Regulation of energy metabolism in skeletal muscle cells by PPARδ activation, *in vitro* exercise and perilipin 2 ablation

Studies in human and mice myotubes

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PPARδ activation in human myotubes increases mitochondrial fatty acid oxidative capacity and reduces glucose utilization by a switch in substrate preference

Archives of Physiology and Biochemistry, 2014. 120(1): p. 12-21.

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Bakke SS, **Feng YZ**, Nikolić N, Kase ET, Moro C, Stensrud C, Damlien L, Ludahl MO, Sandbu R, Solheim BM, Rustan AC, Hjelmesæth J, Thoresen GH and Aas V.

Myotubes from severely obese type 2 diabetic subjects accumulate less lipids and show higher lipolytic rate than myotubes from severely obese non-diabetic subjects

PLoS One, 2015. 10(3): p. e0119556

Paper III

Feng YZ, Nikolić N, Bakke SS, Kase ET, Guderud K, Hjelmesæth J, Aas V, Rustan AC and Thoresen GH.

Myotubes from lean and severely obese subjects with and without type 2 diabetes respond differently to an in vitro model of exercise

American Journal of Physiology - Cell Physiology, 2015. 308(7): p. C548-56

Paper IV

Feng YZ, Lund J, Knabenes IK, Bakke SS, Kase ET, Lee YK, Kimmel AR, Thoresen GH, Rustan AC and Dalen KT.

A metabolic shift in energy metabolism from utilization of glucose towards fatty acids in myotubes lacking perilipin 2

Manuscript

ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ACL	ATP citrate lyase
ACSL	acyl-CoA synthetase
AKT/PKB	protein kinase B
AMPK	AMP-activated protein kinase
ANGPTL4	angiopoietin-like protein 4
AS160	Akt substrate of 160 kDa
ASM	acid-soluble metabolites
ATGL	adipose triglyceride lipase
BMI	body mass index
CaMK	calmodulin-dependent protein kinase
CD36/FAT	fatty acid transporter
CE	cholesteryl ester
CGI-58	comparative gene identification-58
CPT	carnitine palmitoyltransferase
CYC	cytochrome c
DAG	diacylglycerol
DGAT	diacylglycerol acyltransferase
EPS	electrical pulse stimulation
FA	fatty acid
FABP	fatty acid binding protein
FATP	fatty acid transport proteins
FCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
G-6-P	glucose 6-phosphate
GLUT	glucose transporter
HSL	hormone-sensitive lipase
IL	interleukin
IMTG	intramyocellular triacylglycerol
IRS	insulin receptor substrate
KO	knockout
LD	lipid droplet
L-nD	lean non-diabetic donor
MAG	monoacylglycerol
MEF	myocyte enhancer factor
MGAT	monoacylglycerol acyltransferase
MHC	myosin heavy chain
MYF5	myogenic factor 5
MYH	myosin heavy chain, gene
MYOD	myogenic differentiation protein
OA	oleic acid
OXPHOS	oxidative phosphorylation proteins
PDC	pyruvate dehydrogenase complex
PDK4	pyruvate dehydrogenase kinase isozyme 4
PGC	peroxisome proliferator-activated receptor gamma coactivator
PI3-kinase	phosphatidylinositol 3-kinase
PL	phospholipids
PLIN	perilipin
PPAR	peroxisome proliferator-activated receptor
SO-nD	severely obese non-diabetic
SO-T2D	severely obese with established type 2 diabetes
TAG	triacylglycerol
T2D	type 2 diabetes
WT	wild type

ABSTRACT

The prevalence of type 2 diabetes (T2D) has increased worldwide during the last decades. Lifestyle factors such as obesity are strongly associated with insulin resistance and T2D. Increasing evidence suggests that dysregulations in lipid influx, storage, or triacylglycerol (TAG) lipolysis have significant impact on insulin sensitivity and glucose homeostasis in skeletal muscle. Moreover, it has been suggested that insulin resistance in obesity and T2D is associated with accumulation of lipids in skeletal muscles. Much focus has been on the possibility of increasing lipid utilization by exercise or pharmacologically to avoid ectopic lipid accumulation in muscle. The nuclear receptor peroxisome proliferator-activated receptor δ (PPARδ) is shown to be an important regulator of skeletal muscle lipid metabolism, highlighting the potential utility of this receptor as a therapeutic option for treatment of T2D. This thesis aimed to study regulation of energy metabolism related to obesity and T2D in cultured human skeletal muscle cells by PPARδ activation and *in vitro* exercise. The lipid droplet (LD)-associated protein perilipin 2 (PLIN2) is one of the PPARδ target genes, and to study the functional role of PLIN2 and LDs in skeletal muscle energy metabolism we also examined myotube cultures established from $PLIN2^{+/+}$ and $PLIN2^{-/-}$ mice.

