

Effect of high-fat diet on the expression of proteins in muscle, adipose tissues, and liver of C57BL/6 mice

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In the present study, the effect of a high fat diet on the expression of proteins in insulin target tissues was analyzed using a proteomic approach. Gastrocnemius muscle, white and brown adipose tissue, and liver were taken from C57BL/6 mice either fed on a high-fat or a chow diet. Expression levels of approximately 10 000 polypeptides for all the four tissues were assessed by two-dimensional gel electrophoresis (2-DE). Computer-assisted image analysis allowed the detection of 50 significantly ($p < 0.05$) differentially expressed proteins between obese and lean mice. Interestingly, more than half of these proteins were detected in the brown adipose tissue. The differentially expressed proteins were identified by tandem mass spectrometry. Several stress and redox proteins were modulated in response to the high-fat diet. A key glycolytic enzyme was found to be downregulated in adipose tissues and muscle, suggesting that at elevated plasma fatty acid concentrations, fatty acids compete with glucose as an oxidative fuel source. Furthermore, in brown adipose tissue there were significant changes in mitochondrial enzymes involved in the Krebs tricarboxylic acid (TCA) cycle and in the respiratory chain in response to the high-fat diet. The brown adipose tissue is an energy-dissipating tissue. Our data suggest that the high-fat diet treated mice were increasing energy expenditure to defend against weight gain.

Keywords: Diet-induced obesity / Differentially expressed proteins / Insulin resistance / Mice / Type 2 diabetes

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1 Introduction

Obesity, defined as an increase in adipose tissue mass, is now so common within the world's population that it is beginning to replace undernutrition and infectious diseases as the most significant contributor to human diseases. Obesity causes many health problems, both independently and in association with other diseases [1]. In

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Abbreviations: **BAT**, brown adipose tissue; **FFA**, free fatty acid; **OGTT**, oral glucose tolerance test; **PKC**, protein kinase C; **TCA**, tricarboxylic acid; **T2DM**, type 2 diabetes mellitus; **WAT**, white adipose tissue

particular, obesity is associated with the development of type 2 diabetes mellitus (T2DM). In attempts to elucidate the pathogenesis of obesity, genetic predispositions have received great attention. Within the past 20 years, molecular genetic approaches have led to the discovery of a number of disease-related genes [2]. However, it is more likely that a change in environmental factors is the underlying cause of the global epidemic of obesity and T2DM in industrialized countries that has occurred within the last few decades [3]. A genetic program that has evolved over millions of years to permit more efficient food utilization, fat deposition, and rapid weight gain during sporadic times of food abundance, is faced today with the opposite challenges. That is, excess high-calorie food intake combined with a sedentary lifestyle and minimal physical exercise. The hypothesis, that "thrifty genes", selected during periods of food scarcity, underlie the high prevalence of obesity and T2DM was first described by James Neel [4].

Obesity is a strong risk factor for the development of T2DM. T2DM is characterized by an abnormal glucose homeostasis which leads to hyperglycemia. The disease results from a combination of insulin resistance and defects in insulin secretion. Insulin resistance is a dominating factor in obesity. Increasing amounts of adipose (fat) tissue have detrimental effects on the sensitivity of the whole body to the actions of insulin. Elevated rates of fat breakdown (lipolysis) lead to a release of free fatty acids (FFAs). These have emerged as a major link between obesity, insulin resistance, and T2DM. In skeletal muscle, it has been hypothesized that FFAs interfere with normal insulin signaling by activating protein kinase C (PKC) [5, 6]. In the liver, insulin suppresses hepatic glucose production. In a state of insulin resistance, FFA-induced increases in basal glucose production can be observed [7]. In the β -cell, however, FFAs acutely potentiate glucose-stimulated insulin secretion but chronically lead to an impairment of secretion. In addition to the release of FFAs, adipocytes also secrete a variety of proteins into the circulating blood that antagonize the action of insulin. These proteins, collectively known as adipokines, include, for example, TNF- α , leptin, resistin, and adiponectin [8–10]. Obesity is not the only cause for insulin resistance. Mutations have been identified in candidate genes which include the insulin receptor, insulin receptor substrates, glucose transporters, glycogen synthase, β -3-adrenergic receptor, hexokinase II, fatty acid-binding protein, leptin, peroxisome proliferator-activated receptor γ , prohormone convertase 2, tumor necrosis factor- α , and Ras associated with diabetes [11]. However, in the majority of the patients these mutations are not the cause of insulin resistance.

Animal models of both obesity and T2DM are widely used to study the biochemical pathways involved in the regulation of energy balance, insulin sensitivity, and insulin secretion. The *Lep^{ob}/Lep^{ob}* mouse, for example, carries a single defect on the leptin gene and exhibits severe early onset obesity, insulin resistance, and mild T2DM. This model has been extensively used to study obesity and diabetes [12–14]. Diet-induced obesity is another model and may be a better representation of the physiology of the majority of human obese patients than the genetic models of obesity with defects in leptin or leptin signaling [15]. There are only a few patients reported to have obesity as a result of mutations in the leptin or the leptin receptor gene. Dietary factors such as high-fat feeding are known to induce insulin resistance [16], but it is generally assumed that, without a genetic predisposition, diet alone will not suffice to induce diabetes [17]. The inbred mouse strain C57BL/6 fed on a high-fat diet is an established model for diet-induced obesity [18, 19] and diet-induced alterations in gene expression of C57BL/6 mice

have been described in both adipose tissue and the liver using microarrays [15, 20]. However, corresponding global approaches on the protein level are lacking for this model.

In summary, obesity results from an interaction between environmental and genetic factors. Obesity can develop when energy intake exceeds energy expenditure, independently of the genetic or environmental factors leading to this condition. However, the precise molecular events leading to the obese phenotype remain unclear. In the present study, the global protein expression between mice fed on a high-fat diet and mice fed on a chow diet was compared. Tissues essential for glucose homeostasis were included in the analysis: white and brown adipose tissue (WAT and BAT) liver, and skeletal muscle. These tissues are also involved in impaired insulin signaling. Our goal was to achieve a more detailed understanding of the molecular changes in response to dietary obesity and insulin resistance through identification of differentially expressed proteins.

2 Materials and methods

2.1 Reagents, apparatus, and mice treatment

All reagents and apparatus used have been described in detail elsewhere [21]. All animal husbandry and experimental procedures were undertaken in accordance with UK Government Guidelines and Procedures. Female mice were used in this study and were housed on a 12 h light cycle (light on at 08:00) at 21°C. At the age of 6 weeks, mice were divided into two groups: one with free access to a high-fat diet, with 60% of calories coming from fat (Charles River UK, Ltd.) and another group with free access to a rat and mouse standard chow diet (B+K Universal, Hull, UK). The mice were fed for 19 weeks. Insulin resistance was established by measurements of blood glucose and plasma insulin during oral glucose tolerance tests. 5 h fasted mice were given glucose (3.0 g/kg body weight, p.o.) for oral glucose tolerance tests. Blood from the tail (20 μ L) was taken every 30 min (from 0 to 120 min after glucose load). Blood glucose concentrations were measured by the glucose oxidase method.

