Doctoral studies in Biomedicine

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Abstract of doctoral thesis sent:

Defence of PhD thesis:

The full text of doctoral thesis is available at Charles University in Prague, First Faculty of Medicine

SUMMARY

Obesity represents a predominant risk factor for the development of metabolic diseases like type 2 diabetes mellitus and it is characterized by adipocyte hypertrophy. The size of adipocytes influences the adipocytes biology and secretory functions of adipose tissue in general. Understanding the mechanisms regulating growth and secretory activity of adipose tissue is of paramount importance. Moreover, obesity represents a chronic subclinical inflammatory state linking obesity to insulin resistance and hypertrophic adipocytes contribute to this phenomenon. An improvement of strategies used in the prevention and treatment of inflammation associated with obesity is therefore urgently needed.

The four studies described in this thesis address several topics related to adipose tissue biology, and thus contributing to the understanding of the integrating role of adipose tissue secretory functions in response to dietary and pharmacological treatments. This thesis demonstrates a possible role of white adipose tissue thyroid hormones (TH) metabolism in the modulation of its function under conditions of changing adiposity (Publication A); a unique role of leptin, secreted from adipose tissue, in the complex control of energy homeostasis of the organism (Publication B); beneficial effects of the combination treatment of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and rosiglitazone (Publication C); and beneficial effects of the chemical DHA derivatives (Publication D).

Results of all presented studies support the concept of an integrating role of adipose tissue secretory functions in the whole-body responses to dietary constituents as well as to pharmacological agents, which ameliorate obesity and associated insulin resistance.

SOUHRN

Obezita je hlavním faktorem, který zvyšuje riziko rozvoje metabolických onemocnění jako je diabetes mellitus druhého typu. Během obezity dochází k výraznému zvětšení adipocytů a tato změna ovlivňuje jak biologii adipocytů samotných, tak i celkovou sekreční funkci tukové tkáně. Porozumění mechanismů, které regulují růst a ovlivňují sekreční aktivitu tukové tkáně, proto patří mezi nejvýznamnější cíle. Nárůst tukové tkáně navíc vede ke stavu chronického zánětu, který spojuje obezitu s inzlínovou rezistencí, a hypertrofované adipocyty mají na tomto fenoménu výrazný podíl. Z těchto důvodů je nezbytné zdokonalovat strategie prevence a léčby zánětu spojeného s obezitou.

Studie popsané v této práci jsou zaměřené na několik témat souvisejících s biologií tukové tkáně, tím tato dizertační práce přispívá k pochopení a porozumnění integrující role sekreční funkce tukové tkáně v odpovědi na dietu a farmakologickou léčbu. Práce demonstruje možnou roli metabolismu thyroidálních hormonů v bílé tukové tkáni a tím i možné ovlivnění funkce tukové tkáně za stavu, kdy se mění adipozita (Publikace A); studuje specifickou roli leptinu, sekretovaného tukovou tkání, ve vztahu k celkové kontrole energetické homeostázy organismu (Publikace B); zabývá se benefičním efektem kombinační léčby eicosapentaenové kyseliny (EPA), dokosahexaenové kyseliny (DHA) a rosiglitazonu (Publikace C); a benefičním efektem chemických derivátů DHA (Publikace D).

Výsledky uvedených prací podporují představu integrující role sekreční funkce bílé tukové tkáně v odpovědi celého organismu na složení diety stejně jako v odpovědi na farmakologické působení, vedoucí k zlepšení stavu obezity a s ní spojené inzulínové rezistence.

CONTENT

1 INTRODUCTION

1.1 Energy balance and body weight homeostasis

Life exists in a flux of energy transformations that are governed by the laws of thermodynamics. Energy can neither be created nor destroyed but can be transformed only from one form to another. Biological systems depend on the transformation of chemical energy in other forms of energy to perform chemical, mechanical or electrical work. Principles of energy balance are embodied in the following equation:

energy intake = energy expenditure + Δ energy stores

In the state of energy balance energy expenditure is equal to energy intake, according to the first law of thermodynamics. When the intake and expenditure of energy are not equal, then a change in body energy content will occur. Negative energy balance results in a degradation of energy stores while positive energy balance results in an increase in energy stores that leads to the obesity. In other words, the increased prevalence of obesity results mostly from overeating (excess of energy intake) and/or a lack of physical activity (decrease of energy expenditure).

1.2 Central role of adipose tissue

Adipose tissue has been considered for years to be a simple lipid reservoir. The increasing knowledge, concerning the role of adipose tissue in energy balance and in the pathophysiology of metabolic disorders, have focused attention to this tissue.

Adipose tissue is a complex, essential, and highly active metabolic and endocrine organ that is found in mammals in two different forms: white adipose tissue and brown adipose tissue. These tissues are characterized by different anatomic location, morphological structures, functions, and regulation. Both types of adipose tissues are able to store energy as triacylglycerols (TG), but whereas white fat releases this energy in the form of

free fatty acids according to the needs of the organism, brown fat can convert it into a heat.