A reduced insulin response was observed in myotubes from severely obese donors (BMI ≥ 40 kg/m^2) with established T2D confirming that myotubes maintain their diabetic phenotype in culture. Diabetic myotubes showed also lower lipid accumulation and fatty acid (FA) incorporation into TAG, as well as higher lipolysis with lower ability to increase oxidation of lipids with increased FA availability, compared to myotubes from non-diabetic donors with similar BMI. Chronic low-frequency electrical pulse stimulation (EPS), as an *in vitro* model of endurance exercise, was able to improve insulin sensitivity in insulin-resistant myotubes from diabetic donors. EPS increased lipid oxidation and mitochondrial content in myotubes from lean non-diabetic subjects, but not in myotubes from severely obese subjects. Furthermore, EPS increased the mRNA expression of the myokine interleukin-6 in myotubes from both lean and severely obese non-diabetic subjects. The principal effect of PPARδ activation was to increase mitochondrial FA oxidative capacity, and in contrast to the effect of EPS, the effect of PPARδ on lipid metabolism was not different between myotubes from the various donor groups. Increase in PLIN2 gene expression after PPARδ activation was not accompanied with an effect on number of LDs or on lipolysis, while ablation of PLIN2 resulted in myotubes with reduced number of LDs and reduced

accumulation of TAG and higher lipolysis. Both PPARδ activation and ablation of PLIN2 resulted in a metabolic shift in energy metabolism from utilization of glucose towards FAs. Furthermore, neither ablation of PLIN2 nor activation of PPARδ had any impact on insulin-stimulated responses despite of increased oxidative capacity for FAs.

The results presented in this thesis suggest that myotubes to some extent retain the phenotype of their donors, and that responses to EPS, but not to PPARδ, reflected the *in vivo* characteristics of the donors. While both exercise *in vitro* (EPS), activation of PPARδ and lack of PLIN2 increased lipid oxidation, only EPS had any impact on the insulin-stimulated responses, whereas both PPARδ activation and increased FA availability through PLIN2 ablation shifted the cells from glucose to lipid metabolism.

INTRODUCTION

Energy metabolism in skeletal muscle

Skeletal muscle constitutes about 40% of the body weight in non-obese individuals, and is the largest insulin-sensitive organ, accounting for more than 80% of insulin-stimulated glucose disposal, and is therefore the quantitatively the most important site for insulin resistance [1-3]. At resting, skeletal muscle accounts for about 30% of the metabolic rate [4]. Moreover, it is also the quantitatively most dominant tissue with respect to lipid metabolism and the largest glycogen storage organ [5, 6]. Factors regulating fatty acid (FA) oxidation and mitochondrial function capacity are likely to directly affect muscle metabolic function and, because of its large contribution to total body mass, to have a significant impact on whole-body energy metabolism. Thus, the critical role that skeletal muscle plays in glycemic control and metabolic homeostasis makes it an organ of particular interest in obesity and type 2 diabetes (T2D).

Under resting conditions, glucose uptake is considered as the rate-limiting step for glucose utilization [7], and is mainly mediated by glucose transporter (GLUT) 1 and 4 in skeletal muscle [8, 9]. GLUT1 appears to be involved mainly in basal glucose uptake [10], whereas in response to insulin stimulation or contraction, GLUT4 is translocated from intracellular vesicles to the cell surface of skeletal muscle cells, resulting in an increase in glucose uptake [11-14]. The canonical insulin-signaling pathway is triggered by activating of insulin receptor tyrosine kinase, leading to phosphorylation of substrate proteins and their recruitment and activation of phosphatidylinositol 3-kinase (PI3-kinase), which in turn triggers phosphorylation of Akt (PKB) [15]. Thus, Akt is the principal insulin-regulated signal transducer for GLUT4 translocation in response to insulin [16]. Once glucose has been transported across the plasma membrane, it is phosphorylated to glucose 6-phosphate (G-6-P) by hexokinase and proceed to glycolysis, generating pyruvate, ATP and NADH (**Figure 1**). Alternatively, G-6-P can be converted to glycogen for storage, which is mediated by glycogen synthase [17]. In the case of excessive energy supply and the limited ability to store glycogen in skeletal muscle, most excess glucose is converted to lipids through lipogenesis [18]. *De novo* lipogenesis occurs in skeletal muscle, but only to a small extent [19]. Pyruvate, either from plasma glucose or from stored glycogen, can proceed to oxidation in mitochondria via decarboxylation to acetyl-CoA, which is mediated by the pyruvate dehydrogenase complex (PDC) [20].