2.2 Tissues preparation and solubilization

Gastrocnemius muscle, white and brown adipose tissue, and liver tissue were taken from the animals. The tissue samples were then snap-frozen between tongs in liquid nitrogen, lyophilized for 48 h, and crushed in a mortar in presence of liquid nitrogen. The resulting dried powder was stored at -80°C until analysis. Duplicates analyses

of each sample were performed. Liver (200 µg for analytical gels or 2 mg for preparative gels), gastrocnemius muscle (200 µg or 2 mg), white adipose tissue (16 mg or 160 mg), or brown adipose tissue (400 µg or 4 mg) were mixed with 60 µL of a solution containing urea (8 M), CHAPS (4% w/v), Tris (40 mM), dithioerythritol (DTE; 65 mM), SDS (0.05% w/v) and a trace of bromophenol blue. The whole final diluted sample was cup-loaded at the cathodic end of the IPG gels.

2.3 Two-dimensional gel electrophoresis

A commercial sigmoidal immobilized pH gradient (IPG) from pH 3.5 to 10.0 was used for first-dimensional separation [22, 23]. After equilibration, the IPG gel strips were transferred for the second dimension onto vertical gradient slab gels (9–16% T, 2.6% C) and run with the Laemmli-SDS-discontinuous system [24–26]. Proteins were detected using a sensitive ammoniacal silver stain [22, 27] or a less sensitive mass spectrometry-compatible silver stain [28].

2.4 Image acquisition and data analysis

Gels were scanned using a laser densitometer (APB, Uppsala, Sweden). For each tissue, 2-DE maps from obese littermates ($n = 4$) were compared to 2-DE maps from lean littermates ($n = 4$). Computer-assisted image analysis was performed using the MELANIE 3 software package (GeneBio, Geneva, Switzerland) [29]. The relative spot volume was directly related to protein concentration. Differential analysis and a Student's *t*-test were used to find differentially expressed proteins. A $p < 0.05$ was considered to be significant.

2.5 Protein identification by tandem mass spectrometry

Silver-stained 2-DE spots were excised from the 2-D gel, destained, and digested overnight with trypsin [30]. Resulting peptides were extracted using a series of acetonitrile and aqueous washes. The pooled extracts were lyophilized, resuspended in 50 mM ammonium bicarbonate and analyzed by LC-MS/MS. Chromatographic separations were performed using an Ultimate LC system (Dionex, Camberley, UK). Peptides were separated by reversed-phase chromatography on a 75 µm C18 Pep-Map column. A gradient of acetonitrile in 0.05% formic acid was used to elute the peptides at a flow rate of 200 nL/min. Peptides were ionized by electrospray ionization using a Z-spray source fitted to a Q-TOF micro mass spectrometer (Micromass, Manchester, UK). The instru-

ment was set to run in automated switching mode, selecting precursor ions based on their intensity, for sequencing by collision-induced fragmentation. Acquired fragment ion spectra were searched against the Swiss-Prot, NCBI nonredundant, and Mouse EST databases using Mascot software (Matrix Science, UK).

3 Results

3.1 Oral glucose tolerance test

Nineteen weeks of feeding with the high-fat diet induced moderate obesity and insulin resistance in the C57BL/6 mice. Differences in body weight gain between the mice fed on the high-fat diet and the mice fed on the chow diet became noticeable after four weeks of the diet and became significant after 15 weeks. Oral glucose tolerance tests (OGTTs) performed on the two groups of mice in the course of the 19 weeks revealed an initial increase in the insulin concentration in the high-fat diet mice (data not shown). This increase compensated for the developing insulin resistance and helped to maintain normal blood glucose levels. However, after 17 weeks, the mice fed on the high-fat diet showed fasting hyperglycaemia and impaired glucose tolerance. The OGTT revealed a significant increase in the fasted blood glucose levels and in the area under the blood glucose concentration curve for the mice fed on the high-fat diet (Fig. 1).

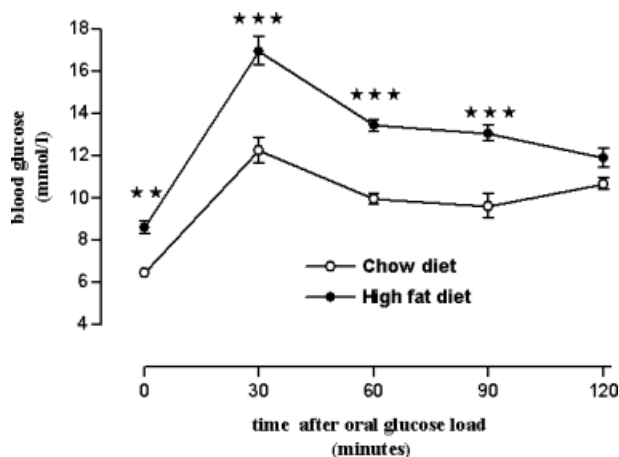


Figure 1. OGTT. C57BL/6 mice were fed on a high-fat diet ($n = 4$) and a standard chow diet ($n = 4$), respectively. After feeding with the two diets for 17 weeks, 5 h fasted mice from both groups were given glucose (3.0 g/kg body weight, p.o.) for an OGTT. Blood from the tail (20 µL) was taken every 30 min (from 0 min to 120 min after glucose load). Blood glucose concentrations were measured by the glucose oxidase method. Results are given as mean \pm SEM. Significant differences between blood glucose concentrations in high-fat fed mice relative to chow fed control mice are indicated by **, $p < 0.01$; ***, $p < 0.001$.

3.2 High-fat diet and insulin resistance associated markers

In order to detect high-fat diet and insulin resistance associated markers, tissues (white and brown adipose tissue, muscle, and liver) from obese ($n = 4$) and lean ($n = 4$) control C57BL/6 mice were arrayed using 2-DE. Differentially expressed proteins were identified using tandem mass spectrometric analysis (Q-TOF-MS) (Fig. 2, 4, 6, and 8). Image analysis and further statistical analysis allowed the detection and identification of 20 proteins whose expression was modulated in response to a high-fat diet. The analysis of the WAT samples resulted in the identification of two proteins downregulated upon high-fat feeding (Fig. 3). These were α -enolase and ubiquinol-cytochrome C reductase complex core protein I.

The analysis of the BAT samples resulted in the identification of four downregulated and six upregulated proteins upon high-fat feeding (Fig. 5). Downregulated proteins were identified as trifunctional enzyme α -subunit, malate dehydrogenase, α -enolase, and the 75 kDa glucose-regulated protein. Upregulated proteins were ATP-specific succinyl-CoA synthetase β -subunit, dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehy-

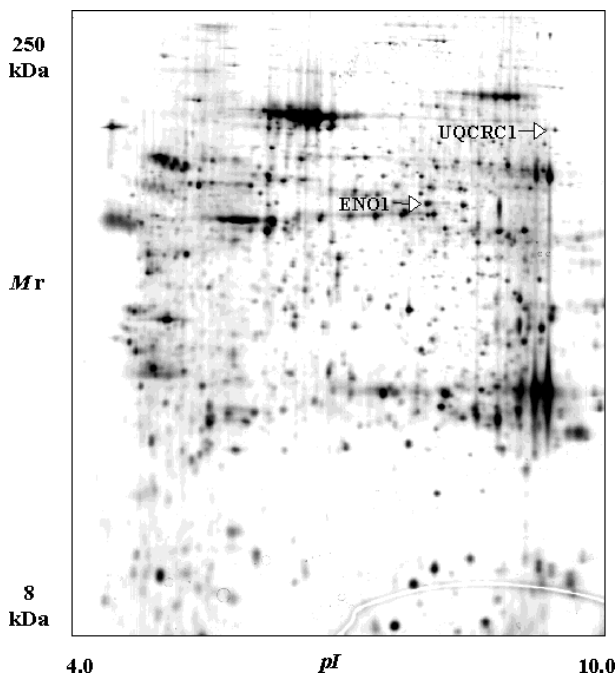


Figure 2. Silver-stained mouse WAT 2-DE image. Sixteen mg were loaded on an IPG gel (3.5–10 NL IPG, 18 cm). Second dimension was a vertical gradient slab gel (9–16% T). The gene names mark the location of the corresponding differentially expressed proteins: UQCRC1, ubiquinol-cytochrome C reductase complex core protein 1; ENO1, α -enolase. White arrowheads indicate downregulation upon high-fat feeding.

