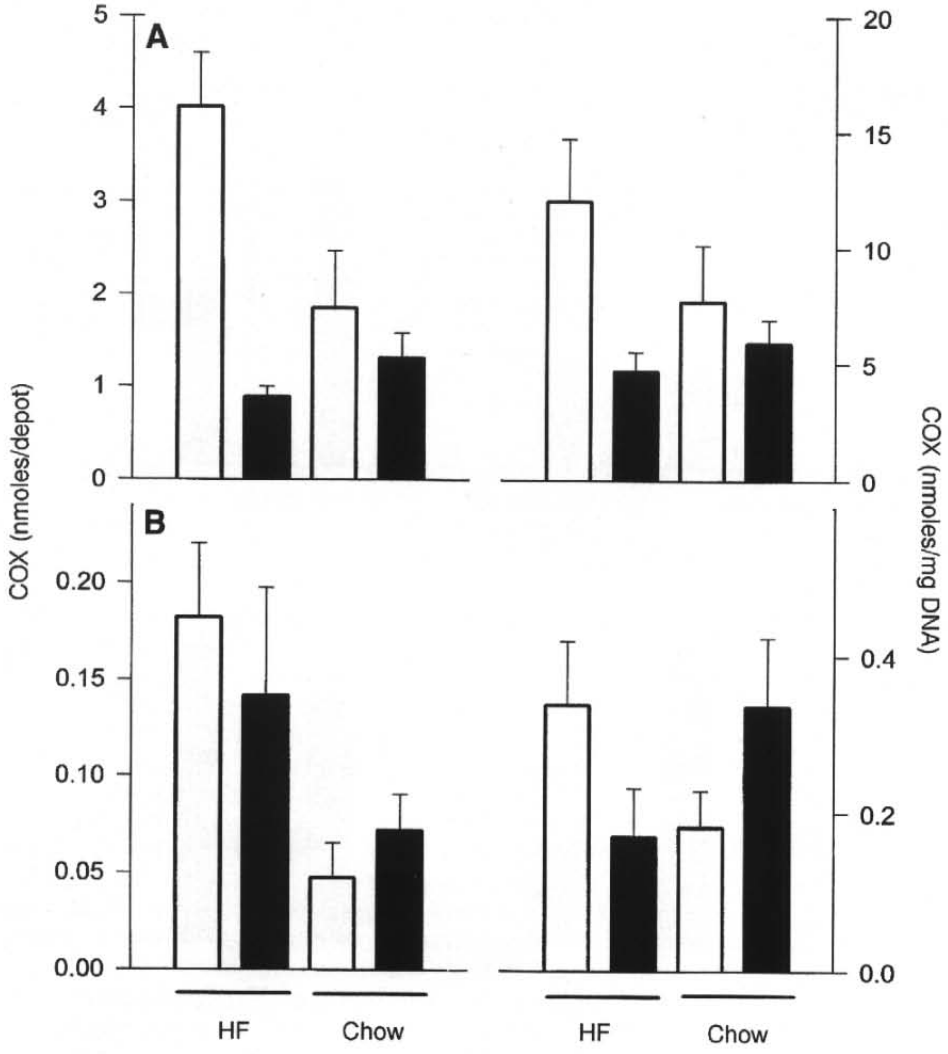

Fig. 5. Quantification of COX in interscapular brown adipose tissue (*A*) and epididymal fat (*B*) in male mice. Analysis was performed by immunoblotting with antiserum against subunit IV of COX, as described in MATERIALS AND METHODS. Part of the blots described in Fig. 4 were used. See legend to Fig. 4 for other details. Significance levels are referred to in the text.

HF diet, without any major effect of the transgene. Thus, in both white fat depots studied, total LPL activity changed similarly to adiposity (see Table 2 of Ref. 16) in response to both the diet and the transgene. Also, the specific LPL activity expressed on the tissue DNA basis correlated very well with the adiposity within each type of white fat depot. In all of the subgroups of mice, LPL activity expressed on the tissue DNA basis was four- to eightfold higher in epididymal than in subcutaneous fat.

DNA content and cell morphology in adipose tissue depots. Measurements of DNA in fat depots of male mice (Table 3) indicated that the differences in adiposity of the interscapular brown and the subcutaneous white fat [see Table 2 of the companion paper (16)] were closely paralleled by differences in total DNA content, mainly in the HF diet-fed mice. Accordingly, only marginal differences in tissue DNA concentration among all four subgroups of mice were found (Table 3). Overall, DNA concentrations were higher in brown fat

Table 2. *Activity of LPL in adipose tissue depots of male mice*

	Fat Depot					
	Dorsolumbal			Epididymal		
	TA	DNA	WW	TA	DNA	WW
HF/non-tg	$1,120 \pm 224^a$	0.41 ± 0.04^a	0.42 ± 0.07^a	787 ± 161^a	1.38 ± 0.5	0.36 ± 0.09^a
HF/tg	236 ± 66^b	0.24 ± 0.05^b	0.30 ± 0.06^a	729 ± 113^a	0.89 ± 0.25	0.62 ± 0.19^b
Chow/non-tg	57 ± 13^c	0.11 ± 0.02^c	0.17 ± 0.03^b	148 ± 29^b	0.56 ± 0.31	0.23 ± 0.06^a
Chow/tg	39 ± 7^c	0.09 ± 0.01^c	0.14 ± 0.02^b	209 ± 52^b	0.82 ± 0.24	0.23 ± 0.04^a

Values are means ± SE. Activities were estimated as described in MATERIALS AND METHODS and are expressed as total lipoprotein lipase (LPL) activity [TA; nmol fatty acid (FA) · min^{-1} · $depot^{-1}$] related to DNA content (DNA; μmol FA · min^{-1} · mg^{-1}), or wet weight of the depot (WW; nmol FA · min^{-1} · mg $tissue^{-1}$). For no. of animals/group, see Table 1. For DNA contents see Table 3. For comparison among subgroups, within each column a different superscript indicates a significant difference between subgroups.

Table 3. *Content and concentration of DNA in adipose tissue depots of male mice*

	Fat Depot					
	Interscapular		Dorsolumbal		Epididymal	
	Content	Concn	Content	Concn	Content	Concn
HF/non-tg	409 ± 106^a	1.49 ± 0.24^a	$2,668 \pm 445^a$	1.00 ± 0.14^a	569 ± 80^a	0.25 ± 0.02^a
HF/tg	196 ± 17^b	1.44 ± 0.15^a	938 ± 82^b	1.24 ± 0.12^a	816 ± 94^b	0.88 ± 0.20^b
Chow/non-tg	174 ± 19^b	1.78 ± 0.18^b	495 ± 78^c	1.44 ± 0.14^b	263 ± 39^c	0.38 ± 0.08^c
Chow/tg	222 ± 20^b	2.25 ± 0.18^b	414 ± 44^c	1.53 ± 0.10^b	395 ± 60^d	0.46 ± 0.07^c

Values are means ± SE for nos. of animals/subgroup as in Table 1. DNA was estimated in tissue homogenates fluorometrically (18) and expressed as total content in fat depot (μg DNA/depot) or as DNA concentration in the tissue (concn; μg DNA/mg tissue). For comparison among groups, within each column a different superscript indicates a significant difference between subgroups.

than in the subcutaneous white adipose tissue, and they were still much higher in subcutaneous than in epididymal fat. Importantly, the lower weight of the epididymal fat in the transgenic compared with the nontransgenic HF diet-fed mice (see Table 2 of Ref. 16) was not associated with a lower DNA content. Instead, the total DNA content was significantly (1.4-fold) higher in the transgenic than in the nontransgenic male mice, as in Chow diet-fed mice (1.5-fold difference). In the HF diet-fed transgenic mice, tissue DNA concentration increased 3.5-fold compared with the nontransgenic mice (Table 3). Also, in the epididymal fat of the Chow diet-fed mice, the DNA concentrations tended to be higher in the transgenic vs. nontransgenic mice; however, this difference was not statistically significant (Table 3). These changes in the epididymal fat pad closely reflected the proliferation of novel small cells in the transgenic mice (see Fig. 7).

Histological analysis of paraffin-embedded interscapular brown fat (Fig. 6) and epididymal white fat (Fig. 7) provided direct evidence of the change of morphology due to dietary conditions and/or the aP2-

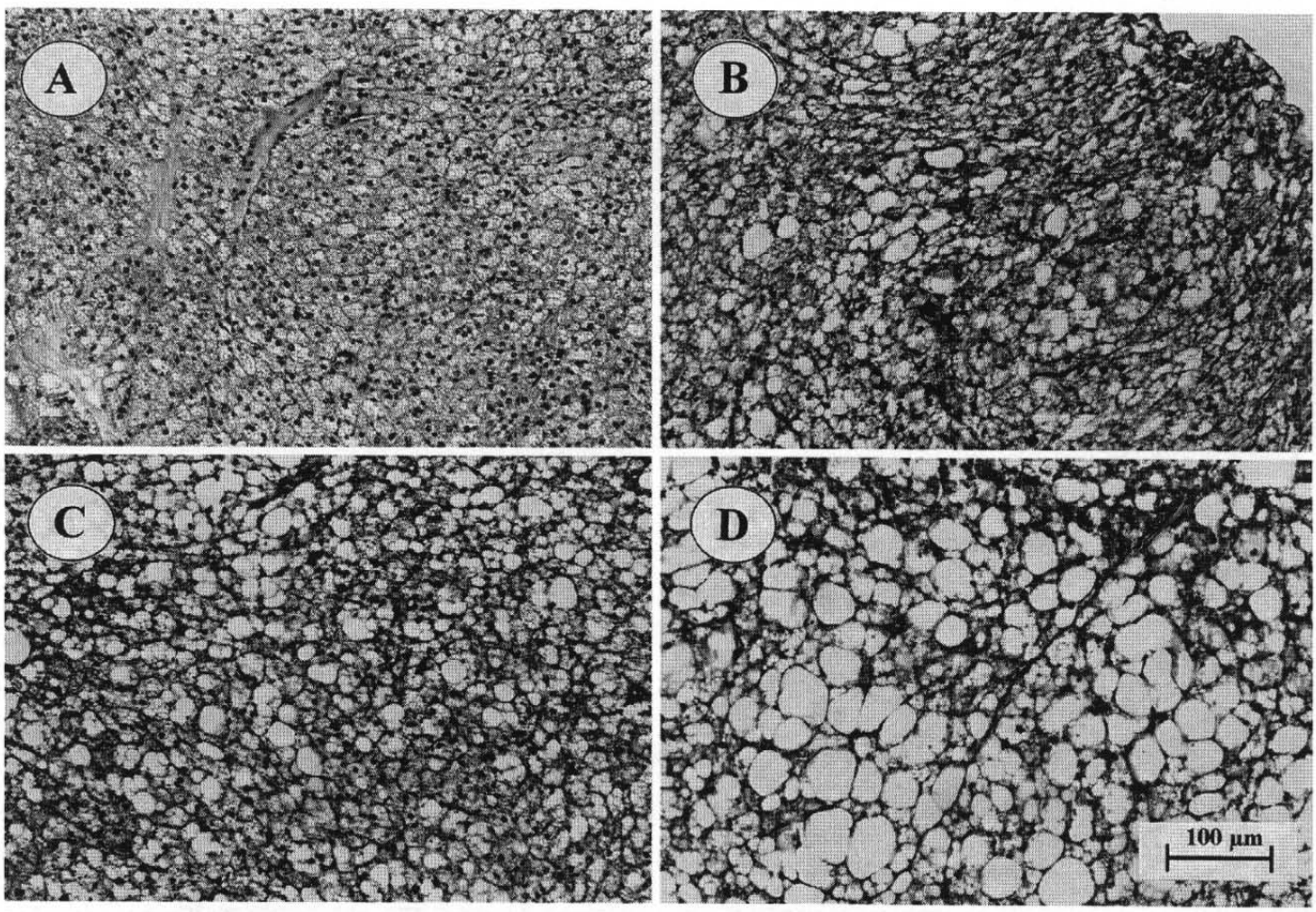

Fig. 6. Morphology of interscapular brown fat. Tissue was dissected from 9-mo-old male mice fed by a Chow (*A* and *B*) or HF (*C* and *D*) diet. Samples from nontransgenic (*A* and *C*) or transgenic (*B* and *D*) mice were stained by hematoxylin and eosin.

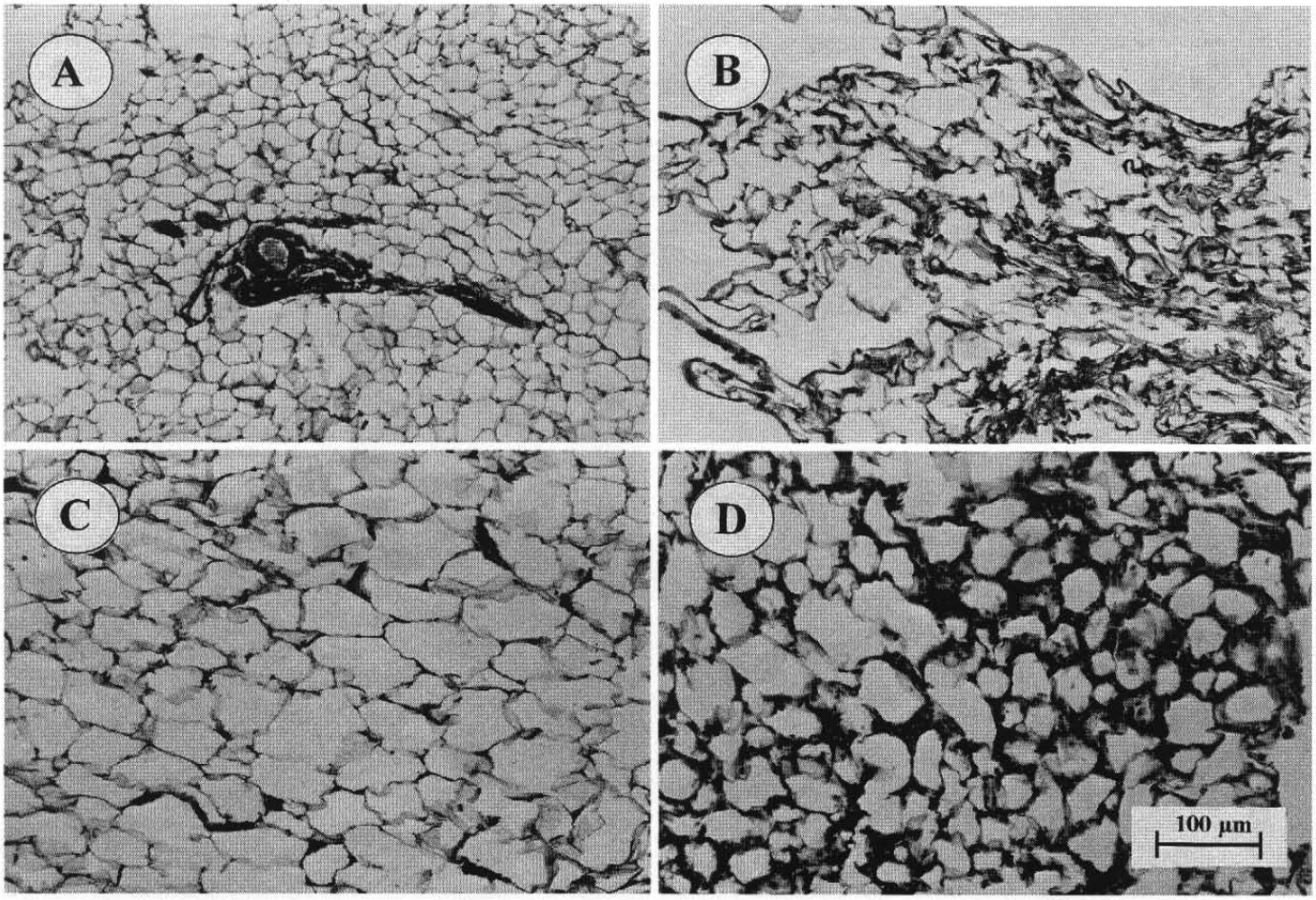

Fig. 7. Morphology of epididymal white adipose tissue. Samples were isolated and processed as described in legend to Fig. 6.

Ucp transgene. In brown fat, both the transgene and HF diet induced the enlargement of some adipocytes (compare Fig. 6, *A*, *B*, and *C*), which was associated with a loss of their multilocular appearance. On a morphological basis, these unilocular cells could not be distinguished from typical white fat adipocytes. The effects of the HF diet and the transgene on brown fat morphology were additive, resulting, in the case of transgenic mice fed the HF diet (Fig. 6*D*), in the formation of the largest unilocular cells and almost complete disappearance of the typical multilocular brown adipocytes. Small cells without lipid droplets and prominent nuclei formed stroma of the tissue.

In gonadal fat pads, the HF diet also induced a significant enlargement of unilocular adipocytes, the most abundant type of white fat cell in nontransgenic mice (compare Fig. 7, *A* and *C*). The major change associated with the transgene expression was appearance of a large number of small cells, which were interspread throughout the gonadal depot (Fig. 7, *A* vs. *B* and *C* vs. *D*). These small cells did not accumulate significant amounts of lipids and tended to be clustered around unilocular adipocytes. They were much more abundant in the transgenic HF diet-fed than in the Chow diet-fed mice (Fig. 7, *B* vs. *D*). In some sections of the gonadal fat pad from the transgenic HF diet-fed mice, the new small cells formed the majority of the tissue (not shown).

DISCUSSION

The results of this report strongly suggest that UCP, ectopically synthesized in white adipocytes of the aP2-*Ucp* transgenic mice is thermogenically active and that energy dissipation in mitochondria of white and not brown adipose tissue determines the phenotype of the transgenic mice.

The quantification of UCP in brown fat (Fig. 4 of this report and Table 1 of Ref. 15) showed identical UCP contents in the transgenic and the nontransgenic mice fed the Chow diet, both in the C57BL6/J and in the genetically obese C57BL6/J-A^{vy} animals. The HF diet induced an increase in UCP content in brown fat of the nontransgenic mice that was reminiscent of the activation of brown fat thermogenesis by cafeteria diets (9, 11) or as a result of gold thioglucose-induced obesity (11). In fact, low-protein diets are also known to activate brown fat (for review see Ref. 27). Because the HF diet used in our study contained less energy from protein than the Chow diet (16), further experiments are required to distinguish whether the increased UCP content resulted from differences in fat or protein

intake. This diet-induced increase of UCP in brown fat was absent in the transgenic mice.

Primer extension assay, which enabled one to distinguish between the endogenous *Ucp* gene and the aP2-*Ucp* transgene products, indicated that whereas the aP2-*Ucp* transgene was constitutively expressed both in brown and white fat, the endogenous *Ucp* gene in brown fat was downregulated (15). However, the UCP mRNA levels were still higher in the brown fat of the transgenic vs. the nontransgenic mice (Fig. 3), indicating a posttranscriptional control of *Ucp* expression (see also Ref. 14). It is conceivable that a decrease of centrally mediated stimulation of brown fat thermogenesis in the transgenic mice compensates for the UCP-induced thermogenesis (caloric drain) in white fat. The sympathetic stimulation of brown fat mediated by a local release of norepinephrine (9) may be depressed.

Norepinephrine is known to control not only *Ucp* expression and function of UCP but also mitochondrial biogenesis in brown fat (9, 10). A significant decline of COX activity (and COX/DNA) was observed in the transgenic vs. the nontransgenic mice (Table 1 and Fig. 5), indicating a lower oxidative capacity in each cell. Morphological changes in brown fat, mainly the appearance of large unilocular cells in the transgenic, and even in the Chow diet-fed mice, also suggest a low oxidation of endogenous triglycerides.

Direct proof of the depressed energy dissipation in the brown fat, and the first functional evidence of the thermogenic effect of the ectopically synthesized UCP in white fat of the transgenic mice, come from the respirometry experiments (Fig. 2). When adjusted to number of cells in the tissue using DNA as a marker (see Table 3), a 1.5-fold higher rate of endogenous oxygen consumption by epididymal fat from the transgenic compared with the nontransgenic mice was observed, whereas an inverse effect of the transgene was detected in brown fat. Similar differences were also noticed when the oxygen uptake was expressed relative to the tissue wet weight or COX activity (not shown; compare with the data in Tables 1 and 3). Overall, the data demonstrate UCP-induced energy dissipation in white fat mitochondria of the transgenic mice. On the other hand, the difference between the oxidative capacity of brown and epididymal fat was probably higher than indicated by the respirometry experiment, because the diffusion barrier of tissue fragments for oxygen would more likely affect the activity of the tissue exerting a higher respiratory rate.

The total content, as well as DNA-adjusted UCP content, was one order of magnitude lower in the epididymal than in the brown fat. The molar UCP/COX, calculated from the immunoblotting data using the COX subunit IV as a marker of the content of the enzyme complex (Figs. 4 and 5), varies from 5 to 11 in brown fat and from 0.7 to 1.0 in epididymal fat of the transgenic mice. In isolated mitochondria from brown adipose tissue of rats, a molar UCP/COX of 3.3 was evaluated (6), giving credit to our measurements in crude cell membranes. According to Lin and Klingenberg (20), capacity of UCP to translocate protons across the inner membrane of brown fat mitochondria exceeds severalfold the activity of COX. Therefore, even at the molar UCP/COX of ~1.0, oxidative phosphorylation in the white fat mitochondria may be partially uncoupled.

Despite the 25- to 50-fold lower content of COX in white compared with brown adipocytes (see COX/DNA in Table 1 and Fig. 5), total oxidative capacity of white adipose tissue is substantial. Assuming that the interscapular depot represents approximately one-half of all brown fat, and that the specific activities of COX in Table 1 are representative of all brown, white subcutaneous, and abdominal fat, respectively, it can be approximately calculated (see also Fig. 3 of Ref. 16) that the total COX activity of white fat represents ~30–50% of the brown adipose tissue COX activity in all subgroups of mice. Therefore the total oxidative capacity of white fat may be sufficiently high to influence total energy balance when unmasked in the aP2-*Ucp* transgenic mice.

A remarkable feature of the aP2-*Ucp* transgene expression is the effect on regional fat distribution and induction of a resistance against the development of obesity. Although the changes in regional adiposity are sexually dimorphic (see DISCUSSION in Ref. 16), in both genders the gonadal fat enlarges, and the subcutaneous white fat is reduced in the transgenic compared with the nontransgenic mice, especially under the conditions inducing development of obesity; in fact, development of obesity in the aP2-*Ucp* transgenic A^{vy} (15) or the HF diet-fed C57BL6/J mice (16) is impaired mainly because of a low accumulation of the subcutaneous white fat. These changes in adipose tissue mass are accompanied by changes in cellularity (Table 3 and Fig. 7). That the changes in adiposity in subcutaneous and abdominal depots did not correlate with aP2-*Ucp* expression at both the UCP mRNA (Fig. 3) and the UCP (Table 1 of Ref. 15) levels was observed, indicating that the changes in adiposity did not depend exclusively on the transgene expression. It can be assumed that the changes are somehow adjusted at the systemic level (see Ref. 15), or that a site-specific component other than the UCP-induced uncoupling of mitochondrial oxidative phosphorylation is also involved.

Concerning the metabolism of the adipocyte itself, a possible involvement of COX and LPL was evaluated. No major effect of transgene, diet, or region on the specific activity of COX, expressed on a tissue DNA content or a weight basis (Table 1), was observed (except for the effect of the transgene in subcutaneous fat of the Chow diet-fed mice and also in epididymal fat of the HF diet-fed mice). These data suggest that mitochondrial oxidative capacity, although essential for UCP-induced energy dissipation, is probably not the major variable determining the effect of transgene on adiposity. However, the unexplained influence of the transgene on COX activity, as well as the effects of the transgene and the diets on COX/DNA in epididymal fat, will be further studied. It is likely that the immunoblotting analysis, rather than the COX activity measure-

ments in the whole tissue homogenate, may reveal small changes in the enzyme protein content.