Free fatty acids (FFAs) are taken up from the circulation into muscle either by passive diffusion or by protein-mediated transport (**Figure 1**). The major proteins regulating muscle FA uptake are FA translocase (FAT/CD36), plasma membrane-associated FA-binding protein (FABPpm) and a family of FA transport proteins (FATP1-6) [21, 22], where CD36 and FATP4 are considered quantitatively the most important in skeletal muscles [23, 24]. Once inside the cells, FAs are reversibly bound to the abundantly expressed cytoplasmatic FABP, which acts as a sink for the incoming FAs to protect against lipotoxic accumulation of free FAs and traffics FAs throughout cellular compartments [25]. FAs are activated to FA-CoA (acyl-CoA) through a reaction mediated by acyl-CoA synthetase (ACSL) [26]. Acyl-CoA-binding protein (ACBP) acts as an intracellular carrier of FA-CoA, where FA-CoA can be oxidized in mitochondria for ATP production, esterified to monoacylglycerol (MAG) and diacylglycerol (DAG) and stored as triacylglycerol (TAG) in lipid droplets (discussed in more details under **"Dynamics in skeletal muscle lipid pools"**, page 9), incorporated into phospholipids or metabolized to lipid second messengers [27, 28]. The fate of FA is influenced by the concentration of the incoming FA, the type of FA, the muscle fiber type, the hormonal milieu, and the energy requirements of the muscle [25]. Long-chain FA-CoA can be oxidized after mitochondrial transport as acyl-carnitine, which is facilitated by carnitine palmitoyltransferase 1 (CPT1) located on the outer mitochondrial membrane and CPT2 located on the inner mitochondrial membrane [29]. CD36 is also found in the mitochondrial membrane, and have been suggested to work in cooperation with CPT1 [30, 31]. Inside the mitochondrial matrix, FA-CoA is metabolized through the β-oxidation pathway to acetyl-CoA. Thereafter, acetyl-CoA from both β-oxidation and glycolysis enters the TCA-cycle (**Figure 1**). The regulation of FA oxidation has previously been attributed primarily to transport of FAs across the mitochondrial membranes and, specifically, by reduced malonyl-CoA inhibition of CPT1 derived from acetyl-CoA from glycolytic pathway catalyzed by acetyl-CoA carboxylase (ACC2) in oxidative tissues [29]. However, recent work has challenged this dogma, suggesting that the regulation of skeletal muscle FA oxidation is a more complicated process and involves multiple regulatory sites, including FA transport across the membrane, binding and transport of FAs in the cytoplasm, LD formation and degradation, FA transport across the mitochondrial membranes and potential regulations within the β-oxidation pathway, TCA cycle and electron transport chain [32-34].