White adipose tissue can expand by increasing intracellular lipid accumulation leading to greater adipocyte size (hypertrophy) and/or by increasing the numbers of adipocytes (hyperplasia). Adipocyte hypertrophy, evident in obese subjects, was originally considered to be the only way whereby adipose tissue mass expands. However, adipocytes hyperplasia (adipogenesis), which occurs most significantly in response to energy imbalance in young age, is now recognized to contribute to the increased adipose tissue mass in obesity. Moreover, it was discovered recently that approximately 10 % of fat cells are renewed annually (1). Furthermore, there are also differences between adipose tissue depots. Whereas visceral fat mass increase primarily due to hypertrophy, subcutaneous fat exhibits both adipocyte hypertrophy as well as hyperplasia.

1.2.1 Endocrine function of adipose tissue

Adipose tissue plays a critical role in energy homeostasis by mobilizing TG reserves in a process of lipolysis in order to provide fatty acids. Fatty acids are important oxidative fuels for other tissues during times of energy deprivation such as fasting and exercise. During the lipolytic process, TG can be quickly hydrolysed by a series of specific lipases and free fatty acids are released into the circulation to be taken up by other tissues. Lipolysis is under tight regulation by insulin and catecholamines. Higher concentration of circulating fatty acids and TG are associated with lipid accumulation in multiple tissues, including liver and muscle, which leads to insulin resistance.

Besides this point of view, white adipose tissue also secretes a large variety of bioactive molecules, known as adipokines with endo-, auto-, or paracrine signal functions (2).

1.2.1.1 Leptin

The first characterised adipokine, leptin, was discovered in 1994 (3). Identification of this hormone, mainly produced by adipocytes, has uncovered a new endocrine system of body weight regulation. Leptin circulates in plasma of mammals, and its concentration increases with accretion of adipose tissue mass and decreases when adipose tissue is

reduced (4). Thereby leptin serves as a signal of energy sufficiency and of the amount of fat and provides this information to leptin-sensitive neurons in hypothalamus to regulate food intake (for review see (5)).

Moreover, leptin acts also directly in peripheral tissues. In the skeletal muscle, leptin selectively stimulates phosphorylation and activation of the alpha 2 catalytic subunit of AMP-activated protein kinase (α2 AMPK), which subsequently suppresses the activity of acetyl-CoA carboxylase and thereby stimulates fatty acid oxidation (6).

1.2.1.2 Adiponectin

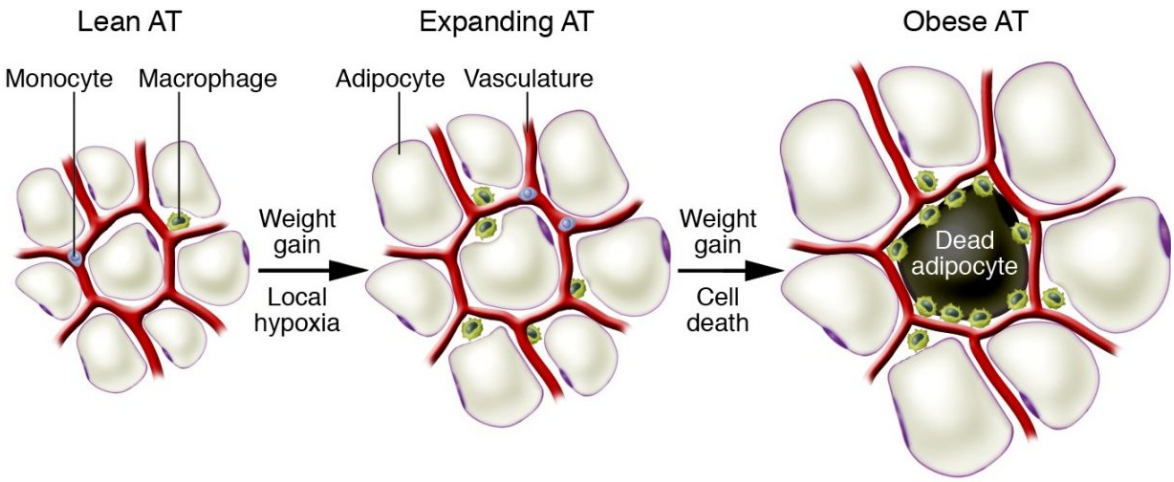
Adiponectin is a protein messenger, synthesized mainly by adipocytes, which improves insulin sensitivity and changes energy metabolism of target tissues (7). Adiponectin circulates in plasma and has a wide range of biological effects. Transcription of adiponectin gene is controlled by the transcription factor PPAR gamma and could be induced by insulin or thiazolidinediones (TZD) (8). Serum adiponectin levels are decreased in obese subjects, particularly in individuals with visceral obesity and insulin resistance (9). Adiponectin seems to have an anti-inflammatory effect through inhibition of tumor necrosis factor-α (TNF-α) expression and reduction of its effects on the endothelial inflammatory response or through induction of the anti-inflammatory cytokines, such as interleukin-10 (IL-10), produced by immune cells (10).

1.2.2 Inflammation of adipose tissue and insulin resistance in obesity

Obesity induces a state of low-grade inflammation that is associated with the development of type 2 diabetes (11). Hypertrophy of adipocytes leads to adipose tissue macrophage (ATM) infiltration, observed primarily in visceral white adipose tissue (12), possibly as a response to the hypoxia. ATM content positively correlates to amount of visceral fat and adipocytes size (13). Increased infiltration of adipose tissue with immune cells could lead to insulin resistance via increased levels of circulating cytokines, such as interleukin-6 (IL-6) and TNF-α, which contributes to systemically decreased insulin sensitivity via endocrine signalling (14).