In contrast to COX, the specific activity of LPL expressed on a tissue DNA basis was much higher in the epididymal than in the subcutaneous fat depot (Table 2) and closely matched the weights of the depots. When LPL activity was expressed on a tissue weight basis, the differences between the depots, as well as the differences among the subgroups of mice within each category of fat depots, were much smaller, except for the epididymal fat of the HF diet-fed transgenic mice, which showed significantly higher LPL activity compared with the other subgroups. It is known that adipocyte metabolism is a function of the size of the fat cell and that the LPL activity per cell increases in obesity (3). Higher DNA concentration in the subcutaneous compared with the gonadal fat pads in all subgroups of mice (Table 3) suggests larger adipocytes in the gonadal fat depots in the aged animals (see also Refs. 18, 19). The LPL data might be interpreted to indicate that intrinsic differences in lipid metabolism between the subcutaneous and abdominal fat depots, already observed (2, 16, 28), are important for determining the mass of the fat depots.

The novel small cells induced by the aP2-*Ucp* expression preferentially (15) in the gonadal fat pad represent another variable. These cells, with still completely unknown metabolism, resemble the interstitial cells identified as brown adipocyte progenitors by Bukowiecki et al. (4). However, these cells are different adipocyte derivatives, because they contain UCP synthesized exclusively from the aP2 promoter and not from the endogenous *Ucp* promoter (15). They also express the fat-specific marker, the aP2 gene (15). Evidently, because of proliferation of these cells (Fig. 7), the total DNA content of the epididymal fat is ~1.5-fold higher in the transgenic than in the nontransgenic mice fed either diet (Table 3), and the DNA concentration is higher in the transgenic HF diet-fed than in the Chow diet-fed mice (Table 3).

The effect of aP2-*Ucp* expression on cellularity indicates a site-specific link between energy conversion in white adipocytes and preadipocyte replication. Differences in the replicative potential in vitro of preadipocytes isolated from anatomically separate regions in the rat were noticed (31). Stimulation of adipose tissue hyperplasia in vivo by dietary FA (26), as well as by increased glucose transport into the adipocytes (25), suggests that the transgene may affect a common regulatory step involved in the control of adipose tissue cellularity by energy metabolism.

Concerning the manifestation of the transgene effect on the total energy balance exclusively under conditions inducing obesity, no definitive explanation is yet available. A few observations indicate that UCP-induced energy dissipation in the gonadal fat may be augmented by the obese state, mainly the increased DNA concentration in the transgenic vs. nontransgenic HF diet (Table 3 and Fig. 7) and A^{vy} mice (15), accompanied by elevated specific activity of LPL. Such a change could not be detected in the lean Chow diet-fed mice.

A possibility was disproved (see Figs. 3 and 4) that aP2-*Ucp* expression was stimulated by elevated levels of FA in white adipocytes accumulating endogenous triglycerides because of an interaction between the FA and the *trans*-elements on the aP2 gene promoter (29). On the other hand, a direct activation of the proton channel in UCP molecules by FA (9, 20) may be involved. However, direct in vivo measurements of lipid metabolism in different fat depots, as well as characterization of adipose tissue mitochondria isolated from the normal and the transgenic mice under different sets of feeding conditions, are still required.

In summary, the results indicate that UCP formed in the white fat of the aP2-*Ucp* transgenic mice is thermogenically active, whereas the endogenous UCP-mediated thermogenesis in brown fat is suppressed. Oxidative capacity of mitochondria in white adipose tissue of adult mice is comparable to that in brown fat. Redistribution of adipose tissue in the transgenic mice suggests the existence of a yet unidentified site-specific link between energy metabolism in white adipocytes and cellularity.

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Publication B

Rossmeisl M, Syrovy I, Baumruk F, Flachs P, **Janovska P**, Kopecky J.

Decreased fatty acid synthesis due to mitochondrial uncoupling in adipose tissue

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Decreased fatty acid synthesis due to mitochondrial uncoupling in adipose tissue

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ABSTRACT **Synthesis of fatty acid (FA) in adipose tissue requires cooperation of mitochondrial and cytoplasmic enzymes. Mitochondria are required for the production of ATP and they also support the formation of acetyl-CoA and NADPH in cytoplasm. Since cellular levels of all these metabolites depend on the efficiency of mitochondrial energy conversion, mitochondrial proton leak via uncoupling proteins (UCPs) could modulate FA synthesis. In 3T3-L1 adipocytes, 2,4-dinitrophenol depressed the synthesis of FA 4-fold while increasing FA oxidation 1.5-fold and the production of lactate 14-fold. Inhibition of FA synthesis in 3T3-L1 adipocytes was proportional to the decrease in mitochondrial membrane potential. FA synthesis from D-[U-^{14}C] glucose 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 and the reduction of adiposity. Transcript levels for lipogenic enzymes were lower in the white fat of the transgenic mice than in the control animals. Our results show that uncoupling of oxidative phosphorylation depresses FA synthesis in white fat. Reduction of adiposity via mitochondrial uncoupling in white fat not only reflects increased energy expenditure, but also decreased *in situ* lipogenesis.—Rossmeisl, M., Syrový, I., Baumruk, F., Flachs, P., Janovská, P., Kopecký, J. Decreased fatty acid synthesis due to mitochondrial uncoupling in adipose tissue. *FASEB J.* 14, 1793–1800 (2000)**

Key Words: UCP · lipogenesis · C57BL/6J mice · 3T3-L1 · obesity

De novo FATTY acid (FA) synthesis in adipocytes is an important mechanism involved in the control of fat content. This mechanism is relevant not only to rodents, but also to humans, where adipose tissue may account up to 40% of whole-body lipogenesis (1). The biosynthesis of FA requires cooperation between mitochondrial and cytoplasmic enzymes and involves fluxes of metabolites across mitochondrial membranes (2–4). Mitochondria are engaged in several pathways that are essential for FA synthesis, namely, the generation of ATP, and they also support the formation of NADPH and acetyl-CoA in cytoplasm.

That lipogenesis in white fat requires coupled mitochondria was suggested by Rognstad and Katz (2), who studied the effect of 2,4-dinitrophenol (DNP) on glucose metabolism in epididymal fat from rats. DNP, an uncoupler of oxidative phosphorylation, acts as a proton shuttle to abolish the proton motive force and ATP synthesis in isolated mitochondria. In intact cells, DNP decreases phosphate potential and ATP-consuming processes (5). The addition of DNP to fat fragments resulted in depressed synthesis of FA and increased production of lactate (2). However, it has also been proposed (6) that production of ATP in adipocytes limits lipogenesis and partial uncoupling of oxidative phosphorylation enhances FA synthesis.

Because of the central role that mitochondrial proton conductance plays in the energetic state of the cell (7), it is tempting to speculate that it may contribute to the control of FA synthesis in lipogenic tissues via mitochondrial uncoupling proteins (UCPs). UCP1, which is specifically expressed in thermogenic brown fat, functions as a regulated proton transporter (8). Recently, two UCP1 homologues, UCP2 (9, 10) and UCP3 (11, 12), have been discovered that are expressed in brown fat and in other organs of mammalians as well. Novel UCPs probably transport protons, similar to UCP1. However, the biochemical activities of these novel UCPs are not understood in detail and may differ among the UCPs (8, 13). UCP2 and UCP3 may play an important role in controlling basic metabolic rate and obesity (9–12, 14–17). An excellent review study on UCPs has been published recently (18).

We have previously reported that expression of UCP1 gene from aP2 gene promoter in white and brown fat of transgenic (aP2-*Ucp1*) mice affected fat distribution in the body and prevented development

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of genetic or dietary obesity (19–21). Resistance to obesity resulted from genetic manipulation of white fat (21, 22), where it was induced by mitochondrial uncoupling in adipocytes, as documented by measurements of mitochondrial membrane potential ($\Delta\psi_m$) in isolated fat cells (23) and of oxygen uptake by adipose tissue fragments (21). Preliminary results (24) suggest that transgenic UCP1 affects body weight not only by increasing *in situ* FA oxidation, but also by decreasing lipogenesis.

This study was designed to analyze in detail the link between efficiency of energy conversion and the rate of FA synthesis in adipocytes and to verify the hypothesis that UCP1 could modulate adiposity via effect on FA synthesis. The effects of chemical uncouplers were studied in 3T3-L1 adipocytes differentiated in cell culture. Transgenic aP2-*Ucp1* mice were used as an *in vivo* model of mitochondrial uncoupling induced by UCP1.

MATERIALS AND METHODS

Measurements of FA oxidation, FA synthesis, and lactate production in 3T3-L1 adipocytes

Cells of 3T3-L1 clonal line (25) were plated at a density of 8000 cells/cm^2 (10 cm^2 dish) and grown in Dulbecco's modified Eagle's medium at 37°C in 10% CO_2 in air. The composition of the growth medium and the differentiation medium (added to confluent cells) ~4–5 days after plating were as described before (26), except that the differentiation medium also contained 2 μM dexamethasone (present only during the first 48 h after the switch to the differentiation medium) and 100 nM 5-(4-[2-(N-methyl-N-(2-pyridyl)amino) ethoxy]benzyl)thiazolidine-2,4-dione maleic acid salt (BRL 49653; in dimethyl sulfoxide). When used for experiments (12–14 days after confluence), cultures contained 50–60% of differentiated adipocytes. Before each measurement, a complete change of the medium was performed. DNP (dissolved in 0.1% KOH) or carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; in 70% ethanol) were also added in some experiments. FA oxidation was measured using a modification of the procedure described previously (27) during a 4 h incubation in the presence of 0.2 mM oleate, 0.4% bovine serum albumin (BSA), and 5 μCi (9, 10[n-^{3}H] oleic acid (10.3 Ci/mmol; Amersham, Little Chalfont, U.K.)/ml. The DNA content of cells was measured fluorometrically (28) after digestion with 150 μl proteinase K solution for 15 min at 20°C (22). FA synthesis was measured (29) by the incorporation of ^{14}C from acetate into saponifiable FA. Cells incubated 4 h in the presence of 1 μCi/ml [2-^{14}C]acetic acid (sodium salt, 50 mCi/mmol; Amersham) were washed twice with phosphate-buffered saline (pH 7.4) and solubilized by proteinase K (see above). Aliquots were used for DNA measurements (30 μl; see above) and incorporation of radioactivity into FA (90 μl) (30). Lactate in the medium was measured using a coupled enzyme assay (kit 139 084 from Boehringer, Mannheim, Germany) after 24 h of culture in the presence of DNP. Lactate contributed to the medium by fetal bovine serum (around 0.2 mM) was subtracted. All samples were run in triplicate.

Correlation of FA synthesis and $\Delta\psi_m$ in 3T3-L1 adipocytes

3T3-L1 adipocytes were maintained for 4 h (in 25 cm^2 dish) with or without DNP or FCCP, and FA synthesis was measured using [2-^{14}C]acetic acid as described above. The $\Delta\psi_m$ was estimated in cells maintained under identical conditions (except for the absence of [2-^{14}C]acetic acid). These cells were harvested using trypsin-EDTA solution, washed in physiological buffered saline (23), and suspended in Krebs Ringer bicarbonate buffer (KRB) containing 5 mM glucose and various concentrations of DNP or FCCP. Estimation of $\Delta\psi_m$ was performed similarly as before (23), except that a minimum of 5000 whole cells (instead of digitonin-permeabilized cells) were used for each measurement. Tetramethylrhodamine methyl ester (TMRM) was added to a final concentration of 50 nM. TMRM fluorescence (reflecting $\Delta\psi_m$) was recorded using FACSort flow cytometer (Becton Dickinson, San Jose, Calif.) at 5 min intervals and expressed as mean fluorescence intensity (IFL). Fluorescence intensity increased during incubation, reaching a maximum at ~30 min (not shown). Therefore, the IFL value at 30 min was taken as a measure of $\Delta\psi_m$.

Animals

Control C57BL/6J male mice and their hemizygous aP2-*Ucp1* transgenic littermates were identified by Southern blot analysis (19). The mice were maintained at 20°C with a 12 h light-dark cycle. After weaning at 4 wk of age, mice were housed 4–5 per cage and had free access to a standard chow diet (20) and water. Adult (6- to 8-month-old) animals were killed by decapitation in diethylether anesthesia. Subcutaneous (s.c.) dorsolumbar white fat (20) and epididymal fat were used for experiments. Samples for isolation of total RNA were frozen and stored in liquid nitrogen.

FA synthesis in animals and tissue fragments

Mice were injected intraperitoneally with 50–100 μl of 0.15 M NaCl containing ^3H_2O (35 μCi/g body weight). After 60 min at 22°C, mice were killed and the rate of FA synthesis in various tissues *in vivo* was measured by incorporation of ^3H_2O into saponifiable FA (30). The activity of FA synthesis was expressed as dpm ^{3}H incorporated to FA/mg tissue per hour. In other experiments, FA synthesis was measured *in vitro* (1). Adipose tissue fragments (70 mg) were incubated for 2 h at 37°C (in an atmosphere of 5% CO_2 and 95% O_2) in total volume of 0.5 ml KRB containing 4% BSA (Serva, Cat. No. 11930), 1.1 μCi D-[U-^{14}C] glucose (4 mCi/mmol; Amersham), 0.5 nmol nonradioactive glucose, and 40 μU insulin (Actrapid MC, Novoindustri, Denmark). ^{14}C incorporation into saponifiable FA was estimated (30) and expressed as pmol of glucose converted to FA/g tissue per hour.

RNA analysis

Total RNA was isolated from adipose tissue and analyzed on Northern blots using a full-length cDNA probe for mouse UCP1 (21). Hybridization with a ribosomal 18S RNA probe was used to correct for inter-sample variations. Total RNA isolated from interscapular brown fat of cold-acclimated mice served as a standard. Radioactivity was evaluated by PhosphorImager SF (Molecular Dynamics, Sunnyvale, Calif.). Semiquantification of the transcripts for acetyl-CoA carboxylase (ACC) and FA synthase (FAS) was performed by reverse transcription of RNA, followed by the polymerase chain reaction (RT-PCR), as described before (31, 32). Total RNA was treated with RNase-free DNase and first-strand cDNA was generated from 1 μg of RNA in a 10 μl volume by using

TABLE 1. *Sequences of PCR primers*

Gene	Sense primer (5′-3′)	Antisense primer (5′-3′)	GeneBank Accession no. for cDNA
ACC	CAGATCCAGGCCATGTTGAGACG	TCGCTGGGTGGGTGAGATGTG	AA451016
FAS	GGCTGCCTCCGTGGACCTTATC	GTCTAGCCCTCCCGTACACTCACTCGT	X13135
β-actin	GAACCCTAAGGCCAACCGTGAAAAGAT	ACCGCTCGTTGCCAATAGTGATG	X03765

Moloney murine leukemia virus reverse transcriptase (Top-Bio, Czech Republic) and the oligo (dT) primers. cDNA (1 μl of the reverse transcription mix) was amplified by hot-start PCR with primers specific for mouse ACC, FAS, and β-actin (**Table 1**). A reaction cycle consisted of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. Four aliquots (12, 12, 8, and 8 μl, respectively) were removed at 3-cycle intervals between cycles 16–34 (log-linear phase of the reaction) and examined on 1.5% agarose gels stained with ethidium bromide. Fluorescence was evaluated by LAS-1000 (Fuji, Japan) and quantified by using AIDA 2.11 software (Raytest, Germany). Levels of the transcripts were expressed relative to that of β-actin.

Statistics

A two- or three-way analysis of variance (ANOVA) with post hoc multiple comparisons was used as described before (20). Statistical significance was evaluated using Student's *t* test. All the comparisons were judged to be significant at $P<0.05$.

RESULTS

Effects of chemical uncouplers on metabolic fluxes and $\Delta\psi_m$ in 3T3-L1 adipocytes

To characterize in detail the effect of mitochondrial uncoupling on metabolism of adipocytes, experiments were performed on 3T3-L1 adipocytes differentiated in cell culture (**Fig. 1**). Adipocytes were maintained for 4–24 h in the presence of different concentrations of DNP. FA oxidation and lactate production increased with increasing concentrations of DNP (2, 33), whereas activity of FA synthesis became inhibited. All three metabolic parameters were affected at similar concentrations of the uncoupler, with 300 μM DNP exerting 80–90% effect. Most pronounced was the effect on lactate production, which was stimulated ~14-fold by DNP. Maximum inhibitory effect of DNP (at 700 μM concentration) on FA synthesis was ~4-fold, whereas oxidation of FA was stimulated only ~1.5-fold under the same experimental conditions (Fig. 1). It is apparent that decreased ATP production uncoupled by DNP from substrate (FA) oxidation in mitochondria resulted in stimulation of glucose utilization by adipocytes (33). Moreover, these experiments support the notion (2, 34) that mitochondrial uncoupling decreases FA synthesis in adipocytes.

The *in vitro* model of 3T3-L1 adipocytes allowed us to analyze directly the link between mitochondrial energetics and FA synthesis. In a separate set of experiments, 3T3-L1 adipocytes differentiated in cell culture were incubated with uncouplers (DNP or FCCP), as above, and FA synthesis was evaluated in parallel with estimation of the $\Delta\psi_m$ in whole cells (**Fig. 2**). The latter type of measurement was performed by flow cytometry using a potential-sensitive fluorescent dye, TMRM. Under these conditions, TMRM fluorescence intensity (IFL) reflects $\Delta\psi_m$ (23). FA synthesis decreased with increasing concentrations (100–600 μM) of DNP, as in the previous experiment (Fig. 1). The lowest activity was observed in the presence of 50 μM FCCP (Fig. 2). Addition of the uncoupler also resulted in a decrease of IFL values. Positive correlation was found between decreased FA synthesis and decreased IFL value, indicating a close link between the magnitude of $\Delta\psi_m$ and activity of FA synthesis in adipocytes.

Gene expression in adipose tissue of control and aP2-*Ucp1* transgenic mice

In further experiments, aP2-*Ucp1* transgenic mice were used as a model of mitochondrial uncoupling

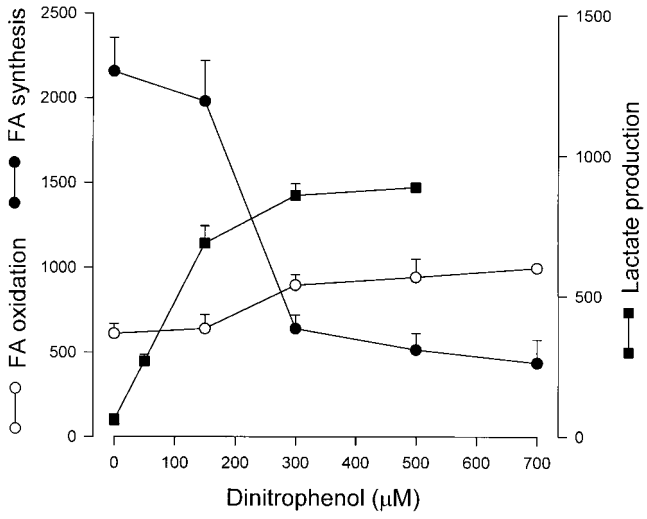

Figure 1. Effect of a chemical uncoupler on metabolic fluxes in 3T3-L1 adipocytes. Adipocytes were incubated for 4 h in a cell culture dish with or without different concentrations of DNP for measurements of FA oxidation (pmol oleate oxidized/dish per hour) or FA synthesis (pmol acetate × 10 converted to FA/dish per hour), or for 24 h for measurements of lactate production (nmol lactate produced/dish per hour). DNA measurements confirmed that no cells had been lost during the procedure (see Materials and Methods). Values are means ± SE of three independent experiments.

induced *in vivo* by ectopic UCP1 in white fat. As observed before (19, 20), the aP2-*Ucp1* transgene affected differentially the size of various fat depots (see legend to **Table 2**). Thus, the weight of s.c. white fat was twofold lower in transgenic compared with control mice and the size of epididymal fat was marginally increased (19, 20).

The aP2-*Ucp1* transgene induced ectopic expression of UCP1 gene in white fat depots of adult mice, whereas no expression could be detected in control animals (**Fig. 3** and refs 19, 21). The levels of UCP1 transcript in s.c. white fat were significantly (~twofold) higher than in epididymal fat (Fig. 3 and Table 2). The expression of UCP1 in the white fat of transgenic mice was at least one order of magnitude lower than that in interscapular brown fat of control, cold-acclimated mice (Table 2 and refs 19, 20). Expression of the genes for ACC and FAS, cytoplasmic enzymes engaged in the synthesis of C_2 moiety of FA, was significantly depressed by the transgene in both white fat depots studied (Fig. 3 and Table 2).

TABLE 2. *Quantification of gene expression in white fat depots of control and transgenic mice*[a]

	mRNA level (arbitrary units)		
Tissue	UCP1	ACC	FAS
Sc-WF			
+/+	0.000 ± 0.000	0.36 ± 0.04	1.28 ± 0.20
tg/+	0.050 ± 0.010[a]	0.15 ± 0.05*	0.63 ± 0.08*
Epid-WF			
+/+	0.000 ± 0.000	0.42 ± 0.12	1.13 ± 0.05
tg/+	0.023 ± 0.001*,†	0.08 ± 0.02*	0.55 ± 0.08*

[a] Control (+/+) and transgenic (tg/+) mice with similar body weights (29.2 ± 0.5 and 28.5 ± 0.6 g, respectively) were used. Weights of subcutaneous white fat (Sc-WF) of control mice (284 ± 18 mg) were significantly (P=0.001) higher than in transgenic (160 ± 24 mg) mice. Weights of epididymal fat (Epid-WF) of control (386 ± 23 mg) and transgenic (427 ± 55 mg) mice were not significantly different (P=0.50). Gene expression was analyzed as in Fig. 3. Levels of UCP1 mRNA are expressed relative to that in interscapular brown fat of cold-acclimated control mice and levels of ACC and FAS mRNAs relative to β-actin mRNA.

* Significant differences between genotypes, † significant differences between fat depots. Data are means ± SE from four (ACC and FAS) to seven (UCP1) measurements.

Effect of transgenic UCP1 on FA synthesis in adipose tissue

FA synthesis in white fat depots of control and transgenic mice was evaluated (**Fig. 4**) *in vivo* by measuring incorporation of injected ^3H_2O into saponifiable FA and *in vitro* as the incorporation of ^{14}C into FA extracted from tissue fragments incubated in the presence of D-[U-^{14}C] glucose and insulin (see Materials and Methods). ^3H incorporation was similarly high in both s.c. and epididymal white fat of control mice (Fig. 4, upper part), which was ~5- to 10-fold lower compared to that in brown fat or liver and ~4-fold higher than that in skeletal muscle (not shown). ^3H incorporation in both white fat depots was decreased to a similar extent by the transgene (~1.6- to 1.8-fold; Fig. 4, upper part). This suppression was found only in adipose tissue and not in other tissues (not shown), reflecting the fat-specific expression of UCP1 from the aP2 gene promoter contained in the transgene (19, 35).

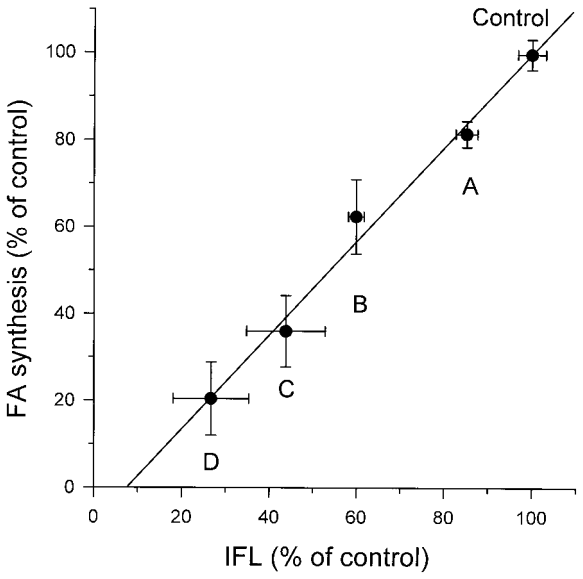

Figure 2. Correlation between $\Delta\psi_m$ and FA synthesis in 3T3-L1 adipocytes. *A)* 100 μM DNP; *B)* 300 μM DNP; *C)* 600 μM DNP; *D)* 50 μM FCCP. Values are expressed in % of the values estimated in the absence of the chemical uncouplers (Control). Values are means ± SE of four independent experiments.