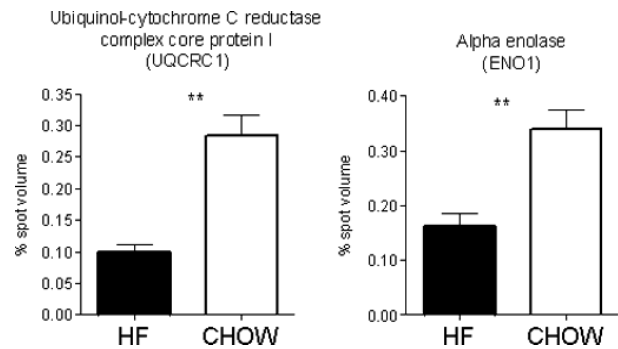


Figure 3. Bar charts representing the relative spot volume detected by the differential expression analysis of the WAT samples. Two proteins were downregulated in response to the high-fat diet. These were UQCRC1 and ENO1. Results are given as mean \pm SEM. Significant differences between protein expression levels in high-fat fed mice ($n = 4$) relative to chow fed control mice ($n = 4$) are indicated by **, $p < 0.01$.

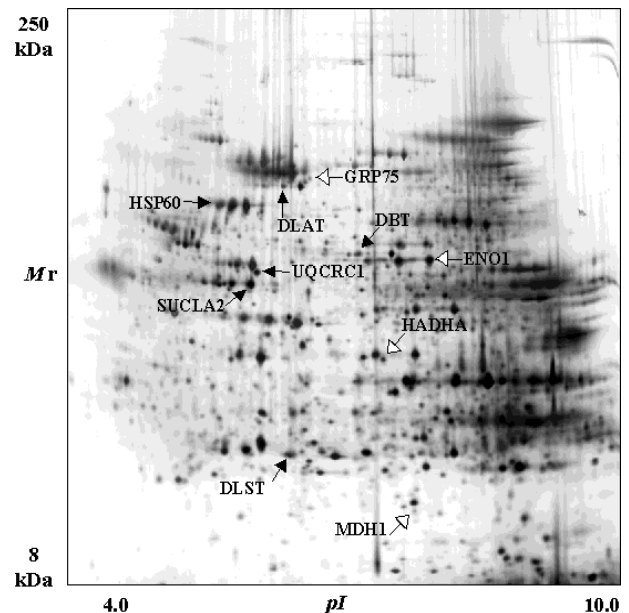


Figure 4. Silver-stained mouse BAT 2-DE image. Four hundred μ g were loaded on an IPG gel (3.5–10 NL IPG, 18 cm). Second dimension was a vertical gradient slab gel (9–16% T). The gene names mark the location of the corresponding differentially expressed proteins: GRP75, 75 kDa glucose-regulated protein; HSP60, 60 kDa heat shock protein; DLAT, dihydrolipoamide S-acteyltransferase; DBT, lipoamide acyltransferase component of branched-chain α -keto acid dehydrogenase complex; UQCRC1; ENO1; SUCLA2, ATP-specific succinyl-CoA synthetase β -subunit; HADHA, trifunctional enzyme α -subunit; DLST, dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex; MDH1, malate dehydrogenase. White arrowheads indicate downregulation, black arrowheads indicate upregulation upon high-fat feeding.

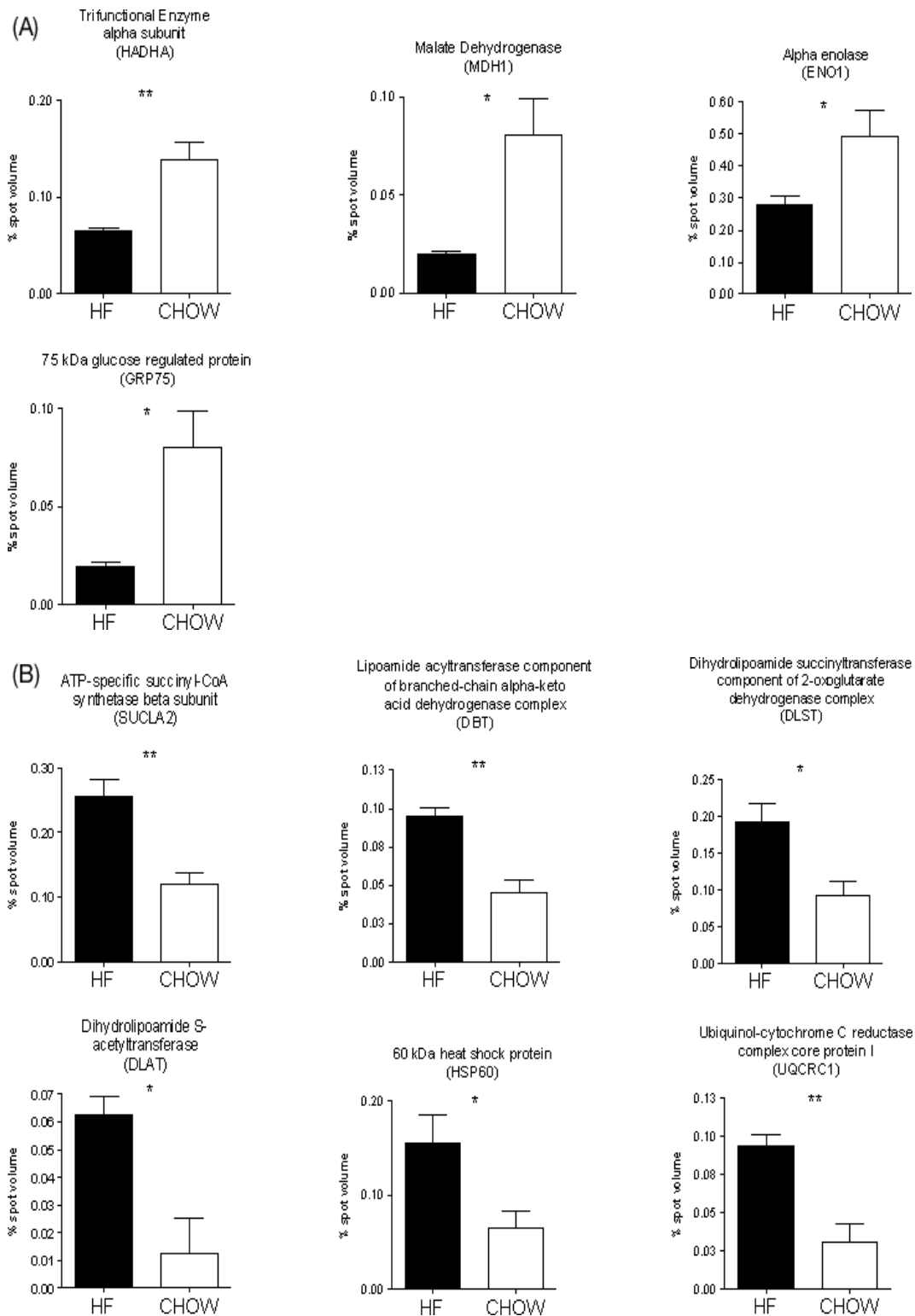


Figure 5. Bar charts representing the relative spot volume detected by the differential expression analysis of the BAT samples. (A) Four proteins were downregulated in response to the high-fat diet. These were HADHA; MDH1; ENO1 GRP75. (B) Six proteins were upregulated in response to the high-fat diet. These were SUCLA2; BBT; DLST; DLAT; HSP60; UQCRC1. Results are given as mean \pm SEM. Significant differences between protein expression levels in high-fat fed mice ($n = 4$) relative to chow fed control mice ($n = 4$) are indicated by *, $p < 0.05$; **, $p < 0.01$.

