Doctoral studies in Biomedicine

Charles University in Prague

and Academy of Sciences of the Czech Republic, v.v.i.

Section:	Biochemistry and pathobiochemistry
Section chairman:	prof. MUDr. Jiří Kraml, DrSc. Institute of Medical Chemistry and Biochemistry First Faculty of Medicine, Charles University in Prague Kateřinská 32, 121 08 Praha 2
Workplace:	Department of Adipose Tissue Biology Institute of Physiology of the Academy of Sciences of the Czech Republic, v.v.i. Videnska 1083, 14220 Praha 4
Author:	Daša Medříková
Supervisors:	Jan Kopecký, MD, DSc. Department of Adipose Tissue Biology Institute of Physiology of the Academy of Sciences of the Czech Republic, v.v.i. Videnska 1083, 14220 Praha 4

Opponents:

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 and associated metabolic disorders, e. g. metabolic syndrome, represent a considerable health threat for modern society. Due to sedentary lifestyle, high caloric intake and changes in composition of diet, prevalence of obesity is increasing worldwide.

One of the possible causes contributing to higher prevalence of obesity in recent population could be the change of fatty acids (FA) composition of dietary lipids, with the shift in the content of n-6 and n-3 FA toward n-6 FA. In contrast to n-6 FA, n-3 FA are known for their anti-atherogenic, anti-obesogenic and anti-inflammatory properties. In our experiments in mice, the capability of naturally occurred and chemically modified n-3 long chain polyunsaturated fatty acids (LC-PUFA) in prevention and reversal of specific parts of metabolic syndrome was demonstrated. A specific chemical derivative of docosahexaenoic acid was proven to be very effective in preventing and improving metabolic conditions of animals exposed to high-fat (HF) diet challenge. Further, the involvement of AMP-activated protein kinase (AMPK), a master regulator of lipid metabolism, in skeletal muscle thermogenesis induced by HF-feeding was investigated. Activation of AMPK in the HF-fed mice is most possibly caused by increased leptin levels and represents an important link in induction of skeletal muscle thermogenesis as possible mechanism protecting from obesity caused by HF diet administration. Besides leptin, LC-PUFA are also involved in AMPK activation, however, in an indirect way through increasing blood levels of adiponectin. Thus, these lipids contribute to preserving insulin sensitivity under the condition of HF-feeding. Insulin resistance, a state characterised by impaired response to insulin action, is at least in part caused by ectopic lipid storage and obesity-associated low-grade adipose tissue inflammation. It is well known, that the difference in the response to HF administration in dependence on the sex exists. In our experiments, we have discovered that in spite of higher adiposity, female mice show milder phenotype and later onset of metabolic disorders than their male counterparts. Better insulin sensitivity in females could be at least in part attributed to higher adipose tissue expandability resulting in decreased extra-adipose tissue lipid storage and lower fat inflammation.

SOUHRN

Obezita a přidružené metabolické poruchy jsou velkým problémem moderní společnosti. Příčinami celosvětového nárůstu obezity jsou sedavý životní styl, vyšší kalorický příjem a v neposlední řadě změna ve složení potravy.

Jednou z příčin vysoké prevalence obezity v současné populaci může být posun poměru mezi obsahem n-6 a n-3 mastných kyselin (MK) v dietě směrem k vyššímu obsahu n-6 MK. Na rozdíl od n-6 MK, se n-3 MK vyznačují antiatherogenními, antiobezogenními a protizánětlivymi účinky. Podařilo se nám prokázat pozitivní schopnosti přírodních i chemicky modifikovaných n-3 vícenenasycených mastných kyselin s dlouhým řetězcem v prevenci i reverzi metabolického syndromu u zvířecího modelu, u myší vystavených působení diety s vysokým obsahem tuku. Další studium bylo zaměřeno na průkaz významu jednoho z hlavnich regulačních mechanizmů lipidového metabolizmu, enzymu AMP-aktivované proteinové kinázy (AMPK), pro indukci netřesové tvorby tepla ve svalu vlivem vysokotukové diety. Zjistili jsme, že zvýšení krevních hladin leptinu v důsledku vysokého příjmu tuků vyvolá aktivaci AMPK ve svalu. To vede k indukci katabolizmu lipidů a částeční ochraně organizmu před obezitou. Kromě leptinu působí jako aktivátory AMPK také vícenenasycené MK, i když ty jsou do procesu aktivace zapojené nepřímo, přes zvýšení krevních hladin adiponektinu. Tento mechanizmus napomáhá zachovat citlivost k inzulínu za podmínek zvýšeného příjmu tuků. Inzulinová rezistence je stav charakterizovaný nedostatečnou odpovědí organizmu na působení inzulinu. Je částečně způsobená ektopickým ukládáním lipidů a chronickým zánětem tukové tkáně a tyto pochody také zavisí na pohlaví. V pokusech na myších se nám podařilo potvrdit, že samice jsou méně náchylné k celkovému metabolickému rozvratu v důsledku obezity, než je tomu u samců. Ukázali jsme, že lepší inzulinová citlivost u samic může souviset s vyšší schopností jejich adipocytů zvětšovat objem a ukládat lipidy. Schopnost většího zvětšení adipocytů u samic než u samců přispívá k nižšímu ektopickemu hromadění lipidů a také k menší míře zánětlivých změn tukové tkáně při obezitě. To vše se projevuje menšími poruchami inzulinové signalizace při obezitě samic u než u samců.

CONTENT

1 INTRODUCTION

1.1 Energy homeostasis

Overweight and obesity represent a considerable threat for modern society, as they are associated with a markedly increased risk of developing many severe health disturbances, such as atherosclerosis, non-alcoholic fatty liver disease, certain types of cancer and metabolic syndrome (1).

