

Nutrient regulation of energy metabolism in relation to obesity and type 2 diabetes

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Andreas J Wensaas

1. Abbreviations

Type 2 diabetes (T2D)
Peroxisome proliferator activated receptors (PPARs)
Liver x receptors (LXRs)
Triacylglycerols (TAGs)
Eicosapentaenoic acid (EPA)
Tetradecylthioacetic acid (TTA)
Acid soluble metabolites (ASM)
White adipose tissues (WATs)
Uncoupling protein 3 (UPC3)
Tumor necrosis factor alpha (TNF α)
Body mass index (BMI)
International Diabetes Federation (IDF)
C-reactive protein (CPR)
Interleukin-6 (IL-6)
Plasminogen activator inhibitor-1 (PAI-1)
World Health Organization (WHO)
Central nervous system (CNS)
Cholecystokinin (CCK)
Peptide YY (PYY)
Glycogen synthase (GS)
Insulin receptor substrate-1 (IRS-1)
Glucose transporter 1 (GLUT1)
Transcription factor 7-like 2 (TCFL2)
Peroxisome proliferator-activated receptor gamma, co activator 1 alpha (PGC1 α)
Fatty acid synthase (FAS)
Glucokinase (GK)
Phosphoenolpyruvate carboxykinase (PEPCK)
protein kinase B (PBK)
AMP-activated protein kinase (AMPK)
Coenzyme A (CoA)
Brown adipose tissue (BAT)
Messenger RNA (mRNA)
Deoxyribonucleic acid (DNA)
Ribonucleic acid (RNA)
Nuclear receptors (NRs)
9-cis retinoic acid receptor α (RXR α)
Direct repeat 4 (DR4)
Cholesterol 7 α -hydroxylase (Cyp7 α)
ATP-binding cassette transporter 1 (ABC1)
Cholesteryl ester transfer protein (CETP)
Sterol regulatory element-binding protein 1c (SREBP1c)
Acetyl-CoA carboxylase (ACC)
Carbohydrate responsive element-binding protein (ChREBP)
Thiazolidinedione (TZD)
fFatty acid (FA)
Saturated FA (SAFA)
Monounsaturated FA (MUFA)

Polyunsaturated FA (PUFA)
Docosahexaenoic acid (DHA)
Arachidonic (ARA)
Lipoprotein lipase (LPL)
Very low-density lipoprotein (VLDL)
Toll-like receptors (TLRs)
Free fatty acid receptors (FFARs)
C-Jun N-terminal kinase (JNK)
Nuclear factor kappa B (NFκB)
Carnityl palmitoyltransferase 1 (CPT1)
Acyl-CoA oxidase (AOX)
Pyruvate kinase (liver) (L-PK)
Free fatty acids (FFA)
Phosphoinositide 3-kinases (PI3K)
Protein kinase c – theta (PKC-θ)
IκB kinase β (IKKβ)
Knock-out (KO)
Diacylglycerol acyltransferase 1 (DGAT1)
Adipose triglyceride lipase (ATGL)
Pyruvate dehydrogenase (PDH)
Phosphofrukrokinase (PFK)
Hexokinase (HK)
Tricarboxylic acid cycle (TCA)
Electron transport chain (ETC)
Reactive oxygen species (ROS)
Hepatocyte nuclear factor 4α (HNF4α)
Endoplasmatic reticulum (ER)
Scintillation proximity assays (SPA)
Palmitic acid (PA)
Cluster of differentiation 36/Fatty acid transporter (CD36/FAT)
High-density lipoproteins (HDLs)

2. List of publications

Paper I

Kase ET, Wensaas AJ, Aas V, Højlund K, Levin K, Thoresen GH, Beck-Nielsen H, Rustan AC, Gaster M. *Skeletal muscle lipid accumulation in type 2 diabetes may involve the liver X receptor pathway*. *Diabetes*. 2005; 54:1108-15.

Paper II

Wensaas AJ, Rustan AC, Just M, Berge RK, Drevon CA, Gaster M. *Fatty acid incubation of myotubes from humans with type 2 diabetes leads to enhanced release of beta-oxidation products because of impaired fatty acid oxidation: effects of tetradecylthioacetic acid and eicosapentaenoic acid*. *Diabetes*. 2009; 58:527-35.

Paper III

Wensaas AJ, Rustan AC, Löfstedt K, Kull B, Wikström S, Drevon CA, Hallén S. *Cell-based multiwell assays for the detection of substrate accumulation and oxidation*. *J Lipid Res*. 2007; 48:961-7.

Paper IV

Wensaas AJ, Rustan AC, Rokling-Andersen MH, Caesar R, Jensen J, Kaalhus O, Graff BA, Gudbrandsen OA., Berge RK., Drevon CA. *Dietary supplementation of tetradecylthioacetic acid increases feed intake but reduces body weight gain and adipose depot sizes in rats fed high-fat diets*. *Diabetes Obes Metab*. Accepted for publication 2009 May 9.

3. Abstract

The study of how nutrient intake may influence health and disease is an important and fast growing field in research. Different fatty acids and other lipids like cholesterol and its derivatives are examples of nutrients capable of regulating their own metabolism as well as general energy turnover in many organs. In pharmacology, drugs have been developed to combat dyslipidemia, insulin resistance and type 2 diabetes (T2D) that work through nutrient mimicking, stimulating a set of fatty acid sensors called peroxisome proliferator activated receptors or PPARs.

In this thesis we have studied how nutrients may interact with human skeletal muscle cells (myotubes) from a T2D background to explain certain traits commonly observed in skeletal muscle of people with insulin resistance and T2D. We have showed that mimicking elevated levels of cholesterol derivatives (oxysterols) by incubating myotubes with a synthetic ligand (T0901317) for the nuclear transcription factors liver x receptors (LXRs), enhanced accumulation of fatty acids as triacylglycerols (TAGs) in myotubes, and particularly more in myotubes of T2D origin. Furthermore, the allover uptake of fatty acids was strongly increased after pretreatment with the LXR activator, whereas fatty acid complete oxidation was only raised in the control cells, compensating for the increased fatty acid influx. Moreover, preincubating myotubes with the n-3 omega fatty acid eicosapentaenoic acid (EPA) likewise increased fatty acid accumulation as TAGs more potently in myotubes derived from T2D individuals. We also observed that myotubes from obese type 2 diabetics had lower fatty acid oxidation as compared to myotubes derived from obese individuals without T2D. The sulfur modified fatty acid analogue tetradecylthioacetic acid (TTA) augmented mitochondrial carbon dioxide (CO₂) production in myotubes derived from obese persons with or without T2D. However, preincubation with TTA did not fully rescue complete fatty acid oxidation in T2D myotubes as their level remained below the average of the controls. Preincubation with fatty acids *per se* all increased fatty acid uptake in myotubes with a concomitant increase in fatty acid β -oxidation measured as acid soluble metabolites (ASM) released into the medium during incubation with a radiolabeled fatty acid tracer. In T2D myotubes this marker of fatty acid β -oxidation was increased more after stimuli than in myotubes from obese controls. This observation suggests that reduced downstream catabolism in T2D myotubes was compensated by increased release of excess β -oxidation products.

In this thesis we also investigated the effects of feeding TTA, which may activate all PPAR subtypes, on rats fed a high fat diet. The results showed a marked reduction in weight gain during a 7 week feeding trial, with a slight increase in feed intake during the last 3 weeks resulting in markedly reduced energy efficiency. White adipose tissues (WATs), in particular the visceral depots, were reduced by 30-40%. The results may be explained by increased futile energy consumption caused by a strong augmentation in β -oxidation by the heart and liver, and possibly caused by enhanced energy dissipation due to ectopically expressed hepatic uncoupling protein 3 (UPC3). Moreover, *ucpl* expression was enhanced in the visceral WATs, and may suggest a low grade energy uncoupling in these tissues contributing to the reduction in weight gain and dietary energy efficiency.

We have finally devoted a part of this work to the development of two methods for measuring cell-associated radiolabeled CO₂ production and substrates accumulation. We demonstrated accumulation as well as subsequent oxidation of ¹⁴C-labeled substrates in cultured human myotubes, adipocytes, and hepatocytes. Both methods are adaptable for compound screening and provide easy-to-use and time-saving methods for in vitro studies of cellular fuel handling.

4. Introduction

Possible causes for insulin resistance and type 2 diabetes

There is a relationship between obesity and risk of developing insulin resistance and T2D, although some individuals seem more protected against obesity-driven T2D than others. Current research on the etiology of insulin resistance has revealed two pathways that may connect obesity with development of T2D. Firstly, insulin resistant subjects may have reduced capacity for complete mitochondrial fatty acid oxidation in the liver and especially skeletal muscle. In obese individuals, in particular those with high levels of plasma lipids, this can lead to an unfavorable balance between fatty acid catabolism to CO₂ and production of β -oxidation products as well as higher intracellular levels of other potentially cytotoxic fatty acid intermediates such as acyl-CoAs, diacylglycerols (DAGs) and ceramides causing insulin resistance. Secondly, insulin resistance may be caused or exacerbated by high circulating levels of different cytokines/adipokines like tumor necrosis factor alpha (TNF α) and resistin derived from inflammatory processes in adipose tissue due to macrophage invasion. Ample evidences connect local and circulating inflammatory cytokines to development of some form of insulin resistance. Such low-grade inflammation can result from damaged adipocytes when exceeding their normal storage capacity, either due to increased risk of physical damage to their integrity or lack of proper microvascularization leading to hypoxia. Some data indicate that adipocyte hyperplasia rather than hypertrophy might increase the storage capacity for fatty acids without the association of low-grade inflammation and consequently insulin resistance. Whether this holds for human adiposity is uncertain, but a correlation between adipocyte size and degree of insulin resistance might partly explain why not all obese individuals are similarly affected with insulin resistance and T2D. By combining the first and second mechanistic approach one may envision that subjects with both reduced capacity for mitochondrial fatty acid oxidation and reduced capacity for fatty acid storage by adipocyte hyperplasia would be more prone to develop insulin resistance and T2D when becoming obese. On the other hand subjects with both high fatty acid oxidative and high storage capacity would be better protected against insulin resistance and T2D at high body mass index (BMI).

Metabolic syndrome

Overweight, obesity, T2D and insulin resistance are interconnected so strongly that the use of the term metabolic syndrome has become very common. Hyperglycemia is often observed together with dyslipidemia and hypertension, and these abnormalities appear to be associated with central obesity, insulin resistance, inflammation and thrombosis. This clustering of signs is further associated with increased risk of developing cardiovascular diseases and T2D, with huge social and economical burdens. Recently, The International Diabetes Federation (IDF) introduced a new clinical definition(1). For a person to be classified with metabolic syndrome he or she must display central obesity together with at least two of four additional features presented in table 1.

In scientific studies, other metabolic factors should be included like measurement of central fat distribution with CT/MRI, insulin resistance (other than raised fasting glucose) or levels of plasma leptin, adiponectin, c-reactive protein (CPR), TNF α , interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), etc(1).

The age-adjusted prevalence of the metabolic syndrome among United States (US) adults was recently estimated to be ~24%, suggesting that about 47 millions Americans shared this phenotype by the year 2000(2).

Table 1) Criteria for the metabolic syndrome.

Primary - must be included for definition	Secondary - 2 of 4 necessary for definition
Central obesity (waist circumference) ≥94 cm (M) (≥90 cm for Asian men), ≥80 cm (F) There are some ethnic differences in risk based that may allow for improved cut-points when specific data become available.	Elevated triglycerides ≥1.7 mmol/L or [§]
	Reduced HDL cholesterol <1.03 mmol/L (M), <1.29 mmol/L (F) or [§]
	Elevated blood pressure ≥130 mmHg (systolic), ≥85 mmHg (diastolic) or [#]
	Elevated fasting plasma glucose ≥ 5.6 mmol/L or [□]

[§]Specific treatment for this lipid abnormality. [#]Treatment of previously diagnosed hypertension. [□]Previously diagnosed T2D. Adapted from(1).

Obesity

One major common feature of metabolic syndrome is obesity especially located in the visceral depots. The prevalence of obesity has been rising globally during the last century, and prevalence of obesity (BMI ≤ 30 kg/m²) in the US increased by more than 60 %, from ~ 12 % of total population in 1991 to ~ 20 % in 2000(3). Another more recent report, estimated the prevalence of obese US adults in 2004 to more than 32 %(4). According to the latest projections by the World Health Organization (WHO) approximately 2.3 billion adults will be overweight and more than 700 million obese world wide by the year 2015(5).