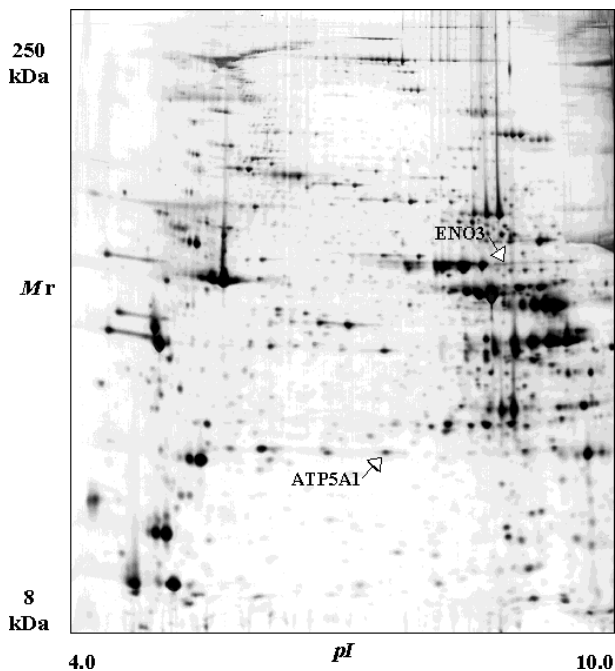


Figure 6. Silver-stained mouse muscle 2-DE image. Two-hundred μg were loaded on an IPG gel (3.5–10 NL IPG, 18 cm). Second dimension was a vertical gradient slab gel (9–16% T). The gene names mark the location of the corresponding differentially expressed proteins: ENO3, β -Enolase; ATP5A1, ATP synthase α -chain. White arrowheads indicate downregulation upon high-fat feeding.

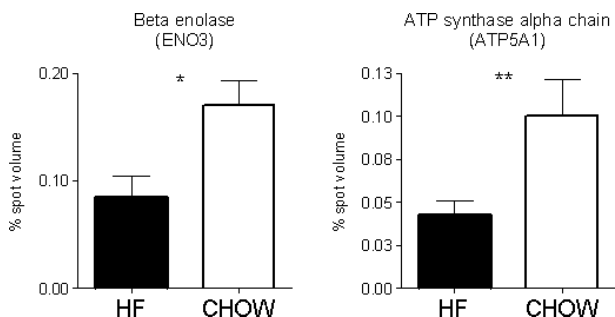


Figure 7. Bar charts representing the relative spot volume detected by the differential expression analysis of the muscle samples. Two proteins were downregulated in response to the high-fat diet. These were ENO3 and ATP SA1. Results are given as mean \pm SEM. Significant differences between protein expression levels in high-fat fed mice ($n = 4$) relative to chow fed control mice ($n = 4$) are indicated by *, $p < 0.05$; **, $p < 0.01$.

drogenase complex, dihydrolipoamide S-acetyltransferase component of pyruvate dehydrogenase complex, lipoamide acyltransferase component of branched-chain α -keto acid dehydrogenase, 60 kDa heat shock protein, and the ubiquinol-cytochrome C reductase complex

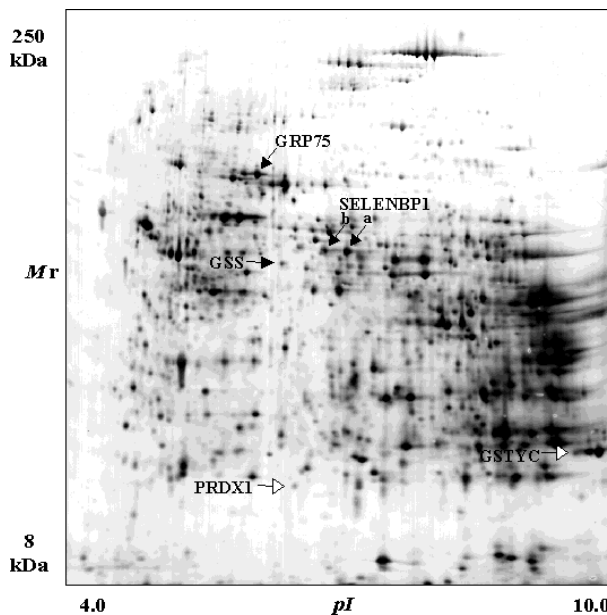


Figure 8. Silver-stained mouse liver 2-DE image. Two-hundred μg were loaded on an IPG gel (3.5–10 NL IPG, 18 cm). Second dimension was a vertical gradient slab gel (9–16% T). The gene names mark the location of the corresponding differentially expressed proteins: 75 kDa glucose regulated protein (GRP75); SELENBP1 a and b, isoforms of selenium-binding protein 1; GSS, glutathione synthetase; PRDX1, peroxiredoxin 1; GSTYC, glutathione S-transferase Y_C. White arrowheads indicate downregulation, black arrowheads indicate upregulation upon high-fat feeding.

core protein I. The analysis of the skeletal muscle samples identified two downregulated proteins upon high-fat feeding (Fig. 7). These were β -enolase and ATP synthase α -chain. The analysis of the liver samples resulted in the identification of two downregulated and four upregulated proteins upon high-fat feeding (Fig. 9). Downregulated proteins were identified as glutathione S-transferase Y_C and peroxiredoxin 1. Upregulated proteins identified were glutathione synthetase, 75 kDa glucose-regulated protein, and two isoforms of the selenium-binding protein 1.