Histological analyses reveal that the vast majority (over 90 %) of macrophages of white adipose tissue form structures that are denominated crown-like structures (CLS) (15).

Figure 1 Adipose tissue expansion



Adipose tissue expansion during weight gain leads to recruitment of macrophages through a variety of signals, which may include local hypoxia. These macrophages predominantly localize around dead adipocytes (adapted from (16)).

The macrophage infiltration of adipose tissue in obese subjects is tightly linked to adipocytes death. By immunohistochemistry was demonstrated that CLS dead adipocytes surrounded by ATM are perilipin (the marker of viable adipocytes) negative, while all viable adipocytes not surrounded by ATM are perilipin positive. The most consistent residual part of the dead adipocyte is the free lipid droplet that must be reabsorbed by the organism. Thus the residual cell debris and free lipid droplet could act as a foreign body requiring a chronic and persistent activity of macrophages (15).

The amelioration of inflammatory state in white adipose tissue leads to improvement of the obesity-related pathologies, in particular to improvement of insulin sensitivity (17;18). For instance, a weight loss induced by negative energy balance reduces macrophage activation and infiltration in adipose tissue. Moreover, the systemic inflammation is improved by reduction of inflammatory cytokines and chemokines produced by adipose tissue (19), leading to improved insulin sensitivity.

1.2.3 The amelioration of inflammatory state in white adipose tissue

1.2.3.1 Thiazolidinediones

Antidiabetic drugs of the TZD class are potent and selective activators of PPAR gamma, known as one of the key regulators of glucose homeostasis, able to promote adipocyte differentiation and lipid storage. PPAR gamma determines the differentiation process of preadipocytes into mature adipocytes and could also induce apoptosis of mature adipocytes. TZD are used in the treatment of type 2 diabetes, since these drugs improve insulin sensitivity and reduce hyperinsulinemia, hepatic steatosis, and inflammation (18). Both in vitro and in vivo studies provide evidence that TZD have anti-inflammatory properties (20). TZD inhibit macrophage activation and decrease inflammatory cytokine expression and release in macrophage and monocyte. Treatment with TZD reduces secretion of resistin, IL-6, monocyte chemoattractant protein-1 (MCP-1) and TNF-α while increasing the insulin-sensitizing hormone adiponectin(21;22).

1.2.3.2 n–3 polyunsaturated fatty acids

Polyunsaturated fatty acids of n–3 (ω−3) family (n-3 PUFA), namely Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), are important essential fatty acids, naturally occurring for example in sea fish. n–3 PUFA are components of phospholipids in cellular membranes, while also acting as regulatory ligands in gene transcription, where the effects of n–3 PUFA are mostly mediated by PPAR alpha (23).

Dietary n-3 PUFA seem to postpone diabetes development (24) and have considerable effect on gene expression in a variety of tissues, including adipose tissue, where they regulate the expression of genes involved in adipocyte differentiation and lipid metabolism (25). Hypolipidaemic and anti-obesogenic effects of n–3 PUFA probably depend on the in situ suppression of lipogenesis and increase of fatty acid oxidation in several tissues (26). This metabolic switch might reduce accumulation of toxic fatty acid-derivatives and thereby protect the insulin signaling in the liver and skeletal muscle (27). Furthermore, n-3 PUFA prevent high-fat diet-induced matrix remodeling, adipocyte enlargement in adipose tissue of obese subjects (28) and adipose

tissue inflammation (17;29). In addition, n-3 PUFA exert immunomodulatory effects by induction of adiponectin (30) and by a decrease in the production of classic inflammatory mediators such as arachidonic acid-derived eicosanoids and inflammatory cytokines (31).

1.2.4 Thyroid hormones metabolism in white adipose tissue

Thyroid hormones (TH) play the major role in development of many tissues as well as in the regulation of many physiological processes. White adipose tissue represents an important target for TH, (32-34). Multiple biological effects of TH depend on intracellular levels of T_3, which binds to thyroid hormone receptor and is mostly generated in peripheral tissues by outer–ring deiodination of T_4. Type I and type II iodothyronine 5'-deiodinase (D1 and D2, respectively) could catalyse the reaction. D1 exerts on relatively broad substrate specificity, while also catalysing inner-ring deiodination of T_4 to produce reverse T_3, an inactive form of TH, as well as deiodination of other TH derivatives (35). D2 also catalyses conversion of T_3 into 3,3'-diiodothyronine (T_2). Type III iodothyronine 5-deiodinase (D3), catalyses inner-ring deiodination of T_4 and T_3, to produce reverse T_3 and T_2. D1 is mainly present in the liver, kidneys, thyroid gland, and pituitary. Due to its high activity, the hepatic D1 is traditionally regarded as being an important source of circulating T_3, while D2, which is mainly present in the brain, brown fat, placenta, pituitary, and muscle, is essential to the local generation of T_3 in the tissues (36). In brown fat, D2 activity is required for tissue differentiation and thermogenic function (32). In white adipose tissue, T_3 regulates both lipolysis and lipogenesis by induction of the key lipogenic enzymes such as ACC, malic enzyme and fatty acid synthase (33). D3 has been suggested to stimulate the proliferation of white fat cells, while D2 could be linked to the differentiation programme of adipocytes, as revealed by in vitro experiments (32). Furthermore, thyroid hormone action is mediated by the family of thyroid hormone receptors. Recent finding suggests that thyroid hormone receptor TRα1 could contribute to subcutaneous adipose tissue expandability in obese subjects (37). However, the metabolism and biological role of TH in white adipose tissue is only poorly described.

