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Kamil Borkowski

The interplay between cyclic AMP and insulin during obesity development

Academic advisers: Karsten Kristiansen and Lise Madsen
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Copenhagen University, Department of Biology

Copenhagen, Denmark

Author: Kamil Borkowski

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Academic advisors:

Karsten Kristiansen, Department of Biology, Copenhagen University, Copenhagen, Denmark

Lise Madsen, National Institute of Nutrition and Seafood Research, Bergen, Norway

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1. List of Abbreviations

007 8-pCPT-2’-O-Me-cAMP
ATF6 activating transcription factor-6
BIP glucose-regulated protein 78
BMI Body mass index
BMP7 Bone-morphogenetic protein 7
C/EBPα CCAAT/enhancer binding protein α
CAP Cbl- associated protein
CREB cAMP responding element binding
EPAC exchange factor directly activated by cAMP
GR glucocorticoid receptor
HSL Hormone sensitive lipase
IBMX isobutylmethylxanthine
IGF insulin like growth factor
IRE1a inositol requiring enzyme-1
IRS Insulin receptor ligand
JNK1 Jun-N-terminal kinase 1
MB N6-monobutyryl-cAMP
MEFs mouse embryo fibroblasts
PDEB3 phosphodiesterase 3B
PERK PKR-like ER kinase
PI3 K phosphatidylinositol 3 kinase
PIP3 trisphosphorylated inositol
PKA Protein kinase A
PKC protein kinase C
PPARγ proliferator-activated receptor γ
pref-1 preadipocyte factor-1
ROCK Rho associated kinase
SOS Son-of-sevenless
TNF-α tumour necrosis factor α
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2. Abstract

Insulin and cAMP signalling are related to two opposite metabolic responses. Insulin secretion is elicited in response to food availability and trigger catabolic processes like lipogenesis and glycogen synthesis with a purpose of energy storage. On the other hand cAMP signalling is associated with stress and starvation and stimulates processes like glycogen degradation and lipolysis to distribute the stored energy through the body. Although in energy preserving tissues insulin inhibit cAMP signalling, in fat precursor cells cAMP potentiates insulin action and promote adipogenesis. Activation of exchange factor directly activated by cAMP (Epac) has been shown to be crucial for cAMP mediated potentiation of insulin signaling. In the current study I am trying to answer the question as to how cAMP accelerates adipogenesis in vivo as well as what is the role of Epac in cAMP mediated potentiation of insulin signalling. Moreover, I am investigating how increased insulin secretion caused by sucrose consumption, affects insulin signaling in peripheral tissues.
3. Introduction

3.1. Purpose of review

The following review summarises current information regarding the metabolic and molecular actions of insulin and cyclic AMP and their role in adipocyte metabolism and differentiation.

3.2. Obesity as a worldwide problem

According to the World Health Organisation, “overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health”. Body mass index (BMI) is a commonly used indicator of obesity, which describes the relationship between height and body mass of an individual. A person with BMI higher than 25 is considered overweight, whereas a person with a BMI higher than 30 is considered obese. According to the data from 2008, more than 1.4 billion adults above the age of 20 are overweight and more than 500 million obese, which is almost 10% of the world population. The prevalence of obesity differs by gender. There are 50% more obese women than men, and the obesity ratio is usually much higher in Westernized countries. In 2008, countries like USA, Kuwait, Czech Republic, and United Arab Emirates reached more than a 30% obesity ratio and are placed in top 20 the most obese countries. On the other hand countries like Ethiopia, Nepal and India are on the bottom of the list with the obesity ratio smaller than 2 [1]. Obesity is a global health concern, as it increases the risk of several diseases, like type 2 diabetes, coronary heart disease and stroke. Additionally, obesity has been related to gallbladder disease, musculoskeletal disorders, respiratory problems and several types of cancers like colon, prostate or pancreas cancers [2, 3].

3.3. Adipogenesis and obesity

There are several hypotheses surrounded the mechanisms underlying obesity and the generation of new fat cells. Recently, hypertrophic and hyperplastic type of obesity has been defined. In hypertrophic type of obesity, fat organ mass is increased due to enlargement of the fat cell size. In hyperplasic type of obesity, fat organ mass is increased due to the
increase in fat cells number [4]. Obesity caused by hyperplasia reminds still an unexplored area of research. A substantial amount of published data supports the hypothesis that high energy diets can promote the development of new fat cells [5]. There is significant amount of human studies showing very significant correlations between severity of obesity and fat cell hyperplasia, as well as negative correlations between adipocyte size and adipocyte number in obese patients [6-8]. The relationship between adipocyte size and number to obesity state is illustrated in Figure 1. However, the physiology behind the origins of development of adipose tissue, as well as new fat cell development throughout human life, is not fully understood. A recent study demonstrated that the number of fat cells in humans increases during childhood and adolescence (until approximately 20th year of life) and reminds constant during adulthood in obese as well as in lean subjects [8]. A similar situation has been observed in rats where high caloric diets can induce hyperplasia of fat cells when rats are subjected to it in the early time of life. A high caloric diet had no effect on adipose cell number in adult rats and caused only hypertrophic type of obesity [9]. Another human study demonstrated a strong negative correlation between fat tissue hyperplasia and the age of onset of obesity [6, 10]. On the other hand, the same studies demonstrated that even obesity developed during adulthood can be hyperplasic. One of the arguments for adulthood adipogenesis can be the fact of limited size of the fat cells. The relationship of the obesity severity and the fat cell size is a saturation curve [6]. This means that at some point during weight gain, the adipose cell size reaches a plateau and the only reasonable possibility of further increase of the fat depot mass is by increase of the cell number.

![Figure 1](image.png)

Figure 1. The increase of fat cell size (A) and cell number (B) in relation to obesity ratio. Figure taken from [6].
3.4. Insulin

Insulin, together with the glucagon, is the key factor regulating the blood levels of glucose, metabolism, energy expenditure and adipogenesis [11, 12]. It coordinates processes related to glucose uptake, lipolysis and gluconeogenesis. Insulin levels increase very shortly after a meal, due to the increase of glucose and free amino acids [13]. Skeletal muscle followed by adipose tissue is the primary organ responsible for blood glucose clearance after the meal followed by liver and adipose tissue [14]. Insulin stimulates glucose uptake through the glucose transporter, GLUT4, in contrary to GLUT-1, insulin independent basal glucose transport [15]. GLUT4 is abundant in skeletal muscle and adipose tissue whereas liver expresses GLUT2, which is insulin insensitive [16]. Upon insulin stimulation, intracellular vesicles containing GLUT4 fuse with the plasma membrane using the SNARE (soluble NSF attachment protein receptor (where NSF is N-ethylmaleimide-sensitive fusion protein) complex [17]. Apart from glucose uptake, insulin can increase synthesis of glycogen and certain metabolic enzymes, as well as enhance DNA and RNA synthesis [18]. Insulin has been reported to regulate the expression of more than 150 genes [19]. Among them is glucose 6 phosphatase in the liver or glucagon in the alpha cells of pancreatic islets. In adipose tissue and muscle, insulin stimulates the expression of hexokinase 2, glycogen synthase, GLUT4, Insulin receptor ligands (IRS) 1 and p85 alpha regulatory subunit of phosphatidylinositol 3 kinase (PI3K) [20]. Insulin has been shown to regulate multiple processes related to homeostasis and acts on many tissues. Recently its action in the central nervous system attracted attention. Insulin stimulation in the central nervous system promotes satiety and decreases food intake. This action is thought to be mediated by arcuate nucleuses of hypothalamus, where the insulin receptor is highly expressed. Here, insulin has been demonstrated to act via stimulation of expression of causing loss of appetite neuropeptides like proopiomelanocortin, which is a precursor of α-melanocyte-stimulating hormone, and cocaine and amphetamine regulated transcript, as well as with the orexigenic neuropeptide Y and the agouti-related peptide [21]. A recent study highlighted a possible role for insulin in the promotion of an obese phenotype based on carbohydrate quality in a high fat diet. Nifedipine, an insulin secretion inhibitor, was able to protect mice from diet induced obesity from a high fat high sucrose diet [22]. Moreover, Ins1+/−:Ins2−/− mice which display reduced insulin secretion and are protected against chronic hyperinsulinemia, are also protected against high fat diet induced obesity [23]. Moreover, elevated plasma insulin is correlated with insulin resistance in humans [24].

3.4.1. Insulin and insulin-like growth factor receptor signalling cascades

Insulin consists of two short peptides bound together by two disulphate bridges and binds to the insulin receptor as its main target. The insulin receptor is necessary to maintain insulin action. Both humans and mice lacking insulin receptor are born alive, but cannot survive, which suggests a critical role of insulin action during early development, but not for
foetal metabolism [25]. The insulin receptor is a heterotetrameric membrane glycoprotein, consisting of two α and two β subunits, bound together with disulfate bridges [26]. It belongs to the receptor tyrosine kinase family. Alpha subunits create an extracellular part of the receptor and binds insulin, whereas beta subunits create transmembrane and the intracellular part of the receptor. From the many of phosphorylated tyrosines on the intracellular part of the insulin receptor, the juxtamembrane autophosphorylation site plays a critical role in interaction between the insulin receptor and the intracellular substrates [26]. The insulin receptor can also bind insulin like growth factors (IGFs) and peptide hormones that have structures similar to insulin. The affinity of binding of IGF is approximately 100 to 1,000 fold lower than for insulin; however, the physiological concentration of IGF is approximately 100 folds higher than insulin. There are two splicing variants of alpha subunit of insulin receptor A and B. Variant A lacks an amino acids fragment of 12 residues on the C terminal [27]. There is no noticeable difference in affinity for insulin between the two isoforms, however, variant A has higher affinity towards IGF-2. Insulin can also bind to IGF receptors, which also belongs to receptor tyrosine kinase family [28]. In pancreatic beta islets, the action of insulin is conducted by IGF receptor 1 which require higher insulin concentration than insulin receptor [29]. Insulin receptors are expressed at high levels predominantly in white and brown adipocytes and skeletal muscle and heart [26].

Insulin receptor ligands are scaffolding proteins that bind to phosphorylated insulin receptor or IGF receptors. IRS consists of a placstrin homolog domain, a phosphotyrosine binding domain at the N terminal and a large C terminal domain. After phosphorylation by insulin or IGF receptor, the large C terminal can bind multiple signalling proteins leading to activation of multiple signalling cascades [30]. All IRS domains have several phosphorylation sites, which can be targeted by multiple kinases, other than insulin or IGF. Those phosphorylations can affect IRS function by promotion of its degradation or prevention of binding to the receptor [31]. There are 6 IRS isomers, named IRS1 to IRS6. All isomers have different functions as well as different tissue specificity. The IRS-1 deficient mice show peripheral insulin resistance and some growth aberrations. In adipose tissue and skeletal muscles IRS-1 is the primary conductor of insulin receptor signalling, and is crucial for glucose transport, glycogen, lipid and protein synthesis, mitogenesis and gene expression [32]. IRS-1 can directly bind the regulatory subunit of PI3K, as well as protein kinase B and C and mitogen activated protein kinase. IRS-2 deficient mice are insulin intolerant and have impaired β islets growth. IRS-2 is also associated with type 2 diabetes. IRS-3 has been shown to conduct insulin signalling in mature brown fat cells [30]. Depletion of IRS-4 causes only modest developmental disorder and insulin intolerance [26]. Regulation of IRS is the crucial point in regulation of entire insulin or IGF receptor signalling cascade. IRS can be regulated in the many different ways involving modification of the protein itself as well as the cytoplasmic part of the receptor. While generally tyrosine phosphorylation of IRS positively stimulates the receptor signalling, serine/threonine phosphorylation typically serves as a negative
regulator of the receptor signalling. There are two groups of the kinases that negatively regulate IRS. One group, like mTOR/S6K1, MAP kinase and protein kinase C (PKC) are the kinases activated by IRS signalling and working as a negative feedback loop. The second group consists of kinases activated by other signalling pathways to control IRS stimulation. Those are glycogen synthase kinase IKKβ, c-Jun NH2-terminal kinase, mouse Pelle-like kinase and AMPK. Regulatory serines are distributed across entire IRS sequence. Phosphoserines located on plakstrin homology domain inhibits plasma membrane – IRS interaction. Phosphoserines located on phosphotyrosine binding domain disturb IRS interaction with juxtamembrane domain of the receptor and can also promote degradation of IRS. Phosphoserines on the C-terminal domain can both inhibit the interaction of IRS with SH2 domains containing effector proteins as well as promote the degradation of IRS. Not all serine/threonine phosphorylations of IRS protein negatively regulate it function. Phosphorylation of Ser1223 or Ser629 (human numbering) by PKB, results in enhanced Tyr phosphorylation of IRS protein [31]. Apart from IRS proteins, at least 6 others factors, Gab-1, three isoforms of Shc, p62dok and APS (adapter protein containing a PH and SH2 domain) can directly bind to insulin receptor and activate signalling cascades [33].

4.4.1.1. Phosphatidylinositol 3-kinase pathway

The majority of insulin- or IGF receptor mediated action is mediated through PI3K or the MAP kinase pathways (figure 2). PI3K is composed of a regulatory subunit and a 110 kD catalytic subunit. The regulatory subunit has four isoforms, (p85-a, p85-b, p55/AS53, p55PIK, and p50) which can either stimulate or suppress some of the activity of PI3K. For example, deletion of p85 alpha subunit causes hypoglycaemia by increase of the basal level of insulin uptake in several insulin responsive tissues. PI3K is required for insulin stimulated glucose uptake; however, it is not sufficient for insulin action. It is known that several factors can activate PI3K; however, only insulin has the ability to stimulate GLUT4 translocation [26]. Studies conducted on 3T3 L1 adipocytes showed that Cbl-associated protein (CAP) may also be necessary for insulin stimulated glucose transport. CAP has been shown to bind directly to the insulin receptor [33]. The direct response to PI3K activation is an increase in trisphosphorylated inositol (PIP3) concentration. PIP3 can further activate PI-dependent protein kinase-1 and-2, Akt (a product of the akt protooncogene), salt- and glucocorticoid-induced kinases, protein kinase C or wortmannin-sensitive and insulin-stimulated serine kinase. Among those Akt seems to be crucial for and glycogen synthesis and together with protein kinase C, for insulin stimulated glucose uptake. In the case of glucose transport, Akt has been shown to act through AS160 protein [27].

4.4.1.2. MAP kinase pathway

In contrary to the PI3K pathway, the MAP kinase pathway mediates most of the insulin gene expression regulation. This signalling cascade starts with recruitment of the Grb2 adaptor
protein by both IRS-1 and by the insulin/IGF receptor itself. Grb2 recruits Son-of-sevenless (SOS) exchange factor to the plasma membrane, which in turns activates protein Ras. Activation of protein phosphatase SHP2 by the interaction with IRS 1/2 or Gab-1 is also required for activation of Ras. Activated Ras initiates the cascade of kinases acting through Raf, MEK and ERK. Phosphorylated ERK then translocates to the nucleuses where it can perform action on transcription factors such as p62TCF. Activation of ERK generally leads to stimulation of proliferation and differentiation. [34]

4.4.1.3. The CAP/Cbl pathway
As mentioned before, activation of the PI3 pathway is not sufficient to stimulate glucose uptake. Based on a study using 3T3-L1 adipocytes, Cbl protein was proposed to work parallel with PI3K signalling. Cbl can be directly phosphorylated by the tyrosine kinase of the insulin receptor. Upon phosphorylation, Cbl binds adaptor protein CAP, which brings it to the plasma membrane by binding flotilin, protein associated with the lipid rafts. Phosphorylated Cbl binds another adaptor protein Crk2, which in turns binds exchange factor C3G. C3G activates small G protein TC10. Activation of TC10 seems to cooperate with PI3K signalling to stimulate GLUT4 translocation to the plasma membrane. The mechanism of how TC10 stimulate GLUT4 translocation is still unclear, but it has been suggested that TC10 action is through stabilization of actin filaments. [34]
Figure 2. Signal transduction in insulin action. The insulin receptor is a tyrosine kinase that undergoes autophosphorylation and catalyses the phosphorylation of cellular proteins such as members of the IRS family, Shc and Cbl. Upon tyrosine phosphorylation, these proteins interact with signalling molecules through their SH2 domains, resulting in a diverse series of signalling pathways, including activation of PI(3)K and downstream PtdIns(3,4,5)P3-dependent protein kinases, Ras and the MAP kinase cascade, and Cbl/CAP and the activation of TC10. These pathways act in a concerted fashion to coordinate the regulation of vesicle trafficking, protein synthesis, enzyme activation and inactivation, and gene expression, which results in the regulation of glucose, lipid and protein metabolism. Figure adapted from [34].

3.4.2. Defects in insulin signalling

The most common outcome of dysfunction of insulin receptor signalling is insulin resistance. Insulin resistance is a state where insulin responsive tissue like liver, muscle and fatty tissue, have lower response to insulin and therefore have lower ability to decrease blood glucose level. Insulin resistance has been pointed as one of the major cause of development of type 2 diabetes [35, 36]. As was described previously, insulin signalling via PKB stimulates glucose uptake by GLUT4 glucose transporter. It has been shown that reduced insulin stimulated glucose transport/phosphorylation activity is an early event of type 2 diabetes [37]. Skeletal muscle has been shown to have the biggest glucose storage capacity and to be the most efficient organ in insulin stimulated glucose uptake process in human body [38]. The ability
of skeletal muscle to take up glucose under insulin stimulation decreases by 50% in patients with type 2 diabetes [38]. On the other hand, liver is responsible for maintaining blood glucose levels during fasting. Insulin signalling during the meal stimulates glycogen synthesis in the liver and inhibits gluconeogenesis. During fasting, degradation of glucagon and gluconeogenesis are promoted, and glucose is secreted from liver into the blood stream. Therefore, one of the outcomes of insulin resistance in liver is increased fasting blood glucose levels [16].

3.4.2.1. Fat Induced insulin resistance

Insulin resistance together with type 2 diabetes has been very highly correlated with obesity, therefore triglycerides accumulation in insulin sensitive tissues has been proposed as one of the causes of impaired insulin signalling (for review see [39]). It has been shown, that elevated fasted blood fatty acid concentration as well as triglycerides accumulation in muscle are good predictors for insulin resistance in humans [37]. Elevated fasted plasma fatty acids has been observed in insulin resistant offspring of type two diabetic parents, which otherwise did not display conditions like obesity or hyperglycaemia [37].

