Regulation of energy metabolism in human skeletal muscle cells

Effects of fatty acids, *in vitro* exercise and extreme obesity with and without type 2 diabetes

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Dissertation for the degree of Philosophiae Doctor (Ph.D.)

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2013

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
No. 1319

ISSN 1501-7710

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

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Acknowledgements

The work presented in this thesis was carried out at Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo during the period 2009-2013 and I would like to thank for the opportunity to do this PhD. During my 4 years doctorate, many people have influenced my life. First of all, I would like to thank my supervisors Arild C. Rustan and G. Hege Thoresen for teaching me a lot and who always were there with an open door and offered help. Thanks to Arild for his sometimes crazy ideas and to Hege for making things possible. Thanks to you both for always listening to me and to allowing me to do almost all I wanted.

Thanks to Camilla Stensrud, Nataša Nikolić, Nina P. Hessvik, Xiang Y. Kong, Eili T. Kase and Yuan Z. Feng for both academic collaboration and for your friendships. Camilla and Nataša, thanks for that I always could burge into your offices with my news or concerns. Thank you Vigdis Aas for the collaboration on the "EO project" and for being the good person that you are. Furthermore, I would like to thank all the members of "Muskelbunten" throughout the years 2009-2013 including many talented master students and a special thanks to Lisbeth Damlien and Line Lauvhaug for their contribution also after their master thesis. Thanks to Arild, Hege, Vigdis and Eili for helpful comments on this thesis.

I would also give my gratitude to my co-authors, especially Cedric Moro who teached me a lot, and to our collaborators at Centre of Morbid Obesity, especially Jøran Hjelmesæth, Department of Nutrition and Institute of Molecular Biosciences, especially Gerbrand Koster.

Furthermore, I would like to thank "4 etasje" at Pharmacy; you all are such good and nice people that always meet me with a smile in the morning and thanks for making these 4 years go by so fast. Especially I would like to thank Robert, my "room-mate" that always helped me with small and big things and Ida for stopping by and providing an energy boost – without the two of you my thesis-writing period had not been as bright.

Finally I would like to thank my family for help and believing in me, and Amin for his support and encouragements. Finally, I would also thank all my friends, especially Shiva, for all their support these years and my cousins Marit and Maria for support and valuable discussions. I love you all!

Oslo, December 2012

Siril S. Bakke

Abbreviations

ACC	acetyl-CoA carboxylase	**IMCL**	intramyocellular lipids
ACBP	acyl-CoA binding protein	**IMTG**	intramyocellular triacylglycerol
ACSL	acyl-CoA synthetase	**KO**	knockout
Akt/PKB	protein kinase B	**LA**	linoleic acid
AMPK	AMP-activated protein kinase	**LCFA**	long chain FA
ANGPTL4	angiopoietin-like protein 4	**LD**	lipid droplet
AS160	Akt substrate of 160 kDa	**LDL**	lipoprotein lipase
ATGL	adipose triacylglycerol lipase	**LXR**	liver X receptor
BMI	body mass index	**LMM**	linear mixed model
CACT	carnitine-acylcarnitine translocase	**MAG**	monoacylglycerol
CD36/FAT	FA translocase	**MGAT**	monoacylglycerol acyltransferase
CPT	carnitine palmitoyltransferase	**MGL**	monoacylglycerol lipase
CrAT	carnitine acetyltransferase	**MUFA**	monounsaturated FA
CVD	cardiovascular disease	**MYH**	myosin heavy chain
Cyc	cytochrome c	**OA**	oleic acid
DAG	diacylglycerol	**OGTT**	oral glucose tolerance test
DGAT	diacylglycerol acyltransferase	**PA**	palmitic acid
DHA	docosahexaenoic acid	**PDH**	pyruvate dehydrogenase
EPA	eicosapentaenoic acid	**PDK**	pyruvate dehydrogenase kinase
EPS	electrical pulse stimulation	**PGC-1α**	PPAR gamma coactivator 1-alpha
ETC	electron transport chain	**PKA**	protein kinase A
(F)FA	(free) fatty acid	**PKC**	protein kinase C
FABP	FA binding protein	**PLIN**	perilipin
FADS	FA desaturase	**PPAR**	peroxisome proliferator-activated receptor
FASN	FA synthase	**PUFA**	polyunsaturated FA
FATP	FA transport protein	**RQ**	respiratory quotient
GLUT	glucose transporter	**SCD**	stearoyl-CoA desaturase
GSEA	gene set enrichment analysis	**SFA**	saturated FA
HbA_{1c}	glycated hemoglobin	**SPA**	scintillation proximity assay
HDL	high density lipoprotein	**T2D**	type 2 diabetes
HOMA	homeostatic model assessment	**TAG**	triacylglycerol
HSL	hormone sensitive lipase	**TCA**	tricarboxylic acid cycle
IL	interleukine	**UFA**	unsaturated fatty acid
IR	insulin receptor	**WHR**	waist-hip-ratio
IRS	insulin receptor substrate		

Abstract

Obesity and type 2 diabetes (T2D) are associated with reduced metabolic flexibility and the development of these conditions are positively associated with dietary saturated fatty acids (e.g. palmitic acid) and negatively associated with poly- and monounsaturated fatty acids (e.g. ω-3 eicosapentaenoic acid and oleic acid). Furthermore, obesity is strongly associated with insulin resistance and T2D, however some extremely obese individuals may possess certain characteristics that protect them against developing T2D. Moreover, physical activity plays a central role in both prevention and improvement of these conditions and we wanted to establish an *in vitro* model for exercise (electrical pulse stimulation) to study these effects. This thesis aimed to explore some aspects of energy metabolism, especially lipid storage and turnover and metabolic flexibility in human skeletal muscle cells.

Eicosapentaenoic acid increased metabolic flexibility and lipid accumulation and upregulated interleukin-6 (IL-6) mRNA expression in myotubes from lean individuals. Palmitic acid had a lower lipid accumulation and upregulated adipose triacylglycerol lipase protein expression compared to oleic acid in myotubes. Eicosapentaenoic acid co-incubation with palmitic and oleic acid eliminated the differences by increasing palmitic acid accumulation. Furthermore, myotubes showed a higher palmitic acid oxidation than oleic acid oxidation, and palmitic acid upregulated the β-oxidation pathway. Furthermore, a reduced insulin response was observed in myotubes isolated from T2D subjects. Mitochondrial content and lipid accumulation was higher, while lipolysis was lower in extremely obese non-diabetic myotubes compared to cells from extremely obese diabetics. Besides, the metabolic flexibility parameters adaptability was higher and substrate-regulated flexibility tended to be higher in non-diabetics. However, myotubes from extremely obese subjects had a higher fatty acid oxidation, but a lower suppression of glucose on fatty acid oxidation than the myotubes from lean subjects. After electrical pulse stimulation of the myotubes derived from lean, healthy donors, number of lipid droplets and IL-6 mRNA expression tended to be higher, while oxidation, mitochondrial content,

muscle fiber type I marker expression and insulin sensitivity was higher than in unstimulated myotubes.

The results presented in this thesis, from extremely obese diabetic cells and electrical pulse stimulated/fatty acid-treated cells, suggest a favorable role of a higher capacity for intramyocellular lipid accumulation and increased metabolic flexibility with regards to improved insulin sensitivity and glucose metabolism in human skeletal muscle.

Introduction

Obesity

Lifestyle diseases as obesity and diabetes type 2 (T2D) are rapidly increasing worldwide (see Figure 1). In 2008 it was revealed that 40 % of the Norwegian population was overweight and that 10 % of these were obese [1].

Overweight and obesity is usually defined either by body mass index (BMI), waist circumference or waist-hip-ratio (WHR). BMI $\geq$ 25 kg/m^2 is considered overweight and BMI $\geq$ 30 kg/m^2 is considered obese, whilst a BMI $\geq$ 40 kg/m^2 is considered morbidly or extremely obese [2, 3]. Another method to define obesity is to consider the waist circumference or the WHR, which are considered above normal for European females/males if higher than 80/94 cm and 0.85/0.90 respectively [4, 5]. The reason for using WHR or circumference is that several studies have shown that visceral obesity poses a great health risk [6-10]; despite this, BMI is still the most used clinical measure. Some obesity scales also considers age, gender and ethnicity, although this is still controversial [11]. In most cases, obesity is caused by a combination of inactivity and excessive food energy intake (diet), although genetics and stress are factors that also may be involved [12-15]. The gut microbiota may also influence metabolic processes and should also be considered as an environmental factor that could contribute to obesity and its comorbidities [16].

A person that has accumulated so much fat that it negatively influences their health may be considered as overweight or obese [9, 17]. Overweight and obesity are strongly associated with insulin resistance and T2D [18, 19], and the majority of individuals with T2D are also classified as overweight or obese [2]. This association is complex and involves increased fatty acid release from visceral adipose tissue, a raised level of plasma free FAs (FFA) and an increased storage of ectopic fat that contributes to insulin resistance

in other peripheral tissues [20-22]. Adipose tissue is also an endocrine organ and secretes numerous bioactive peptides collectively known as adipokines [23, 24]. Examples include peptides such as adiponectin and resistin, hormones like leptin, chemokines such as monocyte chemotactic protein 1 and interleukine 8 (IL-8), other pro-inflammatory cytokines such as IL-6, IL-1 and tumor necrosis factor -α, and anti-inflammatory peptides such as IL-10 [25]. Adipokines are proposed to have a role in the interplay between adipose tissue and muscle [26, 27]. Dysfunctional adipose tissue has high triacylglycerol (TAG) degradation (lipolysis) that releases more FFA into the circulation. In total, this imbalance results in adipose tissue hyperplasia and hypertrophy, which in turn lead to inflammation and oxidative stress [28]. Obesity is associated with a chronic low-grade inflammation in the adipose tissue [29, 30], perhaps due to an imbalance between secretions of pro- and anti-inflammatory adipokines caused by this excessive TAG accumulation. In addition, obesity-induced inflammation may therefore be an important contributor to the induction of insulin resistance [31] and as part of the negative crosstalk between adipose tissue and skeletal muscle; obesity-associated adipokines may promote skeletal muscle insulin resistance [32, 33].

Type 2 diabetes

Diabetes increases in concert with obesity (see Figure 1). T2D comprises about 90 % of people with diabetes around the world [34] and in addition, undiagnosed T2D is quite common [35]. In Norway it is estimated that about 355 000 persons have T2D [36].

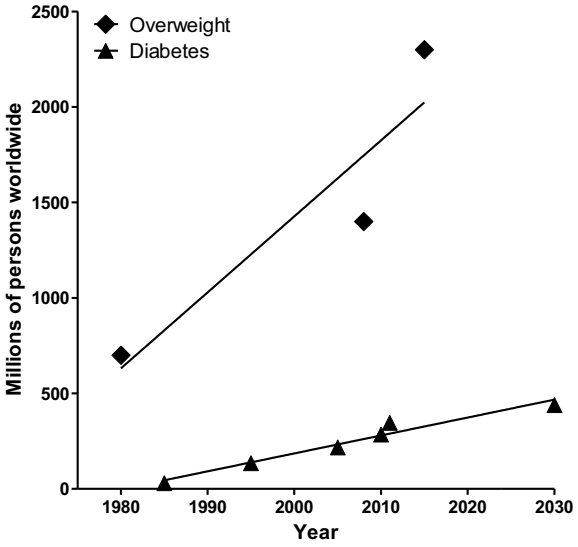

Figure 1. Incidence of overweight and diabetes in adults worldwide over time and predictions of the time to come [2, 37, 38] .

T2D is a chronic metabolic disorder characterized by elevated blood glucose values (hyperglycemia) and insulin resistance and/or deficiency. T2D is diagnosed by fasting blood ≥7 mmol/l for fasting blood glucose test and/or ≥11.1 mmol/l for a two hours oral glucose tolerance test (OGTT) [39]. In addition, glycated hemoglobin levels in blood ($HbA_{1c} \geq 6.5$ %) may also be determined [40, 41]. The probability of having an $HbA_{1c} \geq$ 6.5 % among cases of T2D based on OGTT criteria varies dramatically across ethnicities [42]. This indicates that T2D definitions should be adjusted for ethnicity. Furthermore,

homeostatic model assessment (HOMA) is a method used to quantify insulin resistance and β-cell function with insulin and glucose fasting values and this and hyperinsulinemic-euglycemic clamp may also be used to measure insulin resistance [43].

Development of T2D is believed to be caused by a combination of lifestyle factors such as physical inactivity, diet, stress, smoking, obesity and alcohol consumption. In addition, genetics, age, ethnicity, gender and some medications may predispose people for diabetes [44-46]. Treatments against obesity and T2D are mainly lifestyle intervention (physical activity and diet), but anti-obesity drugs and weight loss surgery are possibilities and in most T2D cases it is necessary to use drugs to normalize blood glucose levels [47].

As of year 2011, 36 diabetes-associated genes have been found that may contribute to the risk of development of T2D [48]. On a cellular level T2D is caused by insulin resistance in liver, adipose tissue and muscle, increased lipolysis from adipose tissue, increased glucose production in liver and a reduced insulin secretion from β-cells in the pancreas [49]. Insulin stimulates glucose utilization and storage of carbohydrates (glycogen), lipids and proteins, while inhibiting their degradation and release into the circulation. Further, insulin stimulates the uptake of glucose, amino acids and fatty acids into cells [50]. Insulin resistance is the inability of cells to respond sufficiently to normal levels of insulin.

Increased fatty acid release from adipose tissue and a raised level of FFA in blood in overweight persons is proposed to contribute to insulin resistance in other peripheral tissues [51-53]. Insulin resistance and T2D are strongly associated with overweight and obesity. Obesity is similarly characterized by reduced glucose uptake in adipose tissue [54], nevertheless fat tissue merely contributes to a small part of the total body glucose uptake and this alone cannot be the full explanation to the connection between obesity and insulin resistance. Moreover, induction of insulin resistance may also be initiated by obesity-induced inflammation and its corresponding cytokines [31]. There are still much that are not fully understood in the relationship between obesity and development of insulin resistance.

Metabolic syndrome

T2D increases the risk of cardiovascular diseases (CVD), microvascular complications – and even increases mortality [9, 45, 47]. In addition, overweight and obesity also increases the mortality and CVD rates, in addition to diabetes, musculoskeletal disorders, sleep apnea as well as some cancers [13].

Metabolic syndrome is a combination of disorders that increase the risk of developing CVD. Obesity or central obesity and insulin resistance or T2D are parts of the definition of metabolic syndrome. To diagnose metabolic syndrome the patient must have three conditions of the following: elevated waist circumference with regards to ethnic origin, dyslipidemia with raised triacylglycerol levels or specific treatment for this lipid abnormality, reduced high density lipoprotein (HDL)-cholesterol or specific treatment for this lipid abnormality, raised blood pressure or treatment of previously diagnosed hypertension or raised fasting plasma glucose or treatment of diagnosed T2D [55]. Causes discussed for developing metabolic syndrome are obesity (especially abdominal) and/or insulin resistance in addition to physical inactivity, age, hormonal imbalance or genetic abnormalities [56]. A study in 2002 estimated that approximately 22 % of American adults have the metabolic syndrome [57], indicating that metabolic syndrome is a quite common condition.

Dietary fat

Dietary fat is associated with development of obesity, insulin resistance and T2D. Intake of saturated fatty acids (SFAs) is positively correlated, while mono- and especially polyunsaturated fatty acids (MUFAs and PUFAs, essential fatty acids) have shown beneficial effects [58-60]. In a study performed in rats, a diet rich in SFAs caused insulin resistance, whereas a diet rich in ω-6 PUFAs resulted in increased insulin sensitivity [59, 61]. However, replacement of the ω-6 fatty acid (FA) linoleic acid (LA) with ω-3 FAs prevented the development of insulin resistance in high-fat fed rats [62]. Haugaard et al showed that changes in HOMA-IR were inversely correlated to changes in membrane concentrations of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and total ω-3 FAs, as well as the ratio of ω-3/ ω-6 PUFA in human skeletal muscle [63]. One meta study concludes that intake of ω-3 PUFAs might be weakly positively associated with the T2D risk [64], and two other meta studies did not find any effect of ω-3 FAs on development of T2D or insulin sensitivity [65, 66]. A third meta study reveals an ethnicity difference in the response [67]. However, a different meta study found that diets higher in ω-3 long chain PUFA may reduce cardiovascular risk in diabetes [68]. Thus, the mechanism by which UFAs (unsaturated FAs)/PUFAs exert positive effects on insulin sensitivity are yet unclear [69, 70]. Besides, ω-3 may not have an effect on body weight [71-73], however ω-3/UFA intake is reported to decrease inflammation in humans [74, 75]. An explanation for this may be that PUFAs are precursors for leukotrienes and prostaglandines that act pro-inflammatory and lipoksines and resolvines that act anti-inflammatory, in addition docosanoides [76, 77] and ω-3, as EPA, is precursor for the anti-inflammatory substances [78]. In addition, the beneficial effects of PUFAs might be explained in their role in the cellular membranes or acylation of proteins that alter membrane plasticity or protein function [79].

Energy metabolism in skeletal muscle

Many organs in the body might be involved in development of obesity and T2D, but skeletal muscle is one of the organs where insulin resistance are most prominent. Skeletal muscle constitutes about 40 % of human body mass in non-obese individuals and has been reported to account for 75-80 % of the body's insulin-stimulated glucose uptake [49, 80]. Skeletal muscle also stores fat, as TAG in lipid droplets (LDs) and both storage and/or intermediates in FA metabolism (LCFA-CoAs (long-chain FA-CoAs), diacylglycerol (DAG), ceramides etc.) has been shown to be involved in regulation of insulin sensitivity [81-84]. Fat is also oxidized in the mitochondria to generate energy and another theory for impaired glucose metabolism is apparent mitochondrial dysfunction [85]. Furthermore, skeletal muscle metabolism depends on fiber type, flexibility, fitness, the involvement of important nuclear receptors and its role as an endocrine organ. In this thesis energy metabolism was examined in human myotubes.

Glucose uptake and glucose transporters

Glucose is a fundamental energy source for all eukaryotic cells and the brain uses 80 % of the dietary glucose ingested. In skeletal muscle cells, glucose may be oxidized in mitochondria for energy (ATP) production, stored as glycogen or contribute as a precursor for lipid synthesis.

Muscle cells take up glucose in the basal, insulin-stimulated or contraction state. Insulin-stimulated glucose uptake is initiated by insulin binding to the insulin receptor (see Figure 2). Rab8A and Rab13 are proposed as responsible Rabs that regulate glucose transporter 4 (GLUT4)-translocation in muscle cells [86]. In addition, TBC1D1 is another Rab GTPase-activating protein that also might be involved in regulation of glucose transport [87, 88], however this mechanism is not clear.

Muscle insulin resistance is most likely due to dysfunctional GLUT4 translocation [89, 90]. However, GLUT4 overexpression in skeletal muscle was shown to increase insulin-

and contraction-stimulated glucose transport and glucose metabolism [91]. Human myotubes express GLUT1, GLUT3 and GLUT4 (preliminary data from our lab). The physiological role of GLUT3 is still unclear [92]. GLUT1, which has been proposed to be responsible for basal glucose uptake in human muscle cells [93-95], was reduced in cells from T2D individuals, while mRNA levels were similar [95]. Nevertheless, GLUT4 levels in skeletal muscle are largely regulated at the level of transcription and this might as well be the case for GLUT1 as well [96].

The dysfunctional insulin-stimulated GLUT4 translocation seen in diabetic muscle might be a result of abnormal FA accumulation or disruptions in the lipid storage dynamics and consequent interaction with protein kinase C (PKC) or ceramide on insulin signaling [83, 97-101]. Ceramides may inhibit serine/threonine phosphorylation of Akt/PKB (protein kinase B) [102-104] and muscle ceramides are observed elevated in obese [105], insulin resistant [106] or obese insulin resistant [107] subjects. SFAs (such as palmitic acid, PA) may induce insulin resistance in muscle, most likely via formation of ceramides [108] and phosphorylation of Akt substrate of 160 kDa (AS160) is observed reduced in myotubes after PA treatment [109] (see Figure 2).

PKC may disturb the GLUT4 translocation by serine phosphorylation of insulin receptor substrate 1 (IRS-1) [101, 110]. DAG may activate PKCε and PKCθ (see Figure 2). DAGs are intracellular second messengers and an intermediate in TAG synthesis and degradation (lipolysis) [101] and has been seen to be positively associated [111, 112] with obesity/insulin resistance in human muscle. In contrast, there are also studies showing inverse association with insulin resistance after high-fat diets in rats [113]. Similar DAG content in obese/insulin resistant subjects [106, 114] or increased DAG content in athletes [107] has also been found. The subcellular location, saturation and different stereoisomers of DAG may play a role and might be the reason for these conflicting results. For instance, Bergman et al showed that membrane DAG was associated with PKCε activation and negatively associated with insulin sensitivity and more abundant in diabetics, while cytoplasmic DAG was not. Furthermore, disaturated DAG species were negatively correlated with insulin sensitivity [111].

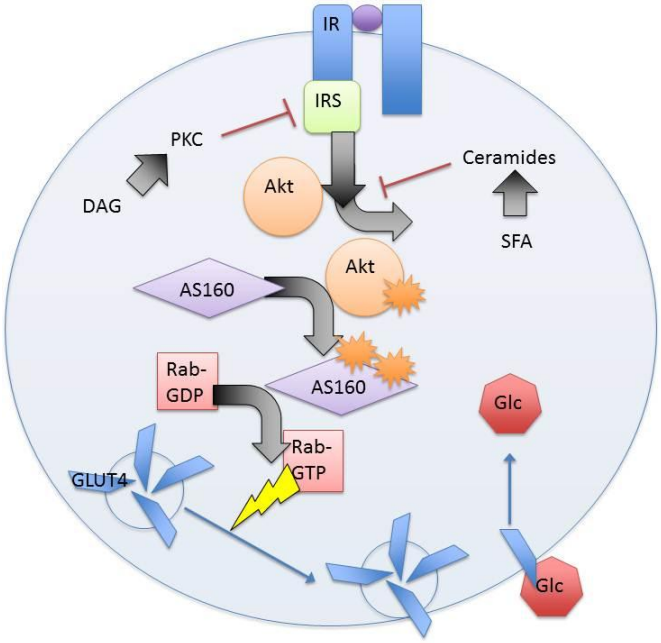

Figure 2. Insulin-stimulated glucose uptake. Insulin-stimulated glucose uptake is initiated by insulin (purple circle) binding to the insulin receptor (IR) at the plasma membrane followed by activation of insulin receptor substrate (IRS) and an insulin-signaling cascade involving the activation by phosphorylation of Akt (protein kinase B (PKB)) [50, 115]. Akt phosphorylates Akt substrate of 160 kDa (AS160 or TBC1D4), which is identified as a regulator of the insulin-stimulated glucose transporter 4 (GLUT4) trafficking and glucose uptake in muscle cells [116-118]. AS160 has a GTPase-activating protein domain that is thought to preserve Rab's inactive GDP form and phosphorylation of AS160 will result in an active Rab-GTP form which facilitates glucose transporters (GLUT4)-containing vesicle exocytosis and consequently glucose (Glc) uptake mediated by GLUT4 transporters [119-121]. Saturated fatty acids (SFA) may result in ceramide influence on Akt phosphorylation and diacylglycerol (DAG) may activate protein kinase C (PKC) that may inhibit IRS. The star represents a phosphorylation.

Fatty acid uptake, transporters and binding proteins

FAs are important energy source, and for instance heart and skeletal muscle prefer FAs as an energy source. Once inside a cell, FAs will be activated to FA-CoA and may be esterified to TAG for storage (LDs) or incorporated into phospholipids for use in cellular membranes. They can also be metabolized to lipid second messengers, such as eicosanoids, oxidized in the mitochondria for energy (ATP) production or alter gene expression. FAs may be supplied by diet or synthesized from glucose (*de novo* lipogenesis). FAs are transported to muscle as free FAs bound to albumin, or derived from TAG in chylomicrons or very low density lipoproteins in the plasma, where the FAs are liberated by (LPL) lipoprotein lipase before cellular uptake [122].

FA uptake can occur through passive diffusion or via protein-mediated mechanisms using transporters such as FA translocase (CD36/FAT), FA binding protein (FABPpm) and FA transport proteins (FATP1-6) [123, 124]. FATP1 and FATP4 are the most abundant FATP expressed in muscle [125]. Insulin has also been observed to induce translocation of intracellular stored CD36 and FATP1 to the plasma membrane in muscle [126-129], while FATP1-KO mice were protected against diet-induced insulin resistance in skeletal muscle [128, 130]. FABPpm translocation, however, is not induced by insulin, although overexpression modestly increased FA uptake. In addition, FABPpm and CD36 are found to coimmunoprecipitate, indicating direct interaction and a potential collaboration [131]. Muscle contraction may also facilitate FA uptake through both FABPpm and CD36 translocation [123, 132] (see Figure 3). However, contraction-induced CD36 trafficking and FA uptake is proposed to be AMP-activated protein kinase (AMPK) independent [132]. In cultured human myotubes, FATP1 is found in the cytosol, while CD36 is overtly present in the plasma membrane [133]. An increase in FA uptake is hence not associated with an increase of total expression of transporters, but rather an increase in transporters at the plasma membrane [132, 134]. Once the FAs enter the cell, FABPc transports them to their destination or they may react with acyl-CoA synthetase (ACSL) and become FA-CoA and is then transported by acyl-CoA binding protein (ACBP) [135].

