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Energy metabolism of inbred mouse strains and its modulation by diet

PhD Thesis

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Statement of authorship

I certify that the thesis represents valid work elaborated under the supervision of Jan Kopecky, MD, DSc, and that neither this manuscript nor one with substantially similar content under my authorship has been submitted in support of an application for any other academical degree. My participation in the published papers is specified at the end of the comments to each paper.

In Prague …….

M.Sc. Vladimír Kůs

Statement of co-authors

I certify that Vladimír Kůs substantially contributed to the formation of the papers used as a basis of this thesis, and that his participation specified at the end of the comments to each paper is correct.

In Prague ……..

Jan Kopecký, MD, DSc

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Abstract

Obesity and associated metabolic disorders, called as “metabolic syndrome”, currently represent a major social and economical problem of public health. From the energy balance point of view, long-lasting energy surplus leads eventually to massive accumulation of energy stores resulting in various adverse effects on metabolism and health. General goal of the thesis was to examine these metabolic disorders at cellular and whole-body level using suitable mouse models. The main focus was on the most metabolically active tissue, namely skeletal muscle, liver and adipose tissue and on the regulatory roles of AMP-activated protein kinase (AMPK) and leptin in the energy metabolism.

The whole thesis is based on four published studies. Two studies were focused on skeletal muscle. In the first study, we proved the involvement of leptin and AMPK in the metabolic response to high-fat diet-feeding. We described a mechanism of muscle non-shivering thermogenesis based on enhanced lipid catabolism, which contributes to the genetically-determined resistance of inbred A/J mice to obesity. Such mechanism was not operating in obesity-prone C57BL/6 mice. In the second study, performed using C57BL/6 mice, we have described beneficial effect of combination treatment using n-3 polyunsaturated fatty acids (n-3 PUFA) of marine origin and anti-diabetic drug rosiglitazone, We have found that synergistic induction of muscle insulin sensitivity by the two interventions was responsible for marked beneficial effects of the combination treatment on both whole-body glucose homeostasis and metabolic flexibility. The third study was focused on the liver and the involvement of AMPK in beneficial treatment by the marine lipids. Using C57BL/6 mice with genetic disruption of one of the catalytic subunits of AMPK (AMPKα2 knock-out mice), we have revealed that AMPK is required for preservation of hepatic insulin sensitivity by n-3 PUFA in the context of high-fat-feeding. In the last study, conducted using C57BL/6 mice, 5′-iodothyronine deiodinase (deiodinase 1) activity in adipose tissue was found to be stimulated by leptin, and, therefore, a novel regulatory mechanism controlling lipid metabolism in adipose tissue and possibly also accumulation of the tissue was described.

In conclusion, this thesis provides new findings in the field of obesity and regulation of energy metabolism, and it also supports the importance and power of using specific mouse strains in the field of experimental obesitology, as well as the requirement of proper choice of the right strain for studying specific topics and hypothesis.

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

A: Kus V, Prazak T, Brauner P, Hensler M, Kuda O, Flachs P, Janovska P, Medrikova D, Rossmeisl M, Jilkova Z, Stefl B, Pastalkova E, Drahota Z, Houstek J, Kopecky J. Induction of muscle thermogenesis by high-fat diet in mice: association with obesity-resistance.
Am J Physiol Endocrinol Metab 295:E356-67. 2008 (IF = 4.129)

B: Kuda O, Jelenik T, Jilkova Z, Flachs P, Rossmeisl M, Hensler M, Kazdova L, Ogston N, Baranowski M, Gorski J, Janovska P, Kus V, Polak J, Mohamed-Ali V, Burcelin R, Cinti S, Bryhn M, Kopecky J. n-3 fatty acids and rosiglitazone improve insulin sensitivity through additive stimulatory effects on muscle glycogen synthesis in mice fed a high-fat diet. *Diabetologia* 52: 941-51. 2009 (IF = 6.328)

C: Jelenik T, Rossmeisl M, Kuda O, Jilkova ZM, Medrikova D, Kus V, Hensler M, Janovska P, Miksik I, Baranowski M, Gorski J, Hébrard S, Jensen TE, Flachs P, Hawley S, Viollet B, Kopecky J. AMP-activated protein kinase {alpha}2 subunit is required for the preservation of hepatic insulin sensitivity by n-3 polyunsaturated fatty acids.
Diabetes 59:2737-46. 2010 (IF = 8.261)

D: Macek Jílková Z, Pavelka S, Flachs P, Hensler M, Kůs V, Kopecký J. Modulation of type I iodothyronine 5'-deiodinase activity in white adipose tissue by nutrition: possible involvement of leptin.
Physiol Res 59:561-569. 2010 (IF = 1.739)

The above papers are included in full in this PhD thesis. For the complete list of my published articles, see List of publications.

LIST OF ABBREVIATIONS

AICAR	aminoimidazole carboxamide ribonucleotide
AMPK	AMP–activated protein kinase
BAT	brown adipose tissue
B/6J	C57BL/6J mouse strain
cHF	corn oil-based composite high-fat diet
cHF+F	cHF diet supplemented with fish oil
cHF+F+TZD	cHF+F diet supplemented with thiazolidinedione
cHF+TZD	cHF diet supplemented with thiazolidinedione
CLS	crown-like structures
D1	type I iodothyronine 5'-deiodinase
D2	type II iodothyronine 5'-deiodinase
D3	type III 5-deiodinase
DHA	docosahexaenoic acid (22:6 n–3)
EPA	eicosapentaenoic acid (20:3 n–5)
FA	fatty acid
HMW	high molecular weight form of adiponectin
IR	insulin resistance
LF	Low Fat standard chow diet
LMW	low molecular weight form of adiponectin
MMW	medium molecular weight form of adiponectin
NEFA	non-esterified fatty acids
PPAR	peroxisome proliferator–activated receptor
PRCF	percent relative cumulative frequency
PUFA	polyunsaturated fatty acids
RER	respiratory exchange ratio (=respiratory quotient)
SCD-1	stearoyl-CoA desaturase
SM	skeletal muscle
T_3	3, 5, 3′, - triiodothyronine
T_4	thyroxine
TH	thyroid hormones
TG	triacylglycerols
TNF	tumor necrosis factor

TR	thyroid receptor
TZDs	thiazolidinediones
UCP-1	uncoupling protein - 1
VO_2	oxygen consumption
WAT	white adipose tissue

CONTENTS

1 INTRODUCTION

Obesity and associated metabolic disorders - joined in the term “metabolic syndrome” - currently represent a relevant social and economical problem of public health. The prevalence of obesity is rapidly rising and has already been proclaimed by the WHO to be a pandemic disease. In principal, from an energy balance point of view, obesity is a consequence of long-lasting and massive accumulation of energy stores resulting in lots of adverse effects on metabolism and health. Experiments using mouse models of obesity provide a strong and advantageous research approach to deeper understanding of the regulation of metabolism and metabolic pathways as well as a unique opportunity to manipulate specific genes and create various mouse strains and knockouts.

1.1 Energy homeostasis

1.1.1 Principles of energetics

Organisms work on the principle of an open thermodynamic system, they interact with surroundings and transform accepted energy to work and heat. The whole-body energy balance is dependent on the ratio between energy intake and energy expenditure. When these two components are equal the system is in balance. In the case of an imbalance of one of the components the system starts to degrade or accumulate energy stores. Thus obesity represents positive- and, for example, fasting, negative energetic status. Both energy expenditure and energy intake are controlled centrally by specific regions in the brain. Regulation of energy intake is based on response of these regions to specific signals from peripheral tissues, mainly represented by hormones secreted by intestine, adipose tissue, liver and other tissue. The hypothalamus has long been appreciated to be fundamental in the control and coordination of peripheral homeostatic activity. It is composed of a few specific nuclei with different roles in the regulation of energy homeostasis. The regulation is mediated by neuroendocrine and neuronal pathways. Nowadays the supra-chiasmatic nucleus (SCN) is the centre of attention because it is where the mammalian biological clock resides. It is already known that whole body glucose metabolism and lipid metabolism in the liver and WAT are controlled by the circadian timing system (for details see review (1)).

1.1.2 Energy expenditure

Energy expenditure is under the control of specific nuclei of the hypothalamus, releasing the signals to the peripheral tissue mainly via the sympathetic nervous system. Muscular work, skeletal muscle shivering thermogenesis and non-shivering thermogenesis are the dominant mechanisms of the energy expenditure. Basal metabolic rate is measured under the conditions when none of the above mechanisms is activated and the organism is at rest. Further energy spent on different kinds of stimuli like food, cold or stress is defined as adaptive thermogenesis. Adaptive thermogenesis is regulated not only by the sympathetic nervous system but also by thyroid hormones and insulin. Adaptive thermogenesis is mainly based on lipid catabolism (2).

1.1.3 Adaptive thermogenesis

In small mammalian species, hibernators, and mammalian neonates, adaptive thermogenesis depends on brown adipose tissue and in mitochondria highly expressed uncoupling protein-1 (UCP1). Thermogenesis in brown fat can be activated in response to both cold exposure and a meal (3). Brown fat thermogenesis serves to maintain both body temperature and energy balance. However, the capacity does not exceed 60 % of total adaptive non-shivering thermogenesis (reviewed in (2)), suggesting a role for other organs in this process [(4), (5), (6), (7)] Also, skeletal muscle is an important site of whole body energy expenditure. Differences in resting muscle metabolism explain part of the variance in resting metabolic rate among adult humans and may play a role in the pathogenesis of obesity (8).

Adipocyte hormone leptin plays a unique role in the complex control of energy homeostasis and thermogenesis acting both centrally in the hypothalamus and also directly in the peripheral tissues (see chapter 1.3.2.1.1). The administration of leptin reverses reduced metabolic rate, depression of body temperature, and excessive adiposity in genetically obese ob/ob mice lacking functional leptin (9). Even in normal mice, leptin induces the capacity for UCP1-mediated thermogenesis (10), and it also stimulates lipid oxidation and uptake of glucose in skeletal muscle by activating AMP-activated protein kinase (AMPK). The direct thermogenic effect of leptin was also demonstrated in murine skeletal muscle, where exogenous leptin stimulated oxygen consumption (11).

1.1.4 Thyroid hormones

Hormones synthesized in the thyroid gland play a key role in the control of metabolic rate. Thyroid hormones (TH) increase the rate of aerobic metabolism, especially by accelerating ATP turnover and heat production. TH are thus responsible not only for obligatory thermogenesis but also act in facultative thermogenesis especially in BAT (12). It is documented in hypothyroid rats, which do not survive cold exposure and fail to increase BAT thermal production (13). The interaction between TH and the sympathetic nervous system is mediated via α and β adrenergic receptors (14). In BAT and other tissue the optimal action of TH is controlled not only by regulation of TH entry through cell membrane but also, and more importantly, by the generation of active TH. Enzymes called deiodinases are responsible for the conversion of T4 to active form. Deiodinase 1 and 2 (D1, D2) are 5′-iodothyronine deiodinases that catalyze TH activation by converting thyroxine (T4) to triiodothironone (T3). D3, a 5-deiodinase, is the main TH inactivator through conversion of T4 to rT3 and T3 to T2. Deiodinases are differentially expressed in tissues (in BAT and the liver mainly D2 and in WAT D1) and are further regulated at the level of transcription, translation, and metabolism by alterations in the intracellular environment (15). For adaptive thermogenesis in BAT based on UCP1 activated by noradrenalin, TRα-mediated pathway is responsible, whereas TRβ activated an alternative facultative thermogenesis with less potential [(16), (17), (18)].