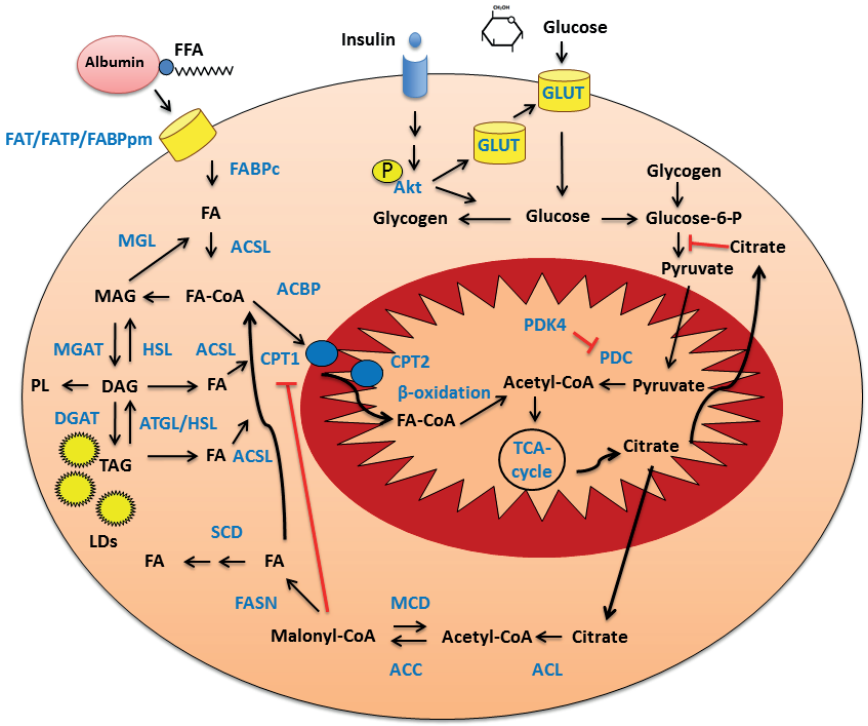

Figure 1. Energy metabolism in skeletal muscle. Glucose is transported into cells through glucose transporters (GLUT) and is stored either as glycogen or utilized through process of glycolysis to yield pyruvate. GLUT4 is translocated from intracellular vesicles to the cell surface through activation of the insulin pathway. Uptake of fatty acids (FAs) is facilitated by different transport proteins (FAT, FATP and FABPpm). Intracellular FAs are bound to cytosolic FA binding proteins (FABPc) and activated to FA-CoA by acyl-CoA synthetase (ACSL). Acyl-CoA-binding protein (ACBP) acts as an intracellular carrier of FA-CoA. Under conditions of excess energy supply, FA-CoAs may 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 monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT). Upon energy demand, TAG, DAG and monoacylglycerol (MAG) are hydrolyzed by adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) to FAs. FA-CoAs from both exogenous and endogenous derived FAs are used as fuel and transported into mitochondria as acyl-carnitine via carnitine palmitoyltransferase (CPT) 1 and 2, and thereafter metabolized through β-oxidation, yielding acetyl-CoA, which then enters the TCA-cycle. Pyruvate, either from plasma glucose or from stored glycogen, can enter the mitochondria via decarboxylation to acetyl-CoA through the action of pyruvate dehydrogenase complex (PDC). Citrate that escapes from TCA-cycle can be converted to acetyl-CoA in the cytosol by ATP citrate lyase (ACL), and thereafter to malonyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA decarboxylase (MCD) catalyzes the reverse reaction and converts malonyl-CoA to acetyl-CoA. Malonyl-CoA can be converted to FAs by the action of FA synthase (FASN) and FAs can be further elongated and desaturated by elongases and stearoyl-CoA desaturases (SCD), respectively. Malonyl-CoA is a potent inhibitor of CPT1, and can therefore inhibit entry and oxidation of FAs in the mitochondria. FAs are able to suppress glucose oxidation through inhibition of PDC by pyruvate dehydrogenase kinase isozyme 4 (PDK4) and by acetyl-CoA, as well as inhibition of glycolytic enzymes by cytosolic citrate. The mutual inhibition of substrate metabolism is often referred as Randle cycle (FA suppresses glucose oxidation) and reverse Randle cycle (glucose suppresses FA oxidation).