As described previously, insulin activates Pi3 kinase via IRS-1 which leads to GLUT4 translocation to the plasma membrane and glucose uptake in insulin sensitive tissues. It has been demonstrated that increased plasma fatty acids leads to attenuation of PI3 kinase activation by insulin in skeletal muscle. This action has been demonstrated to be conducted by blocking of insulin receptor conducted tyrosine phosphorylation of IRS-1 (see Figure 3) [37, 39]. Decreased tyrosine phosphorylation of IRS-1 has been demonstrated to be mediated by IRS-1 serine phosphorylation. Pharmacological inhibition or knock down of certain protein kinases (c-Jun NH2-terminal kinase, inhibitor of nuclear factor kappa beta kinase β subunit, S6 kinase 1, and protein kinase C-θ) have been demonstrated to prevent high fat induced insulin resistance in certain rodent models [37]. Prevention of high fat induced insulin resistance has been achieved also by muscle specific serine to alanine mutation of 302, 307 and 612 IRS-1 residues [37]. Increase of serine phosphorylation of IRS-1 in muscle has been shown to be secondary to intracellular increases in long-chain fatty acids and diacylglycerol [37]. Triacylglycerol and acyl CoAs intracellular accumulation however, does not cause insulin resistance. Therefore regulation of diacylglycerol content via enzymes involved in diacylglycerol metabolism plays a significant role in insulin resistance development [39]. Diacylglycerol can directly activate novel PKCs, which unlike conventional PKCs does not require calcium ions as a co-activator. In skeletal muscles, diacylglycerol action is mediated through PKC θ, which further phosphorylates serine residues of IRS-1 (see Figure 3), whereas in the liver diacylglycerol activates PKC ε, which in turns phosphorylates serine residues of insulin receptor (see Figure 4). Another member of the atypical PKC family, PKC δ has been demonstrated to play a role in development of hepatic insulin resistance, however unlike PKC ε, PKC δ has been suggested to be activated
by inflammatory signalling. PKC δ regulates expression of genes involved in lipogenesis, therefore PKC δ involvement in development of hepatic insulin resistance has been suggested to be conducted via increase hepatic lipid accumulation. [39]

Ceramides are another group of lipids associated with insulin resistance. Ceramides are synthesized from serine and acyl-CoA.

Ceramides mediate only saturated fat induced insulin resistance, since inhibition of ceramid synthesis pathway in rodents prevents insulin resistance development by perfusion of saturated fatty acids, but not by unsaturated fatty acids. There is a line of evidence suggesting involvement of Toll-like receptor 4 in ceramide accumulation and ceramide induced insulin resistance. Ceramide mediated inhibition of insulin signalling has been shown to be downstream from IRS and does not impair IRS tyrosine phosphorylation. Ceramides have been shown to impair Akt2 activation, however, the exact mechanism of this inhibition is not known. There are several implications suggesting that inhibition of Akt2 by ceramics is mediated by activation of protein phosphatase 2 A, which in turns dephosphorylates and by that deactivates Akt2. Another suggested mechanism is via PKC ζ isoform, since ceramides prevent dissociation of PKC ζ and Akt2 complex. [39]

The role of hepatic fat accumulation in development of insulin resistance, besides the mechanism described above, has been associated with activation of unfolded protein response, known also as endoplasmic reticulum stress. Endoplasmic reticulum stress is characterized with accumulation of unfolded proteins, which attracts glucose-regulated protein 78 known as BIP. BIP in the basal state is bind to endoplasmic reticulum membrane proteins inositol requiring enzyme-1 (IRE1a), PKR-like ER kinase (PERK), and activating transcription factor-6 (ATF6), suppressing their activity. After activation, those proteins work to reduce unfolded proteins with the ER lumen by increasing membrane biogenesis, suppressing protein translation and enhancing expression of ER chaperones. IRE1a has been shown also to activate Jun-N-terminal kinase 1 (JNK1), which can phosphorylate serine residue of IRS -1, and therefore inhibit insulin signalling. Endoplasmic reticulum stress has been demonstrated to increase hepatic lipid accumulation by upregulation of SREBP1c and ChREBP, the main transcription factors of lipogenesis. The expression of lipogenic genes is also enhanced by XBP1s transcription factor, which is an alternative splicing form of XBP1 gene. IRE1a has been shown to conduct splicing of XBP1s and by that increase lipogenesis. On the other hand activated PERK increases gluconeogenesis which contributes to fasting hyperglycemia. [39]
Figure 3. The mechanism of insulin resistance caused by fatty acids in muscle. Insulin activates the insulin receptor (IR) tyrosine kinase, which subsequently tyrosine phosphorylates IRS1. Through a series of intermediary steps, this leads to activation of Akt2. Akt2 activation, via AS160 and Rab-GTPase (not shown), promotes the translocation of GLUT4-containing storage vesicles (GSVs) to the plasma membrane, permitting the entry of glucose into the cell, and promotes glycogen synthesis via glycogen synthase (GS). This central signalling pathway is connected to multiple other cellular pathways that are designated by numbers 1–3. (1) The green shaded areas represent mechanisms for lipid induced insulin resistance, notably diacylglycerol (DAG)-mediated activation of PKCq and subsequent impairment of insulin signalling, as well as ceramide-mediated increases in PP2A and increased sequestration of Akt2 by PKCz. Impaired Akt2 activation limits translocation of GSVs to the plasma membrane, resulting in impaired glucose uptake. Impaired Akt2 activity also decreases insulin-mediated glycogen synthesis. (2) The yellow areas depict several intracellular inflammatory pathways—notably, the activation of IKK, which may impact ceramide synthesis, and the activation...
of JNK1, which may impair insulin signalling via serine phosphorylation of IRS1. (3) The pink area depicts activation of the unfolded protein response (UPR), which under some instances (such as acute extreme exercise) may lead to activation of ATF6 and a PGC1α-mediated adaptive response. The endoplasmic reticulum membranes also contain key lipogenic enzymes and give rise to lipid droplets. Proteins that regulate the release from these droplets (e.g., ATGL and PNPLA3) may modulate the concentration of key lipid intermediates in discrete cell compartments. CS, ceramide synthase; G3P, glycerol 3-phosphate; LPA, lysophosphatidic acid; SPT, serine palmitoyl transferase; TAG, triacylglycerol. Figure and figure description adapted from [39].
Figure 4. The mechanism of insulin resistance caused by fatty acids in liver. Pathways Involved in Hepatic Insulin Resistance Insulin activates the insulin receptor (IR) tyrosine kinase, which subsequently tyrosine phosphorylates IRS1 and 2. Through a set of intermediary steps, this leads to activation of Akt2. Akt2 can promote glycogen synthesis (not shown), suppress gluconeogenesis, and activate de novo lipogenesis (DNL). This central signalling pathway is connected to multiple other cellular pathways that are designated by numbers 1–3. Key lipid synthesis pathways are juxtaposed within this domain and are regulated by them. (1) The green shaded areas represent mechanisms for lipid-induced insulin resistance—notably, diacylglycerol-mediated activation of PKCε and subsequent impairment of insulin signalling, as well as ceramide mediated increases in PP2A and increased sequestration of Akt2 by PKCζ. Impaired Akt2 activation limits the inactivation of FOXO1 and allows for increased expression of key gluconeogenesis enzymes. Impaired Akt2 activity also decreases insulin-mediated glycogen synthesis (not shown). (2) The yellow areas depict several intracellular inflammatory pathways—notably, the activation of IKK, which may impact ceramide synthesis, and the activation of JNK1, which may impair lipogenesis. (3) The pink area depicts activation of the UPR that can lead to increased lipogenesis via XBP1s and also increased gluconeogenesis via C/EBP. The ER membranes also contain key lipogenic enzymes and give rise to lipid droplets. Proteins that regulate the release from these droplets (e.g., ATGL and PNPLA3) may modulate the concentration of key lipid intermediates in discrete cell compartments. CS, ceramide synthase; DNL, de novo lipogenesis; FA-CoA, fatty acyl CoA; G3P, glycerol 3-phosphate; LPA, lysophosphatidic acid; SPT, serine palmitoyl transferase; TAG, triacylglycerol; TCA, tricarboxylic acid cycle; PEP, phosphoenolpyruvate. Figure and figure description adapted from [39].

3.4.2.2. Glucose induced insulin resistance

Insulin resistance induced by glucose is less explored than fatty acid induced insulin resistance. Skeletal muscle and adipose tissue are responsible for glucose clearance; therefore most investigations were trying to link glucose with peripheral insulin resistance. It has been demonstrated that hyperglycaemia impairs glucose transport in vivo [40]. The ex vivo experiment with perfused rat skeletal muscle demonstrated that glucose is able to inhibit insulin stimulated glucose uptake. The effect of glucose is highly potentiated when glucose is administrated together with insulin, although insulin by itself does not impair insulin stimulated glucose uptake [41]. A similar observation has been noticed with human subcutaneous adipose tissue. Ex vivo treatment of human adipocytes with high concentration of insulin, alone or in combination with high concentration of glucose, decreased both basal and insulin stimulated glucose uptake. Moreover pretreatment of human adipocytes with high concentration of insulin in combination with high concentration of glucose decreased quantity of IRS-1 proteins and furthermore decreased ability of insulin to activate PKB. Surprisingly, administration of high concentration of insulin alone did not reduce IRS-1 quantity or insulin stimulated PKB activation, yet glucose uptake was decreased [42]. Glucose and insulin induced insulin resistance has been proposed to be mediated through increased intracellular protein modification by O-linked N-acetyl-glucosamine (process known as O-GlcNAcylation). However, a study linking O-GlcNAcylation and insulin resistance seems to be contradictory. N-acetyl-glucosamine is produced from
glucose in hexosamine biosynthetic pathway. An increase in glucose intracellular content has been reported to increase O-GlcNAcylation and increased O-GlcNAcylation has been demonstrated to induce insulin resistance in skeletal muscle and adipose tissue [43]. In primary adipocytes culture, inhibition of rate limiting enzyme in hexosamine biosynthetic pathway (glutamine:fructose-6-phosphate amidotransferase) prevented glucose and insulin induced insulin resistance. This effect was diminished by administration of glucosamine, a carbohydrate that enters the hexosamine biosynthetic pathway downstream from glutamine:fructose-6-phosphate amidotransferase [44]. However, in another study inhibition of the rate limiting enzyme in N-acetyl-glucosamine synthesis did not prevent the reduction in glucose intake caused by high glucose combined with high insulin treatment of isolated rat muscle [45]. The same study demonstrated that inhibition of protein synthesis completely abolished the inhibitory effect of glucose and insulin on glucose uptake. Taken together, more studies are required in order to evaluate the possible role of O-GlcNAcylation in glucose induced insulin resistance. Glucose as well as glucosamine can induce endoplasmic reticulum stress, which, as described above, can induce insulin resistance [46]. N-acetyl-glucosamine is covalently attached to serine or tyrosine residue. One of the proteins undergoing O-GlcNAcylation is IRS. O-GlcNAcylated residues of IRS can no longer be phosphorylated by the insulin receptor and this action can explain how O-GlcNAcylation decrease insulin signalling [43].

3.5. Cyclic AMP

cAMP is a secondary messenger molecule produced by a membrane enzyme adenylyl cyclase. cAMP is synthetized from ATP and is degraded to AMP by phosphodiesterase. Adenylyl cyclasecyclase is attached to the plasma membrane by two hydrophobic domains. Two cytoplasmic domains are responsible for the catalytic activity and binding of G protein. There are at least 9 different adenylyl cyclase types encoded by at least 9 different genes, and more than 40 types of phosphodiesterases. Adenylyl cyclases are expressed at different levels and are differently regulated in different tissues. Almost all adenylyl cyclases can be directly activated by Gs alpha protein coupled receptors, like beta adrenergic receptors or by forskolin. Additionally, depending on the type, adenylyl cyclase can be regulated by Gαi and Gβγ G proteins coupled receptors, calcium ions as well as by some kinases and phosphatases like PKA, PKC calmodulin kinase and calcineurin. All Adenylyl cyclases are expressed in some part of the brain [47]. Phosphodiesterases can target cGMP or cAMP or both of them. As a result, cAMP activity of some phosphodiesterases can be regulated by the cGMP concentration, in a competition like manner. Additionally, cAMP by itself can regulate activity of cAMP specific phosphodiesterases. It has also been reported that insulin, histamine and calcium ions regulates phosphodiesterase activity [48, 49]. cAMP is involved
in many processes in almost every tissue in our body. Many hormones, like vasopressin, dopamine, histamine or prostacyclin works through this secondary messenger. cAMP plays a crucial role in glucose homeostasis and energy balance by mediating both stress and hunger signalling via adrenalin and glucagon receptors. Adrenalin and glucagon signalling have the most robust effect in adipocytes and liver where they counteract the effect of insulin by stimulating of lypolysis and gluconeogenesis [50, 51]. So far there are two known mammalians proteins with cAMP binding domain: protein kinase A (PKA) and exchange factor directly activated by cAMP (EPAC).

3.5.1. Epac
Epac was discovered in 1998 by the genome database search for new cAMP binding proteins, and is a guanine nucleotide exchange factor for small G protein Rap [52]. There are two Epac isoforms, Epac 1 and 2. Epac 2 has two splicing variants named Epac 2A and 2B. Also Epac 1 exists in at least two splicing variants. Both Epac 1 and 2 contains dishevelled egl-10 pleckstrin domain attaching the protein to the cellular membranes, followed by a cAMP binding domain, a Ras exchanger motif, a Ras associated domain and a guanine nucleotide exchange domain (CDC25-homology domain). Epac 2 has an additional cAMP binding domain at the N terminal of the protein (see Figure 5). The cAMP binding domain of Epac is approximately 40 times less sensitive to cAMP than the regulatory subunit of PKA and PKA is therefore considered as the primary cAMP target. Epac is thus considered as a sensor of strong cAMP signalling. Epac 1 is ubiquitously expressed whereas Epac 2 seems to be specific for certain brain region and adrenal glands. Epac 2B is expressed in pancreatic islets. Epac 1 has different cellular localisation and has been observed in both in nucleuses and mitochondria as well as on mitotic spindle during the mitosis. Both Epac isoforms have ability to activate Rab 1 and 2. The Epac Rab1/2 signalling is very poorly describes. Several reports point phosphor lipase C, Akt, mTORC2, inositol 1-4-5 trisphosphate, ERK, Ca2+ /calmodulin-dependent protein kinase II and PKC as the Epac downstream targets [53-55]. There are several reports suggesting regulation of cell adhesion via integrin signalling by Rab1. Epac 2 on the other hand was shown to play an important role in insulin secretion by controlling insulin containing vesicles trafficking [56]. For 15 years after its discovery, Epac has been demonstrated to be involved in regulation of leptin secretion and signalling in hypothalamus [57], cardiac Ca2+ signalling [58], cancer cell migration [59], learning and social interactions [60], exocitosis [61], neuronal differentiation and neurit outgrowth, cardiac hypertrophy [55], prevention of tissue fibrosis [62], smooth muscles relaxation [63] and neuronal apoptosis [64].
3.5.2. cAMP action in adipocytes

Adipocytes express multiple adrenergic receptors (β1, β2, β3, α1 and α2). As described previously, activation of β adrenergic receptors stimulates cAMP signalling, whereas stimulation of α adrenergic receptors decreases cAMP signalling [65]. Expression of adrenergic receptors makes adipocytes sensitive to action of catecholamines, like noradrenaline and adrenaline.

Adipocytes are the main fat storage cells in our body. The primary purpose of this organ is to provide free fatty acids from stored energy when the food is limited and energy required. Glucose is the primary cellular fuel, however when glucose is limited, free fatty acids are secreted from adipose tissue to the blood stream and are oxidised by the liver, skeletal muscle and heart. Moreover, fatty acids are used as a fuel in the situations of stress and increased exercise. Therefore, processes responsible for lipid accumulation as well as for lipolysis need to be strictly controlled by the mechanism regulated by energy status of the organism. Hormone sensitive lipase (HSL) is the crucial enzyme in the lipolysis process. It initiates triglycerides degradation by cleaving off the first fatty acids chain from triacylglycerol. HSL upon activation translocates from the cytoplasm to the lipid droplets, where the first step of lipolysis occurs. The best known mechanism promoting lipolysis is by activation of cAMP pathway (see Figure 6). PKA is the main enzyme regulating HSL activity. PKA phosphorylates HSL on serine Ser-563 (site 1) and Ser-565 (site 2) (numeration according to human protein). Side 1 is phosphorylated upon lipolitic stimulation, whereas side 2 is phosphorylated at the basal state and when phosphorylated, prevents phosphorylation of the side 1 which decreases lipolysis. Other kinases, like glycogen synthase kinase, AMP dependent protein kinase and Ca2+/calmodulin dependent protein kinase II can also phosphorylate HSL on the side 2. However, phosphorylation of side 1 is not crucial for enzyme activity. Further studies discovered that Ser-659 and 660 are phosphorylation targets of cAMP signalling and regulators of the enzyme activity. Perlipins are the lipofilic, lipid droplets associated proteins which are abundant on the surface of the lipid droplets. The function of those proteins is prevention of interaction of lipolitic enzymes with the lipids insight lipid droplets. Perlipins have multiple phosphorylation sides which are
also targets of PKA. Upon phosphorylation, perlipins lose their blocking ability, therefore cAMP signalling enables HSL translocation from cytoplasm to the lipid droplets. cAMP is not the only factor regulating perlipins and HSL in adipocytes. TNF-α also induces lipolysis in adipocytes, regulating HSL and perlipins. [66]

All cAMP action described in this paragraph can ascribed to PKA activity. However, recent studies indicated that Epac may be involved in regulation of AMP-activated protein kinase, the main energy sensor in the cell. AMP-activated protein kinase is activated when AMP/ATP ratio increases due to cell energy deprivation. Activated AMP-activated protein kinase promotes energy delivering processes like for example adipocyte lipolysis. AMP-activated protein kinase is regulated by phosphorylation of tyrosine 172 (human protein numeration) and specific Epac agonist has been shown to increase this phosphorylation and activity of AMP-activated protein kinase. However, Epac specific agonist is not able to increase glycolysis alone. [67]

cAMP signalling in adipocytes is regulated in multiple ways. Testosterone stimulates expression of β adrenergic receptors, and thus sensitises adipocytes to adrenaline and noradrenaline stimulation. TNF-α, on the other hand decreases the level of β-3 adrenergic receptor, however, increases the level of β-2 receptors. Adenosine receptor is conjugated with Gi protein activating phosphodiesterase, and it has been shown that TNF-α downregulate the expression of Gi protein coupled with adenosine receptor. The insulin action promotes utilisation of excess of nutrience in the blood stream, therefore insulin also decreases lipolysis. Insulin inhibits lipolysis, stimulated by cAMP signalling, by activation of phosphodiesterase 3B (PDEB3). Insulin activation of PDEB3 is conducted via phosphorylation of Ser 302 residue (human protein sequence) by Akt. On the other hand thyroid hormones and Tnf-α decrease activity of PDEB3. [66]

Increase in intracellular calcium level, like insulin, also increases activity of PDEB3 and decreases lipolysis. Recent investigation demonstrated that the calcium-sensing receptor, which plays a crucial role in regulation of plasma calcium level, is wildly expressed in adipocytes. Activation of the calcium-sensing receptor leads to increase in intracellular adipocyte calcium level, and by that inhibition of cAMP induced lipolysis. [68]
3.6. The role of insulin and cAMP in adipogenesis

3.6.1. Transcription factors of adipogenesis

While early events in adipocyte differentiation are still unclear, the final step of adipocyte differentiation is far more characterised proliferator-activated receptor γ (PPARγ). PPARγ activates expression of CCAAT/enhancer binding protein α (C/EBPα), which in turn feeds back on PPARγ. PPARγ and C/EBPα activates genes necessary for the final development of the mature, insulin-responsive adipocytes (see figure 7) [69, 70]. A few genes have been reported to play a unique role for differentiation white or brown adipose tissue. Studies conducted in mice have shown that C/EBPα is necessary for WAT origin and its lack does not affect the BAT development. Bone-morphogenetic protein 7 (BMP7) has been reported to induce BAT differentiation whereas two others members of BMP family, BMP 2 and 4 suppress brown adipocyte differentiation by inhibiting of UCP1 expression [69]. Most knowledge about molecular mechanism of adipocyte differentiation comes from cell culture studies. 3T3 L1 mouse fibroblasts, mouse embryo fibroblasts (MEFs) or 3T3 F442A are most frequently used preadipocyte models. Adipocyte differentiation of those cells comes out after 4 to 6 days of growth in medium containing foetal calf serum, high concentration of insulin or physiological concentration of IGF-1 and glucocorticoids. Factors that elevate
cellular concentration of cAMP strongly enhance differentiation. In the standard differentiation method, cells are treated with insulin, dexamethason and isobutylmethylxanthine (IBMX) [71]. IBMX act as an inhibitor of phosphodiesterase and when is present in the cytoplasm, elevates cAMP level [71]. Insulin or IGF-1 binds to IGF receptor on the preadipocytes membrane surface. As it was described previously, activated IGF receptor works through MAP and PI3K pathways, resulting with activation of PKB and Erk. Both PKB and Erk can activate transcription factor cAMP responding element binding (CREB), which initiate expression of adipocyte differentiation related genes [70, 71].