The *in vitro* experiments (Fig. 4, lower part) confirmed the inhibition of FA synthesis by ectopic UCP1 in white fat. However, the inhibition was much more pronounced in s.c. fat (~3.8-fold difference between the genotypes) than in epididymal fat (1.5-fold difference; not significant, P=0.08). The differential effect of the transgene on FA synthesis in s.c. and epididymal fat is in accordance with the relatively higher expression of the aP2-*Ucp1* transgene in the former tissue (Fig. 3 and Table 2). The results also suggest that the *in vitro* measurements of the incorporation of ^{14}C from D-[U-^{14}C] glucose better reveal the link between mitochondrial energetics and FA synthesis than does the estimation of lipogenesis in whole animals injected by ^3H_2O (see Discussion).

DISCUSSION

Mechanism of decreased lipogenesis

This report demonstrates that uncoupling of oxidative phosphorylation, while reducing adiposity in

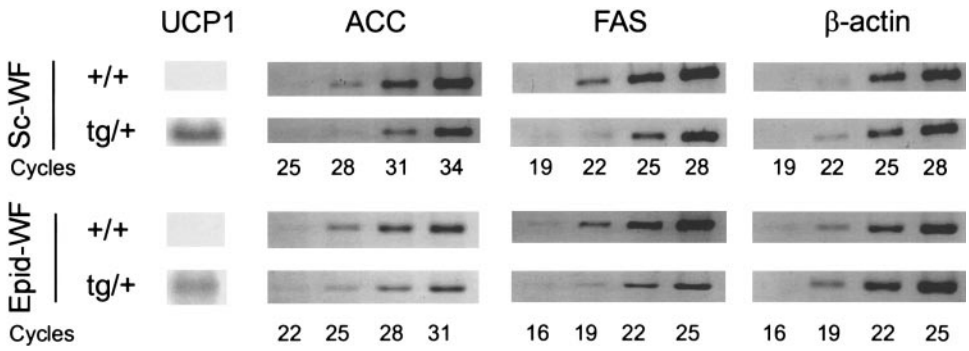

Figure 3. Expression of various genes in s.c. (Sc-WF) and epididymal (Epid-WF) white fat of control (+/+) or transgenic (tg/+) mice. UCP1 transcript was detected using Northern blots; expression of ACC, FAS, and β-actin was evaluated by RT-PCR (inverted gray scale).

white fat, depresses *in situ* FA synthesis. Experiments on adipocytes differentiated in cell culture confirmed previous findings on the effect of DNP in adipose tissue (2, 34) and showed a strong correlation between decreased FA synthesis and decreased $\Delta\psi_m$. The experiments on aP2-*Ucp1* mice document the ability of UCP1 to decrease FA synthesis in white fat *in vivo* while excluding possible side-effects of chemical uncouplers on cell metabolism.

FA synthesis, which requires (2–4) production of extramitochondrial acetyl-CoA, proceeds via 'pyruvate cycle', i.e., conversion of pyruvate into oxalacetate and citrate in mitochondria, transport of citrate to the cytoplasm, and cleavage to acetyl-CoA and oxalacetate. Cytosolic oxalacetate is reduced to malate by NADH, and the malate is decarboxylated to pyruvate with generation of NADPH (in a reaction catalyzed by malic enzyme). Pyruvate returns to the mitochondrion, where it serves to reform citrate. Operation of the cycle not only provides acetyl units for FA synthesis, but also contributes about half the NADPH required, with the rest supplied by the pentose cycle (2). For each molecule of acetate incorporated into long-chain FA, two molecules of NADPH are necessary in the reaction catalyzed by FA synthase. Mitochondrial production of ATP is required for synthesis of oxalacetate by carboxylation of pyruvate and subsequent synthesis of citrate in mitochondria and for FA synthesis and esterification in cytoplasm (2–4).

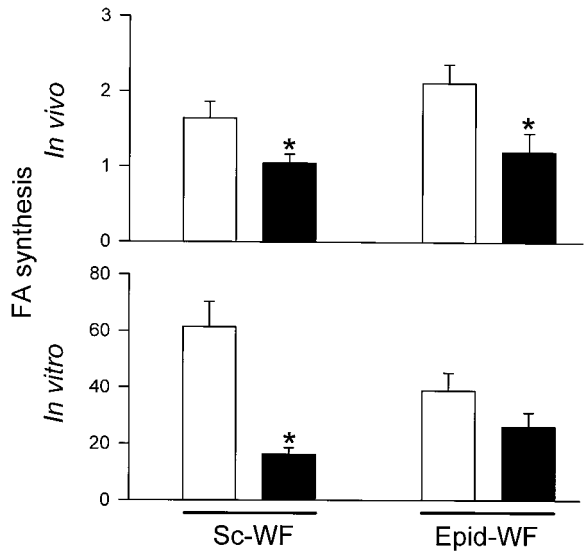

Figure 4. Synthesis of FA in s.c. (Sc-WF) and epididymal (Epid-WF) white fat of control (empty bars) or transgenic (solid bars) mice. Incorporation of radioactivity into saponifiable FA was measured using ^3H_2O *in vivo* (n=6; upper part) or D-[U-^{14}C] glucose *in vitro* (n=15; lower part). Activities are expressed as dpm ^{3}H incorporated to FA/mg tissue/h or as pmol glucose converted to FA/g tissue/h, respectively (see Materials and Methods). Values are means ± SE. Asterisks indicate statistically significant differences between genotypes. The difference between s.c. and epididymal fat of control animals *in vitro* was also significant; other differences were not.

A 4- to 5-fold reduction of FA synthesis was detected in both adipocytes incubated with uncoupler and in fragments of s.c. white fat of transgenic mice, whereas substrate oxidation increased only ~1.5-fold (Fig. 1 and ref 21, respectively). Inhibition of FA synthesis by mitochondrial uncoupling probably results from limited availability of intramitochondrial ATP for the carboxylation of pyruvate (2, 3) whereas ATP level in cytoplasm remains relatively high (2, 33). Activity of pyruvate carboxylase is threefold greater in adipose tissue than in liver mitochondria, and the ATP:ADP ratio directly affects the activity of the enzyme (3, 4). This suggests an important regulatory role of the enzyme in lipogenesis in white fat. The inhibition of pyruvate carboxylation probably slows down FA synthesis as a result of limited supply of acetyl units (2, 3).

When measured in tissue fragments (as the incorporation of D-[U-^{14}C] glucose in the presence of insulin) but not *in vivo* (in mice injected by ^3H_2O), FA synthesis in s.c. fat was more sensitive to inhibition by the transgene than that in epididymal fat. The levels of UCP1 transcript in transgenic mice were significantly higher in s.c. than in epididymal fat, in accordance with the fat depot-specific difference in the activity of the aP2 gene promoter (35). Therefore, inhibition of FA synthesis in tissue fragments correlated with the levels of UCP1 transcript in different depots. The results support the link between ectopic UCP1 and FA synthesis in white fat, providing that the metabolic flux through the path-

way of FA synthesis is higher under the *in vitro* conditions compared to *in vivo* measurements (36) and therefore relatively more sensitive to the alteration of mitochondrial functions. Moreover, systemic control of lipid metabolism in the animal may counteract and equalize the effect of UCP1 in various fat depots. However, it is likely that FA synthesis in various fat depots is also differentially affected by the transgene *in vivo* and the difference is too small to be detected. Also, the steady-state levels of the transcripts for lipogenic enzymes (ACC and FAS) were similarly depressed in both white fat depots of the transgenic mice. The differential effect of the transgene on FA synthesis *in vivo* may explain, at least in part, why the size of s.c. white fat in transgenic mice is reduced whereas that of epididymal fat is not (19, 20). Low accumulation of s.c. and not epididymal fat counteracts development of obesity in the transgenic mice (19, 20).

Biological significance

FA synthesis in white fat is inhibited by starvation (37–39) and by high-fat diets (40) and potentiated by glucose, high-sugar meals, and insulin. These effects result in part from the regulation of ACC activity (40) by insulin and glucagon. However, starvation in both humans (41, 42) and mice (J. Kopecký et al., unpublished data), and high-fat diets in mice (9, 15) also up-regulate UCP2 in white fat. Thus, the same factors that negatively affect lipogenesis in white fat would also up-regulate the expression of UCP2. This suggests that UCP2 may be involved in physiological control of FA synthesis in white fat.

Expression of UCP2 in white adipose tissue is relatively high (9, 10) whereas that of UCP1 is very low in both rodents (32, 43–46) and humans (16). UCP3 is normally absent (47). However, both UCP1 (43–46) and UCP3 (47) can be induced in white fat depots by pharmacological treatments that reduce adiposity. Whether these effects reflect transdifferentiation of white adipocytes or recruitment of brown fat precursor cells remains an important question (35, 43, 46). Our findings suggest that UCP1 has a significant role in the regulation of lipogenesis in brown fat. Indeed, short-term (2 h) cold exposure, which increases adrenergic stimulation and activates UCP1-mediated proton transport via FA released from intracellular triglycerides (8, 13), results in a threefold reduction in FA synthesis in brown fat (38). Norepinephrine or exogenous FA inhibited synthesis of FA in isolated brown adipocytes (48). Therefore, activation of UCP1 in brown fat during acute cold stress probably results not only in thermogenesis, but also in depression of FA synthesis. However, prolonged cold exposure is accompanied by the increase in FA synthesis and increased ACC and FAS activities (38).

The high lipogenic rate in adipose tissue may contribute to development of obesity (49) and may explain the high rate of relapse in patients treated by caloric restriction (32). Compared with lean controls, morbidly obese subjects displayed lower levels of both UCP1 (16) and UCP2 (17) transcripts in intraabdominal fat, and UCP2 expression remained low subsequent to weight-reducing surgery (17). Induction of UCP2 by a high-fat diet correlated with obesity resistance in different strains of mice (9, 15). Lipogenesis in adipose tissue has not been measured in these studies. However, in rats with hyperleptinemia induced by adenovirus transfer of the leptin gene, body fat is depleted whereas expression of UCP1 and UCP2 in white adipose tissue is up-regulated, FA oxidation is increased, and expression of genes for enzymes engaged in lipogenesis (like ACC and FAS) is profoundly suppressed (32). In aP2-*Ucp1* mice, resistance against obesity was also accompanied by down-regulation of genes for lipogenic enzymes (ACC and FAS) and by increased *in situ* oxidation of FA in adipose tissue, which would suggest that the depression of lipogenesis in white fat in both models of obesity resistance results from up-regulation of UCP1 and/or UCP2. The changes in the expression of genes for lipogenic enzymes probably reflect decreased metabolic flux through the pathway of FA synthesis. Tumor necrosis factor α also down-regulates lipogenic genes and prevents their induction by insulin in adipocytes (50). It may be hypothesized that all these effects result from the inhibition of mitochondrial energy conversion by tumor necrosis factor α (51).

In summary, our 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 not only due to increased energy expenditure, but also by depression of *in situ* lipogenesis. The measurement of FA 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 FA synthesis may be a superior target for treatment strategies for obesity. FJ

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Expression of the uncoupling protein 1 from the *aP2* gene promoter stimulates mitochondrial biogenesis in unilocular adipocytes *in vivo*

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Mitochondrial uncoupling protein 1 (UCP1) is a specific marker of multilocular brown adipocytes. Ectopic UCP1 in white fat of aP2-*Ucp1* mice mitigates development of obesity by both, increasing energy expenditure and decreasing *in situ* lipogenesis. In order to further analyse consequences of respiratory uncoupling in white fat, the effects of the ectopic UCP1 on the morphology of adipocytes and biogenesis of mitochondria in these cells were studied. In subcutaneous white fat of both aP2-*Ucp1* and young control (5-week-old) mice, numerous multilocular adipocytes were found, while they were absent in adult (7- to 9-month-old) animals. Only unilocular cells were present in epididymal fat of both genotypes. In both fat depots of aP2-*Ucp1* mice, the levels of the *UCP1* transcript and UCP1 antigen declined during ageing, and they were higher in subcutaneous than in epididymal fat. Under no circumstances could ectopic UCP1 induce the conversion of unilocular into multilocular adipocytes. Presence of ectopic UCP1 in unilocular adipocytes was associated with the elevation of the transcripts for UCP2 and for subunit IV of mitochondrial cytochrome oxidase (COX IV), and increased content of mitochondrial cytochromes. Electron microscopy indicated changes of mitochondrial morphology and increased mitochondrial content due to ectopic UCP1 in unilocular adipocytes. In 3T3-L1 adipocytes, 2,4-dinitrophenol increased the levels of the transcripts for both COX IV and for nuclear respiratory factor-1. Our results indicate that respiratory uncoupling in unilocular adipocytes of white fat is capable of both inducing mitochondrial biogenesis and reducing development of obesity.

Keywords: mitochondria; mice; white fat; brown fat; NRF-1.

Increasing evidence suggests that respiratory uncoupling in white adipose tissue could prevent excessive accumulation of body fat. Part of the evidence comes from studies of mitochondrial uncoupling protein 1 (UCP1), an integral protein of the inner mitochondrial membrane and a well-established protonophore [1–3]. This protein is typically present only in brown fat [4–6] where it dissipates the energy of mitochondrial proton gradient and is essential for regulatory thermogenesis [1,7,8]. However, expression of *UCP1* gene could be also induced in white fat depots of experimental animals by pharmacological compounds that reduce adiposity, e.g. β_3-adrenoreceptor agonists [9–11], nicotine [12], or leptin [13]. Even in adult humans, relatively low levels of the *UCP1* transcript could be detected in various fat depots. In abdominal fat, *UCP1* mRNA levels are negatively correlated with obesity [14]. Accordingly, the expression of *UCP1* gene from a highly fat-specific [15] *aP2* gene promoter in transgenic aP2-*Ucp1* mice [16] resulted in resistance against genetic [16] or dietary [17] obesity. The obesity resistance is induced by transgenic modification of white but not brown fat [3,8,18], and reflects reduction of all fat depots except for gonadal fat [8,16,18]. Ectopic UCP1 induces depression of mitochondrial membrane potential in adipocytes [19], increased energy dissipation [8,18] and depression of *in situ* lipogenesis [20]. The latter mechanism probably reflects insufficient supply of ATP by mitochondrial oxidative phosphorylation [20].

Besides UCP1, efficiency of oxidative phosphorylation in adipocytes may be also controlled by recently discovered UCP1 homologues, i.e. UCP2, UCP3, UCP5 [2,21–23], and even by an adenine nucleotide transporter [24,25]. All these proteins are probably present in mature brown adipocytes, while white adipocytes do not typically contain either UCP1 (see above), or UCP3 [2,26]. However, treatment with β_3-adrenoreceptor agonists is capable of inducing not only UCP1 (see above) but also UCP3 [27] in white fat. In an obesity-prone strain of mice, *UCP2* mRNA levels in white adipose tissue were lower than in mice resistant to diet-induced obesity [28,29] and a similar difference in *UCP2* gene expression was observed in abdominal fat of normal and obese humans [30]. Moreover, a negative correlation between heat production in adipocytes and body fat has been found in humans [31].

Some aspects of the relationships between UCPs in white fat and adiposity remain to be clarified, namely the identification of the adipose cell type involved, and the underlying biochemical mechanisms. The first aspect relates to the occurrence of multilocular cells expressing UCP1

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Abbreviations: aP2, adipocyte lipid binding protein; aP2-*Ucp1* transgenic mouse, mouse with the expression of UCP1 from the fat-specific *aP2* gene promoter; COX IV, subunit IV of mitochondrial cytochrome *c* oxidase; UCPs, mitochondrial uncoupling proteins; NRF-1, nuclear respiratory factor-1.

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that are interspersed in white fat [9,10,32–38]. In large mammals, such as humans, typical brown fat depots do not exist in adults, however, some adipocytes equipped with UCP1 and containing many mitochondria probably remain present in white fat during adulthood [14,36–38]. However, developmental studies on these cells are scarce [37]. The induction of UCP1 in white fat by β_3-adrenoreceptor agonists [9–11], or by cold exposure of animals [32,39–41], occurs in multilocular cells interspersed in white fat depots. Such cells may arise from transdifferentiation of unilocular white adipocytes, or reflect recruitment of brown fat precursor cells [9,10]. The possible role of UCP1 in conversion of unilocular into multilocular cells has not been studied.

Reduction of adiposity by respiratory uncoupling in adipocytes may be limited by mitochondrial oxidative capacity. Importantly, it has been shown *in vitro* [42] that the uncoupling, induced by ectopic UCP1 in HeLa cells, could induce mitochondrial biogenesis and upregulate its co-ordinating factor, the nuclear respiratory factor-1 (NRF-1). In animals treated with β_3-adrenoreceptor agonists [43], the metabolic rate was relatively high and the treatment induced formation of mitochondria in the multilocular cells in white fat depots [10]. Also cold acclimatization induces mitochondrial biogenesis in brown fat, reflecting increased sympathetic stimulation of this tissue [32,40,41,44,45]. These data suggest that respiratory uncoupling in adipocytes is associated with mitochondrial biogenesis. However, possible existence of a causal link between these two processes requires further clarification.

The aim of this work was to characterize further the mechanism by which respiratory uncoupling in white fat reduces adiposity, namely with respect to morphology of adipocytes and mitochondrial biogenesis. It has been investigated whether ectopic UCP1 in white fat of aP2-*Ucp1* mice can induce formation of multilocular cells depending on the age of the animals. The possibility that respiratory uncoupling may activate mitochondrial biogenesis has been also explored both in the transgenic mice and in 3T3-L1 adipocytes differentiated in cell culture.

MATERIALS AND METHODS

Animals and tissues

Control C57BL/6J male mice and their hemizygous aP2-*Ucp1* transgenic littermates were identified by Southern blot analysis [16]. The mice were born and maintained at 20 °C with a 12-h light/dark cycle. After weaning at 4 weeks of age, mice were housed four or five per cage and had free access to a standard chow diet [17] and water. If not specified otherwise, animals were killed at 5 weeks (young mice) or at 7–9 months (adult mice) of age by cervical dislocation. Interscapular brown adipose tissue, subcutaneous dorsolumbar white fat [17], and epididymal fat were used for the experiments. Samples were stored at −70 °C for immunoblotting analysis, and in liquid nitrogen for isolation of total RNA.

Morphological studies

The animals were anaesthetized by intraperitoneal injection of thiopental (80 μL of 5% thiopental/animal) and whole animals were fixed by perfusion with paraformaldehyde (4% solution in 0.1 M phosphate buffer, pH 7.4) through the left ventricle (after the right atrium was opened). After perfusion, the tissues (see above) were dissected and fixed overnight by immersion in the same fixative for light microscopy and immunohistology, and in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 4 h, for ultrastructural study. Tissues for light microscopy and immunohistology were embedded in paraffin blocks. For ultrastructural studies small fragments were postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in an Epon/Araldite (Epon, Multilab Supplies, Fetcham, UK; Araldite, Fluka Chemie AG, Buchs, Switzerland) mixture. Semithin sections (2 μm) were stained with toluidine blue; thin sections were obtained with a Reichert Ultracut E (Reichert, Wien, Austria), stained with lead citrate, and examined in a transmission electron microscope, Philips CM10 (Eindhoven, the Netherlands). Immunohistological demonstration of UCP1 was carried out by the avidin–biotin peroxidase (ABC) method. De-waxed sections (3 μm) were processed through the following incubation steps: (a) 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase; (b) 0.02 M glycine for 10 min; (c) normal rabbit serum 1 : 75 for 20 min to reduce nonspecific background staining; (d) polyclonal sheep antibodies against UCP1 isolated from rat brown adipose tissue, diluted 1 : 8000 in $NaCl/P_i$, overnight at 4 °C; (e) biotinylated rabbit anti-(sheep IgG) Ig 1 : 300 (secondary antibody) for 30 min (Vector Laboratories, Burlingame, CA); (f) ABC complex for 1 h (Vectastain ABC kit, Vector Laboratories); and (g) histochemical visualization of peroxidase using 3′,3′-diaminobenzidine hydrochloride chromogen (Sigma). The specificity of the method was tested by the omission of the primary antibody in the staining, and the use of preimmune serum instead of the first antiserum. Furthermore, tissues known to contain UCP2 and UCP3 (skeletal muscle, white adipose tissue, spleen, and kidney) but not UCP1 were tested. All tissues containing UCP2 and UCP3 showed negative results. The specificity of the anti-UCP1 Ig has been recently confirmed [23]. For immunohistochemical studies, three mice for each type of condition were used.

Morphometry

Morphometric evaluation of subcutaneous white fat of nine control and eight transgenic animals was performed both with light microscopy (semithin sections) and at the ultrastructural level. In case of light microscopy the surface area of about 130–170 cells for each animal was measured by an Image Analyzer KS100 IBAS Kontron (Karl Zeiss Jena, Germany), in order to calculate the diameter of the adipocytes. In the ultrastructural study four to six pictures for each animal (nine control and eight transgenic mice) were taken randomly at a final magnification of 11 300× by a CM10 PHILIPS EM (see above). The images were analysed by the IBAS morphometer in order to measure the lipid-free cytoplasmic surface area, the surface area of the mitochondria (μm^2), mitochondrial density (i.e. number of mitochondria per 100 μm^2 cytoplasmic area) and cristae density [i.e. total cristae length (pm) per mitochondrial surface area, per 100 μm^2 cytoplasmic area].

Evaluation of UCP1 and cytochrome content, protein, and DNA concentration

Crude cell membranes (100 000 ***g***) were prepared from tissue homogenates and used for quantification of the UCP1 antigen by immunoblotting using rabbit anti-(hamster UCP1) serum [46] and a standard consisting of mitochondria isolated from brown fat, as described previously [18]. As a second antibody, ^{125}I-labelled donkey antibody against whole rabbit IgG (Amersham) was used, and radioactivity was evaluated using PhosphorImager SF (Molecular Dynamics). Protein concentration was measured using the bicinchoninic acid procedure [47] and BSA as standard. The membrane fraction was solubilized in the presence of 2% *n*-dodecyl β-D-maltoside (Sigma) and used for evaluation of mitochondrial cytochromes using a pseudo-dual-wavelength spectrophotometry [19]. Tissue DNA was estimated as described previously [8].

Isolation of adipocytes

Adipocytes were isolated from subcutaneous white fat of adult mice according to Rodbell [48]. Modified Krebs-Ringer bicarbonate (KRB) buffer was used, containing 118.5 mM NaCl, 4.8 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.1 mM $MgSO_4{\cdot}7H_2O$, 25 mM $NaHCO_3$, 5 mM glucose and 4% (w/v) bovine serum albumin (fraction V; BSA); pH 7.4. Adipose tissue (1–2 g) was collected from four mice, minced with scissors and digested in 5 mL KRB buffer containing 3 mg·mL^{-1} type II collagenase (C-6885, Sigma) while shaking at 37 °C for 90 min. The tissue was then filtered (250 μm) and floating adipocytes were washed three times in the KRB buffer in the absence of collagenase by centrifuging at 400 ***g*** for 1 min at 20 °C.

Differentiation of 3T3-L1 adipocytes

Cells of 3T3-L1 clonal line were differentiated in cell cultures as described previously [20]. When used for experiments (12–14 days after confluence), cultures contained 50–60% of differentiated adipocytes. Ten hours before use for RNA isolation (see below), a complete change of the medium was performed. 2,4-dinitrophenol (dissolved in 0.1% KOH) was added in some dishes at a 150-μM final concentration.