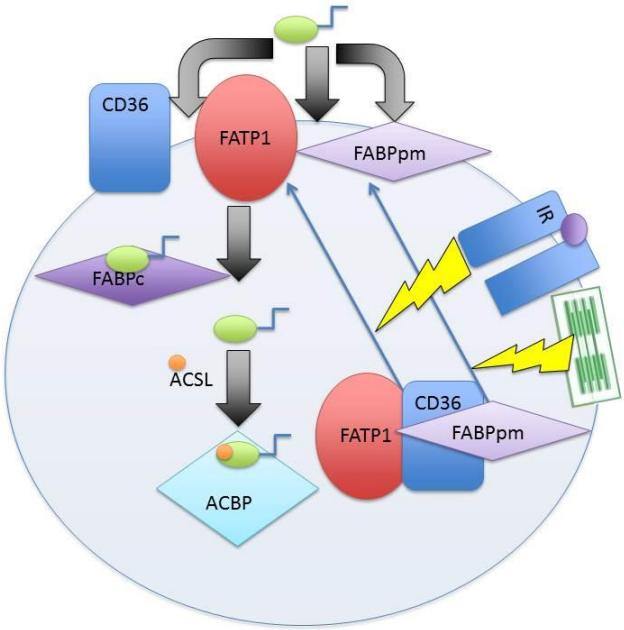

Figure 3. Fatty acid cell uptake. Fatty acid (FA, green oval circle with blue tale) may be taken up by the cell via passive diffusion or via FA translocase (CD36/FAT), FA binding protein (FABPpm) and FA transport proteins (FATP1-6). FATP1 and CD36 translocate to plasma membrane in response to insulin or and CD36 and FABPpm translocate in response to contraction. Once the FAs enter the cell they are transported to their destination by FABPc or they may react with acyl-CoA synthetase (ACSL) and become (LC)FA-CoA (long-chain FA – CoA) which is then transported by acyl-CoA binding protein (ACBP). Orange circle is a CoA molecule.

Malfunctioning of recycling of FA transporters may lead to increased intracellular TAG accumulation and cellular insulin resistance. Earlier studies have shown a higher FA uptake in obese and/or diabetic muscle [136-138]. One *in vivo* study found that FABPc was not regulated by insulin in muscle [127], whereas other studies have found a correlation between FABPc and weight loss in human muscle biopsies [139]. Beside this, the concentration of ACBP was higher in muscles from insulin-resistant obese Zucker rats than in muscles from lean Zucker rats and it is proposed that the concentration of acyl-CoA (FA-CoA) in the cytosol might have an influence on insulin resistance [140]. Bell et al

found a higher protein expression of CD36 in myotubes from morbidly obese compared to lean subjects [138]. There has also been observed that CD36 mRNA expression in muscle biopsies was reduced after a two-month bed rest (inactivity) in humans [141]. Several studies have concluded that CD36 in relation to obesity and T2D were dependent on translocation to the plasma membrane [134, 142, 143]. In accordance to this, a study in muscles from obese Zucker rats proposed that these rats were insulin resistant with respect to CD36 translocation and FA uptake, whilst FABPpm was unchanged [144]. Furthermore, diabetic Zucker rats fed a high-fat diet had an increased total and sarcolemmal CD36 protein expression, but with exercise, a reduction was seen [145]. Bell et al. wanted to mimic the obese situation by overexpression of CD36 in myotubes and although this increased FA uptake, it did not impair insulin signal transduction [138]. Furthermore, GLUT4 and CD36 seem to have quite similar regulation, at it remains to be elucidated [146]. Altogether, this indicates a complex regulation of FA uptake and CD36 function in skeletal muscle dependent on status. The majority of the FAs taken up by muscle (up to 90 % in soleus muscle) are either oxidized or stored as TAG, while the rest ends up in phospholipids, monoacylglycerol (MAG), DAG and cholestryl ester pools [147].

Lipid storage in cells - lipid droplets (LDs) and PLINs

FAs entering the cell are mainly partitioned between mitochondrial oxidation and TAG synthesis and LD storage [123]. In skeletal muscle about 50 - 60 % of the FAs taken up are stored as TAG in LDs [148], referred to as intramyocellular lipid (IMCL) or intramyocellular triacylglycerol (IMTG). LDs are dynamic organelles that interact with almost all other organelles in the cell, and are involved in signaling and lipid trafficking, while they also contain a fuel source for mitochondria. LDs contain mainly TAG, but also DAG, cholesteryl ester and free cholesterol, surrounded by a monolayer of phospholipids and proteins [149, 150].

LDs are coated by LD-binding proteins (PLINs/perilipins) that regulate LD biogenesis [151-153], and in human skeletal muscle all five PLINs are present. In cultured human myotubes PLIN2 and 3 were found to be dominant, whereas expression of PLIN4 and

PLIN1 were very low and no expression of PLIN5 was found [154]. PLIN2 covers 61 % of IMCL in human muscle biopsies [155] and is anticipated to protect the LD against lipolysis [156]. In addition, PLIN2 mRNA expression is positively associated with FA exposure in human skeletal muscle cell [154] or high-fat diet in mice [157], however oleic acid (OA) is shown to be a more potent inducer than PA in C2C12 cells [158]. PLIN3 suppression in HeLa cells resulted in a blocked LD maturation and decreased incorporation of TAG into LDs and PLIN3 is thought to be involved in LD synthesis [159]. PLIN3 increase with OA exposure, but not EPA exposure, in human skeletal muscle cells [154].

Several studies have shown a positive relation between LD or IMCL accumulation and obesity/T2D in muscle [145, 160-162]. Surprisingly, it has been shown that also subjects who exercise regularly (athletes) have elevated IMTG levels, despite a higher insulin sensitivity and higher oxidative capacity, compared to untrained persons [107, 162, 163], even when compared to T2D muscle fibers [163]. This is referred to as the "athlete's paradox". In addition, IMTG level has been found to be similar in muscle from T2D and lean individuals [164, 165] and increasing IMTG did not necessarily increased insulin resistance [100, 166]. Altogether, more recently, researchers believe that IMTG/IMCL accumulation within skeletal muscle *per se* may not be directly linked to insulin resistance [167], but rather a high IMTG turnover protects the cells and prevents accumulation of lipotoxic intermediates as PKC and ceramides [83, 99, 168-170].

To elaborate this theory, regulation of LD biogenesis and turnover in skeletal muscle needs to be further elucidated and in this context; the roles of the PLINs are important. PLIN2 overexpression *in vitro* in C2C12 cells and *in vivo* in rats increased IMTG and was paralleled with improved insulin sensitivity as a result of rescued PA or high fat diet induced impairment of insulin stimulated glucose uptake. PLIN2 knockdown in C2C12 myotubes, however, decreased LD formation and TAG storage [157]. In addition, a lower PLIN2 mRNA expression has been seen in muscle biopsies from insulin resistant compared to insulin sensitive subjects [106]. In contrast to this, some human studies could not see a correlation between PLIN expressions and insulin sensitivity [154, 171] and results from muscle biopsies has also shown a similar PLIN2 protein expression between obese non-diabetics and obese diabetics or obese and lean subjects [172, 173].

Furthermore, an increased PLIN2 protein content is observed with weight loss in non-diabetics [171, 173]. Despite all this, PLIN2-KO mice have normal body weight and fat mass [174]. PLIN3 protein expression has been found to be lower or similar in muscle biopsies of obese compared to non-obese individuals [145, 171] and reduction of PLIN3 in liver increased glucose uptake in skeletal muscle of mice [175].

In adipocytes, PLIN1 is highly expressed and is phosphorylated by the cAMP-dependent kinase protein kinase A (PKA) to initiate lipolysis via interaction with hormone sensitive lipase (HSL) [176-178] (see Figure 4). Although this is not yet thoroughly examined in PLINs from skeletal muscle, researchers speculate that PLIN2 may have some of the same properties [157, 179]. Accordingly, phosphorylations and translocations of PLIN2 might be important [177, 180]. In addition, so far it has been discovered 4 isoforms of PLIN1 and this might as well be the case for the others PLINs [181].

Lipid droplet turnover - esterification and lipolysis

LDs consist mainly of TAG. The FAs taken up by the cell destined for storage react with ACSL and become FA-CoA that may be converted into MAG via monoacyglycerol acyltransferase (MGAT) to form DAG. DAG is converted to TAG catalyzed by diacylglycerol acyltransferase (DGAT) 1 or 2 [182] (See Figure 6). DGAT1 overexpression in rats has resulted in a higher level of IMCL, DAG content and glucose uptake in skeletal muscle. In addition, rats on high-fat diets overexpressing DGAT1 showed increased PLIN2, PLIN5, adipose triacylglycerol lipase (ATGL), CGI-58 protein expression and had larger and more LDs in their muscle [113]. However, Bergman et al found that DGAT1 protein expression was similar in muscle biopsies in athlete, obese and obese T2D individuals [111]. In myotubes, DGAT2 mRNA expression was found to be lower in obese T2D compared to obese [183]. DGAT1 protein expression has been seen to be lower in muscle biopsies from obese compared to non-obese individuals, while DGAT2 and glycerol phosphate acyltransferase 1 protein as well as DGAT and glycerol phosphate acyltransferase activity was not different between the groups [145]. This indicates a discrepancy between expression and activity due to modification of these enzymes.

Degradation of TAG by lipolysis after stimuli for energy demand is driven by the lipases ATGL, HSL and monoacylglycerol lipase (MGL). ATGL is expressed in human skeletal muscle [184-188] and is proposed to be the major lipase that initiates lipolysis by hydrolyzing TAG to DAG [189]. HSL is also expressed in human skeletal muscle [190, 191] and has the function to hydrolyze DAG to MAG and, to a smaller extent, TAG to DAG [192]. In the end, MGL hydrolyzes MAG to glycerol (see Figure 4 and 6). β-adrenergic stimuli with catecholamines may increase lipolysis by phosphorylation of PLIN1 and HSL via PKA in adipose tissue [176, 193-195], and by phosphorylation of HSL in skeletal muscle [196-198]. In adipose tissue, HSL needs to be phosphorylated by PKA at least at two sites before translocation to the LD membrane [195], and in Drosophila it is suggested that PLINs regulate this HSL translocation [195]. AMPK activation is also reported to phosphorylate HSL and even override β-adrenergic stimuli on HSL activity [199, 200]. ATGL has a cofactor comparative gene identification-58 (CGI-58) [201] and overexpression increases TAG hydrolase activity by 2-fold in skeletal muscle cells [184]. One theory is that ATGL and CGI-58 is bound in cytoplasma, while ATGL is liberated when bound to the LD activating lipolysis [202-204]. In addition a ATGL inhibitor G(0)/G(1) switch gene 2 (G0S2) is identified in adipose tissue [205], non-competitive of CGI-58 [204], but not yet identified in skeletal muscle. Nevertheless, little is known about this regulation involving PLINs, phosphorylations, cofactors and translocations of lipases in skeletal muscle. A model of lipolysis is presented in Figure 4 [206].

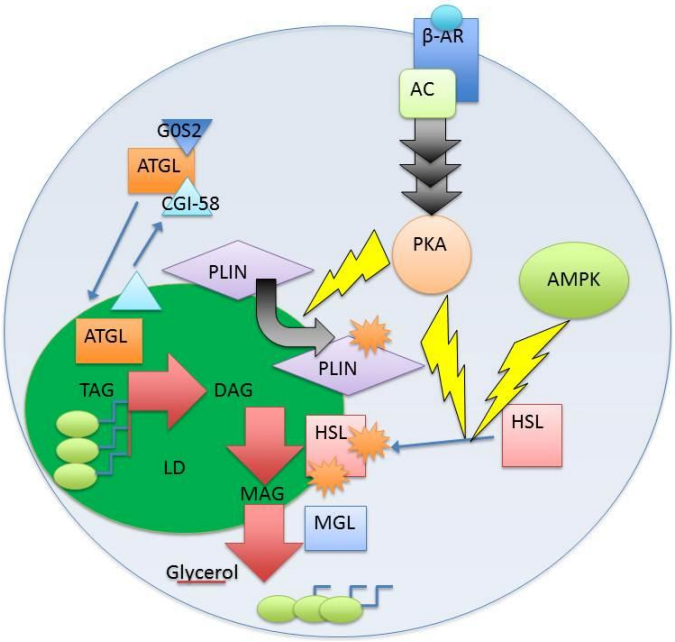

Figure 4. Model of lipolysis. β-adrenergic receptors (β-AR) are activated by the binding of catecholamines (blue circle). This stimulation results in activation of adenylyl cyclase (AC) that subsequently activates protein kinase A (PKA), which phosphorylates hormone-sensitive lipase (HSL) and PLIN/perilipin. Phosphorylation of PLIN facilitates both interactions between phosphorylated HSL and its lipid substrate, and releases comparative gene identification-58 (CGI-58) from the lipid droplet (LD). Then, CGI-58 translocates to the cytosol where it interacts with adipose triacylglycerol lipase (ATGL). The translocation of ATGL to the LD allows for the hydrolysis of triacylglycerol (TAG) to diacylglycerol (DAG). Phosphorylated HSL translocates to the LD to hydrolyze DAG. Monoacylglycerol lipase (MGL) finally hydrolyzes monoacylglycerol (MAG) to glycerol (red line) and FA (green oval circle with blue tale). G(0)/G(1) switch gene 2 (G0S2) might contribute as an inhibitor of ATGL. AMPK activation is also reported to phosphorylate HSL. The star represents a phosphorylation event. An average human cultured muscle fiber has about 30-100 LDs per nuclei.

Studies showing TAG accumulation in subjects suffering from neutral lipid storage disease, where ATGL function is impaired, clearly demonstrate an important role of ATGL in skeletal muscle lipid metabolism [207]. ATGL-KO mice had a lower lipolysis, higher accumulation of TAG and a higher glucose uptake in skeletal muscle [208], while ATGL-deficient animals showed improved glucose tolerance and enhanced insulin sensitivity

despite TAG accumulation in various tissues [209]. On the other hand, HSL-deficient mice had increased DAG levels in adipose tissue, cardiac and skeletal muscle [210]. HSL null mice have shown to be insulin resistant [195] or showed signs of impaired insulin sensitivity [211]. Supply of glucose may play a role in mediating lipase activity in skeletal muscle [212] as well as β-adrenergic induced lipolysis was blunted in muscle of obese individuals [213]. In muscle biopsies from obese/insulin resistant subjects, Jocken et al observed a lower lipolysis and a lower HSL protein expression and phosphorylation compared to lean healthy subjects [198, 214, 215]. In addition, they observed a higher ATGL protein level in obese subjects compared to lean [215] and a lower level in non-obese diabetics and higher in obese diabetics compared to healthy lean controls [214]. This may indicate that the ratio between these lipases is important and that the obesity and diabetic effects are different. These results are in accordance with that several theories regarding development of insulin resistance/T2D in muscle involve a dysregulation of LD turnover rather than the LD accumulation itself [83, 99, 168-170].

Mitochondrial oxidation and function

Once the FAs are stored in LDs as TAG, they can, on stimuli for energy demand, undergo lipolysis and become available substrates for oxidation. To get there, ACSL first catalyzes the reaction of FA to FA-CoA esters, which are substrate for carnitine palmitoyltransferase 1 (CPT1) on the outer mitochondrial membrane [216] and are transported over the mitochondrial membrane (see Figure 5). FAs may also enter the cell and be transported directly to the mitochondria, without entering LDs first. Incomplete oxidation occurs when β-oxidation rates are higher than TCA-cycle or ETC rates [217, 218]. Carnitine O-acetyltransferase (CrAT) is a mitochondrial matrix enzyme that plays a key role in the synthesis and efflux of short-chain carnitine conjugates, such as acetyl-carnitine [219].

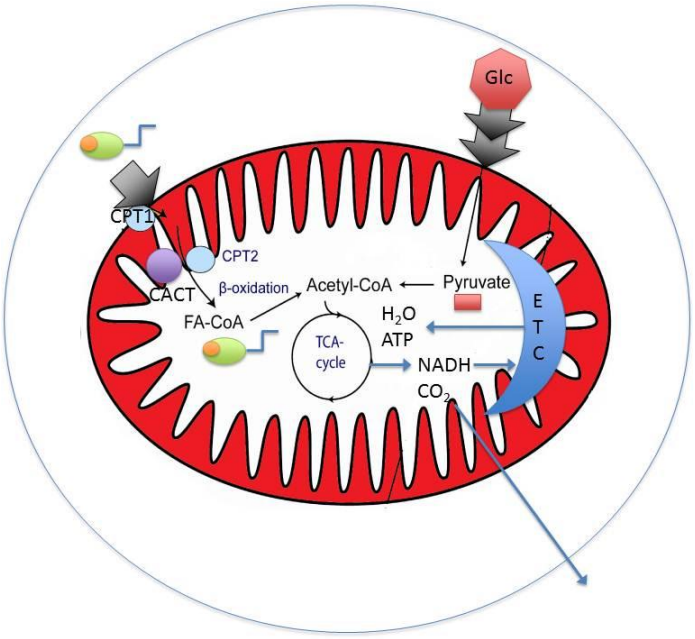

Figure 5. Mitochondria function. To cross the inner mitochondrial membrane, FA-CoA (green oval circle with blue tale) react with carnitine palmitoyltransferase 1 (CPT1) and carnitine-FA is transported via carnitine/acylcarnitine translocase (CACT) [220], and the acyl-CoA form is regenerated by CPT2 on the inner mitochondrial membrane [219]. Once inside the mitochondria, FA-CoA goes through β-oxidation with several chain-shortening reactions that are catalyzed by mitochondrial trifunctional protein [221], which results in acetyl-CoA that enters the tricarboxylic acid cycle (TCA, Kreb's). TCA cycle reduces NAD^+ to NADH and produces carbon dioxide (CO_2). The NADH generated is fed into the oxidative phosphorylation pathway. The electron transport chain (ETC) is the site of oxidative phosphorylation in eukaryotes. ETC couples electron transfer between an electron donor (NADH) and an electron acceptor (O_2) with the transfer of protons across a membrane with the help of several components, as complex I-V and cytochrome c (Cyc) that passes electrons from complex III to IV, and ATP synthase. The end products from these processes are water and energy in the form of ATP [222].

Once glucose enters the cell, it may undergo glycolysis, which results in pyruvate that enters the mitochondria via different transporters. The pyruvate dehydrogenase (PDH) complex converts pyruvate to acetyl-CoA that can enter the TCA cycle and thereby competing with FAs as substrate for energy production [219] (see Figure 5 and 6). Pyruvate dehydrogenase kinase isozyme 4 (PDK4) is an inhibitor of the PDH complex and

is therefore an important factor in switching oxidation towards FAs [223]. Furthermore, citrate, an intermediate in TCA cycle may escape the mitochondria and be converted to acetyl-CoA in cytosol. Lipogenesis is the process by which acetyl-CoA is converted to fats. First, acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC) [224] and ACC2 is the dominant isoform expressed in skeletal muscle [225]. Thereafter, malonyl-CoA can be used for FA synthesis by the action of FA synthase (FASN), and FAs can be further elongated and desaturated by elongases, stearoyl-CoA desaturases (SCDs) and FA desaturases (FADSs) (see Figure 6) [224]. Insulin stimulates this pathway by increasing the pool of malonyl-CoA via PDH and ACC dephosphorylation [226] and LCFA-CoAs might directly affect glucose utilization by altering the activity of glycolytic enzymes and is elevated in obesity and extreme obesity [227].

Dietary short-chain and UFAs are more rapidly oxidized than dietary long-chain and SFA in whole-body of healthy subjects [228]. In contrast to this, in human skeletal muscle cells, it has been reported that palmitic acid (PA, SFA) oxidation was greater than oleic acid (OA, MUFA) oxidation. Human myotubes incubated with PA and OA for 16 h showed higher incomplete and complete oxidation of FAs when exposed to PA compared to OA [133]. In total, saturation of FAs and hence diet may affect mitochondrial oxidation, which is a central component when discussing obesity. The current consensus is that obesity and T2D are associated with reductions in oxidation in skeletal muscle [227, 229-232]. In addition, studies performed on human skeletal muscle cells are few, and those from obese T2D individuals (BMI $\geq$ 30 kg/m^2) have shown similar or reduced ability to oxidize FAs compared to obese/lean individuals [183, 233-236]. There are also studies in extremely obese individuals (BMI $\geq$ 40 kg/m^2) were they found that the extremely obese subjects had a reduced complete FA oxidation related to lean subjects [138, 237, 238]. However, increasing FA oxidation does not prevent insulin resistance [239], and decreasing FA oxidation has even shown improved insulin sensitivity in diet-induced obese mice [240]. There are also reports on unaltered and/or increased FA oxidation in human skeletal muscle of obese or insulin resistant individuals [136, 241-245]. An increased ability to oxidize FAs has been seen in isolated mitochondria from muscle of obese T2D individuals [246]. Furthermore, high-fat feeding has been associated with increased incomplete oxidation [247], mitochondrial DNA copy number and citrate

synthase [248, 249] in rat muscle. This is in line with the observed increased citrate synthase activity in human muscle tissue of obese compared to lean [142]. Thus, the aspect of FA oxidation in relation to insulin resistance and obesity remains controversial.

Mitochondrial deficiency may also be a contributing factor towards development of T2D [164, 165, 250, 251]. The above mentioned reduced oxidation in obese/T2D muscle has been attributed to impaired mitochondrial capacity [250, 252, 253] or lower mitochondrial content [231]. Furthermore, several studies conclude that the observed reduced mitochondrial function in T2D is due to, and secondary to, a lower mitochondrial content in muscle [250, 254-256]. A reduced mitochondrial content has been observed in obese muscle [231], extremely obese myotubes [238], as well as in insulin resistant skeletal muscle [85, 250, 252, 257-261]. Insulin has emerged as a regulator of mitochondrial biogenesis [262, 263], and it might be that a blunted insulin signaling contributes to a decreased mitochondrial content rather than reverse [264]. At the same time, mitochondrial content has in different studies been shown to correlate both negatively and positively with FA oxidation in myotubes [236, 265]. So far, it is not clear whether mitochondrial dysfunction represents a cause or a consequence of T2D or obese phenotype.

Together with reduced FA oxidation, a higher lipid uptake is observed in extremely obese myotubes [138]. However, isolated mitochondria from obese individuals do not have lower CD36 protein level or PA oxidation than lean individuals; nevertheless in lean and obese subjects together, these parameters correlated, suggesting a regulatory role for CD36 at the level of the mitochondria [241, 266]. There is also a study in which colocalization between FATP1 and CPT1 was found in L6 myotubes and an interaction is proposed [267]. Although another study by Garcia-Martinez [133] in human myotubes found no difference in incomplete oxidation and reduced complete oxidation when overexpressing FATP1 and CD36. They propose a closer relationship to lipid storage, but this needs to be further elucidated. Figure 6 reveals the full picture of energy metabolism in skeletal muscle.