In an organism, energy is stored in the form of glycogen in liver and skeletal muscle, and in the form of triacylglycerols (TAG) in adipose tissue. Mammalian species receive required energy for their vital processes as food. This form of chemical energy is converted by metabolic processes in the body to physical work or heat and enables survival of an organism. Unused energy is stored and can be reused in conditions of negative energy balance. Food intake, energy expenditure and preservation of body energy stores are subjected to complex regulation (2).

Total energy expenditure consists of three main components: energy expenditure required for 1) basal metabolism; 2) physical work and 3) adaptive thermogenesis. Adaptive thermogenesis can be classified as shivering and non-shivering thermogenesis. Shivering thermogenesis, caused by the activation of muscle contractions, protects an organism from cold by dissipation of energy in the form of heat. Non-shivering thermogenesis occurs after adaptation to cold, when brown adipose tissue (BAT) and skeletal muscle undertake the heat production by the mechanism of mitochondrial uncoupling. To the non-shivering thermogenesis belongs diet-induced thermogenesis as well, as feeding is known to increase energy expenditure (3). Mechanism of mitochondrial uncoupling leading to heat production was believed to be present only in mammalian neonates and small mammals; however, recent observations suggest its relevance in adults, as it can importantly contribute to energy expenditure (4).

1.2 Linking obesity to insulin resistance and type 2 diabetes

Obesity is a key factor in developing insulin resistance, a state characterised by improper response of insulin target tissues to insulin action. Impaired insulin sensitivity is characterised by decreased efficiency of insulin to stimulate insulin-responsive signalling

pathways, which eventually results in hyperglycaemia and/ or dyslipidaemia. Deficient insulin efficacy can be partially compensated by increased insulin secretion; however, chronically enhanced insulin secretion can lead to β-cells exhaustion and the onset of type 2 diabetes.

1.2.1 Role of adipose tissue

Adipose tissue has been considered a passive storage place for excessive energy in a form of TAG, protecting an organism from lipotoxicity, providing insulation and mechanical support for other organs. However, accordingly to recent studies, adipose tissue is a metabolically active organ that considerably influences metabolic homeostasis and secretes numerous hormones and cytokines (5).

During the obesity development, adipose tissue expands predominantly by hypertrophy (adipocyte enlargement). Hypertrophied adipocytes are resistant to antilipolytic effect of insulin and are associated with insulin resistance and type 2 diabetes (6). However, also adipocyte hyperplasia (formation of new adipocytes) can significantly contribute to a progression of obesity (7).

Adipocytes cannot expand beyond a "critical size". After reaching it, the capacity of adipose tissue for storing excessive lipids is depleted, so the surplus fat is accumulated ectopically in organs such as liver, skeletal muscle and heart. This negatively affects the normal metabolic response of these tissues, as they are not adapted to store large amounts of fat (8). Hence, excessive lipid accumulation together with decreased fatty acid oxidation leads to hepatic steatosis, impaired insulin signalling and accumulation of various harmful lipid metabolites in muscle and liver. Thus, the expandability of adipose tissue could be important for at least partial protection from ectopic lipid accumulation and subsequent insulin resistance.

1.2.2 AMP-activated protein kinase

Obesity-related insulin resistance and type 2 diabetes are characterised by defective energy metabolism. Adenosine monophosphate (AMP)-activated protein kinase (AMPK) controls whole body glucose and lipid homeostasis by regulating metabolism in liver, skeletal muscle, adipose tissue and pancreatic β-cells (9) – key tissues in the pathogenesis

of type 2 diabetes. AMPK represents an energy sensor and master regulator of metabolism, placing it in the center of studies of obesity and metabolic syndrome (10). In general, AMPK activation triggers catabolic pathways that produce ATP and represses ATP consuming processes in order to restore cellular energy stores. Thus, it supports fatty acid oxidation and glucose uptake together with repression of gluconeogenesis and lipolysis. The ability of AMPK to induce lipid oxidation and thereby decrease muscle and liver TAG content is considered an important feature of its insulin-sensitising effect and thus its activation could be beneficial for type 2 diabetes treatment.

1.3 Secretory function of adipose tissue

By now, various products of adipose tissue, termed adipocytokines, have been characterised (5;11). Adipocytokines are bioactive peptides and proteins mainly synthesised and secreted by adipose tissue, although not exclusively by adipocytes. Immune cells infiltrated in fat tissue, such as macrophages, largely contribute to the production of adipocytokines. Adipocytokines are important signalling mediators, fundamental to the pathogenesis of the metabolic syndrome (12). Magnitude of their production depends highly on the body fat mass, subsequently on the degree of obesity and adipocyte size.

1.3.1 Adipocytokines

Leptin is an anorexigenic hormone secreted mainly from differentiated adipocytes. It regulates food intake and energy expenditure by acting at central nervous system (13). Leptin secretion and its circulating levels are positively correlated with total body fat mass (14). Thus, obesity is characterised by hyperleptinaemia. However, leptin and its postreceptor signalling are impaired in obesity. Besides acting at neural tissues, leptin has also direct pleiotrophic effects in peripheral tissues. It stimulates fatty acid oxidation by activating of AMPK (15) and activates skeletal muscle thermogenesis (16).

Adiponectin is an abundant serum protein expressed in mature adipocytes. In contrast to majority of adipocytokines, its circulating levels are negatively correlated with body fat (14). Adiponectin is associated with insulin sensitivity (17), it enhances insulin-dependent suppression of hepatic glucose production and thereby has a lowering effect on

glycaemia. Moreover, it increases glucose uptake and fatty acid oxidation in several tissues via AMPK activation (18) and poses anti-inflammatory properties.

Tumour necrosis factor α (TNF-α) is a pro-inflammatory cytokine, which expression is elevated in an obese state (11). Adipose tissue-derived TNF-α originates to a great extent from macrophages infiltrating fat tissue (19). It represents a promising link between obesity, inflammation and impaired insulin signalling (11). Further, TNF-α is implicated in the pathogenesis of obesity by modulating the gene expression (20).