RNA analysis

Total RNA was isolated from adipose tissue or adipocytes and analysed on Northern blots as described before [8]. Filters (GeneScreen^{TM}; NEN Life Science Products, Boston, MA) were subsequently hybridized with full-length cDNA probes for mouse UCP1 [8], UCP2 [8], human liver subunit IV of mitochondrial cytochrome oxidase (COX IV; ATCC, Rockville, MD), and aP2 [8]. Final hybridization with a ribosomal 18S RNA probe was used to correct for possible intersample variations within individual blots. Radioactivity was evaluated by PHOSPHORIMAGER SF. Total RNA isolated from brown fat of cold acclimatized mice served as a standard. In the case of total RNA isolated from adipocytes, levels of the transcripts for COX IV and for NRF-1, respectively, were evaluated using real time quantitative RT-PCR [20], using the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) and LightCycler-RNA Amplification Kit SYBR Green I (Roche; cat. no. 2015137). Each PCR cycle consisted of 0 s at 94 °C, 8 s at 60 °C, and 20 s at 72 °C. Transcript levels were expressed relative to that of β-actin. Primers used for RT-PCR are specified in Table 1.

Statistical analysis

A two-way analysis of variance (ANOVA) with post hoc multiple comparisons was used as described before [17]. Otherwise, statistical significance was evaluated using Student's *t*-test. The morphometric measurements were evaluated using the Kruskal–Wallis nonparametric test. All comparisons were judged to be significant at $P < 0.05$.

RESULTS

Fat-depot- and age-dependent differences of adipocytes' morphology in white fat

Morphology of adipocytes (Fig. 1) and their UCP1 content (see below) were characterized in semithin sections of subcutaneous white fat and epididymal fat (not shown) of control and transgenic animals during ageing. In both fat depots of all the animal subgroups studied, unilocular adipocytes represented the most abundant cell type. Only in subcutaneous fat of young mice multilocular adipocytes were also detected, and these cells formed a substantial portion of mature adipocytes, with the ratio between multilocular and unilocular adipocytes of about 1 : 4 to 1 : 5 (Fig. 1). No multilocular cells were detected in either subcutaneous fat of adult mice (Fig. 1), or in epididymal fat of both age groups (not shown). Transgene had no effect on the ratio between multilocular and unilocular cells in subcutaneous fat of young animals, neither induced multilocular cells in white fat depots of adult mice [16]. The mean diameter of the unilocular cells present in subcutaneous fat of adult transgenic and control mice were 56 ± 4 μm and 63 ± 5 μm, respectively; the difference was not statistically significant.

Age-related changes in the expression of *UCP1* gene in white fat depots

The expression of UCP1 in subcutaneous and epididymal fat depots of control and transgenic mice of different ages

Table 1. Sequences of PCR primers.

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	GenBank acc. no.
COX IV[a]	AGAAGGCGCTGAAGGAGAAGGA	CCAGCATGCCGAGGGAGTGA	NM_009941
NRF-1	ATGGGCCAATGTCCGCAGTGATGTC	GGTGGCCTCTGATGCTTGCGTCGTCT	AF098077
β-actin	GAACCCTAAGGCCAACCGTGAAAAGAT	ACCGCTCGTTGCCAATAGTGATG	X03765

[a] The primers are specific for the isoform 1 of subunits IV of cytochrome *c* oxidase.

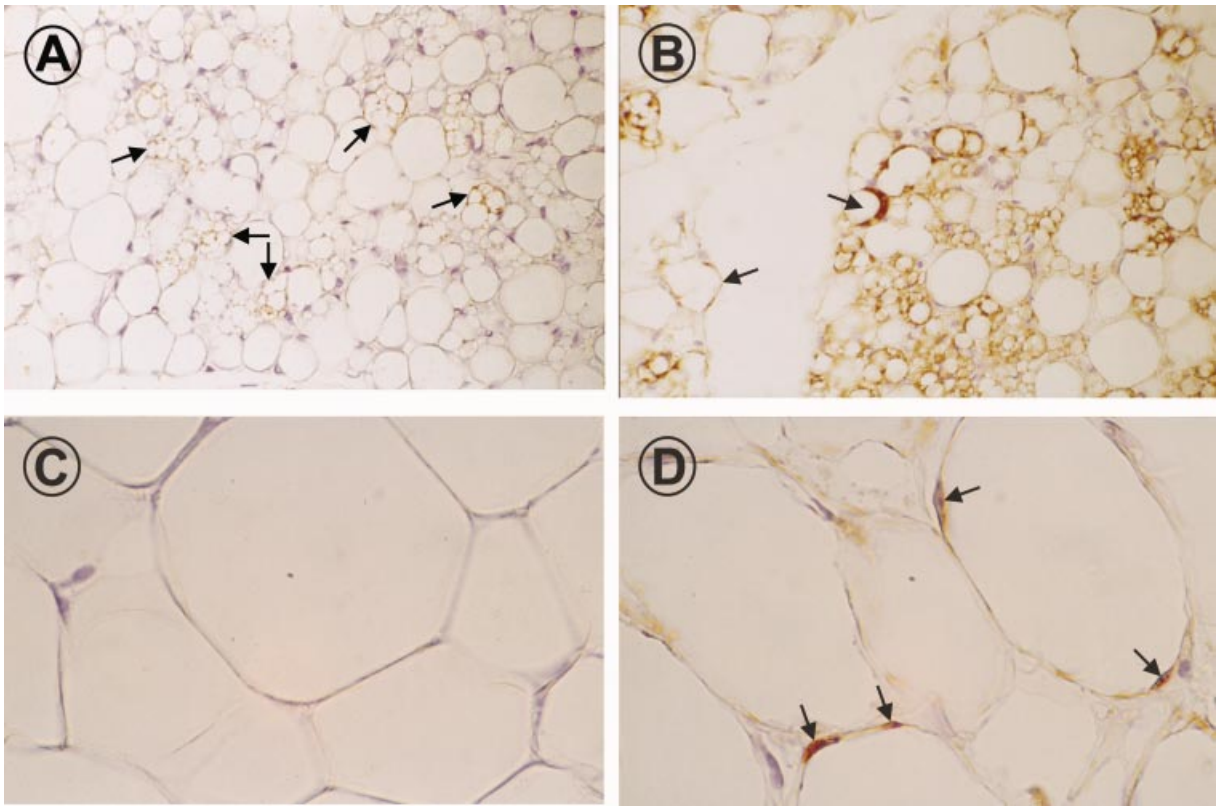

Fig. 1. Immunohistology of subcutaneous white fat from young (A and B) and adult (C and D) control (A and C) and transgenic (B and D) mice. (A) The depot is composed of unilocular and multilocular adipocytes. Only multilocular cells are weakly stained for UCP1 (arrows). (B) The depot is composed of unilocular and multilocular adipocytes. Most of the multilocular and some of the unilocular cells (arrows) are intensely stained. (C) Only unilocular cells are present and they do not contain UCP1 antigen. (D) Only unilocular cells are present and most of them are intensely stained for UCP1; areas of cytoplasmic rim stained for UCP1 are thickened (arrows).

was analysed by immunohistochemistry (Fig. 1) and by biochemical techniques, at both mRNA and protein level (Fig. 2). Immunohistochemistry revealed that multilocular cells found in subcutaneous fat of young mice of both genotypes contained UCP1. The intensity of immunohistochemical staining of brown fat cells was stronger in transgenic than in control mice, in agreement with expression of both UCP1 endogen and aP2-*Ucp1* transgene in these cells [16]. In adult control mice, the unilocular cells in both subcutaneous (Fig. 1) and epididymal fat (not shown) lacked UCP1, while they were UCP1-positive in the transgenic mice. All unilocular adipocytes in transgenic mice contained UCP1. These findings thus confirmed our previous observations in aged transgenic animals [16]. The staining for UCP1 was always restricted to the cytoplasmic area in the vicinity of the plasma membrane, which was thicker in transgenic than in nontransgenic mice. Electron microscopy revealed that these thicker parts of the cytoplasm were rich in mitochondrial content (see below).

Both Northern blot analysis and immunoblotting (Fig. 2) detected UCP1 expression in subcutaneous white fat of 3-week- to 2-month-old-control animals and in both fat depots of transgenic mice, regardless of age of the animal. The levels of *UCP1* mRNA in subcutaneous fat of control mice were by one order of magnitude lower than in transgenic mice, while the corresponding difference in the specific content of UCP1 antigen (expressed relative to adipose tissue membrane protein) was only about twofold. In both fat depots of the transgenic mice, the levels of the *UCP1* transcript and UCP1 antigen declined substantially during ageing (5- to 10-fold), and they were twofold to fourfold higher in the subcutaneous than in epididymal fat. In 3-week- to 2-month-old transgenic mice, levels of *UCP1* transcript in subcutaneous white fat were approximately 30% of those in interscapular brown fat, while in the case of UCP1 antigen this value was about 10% (not shown). No *UCP1* mRNA or antigen could be detected either in white fat depots of adult (4- to 7-month-old) control mice [16,18], or in epididymal fat of younger nontransgenic animals (Fig. 2).

The results document the absence of multilocular adipocytes in the epididymal fat in all the age groups studied, while in subcutaneous fat these multilocular cells completely disappear as the animals age. These results also indicated a higher content of transgenic UCP1 in unilocular adipocytes in subcutaneous than in epididymal fat and suggest that UCP1 is not capable of inducing conversion of a unilocular into a multilocular adipocyte.

UCP1-induced increase of mitochondrial biogenesis

Several independent approaches were used to investigate whether ectopic UCP1 could induce biogenesis of mitochondria in white fat. First, the transcript level of *COX IV*,

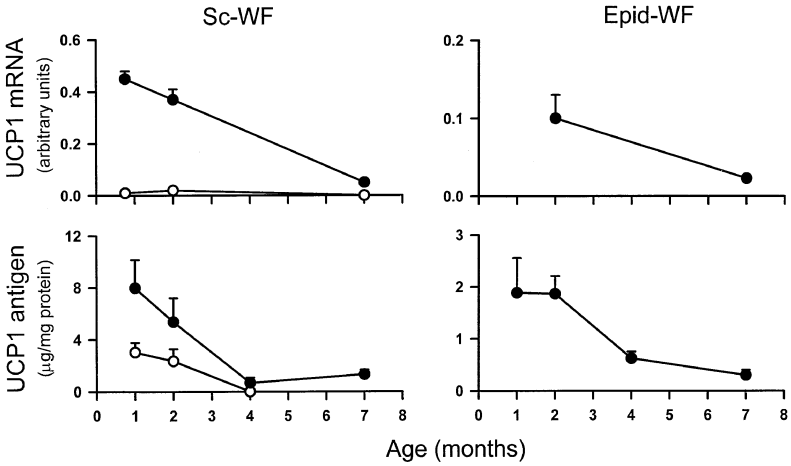

Fig. 2. Quantification of UCP1 expression in white adipose tissue depots during ageing. Analysis was performed in subcutaneous white fat (Sc-WF) and epididymal fat (Epid-WF) of control (open symbols) and transgenic (full symbols) mice of indicated ages ($n = 6$–8). Values are means ± SE. (Top) Results of Northern blot analysis of *UCP1* transcript (1.4 kb). Analysis was not performed in epididymal fat of 3-week-old mice, due to the relatively low amount of the tissue (19 ± 6.0 and 19 ± 4.3 mg in control and transgenic mice, respectively), as compared with subcutaneous white fat (54 ± 6.5 and 49 ± 6.7 mg, respectively). In control mice, the *UCP1* transcript could be detected only in subcutaneous white fat of 3-week- and 2-month-old mice (0.010 ± 0.005 and 0.020 ± 0.010 arbitrary units of *UCP1* transcript, respectively). In 7-month-old transgenic mice, the values were 0.05 ± 0.01 and 0.023 ± 0.001 arbitrary units of *UCP1* transcript in subcutaneous and epididymal white fat, respectively. Evaluation of the aP2 transcripts (0.65 kb) in adult control mice (not shown) indicated significantly higher levels in interscapular brown fat (0.78 ± 0.08 arbitrary units) than in white fat (0.19 ± 0.06, and 0.214 ± 0.02 arbitrary units, in subcutaneous and epididymal fat, respectively). (Bottom) Results of immunoblotting experiments with membrane fractions isolated from fat depots. In control mice, UCP1 could be detected in subcutaneous white fat of 1- and 2-month-old mice. The content of UCP1 in subcutaneous white fat and epididymal fat of 7-month-old transgenic mice was 1.31 ± 0.36 and 0.30 ± 0.1 μg UCP1 per mg membrane protein, respectively. All the differences between genotypes were significant.

a nuclear gene for one of the subunits of mitochondrial cytochrome *c* oxidase, was evaluated in total RNA isolated from subcutaneous and epididymal fat during ageing in mice (Fig. 3). Except for a decrease of *COX IV* mRNA in subcutaneous fat between the first and second month of age, the level of the transcript did not change significantly during ageing in either genotypes. However, as indicated by ANOVA, there was a main effect of genotype in both depots, with transgenic animals showing higher levels of the transcripts. Within different ages and depots, most differences (over 1.5-fold; Fig. 3) were statistically significant. Interestingly, also the levels of the transcript for UCP2 were higher in transgenic than in control mice (Fig. 3). With both, COX IV and UCP2, the highest differences (up to threefold) were observed in epididymal fat. It is known that composition of subcutaneous white fat is quite heterogenous and mature adipocytes represent less than 50% of all cells contained in this fat depot [45]. Therefore, gene expression was also characterized in mature adipocyte fractions isolated from subcutaneous fat of adult mice. The upregulation of both *COX IV* (Table 2) and *UCP2* (not shown) genes by UCP1 was confirmed. A possible effect [42] of the transgene on *NRF-1* mRNA levels was also tested but no significant difference between the adipocytes isolated from control and transgenic mice could be observed (Table 2).

Further experiments were focused only on subcutaneous fat, as the size of this fat depot but not of the epididymal fat

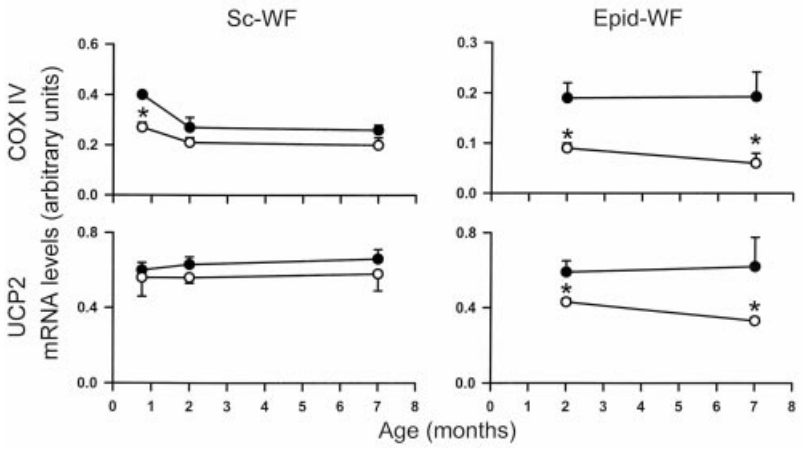

Fig. 3. Quantification of mRNA for mitochondrial markers in white adipose tissue depots during ageing. Analysis of the transcripts for COX IV (0.9 kb), and UCP2 (1.7 kb) was performed using Northern blots in control and transgenic mice. For details and symbols, see Fig. 2. There was a main effect of genotype (ANOVA) within each fat depot and type of transcript. Asterisks indicate significant differences between genotypes within the same age group.

Table 2. Quantification of gene expression in adipocytes. Levels of the transcripts were quantified by real time RT-PCR in adipocytes isolated from subcutaneous white fat of 7-month-old control (+/+) and transgenic (tg/+) mice and from 3T3-L1 adipocytes differentiated in cell cultures. 3T3-L1 adipocytes were incubated for 10 h in a cell culture dish with or without 150 μM 2,4-dinitrophenol before RNA isolation. Values are means ± SE ($n = 6$).

	mRNA level (arbitrary unit)			
	Isolated adipocytes		3T3-L1 cells	
Transcript	+/+	tg/+	Control	2,4-Dinitrophenol
COX IV	0.66 ± 0.08	0.95 ± 0.15*	0.30 ± 0.05	0.40 ± 0.05*
NRF1	0.016 ± 0.005	0.010 ± 0.004	$3.8 \times 10^{-3} \pm 3.6 \times 10^{-7}$	$6.7 \times 10^{-3} \pm 8.7 \times 10^{-7}$*

* $P < 0.05$.

was reduced by the transgene in adult mice [16,17]. The content of mitochondrial cytochromes *b*, and $a + a_3$, respectively, was established in subcutaneous white fat of young and adult mice (Fig. 4). A highly sensitive quantification of absolute amounts of the cytochromes was performed using a pseudo-dual-wavelength spectrophotometry [19]. While cytochrome *b* is contained in the bc_1 complex, cytochromes $a + a_3$ are integral parts of the cytochrome *c* oxidase in the inner mitochondrial membrane. When the content of the cytochromes was expressed relative to the mass of tissue, there was a main effect (ANOVA) of age on cytochrome *b* content, and a main effect (ANOVA) of the genotype; a higher content of cytochromes was present in young and/or transgenic mice. Within the same age, the only statistically significant difference was found with cytochrome *b* content in young mice (1.7-fold difference between genotypes; see Fig. 4). Similar results were obtained when the values were expressed relative to tissue DNA (not shown).

Mitochondrial morphology was characterized by transmission electron microscopy in subcutaneous white fat of adult animals (Fig. 5), where only unilocular adipocytes were present in both genotypes (Fig. 1). In control mice (Fig. 5A–C), the peripheral rim of adipocytes was always thin with a few 'white-type' mitochondria. These mitochondria were elongated and their cristae were randomly oriented. The presence of ectopic UCP1 in transgenic mice (Figs 5D–F) was associated with increased size of mitochondria contained in a thick periplasmic rim of the adipocyte. Mitochondria were mostly oval or round, and the number of cristae per mitochondrion was relatively high. Some cristae were regularly oriented. Thus, most of the mitochondria in the transgenic mice showed an intermediate morphology between that found in white and brown adipocyte [45]. This suggests an activation of mitochondrial metabolism and induction of mitochondrial biogenesis in white fat of transgenic mice. Changes in the ultrastructural appearance were substantiated further by a morphometric analysis (Fig. 6). Mean surface area of mitochondria, mitochondrial density in lipid-free cytoplasmic area, and density of cristae in mitochondria were bigger in transgenic than in control mice. The differences were 1.48-, 1.53-, and 1.22-fold, respectively, and they were statistically significant (see legend to Fig. 6). Calculations based on the morphometric data indicated that 20.3% of the cytoplasmic area of unilocular white adipocytes in transgenic animals was occupied by mitochondria, as compared with only 9.6% in control animals.

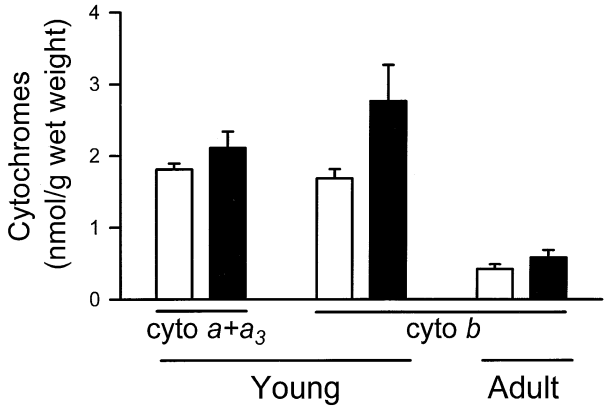

Fig. 4. Content of mitochondrial cytochromes in subcutaneous white fat during ageing. Cytochromes $a + a_3$, and cytochrome *b*, were quantified in control (open bars) and transgenic (solid bars) mice of indicated age group ($n = 6$–7). Values are means ± SE. In the adult animals, the content of cytochromes $a + a_3$ could not be measured due to the limited sensitivity of the method [19]. See text for details.

Finally, in order to confirm that respiratory uncoupling in adipocytes may stimulate mitochondrial biogenesis, 3T3-L1 adipocytes differentiated in cell culture were used (Table 2). Some adipocytes were incubated with 2,4-dinitrophenol that was added to cell culture medium at a final 150 μM concentration. Previously, under similar conditions, a near maximal stimulation of fatty acid oxidation by 2,4-dinitrophenol was observed [20]. In the present experiments, 2,4-dinitrophenol induced a significant increase of the levels of transcripts for both *COX IV* and *NRF-1*.

DISCUSSION

It was found that ectopic expression of UCP1 in white fat depots of aP2-*Ucp1* mice occurs in both forms of mature adipocytes, in multilocular and in unilocular cells. The multilocular adipocytes could be detected only in subcutaneous white fat of young but not adult mice, and they were absent from epididymal fat, regardless of either the age of the animals, or the genotype. Therefore, the results document further that the resistance against obesity brought by ectopic UCP1 in white fat of adult mice [16–18] reflects respiratory uncoupling [19] in unilocular white adipocytes [16]. A higher content of UCP1 in subcutaneous white fat compared with epididymal fat of the transgenic mice helps

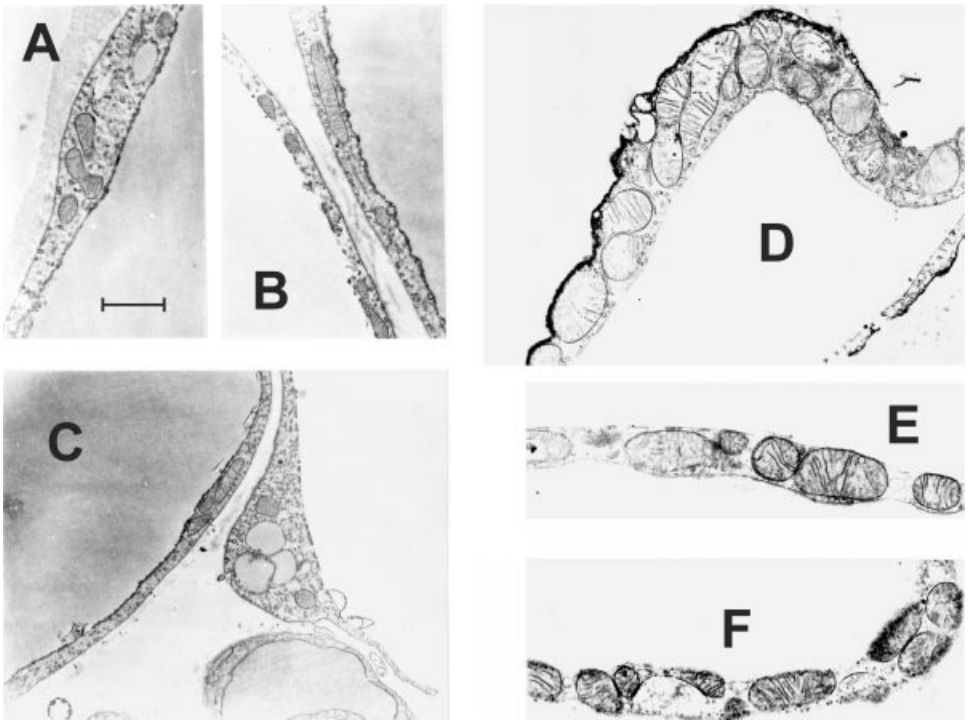

Fig. 5. Transmission electron microscopy of subcutaneous white fat in adult mice. Parts of unilocular adipocytes containing cytoplasmic compartment with mitochondria are shown (bar corresponds to 1 μm). (A–C) Control mice; (D–F) transgenic mice.