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2 AIMS OF THE THESIS

The general goal of this thesis was to deepen the knowledge of adipose tissue biology and to understand the integrating role of adipose tissue in modulation of lipid metabolism and adiposity by leptin and thyroid hormones (see the specific aims 1 and 2, below). Reflecting the prominent role of adipose tissue secretory function in the etiopathogenesis of the insulin resistance, and in the regulation of whole-body inflammatory response by adipokines, a part of the work was focused on the improvement of strategies for the prevention and treatment of inflammation associated with obesity, while focusing on the beneficial effects of a specific composition of dietary lipids combined with and anti-diabetic drugs TZD (see the specific aims 3 and 4, below). All the experiments were performed in mice.

The specific aims of this thesis were:

1. to characterise metabolism of thyroid hormones in white adipose tissue, especially its modulation in response to physiological changes of fat mass, and stimulation of D1 activity by leptin;

2. to characterise plasma levels of leptin and thyroid hormones in response to a high-fat diet, using a model of obesity-resistant (A/J) and obesity-prone (B6) strains of mice;

3. to evaluate possible additive beneficial effects of the combination treatment by n–3 PUFA and rosiglitazone on adipose tissue inflammatory state and insulinemia in the prevention and reversal of diet-induced obesity; and

4. to investigate the effect of DHA-derivatives on adipose tissue biology and inflammation in the prevention and reversal of diet-induced obesity.

3 RESULTS TO SELECTED PUBLICATIONS

Publication A: Modulation of type I iodothyronine 5'-deiodinase activity in white adipose tissue by nutrition: possible involvement of leptin:

Macek Jilkova Z, Pavelka S, Flachs P, Hensler M, Kus V, Kopecky J. *Physiol Research (in press)*, (IF = 1.739)

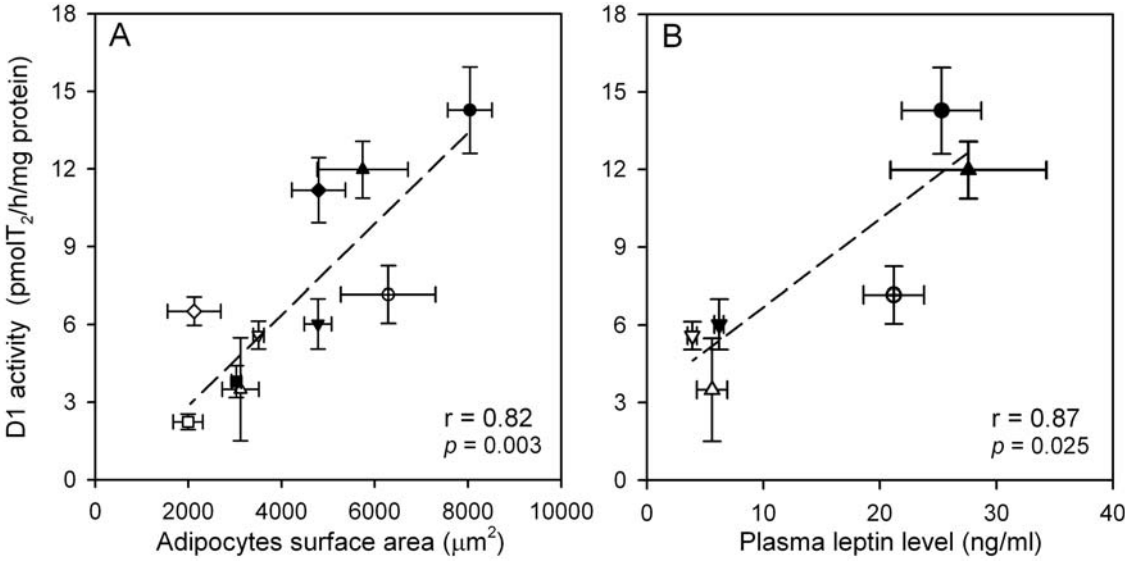
Our objective was to describe possible changes in the activities of TH-metabolising enzymes in white adipose tissue, and the role of TH metabolism in adipose tissue during obesogenic treatment, caloric restriction and, in response to leptin treatment in mice.

First, an "**obesogenic treatment**" was performed to characterise effect of adipose tissue expansion on metabolism of TH in white adipose tissue. Male mice born and maintained at 30 °C were weaned at four weeks of age to either the low-fat (LF) or high-fat (HF) diet. The LF diet contained 25 %, 9 %, and 66 % calories in the form of protein, fat, and carbohydrate, respectively. The HF diet, proven to be obesogenic in C57BL/6J mice, contained 15 %, 59 %, and 26 % calories in the form of protein, fat, and carbohydrate. All the analyses were performed two or eight weeks after the weaning. After eight weeks of dietary treatment, only body weight gain but not body weight was significantly higher in the HF-diet group. However, already after two weeks, the weights of both epididymal (visceral) and dorsolumbar (subcutaneous) fat depots were significantly higher in the HF-diet group, as compared with the LF diet-fed mice. As revealed by histological analysis, the size of adipocytes in both white fat depots also increased in response to the HF diet. Plasma concentration of leptin increased significantly after two weeks of HF feeding, and after eight weeks, leptin levels were even more profoundly elevated. While there were no differences between the LF and HF groups in plasma levels of free T_4 and T_3 levels, the total levels of these hormones were significantly increased after two weeks of HF diet-feeding. However, after eight weeks, only total T_3 remained increased.