Glucocorticoids, like dexamethasone, are reported to be increased in several models of obesity and to induce insulin resistance. In humans, excess of these hormones leads to modification of fatty tissue distribution and to central obesity [72]. Unfortunately, it is still unclear why dexamethasone is necessary for adipogenesis. As others glucocorticoids, dexamethasone diffuses through the cellular membrane and binds to the glucocorticoid receptor (GR). GR is a nuclear transcription factor from the same superfamily like PPARγ. One of the transcriptional targets of GR is C/EBPα, the main proadipogenic transcription factor. It was reported, that operating through C/EBPα, dexamethasone promotes transcription of insulin sensitive glucose transporter GLUT4 [73]. C/EBPα expression is suppressed by TGF-β. Treatment of primary rat preadipocytes with TGF-β prevents adipocyte differentiation, whereas co-treatment with dexamethasone counteracts effect of TGF-β and reverses suppression of C/EBPα expression [74]. However, cells which overexpress C/EBPα still require dexamethasone stimulation to undergo adipocyte differentiation [70], what suggests other way of stimulation of adipogenesis by dexamethasone. GR can also act as a genes expression inhibitor. It was reported, that dexamethasone significantly reduces expression of tumour necrosis factor α (TNF-α) [72] and preadipocyte factor-1 (pref-1) [70], the two potent antiadipogenic agents, and this acting is believed to be crucial for dexamethasone proadipogenic properties.[75].

CCAAT/enhancer binding protein β and δ (C/EBPβ, C/EBPδ) are the earliest transcriptional factors observed during adipocyte-like differentiation. One of the target genes of those two factors encodes peroxisome

Elevation of the cAMP level activates PKA. PKA inactivates small G protein RhoA by phosphorylation. RhoA is an activator of Rho associated kinase (ROCK), which is serine/threonine specific. ROCK was found to regulate insulin/IGF receptor pathway via phosphorylation of IRS protein. At high activity, ROCK phosphorylates serine residue 612 of IRS-1 which cause inhibition of IGF receptor signalling. On the other hand, at low activity, ROCK induces IGF receptor signalling by phosphorylation of serine residues 632 and 635. However, blocking of ROCK, by specific inhibitor Y27632, can replace the action of PKB in adipocyte differentiation, suggesting that ROCK activity is not necessary for adipocyte differentiation [71]. In 2008, Petersen et al. [71] discovered that activation of both cAMP targets is required for adipocyte like differentiation. The studies were carried out using 3T3
L1 mouse fibroblast preadipocytes and two cAMP analogues - 8-pCPT-2′-O-Me-cAMP (007) and N6-monobutyryl-cAMP (MB) which specifically activates only Epac or PKA respectively. 007 and MB have been used to induce adipocyte like differentiation instead of IBMX.

Figure 7. Molecular mechanism of adipogenesis induction in 3T3 L1 Mouse fibroblasts. IGF receptor activation, as well as cAMP signalling and glucocorticoids stimulation is necessary for adipogenesis. Activation of IGF receptor by insulin or IGF triggers two intracellular signalling cascades leading thru PI-3 kinase pathway and MAP kinase pathway. CREB is believed to stimulate expression of initial adipogenic transcription factors C/EBPβ and C/EBPδ which afterwards induce expression of PPARγ. One of the PPARγ target genes is C/EBPα which feeds back on PPARγ. Products of genes activated by PPARγ and C/EBPα are necessary for fibroblasts differentiation into adipocytes. CREP can be activated by three different enzymes: Erk, known also as a MAP kinase, PKB, activated in PI-3 kinase pathway, and by PKA. However, in adipogenesis, CREB activation seems to be Erk dependent. Glucocorticoids, also necessary for adipocyte differentiation, bind to their soluble receptor which acts as a negative regulator of antiadipogenic Pref-1 and TNF-α genes. Schema based on [70-72].
This study demonstrated that stimulation of only one of the cAMP target proteins does not potentiate dexamethasone and insulin induced adipocyte like differentiation in 3T3 L1 cells. On the other hand, differentiation was strongly enhanced when both Epac1 and PKA were activated. Those findings suggest that activation of Epac1 may counteract the negative impact of PKA on insulin signalling transition in still unknown manner. Illustration of insulin, cAMP and glucocorticoids signalling in preadipocytes is presented in Figure 7.

4. The aim of the study

The present thesis focuses on the role that in obesity play one of the major metabolism regulating factor – insulin, and molecular regulator of cell metabolism – cAMP.

The purpose of the first part is to provide information about specific action of two cAMP activated factors, Epac and PKA which together with insulin are the driving force of adipogenesis in vitro.

The second part investigates how PTP1B, the phosphatase reported to regulate insulin secretion, impacts body function in different types of diets.

The third part demonstrates how the model of in vitro preadipocyte cell differentiation, driven by the combined action of insulin and cAMP, can be observed in vivo.
5. List of manuscripts:


2. **Borkowski K**, Bettaieb A, Bakke J, Xi Y, Myrmel LS, Haj F, Madsen L, Kristiansen K. Perturbation of first phase insulin secretion in mice with pancreas-specific ablation of Ptpn1 attenuates sucrose induced peripheral insulin resistance. (Manuscript)

6. Discussion of results

Insulin and cAMP signalling play significant roles in sensing energy condition in our body. Insulin is associated with food availability and promotes processes of food storage such as glucose uptake, lipogenesis and glycogen synthesis. On the other hand, cAMP signalling is associated with nutrition deprivation, promoting release of stored energy by activation of lipolysis and glycogen degradation. Gluconeogenesis is not release of energy, it consumes energy [76]. More importantly, both signalling cascades directly interfere with each other. In insulin sensitive tissue, like adipocytes, muscle cells and liver, Insulin inhibits cAMP signalling by activation of phosphodiesterase. On the other hand, in differentiating adipocytes and muscle, the Epac branch of cAMP signalling tends to potentiate insulin/Insulin like growth factor signalling.

6.1. The role of cAMP in the adipocyte differentiation

Our group has demonstrated that activation of Epac is necessary for cAMP-induced acceleration of adipocyte differentiation [71]. The microarray analysis (data not published) demonstrated that Epac by itself is unable to induce major transcriptional responses. Epac has been demonstrated to play an important role in regulation of leptin signalling in hypothalamus [57], cardiac Ca\textsuperscript{2+} signalling [58], cancer cell migration [59], learning and social interactions [60], exocytosis [61], neuronal differentiation and neurit outgrowth, cardiac hypertrophy [55], prevention of tissue fibrosis [62], smooth muscles relaxation [63] and neuronal apoptosis [64]. In the first manuscript we have identified 7 novel targets of Epac, which have not been previously reported. Only one of the target proteins displayed changes at the mRNA level as well, demonstrating that the majority of the observed differences could not be detected by gene expression analyses.

As was pointed previously, Epac is involved in calcium signalling in β-cells, and high levels of intracellular calcium are known to inhibit adipocyte differentiation [77]. Three of the proteins down-regulated in our analysis are calcium regulated, and changes of two of them are Epac specific. One of them is an enzyme, lysosomal alpha glucosidase, which is involved in degradation of glycogen to glucose. In mature adipocytes, cAMP signalling is involved in activation of energy deriving processes, like glycogen degradation, however, this action is associated with the PKA branch of cAMP signalling.

In vitro studies have shown that steroid hormones, like glucocorticoids, accentuate adipogenesis in many settings [78]. All steroid hormones are derived from cholesterol. In our analysis we have demonstrated that Epac stimulates the expression of a key enzyme in cholesterol synthesis - Hydroxymethylglutaryl-CoA synthase at both the transcriptional and the translational level. This action is not counteracted but rather potentiated by PKA. Moreover, PKA alone does not induce Hydroxymethylglutaryl-CoA synthase expression.
cAMP is known to regulate testosterone production [79], however, this regulation appears downstream from cholesterol synthesis. In vitro studies have demonstrated that cholesterol rich very low density lipoprotein stimulates adipogenesis. Surprisingly, other reports have demonstrated a suppressive role of cholesterol on mouse adipose-derived stromal cells differentiation [80]. This action was accompanied by down-regulation of PPARgamma2 expression.

6.2. The role of insulin in mediating the negative effect of sucrose in high fat diet

There is a considerable body of evidence that sucrose potentiates the obesogenic effect of high fat diets. Sucrose supplementation of high fat diets causes an increase in body weight together with an augmentation of fat depots in mice models, when compared to protein supplementation [81-83]. Studies with chemical inhibitors of insulin secretion linked insulin to the obesogenic effect of sucrose in high fat diets [22]. It was recently demonstrated, that glucose-stimulated insulin secretion is highly dependent on communication between β cells in the pancreas and this phenomenon is mediated by ephrins [84]. Both ephrin A5 and EphA5 can differentially effect insulin secretion. Signalling from EphA5 attenuates insulin secretion, while signalling by ephrin A5 enhances insulin secretion [84]. EphA5 is active when phosphorylated and protein tyrosine phosphatase 1B (PTP1B) is responsible for its dephosphorylation (unpublished data). Therefore, PTP1B was proposed to regulate insulin secretion in pancreatic beta cells. In the study presented in second manuscript we have used Ptpn1 pancreas specific deletion to further investigate the role of insulin in the obesogenic effect of sucrose supplementation in high fat diet.

Sucrose has been demonstrated to induce peripheral insulin resistance in animal models [85]. Series of ex vivo experiments have demonstrated that insulin is necessary for glucose to induce insulin resistance in skeletal muscle and fat tissue [41, 42]. Increased insulin secretion has been previously suggested to be a response of β cells to the development of peripheral insulin resistance [86]. On the other hand, some studies have demonstrated that hyperinsulinemia can induce insulin resistance [87]. High sucrose diets are known to increase the first phase insulin secretion in animal models [88]. It has been previously suggested that developing insulin resistance is counteracted by an increase in first phase insulin secretion [86]. On the other hand elevated insulin level combined with high glucose has been shown to induce insulin resistance in human adipose tissue [89].

In the second manuscript we show as expected that animals fed with a high fat diet supplemented with sucrose displayed an increased first phase insulin secretion when compared to the low fat fed animals. The first phase insulin secretion was not elevated when sucrose was replaced with protein in the high fat diet. Of note, the effect of sucrose on first phase insulin secretion was blunted in mice with pancreas specific Ptpn1 deletion, supporting the hypothesis of an involvement of PTP1B in insulin secretion. On the other
hand, pancreas specific *Ptpn1* deletion influenced neither fasting nor fed, plasma glucose and insulin levels in mice fed sucrose or protein supplemented high fat diets. This suggests that PTP1B may be involved in the rapid first phase insulin secretion, but has no or minor impact on insulin secretion caused by prolonged glucose stimulation of β cells.

Pancreatic specific *Ptpn1* deletion prevented induction of peripheral insulin resistance in high fat high sucrose fed mice, linking first phase insulin secretion with sucrose induced peripheral insulin resistance.

It is important to mention that sucrose supplementation of high fat diets significantly increased the total mass of white fat depots, as well as the mass of the intrascapular brown adipose depot. Although the difference in adipose tissue mass of wild type and KO mice was not significantly different, pancreas specific *Ptpn1* deletion blunted the difference in fat depots mass between the mice fed a high fat diet supplemented with sucrose and mice fed a high fat diet supplemented with protein.

### 6.3. Combine action of insulin and cAMP accelerate adipocyte differentiation in vivo.

*In vitro* adipogenesis is induced by the combined action of insulin and cAMP [11]. Insulin and cAMP signalling are usually stimulated in cell under different conditions. Insulin is and food availability signal, promoting glucose intake, lipogenesis and glucagon synthesis. On the other hand cAMP signalling is associated with glucagon and adrenalin stimulation, meaning that it mediates responses to food deprivation and stress [76]. In the third manuscript we hypothesized that so-called yoyo dieting (circles of caloric restriction and refeeding) would promote an increase in fat tissue mass by stimulation of adipogenesis.

The results demonstrated that mice subjected to yoyo diet gained significantly more weight compared to the mice fed *ad libitum*. Moreover, the total caloric intake was not different between those two groups suggesting that yoyo dieting increases feed efficiency.

Calculation of the epididymal and inguinal fat cellularity demonstrated a very strong tendency towards an increase of the total cell number in the inguinal adipose depot in the yoyo dieting group when compared to the *ad libitum* fed group. The same tendency, however, less significant, was observed in the epididymal adipose depot. The increase in adipose cell mass could then explain the difference in the weight gain between yoyo dieting and *ad libitum* fed mice. It is worth to mention that the masses of neither liver nor muscle were different in the two groups. Despite the increased body weight, yoyo dieting mice remained glucose tolerant. Also, plasma triglycerides and fatty acids were lower and adiponectin levels were higher in the yoyo dieting group compared to the *ad libitum* group. These finding may suggest an increase capacity of fat storage, which can be explained by an increase in adipocyte cell number in the fat depots.
There is emerging evidence for a key role of clock genes in energy homeostasis [90]. The aberration of circadian rhythm by night shift work is associated with an increased risk of obesity and metabolic syndrome [91]. Of interest in this context, we describe in the third manuscript a possible link between cAMP signalling and expression of circadian rhythm genes in adipose tissue. Thus, we show that mice undergoing yoyo dieting have decreased expression of several circadian genes in white adipose tissue, when compare to the *ad libitum* fed mice. In our experiment, two circadian genes, albumin D box-binding protein (Dbp) and thyrotroph embryonic factor (Tef) has been found to be down-regulated in inguinal and epididymal adipose tissue of the yoyo group compare to the *ad libitum* fed mice. Moreover, we have demonstrated that in 3T3-L1 cell model of adipocyte, cAMP decrease dramatically expression of Tef and Dbp, which may suggests that similar mechanism is activated *in vivo* in preadipocytes during conditions where cAMP signalling is elevated adding another aspect to the many physiological roles of cAMP-dependent processes.

### 6.4. Conclusions
In the current study we have demonstrated that cAMP signalling can regulate the circadian rhythm genes, and we hypothesized that by down-regulation of circadian genes, cAMP stimulates adipogenesis in vivo. Moreover, we have identified a number of new Epac regulated proteins. The nature of those regulations prevented them from being identified using microarray data analysis. Our results provide a solid base for further investigations of the involvement of Epac in biological processes previously not related to this cAMP-activated factor.

We have demonstrated that pancreas specific *Ptpn1* deletion blunts the increase in first phase insulin secretion induced by sucrose in the context of a high fat diet, however, but has no effect on insulin secretion caused by prolonged glucose stimulation of b cells. Moreover, we suggest that a decrease in the first phase insulin secretion is linked to the prevention of peripheral insulin resistance.

Moreover, we have shown that yoyo dieting increases feed efficiency and promotes obesity in rodents partially by increasing fat cell number. However, the increase in obesity is not accompanied with a deterioration of metabolic parameters.
7. Literature


41. Torii N: [The role of Ca ions and cAMP in ACTH lipolysis]. *Nihon Naibunpi Gakkai zasshi* 1985, 61(9):859-871.


8. Annex
Proteomic analysis of cAMP-mediated signaling during adipocyte differentiation of 3T3-L1 preadipocytes

Kamil Borkowski1, Krzysztof Wrzesinski2, Adelina Rogowska-Wrzesinska2, Karine Audouze3, Jesse Bakke4, Rasmus Koefoed Petersen1, Fawaz Gaj Haj4, Lise Madsen1,5, Karsten Kristiansen1

1 - Department of Biology, University of Copenhagen, Ole Maaløes Vej 5
DK-2200 Copenhagen N, Denmark

2 – Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55
DK-5230 Odense M, Denmark

3 - Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark,
DK-2800 Kgs Lyngby, Denmark

4 - Departments of Nutrition, University of California Davis, Davis, CA 95616, USA

5 - National Institute of Nutrition and Seafood Research (NIFES), N-5817 Bergen, Norway

Corresponding authors:

Karsten Kristiansen, Department of Biology, Copenhagen University, Ole Maaløes Vej 5, 2200
København N, Copenhagen, Denmark. Phone: +45 353-24443, E-mail: kk@bio.ku.dk

Lise Madsen, National Institute of Nutrition and Seafood Research (NIFES), Nordnesboder 2,
5005 Bergen, Norway. E-mail Lise.Madsen@nifes.no
ABSTRACT

Initiation of adipocyte differentiation is promoted by the synergistic action of insulin/insulin-like growth factor and cAMP-dependent signaling. The action of cAMP is mediated via PKA and Epac, where PKA acts by repression of tRho-kinase activity, whereas Epac counteracts the PKA-Rho-kinase dependent reduction in insulin/insulin-like growth factor signaling by restoring and enhancing insulin/insulin-like growth factor signaling. The action of PKA per se in adipocyte differentiation is well described, but detailed knowledge of the Epac-dependent branch and the interplay with PKA in the context of adipocyte differentiation is still scarce. In the present study we present a comprehensive study of Epac-mediated processes and their interplay with PKA during the initiation of adipocyte differentiation of 3T3-L1 preadipocytes using a combination of proteomics, molecular approaches and bioinformatics. Proteomic analyses revealed 7 proteins regulated uniquely in response to Epac activation, 4 in response to PKA activation, and 11 in response to the combined activation by Epac and PKA. Network analyses indicated that these proteins are involved in pathways of importance for glucose metabolism, inositol metabolism and of calcium-dependent signaling adding a novel facet to our understanding of cAMP-mediated signaling during initiation of adipocyte differentiation.