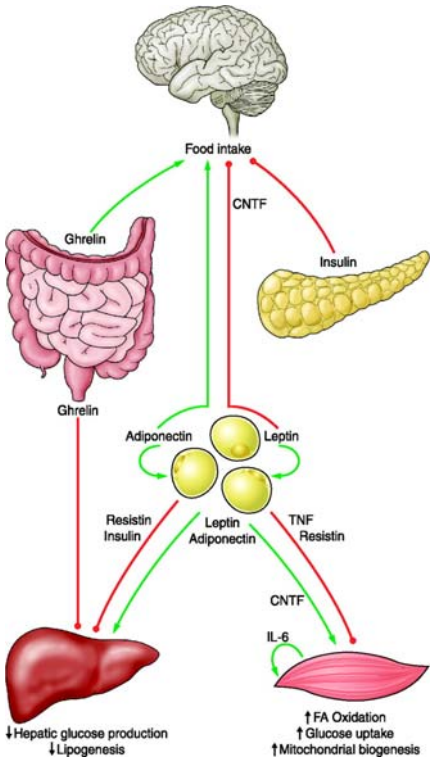
1.2 Central regulation of energetic metabolism in the cell

1.2.1 AMP–activated protein kinase

One of the most important enzymes responsible for the regulation of energy homeostasis at the cellular and whole body level is enzyme sensitive to ATP/AMP ratio, AMP-activated protein kinase (AMPK). Currently AMPK is the centre of attention in studies focused on metabolic syndrome and accompanying diseases. AMPK is a heterotrimeric protein consisting of a catalytic α subunit and regulatory β and γ subunits (19). Tissue specific forms of AMPK can differ in subunit composition. In the liver β1 is mainly expressed and an approximately similar amount of α1 a α2 whereas in the muscle α2 and β2 subunits predominate. This enzyme is activated by energy-consuming pathways like exercise (muscle contraction), starvation or hypoxia i.e. in states when ATP/AMP ratio is changing towards AMP (20). The activity of AMPK is regulated not only by AMP but also by its upstream kinases. One of the most important kinases is LKB1 which is responsible for AMPK activation during muscle

contraction (21). Another important upstream kinase is CaMKK. CaMKK is activated by release of Ca^{2+} in cytoplasm (22). Furthermore, it is activated by specific hormones e.g. adipokines leptin and adiponektin [(23), (24)], n-3 polyunsaturated fatty acids (PUFA) and antidiabetic drugs like thiazolidinediones (25) and metformin (26). Already a few specific activators of AMPK exist; the most known and used is AICAR and more specifically Abbott compound.

Figure 1 Hormonal regulation of AMPK signaling



In the brain, increased activity of AMPK promotes food intake. In skeletal muscle, AMPK activation promotes energy expenditure through upregulation of fatty acid oxidation, glucose uptake, and mitochondrial biogenesis. In the liver, AMPK suppresses hepatic glucose production and lipogenesis. Green arrow, stimulation/activation of AMPK; red arrow, inhibition/deactivation. Adapted from (27)

1.2.2 Role of AMP-activated protein kinase in carbohydrate and lipid metabolism

1.2.2.1 Carbohydrate metabolism

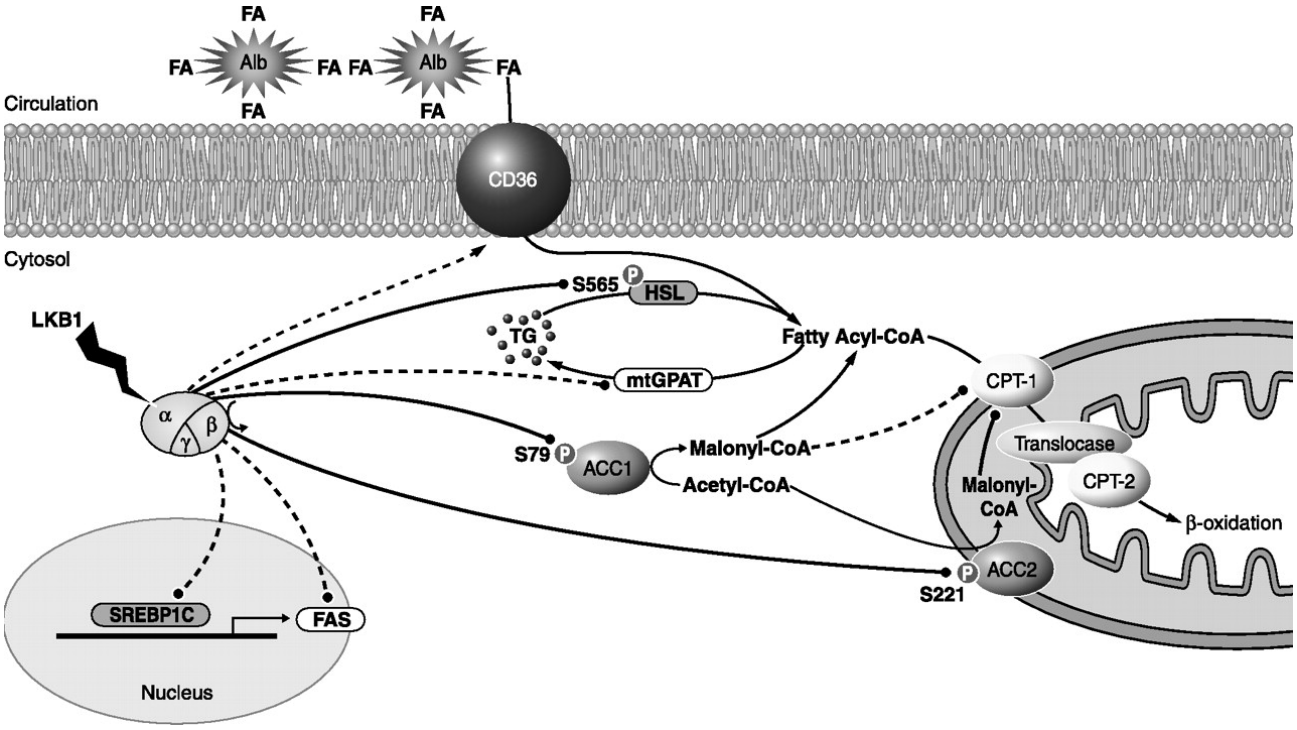
In the skeletal muscle, the major mechanism regulated by AMPK is glucose uptake. AMPK increases expression and translocation of glucose transporter CLUT4 and hexokinase therefore increases uptake of glucose (28). Regulation of gene expression is mediated via PPAR gamma coactivator (PGC1α) (29).

In the liver, AMPK suppresses glucose production via supression of key enzymes of gluconeogenesis phosphoenol-pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6P) [(30), (31)]. Furthermore AMPK regulates glycogen metabolism in the liver. Enzymes glycogen synthase and glycogen phosphorylase are under the control of AMPK (32).

1.2.2.2 Lipid metabolism

AMPK regulates uptake of fatty acids (FA) uptake mainly via gene expression of transporter FAT/CD36, fatty acid binding protein (FABP) (detailed in review (33)). Fatty acid oxidation is also under the positive control of AMPK (see more in the chapter 1.2.3.1). AMPK, when activated, inhibits fatty acid synthesis by inhibition of fatty acid synthase (FAS) (34). Further AMPK also regulates synthesis and turnover of triacylglycerols. For a representative scheme, see Fig. 2.

Figure 2 Lipid metabolism and the role of AMPK



AMPK controls the fate of fatty acids in the cell by controlling rates of uptake by inducing the translocation of CD36 to the plasma membrane. AMPK suppresses malonyl-CoA content by phosphorylating and inhibiting ACC1 and therefore suppressing fatty acid synthesis and increasing mitochondrial β-oxidation, respectively. Futile cycling of fatty acids is suppressed by AMPK inhibition of TG synthesis and TG hydrolysis through the phosphorylation of GPAT and HSL, respectively. AMPK also reduces FA synthesis by inhibiting the transcription factor SREBP1c, which controls the entire synthesis pathway or by directly inhibiting the activity of FAS.

Adapted from (27)

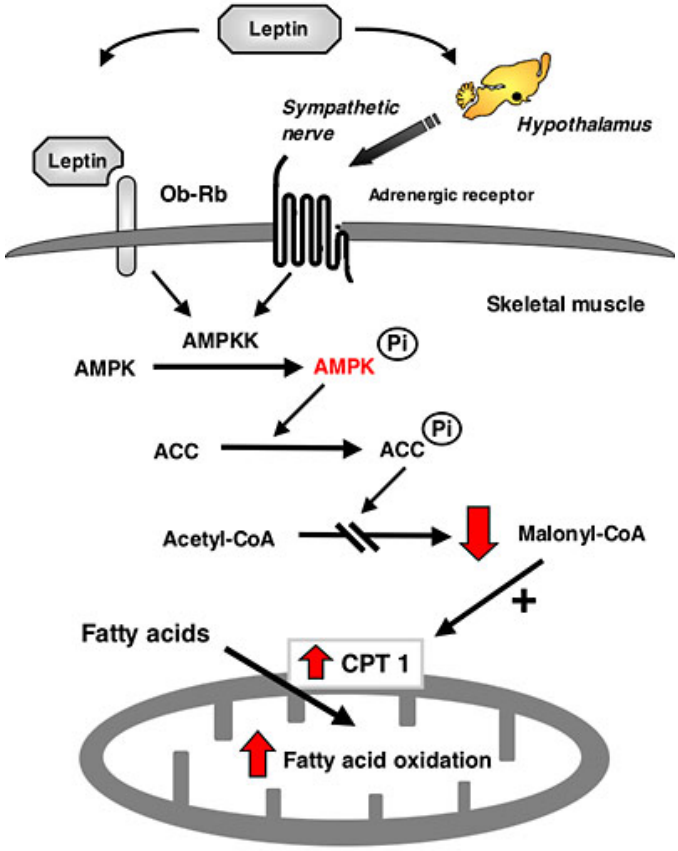
1.2.3 Modulation of AMP-activated protein kinase activity

1.2.3.1 Leptin – AMPK axis

In peripheral tissues like muscle, the adipocyte-secreted hormone leptin directly activates AMPK and fatty acid oxidation. The binding of leptin on the receptor activates a signaling pathway which selectively stimulates phosphorylation of catalytic α2 subunit of AMPK in muscle (23).

AMPK phosphorylates acetyl-CoA carboxylase (ACC), which is a rate-limiting enzyme in fatty acid synthesis, and therefore the amount of malonyl-CoA decreases. It leads to activation of carntine palmitoyl transferase-1 (CPT-1) which is inhibited by malonyl-CoA. CPT-1 mediates the transport of long-chain fatty acids across the membrane by binding them to carnitine. Therefore AMPK activates flux of fatty acids into mitochondria (see Fig.3).

Figure 3 Direct activation of β-oxidation by leptin in skeletal muscle



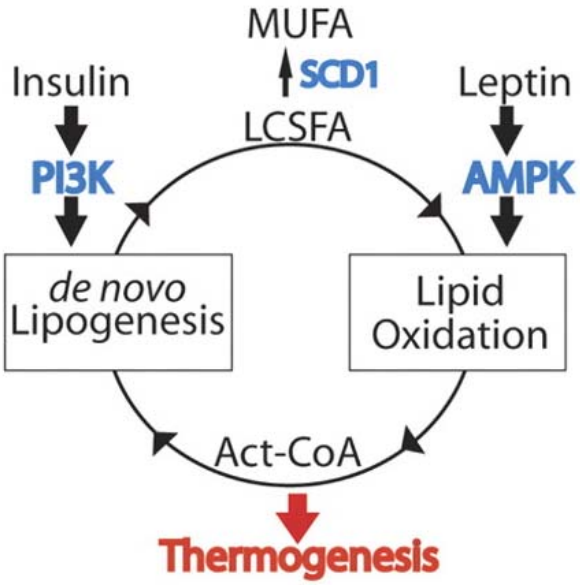
Adapted from www.biochem.arizona.edu website

1.2.4 Futile cycling

The substrate cycle that occurs when two metabolic pathways run simultaneously in opposite directions and have no overall effect except heat production, contributes to the total energy homeostasis. In principle" the results of this so called "futile substrate cycle" are that ATP energy is depleted, heat is produced and no net substrate-to-product conversion is achieved. Examples of substrate cycling are cycling of gluconeogenesis and glycolysis pathways and

cycling of the triglycerides and fatty acid pathways. In skeletal muscle, futile cycling between de-novo lipogenesis and FA oxidation was found, with the main regulators represented by leptin, AMPK, PI3 kinase and SCD-1 [(11), (35)]. This mechanism results in non-shivering muscle thermogenesis and it may importantly contribute to differential resistance to obesity among individuals.