The cause for this obesity epidemic seems to be related to a combination of increased food supply (6; 7) and reduced physical activity(8; 9). The palatability and energy density of the modern food products also contribute to overnutrition, and the availability of these products makes it difficult to prevent hyper-alimentation(10). Regulation of food intake, energy expenditure and adiposity is governed by many signal molecules designed to maintain energy homeostasis. Circulating concentrations of leptin and insulin increase with energy accumulation in adipose tissue and bind to receptors in the central nervous system (CNS) reducing the energy intake, whereas gastrointestinal peptides like cholecystokinin (CCK) and peptide YY (PYY) are released into the blood during a meal and may reduce food intake(11). The peptide hormone ghrelin on the other hand may function to stimulate appetite(12). CNS may also influence energy expenditure via peripheral nervous sympathetic signaling, and recently hypothalamic malonyl-CoA has been implicated in controlling feed intake and energy consumption in rodents(13). When it comes to who will become obese, there seems to be substantial individual variance, probably reflecting genetic and epigenetic differences, either protecting against or exacerbating the drive towards obesity(6; 9). E.g. increased non-exercise (heat producing) energy consumption in response to overeating may constitute a protective mechanism against obesity(14). Reduced physical activity also greatly contributes to obesity and several associated metabolic diseases like insulin resistance and T2D(15).

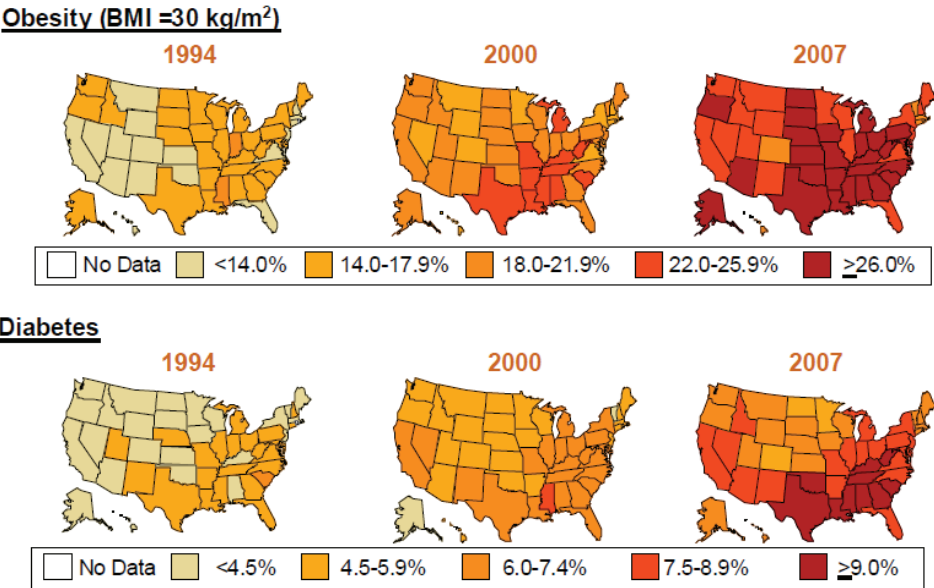
Diabetes mellitus type 2

Paralleling the rise in obesity is the prevalence of T2D (fig 1), and among US adults the estimated prevalence of T2D increased from 4.9% in 1990 to 6.5% in 1998(16). Worldwide,

the estimated adult population with diabetes mellitus was ~170 million in 2000, and more than 370 million adults are projected to be afflicted globally by the year 2030(17). Similar trends also seem to apply in Norway, with increasing prevalence of obesity and diabetes(18). T2D was formerly known as non-insulin dependent diabetes mellitus, and is the predominant form of diabetes mellitus, accounting for about 80% of all diabetic cases(19). The etiology of T2D is not well understood, and probably includes several different genetic and environmental factors working in concert(19). Studies on the Pima Indians in Arizona have shown a marked connection between developments of T2D and degree of obesity(20). The genetic predisposition of T2D is strong with concordance rates between monozygotic twins older than 60 years of 35-58%, as compared to 17-20% for dizygotic twins(19). First degree relatives of individuals with T2D have increased risk of developing the disease as well as being insulin resistant. Many putative candidate genes have been investigated for disruptive mutations and polymorphisms, but only a few, including genes encoding PPAR γ , glycogen synthase (GS), insulin receptor substrate 1 (IRS-1), glucose transporter 1 (GLUT1), transcription factor 7-like 2 (TCFL2) and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1 α) have been identified, but accounting for only moderate increased risks of developing T2D(19). T2D typically includes a combination of insulin resistance and insufficient insulin secretion by pancreatic beta-cells(19), either feature may dominate, but both are usually present when clinically diagnosed(21). Insulin resistance is a strong predictor of development of T2D (22) and obesity may cause or aggravate insulin resistance(23; 24).

Fig 1. Prevalence of obesity and T2D in US 1994-2007.

Age-adjusted Percentage of U.S. Adults Who Were Obese or Who Had Diagnosed Diabetes



Adapted from CDC's Division of Diabetes Translation. National Diabetes Surveillance System available at <http://www.cdc.gov/diabetes/statistics>.

Insulin resistance

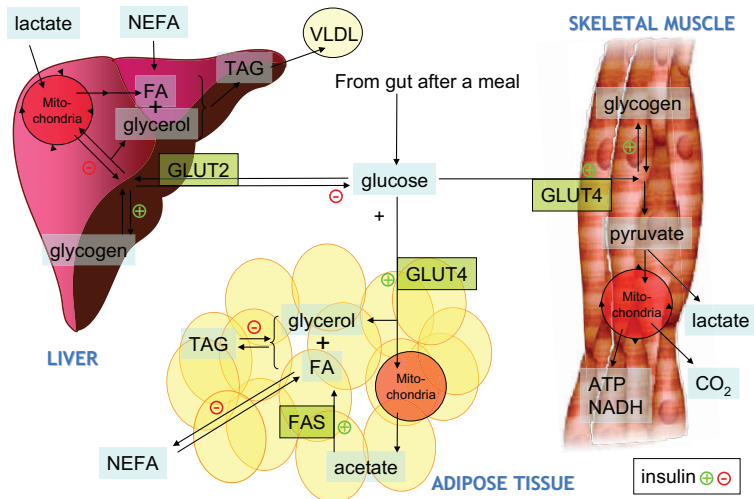
Insulin resistant individuals are normally characterized as having normal or slightly elevated fasting plasma glucose in the presence of elevated insulin concentrations, and they often display prolonged postprandial hyperglycemia(25). Reduced response to insulin (i.e. insulin resistance) may affect several organs, but skeletal muscles, liver and adipose tissues seem to play the major roles in insulin-induced glucose clearance. In effect, insulin resistance reduces insulin-stimulated glucose uptake in skeletal muscle and adipose tissue together with reduced insulin-mediated inhibition of hepatic glucose output(26). Hyperglycemia due to insulin resistance can be avoided as long as the insulin-producing pancreatic beta cells can compensate by releasing more insulin(27). By reasons yet not fully elucidated, this compensatory mechanism tends to break down over time, thus moving prediabetic individuals into overt diabetes mellitus.

Insulin-regulated glucose metabolism

In skeletal muscle and adipose tissue, insulin enhances glucose uptake by translocation and fusion of intracellular vesicles containing the specific glucose transporter GLUT4(28). Skeletal muscle may further accumulate the incoming glucose as glycogen or catabolize it partly to lactate, or completely to CO₂ by mitochondrial oxidation. Adipocytes on the other hand do not generate glycogen or oxidize glucose to a significant extent; instead glucose is utilized to produce the glycerol backbone of TAGs, or broken down to acetate in mitochondria and to be further utilized as substrates for *de novo* fatty acid synthesis via the enzyme fatty acid synthase (FAS) located in the cytosol. Insulin also increases fatty acid uptake in skeletal muscle and adipocytes, and inhibits lipolysis of TAG stored in adipose depots. In the liver, however, insulin functions differently as the liver cells do not exhibit GLUT4-mediated insulin-stimulated glucose uptake. Instead glucose uptake is regulated downstream of membrane transport (facilitated by GLUT2) by a liver specific hexokinase (also called glucokinase; GK) (29; 30) and GS, the rate limiting step in glycogen synthesis(31). Glucose entering the hepatocyte may become incorporated into glycogen, oxidized or used as substrate for *de novo* fatty acid synthesis(31). Another important hepatic function is regeneration of glucose from lactate and alanine via gluconeogenesis, regulated by insulin via the rate limiting enzyme phosphoenolpyruvate carboxykinase (PEPCK)(32). Insulin regulates glycogen synthesis positively and gluconeogenesis negatively, thereby increasing storage and reducing output of hepatic glucose into the blood stream, respectively(31; 33).

Figure 2. Insulin-regulated glucose metabolism.

Insulin regulated glucose metabolism



The fate of glucose entering the blood after a meal and responses to insulin. After entering the cell, glucose may either be stored as glycogen (mainly in skeletal muscle and liver), catabolized partly to lactate by working muscle exceeding its oxidative capacity, converted to glycerol to form the backbone of TAG (mainly adipocytes), catabolized completely to CO₂ to produce energy carriers like ATP and NADP (most organs including brain). In adipose tissue glucose may also be oxidized to acetate and be further utilized as substrates for de novo fatty acid synthesis via FAS. Glucose transporter 1,2 (GLUT1,2), fatty acid synthase (FAS), fatty acid (FA), triacylglycerol (TAG), non-esterified fatty acid (NEFA), very low density lipoprotein (VLDL), adenosine triphosphate (ATP).

Skeletal muscle

Skeletal muscle constitutes the largest organ in the body accounting for approximately 40% of body weight in normal individuals (34). The share mass of this organ renders its importance for energy homeostasis, and even at rest it utilizes approximately 30% of total body energy expenditure, whereas during hard physical activity energy expenditure rises several-fold(35). Lack of muscle activity has been associated with increased risk of developing obesity, T2D and cardiovascular diseases(36), and reduced muscle energy turnover is associated with development of both obesity (35) and T2D(15).

Skeletal muscle energy metabolism

Glucose and fatty acids are quantitatively the primary energy sources for skeletal muscle, with resting muscles consuming more fatty acids during fasting, and more glucose when carbohydrate uptake increases postprandially(37). Functionally, skeletal muscle can be divided in either slow-twitch (type 1) or fast-twitch (type 2) fibers. Differences in fiber type

compositions direct preferences for either glucose or fatty acids as the primary fuel. Type 1 fibers are better adapted for endurance activity, contain more mitochondria and myoglobin (which makes them redder) and show a preference for fatty acids as primary fuel. On the contrary, type 2 fibers appear whiter, have lower capacity for fatty acid oxidation and generate energy more anaerobically via glycolysis during contraction(38; 39). All over, skeletal muscles are responsible for more than 80% of insulin-stimulated glucose disposal(40), and type 1 fibers are more insulin sensitive than type 2 fibers and have higher capacity for insulin-stimulated glucose uptake(41). During physical activity, skeletal muscle contractions increase glucose uptake via an insulin-independent pathway. Like insulin, contraction enhances translocation and budding of small vesicles, containing the glucose transporter (GLUT4), with the skeletal muscle plasma membrane, promoting influx of glucose from interstitial fluids and thus plasma. Each stimulus generates a unique signaling cascade that probably converges on a junction protein called AS160. When phosphorylated by upstream AKT/protein kinase B (PKB) (insulin) or AMP-activated protein kinase (AMPK) (contraction), AS160's Rab-GTPase activity is inhibited and conversely more Rab-GTP is retained, allowing GLUT4 rich vesicles to fuse with the plasma membrane(42). In skeletal muscle fatty acids are taken up and esterified with coenzyme A (CoA) and substantial amounts of this seem to undergo a cycle into imTAG before being utilized for oxidation(43). Alternatively, fatty acyl-CoAs may be re-esterified with glycerol or other lipid intermediates to produce mono-, di- and triacylglycerols. TAG is the final product of this chain of anabolic processes designed for storage of energy, and this storage occurs in small TAG-containing lipid droplets. The fatty acids stored as imTAG may then be released and utilized as fuel by mitochondria and peroxisomes.

Myokines

The discovery of adipose tissue as an important source of peptide hormones (adipokines) like leptin, adiponectin and resistin(44), has paved the way for the possibility that skeletal muscle may produce and release its own myokines(45). Examples like IL-6, IL-8 and IL-15 have demonstrated that at least a limited number of these myokines exist, although the functions of these factors need to be further studied(46), e.g. with focus on fiber type differences(47). Potential, new myokines have been hypothesized(48), and many will probably be characterized in the near future that may help explain the connection between skeletal muscle activity and disease development.

Adipose tissue

There are two main types of adipose tissue, namely white (WAT) and brown (BAT) that differ markedly in function and localization. WAT is found in subcutaneous layers and around the inner body organs (visceral), whereas BAT is mostly located in the neck area of newborn children and rodents, but may also be scattered around in association with subcutaneous and visceral WAT. The purpose of BAT is to produce heat through a process called non-shivering thermogenesis. BAT contains highly specialized brown adipocytes that have large amounts of mitochondria enabling them to oxidize fatty acids to produce energy. This energy is then released as heat, as BAT mitochondria express a specific uncoupling protein called UCP1, allowing protons to flow "freely" through the inner mitochondrial membrane(49). WAT on the other hand functions as thermal and mechanical insulation, an energy reservoir for storage of TAG and as an endocrine organ secreting adipokines that are involved in regulation of energy balance and immune system among other.