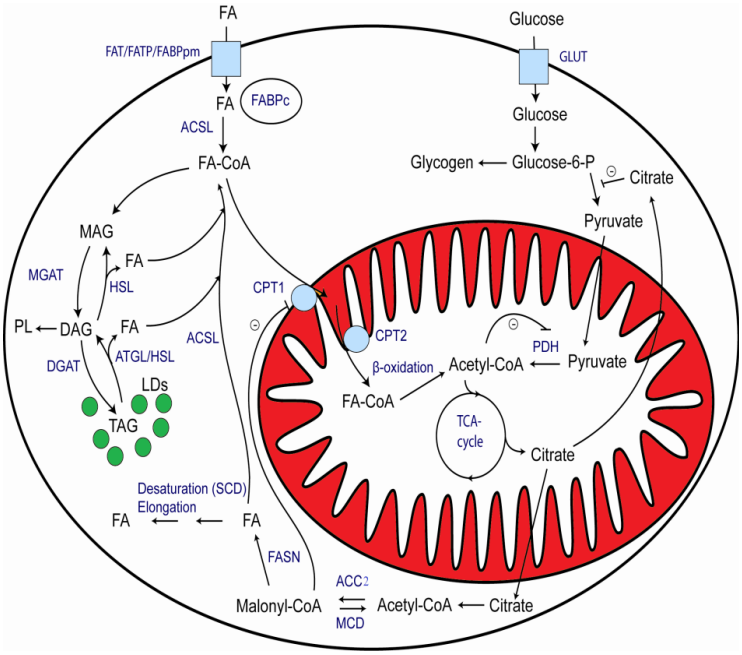

Figure 6. Energy metabolism in skeletal muscle. Glucose is transported into cells through transporters and either stored as glycogen or utilized through glycolysis to yield pyruvate. Uptake of fatty acids (FAs) is facilitated by different transport proteins (FAT/CD36, FATP, FABPpm). Intracellular FAs are bound to cytosolic FA binding proteins (FABPc) and activated by acyl-CoA synthetase (ACSL). FA-CoAs can be incorporated into complex lipids as diacylglycerol (DAG), triacylglycerol (TAG) and phospholipids (PL), and assembled in lipid droplets (LDs) for storage via the action of diacylglycerol acyltransferase (DGAT) and monoacylglycerol acyltransferase (MGAT). TAG and DAG are hydrolyzed by adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). FA-CoAs are transported into mitochondria via carnitine palmitoyltransferase (CPT) 1 and 2, and thereafter metabolized through β-oxidation, yielding acetyl-CoA, which enters the TCA-cycle. Pyruvate derived from glucose may also enter the mitochondria and the TCA-cycle through the action of the pyruvate dehydrogenase (PDH) complex. Citrate that escapes TCA-cycle is converted to acetyl-CoA in cytosol, and thereafter to malonyl-CoA by acetyl-CoA carboxylase 2 (ACC2). Malonyl-CoA decarboxylase (MCD) catalyzes the reverse reaction. Malonyl-CoA can be used for FA synthesis by the action of FA synthase (FASN), which can be further elongated and desaturated by elongases and stearoyl-CoA desaturases (SCDs), respectively. Malonyl-CoA inhibits CPT1, acetyl-CoA inhibits the PDH complex and cytosolic citrate inhibits glycolytic enzymes. Figure adapted and modified from [268].

Fiber types

Skeletal muscle consists of different muscle fiber types; fiber type I, IIa, IIx and IIb. Type I are slow twitch fibers, while type II are fast twitch fibers. In addition, type I are more oxidative and have more mitochondria, while type II are more glycolytic. Type I are more suited for endurance training, while type II for strength training. Type II fibers have mainly three subtypes, a, x and b, where IIb is the fastest fiber and has fewest mitochondria/lowest oxidative capacity. Type I, IIa, IIx and IIb are characterized by myosin heavy chain proteins (MYH) encoded by the genes MYH7, MYH2, MYH1, MYH4 respectively. Human skeletal muscle contains fiber type I and IIa, while to a smaller extent also type IIx [269, 270] and different muscles have different composition of the fiber types [271].

The gene expression of genes involved in lipid storage (IMCL, PLIN2, PLIN5) and lipid turnover (HSL and ATGL) have been reported to be more expressed in type I muscle fibers than in type II muscle fibers [155, 188, 272-275]. A study by Lillioja [276] performed decades ago showed a positive correlation between *in vivo* insulin sensitivity and type I fiber and an inverse correlation between *in vivo* insulin sensitivity and type IIx fiber from muscle biopsies. Other studies showed a lower fiber type I in obese T2D muscle than in obese and lean [277] or lower fiber type I in the insulin resistant group [272]. Type IIa and IIx was found to be higher in muscle of obese diabetic, diabetic and insulin resistant groups compared to the lean, obese, insulin sensitive groups [272, 277, 278]. A higher level of IIx was found for obese compared to lean muscle, and in support a positively correlation was found with BMI and fiber type IIx [279] and an inverse correlation between BMI and type I fibers was found [277]. All this indicates a strong connection between fiber type, obesity and insulin sensitivity.

Metabolic flexibility

Skeletal muscle use both FAs and carbohydrate as fuel, but FAs predominates during fasting, while glucose dominates in the postprandial period (see Figure 7). Metabolic flexibility is defined as the muscle's ability to change between predominantly FA oxidation in the fasting state and carbohydrate oxidation in the fed (insulin-stimulated) state. Loss of this capacity to switch easily between glucose and lipid oxidation was termed metabolic inflexibility by Kelley et al [280]. Obesity, insulin resistance, and T2D are associated with impaired postprandial switch from lipid to glucose oxidation [281, 282] (Figure 7). A reduced flexibility is thus been observed in association with insulin resistance and T2D *in vivo* [281-285]. Galgani et al [286] propose that metabolic inflexibility to glucose in type 2 diabetic subjects is mostly related to defective glucose transport, but that impaired metabolic flexibility might be responsible for the accumulation of intramyocellular lipid and insulin resistance. Furthermore, high-fat feeding has also been associated with reduced metabolic flexibility in skeletal muscle from rats [247] and lower metabolic flexibility has been seen *in vivo* in obese individuals [281] and after three-week high fat diet in overweight men [287]. Clinical studies have shown that postprandial impairments in metabolic flexibility can be improved by weight loss [282, 284, 288, 289]. Further, ω-3 FAs (e.g. EPA) have been observed to have a preservative effect on metabolic flexibility when co-administrated with thiazolidinediones in high-fat fed mice [290]. Subsequently, metabolic inflexibility could be due to both intrinsic and extrinsic factors.

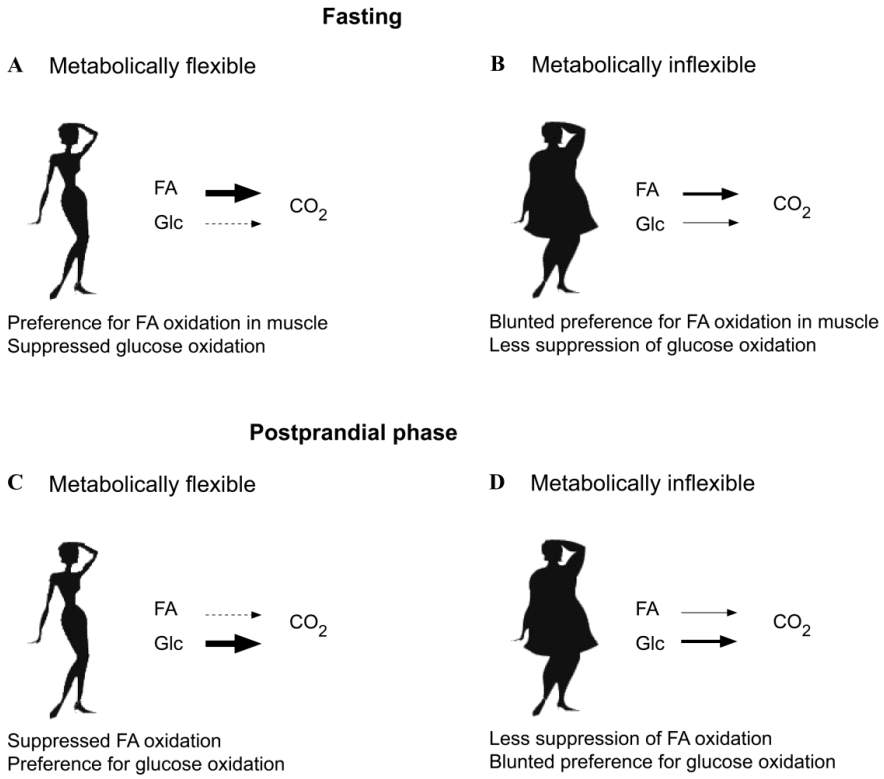

Figure 7. Metabolic flexibility. Healthy skeletal muscle of lean subjects is metabolically flexible and has the ability to switch easily between oxidation of FAs (FAs) and glucose (Glc). During fasting, skeletal muscle of healthy, lean individuals (A) has a preference for FA oxidation, whereas glucose oxidation is suppressed. On the other hand, skeletal muscle of obese, metabolically inflexible individuals (B) has a blunted preference for FA oxidation and reduced suppression of glucose oxidation during fasting. After a meal, skeletal muscle of healthy, lean individuals (C) shifts to a preference for glucose oxidation, whereas FA oxidation is suppressed. In this situation, skeletal muscle of obese, metabolically inflexible individuals (D) shows less suppression of FA oxidation and blunted preference for glucose oxidation. Modified from [268, 283].

Interestingly, it has been observed that muscle cells isolated from healthy lean subjects and overweight/obese patients with/without T2D maintain these characteristics in culture [235, 236, 285, 291-293]. Ukropcova et al. [285] described metabolic switching *in vitro* in human myotubes as adaptability and suppressibility. Adaptability was defined as the

capacity of the cell to increase FA oxidation upon increased FA availability. Suppressibility was defined as the ability of acutely added glucose to suppress FA oxidation and may reflect the reverse Randle's cycle [285].

Randle's cycle describes the biochemical mechanism by which FA oxidation inhibits glucose utilization and vice versa in muscle [229]. Malonyl-CoA inhibits CPT1, thereby inhibiting entry and oxidation of FAs in mitochondria, explaining the inhibitory effect of glucose on FA oxidation (see Figure 9). The ability of FAs to suppress glucose oxidation may be mediated through inhibition of PDH by acetyl-CoA, as well as inhibition of glycolytic enzymes by cytosolic citrate (see Figure 6). Furthermore, AMPK may inhibit ACC and therefore decrease malonyl-CoA and its inhibitory effect on CPT1 [294] (see Figure 9). An old theory is that insulin resistance occurs due to a defective Randle's cycle [229]. However recent studies demonstrate that Randle's cycle does not completely explain the effects of FA on glucose metabolism indicating that other mechanisms are also involved in the FA-induced insulin resistance [294, 295]. A new factor in regulating substrate switching and glucose tolerance is identified, CrAT. CrAT converts acetyl-CoA to acetylcarnitine ester, and seems to regulate mitochondrial and intracellular carbon trafficking [296]. Muoio et al proposed that CrAT eases Randle's cycle, promotes metabolic flexibility, and enhances insulin action by permitting mitochondrial efflux of excess acetyl moieties that otherwise inhibit key regulatory enzymes such as PDH.

The role of PPAR and LXR

Peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs) both belong to the RXR heterodimer subfamily of nuclear receptors which act as transcription factors. PPARs play essential roles in the regulation of energy metabolism, while LXRs are important regulators of cholesterol, FA, and glucose homeostasis [297, 298]. LXRs and PPARs may also interact with each other (crosstalk) [299, 300].

PPARs have three different subtypes; α, δ/β and $\gamma_{1,2}$ and the subtypes display different tissue specific expression and gene regulatory profiles [301]. PPARδ and PPARα-regulated

genes are involved in FA oxidation, whereas PPARγ is a key regulator of adipose development and lipogenesis [301] and whole-body insulin sensitivity. PPARδ is the most abundant subtype in skeletal muscle [302, 303], and activation of PPARδ is reported to improve hyperglycemia and insulin resistance in humans [304, 305]. PPARδ may be activated by all FAs [305, 306], however they might be more strongly activated by UFAs than by SFAs [307, 308]. PPARγ may also be activated by all FAs [305], perhaps to a higher degree by UFAs [307, 308] or PUFAs [309]. PPARα is most likely activated by all FAs [305, 307, 308]. Furthermore, PPARs may also be activated by eicosanoids [305, 306, 309]. Target genes of PPAR are e.g. genes involved in FA uptake and transport (FATP, FABPpm, CD36, ACSLs) and glucose uptake (GLUT4). In addition genes involved in FA oxidation (CPT1, Cyc1, PDK4), lipid storage (PLIN2, PLIN4, PLIN5), lipogenese (SCD1, FADS2), lipid turnover (DGAT, ATGL, HSL) and angiopoietin-like protein 4 (ANGPTL4) [310-316] are also found. However, not all of these are found as targets in muscle or in humans yet. ANGPTL4 is believed to be involved in regulating glucose homeostasis and lipid metabolism, in addition to angiogenesis, however little is known how exactly this occurs [311, 317, 318]. PPARδ activation with a selective agonist increased OA oxidation, PDK4, ANGPTL4, CPT1a, PLIN2 and CD36 expression; on the other hand, it decreased glucose oxidation in human myotubes [316].

PPAR gamma coactivator 1-alpha (PGC-1α) is an important regulator of gluconeogenesis, FA oxidation and adaptive thermogenesis [319] and is a "master regulator" of the coordination of mitochondrial biogenesis [320, 321]. PGC-1α is observed downregulated in skeletal muscle of prediabetic and diabetic individuals [322], while high-fat feeding has been associated with increased PGC-1α expression [249, 323] in rat muscle. Overexpression of this gene resulted in an increase in GLUT4 expression [324] and treatments with a PPARγ agonist increased both PGC-1α and GLUT4 expression in myotubes [325]. Another study revealed that PGC-1α overexpression increased the oxidation rate of PA and mRNA expression of genes regulating lipid metabolism, mitochondrial biogenesis, and function in human myotubes. However, basal and insulin-stimulated glucose uptake was decreased [326]. PPARδ may be a key regulator of skeletal muscle fiber type towards a higher expression in type I muscle compared with type II muscle fibers [327-329] and PGC-1α overexpression in human myotubes resulted in a

decreased expression of fiber-type gene marker for type IIA (MYH2) [326]. Adaptation to increased contractile activity involves conversion from type II to type I fibers [330, 331], is proposed to be a process driven by PGC-1α [332].

LXRs are important regulators of cholesterol, lipid and glucose metabolism [333]. The LXRβ isoform is ubiquitously expressed in adults [334], whereas the expression of LXRα is mainly restricted to tissues known to play an important role in lipid metabolism [335]. Agonists for LXRs include naturally occurring oxysterols [336] and the synthetic compound T0901317 [337], whereas the synthetic 22(S)-hydroxycholesterol has been shown to act as a LXR modulator on certain genes in myotubes [338]. In myotubes several lipogenic genes (SCD, FASN, ACSLs), lipogenesis, FA and glucose uptake (CD36 and GLUT4 expressions) and oxidation were increased after long-term T0901317 incubation. In addition, the number of LDs, as well as cell content of DAG and TAG, was increased after exposure to T0901317 in myotubes [338, 339], indicating an important role of LXR in energy metabolism in skeletal muscle.

Exercise and skeletal muscle

Exercise *in vivo* has many beneficial effects, for instance in prevention of development of T2D and in improvement of insulin sensitivity in T2D patients already suffering from insulin resistance [289, 340, 341]. These positive effects are probably caused by an increased glucose disposal and glycogen storage in skeletal muscle tissue, as a single bout of exercise increases glucose uptake into contracting muscle via GLUT4 [341]. This benefit can be maintained in those who exercise regularly throughout their lifespan. Exercise-mediated enhancement of substrate oxidation is especially important in the treatment of obesity and T2D and an acute bout of exercise increases lipid oxidation in overweight and obese subjects [243, 342].

Regular physical activity also increases the GLUT4 expression [343] and GLUT4 overexpression in skeletal muscle has been found to increase both insulin- and contraction-stimulated glucose transport and metabolism [91]. However, exercise may affect GLUT4-regulated glucose uptake through a different signaling pathway than insulin involving AMPK activation [87, 96, 116]. There are indications that AS160 and TBC1D1 are regulated by exercise/muscle contraction through protein kinases, involving activation by AMPK, but not by Akt [87, 88] and glucose inactivates AMPK, perhaps as a feedback control [344] (see Figure 8). Further, a study observed an increased AMPK activity, AS160-Ser711, TBC1D1-Ser231/660 phosphorylation, but no increase in Akt or TBC1D1-Ser700/Thr-590 phosphorylation, confirming the results that exercise-effects are not using the same pathway as insulin [345, 346]. Aguer et al found that AMPK protein level was lower in obese skeletal muscle compared to lean and obese with T2D skeletal muscle, while both Aguer et al and Bikman et al found that AMPK phosphorylation was similar in obese and lean muscle/myotubes [109, 143]. Furthermore, AMPK may exert stimulatory effects on different enzymes resulting in a less inhibited IRS and thereby also improving insulin signaling in that matter [347].

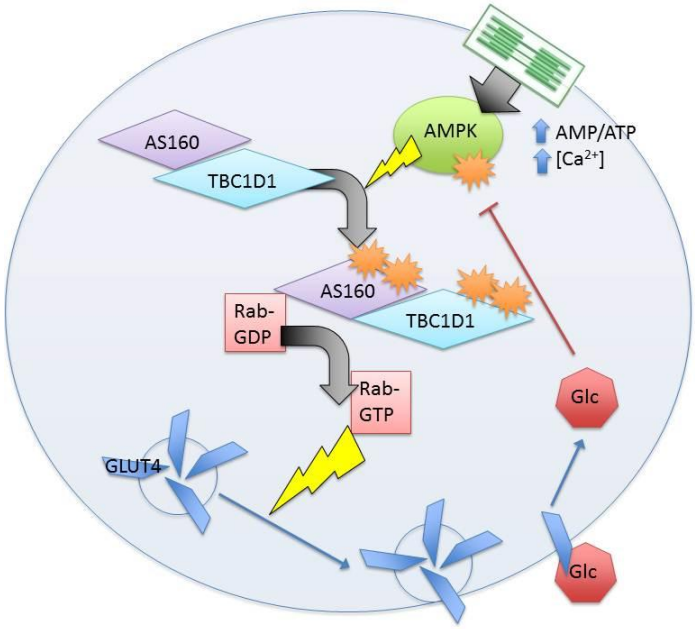

Figure 8. Model for contraction-stimulated glucose transporter 4 (GLUT4) translocation in muscle. Contraction leads to energy depletion (i.e., an elevated AMP/ATP ratio) and elevated intracellular [Ca^{2+}] that again leads to activation of AMP-activated protein kinase (AMPK). Activated AMPK leads to Akt substrate of 160 kDa (AS160) and TBC1D1 phosphorylation at multiple phosphorylation sites and allows the conversion of less active GDP-loaded Rab to more active GTP-loaded Rab. The more active GTP-loaded Rab then allows GLUT4 storage vesicles to move to and fuse with the plasma membrane. Glucose may inhibit AMPK. The star represents a phosphorylation event.

Another effect of endurance exercise is an increased proportion of type I muscles [107, 167, 348], and PPARδ and PGC-1α may partially mediate these positive adaptions in skeletal muscle in response to exercise [301, 321, 326-329, 349, 350]. After exercise, PPARα, PPARδ, PGC-1α and PPAR targets such as PDK2 and PDK4 are increased [199]. Surprisingly, it has been shown that also subjects who exercise regularly (athletes) have elevated IMTG levels, despite a higher insulin sensitivity and higher oxidative capacity, compared to untrained persons [107, 162, 163], even when compared to T2D muscle fibers [163]. This is referred to as the “athlete’s paradox”. Some studies have also demonstrated

that training increases IMTG level [167, 289, 341, 348, 351]. In an *in vivo* study, exercise restored mitochondrial function, improved insulin-mediated glucose disposal and metabolic flexibility in type 2 diabetic subjects in the face of near-significantly increased IMCL content [289]. Moderate exercise also increased IMCL, oxidative capacity of muscle, and insulin sensitivity in previously sedentary overweight to obese, insulin-resistant, older subjects [167]. The current paradigm is that regular exercise increases turnover and content of the IMTG pool, which prevents accumulation of lipotoxic intermediates [99, 107, 352]. In correspondence to this, DGAT1 expression increase in mouse and human skeletal muscle after exercise [353, 354]. However, in other studies this could not be reproduced [107, 186]. A six-week exercise study in obese subjects discovered both increased insulin sensitivity and IMCL in muscle biopsies post training, while DAG and ceramide levels decreased [167]. However, contradictory results from athletes on DAG level are reported [107, 111]. Moreover, athlete muscle was found to have more PLIN5 [107] and SCD protein expression [107, 111] and higher mitochondrial content [107]. Peters et al also found more PLIN5 in exercised muscle, which correlated with IMCL in lean and obese muscle and with mitochondrial density in obese muscle [171]. In contrast to endurance training, strength training does not lead to an increase in mitochondrial content in the skeletal muscle, yet insulin sensitivity increases also with this training modality [355].

In addition to increased mitochondrial content after exercise [331], exercise also increases FA oxidation from IMTG stores during exercise [302, 341, 356]. ACC2 may be phosphorylated by AMPK and acetyl-CoA is converted to malonyl-CoA by ACC, and thereafter malonyl-CoA may inhibit CPT1 [294] (See Figure 9). In addition, AMPK protein level is highest in fiber type I muscle [357]. Contraction may also increase lipolysis by phosphorylation of HSL in skeletal muscle [190, 197] and AMPK activation is also reported to phosphorylate HSL and even override β-adrenergic stimuli on HSL activity [199, 200]. On the other side, this phosphorylation is observed not to influence HSL activation in skeletal muscle [358]. After exercise several genes were observed increased, however, Watt et al speculate that this increase might be a secondary effect due to increased epinephrine levels, not due to contraction [199], and this might also be the cause for HSL activation as well. In skeletal muscle, no effect on HSL protein content and

decreased HSL Ser565 phosphorylation after exercise has been found, but instead an increase in ATGL protein expression was observed. However, the level of CGI-58 was not changed by endurance exercise [186]. The upregulation of ATGL by exercise/in athletes has later been reproduced [107].

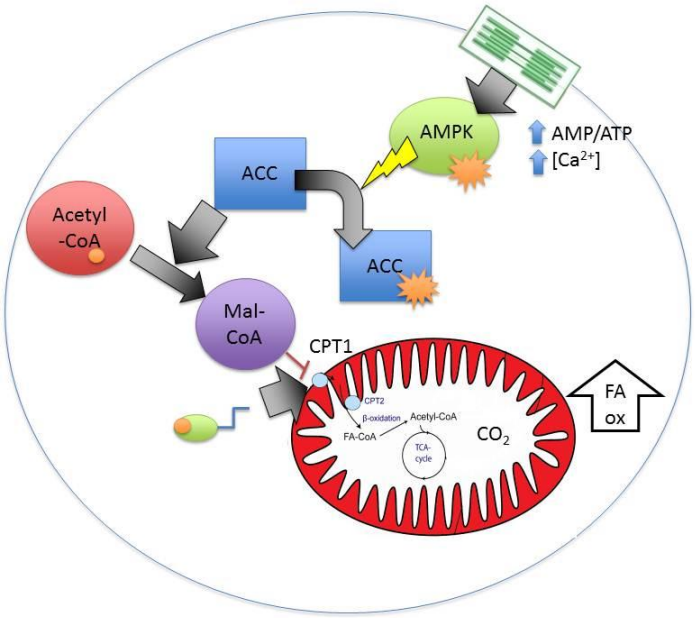

Figure 9. Proposed signaling pathways for contraction-stimulated fatty acid (FA-CoA, green oval circle with blue tale) oxidation in muscle. Contraction through both energy depletion (i.e., an elevated AMP/ATP ratio) and elevated intracellular [Ca^{2+}] leads to activation of AMP-activated protein kinase (AMPK). Activated AMPK leads to phosphorylation and deactivation of acetyl-CoA carboxylase (ACC). Activated ACC should have converted acetyl CoA to malonyl-CoA that inhibits carnitine palmitoyltransferase 1 (CPT1). However, this inhibition is prevented with phosphorylation of ACC and FA oxidation is increased ("FA ox"). The star represents a phosphorylation event.

Skeletal muscle as an endocrine organ

During the past decade, skeletal muscle has been identified as a secretory organ. The muscle secretome consists of several hundred secreted peptides [359], and this introduces a

new paradigm for understanding how muscles communicate with other organs. Myokines are peptides that are released by muscle fibers exerting autocrine, paracrine or endocrine effects. Myokines may balance and counteract the effects of adipokines and may mediate protective effects of muscular exercise with regard to diseases associated with a physically inactive lifestyle [360]. Some myokines exert their effects within the muscle itself, and for instance myostatin, IL-6 and IL-7 are involved in muscle hypertrophy and myogenesis [361, 362] and IL-6 are involved in AMPK-mediated fat oxidation [363, 364] (See Figure 9). Furthermore, IL-6 from muscle may stimulate lipolysis in adipose tissue, while IL-6 from adipose tissue may induce insulin resistance in muscle [365].

By elevating circulating leptin concentrations to concentrations comparable with those of obese individuals, *in vivo* skeletal muscle signaling through the AMPK pathway was increased and caused an increase in FA oxidation in human and rodent skeletal muscle [366]. Steinberg et al 2006 [367] also found that leptin stimulates AMPK activity and FA oxidation in myotubes from lean subjects. In myotubes of obese subjects there was a blunted leptin-dependent activation of AMPK signaling. Furthermore, incubation of human skeletal muscle cells with adipocyte-conditioned media increased PA uptake, TAG content and DAG species, while decreased PA oxidation. Adipocyte-conditioned media alone increased CD36 protein levels and reduced insulin-stimulated phosphorylated Akt. Taube et al concluded that adipokines play a role in the pathogenesis of T2D due to an increased lipotoxic potential of PA [368].