Figure 4 Futile substrate cycling



Scheme illustrating repeated recycling of acetyl-CoA through the flux of substrates across de novo lipogenesis followed by mitochondrial β-oxidation constitutes an energy dissipating "substrate cycling" in skeletal muscle of mice fed HF diet with PI3K, AMPK, and SCD1 as control points in this effector of thermogenesis.
Adapted from (35)

1.3 Biology of the adipose tissue

Adipose tissue is no longer considered to be an inactive tissue with minor impact on whole body metabolism. Increasing interest in adipose tissue role in obesity and diabetes research more and more highlights the importance of adipose tissue in the regulation of energy metabolism and in metabolic syndrome development. There are two different types of adipose tissue with unique properties:

1.3.1 Brown adipose tissue

Brown adipose tissue (BAT) has a different origin than white adipocytes; brown adipocytes share a common origin with skeletal muscles in the paraxial mesoderm (36). Brown adipocyte is characterized by its typical morphology, with triacylglycerols stored multilocullary. These cells are rich in mitochondria, which are equippped by thermogenic uncoupling protein 1 (UCP1). BAT burns fat and it is specialized in energy expenditure. It is a key thermogenic organ of newborn mammals and hibernating animals, as brown adipocytes convert nutrients into heat by uncoupling respiration from ATP synthesis.

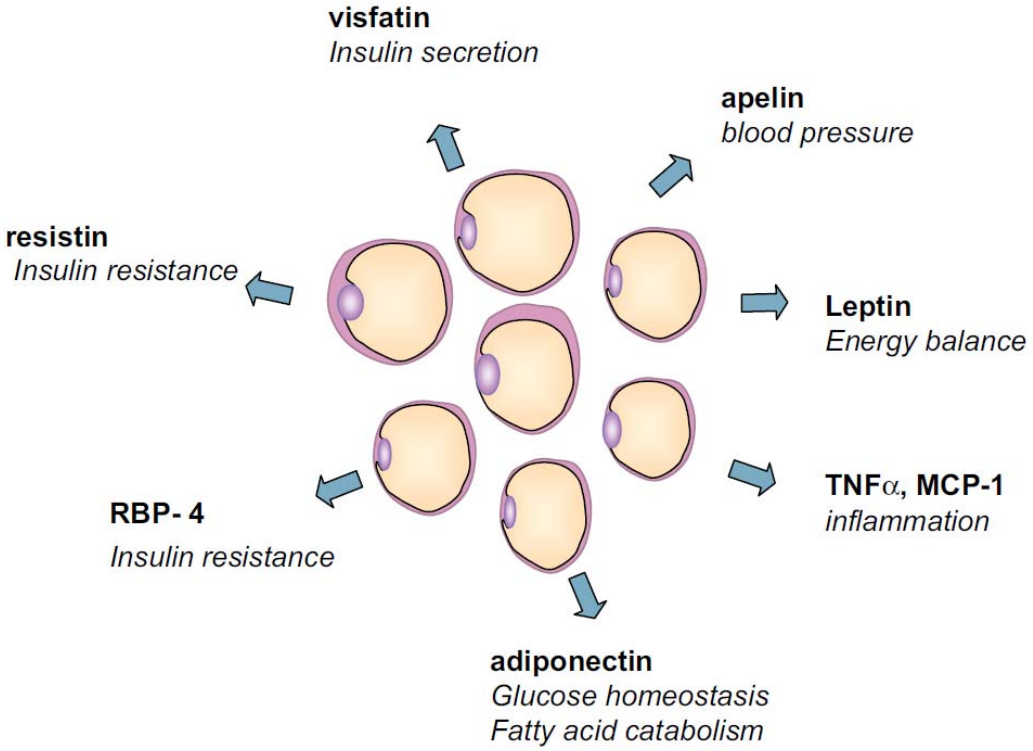
1.3.2 White adipose tissue

White adipose tissue consists of several different types of cells. Most abundant are adipocytes, which are suited for the prominent role of this tissue: the storage of triacylglycerols in one large lipid vacuole filling most of the intracellular space. Other cells contained in WAT are pre-adipocytes, fibroblasts, endothelial cells and immune cells – resident macrophages (37).

1.3.2.1 Endocrine function of white adipose tissue

Although the main function of WAT is to store triaclyglycerols and release them into blood as a substrate for other tissues, WAT is not only an energy storage organ. Adipose tissue secretes circulating hormones (see Fig.5) and adipokines that act as systemic inflammatory mediators and signals of the organism's nutritional status. Therefore the old paradigm that adipose tissue just slightly contributes to energetic metabolism is no longer valid. Furthermore, fat deposition within organs and around blood vessels appears to have important metabolic consequences. These mechanisms involve both a nutrient-sensing mechanism within adipocytes and other cells (autocrine effects), and intercellular (paracrine) or interorgan (endocrine) cross talk, representing a propagation of signals, particularly from fat, to entrain the metabolic cooperation with other target organs such as liver and skeletal muscle (38). Secretion profile of adipokines changes in obesity and is highly dependent on the adiposity.

Figure 5 Most important adipose tissue-secreted molecules



Adipocytokines modulate energy balance, insulin sensitivity, cardiovascular function and inflammation. MCP-1, monocyte chemoattractant protein-1; PAI-1, RBP-4, retinol binding protein-4; TNF-a, tumour necrosis factor-a; adapted from (39)

1.3.2.1.1 Leptin

Leptin, the first adipokine (i.e., a hormone synthesized in adipocytes) described, exerts anorexigenic effects (40). It is synthetised by mature adipocytes, released in the blood flow and thus acts in the regulation of appetite and body weight. Leptin level in plasma is a marker of adipose tissue amount, the more body fat content is the higher leptin concentration in plasma is detected (41). This information is processed in the central nervous system especially in the hypothalamus (42). The hypothalamus and specifically the arcuate nucleus contains leptin-sensitive neurons which regulate food intake and energy homeostasis by regulating the sympathetic action (43). But not only leptin but also insulin regulates activity of these neurons (see review (44)). An important role in this regualtion is played by orexigenic neuropeptide Y (NPY)-, agouti related protein (AGRP)-containing neurons. ARC also contains a population of anorexigenic pro-opiomelanocortin (POMC)-containing neurons (1).

Obesity is characterized by high concentration of leptin in plasma, and the absence of the effect of leptin on body weight or food consumption. This paradox is described as leptin resistance. Both leptin transport across the blood-brain barrier, and leptin signaling in neurons are impaired in obesity [(45), (46)]. Therefore, for therapeutic use of leptin, a few alternative approaches have been tested: (i) the use of new leptin analogues, which have longer half-lives and are freely transported across the blood-brain barrier (low molecular weight, high solubility in lipids), (ii) bypassing the the blood-brain barrier (intrathecal injection of leptin), or (iii) modulation of activities of leptin transporter systems by drugs [(47), (48)]

1.3.2.1.2 Adiponectin

Adiponectin as another major adipokine besides leptin, plays an important role in insulin sensitivity (49). In contrast to leptin and the majority of other adipokines, adiponectin levels in plasma negatively correlate with adiposity. Adiponectin is a 35 kDa protein which forms several isoforms with different response in tissues. It can act as a monomer, trimer, hexamer or multimer (12-18 units), described as LMW (low molecular weight, trimer), MMW (medium molecular weight, hexamer) or HMW (high molecular weight, ten or more units) (50). Adiponectin improves insulin sensitivity of peripheral tissue like skeletal muscle and liver via AMPK activation leading to enhanced fatty acid oxidation (24). Moreover, adiponectin facilitates glucose uptake in skeletal muscle and liver (50). This action is mediated by HMW form whereas LMW and MMW act centrally and increase food intake and decrease energy expenditure (51). These tissue-specific differences are detected also at the adiponectin receptors level. HMW and MMW forms interact preferentially with AdipoR2 receptors, whereas AdipoR1 receptors preferentially interact with MMW or LMW adiponectin.

1.4 Positive energy balance and obesity

When the regulatory mechanisms described above fail to maintain stable body weight, positive energetic balance leads to a massive triglyceride accumulation (obesity), which is frequently associated with insulin resistance. WAT serves as an exclusive tissue for storing triglycerides. When the TG content exceeds the storage capacity of this tissue, TG are massively stored also in other tissues and organs, namely in the liver, skeletal muscle, kidney and pancreas. Accumulation of TG in the tissues that are not suited for this is followed by

adverse metabolic consequences represented mainly by induction of insulin resistance (IR). The major pathologies in each tissue are described below.

1.4.1 White adipose tissue

Adipose tissue lipid overload is accompanied by structural and molecular changes in the tissue. Firstly, by the hypertrophy of adipocytes, followed by the infiltration of immune cells (mainly macrophages) activated by adipocytes death, induction of endoplasmic reticulum stress and hypoxia [(52), (53), 54)]. Furthermore, the profile of secreted adipokines is changed, which contributes to type 2 diabetes development (for the characteristics of adipokines, see chapter 1.3.2.1). Non-responsivness of adipose tissue to insulin action leads to hyperlipidaemia because insulin is not able to inhibit lipolysis in adipocytes and fatty acids and glycerol are released into circulation.

1.4.2 Skeletal muscle

Fatty acids are a major fuel in oxidative skeletal muscle and the heart. FA are directed towards either the synthesis of lipid metabolites or β-oxidation. When the FA uptake exceeds the rate of β-oxidation, intramuscular lipids are accumulated leading to deleterious effects on insulin resistance. Some of the lipid signaling metabolites, namely diacylglycerols (DAG), ceramide or TG, are associated with the activation of specific signaling pathways modulating insulin action, content of glucose transporter GLUT4 and induction of insulin resistance [(55) (56), (57)]. Insulin resistant muscle is not able to switch between substrates (between glucose and FA), this state is called metabolic inflexibility (58). Increase of β-oxidation rate leads to decrease of intramuscular lipids in the muscle, but its beneficial role in improving insulin resistance is still a subject of debate. The reason for this debate is observation of the incomplete fatty acid oxidation. High rate of β-oxidation leads to failure of muscle to completely oxidize FA and these acid soluble metabolites are accumulated (59). The mechanism by which incomplete β-oxidation leads to IR is still not fully understood.

1.4.3 Liver

Ectopic lipid accumulation in the liver results in pathology known as non-alcoholic fatty liver disease or hepatic steatosis. Mechanisms of insulin resistance development are similar to skeletal muscle. Lipid metabolites DAG, ceramide or TG affect the insulin signaling pathway.

Lack of the insulin action leads to inability to activate hepatic glycogen synthesis and suppress of hepatic glucose production (60). Protein kinase (PKCε) is the key mediator hepatic steatosis- induced insulin resistance (61).