Monocyte chemoattractant protein-1 (MCP-1) is secreted by adipocytes and adipose tissue macrophages and its release is in proportion to progressive accumulation of fat and adipose tissue inflammation (21). It is a potent chemotactic factor responsible for recruiting monocytes/ macrophages into adipose tissue (22), which is the major cause of obesity-associated low-grade inflammatory state. Further, it possibly contributes to insulin resistance as well (22).

1.3.2 Adipose tissue inflammation

Progressive overnutrition and obesity is characterised by adipocyte hypertrophy and/ or hyperplasia, increased macrophage infiltration, higher propensity of enlarged adipocytes to necrotic cell death (23) and dysregulation of adipocytokine expression, shifted toward dominance of pro-inflammatory adipocytokines. Enhanced secretion of chemoattractants in obese state promotes migration of macrophages into adipose tissue, accompanied by the phenotypic switch of macrophages to a pro-inflammatory state (24). Moreover, higher release of pro-inflammatory cytokines into the circulation, may disrupt insulin signalling in other tissues (22). Large adipocytes are less insulin sensitive in comparison to smaller ones, and are associated with a chronic low-grade adipose tissue inflammation that represents a key factor linking obesity with insulin resistance (12).

1.4 Attenuation of obesity-associated pathogenesis by n-3 PUFA

Long chain polyunsaturated fatty acids (LC-PUFA) of n-3 series are essential for many mammalian species. They are abundant in fish oils, especially of marine origin; act as hypolipidemics, reduce the incidence of cardiac events, prevent progression of

atherosclerosis, have immunomodulatory effects and can prevent obesity and impaired glucose tolerance (25;26).

Metabolic actions of n-3 LC-PUFA are largely mediated through the altered expression of genes involved in carbohydrate and lipid metabolism. n-3 LC-PUFA regulate gene expression in various tissues by acting as ligands to different transcription factors, i.e. peroxisome-proliferator activated receptors (PPARs), retinoid X receptors, liver X receptors, hepatic nuclear factor-4 and SREBPs (27). Besides direct actions, n-3 LC-PUFA act through their active metabolites including resolvines, protectines, eicosanoids, and docosanoids (28).

1.5 Aspect of sex in control of insulin sensitivity

Human and animal studies proved that female subjects, although having higher total body fat mass, demonstrate milder phenotypes concerning obesity-related metabolic disorders and/ or showed later onset of metabolic impairment (29;30) in comparison to their male counterparts. Underlying mechanisms of this phenomenon remain largely unknown; however, they could be partially associated with differences in fat accumulation and adipose tissue inflammation.

It has been shown that female mice fed a HF diet demonstrated lower adipose tissue inflammation compared to males (31). Furthermore, females present higher circulating adiponectin concentrations (32). As adiponectin exerts anti-inflammatory effects (33) and activates AMPK (18), higher adiponectin levels in females can contribute to lower infiltration of their adipose tissue by macrophages and/ or can promote macrophage polarization towards an anti-inflammatory phenotype (33) and decreased tendency to ectopic lipid accumulation.

However, since the protective effect is apparent only until the menopause, beneficial effects of oestrogens on metabolism cannot be ruled out. Some studies showed anti-inflammatory oestrogen action (35), but further research is needed to elucidate these mechanisms.

2 AIMS OF THE THESIS

The general aim of the thesis was to evaluate metabolic changes of an organism after challenge with HF-feeding with respect to development of specific components of the metabolic syndrome. Part of the work focused on the role of lipid composition in the diet, namely the beneficial effects of partial replacement of dietary lipids with n-3 LC-PUFA concentrate (specific aims 1 and 3). Furthermore, this thesis was aimed to contribute to the elucidation of the role of widely expressed enzyme AMPK in the protection from obesity and insulin resistance (specific aims 2 and 3). And finally, regarding significant differences in the incidence, prevalence and severity, by which males and females experience diseases associated with the metabolic syndrome, this thesis attempted to elucidate the disparity in the development of obesity in males and females by evaluating adipose tissue function at the onset of insulin resistance using a model of HF-feeding in mice.

The specific aims of this thesis were:

1. to characterise a role of lipid composition, namely with respect to n-3 LC-PUFA and their derivatives, in the induction of obesity and insulin resistance associated with the metabolic syndrome;

2. to examine the involvement of leptin-AMPK axis in lipid metabolism of skeletal muscle in response to HF-feeding, using a model of obesity-resistant (A/J) and obesity-prone (C57BL/6) strain of mice;

3. to investigate the role of AMPK in preservation of insulin sensitivity induced by n-3 LC-PUFA feeding using a model of AMPKα2 knock-out mouse model;

4. to investigate sex-dependent changes in adipocyte hypertrophy, low-grade inflammation and secretory function of adipose tissue with respect to the development of insulin resistance induced by HF-feeding in C57BL/6 mice.

3 RESULTS TO SELECTED PUBLICATIONS

3.1 Publication A: Prevention and reversal of obesity and glucose intolerance in mice by DHA derivatives

The aim of this work was to determine the efficiency of four different chemical α-derivatives of DHA compared to pure, non-modified DHA in the prevention and reversal of obesity and associated metabolic disorders.

The *"Prevention study"* was performed on male mice of C57BL/6 strain. Three-month-old mice were randomly assigned either to high-fat diet (35 % wt/wt; cHF) or to cHF diet, where 1.5 % of total lipids was 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). The experimental diets were administered for a period of four months. In the *"Reversal study"*, the effect of Substance 2 was evaluated in C57BL/6 mice with diet-induced obesity. After 4 months of cHF-feeding, mice were administered cHF diet supplemented with Substance 2 (1.5 % of total lipids) for 2 months. To evaluate the effect of a concentrate of DHA/EPA and a pure DHA, the separate experiment was performed in the same experimental setup as the *"Reversal study"*.