IL-6 is a pleiotropic hormone and cytokine that has both pro- and anti-inflammatory actions and is up to 100-fold upregulated and released into the circulation by contracting muscle [365, 369, 370]. Acute treatment of rat L6 muscle cells with IL-6 increased both insulin-stimulated and basal glucose uptake [364] and contraction by electrical pulse stimulation (EPS) in C2C12 muscle cells increased IL-6 [371]. Furthermore, both IL-6 mRNA expression [372] and protein release [372] were enhanced when glycogen levels were low, which suggests that IL-6 works as an energy sensor [359]. Interestingly, IL-6 KO mice develop late-onset obesity and glucose intolerance [373]. However, whole-body glucose disposal, glucose uptake or endogenous glucose production was not affected by IL-6 infusion [363, 374].

Aims

The overall aim of this thesis was to expand our knowledge on energy metabolism in human skeletal muscle cells in relation to type 2 diabetes (T2D), obesity and effects of dietary fatty acids and exercise *in vitro*. In particular the study concentrated on the elucidation of regulatory aspects of accumulation of fatty acids, and the connection with metabolic flexibility and glucose metabolism in skeletal muscle cells. The overall aim was broken down into four subthemes and the resulting data are presented as published papers and manuscript.

- A study of the effect of different fatty acids on energy metabolism and metabolic flexibility.

- A study on how a saturated fatty acid (palmitic acid) is metabolized differently from a monounsaturated fatty acid (oleic acid) with focus on lipid turnover

- A study on the effect of extreme obesity and T2D on energy metabolism, lipid turnover and metabolic flexibility.

- Finally, a study to establish and evaluate an *in vitro* model for exercise using electrical pulse stimulation.

Summary of papers

Paper I: *Metabolic switching of human myotubes is improved by n-3 FAs.*

Skeletal muscle in lean, healthy individuals is characterized by metabolic flexibility; the ability to switch from predominantly lipid oxidation during fasting conditions to suppression of lipid oxidation and increased glucose oxidation in response to insulin. Obesity, insulin resistance, and T2D are associated with reduced lipid oxidation during fasting, impaired postprandial switch from lipid to glucose oxidation, and reduced capacity to increase lipid oxidation during exercise. The aim of the present study was to examine whether pretreatment with different FAs (PUFA, MUFA, SFA), as well as the LXR agonist T0901317, could modify metabolic switching of human myotubes.

Myotubes were established from biopsy samples from *M. obliquus internus abdominis* of lean healthy individuals. Substrate-regulated flexibility was defined as the ability of the cells to increase the OA oxidation while changing from the "fed" (low FA, high glucose) to the "fasted" (high FA, no glucose) condition. Treatment with EPA increased suppressibility, the ability of glucose to suppress FA oxidation. Treatment with EPA, DHA and alpha-linolenic acid (ω-3 FAs) increased substrate-regulated flexibility of FA metabolism. Adaptability, the capacity to increase FA oxidation with increasing FA availability, was enhanced after pretreatment with EPA, LA (ω-6), and PA. T0901317 counteracted the effect of EPA on suppressibility and adaptability, but it did not affect these parameters alone. EPA *per se* accumulated less in the cells; however, EPA, LA, OA, and T0901317 treatment increased the number of LDs in myotubes. Mitochondrial staining was independent of FA pretreatment. Microarray analysis showed that EPA regulated more genes than the other FAs (24 up, 98 down) and gene set enrichment analysis (GSEA) showed that only EPA induced pathways involved in carbohydrate metabolism and cholesterol biosynthesis.

The present study suggests a favorable effect of ω-3 FAs, compared to other FAs, on skeletal muscle metabolic switching that might contribute to the beneficial effects observed after dietary intake of ω-3 FAs. We also suggest the use of the new parameter

substrate-regulated flexibility in functional studies of fuel selection and energy metabolism in cell cultures in addition to suppressibility and adaptability.

Paper II: *Palmitic acid follows a different metabolic pathway than oleic acid in human skeletal muscle cells; lower lipolysis rate despite an increased level of adipose triglyceride lipase.*

Development of insulin resistance and obesity is positively associated with dietary SFAs and negatively associated with MUFAs. To clarify aspects of this difference we have compared the metabolism of OA (MUFA) and PA (SFA) in human myotubes.

Myotubes were established from biopsy samples from *M. obliquus internus abdominis* and *M. vastus lateralis* of lean healthy individuals. We observed that PA had a lower lipolysis rate than OA, despite a more than two-fold higher protein level of ATGL after 24 h incubation with PA. PA was less incorporated into TAG and LDs, had a higher DAG/TAG ratio and more incorporated into phospholipids and cholesteryl ester after 24 h. In support, incubation with compounds modifying lipolysis and reesterification pathways suggested a less influenced PA than OA metabolism. In addition, PA showed a lower accumulation than OA, though PA was oxidized to a relatively higher extent than OA. Microarray based on mRNA samples and GSEA revealed that 24 h of PA treatment upregulated lipogenesis and FA β-oxidation and downregulated oxidative phosphorylation compared to OA. In addition, the DGAT-1 inhibitor A922500 was used, as far as we know, for the first time and reduced TAG formation by about 90 % and increased oxidation of OA about 2-fold. Mitochondrial staining was independent of OA or PA pretreatment. The differences in lipid accumulation and lipolysis between OA and PA were eliminated in combination with EPA. EPA upregulated cholesterol biosynthesis and pathways involved in carbohydrate metabolism in comparison with OA.

In conclusion, this study reveals that the two most abundant dietary FAs are partitioned toward different metabolic pathways in muscle cells, and this may be relevant to understand the link between dietary fat and skeletal muscle insulin resistance.

Paper III: *Myotubes from extremely obese non-diabetic donors have higher ability for lipid accumulation and lower lipolysis compared to extremely obese type 2 diabetic donors.*

Obesity is strongly associated with insulin resistance and T2D and about 80 % of patients with T2D are classified as overweight. However, a study at The Morbid Obesity Center in Tønsberg, Norway revealed that only about 1/3 of extremely obese patients undergoing bariatric surgery have T2D. This indicates that many extremely obese individuals possess certain characteristics that protect them against developing T2D. We hypothesized that some of these characteristics could be related to differences in skeletal muscle energy metabolism.

Myotubes were established from biopsy samples from *M. obliquus internus abdominis* of lean healthy (BMI 22 ± 3 kg/m^2), extremely obese non-diabetic (BMI 44 ± 7 kg/m^2) and extremely obese subjects with T2D (BMI 43 ± 6 kg/m^2). Decreased insulin sensitivity was observed in myotubes from extremely obese subjects with T2D, assessed as decreased deoxyglucose accumulation and near-significant decreased phosphorylation of Akt. Lipolysis was about 60 % lower, and FA accumulation, mitochondrial staining and adaptability was higher in myotubes from extremely obese non-diabetic subjects compared to extremely obese subjects with T2D. A two-fold increase in FA oxidation and a lower glucose suppression of FA oxidation was observed in cells established from extremely obese subjects compared to cells from lean controls.

Myotubes from extremely obese non-diabetic donors had higher ability for lipid accumulation and lower lipolysis compared to diabetic donors. Our hypothesis is that by storing more lipids, the formation of lipotoxic intermediates that can interfere with insulin signaling could be prevented. Myotubes from extremely obese non-diabetic donors had higher FA oxidation and mitochondrial staining, whilst a lower suppressibility of FA oxidation than lean donors. This might be caused by a compensatory reaction *in vivo* to handle a higher availability of lipids in the extremely obese condition and this occurs at the expense of suppressibility. This study provides results that might contribute to more knowledge of how some extremely obese are protected against development of obesity-related T2D.

Paper IV: *Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise.*

Physical exercise leads to substantial adaptive responses in skeletal muscles and regular physical activity plays a central role in both prevention and improvement of many chronic diseases, as T2D and obesity, and increased life expectancy. Since exercise induces major systemic responses, underlying cellular mechanisms are difficult to study *in vivo*. It was therefore desirable to develop an *in vitro* model that would resemble training in cultured human myotubes.

Myotubes were established from biopsy samples from *M. obliquus internus abdominis* of lean healthy individuals. Electrical pulse stimulation (EPS) was applied to adherent human myotubes for 5 min - 48 h. High-frequency, acute EPS increased glucose uptake and lactate production, while cell contents of both ATP and PCr decreased. Chronic, low-frequency EPS increased oxidative capacity of cultured myotubes by increasing glucose metabolism (uptake and oxidation) and complete OA oxidation. Mitochondrial staining was doubled after 48 h of chronic, low-frequency EPS. Gene expression level of PDK4 was significantly increased in EPS-treated cells, while mRNA expressions of IL-6, Cyc and CPT1b tended to increase. Protein expression of a slow fiber type I marker (encoded by MYH7) was increased in EPS-treated cells.

Even though it is difficult to directly compare effects of *in vivo* exercise to the observed effects in our model of EPS in cultured human myotubes, several of our observations display important aspects of the *in vivo* effects of exercise. We observed important functional changes in cell culture: improved lipid oxidation and glucose metabolism, as well as a fiber-type switch. Our results imply that *in vitro* EPS (acute, high-frequent as well as chronic, low frequent) of human myotubes may be used to study the effects of exercise on the cellular level.

Methodological considerations

Skeletal muscle cells (myotubes)

Satellite cells can be isolated from skeletal muscle biopsies, activated to proliferating myoblasts and differentiated into multinuclear myotubes in culture [375]. These cell cultures represent the best available model system to intact human skeletal muscle, which can be modulated *in vitro*. In the *in vitro* system, the influence of physiological factors as hormonal concentrations, physical activity and neural input are removed, and remaining differences should be attributed to genetic or epigenetic origins [376]. Advantages of this system include having the extracellular environment that can be precisely controlled and that the cells are not immortalized, thereby offering the possibility of studying innate characteristics of the donor. Moreover, it has been shown that human myotubes display the same phenotype as intact muscle *in vivo* [377, 378], perhaps due to epigenetic changes as DNA/mRNA methylation, histone modifications or differentiation patterns. The muscle cells used in this thesis are derived from biopsies from lean healthy donors at Oslo University Hospital, Oslo, Norway, taken during kidney donation surgery. The muscle cells derived from extremely obese donors came from The Morbid Obesity Center in Tønsberg, Norway, and are from biopsies retrieved during bariatric surgery.

The fast and slow fibers are present in skeletal muscle *in vivo* [379], and when grown in culture, human satellite cells mature to myotubes that express fiber types I, IIa and IIx (**Paper IV,** [380]). However, they seem to depend more on glycolytic (type II fiber) than oxidative metabolism (type I fiber) [377, 379]. Compared with myoblasts, the protein expression pattern of myotubes exhibits a higher resemblance to adult skeletal muscle, and myotubes are therefore preferred for experimental use [381]. Furthermore, the ratio GLUT1/GLUT4 is increased in human myotubes compared with skeletal muscle [94, 382], resulting in a decreased insulin responsiveness of glucose transport. Even though the effect of insulin is decreased in myotubes, the responsible molecular mechanisms of glucose transport are observed to remain the same as *in vivo* [94]. Muscle contraction also

stimulates translocation of GLUT4 and increases glucose uptake (see Figure 8), and there have been some studies on human myotubes that have observed a response to electrical pulse stimulation (EPS) in a similar way as to exercising muscle *in vivo* (**Paper IV**, [380]). Insulin stimulation also increases glycogen synthesis about 2-fold in human myotubes, which is similar to *in vivo* observations [94, 325, 383]. Together, these observations imply that glucose metabolism, insulin signaling and responses to exercise are very similar in human myotubes as in skeletal muscle *in vivo*, confirming the dependability of human myotubes as a model system. Furthermore, the capacity to oxidize fat has been compared between homogenates of donor muscle biopsies from healthy subjects and their corresponding derived muscle cell cultures [384, 385]. The oxidation rate of PA [384, 385] and the activity of citrate synthase [385] were comparable. In another study it was observed that a low lipid oxidation *in vivo*, represented by a high respiratory quotient (RQ) measured during fasting, was reflected by a low lipid oxidation *in vitro* [236]. Moreover, in myotubes established from young healthy subjects, *in vitro* adaptability was related to a change in RQ during hyperinsulinemic euglycemic clamp (a measure for metabolic flexibility *in vivo*) [285].

There are also limitations to the model. As previously mentioned, compared to the *in vivo* situation, the insulin-stimulated glucose uptake in primary human myotubes is relatively low and there is a dominant fiber type II expression in myotubes. A cell model that depend more on oxidative metabolism would therefore be desirable to resemble *in vivo* situation to a higher degree. Cultivation conditions may contribute to this, as for instance by EPS, PPAR activation, or by exchanging glucose with galactose in the cultivation media (**Paper IV**, [316, 380, 386]). Moreover, a direct comparison of mRNA expression in muscle biopsy materials with myotube cultures show that most genes studied was markedly higher expressed in biopsies than in cells (**Paper III**), the only exception were the LD protein PLIN3. In addition, a study revealed that in myotubes PLIN2 and PLIN3 dominates, while in biopsy PLIN2, PLIN4 and PLIN5 dominates [154]. Moreover, in myotubes established from young healthy subjects, the suppressibility *in vitro* was inversely related to insulin sensitivity and change in RQ during a hyperinsulinemic euglycemic clamp (measure of metabolic flexibility) *in vivo*, despite a positively correlation with adaptability. These observations might be inappropriate to compare with

the studies in this thesis, as the *in vivo* experiments were done in the presence of insulin [285], whereas the *in vitro* experiments were done in the absence of insulin. However, more studies need to be performed to conclude on this. Finally, taking these limitations into consideration, there are many studies to be performed in cultured skeletal muscle cells, especially on genetic and epigenetic changes.

Donor characteristics

Donors that have contributed with biopsies were adults of different ages (26-70 years), both genders, as well as they may have possessed different diseases or conditions. They were on average 40-50 years old with majority of women (≈ 70 %). The lean donors used in this thesis had an average BMI of 24 kg/m^2, while the extremely obese donors had an average BMI of 44 kg/m^2 and a lower HDL, whilst higher TAG values and blood pressure than the lean. On average fasting glucose and HbA_{1c} was normal for the lean and the extremely obese non-diabetic donors, while for the extremely obese T2D donors the average value was above reference values (see Table 1). In addition, 72 % of the extremely obese and 5 % of the healthy lean donors had the metabolic syndrome.

Table 1. Donor characteristics for the three donor groups Lean (healthy), EO-nD (extreme obese non-diabetics) and EO-T2D (extreme obese with type 2 diabetes) used in this thesis. Mean are presented. $^{\#}$Significantly different from L. *Significantly different from EO-nD. BMI; body mass index, HbA_{1c}; glycosylated haemoglobin, HDL; high-density lipoprotein, LDL; low-density lipoprotein, - ; no data, TAG; triacylglycerol.

	Age (yrs)	BMI (kg/m^2)	Fasting glucose (mmol/l)	HbA_{1c} %	Insulin (pmol/l)	Blood pressure (mmHg)	HDL (mmol/l)	LDL (mmol/l)	TAG (mmol/l)	Total cholesterol (mmol/l)
Lean	48	24	5.0	-	-	130/75	3.0	-	0.9	5.1
EO-nD	40	44$^{\#}$	5.0	5.4	97	140/84$^{\#}$	2.8$^{\#}$	1.2	1.6$^{\#}$	4.7
EO-T2D	49*	43$^{\#}$	7.6*	7.1*	78	139/80$^{\#}$	2.4$^{\#}$	1.1	1.8$^{\#}$	4.4

Age influence

Increasing age is associated with muscle fatigue, atrophy or sarcopenia [387, 388], oxidative damage to mitochondrial DNA [389-391], reductions in mitochondrial content [392], function [393], in addition to a reduced insulin sensitivity [388, 394, 395]. Exercise may prevent age-related effects and perhaps increase life span [395-398] and an age-related decline in mitochondrial oxidative capacity or content and DNA was absent in endurance-trained old subjects [396, 398]. However, Lanza et al [396] concluded that old age, rather than lack of exercise or physical activity, is the cause for mitochondrial changes and Karakelides et al [399] stated that age-related decrease in muscle mitochondrial function is not related to obesity or insulin sensitivity. In contrast, it is hypothesized that obesity rather than age *per se* is the primary determinant of age-related declines in insulin sensitivity [394]. Moreover, a few studies has shown that IMTG stores increase with increasing age [400] and that total fiber area and number of type II fibers are inversely related to age [401], in addition to that skeletal muscle PPARδ decline with age [402]. In this thesis, the donors were mostly under the age of 60 years and donors of both old and young age were merged.

Furthermore, adult stem cells are known to have a finite replication potential and human myotubes from myoblast cultures undergoing senescence exhibit defects in glucose and lipid metabolism. In a study muscle biopsy-derived human satellite cells were grown at different passages and differentiated to human myotubes in culture to analyze the functional state of various carbohydrate and lipid metabolic pathways. A number of cellular functions were altered as the proliferative potential of myoblasts decreased with increasing passage number. The capacity of myoblasts to fuse and differentiate into myotubes was reduced, and metabolic processes in myotubes such as glucose uptake, glycogen synthesis, glucose oxidation and FA β-oxidation became gradually impaired. Late-passage, non-proliferating myoblasts cultures showed strong senescence-associated β-galactosidase activity, indicating that the observed metabolic defects accompany the induction of a senescent state [403]. However, the numbers of passages were kept within the normal response in this thesis.

Gender influence

Whole-body resting energy expenditure and the mass of skeletal muscle are greater in men than in women [404] and *in vivo* whole body differences in glucose/lipid metabolism does exist between men and women [405]. Women are found to have higher expression levels of genes involved in lipid storage and turnover (IMCL, PLIN2-5, HSL), fatty acid uptake (CD36), insulin sensitivity (GLUT4, IR (insulin receptor), Akt, phosphorylation of Akt) and higher level of muscle fiber type I than the men [171, 406-414]. However, a study exploring the protein expression of PPARδ and genes that are regulated by or related to PPARδ (PPARα, PGC-1α, AMPKα, CD36, CPT1, COXIV)[415], as well as enzyme activity of citrate synthase and cytochome c oxidase [416] after exercise; no gender differences were observed. Gene expression of several genes involved in glucose and lipid metabolism has also been observed to be higher in skeletal muscle biopsies from female versus male donors, but these were unaltered in cultured myotubes in the same study, indicating the importance of hormones on these processes [417]. An important additional observation is that the expression patterns of fiber type markers seem to be the same in myotubes from male and female donors [379]. Thus, in the studies included in this thesis, the donors of both genders were merged.

Methods used to measure energy metabolism

The main methods in this thesis used radiolabelled energy substrates, mainly glucose and different FAs, added to the cell culture media to study different aspects of energy metabolism. Wensaas et al have previously developed methods for studying live substrate accumulation (scintillation proximity assay, SPA) and oxidation (substrate oxidation assay) with cultured cells [418]. Substrate oxidation assay was also used to measure *in vitro* metabolic flexibility [419]. Wensaas et al and Corpeleijn et al have previously used the SPA method in human skeletal myotubes and similar results in lipid accumulation is observed if cells are lyzed and remaining radioactivity is measured (**Paper II and III,** [236, 418]). In this thesis SPA was used with both lipid accumulation and lipolysis

experiments. SPA was first performed with radiolabelled FA present where the accumulation increased until steady state level after 24 h [236]. Therefore, the experiments in this thesis were performed using the same concentration of FA and a pre-incubation period up to 24 h. Afterwards SPA was performed without FA present in the cultivation media where the decline in radiolabelled FA represented a measure of lipolysis. This corresponded with incline in radiolabelled FA released to the cultivation media measured as remained radioactivity in the cultivation media. The assumption for this method is that most of the lipids accumulate in LD. An inhibitor of ACSL, triacsin C, was used to inhibit reesterification and it would therefore reflect more selectively lipolysis. Earlier reports in human skeletal myotubes have shown that TAG synthesis is efficiently blocked with incubation of 10 μM triacsin C [184, 420]. However, triacsin C may inhibit several ACSLs and ACSLs may also catalyze other reactions. An example for this is shown as decreased OA oxidation with triacsin C incubation is presented in **Paper II**. However, triacsin C increases lipolysis about 2-fold (**Paper II and III**). Incubation with triacsin C and measured TAG or fluorescent staining and quantification of LDs after OA exposure, revealed a triacsin C-induced reduction of 25 % and 70 %, respectively (Figure 10). Reesterification was measured as the difference between with and without triacsin C incubation [421].

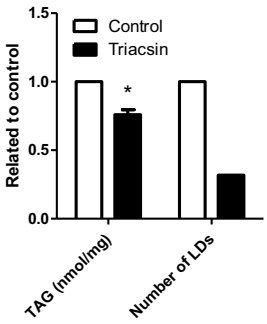

Figure 10. Incorporation of OA into triacylglycerol (TAG) (measured by Thin Layer Chromatography, N=3, *p<0.05 vs control) and number of lipid droplets (LDs) per nucleus (measured by live cell imaging, N=1) in human skeletal muscle cells after exposure to triacsin C after 24 h of incubation with 100 μM oleic acid.

The cells were also incubated with fluorescent substances that are known diffuse through the plasma membrane and bind to intracellular organelles. After excitation of the fluorophore, the emitted light can be used as a quantitative measure. In this thesis, the live human skeletal muscle cells have been labeled with Hoechst 33258, MitoTracker®Red FM and Bodipy 493/503. Hoechst 33258 binds to double stranded DNA, and is well known as a quantitative measure for DNA. Bodipy 493/503 is a lipophilic dye that accumulates within non-polar neutral lipids and is rather specific for LDs in cells [422]. MitoTracker®Red FM is a fluorescent dye that contain a mildly thiol chloromethyl moiety for accumulation within active mitochondria. There are, however, controversies regarding the latter dye's limitation as a specific marker, for instance its dependence on membrane potential and oxidative capacity, and interpretations should be carried out with caution [423]. In **Paper III** and **Paper IV** we observed an increased staining of MitoTracker®Red FM in parallel with higher OA oxidation. At the same time in **Paper III** we show that there is less MitoTracker®Red FM regardless of similar oxidation and in **Paper I and II** treatment with different FAs did alter oxidation but not MitoTracker®Red FM staining. Thus, this dye seem to stain mitochondria independent of oxidation status in skeletal muscle cells and therefore possibly independent of membrane potential, implying that, limited to these cells and our conditions, it is a good measure of mitochondrial content. Mitochondrial content may also be measured as citrate synthase activity, cardiolipin content or as mitochondrial DNA content [424]. In support of our conclusion, citrate synthase activity and MitoTracker®Red FM intensity have the same pattern in our results (**Paper IV**). These dyes have been widely used in cell lines and tissue, but as far as we know, the use of these mitochondria and LD dyes for live cell imaging have not been reported previously in cultured human skeletal muscle cells (representative images Figure 11).

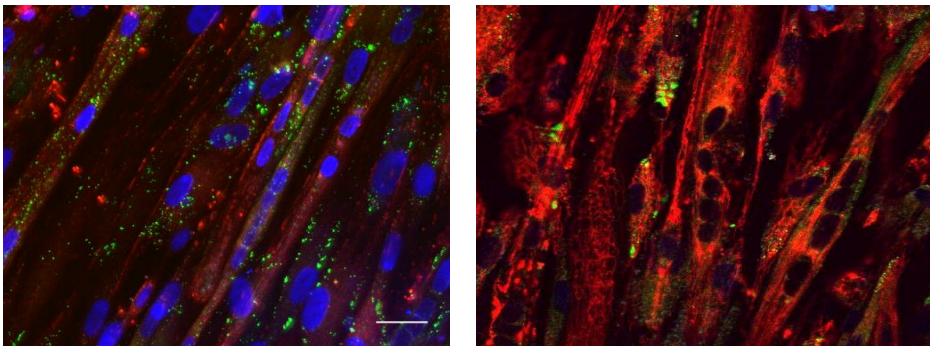

Figure 11. A representative image of mitochondria, LDs and nuclei in human skeletal muscle cells. Mitochondria are stained by MitoTracker®Red FM (red), LDs by Bodipy 493/503 (green) and nuclei by Hoechst 33258 (blue). The image to the left is acquired by a fluorescence microscope (20 x objective, scale bar 50 µm), while the image to the right is from another experiment using a confocal fluorescence microscope (40 x objective) ant there is no nuclear staining (nuclei as "black holes").

The image acquisition and analysis was automated and the images are taken randomly, avoiding the potentially biased human factor. The autofocus was set for the Hoechst 33258 channel to orient around the middle of the cells. The microscope set up had a temperature and a CO_2 enrichment incubator for long-term live imaging so the cells kept healthy during image acquisition. Standard filter sets optimized for Hoechst 33258 (350 nm), Bodipy 493/503 (488 nm), and MitoTracker®Red FM (594 nm) were used so that the excitation and emission should not overlap for the chosen dyes and minimizing bleed-through. Images were made at 5x5 or 6x6 positions from the middle of the well to cover most of the well surface (25-36 positions per well). We used the background-subtracted maximal intensity projection from 7-12 images taken in z-direction, 1 µm apart, to include the whole cell. The images were analyzed and quantified using analysis software, which used an edge detection algorithm for object segmentation (see Figure 12) and the analysis was optimized for each experiment after examining at least 10 images. After analysis the images were visually examined and obvious artifactual image abnormalities were excluded. In the end about 200-400 images were used per parameter, giving good statistical data. The main possible source of error with this technology remains specificity of the staining agents and therefore we also used different biochemical methods to back up the fluorescence data.