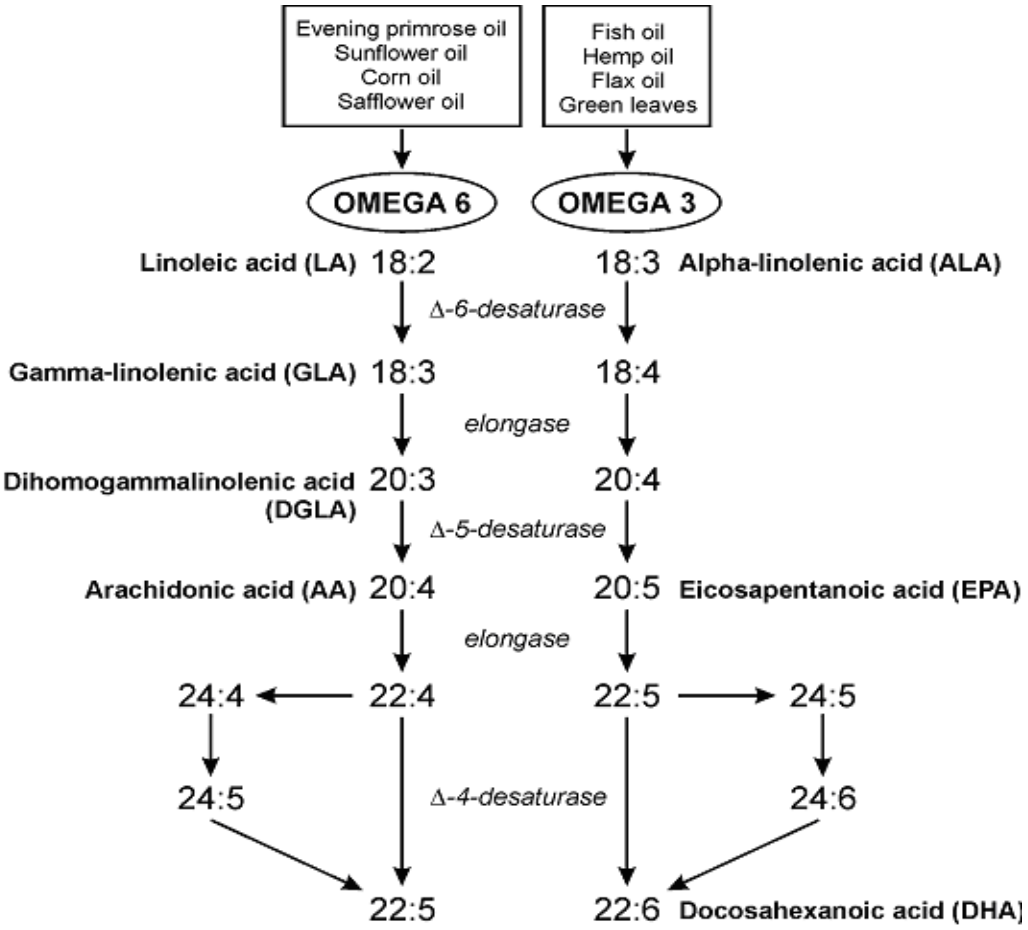
Insulin resistance of the most metabolically relevant tissues is a basis for type 2 diabetes development. Still increasing demands on insulin to retain stable glucose concentration in plasma lead eventually to the exhaustion and failure of pancreatic β-cells (62).

1.5 Important modulators of metabolism and insulin resistance

1.5.1 Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFA) of n–3 and n-6 family are biologically important fatty acids, which contain double bonds at C-3 and C-6 respectively, from the methyl end of the molecule. They are synthesized mainly by phytoplankton and found in high concentration in sea fish and some plants. For many mammalian species, including humans, they are essential. n–3 PUFA have a fundamental structural function in phospholipid membranes and act as regulatory ligands in gene transcription and a source of active lipid metabolites. As precursors, dietary-derived n–3 α–linolenic acid and n-6 linoleic acid are used, which are then elongated and desatureted by specific enzymes (for details, see Fig. 6). The most efficient and currently highly studied are members of the n-3 family of PUFA; eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA). The major n-3 PUFA of plant origin, alpha-linoleic acid (ALA) is rapidly oxidised in the organism and its conversion to EPA and DHA is quite inefficient (63). Hence, fish oil is the most effective source of EPA and DHA for mammalians.

Figure 6 Metabolic pathways of long-chain PUFA



1.5.1.1.1 Molecular mechanism of polyunsaturated fatty acids action

Incorporation of n-3 and n-6 PUFA into plasmatic membranes increases fluidity of membrane and can therefore influence activity of membrane-associated enzymes. Already a few molecular targets of n-3 PUFA have been identified. There are mainly nuclear transcriptions factors. It results in an up-/down regulation of gene expression of key enzymes of lipid and carbohydrate metabolism. This action is mediated mainly by the following transcription factors: peroxisome-proliferator activated receptors (PPARs) α, β/δ, γ; retinoid X receptors; liver X receptors, hepatic nuclear factor-4 and SREBPs, ChREBP [(64), (65), (66), (67)].

n-3 and n-6 PUFA are also a target for specific enzymes creating active lipid molecules. By the action of lipoxygenase and cyclooxygenase, eicosanoids and docosanoids are formed, while targeted enzymatic synthesis provides resolvines and protectins. These active metabolites have strong anti-inflammatory effects. (68)

All the mechanisms involved in the beneficial effects of n-3 PUFA have been shown to involve AMPK activation. Some studies have already shown that feeding of n-3 PUFA increased activity of AMPK in the liver, adipose tissue and intestine [(69), (70), (71)].

1.5.1.2 n-3 polyunsaturated fatty acids and insulin resistance

n-3 PUFA have a beneficial role in the prevention of obesity and insulin resistance. Intake of n-3 PUFA results in a decrease of adiposity and prevention of diet-induced obesity (72). Prevention of insulin resistance by n-3 PUFA is mainly based on its ability to suppress lipogenesis and triaclyglycerol accumulation in skeletal muscle and thus eliminating the lipotoxicity effect (73).

1.5.2 Thiazolidinediones

Thiazolidinediones (TZDs), like roziglitazone, pioglitazone or troglitazone are potent anti-diabetic medicaments, which increase insulin-sensitivity. The members of the TZD family differ in side-chain arm. TZDs improve glycaemic control mostly by repartitioning fat away from skeletal muscle (74), while augmenting insulin action in liver, adipose tissue and skeletal muscle [(75), (76)]. Clinical studies have demonstrated that TZDs reduce accumulation of hepatic lipids (steatosis), which is frequently associated with systemic IR, and can be also used for the treatment of nonalcoholic steatohepatitis (77). Treatment with TZDs reduces adipocyte hypertrophy and low-grade inflammation of adipose tissue in obesity, while inducing secretion of insulin-sensitizing hormone adiponectin (78). TZDs act as potent PPARγ ligands; they bind directly, and as a consequence change gene expression, or stimulate AMPK in liver, skeletal muscle and other tissues (79). TZDs are also known to have adverse side-effects, like body weight gain, water retention and increased risk of heart failure (80).

2 AIMS OF THE THESIS

The general goal of the thesis was to examine metabolic disorders caused by energy surplus at cellular and whole-body level using mouse models suitable for investigations on the pathophysiology of obesity and associated diseases. The main focus was on disorders of the most metabolically active tissues, namely skeletal muscle, liver and adipose tissue. The keystones of this thesis are energy metabolism, and the regulatory roles of AMPK and leptin.

The specific aims were:

A. To learn, using obesity-prone C57BL/6J and obesity-resistant A/J of mice, whether muscle non-shivering thermogenesis could contribute to differential susceptibility to obesity of these two strains. Namely whether induction of lipid catabolism in the muscle by HF diet-feeding *via* the leptin-AMPK axes could be involved in the obesity-resistant phenotype of mice of the A/J strain.

B. To evaluate, using the C57BL/6J mouse model of dietary obesity, whether beneficial metabolic effects of n–3 PUFA could be augmented by combination treatment using TZD, with the main focus on changes in muscle metabolism, its sensitivity to insulin, and whole-body metabolic flexibility.

C. To investigate the role of AMPK in preservation of insulin sensitivity in response to n-3 PUFA admixed to HF diet, using the obesity-prone C57BL/6J mice with genetically disrupted gene for the α2 catalytic subunit of AMPK and their wild-type littermates, with the main focus on the hepatic metabolism and its insulin sensitivity.

D. To characterize the involvement of the metabolism of thyroid hormones in white adipose tissue, namely the conversion of T4 into T3 mediated by D1, in the adaptive response to HF diet-feeding in the obesity-prone C57BL/6J mice, namely the role of D1 in the control of adiposity by leptin and the involvement of D1 in adipocytes in this mechanism.

3 RESULTS OF SELECTED PUBLICATIONS

3.1 Publication A: Leptin, AMPK and muscle non-shivering thermogenesis

Synopsis of results of the article "**Induction of muscle thermogenesis by high-fat diet in mice: association with obesity-resistance"**, by Kus et al, published in American Journal of Physiology 2008.

The objective of this study was to learn, using obesity-prone C57BL/6J and obesity-resistant A/J of mice, whether muscle non-shivering thermogenesis could contribute to differential susceptibility to obesity of these two mouse strains and especially whether induction of lipid catabolism in oxidative skeletal muscle by HF diet-feeding *via* the leptin-AMPK axes could be involved in the obesity-resistant phenotype of mice of the A/J strain.

Experiments were performed on male mice of B/6J and A/J strains, born and maintained at a temperature of 30 °C, temperature closed to thermoneutrality, to eliminate the effect of cold stress. At the age of four weeks, mice were randomly weaned to a low fat (LF/chow), containing 25, 9, and 66 % calories as protein, fat, and carbohydrate, respectively or a high fat (HF) diet, proved to be obesogenic, containing 13, 60, and 27 % calories as protein, fat, and carbohydrate, respectively. The majority of the measurements were performed following 2 weeks after the differential feeding began, i.e. at the age of 6 weeks.

Obesity resistance in A/J compared to B/6J mice was clearly seen during prolonged feeding by the diets (Fig. 1 of the publication A). But at the time of dissection no difference in body weight was observed (Tab. 1). However, weight of WAT depots was significantly increased by HF diet feeding in both strains. A strong effect on leptinaemia was found in A/J mice fed an HF diet (Tab. 1). There was a big difference in the thermogenic capacity between the mice of the two strains. During cold exposure (4 °C), the A/J LF mice became hypothermic, but the HF diet feeding reverted this negative phenotype and the mice maintained a stable core body temperature similarly to B/6J where no effect of diet was found. HF diet induced UCP1-mediated thermogenesis in both strains, with stronger induction in A/J mice.