Specific activity of D1 increased in both white fat depots in response to the HF diet, the strongest effect (~3.4-fold induction) being on epididymal fat after eight weeks.

On the other hand, the dietary treatment had no effect on specific D1 activity in interscapular brown fat. In the liver, specific D1 activity was several orders of magnitude higher than in adipose tissue and it was stimulated by the HF diet. Specific activity of D1 in white adipose tissue increased together with the size of adipocytes (Figure 2A), as well as with the plasma leptin level (Figure 2B), suggesting correlative relations. Compared with D1, specific activities of both D2 and D3 in white fat depots were ~50- (epididymal fat) to ~100-fold (dorsolumbar fat) lower and did not change in response to the HF diet.

Figure 2 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.

Second, **caloric restriction** was applied in adult mice fed the HF diet to find out whether the HF diet-induced elevation of D1 activity in white adipose tissue could be reversed when fat accumulation is compromised without changing the diet composition.

Male mice born and maintained at 22 °C were fed the LF diet after weaning and then fed the HF diet for seven weeks, beginning at the age of three month. 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. Compared with ad libitum fed HF-AL mice, a five-week-caloric restriction in HF-CR mice resulted in a reduction in body weight gain, a decrease in the weight of adipose tissue depots, and the reversal of adipocytes hypertrophy in epididymal fat pad. In association with the changes in white fat content, plasma leptin levels and leptin gene expression in epididymal fat were significantly reduced by caloric restriction. Compared with the HF-AL mice, the HF-CR mice exhibited ~2.0-fold lower specific activity of D1 in epididymal adipose tissue. Expression of the SCD-1 gene, a marker of the leptin metabolic effect, in epididymal adipose tissue increased in response to caloric restriction.

Third, **leptin treatment** was used to reveal whether the D1 activity in white fat could be increased by leptin secreted from hypertrophic adipocytes. To verify this hypothesis, mice weaned to the LF diet were subcutaneously injected with three doses (3 mg/kg) of recombinant mouse leptin or saline. D1 gene transcript levels and D1 activity were increased in epididymal fat 16 hours after the last leptin injection. As expected, the expression of the SCD-1 gene was substantially, suppressed by leptin (for data see publication A).

Our results demonstrate for the first time changes in D1 activity in white adipose tissue under conditions of changing adiposity, and a stimulatory effect of leptin on D1 activity in white adipose tissue. We suggest that D1 has a functional role in white adipose tissue with D1 possibly being involved in the control of adipose tissue metabolism and/or accumulation of the tissue.

Publication B: Induction of muscle thermogenesis by high-fat diet in mice: association with obesity-resistance:

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. *Am J Physiol Endocrinol Metab* 295: E356-E367, 2008, (IF = 4,129)

The aim of this study was to reveal whether muscle non-shivering thermogenesis could be stimulated by a HF diet, especially in obesity resistant A/J mice compared with obesity-prone C57BL/6J (B/6J) mice.

Experiments were performed on male mice born and maintained at 30 °C. Four-week-old mice were randomly weaned onto a LF or HF diet for 2 wk. At weaning, as well as at the time of sacrifice, mice of both strains had similar body weights, independent of the type of diet. Accordingly, body weight gains during the 2-week post-weaning period and caloric intake were similar in all animal subgroups (Table 1). Weight of white fat depots (subcutaneous and epididymal) was increased by HF diet in both strains. As expected, HF diet strongly increased leptin levels in A/J mice, while no significant induction of leptin was detected in B/6J mice (Table 1). In contrast, plasma levels of both T_4 and T_3 were not affected by HF diet in A/J mice, but they were increased in B/6J mice, indicating the stimulatory effect of HF diet on thyroid function in B/6J but not in A/J mice (Table 1).

In both strains, the HF diet induced UCP-1-mediated thermogenesis, with a stronger induction in A/J mice. Only in A/J mice: 1) the HF diet augmented activation of whole body lipid oxidation by cold; and 2) at 30°C, oxygen consumption, total content, and phosphorylation of AMPK, and AICAR [aminoimidazole carboxamide ribonucleotide (AICAR), a pharmacological activator of AMPK] -stimulated palmitate oxidation in soleus muscle was increased by the HF diet in parallel with significantly increased leptinemia (for data see publication B). Gene expression data in soleus muscle of the A/J HF mice indicated a shift from carbohydrate to fatty acid oxidation (for data see publication B).

Table 1 Growth characteristics and plasma hormone levels

	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
Weight of fat depots (mg)				
BAT	90 ± 3	57 ± 3*	69 ± 3†	61 ± 3*
DL	152 ± 4	180 ± 10*	167 ± 9	252 ± 14*†
EPI	160 ± 8	239 ± 21*	163 ± 14	292 ± 26*
Plasma levels				
Leptin (ng/ml)	4.48 ± 0.38	5.21 ± 0.54	3.35 ± 0.34†	9.42 ± 1.15*†
T4 (nmol/L)	37 ± 2	46 ± 2*	45 ± 1†	45 ± 3
T3 (nmol/L)	1.5 ± 0.2	1.9 ± 0.2*	1.9 ± 0.2†	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; BAT, interscapular brown fat; DL, dorsolumbar white fat; EPI, epididymal white fat. Data are means ± S.E. (n = 11-14). *Significant effect of diet; †significant effect of genotype.