Substance 2 had the most pronounced effect in body weight gain reduction from all DHA-derivatives in the *"Prevention study"*. It reduced weight of subcutaneous (scWAT) and gonadal (gWAT) fat by 73 % and 42 % respectively, and the effect on reduction of food intake was demonstrated as well. Since Substance 2 was proven as the most effective derivative in the suppression of obesity and its detrimental metabolic consequences, it was also tested in the *"Reversal study"* using obese mice with impaired glucose tolerance.

In both types of experiments, Substance 2 prevented or normalised adipocyte hypertrophy and macrophage infiltration to WAT and formation of CLS. The immunohistochemical analysis was confirmed by real-time PCR (RT-PCR), showing that Substance 2 reduced the expression of macrophage markers CD68 antigen and MCP-1 in

WAT by 91 % and 56 % respectively in *"Prevention study"* and by 32 % and 50 % respectively in *"Reversal study"*.

In liver, genes implicated in fatty acid oxidation were influenced by the treatment. Acyl-CoA oxidase-1 (*Aox-1*) and carnitine palmitoyltransferase-1α (*Cpt-1α*) were strongly upregulated by Substance 2, in agreement with a marked increase in mRNA expression of their regulatory transcription factor *Pparα*. Pure DHA and DHA in combination with EPA also increased transcription of *Cpt-1α,* but not *Aox-1*, however, the observed changes were much smaller than changes caused by the ingestion of Substance 2. The differences are striking, especially when we take into account a 10-fold lower concentration of Substance 2 in comparison with n-3 LC-PUFA (EPA/DHA) concentrate. Lipogenic genes, such as stearoyl-CoA desaturase (*Scd-1*), thyroid hormone responsive SPOT 14 (*Spot 14)*, farnesyl diphosphate synthase (*Fdps*), are known to be downregulated by n-3 LC-PUFA. In accordance, DHA and EPA/DHA decreased the expression of *Scd-1*and *Spot 14*, however, Substance 2 increased mRNA expression of *Scd-1*and *Fdps*. Effect of Substance 2 on skeletal muscle was insignificant.

To conclude, from the four tested DHA derivatives, Substance 2 appeared as most effective in obesity prevention as well as in the treatment of dyslipidaemia and insulin resistance associated with dietary obesity. It showed similar range of beneficial effects as naturally occurring n-3 LC-PUFA, but exerted much higher efficiency. Substance 2 also prevented macrophage accumulation in adipose tissue, which can have beneficial systemic effects on inflammatory state associated with obesity and consequently on insulin resistance. Furthermore, Substance 2 reduced ectopic lipid accumulation via enhanced lipid catabolism and possibly futile substrate cycling, as indicated by simultaneous activation of lipogenic genes (*Scd-1*, *Fdps*) and genes responsible for lipid oxidation (*Cpt-1α, Aox-1*) in the liver. Therefore, Substance 2 could be qualified as a novel drug for obesity treatment.

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

The main goal of this study was to elucidate, whether HF diet could stimulate muscle non-shivering thermogenesis and determine possible involvement of leptin-AMPK axis in this process.

The experiments were performed on male mice of obesity prone (C57BL/6) and obesity resistant (A/J) strain, born and maintained at nearly thermoneutral temperature of 30°C, to eliminate the possible confounding effect of shivering thermogenesis and adaptation to cold. At the age of four weeks, mice were randomly assigned to low fat (LF) or HF diet and maintained on these diets for two more weeks until sacrifice.

After 2 weeks on diets, no differences either in body weight, body weight gain or food consumption were observed. However, HF diet increased the weight of fat depots with a more pronounced effect in A/J mice, which was accompanied by a higher induction of plasma leptin levels in these mice. Different propensity to dietary-induced obesity was apparent only after longer exposure to HF diet and did not depend on stronger induction of energy expenditure of A/J mice in response to cold. A/J and C57BL/6 mice strains differed in their thermogenic capacity. After cold exposure (4°C), A/J LF mice became hypothermic, while the activation of shivering was similar as in C57BL/6 LF mice. HF diet reversed this phenotype and A/J HF mice were able to maintain their body temperature in cold. Measurement of norepinephrine-stimulated metabolic rate revealed stronger inducibility of UCP1-mediated thermogenesis by HF-diet in A/J compared to C57BL/6 mice. Protein content of UCP1 in interscapular BAT demonstrated induction by HF diet, while the increase was significantly higher in A/J when compared to C57BL/6 mice (Table 1). Furthermore, protein levels of UCP1 and its mRNA expression were measured in subcutaneous fat depot and skeletal muscles, respectively (Table 1); however, UCP1 levels were too low to be physiologically important for thermogenesis.

Table 1 Quantification of UCP1 and UCP2 expression in fat depots

Fat depot	C57BL/6		A/J	
	LF	HF	LF	HF
BAT				
UCP1-protein	9.6 ± 0.7	32.0 ± 4.1*	6.0 ± 0.8†	63.8 ± 10.5*†
UCP2-transcript	1.26 ± 0.30	0.81 ± 0.27	0.61 ± 0.17	0.69 ± 0.39
DL				
UCP1-protein	0.016 ± 0.005	0.043 ± 0.007*	0.043 ± 0.013†	0.065 ± 0.011
UCP2-transcript	0.62 ± 0.10	1.08 ± 0.36	0.95 ± 0.20	1.53 ± 0.16
EPI				
UCP2-transcript	1.23 ± 0.22	1.00 ± 0.10	2.17 ± 0.69	1.59 ± 0.34

Mice adapted to 30 ^{o}C and fed either LF of HF diet for 2 weeks after weaning were studied. UCP1 protein (mg/g membrane protein) was quantified using Western blots in 100,000 x g membranes isolated from adipose tissue homogenates. UCP2 transcript levels (AU) were measured using qRT-PCR in total RNA isolated from fat depots. BAT, interscapular brown fat; DL, dorsolumbar white fat; EPI, epididymal white fat. Data are means ± S.E. (n = 4-8). *Significant effect of diet; †significant effect of genotype. (See Table S2 in online Appendix of the respective publication).