Table 1 Growth characteristics and plasma parameters

Fat depot	B/6J		A/J	
	LF	HF	LF	HF
BW (g)	18.6 ± 0.3	18.4 ± 0.5	17.0 ± 0.7	18.4 ± 0.7
BWG (g)	5.63 ± 0.39	4.91 ± 0.20	4.71 ± 0.50	5.31 ± 0.28
FC (kcal/day)	7.74 ± 0.8	7.78 ± 0.4	8.06 ± 0.7	8.11 ± 0.4
Weight of fat depots (mg)				
BAT	90 ± 3	57 ± 3[a]	69 ± 3[b]	61 ± 3[a]
DL	152 ± 4	180 ± 10[a]	167 ± 9	252 ± 14[ab]
EPI	160 ± 8	239 ± 21[a]	163 ± 14	292 ± 26[a]
Plasma levels				
TG (mg/dl)	148 ± 12	133 ± 19	139 ± 11	130 ± 7
NEFA (μM)	264 ± 2	346 ± 18[a]	274 ± 10	273 ± 18
Leptin (ng/ml)	4.48 ± 0.38	5.21 ± 0.54	3.35 ± 0.34[b]	9.42 ± 1.15[ab]
T4 (nmol/L)	37 ± 2	46 ± 2[a]	45 ± 1[b]	45 ± 3
T3 (nmol/L)	1.5 ± 0.2	1.9 ± 0.2[a]	1.9 ± 0.2[b]	1.9 ± 0.2

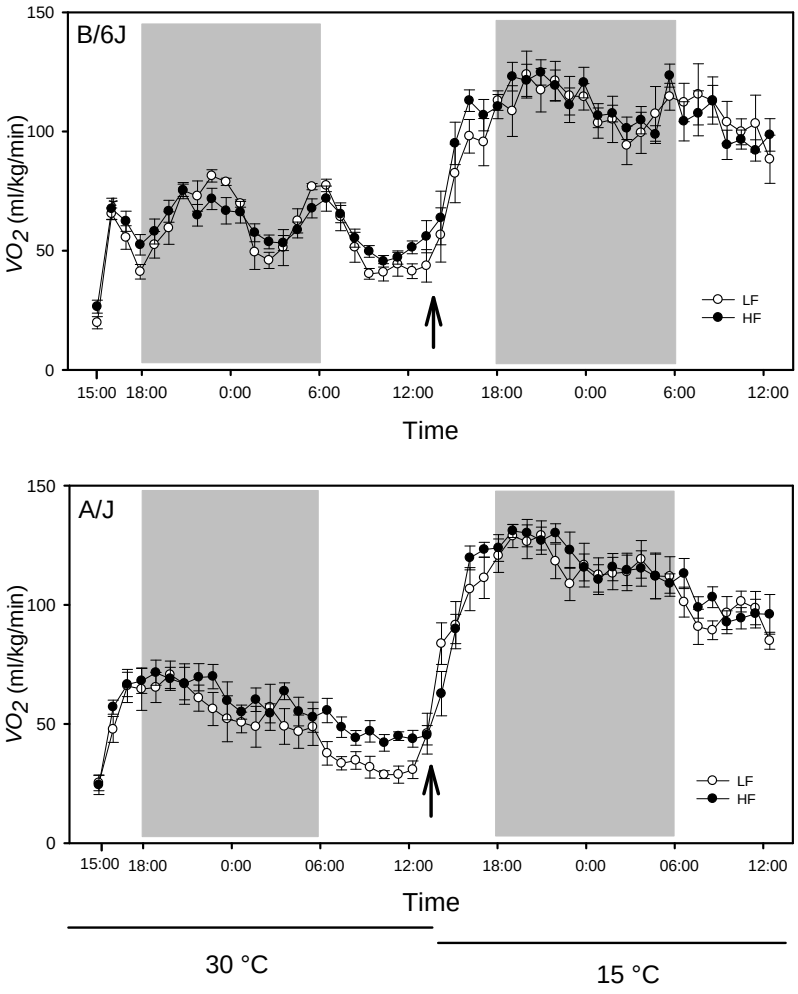
Six-week-old mice reared at 30 ^{o}C and weaned at 4 weeks after birth onto LF or HF diets were analyzed. Mean body weight at the time of weaning was similar in all subgroups of mice (12.3 – 13.5 g). BW, body weight at 6 weeks of age; BWG, gain of body weight during a period of 2 weeks after weaning; FC, mean food consumption measured at day 2, 4, 9, and 13 after weaning; BAT, interscapular brown fat; DL, dorsolumbar white fat; EPI, epididymal white fat. Data are means ± S.E. (n = 11-14). [a]Significant effect of diet; [b]significant effect of genotype.

Skeletal muscle was found to be most influenced by HF diet feeding in A/J mice. Especially oxidative type of skeletal muscle, *soleus musculus*. Muscles dissected from the A/J mice fed the HF diet showed significantly increased ex-vivo respiration as compared to the LF diet group. Further measurements of fatty acid oxidation showed that only in A/J mice HF diet increased the rate of fatty acid catabolism, especially after activation by AMPK-activator AICAR (aminoimidazole carboxamide ribonucleotide). This finding was supported by the measurement of expression of the genes involved in lipid and carbohydrate metabolism in the muscle. The major findings are that HF diet upregulated the expression of pyruvate dehydrogenase kinase 4 gen (*Pdk-4*) that is associated with suppression of glucose oxidation. Moreover, HF diet downregulated the expression of *Scd-1* in all studied subgroups with the most potent suppression in *soleus* muscle of the A/J HF mice. This is in agreement with higher plasma leptin concentrations in these mice, since *Scd-1* is suppressed by leptin. Finally changes in key enzyme of cell energetics – AMPK were assessed using western blotting. In

the A/J HF mice, both the total content of AMPK and its phospohorylation increased (see Fig. 8 of the Publication A). .

The changes in the metabolism described above were confirmed at the whole body level using indirect calorimetry. In A/J mice, HF diet feeding led to increase of metabolic rate (Fig. 7), measured as oxygen consumption, and during cold exposure shift towards lipid partionning was observed (Fig. 8).

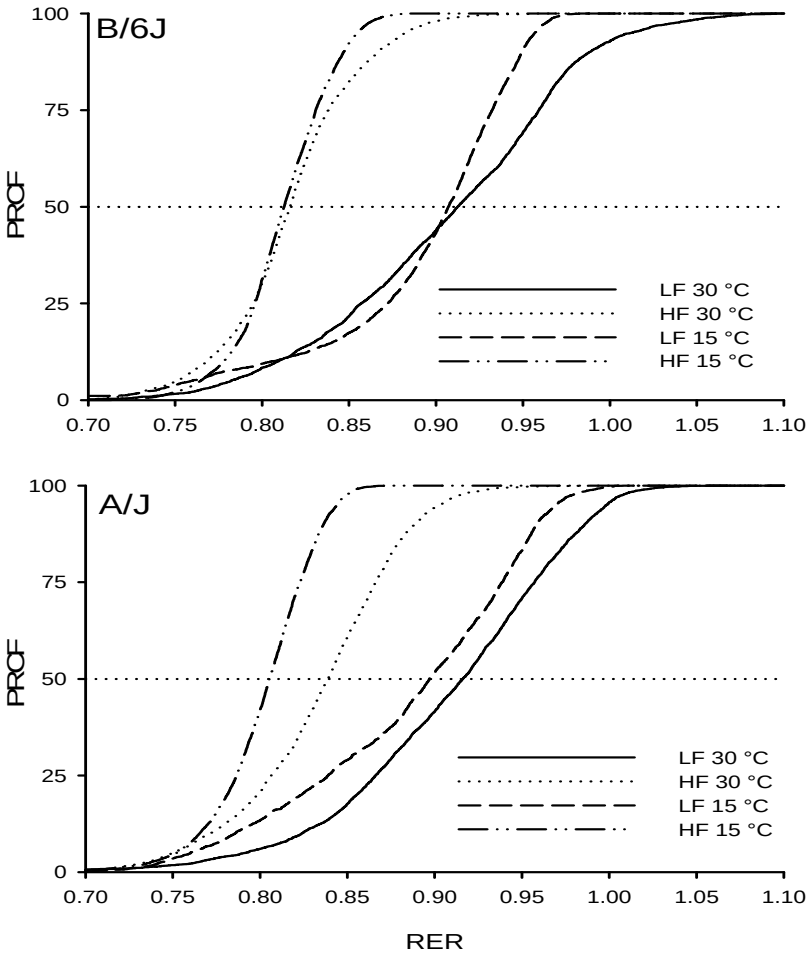
Figure 7 Time course of VO_2 measurements



Time course of VO_2 measurements in mice maintained at 30 °C and fed either LF or HF diet for 2 weeks after weaning. Indirect calorimetry was performed on singly-caged mice with a free access to water and diet, initially at 30 °C for a period of 23 h, followed by 23 h at 15 °C. The *VO2* data were sampled at 60 sec for every 2 min, however, only mean values of single recordings at 1-h intervals are shown. Arrows indicate the beginning of a 30-min period during which the temperature was dropped. Gray areas represent the dark phases of diurnal cycle. A biphasic circadian rhythm of VO_2 has been observed in B/6J mice, with 2 maxima, one in the middle of the day and the other at the end of the dark phase. This pattern has been only marginally affected by the diet. In contrast, in the A/J mice, a monophasic rhythm of

VO2 was recorded, with a maximum around the beginning of the dark phase of the day and lower VO2 values recorded during the light phase. Data are means ± S.E. (n = 6-8)

Figure 8 Plots of PRCF of RQ values obtained by indirect calorimetry



RQ data from the experiment were used to construct PRCF curves (Percent Relative Cumulative Frequency), each of which represents the data pooled from all mice (n = 6-8) within a given subgroup (~4,200-5,600 RQ measurements per curve). For the statistical analysis, see Methods in the publication. Values of logEC50 (50th percentile values) are significantly different between the HF- and LF-fed mice within each genotype and irrespective of the experimental temperature (p< 0.001). Only in the HF A/J mice, values of logEC50 at 15 °C and 30 °C are also significantly different (p = 0.004). At 15 °C, the Hill-slope values are significantly different between the HF- and LF-fed mice within each genotype, while at 30 °C, a significant difference between the HF- and LF-fed mice was found only in B/6J mice.

All the results indicated increased fatty acid oxidation, enhanced lipid catabolism and thermogenesis in oxidative skeletal muscle in response to HF diet in the obesity-resistant A/J mice, with involvement of leptin-AMPK axis, whereas in B/6J mice, none of these effects

was observed. Together with increased UCP1-mediated thermogenesis in BAT, both mechanisms contribute to the obesity-resistant phenotype of A/J mice.

My contribution to this work was the management and coordination of the experiments, and the in vivo and ex vivo experiments, except for the meaurements of the shivering- and NE-induced thermogenesis.

3.2 Publication B: n-3 fatty acids and thiazolinediones in combination

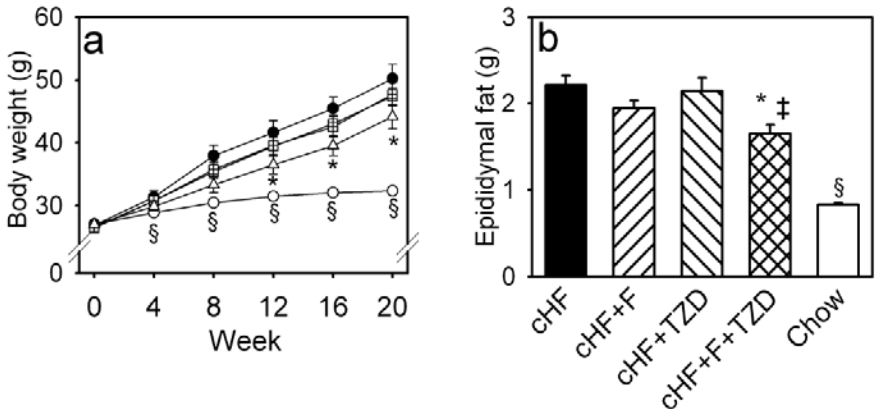
Synopsis of results of the article "**n–3 fatty acids and rosiglitazone improve insulin sensitivity through additive stimulatory effects on muscle glycogen synthesis in mice fed high-fat diet"**, by Kuda et al, published in Diabetologia 2009.

The objective of this study was to evaluate, using the C57BL/6J mouse model of dietary obesity, whether beneficial metabolic effects of n–3 PUFA could be augmented by a combination treatment using TZD, with the main focus on changes in muscle metabolism, its sensitivity to insulin, and whole-body metabolic flexibility. Thus, this study tested the effects of (i) EPA/DHA (replacing 15 % of dietary lipids), (ii) low dose of rosiglitazone admixed to the diet, and (iii) combination of both. In fact, two separate studies were performed, which differed in the phase of development high fat diet-induced obesity.