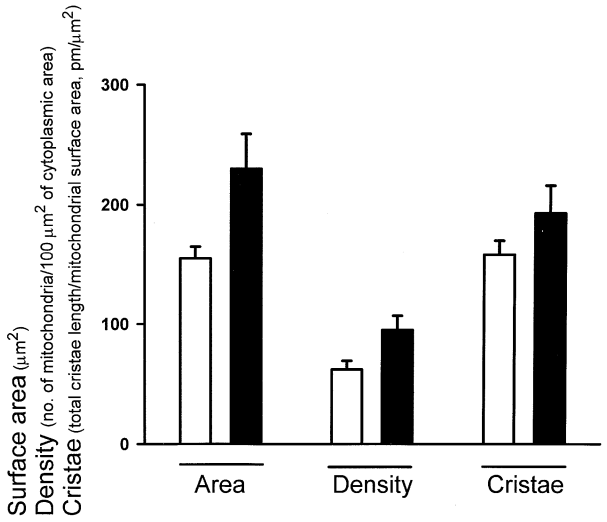

Fig. 6. Mitochondrial morphometry in subcutaneous white fat of adult mice. Morphometric analysis of surface area of the mitochondria, mitochondrial density, and cristae density was performed in control (empty bars) and transgenic (solid bars) mice. Values are means ± SE. All the differences between genotypes were statistically significant ($P = 0.023$, $P = 0.026$, and $P = 0.008$ in the case of the mitochondrial area, mitochondrial density and cristae density, respectively).

to explain the lack of the effect of the transgene on the size of the latter depot [16,17]; this is also associated with the differential effect of the transgene on *in situ* fatty acid synthesis in the two fat depots [20]. The results are in agreement with the hypothesis that induction of endogenous UCP1 acts locally, in concert with adrenergic stimulation [9], to reduce to a greater extent the adiposity of fat depots with high induction of UCP1 than in depots with low induction.

During mammalian ontogeny, recruitment of brown adipose tissue precedes the first appearance of white fat, and the timing of these events during perinatal development varies in different species [49]. Mice belong to a group of the altricial species, with the recruitment of brown fat during late period of the fetal development [46,49,50]. This study shows a dramatic decrease of the content of multilocular adipocytes expressing the *UCP1* gene in subcutaneous white fat depot during ageing in mice. Also UCP1 expression in numerous fat depots of some other species (e.g. bovine [37] and human [51]) is restricted to early stages of development. Therefore, the disappearance of UCP1-producing cells from subcutaneous white fat of mice during ageing reflects a general trend for a localization of UCP1-based thermogenesis into a limited number of anatomical sites in adult animals.

It has been suggested that white adipocyte precursors might belong to brown fat lineage [9]. Inversely, most multilocular cells in white adipose tissue of rats treated with β_3-adrenergic agonists originated from unilocular adipocytes and contained UCP3, while only a small fraction of novel multilocular adipocytes contained UCP1 [10]. As reported in this study, the expression of functional UCP1 in unilocular adipocytes of animals between 5 weeks and 9 months of age was not accompanied by the conversion of these cells into multilocular adipocytes. After prolonged (over 1 week) stimulation with β_3-adrenergic agonists, the number of multilocular adipocytes containing UCP1 in rat white fat is still increasing, without further changes of the ratio between unilocular and multilocular cells (Zingaretti, M. C., Ceresj, E.,

Barbatelli, G. & Cinti, S., unpublished observation). All these experiments suggest that the expression of UCP1 (or UCP3) in unilocular adipocytes, in the absence of a contribution by other controlling factor(s), cannot convert unilocular into multilocular adipocytes. This is in agreement with the experiments on the emergence of brown adipocytes in white fat depots of mice, indicating involvement of at least four different genes [9].

In contrast with the inability of UCP1 to induce multilocular cells in white fat, the morphology of mitochondria and the mitochondrial content of the unilocular cells were affected by the transgene. 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. Thus, the cytoplasmic area occupied by mitochondria was about twofold larger in the adipocytes of transgenic than control mice. The results of the morphometric analysis indicated induction of mitochondrial biogenesis by ectopic UCP1 in the unilocular adipocytes.

The stimulatory effect of UCP1 on mitochondrial content and biogenesis was also supported by differences in the level of the transcripts for COX IV, in both whole adipose tissue and isolated adipocytes, as well as by differences in the content of mitochondrial cytochromes between two genotypes. That UCP2 was upregulated in aP2-*Ucp1* mice was somehow surprising and suggested that UCP1 and UCP2 function differently in adipocytes. This supports the idea that both UCP2 and UCP3 are linked to fatty acid oxidation [53] that is elevated by respiratory uncoupling in adipocytes [19]. It is not clear why the *COX IV* and *UCP2* transcript levels in both white fat depots of transgenic mice change very little with age whereas the UCP1 antigen content strongly decreases during the same time. Nevertheless, all the approaches indicated a moderate induction of mitochondrial biogenesis by ectopic UCP1 in unilocular adipocytes. The resulting increase of mitochondrial content was evidently smaller than that induced in multilocular adipocytes by β_3-adrenoreceptor agonists [10,44], or due to adrenergically mediated stimulation of mitochondrial biogenesis that occur in cold acclimatized animals [32,39–41]. The relatively high potency of the adrenergic stimulation could be explained by the complex effect on gene expression in adipocytes. It may be also speculated that the effect of adrenergic system on mitochondrial biogenesis represents a compensation for decreased efficiency of energy conversion in adipocytes with upregulated *UCP1* gene expression.

It has been found by Zhou *et al.* [13] that adenovirus-mediated hyperleptinemia in rats depletes adipocyte fat while upregulating UCP1, UCP2, and genes for enzymes of fatty acid oxidation. On the other hand, genes for lipogenic enzymes, aP2, and the transcription factor PPARγ were downregulated. To achieve such a transformation of adipocytes may be useful for treatment of obesity [13]. Results of our present and the previous [20] study on white fat of adult mice suggest that UCP1 alone could initiate the 'transdifferentiation' program, including an increased expression of the genes controlling oxidative capacity (COX), as well as that of UCP2, and depression of genes engaged in fatty acid synthesis.

The molecular mechanism for the induction in mitochondrial biogenesis by ectopic UCP1 in HeLa cells was shown to involve up-regulation of NRF-1 [42]. In our experiments, an increase of *NRF-1* mRNA level was detected in 3T3-L1 adipocytes incubated with 2,4-dinitrophenol but not in adipocytes isolated from white fat of transgenic compared to control mice. Therefore, NRF-1 may function as a critical component of the energy-sensing mechanism that co-ordinates expression of mitochondrial genes in adipocytes. However, stimulation of NRF-1 expression in mice may be only transient and can already have taken place before the experiments are carried out.

The levels of *UCP1* transcript in white fat depots of adult transgenic mice were expected to reflect the activity of *aP2* gene promoter that is contained in the aP2-*Ucp1* transgene. However, in both subcutaneous and epididymal white fat of control adult mice, the *aP2* gene transcript levels were quite similar, and they were about fourfold lower than in their interscapular brown fat (see legend to Fig. 2). This suggests a differential postranscriptional control of the transgene expression in various white fat depots, resulting in higher UCP1 content in subcutaneous than in epididymal fat. Differential post-transcriptional control of the endogenous *UCP1* gene and the transgene, respectively, may also explain why the difference in *UCP1* mRNA levels between transgenic and control mice is much higher than that in UCP1 antigen levels (see Fig. 2). Our results showed the profound fat-depot- and age-dependent differences in transgene expression that may be relevant for other studies, where the *aP2* promoter is used to direct the expression of various genes into adipose tissue in mice (see also patent no. US5476926).

In conclusion, our results indicate that respiratory uncoupling *per se* is capable of inducing mitochondrial biogenesis *in vivo*. They also support the hypothesis that respiratory uncoupling in unilocular adipocytes of white fat depots may reduce adiposity and prevent the development of obesity.

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Publication D

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Synergistic induction of lipid catabolism and anti-inflammatory lipids in the white fat of dietary obese mice in response to calorie restriction and n-3 fatty acids

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ARTICLE

Synergistic induction of lipid catabolism and anti-inflammatory lipids in white fat of dietary obese mice in response to calorie restriction and *n*-3 fatty acids

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Abstract
Aims/hypothesis Calorie restriction is an essential component in the treatment of obesity and associated diseases. Long-chain *n*-3 polyunsaturated fatty acids (LC *n*-3 PUFA) act as natural hypolipidaemics, reduce the risk of cardiovascular disease and could prevent the development of obesity and insulin resistance. We aimed to characterise the effectiveness and underlying mechanisms of the combination treatment with LC *n*-3 PUFA and 10% calorie restriction in the prevention of obesity and associated disorders in mice.
Methods Male mice (C57BL/6J) were habituated to a corn-oil-based high-fat diet (cHF) for 2 weeks and then randomly assigned to various dietary treatments for 5 weeks or 15 weeks: (1) cHF, ad libitum; (2) cHF with LC *n*-3 PUFA concentrate replacing 15% (wt/wt) of dietary lipids (cHF+F), ad libitum; (3) cHF with calorie restriction (CR; cHF+CR); and (4) cHF+F+CR. Mice fed a chow diet were also studied.
Results We show that white adipose tissue plays an active role in the amelioration of obesity and the improvement of glucose homeostasis by combining LC *n*-3 PUFA intake and calorie restriction in cHF-fed mice. Specifically in the epididymal fat in the abdomen, but not in other fat depots, synergistic induction of mitochondrial oxidative capacity and lipid catabolism was observed, resulting in increased oxidation of metabolic fuels in the absence of mitochondrial uncoupling, while low-grade inflammation was suppressed, reflecting changes in tissue levels of anti-inflammatory lipid mediators, namely 15-deoxy-$\Delta^{12,15}$-prostaglandin J_2 and protectin D1.
Conclusions/interpretation White adipose tissue metabolism linked to its inflammatory status in obesity could be modulated by combination treatment using calorie restriction and dietary LC *n*-3 PUFA to improve therapeutic strategies for metabolic syndrome.

Keywords 15-Deoxy-$\Delta^{12,15}$-prostaglandin J_2 · DHA · EPA · Fish oil · Metabolic syndrome · White adipose tissue

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Abbreviations

15d-PGJ_2	15-Deoxy-$\Delta^{12,15}$-prostaglandin J_2
AA	Arachidonic acid
AMPK	AMP-activated protein kinase
cHF	Corn-oil-based high fat diet
cHF+F	cHF diet supplemented with LC *n*-3 PUFA concentrate (15% [wt/wt] of dietary lipids)
CLS	Crown-like structure
COX	Cyclooxygenase
CyOX	Cytochrome *c* oxidase
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FASTED	Fasted state, specifically defined
FCCP	Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone
LC *n*-3	Long-chain *n*-3
LOX	Lipoxygenase
NF-κB	Nuclear factor κ light-chain enhancer of activated B cells
MAC-2	β-Galactoside-binding lectin
PD1	Protectin D1
PGC-1α	PPARγ coactivator 1α
PLS-DA	Partial least squares-discriminant analysis
PPARγ	Peroxisome proliferator-activated receptor γ
PUFA	Polyunsaturated fatty acids
RE-FED	Re-fed state, specifically defined
SIRT1	Sirtuin 1 (silent mating type information regulation 2, homologue) 1
TG	Triacylglycerol
UCP1	Uncoupling protein 1
WAT	White adipose tissue

Introduction

Intrinsic metabolic properties and secretory functions of white adipose tissue (WAT) have a major impact on the development of chronic morbidities associated with obesity, including type 2 diabetes, dyslipidaemia and hypertension. When (post)prandial plasma levels of NEFA and triacylglycerol (TG) exceed the storage capacity of WAT [1], other tissues including the liver and muscle become overloaded with lipids, which results in insulin resistance, the key event in the pathophysiology of metabolic syndrome [2]. The important role of WAT in energy homeostasis is underscored by the findings that WAT is one of the key organs affected by calorie restriction, the most effective strategy to prolong a healthy life in several species [3], and by the fact that accumulation of body fat can be reduced through upregulation of lipid catabolism in WAT [4–10]. The metabolism and secretory functions of WAT are also modulated by long-chain (LC) *n*-3 polyunsaturated fatty acids (PUFA), namely eicosapentaenoic acid (EPA; 20:5 *n*-3) and docosahexaenoic acid (DHA; 22:6 *n*-3), which exert numerous beneficial effects on health, including improvements in lipid metabolism and prevention of obesity and diabetes [11], while decreasing the rate of fatal coronary heart disease in diabetic patients who have had a myocardial infarction [12]. The metabolic changes induced by both calorie restriction and LC *n*-3 PUFA include induction of mitochondrial biogenesis and lipid catabolism in WAT [3, 13].

Importantly, LC *n*-3 PUFA also decrease inflammation [14], including obesity-associated low-grade inflammation of WAT [15, 16], characterised by altered secretion patterns of adipokines, which contributes to insulin resistance [1]. The anti-inflammatory effects of LC *n*-3 PUFA probably depend on the formation of their active metabolites. These lipid mediators originate from either targeted enzymatic synthesis, such as resolvins and protectins [17, 18], or from non-enzymatic oxidation reactions [19, 20]. They can act as ligands for surface receptors or can interact with signalling proteins including the transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and nuclear factor κ light-chain enhancer of activated B cells (NF-κB) [19]. Notably, resolvins and protectins mediate the anti-inflammatory and protective actions of LC *n*-3 PUFA in obesity-induced insulin resistance and hepatic steatosis [17, 21]. As LC *n*-3 PUFA and LC *n*-6 PUFA compete for common enzymatic pathways, a relatively small increase in the LC *n*-3 PUFA intake usually slows down synthesis of pro-inflammatory metabolites derived from arachidonic acid (AA; 20:4 *n*-6) [17, 18]. Given the complexity of factors contributing to the development of metabolic syndrome, its prevention and treatment requires strategies combining several approaches. Clinical studies suggest [22–24] that combining calorie restriction and LC *n*-3 PUFA intake may be helpful. As both treatments could promote fatty acid (FA) oxidation in WAT (see above), we have endeavoured in this study to establish whether lipid catabolism in WAT could be augmented by calorie restriction combined with LC *n*-3 PUFA intake. Our results in dietary obese mice show additive effects of calorie restriction and LC *n*-3 PUFA in the induction of mitochondrial biogenesis and lipid catabolism, occurring with a surprising specificity in intra-abdominal WAT, which could contribute to the beneficial systemic effects of the combination treatment. The tissue-specificity could be explained by formation of anti-inflammatory lipid mediators derived from both LC *n*-3 and LC *n*-6 PUFA.

Methods

Animal treatment Male mice (C57BL/6J; Jackson Laboratory, ME, USA) were weaned onto standard laboratory chow (Chow; extruded R/M-H diet; Ssniff Spezialdiäten, Soest, Germany). Singly caged mice were habituated to a corn-oil-based high-fat diet (cHF; lipid content 35%, wt/wt; [16]) for 2 weeks starting at 2 months of age and then randomly assigned for 5 weeks (four independent experiments) or 15 weeks (one experiment) to various dietary treatments (see electronic supplementary material [ESM] Fig. 1): (1) cHF, ad libitum; (2) cHF supplemented with LC *n*-3 PUFA concentrate (46% wt/wt DHA, 14% wt/wt EPA; product EPAX 1050 TG; EPAX, Alesund, Norway) replacing 15% wt/wt of dietary lipids (cHF+F), ad libitum; (3) cHF with restriction of energy intake—the ration was reduced by 10% wt/wt compared with mice fed ad libitum with the same type of diet (CR; cHF+CR); and (4) cHF+F+CR. When indicated, plasma was collected by using tail bleeds during the FASTED to RE-FED transition (for the detailed definition of FASTED and RE-FED state, see ESM). An OGTT was performed in overnight-fasted mice as in Kuda et al. [16], except that glucose was administered by oral gavage. At the end of the treatment, mice were killed in a random fed state (between 08:00 hours and 10:00 hours).

Experiments were conducted according to the guidelines for the use and care of laboratory animals of the Institute of Physiology.

Tissue lipid content The tissue content of TG was estimated in ethanolic KOH tissue solubilisates [25].

Energy expenditure and metabolic flexibility Energy expenditure and fuel partitioning were evaluated using the indirect calorimetry system INCA (Somedic, Horby, Sweden) at 30°C [26]. Metabolic flexibility was assessed as a maximal induction in RQ ($\dot{V}CO_2/\dot{V}O_2$) values in response to a glucose load (0.45 ml of 50% [wt/vol.] D-glucose) administered by intragastric gavage to overnight (12 h) fasted animals. The induction was calculated from RQ values averaged over 60 min intervals before and after the gavage (ESM Fig. 2).

Physical activity Animal behaviour was recorded by video camera and analysed off-line (see ESM).

Plasma variables Levels of glucose, NEFA, TG, leptin, total adiponectin, IL-6, β-hydroxybutyrate and insulin were determined in plasma [16, 27].

Gene expression Transcript levels were evaluated using quantitative real-time RT-PCR (see ESM).

Ex vivo biochemical analysis FA oxidation was measured using [1-^{14}C]palmitate in fragments of epididymal fat or gastrocnemius muscle or whole soleus muscle [28], in isolated adipocytes [29], and in hepatocytes isolated from mice following the in vivo treatment [30]. The rate of FA synthesis in epididymal fat was measured by incorporation of ^3H_2O into saponifiable FAs [9]. Mitochondrial respiration was evaluated using high-resolution respirometry in digitonin-permeabilised adipocytes [29] using OROBOROS Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria; see Fig. 4c,d legend).

Activity of cytochrome c oxidase and cytochrome b and protein content Crude membrane fraction was used to evaluate activity of cytochrome *c* oxidase (CyOX) spectrophotometrically [10] and to quantify cytochrome *b* using a pseudo-dual-wavelength spectrophotometry [29].

Immunohistochemical analysis Morphometry of adipocytes was performed (5 μm sections) using Lucia IMAGE software (Laboratory Imaging, Prague, Czech Republic). In the case of epididymal fat, sections were processed to detect β-galactoside-binding lectin (MAC-2) and perilipin [16].

Lipidomic analysis In total, 24 lipid molecular species were quantified in epididymal fat and liver extracts using HPLC MS-MS analysis [31] (see ESM).

Statistical analysis All values are presented as means±SEM. Comparisons were judged to be significant at $p \leq 0.05$ (see ESM).

Results

Combination treatment counteracts development of obesity and accumulation of ectopic fat Feeding mice a cHF diet increased their body weight (Fig. 1a). Either cHF+F or cHF+CR treatment partially prevented the cHF-induced obesity, while the combination treatment (cHF+F+CR) provided full protection, as shown by the treatments lasting either 5 weeks (Fig. 1a and Table 1) or 15 weeks (see legend to Fig. 1). As already described [13, 30], food consumption was not affected by dietary LC *n*-3 PUFA (Table 1). Physical activity, evaluated after 3 weeks of the treatment (ESM Fig. 1) as total distance travelled and total duration of moving per 4 h, was similar in the cHF, cHF+F and cHF+CR mice, while it was lower in the cHF+F+CR mice (Table 1). Prevention of dietary obesity by various treatments correlated with reduction of both adiposity and fat cell size in all fat depots analysed at

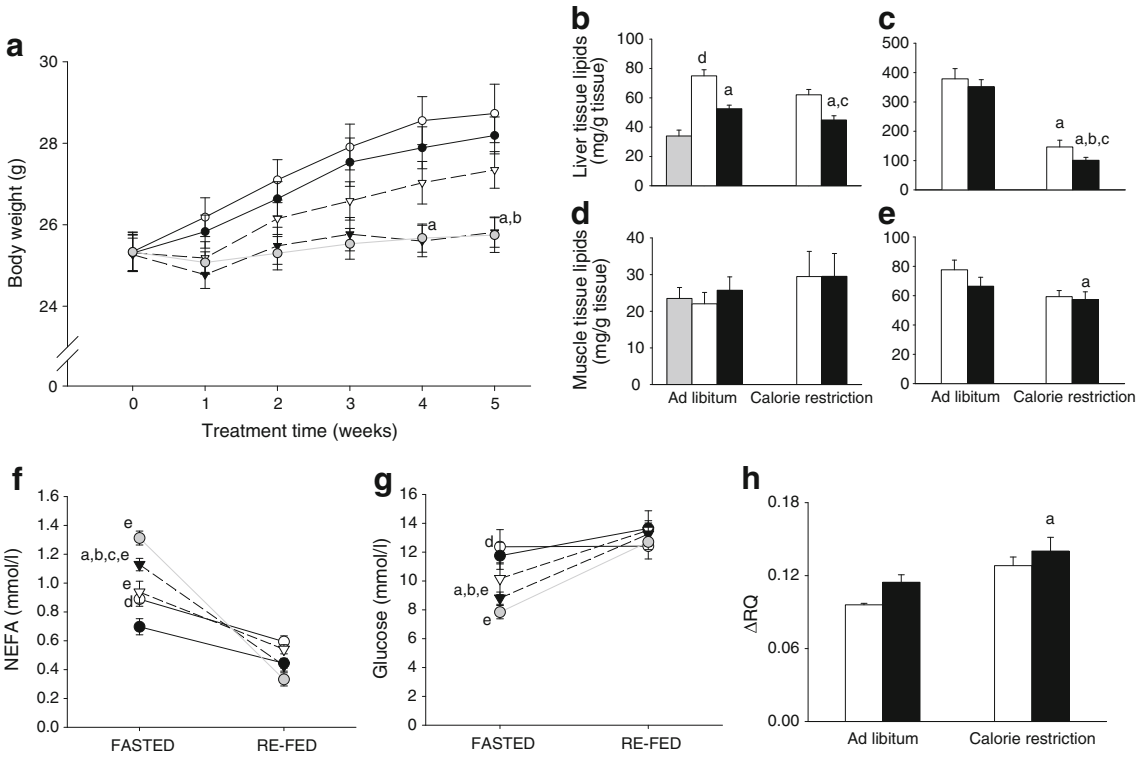

Fig. 1 Prevention of diet-induced obesity, hepatic steatosis, and metabolic inflexibility. **a** Body weight of mice; week 0, start of the differential dietary treatment. Results from a typical experiment (n=10; see also Table 1). White circles, cHF; black circles, cHF+F; white triangles, cHF+CR; black triangles, cHF+F+CR; grey circles, Chow. Five independent short-term (5 weeks) and one long-term (15 weeks) experiments were performed with similar results (not shown). After 15 weeks, body weights in mice fed different diets were as followed: cHF, 42.7±1.2 g; cHF+F, 36.8±1.9 g; cHF+CR, 34.7±2.5 g; cHF+F+CR, 33.1±0.8 g; p<0.05 vs cHF for all the treatments. **b–e** Ectopic lipid accumulation in liver (**b**, **c**) and gastrocnemius muscle (**d**, **e**) after 5 weeks (**b**, **d**) or 15 weeks (**c**, **e**) of the treatment; n=10. Data for the Chow-fed mice are shown only for week 5, but similar values at week 15 should be expected [16]. NEFA (**f**) and (**g**) glucose levels in plasma after 4 weeks of the treatment, measured in either the FASTED or RE-FED state (see ESM; n=10). White circles, cHF; black circles, cHF+F; white triangles, cHF+CR; black triangles, cHF+F+CR; grey circles, Chow. **h** During the fourth week of the treatment, metabolic flexibility was assessed as a maximal induction in RQ values in response to a glucose load administered by intragastric gavage to animals fasted overnight (12 h) (n=9–10). White bars, cHF or cHF+CR diet; black bars, cHF+F or cHF+F+CR. Data from an independent experiment using the Chow-fed mice showed ΔRQ= 0.15±0.01 (n=9). For details, see ESM Fig. 2 and ESM Table 1. Data are means±SEM. [a,b,c]Significant difference (ANOVA) compared with cHF, cHF+F, and cHF+CR, respectively; [d]significant difference (t test) compared with Chow-fed mice [e]significant difference (repeated-measures ANOVA) between the FASTED and RE-FED states

5 weeks (Table 1). Significant differences in both variables were observed between the cHF mice and the cHF+F+CR mice, with the strongest effects elicited in the epididymal fat (Table 1). Feeding of the cHF diet induced accumulation of lipids in the liver and skeletal muscle. In the liver, all the treatments decreased TG accumulation and the effect of LC n-3 PUFA was stronger at 5 weeks (Fig. 1b) than at 15 weeks (Fig. 1c). In response to the longer treatment, the maximal reduction of TG accumulation in skeletal muscle was observed in the case of cHF+F+CR mice (Fig. 1e).