INTRODUCTION

The development of obesity is related not only to increased fat cell mass, but also increased fat cell number as the result of fat cell differentiation [1]. Much data on preadipocyte differentiation has been acquired from cell culture studies, where 3T3-L1 and 3T3-F442A mouse fibroblasts as well as mouse embryo fibroblasts have been used as models. In the standard differentiation procedure, mouse fibroblasts are treated with high concentrations of
insulin, glucocorticoid and a cAMP elevating agents such as 3-isobutyl-1-methylxanthine (IBMX) or forskolin [2, 3]. Factors that elevate cellular concentrations of cAMP strongly enhance differentiation. In the cytoplasm, cAMP activates protein kinase A (PKA) and exchange factor directly activated by cAMP (Epac). PKA inactivates Rho kinase by phosphorylation, and this action has been previously shown to be crucial for preadipocyte differentiation [3, 4]. Rho kinase was found to regulate the insulin/insulin like growth factor receptor (IGFR) signaling pathway via phosphorylation of the IRS protein. When Rho kinase is highly active, it phosphorylates serine residue 612 of IRS-1, which causes inhibition of IGFR signaling. On the other hand, at low activity, Rho kinase induces IGFR signaling by phosphorylation of serine residues 632 and 635 [5, 6]. Epac has been reported to activate the G protein Rap, which induces important changes in cytoskeleton organization and cell adhesion [7]. In 2008, Petersen et al. [3] reported that activation of both of these cAMP targets is required for differentiation of mouse fibroblasts by employing the two cAMP analogues 8-pCPT-2′-O-Me-cAMP (007) and N6-monobutyryl cAMP (MB) which specifically activate Epac or PKA, respectively. Similarly, it was show that synergistic activation of PKA and Epac also is required for adipocyte differentiation of human hMADS cells [8].

The aim of the present study was to examine the role of cAMP-mediated signaling via Epac and PKA during adipocyte differentiation of 3T3-L1 preadipocytes using proteomics in combination with molecular approaches and network analyses. The cells were induced to differentiate using 007 or MB or mixture of 007 and MB. Samples were collected at 0, 8, 24 and 48 hours of the treatment and at day 8. The samples were submitted to proteomic analysis using two dimensional gel electrophoresis combined with protein identification by mass spectrometry.
MATERIALS AND METHODS

Cell culture and differentiation

3T3 L1 mouse fibroblasts were obtained from the Eukaryotic Gene Expression and Differentiation Group, Department of Biochemistry and Molecular Biology, University of Southern Denmark. Cells were cultured up to the third passage before start of differentiation. Cells were maintained and induced for differentiation as described (Petersen 2008 [3]). In short 3T3-L1 cells were cultured to confluence in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen™: Cat. No. 41966029) supplemented with 10% calf serum. Two-days postconfluent (designated day 0) cells were induced to differentiate with DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal bovine serum (FBS), 1 μM dexamethasone (dex)(Sigma) 1 μg/ml insulin (ins) (Sigma), and 0.5 mM isobutylmethylxanthine (IBMX) (Sigma). For analyses of the roles of PKA and Epac, IBMX was replaced with 200 μM of 8-pCPT-2’-O-Me-cAMP (Biolog) (007 treatment), 100 μM of N6-monobutyryl-cAMP (Biolog) (MB treatment) or 200 μM of 8-pCPT-2’-O-Me-cAMP + 100μM of N6-monobutyryl-cAMP (007MB treatment). After 48 h media were replaced with DMEM supplemented with 10% FBS and 1 μg/ml insulin. The cells were subsequently refeed every 48 h with DMEM supplemented with 10% fetal bovine serum. The cells were cultured up to day 8 of differentiation. At day 8 triglycerides presents in differentiated cells were visualized by Oil-Red-O-staining [9].

Samples collection for two dimensional gel analysis

Cells were washed three times with HANK’s buffer and collected by scraping. After collection cells were immediately frozen in liquid nitrogen. The cell lysates were prepared by
addition of IPG lysis buffer and shaking overnight. Each sample was centrifuged at 1500 rcf for 15min at 10oC and the supernatants were transferred to new tubes. Protein concentration was determine by the Bradford method.

Two dimensional gel electrophoresis

For the first dimension 18cm IPG strips, covering a pH gradient from 4 to 7 (IPG 4-7) were used (Ge Healthcare Cat. No. 17-1233-01). In-gel rehydration was performed in two steps. First step strips were rehydrated overnight in 200 μl of sample diluted in IPG lysis buffer (300μg of protein per gel were loaded). Next, strips were rehydrated for 6 hour in 100 μl of lysis buffer. Isoelectric focusing was performed using a linearly increasing profile: 0 V to 600 V for 2:15 h, f600 V to 3500 for 8 h, and 3500 V for 9:25 h. After focusing the strips were incubated in equilibration buffer for 15 min. and then frozen at -80°C. After thawing gels were incubated in equilibration buffer for 15 min. The Second dimension SDS PAGE was performed using a vertical electrophoresis system Protean II™ (BioRad) and 12.5% (w/v) laboratory made acrylamide gels (acrylamide: N,N’-ethylene-bis-acrylamide ratio 200:1). The gels were run overnight at 20oC. 6 mA per gel was applied for the first 2 hours of electrophoresis, then 12 mA per gel. The running buffer contained 0.67% Tris-Base, 1.44% of glycine and 1% SDS. Additionally, buffer recirculation was applied. The gels were stained with Sypro Ruby (Invitrogen™ Cat. No. S21900) and scanned on the Typhoon scanner with an excitation wave length of 488nm and emission filter at 610nm.
Gels images analysis with DECODON Delta 2D, version 3.6 software

Gel images were analyzed using the DECODON Delta 2D software, version 3.6. Differences were considered as significant when the average intensity ratio of a spot from the experimental treatment group compared to the average intensity ratio of a spot of the control group was bigger than 1.5 or smaller than 0.67. All found differences were evaluated by a t-test with a level of confidence p<0.05.

Mass spectrometry

In gel digestion. Spots exhibiting significant changes were cut out from the gel. The gel plugs were washed with 100 μl of sterile water for 5 min. Water was removed and the gel plugs were washed with 100 μl of 100% acetonitrile (ACN) for 20 min. ACN was removed and gel plugs were dried in a Speed Vac. Dry gel plugs were rehydrated with 10μl of trypsin solution (10ng/ml, dissolved in 50 mM NH4HCO3, pH 7.8) for 20 min on the ice. Unabsorbed trypsin was removed and the gel plugs were covered with 20 μl of 50 mM NH4HCO3, pH 7.8. Digestion was carried out overnight at 37°C.

Target loading. Desalting and concentration of peptides mixtures were done using laboratory made microcolumns, prepared from GELoader micropipette tips, packed with POROS R2. The columns were pre-washed with 10 μl of 0.1% trifluoroacetic acid (TFA). 10 μl of peptides mixture from the digested protein were loaded onto microcolumns. The columns were then washed with 10 μl of 0.1% TFA. Peptides were released from the column with 3μl of CHCA diluted in 70% ACN/0.1% TFA and placed on the MALDI plate. Trypsin digested β-galactosidase was used as a standard for instrument calibration and was placed on every third spot on the MALDI plate.
**Sample analysis.** A 4800 MALDI TOF/TOF Analyzer from Applied Biosystems was used for recording of positive ion MS and MS/MS spectra. For Ms analysis the mass range 700 to 3500 Da was selected. Total laser shots number was set to 800 and a fixed laser intensity of 3100 was selected. For Ms/MS analysis, the total number of shots was set to 1280. For each Ms spectrum at least three of the most intensive peaks were selected for Ms/MS analysis. MS and associated MS/MS data were combined into a single mass list in the mgf. file format using an in-house developed script developed by Jakob Bunkenborg, University of Southern Denmark. Mass lists were searched in Swiss-Prot database using an in-house MASCOT server. The MASCOT server settings were: maximal number of missed cleavages: 1; significant threshold p<0.05; MS mass accuracy: 50 ppm; MS/MS 0.6 Da: partial modification: methionine oxidation.

**Data analysis**

The Ingenuity pathway analysis software was used for network analysis. To perform gene enrichment and facilitate further data integration, the selected mouse genes were mapped to their human homologs (EntrezGene identifiers). A total of 19 genes were identified. The list of genes was expanded by including their known first-order protein-protein interaction partners using a high confidence human interactome containing 428,429 unique protein-protein interactions consisting of refined experimental proteomics data (Lage). This step result in a second gene set containing 81 genes. Both gene sets were investigated for functional annotation based on the three Gene Ontology categories (molecular function, biological process and cellular components). We further explored pathways integrating KEGG pathway information in both sets. All p-values obtained were calculated using hypergeometric testing, and were corrected for multiple testing with Bonferroni correction.
The significance cutoff for the corrected p-values was set to 0.05. Cytoscape open source software was used for data visualization.

**Quantitative PCR**

For mRNA isolation cells were harvested in TRIsol reagent (Invitrogen) followed by chloroform/isopropanol extraction and precipitation. cDNA synthesis was performed using the First Strand cDNA Synthesis Kit (Ferments). Quantitative PCR reactions were performed using SYBR green assay with FAST qPCR MasterMix Plus Kit (Eurogentec) and a Mx3000P™ Real-Time PCR System from Stratagene. TATA box binding protein was used as a reference gene. Data were analyzed using the MxPro software from Stratagene.

**Short hairpin RNA transfection**

shRNA against Isyna1 was transduced into 3T3-L1 cells using the lentivirus pGIPZ vector. 293FT cells were used for virus production. In short, 80% confluent 293FT cells were using Lipofectamine 2000 (Invitrogen) transfected with a pGIPZ vector containing shRNA or with a pGIPZ empty vector, packaging plasmid psPAX2 and envelope plasmid 2.G. Two days after transfection, the medium containing viruses was collected and added to 40% confluent 3T3-L1 cells. Two days after transfection, the cells were selected using 2µg/ml puromycin (InviveGen). After selection cells were maintained in medium containing 1µg/ml puromycin.
Western blot analysis

The Mini Format 1-D Electrophoresis Systems (Bio-Rad) and homemade precast 8% acrylamide gels were used for western blot analyses. The Chemiluminescence Detection Kit for HRP (Biological Industries) and a Fusion FX5 Chemiluminescence imaging system (Montreal Biotech. Inn) were used. The acquired data was quantified using the ImageJ open source software.

RESULTS

Two dimensional gel analysis

In order to determine protein expression and post translational alterations in 3T3-L1 cells following treatment with cyclic AMP (cAMP) analogues targeting either PKA or Epac, we analyzed cells during different time points of differentiation using two dimensional (2D) gel electrophoresis. As described in Materials and Methods, two cAMP analogues were used to replace IBMX used as cAMP-elevating agents in the standard differentiation protocol. Results from 2D gels were analyzed to identify alterations in protein expression and/or modification. The most conspicuous changes in protein expression during the differentiation were observed at 48 hours (graphs summarizing expression of all proteins, where alterations were observed are presented in Figure S1). In order to obtain statistically significant results, we extensively analyzed the 48 hour time point in terms of differences between individual treatments and dexamethasone plus insulin stimulation (reference treatment) (see Materials and Methods). The experimental design together with a list of proteins exhibiting significant changes at the 48 hour time point are presented in Figure 1.
Twenty-five spots were significantly different from the reference treatment at 48 hours. Nineteen proteins were identified from the original 25 spots and two spots remained unidentified. Eight proteins (Hydroxymethylglutaryl-CoA synthase, Pyruvate carboxylase, Phosphoglycerate mutase, Transaldolase, Laminin subunit gamma-1, Endoplasmin, Ras-related protein Rap and Guanine deaminase) reached a 2 fold up-regulation and one protein, Importin subunit beta, was more than 2-folds down-regulated (see table 1). Four proteins, Inositol-3-phosphate synthase 1, Phosphoglycerate mutase, Pyruvate carboxylase and Laminin subunit gamma-1 were identified in more than one spot. The Spots identified as inositol-3-phosphate synthase 1, phosphoglycerate mutase 1/2, guanine deaminase, hydroxymethylglutaryl-CoA synthase and hippocalcin-like protein 1 are shown in the figure 2. Compared with the reference treatment, Epac activation induced the change of 10 proteins (8 up and 2 down – regulations), from which 9 was also observed in companied Epac and PKA stimulation (Figure 3). In addition, activation of PKA changed intensity of 7 proteins (6 up and 1 down – regulations), from which 5 were common with combined Epac and PKA activation. Finally in response to the simultaneous activation of Epac and PKA we observed a significant change of 22 different proteins (17 up and 5 down – regulations) from which 11 were unique for the combined stimulation of Epac and PKA. Surprisingly, 3 protein changes were common for both Epac and PKA activation. All differences have been summarized in the protein identification table (Table 1).

**Analysis of mRNA expression**

We examined mRNA expression of proteins identified by proteomics using quantitative PCR to investigate whether or not observed changes involved transcriptional regulation. Levels of mRNA encoding 9 proteins were significantly changed during the differentiation process
(Figure 4). The mRNA level of Inositol-3-phosphate synthase 1 increased during differentiation in response to 007MB treatment and reached an approximate 2.5-fold change at 48 hours. In mature adipocytes (Day 8) the mRNA level of Inositol-3-phosphate synthase 1 was half that of preadipocytes (day 0). At 48 hours, the mRNA level of Inositol-3-phosphate synthase 1 was significantly different from the reference treatment for both 007MB and 007 treatments. However, the fold change of 007MB treatment was 2 times larger than the fold change of 007 treatment alone. The mRNA levels of three proteins Hydroxymethylglutaryl-CoA synthase, Phosphoglycerate mutase and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 increased during differentiation and appeared to be significantly different in mature adipocytes (day 8 007MB treatment) compared to both day 0 and the reference treatment. The mRNA level of Hydroxymethylglutaryl-CoA synthase and Phosphoglycerate mutase were significantly up-regulated at the 48 hour time point in case of both 007 and 007MB treatments compared to the control treatment. Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 mRNA levels were significantly increased by every treatment together with the reference at the 24 hour and 48 hour time point compared to day 0. However, no significant differences at those two time points were observed between cAMP analogues and the reference. A similar situation was observed in the case of Laminin subunit gamma-1, except that no significant difference was observed between the treatments at day 8. The mRNA level of three proteins, Lysosomal alpha-glucosidase, Beta-hexosaminidase subunit alpha and Hippocalcin-like protein 1 (for proteomic data see figure 2) were down-regulated during differentiation. In all three cases, the mRNA levels in cells treated with 007MB were significantly down-regulated compared to cells receiving the reference treatment at the 48 hour and the day 8 time point. In mature adipocytes, the mRNA levels of all three proteins were also significantly down-regulated compared to preadipocytes (not shown). The mRNA
level of Importin subunit beta-1 was significantly different only in cells treated with 007MB compared to the other treatments at day 8.

Data analysis

Gene ontology and protein-protein interaction analysis

In order to obtain an overview of the functions of identified proteins, we performed gene ontology (GO) as well as Reactome Main Pathway analyses (table S1). These analyses indicated relationships of identified proteins with alcohol metabolic processes (G6PD;GAA;HMGCS1;ISYNA1;PGAM1;PGAM2;TALDO1) as well as carbohydrate catabolic processes (G6PD;GAA;PGAM1;PGAM2;TALDO1). Detailed involvement of identified proteins described above in carbohydrate (particularly glucose) catabolic processes is shown in the figure 5. Most of the proteins, G6PD, ISYNA1, PGAM1, PGAM2, TALDO1, were regulated by the combined action of Epac and PKA (see table S1); whereas Lyag and HMGCS1 were changed in response to Epac activation as well as simultaneous activation of both Epac and PKA. Due to the small number of identified proteins, we performed gene enrichment analyses together with protein-protein interaction network analyses (Figure 6 and Table S2). Protein-protein interaction analyses were restricted to highly significant interactions. Nineteen proteins were recognized and submitted to the network analyses. Fourteen of them formed an interaction network. The entire network consists of 81 proteins. Six proteins, identified by proteomics, Importin subunit beta-1, Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, Cofilin, Beta-hexosaminidase subunit alpha, Endoplasmin and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 exhibit direct interactions, whereas Endoplasmin and Ras-related protein Rap have a common interaction protein – von Willebrand factor (VWF). GO analysis for gene enrichment analysis indicated a possible role
of the identified proteins in hexose catabolic processes, monosaccharide catabolic processes, alcohol catabolic processes, small GTPase mediated signal transduction, carbohydrate metabolic processes, NADP metabolic processes, cellular carbohydrate catabolic process and glucose catabolic processes. The Reactom Main Pathway analysis (Figure 6 and Table S2) indicated a possible connection of the identified proteins in relation to integrin cell surface interactions, metabolism of carbohydrates and homeostasis.

**Ingenuity pathway analysis**

We used the Ingenuity pathways analysis™ software to show indirect relationships between identified proteins as well as to illuminate key regulators of identified proteins. Relationships are presented in Figure 7. The identified proteins created two independent networks. TGFB1 is a central part of first network and is involved in indirect interactions with 3 proteins the exhibited changes in expression in response to a combined activation of Epac and PKA (Glucose-6-phosphate 1-dehydrogenase, Importin subunit beta-1, Ras-related protein Rap) and two proteins changed by Epac activation alone (Endoplasmin and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1). Inositol-3-phosphate synthase 1 and Transaldolase have common binding partner - inhibitor of kappaB kinase epsilon (IKBKE). Calcium ions are central regulators of the second network. It regulates directly or indirectly three proteins down-regulated during the differentiation process. The proteomics analyses demonstrated that the expression of two of them (beta-hexosaminidase subunit alpha and lysosomal alpha-glucosidase) was altered in response to Epac activation (see also figure 5). Platelet-derived growth factor (PDGF) has been reported to regulate Cofillin (changed by PKA activation in our study) as well as Guanine deaminase and Hydroxymethylglutaryl-CoA synthase, which were up-regulated by Epac activation.
We further investigated the change in myo inositol synthase expression observed at the 48 hour time point of 3T3-L1 cells differentiation. Western blot analysis showed two species of the protein present in the cells, clearly separated by SDS PAGE (see Figure 8). The specificity of the antibody was confirmed by shRNA knockdown of the Ino1 mRNA (see Figure 8). While the intensity of lower band did not change during first 48 hours of differentiation, the intensity of the upper band increased markedly during the first 48 hours of differentiation in response to the combined action of 007 and MB. Replacement of cAMP analogues with IBMX gave a similar effect with an even more pronounced increase in the intensity of the upper band at the 24 hour time point. Total Ino1 protein was more than 2 times down-regulated in mature adipocyte compared to preadipocytes.

Western blot analysis of different mouse fat depots showed that most of the Ino1 protein migrated as the upper band form (Figure 8). High fat feeding had no effect on abundance of Ino1 protein in epididymal and inguinal fat depots (Figure S2). Regular Dulbecco's Modified Eagle Medium contains 40µM of inositol. Mammalian cells can utilize extracellular inositol employing two classes of transporters both of which use sodium or protons to co-transport inositol. To investigate the ability of 3T3-L1 cells to utilize inositol from the culture medium, we measured mRNA expression of inositol transporters. The mRNA of solute carrier family 5 member 3 (SLC5a3) was detected in both preadipocytes and mature adipocytes. Other inositol transporters were not detected. During the differentiation, the mRNA expression of SLC5a3 was strongly down-regulated when IBMX was present in differentiation medium.
Since its discovery, Epac has been implicated in numerous cellular processes (for review see [10]). We have shown that activation of Epac is necessary for cAMP-induced acceleration of adipocyte differentiation of 3T3-L1 preadipocytes, mouse embryo fibroblast [3] and human hMADS cells [8]. Here we have examined protein changes induced by cAMP-mediated signaling driven by Epac, PKA or both factors during differentiation of 3T3-L1 preadipocytes.