Regulation of gene expression by nuclear transcription factors

Expression of a gene to its corresponding messenger ribonucleic acid (mRNA) and finally as its protein product is a process including several steps that may be subject to regulation. The cells need to tightly regulate their transcriptional machinery to balance the synthesis and degradation of the protein products to maintain a steady state within the concentration boundaries appropriate for normal function. Regulation of gene expression takes place at several levels, from chromatin blocking of transcriptional access to deoxyribonucleic acid (DNA) to degradation of protein via proteasomes. One important regulatory step determining gene expression is the transcription of genes to their corresponding mRNA. To regulate mRNA transcription, the cell is equipped with many receptors that sense chemical and molecular changes in the extra- and intracellular milieu. These receptors may transfer their signals to downstream transcription regulating factors, or may themselves be transcription factors. Nuclear transcription factors may enhance or repress the transcriptional process by binding to specific DNA sequences associated with the gene, and thereby helping to stabilize or destabilize the DNA binding of RNA polymerase II to the core promoter region containing the sequence for initiation of transcription. The nuclear receptors (NRs) constitute a large superfamily within the category of nuclear transcription factors, with members including vitamin D receptor, thyroid hormone receptor, retinoic acid receptor and steroid receptors(50). Despite decades of research, the endogenous ligands of many NRs still remain unsettled and thus these NRs are collectively called orphan receptors. Besides the NRs, several co-activators and co-repressors work in concert to regulate the functions of e.g. the LXR and PPAR transcription factors. The relative importance of these cofactors is starting to be unveiled but the field is still young and much is not settled.

Liver X receptors

LXRs were first described in liver, and include two highly conserved isoforms, namely LXR α (NR1H3) and LXR β (NR1H2). Both heterodimerize with 9-cis retinoic acid receptor α (RXR α), and bind to DNA upon activation by ligands via a LXR response element consisting of two direct repeats of the sequence AGGTCA with 4 indifferent nucleotides in between; so called direct repeat 4 (DR4) type (51). LXR α is mainly expressed in the liver, adipose tissue, macrophages, skeletal muscle, small intestine and kidney(52), whereas LXR β is more ubiquitously expressed(51). LXRs were previously known as orphan receptors, because no known endogenous ligands were identified. Over the years, however, several naturally occurring oxysterols (cholesterol metabolites) have been shown to activate the LXRs(53; 54), and more recently glucose was demonstrated to activate LXRs at physiological concentrations(55). The finding that cholesterol derivatives, but not cholesterol itself, could act as a ligand for LXRs prompted the search for LXR target genes in cholesterol metabolism, and the rate limiting enzyme in conversion of cholesterol to bile acids, cholesterol 7 α -hydroxylase (Cyp7 α), was one of the first genes to be identified(56). Later, several genes involved in cholesterol, lipid and glucose metabolism have been identified as LXR targets, including ATP-binding cassette transporter 1 (ABC1) (57) and cholesteryl ester transfer protein (CETP) (58) regulating reverse cholesterol transport, FAS (59) controlling fatty acid synthesis, and GLUT4 (60) regulating glucose transport in skeletal muscle and adipocytes. LXRs have been extensively studied in regulation of cholesterol homeostasis, stimulating reverse cholesterol efflux from peripheral tissues as well as hepatic cholesterol excretion(61), but less is known about their impact on skeletal muscle fatty acid and glucose metabolism. LXRs may positively regulate the mRNA levels and activity of another transcription factor

called sterol regulatory element-binding protein 1c (SREBP1c)(62), which is involved in promoting insulin-stimulated fatty synthesis via up-regulation of acetyl-CoA carboxylase (ACC) and FAS(63). Insulin may increase the levels of hepatic LXRs, and insulin-stimulated fatty acid synthesis is dependent on functional LXREs in the promoter region of the SREBP1c gene(64). LXRs can also up-regulate the expression of carbohydrate responsive element-binding protein (ChREBP)(65), which is a transcription factor that is believed to be indirectly sensitive to glucose via the metabolite xylose-5-phosphate generated by the pentose shunt(66). ChREBP may increase the transcription of genes involved in glucose-derived fatty acid synthesis(67). Thus, activating LXRs may directly or indirectly enhance *de novo* fatty acid synthesis as demonstrated by feeding the synthetic LXR-agonist T0901317 to rodents, leading to hepatic steatosis and hypertriglyceridemia(68). Furthermore, Kalaany et al. demonstrated that LXR-null mice were protected against hypertriglyceridemia and insulin resistance induced by high-fat/high-cholesterol feeding through a cholesterol-dependent pathway, implicating that LXRs take part in the complex regulation between storage and oxidation of fatty acids in hepatic and peripheral tissues(69). However, activation of LXRs may produce beneficial effects besides reduction of cholesterol synthesis, as demonstrated by improved glucose tolerance in a rodent model of insulin resistance, where addition of the LXR-activator GW3965 reduced hepatic gluconeogenesis and improved hepatic glucose utilization, and increased glucose uptake in adipose tissue(70). The finding that GLUT4 expression could be regulated by LXR α in adipocytes(60; 70), together with a possible functional role in skeletal muscle, increasing reverse cholesterol efflux(71), has called for further investigations into LXRs functional roles in skeletal muscle.

Peroxisome proliferator-activated receptors (PPARs)

The PPARs are ligand-activated nuclear transcription factors that can be divided into three distinct isoforms, α , γ and δ/β . Like LXRs, they heterodimerize with RXR and bind to DNA at specific response elements (PPREs of DR1 type) in the promoter regions of their target genes. The PPARs serve critical roles by “sensing” the presence of fatty acids and eicosanoids, thus reciprocally regulating lipid metabolism(72).

PPAR α

The first characterized member of the PPARs was the alpha subtype, which was cloned from liver of mice and described as the factor responsible for the peroxisome proliferation induced by various carcinogenic xenobiotics(73). PPAR α was further found to be predominantly expressed in liver, but also resides in the heart and skeletal muscle where it influences fatty acid oxidation(74). The physiological relevance of PPAR α in mice has been unraveled by the development of knock-out animals, showing profound reductions in expression of several genes involved in fatty acid oxidation(75). Moreover, PPAR α seems to mediate the adaptive responses to prolonged fasting, with increased hepatic fatty acid β -oxidation and ketone body production(76; 77). Synthetic ligands have been developed that activate PPAR α , including the fibrate class frequently used in human medicine as lipid lowering drugs(78). The search for endogenous ligands have demonstrated that several long-chain fatty acids may activate PPAR α at micromolar concentrations(79; 80). However, other more specific ligands with binding properties in the nanomolar range have been sought that may fit better with the concentrations presumed to exist inside the cells. E.g. the high-affinity lipoxigenase metabolite 8(S)-HETE has been proposed as an endogenous PPAR α activator(80-82), but its subcellular concentrations in relevant tissues have been found to be too low. Thus, it has been proposed that PPAR α may sense the total flux of fatty acids rather than single components(80;

83). Alternatively oleylethanolamide, a naturally occurring fatty acid derivative, was recently shown to be a potent selective activator of PPAR α within physiological concentrations(84). Administration of fibrates promotes lowering of serum triacylglycerols, presumably due to reduced VLDL-formation due to increased fatty acid oxidation and reduced expression and subsequently lower serum levels of apoC-III(85), an inhibitor of VLDL clearance(86). Furthermore, activation of PPAR α with different ligands is associated with protection against weight gain (87) and insulin resistance induced by feeding high-fat diets(88), but apparently this may be at least partly explained by a parallel reduction in feed intake(84; 89). Surprisingly, selective overexpression of PPAR α in skeletal muscle was associated with reduced insulin-stimulated glucose uptake and glucose intolerance despite increased fatty acid oxidation and protection against diet-induced obesity. Moreover, PPAR α knock-out (KO) animals became excessively obese, but remained more glucose tolerant on a high-fat diet than wild type animals(90).

PPAR γ

The gamma subtype is the best studied of the three PPARs, and evidence suggests that PPAR γ is the master regulator of adipocyte generation and function(91). Forced expression of PPAR γ in non-adipogenic cells converts them into mature adipocytes(92), and in knock-out mice, although lethal, newborn pups exhibit no adipose tissue development(93). PPAR γ may be activated by endogenous fatty acids (in particular polyunsaturated) (82; 83) or eicosanoids(94). Interestingly from a pharmacologically view, activation of PPAR γ with the glitazone class of drugs, leads to improved insulin sensitivity (95) as well as reduced plasma lipids in animals and humans(96). Furthermore, dominant negative mutations of the PPAR γ gene in humans are associated with insulin resistance, diabetes mellitus and hypertension(97). The leading hypothesis underlying these effects are that increased adipose tissue expansion and consequently storage of TAG remove plasma lipids, thus relieving liver and skeletal muscle from fatty acid-mediated insulin resistance(98-100). Furthermore, activation of PPAR γ enhances plasma levels of the antidiabetic adipokine adiponectin, whereas plasma levels of the prodiabetogenic resistin and TNF α are reduced(98). Conditional knock-out of PPAR γ in mature adipocytes resulted in hepatic steatosis and insulin resistance with progressively reduced amounts of fat stored in adipose tissue. As long as some fat remained, however, whole body glucose tolerance and skeletal muscle insulin sensitivity were unchanged(101). However, in another Cre/Lox mediated adipose tissue specific knock-down of PPAR γ , overall insulin resistance was improved on a high-fat diet due to enhanced hepatic glucose metabolism(102). Although PPAR γ is predominantly expressed in adipose tissue, conditional hepatic knock-down resulted in hyperlipidemia, hyperglycemia and hyperinsulinemia besides increased fat storage in adipose tissue, all probably reflecting reduced hepatic TAG removal(103). PPAR γ may regulate the expression of GLUT2 and glucokinase in liver and pancreas, thus affecting hepatic glucose uptake and pancreatic glucose-stimulated insulin release(104). Intriguingly, despite very low expression, knocking down PPAR γ in skeletal muscle resulted in profound insulin resistance, which was non-responsive towards thiazolidinedione (TZD) treatment(105). Hence, the insulin-sensitizing and other effects of TZD administration seem to depend at least partly on direct activation of PPAR γ in the target tissues, thus challenging the hypothesis of adipocyte-mediated plasma lipid removal and adipokine regulation causing the beneficial effects of TZD treatment. Despite the beneficial effects of TZD on insulin resistance, one major negative side effect is weight gain (106) due to a combination of enhanced nutrient intake (107) and increased energy efficiency(89; 100; 108; 109). Thus, several drug companies are currently searching

for combined or partial agonists for the different PPARs that may prove to have a more favorable functional profile than the pure agonists(110).

PPAR δ

The third member of the PPAR family, the delta subtype, has traditionally been less studied than the other partly because of lack of specific activators like the fibrates and the glitazones. PPAR δ is ubiquitously expressed with relatively high expression in skeletal muscle(111). Like the two other PPAR members its endogenous ligands are believed to be fatty acids or their derivative eicosanoids(80; 81; 83). The functional roles of PPAR δ are currently being unraveled, and recent studies suggest that it might play a major role in energy turnover and obesity regulation. Wang et al. identified PPAR δ as an important regulator of energy consumption, by increasing fatty acid combustion and energy uncoupling in tissues when over-expressing the receptor or when activating it with a selective ligand. Mice lacking PPAR δ on the other hand showed less adaptive uncoupling response when challenged with a high-fat diet, and were more prone to obesity(112). Several studies have implicated PPAR δ in regulation of fatty acid oxidation in skeletal muscle(113-115). Recently, over-expressing a constitutively active a PPAR δ variant in skeletal muscle was shown to elicit effects similar to those seen during endurance training, with conversion of type 2 to type 1 fibers(116). In another recent study, addition of the delta-specific ligand GW1516 to exercising mice further increased their exercise-induced generation of fatigue-resistant type 2 fibers and synergistically improved their running endurance(117).