Combination treatment improves lipid and glucose homeostasis and preserves metabolic flexibility After 5 weeks of the treatment and in the ad libitum fed state, plasma TG levels were strongly reduced through either LC n-3 PUFA or calorie restriction alone, and even more with the combination treatment (Table 1). This hypolipidaemic effect was negatively correlated with the effect of the treatments on plasma β-hydroxybutyrate, the marker of lipid catabolism. Glucose levels in plasma were similar in all the groups; however, insulin levels were decreased by cHF+CR treatment and even more by the cHF+F+CR treatment (Table 1). In this respect, cHF+F exerted a smaller but still significant effect. In the fasted state (Fig. 1g), the plasma concentration of glucose was lowered in response to calorie restriction, independent of dietary LC n-3 PUFA, with a trend for the lowest value in the cHF+F+CR mice. Levels of adiponectin were increased by cHF+F to a similar extent in mice with free access to food and in the mice with restricted energy intake (Table 1), also in agreement with our previous findings [27]. As expected from the effects of treatments on adiposity, leptin levels tended to be reduced by cHF+F and were significantly lowered by calorie

Table 1 Body weight gain, food consumption, physical activity, organ weights, size of adipocytes and plasma variables in mice

Variable	cHF	cHF+F	cHF+CR	cHF+F+CR
Body weight gain (g)	3.7±0.5	2.9±0.6	2.1±0.3[a]	0.8±0.2[a,b,c]
Food consumption (kJ/day per animal)	74.6±0.6	73.7±1.8	67.1±0.5[a,b]	66.3±1.6[a,b]
Physical activity				
Total distance moved (m)	236±22	231±65	200±19	152±19[a]
Moving time (s)	3668±432	3424±775	2974±359	2204±362[a]
Epididymal fat				
Weight (mg)	624±74	566±90	408±58	262±26[a,b]
Adipocyte area (μm^2)	2395±147	1920±160	1851±300	1690±152[a]
Mesenteric fat				
Weight (mg)	298±31	277±19	268±22	205±16[a,b]
Adipocyte area (μm^2)	1217±84	1136±136	1315±153	997±108[a,c]
Subcutaneous fat				
Weight (mg)	284±26	269±21	233±20	208±10[a]
Adipocyte area (μm^2)	1356±60	1055±69[a]	1188±2	1069±124[a]
Interscapular brown fat				
Weight (mg)	173±10	121±5[a]	119±4.8[a]	76±5[a,b,c]
Liver				
Weight (mg)	1148±59	1233±18	1134±26	1009±36[b]
Plasma				
TG (mmol/l)	1.24±0.11	0.62±0.06[a]	0.65±0.06[a]	0.35±0.05[a,b,c]
β-Hydroxybutyrate (mmol/l)	0.09±0.01	0.13±0.02[a]	0.20±0.01[a,b]	0.28±0.02[a,b,c]
Glucose (mmol/l)	17.2±0.5	17.0±0.4	17.4±0.5	15.5±0.8
Insulin (pmol/l)	300±35	219±37[a]	57±5[a,b]	35±9[a,b,c]
Adiponectin (μg/ml)	8.9±0.3	12.1±0.5[a]	9.3±0.2[b]	11.1±0.5[a,c]
Leptin (ng/ml)	77.2±7.6	66.2±8.3[a]	15.5±1.7[a,b]	5.3±0.7[a,b,c]

All the variables were evaluated after 5 weeks of the treatment, except for physical activity, which was evaluated during week 4 and is represented by the total distance moved and moving time during both time intervals of the measurement (2 h during light phase and 2 h during dark phase, see Methods). In the case of WAT, subcutaneous dorsolumbar, epididymal and mesenteric depots were collected. Tissue dissection and plasma collection were performed in random-fed state (between 08:00 hours and 10:00 hours)

Data are means±SEM; $n=10$

[a,b,c] Significant differences (ANOVA) compared with cHF, cHF+F, and cHF+CR, respectively

restriction, with the cHF+F+CR mice showing the lowest leptin levels (Table 1).

To characterise whole-body metabolic flexibility, which is associated with insulin sensitivity [2, 32], the metabolic response to the FASTED/RE-FED transition was also analysed by assessing plasma levels of NEFA and glucose after 4 weeks of the treatment (Fig. 1f,g and ESM Fig. 1). Only in the FASTED, and not in the RE-FED state, did the metabolite levels differ between groups. Among the mice fed cHF-based diets, the cHF+F+CR mice showed the highest NEFA and the lowest glucose levels, while exhibiting the largest differences in plasma levels of both metabolites in response to the re-feeding. In fact, the cHF+F+CR treatment normalised the metabolic response when compared with the Chow-fed mice (Fig. 1f,g). In contrast, the cHF mice showed the lowest NEFA and the highest glucose levels in fasted state, with a minimum response to the FASTED/RE-FED transition observed with both metabolites (Fig. 1f,g). Indirect calorimetry was performed after 3 weeks of the treatment (ESM Figs 1 and 2) to characterise metabolic flexibility, as well as whole-body capacity to use carbohydrate and lipid fuels (Fig. 1h). When metabolic flexibility was expressed as maximal induction of RQ in response to the intragastric gavage of glucose, the combination treatment preserved metabolic flexibility better than any of the treatments applied separately. No effect of any of the treatments on energy expenditure, assessed as oxygen consumption ($\dot{V}O_2$)/animal, was observed either before or after the glucose gavage (ESM Fig. 2 and ESM Table 1).

To further characterise changes in glucose homeostasis, fasted plasma was analysed (Fig. 2a,b) and an OGTT was performed (Fig. 2c and ESM Fig. 3) after 3–4 weeks of treatment (ESM Fig. 1). Compared with cHF diet, only the combination treatment significantly decreased fasted insulinaemia and HOMA index (Fig. 2a,b). Specifically in mice subjected to calorie restriction, glucose levels were increased in response to overnight fasting, which preceded the glucose tolerance test (ESM Fig. 3). Feeding cHF diet resulted in glucose intolerance (Fig. 2c), while the deterioration of glucose homeostasis was prevented by the combination treatment with the other treatments tending to exert similar effects. In addition, insulin levels estimated in plasma during the test, 30 min after the glucose gavage, revealed the protective effect of the combination treatment (220±51, 296±37, and 253±41 pmol insulin/l in the Chow-fed, cHF and cHF+F+CR mice, respectively). Ex vivo analysis of insulin-stimulated de novo FA synthesis in

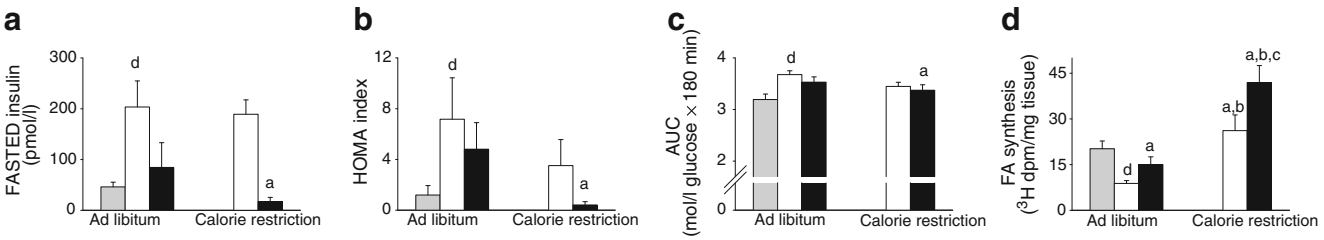

Fig. 2 Improvement of glucose homeostasis. **a** Insulin levels in plasma and **b** HOMA index of insulin resistance after 4 weeks of the treatment, calculated (see ESM) from the insulin levels (see **a**) and glycaemia measured in the fasted state (see Fig. 1g); **c** OGTT after 3 weeks of treatment (0.5 ml 30% [wt/vol.] D-glucose in water; see ESM Fig. 3). The results are expressed as AUC values; **d** ex vivo analysis of de novo FA synthesis in epididymal fat after 5 weeks of the treatment, measured in the presence of insulin (80 μU/ml) and expressed as dpm ^{3}H incorporated to saponifiable FAs/mg tissue. White bars, cHF or cHF+CR; black bars, cHF+F or cHF+F+CR; grey bars, Chow. Data are means±SEM. [a,b,c]Significant difference (ANOVA) compared with cHF, cHF+F, and cHF+CR, respectively; [d]significant difference (*t* test) compared with Chow-fed mice

epididymal fat after 5 weeks of treatment showed an increased rate of FA synthesis due to either LC *n*-3 PUFA or calorie restriction alone, and a synergistic induction in response to the combination treatment (Fig. 2d).

Synergistic induction of genes of mitochondrial biogenesis and lipid catabolism in epididymal fat Based on the previous results showing induction of mitochondria and lipid catabolism by LC *n*-3 PUFA [13] and calorie restriction [3], we investigated gene expression in WAT, interscapular brown fat, liver and muscle (Fig. 3a–e). In epididymal WAT (Fig. 3a), the analysis revealed a strong induction of PPARγ co-activator 1α (*Pgc-1α* [also known as *Ppargc1a*]), peroxisome proliferator-activated receptor α

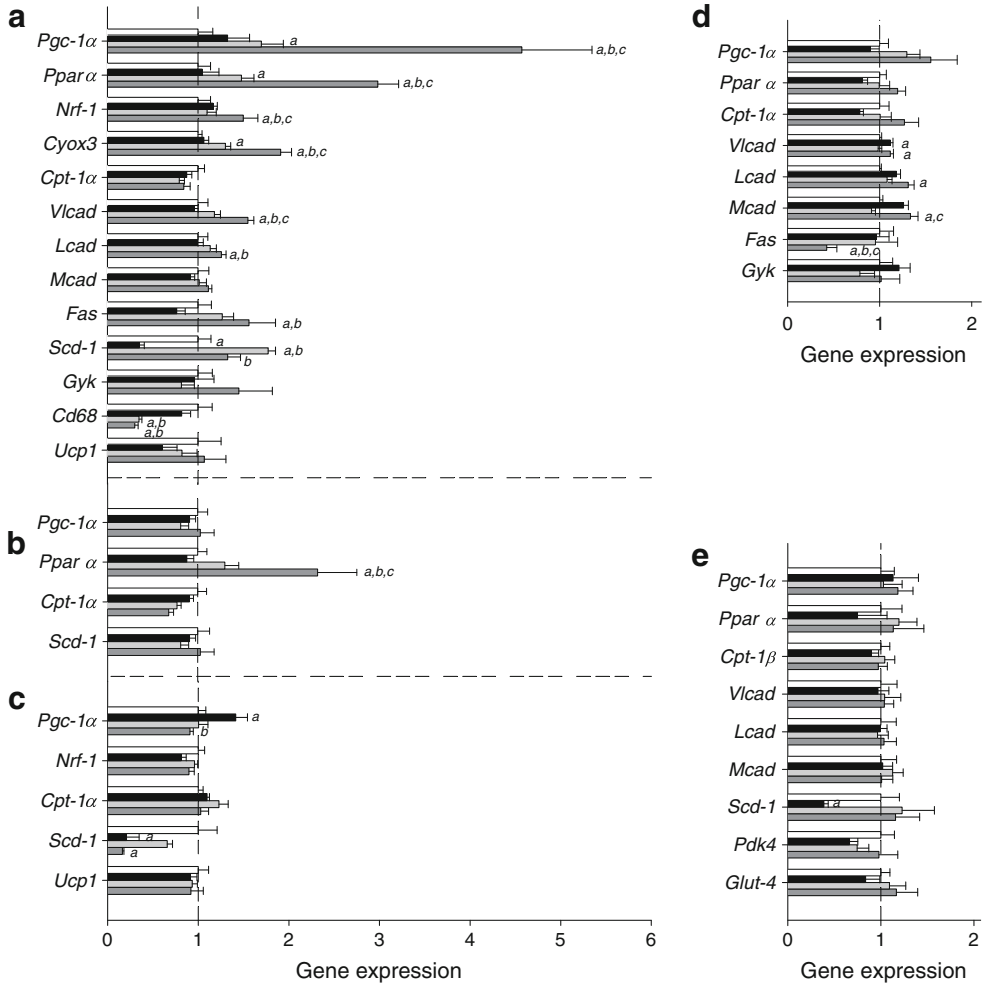

Fig. 3 Specific induction of mitochondrial genes in epididymal fat. Quantitative real-time RT-PCR data showing relative levels of gene expression (cHF=1) in epididymal (**a**) and subcutaneous (**b**) WAT, interscapular brown fat (**c**), liver (**d**), and gastrocnemius muscle (**e**) after 5 weeks of the treatment. Data are means±SEM; $n=10$; white bars, cHF; black bars, cHF+F; light grey bars, cHF+CR; dark grey bars, cHF+F+CR; [a,b,c]Significant difference (ANOVA) compared with cHF, cHF+F and cHF+CR, respectively. An independent experiment showed no difference in either *Pgc-1α* or *Pparα* expression in epididymal fat between the cHF and the Chow-fed mice (the cHF/Chow ratio of the *Pgc-1α* and *Pparα* transcript levels was 1.08±0.05 and 1.04±0.08, respectively; $n=6$). All analyses were performed after 5 weeks of treatment

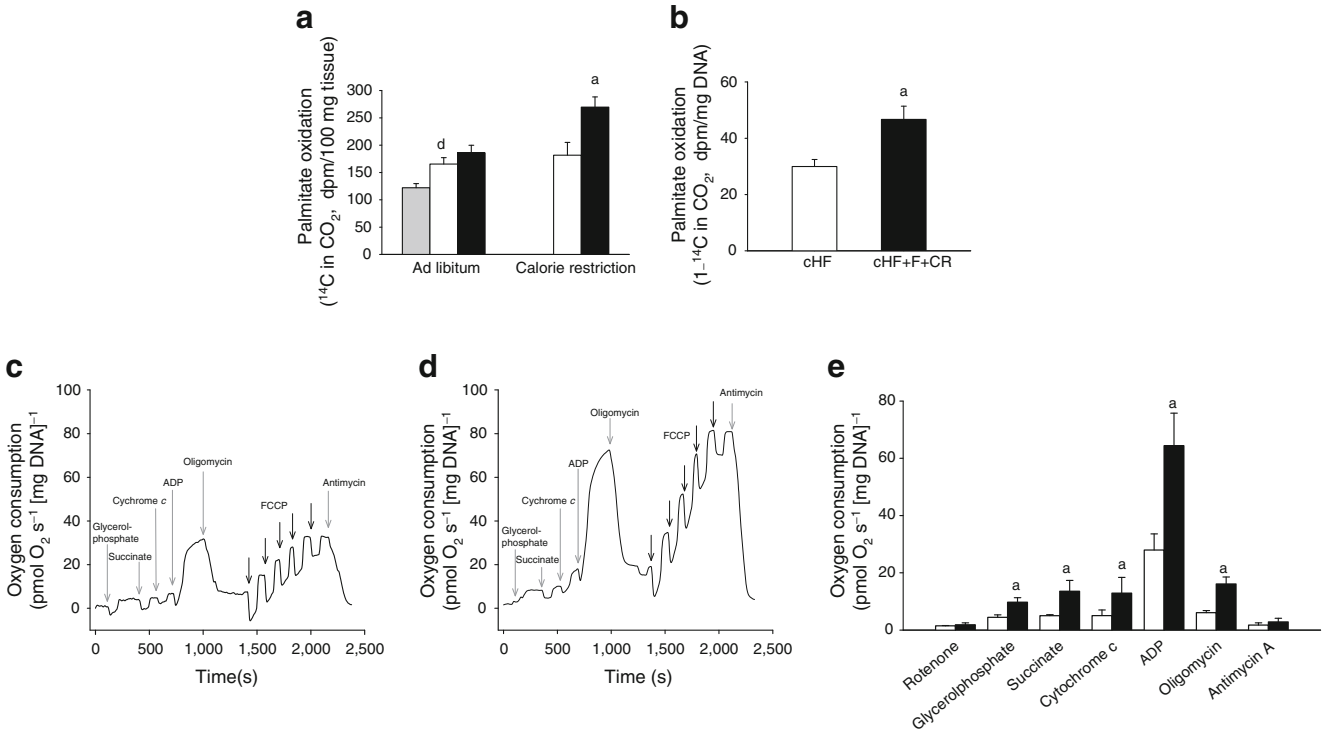

Fig. 4 Induction of fatty acid oxidation and mitochondrial oxidative capacity in epididymal fat. **a** Oxidation of [1- ^{14}C]palmitate into CO_2 by tissue fragments. Data are means±SEM; $n=10$. White bars, cHF or cHF+CR; black bars, cHF+F or cHF+F+CR; grey bars, Chow. [a]Significant difference (ANOVA) compared with cHF; [d]significant difference (*t* test) compared with Chow-fed mice. **b** Oxidation of [1-^{14}C]palmitate into CO_2 by freshly isolated adipocytes. Data are means±SEM; $n=8$. [a]Significant difference (*t* test). **c, d, e** Evaluation of mitochondrial respiratory capacity and oxidative phosphorylation in permeabilised adipocytes isolated from epididymal fat of the cHF and cHF+F+CR mice using multiple substrate-inhibitor titration respirometry. **c, d** Representative oxygen flux curves (**c** cHF; **d** cHF+F+CR). Adipocyte added in suspension (0.2 ml) to 2 ml incubation medium (10 mmol/l Tris-HCl, 80 mmol/l KCl, 3 mmol/l $MgCl_2$, 5 mmol/l KH_2PO_4, 1 mmol/l EDTA and 1% wt/wt fatty-acid-free BSA, pH 7.4) were permeabilised with the addition of 3 μl digitonin (10 mg/ml DMSO), mitochondrial complex I was inhibited by 2 μl of 1 mmol/l rotenone, and respiration was stimulated by successive additions of: (1) 20 μl 1 mol/l glycerol 3-phosphate; (2) 20 μl 1 mol/l succinate; and (3) 10 μl 0.3 mol/l ADP (before the ADP addition, mitochondrial integrity was tested by addition of 10 μl of 4 mmol/l reduced cytochrome *c*). Subsequently, ATP synthase was inhibited by 2 μl oligomycin (4 mg/ml), respiration was re-activated by additions of uncoupler of oxidative phosphorylation (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; FCCP; 0.5 μl of 1 mmol/l FCCP at each addition), and finally, respiration was inhibited by 2 μl of 5 mmol/l antimycin A (inhibitor of complex III). **e** Averaged values from respirometry ($n=4$; pooled samples from two animals). White bars, cHF; black bars, cHF+F+CR. Data are means±SEM. [a]Significant difference (paired *t* test) between the groups. All analyses were performed after 5 weeks of treatment

(*Pparα* [also known as *Ppara*]), nuclear respiratory factor 1 (*Nrf1*) and CyOX subunit 3 (*Cyox3* [also known as *Cox3*]) by the combination treatment, suggesting increased biogenesis and oxidative capacity of mitochondria, independent of the type of diet (see legend for Fig. 3). Only *Pparα* expression was upregulated in subcutaneous WAT by the combination treatment, while no significant changes in the expression of the above gene cluster was observed in interscapular brown fat (Fig. 3c), liver (Fig. 3d) or skeletal muscle (Fig. 3e). Concerning the genes of β-oxidation such as very long-chain, long-chain and medium-chain acyl-CoA dehydrogenases (*Vlcad* [also known as *Acadvl*], *Lcad* [also known as *Acadl*] and *Mcad* [also known as *Acadm*], respectively), additive induction of *Vlcad* and *Lcad* was noted in the epididymal WAT (Fig. 3a), and a slight increase in the expression of the β-oxidation genes observed in response to cHF+F or cHF+F+CR treatments in the liver (Fig. 3d) but not in the muscle (Fig. 3e). FA synthase (*Fas*) was upregulated in epididymal WAT of the cHF+F+CR mice (Fig. 3a), but significant downregulation of *Fas* was observed in the liver of these animals (Fig. 3d). No induction of uncoupling protein 1 (*Ucp1*) by any of the treatments either in epididymal fat or in interscapular brown fat was detected (Fig. 3a,c).

Combination treatment induced FA oxidation and mitochondrial oxidative capacity in epididymal fat To confirm the synergistic induction of mitochondrial FA oxidation by LC *n*-3 PUFA and calorie restriction in epididymal fat, biochemical assessment was performed ex vivo after 5 weeks of the dietary treatment. First, [1-^{14}C]palmitate oxidation was measured in fragments of epididymal fat. Feeding the cHF diet stimulated palmitate oxidation compared with Chow feeding, and this activity was further

significantly stimulated only by the combination treatment, documenting a synergism between LC *n*-3 PUFA and calorie restriction in the induction of FA oxidation (Fig. 4a). Accordingly, adipocytes isolated from epididymal fat of the cHF+F+CR mice also exerted a higher DNA-adjusted rate of palmitate oxidation compared with cHF mice (Fig. 4b). This analysis, as well as the analysis of mitochondrial function described below, was restricted to the comparison between the cHF mice and the cHF+F+CR mice, i.e. the two groups supposed to exhibit the largest differences in the metabolic variables.

Second, the activity of CyOX, the terminal component of the mitochondrial respiratory chain, and the content of cytochrome *b* were evaluated in epididymal fat. In the cHF+F+CR mice, the CyOX activity was higher compared with cHF mice (4.5±0.3 vs 3.5±0.4 μmol cytochrome *c* oxidised min^{-1}mg $protein^{-1}$, $p<0.05$), in accordance with the difference in the cytochrome *b* content (926±116 vs 590±34 pmol/mg protein, $p<0.05$).

Third, mitochondrial oxidative capacity was characterised in adipocytes using respirometry and adjusted to DNA content (Fig. 4c–e). Adipocytes from the cHF+F+CR mice exerted an approximately twofold higher rate of ADP-dependent oxygen consumption in the presence of respiratory substrates glycerol-3-phosphate and succinate (state 3 respiration) compared with the cHF mice. This indicates a much higher oxidative capacity of adipose tissue mitochondria in the cHF+F+CR mice. In addition, a similar degree of inhibition of state 4 respiration by oligomycin (Fig. 4c–e), similar respiratory control indexes (state 3/state 4; cHF+F+CR, 5.54±1.13 vs cHF, 5.57±1.15), and a reversal of the inhibitory effect of oligomycin by carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, see Fig. 4c,d) indicated tight coupling between respiration and phosphorylation in the epididymal fat mitochondria of both groups.

To assess changes in lipid catabolism in the other tissues, palmitate oxidation was measured in isolated hepatocytes and in skeletal muscles ex vivo, following the 5 week treatment (ESM Table 2). The combination treatment, but not the other treatments, increased the hepatic FA oxidation compared with the cHF mice, but it had no effect on FA oxidation in muscle.

Combination treatment ameliorates low-grade adipose tissue inflammation induced by high-fat diet As LC *n*-3 PUFA prevented obesity-associated low-grade inflammation of WAT in mice [15, 16], we analysed the anti-inflammatory action of LC *n*-3 PUFA in the context of mild restriction of energy intake. Immunohistochemical analysis of epididymal fat of various treatments revealed a reduced content of macrophages aggregated in crown-like structures (CLS) surrounding dead adipocytes [33] compared with the cHF mice. The macrophage infiltration was maximally reduced in mice subjected to the combination treatment (Fig. 5a–c) and mRNA levels for the macrophage marker CD68 were decreased in the epididymal fat of all treatment groups compared with the cHF controls (Fig. 3a). Moreover, plasma levels of IL-6, a marker of systemic inflammation, were significantly reduced only by the combination treatment (Fig. 5d).