Our results suggest that muscle non-shivering thermogenesis and lipid oxidation can be stimulated by HF diet in the obesity-resistant phenotype of A/J mice. Furthermore they indicate that a HF diet could induce non-shivering thermogenesis in oxidative muscle, possibly via the leptin-AMPK axis. Thus, white adipose tissue, by secreting leptin, played an integrating role in the adaptive induction of thermogenesis in response to increased consumption of dietary fat, and also in protection against development of obesity.

Publication C: n–3 fatty acids and rosiglitazone improve insulin sensitivity through additive stimulatory effects on muscle glycogen synthesis in mice fed high-fat diet:

Kuda O, Jelenik T, **Jilkova Z**, Flachs P, Rossmeisl M, Hensler M, Kazdova L, Ogston N, Baranowski M, Gorski J, Janovska P, Polak J, Mohamed-Ali V, Burcelin R, Cinti S, Bryhn M, Kopecky J. *Diabetologia* 52: 941-951, 2009, (IF = 6.328)

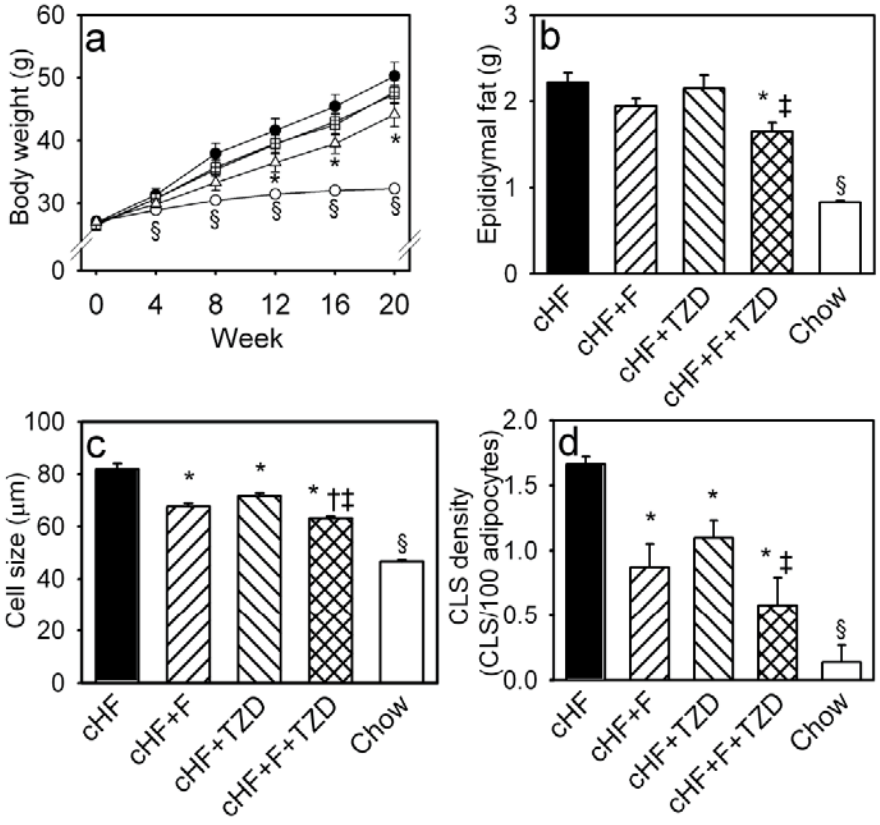
Based on preliminary results we hypothesized that the partially overlapping mechanisms of action of n–3 PUFA and TZD could have additive or synergistic effects in a combination treatment leading to an improvement of lipid and glucose homeostasis in obesity.

In initial analysis, a "**prevention study**" was performed to characterise the effects of n–3 PUFA, rosiglitazone and their combination, on developing obesity and impaired glucose tolerance (IGT) in mice fed high-fat diet. After weaning, male C57BL/6N mice were fed standard chow diet. At 3 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 increased body weight and the size of adipocytes was modulated in accord to reduced weight of epididymal fat tissue after combination treatment (Figure 4 b-c). Histological analysis combined with immunodetection of macrophages revealed cHF diet-induced hypertrophy of adipocytes in epididymal fat (Figure 4 c), accompanied by increased content of macrophages aggregated in crown-like structures surrounding individual adipocytes (Figure 4 d), indicating induction of low-grade inflammation of adipose tissue in cHF-mice. The induction of adipocyte hypertrophy and macrophage

infiltration was significantly counteracted by cHF+F and cHF+TZD diets, with cHF+F+TZD diet exerting the strongest effect.

Figure 3 Characterisation of the model – "*prevention study*"



Body weight (**a**), weight of epididymal fat (**b**), size of adipocytes (**c**), and macrophage infiltration of adipose tissue (**d**). 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). **b** Epididymal fat at 8 weeks, weight of fat depot. **c** Size of adipocytes. **d** Relative count of crown-like structures (CLS), formed by MAC-2-positive macrophages surrounding adipocytes. The morphometry data are based on measurements of more than 1,000 cells taken randomly from six different areas per animal (n=3-4); (**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).