To assess skeletal muscle metabolism and its possible involvement in non-shivering thermogenesis, gene expression in glycolytic *gastrocnemius* and oxidative *soleus* muscles were determined. HF diet upregulated the expression of pyruvate dehydrogenase kinase 4 (*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 A/J HF mice. This is in agreement with higher plasma leptin concentrations in this muscle, since *Scd-1* is suppressed by leptin. In addition, aminoimidazole carboxamid ribonucleotide-stimulated fatty acid oxidation was elevated in *soleus* of A/J HF mice, reflecting higher activation of AMPK (see publication).

These results indicated increased fatty acid oxidation and enhanced thermogenesis in oxidative skeletal muscle in response to HF diet in A/J mice, with a likely involvement of leptin-AMPK axis. Together with increased UCP1-mediated thermogenesis in BAT, both mechanisms could lead to obesity resistance in A/J mice fed HF diet.

3.3 Publication C: AMP-activated protein α2 subunit is required for the preservation of hepatic insulin sensitivity by n-3 polyunsaturated fatty acids.

Our objective was to test the hypothesis, whether AMPK is implicated in the beneficial effects of n-3 PUFA in the prevention of obesity, dyslipidaemia and insulin resistance.

Four-month-old whole-body AMPKα2 knock-out (AMPKα2$^{-/-}$) mice on C57BL/6J background and their wild-type littermate controls were used in this study. Mice were fed 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 LC-PUFA concentrate (cHF+F) for 9 weeks.

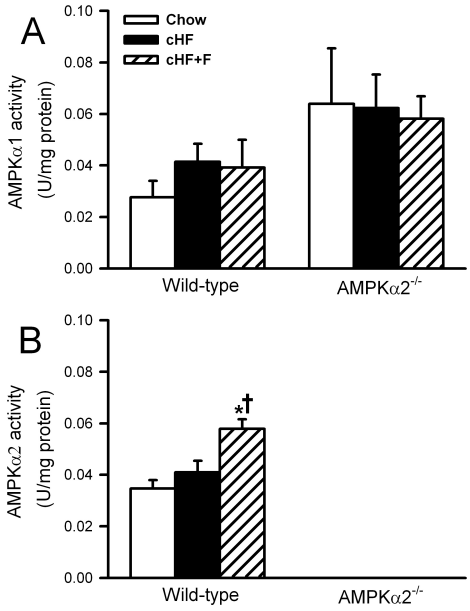

Figure 1 Liver AMPKα1 (A) and AMPKα2 (B) activity in wild type and AMPKα2$^{-/-}$ mice. The data are the means ± SE (n=5-8). In the AMPKα2$^{-/-}$ mice, AMPKα2 activity was below the detection limit. *P < 0.05 vs. genotype Chow; † P < 0.05 vs. genotype cHF. (See Figure 1 in the respective publication).

When specific activities of hepatic AMPKα1 and AMPKα2 subunits were evaluated, an increase in the activity of AMPKα2 subunit in response to n-3 LC-PUFA supplementation was observed. AMPKα1 activity did not show any significant compensatory changes in AMPKα2$^{-/-}$ mice, however, its activity tended to be increased (Fig. 1).

Administration of cHF diet resulted in higher body weight gain in wild-type and AMPKα2$^{-/-}$ mice when compared to chow-fed animals. However, the effect was less pronounced in AMPKα2$^{-/-}$. cHF+F diet induced smaller body weight gain regardless of the genotype. Similarly, cHF+F diet decreased plasma lipids independently of AMPKα2 subunit. However, n-3 LC-PUFA-containing diet prevented high plasma insulin levels associated with cHF diet feeding only in wild-type animals. Assessment of insulin sensitivity by hyperinsulinemic-

euglycaemic clamp revealed protective effects of n-3 LC-PUFA from detrimental effects of cHF feeding only in wild-type mice, while no beneficial effects were observed in AMPK$\alpha 2^{-/-}$ mice. In line with these observations, plasma levels of insulin-sensitising hormone adiponectin, were increased only in wild-type animals in response to n-3 LC-PUFA (Fig. 2).

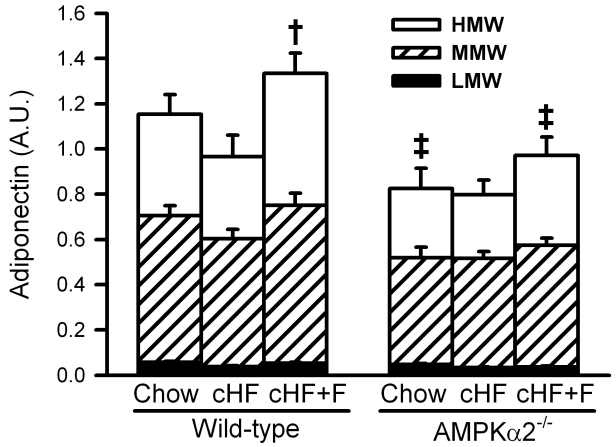

Figure 2 Adiponectin levels in plasma of wild-type and AMPKα2$^{-/-}$ mice fed either a Chow diet or corn oil-based high-fat diets without (cHF) or with 15% of the lipids in the form of n-3 LC-PUFA concentrate (cHF+F) for 9 weeks, and killed in *ad libitum* fed state. The total adiponectin levels and the distribution of adiponectin multimeric complexes were determined using Western blotting. The data are the means ± SE (n = 13-15). **P* < 0.05 vs. genotype Chow; †*P* < 0.05 vs. genotype cHF; ‡*P* < 0.05 vs. wild-type on respective diet. Significance evaluated for the total adiponectin levels. A.U., arbitrary units; LMW, low molecular weight; MMW, medium molecular weight; HMW, high molecular weight.