4 Discussion

The information gained from the pathophysiological analyses of human obesity is not sufficient to unravel the complex mechanisms of its pathogenesis. Obese individuals are characterized by high rates of lipid deposition in adipose tissue, reduced insulin sensitivity of muscle and adipose tissue, increased insulin levels (hyperinsulinemia), and alterations in leptin levels. Obesity is a disease where animal models have been widely used to investigate the fundamental mechanisms of ingestive behavior, metab-

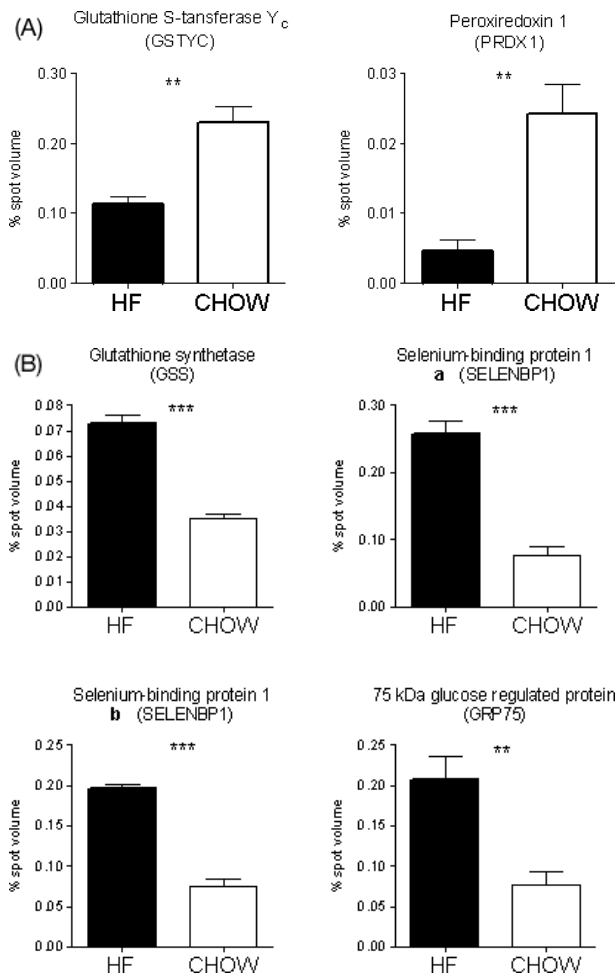


Figure 9. Bar charts representing the relative spot volume detected by the differential expression analysis of the liver samples. (A) Two proteins were downregulated in response to the high-fat diet. These were GST YC and PRDX1. (B) Four proteins were upregulated in response to the high-fat diet. These were GSS; GRP75; and SELENBP1 a and b. Results are given as mean \pm SEM. Significant differences between protein expression levels in high-fat fed mice ($n = 4$) relative to chow fed control mice ($n = 4$) are indicated by **, $p < 0.01$; ***, $p < 0.001$.

olism, energy expenditure, and energy storage in adipose tissues. Such models provide an opportunity to quickly perform experiments that are not feasible in humans. Animal models, which spontaneously reproduce one or several characteristics of the disease, are crucial for genomic and proteomic investigations. Major advantages are that inbred animals are genetically homogenous and environmental factors such as dietary intake can be tightly controlled. In the current study, an established mouse model for diet-induced obesity (C57BL/6) was used to detect fat markers and insulin resistance associated targets. To our knowledge, this is the first proteomic study in which diet-

induced alterations of protein expression were analyzed in parallel in four tissues essential for glucose homeostasis.

4.1 Adipose tissue

Adipose tissue is an important component of the whole-body energy balance. Therefore, it is of particular interest in studying diseases such as obesity and T2DM. Adipose tissue, commonly called fat, is a type of loose connective tissue comprised of lipid-filled cells, (adipocytes), surrounded by a matrix of collagen fibers, blood vessels, fibroblasts and immune cells. The view of the role of the adipose tissue has changed from the notion of an inert fat storage vessel to that of an endocrine organ [8]. Among the proteins secreted by adipocytes is leptin. This protein provides information about the size of the energy reserves to systems that regulate feeding, substrate utilization, and energy balance. Mammals have two terminally differentiated adipose cell types that compose the WAT and the BAT. WAT is an energy storage tissue and is the predominant type of adipose tissue in humans. BAT is an energy dissipating tissue (thermogenesis) present in small and newborn mammals. In humans, it is found mainly in neonates, and the number of sites where BAT is found decreases with age.

Obesity is defined as an increase in adipose tissue mass. This can be the result of the production of new adipocytes and/or the accumulation of increased amounts of fat per adipocyte. Fat accumulation is determined by the balance between fat synthesis (lipogenesis) and fat breakdown (lipolysis/fatty acid oxidation). Lipogenesis is responsive to dietary changes, for example, polyunsaturated fatty acids decrease lipogenesis [31]. Adipogenesis, the differentiation of pre-adipocytes into mature fat cells, is characterized by morphological changes such as cessation of cell growth, expression of many lipogenic enzymes, extensive lipid accumulation, and the establishment of sensitivity to key hormones such as insulin. The molecular regulation of adipogenesis has been intensively studied, with the hope that manipulation of this process in humans might one day lead to a reduction in the burden of obesity and T2DM [32].

Since BAT serves primarily to dissipate energy, its physiological role is diametrically opposed to that of WAT, which is an energy storage tissue. The energy wasting in the BAT occurs *via* increased mitochondrial biogenesis and the expression of the BAT-specific uncoupling protein (UCP1). UCP1 dissipates the proton gradient across the inner mitochondrial membrane [33, 34]. This proton gradient is normally coupled to ATP production. Uncoupling the proton gradient from ATP production results in the production of heat. Heat production in response to envi-

ronmental factors such as cold or diet is defined as adaptive thermogenesis [35]. BAT is therefore thought to function in two major contexts: defense against cold and obesity.

4.1.1 WAT fat markers

The analysis of the WAT samples from high-fat vs. chow fed mice resulted in the identification of two differentially expressed proteins. α -Enolase was downregulated in WAT of high-fat fed mice. The enzyme participates in the conversion of glucose to pyruvate. This pathway is called glycolysis and occurs in the cytoplasm. α -Enolase catalyzes a reaction giving rise to phosphoenolpyruvate from 2-phosphoglycerate. Phosphoenolpyruvate is then converted to pyruvate by dephosphorylation. Downregulation of a key enzyme of the glycolysis pathway upon high fat feeding suggests that there is a competition between fatty acids and glucose as an oxidative fuel source. Such a relationship has been reported to exist in muscle [36, 37]. The enolase enzyme, (called β -enolase in muscle), was also downregulated in skeletal muscle upon high-fat feeding, hence the effect of lipid oxidation on glucose utilization will be discussed below in more detail.

Ubiquinol-cytochrome C reductase complex core protein I was downregulated in WAT upon high fat feeding. The ubiquinol-cytochrome C reductase complex (also known as complex III or cytochrome B-C1 complex) is part of the mitochondrial respiratory chain. The complex catalyzes electron transfer from ubiquinol (also known as Coenzyme Q) to cytochrome C [38]. Cytochrome C oxidase then catalyzes the transfer of electrons to O₂, the final electron acceptor in the chain. During the passage of electrons along the respiratory chain, a proton gradient is established across the inner mitochondrial membrane. Dissipation of the proton gradient is coupled to ATP production. Downregulation of an enzyme involved in the respiratory chain suggests that ATP synthesis is decreased in WAT. In contrast, ubiquinol-cytochrome C reductase complex core protein I was upregulated in BAT (see below). The opposite regulation of this protein in the two types of adipose tissue may reflect the opposite roles of the two tissues. On the one hand, energy storage with decreased ATP production in the WAT, and on the other hand, energy dissipation with increased ATP production in the BAT.