Dynamics of skeletal muscle lipid pools

Approximately 50-60% of the FAs taken up by the muscle cells are stored as TAG in lipid droplets (LDs), referred as intramyocellular TAG (IMTG) [35]. The conversion of FA-CoA and MAG to DAG is mediated by monoacylglycerol acyltransferase (MGAT), and conversion of DAG to TAG is mediated diacylglycerol acyltransferase (DGAT) 1 or 2 [36] (**Figure 1**). Besides TAG, LDs also contain DAG, cholesteryl ester (CE) and free cholesterol, and they are surrounded by a monolayer of phospholipids (PL) and LD-associated proteins (e.g. perilipins) [37, 38]. The mammalian genome encodes for five perilipin (PLIN) genes, and additional splice variants, with different tissue expression patterns [39]. In addition, PLINs differ also in size, affinity to LDs, stability when not bound to LDs, and transcriptional regulation. Positioned at the lipid LD surface, PLINs manage access of lipases to the lipids within the LD core and thereby regulating LD size and turnover [38]. In human skeletal muscle, all five PLINs are present at mRNA level where PLIN2 (also known as adipocyte differentiation-related protein or adipophilin) is one of the most abundantly LD-associated proteins expressed [40]. Further, in human muscle biopsies, the majority of the LDs (~ 60%) were shown to be covered by PLIN2, and PLIN2 content was also higher in oxidative type I muscle fibers compared to glycolytic type II fibers [41]. Furthermore, PLIN2 is shown to be more abundant in woman than in men, consistent with higher IMTG content observed in female skeletal muscle [42].

Upon energy demand e.g. during exercise, the enzymatic degradation of the esterified neutral lipids in the LD-core into single lipid species such as FAs or glycerol depends on active recruitment of lipases to the LD surface. Adipose triglyceride lipase (ATGL) is considered to be the first step in TAG catabolism [43], generating DAG, which is subsequently degraded by hormone-sensitive lipase (HSL) [44]. In the final step, MAG is degraded to glycerol and FA by monoacylglycerol lipase (MGL) (**Figure 1**) and thus providing FAs that can be oxidized in mitochondria. Moreover, lipase-mediated TAG hydrolysis can also generate lipid ligands for peroxisome proliferator-activated receptors (discussed in more details under **"Role of peroxisome proliferator-activated receptors (PPARs) on energy metabolism in skeletal muscle"**, page 12). Other potentially important proteins in the regulation of breakdown of IMTG are CGI-58 [45] and G0/G1 switch genes 2 (G0S2) [46], which are identified as coactivator and inhibitor of ATGL, respectively. Interestingly, during contraction-induced muscle lipolysis, ATGL and CGI-58 are strongly associated and they work together with PLIN proteins to regulate lipolysis [47]. HSL

activity is mostly regulated by phosphorylation such as phosphorylation on serine 660 [48]. LDs in oxidative muscle are often close to mitochondria, thereby maintaining coupling of lipid storage with consumption of lipids as fuel which appears to be important for efficient energy utilization [37].

Metabolic flexibility of skeletal muscle

The ability of skeletal muscle to switch between substrates for fuel depending on substrate availability, exercise intensity and physiological conditions represent an important feature in healthy skeletal muscle and is called metabolic flexibility [49, 50]. In the fed state, increased availability of plasma glucose stimulates glucose oxidation and FA synthesis, whereas FA oxidation increases both during fasting and sustained exercise [51, 52]. The inhibition of glucose oxidation by FAs is often referred as "Randle cycle" [53]. This is mediated by inhibition of several glycolytic steps. Pyruvate dehydrogenase kinase isozyme 4 (PDK4), the dominant isoform in skeletal muscle inhibits PDC by phosphorylation, and thereby switching the fuel source from glucose to FA [54] (**Figure 1**). Further, excess production of citrate from enhanced FA oxidation escapes from mitochondria and inhibits the rate-limiting enzyme of glycolysis, 6-phosphofructo-1-kinase, leading to an increase in G-6-P, which eventually inhibits hexokinase and leads to reduced glucose uptake and oxidation [32, 55]. The opposite situation, where glucose suppresses FA oxidation, is often referred as "reverse Randle cycle" [56]. Citrate escaped from oxidation of glucose is transported back to the cytosol, where it is converted to acetyl-CoA by ATP citrate lyase (ACL), which in turn is converted to malonyl-CoA by ACC. As stated above, malonyl-CoA inhibits CPT1 and thereby entry and oxidation of FAs in mitochondria [56, 57] (**Figure 1**).

Loss of ability to switch easily between glucose and lipid oxidation is termed metabolic inflexibility [50], and is associated with reduced lipid oxidation, and thereby promotes accumulation of lipids in skeletal muscle [58], which can interfere with insulin signaling and function (discussed in more details under **"Insulin resistance, obesity and type 2 diabetes"**, page 19). Obesity, insulin resistance and T2D are linked to reduced lipid oxidation during fasting and impaired postprandial switch from lipid to glucose oxidation [59], and this inflexibility is also observed in individuals with impaired glucose tolerance [60], suggesting that inflexibility plays an role in the early development of T2D. In fact, cultured skeletal muscle cells (myotubes) established from subjects with T2D, as well as those from obese subjects, have reduced capacity to oxidize FAs compared to cells from lean subjects [61, 62].