Fatty acids

Structure

Fatty acids consist of straight hydrocarbon chains of varying length with a carboxyl acid group in one end and a methyl group in the other, where the carbon atoms may be numbered (C1, C2, C3,...) from the methyl end. In general, naturally occurring fatty acids have a paired number of carbon atoms in their backbone, and they can further be subdivided by the number and position of double bounds (unsaturation) in their acyl chain. Fatty acids with no, one or more double bounds are termed saturated (SAFA), monounsaturated (MUFA) or polyunsaturated (PUFA), respectively. Based on the number of carbon atoms, double bounds as well as the position of the first double bound, a systematic nomenclature has been constructed. E.g. palmitic acid, a SAFA of 16 carbons, is described as (16:0); oleic acid, a MUFA with 18 carbons and one double bound between carbons 9-10, is written (18:1, n-9); and the PUFA linoleic acid, with an extra double bound between carbons 6-7 as compared to oleic acid, is (18:2, n-6). Ruminant animals contain mostly SAFA and some MUFA, plants have more MUFA and PUFA of 16-18 carbons, whereas marine animals may contain substantial amounts of long-chain PUFAs (20-22 carbons), because of their food chain is based on EPA- (20:5, n-3) and docosahexaenoic acid (DHA)- (22:6, n-3) producing algae(118). Mammals may synthesize fatty acids from acetate precursors, but can only produce SAFA, MUFA or PUFA of the n-9 type. Thus, because n-6 and n-3 PUFAs have essential functions(119), these fatty acids must be obtained via the diet as linoleic (18:2, n-6) and α -linolenic (18:3, n-3) acids or other fatty acids of the n-6 and n-3 fatty acid families. The C18 fatty acids of the n-6 and n-3 fatty acid families can be further elongated and desaturated to arachidonic (ARA, 20:4, n-6) and EPA/DHA, respectively(120).

Functions

Fatty acids serve several important biological functions including energy source, phospholipids building blocks in cell membranes, thermal and mechanical insulation (when stored as TAG in adipose tissues), as substrates for eicosanoid synthesis (e.g. ARA and EPA), protein acylation and ligands for nuclear receptor-regulated gene transcription. Dietary fatty acids are absorbed from the gut and re-esterified into TAG in the enterocytes producing chylomicrons that enter the blood via lymph drainage. The TAG in chylomicron particles are hydrolyzed by lipoprotein lipase (LPL) in the capillaries and the released fatty acids are taken up by skeletal muscle, adipose tissue and liver. The liver may utilize fatty acids to produce TAG-rich very low-density lipoprotein particles (VLDL) for redistribution of fatty acids to peripheral organs. Adipocytes take up fatty acids and glycerol and synthesize TAG for long term storage in intracellular lipid droplets, whereas skeletal muscle preferentially utilize fatty acids as fuel, but may also store minor amounts as TAG. The liver may also utilize fatty acids released from adipocytes during starvation to produce ketone bodies as an alternate fuel source to glucose and fatty acids.

Fatty acids and their ketone body derivatives constitute a major fuel source, particularly after 24 h fasting, when an estimated ~80% of body energy consumption is provided by lipid catabolism(121). Despite their nutritional and essential functions, evidences are pointing towards increased fatty acid consumption as an important player in development of obesity, insulin resistance and T2D. General lipid overload may result in these negative effects, but fatty acid composition likely modulated the responses(122). Diets high in saturated fat are associated with increased risk of developing insulin resistance and T2D(123), whereas diets rich in monounsaturated fat, like the Mediterranean diet, may prove less prone to such detrimental effects(124).

Different types of fatty acids and derivatives may regulate gene transcription by activating or inhibiting nuclear transcription factors, but they may also activate receptors in the cell membrane like toll-like receptors (TLRs) and free fatty acid receptors (FFARs). In skeletal myotubes, activation of TLR2 was shown to be important for palmitic acid-mediated insulin resistance by activating an inflammatory signal cascade involving c-Jun N-terminal kinase (JNK) and nuclear factor kappa B (NFκB) activity(125). The FFAR, GPR40, is a G-protein coupled receptor that can be activated by medium to long-chain fatty acids and thereby induce insulin secretion by pancreatic beta cells(126). Fatty acids are also involved in more direct regulation of enzyme activity by allosteric activations or repressions, e.g. fatty acyl-CoA inhibition of malonyl-CoAs repression of carnityl palmitoyltransferase 1 (CPT1) activity, thus promoting fatty acid β-oxidation(127). Several proteins and peptide hormones are acylated on specific amino acid residues, which is important for their proper function. E.g. the gastric peptide ghrelin that may regulate appetite, is acylated on a serine residue essential for activity, and the acyl moieties used are reflected by the available dietary fatty acids(128).

Long-chain omega-3 polyunsaturated fatty acids

Long-chain n-3 PUFAs like EPA and DHA are one of the best-studied types of fatty acids. Marine oils (e.g. cod liver oil) are rich in these n-3 fatty acids, and supplementation with fish oils have been associated with reduced insulin resistance and glucose intolerance in animals fed high-fat or high-sucrose diets(129-133), although these beneficial effects are not always observed in animals fed high-fat diets(124; 134-136). A meta-analysis comparing the effects of fish oil on humans with T2D found no improvements in plasma glucose or HbA_{1c} levels, but confirmed the effect of fish oils in lowering plasma triacylglycerols(137). The effect of n-

3 fatty acids on plasma TAG is probably caused by inhibition of hepatic TAG synthesis and VLDL release(138), that most likely is related to reduced hepatic fatty acid synthesis and increased fatty acid oxidation(139). Studies have demonstrated that feeding long-chain n-3 PUFA may activate hepatic PPAR α (140) that leads to enhanced expression of genes involved in fatty acid β -oxidation like CPT1 and acyl-CoA oxidase (AOX) (141). The reduced expression of ACC, FAS and pyruvate kinase (liver) (L-PK) involved in fatty acid synthesis may be attributed to negative influence by long-chain n-3 PUFAs on SREBP-1c and ChREBP; transcription factors controlling the expression of these genes. PUFAs have been shown to negatively affect SREBP-1c mRNA levels(142), possibly via inhibition of LXRs(143), and PUFAs may also inhibit the maturing cleavage, and thus activation of SREBP-1c(144). Furthermore, PUFAs were shown to negatively influence ChREBP activity by reduced mRNA expression and reduced nuclear translocation(145). In PPAR α knock-out mice the effects of PUFAs on fatty acid oxidation were abolished, whereas the inhibition of fatty acid synthesis was not(140; 146).

Fish-oils also may also reduce size of retroperitoneal and epididymal fat depots in rats fed high fat diets(147), and these effects are likely due to reduced adipocyte size and not number(148). As shown recently, the reduced perirenal and epididymal depots seem to involve a redistribution of fat rather than a net reduction, as both total lipid amount and body weight remained similar for rats fed high-fat diets with or without fish-oils(149).

Eicosapentaenoic acid

The exact physiological role of EPA in the body or specific organs is less studied as compared to fish-oils where EPA is one major constituent. The plasma lipid reducing properties of marine n-3 PUFAs may be explained by the presence of EPA, as incubation of cultured rat hepatocytes with this fatty acid reduces cholesterol (150) and TAG (151) esterification as well as increases palmitoyl-CoA oxidation(152), which in turn reduces synthesis and secretion of VLDL (152; 153). In primary human adipocytes preincubation with EPA induced the expression of PPAR γ (154) which is important for adipocyte differentiation(100). PPAR γ activation may promote fat accumulation in subcutaneous depots, with reduced or unchanged visceral storage(155), that may explain the effects of fish-oils on possible fat tissue redistribution mentioned previously. Preincubation of human skeletal myotubes with EPA also induced mRNA expression of PPAR γ and increased both glucose and fatty acid uptake(156).

Tetradecylthioacetic acid

The sulfur-substituted fatty acid analogue TTA is a saturated fatty acid containing 16 carbon atoms and a sulfur atom inserted between the second and the third carbon counting from the carboxyl acid end, making TTA resistant to fatty acid β -oxidation(157). TTA may reduce plasma lipids, probably by increasing hepatic mitochondrial β -oxidation of fatty acids(158; 159). TTA reduced epididymal fat in young obese Zucker (fa-fa) rats, as well as epididymal and retroperitoneal fat in male Wistar rats fed high-fat diets (70% energy) for three weeks(160).

TTA is a pan-PPAR-activator in both rodents and humans(80; 161-164), and dual and pan-PPAR-agonists are currently being developed for treatment of T2D(110), and TTA has been shown to improve glucose metabolism in insulin resistant rats(160) as well as stimulate mitochondrial proliferation in rat skeletal muscle(165). We have recently demonstrated that TTA may increase fatty acid oxidation in human myotubes similar to the PPAR δ specific agonist GW501516(159).

Obesity and IR/T2D

Development of insulin resistance, and consequently T2D, may have several distinct but related causes that are more or less prominent in each afflicted subject. The obese phenotype with raised plasma lipids may cause or aggravate insulin resistance and T2D through different mechanisms.

Plasma free fatty acids

Insulin resistance and T2D are associated with elevated plasma free fatty acids (FFA) levels (166; 167), and experimental lowering of plasma FFA improved insulin sensitivity in obese diabetic and non-diabetic individuals(168). The relevance of fatty acid overload in the etiology of T2D is a central feature of lipid-induced insulin resistance, where increased levels of intracellular lipid intermediates like acyl-CoAs, DAGs and ceramides interfere with intracellular insulin signaling. During experimental infusions of FFA, skeletal muscles of healthy individuals become insulin resistant(169; 170), most likely mediated by fatty acid interference with INS-1 related activation of phosphoinositide 3-kinases (PI3K), thereby inhibiting insulin-stimulated glucose uptake(171-173). Acute high plasma lipid concentrations may negatively affect insulin sensitivity through elevation of intracellular acyl-CoA and DAG (173) activating protein kinase ϵ - theta (PKC- θ) (172) and I κ B kinase β (IKK β) (174). Mice with PKC- θ specific KO were protected against fat-induced skeletal muscle insulin resistance(175), showing the importance of this serine kinase in linking high lipid intake with disruption of the insulin signaling cascade. Alternatively, increased palmitic acid availability may cause insulin resistance through enhanced production of intracellular ceramides interfering with insulin signaling possibly by activation of stress-induced serine phosphorylation of IRS-1(176).

Ectopic storage of fat in liver and skeletal muscle

A common consequence of obesity is ectopic storage of TAG in tissues other than adipose tissue, like liver and skeletal muscle. Intramyocellular TAG (imTAG) storage in untrained individuals is a strong predictor of insulin resistance and development of T2D(177-179). Ectopic storage of TAG in lipid droplets, however, may not by itself be harmful for the cell functions, as endurance-trained athletes often have high imTAG levels but at the same time show superior insulin-sensitivity compared to sedentary individuals(180; 181). The combination of higher capacity for mitochondrial fatty acid oxidation as well as increased short term storage of excess TAG, may reduce intracellular lipid intermediates that interfere negatively with insulin signaling, thereby relieving insulin resistance(182-185). In fact, increased sequestering of incoming fat into imTAG have been shown to decrease lipid intermediates and improve insulin sensitivity in transgenic animals either over-expressing diacylglycerol acyltransferase 1 (DGAT1) in skeletal muscle(186), or lacking the lipolytic enzyme adipose triglyceride lipase (ATGL)(187).

Skeletal muscle and dysfunctions in relation to T2D

The underlying mechanisms explaining skeletal muscle insulin resistance is not settled, and may involve more than one process. Different lipotoxic mechanisms have been suggested where increased levels of e.g. fatty acyl-CoAs, ceramide, DAG or acylcarnitines negatively influence insulin signaling via activation of different serine kinases(26). The mechanisms

behind the increased levels of lipid intermediates probably involves the high lipid availability observed in the obese state, but other factors likely influence the intracellular lipid milieu in a way that promote insulin resistance.

Metabolic inflexibility of skeletal muscle

One central feature observed in many insulin resistant individuals is a lack of flexibility of switching from carbohydrate to fatty acid metabolism in skeletal muscles during a fasting condition, and likewise to increase skeletal muscle utilization of glucose in a response to insulin(188; 189). The Randle cycle envisions that increased fatty acid oxidation in skeletal muscle may inhibit glucose utilization through feedback inhibition of key enzymes such as pyruvate dehydrogenase (PDH) and phosphofruktokinase (PFK) leading to increased levels of glucose-6 phosphate and further inhibition of hexokinase (HK) (190). Likewise, it has been suggested an inverse Randle cycle where increased glucose influx generates malonyl-CoA at a level that inhibits CPT1, and thus reduce fatty acid influx into mitochondria. In the presence of high plasma TAG and free fatty acid levels such inhibition of fatty acid catabolism might generate enough lipid intermediates and byproducts to interfere with insulin signaling(191).

Mitochondrial dysfunction – implications of PGC1 α

Several lines of evidence point at mitochondrial dysfunction as important in development of skeletal muscle insulin resistance. Reduced mitochondrial number and/or function have been observed in studies of skeletal muscle in elderly (192) obese insulin resistant (193) and T2D subjects (193; 194) as well as in first degree insulin resistant relatives of people with T2D(195; 196). Skeletal muscle mitochondrial oxidative capacity was also found to be a better predictor of insulin sensitivity than imTAG or long-chain acyl-CoAs(197). Reduced skeletal muscle capacity for fatty acid oxidation may result in harmful levels of lipid intermediates(198), and has been associated with increased imTAG levels(192; 195). Reduced complete mitochondrial oxidation of fatty acid to CO₂ may also promote higher levels of β -oxidation intermediates interfering negatively with insulin signaling.(199-201).