Liver TAG under the clamp conditions were decreased by n-3 LC-PUFA in AMPKα2-dependent manner. Moreover, experiments on isolated hepatocytes confirmed AMPK-dependent induction of fatty acid oxidation by n-3 LC-PUFA in the liver (see publication). The composition of hepatic lipids is very important for insulin sensitivity. While hepatic ceramides and phospholipids were not affected, the content of polyunsaturated DAG was modified by both diet and genotype. Dietary supplementation by n-3 LC-PUFA prevented accumulation of total DAG in wild-type mice compared to mice without functional AMPKα2 subunit (for details see publication).

To summarise, our results suggest that regulatory α2 subunit of AMPK is not necessary for anti-obesity and hypolipidemic effects of n-3 LC-PUFA, however, it is required for the preservation of insulin sensitivity, especially in the liver. Nevertheless, the activation of AMPKα2 by n-3 LC-PUFA is likely independent of their on direct interaction since it is most probably mediated by n-3 LC-PUFA-dependent increase in adiponectin plasma concentration and subsequent AMPK activation in target tissues.

3.4 Publication D: Sex differences during the course of diet-induced obesity in mice: adipose tissue expandability and glycaemic control

Our objective was to evaluate sex-based differences in adipose tissue plasticity with respect to adipose tissue inflammation and propensity to deterioration of insulin signalling between male and female mice.

This study was performed on male and female mice of C57BL/6N strain fed a control (3.4 % wt/wt of fat; ST) or high fat (35 % wt/wt of fat; HF) diet from four weeks of age for 15 or 35 weeks, to compare different states of body weight development after long-term HF diet administration. ST-fed mice served as a control for the obesogenic effect of HF-treatment (most of the parameters analysed in the study were differentially affected by ST and HF diet; for results from ST-fed mice see publication). HF-fed males weighing more than females until week 20. From that week onwards, body weights of both sexes equalised.

HF-fed females tended to have bigger gonadal (gWAT) and subcutaneous (scWAT) depots than males at week 15 (see publication) and the differences became statistically significant at week 35 (5989 ± 470 vs. 1565 ± 95 mg for gWAT; 1590 ± 133 vs. 1297 ± 113 mg for scWAT, $p<0.05$). Higher adiposity in females at week 35 was also reflected in higher leptinaemia (89.6 ± 2.8 vs. 71.9 ± 4.7 ng/ml at week 35, $p<0.05$). HF-feeding induced accumulation of TAG in the livers of both sexes with a more pronounced effect in males (197 ± 27 vs. 121 ± 8 mg/g of tissue in males and females, respectively; $p<0.05$). Higher TAG accumulation in the liver together with smaller gWAT and scWAT suggested decreased capacity for adipose tissue enlargement and thereby higher ectopic lipid deposition in males as compared to females.

At week 15, HF-fed females showed the ability to decrease plasma glucose levels in response to fasting, in contrast to HF-fed males, indicating metabolic inflexibility in males (Fig. 3a). Glucose homeostasis was evaluated by three subsequent intraperitoneal glucose tolerance tests (IP GTT) and fasting blood glucose (FBG) levels determination at weeks 10, 25 and 33. HF-feeding exacerbated glucose tolerance associated with aging in both sexes, however, males were affected earlier (Fig. 3b,c). At week 33 plasma insulin levels were determined at the baseline and 30 minutes after glucose load during IP GTT.

Although the fasted insulin did not differ between the sexes, a higher increase in response to glucose load was observed in males (Fig. 3d). This suggests that females need lower insulin levels to maintain the same glucose tolerance as males. Since adiponectin possess substantial insulin-sensitising effect mediated through activation of AMPK, better sensitivity to insulin action in females can be related to constantly higher plasma adiponectin levels (1.25 ± 0.05 vs. 0.78 ± 0.03 A.U. at week 15; 1.44 ± 0.13 vs. 0.92 ± 0.07 A.U. at week 35 in females and males, respectively; $p<0.05$).

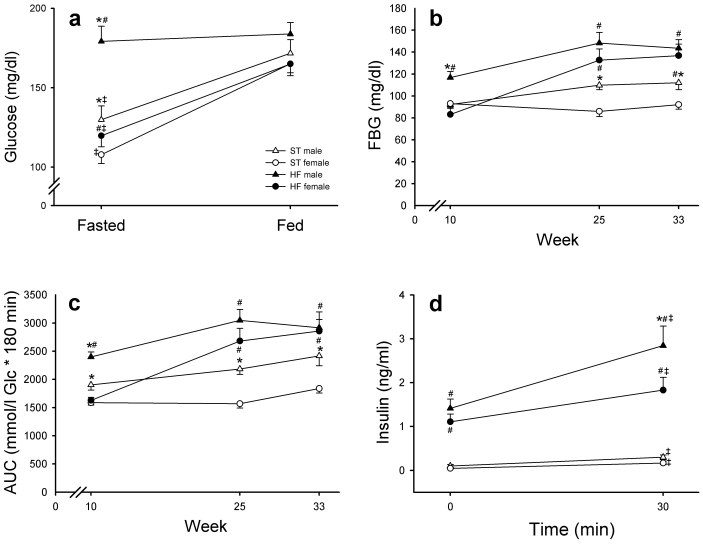

Figure 3 Glucose homeostasis. (a) Plasma glucose levels in fasted and fed mice at week 15. **(b-d)** Intraperitoneal glucose tolerance (GTT) test was performed at week 10, 25 and 33, respectively, after weaning to either ST or HF diet. **(b)** Fasting blood glucose (FBG) at time 0 of the GTT performed at week 10, 25 and 33, respectively. **(c)** Area under the glycaemic curve (AUC) of the tests performed at week 10, 25 and 33, respectively. **(d)** Plasma insulin levels at time 0 and 30 min of the test performed at week 33. Data are means ± SE (*n=10*; at week 10, only part of mice was randomly selected for the testing). *significant difference between sexes within diet, #significant difference between diets within sexes (ANOVA), ‡significant difference before and after respective treatment (RM ANOVA).