Furthermore, metabolic flexibility of substrate oxidation is also preserved in cells when grown in culture, suggesting that metabolic switching is an intrinsic property of skeletal muscle [63]. Metabolic switching of myotubes *in vitro* is referred as suppressibility, adaptability and substrate-regulated flexibility [63, 64]. Suppressibility is defined as the ability of the cells to suppress FA oxidation by acute addition of glucose, and adaptability is defined as the capacity of the cells to increase FA oxidation upon increased FA availability [63], whereas substrate-regulated flexibility is defines as the ability to increase FA oxidation when changing from a high glucose, low FA condition ("fed state") to a high FA, low glucose condition ("fasted state") [64]. Nevertheless, metabolic inflexibility could be due to both intrinsic and extrinsic (induced) factors and the molecular mechanism underlying metabolic inflexibility remains to be revealed.

Skeletal muscle fiber types

Skeletal muscles comprise a variety of muscle fiber types with different contractile and metabolic properties [65, 66]. Based on the determination of predominant myosin heavy chain (MHC) isoforms, it has been established that rodents have four fiber types termed I, IIa, IIx and IIb (respective genes, MYH7, MYH2, MYH1 and MYH4), while human muscles contain three fiber types; I, IIa, and IIx [67, 68] where the slow-twitch type I fibers are associated with higher mitochondrial content compared to fast-twitch type II fibers [69], and higher GLUT4 protein expression [70, 71]. Furthermore, type I fibers mainly depend on oxidative (aerobic) and type IIx fibers on glycolytic (anaerobic) pathways for ATP production, while type IIa fibers display an intermediate phenotype [67]. Therefore, a higher composition of type I fibers in muscle have been reported to be associated with increased insulin responsiveness [72]. In fact, individuals with insulin resistance or T2D have a distinct muscle phenotype with decreased type I fibers [73, 74], accompanied by decreased GLUT4 expression within the type I fibers [75]. However, this view has been challenged by the finding that altering the fiber composition to muscles towards type IIb fibers in mice also improves glucose homeostasis and insulin action [76]. The mechanisms involved in muscle fiber type switching are complex and not known in detail, but transcription factors such as myocyte enhancer factor 2 (MEF2) [77] and its target gene, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) [78], have been shown to be implicated in control of slow fiber type program (discussed in more details under **"Exercise and energy metabolism in skeletal muscle"**, page 14).

Role of peroxisome proliferator-activated receptors (PPARs) in energy metabolism in skeletal muscle

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent nuclear receptors that belong to the superfamily of nuclear transcription factors. PPARs are activated by a diversity of molecules including FAs and FA metabolites. The PPAR family consists of three subtypes encodes by three genes; PPARα, PPARδ/β, and PPARγ [79]. The PPARs share a high degree of functional and structural similarities, and specific activities of the PPARs is depending on their tissue distribution, ligand binding and the recruitment of coactivators and corepressors [80]. PGC1α, which is one of the best described coactivators of PPAR, is able to activate all three PPAR subtypes, as well as other transcription factors such as MEF2 [81]. A heterodimer of retinoid X receptor and PPAR bind to the PPAR-responsive element in specific target genes, but also interacts with other transcriptional regulatory pathways [65]. PPARs regulate transcription of a large variety of genes involved in energy metabolism, proliferation and differentiation [65, 82]. Additionally, PPARs have emerged as key regulators of inflammatory and immune responses [80].

PPARα is abundantly expressed in tissues with high FA catabolism such as liver, heart and skeletal muscle [65, 82, 83]. PPARα in the liver upregulates genes involved in FA uptake, FA activation and FA transport into the mitochondria, and mitochondrial FA oxidation, reducing FAs ability to become integrated into plasma lipoproteins carrying TAG. Consequently, PPARα agonists such as fibrates are used to treat dyslipidemia [84]. PPARγ is primarily expressed in white adipose tissue (WAT) promoting adipogenesis and lipid synthesis, but it is also expressed in immune cells [79]. PPARγ plays a critical role in the differentiation of pre-adipocytes to adipocytes, where WAT serves as a safe place to store excess energy as TAG to avoid lipid deposition in other tissues, and thus contributes to preserve insulin sensitivity. In addition, PPARγ plays an important anti-inflammatory role in macrophages [80]. Thus, synthetic ligand PPARγ agonists (thiazolidinediones or glitazones) work as insulin sensitizers and are used in the treatment of T2D [84].