Specific induction of anti-inflammatory lipid mediators in epididymal fat To investigate the possible involvement of bioactive lipids derived from PUFA and modulating inflammation (see Introduction and Fig. 6), lipidomic analysis was performed in epididymal fat and in the liver. In total, 24 lipid species were quantified (ESM Table 3). Partial least-squares-discriminant analysis (PLS-DA) revealed the major discriminating lipid species between the groups. In WAT, but not in the liver, the first two PLS-DA components separated mice into four distinct groups corresponding to dietary treatments (ESM Figs 4 and 5). Following contribution-score analysis, the most important lipids were identified for each of the intervention groups. As shown in Fig. 7a,c,e and in ESM Table 3, the levels of AA, EPA and DHA in the free FA fraction of WAT were significantly higher in all treated groups compared with the cHF mice (except for AA in the ad libitum mice treated by LC *n*-3 PUFA). The cHF+F+CR treatment exerted the most

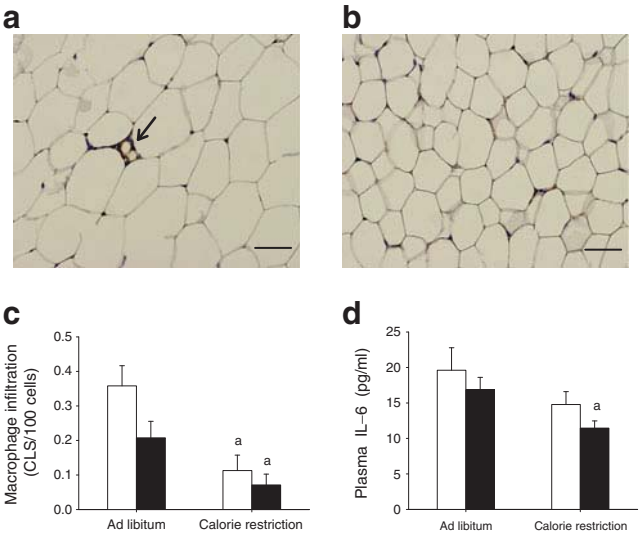

Fig. 5 Prevention of adipose tissue and systemic inflammation. Immunohistochemical analysis of epididymal fat of the cHF mice (**a**) and the cHF+F+CR mice (**b**) with visualised MAC-2 antigen (expressed on activated macrophages); arrow indicates aggregates of macrophages forming CLS, which surround dead adipocytes. **c** Relative count of CLS; $n=6$. **d** Plasma level of IL-6, marker of systemic inflammatory status. Data are means±SEM; $n=10$; white bars, cHF or cHF+CR; black bars, cHF+F or cHF+F+CR; [a]Significant difference (ANOVA) compared with the cHF mice. The morphometry data are based on more than 1,000 cells taken randomly from six different areas per animal. All analyses were performed after 5 weeks of the treatment

pronounced effect. In the liver, free EPA and DHA levels were significantly affected only by LC *n*-3 PUFA, independent of calorie restriction (Fig. 7d,f). In both tissues, the levels of primary monohydroxy metabolites derived from linoleic (HODE), AA (HETE), EPA (HEPE) and DHA (HDoHE) correlated with those of the corresponding FAs (Fig. 6). The lipoxygenase (LOX) products were the most abundant autacoids in both tissues of the cHF controls. Dietary supply of EPA and DHA triggered formation of LC *n*-3 PUFA-derived metabolites in both tissues. The WAT-specific liberation of FAs from membrane phospholipids in response to calorie restriction resulted in the formation of lipid mediators derived from both LC *n*-3 and LC *n*-6 PUFA (Fig. 6). Only in WAT, dietary LC *n*-3 PUFA combined with calorie restriction synergistically increased the levels of protectin D1 (PD1; Fig. 7g), a well described anti-inflammatory lipid mediator derived from DHA, or PD1 isomers such as PDX. In accordance with a previous finding [18], resolvin E1 (derived from EPA) was below the level of detection. Unexpectedly, 15-deoxy-$\Delta^{12,15}$-prostaglandin J_2 (15d-PGJ_2), an anti-inflammatory mediator and potent PPARγ agonist derived from AA [34], was also synergistically upregulated by the combination treatment specifically in WAT (Fig. 7i).

Discussion

We show here that the combination treatment of dietary LC *n*-3 PUFA and mild calorie restriction is more effective than each of the treatments applied separately in the protection against obesity in mice. Pronounced induction of mitochondrial lipid catabolism and synergistic induction of anti-inflammatory lipid mediators in WAT could contribute the systemic effects of the combination treatment (Fig. 8).

Our results demonstrate an improvement of metabolic flexibility by the combination treatment, based on both the

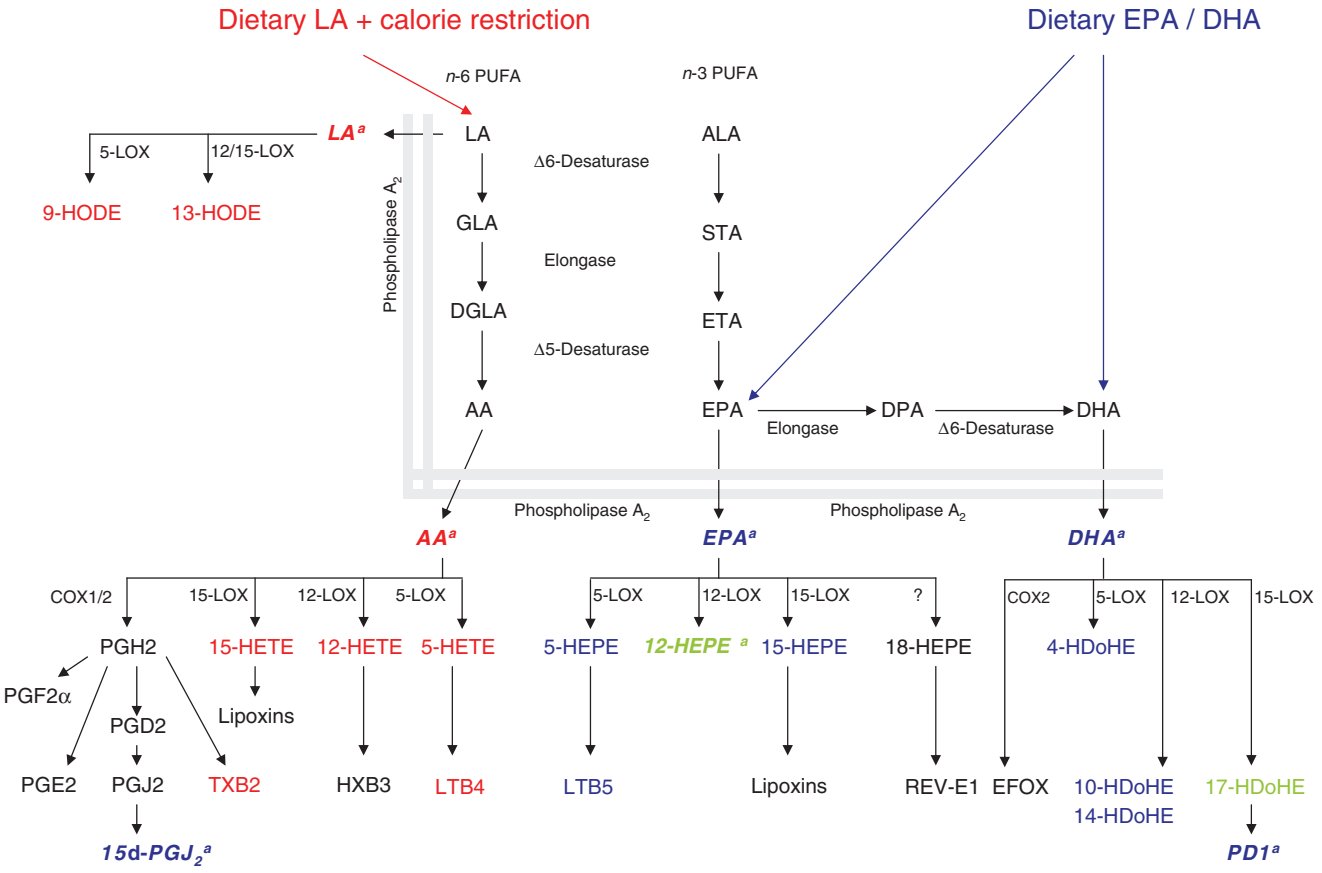

Fig. 6 Overview of the effects of calorie restriction, LC *n*-3 PUFA and the combination treatment on the levels of lipid mediators in epididymal fat. Scheme is based on the results of lipidomic analysis (see Fig. 7, ESM Table 3 and ESM Fig. 3). Significantly upregulated metabolites are marked by different colours; red colour, upregulated by calorie restriction; blue colour, upregulated by LC *n*-3 PUFA; green colour, upregulated by each of the treatments; [a]synergistic upregulation by the combination treatment. Some pathways mentioned in this figure are speculative and not conclusively described. The scheme is inspired by Larsson et al. [50]. ALA, α-linolenic acid (18:3 *n*-3); DGLA, dihomo-γ-linolenic acid (20:3 *n*-6); EFOX, electrophilic oxo-derivatives from DHA [19]; GLA, γ-linolenic acid (18:3 *n*-6); HXB3, hepoxilin B3; LA, linoleic acid (18:2 *n*-6); 5-,12-,15-LOX, 5,12,15-lipoxygenase; LTB4/B5, leukotriene B4/B5; PD1, protectin D1; PG, prostaglandin; PLA_2, phospholipase A_2; Rev-E1, resolvin E1; TXB2, thromboxane B2

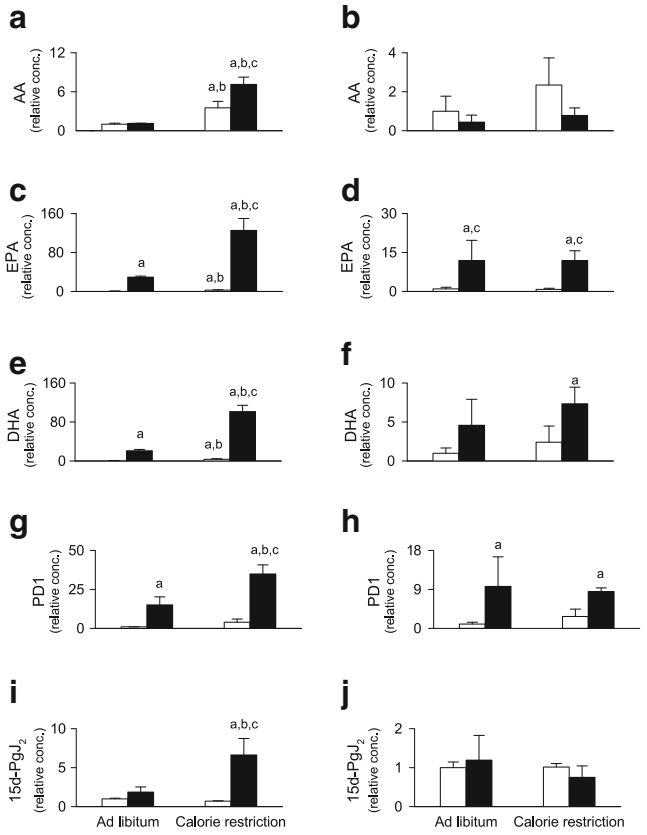

Fig. 7 Synergistic induction of anti-inflammatory lipid mediators in adipose tissue. **a–j** Selected results of lipidomic HPLC MS-MS analysis of epididymal fat (**a**, **c**, **e**, **g**, **i**) and liver (**b**, **d**, **f**, **h**, **j**) after 5 weeks of treatment. **a**, **b** AA; **c**, **d** EPA; **e**, **f** DHA; **g**, **h** PD1; **i**, **j** 15d-PgJ_2. Data are expressed as relative concentration of individual lipids adjusted to wet weight (cHF=1) and expressed as means±SEM; n=3. White bars, cHF or cHF+CR; black bars, cHF+F or cHF+F+CR. [a,b,c]Significant differences (ANOVA) compared with cHF, cHF+F, cHF+CR, respectively. For complete results, see ESM Table 3

response of plasma levels of NEFA and glucose to FASTED to RE-FED transition and the glucose-induced increase in RQ. This improvement of metabolic flexibility correlated with changes in body weight, adiposity and glycaemic control in accordance with the notion that metabolic flexibility is closely associated with insulin sensitivity [2, 32] and that impaired flexibility represents an early defect in the development of type 2 diabetes [35].

Metabolic syndrome is linked to inflammatory changes in both WAT and liver [1]. In this study, in accordance with the previous findings [17, 18, 21], dietary LC n-3 PUFA supplementation resulted in the inhibition of formation of various LC n-6 PUFA-derived pro-inflammatory eicosanoids in both tissues as well as in the induction of the anti-inflammatory molecules. In WAT, in contrast to the liver, the levels of EPA, DHA, AA and their active metabolites, including the anti-inflammatory molecules PD1 and prostaglandin 15d-PGJ_2, were increased in a synergistic manner by the combination treatment (Fig. 7). A key role of the enzyme involved in PD1/PDX formation, 12/15 LOX, in adipose tissue inflammation and insulin resistance has been described [36, 37]. The induction of prostaglandin 15d-PGJ_2 was an unexpected finding as 15d-PGJ_2 is derived from the metabolism of LC n-6 PUFA, and enzymatic formation of LC n-6 PUFA metabolites is, in general, inhibited upon LC n-3 PUFA supplementation. This suggests that dietary LC n-3 PUFA, especially in combination with calorie restriction, selectively activates formation of 15d-PGJ_2 from prostaglandin D2 (PGD2), the major product of cyclooxygenase (COX) in many tissues [34],

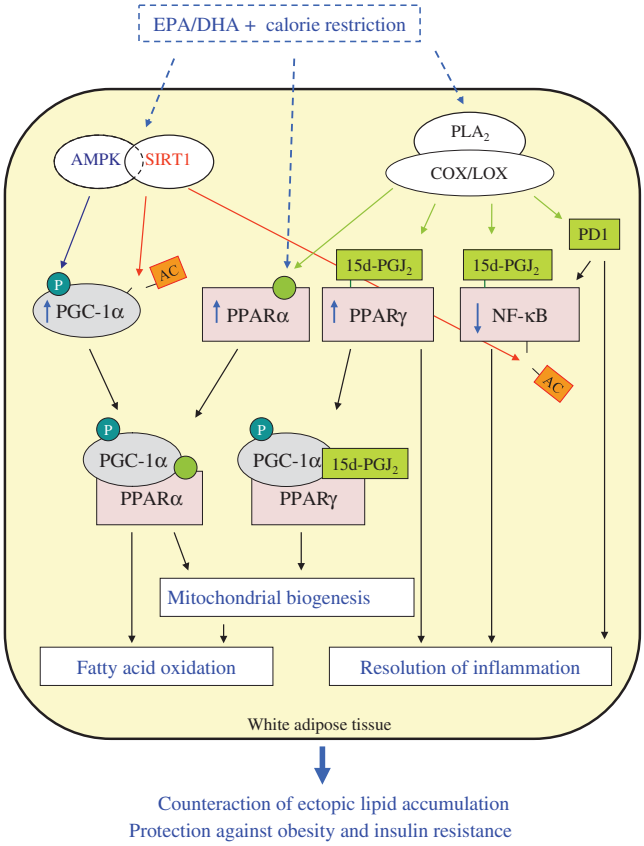

Fig. 8 Putative involvement of AMPK/SIRT1 signalling, 15d-PGJ_2 and PD1 in the additive metabolic and anti-inflammatory effects of the combination of LC n-3 PUFA and calorie restriction in adipose tissue. Dietary intake of LC n-3 PUFA combined with calorie restriction activates AMPK/SIRT1 signalling pathway and synergistically induces production of anti-inflammatory and pro-resolving lipid mediators by COX/LOX enzymatic systems. AMPK and SIRT1 can increase activity of transcriptional co-activator PGC-1α by phosphorylation and de-acetylation, respectively. PGC-1α can enhance transcriptional activity of PPAR nuclear receptors, resulting in higher mitochondrial oxidative capacity and induction of FA catabolism in situ in white fat. Simultaneously, unsaturated FAs (light green circle) as well as their active metabolites (lipid mediators) can also activate directly PPARα and PPARγ (e.g. 15d-PGJ_2). Lipid mediators are formed after the release of FAs from membrane phospholipids and can inhibit the NF-κB signalling pro-inflammatory pathway (15d-PGJ_2, PD1) or induce the resolution phase of inflammation (PD1). Of note, SIRT1 can also interfere with NF-κB signalling. AC, acetyl group; P, phosphate

reflecting possibly the ability of EPA- and DHA-derived peroxyl radicals to favour the formation of 'less pro-inflammatory' peroxidation products derived form AA [38].

Here we suggest for the first time that 15d-PGJ_2 represents an important mediator of LC *n*-3 PUFA effects. 15d-PGJ_2 can modulate the activity of various signalling molecules by covalent binding and their subcellular localisation [34], including various components of the NF-κB signalling pathway [19, 39]. Moreover, 15d-PGJ_2 is the most potent endogenous ligand for PPARγ [40, 41]. Interestingly, DHA is a precursor for anti-inflammatory electrophilic cyclopentenone neuroprostanes in macrophages [20] and electrophilic oxo-derivatives generated by COX-2 reactions [19], which can act similarly to 15d-PGJ_2. Thus, the activation of PPARγ via 15d-PGJ_2 binding could be responsible for the additive effects of the combination treatment on adipose tissue energy and lipid metabolism. This idea is supported by the fact that rosiglitazone, a PPARγ ligand, increases PPARγ coactivator 1α (PGC-1α) production, mitochondrial mass, palmitate oxidation and mitochondrial uncoupling protein 1 (UCP1) in the adipose tissue of genetically obese *ob*/*ob* mice [42].

It has been found that brown adipocyte-like cells originating from rosiglitazone-treated epididymal WAT cell precursors represent a new subtype of adipocytes, called 'brite' cells, which differ from classic white and brown adipocytes, but possess mitochondrial UCP1-mediated thermogenesis [43]. Recent studies [44, 45] demonstrated the involvement of COX-2 in the induction of these fat-burning cells and the importance of the COX-2-mediated mechanisms for the resistance to dietary obesity in mice. Our results convincingly demonstrate a marked induction of mitochondrial oxidative capacity in permeabilised adipocytes isolated from epididymal fat in response to mild calorie restriction combined with LC *n*-3 PUFA intake, using succinate as the optimum fuel [46]. Moreover, changes in palmitate oxidation in both intact isolated adipocytes and in fat fragments document induction of energy expenditure by the combination treatment. Importantly, these changes occurred even in the absence of UCP1, depending probably on the simultaneous activation of PPARα/PGC-1α and PPARγ signalling (Fig. 8). Induction of futile substrate cycling in adipocytes by this mechanism [7] might explain the increased lipid catabolism in the absence of mitochondrial uncoupling. This idea is consistent with our current findings that the combination treatment increases capacity for de novo lipogenesis in epididymal fat, both at the level of gene expression and biochemical activity. In adult mice reared at 20°C, total oxidative capacity in WAT represents ~30–50% of brown adipose oxidative capacity [10] indicating that energy expenditure in WAT may influence total energy balance (see Introduction). Even small but persistent changes in WAT energy expenditure, undetectable using indirect calorimetry, could influence body weight substantially. Fat-depot-specific differences affecting the inducibility of energy-dissipating adipocytes should be explored further. In addition to the stimulation of lipid catabolism in WAT, FA oxidation in liver—but not in skeletal muscle—could contribute to the whole-body effects of the combination treatment.

We have demonstrated previously that AMP-activated protein kinase (AMPK), a cellular energy sensor, is involved in the effects of LC *n*-3 PUFA on hepatic lipid and glucose metabolism [30] and that it is activated in WAT by LC *n*-3 PUFA [47]. AMPK activation in adipocytes results in increased mitochondrial biogenesis and lipid catabolism [4, 48]. Moreover, AMPK closely interacts with deacetylase sirtuin 1 (SIRT1), which controls metabolic processes in response to calorie restriction and exerts anti-inflammatory effects in WAT [49]. Therefore, AMPK/SIRT1 signalling is probably involved in the additive metabolic and anti-inflammatory effects of the combination treatments in epididymal WAT (Fig. 8).

In summary, dietary LC *n*-3 PUFA augment the anti-obesity effects of mild calorie restriction in mice while improving lipid metabolism and glucose homeostasis. These effects are probably reflected by the large synergistic induction of mitochondrial FA oxidation in WAT, linked to a suppression of low-grade inflammation of this tissue. The synergistic induction of specific anti-inflammatory lipid mediators, namely 15d-PGJ_2, the LC *n*-6 PUFA metabolite, and PD1, the LC *n*-3 PUFA metabolite, may underlie both the anti-inflammatory and metabolic effects of the combination treatment in WAT. Further exploration of the strategy to target WAT by combining two complementary and physiological approaches, i.e. dietary intake of LC *n*-3 PUFA and mild restriction of energy intake, may be valuable for the prevention and treatment of metabolic syndrome.

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P.F. contributed to the conception and design of experiments, analysis and interpretation of data, and drafted the manuscript; R.R. and M.R. contributed to the conception and design of experiments, analysis and interpretation of data, and revising the manuscript; M.H., P.J., P.Z., V.K., Z.M.J., E.P., O.K., M.S., G.T. and V.M-A. contributed to the analysis and interpretation of data, and revising the manuscript; and J.K. coordinated the research, contributed to the conception and design of experiments, and drafted the manuscript. All authors approved the final version of the manuscript.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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Publication E

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Anti-obesity effect of n-3 polyunsaturated fatty acids in mice fed high-fat diet is independent of cold-induced thermogenesis

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Anti-Obesity Effect of *n*-3 Polyunsaturated Fatty Acids in Mice Fed High-Fat Diet Is Independent of Cold-Induced Thermogenesis

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Summary

Long-chain *n*-3 polyunsaturated fatty acids (LC *n*-3 PUFA) exert beneficial effects on health and they could help to prevent development of obesity and associated metabolic disorders. In our previous studies in mice fed high-fat (cHF; ~60 % calories as fat) diet and maintained at 20 °C, dietary LC *n*-3 PUFA could counteract accretion of body fat, without inducing mitochondrial uncoupling protein 1 (UCP1) in adipose tissue, suggesting that the anti-obesity effect was not linked to adaptive (UCP1-mediated) thermogenesis. To exclude a possible dependence of the anti-obesity effect on any mechanism inducible by cold, experiments were repeated in mice maintained at thermoneutrality (30 °C). Male C57BL/6J mice were fed either cHF diet, or cHF diet supplemented with LC *n*-3 PUFA, or standard diet for 7 months. Similarly as at 20 °C, the LC *n*-3 PUFA supplementation reduced accumulation of body fat, preserved lipid and glucose homeostasis, and induced fatty acid re-esterification in epididymal white adipose tissue. Food consumption was not affected by LC *n*-3 PUFA intake. Our results demonstrated anti-obesity metabolic effect of LC *n*-3 PUFA, independent of cold-induced thermogenesis and they suggested that induction of fatty acid re-esterification creating a substrate cycle in white fat, which results in energy expenditure, could contribute to the anti-obesity effect.

Key words

Marine lipids • Obesity • Thermoneutrality • Indirect calorimetry • Metabolic syndrome

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Introduction

Epidemy of obesity triggered intense research of inducible metabolic mechanisms, which could counteract accumulation of body fat. Thus, traditional research of adaptable cold- and diet-induced thermogenesis mediated by mitochondrial uncoupling protein 1 (UCP1) (Nicholls and Locke 1984, Klaus *et al*. 1991, Nedergaard *et al*. 2005) has been revived reflecting also the discovery of functional brown adipose tissue (BAT) in adult humans (Cypess *et al*. 2009, van Marken Lichtenbelt *et al*. 2009, Virtanen *et al*. 2009, Zingaretti *et al*. 2009, Nedergaard and Cannon 2010), as well as the negative correlation between BAT content and body weight in humans (Saito *et al*. 2009, Zingaretti *et al*. 2009). Nevertheless, several studies suggest that UCP1-independent thermogenesis also exists, which could be recruited by various treatments reducing obesity (Guan *et al*. 2002, Granneman *et al*. 2003, Cannon *et al*. 2004, Kus *et al*. 2008, Summermatter *et al*. 2008, Chen *et al*. 2010, Kozak 2010, Langin 2010, Meyer *et al*. 2010).