4.1.2 BAT fat markers

The analysis of the BAT samples from high-fat vs. chow fed mice resulted in the identification of ten differentially expressed proteins. The expression of ATP-specific suc-

cynyl-CoA synthetase β -subunit was increased in BAT for high-fat feeding. Succinyl-CoA synthetase (also known as succinate thiokinase) participates in the mitochondrial Krebs tricarboxylic acid (TCA) cycle. It catalyzes the breakdown of succinyl-CoA to succinate thereby forming ATP from ADP. Both ATP-specific and GTP-specific forms of the enzyme are known and the different forms are encoded by different genes. Changes in the ratio of the two forms observed during experimentally induced diabetes and porphyria suggested that the ATP-specific version is important for succinyl-CoA degradation, whereas the GTP-specific form catalyzes the reverse reaction in support of heme biosynthesis and ketone body activation [39, 40]. Both enzyme forms are known to be present in mammalian tissues. In mouse, expression of ATP-specific succinyl-CoA synthetase β -subunit was shown to be highest in liver, heart, brain, and testis [41]. To our knowledge, our results provide the first evidence for the expression of ATP-specific succinyl-CoA synthetase in mouse BAT. Upregulated expression of ATP-specific succinyl-CoA synthetase β -subunit suggests an increased capacity of the TCA cycle or an increased abundance of mitochondria in BAT in response to high-fat feeding. This is supported by the finding that the enzyme 2-oxoglutarate dehydrogenase, which acts directly upstream of the ATP-specific succinyl-CoA synthetase in the TCA cycle, was also upregulated in mice fed on the high-fat diet (see below).

The E2 components of three members of the 2-oxoacid dehydrogenase family were found to be upregulated in BAT of mice fed a high-fat diet: 2-oxoglutarate dehydrogenase, pyruvate dehydrogenase and branched-chain α -keto acid dehydrogenase. These enzymes are all located in the mitochondrion, reinforcing the idea that a high-fat diet leads to a higher cell mitochondrial content in BAT. 2-Oxoacid dehydrogenase complexes are a ubiquitous family of multienzyme systems that catalyze the oxidative decarboxylation of various 2-oxoacid substrates. The complexes have a common architecture consisting of multiple copies of three enzymatic components: α -ketoacid decarboxylase (E1), lipoamide acyltransferase (E2), and lipoamide dehydrogenase (E3). A common mechanism might be involved in the regulation of the expression of these E2 components in BAT upon high-fat feeding. Although the other enzymatic components (E1, E3) of the complex were not among the proteins identified, upregulation of the E2 subunit suggests, that the corresponding 2-oxoacid dehydrogenases were upregulated as a whole.

The dihydrolipoamide succinyltransferase component (E2) of the 2-oxoglutarate dehydrogenase complex was upregulated in BAT under high-fat feeding. The 2-oxoglu-

tarate dehydrogenase complex participates in the mitochondrial TCA cycle directly upstream of the ATP-specific succinyl-CoA synthetase (discussed above). The 2-oxoglutarate dehydrogenase catalyzes the conversion of 2-oxoglutarate to succinyl-CoA and CO₂. Succinyl-CoA is then processed further to form succinate, ATP and CoA. The discrepancy of the theoretical molecular weight with the molecular weight estimated from the position on the 2-DE gel, suggested that we were dealing with a fragment of the dihydrolipoamide succinyltransferase component (E2) of 2-oxoglutarate dehydrogenase complex. Based on the mass spectrometric data, the protein spot on the gel could correspond to a C-terminal fragment of the protein.

The dihydrolipoamide S-acetyltransferase component (E2) of the pyruvate dehydrogenase complex was upregulated in BAT upon high-fat feeding. The pyruvate dehydrogenase complex catalyzes the oxidative decarboxylation of pyruvate and links glycolysis to the TCA cycle and ATP production. In a previous study dihydrolipoamide S-acetyltransferase transcripts have been shown to be upregulated in adipose tissue of diet-induced obese rats [42], supporting the upregulation we observed in the present study at the protein level. The pyruvate dehydrogenase complex plays a pivotal role in fuel cross-talk [43]. It is a target for the substrate competition between glucose and fatty acids. Activation of pyruvate dehydrogenase promotes glucose disposal, whereas suppression of pyruvate dehydrogenase is crucial for glucose conservation. Since the activity of the pyruvate dehydrogenase is regulated via phosphorylation of the E1 component, with phosphorylation resulting in pyruvate dehydrogenase inactivation, it is difficult to interpret the effect of an upregulation of the E2 component on pyruvate dehydrogenase activity. The kinase responsible for pyruvate dehydrogenase phosphorylation has been shown to be upregulated in a state of insulin resistance [44], suggesting a decreased pyruvate dehydrogenase activity upon high-fat feeding.

The lipoamide acyltransferase component (E2) of the branched-chain α -keto acid dehydrogenase complex was upregulated in BAT under high-fat feeding. This complex is the main enzyme of the branched-chain amino acid metabolism. It catalyzes the first irreversible and rate limiting step in oxidation of leucine, isoleucine, and valine. The activity of the branched-chain α -keto acid dehydrogenase complex is regulated through the action of the complex-specific kinase. Both insulin and leucine are known to stimulate branched-chain α -keto acid dehydrogenase activity in adipose tissue [45]. Interestingly, plasma concentrations of leucine and other branched-chain amino acids are significantly elevated in both human and rodent forms of obesity [46, 47].

The trifunctional enzyme α -subunit was downregulated in BAT of mice fed a high-fat diet. The trifunctional enzyme is composed of four α and four β -subunits, with two enzymatic activities in the α -subunit and one in the β -subunit. The α -subunit contains the long-chain enoyl-CoA hydratase in the N-terminal domain and the long-chain 3-hydroxyacyl-CoA dehydrogenase in the C-terminal domain. The long-chain thiolase is contained in the β -subunit. The trifunctional enzyme is involved in the β -oxidation of long-chain fatty acids in the mitochondrial matrix. Since the BAT is an energy dissipating tissue, fatty acid β -oxidation is expected to be increased upon high-fat feeding. Depending on the length and degree of unsaturation, specific enzymes are involved in the metabolism of fatty acids. Nadler *et al.* [48] observed a decreased mRNA expression of enoyl-CoA hydratase in WAT of obese vs. lean mice. The discrepancy of the theoretical molecular weight of the α -subunit with the molecular weight estimated from the position on the 2-DE gel suggested that we were dealing with a fragment of the trifunctional enzyme α -subunit. Based on the mass spectrometric data, the protein spot on the gel could correspond to a C-terminal fragment of the protein.

Ubiquinol-cytochrome C reductase complex core protein I was upregulated in BAT upon high-fat feeding. The ubiquinol-cytochrome C reductase complex catalyzes electron transfer from ubiquinol to cytochrome C in the mitochondrial respiratory chain. In contrast to the upregulation in BAT, ubiquinol-cytochrome C reductase complex core protein I was downregulated in WAT. Implications of this opposite regulation were discussed in Section 4.1.1.

Cytoplasmic malate dehydrogenase was downregulated in BAT in mice fed on the high-fat diet. This enzyme catalyzes the oxidation of cytoplasmic malate to oxaloacetate and thereby produces reducing equivalents. The same reaction also occurs in the TCA cycle, where the oxidation is catalyzed by mitochondrial malate dehydrogenase. These two different forms of malate dehydrogenase are encoded by different genes. The cytoplasmic form is involved in *de novo* fat synthesis by providing extra-mitochondrial reducing equivalents from the oxidation of malate. Its activity was found to be significantly lower in adipose tissue of fat compared to lean mice [49]. Belfiore *et al.* [50] also observed a reduction in NADPH-forming enzymes, including malate dehydrogenase, in adipose tissue of diabetic subjects, which suggested depressed lipogenesis. Moreover, downregulation of malate dehydrogenase in BAT is in agreement with the findings of a previous study, where we observed a 3-fold lower expression of this enzyme in BAT of genetically obese *Lep^{ob}*/*Lep^{ob}* vs. lean mice [51].