Peroxisome proliferator-activated receptor δ/β

PPARδ (also known as β) is the least characterized of the three PPAR subtypes. It is ubiquitously expressed, and is the most abundant subtype in skeletal muscle [85]. Furthermore, PPARδ also shows a higher expression in oxidative type I fibers compared to

glycolytic type II fibers [86], while similar PPARδ gene expressions were observed in non-diabetic and diabetic muscle [87, 88]. PPARδ signalling pathway plays a central role in regulation of skeletal muscle energy metabolism and especially in the adaptive responses to metabolic challenges, including sustained exercise training and during fasting [27, 65]. Endurance exercise and fasting both lead to increased FAs, and thereby activation of PPARδ [82]. As also stated above, both fasting and sustained exercise are characterized by a shift from glucose to lipid utilization to cope with energy demand [89]. Expression of PPARδ in skeletal muscle is also increased after exercise [90-92]. During fasting, the findings on PPARδ expression has been inconsistent, varying from increased [93, 94] to decreased [95] expression.

PPARδ activation, either by FAs or the PPARδ specific agonists GW501516 and GW0742, resulted in an increase in FA catabolism and promoted induction of several important genes involved in skeletal muscle lipid metabolism such as CD36, PLIN2, CPT1, angiopoietin-like protein 4 (ANGPTL4) and PDK4 [64, 85, 96-99]. Many of PPARδ target genes are also induced by exercise, confirming the important role of PPAR mediating adaptive responses to exercise (discussed in more details under **"Exercise and energy metabolism in skeletal muscle"**, page 14). In skeletal muscle cells, GW501516 was also able to prevent palmitate-induced inflammation and insulin resistance [100].While PPARδ is a well-established regulator of lipid metabolism, the direct effect of PPARδ activation on glucose utilization in skeletal muscle is conflicting, ranging from stimulation of glucose uptake and enhanced insulin-mediated effects [101, 102] to impaired glucose utilization [103]. In addition to be implicated in energy metabolism in skeletal muscles, PPARδ is also involved in the regulation of lipid, lipoprotein and glucose metabolism in other tissues such as adipose tissue and heart [79].

Taken together, there is increasing evidence that PPARδ is an important regulator of skeletal muscle energy metabolism, highlighting the potential utility of this isoform as a therapeutic option for treatment of metabolic disorders such as T2D. In fact, activation of PPARδ by synthetic agonists is reported to ameliorate hyperglycemia, insulin resistance and dyslipidemia both in animals [98, 104] and humans [105-108].

Exercise and energy metabolism in skeletal muscle

An acute bout of exercise improves glucose homeostasis by increasing skeletal muscle glucose uptake, while regular exercise induces alterations in expression of metabolic genes such as those involved in mitochondrial activity, muscle fiber type or GLUT4 at protein levels [109]. The functional consequences of these adaptations are determined by duration, intensity, frequency and mode of exercise [110]. The ability to adapt and remodel in response to contractile activity in skeletal muscle allows the muscle to more efficiently utilize substrates for ATP production and thus become more resistant to fatigue [110, 111].

Endurance exercise-induced adaptations in energy metabolism are reflected as increases in mitochondrial content, both in size and number and improved lipid oxidative capacity [111-113]. Regular exercise has also been shown to enhance both lipid synthesis and lipid oxidation [5, 114] and thereby resulting in an increase in IMTG [115]. As a consequence of these metabolic adaptations, trained skeletal muscle takes more of its required energy from lipids and less from glucose compared to untrained muscle during submaximal work [57, 113, 116], and this is accounted for by an increase in IMTG utilization in the trained muscle [117, 118]. In skeletal muscle, HSL has previously been accepted to be the principal enzyme responsible for lipolysis of IMTG during exercise [119, 120], but recently, ATGL has emerged as the major player in lipolysis of IMTG during muscle contraction [47, 121]. Furthermore, enhanced FA oxidation after regular exercise is closely associated with the genes and proteins involved in regulating FA uptake across the plasma membrane (CD36) [122] and across the mitochondrial membrane (CPTI) [123, 124]. In addition, muscle contraction also promotes relocation of CD36 to both plasma membrane [125, 126] and the outer mitochondrial membrane [31, 127] thereby playing a role in FA uptake both into the cell and into the mitochondria (**Figure 2**). Moreover, several important mediators regulating mitochondrial activity and biogenesis are upregulated after regular exercise training, including PGC1α [128, 129], cytochrome c [130, 131], and the TCA enzyme citrate synthase [132-134].