It has been reported that cAMP elevation in preadipocytes dramatically increases glucose uptake and intracellular glucose levels [11]. A number of identified proteins exhibiting changes in abundance in response to the combined activation of Epac and PKA were enzymes involved in glucose metabolism (Figure 5 and table S1 and S2). Regulation of three distinct pathways could be observed. Glucose 6-phosphate dehydrogenase is the first enzyme in the pentose phosphate pathway providing precursors for nucleic acids synthesis. Glucose 6-phosphate dehydrogenase also plays a role in production of NADPH, which is important for fatty acids and cholesterol synthesis. Regulation of the pentose phosphate pathway during the early stage of 3T3-L1 differentiation has previously been reported [12]. Inhibition of glucose 6-phosphate dehydrogenase inhibits differentiation of 3T3-L1 preadipocytes and lipid accumulation [12, 13]. Transaldolase, the enzyme that links the pentose phosphate pathway and glycolysis was in the present study also found to be up-regulated at the protein level. A microarray study conducted on 3T3-L1 cells showed a decrease in mRNA expression of this enzyme during first 4 days of differentiation and an increase of its expression in the later phase of differentiation [14]. Transaldolase directs carbohydrate metabolites derived from glucose to the glycolysis pathway (see figure 5). An increase in the activity of this enzyme may be needed for switch from nucleic acids synthesis (needed during the clonal expansion phase) to glycolysis and subsequent to de novo fatty acids synthesis.
However, in contrast to the microarray study, we did not observed changes in transaldolase mRNA levels. Phosphoglycerate mutase, an enzyme catalyzing eight step of glycolysis was also found to be up-regulated in the present study both at protein and at the mRNA level (see figure 2 and 4), but has not so far been reported to play a role in adipogenesis. Phosphoglycerate mutase was identified on 2D gels as two different spots displaying different isoelectric point, suggesting that two different protein species may be involved in 3T3-L1 cells differentiation. Inositol-3-phosphate synthase 1 (Ino1) is another protein involved in glucose metabolism. Increase in Ino1 expression was observed at both the protein and the mRNA level (see figure 2 and 4) in response to combine action of Epac and PKA. Ino1 converts glucose 6-phosphate into myo inositol, a precursor of all phospho inositol species covalently bound to phospholipids. An increase in Ino1 mRNA levels during adipocyte differentiation was previously reported [14], however the function of this enzyme as well as myo inositol during 3T3-L1 preadipocyte differentiation has not been studied. Western blot analysis of Ino1 showed two species of the protein present in the cells (Figure 8A and B). The protein species represented by upper band on the western blot seems less abundant in preadipocytes than in mature adipocytes. Band of the same molecular mass constitutes the majority of the Ino1 protein in mouse fat tissue (see figure 8D), suggesting the biological importance of this protein species. The level of the lower band present in preadipocytes did not changed during the differentiation process. The possibility that the upper band represented a phosphorylated state of the Ino1 protein is unlikely, as phosphatase treatment did not alter the intensity of the upper band (data not shown). Cell can obtain inositol in two ways. One is de novo synthesis from glucose and the second is by transport across the plasma membrane from the extracellular space. Mammalian cells express two types of inositol transporters, Slc5a3 and Slc5a11, which both are sodium co-transporters, and Slc5a13 which is a H+ co-transporter [15]. 3T3-L1 fibroblasts and mature adipocytes express only the
Slc5a3 transporter, the expression of which is strongly down-regulated during the first 48 hours of differentiation in response to IBMX treatment (figure 8E). Down-regulation of inositol transport together with up-regulation of inositol producing enzymes seem to be contradictory and suggest that further study on the role of inositol in adipocyte differentiation need to be conducted.

Epac has not been observed to transcriptionally regulate a broad spectrum of genes, suggesting that its action may be mostly related to the posttranscriptional regulations. In our proteomic analysis, 7 different proteins were found to be regulated by Epac (see table 1). This regulation did not required activation of PKA. However, only one protein, 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) was observed to be regulated by Epac at the level of transcription level (see figure 4). Two proteins were observed to be down-regulated at the protein level in response to Epac activation, Beta-hexosaminidase subunit alpha (Beta-hexosaminidase) and Lysosomal alpha-glucosidase (Lyag), were observed to be down-regulated at the mRNA level only when Epac signaling was supported by PKA signaling (see figure 4), suggesting a different regulation at the transcriptional, translational and posttranslational level by Epac alone or the combination with PKA.

Epac has been reported to regulate calcium signaling in cardiac muscle cells and in pancreatic β-cell islets [16, 17]. Ingenuity pathway analyses revealed calcium ions as one of the central regulators of the network created by the identified proteins (see figure 7) Two of the proteins in the network, beta-hexosaminidase and Lyag, were observed to be regulated by Epac alone. Lyag is one of the glycogen degrading enzymes, shown to play a role in insulin secretion by β-cells and to be regulated by calcium ions [18, 19]. Hexosaminidase is an enzyme taking part in degradation of glycoconjugates such as glycoproteins or glycolipids. Both enzymes are located in lysosomes and there role in adipogenesis has never been tested. Adipocyte differentiation has been shown to be inhibited by high intracellular level of calcium in several
cells models [20, 21] and beta-hexosaminidase and Lyag may be potential mediators of calcium-mediated inhibition of adipocyte differentiation.

Acknowledgments

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REFERENCES


[21] Ntambi JM, Takova T. Role of Ca2+ in the early stages of murine adipocyte differentiation as evidenced by calcium mobilizing agents. Differentiation; research in biological diversity 1996;60:151-8.
**Figure legends**

**Figure 1.** Experimental design and identification of proteins significantly changed at the 48 hour time point of 3T3-L1 adipocyte differentiation in response to cAMP signaling via the Epac, the PKA branch or both. The protein identification map shows proteins changed by activation of Epac or PKA and by the combined action of both factors.

**Figure 2.** Changes of spots identified as ISYNA1 - Inositol-3-phosphate synthase 1, PGMA1/2 - Phosphoglycerate mutase 1/2, GUAD - Guanine deaminase, HMCS1 - Hydroxymethylglutaryl-CoA synthase, HPCL1 - Hippocalcin-like protein 1 in 48 hours time point of 3T3L1 cells differentiation. A – Representative fragments of the gels with the spots of interest. B – Quantification of the intensity of the spots of interest. * - intensity significantly different (p<0.05) from the reference treatment.

**Figure 3.** Venn diagram of up- or down-regulated proteins in response to activation of Epac, PKA or both Epac and PKA at the 48 hour time point of 3T3-L1 adipocyte differentiation.

**Figure 4.** Quantitative PCR measurement of mRNA expression of nine genes identified as changed by proteomics analysis. * - intensity significantly different (p<0.05) from the reference treatment. Isyna1 - Inositol-3-phosphate synthase 1, Hmgcs1 - Hydroxymethylglutaryl-CoA synthase, Lyag - Lysosomal alpha-glucosidase, Pgam1 -
Phosphoglycerate mutase 1, Lamc1 - Laminin subunit gamma-1, Hpcl1 - Hippocalcin-like protein 1, Kpnb1 - Importin subunit beta-1, Plod1 - Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1, Hexa - Beta-hexosaminidase subunit alpha.

**Figure 5.** The role of identified proteins in pathways of glucose metabolism, cholesterol biosynthesis and calcium signaling pathway.

**Figure 6.** Protein - protein interaction network between proteins identified as up- or down-regulated due to stimulation of Epac, PKA or both Epac and PKA at the 48 hour time point of 3T3L1 adipocyte differentiation. Blue nodes represent proteins identified as changed in one or more of the treatments when compared to the reference treatment, at 48 hour time point of 3T3-L1 adipocyte differentiation. Green nodes represent interacting proteins. Connections represent physical interactions between proteins. Arrows indicate up- or down-regulation. Labels below nodes represent treatment group where the difference has been observed.

**Figure 8.** Impact of cAMP on the presence of Ino1 species during 3T3-L1 adipocyte differentiation. Western blot analysis of Ino1 protein during 3T3-L1 differentiation with different cAMP analogues (A) or with IBMX (D). B) Western blot analysis of 3T3L1 cells with shRNA knockdown of Ino1. C) Western blot analysis of Ino1 protein in 3T3-L1 cells and mouse epididymal white adipose tissue. E) Quantitative PCR analysis of Slc5a3 mRNA levels
during 3T3-L1 adipocyte differentiation with or without IBMX. DI- Dexamethasone, insulin.

DMI- Dexamethasone, insulin and IBMX.

Figure S1. Proteins level changes during 3T3L1 cells differentiation caused by stimulation of Epac, PKA, or both Epac and PKA.

Figure S2. Ino1 protein level and modification state in subcutaneous and epidydymal fat under different feeding conditions.
Figure 1
**Figure 2**

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B

![Bar charts for ISYNA1, PGMA1/2, Cofilin 1, GUAD, HMCS1, HPCL1](image)
Figure 3

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Figure 4
Figure 5
Figure 8
Figure S1
Figure S2
Table S1. Gene Ontology and Reactome Main Pathways analysis of proteins identified as up or down-regulated due to stimulation of Epac, PKA or both Epak and PKA in 48 hours time point of 3T3L1 cells differentiation.

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Table S2. Gene enrichment analysis of proteins identified as up or down-regulated due to stimulation of Epac, PKA or both Epak and PKA in 48 hours time point of 3T3L1 cells differentiation. Identified proteins together with their interaction partners have been submitted to Gene Ontology, KEGG pathways and Reactome Main Pathways analysis.

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Perturbation of first phase insulin secretion in mice with pancreas-specific ablation of \textit{Ptpn1} attenuates sucrose induced peripheral insulin resistance

Kamil Borkowski\textsuperscript{1}, Ahmed Bettaieb\textsuperscript{2}, Jesse Bakke\textsuperscript{2}, Yannan Xi\textsuperscript{2}, Lene Secher Myrme\textsuperscript{1,3}, Fawaz Haj\textsuperscript{2}, Lise Madsen\textsuperscript{1,3}, Karsten Kristiansen\textsuperscript{1}

\textsuperscript{1} - Department of Biology, University of Copenhagen, DK-2200 Copenhagen, Denmark
\textsuperscript{2} - Departments of Nutrition, University of California Davis, Davis, CA 95616, USA
\textsuperscript{3} - National Institute of Nutrition and Seafood Research (NIFES), N-5817 Bergen, Norway

Corresponding authors:

Karsten Kristiansen, Department of Biology, Copenhagen University, Ole Maaløes Vej 5, 2200 København N, Copenhagen, Denmark. Phone: +45 353-24443, E-mail: kk@bio.ku.dk

Lise Madsen, National Institute of Nutrition and Seafood Research (NIFES), Nordnesboder 2, 5005 Bergen, Norway. E-mail Lise.Madsen@nifes.no
ABSTRACT

A considerable body of evidence indicates that sucrose potentiates the obesogenic effect of a high fat diet in a dose-dependent manner and induces insulin resistance. We have previously suggested that the obesity-promoting effect of sucrose at least in part is mediated by insulin. Pharmacological inhibition of insulin secretion diminishes the effect of elevated sucrose content in high fat diets on body fat mass accumulation. Protein tyrosine phosphatase 1B was recently shown to positively regulate insulin secretion. In the current study we have investigated the effect of pancreas specific Ptpn1 deletion on the ability of sucrose to increase fat mass and accentuate insulin resistance. We show that pancreas specific deletion of Ptpn1 did not influence fed or fasted insulin levels however, mice with pancreas-specific deletion of Ptpn1 exhibited decreased sucrose-induced first phase insulin secretion in the context of a high fat diet. Interesting, pancreas specific deletion of Ptpn1 attenuated peripheral insulin resistance in mice fed a high fat-high sucrose diet.

INTRODUCTION

Animals studies have demonstrated that the carbohydrate:protein ratio determines the obesogenic effect of high fat diets. This is supported by research showing that weight gain is increased when isocaloric high fat diets are rich in sucrose or high-glycemic index carbohydrates rather than in protein [1-3]. Insulin signaling is crucially involved in fat cells differentiation [4] as well as an important regulator of metabolism and energy expenditure [5, 6]. Defects in insulin signaling have been associated with the metabolic syndrome and
obesity [7-9]. A possible mechanism, by which high fat diets promote obesity when combined with sucrose, but not proteins, may relate to the effect of sucrose on insulin secretion and enhanced insulin signaling. This finding is underscored by the finding that nifedipine, a dihydropyridine calcium channel blocker that inhibits insulin secretion, was able to protect mice from diet-induced obesity elicited by a high fat high sucrose diet (HF/HS) [2]. Dietary proteins and sucrose differ in their ability to stimulate insulin secretion. Animals fed a HF/HS diet have elevated fasting insulin levels compared to animals on a high fat/low sucrose diet. Ins1+/−:Ins2−/− mice which display reduced insulin secretion and are protected against chronic hyperinsulinemia, are also protected against high fat diet-induced obesity [9]. Moreover, elevated plasma insulin is correlated with insulin resistance in humans [10]. It is worth noting that even though a high protein:sucrose ratio in the diets prevents high fat diet induced obesity, the mice are still glucose intolerant [1]. However, as both fasting glucose and fasting insulin levels are as low as in low fat fed control mice it is likely that insulin sensitivity is increased. High protein diets may reduce the capacity of pancreatic β-cells to secrete insulin in response to glucose. It was recently demonstrated that glucose-stimulated insulin secretion is highly dependent on communication between β-cells in the pancreas, and this phenomenon is mediated by ephrins [11]. Both ephrin A5 and EphA5 can differentially effect insulin secretion. Signaling from EphA5 attenuates insulin secretion, while signaling by ephrin A5 enhances insulin secretion [11]. EphA5 is active when phosphorylated and protein tyrosine phosphatase 1B (PTP1B) is responsible for its dephosphorylation (unpublished data). Therefore, PTP1B is proposed to regulate insulin secretion in pancreatic β-cells. Whole body PTP1B deficiency Increases energy expenditure, decreases adiposity, and enhances tissue-specific insulin sensitivity [6]. In the current study we used pancreas specific Ptpn1 knockout mice to further investigate the role of insulin secretion in relation to intake of high fat diets rich in sucrose.
MATERIALS AND METHODS

Ethics statement

All mouse studies were conducted according to federal guidelines and were approved by the Institutional Animal Care and Use Committee at University of California Davis.

Animals and diets

Mice carrying the floxed alleles for Ptpn1, in which exons 6-8 (encoding the active site of the enzyme) are flanked with loxP sites (Ptpn1 fl/fl), were generated [12] and used to generate Ptpn1 fl/fl mice on a 129Sv/J x C57Bl/6J background. Pdx1-Cre mice on a C57Bl/6J background were obtained from Dr. D. Melton (Harvard University, Boston, MA). All mice were maintained on a 12-hour light-dark cycle in a temperature-controlled facility, with free access to water and food. Genotyping for Ptpn1 floxed allele and the presence of Cre was performed by PCR, using DNA extracted from tail tips. Approximately 8 week old male mice with pancreas specific Ptpn1 knock out (KO) and wild type (WT) controls were fed ad libitum a low fat, high fat high protein (HF/HP) or a high fat high sucrose (HF/HS) diet for ten weeks (n=8-10 per diet). The composition of the diets is shown in table 1. Diets were obtained from Ssniff Spezialdiaten GmbH, Soest, Germany. In order to control feed-intake and calculate feed-efficiency for each individual mouse, the mice were housed single caged.
Glucose tolerance test (GTT), insulin tolerance test (ITT) and Glucose stimulated insulin secretion test (GSIS).

GTT was performed after 4 weeks of feeding. After one week of recovery from the GTT, the ITT was performed. Both assays were repeated starting from week 8. Additionally, at week 10 GSIS was determined. For GTT and GSIS mice were fasted overnight before an intraperitoneal injection of 2 g/kg glucose in saline. For ITT mice were fasted 4 hours before an intraperitoneal injection of 0.5 Unit/kg human recombinant insulin in saline. Blood was collected from the tip of the tail at indicated time points. Glucose concentrations were measured with Bayer Contour glucometer. Insulin concentrations were measured using Insulin (Mouse) ELISA kit (DRG Diagnostics).

Statistics and calculations

HOMA IR was calculated using fasting blood glucose and insulin values using the following equation: plasma glucose (mmol/l)×plasma insulin (μU/ml)/ 22.5. Two tail distribution Student t-test analysis for unequal variance for area under the curve in GTT and ITT, and log10 transformed GSIS. Repeated measures ANOVA was used for body weight, and two ways ANOVA for tissue masses. Data were considered statistical significant when P <0.05.
RESULTS

Pancreas specific Ptpn1 deletion attenuates the first phase insulin secretion in mice fed a high fat-high sucrose diet

To investigate the effect of Ptpn1 deletion on first phase insulin secretion, a glucose-stimulated insulin secretion test was performed after 10 weeks of feeding. As expected, the first phase insulin secretion (measured 5 min after glucose injection) was higher in mice fed the HF/HS diet compared with low fat fed mice (Figure 1). This increase was blunted in the pancreas specific Ptpn1 knockout mice. Compared with low fat fed mice, insulin levels 5 min after glucose injection were not increased in mice fed a HF/HP diet, and insulin levels were similar in wild type and KO mice fed the HF/HP diet.

Pancreatic Ptpn1 deletion does not influence body mass in high fat fed mice irrespective of the sucrose content in the diet.

As expected, wild type mice fed a HF/HS diet gained more weight compared with mice fed the HF/HP diet (Figure 2A) even though, no significant difference in caloric intake was observed (Figure 2B). The masses of retroperitoneal, gonadal, mesenteric and brown interscapular fat depots as well as in total fat mass were significantly increased in the HF/HS fed mice when compared to HF/HP fed mice (Figure 3) in agreement with our previous findings. The same strong tendency was observe in the case of liver mass of the liver (p<0.056) comparing HF/HS with HF/HP fed mice.
Total body weight was not influenced by pancreas specific Ptpn1 deletion regardless whether or not sucrose was present in the diet. Similarly, no significant differences between KO and WT mice were observed regarding fat depot masses as well as total fat mass (Figure 3).

Pancreatic Ptpn1 deletion does not affect blood glucose and insulin parameters. Mice fed the HF/HS diet showed elevated fed plasma glucose level in comparison mice on the HF/HP diet (Figure 4). On the other hand, HF/HS diet did not change fasting glucose as well as fed or fasting insulin parameters compared with the HF/HP diet. None of the blood parameters were changed in response to pancreas specific Ptpn1 deletion. The calculated HOMA IR was influenced neither by sucrose content of high fat diet nor by the mice genotype.