PGC1 α is a transcription factor important for mitochondrial biogenesis and formation of slow twitch type 1 oxidative fibers(202), and its activity seems to control complete mitochondrial fatty acids oxidation by regulating the expression of several key enzymes in the tricarboxylic acid cycle(TCA) and electron transport chain (ETC) (203). Fatty acid infusion in humans was associated with reduced expression of PGC1 and other genes involved in mitochondrial metabolism(204). Several studies have observed a correlation between insulin resistance/T2D and reduced levels of the transcription factor PGC1 α (199; 205; 206). Whereas activation of PPARs in skeletal muscle may increase fatty acid β -oxidation, activation of PGC1 α , either alone or coordinated with APMK activation, seems necessary for optimal stimulation of complete fatty acid oxidation (117).

Induction of β -oxidation capacity without concomitant increase in complete oxidation via the electron transport chain, may lead to skeletal muscle insulin resistance(200). This was also demonstrated by muscle-specific PPAR α overexpression, increasing fatty acid β -oxidation, but at the same time reducing insulin sensitivity(90). Koves *et al.* have proposed that high levels of plasma lipids observed together with obesity, may induce skeletal muscle β -oxidation of fatty acids by activation of PPARs, but without a coordinated down-stream induction of PGC1 α -dependent complete oxidation via mitochondrial Krebs cycle and electron transport chain/ATP synthase machinery(200). This imbalance between mitochondrial β -oxidation and complete oxidation may lead to increased mitochondrial stress with enhanced production of reactive oxygen species (ROS) and release of excess acyl-

carnitines, causing insulin resistance(203). Also inherent dysfunctions in complete oxidation (possibly related to deficits in PGC1 α function and other related transcriptional partners like hepatocyte nuclear factor 4 α (HNF4 α) and PPAR γ), would result in such mitochondrial stress, and be potentially worsened by obesity and high-fat intake.

Whether PGC α -associated mitochondrial dysfunction can explain all acquired and inherent forms of skeletal muscle insulin resistance is presently unknown, but may be unlikely because of all the evidence connecting different adipokines and inflammatory cytokines with insulin resistance in muscle. In a recent review it was proclaimed that “Skeletal muscle “mitochondrial deficiency” does not mediate insulin resistance”, enlisting several arguments against the concept of mitochondrial dysfunction being the culprit of insulin resistance(207). Moreover, a study with mice fed a high-sucrose and -fat diet, glucose intolerance and insulin resistance preceded the development of mitochondrial damage, mediated by augmented ROS production(208). These latter examples display the complexity of understanding the basis of insulin resistance, but they do not entirely exclude the compelling possibility of inherent mitochondrial defects working in concert to increase the likelihood of developing skeletal muscle insulin resistance when the energy balance tips in favor of obesity, thus the issue is not yet settled.

Adipokines

Adipose tissues release potent hormone signals called adipokines that may promote insulin resistance in obese individuals. Leptin is an adipokine that regulates body weight by reducing nutrient intake and increasing energy expenditure, and adipocyte mRNA abundance as well as plasma levels of leptin are increased with obesity, providing a feed-back signal from the fat stores to the CNS via hypothalamus(209). Disruption of leptin function leads to severe hyperphagy, obesity and insulin resistance(210), whereas infusion of leptin may reverse insulin resistance and hepatic TAG accumulation in ob/ob and lipodystrophic mice(211). The adipokine adiponectin was demonstrated to improve insulin resistance associated with obesity and lipoatrophy(212). Disrupting adiponectin resulted in severe diet-induced insulin resistance(213), and adipocyte expression of adiponectin was furthermore found to be reduced with obesity(214), along with reduced plasma levels(215). Later adiponectin was shown to improve insulin resistance by activation of skeletal muscle and hepatic AMPK, stimulating fatty acid oxidation and glucose uptake in muscle and reducing gluconeogenesis in liver(216). Another adipokine, resistin, was identified as an insulin resistance-promoting adipokine, with circulating plasma levels positively correlated with adipose tissue size(217). Expression of the cytokine TNF α in adipose tissue increases with obesity and increased plasma levels of TNF α may promote insulin resistance(218). In models of obesity, knocking out TNF α improved insulin sensitivity(219). In humans, obesity was associated with increased mRNA levels of TNF α in fat tissue that was positively correlated with a measure of insulin resistance, and weight reduction reduced insulin resistance as well as TNF α adipose tissue gene expression(220).

Adipose tissue inflammation

Obesity may promote inflammatory processes in adipose tissue and other organs like liver, skeletal muscle and pancreas and thus be involved in development of insulin resistance and T2D(26). During obesity, adipose tissue may expand either by proliferation of new adipocytes (hyperplasy) or enlargement of old ones (hypertrophy), although the total number seems to change little during adulthood(221). Adipocyte enlargement correlates well with degree of insulin resistance (222) and predicts development of T2D better than obesity itself(223).

When adipocytes enlarge above a certain size they tend to generate inflammation that may be linked to microhypoxia (224) and endoplasmatic reticulum (ER) stress(225). Inflamed adipose tissue may further release chemokines and attract macrophages that remove damaged cells and other debris, forming crown-like structures around necrotic adipocytes(226). Obesity and insulin resistance are associated with increased macrophage invasion and inflammation of adipose tissue in rodents as well as humans(227; 228). The proinflammatory signaling pathways mediated by activation of JNK1(229) and IKK/NF- κ B(230) seem to play important roles. Disrupting the proinflammatory activation of JNK1 and IKK β resulted in improvement of obesity-related insulin resistance (231; 232). Furthermore, conditional knock-downs of JNK1 and IKK β in the myeloid cell lineage (including macrophages) protected against high-fat diet-induced insulin resistance without affecting obesity(229; 230).

Insulin resistance – adipocyte hyperplasia better than hypertrophy?

In transgenic ob/ob mice moderately over-expressing adiponectin Kim et al. (55) observed that although the mice grew morbidly obese they were protected against obesity-induced insulin resistance, possibly because of adipose tissue hyperplasia rather than hypertrophy(233). Tissue inflammation and macrophage invasion were also markedly reduced, suggesting that these processes are not merely functions of adipose tissue expansion, but possibly related adipocyte size(233). This notion is further supported by the fact that treatment of diabetes with the glitazone type of antidiabetic drugs (activating PPAR γ), promotes weight gain and enlarged fat tissues, with a reduction in adipocyte size(98; 100; 234). PPAR γ activation also reduces proinflammatory macrophage invasion in adipose tissue(235), and increases plasma concentration of adiponectin(98).

Ectopic fat storage seems to be dictated by dietary fatty acid availability and storage capacity of TAG in adipose tissues, as well as fatty acid uptake and catabolism in target tissues. E.g. lipodystrophy is a condition with reduced or abolished adipose tissue fat mass. Consequently, reduced fat storage capacity is associated with severe insulin resistance probably due to massive ectopic fat storage and increased fatty acid metabolism in skeletal muscle and liver(236). Strategies of increasing fatty acid storage capacity of adipose tissues may reduce plasma lipids and ectopic TAG storage, thus improving insulin resistance(98; 233).

5. Aims of the present study

The aims were to:

1. Study changes in nutrient metabolism of myotubes derived from individuals with type 2 diabetes (T2D).
2. Study the role of LXR and interaction with specific fatty acids such as EPA and TTA in regulating these changes.
3. Develop more high-throughput and low-cost methodology for assessing cellular uptake and CO₂-production from radiolabeled energy substrates.
4. study the impact of feeding TTA to rats on a high-fat background diet, to investigate changes in feed intake, body weight gain and sizes of five different adipose tissue depots

The thesis includes four papers:

- 1) The nuclear transcription factors LXR α/β have been shown to play important roles in regulation of cholesterol metabolism, fatty acid biosynthesis and glucose metabolism. In **paper I** data are presented on the metabolic effects of chronic preincubation with a LXR activator (T0901317) in human myotubes derived from T2D subjects and lean controls. We evaluated whether activation of LXRs induced differential metabolic responses in the two types of skeletal muscle cells.
- 2) Myotubes from individuals with T2D display several differences in glucose and fatty acid metabolism. In **paper II** we investigated whether fatty acids like EPA and TTA may influence energy metabolism of cultured myotubes, and if these substances may ameliorate the reduced fatty acid oxidation previously observed in myotubes derived from obese T2D as compared to cells from obese non-diabetic subjects.
- 3) The lack of efficient low-cost methods for screening cellular uptake and CO₂-formation from nutrients such as fatty acids and glucose, prompted us to develop two such “fuel handling” systems. **Paper III** describes the basic principles behind our approaches for quantitative measurements of nutrient uptake by scintillation proximity assays (SPA) and oxidation to CO₂ in cells grown in 96-well standard tissue-culture plates.
- 4) Dual- and pan-PPAR ligands are currently being developed as potential drugs for treatment of T2D with associated coronary heart diseases, and TTA has been shown to possess pan-PPAR-activation in several studies. However, activators of PPAR α seem to reduce weight gain and feed intake in rodents, whereas PPAR γ activators may enhance feed intake and increase body weight. In **Paper IV** we investigated the effects of TTA on body weight gain and feed intake in rats fed a lard-based high-fat diet, focusing on depot-specific changes in adipose tissue sizes and gene expression as well as metabolic changes in plasma, liver, heart and skeletal muscle.

6. Summary of papers

Paper I: Skeletal muscle lipid accumulation in type 2 diabetes may involve the liver X receptor pathway

This article explores the possible metabolic roles of LXRs in skeletal muscle established from obese T2D and lean non-diabetic control subjects. Previous work had shown that activation of LXRs constitutes an important regulatory pathway for controlling hepatic cholesterol synthesis and lipogenesis. However, some recent studies had also shown that LXR activation promoted up-regulation of the muscle and adipocyte specific glucose transporter gene, GLUT4. Thus, we conducted this study to investigate the impact of chronic pretreatment with the LXR activator T0901317, on glucose and fatty acid intermediary metabolism in myotubes from individuals with or without T2D.

We observed enhanced cellular palmitic acid (PA) accumulation, especially as non-esterified PA, DAG and TAG. Interestingly, T2D myotubes responded more to LXR-activation as compared to control cells, resulting in increased accumulation of DAG and TAG. Some myotubes were co-incubated acutely with insulin during PA uptake, which resulted in further enhancement of uptake and accumulation of in particular TAG. The effect of LXR-stimulation was additive to the effect of insulin increasing TAG accumulation by 3-fold as compared to non-stimulated cells. β -oxidation of PA, measured as ASM, was slightly increased by LXR-activation in T2D cells, whereas complete oxidation to CO₂ only increased in control cells. The stronger increase in cellular PA accumulation, but relatively unchanged PA oxidation in T2D myotubes relative to control cells, indicated that LXR-activation enhanced partitioning of PA to TAG and other lipids profoundly more in T2D cells. We investigated the expression several genes related to lipid metabolism and found LXR α , SREBP-1c, PPAR γ , cluster of differentiation 36/Fatty acid transporter (CD36/FAT), CPT1 and UCP2 to be up-regulated in myotubes from both donor groups after pretreatment with T0901317. There were no significant differences in gene expression between groups, although the expression of both LXR β and UCP3 was significantly enhanced in control, but not in T2D myotubes after chronic LXR-activation. We observed a dose-responsive increase in insulin-stimulated glucose uptake after preincubation of myotubes with increasing concentrations of T0901317. Similarly, glucose oxidation was enhanced in myotubes after preincubation with the LXR agonist. T2D myotubes did not display any significant differences in glucose uptake or oxidation as compared to control cells, and thus showed similar responses to LXR-stimulation. Abundance of GLUT1 and GLUT4 mRNA was increased by LXR activation in the myotubes, probably explaining the enhanced insulin-stimulated glucose uptake. However, despite a 5-fold increase in GLUT4 mRNA abundance, we observed only about 40% increase in insulin-stimulated glucose uptake when pooling the results from both groups, suggesting that other mechanisms besides GLUT4 gene expression is important for insulin-regulated glucose transport, like intracellular distribution of the transporter.

Skeletal muscles from T2D individuals often display reduced fatty acid oxidation with increased deposition of imTAG. An imbalance in uptake and oxidation of fatty acids may promote insulin resistance by accumulation of lipotoxic intermediated such as acyl-CoAs, DAG and ceramides. We demonstrate a possible role of LXR-activation in accumulation of imTAG in skeletal muscle from subjects with T2D. The finding that incubation with T0901317 increased DAG as well as TAG in T2D myotubes, may suggest adverse effects of activating LXRs on skeletal muscle insulin sensitivity. It is possible that enhanced deposition of fatty acids as imTAG may constitute a protective mechanism against high levels of free fatty acids known to promote insulin resistance through increased level of intracellular fatty acid intermediates. The fact that incubation of myotubes with T0901317 enhanced GLUT4

mRNA abundance and promoted enhanced insulin-stimulated glucose uptake, suggests that LXR-activation may even prove beneficial in T2D cells.