Long-chain *n*-3 polyunsaturated fatty acids (LC *n*-3 PUFA) of marine origin, namely eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) exert numerous beneficial effects on health, including improvements in lipid metabolism and prevention of obesity and diabetes (reviewed in Flachs *et al*. 2009). These effects are well documented in our previous studies, using a model of metabolic syndrome in dietary obese

mice (Kuda *et al*. 2009, van Schothorst *et al*. 2009, Jelenik *et al*. 2010, Flachs *et al*. 2011, Hensler *et al*. 2011, Kus *et al*. 2011, Rossmeisl *et al*. 2012), which have also demonstrated that LC *n*-3 PUFA could increase mitochondrial oxidative capacity specifically in white adipose tissue (WAT) and not in BAT, skeletal muscle or liver (Flachs *et al*. 2005). This induction was augmented by calorie restriction (Flachs *et al*. 2011). Importantly, no up-regulation of UCP1 gene in adipose tissue could be observed (Flachs *et al*. 2005, 2011). Instead, our results suggested the involvement of fatty acid (FA) re-esterification in WAT in the anti-obesity effect of the combined use of LC *n*-3 PUFA and calorie restriction (Flachs *et al*. 2011). All the above studies were conducted in mice maintained at 20 °C, i.e. under the conditions activating inherent mechanisms of metabolic cold defense, since thermoneutral zone in mice is close to 30 °C (Cannon *et al*. 2004, Alberts *et al*. 2005). Therefore, a possibility existed that the induction of the catabolic processes by LC *n*-3 PUFA, which resulted in energy expenditure and obesity resistance, reflected mechanisms independent of UCP1, but activated by the cold exposure. Results of this study document, that dietary intervention with LC *n*-3 PUFA could counteract accumulation of body fat even at thermoneutrality, independent of the mechanisms underlying cold-induced thermogenesis.

Methods

Animals and treatments

C57BL/6J (B/6J) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred at the Institute of Physiology for several generations. Male mice born and maintained at 20 °C on a 12:12-h light-dark cycle were weaned at 4 weeks of age to either the standard low-fat (ST) or high-fat (cHF) diet, while the ambient temperature was increased to 30 °C and this temperature was maintained until the end of the experiment (with 4 mice per cage). ST diet (Velaz, Prague, Czech Republic) contained 21, 3, and 56 % calories as protein, fat, and carbohydrate, respectively. The cHF diet, proven to be obesogenic in B/6J mice, contained 15, 59, and 26 % calories as protein, fat, and carbohydrate, respectively (see Kuda *et al.* 2009). In some animals, cHF diet was supplemented with EPA and DHA (cHF+F), added as a concentrate of LC *n*-3 PUFA (46 % DHA, 14 % EPA; EPAX 1050 TG, EPAX a.s., Lysaker, Norway), which replaced 15 % of dietary lipids (specifically, 5.25 g of corn oil/100 g cHF diet). Thus, 5.3 % of total energy content in the LC *n*-3 PUFA-supplemented diet came from EPA and DHA. In contrast with cHF+F, both ST and cHF diet were virtually free of any LC *n*-3 PUFA (see our previous publication Kuda *et al.* 2009 for the composition of FA in lipids in both cHF-based diets).

Body weight of each mouse was monitored weekly. Food intake of the group of 4 mice in each cage during a 24-h period was assessed four times per experiment (at 2, 3, 4 and 7 months of age), and averaged per mouse for the whole period of the dietary intervention (i.e., from the time of weaning to 8 months of age). Mice were killed at 8 month of age in *ad libitum* fed state, by decapitation between 10:00 and 12:00 a.m. EDTA-plasma was prepared from truncal blood and stored at −70 °C. Subcutaneous (dorsolumbar) and epididymal WAT were dissected.

All experiments were performed in accordance with the guidelines for the use and care of laboratory animals of the Institute of Physiology, the directive of the European Communities Council (2010/63/EU), and the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985).

Glucose tolerance test

Two weeks before the end of the experiment, an intraperitoneal glucose tolerance test (IP GTT) was performed at ambient temperature of 30 °C, in overnight-fasted mice as described before (Rossmeisl *et al.* 2009).

Indirect calorimetry

To evaluate energy expenditure, indirect calorimetry was performed using a system from Somedic (Horby, Sweden; refs. Kus *et al.* 2008, Flachs *et al.* 2011) at 6 months of age. Briefly, the measurements were performed in individually caged mice (Eurostandard type II mouse plastic cages; ~ 6,000 ml; Techniplast, Milan, Italy), with the cages placed in a sealed measuring chamber equipped with thermostatically controlled heat exchangers at 30 °C. Oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were recorded every 2 min under a constant airflow rate (1000 ml/min) for 22 h, starting at 3:00 p.m. The level of substrate partitioning was estimated by calculating respiratory exchange ratio (RER; i.e., $\dot{V}CO_2/\dot{V}O_2$ ratio). Percent relative cumulative frequency (PRCF) curves were constructed based on RER values pooled from all the animals within a given dietary group (6-8 animals per group) during the whole measurement period (Kus *et al.* 2008).

Table 1. Growth characteristics, adiposity, and plasma parameters.

	ST	cHF	cHF+F
Body weight (g)			
Initial (at weaning)	12.44 ± 0.45	12.61 ± 0.37	11.38 ± 0.81
Final	29.55 ± 0.39	40.11 ± 2.21#	37.17 ± 2.29#
Gain	16.98 ± 0.61	27.73 ± 2.09#	25.50 ± 1.76#
Food consumption (kJ/mice/day)	36.5 ± 1.6	40.6 ± 1.1#	40.5 ± 1.6#
Weight of fat depots (mg)			
EPI	393 ± 36	2208 ± 144#	1534 ± 228*#
DL	146 ± 14	738 ± 88#	531 ± 76#
Plasma levels			
TG (mmol/l)	1.40 ± 0.07	1.73 ± 0.20	1.19 ± 0.15*
NEFA (mmol/l)	0.96 ± 0.05	1.32 ± 0.05#	0.94 ± 0.07*
Glucose homeostasis			
Fasting glucose (mmol/l)	3.66 ± 0.07	4.63 ± 0.31#	3.63 ± 0.18*
Incremental AUC (glucose mmol/l)	655 ± 53	1129 ± 52#	842 ± 41*#

Four weeks after birth, mice were weaned onto standard low-fat diet (ST), or high-fat diet (cHF) diet, or cHF diet supplemented with LC *n*-3 PUFA (cHF+F) and maintained at 30 °C (*n*=8). Mice were killed at 8 months of age in *ad libitum* state and plasma levels of NEFA and TG and adiposity were analyzed. Glucose homeostasis was assessed using IP GTT in mice fasted overnight 2 weeks before killing (see Fig. 1). Food consumption (expressed as kJ/day per animal) was measured four times during the whole differential dietary treatment protocol. EPI - epididymal fat, DL – subcutaneous WAT in dorsolumbar region. Data are means ± SE. * $p<0.05$ for the effect of cHF+F compared to cHF; # $p<0.05$ for the effect of cHF-based diet compared to ST.

Metabolite quantification

Non-esterified FA (NEFA) and triacylglycerols (TG) in EDTA-plasma were assessed as described before (Ruzickova *et al.* 2004).

Ex vivo metabolism of adipose tissue

Basal and insulin-stimulated TG synthesis was quantified as previously described (Pravenec *et al.* 2006). Briefly, distal parts of epididymal adipose tissue (~200 mg aliquots) were incubated in modified Krebs-Ringer bicarbonate buffer containing 4 % bovine serum albumin (Fraction V), 5 mM glucose, and 0.1 μCi/ml ^{14}C-glucose in gas phase of 95 % O_2 and 5 % CO_2 at 37 °C. After 2 h incubation without or with insulin (250 μU/ml), the tissue fragments were washed by saline, homogenized in chloroform and thereafter methanol was added in a 2:1 ratio (chloroform : methanol). The lipid extraction proceeded during night at 4 °C. For the chloroform phase separation, KH_2PO_4 was added (Folch *et al.* 1957). Water phase of the extract was used for quantification of the incorporation of glucose into total neutral lipids and expressed as nmol of glucose converted into lipid per gram of adipose tissue. Aliquot of the chloroform phase (which was saponificated and subsequently extracted by petrol ether) was used for the determination of ^{14}C-glucose incorporation into acyl groups and was expressed as nmol of glucose converted into lipid per gram of adipose tissue (Pravenec *et al.* 2006). The amount of ^{14}C-glucose incorporated into glycerol residues was calculated as the difference between the total incorporation into neutral lipids and the incorporation into acyl groups, separately for each sample.

Statistics

All values are expressed as means ± SE. Logarithmic transformation was used when necessary. Data were analyzed using Student's t-test or ANOVA (one-way or two-way) with Holm-Sidak method using SigmaStat statistical software. The PRCF curves were analyzed by Nonlinear Regression using SigmaPlot and 50th percentile value (EC_{50}) and Hillslope values were compared in a one-way ANOVA. Statistical significance was defined as $p\leq0.05$.

Results

Mice, which were maintained at 30 °C since weaning (at 4 weeks of age) showed different final body weight at 8 months of age, depending on the type of diet

fed during the post-weaning period. Thus, the final body weight of the cHF diet-fed mice was significantly higher as compared with the ST diet-fed mice, while supplementation of the cHF diet with LC *n*-3 PUFA (cHF+F) tended to counteract the cHF diet-induced obesity (Table 1). The differences in body weight could be explained by differences in adiposity. Thus, weight of epididymal fat depot was significantly lower and the weight of dorsolumbar fat depot tended to be lower in the cHF+F group, as compared with the cHF-diet fed mice (Table 1). Calorie intake (measured in groups of 4 mice caged together; see Methods) was significantly higher in the cHF diet-fed as compared with the ST diet-fed mice. However, it was not affected LC *n*-3 PUFA admixed to the cHF diet (Table 1). While plasma levels of TG tended to be elevated and levels of NEFA were significantly increased in response to the cHF diet-feeding, the supplementation of the cHF diet with LC *n*-3 PUFA exerted a protective, anti-hyperlipidaemic effect (Table 1).

To evaluate the effect of the differential dietary treatment on glucose homeostasis, IP GTT was performed at 30 °C, two weeks before killing of the mice (Fig. 1). The cHF diet-feeding resulted in increased fasting blood glucose levels, as measured at the beginning of the test, and in deterioration of glucose tolerance, assessed as an incremental area under the curve (AUC), which increased ~1.7-fold (Fig. 1 and Table 1). The supplementation of cHF diet with LC *n*-3 PUFA prevented the adverse effect of cHF diet-feeding on glucose homeostasis, as documented by the normalization of blood glucose levels, and by almost complete prevention of the AUC increase (Fig. 1 and Table 1).

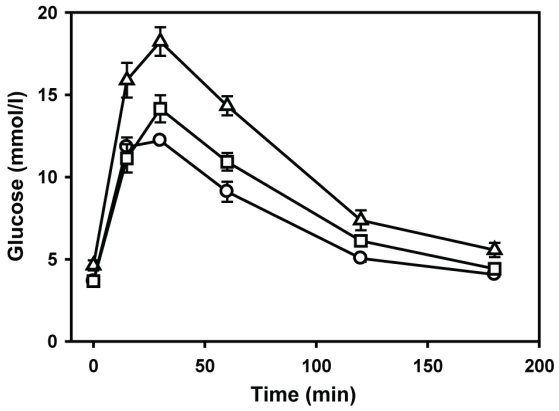

Fig. 1. Glucose tolerance test. Four weeks after birth, mice were weaned onto ST diet (circle), or cHF diet (triangle), or cHF+F diet (square) and maintained at 30 °C (n=8) during the whole experiment. Two weeks before mice killing (at 8 month of age) IP GTT was performed. Data are means ± SE; for incremental AUC and fasting glucose (see Table 1).

Table 2. Indirect calorimetry.

	ST	cHF	cHF+F
VO_2 *(ml/min)*	1.07 ± 0.04	1.25 ± 0.07	1.19 ± 0.05
RER	0.904 ± 0.010	0.792 ± 0.008#	0.805 ± 0.007#

Four weeks after birth, mice were weaned onto different diets and maintained at 30 °C as described in Table 1. At 6 month of age, indirect calorimetry was performed. Data are means ± SE. # p<0.05 for the effect of cHF-based diet.

To characterize whole-body metabolism and its changes in response to different diets, indirect calorimetry was used in *ad libitum* fed mice. To avoid any cold stress and similarly as in the case of IP GTT (see above), the measurements were performed at 30 °C. The measurements were carried over a 22 h period, i.e., during almost complete light-dark cycle of the day. In the case of VO_2, no significant differences between groups were observed (Table 2). As expected, RER values were lower in both cHF and cHF+F groups as compared with mice fed ST diet (Table 2), in agreement with a relatively high content of lipids in the cHF-based diets and the preferential oxidation of lipid over carbohydrate fuels under these conditions. This analysis also suggested an increase in RER in response to the supplementation of the cHF diet with LC *n*-3 PUFA (Table 2), in agreement with the beneficial effect of LC *n*-3 PUFA on glucose homeostasis and insulin sensitivity (see above). Therefore, a robust analysis of RER was used, while constructing PRCF curves based on all the data pooled from each dietary group (see Methods and Fig. 2). This quantitative approach is capable to detect small differences in fuel partitioning. Provided that PRCF curves represent the normally distributed data, the values of log EC_{50} of PRCF (50^{th} percentile value) correspond to RER values (Kus *et al.* 2008). The PRCF curves shifted to the left in response to both cHF-based diets, and a trend for a difference between the EC_{50} value of the cHF and cHF+F curves was observed, supporting a shift from lipid to carbohydrate oxidation in response to the LC *n*-3 PUFA supplementation. That the cHF curve was significantly steeper than both the cHF+F and the ST curves suggests (i) a relatively homogeneous distribution of RER values in the cHF diet fed-mice (Kus *et al*. 2008), reflecting a strong drive for oxidation of abundantly supplied dietary lipids, and (ii) that the supplementation of the cHF diet with LC *n*-3 PUFA could unmask an inherent heterogeneity of the mice with respect to the

preservation of glucose homeostasis by the LC *n*-3 PUFA supplementation (Fig. 2). In any case, concerning the subtle effects of the LC *n*-3 PUFA supplementation, unequivocal interpretation of the data would require measurements using a larger cohort of the experimental animals.

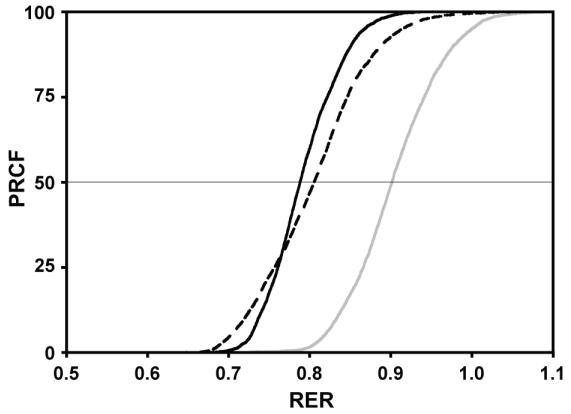

Fig. 2. Evaluation of fuel partitioning using indirect calorimetry. At 6 months of age, the measurements were performed at 30 °C for a period of 22 h, on a 12:12-h light-dark cycle, while mice had free access to ST diet (gray line), or cHF diet (black line), or cHF+F diet (black dash line) and water. RER data (their means ± SE are shown in Table 2) pooled from all the mice of the same dietary group (n=6-8; ~3,600 RER measurements per each curve) were used to construct PRCF curves. Both EC_{50} and Hillslope values were significantly different between ST and cHF-based diets (cHF, cHF+F), while only the Hillslope values differed between cHF and cHF+F diets (not shown).

The above results document that similarly as at 20 °C (Kuda *et al*. 2009, van Schothorst *et al*. 2009, Jelenik *et al*. 2010, Flachs *et al*. 2011, Hensler *et al*. 2011, Kus *et al*. 2011, Rossmeisl *et al*. 2012), also at the thermoneutral temperature of 30 °C, the LC *n*-3 PUFA supplementation exerts anti-obesity effect, while preserving healthy plasma lipid profile and glucose homeostasis in the animals exposed to obesogenic environment. Since our previous results indicated a surprisingly tissue specific involvement of FA re-esterification in WAT in the anti-obesity effect of LC *n*-3 PUFA in the combination with calorie restriction (Flachs *et al*. 2011), we sought to characterize the effect of the LC *n*-3 PUFA supplementation on WAT metabolism also in this study. Incorporation of ^{14}C-glucose into total lipids (Fig. 3A), as well as into the acyl groups (Fig. 3B) in epididymal WAT, were significantly decreased in association with the cHF diet-feeding, in agreement with the impairment of *de novo* FA synthesis in response to high intake of dietary fat (Flachs *et al*. 2011). Also in agreement with the results in mice maintained at 20 °C (Flachs *et al*. 2011), this decrease was partially prevented by the LC *n*-3 PUFA supplementation, namely under the insulin-stimulated conditions (Fig. 3B). That LC *n*-3 PUFA support the metabolic effect of insulin is consistent with their beneficial effect on glucose homeostasis (see above). Moreover, as suggested by the changes in incorporation of radiolabeled glucose into glycerol residues (Fig. 3C), i.e., the marker of *de novo* glycerol synthesis and FA re-esterification (Pravenec *et al*. 2006), cHF-feeding depresses FA re-esterification in WAT, while LC *n*-3 PUFA could preserve this activity, namely under the basal conditions, in the absence of the insulin stimulation.

Discussion

The principal finding of this report is a moderate protection against accumulation of body fat by LC *n*-3 PUFA admixed to high-fat diet, namely in the abdominal WAT, which occurred in mice maintained at thermoneutral conditions of 30 °C, i.e. independent of cold-induced thermogenesis. This observation is consistent with a lack of any up-regulation of UCP1 gene neither in WAT nor in interscapular BAT in response to dietary LC *n*-3 PUFA under the conditions similar to this

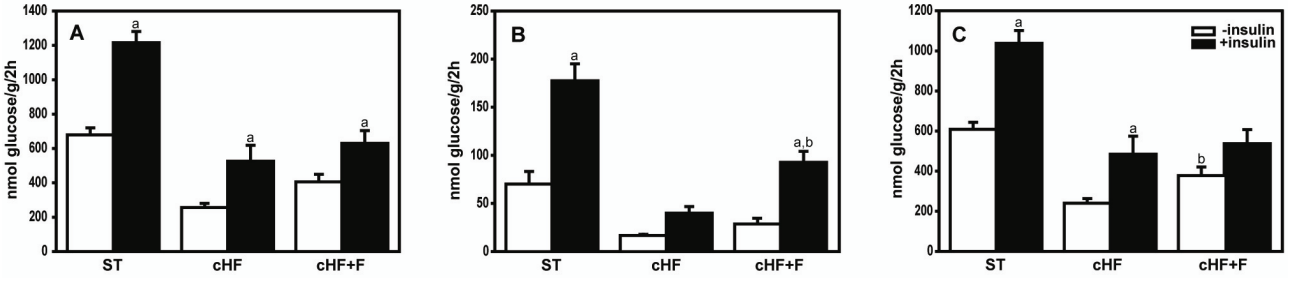

Fig. 3. Lipid metabolism in adipose tissue at 8 months of age. Incorporation of ^{14}C- glucose into neutral lipids (TG synthesis); (**A**), and incorporation of ^{14}C-glucose into acyl groups (*de novo* FA synthesis); (**B**) was evaluated *ex vivo* in fragments of epididymal fat. Incorporation of ^{14}C- glucose into glycerol residue (FA re-esterification); (**C**) was calculated based on the data in **A** and **B.** [a] p<0.05 for the effect of insulin, [b] p<0.05 for the effect of cHF+F compared to cHF diet.

experiment, except that the previous studies (Flachs *et al*. 2005, 2011) were performed in mice maintained at 20 °C, i.e. under the conditions, which should augment UCP1 gene expression. Similarly to our previous studies of this animal model, all of which were performed using mice maintained at 20 °C (Kuda *et al*. 2009, van Schothorst *et al*. 2009, Jelenik *et al*. 2010, Flachs *et al*. 2011, Hensler *et al*. 2011, Kus *et al*. 2011, Rossmeisl *et al*. 2012), reduced accumulation of body fat in response to LC *n*-3 PUFA in this study could not be attributed to changes in food intake, supporting the notion that UCP1-independent energy expenditure was involved (see Introduction). However, it cannot be excluded that the magnitude of LC *n*-3 PUFA response is affected to some extent by ambient temperature. To test this possibility, the effect of dietary LC *n*-3 PUFA on adiposity might be studied in mice maintained at various temperatures within the same experiment.

While in many of the previous studies in rodents fed a high-fat diet, LC *n*-3 PUFA prevented development of obesity, dyslipidemia (Ikemoto *et al*. 1996, Ruzickova *et al*. 2004, Flachs *et al*. 2005, 2011, Kuda *et al*. 2009) and impaired glucose tolerance (Storlien *et al*. 1987, Jucker *et al*. 1999, Neschen *et al*. 2007, Kuda *et al*. 2009, Jelenik *et al*. 2010), depending possibly in part on the dietary macronutrient composition (Hao *et al*. 2012), only few studies in obese humans demonstrated reduction of adiposity after LC *n*-3 PUFA supplementation (Couet *et al*. 1997, Mori *et al*. 1999, Kunesova *et al*. 2006). Thus, the metabolic effect of LC *n*-3 PUFA could differ in part between rodents and humans, and the mechanisms underlying possible induction of energy expenditure (thermogenesis) and protection against fat accumulation remain to be clarified. As found in mice, the anti-obesity effect could reflect in part the inhibition of fat cell proliferation (Ruzickova *et al*. 2004, Hensler *et al*. 2011), while the metabolic effects could depend on increased lipid catabolism in the liver (Jelenik *et al*. 2010) and the intestine (van Schothorst *et al*. 2009). In contrast, muscle energy metabolism is relatively little affected (Horakova *et al*. 2012). Moreover, as we have shown previously (Flachs *et al*. 2005, 2011), specific modulation of WAT metabolism, namely the induction of FA re-esterification (Flachs *et al*. 2011) could also contribute. Thus, somehow paradoxically with respect to the reduction of weight of epididymal fat in response to the LC *n*-3 PUFA supplementation, induction of *de novo* lipogenesis by LC *n*-3 PUFA in this WAT depot was observed in mice maintained both at 20 °C (Flachs *et al*. 2011) and 30 °C (this study). Since FA re-esterification creates a substrate cycle, its activation results in energy expenditure (Kalderon *et al*. 2000, Langin 2010). Moreover, the induction of energy-demanding FA re-esterification in WAT in response to the LC *n*-3 PUFA supplementation could help to explain both, the induction of mitochondrial biogenesis (Flachs *et al*. 2005, 2011) in WAT and the suppression of NEFA levels in plasma (results of this study and refs. Flachs *et al*. 2006, 2011, Kuda *et al*. 2009, Jelenik *et al*. 2010, Kus *et al*. 2011).

Our results contribute to understanding of the basic mechanisms regulating energy metabolism. As already discussed by Cannon and Nedergaard (2004), metabolic mechanisms enhancing energy expenditure independent of UCP1 probably exist, with a significant characteristic that they are not augmented by cold. Our results are in favor of this concept, while suggesting that the anti-obesity effect of LC *n*-3 PUFA in rodents depends on the activation of the UCP1-independent thermogenesis, using mechanisms distinct from those mediating classical adaptive thermogenesis. Because of the enormous capacity of cold-induced thermogenesis in small rodents (Cannon and Nedergaard 2004), the demonstration of the anti-obesity effect of LC *n*-3 PUFA in mice under the thermoneutral conditions suggests that at least some of the underlying mechanisms could serve as a target for treatment of human obesity.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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