In the first, a "prevention study," non obese male mice (C57BL/6J) were used. As demonstrated by the results of the project described above (Publication A), this mouse strain represents a suitable and favorable model for studying the obesity development or reversal. The purpose of this experiment was to characterize effects of n–3 PUFA, rosiglitazone and their combination on developing obesity and impaired glucose tolerance against a background of a high-fat diet. After weaning, mice were fed by a standard chow diet and at three months of age they were randomly assigned to a corn oil-based high-fat diet (cHF; lipid content ~35.2% wt/wt, mainly corn oil) or to the following treatments: (i) cHF diet supplemented with EPA and DHA (cHF+F) as concentrate of n–3 PUFA (46% DHA, 14% EPA; 1050TG; EPAX, Lysaker, Norway) replacing 15 % of dietary lipids; (ii) cHF diet supplemented with rosiglitazone (cHF+TZD) (10 mg/kg diet); and (iii) cHF diet supplemented with EPA, DHA and rosiglitazone (cHF+F+TZD). One group of mice was maintained on the standard chow diet. Various analyses were performed at different time points between 5 to 20 weeks after initiation of treatments.

cHF diet induced development of obesity. Already at the 4 week time point there was a significant difference compared to the chow diet (Fig. 9). It was observed that a 12 week combinational treatment cHF+F+TZD resulted in significantly lowered body weight gain compared to the control cHF fed group (Fig. 9a). This trend was clearly seen also at the weight of adipose tissue depot at dissection (Fig. 9b). This was accompanied by adipocyte hypertrophy and increased content of macrophages, indicating inflammation of adipose tissue, all treatments (i.e. cHF+F, cHF+TZD and cHF+F+TZD) lowered the inflammation compared to the cHF, with strongest effect observed with the cHF+F+TZD mice. (see Fig. 1 of the Publication B).

Figure 9 Body weight and weight of epididymal fat



Body weight (**a**) and weight of epididymal fat (**b**). Three-month-old mice were placed on cHF diet or various cHF-based diets (cHF+F, cHF+TZD and cHF+F+TZD), or maintained on a chow diet; this treatment lasted for up to 20 weeks. **a** Body weights during 20-week treatment by cHF (black circles), cHF+F (white squares), cHF+TZD (crossed squares), cHF+F+TZD (white triangles) or chow (white circles) diet (n=16). (**a–b**) (n=7–8). Data are means±SE *$p\leq$ 0.05 for difference from cHF; $^{\dagger}p\leq$ 0.05 for difference from cHF+F; $^{\ddagger}p\leq$ 0.05 for difference from cHF+TZD (ANOVA); $^{\S}p\leq$ 0.05 for difference from cHF (t test).

Indirect calorimetry at 8 weeks did not reveal any effect of the treatments on energy expenditure or respiratory quotient in mice with free access to either diet. But there were effects on metabolic flexibility tested using "diet switch protocol" (for details see Tab. 2). This protocol was developed to test the ability to switch metabolism between lipid and sugar combustion. An increase of respiratory quotient (RQ) towards glucose oxidation is a marker of metabolic flexibility. In the first period, animals were analyzed on their HF-based diets (3 p.m.-8 a.m. of the following day), then food was removed, and at 6 p.m. all the animals were

re-fed by standard chow diet until end of the measurement (6 p.m. - 8 a.m.). It was seen that mice from the combination treatment group increased RQ values, showing thus a similar phenotype as the mice fed chow diet, and indicating that the combination treatment promoted normal metabolic flexibility. These results have not been published so far.

Table 2 VO_2 and RQ

	Dietary treatment			
	cHF	**cHF+F**	**cHF+TZD**	**cHF+F+TZD**
VO_2 (ml/min)				
Original cHF-diets				
Whole period	2.01 ± 0.07	2.01 ± 0.07	1.98 ± 0.07	1.99 ± 0.06
Light	1.85 ± 0.08	1.86 ± 0.07	1.92 ± 0.12	1.87 ± 0.07
Dark	2.15 ± 0.06	2.17 ± 0.08	2.03 ± 0.07	2.12 ± 0.04
Re-feeding STD diet				
Whole period	1.81 ± 0.10	1.86 ± 0.08	1.85 ± 0.08	1.80 ± 0.10
Light	1.54 ± 0.07[c]	1.63 ± 0.04[c]	1.63 ± 0.05[c]	1.53 ± 0.09[c]
Dark	2.09 ± 0.06	2.09 ± 0.05	2.04 ± 0.03	2.06 ± 0.06
RQ				
Original cHF-diets				
Whole period	0.81 ± 0.01	0.81 ± 0.01	0.80 ± 0.01	0.84 ± 0.01
Light	0.77 ± 0.01[c]	0.78 ± 0.01	0.78 ± 0.01	0.81 ± 0.01[ab]
Dark	0.82 ± 0.01	0.82 ± 0.01	0.81 ± 0.01	0.86 ± 0.01[ab]
Re-feeding STD diet				
Whole period	0.80 ± 0.01	0.86 ± 0.02[acd]	0.82 ± 0.01	0.91 ± 0.01[abcd]
Light	0.81 ± 0.01	0.85 ± 0.02[cd]	0.83 ± 0.01[c]	0.92 ± 0.01[abcd]
Dark	0.79 ± 0.02	0.86 ± 0.01[ab]	0.80 ± 0.01	0.88 ± 0.01[ab]

At 3 months of age, mice were randomly assigned to various diets, and 6 weeks after initiation of this treatment, oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were recorded every 2 min using indirect calorimetry . The measurements were performed following the "Diet-switch protocol" in individual mice. During the first part of the measurements, lasting for 17 hours, animals had ad libitum access to water and their "Original cHF-based diets". After that period, the original diets were removed and the animals fasted for 10 hours. In the beginning of the dark cycle at 18:00, all subgroups were switched to STD diet, and the measurements continued for 20 more hours ("Re-feeding STD diet"). The measurements were performed under the 12-hour light-dark cycle (light from 6:00 a.m.) at ambient temperature of 22 °C. Data are means ± SE (n=5) expressed for the whole period on respective diets, or during the light or dark phase within different diets, respectively. [a]Significantly different from cHF diet; [b]significantly different from cHF+TZD diet; [c]significantly different from mice consuming cHF-based diets during calorimetry; [d]significantly different from cHF+F (2-way ANOVA, $p<0.05$).

As expected the HF diet feeding resulted in the accumulation of triaclyglycerols in the liver and muscle (representative mixed-fibre *m. gastrocnemius*). Except from cHF+TZD

which even increased liver triacylglycerol content in the liver, all treatments decreased accumulation compared to the cHF (see Tab. 1 of the Publication B). In plasma, adipokines secretion profile was changed. All treatments reduced hyperinsulineamia observed in the cHF group with strongest effect in the combinational group. Multimeric adiponectin complexes in plasma were also analyzed. The ratio between high molecular weight (HMW) and total adiponectin was increased by all the treatments, again with the highest additive effect observed in the cHF+F+TZD group (see Tab. 2 of the Publication B). To test more precisely changes in whole-body insulin sensitivity, euglycaemic–hyperinsulinaemic clamp was performed. All the treatments significantly decreased the glucose infusion rate, which means the amount of exogenous glucose required to maintain euglycemia, with strongest effect in the combination cHF+F+TZD. Whole body glycogen synthesis with stimulation was also detected, strongest in cHF+F+TZD. These results were further confirmed at the muscle level using assay for measurement incorporation of radiolabeled glucose into the glycogen of *diaphragm* in absence or presence of insulin (see Fig. 4 of the Publication B).

Beneficial effects described above were also supported by experiments using another model, a model of "reversal" of dietary obesity.. This means that male mice were fed by cHF diet for one moth before the beginning of the study. All the treatments significantly affected body weight, glucose tolerance and changed metabolic profile with additive effect in cHF+F+TZD group (see Tab. 3 of the Publication B).

In summary, combination of n-3 PUFA and low dose of TZDs can be used as therapy to counteract dyslipidaemia and insulin resistance. Additive effect of these treatments on changes in lipid profile, triacylglycerol accumulation, insulin sensitivity, muscle glycogen synthesis stimulation and whole-body metabolic flexibility were found.

My contributions to this work were indirect calorimetry measurement and most of the ex-vivo experiments.

3.3 Publication C: n-3 PUFA, AMPK and insulin sensitivity

Synopsis of results of the article "**AMP-activated protein kinase α2 subunit is required for the preservation of hepatic insulin sensitivity by n-3 polyunsaturated fatty acids"**, by Jeleník et al, published in Diabetes 2010.

The objective of this study was to investigate the role of AMPK in preservation of insulin sensitivity in response to n-3 PUFA admixed to HF diet, using the obesity-prone C57BL/6J mice with genetically disrupted gene for the α2 catalytic subunit of AMPK and their wild-type littermates, with the main focus on the hepatic metabolism and its insulin sensitivity.

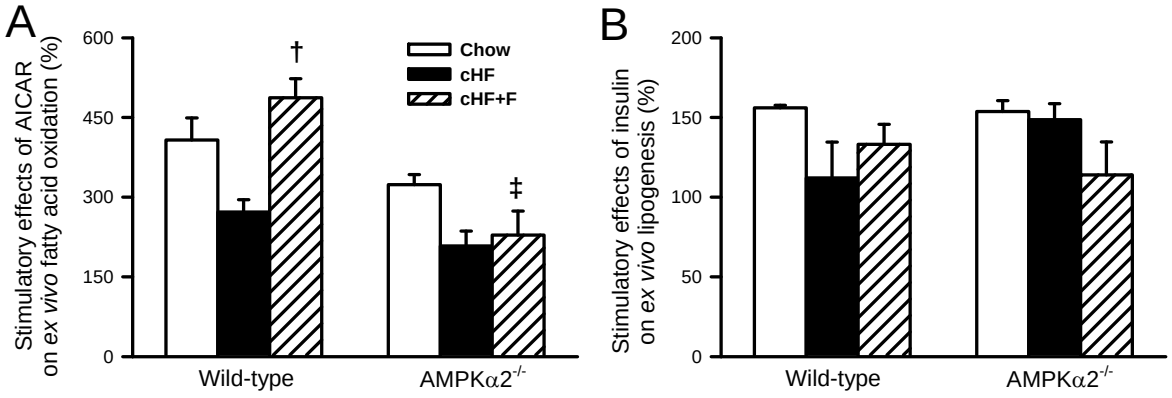
To answer this question, whole-body AMPKα2 knock-out (AMPKα2$^{-/-}$) mice on B/6J background and their wild-type littermate controls were used. Experiments were done on four-month-old mice fed on either a low-fat chow diet (chow), high-fat diet (cHF), or cHF diet, in which 15 % of total lipids were replaced with n-3 PUFA concentrate (cHF+F). These diets were fed for 9 weeks.

HF diet feeding induces significant body weight gain compared to chow diet, independently of the genotype. Presence of n-3 PUFA in the diet resulted in smaller body weight gain, prevention of dyslipidemia and hepatic triacylglycerols accumulation in wild-type animals as well as AMPKα2$^{-/-}$ mice in fed state. Only in wild-type animals, n-3 PUFA eliminated hyperinsulineamia observed in mice fed HF diet. It nicely correlated with the increase of adiponectin levels in plasma in the response to n-3 PUFA, which was missing in knock-out animals (see Tab. 1 of the Publication C).