Insulinemia increased between 8 and 20 weeks of high-fat feeding, suggesting development of insulin resistance and was prevented to a similar extent by cHF+TZD and cHF+F+TZD, while cHF+F exhibited also a significant but smaller effect. Both at 8 and 20 weeks, cHF+F and cHF+TZD increased total immunoreactive adiponectin in plasma. Even a stronger induction was observed by a combination of the two treatments. Other effects of treatment on metabolic markers as well as liver and muscle gene expression and metabolism were also analysed. Euglycaemic–hyperinsulinaemic clamps were used to characterize the changes in insulin sensitivity (for data see publication C).

Second, in a "**reversal study**", obesity and IGT were induced by feeding male C57BL/6J mice the cHF diet between 3 and 7 months of age. Then, they were randomly assigned to eight-week-long treatment as above; i.e. with cHF, cHF+F, cHF+TZD and cHF+F+TZD, respectively. Compared with cHF, body weight gain was suppressed by cHF+F, while cHF+TZD significantly decreased plasma insulin levels. Only cHF+F+TZD affected all the parameters studied, showing additive effects of DHA/EPA and rosiglitazone in the reversal of obesity. The combination treatment also reversed dyslipidaemia and IGT (for data see publication C).

The combined treatment using n–3 PUFA and a low-dose rosiglitazone generated additive effects in the prevention as well as reversal of adipose tissue hypertrophy induced by dietary fat. n–3 PUFA and rosiglitazone can be therefore used as complementary therapies to counteract dyslipidaemia and insulin resistance. The combination treatment may reduce dose requirements and hence the incidence of adverse side effects of thiazolidinedione therapy. The beneficial effects of the combination treatment were in large based on the amelioration of adipose tissue inflammation associated with obesity and modulation of the pattern of secretion of adiopokines.

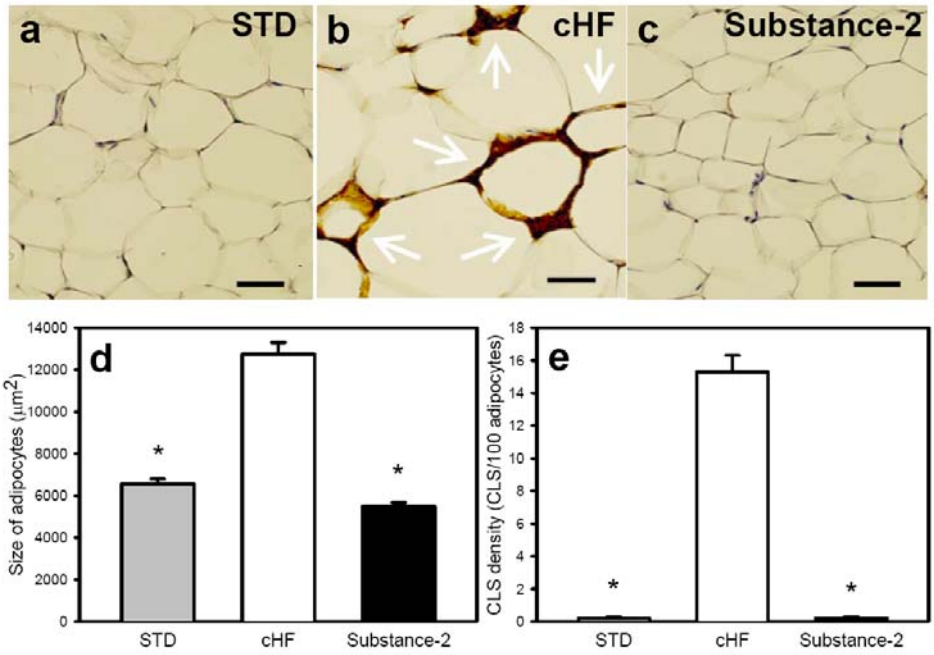
Publication D: DHA-derivatives in the prevention and reversal of obesity and glucose intolerance in mice: Rossmeisl M, Jelenik T, **Jilkova Z**, Slamova K, Kus V, Hensler M, Medrikova D, Povysil C, Flachs P, Mohamed-Ali V, Bryhn M, Berge K, Holmeide K, Kopecky J. *Obesity* 17: 1023–1031, 2009, (IF =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

Our objective was to determine the efficacy of α-substituted DHA derivatives as lipid-lowering, antiobesity, and antidiabetic agents.

C57BL/6 mice were given a corn oil–based high-fat (35% weight/weight) diet (cHF), or cHF with 1.5% of lipids replaced with α-methyl DHA ethyl ester (Substance 1), α-ethyl DHA ethyl ester (Substance 2), α,α-di-methyl DHA ethyl ester (Substance 3), or α-thioethyl DHA ethyl ester (Substance 4) for four months. Compared to the cHF-fed mice, all DHA derivatives except Substance 3 reduced weight gain and Substance 2 exerted the strongest effects. Mice fed Substance 2 had a reduced food intake and reduced the weight of subcutaneous and epididymal white adipose tissue by 73 and 42 %, respectively, while the remaining DHA derivatives had less effect on adiposity.