Lower ectopic fat storage and better metabolic profile of females prompted us for further analysis of adipose tissue, as insulin resistance is closely associated with adipose tissue inflammation and correlates with adipocyte size. Histological analysis of gWAT and scWAT combined with morphometry of adipocytes showed that HF-feeding increased adipocyte size as well as macrophage infiltration in gWAT and scWAT of both sexes. In agreement with a widely accepted concept that adipocyte size correlates with adipose tissue inflammation, males demonstrated adipocyte hypertrophy accompanied by a markedly increased number of CLS especially in gWAT both at week 15 and 35. Surprisingly, the dissociation of adipocyte size from macrophage infiltration was observed in females, where in spite of bigger adipocytes, adipose tissue macrophage infiltration remained considerably lower (see publication)

4 CONCLUSIONS

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

1. Naturally occurring n-3 LC-PUFA exhibited beneficial effects in the prevention and treatment of obesity and metabolic syndrome. However, chemical DHA-derivatives manifested the same range of health benefits, but with a higher efficacy. Substance 2 (α-ethyl DHA ethyl ester) profoundly reduced adipocyte size and adipose tissue inflammation in obese mice, which was associated with improved insulin sensitivity.

2. Differences in plasma leptin levels between obesity resistant A/J and obesity prone C57BL/6 mice were identified. A/J mice exhibited a cold sensitive phenotype when fed LF diet. However, HF diet normalised cold tolerance by inducing both, UCP1 mediated thermogenesis and non-shivering thermogenesis in oxidative skeletal muscle. Muscle thermogenesis was accompanied by a preferential oxidation of lipids, suggesting the involvement of leptin-AMPK axis.

3. α2 subunit of AMPK was required for the preservation of hepatic insulin sensitivity mediated by n-3 LC-PUFA in mice fed HF diet. Dietary n-3 LC-PUFA modulated the type of accumulated hepatic DAG and completely prevented an increase of polyunsaturated DAG in AMPKα2-dependent manner.

4. Sex-dependent differences in adipose tissue in response to HF-feeding were described. Female mice demonstrated higher adiposity, however, in spite of larger adipocytes, the frequency of CLS was significantly lower than in males. Females accumulated less fat in non-adipose tissues, which together with lower inflammation of WAT and constantly increased adiponectin levels corresponded with their improved sensitivity. Thus, adipose tissue expandability and its capability to handle energy surplus rather than total body fat content contributed to a resistance of female mice to metabolic derangements associated with obesity.

5 LIST OF PUBLICATIONS

5.1 Thesis based on articles:

- 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-E367, 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)
- Jelenik T, Rossmeisl M, Kuda O, Macek Jilkova Z, **Medrikova D**, Kus V, Hensler M, Janovska P, Miksik I, Baranowski M, Gorski , Jensen TE, Flachs P, Viollet B, Kopecky J. AMP-activated protein kinaṣe α2 subunit is required for the preservation of hepatic insulin sensitivity by n-3 polyunsaturated fatty acids. *Diabetes.* 59:2737-2746, 2010 (IF=8.261)
- **Medrikova D,** Macek Jilkova Z, Bardova K, Janovska P, Rossmeisl M, Kopecky J. Sex differences during the course of diet-induced obesity in mice: adipose tissue expandability and glycaemic control. Accepted in: *Int J Obes*. (IF=4.343)

5.2 Other published articles:

- Flachs P, Sponarova J, Kopecky P, Horvath O, Sediva A, Nibbelink M, Casteilla L, **Medrikova D**, Neckar J, Kolar F, Kopecky J. Mitochondrial uncoupling protein 2 gene transcript levels are elevated in maturating erythroid cells. *FEBS Lett*. 581:1093-1097, 2007 (IF = 3.263)
- Benes J, Kazdova L, Drahota Z, Houstek J, **Medrikova D**, Kopecky J, Kovarova N, Vrbacky M, Sedmera D, Strnad H, Kolar M, Petrak J, Benada O, Skaroupkova P, Cervenka L, Melenovsky V. The effect of metformin therapy on cardiac function and survival in volume-overload model of heart failure in rats. *Clin Sci (Lond.), [Epub ahead of print], 2011* (IF=3.982)

6 REFERENCES

1. Wellen,KE, Hotamisligil,GS: Inflammation, stress, and diabetes. J Clin Invest. 115:1111-1119, 2005
2. Schwartz,MW, Woods,SC, Porte,D, Jr., Seeley,RJ, Baskin,DG: Central nervous system control of food intake. Nature. 404:661-671, 2000
3. Rothwell,NJ, Stock,MJ, Stribling,D: Diet-induced thermogenesis. Pharmacol Ther. 17:251-268, 1982
4. Cypess,AM, Lehman,S, Williams,G, Tal,I, Rodman,D, Goldfine,AB, Kuo,FC, Palmer,EL, Tseng,Y, Doria,A, Kolodny,GM, Kahn,CR: Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 360:1509-1517, 2009
5. Cao,H, Gerhold,K, Mayers,JR, Wiest,MM, Watkins,SM, Hotamisligil,GS: Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. Cell. 134:933-944, 2008
6. Boden,G: Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes. 46:3-10, 1997
7. Spalding,KL, Arner,E, Westermark,PO, Bernard,S, Buchholz,BA, Bergmann,O, Blomqvist,L, Hoffstedt,J, Naslund,E, Britton,T, Concha,H, Hassan,M, Ryden,M, Frisen,J, Arner,P: Dynamics of fat cell turnover in humans. Nature. 453:783-787, 2008
8. Yki-Jarvinen,H: Ectopic fat accumulation: an important cause of insulin resistance in humans. J R Soc Med. 95:39-45, 2002
9. Long,YC, Zierath,JR: AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest. 116:1776-1783, 2006
10. Hardie,DG, Carling,D, Carlson,M: The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem. 67:821-855, 1998