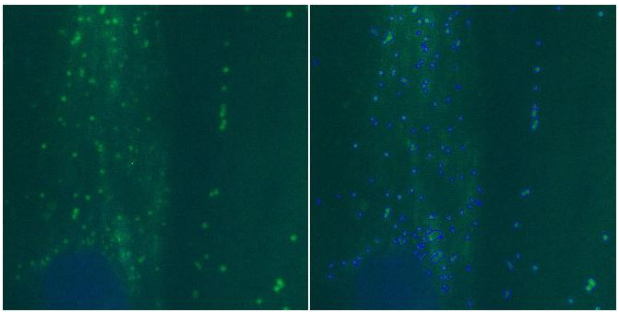

Figure 12. Raw images with background staining (left image) that demonstrate the quantification of LDs (green) in blue circles (right image). Nuclei are stained in blue. These images are taken with a fluorescence microscope, 60 x objective, zoom 2x.

There are two major stimuli that are associated with physical exercise; electrical and mechanical signals. Motor neuron activities generate electrical signals, while stretching of muscle fibers are mechanical signals. Excitation of muscle cells causes contraction and skeletal muscle cells contract when they are stimulated either via a nerve or electrically. Excitation-contraction has many short-and long-term effects on muscle cell function. An *in vitro* model that simulates exercise would be beneficial to explore effects of training and to identify training effects with regards to treatment against T2D and obesity. The model has previously been performed in myotubes from different species giving exercise-like effects [371, 380, 425-427]. In this thesis the human cells were stimulated via carbon electrodes either by applying acute, high-frequency EPS (pulse trains of bipolar pulses 100 Hz for 200 ms given every 5th s, 30 V, for 5-60 min) to simulate a single bout of exercise or by applying chronic, low-frequency EPS (single, bipolar pulses of 2 ms, 30 V, 1 Hz continuously for 24/48 h) to simulate regular exercise (**Paper IV**).

Microarray for gene expression was assessed from isolated RNA samples and Gene Set Enrichment Analysis (GSEA) was performed for functional analysis of changes in gene expression (**Paper I and II**). Arrays for analyzing gene expression generates a huge amount of data, and is therefore highly valuable as a screening tool. However, there is a risk for false positive findings and the data must be carefully interpreted using advanced statistical tools. Significantly regulated genes by the FAs from the microarray analysis

described in **Paper I** are presented in **Appendix I** and all microarray data are submitted to Gene expression omnibus (GSEE18589). GSEA is focused on predefined gene sets, that is, groups of genes that share biological function, chromosomal location, or regulation. The functional catalogue [428] was modified to contain 505 well-defined biochemical, metabolic, and signaling pathways compiled from the following publicly available, curated databases: Biocarta, GenMAPP [429], Kyoto Encyclopedia of Genes and Genomes [430], Sigma-Aldrich pathways, and Signal Transduction Knowledge Environment. The advantage of this method is that it is unbiased because a score is calculated based on all genes in a gene set. In this thesis the microarray data was related to BSA (no fatty acid) (**Paper I, Appendix I**) or OA (**Paper II**) treatment as a control.

Western blotting technology was applied for investigation of amount of expressed protein and phosphorylation of proteins. This allows us to detect post-translational modification, however it is a semi-quantitative technique and highly dependent on the quality of the antibodies. Overall, the methods used in the studies in this thesis are mostly radiolabelled tracer studies and fluorescent staining in addition to gene and protein expression to describe the processes in the cell. This combination is beneficial to cover the many aspects of regulation in a cell.

Data analysis and statistics

The most used statistical method in this thesis is the two-tailed Student´s t-test. The criterion of this test is that the data needs to be normal distributed. It may test if two distributions are equal (unpaired) or if the difference of two responses has a mean value of zero (paired). In the studies in this thesis a two-tailed unpaired or paired test was chosen depending on the experimental set up. Treatments within the same donor were tested with paired t-test, while when comparisons were made across donors or donor-groups they were tested with unpaired t-test. For correlation studies, Spearman correlation analysis was performed. Spearman is a non-parametric measure of statistical dependence between two variables. It assesses how well the relationship between two variables can be described

using a monotonic function. A perfect Spearman correlation of +1 or −1 occurs when each of the variables is a perfect monotone function of the other and the Spearman's coefficient (ρ) defines the strength of the correlation. Linear mixed model (LMM) analysis was used in time-dependent experiments. Furthermore, LMM were used to compare the differences between conditions with within-donor variation and simultaneously compare differences between groups with between-donor variation. The LMM include all observations in the statistical analyses and at the same time take into account that not all observations are independent. For the mRNA expression data, significant difference was defined as two times or half expression of baseline in **Paper III**, but t-test was performed in the other papers. In experiments where there were three experimental groups, data were analyzed using non-parametric Kruskal-Wallis test (one-way ANOVA) (**Paper IV**). In experiments where EPS-treatment versus unstimulated control cells were compared, non-parametric Wilcoxon matched pair tests were performed, since the number of observations were few and normal distribution could not be assumed (**Paper IV**). All values are reported as means ± SEM if not stated otherwise. The value N represents the number of different donors used each with at least duplicate samples. Bonferroni correction was applied for multiple comparisons. Differences were considered significant when $p < 0.05$ and GSEA operated with a FDR < 0.2.

Discussion and Conclusion

In this thesis energy metabolism has been examined in myotubes established from lean healthy, extremely obese non-diabetic and extremely obese T2D donors. Lean myotubes were treated with various fatty acids (SFA, MUFA, and PUFA) to explore possible different effects on metabolism and electrical pulse stimulation (EPS) to simulate exercise.

Lipid storage and turnover

Lipid accumulation was lower in PA-treated human skeletal muscle cells compared to UFA (EPA/LA/OA)-treated cells (**Paper I and II**). It is hypothesized that DGAT expression was dependent on the degree of FA saturation [431] and a study found that DGAT1 appeared to prefer oleoyl-CoA over palmitoyl-CoA [432], however they used other cell models, but this might be part of the explanation for different storage of FAs. In addition DGAT is a PPAR target [310, 316], and UFAs might act as stronger PPAR agonists than SFAs [307, 308]. Finally the DGAT inhibitor increased oxidation of OA to a higher degree than oxidation of PA (**Paper II**), indicating a more influenced OA metabolism.

Lipolysis was lower, while lipid accumulation was higher in non-diabetic cells compared to diabetic while the mRNA expression of PLINs, HSL and ATGL was similar (**Paper III**). The hypothesis was that the cells from non-diabetics stored more lipids and this protected against generation of lipotoxic intermediates that might interfere with insulin signaling. In support of this hypothesis, we observed that PA upregulated ATGL protein content and the DAG/TAG ratio was higher in comparison to OA (**Paper II**). This is in correspondence with that ATGL-KO mice had a lower lipolysis, higher accumulation of TAG but a higher glucose uptake in skeletal muscle [208], while ATGL-deficient animals had enhanced insulin sensitivity [209]. However, in contrast, a higher ATGL protein expression is found in athlete muscle or after exercise [107, 186]. This indicates a complex regulation, and the discrepancy between lipid turnover and mRNA/protein lipase

expression may be due to β-adrenergic stimuli [196-198], translocations [195, 433], post-translational modifications [421] (phosphorylations [198, 215]), in addition to being influenced by presence of co-factors [184] or inhibitors [205] suggesting that regulation of their activity may be on these levels.

MUFAs or PUFAs (as OA and EPA) are found to have positive effects or to counteract PA's negative effects on glucose metabolism in rat or human myotubes [434-437]. Previous studies revealed that PA incubation did not increase IMCL, but reduced phosphorylation of Akt, though OA treatment increased IMCL, but did not affect Akt phosphorylation. Co-incubation with these two FAs resulted in rescued insulin sensitivity and an increased IMCL [437]. Furthermore, EPA co-incubation with OA/PA eliminated the differences between OA and PA treated cells with respect to lipid accumulation and lipolysis (**Paper II**). This was in accordance with previous observations that EPA increased PA or OA-derived TAG in human skeletal muscle [183, 438]. In correspondence with this, EPA pre-treatment increased the number of LDs in lean myotubes compared to PA pre-treatment, despite a lower cellular accumulation. EPA pre-treatment also increased accumulation of OA compared to OA/PA pre-treatment (**Paper I**). Furthermore, incubations of OA/PA/EPA revealed a higher re-esterification rate of OA and PA in the presence of EPA (preliminary data from our lab). Addition of a β-adrenergic agonist increased OA lipolysis, and this indicates that adrenergic stimulus is involved in regulation of skeletal muscle cell lipolysis (**Paper II**), as previously seen for HSL in skeletal muscle [197, 198, 273]. EPA treatment did however down-regulate the *ADRB2* gene in comparison with BSA (no fatty acid) treatment in myotubes (**Appendix I**). The *ADRB2* gene codes for the β2-adrenergic receptor and this might contribute to the explanation on EPA's effect on lipid accumulation. In line with this, a study in cultured rat adipocytes has shown that EPA decreased lipolysis in an AMPK-dependent manner [439]. On the other hand, this might be a mechanism through PPARs, as PUFAs might be stronger agonists than the other FAs [309]. Therefore, EPA's elimination of OA/PA difference might have been caused by increased PA's re-esterification, inhibition of lipolysis and/or via PPARs activation that prefer PUFAs before SFAs as agonists. Additionally, EPA is precursors of anti-inflammatory eicosanoids [78] as well as membrane alterations [79] that may have played a role in EPA´s beneficial effects.

Exercise has been found to increase DGAT1 [353, 354], lipolysis [197, 198, 273] and lipid storage in skeletal muscle [167, 348, 351] and exercise was also associated with involvement of PPARδ [440]. In accordance to this, the number of LDs increased by about 1.9-fold, however not significantly, after EPS in myotubes (Figure 13), despite a similar cellular lipid accumulation (**Paper IV**). Furthermore, a LXR agonist also increased the number of LDs and OA accumulation in lean healthy myotubes ([338, 339], **Paper I**). The beneficial effect of increasing lipid accumulation (or decreasing lipolysis) in myotubes by EPS, EPA or LXR agonists treatments may perhaps be to protect against formation of lipotoxic intermediates. This possibility needs to be further elaborated.

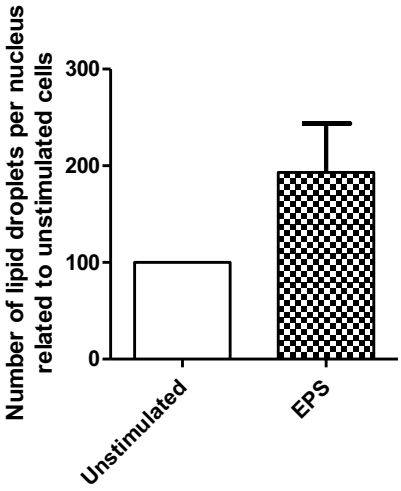

Figure 13. Lipid accumulation in lipid droplets (LDs) in myotubes. Human skeletal muscle cells after 48 h electrical pulse stimulation (EPS) were stained and LDs and nuclei quantified. Results are presented as number of LDs per nucleus, mean±SEM, related to unstimulated, N=6 donors, two-tailed, paired t-test, p=0.12.

PLIN2 and PLIN3 are the most abundant PLINs in cultured skeletal muscle cells [154] and have also been examined in this thesis. Treatment with PA did not differ from treatment with OA with regards to neither mRNA nor protein expression of the two PLINs (**Paper II**). Additionally, no difference in mRNA expression of these PLINs was found

between lean, extremely obese non-diabetics or diabetics (**Paper III**). Though, OA/PA/LA/EPA exposure increased the PLIN2 mRNA expression ([154, 158], **Paper I and III**), which might reflect that these FAs are activators of PPARs, as PLIN2 was observed as a PPARδ target [310, 316]. An "exercise mimetic" treatment of myotubes with forskolin and ionomycin to activate PKA and Ca^{2+} signaling pathways is previously seen to increase PLIN2 and PLIN3 expression in myotubes [441] and endurance training enhanced mRNA expression of PLIN2 and PLIN3 in muscle biopsies [154]. PUFA has also been observed to be more efficient than shorter FAs at stimulating PLIN2 mRNA expression in a human placental choriocarcinoma cell line and in primary human trophoblast [442]. However, microarray/mRNA expression studies indicate that the mRNA expression of PLIN2 after EPA treatment is increased compared to BSA, but not compared to OA ([183], **Paper I, Appendix I**) and that EPS does not alter PLIN2 or PLIN3 mRNA expression (microarray, preliminary data from our lab).

Oxidation and Metabolic flexibility

On energy demand, LDs may undergo lipolysis and make FAs available for oxidation in the mitochondria. PA oxidation was higher than OA oxidation in myotubes, and EPS increased both OA and glucose oxidation (**Paper II and IV**). The EPS-stimulated cells had a tendency towards an upregulation of CPT1b and Cyc1 mRNA expressions, perhaps due to PPARδ activation that could explain the increased oxidation [310, 312, 314], in addition to mechanism shown in Figure 9 (**Paper II and IV**). This was not observed for PA-treated cells (data submitted to Gene expression omnibus (GSEE18589))**,** however EPA and PA did upregulate the β-oxidation pathway (**Paper I and II**). EPA has earlier been reported to increase incomplete PA oxidation in human skeletal muscle cells [183]. In this thesis, EPA- and PA-treated cells had a higher incomplete oxidation compared to OA-treated, perhaps explained by EPA's upregulation of ACC (**Paper I and II)**. EPS did not influence this parameter (**Paper IV**), indicating that the ratio complete/incomplete oxidation is different between these stimuli and might contribute to the explanation of why diet and training have somewhat different outcome. The extremely obese myotubes had a higher

FA oxidation than the lean myotubes (**Paper III**). This might be due to a compensatory mechanism to handle a higher availability/load of lipids in the extremely obese cells. Moreover, PDK4 is an important factor in switching oxidation towards FAs [223] and is also observed to be a PPARδ target gene [310, 312, 314, 316], also in human muscle cells [315]. EPA/LA/PA/OA/EPS treatment upregulated PDK4 mRNA expression in comparison with BSA treatment, and PA tended to upregulate PDK4 mRNA expression compared to OA (**Paper I, II and IV**). This indicates a PPARδ effect that leads to more FA oxidation; however PA might be more potent than OA. PA's upregulation of this gene might explain the higher FA oxidation in comparison with OA; however it does not explain PAs higher incomplete FA oxidation. Though, a study found that palmitoyl-carnitine content was higher than oleoyl-carnitine in rat myotubes [247], indicating a higher PA uptake into the mitochondria than OA. A higher PA than OA oxidation might therefore be due to PA-upregulated PDK4 and/or a CPT1 preference for PA. The LXR agonist also increased OA oxidation, and increased glucose and OA oxidation in combination with EPA (**Paper I),** in accordance with earlier observations [338, 339]. EPS and EPA+LXR agonist in combination were observed to increase glucose oxidation in concert with improved insulin sensitivity (**Paper I, IV**). However, the explanation behind the regulation of complete and incomplete FA oxidation by dietary FAs, extreme obesity and exercise needs further elaboration.

Metabolic flexibility was reduced in association with insulin resistance and T2D [281-284] and in obese individuals [281] *in vivo*. Definitions of metabolic parameters used *in vitro* are presented in **Paper I and III**. EPA increased suppressibility, substrate-regulated flexibility and adaptability compared to OA, suggesting a beneficial response on substrate utilization by EPA in myotubes (**Paper I**). This beneficial effect might be caused by increased lipid storage, or though their role as precursors of eicosanoids [78]. PUFAs are perhaps better agonist of PPARs than other FAs [309]. However no increase inn flexibility parameters with PPARδ agonist was found and LA (ω-6, PUFA) did not increase these parametres (**Paper I**). Furthermore, adaptability was lower in diabetic compared to non-diabetic myotubes (**Paper III**) that might be due to impairment in diabetic muscle mitochondria to exchange substrate on demand. However, PA, LA and EPA-treated cells had a higher adaptability compared to BSA/OA-treated myotubes, indicating a general FA

effect, perhaps via PPAR or upregulation of genes involved in FA oxidation (**Paper I**). The extremely obese myotubes showed however a lower suppressibility than the lean myotubes and this might be a consequence of high FA oxidation and thus high pressure on the mitochondria (**Paper III**). The LXR agonist counteracted EPA's effect on suppressibility and adaptability (**Paper I**), perhaps due to PPAR's ability to act as an LXR antagonist [299, 300]. Furthermore, the new parameter, substrate-regulated flexibility tended to be lower in diabetics compared to non-diabetics (**Paper III**), and we conclude that the new flexibility parameter can be used in this context.

EPS seemed to increase the protein level of type I fiber marker (MYH7) in concert with increased FA oxidation, as has previously been found after chronic exercise or in endurance-trained athletes compared to untrained subjects ([107, 167, 348], **Paper IV, Erratum IV**). EPA treatment did not influence complete FA oxidation, but the results revealed that EPA downregulated mRNA expression of markers for type II fibers (MYH1 and MYH2), compared to BSA treatment (**Appendix I**). The higher protein expression of marker for type I fiber after EPS and lower type II fiber expression after EPA treatment corresponded with a tendency towards higher IMCL observed, and higher insulin sensitivity after EPS (**Paper I and IV**). These effects may also be attributed to PPAR, since PPARδ is found in association with higher expression of type I fibers [440], and PGC-1α overexpression in human myotubes did result in a decreased expression of fiber type IIa marker (MYH2) [326] and is proposed as involved in fiber switch. In contrast, microarray results showed that PA upregulated fiber type IIx marker (MYH1) compared with OA in myotubes (data submitted to Gene expression omnibus (GSEE18589)), as found in insulin resistant or obese muscle [272, 277, 278]. Surprisingly, myotubes from lean, extremely obese non-diabetics and diabetics showed similar protein expression of marker for type I fibers (MYH7, **Paper III**).

Insulin resistance

Acute treatment with IL-6 has shown to increase both insulin-stimulated and basal glucose uptake in rat muscle cells [364]. EPS tended to upregulate, while EPA upregulated IL-6 mRNA expression (**Paper I and IV**). IL-6 is a secretory protein and has also been shown to activate AMPK [363, 364, 443] and exercise-induced effects are proposed to be dependent on AMPK [87, 96, 116, 345, 346](see Figure 8 and 9). Preliminary experiments showed that phosphorylated AMPK might be lower in extremely obese myotubes than in lean (preliminary data from our lab, N=3, p=0.16), indicating a beneficial effect of its activation. An upregulation of IL-6 and subsequently activation of AMPK might therefore explain increased complete FA oxidation after EPS and increased incomplete oxidation after EPA (see Figure 9) and might also indicate a role for AMPK for increased lipid accumulation after EPA/EPS treatments.

A reduced insulin-stimulated glucose uptake and tendency towards reduced insulin signaling (phosphorylation of Akt) was observed in myotubes established from T2D subjects (**Paper III**). A hypothesis for development of insulin resistant muscle was first, a lower lipid accumulation and higher lipolysis without an increase in FA oxidation produce more lipotoxic intermediates that may interfere with insulin signaling pathway (at p-Akt or above). Second, a blunted insulin effect on mitochondrial biogenesis result in lower mitochondrial content. Third, mitochondrial content has previously been correlated with metabolic flexibility [444], and result in adaptability and substrate-regulated flexibility reduction (**Paper III**). Furthermore, a study has shown improved insulin sensitivity after preincubation with EPA in human skeletal muscle cells [438]. However, we did not observe a higher insulin-stimulated glucose uptake, insulin signaling (phosphorylation of Akt) or glycogen synthesis after EPA treatment in lean myotubes. On the other hand, the effect of the LXR agonist on basal glucose uptake was higher with co-incubation with EPA than the other FAs alone (**Paper I**), in accordance with previous findings in myotubes [338, 339], indicating a beneficial effect of LXRs agonist and EPA in combination. Moreover, exercise is well known to improve glucose metabolism [87, 96, 167, 289, 340, 341] and EPS increased the insulin-stimulated glucose uptake in myotubes. However, GLUT1 and GLUT4 mRNA expression were similar (**Paper IV**), indicating an increased translocation

rather than upregulation of GLUTs (see Figure 8). In addition, the insulin effect on glucose uptake and Akt phosphorylation was unaffected by EPS (**Paper IV**), supporting the hypothesis of distinct pathways of exercise- and insulin-induced GLUT4 translocation. The effects resemble changes observed *in vivo* during exercise [289, 340, 341] and we conclude that EPS may be used as an *in vitro* model for exercise.

Final considerations

Finally, it should be noted that the myotubes used in this thesis were from donors of different origin, age, gender, physical activity level and the donors might have different metabolic disorders. In the case of the studies with myotubes from lean, healthy donors this contribution may not be as severe. However, fasting plasma glucose concentrations of the extremely obese donors correlated inversely with insulin-stimulated glucose uptake in the myotubes (**Paper III**). Moreover, TAG values, BMI and blood pressure were higher and HDL lower in extremely obese donors in comparison with lean donors that may also have "left a print" in the myotubes and we observed that the BMI of the donors correlated with the mitochondrial staining in their corresponding myotubes (**Paper III**). Thus, the genetics or epigenetics within the donors does influence the behavior of the myotubes.

The results presented in this thesis, from extremely obese diabetic myotubes and EPS/FA-treated skeletal muscle cells, suggest a favorable role of a higher capacity for intramyocellular lipid accumulation. This promotes increased metabolic flexibility with improved insulin sensitivity and glucose metabolism in human skeletal muscle. Figure 14 presents the working model of this discussion.

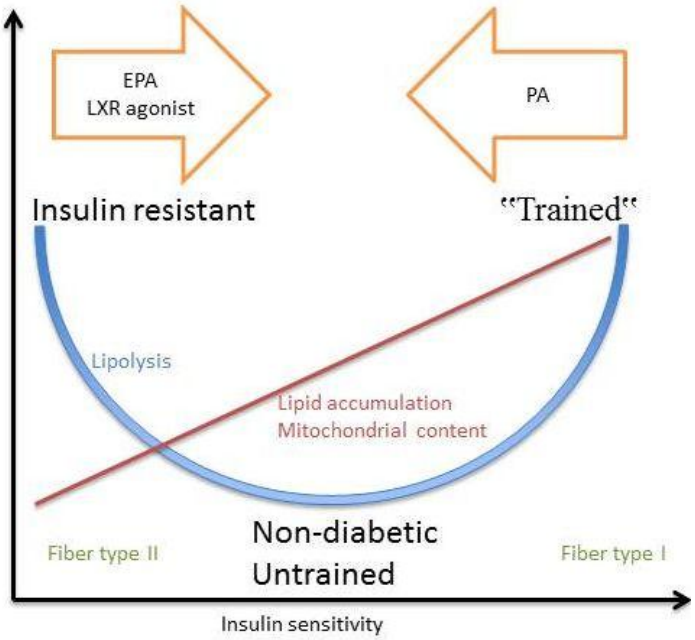

Figure 14. Our working model. In the insulin resistant state, a low intramyocellular lipid accumulation, high lipolysis rate and low mitochondrial content is present. In a non-diabetic state, the mitochondrial content and lipid accumulation is higher, while lipolysis is lower and insulin sensitivity improved (**Paper III**). In "trained" (after EPS) cells or the athlete state, lipid accumulation, mitochondrial content, fiber type I marker expression and insulin sensitivity is higher to meet the increased energy demand created in these muscles (**Paper IV**). However, there are also evidence not explored in this thesis that lipolysis also is higher in athlete/exercised muscle [107, 186, 197, 199, 200], perhaps via AMPK activation [87, 96, 116]. EPA (ω-3 fatty acid) and to some extent OA (monounsaturated fatty acid), and LXR agonist treatments contributed positively to this (**Paper I**), while PA (saturated fatty acid) (**Paper II**), obesity and inactivity contributed negatively. Furthermore, EPA downregulated marker expression for fiber type II, while PA upregulated marker for fiber type IIx.

Future studies

To further clarify the problems in this thesis it would in future studies be of value to examine:

- Regulation of lipases and PLINs expressions, translocations, post-translational modifications, isoforms and presence of co-factors or inhibitors.
- The possible beneficial effect of increasing lipid accumulation (or decreasing lipolysis) in myotubes from obese and diabetic donors.
- Measure lipotoxic intermediates in relation to interference with insulin signaling and lipid accumulation (or lipolysis).
- Metabolic flexibility parameters in EPS- treated cells in obese and diabetic myotubes, and to further study the beneficial effect of EPA.
- Regulation of GLUT4 translocation and expression in relation to the separate effects of EPS and insulin on glucose uptake.
- The role of CD36 versus GLUT4-containing vesicles, translocation and insulin response in the interplay between fatty acid handling and impaired glucose metabolism.