Pancreas specific Ptpn1 deletion prevents insulin resistance in mice fed the high fat-high sucrose diet. Mice fed HF/HS displayed a tendency towards lower glucose tolerance (p<0.075 in area under the curve analysis of the GTT test) compare to mice fed the HF/HP diet (Figure 5). In the same way, HF/HS fed mice exhibited peripheral insulin resistance (measured by IIT test) in comparison to HF/HP fed mice (Figure 5). The effect of the diet was genotype dependent. The effect of the HF/HS diet on glucose tolerance and peripheral insulin resistance was diminished in mice with pancreas specific Ptpn1 KO. Ptpn1 deletion had no effect on glucose tolerance or insulin resistance in HF/HP fed animals.
DISCUSSION

We have previously reported that a HF/HS diet increased total body mass as well as adipose tissue mass compared to a HF/HP diet [1, 2]. The effect of HF/HS diet on adipose tissue mass was diminished by administration of nifedipine, an inhibitor of insulin secretion inhibitor. The current study confirmed the effect of HF/HS diet on mice body weight (Figure 2) and adipose tissue mass (Figure 3). However, pancreas specific Ptpn1 deletion did not significantly change either body or fat depot masses.

Both high sucrose and high fat diets have been reported to induce insulin resistance in animal models [13, 14]. Liver is the primary organ sustaining fasting glucose level through degradation of glycogen and gluconeogenesis. Development of insulin resistance in liver is associated with fasting hyperinsulinemia [15]. In our experiment, all mice fed the high fat diet developed insulin resistance measured by HOMA IR (Figure 3). Replacement of proteins with the sucrose in the high fat diet did not cause an increase in HOMA IR index or fasted glucose or insulin levels (Figure 4). These findings may suggest that all high fat diet fed animals developed hepatic insulin resistance and that the sucrose content did not aggravate this process in our animal model.

High sucrose diets have previously been associated with peripheral insulin resistance [16]. In our experiment mice fed with HF/HS diet displayed higher peripheral insulin resistance, measured by insulin tolerance test in comparison with mice fed with the HF/HP diet (Figure 5). Interestingly, pancreas specific Ptpn1 deletion reduced insulin resistance caused by HF/HS diet, bringing it to the level of that observed in mice fed the HF/HP diet. On the other hand, pancreas specific Ptpn1 deletion had no effect on insulin resistance in HF/HP diet, suggesting that the observed effect is sucrose dependent.
Sucrose, in contrast to fat potently induces insulin secretion. Hyperinsulinemia, the state of elevated blood insulin level coexists with insulin resistance, and it has been demonstrated that elevated insulin level induces insulin resistance in rodents [17]. Studies on ex vivo mouse and human tissues have indicated that the combined action of insulin and glucose is required for development of glucose-induced insulin resistance in peripheral tissues [18, 19]. This suggests that glucose-stimulated insulin secretion may be involved in glucose-induced insulin resistance. Here we provided evidence that the first phase insulin secretion may be involved in sucrose induced peripheral insulin resistance. A HF/HS diet significantly increased the first phase insulin secretion as compared to that observed in the low fat control group (Figure 1). Pancreas specific Ptpn1 deletion blunted HF/HS induced elevation in the first phase insulin secretion. Despite of reduction of the first phase insulin secretion, pancreas specific Ptpn1 deletion did not significantly changed fed or fasted blood insulin and glucose levels (Figure 4) suggesting, that the second phase insulin secretion was maintained. These findings support the hypothesis that the elevated first phase insulin secretion is associated with sucrose-induced peripheral insulin resistance.

Increased fat mass and body weight have been shown to correlate with insulin resistance [20] and a reduction in the body weight has been demonstrated to reduce insulin resistance [21]. In our experimental model, mice fed the HF/HS diet increased body weight compared to that of mice fed the HF/HP diet (Figure 2) as shown previously [1]. The HF/HS diet also increased the mass of retroperitoneal, gonadal, mesenteric and brown interscapular fat depots (Figure 3). Our data support the notion that PTP1B is involved in regulating first phase insulin secretion, however, probably not in the regulation of general overall insulin secretion caused by prolonged elevated plasma glucose levels as indicated by the lack of differences in fed plasma insulin and glucose levels in WT and KO mice. WT mice fed the HF/HS diet exhibited an increase in peripheral insulin resistance in comparison with mice fed the HF/HP
diet. However, it is important to note that insulin sensitivity was increased in Ptpn1 KO mice despite that no significant changes in body weight and fat mass in the WT and Ptpn1 KO mice were observed suggesting that development of HF/HS diet-induced insulin resistance somehow is linked to first phase insulin secretion.

Acknowledgments

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REFERENCES


FIGURE LEGENDS

Figure 1. Insulin levels during a glucose-stimulated insulin secretion test (GSIS). LF – low fat diet, HF/HP – high fat, high protein diet, HF/HS – high fat high sucrose diet, WT – wild type, KO – pancreas specific *Ptpn1* knockout. n=6 per group.

Figure 2. A - body weight of experimental animals with the p values from ANOVA repeated measurements analysis. B - the average calories intake of experimental animals. LF – low fat diet, HF/HP – high fat, high protein diet, HF/HS – high fat high sucrose diet, WT – wild type, KO – pancreas specific *Ptpn1* knockout. (n=7-10).

Figure 3. Tissues mass of experimental animals. LF – low fat diet, HF/HP – high fat, high protein diet, HF/HS – high fat high sucrose diet, WT – wild type, KO – pancreas specific *Ptpn1* knockout (n=7-10).

Figure 4. Plasma glucose and insulin level in fed and 12 hours fasted stats as well as calculated HOMA IR. LF – low fat diet, HF/HP – high fat, high protein diet, HF/HS – high fat high sucrose diet, WT – wild type, KO – pancreas specific *Ptpn1* knockout. n=10 to 7 per group.

Figure 5. Glucose tolerance test and insulin tolerance test of experimental animals. Upper panel shows unmodified data and the lower panel shows calculated area under the curve outlined by the measurement values. LF – low fat diet, HF/HP – high fat, high protein diet, HF/HS – high fat high sucrose diet, WT – wild type, KO – pancreas specific *Ptpn1* knockout. n=10 to 7 per group.
Table 1. Composition of the experimental diets. All values in g/kg of the diet.

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(b) Average calories intake

- LF
- HF/HF WT
- HF/HF KO
- HF/HF WT vs HF/HF WT
- HF/HF KO vs HF/HF WT

Average calories intake from week 0 to week 10 for different groups.
Figure 3
**Figure 4**

Bar charts showing comparisons of fasted and fed glucose and insulin levels across different groups (LF, HF, HP WT, HP KO, NS WT, NS KO). The graphs illustrate statistical differences indicated by asterisks (*) and letters (a, b).
Figure 5

GTT area under the curve

ITT area under the curve
Weight cycling promotes circadian shift and fat gain in C57BL/6J mice

SN Dankel1,2, EM Degerud1,2, K Borkowski3,4, E Fjære3,4, LK Midtbø3,4, C Haugen1,2, MH Solsvik1,2, AM Lavigne2, K Kristiansen4, B Liaset3,4, JV Sagen1,2, G Mellgren1,2*, L Madsen3,4

1 Department of Clinical Science, University of Bergen, N-5021 Bergen, Norway
2 Hormone Laboratory, Haukeland University Hospital, N-5021 Bergen, Norway
3 National Institute of Nutrition and Seafood Research (NIFES), N-5817 Bergen, Norway
4 Department of Biology, University of Copenhagen, DK-2200 Copenhagen, Denmark

*Corresponding author: gunnar.mellgren@med.uib.no

Conceived and designed the experiment: LM, BL, KK, SND, GM
Carried out experiments/collection samples: EMD, KB, EF, LKM, CH, MHS, AML
Analyzed clinical and biochemical data: SND, EMD, KB, EF, LKM, KK, BL, JVS, GM, LM
Analyzed gene expression data: SND, EMD
Wrote the manuscript: SND, EMD
Reviewed manuscript: All co-authors

Abbreviated title: Weight cycling and adipose tissue clock genes

Corresponding author and reprint requests:
Professor Gunnar Mellgren, MD, PhD
Hormone Laboratory, Haukeland University Hospital, N-5021, Bergen, Norway
Phone/Email: +47 55 97 50 00 / gunnar.mellgren@med.uib.no
ABSTRACT

To lose or maintain weight, many individuals initiate periods with low energy intake (‘‘yo-yo dieting’’), resulting in weight cycling which might ultimately result in further gain of body fat. We tested this by a controlled experiment in C57BL/6J mice, and searched for genes with differential expression in adipose tissue that could be involved in an adaptive mechanism to increase fat storage efficiency. Eleven mice were subjected to four weight cycles, followed by a final three-week overfeeding period. As controls, eleven mice were subjected to chronic overfeeding and low-fat diet, respectively. The weight cycled mice gained significantly more weight than chronically overfed mice (mean±SEM 12.4±0.8 compared to 10.0±0.4 grams, p=0.018). Total energy intake was identical in the weight cycled and chronically overfed groups. Weight cycled mice showed increased adipose tissue mass and leptin levels. While chronic overfeeding primarily increased adipocyte size, weight cycled mice gained weight by a normalization of adipocyte recruitment, and were protected from a worsening of circulating lipid, glucose, and adiponectin levels. Global gene expression was analyzed by microarrays. Weight cycled mice were characterized by a down-regulation of several circadian clock genes (Dbp, Tef, Per1, Per2, Per3, Nr1d2) in adipose tissues, which was confirmed by qPCR. In 3T3-L1 cells, we found reduced expression of Dbp and Tef early in adipogenic differentiation, which was mediated by cAMP. Our data suggest that circadian clock transcription factors play a role in the regulation of adipocyte recruitment and fat storage efficiency in response to cyclic feeding patterns.
KEYWORDS

Energy restriction, Adipose tissue, Adipocyte, Clock genes, Weight cycling
INTRODUCTION

Weight cycling results from intermittent overeating and dieting, representing a major challenge for many individuals (22, 51, 56) which could ultimately promote further expansion of adipose tissue (8, 27, 40, 46, 58). Evolutionary conserved molecular mechanisms allow fine-tuning of metabolic processes to environmental cues. Fat storage is a key evolutionary process conducive to survival in organisms ranging from the worm C. elegans to humans (34). The adipocyte, being the primary fat storing cell, responds to changes in energy availability in part by releasing the peptide hormones leptin and adiponectin. These potent hormones exert local, central and peripheral effects, coordinating the systemic response to fasting/refeeding cycles by modulating energy storage/expenditure, appetite, biological rhythms, and other functions. In adipose tissue, signals such as leptin may modulate the capacity for lipogenesis in response to weight fluctuations, predisposing to compensatory fat regain during consequent energy surplus (23, 24, 52). Identification of novel genes associated with weight cycling and weight regain may provide new molecular insight into the evolutionary adaptation to variable energy availability.

Adipocytes are derived from multipotent mesenchymal stem cells, in a process involving commitment to the adipocyte lineage followed by terminal differentiation of the committed preadipocytes. Research using cell lines such as 3T3-L1 or mouse embryo fibroblasts (MEFs) has provided information on terminal adipocyte differentiation. Treatment of these cells with fetal bovine serum, glucocorticoids and high levels of insulin (or physiological concentrations of insulin like growth factor-1 (IGF-1)) initiates differentiation. The fasting signal cyclic adenosine monophosphate (cAMP) and cAMP-dependent processes are pivotal during the early stages of adipocyte differentiation. Factors that increase cellular cAMP, such as isobutylmethylxanthine (IBMX) or forskolin, strongly accelerate the initiation of the
differentiation program in vitro and lead to commitment the cells for terminal differentiation
and lipid-filling (7, 33).

The synergistic actions of insulin/IGF-1 and cAMP signaling during initiation of adipocyte
differentiation is remarkable and contrasts the normal interplay between insulin and cAMP
signaling in liver, muscle, and mature adipocytes. Aiming to recreate this concurrence of
fasting/refeeding adipogenic signals in vivo, we switched C57BL/6J mice to ten days periods
of overfeeding immediately after four days of energy restriction. We hypothesized that
elevated cAMP signaling during periods of energy restriction contributes to development of
new preadipocytes that expand into lipid-filled mature adipocytes when calories again are in
excess. Moreover, we wanted to identify novel genes in adipose tissue that could be involved
in mediating such an effect. To our knowledge, there are no reports on the transcriptomic
responses to weight cycling in adipose tissues. In rats, it has previously been shown that
weight cycling increases feed efficiency (the weight gained relative to the amount of energy
ingested) and resistance to weight loss after repeated weight cycling (5, 18, 45). There are
conflicting reports (13, 29), but these diverging results might be explained by different
protocols for weight cycling. We designed a weight cycling experiment for C57BL/6J mice
based on the protocol used for in vitro differentiation of mouse 3T3-L1 preadipocytes and
MEFs. Using this protocol we found greater increase in total body mass and visceral fat mass
after weight cycling compared to chronic overfeeding, which was associated with a
suppressed expression of circadian clock genes that may be novel regulators of metabolism in
adipose tissue.

MATERIALS AND METHODS

Weight cycling experiment
The study was approved by the Norwegian State Board of Biological Experiments with Living Animals. Thirty-six male C5BL/6J BomTac mice were obtained from Taconic, Europe (Ejby Denmark). At 28 ± 2°C with 50% stainless-steel wire cages with wooden chips and paper peel that were changed every two weeks. At arrival, the mice were six to eight weeks old, weighed 26 ± 1.3 grams and were fed low-fat diet for one week. All mice had free access to tap water during the entire experiment. The mice were fed Monday, Wednesday and Friday morning and feed leftover was removed, weighed and registered. Body mass was measured at start and at sacrifice, as well as every Monday and Friday throughout the experiment.

After one week of acclimatization, the 36 mice were randomly divided into three groups (n=11): low-fat diet fed, chronically overfed (CO), and weight cycled (WC). The low-fat diet (D12450B) contained 20e% protein, 35e% starch, 35e% sucrose, and 10e% fat (Research Diets, USA). The WC mice were overfed for ten days by unlimited access to an energy dense high-fat high-sucrose diet (S8672-E056S), containing 15e% protein, 1e% starch, 37e% sucrose and 47e% fat (ssniff Spezialdiäten GmbH, Germany). This energy-dense diet provided 900 more kilocalories per kilogram of food compared to the low-fat diet, equivalent to approximately 25% more energy. After each of four overfeeding periods, WC mice were energy restricted for four days by limiting their access to the energy dense diet to 70% of the energy ingested during the previous overfeeding period. After the fourth energy restriction phase, the WC mice received the energy-dense diet for three weeks ad libitum before sacrifice.

One week prior to sacrifice, after an overnight fast, a glucose tolerance test (GTT) was performed as previously described (38). Briefly, mice were injected with glucose dissolved in saline in the intraperitoneal cavity (2 gram glucose per kg body weight). Blood was drawn from the tail at 0, 15, 30, 60 and 120 minutes after injection.
The mice were sacrificed in random order between 9 and 12 AM on two separate days. The mice were anesthetized with Isofluran (Isoba-vet. Schering-Ploeg, Denmark) using the Univentor 400 anesthesia Unit (Univentor Limited, Sweden), and euthanized by cardiac puncture in the fed state. Tissues were quickly dissected out, weighed, placed in marked plastic bags, freeze clamped and stored at -80°C.

Overfeeding experiments

Thirteen male C57BL/6J BomTac and 14 male 129S6/SvEvTac were obtained from Taconic, Europe (Ejby Denmark) at 8 weeks of age and acclimated as described above. After acclimatization, the mice were housed individually and randomly assigned to the experimental diets (n=6-7). The low-fat diet (S8672-E050) contained 20e% protein, 35e% starch, 35e% sucrose, and 10e% fat, and the western diet (S8672-E400) contained 18e% protein, 13e% starch, 31e% sucrose, and 38e% fat (ssniff Spezialdiäten GmbH, Germany). The animals were fed the experimental diets described for 10 weeks ad libitum. Throughout the experiment, all mice were weighed once a week and feed intake was assessed every Monday, Wednesday and Friday. GTT, euthanization and tissue collection were performed as described above.

Blood measurements

Blood lipids were measured in 80µl heparin-plasma by a MAXMAT PL instrument (MAXMAT S.A., France). Plasma insulin was measured by the DRG® Mouse Insulin ultrasensitive ELISA kit (EIA-3440, DRG International, Inc., USA), according to the manufacturer’s protocol. Leptin and adiponectin were measured by commercial available
ELISA kits (Mouse and Rat Leptin, RD291001200R, BioVendor – Laboratorní medicína a.s.,
Mouse Adiponectin, EZMADP-60K, Millipore).

**Adipose tissue morphology**

Fat tissue was fixed overnight at 4°C by immersion in phosphate buffered 4% paraformaldehyde. Samples were then dehydrated and embedded in paraffin. 5µm thick sections were taken from two different locations of embedded tissue and were hematoxylin and eosin (H&E) stained. Three to six microscopic images from five mice per group were taken from random places of each section. The area of each cell on each image was measured by drawing the circumference using the ImageJ open source software. Additionally, using the same software the number of cells per surface was estimated by extrapolating the average adipocyte volume (µm³). Total adipocyte number in each fat pad was estimated by dividing total fat pad mass by average adipocyte volume.

**In vitro adipocyte differentiation**

3T3-L1 mouse cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (4.5g/l) containing 10% calf serum (CS) and 1% penicillin and streptomycin (PEST), and were allowed to grow to 100% confluence in 6-well plates. Two days after total confluence (“day 0”), differentiation was induced by a two-day treatment with the synthetic glucocorticoid dexamethasone (0.5mM), insulin (175nM), phosphodiesterase inhibitor methylisobutylxanthine (IBMX) (0.5M), and 10% fetal bovine serum (FBS). On day 2, the medium was changed to DMEM with 10% FBS and 175nM insulin. On day 4 the medium
was changed to DMEM supplemented only with 10% FBS, and the cells were then allowed to
develop until day 10.

To collect cell lysates for qPCR, each well in one 6-well plate was washed with 2ml room
tempered PBS before adding 350µL buffer RLT (Qiagen) or 500µl of TRI Reagent® (Sigma
Aldrich), followed by scraping and transfer to 1.5ml eppendorf tubes. The lysates were
vortexed for ten seconds, spun down and stored at -80°C.

RNA purification

To homogenize the mouse inguinal and epididymal white adipose tissues, approximately
100mg of frozen tissue was transferred to 2ml micro-centrifuge tubes with round bottom. One
ml Qiazol (Qiagen) and a stainless steel bead 5mm in diameter were added, immediately
before shaking in a TissueLyser II (Qiagen) three times at 25Hz for two minutes. RNA was
extracted using the RNeasy Lipid Tissue Mini Kit together with the On-Column DNase
digestion with the RNase-Free DNase set (Qiagen), according to the protocol of the
manufacturer. RNA from the primary human adipose culture was purified in a QIAcube using
the RNeasy Lipid Tissue Mini Kit (Qiagen). The quantity and quality of RNA was analyzed
by the NanoDrop®ND-1000 spectrophotometer and the Agilent 2100 BioAnalyzer (Agilent
RNA 6000 Nano Kit).