Paper II: Fatty acid incubation of myotubes from humans with Type 2 Diabetes leads to enhanced release of beta oxidation products due to impaired fatty acid oxidation: effects of tetradecylthioacetic acid and eicosapentaenoic acid

Different types of fatty acids may have different metabolic effects on skeletal muscle e.g. saturated vs. mono- and polyunsaturated fatty acids, where the saturated fatty acids may be more diabetogenic than the other types. EPA has been associated with improved skeletal muscle insulin response, despite a tendency to enhanced imTAG synthesis and intermediary lipid? storage. Skeletal muscle from individuals diagnosed with T2D frequently shows reduced mitochondrial fatty acid oxidation and increased imTAG deposition possibly related to the development of insulin resistance. TTA is a sulfur-modified fatty acid analogue with possible beneficial effects on skeletal muscle fatty acid oxidation and insulin sensitivity. Previously, Gaster et al.(237) have demonstrated that myotubes derived from obese T2D subjects have reduced capacity for complete oxidation of palmitic acid (PA) to CO₂ as compared to obese control cells, and we have shown that activation of LXRs may promote imTAG synthesis specifically in myotubes from individuals with T2D (Paper I). Thus, this study was performed to monitor the effects of chronically preincubating myotubes from obese T2D and control subjects with EPA and TTA, both bound to fatty acid-free bovine serum albumin (BSA). As controls we used both BSA (fatty acid-free control) and oleic acid (OA, fatty acid control) at the same molar concentrations as for EPA and TTA.

Our main findings were that T2D myotubes from obese subjects showed reduced complete oxidation of ¹⁴C-PA to CO₂ as compared to myotubes from obese controls. Preincubations with fatty acids, in particular EPA, enhanced PA uptake, and both EPA and especially TTA enhanced β-oxidation of PA measured as ASM. TTA also improved complete oxidation of PA to CO₂ in both groups. Thus, after preincubation with TTA, the capacity for complete PA oxidation in T2D myotubes was enhanced comparable to the basal level of control myotubes, but still remained lower as compared to TTA-preincubated controls. T2D cells displayed reduced partitioning of PA into complete mitochondrial CO₂ oxidation relative to ASM, and when incubated with EPA, TTA or insulin they released significantly more ASM to the media than control myotubes. This suggested an impairment of fatty acid oxidation downstream of β-oxidation in myotubes from obese T2D as compared to cells from obese non-diabetic controls. We further looked at the abundance of important genes involved in fatty acid uptake (CD36, FABP3), activation (ACSL1, 3), β-oxidation (CPT1, CPT2, ACADM, HADHB) and complete oxidation (CS, MDH2, UCP2, UCP3). There were no differences between the two groups, but as expected most of the genes involved in fatty acid uptake, activation and β-oxidation (not CPT2 and ACADM) showed increased abundance when myotubes were incubated with fatty acids. On the other hand, genes involved in mitochondrial oxidation/function were basically unchanged by the preincubations, except for a slight (~40%) increase in UCP2 abundance after preincubation with TTA. Preincubation with TTA also increased the mRNA abundance of CD36, FABP3 and ACSL3 significantly more than both OA and EPA. Interestingly, the relative changes in PGC1α and DGAT2 mRNA expression induced by fatty acids (mainly EPA and TTA) were reduced in T2D as compared to control myotubes, and the changes in these two genes seemed to be positively correlated. Besides the enhanced PA oxidation, the enhanced PA uptake promoted by the fatty acid preincubations resulted in accumulation of PA in complex lipids, especially TAG. Both preincubations with OA and in particular EPA profoundly enhanced PA-derived TAG accumulation in the myotubes, whereas preincubation with TTA caused significantly less

TAG synthesis than the other fatty acids. The intracellular presence of TTA or TTA-CoA, seemed to inhibit DGAT activity as demonstrated by a 7-fold increase in DAG/TAG ratio from ^{14}C -acetate without change in *de novo* fatty acid synthesis. Co-incubation with TTA during the last 24 h of OA preincubation repressed the OA-induced increase in cell-associated PA down to baseline (fatty acid-free BSA control). The relative response in TAG synthesis after preincubation with EPA was stronger in T2D cells as compared to obese controls, and was reflected in a negative correlation between EPA-induced change in fatty acid oxidation and change in TAG formation. However, such correlation was not observed after preincubation with OA or TTA. Furthermore, preincubation of myotubes with EPA reduced total acyl-CoA, by a marked reduction in C16:0-CoA, C18:1-CoA and C18:2-CoA. Both total and C18:2-CoA was significantly more reduced in T2D as compared to obese control myotubes, whereas C16:1-CoA was markedly enhanced by preincubation with EPA in both groups of cells. Total acyl-CoA was enhanced after preincubation with TTA, but TTA-CoA probably accounted for the change. However, in T2D myotubes C18:2-CoA and C20:4-CoA both were reduced after TTA-preincubation relative to baseline and obese control myotubes, respectively. The cellular metabolism of glucose was basically unchanged after preincubation with fatty acids, except for glucose oxidation which was increased by $\sim 20\%$ after preincubation with EPA as well as TTA. Both T2D and control myotubes exhibited a similar increase in glucose oxidation, although allover glucose oxidation was about 10% reduced in the diabetic myotubes, further strengthening the notion of a down-stream mitochondrial dysfunction. Glucose uptake or glycogen synthesis (basal and insulin-stimulated) were, however, not significantly different between the two groups. This may be related to the use of control myotubes from obese instead of lean non-diabetic subjects, as used in previous studies. Furthermore, the reduced fatty acid oxidation previously observed by Gaster et al.(237) was obtained by comparing obese T2D with obese control myotubes, as in this paper. We observed that myotubes from obese T2D, as compared to obese control individuals, have reduced capacity for mitochondrial oxidation, by some unknown mechanism probably down-stream of fatty acid β -oxidation. Preincubation with fatty acids stimulate the enzymatic machinery for fatty acid handling (i.e. uptake, activation, β -oxidation and lipid synthesis), but to a lesser extent mitochondrial complete oxidation generating CO_2 . Thus, T2D myotubes exhibit excess β -oxidation activity when challenged by fatty acid preincubations, resulting in enhanced release of β -oxidation intermediates. The reduced capacity for removal of fatty acid intermediates via complete fatty acid catabolism, may result in increased levels of potentially lipotoxic intermediates like acyl-CoAs, DAGs and ceramides, known to negatively affect insulin sensitivity and insulin-stimulated glucose disposal. Temporal storage of excess incoming fatty acids in imTAG may provide protection against acyl-CoAs and DAGs that are substrates for the final enzymatic step in TAG synthesis. In this study we observed that preincubation with EPA enhanced TAG synthesis and reduced acyl-CoAs specifically in T2D myotubes, besides increasing both glucose and PA oxidation. Thus, EPA may prove beneficial for skeletal muscle energy handling in diabetic individuals. Likewise, preincubation with TTA enhanced glucose and fatty acid oxidation, and thereby “restored” the complete fatty acid mitochondrial oxidation in T2D-myotubes to the baseline level of the obese control cells. Preincubation with TTA also opposed the fatty acid-induced increase in TAG synthesis, but whether this is a positive effect is unknown as total acyl-CoA level was elevated in cells exposed to TTA. However, TTA-CoA probably accounted for this elevation, which may not be negative, because we observed that glucose metabolism (basal and insulin-stimulated) was unchanged or increased (oxidation) in myotubes preincubated with TTA. Altogether, our data from cell experiments suggest that both TTA and EPA may improve overall oxidative glucose and fatty acid metabolism in skeletal muscle from T2D as well as obese subjects.

Paper III: Cell based multiwell assays for detection of substrate accumulation and oxidation

Studies investigating cellular metabolism have one feature in common, the difficulty in reliably determining substrate oxidation and accumulation in the least time- and resource-demanding way. Traditionally, substrate oxidation to CO₂ has been carried out in small flasks (12 cm²) by capturing ¹⁴C-labeled CO₂ on a filter soaked with an alkali solution while maintaining a closed environment to avoid leakage and loss of CO₂. This method is far from optimal when it comes to amount of “hands-on” time as well as consumption of resources like cells, media, substrates, scintillation cocktails and other chemicals. It may therefore be safe to say that studies involving measurements of cellular substrate oxidation in relation to compound effects or functional disorders have been seriously hampered by the lack of an efficient and low-cost method for assessment of CO₂ release. Similarly, in many circumstances determining how cellular uptake of particular substrates changes with time and concentration may be of great importance. These types of measurements are labor intensive, time-consuming and usually require a large amount of cells. Thus, there are considerable potentials also for improving these types of studies by real-time measuring cellular accumulation of radiolabeled substrates with so-called scintillation proximity assays. SPAs allow β-emitting substrates to be quantitatively determined depending on their accumulation in adherent cells growing on the surface of scintillating plastic. Cytostar-T™, developed by Amersham, provide a commercially available system for growing cells in 96-well tissue-coated plates, and allowing quantitative detection of radiolabeled substrates accumulated time-dependently in cells in real time. The main benefit is the ability to measure time-dependent accumulation of various substrates in the same cells without harvesting the cells. Although different products have been on the market for many years, we could not find any description of SPA utilized for the study of cellular accumulation of fatty acids and glucose.

In this work we have developed two general principles for quantitative measurements of substrate accumulation and oxidation (to CO₂) in living cells growing in a multi-well system. Our first approach was to show that ¹⁴C-labeled CO₂ could be efficiently and reliably captured in a 96-well system by applying one standard 96-well tissue-culture plate and one 96-well glass fiber filter plate, where each well was sealed with a cover slip. The filters were soaked with an appropriate volume of NaOH (1M), and then inverted to be aligned upon the corresponding wells of the culture plate. To ensure an air-tight system, a ready made silicon gasket with 96 holes was placed between the two plates, each hole aligned with both the bottom and top well. Furthermore, the “sandwich” was placed in a custom made clamp/apparatus designed to fit snugly with the bottom 96-well plate, and a precisely cut flat metal plate was placed on top of the upper filter plate. The system was finally closed tight by applying static pressure on the upper metal plate, delivered by locking a knee-joint handle on the clamp in the upright position. To allow quantitative determination of ¹⁴CO₂ generated from living cells, we had to use a bicarbonate-free buffer during incubation. Thus, PBS with addition of Ca²⁺, Mg²⁺ and HEPES was chosen as standard incubation medium. The incubation times tested varied from 2-6 hours, where 4 h was used most frequently. At termination of incubation the filter plate was removed, and added a scintillation cocktail and counted in 96-well β-counter. With this improved method we could demonstrate a long linearity range, comparable results to the previous flask method mentioned above and precise measurements of known kinetic parameters, generated from both myotubes and HepG2 cells. Our second approach was to investigate the possibility and conditions required for measuring time-dependent cell-accumulation of radiolabeled substrates such as fatty acids and glucose in human myotubes and SGBS adipocytes by utilizing the SPA technique. Our results

demonstrated that the SPA approach produced reliable time-dependent results on accumulation of different fatty acid substrates at various concentrations, as well as on insulin-stimulated glucose uptake.

We use the two methods for quantitative and accurate measurements of accumulation as well as oxidation of radiolabeled fatty acids and glucose in myotubes, adipocytes and liver cells. Our systems are versatile and can be adjusted to encompass different cell types and types of labeled fuels/substrates. One of the main advances is the use of standard 96-well format, which allows scintillation counting in commercial 96-well plate readers found in many laboratories. All equipments are relatively inexpensive, and the design of the plate-clamp should be easy to replicate in a metal workshop, thus making these methods available for most scientists. In this article we describe some basic requirements for our methods to work satisfactory, although careful optimization is required before applying the same principles to other cell types or other substrates.

Paper IV: Dietary supplementation of tetradecylthioacetic acid increases feed intake, but reduces body weight gain and adipose depot sizes in rats fed high-fat diets

Energy balance in complex organisms such as mammals is obtained by an intricate network of hormones, neuropeptides and sympathetic/parasympathetic innervations, controlling energy intake via emotions like hunger and satiety as well as regulating energy expenditure by physical activity or futile energy cycling (e.g. uncoupling). To avoid development of obesity or anorexia, our bodies need to assess the energy density of our diets and balance the energy intake with the expenditure. Carbohydrate, protein and fat contain different amount of extractable energy, with e.g. palmitic acid per molecule containing more than three times the chemical energy compared to a glucose molecule. Thus, fatty acids may signal their presence to the body, and one proposed receptor group are the orphan nuclear receptors PPAR alpha, delta and gamma. Activation of PPARs may have many different effects depending on the organs expressing them, the co-factors associated with their activation and which receptor isoform that gets activated. Feeding rats with agonists specific for one isoform is one way of determining e.g. the metabolic impact of PPAR α activation. Rats fed the specific PPAR α ligand fenofibrate reduce weight and feed intake compared to animals fed a control diet, whereas the PPAR γ specific ligand rosiglitazon enhances feed intake, body weight gain and adipocyte proliferation. Both ligands, however, reduce plasma lipids, but fenofibrate in addition enhances HDL-cholesterol and appears cardio protective, whereas rosiglitazon improves skeletal muscle insulin sensitivity. In an attempt to take advantage of both the cardio protective and insulin sensitizing properties and avoid the adverse effects like weight gain, dual- and pan-PPAR activators have been synthesized.