11. Hotamisligil,GS, Shargill,NS, Spiegelman,BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 259:87-91, 1993

12. Maury,E, Brichard,SM: Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol Cell Endocrinol.* 314:1-16, 2010

13. Morton,GJ, Cummings,DE, Baskin,DG, Barsh,GS, Schwartz,MW: Central nervous system control of food intake and body weight. Nature. 443:289-295, 2006

14. Staiger,H, Tschritter,O, Machann,J, Thamer,C, Fritsche,A, Maerker,E, Schick,F, Haring,HU, Stumvoll,M: Relationship of serum adiponectin and leptin concentrations with body fat distribution in humans. *Obes Res.* 11:368-372, 2003

15. Minokoshi,Y, Kim,YB, Peroni,OD, Fryer,LG, Muller,C, Carling,D, Kahn,BB: Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature. 415:339-343, 2002

16. Dulloo,AG, Stock,MJ, Solinas,G, Boss,O, Montani,JP, Seydoux,J: Leptin directly stimulates thermogenesis in skeletal muscle. *FEBS Lett.* 515:109-113, 2002

17. Hotta,K, Funahashi,T, Arita,Y, Takahashi,M, Matsuda,M, Okamoto,Y, Iwahashi,H, Kuriyama,H, Ouchi,N, Maeda,K, Nishida,M, Kihara,S, Sakai,N, Nakajima,T, Hasegawa,K, Muraguchi,M, Ohmoto,Y, Nakamura,T, Yamashita,S, Hanafusa,T, Matsuzawa,Y: Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol.* 20:1595-1599, 2000

18. Yamauchi,T, Kamon,J, Minokoshi,Y, Ito,Y, Waki,H, Uchida,S, Yamashita,S, Noda,M, Kita,S, Ueki,K, Eto,K, Akanuma,Y, Froguel,P, Foufelle,F, Ferre,P, Carling,D, Kimura,S, Nagai,R, Kahn,BB, Kadowaki,T: Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med.* 8:1288-1295, 2002

19. Weisberg,SP, McCann,D, Desai,M, Rosenbaum,M, Leibel,RL, Ferrante,AW Jr.: Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest.* 112:1796-1808, 2003

20. Ruan,H, Miles,PD, Ladd,CM, Ross,K, Golub,TR, Olefsky,JM, Lodish,HF: Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance. *Diabetes.* 51:3176-3188, 2002

21. Sartipy,P, Loskutoff,DJ: Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A.* 100:7265-7270, 2003

22. Xu,H, Barnes,GT, Yang,Q, Tan,G, Yang,D, Chou,CJ, Sole,J, Nichols,A, Ross,JS, Tartaglia,LA, Chen,H: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest.* 112:1821-1830, 2003

23. Cinti,S, Mitchell,G, Barbatelli,G, Murano,I, Ceresi,E, Faloia,E, Wang,S, Fortier,M, Greenberg,AS, Obin,MS: Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res.* 46:2347-2355, 2005

24. Lumeng,CN, Bodzin,JL, Saltiel,AR: Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest.* 117:175-184, 2007

25. Ruxton,CH, Reed,SC, Simpson,MJ, Millington,KJ: The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. *J Hum Nutr Diet.* 17:449-459, 2007

26. Ruzickova,J, Rossmeisl,M, Prazak,T, Flachs,P, Sponarova,J, Vecka,M, Tvrzicka,E, Bryhn,M, Kopecky,J: Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. *Lipids.* 39:1177-1185, 2004

27. Jump,DB: Fatty acid regulation of gene transcription. *Crit Rev Clin Lab Sci.* 41:41-78, 2004

28. Arita,M, Yoshida,M, Hong,S, Tjonahen,E, Glickman,JN, Petasis,NA, Blumberg,RS, Serhan,CN: Resolvin E1, an endogenous lipid mediator derived from omega-3 eicosapentaenoic acid, protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis. *Proc Natl Acad Sci U S A.* 102:7671-7676, 2005

29. Krotkiewski,M, Bjorntorp,P, Sjostrom,L, Smith,U: Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution. *J Clin Invest.* 72:1150-1162, 1983

30. Hevener,A, Reichart,D, Janez,A, Olefsky,J: Female rats do not exhibit free fatty acid-induced insulin resistance. *Diabetes.* 51:1907-1912, 2002

31. Grove,KL, Fried,SK, Greenberg,AS, Xiao,XQ, Clegg,DJ: A microarray analysis of sexual dimorphism of adipose tissues in high-fat-diet-induced obese mice. *Int J Obes (Lond).* 34:989-1000, 2010

32. Combs,TP, Berg,AH, Rajala,MW, Klebanov,S, Iyengar,P, Jimenez-Chillaron,JC, Patti,ME, Klein,SL, Weinstein,RS, Scherer,PE: Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin. *Diabetes.* 52:268-276, 2003

33. Ohashi,K, Parker,JL, Ouchi,N, Higuchi,A, Vita,JA, Gokce,N, Pedersen,AA, Kalthoff,C, Tullin,S, Sams,A, Summer,R, Walsh,K: Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. *J Biol Chem.* 285:6153-6160, 2010

34. Ghisletti,S, Meda,C, Maggi,A, Vegeto,E: 17beta-estradiol inhibits inflammatory gene expression by controlling NF-kappaB intracellular localization. *Mol Cell Biol.* 25:2957-2968, 2005