Microarray analysis

Global gene expression was measured in iWAT, eWAT and iBAT of WC and CO mice by
Illumina microarray analysis. The microarray data are MIAME compliant and are available in
ArrayExpress (awaiting accession number). Each group had eleven iWAT and iBAT samples
and ten eWAT samples. The microarrays (MouseRef-8 v2.0 Expression BeadChip) contained 25,697 unique probes representing approximately 19,100 unique genes. 400ng of RNA from each sample was reversely transcribed, amplified and labeled with biotin-16-UTP using the Illumina® TotalPrep RNA Amplification Kit Ambion, using an Eppendorf Mastercycler (Applied Biosystems/Ambion, USA). 750ng cRNA of each sample was hybridized to the MouseRef-8 v2.0 Expression BeadChip according to manufacturer’s protocol. Signal detection was performed using the Illumina iScan Reader. All RNA integrity numbers (RIN) were above 7.5. Raw data were imported and analyzed in J-Express (6). Rank product analysis (4) was used to compare global gene expression in the WC and CO groups.

cDNA synthesis and qPCR
cDNA synthesis was synthesized from 300ng of total RNA using the SuperScript® VILO™ cDNA Synthesis Kit. Reaction and enzyme mixtures were added to the samples along with PCR-grade water to adjust to a total volume of 20µl. The samples were then run in the thermal block cycler GeneAmp® PCR System 9700 at 25°C for 10 minutes, 42°C for 60 minutes, and finally at 85°C for five minutes. The samples were diluted 10-fold with PCR-grade water and stored at -20°C.

qPCR was performed using the LightCycler® 480 Probes Master kit and a LightCycler® 480 (Roche). Primers and probes were designed using the Universal Probe Library (UPL) online design center (www.roche-applied-science.com) (Table 1). Primers were obtained from Sigma-Aldrich (www.sigmaaldrich.com/configurator/servlet/DesignCenter). Amplification efficiency of target genes was assessed by running 1:5 or 1:10 dilution standard curves based on concentrated cDNA of mouse or human origin. Mouse and human target gene
concentrations were calculated relative to the mRNA concentration of reference genes Rplp0 and IPO8, respectively.

Statistical analyses

Rank product analysis and Significance of Microarray Analysis (SAM) were performed using J-Express 2009 (6). All other statistical analyses in this study were performed with PASW Statistics 18 for Windows. Statistical hypothesis testing was performed using parametric 2-tailed t-test (for normally distributed data, presented as mean) or non-parametric Mann-Whitney U test (for non-normally distributed data, presented as median). Normality was determined by the Shapiro-Wilk test (p>0.05).

RESULTS

Increased body weight after weight cycling

The weight cycled (WC) mice gained significantly more weight by the end of the study than chronically overfed (CO) mice (measured as the difference in body mass between the last and first day) (mean 12.4±0.8 compared to 10.0±0.4 grams, p=0.018) (Fig. 1A). Since total energy intake was identical in the WC and CO mice at the end of the experiment (mean 1180±14 and 1172±23 kcals, p=0.746), the WC mice had a significantly higher overall feed efficiency (total weight gain relative to total energy intake) compared to CO mice (mean 10.5±0.6 and 8.5±0.3 after 81 days, p=0.013) (Fig. 1B). Comparing CO to low-fat fed mice, the CO mice did not gain significantly more total body mass (mean 10.0±0.4 and 9.4±0.5 grams, p=0.336). However, energy intake was significantly higher in CO mice than in low-fat fed mice (mean 1172±23 vs. 1008±19 kcal, p=2.47E-5).
To determine where energy surplus was stored in response to the different diets, we weighed adipose tissues, muscle and liver. The iWAT mass was on average 1.39-fold higher after WC compared to CO, but this was not significant (mean 0.43±0.05 and 0.31±0.04 grams, p=0.083) (Fig. 1C). The eWAT of the WC group weighed 1.64-fold more than the CO group (mean 1.20±0.14 compared to 0.73±0.11 grams, p=0.018) (Fig. 1C). The iBAT mass was significantly higher after WC than CO (mean 0.094±0.008 and 0.075±0.003 grams, p=0.032), but was not significantly different between WC and low-fat fed mice (mean 0.094±0.008 and 0.083±0.005, p=0.177). There was no significant difference between the WC and CO mice in the weight of liver (mean 1.49±0.07 and 1.39±0.06 grams, p=0.280) or muscle (mean 0.130±0.006 and 0.124±0.004 grams, p=0.466) (Fig. 1C).

The adaptation to fasting/refeeding could involve a change in signals that influence preadipocyte recruitment and/or terminal differentiation of adipocytes. Therefore, we assessed differences in number and size of adipocytes between the groups, based on slides from five mice per group. In iWAT, we found a strong trend towards a reduced number of adipocytes in CO compared to the low-fat group (p=0.058) (Fig. 2B). On the other hand, adipocyte number in WC mice tended to be higher than in CO mice (2.3-fold, p=0.079) (Fig. 2B). Similarly, the relative adipocyte number in eWAT tended towards a reduction in the CO group compared to the low-fat group (p=0.160), but was on average ~2-fold albeit non-significantly higher in WC compared to CO (p=0.240) (Fig. 2E). These consistent tendencies suggest that WC led to a normalization of preadipocyte recruitment relative to chronic overfeeding, which could account for increased fat mass after WC. Regarding size, adipocytes were on average similarly enlarged in iWAT in both WC (mean 2135 µm²) and CO (mean 2347 µm²) mice compared to low-fat fed mice (mean 1224 µm²) (p=0.031 and p=0.028, respectively) (Fig. 2C). In eWAT, average adipocyte size was not significantly higher after WC compared to CO (p=0.253), though the observed ~1.6-fold higher adipocyte size in WC mice was non-
significant compared to the low-fat group (p=0.0505) (Fig 2F), although it should be noted that statistical power was reduced in these analyses. However, the overall consistency in the data support that weight cycled mice gained fat mass by efficient recruitment of new preadipoeytes, which was not evident after chronic overfeeding.

Circulating hormones, glucose and lipids

There were no significant differences in fed state plasma insulin or glucose between WC, CO and low-fat fed mice (Fig. 3). Interestingly, despite the higher fat mass, WC mice were not less glucose tolerant than CO mice relative to low-fat fed mice (mean area under the curve for blood glucose 1387, 2249 and 2479 mmol/liter*120 min for low-fat fed, CO, and WC, respectively (CO and low-fat fed p=1.67E-4; WC and CO p=0.345) (Fig. 3).

The increased fat mass in the WC mice may be reflected in altered circulating levels of lipids and adipokines. As expected, the concentration of free fatty acids (FFA) was significantly increased in CO compared to low-fat fed mice (mean 1.55-fold, p=0.016) (Fig. 3). Interestingly, however, there was no further increase in FFA after WC, rather the FFA tended to be decreased in WC compared to CO mice (mean 1.21-fold, p=0.218). Similarly, plasma triacylglycerol (TG) levels were reduced or similar rather than increased in WC compared to low-fat fed mice (mean 1.53-fold lower, p=0.008) and CO mice (mean 1.3-fold, p=0.260).

There were no significant differences in plasma glycerol between the groups (Fig.3). Total plasma cholesterol levels were increased similarly in both CO and WC compared to low-fat fed mice (mean 1.27 and 1.30-fold, p<0.0001) (Fig. 3).

As expected from the high-energy feeding and reduced glucose tolerance (37), plasma adiponectin levels were significantly decreased after CO compared to the low-fat group (mean 1.46-fold, p=0.006) (Fig. 3). However, despite the increased fat mass after WC, WC mice
tended to have higher adiponectin levels rather than a further suppression relative to CO (1.28-fold, p=0.073). Plasma leptin levels were significantly increased in the WC mice relative to both low-fat diet (4.22-fold, p=0.006) and CO (2.53-fold, p=0.031) (Fig. 3). There was a strong positive correlation between plasma leptin and feed efficiency within the WC group (p=0.006), but not within the CO group (p=0.424) (Table 2). Taken together, the data indicate that WC promotes increased fat storage efficiency although not worsening metabolic health compared to CO.

**Depot-specific transcriptomic responses**

To obtain a global view of the effect of weight cycling on gene expression in adipose tissues, we performed a microarray-based analysis on gene expression in iWAT, eWAT and iBAT using Illumina microarrays. A rank product analysis (4) was performed to identify differentially expressed genes between WC and CO in each fat pad (Table S1-S3). Because eWAT contributed more to the increased fat mass after weight cycling, we searched for genes responding more strongly to weight cycling in eWAT than in iWAT, as these genes may be related to the eWAT mass development. Comparing WC and CO mice, five transcripts (Fgf13, Ubd, Tph2, Sfrp5, Lipf) were more up-regulated and two transcripts (Myc11, Inmt) were more down-regulated after WC in eWAT than in iWAT (≥ 1.2-fold greater WC/CO ratio in eWAT compared to iWAT) (Table 3). The up-regulated genes were positively correlated with feed efficiency within the WC group but not within the CO group, and the correlation coefficients were stronger for eWAT than iWAT (Table 4). Corresponding negative correlations were observed for the down-regulated genes, with stronger correlation coefficients for eWAT (Table 4). In iWAT, three genes were more up-regulated (Apoa1,
Apoa2, Ambp) while none were more down-regulated compared to eWAT after WC (data not shown).

We moreover hypothesized that gene networks altered in eWAT might describe molecular processes underlying the increased feed efficiency after WC. Gene ontology analysis was performed on differentially expressed genes in eWAT that were defined to be unaffected in iWAT (63 transcripts with higher expression (Table S4) and 46 transcripts with lower expression (Table S5) after WC compared to CO, fold change > 1.2, eWAT q-value < 0.05 and iWAT q-value > 0.95). Based on PANTHER gene ontology analysis, there was an over-representation of up-regulated eWAT related genes in the functional categories development, angiogenesis, lipid transporter activity, and inflammation (Table 5). Of particular note, genes encoding markers of pro-inflammatory macrophages were up-regulated after WC in eWAT but not in iWAT, including Ccl2/Mcp-1, Cd68, and Lgals3/Mac-2 (Table S4). For down-regulated eWAT related genes after WC, there was an over-representation of genes involved in defense response to bacterium (Defb11, Defb20, Defb37, Defb38) and oxygen and reactive oxygen species metabolism (Table S5). In comparison, in iWAT there were only two up-regulated genes (Npm3, Eepd1) and 14 down-regulated genes that were defined to be unaffected in eWAT (Table S6 and S7). Most of the down-regulated iWAT related genes not affected in eWAT were related to muscle contraction, muscle organ development, and mesoderm development. Of note, these genes were similarly down-regulated in iBAT. In total, there were 81 up-regulated and 49 down-regulated genes in iBAT after WC compared to CO (q-value < 0.05, fold difference > 1.2) (Table S8 and S9). Among these, 34 up-regulated and six down-regulated genes were defined to be unaffected in eWAT and iWAT (q-value > 0.95) (Table S10 and S11).
Altered clock gene expression in adipose tissues and liver

We next investigated whether WC induced systemic changes that could be detected via common transcriptomic responses in adipose tissues. By rank product meta-analysis (4) of all three fat pads (q-value < 0.05, and fold difference > 1.2 for each depot), we found six transcripts with higher and ten with lower expression after WC compared to CO (Table 3, Table S12). Interestingly, four of the ten common genes exhibiting reduced expression after WC encoded circadian clock transcription factors (Dbp, Per2, Tef, Nr1d2/Rev-Erb beta) (Table 3). These factors act as negative elements in the circadian feedback loop, whereas the Arntl/Bmal1 and Clock represent positive elements that activate the negative elements (12, 39). By qPCR, we verified a significant down-regulation of Dbp, Tef (variant 2) and Nr1d2 in eWAT after WC compared to CO (Fig. 4A). Although qPCR did not verify reduced Per2 expression in eWAT, the expression levels of Per1 and Per3 were significantly decreased. The decreased positive loop clock gene expression in eWAT was generally specific to WC, and not an effect of high-energy feeding per se, since expression in CO was similar (Dbp, Nr1d2) or higher (Per1, Per3) compared to low-fat fed mice, rather than reduced (Fig. 4A). Moreover, as expected, we observed significantly increased expression of the positive loop clock genes Bmal1 and Clock in eWAT of both CO and WC mice compared to the low-fat group (Fig. 4A). In iWAT, there was overall less difference in clock gene expression between WC and CO, where Dbp, Tef, Nr1d2, and Per1 were step-wise but non-significantly lower in WC and CO compared to the low-fat group.

To verify that the reduced expression of negative loop clock genes in eWAT was a specific characteristic of weight cycling, and not a normal response to high fat feeding or eWAT mass, we analyzed independent high-fat feeding experiments in C57BL/6J (obesity-prone) and SV129 (obesity-resistant) mice. We found no reduction in clock gene expression in these independent experiments (Fig. 4A).
Altered clock gene expression has recently been implicated in metabolic regulation in the liver (10, 15). Since we found altered clock gene expression in all fat pads, we examined if these genes were also differentially expressed in liver as part of a systemic effect. There was a tendency towards a similar decrease in the expression of clock genes also in liver, though the data for each gene were not statistically significant (Fig. 4B). Thus, we cannot rule out that weight cycling affected clock gene expression also in other peripheral metabolic tissues.

Clock genes in adipogenesis

Our primary hypothesis was that weight cycling would promote adipogenesis and subsequently fat storage, and we designed our weight cycling protocol to mimic the synergistic action of insulin/IGF-1 and cAMP signaling during initiation of adipocyte differentiation. However, based on the in vivo data we could not determine if clock genes are regulated by these adipogenic signals. We therefore differentiated 3T3-L1 cells in vitro and compared the mRNA expression of Dbp and Tef at different time-points relative to undifferentiated cells. Dbp and Tef are members of the PAR (proline and acidic amino acid-rich) subfamily of basic leucine zipper (bZip) transcription factors together with the third member Hlf (hepatic leukemia factor), which operate as heterodimers (9). These factors have previously been implicated in lipid metabolism in the liver (10), but a function for these genes in preadipocyte recruitment and/or terminal differentiation has not been described. We found reduced expression of Dbp and Tef (variant 2) mRNA in cells treated with differentiation stimuli compared to untreated cells, beginning at about 16 hours after induction (Fig. 5A, 5B) (Tef variant 1 was expressed at a diminished level). These data suggest that a reduction in Dbp and Tef expression could potentially facilitate early adipocyte development. On the other hand, in the later stage of adipogenesis (from day 5-6), expression was higher in the
differentiating compared to the untreated cells. Thus, Dbp and Tef may be dynamically involved in the adipogenic program.

Our morphological data suggested increased preadipocyte recruitment after weight cycling, in which any of the early adipogenic signals could play a role. We therefore assessed which of the individual adipogenic stimuli may have suppressed adipose tissue Dbp and Tef mRNA in the weight cycled mice. After 24 hours treatment of 3T3-L1 cells, we found that IBMX (promoting increased cAMP levels) was the most potent suppressor of Dbp and Tef expression (Fig. 5C, 5D). Dexamethasone increased Tef expression (Fig. 5D); however, this was completely abolished by simultaneous IBMX treatment (data not shown).

DISCUSSION

In the present study, we show that young C57BL/6J mice experience increased feed efficiency (weight gained relative to energy consumed) after repeated cycles of high and reduced energy intake. This obesogenic effect of weight cycling was related to a normalization of adipocyte recruitment compared to chronic overfeeding. We moreover performed a comprehensive analysis of gene expression in adipose tissues in response to weight cycling. The key finding is that weight cycling associated with a consistent alteration in clock gene expression, which was not observed with isocaloric chronic overfeeding. The clock gene expression was measured three weeks after the last energy restriction phase, indicating that clock gene expression in adipose tissue was persistently affected.
There is emerging evidence for a key role of clock genes in energy homeostasis (49). Night-shift work has been found to increase the risk of obesity and metabolic syndrome (31). Importantly, although central and peripheral circadian rhythms are synchronized, peripheral mammalian clocks do not depend on central control (39, 53), which is also the case for adipocytes (12). Feeding pattern is a primary factor influencing the autonomous peripheral clock (57), referred to as the food-entrainable oscillator (FEO) (50). Disruption of the gene encoding the clock transcription factor Arntl/Bmal1 in hepatocytes (28) and beta cells (35) of mice alters glucose metabolism. Members of the period (Per) proteins have also been implicated in lipid metabolism, at least in part via direct regulation of PPARγ (14, 16). More recently, clock genes were for the first time causally implicated adipocyte development, as it was demonstrated that adipocyte-specific knock-out of Bmal1 affects feeding pattern and promotes obesity (44). This effect was explained at least in part by reduced energy expenditure without a change in total energy intake (the mice ate less than normal during the dark phase and more during the light phase). A previous study also showed that knock-down of Bmal1 diminished lipid accumulation, whereas Bmal1 overexpression induced expression of lipogenic genes in 3T3-L1 adipocytes (47). Another very recent study further demonstrated that Bmal1 and Clock deficient mice have increased fat storage and are sensitive to fasting (48). These data support a functional involvement of clock genes in the obesogenic response to weight cycling.

We provide new evidence that the circadian regulators Dbp (albumin D box-binding protein) and Tef (thyrotrph embryonic factor) may play a role in adipose tissue development and function. Dbp and Tef are members of the PAR (proline and acidic amino acid-rich) subfamily of basic leucine zipper (bZip) transcription factors together with the third member Hlf (hepatic leukemia factor) (9). Previously, it has been shown that complete knock-out of all three PAR members display differential expression of genes involved in lipid metabolism in
the liver (10), and that Tef knock-out mice lose more weight during energy restriction than
wild-type animals (20). We observed reduced expression of Dbp and Tef mRNA after weight
cycling compared to chronic overfeeding, which fits with the tendency of up-regulated
Arntl/Bmal1 as these genes act as negative and positive regulators of the circadian clock,
respectively (39). Furthermore, our in vitro data show that Dbp and Tef mRNA is negatively
regulated by cAMP, suggesting that a suppression of these genes may promote initiation of
adipocyte differentiation, with subsequent development of the newly recruited preadipocytes
during overfeeding.

It is interesting that we observed altered clock gene expression three weeks after the
last energy restriction cycle. Of note, an effect on clock genes was not observed in our
independent standard diet-induced obesity experiment in C57BL/6J mice. A previous study
showed that high-fat feeding altered the diurnal expression of clock genes in adipose tissue of
mice (25). However, although several of the clock genes were up-regulated after high-energy
diet in SV129 mice (who are resistant to diet-induced obesity) and after chronic overfeeding
relative to low-fat diet, the weight cycled mice were characterized by reduced expression of
negative loop clock genes. This particular pattern of clock gene expression could be a lasting
adaptive response to the cyclic feeding. Conceivably, the weight cycling may have evoked
epigenetic changes, which has been indicated to ensure specificity and plasticity in the
regulation of circadian rhythm in response to metabolic cues (1, 3, 36). Differential histone
H3K9 acetylation was recently demonstrated at the promoters of clock genes such as Dbp,
Per2 and Bmal1 in mouse adipose tissue (17). The altered clock gene expression, in turn, may
have promoted a lasting propensity for fat storage in adipocytes rather than being a response
to the increased fat mass, as recently evidenced by Bmal1/Arnt knock-out mice (44).