One such dual PPAR α/γ activator is ragaglitazar promoting reduced plasma lipids but only moderate effects on weight change and feed intake in rats. TTA is a synthetic fatty acid analogue, which is resistant to fatty acid β -oxidation. Several studies have demonstrated that TTA is a fairly potent activator of all PPAR-isoforms, where the isoform-specific potency may depend partly on cell types and species investigated(158). Besides its beneficial effect on plasma lipids, TTA increases hepatic β -oxidation, enhances mitochondrial and peroxisomal proliferation in several tissues, and may reduce visceral fat pads and enhance glucose tolerance in high-fat fed rats (238). TTA has also shown promising effects on several cancer models(164; 239) and may affect keratinocyte proliferation and differentiation in diseases such as psoriasis(163). Although being a pan-PPAR-activator, TTA may execute some of its

effects via non PPAR-mediated mechanisms like enzymatic competitive inhibition. Whether TTA may affect body weight gain and feed intake in rats fed a high-fat diet similar to other PPAR-activators is currently unknown. Thus, this study was performed to investigate the long-term impact of adding TTA to a lard-enriched diet providing ~ 40% energy from fat to rats up to 7 weeks. The main outcomes measured were body weight gain, adipose tissue depot sizes, feed intake, body composition and mRNA abundance of several genes in different adipose tissues and liver.

Rats fed diets supplemented with TTA drastically reduced their body weight gain from day 10 and onward, as compared to lard-fed controls. Similarly, feed efficiency dropped in TTA-fed rats and remained below lard-fed controls from day 10 and until the end of the feeding experiment. Feed intake fell between day 8 and 10 in rats fed TTA, but steadily increased thereafter, and was significantly higher than in lard-fed animals during the last two weeks of the feeding. Carcass analysis showed reduced amounts of fat, protein and water in rats given TTA, and the ratio between fat and protein content was reduced, suggesting that rats fed TTA became smaller as well as slimmer than the lard-fed controls. Evaluation of the different fat depots with magnetic resonance (MR) imaging and dissection showed that visceral tissues as well as abdominal subcutaneous fat depots were reduced in sizes in rats fed TTA. Relative to body mass, only epididymal and perirenal/retroperitoneal tissues were significantly reduced by TTA feeding, although mesenteric tissues tended to be similarly reduced as well. We measured plasma lipids, and confirmed previous results with marked reductions in TAG, phospholipids, cholesterol and free fatty acids, plasma glucose and insulin on the other hand were unchanged by TTA administration for 7 weeks. However, rats fed TTA had reduced plasma CRP and tended to have reduced plasma leptin and enhanced plasma adiponectin. We further investigated fatty acid β -oxidation, and observed a 3-fold increased oxidation of palmitoyl-CoA in livers and hearts from rats fed TTA, whereas skeletal muscle lipid oxidation surprisingly was unchanged when compared to lard-fed animals. Moreover, neither skeletal muscle glycogen content, nor insulin-stimulated glucose transport was significantly changed by TTA. We measured the abundance of several relevant genes in the different adipose tissues, but observed relatively few changes consistent in more than one adipose depot. One notable exception was enhanced mRNA levels of *Ucp1*, *Fasn* and *Acaca* in visceral adipose tissues, the same tissues that were most reduced relative to body weight in rats fed TTA. UCP1 protein levels in WATs, however, remained below the detection limits of standard western blotting, and may therefore not be of relevance in explaining the reduced depot mass. As expected, however, livers of TTA-fed rats showed marked changes in both liver size and gene expression, with more than 50% increase in mass and altered mRNA levels by more than 50% of the genes examined, respectively. Among others, several genes important for uptake, handling, activation and oxidation of fatty acids had increased abundance in livers of TTA-fed rats, including *Cd36*, *Acs11*, *Cpt1*, *Cpt 2* and ectopic expressions of *Lpl* (12-fold) and *Ucp3* (1900-fold). Hepatic UCP3 expression was also markedly increased in TTA-fed as compared to control rats.

Collectively, these data demonstrate a remarkable effect of TTA on rats fed a high-fat lard-based diet, decreasing weight gain, while concomitantly increasing feed intake. The reduced feed-efficiency and plasma lipids induced by TTA-administration may partly be explained by an increased hepatic fatty acid uptake and β -oxidation, and possibly by increased UCP3-mediated energy dissipation. At the same time, the enhanced levels of *Ucp1* in visceral adipose tissues of rats fed TTA may suggest that energy uncoupling could partly account for the reduced feed-efficiency as well as the smaller sizes of these adipose depots relative to body mass. The time dependent reduction of appetite observed in TTA-fed rats, may relate to increased PPAR γ activity in response to TTA accumulating over time. Alternatively, TTA

seemed to reduce plasma leptin, a well-known regulator of satiety. Other mechanisms might explain TTA's effect on feeding behavior, such as malonyl-CoA inhibition by TTA-CoA in the hypothalamus or changes in secretion of satiety-regulating peptides from the gut or brain. Although the latter possibilities are quite speculative, our study reveals important questions that remain to be answered. Hence, they illustrate the need for further studies to elucidate the mechanisms governing the metabolic impact of this interesting fatty acid analogue.

7. Discussion

In **paper I**, we reported for the first time that chronic activation of LXRs may affect glucose uptake and oxidation as well as promote a strong effect by increasing fatty acid uptake and accumulation as complex lipids in human myotubes. Furthermore, myotubes derived from obese T2D subjects showed an ectopic response towards LXR-activation, with enhanced incorporation of PA into complex lipids and reduced PA-oxidation as compared to control myotubes from lean non-diabetic individuals.

Previously, Muscat et al. have demonstrated that LXR may have functional roles in skeletal muscle and myotubes from mice. They could show enhanced cholesterol efflux from skeletal muscle treated with the LXR-activator T0901317, and observed muscle specific changes in mRNA abundance of several genes involved in reverse cholesterol transport and lipid metabolism. Levels of LXR α mRNA also showed a peak during differentiation of C2C12 myotubes, suggesting a myotube-specific role of this nuclear receptor(71). Later, Dalen et al. demonstrated that GLUT4 expression in adipose tissues was dependent on LXR α (60). We found that chronic activation with T0901317 markedly enhanced GLUT4 and LXR α mRNA levels in human myotubes, but insulin-stimulated glucose transport was only marginally increased. Human myotubes traditionally display a blunt response in glucose transport towards insulin-stimulation as compared to skeletal muscle *in vivo*(240). The reason for this is unknown, but may involve the relatively low expression of GLUT4 with respect to GLUT1 in cultured myotubes (241; 242) as compared to muscle fibers *in vivo*(243). The nutrient metabolism in human myotubes also resembles more type 2 glycolytic muscle fibers, with enhanced basal glucose turnover, reduced insulin responsiveness and reduced fatty acid oxidation as compared to type 1 oxidative fibers(241; 244). Our finding of LXR-activation promoting fatty acid uptake and accumulation could in part be explained by the enhanced mRNA levels of the alleged fatty acid transporter CD36, either via direct transcriptional activation by LXRs or indirectly via LXR-mediated up-regulation of SREBP-1c and PPAR γ mRNA expressions. CD36 promotes fatty acid uptake and may be translocated to the plasma membrane in response to insulin or contraction much like GLUT4(245; 246).

The LXR-mediated effects on lipid accumulation was later confirmed in two other studies, and both demonstrated an increased abundance of ACC, FAS and SCD1 in myotubes incubated with LXR-activators, implying enhanced *de novo* lipid synthesis(247; 248). In myotubes from subjects with T2D we further observed an ectopic response in accumulation of PA as compared to myotubes from lean non-diabetic subjects. Skeletal muscle from insulin resistant and T2D individuals in general display accumulation of more imTAG than matched controls, and this imTAG is thought to relate to the development of these afflictions(177; 249). The mechanisms underlying excess imTAG storage is not clear, but seems to be related to obesity and thus increased availability of fatty acids(249). Storage of incoming fatty acids as imTAG depends on the balance between the rates of uptake and elimination. In skeletal muscle, fatty acids mainly arrive from the blood and are eliminated by oxidative catabolism(250). Activation of LXRs seemed to markedly govern fatty acid accumulation in human myotubes by up-regulation of the CD36 mRNA levels in both groups of myotubes (**paper I**). Bonen et al. also reported enhanced levels of membrane-bound CD36 in association with increased imTAG accumulation of fatty acids in obesity and T2D(251). However, this was not observed in another study by Bruce et al.(197).

In myotubes from lean controls we observed a slight increase in fatty acid oxidation to CO₂ after preincubation with the LXR-activator, whereas cells from subjects with T2D did not. This difference in oxidative response may partly explain the enhanced lipid accumulation, although the differences in magnitudes were quite substantial, with overall changes in lipid-

accumulation being several-fold greater than in lipid oxidation. The discrepancy between oxidation and accumulation responses could be explained by either T2D-specific LXR-activation/inhibition of genes involved in complex lipid synthesis or in lipolysis/re-esterification of fatty acids. Alternatively, T2D myotubes may have an intrinsic enzymatic configuration favoring lipid storage that was just passively reflected by the enhanced fatty acid uptake caused by chronic activation of LXRs.

Skeletal muscle fatty acid uptake and oxidation must be closely regulated to avoid increased intracellular levels of fatty acids and other potential lipotoxic lipids. As we have discussed, LXRs may constitute a transcriptional switch that regulates fatty acid uptake in skeletal muscle and stimulates myotubes from individuals with T2D to deposit more fatty acids as imTAG (**paper I**). Fatty acid uptake and metabolism may however also be determined via another type of nuclear transcription factors, namely the PPARs. Fatty acids may themselves function as ligands for PPARs, and the specificity for any certain isoform may differ between them(80). Previous studies with fish oils (containing high amounts of n-3 PUFA) had shown beneficial effects on skeletal muscle insulin resistance in rat(129; 130). EPA has potentially beneficial effects on glucose uptake in skeletal myotubes, but may enhance imTAG accumulation(156).

In **paper II** we explored the impact of chronic preincubations with naturally occurring fatty acids and the sulfur-substituted fatty acid analogue TTA on intermediary glucose and fatty acid metabolism in myotubes obtained from obese T2D and obese control individuals. We observed that T2D myotubes have reduced capacity for palmitic acid oxidation to CO₂ as compared to obese control cells, and that this dysfunction was improved, but not corrected by preincubation with TTA. Furthermore, preincubation with TTA and EPA enhanced fatty acid β -oxidation, leading to increased release of fatty acid β -oxidation intermediates with a significantly stronger response in T2D myotubes. Our data from human myotubes support the notion that skeletal muscle of insulin resistant and T2D individuals may have reduced mitochondrial capacity at some stage in the TCA cycle or in the electron transport chain/ATP synthase machinery(192; 194; 195), leading to release of excess β -oxidation intermediates during increased fatty acid β -oxidation(200). Very recently, evidence have emerged indicating that indeed reduced TCA flux in T2D myotubes may explain the reduced complete PA oxidation observed by us and others(252). The nuclear transcription cofactor PGC1 α is an important molecular regulator controlling skeletal muscle fiber type composition as well as fatty acid and glucose metabolism(202). Reduced PGC1 α activity may be implicated in the development of insulin resistance and T2D by its effects on mitochondrial biogenesis. Several studies have shown that skeletal muscle of T2D individuals have reduced activity and mRNA levels of PGC1 α (199; 205; 206; 253). However, these data are not entirely conclusive as others have reported no such difference between insulin-resistant offspring of T2D parents and control individuals(196). We observed in **paper II** that fatty acid preincubation increased the abundance of PGC1 α mRNA in human myotubes, whereas T2D myotubes displayed a reduced response as compared to control cells. Several studies have observed that fatty acids may regulate the mRNA abundance of PGC1 α in skeletal muscle, possibly via activation of PPAR δ (254; 255). We also found the relative mRNA levels of PGC1 α and DGAT2 to correlate positively, an effect that could help explaining the finding of increased imTAG storage in endurance trained individuals(256). Endurance training increases the proportion of type 1 fibers in skeletal muscle probably via PGC1 α activity (257) and type 1 fibers generally have more mitochondria, oxidize fatty acids better and show enhanced insulin-stimulated glucose disposal as compared to type 2 fibers(38; 39). In T2D individuals, skeletal muscle were associated with reduced number of type 1 fibers as well as reduced oxidative capacity(193-195).