In the "prevention study," histological analysis of epididymal white adipose tissue (Figure 4) revealed adipocyte hypertrophy in the cHF-fed mice, resulting in an approximately twofold increase in the mean cell size. This effect was completely prevented by Substance 2 (Figure 4d). Moreover, Substance 2 also completely prevented obesity-associated macrophage infiltration of white adipose tissue, as revealed by immunohistochemical detection of Mac-2 (Figure 4b; white arrows). Macrophages aggregate in crown-like structures surrounding individual adipocytes. While the density of crown-like structures was ~77-fold higher in cHF-fed compared with STD-fed mice, Substance 2 completely prevented this effect (Figure 4c,e). Moreover, in epididymal white adipose tissue Substance 2 reduced mRNA levels of CD68 and MCP-1, two factors closely linked to macrophage function, by 91 and 56 %, respectively. In the "reversal study," Substance 2 reduced the accumulation of macrophages in epididymal white adipose tissue by 65 % and expression of CD68 and MCP-1 by 32 and 50 %, respectively. Thus, Substance 2 completely prevents and even partially reverses adipocyte hypertrophy and macrophage infiltration of white adipose tissue, induced by the obesogenic cHF diet.

Figure 4 The effect of Substance-2 on adipose tissue morphology and macrophage infiltration in the „*prevention study*“



The amount of MAC-2 immunoreactive macrophages (brownish color) was analyzed in epididymal fat. Sections were counterstained with hematoxylin-eosin. (**a**) Mice fed a low-fat chow (STD) diet. (**b**) Composite high-fat (cHF) diet. (**c**) Substance-2**.** Arrows indicate crown-like structures (CLS) surrounding individual adipocytes, where the majority of macrophages are localized. Scale bars = 50 µm. (**d**) Size of adipocytes. (**e**) CLS density. The morphometry data are based on more than 1,000 cells taken randomly from 5 different areas per animal ($n = 3$). *$P < 0.05$ vs. cHF diet (ANOVA).

In summary, among the four DHA derivatives tested, Substance 2 (α-ethyl DHA ethyl ester) appeared to exhibit a similar range of beneficial effects on obesity and associated metabolic traits as naturally occurring n-3 PUFA, but with a higher efficacy. These effects reflect extremely efficient reduction of the obesity-associated inflammation of adipose tissue, suggesting important beneficial effect on the secretion of pro- and anti-inflammatory adipokines from the adipose tissue. Therefore, α-ethyl DHA ethyl ester could qualify as a novel drug for the treatment of obesity, obesity associated low-grade inflammation, dyslipidemia, and insulin resistance.

4 CONCLUSIONS

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

1. For the first time, changes in D1 activity in white adipose tissue were demonstrated under conditions of changing adiposity, and a stimulatory effect of leptin on D1 activity in white adipose tissue was also found. We suggest that D1 has a functional role in white adipose tissue with D1 possibly being involved in the control of adipose tissue metabolism and/or accumulation of the tissue.

2. Differences in HF diet induced changes in plasma levels of leptin and thyroid hormones between obesity-resistant A/J and obesity-prone B6 strains of mice were found. Our results suggest a role for muscle non-shivering thermogenesis and lipid oxidation in the obesity-resistant phenotype of A/J mice and indicate that the HF diet could induce thermogenesis in oxidative muscle, possibly via the leptin-AMPK axis. Thus, white adipose tissue, by secreting leptin, plays an integrating role in the adaptive induction of thermogenesis in response to increased consumption of dietary fat, and also in the protection against development of obesity.

3. The combined treatment using n–3 PUFA and a low-dose rosiglitazone generated additive effects in the prevention as well as reversal of adipose tissue hypertrophy induced by HF diet. n–3 PUFA and rosiglitazone can be therefore used as complementary therapies to counteract dyslipidaemia and insulin resistance. The combination treatment may reduce dose requirements and hence the incidence of adverse side effects of thiazolidinedione therapy. The beneficial effects of the combination treatment were mostly based on the amelioration of adipose tissue inflammation associated with obesity and on the modulation of the secretion pattern of adipokines.

4. Among the four chemical DHA derivatives tested in high-fat diet-fed mice, Substance 2 (α-ethyl DHA ethyl ester) appeared to exhibit a similar range of beneficial effects on obesity and associated metabolic traits as naturally occurring n-3 PUFA, but with a much higher efficacy. These effects reflect extremely efficient reduction of the obesity-associated inflammation of adipose tissue, suggesting an important beneficial effect on the secretion of pro- and anti-inflammatory adipokines from the tissue. Therefore, α-ethyl DHA ethyl ester could qualify as a novel drug for the treatment of obesity, obesity-associated low-grade inflammation, dyslipidemia, and insulin resistance.

In conclusion, results of all four studies support the concept of the integrating role of adipose tissue in the whole-body responses to dietary constituents as well as pharmacological agents which ameliorate obesity and associated insulin resistance. Our results suggest that the integrating role strongly depends on a changing profile of adipokines secreted from the tissue.

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6 LIST OF PUBLICATIONS

6.1 Thesis based on articles:

- **Macek Jilkova Z**, Pavelka S, Flachs P, Hensler M, Kus V, Kopecky J. Modulation of type I iodothyronine 5'-deiodinase activity in white adipose tissue by nutrition: possible involvement of leptin. *Physiol. Research, in press,* (IF = 1.739)
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6.2 Other published articles:

- Kopecky J, Rossmeisl M, Flachs P, Kuda O, Brauner P, **Jilkova Z**, Stankova B, Tvrzicka E, Bryhn M. n-3 PUFA: bioavailability and modulation of adipose tissue function. *Proc Nutr Soc,* 2009, *in press,* (IF = 4.304)
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