Leptin is an adipocyte-derived peptide hormone which plays a fundamental role in the plastic
regulation of energy balance (59). We found a close correlation between plasma leptin levels
and feed efficiency within the weight cycling group, supporting increases in adipocyte size.

By our study design we could not determine whether temporal leptin suppression during the energy restriction phases suppressed energy expenditure, thereby explaining the increased feed efficiency in the subsequent periods. However, it is interesting to note that leptin deficient ob/ob mice show blunted expression profiles of clock genes in liver and adipose tissue but not in brain, which can be reversed by leptin treatment (2). Thus, it was proposed that suppressed clock gene expression is a result of leptin deficiency rather than being a secondary effect of metabolic abnormalities (2). Together with our study, this link with leptin supports that peripheral clock genes participate in the adaptive strategy to limit future energy deficiency after weight cycling. Interestingly, a weight cycling study in rats showed suppressed leptin levels even 12 weeks after the last weight cycle, and this was associated with increased adipose tissue expression of lipogenic enzymes (23). Additionally, leptin administration in ob/ob mice has been shown to suppress the protein expression of lipogenic enzymes such as fatty acid synthase (Fas) and ATP citrate lyase (Acl) (19). These data suggest that changes in both energy expenditure and fat storage may have contributed to the increased feed efficiency after weight cycling, in part via leptin-mediated effects on clock genes. Of note, at the end of our study we found increased rather than decreased leptin levels, and no significant correlation with the mRNA expression of clock genes in adipose tissue (data not shown). These observations indicate leptin resistance, which may have caused insufficient suppression of lipogenic enzymes after weight cycling, and further support that the clock gene expression was unrelated to adipocyte size per se.

Despite greater fat mass gain after weight cycling compared to chronic overfeeding, the weight cycled mice had lower levels of plasma triglycerides and fatty acids, and higher adiponectin levels. This phenotype could be due to increased fat storage efficiency in adipocytes. Of particular note, overexpression of adiponectin in leptin deficient mice leads to
extreme obesity while positively affecting lipid levels and glucose homeostasis (21). Moreover, mice with time-restricted feeding (access to a high-fat diet only during 8 hours of the dark phase), compared to isocaloric ad libitum feeding, showed positive effects on energy expenditure and protection against obesity, hyperinsulinemia, and hepatic steatosis (15). Intriguingly, these mice also showed altered expression of clock genes in the liver. Thus, although weight cycling increased fat storage, particularly in eWAT which also showed increased expression of pro-inflammatory markers, the fat gain after weight cycling associated with relatively salutary effects on the whole-body metabolic profile. This is supported by previous studies showing beneficial effects of time-restricted feeding on metabolic disease (15) and of weight cycling on longevity compared to a steady state of obesity (32).

In addition to clock genes, we identified several genes that may promote feed efficiency and fat gain due to weight cycling. Of particular note, a study of genetically identical C57BL/6J mice indicated Sfrp5 as a causal factor in fat mass expansion, since a higher expression in seven-week-old mice before an obesogenic diet strongly correlated with the level of adiposity after eight weeks of the obesogenic diet (26). Moreover, Sfrp5 knock-out mice are protected from diet-induced obesity, an effect which was related to effects on adipocyte size rather than number. Functional analyses further showed that a lack of Sfrp5 increased mitochondrial activity (41). Thus, the increased expression of Sfrp5 we observed after weight cycling particularly in eWAT may have directly contributed to increase adipocyte size and adiposity, at least in part by suppressing energy expenditure. Additionally, Sfrp5 exerts anti-inflammatory effects on inflammatory cells in adipose tissue thereby protecting against glucose intolerance and hepatic steatosis (43), which is in line with the relatively healthy phenotype of our obese weight cycled mice. The metabolic effects of other genes with a
similar expression pattern as Sfrp5, e.g. Tph2, Fgf13, Ubd, and Lipf, should be further investigated.

The human relevance of our findings is of clinical interest. Accumulating evidence indicates that adipose tissue expandability during energy surplus may determine the pathological outcome, by preventing elevated circulating lipids and protecting from ectopic lipid storage in vital organs (55). Experience from clinical practice has shown that avoiding weight regain after fat loss is a major challenge; rather, individuals with a history of weight cycling may regain weight more easily (8). It is therefore debated whether the focus should be on weight maintenance rather than loss, though the long-term effect of weight cycling on disease risk is unclear (8, 30, 54). Our findings highlight the intriguing regulation of feed efficiency, adding to previous evidence that feeding pattern may ameliorate metabolic syndrome without reducing energy intake (15). It remains to be determined to what extent clock genes coordinate metabolic responses in human tissues in the context of weight cycling. While one study of human adipose tissue found no effect of obesity and type 2 diabetes on diurnal clock gene expression (42), another study showed that clock genes in human adipose tissue show a diurnal expression that relates to adiponectin, leptin, and glucocorticoid-related genes (11).

It should be noted that the low-fat group in our experiment gained more weight than anticipated, resulting in no difference between low-fat diet and chronic overfeeding in regards to total body mass. We believe this could be due to the relatively high sucrose content of this low-fat diet. Also, we kept the mice at 28°C (i.e. close to thermoneutrality), which might have facilitated fat gain also in the low-fat group. Nonetheless, the mice on low-fat diet ate considerably less than the chronically overfed and weight cycled mice, and showed a strong tendency towards expected values for several metabolic parameters such as glucose tolerance, hormone levels, and lipid levels. In the additional feeding experiments we used low-fat diet with lower sucrose content, and found the expected increase in body mass compared to high-
fat diet. Regardless of the higher than expected fat gain in the low-fat group of the weight cycling experiment, the down-regulation of negative loop clock genes was a specific feature of weight cycling compared to isocaloric chronic overfeeding.

In conclusion, weight cycling increased weight gain per calorie in C57BL/6J mice compared to chronic overfeeding, which appeared to occur by a normalization of preadipocyte recruitment without worsening metabolic health. This was further associated with a shift in the expression of circadian clock transcription factors in adipose tissues. Our data suggest that weight cycling may lead to a persistent suppression of peripheral clock gene expression, possibly as an adaptive response to elevated cAMP signaling during repeated fasting/refeeding. These molecular changes in adipose tissue may promote development of new preadipocytes that are able to expand during overfeeding.

Acknowledgment

We thank Jan-Inge Bjune, Vivian Veum and Rita Holdhus for expert technical assistance.

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Disclosures

We declare no conflicts of interest.
REFERENCES


49. Torii N: [The role of Ca ions and cAMP in ACTH lipolysis]. Nihon Naibunpi Gakkai zasshi 1985, 61(9):859-871.


Involvement of calcium-sensing receptor in inhibition of lipolysis through intracellular cAMP and calcium pathways in human adipocytes. Biochemical and biophysical research communications 2011, 404(1):393-399.


Fig. 1 Weight cycling led to increased feed efficiency, hyperglycemia, and fat mass. Young male C5BL/6J mice were fed low-fat or high fat/high sucrose with or without intermittent periods of energy restriction, for a total of 11 weeks. A. Body weight development throughout the experiment. B. Feed efficiency (weight gained relative to the amount of energy ingested) for each 10 days period of weight regain after energy restriction. C. Tissues were dissected and weighed on the final day of the experiment. All data are presented as mean ± SEM (n=11 per group). CO, chronic overfeeding; WC, weight cycling.

*, p < 0.05 vs. CO.

Fig. 2 Increased adipocyte number and size after weight cycling. 5µm thick sections of paraffin-embedded fat tissue from two different locations were hematoxylin and eosin (H&E) stained. The photographs are representative of three to six microscopic images that were taken from random places of each section. The average area of each cell on each image was measured by drawing the circumference of each cell (µm²), and based on this the average cell volume (µm³) was estimated. Cell size distribution was assessed by segregating the cell sizes into 20 brackets (given as percent). 

A/D. Adipose tissue morphology. B/E. Relative adipocyte number in each fat pad between the groups was estimated by dividing total fat pad mass by average adipocyte volume. C/F. Average adipocyte size and cell size distribution. Data are presented as mean ± SEM (n=5 per group).

§, p < 0.05 vs. low-fat.
Fig. 3. Weight cycling did not worsen plasma parameters compared to chronic overfeeding despite fat gain. Glucose tolerance test (GTT) was performed one week prior to sacrifice by injecting glucose dissolved in saline into the intraperitoneal cavity (two grams per kg body weight). Blood was drawn from the tail at 0, 15, 30, 60 and 120 minutes after injection. All other parameters were measured in plasma collected at sacrifice. Data are presented as mean ± SEM (n=11 per group). LF, low-fat diet; CO, chronic overfeeding; WC, weight cycling.

§, p<0.05 vs. low-fat; §§, p<0.01 vs. low-fat; *, p<0.05 vs. CO; **, p<0.01 vs. CO.

Fig. 4 Clock genes are down-regulated after WC but not after chronic overfeeding. Tissues from all mice in each group were dissected and weighed on the final day of the experiment, and mRNA levels were measured by qPCR relative to Tbp as a reference gene. A. Clock gene expression in iWAT and eWAT (n=11 per group). As an additional control of a WC specific effect, gene expression was also measured in adipose tissue from another overfeeding experiment in C57BL/6J mice (n=5-7 per group) and an overfeeding experiment of obesity-resistant SV129 mice (n=7 per group). B. Clock gene mRNA expression in liver relative to Tbp mRNA (n=11 per group). Data are presented as median ± SEM. LF, low-fat diet; CO, chronic overfeeding; WC, weight cycling.

§, p<0.05 vs. low-fat; §§, p<0.01 vs. low-fat; *, p<0.05 vs. CO; **, p<0.01 vs. CO.

Fig. 5 Altered expression of Dbp and Tef during adipogenesis. A/B. 3T3-L1 cells were seeded in 6-well plates and induced to differentiate into adipocytes two days post-confluence, by changing from 10% calf serum to 10% fetal calf serum and adding dexamethasone (0.5mM), insulin (175nM) and...
IBMX (0.5M). Lysates were collected at different time-points during differentiation. C/D. 3T3-L1 cells were seeded in 24-well plates, and were treated two days post-confluence with 10% fetal calf serum, dexamethasone (0.5mM), insulin (175nM) or IBMX (0.5M) and lysed after 24 hours. Dbp and Tef (transcript variant 2) mRNA was measured by qPCR. Data are presented as mean ± SEM of triplicate measurements and representative of two independent experiments. FCS, fetal calf serum; DEX, dexamethasone; IBMX, phosphodiesterase inhibitor methylisobutyloxanthine.

### Tables

**Table 1.** Primers and probes used for qPCR.

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<th>Reverse (right) primer</th>
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Table 2. Correlation between feed efficiency and circulating leptin.

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WC, weight cycling; Co, chronic overfeeding.
Table 3. Differentially expressed genes after weight cycling with similar effects in iWAT, eWAT, and iBAT (q-value < 0.05, fold change > 1.2).

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<thead>
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<th>eWAT WC</th>
<th>iBAT WC</th>
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More up-regulated after WC vs. CO in eWAT than in iWAT

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More down-regulated after WC vs. CO in eWAT than in iWAT

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Higher expression after WC vs. CO in all fat pads

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Higher expression after WC vs. CO in all fat pads

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*Lower expression after WC vs. CO in all fat pads*

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<td>353187</td>
<td>218</td>
<td>294</td>
<td>-1.23</td>
<td>242</td>
<td>368</td>
<td>-1.32</td>
<td>178</td>
<td>261</td>
<td>-1.25</td>
</tr>
<tr>
<td>Prodh</td>
<td>19125</td>
<td>336</td>
<td>414</td>
<td>-1.27</td>
<td>311</td>
<td>445</td>
<td>-1.35</td>
<td>256</td>
<td>313</td>
<td>-1.21</td>
</tr>
<tr>
<td>Usp2</td>
<td>53376</td>
<td>321</td>
<td>319</td>
<td>-1.23</td>
<td>520</td>
<td>719</td>
<td>-1.26</td>
<td>464</td>
<td>688</td>
<td>-1.28</td>
</tr>
</tbody>
</table>

A rank product meta-analysis was performed on rank product microarray data for iWAT (n=11), eWAT (n=10) and iBAT (n=11) comparing WC and CO. Genes with log2 fold change > 1.2 q-value < 0.05 and their median signal intensities (quantile normalized Illumina microarray data) are shown. WC, weight cycling; CO, chronic overfeeding.
Table 4. Correlation between feed efficiency and gene expression in eWAT and iWAT.

<table>
<thead>
<tr>
<th>WC</th>
<th>Feed efficiency</th>
<th>Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>eWAT (n=10)</td>
<td>iWAT (n=11)</td>
</tr>
<tr>
<td>Gene</td>
<td>Rho</td>
<td>p-value</td>
</tr>
<tr>
<td>Fgf13</td>
<td>.867</td>
<td>.0012</td>
</tr>
<tr>
<td>Tph2</td>
<td>.818</td>
<td>.0038</td>
</tr>
<tr>
<td>Sfrp5</td>
<td>.685</td>
<td>.0289</td>
</tr>
<tr>
<td>Ubd</td>
<td>.879</td>
<td>.0008</td>
</tr>
<tr>
<td>Lipf</td>
<td>.903</td>
<td>.0003</td>
</tr>
<tr>
<td>Mycl1</td>
<td>-.624</td>
<td>.0537</td>
</tr>
<tr>
<td>Inmt</td>
<td>-.527</td>
<td>.1173</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CO</th>
<th>Feed efficiency</th>
<th>Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>eWAT (n=10)</td>
<td>iWAT (n=11)</td>
</tr>
<tr>
<td>Gene</td>
<td>Rho</td>
<td>p-value</td>
</tr>
<tr>
<td>Fgf13</td>
<td>.418</td>
<td>.2291</td>
</tr>
<tr>
<td>Tph2</td>
<td>.539</td>
<td>.1076</td>
</tr>
<tr>
<td>Sfrp5</td>
<td>.273</td>
<td>.4458</td>
</tr>
<tr>
<td>Ubd</td>
<td>.236</td>
<td>.5109</td>
</tr>
<tr>
<td>Lipf</td>
<td>.164</td>
<td>.6515</td>
</tr>
<tr>
<td>Mycl1</td>
<td>-.382</td>
<td>.2763</td>
</tr>
<tr>
<td>Inmt</td>
<td>-.139</td>
<td>.7009</td>
</tr>
</tbody>
</table>

WC, weight cycling; CO, chronic overfeeding; rho, Spearman’s rho.
Table 5. Over-represented PANTHER gene ontology categories for differentially expressed genes in eWAT or iWAT.

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>REFLIST (26185)</th>
<th># genes</th>
<th># expected</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Up-regulated WC vs. CO, eWAT not iWAT (68)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>11454</td>
<td>11</td>
<td>29.74</td>
<td>0.0002</td>
</tr>
<tr>
<td>Developmental process</td>
<td>2262</td>
<td>19</td>
<td>5.87</td>
<td>0.0006</td>
</tr>
<tr>
<td>mesoderm development</td>
<td>819</td>
<td>10</td>
<td>2.13</td>
<td>0.0085</td>
</tr>
<tr>
<td>system development</td>
<td>1311</td>
<td>10</td>
<td>3.4</td>
<td>0.3420</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>188</td>
<td>6</td>
<td>0.49</td>
<td>0.0018</td>
</tr>
<tr>
<td><strong>Cellular process</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation mediated by chemokine and cytokine signaling pathway</td>
<td>287</td>
<td>6</td>
<td>0.75</td>
<td>0.0187</td>
</tr>
<tr>
<td><strong>Up-regulated WC vs. CO, iWAT not eWAT (2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N/A (only 2 genes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Down-regulated WC vs. CO, eWAT not iWAT (49)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen and reactive oxygen species metabolic process</td>
<td>56</td>
<td>4</td>
<td>0.1</td>
<td>0.0007</td>
</tr>
<tr>
<td>Defense response to bacterium</td>
<td>53</td>
<td>3</td>
<td>0.1</td>
<td>0.0244</td>
</tr>
<tr>
<td><strong>Down-regulated WC vs. CO, iWAT not eWAT (14)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>System process</td>
<td>1851</td>
<td>8</td>
<td>0.99</td>
<td>0.0002</td>
</tr>
<tr>
<td>muscle contraction</td>
<td>337</td>
<td>7</td>
<td>0.18</td>
<td>0.0000</td>
</tr>
<tr>
<td>Developmental process</td>
<td>2262</td>
<td>7</td>
<td>1.21</td>
<td>0.0122</td>
</tr>
<tr>
<td>muscle organ development</td>
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<td>5</td>
<td>0.1</td>
<td>0.0000</td>
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<tr>
<td>mesoderm development</td>
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<td>5</td>
<td>0.44</td>
<td>0.0081</td>
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<tr>
<td>system development</td>
<td>1311</td>
<td>5</td>
<td>0.7</td>
<td>0.0735</td>
</tr>
<tr>
<td>Unclassified</td>
<td>11454</td>
<td>0</td>
<td>6.12</td>
<td>0.0544</td>
</tr>
</tbody>
</table>

WC, weight cycling; CO, chronic overfeeding.
A. iWAT morphology

B. iWAT

C. iWAT

D. eWAT morphology

E. eWAT

F. eWAT
<table>
<thead>
<tr>
<th>B. Liver</th>
<th>Relative Dbp level</th>
<th>Relative Per3 level</th>
<th>Relative Per2 level</th>
<th>Relative Per1 level</th>
<th>Relative Dbp level</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>CO</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>WC</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relative Tef level</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>0.1</td>
</tr>
<tr>
<td>CO</td>
<td>0.2</td>
</tr>
<tr>
<td>WC</td>
<td>0.3</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Relative Per2 level</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>0.4</td>
</tr>
<tr>
<td>CO</td>
<td>0.5</td>
</tr>
<tr>
<td>WC</td>
<td>0.6</td>
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</table>

<table>
<thead>
<tr>
<th>Relative Nrd2 level</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>0.8</td>
</tr>
<tr>
<td>CO</td>
<td>0.9</td>
</tr>
<tr>
<td>WC</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relative Bmal1 level</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>0.1</td>
</tr>
<tr>
<td>CO</td>
<td>0.2</td>
</tr>
<tr>
<td>WC</td>
<td>0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relative Clock level</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>0.4</td>
</tr>
<tr>
<td>CO</td>
<td>0.5</td>
</tr>
<tr>
<td>WC</td>
<td>0.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relative Tef level</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>0.8</td>
</tr>
<tr>
<td>CO</td>
<td>0.9</td>
</tr>
<tr>
<td>WC</td>
<td>1.0</td>
</tr>
</tbody>
</table>

A. White adipose tissue

- Low-fat SW12a
- High-fat SW12a

B. Liver

- Relative Dbp level
- Relative Per3 level
- Relative Per2 level
- Relative Per1 level
- Relative Dbp level

- Relative Tef level
- Relative Nrd2 level
- Relative Bmal1 level
- Relative Clock level
- Relative Tef level

- Low-fat SW12a
- High-fat SW12a