The 60 kDa heat shock protein was upregulated in BAT of high-fat fed mice. This chaperone is involved in mitochondrial protein import, folding and macromolecular assembly. Upregulation of Hsp60 may reflect the increased demand for chaperones in order to prevent protein aggregation in BAT mitochondria that are highly active in response to high-fat feeding.

The mitochondrial resident chaperone Grp75 was downregulated in BAT upon high-fat feeding. This protein mediates the import of translocation-competent proteins into mitochondria and the subsequent assembly of these proteins within the organelle [52]. Induction of glucose-regulated proteins (GRPs) is a ubiquitous intracellular response to stresses such as hypoxia, glucose starvation and acidosis. Their induction offers some protection against these stresses *in vitro*, but the specific role of GRPs *in vivo* remains unclear. The downregulation of Grp75 seems to contrast with the upregulation of another mitochondrial chaperone, Hsp60 (discussed above). However, a moderate decrease in Grp75 expression has also been found in heart mitochondria of diabetic rats [53]. In contrast to the BAT, Grp75 was upregulated in WAT in genetically obese *Lep^{ob}/Lep^{ob}* vs. lean mice [51]. This might reflect an increased need to protect newly translated proteins from aggregation in WAT compared to BAT.

α -Enolase was downregulated in BAT of high-fat fed mice. The protein was also downregulated in WAT and muscle. Implications of the differential expression of this protein are discussed in the corresponding sections.

4.2 Muscle fat markers

Skeletal muscle is the principal site of insulin-stimulated glucose uptake. It accounts for approximately 75% of glucose disposal following glucose infusion. Glucose transport is the rate-limiting step for glucose uptake and metabolism in skeletal muscle [54]. Insulin stimulates glucose transport in skeletal muscle mainly by eliciting translocation of the glucose transporter 4 (GLUT4) from an intracellular pool to the plasma membrane. In insulin-resistant states such as obesity and T2DM, insulin-induced translocation of GLUT4 to the cell membrane is reduced, hence less glucose is taken up. Plasma FFA levels are often elevated in obese individuals and have emerged as a major link between obesity and insulin resistance and T2DM [55]. It has been hypothesized that FFAs interfere with normal insulin signaling by activating PKC in skeletal muscle [5, 6]. FFA-mediated insulin resistance is related to the accumulation of intramuscular triglyceride. Elevated FFA levels also result in a preference for lipids as a muscle fuel substrate, resulting in a de-

crease in the oxidation of carbohydrates in skeletal muscle. This phenomenon was first described by Randle *et al.* [36] in the early 1960s as the so-called glucose-fatty acid cycle. The analysis of the skeletal muscle samples from high-fat vs. chow fed mice resulted in the identification of two differentially expressed proteins.

The skeletal muscle specific enolase, β -enolase was downregulated in muscle of mice fed a high-fat diet. The enzyme participates in the glycolytic conversion of glucose to pyruvate. The isoenzyme α -enolase, which is present in most tissues, was also downregulated in WAT and BAT of the high-fat fed mice (see Section 4.1). The high-fat diet-induced decrease of an enzyme that participates in carbohydrate metabolism concurs with the increase in lipid oxidation (the glucose-fatty acid cycle discussed above).

ATP synthase α -chain was downregulated in muscle upon high-fat feeding. The ATP synthase is part of the mitochondrial respiratory chain and produces ATP from ADP in the presence of a proton gradient across the mitochondrial membrane. The α -chain is part of the water-soluble catalytic core F1, which is composed of five subunits (α - ϵ). Hojlund *et al.* [56] reported a downregulation of the ATP synthase β -subunit in skeletal muscle of patients with T2DM. Several subunits from other complexes of the mitochondrial respiratory chain were found to be decreased in skeletal muscle of diabetic mice [57]. In fact, there is evidence that regulation of mitochondrial function and glucose uptake are tightly coupled [58, 59]. However, the mechanism by which a high-fat diet leads to decreased mitochondrial function in skeletal muscle remains to be elucidated. Gastrocnemius is a fast-twitch muscle that is predominantly glycolytic. Upon high-fat feeding, this glycolytic muscle is “forced” to shift substrate from glucose to lipids, which could explain the reduced mitochondrial function.

4.3 Liver fat markers

The liver is the main source of endogenous glucose production through the breakdown of glycogen (glycogenolysis) and the synthesis of glucose from lactate, alanine, and glycerol (gluconeogenesis). Insulin normally suppresses hepatic glucose production and hepatic insulin resistance results in elevated blood glucose levels. Elevated plasma FFA levels and high-fat diets can increase basal (postabsorptive) glucose production. FFA have been implicated as an important causative link between obesity, insulin resistance, and T2DM. In contrast to glucose metabolism in muscle, the effects of FFA on hepatic glucose metabolism are controversial and the mechanisms that are responsible remain unknown [7]. Depending

on the body's needs, fatty acids can either be stored or used as an energy source. Insulin potently stimulates lipogenesis in the liver. Lipolysis of an increased (visceral) fat mass is resistant to suppression by insulin. Increased production of mitochondrial reactive oxygen species (ROS) appears to be a response to the increased hepatic supply of FFA. Biological antioxidants such as glutathione can protect hepatocytes from damage by ROS. Increased oxidative stress is widely accepted as a participant in the development and progression of diabetes and its complications [60]. The analysis of the liver samples from high-fat vs. chow fed mice resulted in the identification of five differentially expressed proteins.

Glutathione synthetase was upregulated in the liver of high-fat fed mice. This enzyme participates in the biosynthesis of glutathione from γ -L-glutamyl-L-cysteine and glycine. Glutathione is a biological antioxidant that functions as a direct free-radical scavenger, as a cosubstrate for glutathione peroxidase activity, and as a cofactor for many enzymes. Liver mitochondria from obese mice have been reported to contain 25% more glutathione compared to lean mice [61]. Moreover, Guarino *et al.* [62] suggested that hepatic glutathione is critical for hepatic insulin action. The increased levels of glutathione synthetase observed in our high-fat fed mice suggest that in a state of moderate insulin resistance the liver cells attempt to protect themselves from oxidative stress. In addition, elevated glutathione production may render the liver more sensitive to the action of insulin. This adaptive mechanism appears to occur only in the prediabetic state. In human diabetes decreased levels of glutathione in several tissues, including the liver are a common finding [63, 64].

Glutathione S-transferase Y_C was downregulated in liver upon high-fat feeding. This enzyme conjugates glutathione to a wide number of hydrophobic electrophiles and represents a protective mechanism against oxidative stress. Studies of the expression and activity of glutathione S-transferases during diabetes are so far inconclusive. Both increased and decreased hepatic expression of glutathione S-transferases have been reported *in vitro* and *in vivo* [65, 66]. Elevated levels of glutathione synthetase and reduced glutathione S-transferase levels suggest that the role of glutathione as an insulin sensitizer in the liver prevails its role as an antioxidant in a state of moderate insulin resistance induced by a high-fat diet.

Peroxiredoxin 1 was downregulated in liver of high-fat fed mice. Peroxidases probably play an important role in eliminating peroxides generated during metabolism and stimulation of cell surface receptors. Mitochondrial generation of hydrogen peroxide was found to be markedly increased in liver of obese vs. lean mice [61]. Peroxire-

doxin 1 is exclusively found in the cytosol, whereas peroxiredoxin 3 is a mitochondrial protein. There seems to be no requirement for an increase in the level of the cytoplasmic peroxiredoxin in mice fed a high-fat diet.