Data obtained using hyperinsulinemic-euglycaemic clamp, the technique for testing insulin sensitivity, revealed lack of n-3 PUFA beneficial effects on insulin sensitivity in AMPKα2$^{-/-}$ mice compared to the control animals. Hepatic insulin sensitivity was not associated with changes in triacylglycerol content during clamp, however it was closely related to the content of diacylglycerol that are known to affect insulin sensitivity. AMPKα2 was essential for preserving low levels of both hepatic and plasma triglycerides, as well as plasma free fatty acids, in response to the n-3 PUFA treatment (see Fig. 2 of the Publication C).

These results were supported by in-vitro experiments using isolated hepatocytes where the induction of fatty acid oxidation was dependent on presence of functional AMPK. Hepatocytes from cHF-fed mice showed reduced stimulatory effect of AICAR irrespective of the genotype, n-3 PUFA feeding normalized this defect in wild-type but not in AMPKα2$^{-/-}$ hepatocytes, suggesting AMPK-dependent induction of capacity for fatty acid oxidation by n-3 PUFA in the liver. The stimulatory effect of insulin on de novo fatty acid synthesis was reduced in hepatocytes from cHF-fed wild-type mice, whereas it was retained in the hepatocytes from cHF-fed AMPKα2$^{-/-}$ mice. n-3 PUFA feeding tended to restore the stimulatory effect of insulin only in wild-type hepatocytes (Fig. 10 and Tab. 3).

Figure 10 The effect of differential dietary treatment on the regulation of metabolic fluxes in the liver



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AICAR-stimulated fatty acid oxidation (A) and insulin-stimulated de novo fatty acid synthesis (B) in cultured hepatocytes isolated from wild-type and AMPKα2-/- mice fed for 9 weeks either a Chow diet or isocaloric corn oil-based high-fat diets without (cHF) or with 15% of the lipids in the form of n-3 PUFA concentrate (cHF+F). For basal non-stimulated rates of lipid metabolism, see table below. The data are means ± SE (isolated hepatocytes, n = 3 in triplets; hepatic gene expression, n = 5-8). *P < 0.05 vs. genotype Chow; †P < 0.05 vs. genotype cHF; ‡P < 0.05 vs. wild-type on respective diet.

Table 3 The basal rates of lipid metabolism in isolated hepatocytes of wild-type and AMPKα2$^{-/-}$ mice

	Wild-type			AMPKα2$^{-/-}$		
	Chow	cHF	cHF+F	Chow	cHF	cHF+F
Fatty acid oxidation (pmol/h/mg protein)	13 ± 2	8 ± 1*	9 ± 1*	15 ± 2	12 ± 1‡	6 ± 1*†
Lipogenesis (pmol/h/mg protein)	108 ± 9	74 ± 24	43 ± 10*†	79 ± 10	51 ± 6	34 ± 6*

The data are presented as means ± SE (n = 3 analyzed in triplets). Wild-type and AMPKα2$^{-/-}$ mice were fed either a Chow diet or corn oil-based high-fat diets without (cHF) or with 15 % of the lipids in the form of n-3 PUFA concentrate (cHF+F) for 9 weeks. *P < 0.05 vs. genotype Chow; †P < 0.05 vs. genotype cHF; ‡P < 0.05 vs. wild-type on respective diet.

In conclusion, the results indicated the AMPKα2 is not essential for all the beneficial effects of n-3 PUFA. The AMPKα2-dependent acute changes in lipid metabolism and hepatic triglyceride accumulation largely reflect the extrahepatic action of n-3 PUFA but the

preservation of hepatic insulin sensitivity by n-3 PUFA in mice fed a high-fat diet depends on AMPKα2.

My main contribution to this work was the isolation of primary hepatocytes and the measurements of metabolic fluxes in this model, further ex-vivo analysis and indirect calorimetry (data not included in the publication).

3.4 Publication D: Leptin and deiodinase 1 in white adipose tissue

Synopsis of results of the article "**Modulation of type I iodothyronine 5'-deiodinase activity in white adipose tissue by nutrition: possible involvement of leptin"**, by Macek Jílková, published in Physiological Research 2010.

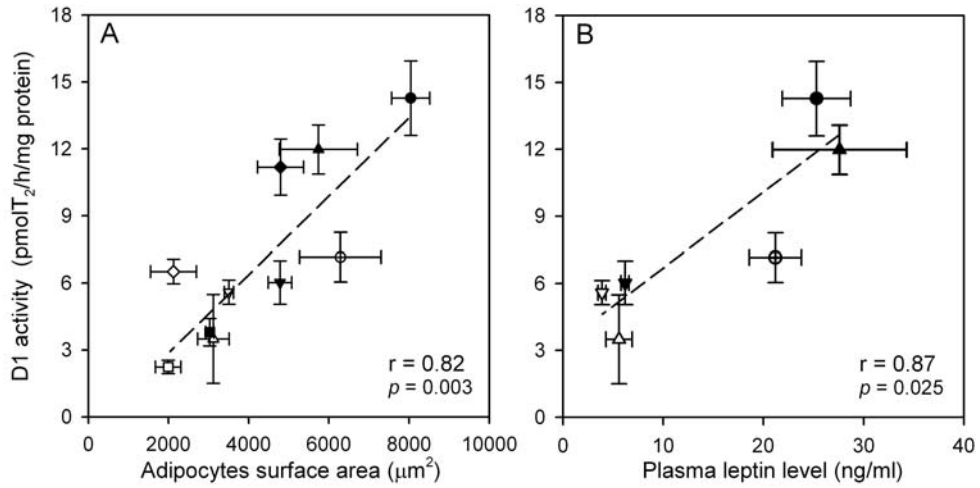
The objective of this study was to characterize the involvement of the metabolism of thyroid hormones in WAT, namely the conversion of T4 into T3 mediated by D1, in the adaptive response to HF diet-feeding in the obesity-prone C57BL/6J mice, namely the role of D1 in the control of adiposity by leptin and the involvement of D1 in adipocytes in this mechanism.

The project was performed on male C57BL/6J mice, which were exposed to (i) the obesogenic HF diet (contained 15 %, 59 %, and 26 % calories in the form of protein, fat, and carbohydrate) to provoke diet-induced obesity (the first study), (ii) calorie restriction (the second study), or (iii exogenous leptin injections (third study). Control animals were fed by standard chow diet containing 25 %, 9 %, and 66 % calories in the form of protein, fat, and carbohydrate, respectively.

In the first study, mice born and maintained at 30 °C were exposed after weaning to HF or LF diet for 8 weeks. All the analyses were performed at 2 weeks and after 8 weeks. Mice fed HF diet gained weight significantly compared to the control animals. Also the weights of the white adipose tissue depots were increased by HF diet, and this was already seen at 2 weeks. It was accompanied by increased size of adipocytes in both WAT depots. Plasma concentration of leptin increased significantly after 2 weeks of HF feeding, and after 8 weeks, leptin levels were even more profoundly elevated. Total levels of T4 and T3 were significantly increased after 2 weeks of HF diet-feeding. However, after 8 weeks, only total T3 remained increased. No differences in levels of free T4 and T3 between the LF and HF group levels of these hormones in plasma levels were found (see Tab.1 of the Publication D).

Activities of D1, D2 and D3 were assessed. Only D1 showed significant changes in response to the obesogenic treatment and correlated with adiposity, i.e. with adipocyte surface area and leptin levels in plasma (Fig. 11).

Figure 11 Correlation of D1 activity and size of adipocytes and plasma leptin levels



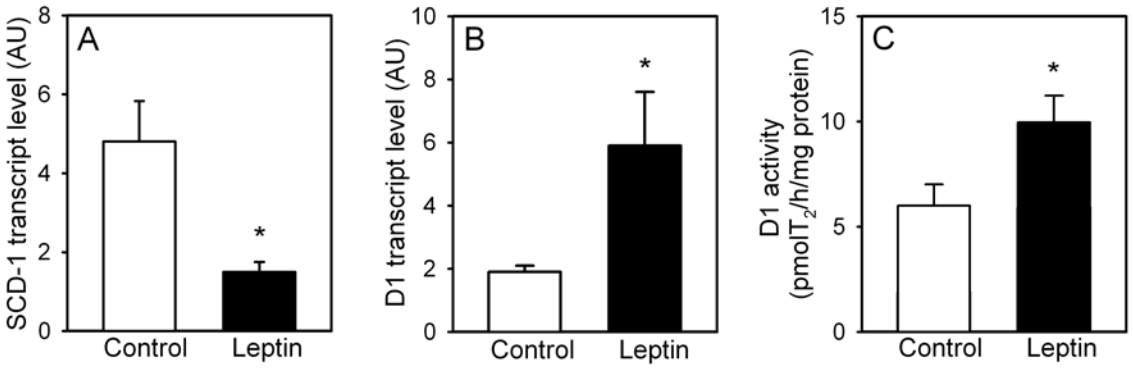
A. Correlation of the white adipose tissue D1 activity (two weeks, n = 14-17; eight weeks n = 3-8) and surface area of adipocytes (n = 3-4). Epididymal fat at two (triangle down) and eight weeks (triangle up), and dorsolumbar fat at two (square) and eight weeks (diamond) from mice fed HF (black) or LF diet (white), following the obesogenic treatment protocol; epididymal fat from the HF-AL-mice (black circle) and HF-CR-mice (crossed circle), following the caloric restriction treatment protocol. **B.** Correlation of the white adipose tissue D1 activity (two weeks, n = 14-17; eight weeks n = 3-8) and plasma leptin levels (n = 7-8). Epididymal fat at two (triangle down) and eight weeks (triangle up) from mice fed HF (black) or LF diet (white), following the obesogenic treatment protocol; epididymal fat from the HF-AL-mice (black circle) and HF-CR-mice (crossed circle), following the caloric restriction treatment protocol. Data are means ± SE.

In the second study, mild calorie restriction was applied to the animals to see whether the observed HF diet-induces increase of D1 activity in white adipose tissue persists when fat accumulation is lowered. Male mice born and maintained at 22 °C were fed by the LF diet from weaning. At the age of 3 months they were transferred onto HF diet for 7 weeks. During the last five weeks of the HF-feeding, one group of mice was fed ad libitum (HF-AL), while the other group of mice was subjected to 10% caloric restriction (HF-CR) compared with the HF-AL mice. Mice exposed to mild caloric restriction significantly reduced weight compared to the cHF-AL fed mice, accompanied with reversal of adipocyte hypertrophy in epididymal fat pad and decrease of the leptin levels in plasma. The HF-CR mice had also relatively low

plasma levels of free T3. The HF-CR mice exhibited significantly lower specific activity of D1. Leptin metabolic effect was assessed at the gene expression level of the SCD-1 gene, known as a marker of leptin action. In epididymal adipose tissue decreased in response to caloric restriction (see Tab. 2 of the Publication D).

The last part of the study was performed to reveal whether leptin secreted from hypertrophic adipocytes controls the activity of D1 in the tissue. Exogenous leptin injected to the LF fed animals was used to test this hypothesis. Males of B/6J mice were injected s.c. with 3 doses (3 mg/kg) of recombinant mouse leptin or saline. D1 gene transcript levels and D1 activity were evaluated in epididymal fat 16 hours after the last leptin injection. As expected, the expression of the SCD-1 gene was substantially suppressed by leptin (see Fig. 12).

Figure12 Effect of leptin on gene expression (A, B) and D1 activity (C) in epididymal fat.



At two weeks after weaning to LF diet, mice were injected with three doses (3mg/kg) of leptin for three days and epididymal adipose tissue was dissected 16 hours after the last injection. Data are means ± SE (n = 4-5). *p<0.05 for the effect of leptin vs. saline-injected mice.