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Appendix I

Table A-1. Genes involved in pathways regulated by EPA, OA, PA or LA related to BSA. Myotubes were incubated with fatty acids (100 μM) for 24 h and then harvested for RNA isolation. Gene expression was measured by Affymetrix human NuGO GeneChip arrays. Genes with q-value < 0.2 (that is, log (q) > 0.7) were considered significantly regulated and fold change (FC) is presented. ADFP/PLIN2, adipose differentiation-related protein/perlipin 2; EPA, eicosapentaenoic acid; OA, oleic acid; PA, palmitic acid; LA, linoleic acid.

Fatty acid-regulated genes, related to BSA, q < 0.2				
EPA				
Upregulated				
Gene name	**EntrezID**	**Mean FC**	**q-value**	**Description**
ADFP	123	2,676775	5,43E-06	adipose differentiation-related protein
ANGPTL4	51129	3,448598	5,71E-05	angiopoietin-like 4
PDK4	5166	4,577311	0,000236	pyruvate dehydrogenase kinase, isozyme 4
CAT	847	1,589811	0,001275	catalase
KLF11	8462	1,467846	0,01299	Kruppel-like factor 11
ACADVL	37	1,439108	0,018239	acyl-Coenzyme A dehydrogenase, very long chain
IMPA2	3613	1,484835	0,018239	inositol(myo)-1(or 4)-monophosphatase 2
SPOCD1	90853	1,596004	0,018239	SPOC domain containing 1
FOSL1	8061	1,566343	0,018239	FOS-like antigen 1
SLC25A20	788	1,394179	0,018239	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20
CXCL2	2920	1,618288	0,019173	chemokine (C-X-C motif) ligand 2
NAMPT	10135	1,527298	0,025021	nicotinamide phosphoribosyltransferase
ABCC3	8714	1,476156	0,044788	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
ECH1	1891	1,508397	0,057607	enoyl Coenzyme A hydratase 1, peroxisomal
OSR1	130497	1,40961	0,057607	odd-skipped related 1 (Drosophila)
IL6	3569	1,544659	0,068279	interleukin 6 (interferon, beta 2)
SLC6A15	55117	1,715871	0,068279	solute carrier family 6, member 15
F2RL1	2150	1,633449	0,068279	coagulation factor II (thrombin) receptor-like 1
FLCN	201163	1,442192	0,091093	folliculin
C2orf18	54978	1,3535	0,092997	chromosome 2 open reading frame 18
RALA	5898	1,267809	0,098387	v-ral simian leukemia viral oncogene homolog A (ras related)
HES2	54626	1,389738	0,098387	hairy and enhancer of split 2 (Drosophila)
MT1X	4501	1,699545	0,099041	metallothionein 1X
HMOX1	3162	1,606951	0,099041	heme oxygenase (decycling) 1
SLC25A34	284723	1,59241	0,122649	solute carrier family 25, member 34
SQLE	6713	1,316659	0,129442	squalene epoxidase
UPP1	7378	1,348286	0,136419	uridine phosphorylase 1
C5orf32	84418	1,297268	0,136419	chromosome 5 open reading frame 32
MT1F	4494	1,577383	0,136809	metallothionein 1F
C10orf12	26148	1,295043	0,148916	chromosome 10 open reading frame 12

ACACB	32	1,408865	0,148916	acetyl-Coenzyme A carboxylase beta
PCIF1	63935	1,382618	0,150552	PDX1 C-terminal inhibiting factor 1
LIF	3976	1,379168	0,154335	leukemia inhibitory factor (cholinergic differentiation factor)
PPP1R15A	23645	1,230676	0,156188	protein phosphatase 1, regulatory (inhibitor) subunit 15A
TNFAIP3	7128	1,290405	0,17038	tumor necrosis factor, alpha-induced protein 3
DLEC1	9940	1,285832	0,173634	deleted in lung and esophageal cancer 1
CTSH	1512	1,226963	0,173634	cathepsin H
TRIM47	91107	1,256264	0,174838	tripartite motif-containing 47
TAC3	6866	1,350827	0,174838	tachykinin 3
SKAP2	8935	1,216654	0,198085	src kinase associated phosphoprotein 2
Downregulated				
STON1	11037	-1,50835	0,010368	stonin 1
MAMDC2	256691	-1,49071	0,018239	MAM domain containing 2
ADRB2	154	-1,74902	0,018239	adrenergic, beta-2-, receptor, surface
ABCA1	19	-2,06969	0,018239	ATP-binding cassette, sub-family A (ABC1), member 1
C5	727	-1,57227	0,018239	complement component 5
RNF144B	255488	-1,70537	0,018239	ring finger 144B
TMEM178	130733	-1,75903	0,018239	transmembrane protein 178
MYH1	4619	-1,72823	0,018895	myosin, heavy chain 1, skeletal muscle, adult
HSPA2	3306	-1,80678	0,019173	heat shock 70kDa protein 2
GDF6	392255	-1,55371	0,019173	growth differentiation factor 6
FRY	10129	-1,59231	0,019173	furry homolog (Drosophila)
VASH1	22846	-1,5968	0,025021	vasohibin 1
GPR126	57211	-1,76963	0,025021	G protein-coupled receptor 126
EBF3	253738	-1,40214	0,025981	early B-cell factor 3
SLC39A10	57181	-1,4292	0,043553	solute carrier family 39 (zinc transporter), member 10
B3GALT2	8707	-1,52979	0,057607	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2
ATP1B4	23439	-1,52371	0,057607	ATPase, (Na+)/K+ transporting, beta 4 polypeptide
XG	7499	-1,36217	0,061487	Xg blood group
TXNIP	10628	-1,27888	0,067245	thioredoxin interacting protein
MTMR4	9110	-1,42535	0,067245	myotubularin related protein 4
ALDH1A1	216	-1,37716	0,067245	aldehyde dehydrogenase 1 family, member A1
SLITRK4	139065	-1,51582	0,067945	SLIT and NTRK-like family, member 4
WDR42A	50717	-1,47639	0,068279	WD repeat domain 42A
NFYB	4801	-1,39585	0,068279	nuclear transcription factor Y, beta
EFNB2	1948	-1,45402	0,068279	ephrin-B2
TLE4	7091	-1,34697	0,075208	transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila)
FBXW7	55294	-1,41743	0,07862	F-box and WD repeat domain containing 7
ERCC5	2073	-1,35139	0,07862	excision repair cross-complementing rodent repair deficiency, complementation group 5
ABCA8	10351	-1,37673	0,092997	ATP-binding cassette, sub-family A (ABC1), member 8
SLC44A1	23446	-1,32982	0,098387	solute carrier family 44, member 1

LPGAT1	9926	-1,36158	0,098387	lysophosphatidylglycerol acyltransferase 1
ADARB1	104	-1,42415	0,098387	adenosine deaminase, RNA-specific, B1 (RED1 homolog rat)
MYH2	4620	-1,45356	0,099041	myosin, heavy chain 2, skeletal muscle, adult
C15orf52	388115	-1,318	0,103494	chromosome 15 open reading frame 52
LYPD6B	130576	-1,36324	0,107867	LY6/PLAUR domain containing 6B
DNAJB6	10049	-1,28972	0,108609	DnaJ (Hsp40) homolog, subfamily B, member 6
CPM	1368	-1,51664	0,108609	carboxypeptidase M
LRRC1	55227	-1,36114	0,108609	leucine rich repeat containing 1
TP53INP2	58476	-1,3418	0,108609	tumor protein p53 inducible nuclear protein 2
RCAN2	10231	-1,36619	0,108609	regulator of calcineurin 2
AMPH	273	-1,51101	0,108609	amphiphysin
ABAT	18	-1,45773	0,110124	4-aminobutyrate aminotransferase
PLK2	10769	-1,3957	0,110674	polo-like kinase 2 (Drosophila)
SORBS2	8470	-1,2938	0,119243	sorbin and SH3 domain containing 2
WDR5	11091	-1,29399	0,119243	WD repeat domain 5
OSAP	84709	-1,40281	0,119243	ovary-specific acidic protein
C1orf83	127428	-1,3601	0,119243	chromosome 1 open reading frame 83
ADAMTS5	11096	-1,43301	0,119243	ADAM metallopeptidase with thrombospondin type 1 motif, 5
PRR15	222171	-1,33503	0,119243	proline rich 15
MFAP3L	9848	-1,32287	0,119243	microfibrillar-associated protein 3-like
PLA2G4A	5321	-1,40289	0,122287	phospholipase A2, group IVA (cytosolic, calcium-dependent)
CLTB	1212	-1,25697	0,122287	clathrin, light chain (Lcb)
MTUS1	57509	-1,37696	0,122385	mitochondrial tumor suppressor 1
C10orf54	64115	-1,29827	0,122605	chromosome 10 open reading frame 54
CAMK2N1	55450	-1,24378	0,122649	calcium/calmodulin-dependent protein kinase II inhibitor 1
DAPK1	1612	-1,36291	0,122649	death-associated protein kinase 1
ANGPT1	284	-1,49822	0,122649	angiopoietin 1
KIAA0430	9665	-1,27518	0,122649	KIAA0430
TEAD1	7003	-1,22736	0,12933	TEA domain family member 1 (SV40 transcriptional enhancer factor)
CCDC136	64753	-1,42117	0,136419	coiled-coil domain containing 136
TEK	7010	-1,43594	0,136419	TEK tyrosine kinase, endothelial
SH3D19	152503	-1,28666	0,136419	SH3 domain containing 19
GYG2	8908	-1,40894	0,136419	glycogenin 2
LOC284023	284023	-1,35869	0,136419	hypothetical protein LOC284023
IVNS1ABP	10625	-1,34122	0,148102	influenza virus NS1A binding protein
SLC6A6	6533	-1,28415	0,148916	solute carrier family 6 (neurotransmitter transporter, taurine), member 6
PPFIBP2	8495	-1,44836	0,148916	PTPRF interacting protein, binding protein 2 (liprin beta 2)
FOLR1	2348	-1,27793	0,148916	folate receptor 1 (adult)
MAP2	4133	-1,37278	0,150552	microtubule-associated protein 2
JSRP1	126306	-1,29924	0,156188	junctional sarcoplasmic reticulum protein 1

TNNT2	7139	-1,23634	0,156188	troponin T type 2 (cardiac)
VGLL2	245806	-1,28626	0,160229	vestigial like 2 (Drosophila)
LBH	81606	-1,26332	0,16476	limb bud and heart development homolog (mouse)
PRTFDC1	56952	-1,39798	0,165333	phosphoribosyl transferase domain containing 1
LOC100129762	100129762	-1,23741	0,165806	similar to KIAA0367
FBXO32	114907	-1,2737	0,165806	F-box protein 32
MEIS1	4211	-1,31548	0,165806	Meis homeobox 1
PTCH1	5727	-1,53641	0,166938	patched homolog 1 (Drosophila)
CCL13	6357	-1,38921	0,166938	chemokine (C-C motif) ligand 13
TMEM119	338773	-1,25072	0,166938	transmembrane protein 119
HEYL	26508	-1,47755	0,166938	hairy/enhancer-of-split related with YRPW motif-like
SLC4A4	8671	-1,41778	0,166938	solute carrier family 4, sodium bicarbonate cotransporter, member 4
OSR2	116039	-1,35493	0,17038	odd-skipped related 2 (Drosophila)
EFHD1	80303	-1,2711	0,17038	EF-hand domain family, member D1
ACAD8	27034	-1,26313	0,172488	acyl-Coenzyme A dehydrogenase family, member 8
MCCC1	56922	-1,2733	0,173634	methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)
OTUD1	220213	-1,26246	0,173634	OTU domain containing 1
STARD13	90627	-1,26388	0,173634	StAR-related lipid transfer (START) domain containing 13
VIT	5212	-1,36047	0,173634	vitrin
TNFSF4	7292	-1,37819	0,173634	tumor necrosis factor (ligand) superfamily, member 4
SPTBN1	6711	-1,23149	0,173634	spectrin, beta, non-erythrocytic 1
CDK5RAP3	80279	-1,23843	0,174838	CDK5 regulatory subunit associated protein 3
SLC46A3	283537	-1,45166	0,174838	solute carrier family 46, member 3
REEP1	65055	-1,27231	0,174838	receptor accessory protein 1
C8orf58	541565	-1,26186	0,174838	chromosome 8 open reading frame 58
ITGA6	3655	-1,20492	0,174838	integrin, alpha 6
NEDD4L	23327	-1,37693	0,174838	neural precursor cell expressed, developmentally down-regulated 4-like
GAS1	2619	-1,26959	0,174838	growth arrest-specific 1
C10orf10	11067	-1,33307	0,174838	chromosome 10 open reading frame 10
EIF4A2	1974	-1,1799	0,174838	eukaryotic translation initiation factor 4A, isoform 2
PLCB4	5332	-1,45635	0,174838	phospholipase C, beta 4
LIMCH1	22998	-1,2803	0,175789	LIM and calponin homology domains 1
MACF1	23499	-1,24536	0,178125	microtubule-actin crosslinking factor 1
SDPR	8436	-1,46712	0,18401	serum deprivation response (phosphatidylserine binding protein)
MYF5	4617	-1,55998	0,18401	myogenic factor 5
MYOM1	8736	-1,32107	0,18401	myomesin 1, 185kDa
DTX4	23220	-1,4527	0,185602	deltex 4 homolog (Drosophila)
WEE1	7465	-1,34847	0,185602	WEE1 homolog (S. pombe)
RNF38	152006	-1,37914	0,186522	ring finger protein 38
FBLN5	10516	-1,212	0,190924	fibulin 5

CEP63	80254	-1,24088	0,199118	centrosomal protein 63kDa
PAPPA	5069	-1,31747	0,199246	pregnancy-associated plasma protein A, pappalysin 1
OA				
Upregulated				
ADFP	123	2,272462	4,46E-05	adipose differentiation-related protein
PDK4	5166	5,971675	4,46E-05	pyruvate dehydrogenase kinase, isozyme 4
ANGPTL4	51129	2,717409	0,000639	angiopoietin-like 4
CAT	847	1,525803	0,004108	catalase
IMPA2	3613	1,475581	0,034696	inositol(myo)-1(or 4)-monophosphatase 2
ACADVL	37	1,416289	0,034696	acyl-Coenzyme A dehydrogenase, very long chain
ECH1	1891	1,631235	0,036468	enoyl Coenzyme A hydratase 1, peroxisomal
SNN	8303	1,448111	0,048225	stannin
SLC25A34	284723	1,867299	0,053586	solute carrier family 25, member 34
OSR1	130497	1,426541	0,081994	odd-skipped related 1 (Drosophila)
CXCL2	2920	1,511862	0,098956	chemokine (C-X-C motif) ligand 2
SPOCD1	90853	1,444781	0,11201	SPOC domain containing 1
KLF11	8462	1,310076	0,122705	Kruppel-like factor 11
SLC25A20	788	1,295436	0,128156	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20
NEURL2	140825	1,506356	0,128156	neuralized homolog 2 (Drosophila)
FOSL1	8061	1,376992	0,193161	FOS-like antigen 1
Downregulated				
INSIG1	3638	-1,49267	0,030974	insulin induced gene 1
SCD	6319	-1,59416	0,034696	stearoyl-CoA desaturase (delta-9-desaturase)
LOC100133348	100133188	-1,82308	0,048225	hypothetical protein LOC100133348
HMGCS1	3157	-1,69	0,048225	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)
LPGAT1	9926	-1,46522	0,053586	lysophosphatidylglycerol acyltransferase 1
FADS2	9415	-1,4497	0,064072	fatty acid desaturase 2
SC4MOL	6307	-1,49538	0,081994	sterol-C4-methyl oxidase-like
ACAT2	39	-1,76328	0,091148	acetyl-Coenzyme A acetyltransferase 2
MVD	4597	-1,59899	0,094947	mevalonate (diphospho) decarboxylase
PRR15	222171	-1,40285	0,109537	proline rich 15
GDF6	392255	-1,43564	0,122705	growth differentiation factor 6
SLC6A6	6533	-1,34866	0,128156	solute carrier family 6 (neurotransmitter transporter, taurine), member 6
XG	7499	-1,33879	0,128156	Xg blood group
TMEM178	130733	-1,54384	0,14222	transmembrane protein 178
LOC100129762	100129762	-1,28934	0,150972	similar to KIAA0367
RNF144B	255488	-1,48682	0,170369	ring finger 144B
NQO1	1728	-1,29801	0,170369	NAD(P)H dehydrogenase, quinone 1
IDI1	3422	-1,45248	0,170369	isopentenyl-diphosphate delta isomerase 1

FDPS	2224	-1,43701	0,180673	farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase,
				dimethylallyltranstransferase, geranyltranstransferase)
SCML2	10389	-1,46026	0,199695	sex comb on midleg-like 2 (Drosophila)
PA				
Upregulated				
PDK4	5166	4,932484	0,000453	pyruvate dehydrogenase kinase, isozyme 4
ADFP	123	1,90402	0,000919	adipose differentiation-related protein
CAT	847	1,607211	0,001519	catalase
ANGPTL4	51129	2,418763	0,002532	angiopoietin-like 4
DHRS3	9249	1,745998	0,01238	dehydrogenase/reductase (SDR family) member 3
ECH1	1891	1,687221	0,030494	enoyl Coenzyme A hydratase 1, peroxisomal
ACADVL	37	1,39974	0,064705	acyl-Coenzyme A dehydrogenase, very long chain
SLC25A20	788	1,342265	0,139183	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20
ASPA	443	1,731415	0,139183	aspartoacylase (Canavan disease)
CD36	948	1,517799	0,192649	CD36 molecule (thrombospondin receptor)
IMPA2	3613	1,373707	0,193087	inositol(myo)-1(or 4)-monophosphatase 2
LA				
Upregulated				
ADFP	123	2,074636	0,000356	adipose differentiation-related protein
PDK4	5166	4,408119	0,000566	pyruvate dehydrogenase kinase, isozyme 4
ANGPTL4	51129	2,419887	0,003266	angiopoietin-like 4
CAT	847	1,508029	0,00635	catalase
CXCL2	2920	1,80077	0,010969	chemokine (C-X-C motif) ligand 2
IMPA2	3613	1,550102	0,012036	inositol(myo)-1(or 4)-monophosphatase 2
ECH1	1891	1,572001	0,10183	enoyl Coenzyme A hydratase 1, peroxisomal
ACADVL	37	1,341482	0,141395	acyl-Coenzyme A dehydrogenase, very long chain
SCG2	7857	1,431784	0,143893	secretogranin II (chromogranin C)
SLC25A34	284723	1,76774	0,143893	solute carrier family 25, member 34
IL8	3576	1,612842	0,169838	interleukin 8
CXCL1	2919	1,494875	0,169838	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
RNF145	153830	1,522137	0,169838	ring finger protein 145
FOSL1	8061	1,415052	0,183635	FOS-like antigen 1
SLC25A20	788	1,296048	0,183635	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20
KLF11	8462	1,286423	0,186328	Kruppel-like factor 11
IRF7	3665	1,375087	0,186328	interferon regulatory factor 7
NAMPT	10135	1,392262	0,19171	nicotinamide phosphoribosyltransferase
SLC6A15	55117	1,632492	0,19171	solute carrier family 6, member 15
GDF15	9518	1,34191	0,193354	growth differentiation factor 15
Downregulated				

SCD	6319	-1,67326	0,014287	stearoyl-CoA desaturase (delta-9-desaturase)
GDF6	392255	-1,49077	0,128727	growth differentiation factor 6
FADS2	9415	-1,43939	0,128727	fatty acid desaturase 2
LOC100133348	100133188	-1,73345	0,133036	hypothetical protein LOC100133348
C5	727	-1,43627	0,169838	complement component 5
ACAT2	39	-1,6866	0,183635	acetyl-Coenzyme A acetyltransferase 2
VASH1	22846	-1,46466	0,186328	vasohibin 1
WDR5	11091	-1,32265	0,186328	WD repeat domain 5
MAMDC2	256691	-1,33878	0,186328	MAM domain containing 2
FDPS	2224	-1,45774	0,186328	farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, geranyltranstransferase)
ANLN	54443	-1,52072	0,186328	anillin, actin binding protein
SLC6A6	6533	-1,33022	0,186328	solute carrier family 6 (neurotransmitter transporter, taurine), member 6
POU2F1	5451	-1,40541	0,186328	POU class 2 homeobox 1
TEAD1	7003	-1,25282	0,186328	TEA domain family member 1 (SV40 transcriptional enhancer factor)
RNF144B	255488	-1,48955	0,186328	ring finger 144B
TTN	7273	-1,89547	0,186328	titin
PCYT2	5833	-1,32698	0,186328	phosphate cytidylyltransferase 2, ethanolamine
KRT34	3885	-1,49517	0,186328	keratin 34
NEDD4L	23327	-1,46331	0,19171	neural precursor cell expressed, developmentally down-regulated 4-like

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IV

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PLoS one

Electrical Pulse Stimulation of Cultured Human Skeletal Muscle Cells as an *In Vitro* Model of Exercise

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Abstract

Background and Aims: Physical exercise leads to substantial adaptive responses in skeletal muscles and plays a central role in a healthy life style. Since exercise induces major systemic responses, underlying cellular mechanisms are difficult to study in vivo. It was therefore desirable to develop an *in vitro* model that would resemble training in cultured human myotubes.

Methods: Electrical pulse stimulation (EPS) was applied to adherent human myotubes. Cellular contents of ATP, phosphocreatine (PCr) and lactate were determined. Glucose and oleic acid metabolism were studied using radio-labeled substrates, and gene expression was analyzed using real-time RT-PCR. Mitochondrial content and function were measured by live imaging and determination of citrate synthase activity, respectively. Protein expression was assessed by electrophoresis and immunoblotting.

Results: High-frequency, acute EPS increased deoxyglucose uptake and lactate production, while cell contents of both ATP and PCr decreased. Chronic, low-frequency EPS increased oxidative capacity of cultured myotubes by increasing glucose metabolism (uptake and oxidation) and complete fatty acid oxidation. mRNA expression level of pyruvate dehydrogenase complex 4 (PDK4) was significantly increased in EPS-treated cells, while mRNA expressions of interleukin 6 (IL-6), cytochrome C and carnitin palmitoyl transferase b (CPT1b) also tended to increase. Intensity of MitoTracker®Red FM was doubled after 48 h of chronic, low-frequency EPS. Protein expression of a slow fiber type marker (MHCI) was increased in EPS-treated cells.

Conclusions: Our results imply that *in vitro* EPS (acute, high-frequent as well as chronic, low-frequent) of human myotubes may be used to study effects of exercise.

Citation: Nikolić N, Skaret Bakke S, Tranheim Kase E, Rudberg I, Flo Halle I, et al. (2012) Electrical Pulse Stimulation of Cultured Human Skeletal Muscle Cells as an *In Vitro* Model of Exercise. PLoS ONE 7(3): e33203. doi:10.1371/journal.pone.0033203

Editor: Maria Moran, Instituto de Investigación Hospital 12 de Octubre, Spain

Received May 10, 2011; **Accepted** February 13, 2012; **Published** March 22, 2012

Funding: This work was funded by the University of Oslo, Oslo University College, the Norwegian Diabetes Foundation, the Freia Chocolade Fabriks Medical Foundation and the Anders Jahre's Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Physical training leads to extensive adaptations in skeletal muscles [1-4]. Regular physical activity plays central role in both prevention and improvement of many chronic diseases, improvement of life-style and increased life expectancy [2]. However, molecular mechanisms underlying these adaptations are still poorly understood, emphasizing the requirement for a cell culture model resembling training *ex vivo*.

Motor neuron activation of muscle fibres can be replaced by electrical pulse stimulation (EPS) of differentiated skeletal muscle cells (myotubes) in culture [5,6]. Metabolic and genetic adaptations caused by EPS *in vitro* have previously been described in murine C2C12 cells [7-9], in L6 cells [10] and in primary rat skeletal muscle cells [11]. *In vivo*, immediate effects of EPS, such as activation of glucose uptake and glycogenolysis [12] can be clearly distinguished from more profound changes in the metabolic and transcriptional phenotypes of muscles occurring as a result of chronically increased contractile activity evoked by chronic, low-frequency EPS [12]. Likewise, acute changes in skeletal muscle after a single bout of exercise *in vivo* differ considerably from those observed after regular training, which, obviously, confers for the most of the beneficial health effects of exercise *in vivo* [2,13].

Adaptations of metabolic properties in skeletal muscles after exercise are reflected by both increased mitochondrial content [14,15] and improved oxidative capacity [4,16,17]. Several signalling pathways have been proposed to be involved in the stimulation of mitochondrial biogenesis in a contracting muscle [18-20]. Exercise has also been shown to enhance both lipid synthesis and lipid oxidation [20-23]. As a consequence of these metabolic adaptations, trained muscle takes more of its required energy from lipids and less from carbohydrate compared to untrained muscle during submaximal work (i.e. work performed below the maximal oxygen utilization capacity) [24]. In addition to fatty acids, trained fibers import and use more glucose than untrained muscle fibers [25,26]. Glucose transporters 1 and 4 (GLUT1 and GLUT4) are the major glucose transporters in the cell membrane of skeletal muscle cells [27], and regular physical

activity improves the ability of insulin to stimulate the uptake of glucose at rest [28-30].