Selenium-binding protein 1 was upregulated in liver of mice fed a high-fat diet. The physiological function of this protein, as well as the significance of selenium binding, remains unknown. Selenium-binding protein 1 is predominantly localized in the cytosol. However, Porat *et al.* [67] observed that a significant amount of the protein is peripherally associated with the Golgi membrane. They suggested a role of the selenium-binding protein 1 in promoting vesicular transport through the Golgi stack. The protein was proposed to be involved in regulating vesicle docking or fusion. The biological significance of the upregulation of the selenium-binding protein 1 in the context of obesity and insulin resistance remains unclear. Interestingly, selenium-binding protein 2, which shows 97% sequence homology to selenium-binding protein 1, was reported to decrease in abundance in liver of mice treated with the peroxisome proliferator ciprofibrate [68]. This class of peroxisome proliferators directly induce the expression of genes involved in fatty acid metabolism [69]. The levels of the selenium-binding proteins 1 and 2 in liver appear to correlate with the levels of fatty acids.

The mitochondrial resident chaperone Grp75 was upregulated in liver upon high-fat feeding. The protein was downregulated in BAT and its general functions were discussed in Section 4.1.2. High-fat feeding leads to an increased hepatic supply of FFAs, resulting in an increased production of mitochondrial ROS. Overexpression of Grp75 may meet the requirement of enhanced protection of proteins from damage and aggregation.

In conclusion, the comparative proteomic analysis of a mouse model of diet-induced obesity enabled us to outline the principal pathways involved in the response to a high-fat diet. Based on the differential expression of proteins observed in this study, a model for the high-fat diet response in BAT is proposed (Fig. 10). Mice fed a high-fat diet increased the cell mitochondrial content in brown adipocytes. In particular, the capacity of the Krebs TCA cycle was increased. This allows to oxidize the high levels of fatty acids taken up by the cell. Consequently, fatty acid synthesis (lipogenesis) from acetyl-CoA was downregulated. Moreover, glycolysis was decreased and there was little acetyl-CoA produced from pyruvate. The acetyl-CoA that entered the TCA cycle resulted mainly from β -oxidation of fatty acids but also from metabolism of the leucine nutrient signal. Increased acetyl-CoA input in the TCA cycle resulted in increased output in the form of reducing equivalents, which served as electron donors in the respiratory chain. Consequently, the capacity of the

BAT Adipocyte

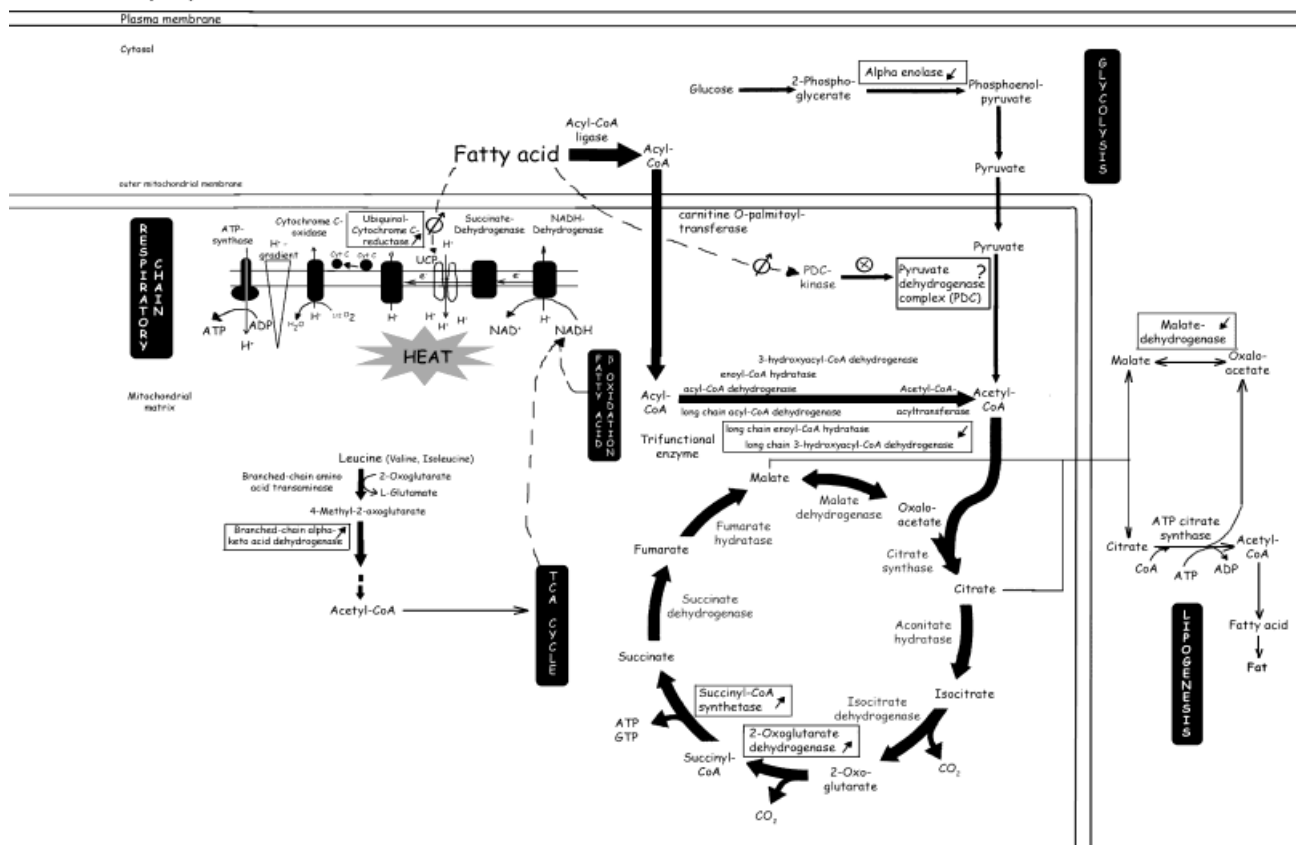


Figure 10. Proposed model of the high-fat diet response in BAT. Increased cell mitochondrial content and increased capacity of the Krebs TCA cycle allowed to metabolize the elevated levels of fatty acids taken up by the cell. Uncoupling of ATP synthesis from proton transport resulted in the production of heat. A mechanism which allowed the cell to dissipate part of the excess in energy which is present upon high-fat feeding. Proteins modulated in response to the high-fat diet are highlighted by a frame. Up- or downregulation is indicated by an arrow.

respiratory chain was also increased. For high-fat feeding, the need of the cell for ATP is smaller than its capacity for ATP production. Therefore, the dissipation of the proton gradient can be uncoupled from ATP synthesis. Elevated fatty acid levels induced the expression of uncoupling proteins, which function as proton channels across the inner mitochondrial membrane. Uncoupling of ATP synthesis from proton transport resulted in the production of heat. This mechanism allowed the cell to dissipate part of the excess energy that is present upon high-fat feeding.

Although the production of heat in response to environmental factors such as cold or diet is described in the literature, our study highlights the power of proteomics as a hypothesis generating tool. However, we also have to face with the limitations of the 2-DE approach. The uncoupling protein (UCP1) in BAT for example was not among the proteins identified. This is not surprising con-

sidering the hydrophobic nature of this transmembrane protein. The analysis of subcellular compartments, particularly mitochondria, could be undertaken in order to validate our results, but also to access lower abundant proteins.

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5 References

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