In summary, the D1 activity is present in WAT. This activity is highly changeable according to the rate of adiposity and it is regulated by leptin. This represents a new control mechanism, which can influence tricylglycerol accumulation in WAT and thus control development of obesity.

My contribution to this work was assistance at the third part of publication where the effect of exogenous leptin was tested.

4 DISCUSSION

Concerning the severe impact of metabolic disorders linked to obesity on public health, better knowledge of the regulatory mechanisms engaged in energy homeostasis is required. All the studies described in this thesis were based on the use of the mouse models/strains suitable for investigations on obesity and associated diseases. The most often used mouse strain C57BL/6J represents an excellent model for studying obesity and insulin resistance. When exposed to HF diet, the mice gain weight and develop obesity with associated comorbities (81). Studying the process of the obesity development is a source of new findings in the regulation of energetic metabolism. There is also a space for targeting a "therapy" represented by dietary intervention (like addition of n-3 PUFA or TZD) or genetically disruption of specific genes. Another experimental approach is research based on the investigation of this mouse strain in comparison with another mouse strain completely different with respect to the propensity to obesity development. For example comparison of C57BL/6J mice with A/J resulted in discovery of a mechanisms contributing to the obesity development and to the resistance against obesity [(82), (83; 84)].

The most important tissues and organs involved in the energetic metabolism (e.g. skeletal muscle, liver and adipose tissue) were included in our studies, although with slight differences concerning the topics of the specific studies. The first two studies were focused on skeletal muscle. In the first study we proved the relationship among HF diet, leptin and AMPK, in the second one, muscle also played a crucial role in the phenotype but the mechanism needs further determination. In the third part of the thesis my effort was focused on the liver and beneficial treatment by marine lipids. Adipose tissue physiology in a relationship with leptin was more deeply evaluated in the last part of this thesis.

Adaptive, and especially diet-induced thermogenesis, was studied against the background of propensity or resistance to development of obesity (Publication A). The strategy of using two opposite phenotypes represented by B/6J and A/J mice was rightly chosen and helped to reveal a mechanism which contributes to the phenotype of resistance to obesity development. Results of the study highlight the importance of hormone leptin in regulation of energy homeostasis. We confirmed the previously published phenotype of both mouse strains and elevated leptineamia in A/J strain (82). Feeding of HF diet resulted specifically in obesity prone A/J mice in activation of energy expenditure-mechanisms. The importance of UCP1-mediated thermogenesis was confirmed (85). Moreover, we described also activation of energy dissipation in skeletal muscle in A/J mice. Increase of oxygen

consumption in oxidative muscle was mainly based on lipid catabolism. The mechanism is based on activation of AMPK by leptin. Taking this data together with the phenotype of this mouse strain, we demonstrate that activation of lipid catabolism in skeletal muscle can contribute to lean phenotype during high-calorie diet feeding. Moreover, the importance of skeletal muscle energetic in energy homeostasis was clearly demonstrated. This conclusion conflicts with statement that only UCP1 can mediate adaptive thermogenesis [(86), (87)]. Our results document that not only UCP1 is involved, also in accordance with other studies [(6; 7)], which support the important role of leptin in the mechanisms discussed. One explanation of this paradox could be the selection of a proper mouse model. This question thus needs to be further investigated.

The importance of skeletal muscle in the regulation of insulin resistance development was confirmed in the second study (Publication B). It has been shown that both n-3 PUFA and TZDs can increase mitochondrial biogenesis and reduce adiposity and dyslipidemia [(88), (89)]. Treatment using the anti-diabetic drugs of the TZD family is associated with lowering of triacylglycerols accumulation in liver and skeletal muscle (74). Experiments were based on feeding mice by diet with a combination of low dose of TZDs and partial replacement of lipids by EPA/DHA concentrate. This combination resulted in many metabolic improvements, with additive interactions between the treatments. Development of dyslipidemia and IR was prevented, further the adipocyte hypertrophy and inflammation were reduced. Moreover accumulation of triacylglycerols in the liver and SM was decreased. Insulin responsiveness of skeletal muscle and liver could be at least partially explained by significant induction of adiponectin secretion [(90; 91)]. The combinatory treatment showed the highest induction compared to other treatments. Data obtained from the measurement of whole body energetic using indirect calorimetry showed significant improvement of metabolic flexibility also refer to improvement of muscle metabolism. It was already shown that the muscle is the key organ of metabolic flexibility (58). All the beneficial effects resulted in better glucose homeostasis and prevented the development of type 2 diabetes. We showed for the first time that combination of n-3 PUFA with low dose TZD is of such a strong potential and this strategy could thus lead to reduce doses of TZD.

Following the research effort to better characterize a molecular effect of n-3 PUFA, the third study was designed (Publication C). Part of the effect of n-3 PUFA was suggested to be mediated by AMPK (69). To reveal if AMPK is crucial for beneficial effects of n-3 PUFA in the prevention of high-fat induced obesity and insulin resistance, specific whole-body AMPKα2 knock-out mice on C57BL/6J background were used. It was shown that AMPKα2

is required for the effect of n-3 PUFA to preserve whole-body, muscle, and especially hepatic insulin sensitivity, as well as to suppress hepatic and plasma triglycerides under insulin-stimulated conditions. In contrast, AMPKα2 was not required for protection by n-3 PUFA from hepatic lipid accumulation and dyslipidemia in ad libitum-fed mice. Our results supported the important role of diacyglycerols rather than triacyglycerols in preservation of insulin resistance [(92; 93)]. Some contradictory results to previous studies (69) were found in the case of data of AMPK activity. This could probably be a result of different dietary intake of n-3 PUFA. Our results also supported the role of adiponectin in beneficial effects of n-3 PUFA. In accordance with (94), plasma adiponectin levels tended to be reduced in knockout mice. Thus, the absence of AMPKα2 may blunt adiponectin-mediated effects of n-3 PUFAs.

As a "byproduct" of studying leptin and its regulation of whole body metabolism, we revealed a new regulatory mechanism of TH metabolism in WAT. Activity of enzyme D1 positively correlated with the size of adipocytes and leptin levels during HF diet feeding. It is known that adipokines have the auto-, para- and endocrine effects. It seems that leptin in adipose tissue acts under physiological conditions in an autocrine manner. This was supported on BAT adipocytes where the D1 activity was not stimulated by HF diet, reflecting the fact that brown adipocytes do not secret leptin (95). In experiments with the administration of exogenous leptin the circulating concentration of leptin was much higher than normal. Leptin caused stimulatory effect on D1 activity in white fat, similarly to other tissues (96). The response of D1 activity was associated with SCD-1 downregulation indicating a role of T_3 in regulation in adipocyte lipid metabolism (97).

In conclusion this thesis not only provides new findings in the field of obesity and regulation of energy metabolism, but also supports the importance and power of using specific mouse strains in the field of experimental obesitology, as well as the requirement of proper choice of the right strain for studying specific topics and hypotheses.

5 CONCLUSIONS

A. Only in the obesity-prone A/J mice, but in the obesity-resistant C57BL/6J mice induction of lipid metabolism by HF diet feeding in oxidative skeletal muscle was found, which was associated with cold tolerance. Thus, our results document the role of muscle non-shivering thermogenesis in the differential susceptibility to obesity of these two strains of mice, moreover, they also suggest the involvement of the leptin-AMPK axis in the inducibility of lipid catabolism in the muscle.

B. The treatment using a combination of low doses of TZDs and n–3 PUFA showed strong additive beneficial effect in the prevention of adipose tissue hypertrophy, dyslipidemia and whole-body metabolic inflexibility in C57BL/6J mouse model of dietary obesity. Synergistic effect on stimulation of muscle glycogen synthesis in the presence of insulin was found. Therefore this combination treatment strategy could be adapted in therapy of type 2 diabetes in humans.

C. α2 subunit of AMPK is required for preservation of hepatic insulin sensitivity. Some of the beneficial effects of n-3 PUFA are extrahepatic and not all of them are mediated via AMPK. Diacylglycerols content changes in the liver seem to be crucial for preservation of insulin sensitivity. AMPKα2 is also essential for hypolipidemic and anti-steatotic effects under insulin stimulated condition.

D. Thyroid hormones in adipose tissue play an important role in adaptive response to HF diet-feeding in the obesity-prone C57BL/6J, with main importance of D1 in the control of adiposity by leptin. Leptin was found to stimulate the D1 activity and, therefore, a novel regulatory mechanism controlling lipid metabolism in adipose tissue and possibly also its accumulation was described.

6 LIST OF ALL MY PUBLICATIONS

- **Kus V**, Prazak T, Brauner P, Hensler M, Kuda O, Flachs P, Janovska P, Medrikova D, Rossmeisl M, Jilkova Z, Stefl B, Pastalkova E, Drahota Z, Houstek J, Kopecky J. Induction of muscle thermogenesis by high-fat diet in mice: association with obesity-resistance. Am J Physiol Endocrinol Metab. 295:E356-67. 2008 (IF = 4.129)

- Rossmeisl M, Jelenik T, Jilkova Z, Slamova K, **Kus V**, Hensler M, Medrikova D, Povysil C, Flachs P, Mohamed-Ali V, Bryhn M, Berge K, Holmeide AK, Kopecky J. DHA-derivatives in the prevention and reversal of obesity and glucose intolerance in mice. *Obesity.* 17:1023–1031, 2009 (IF = 2.798)

- Kuda O, Jelenik T, Jilkova Z, Flachs P, Rossmeisl M, Hensler M, Kazdova L, Ogston N, Baranowski M, Gorski J, Janovska P, **Kus V**, Polak J, Mohamed-Ali V, Burcelin R, Cinti S, Bryhn M, Kopecky J. n-3 fatty acids and rosiglitazone improve insulin sensitivity through additive stimulatory effects on muscle glycogen synthesis in mice fed a high-fat diet. Diabetologia. 52: 941-51. 2009 (IF = 6.328)

- Macek Jílková Z, Pavelka S, Flachs P, Hensler M, **Kůs V**, Kopecký J. Modulation of type I iodothyronine 5'-deiodinase activity in white adipose tissue by nutrition: possible involvement of leptin. Physiol Res. 59:561-569. 2010 (IF = 1.739)

- Jelenik T, Rossmeisl M, Kuda O, Jilkova ZM, Medrikova D, **Kus V**, Hensler M, Janovska P, Miksik I, Baranowski M, Gorski J, Hébrard S, Jensen TE, Flachs P, Hawley S, Viollet B, Kopecky J. AMP-activated Protein Kinase {alpha}2 Subunit Is Required for the Preservation of Hepatic Insulin Sensitivity by n-3 Polyunsaturated Fatty Acids. Diabetes. 59:2737-46. 2010 (IF = 8.261)

Articles in press

- Pico C, Jilkova MZ, **Kus V**, Palou A, Kopecky J. Perinatal programming of body weight control by leptin – putative roles of AMP kinase and muscle thermogenesis. Submitted to: Am J Clin Nutr (IF = 6.307)

Articles in preparation

- Flachs P, Rühl R, Hensler M, Janovska P, Zouhar P, Jilkova MZ, Papp E, Kuda O, Planavila A, **Kus V**, Rossmeisl M, Mohamed-Ali V, Villarroya F, Kopecky J. 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. Submitted to Diabetologia (IF = 6.328)

- **Kus V**, Flachs P, Kuda O, Bardova K, Janovska P, Svobodova M, Macek Jilkova Z, Rossmeisl M, Wang-Sattler R, Yu Z, Illig T, Kopecky J. Differential effects of rosiglitazone and pioglitazone in combination treatment with n-3 fatty acids in mice fed high-fat diet.

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8 PUBLICATIONS ENCLOSED IN FULL (Publications A-D)