Plasticity of skeletal muscles in response to endurance exercise extends beyond the metabolic changes. In human skeletal muscles, three main muscle fiber types, type I (oxidative, slow twitch), IIa (intermediate) and IIx (glycolytic, fast twitch), can be delineated based on histochemical, functional and biochemical properties [31]. Type I fibers are characterized by higher mitochondrial content and increased glucose transport compared to type II fibers, and whole-body insulin-sensitivity has been positively correlated with the proportion of slow-twitch oxidative fibers in humans [32]. Conversion from type II to type I muscle phenotype has been shown in animals [33], but there is little evidence that this type of transition actually happens in adult humans [15,24]. Chronic, low-frequency EPS *in vivo* has been shown to lead to the transformation of fast-twitch glycolytic muscle fibres into slow-type oxidative fibres [12]. Moreover, skeletal muscle has recently been identified as an organ that produces and releases several cytokines, which are termed "myokines", among these are interleukins 6, 8 and 5 (IL-6, IL-8 and IL-5) [34]. It has been demonstrated that plasma concentration of IL-6 increases during muscular exercise [35,36], and IL-6 appears to have positive effects on skeletal muscle glucose metabolism [37,38]. These findings suggest that also the immune system is affected by physical exercise; however, the implications this might have on the metabolic responses are not yet understood.

We have previously reported effects of acute electrical stimulation on glucose metabolism in cultured human skeletal muscle cells, both at high and low glucose concentrations [39]. In the past years, reports of several EPS models applied to cultured skeletal muscle cells have increased in number, suggesting that there is growing interest in establishing a method that would allow to study cellular mechanisms of exercise under controlled conditions *in vitro* [6-9,11,40]. However, to our knowledge, no model of EPS has been applied to primary human skeletal muscle cells. Cell culture systems derived from muscle biopsies have been used to study glucose and lipid metabolism over the past 30 years. Differentiated primary human myotubes represent the best available alternative system to intact human skeletal muscle. They have the most relevant genetic background to study human disease as opposed to rodent culture systems, and they also display the morphological, metabolic and biochemical properties of adult skeletal muscles [41,42].

In the present study, we aimed to develop an *in vitro* model of exercise in cultured human skeletal muscle cells, with main focus on metabolic effects of chronic, low-frequency EPS. This model could be used to study adaptive responses of skeletal muscle cells to different types of contractile activity applied by electrical pulse stimulation (EPS).

Materials and Methods

Materials

Dulbeccòs modified Eaglès medium (DMEM-Glutamax^{TM}), heat-inactivated fetal calf serum (FCS), penicillin/streptomycin (P/S) and amphotericin B were purchased from Gibco (Gibco, Life Technologies Paisley, UK). BSA (Bovine Serum Albumin) (essentially fatty acid-free), L-carnitine, and Dulbecco's phosphate-buffered saline (DPBS; with Mg^{2+} and Ca^{2+}), oleic acid, extracellular matrix (ECM) gel and HEPES were obtained from Sigma (St Louis, MO). Ultroser G was purchased from Ciphergen (Cergy-Saint-Christophe, France), and insulin (Actrapid®) was from NovoNordisk (Bagsvaerd, Denmark). [1-^{14}C]oleic acid (55 mCi/mmol) and D-[^{14}C(U)]glucose (5 mCi/mmol) were from NEN Radiochemicals, PerkinElmer (Boston, MA). [^{3}H]deoxyglucose (10 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO). Ecoscint A scintillation solution was from National diagnostics (Hessle, England, UK). Glass bottom plates were from MatTek (Ashland, MA). Protein assay reagens was purchased from BioRad (Copenhagen, Denmark). Phospho-Akt (Ser473) and Akt antibodies were from Cell Signaling Technology (Beverly, MA), OXPHOS human cocktail antibodies were from MitoSciences (Eugene, OR) and Anti-Myosin, slow muscle (MAB1628) was from Millipore (Billerica, MA). MitoTracker®Red FM and Hoechst 33258 were obtained from Molecular Probes, Invitrogen (Carlsbad, CA). NuPAGE® 4–12% (w/v) Bis-Tris Gel, 1 mm×12 well was from Invitrogen (Carlsbad, CA). Citrate Synthase Activity Assay Kit was from Sigma-Aldrich® (St. Louis, MO). Cytotoxicity Detection Kit Plus (LDH) was from Roche Applied Science, Mannheim, Germany. The primers for TaqMan Real Time PCR were provided by Invitrogen (Carlsbad, CA). SYBR green and TaqMan reverse transcription kit reagents were obtained from Applied Biosystems (Warrington, UK). Agilent Total RNA isolation Kit was purchased from Agilent Technologies (Santa Clara, CA). All chemicals used were of standard commercial high-purity quality.

Ethics statement

The biopsies were obtained with informed written consent and approval by the National Committee for Research Ethics (Oslo, Norway). The research performed in this study was approved, as a part of a larger project, by the National Committee for Research Ethics (Oslo, Norway).

Human skeletal muscle cell cultures

A cell bank of satellite cells was established from muscle biopsy samples of the *Musculus obliquus internus abdominis* of twelve healthy volunteers (10 females, 2 males), age range 34–70 years (50.9±9 years), body mass index (BMI) range 19.6–29.7 kg/m^2 (23.9±0.9 kg/m^2), fasting glucose range range 4.9–6.9 mM (5.2±0.2 mM), and plasma lipids and blood pressure within normal range. Not all donors were used in each experiment. Muscle cell cultures free of fibroblasts were established by the method of Henry et al [42]. Briefly, muscle tissue was dissected in Ham's F-10 medium at 4°C and dissociated by three successive treatments with 0.05% trypsin/EDTA, and satellite cells were resuspended in skeletal muscle DMEM-Glutamax^{TM} with 2% FCS, 2% Ultroser G, 50 U/mL penicillin, 50 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and no added insulin. The cells were grown on culture wells or flasks coated with extracellular matrix gel [43]. After 1–2 weeks, at ~80% confluence, growth medium was replaced by DMEM-Glutamax^{TM} with 2% FCS, 50 U/mL penicillin, 50 µg/mL streptomycin, 1.25 µg/mL amphotericin B and 25 pM insulin to induce the differentiation of myoblasts into multinucleated myotubes. The cells were cultured in a humidified 5% CO_2 atmosphere at 37°C, and medium was changed every 2–3 days. All myotube cultures were used for analysis on day 8 or 9 after the onset of differentiation.

Electrical pulse stimulation of muscle cells

Multinucleated myotubes grown in ECM-coated 6-well plates were stimulated via carbon electrodes either by applying acute, high-frequency EPS (pulse trains of bipolar pulses 100 Hz for 200 ms given every 5th second, 30 V, for 5–60 min), or by applying chronic, low-frequency EPS (single, bipolar pulses of 2 ms, with 30 V and 1 Hz continuously for the last 24 or 48 h of differentiation period). Culturing medium was changed every 12th h during chronic, low-frequency EPS. Electrical pulses were

generated by a muscle stimulator built at the Electronics Lab, Institute of Chemistry, University of Oslo.

Contents of ATP, PCr and lactate

The myotubes were preincubated for 1 h (37°C, 5% CO_2) with DMEM-Glutamax™. Medium was then changed to fresh DMEM-Glutamax™ and acute, high-frequency EPS was applied for 5–60 min. After stimulation, the cells were immediately placed on ice, the media were removed, and the cells were washed three times with ice-cold phosphate-buffered saline (PBS) before being harvested in 200 µL of ice-cold perchloric acid (3 M). Analyses of ATP, PCr and lactate contents were performed at the Institute for Experimental Medical Research, Ullevål University Hospital, Oslo. The cells were analyzed for ATP and PCr levels with luminescence spectrometry, and lactate content in cells was analyzed with fluorescence spectrometry as described by Lowry et al. [44].

Measurement of lactate dehydrogenase (LDH) in culture media from myotubes treated with chronic, low-frequency EPS

Cytotoxic effect of EPS was determined in a colorimetric assay based on the measurement of lactate dehydrogenase (LDH) activity in the supernatant with the use of Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Applied Science, Mannheim, Germany). Multinucleated myotubes grown in ECM-coated 6-well plates were stimulated via carbon electrodes by applying chronic, low-frequency EPS continuously for the last 24 h or 48 h of the differentiation, and LDH activity in the supernatant was determined according to the supplier's protocol. Triton-X-100-lysed cells were used for determination of maximum values.

Glucose metabolism

Initial experiments with frequency-dependent deoxyglucose uptake: The myotubes were starved for 60 min in serum-free DMEM-Glutamax™ (5.5 mM glucose) in a 5% CO_2 incubator at 37°C. Then, the medium was changed to serum-free DMEM-Glutamax™ with [^{3}H]deoxyglucose (1 µCi/mL) +/− cytochalasin B (20 µM), and acute EPS was applied for the first 15 min of the 60 min deoxyglucose uptake period, using frequencies of 2 to 10 Hz. **Deoxyglucose uptake at acute, high-frequency EPS:** The myotubes were starved for 60 min in serum-free DMEM-Glutamax™ (5.5 mM glucose) in a 5% CO_2 incubator at 37°C. Then, the medium was changed to serum-free DMEM-Glutamax™ with [^{3}H]deoxyglucose (1 µCi/mL) +/− insulin (100 nM) and +/− cytochalasin B (20 µM), and acute, high-frequency EPS was applied for 5–60 min. **Deoxyglucose uptake after chronic, low-frequency EPS:** Chronic, low-frequency EPS was applied to myotubes continuously for the last 24–48 h of the differentiation period. After ended EPS, the myotubes were starved for 60 min in serum-free DMEM-Glutamax™ (5.5 mM glucose) in a 5% CO_2 incubator at 37°C, and then exposed to [^{3}H]deoxyglucose (1 µCi/mL) +/− insulin (100 nM) and +/− cytochalasin B (20 µM). Deoxyglucose uptake was measured for 60 min for both acute, high-frequency EPS and chronic, low-frequency EPS. After ended uptake, the cells were immediately placed on ice and washed three times with ice-cold PBS, lysed with 0.05 M NaOH, and radioactivity was counted by liquid scintillation. The protein content of each sample was measured according to Bradford [45]. Non-carrier-mediated uptake was determined in the presence of cytochalasin B and subtracted from all presented values. **Glucose oxidation after chronic, low-frequency EPS:** Chronic, low-frequency EPS was applied to myotubes for 24 or 48 h. Then, the myotubes were incubated with glucose-free DMEM and D-[^{14}C(U)]glucose (2 µCi/mL) (1 mL/well) in a 5% CO_2 incubator at 37°C. After 2 h, 500 µL cell medium was transferred to airtight flasks, and 300 µL of phenyl ethylamine-methanol (1:1, v/v) was added with a syringe to a center well containing a folded filter paper. Subsequently, 100 µL of 1 M perchloric acid was added to the media through the stopper tops using a syringe. The flasks were placed for a minimum of 2 h at room temperature to trap labeled CO_2, and radioactivity was counted by liquid scintillation The protein content of each sample was measured according to Bradford [45].

Fatty acid metabolism after chronic, low-frequency EPS

After 24–48 h of chronic, low-frequency EPS, the myotubes were exposed to 1 mL/well of DPBS supplemented with HEPES (10 mM), $NaHCO_3$ (44 µM), [1-^{14}C]oleic acid (1 µCi/mL, 0.1 mM), 0.24 mM BSA and 1 mM L-carnitine in a 5% CO_2 incubator at 37°C. After 2 h, 500 µL of cell medium was transferred to airtight flasks, and 300 µL of phenyl ethylamine-methanol (1:1, v/v) was added with a syringe to a center well containing a folded filter paper. Subsequently, 100 µL of 1 M perchloric acid was added to the media through the stopper tops using a syringe. The flasks were placed for a minimum of 2 h at room temperature to trap labeled CO_2. To measure ß-oxidation products (acid-soluble metabolites (ASMs)), aliquots of 250 µL of the cell media were precipitated with 100 µL of 6% BSA and 1 mL of 1 M perchloric acid. After centrifugation (20000 *g*, 10 min, 4°C, Heraues Fresco21 Centrifuge, Thermo Scientific), 250 µL of the supernatant was counted by liquid scintillation. No-cell controls were included and subtracted from all presented values. The cells were placed on ice and washed three times with ice-cold PBS, lysed with 0.05 M NaOH, and cell-associated (CA) radioactivity was counted by liquid scintillation to determine the uptake of oleic acid. The protein content of each sample was measured according to Bradford [45].

Immunoblotting after chronic, low-frequency EPS

Aliquots of 40 µg cell protein from total cell lysates prepared in Laemmli buffer were electrophoretically separated on NuPAGE® 4–12%(w/v) Bis-Tris Gel (Invitrogen) followed by immunoblotting with antibodies recognizing total Akt kinase [protein kinase B (PKB)], Akt phosphorylated at Ser478, and protein complexes of the electron transport chain (OXPHOS human cocktail containing antibodies against Complex I subunit NDUFB8, Complex II subunit, Complex III subunit core 2, Complex IV subunit II and ATP synthase subunit alpha) and Myosin, slow muscle (MHCI). Immunoreactive bands were visualized with enhanced chemiluminescence and quantified with Gel-Pro Analyzer (version 2.0) software.

Staining and live imaging of mitochondria and enzyme activity assay after chronic, low-frequency EPS

Myotubes were cultured in ECM-coated 6-well glass bottom plates. Chronic, low-frequency EPS was applied to cultured myotubes for the last 48 h of the differentiation period, and in addition, on day 8 of the differentiation, myotubes were incubated at 37°C and 5% CO_2 with MitoTracker®Red FM (100 nM) for 15 min to stain mitochondria and Hoechst 33258 (2.5 µg/mL) for 15 min to stain nuclei and washed with PBS in between. Automated image acquisition was performed in culture medium without phenol red with an Olympus ScañR platform (Olympus IX81 inverted fluorescence microscope) equipped with a temperature and CO_2-enrichment incubator for long-term live imaging, as described in Hessvik et al [46]. We used a 20X objective and

live images were aquired in 25 positions per well and 3 wells per treatment per donor were examined. The background-subtracted maximal intensity projection from 7 images taken in z-direction (1 µm apart) was used for both color channels at each position. Olympus ScanR software was used for automated image analysis, using edge detection algorithm for object segmentation to quantify the number of nuclei and mitochondrial content(total intensity of MitoTracker®Red) per image. After gating out aggregates and dead cells the results were determined from about 386 images per treatment (average of 39±4 nuclei per image). After 48 h of chronic, low-frequency EPS, citrate synthase (CS) activity was determined spectrophotometrically from cell homogenates prepared from the myotubes according to the supplier's protocol (Citrate Synthase Activity Assay Kit, Sigma-Aldrich®, St. Louis, MO). Citrate synthase activity in cell homogenates from myotubes stimulated for 48 h was compared to activity in homogenates from unstimulated control myotubes.

RNA isolation and analysis of gene expression by TaqMan® Real-Time RT-PCR

Cells were harvested and total RNA was isolated by Agilent Total RNA isolation kit according to the supplier's total RNA isolation protocol. Total RNA was reverse-transcribed with oligo primers using a Perkin-Elmer Thermal Cycler 9600 (25°C for 10 min, 37°C for 1 h 20 min, and 85°C for 5 min) and a TaqMan reverse transcription reagents kit. Two micrograms of total RNA were added per 20 µL of total TaqMan reaction solution. Real-time PCR was performed using an ABI PRISMT 7000 Detection System (Applied Biosystems, Warrington, UK). RNA expression was determined by SYBRT Green, and primers were designed using Primer ExpressT (Applied Biosystems, Warrington, UK). Each target gene was quantified in triplicate and carried out in a 25 µL reaction volume according to the supplier's protocol. All assays were run for 40 cycles (95°C for 12 s followed by 60°C for 60 s. The transcription levels were normalized to the housekeeping control gene 36B4. Another housekeeping control gene tested, GAPDH, gave similar results as 36B4. Following forward and reverse primers were used at concentration of 30 µM: **36B4** (**acc_no M17885**): F:CCATTCTATCATCAACGGGTACAA, R: AGCAAGTGGGAAGGTGTAATCC; **GAPDH** (**acc_no NM002046**): F: TGCACCACCAACTGCTTAGC, R: GGCATGGACTGTGGTCATGAG; **CPT1b** (**acc_no** : **L39211**): F: GAGGCCTCAATGACCAGAATGT, R: GTGGACTCGCTGGTACAGGAA; **cytochrome C** (**acc_no NM001916**): F: CTGCCAACAACGGAGCATT, R: CGTGAGCAGGGAGAAGACGTA; **PGC-1α** (**acc.no NM013261.3**): AAAGGATGCGCTCTCGTTCA, R: TCTACTGCCTGGAGACCTTGATC; **IL-6** (**acc_no NM000600**): F: CGGGAACGAAAGAGAAGCTCTAT, R: AGGCGCTTGTGGAGAAGGA; **PDK4** (**acc_no BC040239**): F: TTTCCAGACCAACCAATTCACA, R: TGCCCGCATTGCATTCTTA; **GLUT1** (**acc_no K03195**): F: CAGCAGCCCTAAGGATCTTCTCA, R: CCGGCTCGGCTGACATC; **GLUT4** (**acc_no M20747**): F: ACCCTGGTCCTTGCTGTGTT, R: ACCCCAATGTTGTACCCAAACT; **MHCI** (**acc_no NM005963**) : F: CCAGACTGTGTCTGCTCTCTTCAG, R: CAGGACAAGCTCATGCTCC-AT; **MHCIIa** (**acc_no NM017534**): F: AAGGTCGGCAATGAGTATGTCA, R: CAACCATCCACAGGAACATCTTC.

Data presentation and statistics

Statistical analysis of the overall effects of acute, high-frequency EPS on the contents of ATP, PCr and lactate was performed using linear mixed models (LMM) (SPSS version 17, SPSS Inc., Chicago, IL). In experiments where effects of 24 h and 48 h EPS were compared to unstimulated control cells, data were analyzed using non-parametric Kruskal-Wallis test, while in experiments where two groups were compared (EPS-treatment versus unstimulated control cells), non-parametric Wilcoxon matched pair tests were performed (GraphPad Prism 5.0 for Windows, GraphPad Software Inc., San Diego, CA). All values in figures are presented as means±SEM, with n representing the number of experiments performed, each experiment were performed with cells from separate (different) donors, with triplicate samples in each experiment. Statistical significance was set at $P<0.05$. In most experiments, results are presented normalized to unstimulated control cells, and the absolute values of unstimulated control cells are stated in the figure text.

Results

Effects of acute, high-frequency EPS on deoxyglucose uptake, cell contents of ATP, PCr and lactate in cultured myotubes

To verify that electrical stimulation of cultured human myotubes leads to expected metabolic changes, the myotubes were exposed to acute, high-frequency EPS, and deoxyglucose uptake and cellular contents of ATP, PCr and lactate were examined. Frequency-dependence of deoxyglucose uptake during EPS is showed in Figure 1A. Acute, high-frequency EPS increased deoxyglucose uptake in cultured myotubes (Fig. 1B). This uptake was specific, since it was inhibited by cytochalasin B (20 µM) (data not shown). In addition, the amount of deoxyglucose taken up by electrically stimulated myotubes correlated positively with the duration of stimulation. Cell contents of ATP and PCr in electrically stimulated myotubes were compared to unstimulated control cells incubated for the same period of time. In response to 5–60 min of electrical stimulation, the contents of both ATP and PCr decreased significantly ($P=0.001$ and $P=0.007$, respectively), while the amount of lactate significantly increased ($P=0.03$) (Figure 1C, overall effect, linear mixed model, SPSS). Together, these findings indicated that the cells were contracting and consuming energy, and that they responded to acute EPS in a similar way as a single bout of exercise *in vivo*.

Effects of chronic, low-frequency EPS on glucose metabolism in cultured human skeletal muscle cells

We were further interested in whether the effects of chronic, low-frequency EPS on cultured human skeletal cells could mimic the effects of regular exercise *in vivo*. A movie showing contractions of cultured skeletal muscle cells under EPS is attached as Video S1.

Deoxyglucose uptake was significantly increased ($P=0.004$) in cultured myotubes after both 24 and 48 h of chronic, low-frequency EPS by 96% and 145%, respectively, compared to unstimulated control cells. The insulin effect was unaffected by EPS (data not shown). As with acute, high-frequency EPS, the observed increase in deoxyglucose uptake after chronic, low-frequency EPS was specific, since it was inhibited by cytochalasin B (20 µM) (data not shown). Glucose oxidation, measured as the amount of CO_2 produced, was also significantly increased ($P=0.008$) after 48 h of chronic, low-frequency EPS compared to unstimulated control (Fig. 2A).

Effects of chronic, low-frequency EPS on fatty acid metabolism in cultured myotubes

A known effect of exercise is to increase oxidative capacity of the cells, resulting in increased oxidation of both glucose and fatty

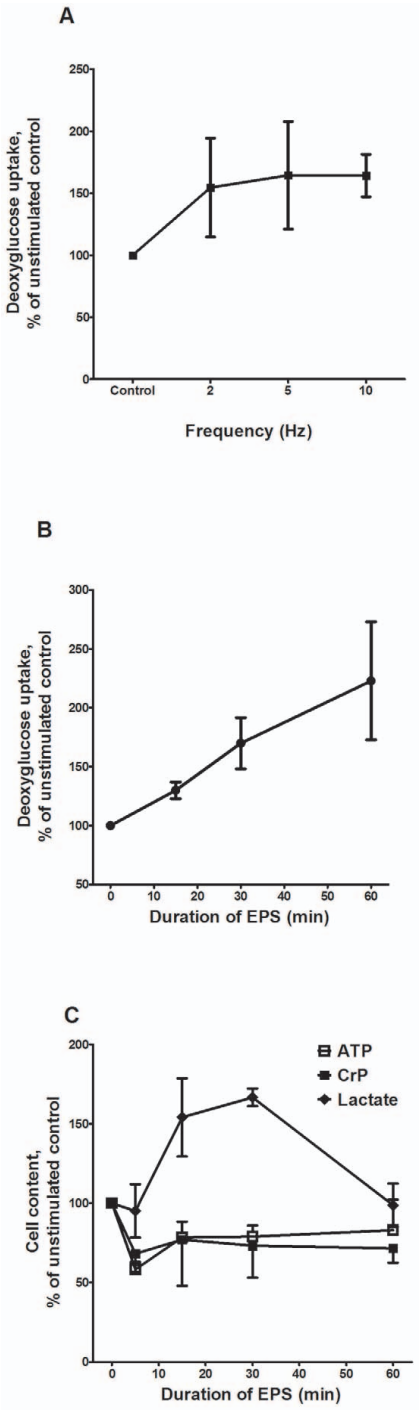

Figure 1. Frequency-dependence of deoxyglucose uptake (A) effects of acute, high-frequency EPS on deoxyglucose uptake (B) and cell contents of ATP, PCr and lactate (C). A: Frequency-dependence of deoxyglucose uptake: Eight days after the onset of differentiation, cultured myotubes were incubated in serum-free DMEM (5.5 mmol/L glucose) for 1 h in a 5% CO_2 incubator at 37°C, before addition of [^{3}H]deoxyglucose (1 vμCi/mL). Electrical pulse stimulation (30 V, pulse trains of 200 ms) was applied to the cells using frequencies of either 2, 5 or 10 Hz for the first 15 min of the deoxyglucose uptake period (60 min). Values are presented as means±SEM of 4 experiments, normalized to unstimulated control cells (absolute values 56.4–333.6 nmol/mg). B:Deoxyglucose uptake: Eight days after the onset of differentiation period, cultured myotubes were incubated in serum-free DMEM (5.5 mmol/L glucose) for 1 h in a 5% CO_2 incubator at 37°C, before addition of [^{3}H]deoxyglucose (1 μCi/mL). Electrical pulse stimulation (100 Hz, 30 V, pulse trains of 200 ms given every 5th second) was applied to the cells the first 5-15-30 min of the deoxyglucose uptake period or during the whole period (60 min) of deoxyglucose uptake. Values are presented as means±SEM of 6 experiments, normalized to unstimulated control cells (57.9–92.5 nmol/mg). C: Cell contents of ATP, phosphocreatine (PCr) and lactate: Eight days after the onset of differentiation period, the myotubes were preincubated for 1 h (37°C, 5% CO_2) with DMEM and high-frequency electrical stimulation (100 Hz, 30 V, pulse trains of 200 ms given every 5th second) was applied for 5–60 min. The cells were analyzed for ATP and PCr levels with luminescence spectrometry, and cell content of lactate was analyzed with fluorescence spectrometry as described in Materials and Methods. Values are presented as means±SEM of 3 experiments, normalized to unstimulated control cells (absolute values: ATP; 42.5–73.9 nmol/mg, PCr; 48.4–169.1 nmol/mg and lactate; 1.9–87.6 nmol/mg). Overall effect of electrical pulse stimulation on cells was statistically significant (linear mixed model, SPSS) compared to unstimulated control cells ($P = 0.001$ for ATP, $P = 0.007$ for PCr and $P = 0.03$ for lactate).
doi:10.1371/journal.pone.0033203.g001

acids. We examined the effect of chronic, low-frequency EPS on oleic acid metabolism by measuring uptake, and production of acid soluble metabolites (ASMs) and CO_2. Complete oleic acid oxidation, measured as the amount of produced CO_2, was significantly increased ($P = 0.04$) after 48 h of EPS by 35% compared to control cells (Fig. 2B). Uptake of oleic acid was unaffected by EPS compared to unstimulated control cells (Fig. 2B). β-oxidation, measured as the amount of ASMs, also remained unchanged after EPS compared to unstimulated control cells (Fig. 2B).