In addition to increased release of β -oxidation intermediates, reduced mitochondrial phosphorylation combined with increased fatty acid uptake, may result in high intracellular levels of ceramides, acyl-CoAs, DAGs and TAGs(182-185). In particular, we observed an enhanced response with TAG accumulation in T2D myotubes as compared to control cells after preincubation with either the LXR activator T0901317 (**paper I**) or EPA (**paper II**), both strongly promoting fatty acid uptake and accumulation. Although insulin resistance is associated with increased imTAG accumulation, it may not be that harmful in itself. In fact, recent studies have shown that genetically modified mice, either over-expressing the TAG-synthesizing enzyme DGAT1 (186) or knocking out the TAG-hydrolyzing enzyme ATGL(187), enhance imTAG storage in addition to improving glucose tolerance. Moreover, enhanced incorporation of incoming palmitic acid into TAG was associated with protection against apoptosis in cultured fibroblasts, and co-incubation with oleic acid promoted TAG partitioning (258) Thus, imTAG storage may rather be protective against high levels of insulin desensitizing lipid intermediates like acyl-CoAs, DAGs and ceramides(259). Incubation with T0901317 also reduced PA-mediated lipotoxicity in primary human arterial endothelial cells, through up-regulation of SCD-1 (converting C16:0 to C16:1), improving incorporation of PA into more inert TAG-containing lipid droplets(260). Furthermore, lipid induced SCD-1 expression may similarly function to protect skeletal muscle from PA induced endothelial reticulum stress as demonstrated in primary human myotubes(261). In **paper II** we also observed that T2D myotubes preincubated with EPA had reduced total, but enhanced C16:1-CoA, with increased capacity for directing incoming fatty acids either into imTAG or oxidation. Furthermore, neither LXR-activation nor EPA-incubation impaired insulin-stimulated glucose uptake, and both improved glucose oxidation in myotubes from subjects with T2D (**papers I and II**).

Preincubation of control and T2D myotubes with the sulfur-substituted fatty acid TTA enhanced mitochondrial fatty acid and glucose oxidation, indicating increased mitochondrial biogenesis (**paper II**). TTA-feeding has been shown to increase mitochondrial content in skeletal muscle, liver and heart of rats(165; 262). However, in rat *gastrocnemius* muscle we did not observe differences in β -oxidation after feeding rats TTA for 7 weeks, whereas both liver and heart experienced a 3-fold increase (**paper IV**). The explanation for this could be the longer feeding time (12 weeks) used in the previous studies, or alternatively the differences between TTA effects on fatty acid β -oxidation in human myotubes and rat skeletal muscle may be attributed to different specificities towards PPAR α and PPAR δ . TTA seems better at activating rat PPAR α than PPAR δ (160; 164), whereas the opposite has been demonstrated for human PPARs(163). This may partly explain why TTA has particularly strong effects on hepatic metabolism in rodents where PPAR α is a major regulator, but relatively modest effects in skeletal muscle where PPAR δ may play a more prominent role(77; 111). In human myotubes, PPAR δ seems to play a dominant role over PPAR α in controlling lipid oxidation(114), and we have demonstrated that preincubation with the PPAR δ selective agonist GW501516 and TTA, but not the PPAR α selective agonists fenofibrate and clofibrate (at PPAR α selective concentrations), could induce increased mitochondrial fatty acid oxidation in human myotubes(263). In **paper II**, however, UCP2 was the only potential candidate explaining the TTA-specific augmentation of mitochondrial fatty acid oxidation as compared to the other fatty acid preincubations. Recently, it has been speculated whether increasing mitochondrial fatty acid β -oxidation may worsen rather than improve insulin resistance in skeletal muscle without a compensatory increase in the capacity for complete oxidation of β -oxidation intermediates(200). Studies have shown improved insulin response when inhibiting fatty acid β -oxidation either chemically with CPT1 inhibitors (264; 265) or genetically by deletion of malonyl-CoA dehydrogenase (MCD) in mice (200) as well as in myotubes by MCD-specific siRNA(266). Thus, the improved mitochondrial fatty acid

oxidation induced by TTA in human myotubes (**paper II**) may not be entirely beneficial, even though we did observe enhanced glucose oxidation and no detrimental effects on glucose uptake and glycogen synthesis. Moreover, endurance trained skeletal muscles are more insulin sensitive despite higher capacity for fatty acid oxidation (267) and this may be related to a better protection against lipid peroxidation. UCP2 is thought to be involved in reducing oxidative stress and lipid peroxidation in mitochondria(268), and PGC1 α may regulate the expression of UCPs(269). Our finding that TTA increased the UCP2 mRNA levels in human myotubes might therefore be a beneficial effect, protecting against mitochondrial stress caused by enhanced fatty acid β -oxidation(200).

In **paper IV**, we further studied the impact of feeding rats a high-fat diet with or without addition of TTA on body weight gain, feed intake and adipose tissue development. Our findings of reduced mass of epididymal and perirenal tissues, enhanced hepatic fatty acid β -oxidation and reduced plasma lipids in rats given TTA was in accordance with previous publications (reviewed in(238)). Furthermore, we observed that TTA-fed rats grew smaller while at the same time consumed more feed during the last two weeks of the study as compared to lard-fed controls. Carcass-data on whole animal composition indicated that TTA-feeding restricted both adipose tissue and whole animal growth. By analyzing mRNA abundance we could show that TTA-feeding induced ectopic expressions of *ucp3* in liver and *ucp1* in visceral adipose tissues. Thus, increased energy dissipation by either UCP3 or UCP1 could explain the reduced feed efficiency induced by TTA. Further analyzes by Western blotting revealed a concomitant increase in hepatic UCP3 protein, whereas visceral WAT UCP1 protein expression remained below the detection limit. The mRNA expression of *ucp1* in WAT may be dependent on PGC1 α activity (257) or activation of PPAR γ , crucial for controlling conversion of WAT to BAT(270). Because the initial mRNA levels of *ucp1* in visceral WATs of the control animals were very low, a 10-100-fold relative increase in TTA-fed rats may not influence UCP1 protein levels and thus energy consumption to a sufficient degree in these tissues. The observed reduced visceral WAT sizes in rats fed TTA may therefore be explained by other factors than uncoupling, such as reduced storage or enhanced release of fatty acids from WAT. In a recent review, Berge et al. put forward a hypothesis where TTA-feeding may induce drainage of peripheral fatty acids by increased fatty acid β -oxidation and ketone body formation in the liver(158). Studies of rats given the PPAR α activator fenofibrate have demonstrated *de novo* hepatic expression of UCP3 associated with enhanced energy uncoupling(271; 272), and TTA has been shown to promote mitochondrial energy uncoupling(273). Therefore it seems plausible that the observed reduction in feed efficiency may involve enhanced energy dissipation, possibly involving hepatic processes including UCP3 expression. Alternatively, other explanations could be reduced fat absorption by the gut, and hence loss of energy in the feces, or perhaps enhanced energy expenditure through increased locomotion and/or shivering. Our daily observations did not indicate such effects, and studies looking at fat in feces observed no difference between TTA and control-fed animals, indicating that such mechanisms are less likely to explain the reduced feed efficiency in our study.

TTA is a pan-PPAR activator, which may explain the somewhat surprising results of reduced body weight gain combined with increased feed intake. Feeding experiments with PPAR α activators have demonstrated both reduced weight gain as well as feed intake(84; 274), whereas addition of rosiglitazone increases feed consumption (107) and weight gain(109). Hence, rats fed dual and pan-PPAR agonists may experience a combination of opposing PPAR effects, as observed with ragaglitazar (109) or TTA (**paper IV**). We cannot, however, exclude the possibility of other mechanisms promoting the increased feed intake caused by

TTA administration, such as reduced leptin concentration in plasma or more direct interactions between TTA and the hypothalamic regulation of feeding behavior.

Paper I and **II** revealed the need for more convenient, recourse and time-saving methods for assaying fuel handling, such as nutrient uptake, accumulation and oxidation to CO₂. The relevance of better screening techniques was also exemplified in a paper by Ukropcova et al(189). They used an improved method for assaying fatty acid oxidation (275) to show important correlations between intrinsic oxidative capacities in human myotubes and metabolic donor-characteristics, like respiratory quotient and insulin sensitivity. This prompted us to develop two new methods for measurement of nutrient handling in cells grown in 96-well plates (**paper III**). Our new methods are versatile, less laborious and more cost-effective than previous assays, and they have successfully been implemented in recent publications(248; 263; 276), including **paper II**. We think that application of these new assays will enable researchers to improve their understanding of nutrient metabolism under different cellular conditions in a cost-effective way.

Cultured human myotubes as a model of skeletal muscle

Cultured human myotubes are morphologically, metabolically and biochemically similar to adult skeletal muscle(277), and thus is a valuable *ex vivo* model to distinguish between genetic and environmental factors in the etiology of e.g. insulin resistance(278). Satellite cells are isolated from skeletal muscle biopsies and then proliferated and differentiated to become mature myotubes. Primary satellite cells from human or animal donors may proliferate for many passages as well as frozen and thawed like immortalized cell-lines. The drawbacks are that isolation of pure satellite cell colonies may not be complete, thus importing minor impurities with different cell types such as fibroblasts. There is also some discussion about whether the satellite population may have more than one lineage, and the composition of this mix is not exactly known, or perhaps not even fixed from one human to another. Furthermore, culturing of primary human myotubes generally takes more time than other cell-lines because of slower growth. Another drawback related to T2D research is that measurements of insulin-stimulated glucose uptake tend to be only about 50% increased as compared to basal uptake, thus making it more difficult to investigate differences in this parameter. Several genes in myotubes including GLUT4, PPAR α and CPT1 β are expressed at lower relative levels than in skeletal muscle *in vivo*.

Cultured human myotubes are the most similar cell system to intact skeletal muscle that can be modulated *ex vivo*. Compared to rodent models they express the right genetic background as well as some of the specific skeletal muscle phenotype. The extracellular environment can be controlled precisely and kept relatively constant over time, without interference by systemic homeostatic compensatory mechanisms. We and others have reported several potential intrinsic deficiencies in myotubes from individuals with T2D including lower basal palmitate oxidation(237), and impaired insulin-stimulated glucose metabolism(240; 278). In a recent paper Ukropcova *et al.* demonstrated that the ability to suppress fatty acid oxidation with glucose in human myotubes *in vitro* was negatively associated with the *in vivo* metabolic flexibility (MF) and insulin sensitivity (IS) of the donors as measured by euglycemic hyperinsulinemic clamp(189). Furthermore, in the same study, the degree of fatty acid oxidative adaption by the myotubes towards increasing concentrations of fatty acid in the medium was positively correlated with individual donor IS and MF(189).

Choosing a proper control is important for what conclusions may be drawn on the etiology of the diabetic phenotype. As we and others have mainly worked with myotubes derived from obese individuals with T2D, perhaps the most proper control would be to use myotubes from

individuals without T2D, but with similar BMI. However, if obesity is such a strong determinant for the development of T2D, obese subjects without signs of T2D might be considered a “super-cohort” that may be especially adapted at tackling the metabolic stresses associated with overnutrition. On the other side, choosing slim subjects without T2D, could mean that they belong to the same cohort as the obese T2D subjects, only that they have not by any reason become obese and consequently have not developed T2D.

In this thesis **paper I** utilized lean control myotubes whereas **paper II** used “obese” ones. There seemed to be a more clear difference in fatty acid complete oxidation to CO₂ between myotubes in **paper II** than in **paper I**, and that may be because skeletal muscle of obese non-diabetic subjects are better suited than average to handle fatty acids without detrimental effects on insulin signaling. This aspect, however, needs to be further clarified in future studies.

8. Conclusion

In this thesis we demonstrate that:

1. Skeletal muscle of insulin resistant and T2D individuals may have reduced mitochondrial oxidative capacity. Thus, when T2D myotubes experience increased fatty acid availability, like during obesity, the enhanced flux through fatty acid β -oxidation may create a surplus generation and consequently release of β -oxidation intermediates, perhaps to avoid cellular damage or disruption of cellular functions.
2. Fatty acids or LXR-agonists may enhance fatty acid uptake in skeletal myotubes, and after preincubation with either T0901317 or EPA imTAG storage was particularly enhanced in T2D relative to control myotubes.
3. Increased storage of fatty acids as imTAG may function as a protective mechanism against high levels of insulin-desensitizing lipid intermediates such as acyl-CoAs and DAG. EPA specifically decreased acyl-CoA levels in T2D myotubes, and preincubations with both EPA and T0901317 improved, rather than worsened glucose handling in control as well as T2D myotubes.
4. TTA improved mitochondrial glucose and fatty acid oxidation in diabetic as well as control myotubes, without detrimental effects on insulin-stimulated glucose uptake and glycogen synthesis. Furthermore, TTA reduced body weight gain but increased feed intake in rats fed a high-fat diet, indicating effects of this hypolipidemic fatty acid analogue on whole body energy consumption.
5. Previous laborious methods for measuring cellular nutrient uptake and CO_2 -catabolism can be scaled down to 96 wells format, allowing the use of SPAs and our novel CO_2 -trapping technique, thereby providing more time and cost-effective analyzes.

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