Effects of chronic, low-frequency EPS on mitochondrial content and citrate synthase activity

To support the results obtained in the metabolic experiments with glucose and oleic acid, we investigated effects of chronic, low-frequency stimulation for 48 h on mitochondrial content in cultured myotubes. Representative images are presented for control, unstimulated myotubes in figure 3A (left), and for electrically stimulated myotubes in figure 3A (right). Total intensity of MitoTracker®Red FM per nucleus was significantly increased (2.2-fold, Fig. 3B, $P = 0.03$), and the citrate synthase activity tended to increase ($P = 0.1$, Fig. 3C) in EPS-treated cells compared to unstimulated control cells.

Effects of chronic, low-frequency EPS on gene and protein expression in cultured myotubes

The observed functional changes in fatty acid and glucose metabolism were accompanied by a range of changes in mRNA expressions (Fig. 4). mRNA expression level of pyruvate dehydrogenase complex kinase 4 (PDK4) was significantly increased ($P = 0.04$) after 24 h of EPS (Fig. 4). mRNA expression levels of following genes also tended to increase: CPT1b ($P = 0.06$),

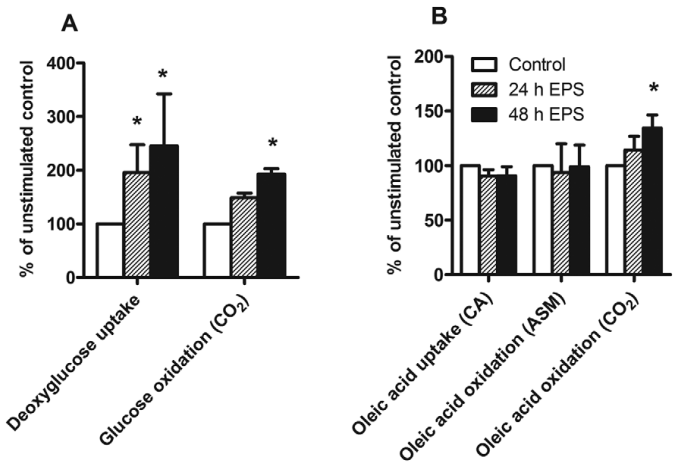

Figure 2. Effects of chronic, low-frequency EPS on glucose (A) and oleic acid metabolism (B). Cultured myotubes were electrically stimulated (1 Hz, 2 ms pulses, 30 V), for the last 24 h or 48 h of the differentiation period. (A) **Deoxyglucose uptake**: After termination of electrical stimulation (day 8 of differentiation), uptake of [^{3}H]deoxyglucose (1 μCi/mL) was measured for 1 h as described in Materials and Methods. Values are presented as means±SEM of 6 experiments. *Significantly different from unstimulated control cells (absolute values 28.0–170.5 nmol/mg protein) ($P = 0.004$, non-parametric Kruskall-Wallis test). **Glucose oxidation**: After termination of electrical stimulation (day 8 of differentiation), the rate of D-[^{14}C(U)]glucose (2 μCi/mL) oxidation was measured as described in Materials and Methods. Values are presented as means±SEM of 4 experiments. *Statistically significant compared to unstimulated control cells (absolute values 2.7–28.4 nmol/mg protein) ($P = 0.008$, non-parametric Kruskall-Wallis test). (B) **Oleic acid metabolism**: Eight or nine days after the onset of differentiation, myotubes were exposed to [1-^{14}C]OA (1 μCi/mL) for 2 h, and CO_2, ASMs and cell-associated (CA) radioactivity were measured as described in Materials and Methods. Values are presented as means±SEM of 8 experiments. *Statistically significant vs. unstimulated control myotubes (absolute values 15.0–166.8 nmol/mg protein for CA, 0.6–4.0 nmol/mg protein for ASMs and 0.2–2.9 nmol/mg protein for CO_2) ($P = 0.04$, non-parametric Kruskall-Wallis test).
doi:10.1371/journal.pone.0033203.g002

Cytochrome c ($P = 0.07$), PGC-1α ($P = 0.2$) and IL-6; a myokine reported to be secreted from contracting skeletal muscles *in vivo* [35,36] ($P = 0.07$) (Fig. 4). GLUT1 and GLUT4 were not affected by EPS (Fig. 4). The results from immunoblots of phosphorylated Akt to total Akt ratio, showed no effect of electrical pulse stimulation (data not shown). Protein expressions of the complexes in the electron transport chain were unchanged by EPS (data not shown).

Effects of chronic, low-frequency EPS on the markers of slow-oxidative (MHCI) and fast glycolytic (MHCIIa) fiber type

To assess the effect of EPS on fiber type markers in myotubes, we further investigated expression of genes and proteins specifically enriched in either type I (slow) fibers (MHCI) or type IIa (fast) fibers (MHCIIa). Expressions of MHCI and MHCIIa appeared to increase and decrease, respectively, thus the MHCI/MHCIIa mRNA ratio tended to increase ($P = 0.06$) in electrically stimulated myotubes (Fig. 5A). Protein expression of MHCI was significantly increased ($P = 0.03$) by 45% after 24–48 h of EPS (Fig. 5B, C).

Evaluation of toxic effects of EPS on cultured myotubes

Protein contents of the cells were unaffected by acute, high-frequency as well as chronic, low-frequency EPS under the conditions used in the experiments (data not shown). Further, lactate dehydrogenase content was unchanged in media from myotubes exposed to chronic, low-frequency EPS for both 24 h and 48 h compared to unstimulated control cells (data not shown). Number of nuclei in myotubes after 48 h of chronic, low-frequency EPS, determined by live imaging of the cells, did not differ from unstimulated control cells (data not shown). After staining with trypan blue, a low percentage (less than 1%) was stained, both in control cells and in electrically stimulated myotubes, with no difference between the two groups (data not shown). In conclusion, EPS did not induce toxic effects to cultured human skeletal muscle cells.

Discussion

Cultured myotubes are a valuable tool for investigation of metabolic processes in skeletal muscles. In the present study, we demonstrate how a cell model of human myotubes can be established as an *in vitro* model of exercise, which can be used to study some of the adaptations seen in trained skeletal muscles. Two different patterns of electrical pulse stimulation (EPS) were applied: acute, high-frequency EPS (bipolar pulse trains of 200 ms, 100 Hz, given every 5th second, 30 V for 5–60 min) to simulate a single bout of exercise; and chronic, low-frequency EPS (single, bipolar pulses of 2 ms, 1 Hz at 30 V for 24 or 48 h) to simulate regular exercise.

By acutely stimulating cultured myotubes with high-frequency EPS, glucose uptake and cell lactate content increased, while ATP and PCr contents decreased. By continuously applying chronic, low-frequency EPS, we successfully increased oxidative capacity of the cells by increasing glucose metabolism and complete oleic acid oxidation. Further, these functional changes in the metabolic processes were accompanied by doubling of mitochondrial content, measured as total intensity of MitoTracker®Red, after 48 h of EPS. Citrate synthase activity tended to increase in EPS-treated myotubes, although not significantly. Increased mitochon-

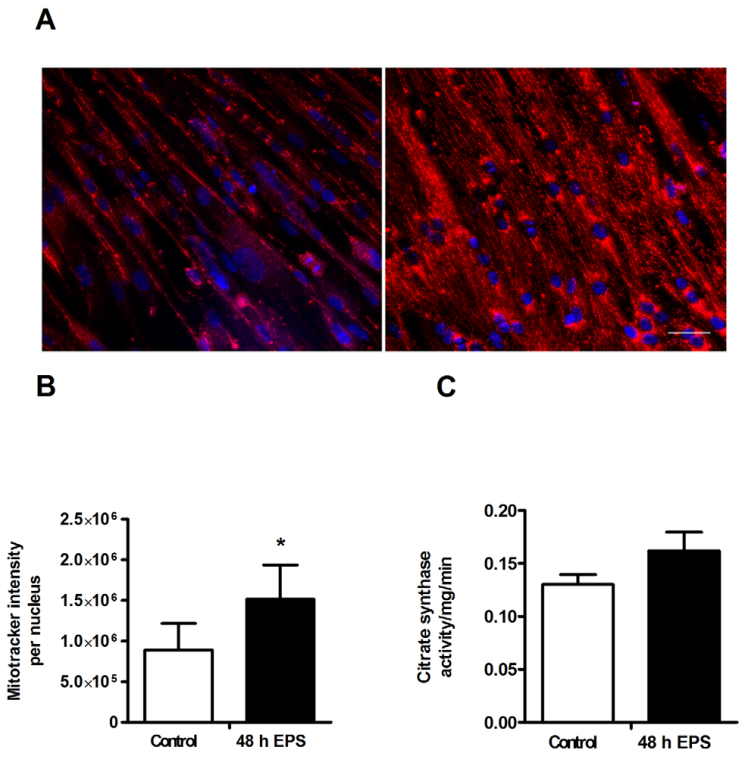

Figure 3. Effects of 48 h of chronic, low-frequency EPS on mitochondrial content and citrate synthase activity. Low-frequency EPS was applied to cultured myotubes for the last 48 h of the eight days differentiation period as described in Materials and Methods. (**A**) **Live imaging of mitochondria:** The cells were stained for nuclei (blue) and mitochondria (red) as described in Materials and Methods. Scale bar is 50 μm. Left: Unstimulated control myotubes. Right: Myotubes after 48 h of chronic, low-frequency EPS. (**B**) **Mitochondrial content in electrically stimulated myotubes**: Mitochondrial content was measured by live imaging after 48 h of chronic, low-frequency EPS. Values are presented as means±SEM of 6 experiments. *Statistically significant vs. unstimulated control ($P = 0.03$, non-parametric Wilcoxon matched pair test). (**C**) **Citrate synthase activity in electrically stimulated myotubes:** Enzyme activity was determined spectrophotometrically from cell homogenates prepared from the myotubes after 48 h of chronic, low-frequency EPS as described in Materials and Methods, and compared to activity in unstimulated control cells. Values are presented as means±SEM of 5 experiments.
doi:10.1371/journal.pone.0033203.g003

drial content in skeletal muscles after exercise is believed to result from the cumulative effects of transient increases in mRNA transcripts encoding mitochondrial proteins after repeated exercise sessions [3,47]. Temporal sequences of molecular effects that occur in human muscle when mitochondrial biogenesis is induced with exercise training were investigated in a study by Perry et al. [48]. Although CS mRNA increased already after first training session, increase in the activity of CS was not observed until the end of the 3rd of the seven performed training sessions. In general, repeated transient bursts of mRNA were shown to occur in the early phases of training, before increases in the activities of mitochondrial proteins, but the time and magnitude of mRNA and protein responses of different transcriptional and mitochondrial proteins also showed considerable variation, depending on the phase of the exercise they were measured in [48]. Thus, the lack of significant increase in CS activity in our experiments after only 48 hours of EPS is in agreement, or at least reflects the complexity and precise time-dependence of molecular events that were described to occur in mitochondrial biogenesis during exercise in human skeletal muscles. At applied conditions, we did show expected functional changes (lipid oxidation and glucose metabolism), as well as changes in mRNA and protein expressions of some factors, but in order to demonstrate other changes, both on mRNA and protein level, we would perhaps have to use different patterns and time periods, and with the present model, this is something that can be done in the future work. The same inconsistency between citrate synthase activity and MitoTracker®Red intensity has also been reported in a recent work presenting a novel, exercise-mimicking approach to remodel lipid metabolism in cultured human myotubes [49]. Interestingly, this was a cell system like ours, but the exercise-mimicking effects were induced by a pharmacological activation. Thus, for future studies, time aspect may be an important factor to be considered when performing exercise-mimicking studies in cell cultures.

Changes in expression levels of a range of genes were also observed after chronic EPS. mRNA level of PDK4 was significantly increased in electrically stimulated cells, while mRNA levels of Cytochrome c and CPT1b also tended to increase.

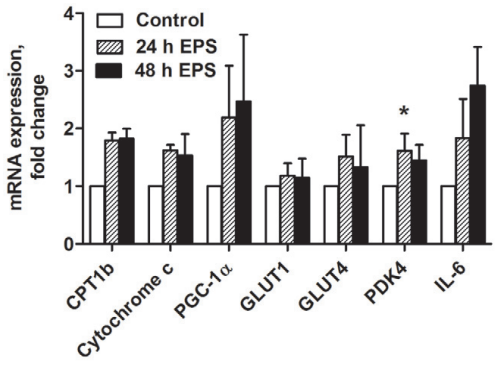

Figure 4. Effects of chronic, low-frequency EPS on gene expression. Low-frequency EPS was applied to cultured myotubes for the last 24 h or 48 h of the eight days differentiation period. mRNA was isolated and expression assessed by real time RT-PCR as described in Materials and Methods, and values are presented as means±SEM of 3–6 experiments, normalized to levels of housekeeping genes 36B4. The ranges of the fold changes of the mRNA expression levels in the control groups normalized to the level of housekeeping gene 36B4 were as follows: 0.3–1.9 for CPT1b, 0.4–1.3 for cytochrome *C*, 0.001–0.6 for PGC-1α, 0.6–1.2 for GLUT1, 0.2–2.6 for GLUT4, 0.7–1.0 for PDK4 and 0.1–1.1 for IL-6. *Statistically significant vs. unstimulated control cells ($P = 0.04$, non-parametric Kruskall-Wallis test).
doi:10.1371/journal.pone.0033203.g004

GLUT1 and GLUT4 did not appear to be affected by EPS. Moreover, the ratio of the mRNA level of MHCI (a gene marker of type I, slow oxidative fiber type), to that of MHCIIa (a gene marker of glycolytic, fast-twitch skeletal muscle fibers) tended to increase in electrically stimulated myotubes, and this finding was also supported by increased protein expression of MHCI in EPS-treated cells. Further, mRNA expression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), a transcriptional cofactor referred to as the master regulator of mitochondrial function and biogenesis [50]; which is frequently considered as an important factor in cellular mechanisms evoked by exercise, tended to increase, although not significantly, as well as mRNA level of IL-6, an interleukin known to be secreted by skeletal muscles after exercise *in vivo*, strengthening the conclusion that our model of EPS may resemble trained muscle.

The relative contribution of fatty acid oxidation to total fuel demand is increased in healthy subjects performing moderate-intensity exercise, and several studies support that exercise reduces the reliance on carbohydrates as an energy source and increases fatty acid oxidation [51,52]. The rate of oleic acid oxidation was significantly increased after 48 h of EPS in our cell culture model, while the uptake of oleic acid was unchanged. Although exercise has been shown to increase uptake of fatty acids in humans, cellular mechanisms of elevated uptake are still not clear, since inconsistencies exist due to different duration and intensity of training studies [53]. A key factor facilitating transport of fatty acids through the carnitine shuttle over the outer mitochondrial membrane, CPT1b, has been shown to be increased in an *in vivo* human training study with moderate-intensity exercise for a shorter duration (2 months), accompanied by an increase in mitochondrial fatty acid oxidation rate [54]. Although not significantly, mRNA expression level of CPT1b tended to increase in our EPS cell model as well.

Our model of chronic, low-frequency EPS showed both increased import and oxidative metabolism of glucose, and these effects are also known from *in vivo* trained fibers [25,26]. However, we did not observe any additional effect of insulin and EPS on glucose uptake, nor was phosphorylation of Akt affected by EPS. These observations are in agreement with suggestions of an insulin-independent pathway to enhance glucose uptake [55,56]. In addition, there are proposals that some key metabolic substances typically triggered by insulin, may also be activated during muscle contractions in the absence of this hormone [26]. mRNA expression levels of GLUT1 and GLUT4 were unaffected by EPS. GLUT4 is often deficient in cultured skeletal muscle cells [57], and in primary human myotubes, basal glucose uptake is generally mediated by other glucose transporters, such as GLUT1 and GLUT3 [58,59]. Inconsistencies between mRNA levels of

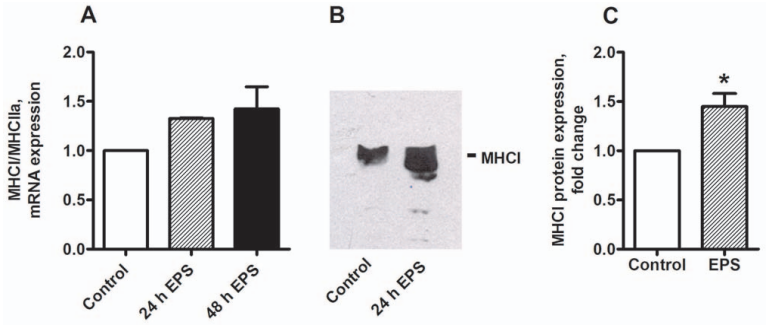

Figure 5. Effects of chronic, low-frequency EPS on markers of slow-oxidative (MHCI) and fast-glycolytic (MHCIIa) muscle fiber types. Low-frequency EPS was applied to cultured myotubes for the last 24 h or 48 h of the eight days differentiation period as described in Materials and Methods before the cells were harvested. (**A**) **MHCI/MHCIIa mRNA ratio:** mRNA was isolated from cultured myotubes after the EPS treatment. Expression was assessed by RT-PCR as described in Materials and Methods, and values are presented as means±SEM of 4 experiments, normalized to levels of housekeeping genes 36B4. (**B**) (**C) Immunoblot analysis of MHCI after 24–48 h of EPS:** Aliquots of 40 μg cell protein from total cell lysates prepared in Laemmli buffer were electrophoretically separated on NuPAGE® 4–12% (w/v) Bis-Tris Gel, followed by immunoblotting with specific antibody for slow-oxidative MHCI. (B) One representative immunoblot. (C) Densitometric analysis of immunoblots, values are presented as means±SEM of 6 experiments. *Statistically significant vs. unstimulated control cells ($P = 0.03$, non-parametric Wilcoxon matched pair test).
doi:10.1371/journal.pone.0033203.g005

GLUTs and functional data have previously been reported in cultured human skeletal muscle cells [60,61]. On the other hand, PDK4, an inhibitor of pyruvate dehydrogenase complex, which is an important factor in switching oxidation towards fatty acids [62], was significantly increased in EPS-treated cells, indicating a possible switch in the fuel preference of the myotubes. When grown in culture, satellite cells mature to myotubes that generally display the characteristics of glycolytic type II muscle fibers [41], and are characterized by low mitochondrial oxidative capacity [63,64], with higher fuel preference for carbohydrates over lipids [65]. This could be due to lack of proliferation of mitochondria *in vitro* in the absence of appropriate environmental signals, such as contractions. Thus, approaches that increase mitochondrial oxidative potential of human myotubes are highly relevant with respect to studies on cellular energy metabolism.

Even though it is difficult to directly compare effects of *in vivo* exercise to the observed effects in our model of EPS in cultured human myotubes, several of our observations display important aspects of the *in vivo* effects of exercise. In summary, by applying our model of chronic continuous, low-frequency EPS, we observed important functional changes in cell culture: improved lipid oxidation and glucose metabolism, which are known effects of exercise *in vivo*. Further, we also demonstrated a possible fiber-type switch, measured by increased protein expression of MHCI in EPS-treated cells. To our knowledge, such changes have not previously been reported in human cell cultures. Thus, we believe that our model of EPS in cultured human skeletal muscle cells represents a unique, physiologically relevant *ex vivo* model, which can be used to further study interrelationship between exercise-induced cellular mechanisms and underlying signalling pathways under controlled conditions. Particularly, the present model might be of great interest in clarifying the potential of contractions on energy metabolism in skeletal muscle cells obtained from different groups of individuals (obese, glucose intolerant, athletes etc.). Currently, contraction-induced effects on energy metabolism in human skeletal muscle cells originating from extremely obese individuals with or without type 2 diabetes are being investigated.

Supporting Information

Video S1 A film showing contractions of cultured human skeletal muscle cells exposed to chronic, low-frequency EPS. Cultured human skeletal muscle cells were exposed to chronic, low-frequency EPS (single, bipolar pulses of 2 ms, with 30 V and 1 Hz continuously for the last 24 or 48 h of differentiation period). The observed contractions were synchronous with the electrical pulses.
(M4V)

Acknowledgments

The authors are thankful to Ph.D. Geir Florholmen for doing the measurements of lactate, ATP and PCr, to Dr. Gerbrand Koster and Professor Oddmund Bakke of the NORMIC-UiO imaging platform, Department of Molecular Biosciences, University of Oslo, for support and use of equipment, and to Åse-Karine Fjeldheim and Robert Smith, for excellent technical assistance.

Author Contributions

Conceived and designed the experiments: NN SSB ETK IR IFH ACR GHT VA. Performed the experiments: NN SSB ETK IR IFH ACR GHT VA. Analyzed the data: NN SSB ETK IR IFH ACR GHT VA. Contributed reagents/materials/analysis tools: NN SSB ETK IR IFH ACR GHT VA. Wrote the paper: NN.

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Erratum to: Electrical Pulse Stimulation of Cultured Human Skeletal Muscle Cells as an *In Vitro* Model of Exercise

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Published 22 March 2012

Erratum to PLoS ONE 7(3): e33203. doi:10.1371/journal.pone.0033203

Unfortunately there was a mistake in Figure 5A. The primer used for mRNA expression was actually MYH1 which regulates expression of MHC type IIx muscle fibers and not MHC type I as stated in the figure. However, we have repeated the experiment with the correct primer MYH7 (acc_no NM000257.2, F: CTCTGCACAGGGAAAATCTGAA, R: CCCCTGGAGACTTTGTCTCATT), and the new Figure 5 is shown here. This makes no difference to the conclusions of the paper. Chronic low frequent electrical pulse stimulation for 48 h increased expression of slow muscle fibers assessed by Western blotting, but there were no significant change in mRNA expression observed at the same time, neither was the MHCI/MHCIIa mRNA ratio significantly increased (p=0.125, non-parametric Wilcoxon matched pair test, n=4).

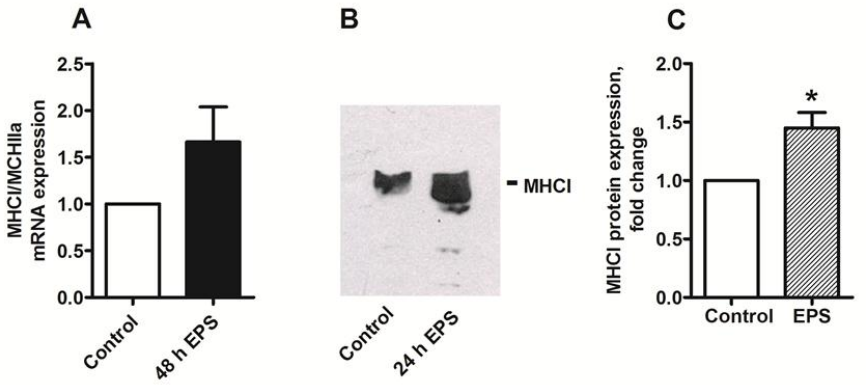

Figure 5
Effects of chronic, low-frequency EPS on markers of slow-oxidative (MHCI) and fast-glycolytic (MHCIIa) muscle fiber types. Low-frequency EPS was applied to cultured myotubes for the last 24 h

or 48 h of the eight days differentiation period as described in Materials and Methods before the cells were harvested. (A) MHCI/MHCIIa mRNA ratio: mRNA was isolated from cultured myotubes after the EPS treatment. Expression was assessed by RT-PCR as described in Materials and Methods, and values are presented as means ± SEM of 4 experiments, normalized to levels of housekeeping genes 36B4. (B) (C) Immunoblot analysis of MHCI after 24–48 h of EPS: Aliquots of 40 µg cell protein from total cell lysates prepared in Laemmli buffer were electrophoretically separated on NuPAGE® 4–12% (w/v) Bis-Tris Gel, followed by immunoblotting with specific antibody for slow-oxidative MHCI. (B) One representative immunoblot. (C) Densitometric analysis of immunoblots, values are presented as means±SEM of 6 experiments. *Statistically significant vs. unstimulated control cells (P = 0.03, non-parametric Wilcoxon matched pair test).