In addition to enhanced FAs utilization, trained fibers import more glucose than untrained muscle fibers at resting state [135]. In the post-exercise period, the muscles display an increased sensitivity to insulin, resulting in increased glucose uptake and glycogen resynthesis [136-138]. It has been proposed that contraction stimulates GLUT4 translocation through a molecular mechanism different from that of insulin as muscle contraction has no

effect on the canonical insulin signaling pathways such as insulin receptor substrate (IRS) phosphorylation and PI3K activity [8, 111, 139]. In contrast, other studies have shown some degree of activation or phosphorylation of Akt in intact skeletal muscles in response to exercise [140, 141] and *in situ* muscle contraction [142, 143]. These discrepant findings suggest that contraction may regulate Akt in an intensity- and time-dependent manner. Although probably trough different mechanisms, signaling molecules involved in GLUT4 translocation such as the Akt substrates TBC1D1 and TBC1D4 (AS160), are activated by both insulin and muscle contraction [144-147] (**Figure 2**).

Contraction-induced molecular signaling is complex and involves a variety of signaling molecules including AMP-activated protein kinase (AMPK) and intracellular calcium. Muscle contraction leads to energy depletion (i.e. an elevated AMP/ATP ratio) and an increase in intracellular calcium that activates AMPK, which in turn increases glucose uptake and FA oxidation (**Figure 2**), suggesting that AMPK may be the primary mechanism mediating the adaptations to exercise (reviewed in [148]). However, data obtained from mouse models of attenuated AMPK activity demonstrated that inhibition of AMPK had little or no effect on contraction-induced glucose uptake [149], CD36 translocation to the plasma membrane and FA uptake [150] and mitochondrial marker (citrate synthase and succinate dehydrogenase) [109], indicating that additional signaling pathways are involved in triggering muscular adaptations. In addition to activate AMPK, prolonged influx of calcium into skeletal muscle during exercise activates calmodulin-dependent protein kinases (CaMK), which may also regulate glucose uptake independent of AMPK signaling [151]. Further, prolonged activation of CaMK in skeletal muscle also promote an increase in mitochondrial content [152].

Plasticity of skeletal muscle in response to regular exercise extends beyond the described metabolic changes. As also mentioned above, some of the key factors implicated in regulation of muscle fiber type and oxidative capacity are also known to be upregulated by exercise, including PPARδ [91, 92], PGC1α [128, 153] and MEF2 [154]. In endurance exercise, prolonged influx of FAs and long-term increase in intracellular calcium are shown to activate PPARδ and MEF2, respectively (reviewed in [27]). Furthermore, MEF2 also induces PGC1α [155] which in turn amplifies the activity of MEF2 and PPARδ by acting as their coactivator, thus PGC1α plays a central role in adaptation of skeletal muscle to exercise and is considered as a master transcriptional regulator of mitochondrial biogenesis and is also involved in increased GLUT4 expression [145, 153, 156-158]. Although the proportion

of oxidative type 1 fibers is reported to be increased after endurance exercise [69, 159, 160], it remains unclear whether regular exercise can switch type II fibers completely to type I fibers. Moreover, some of the known PPARδ target genes, such as PDK4 [161] and ANGPTL4 [162] are also increased in the post-exercise period. Increase of ANGPTL4 by acute exercise is proposed to coordinate lipid uptake between exercising and non-exercising muscles [162]. Further, up-regulation of PPARδ has been linked to improvements in clinical variables in diabetic subjects following exercise intervention. PPARδ expression was unchanged in subjects who did not improve their clinical profile after exercise [91] suggesting a direct implication of PPARδ in the muscle adaptations. Therefore, with all the evidence linking activation of PPARδ to muscle performance, it is interesting to speculate whether pharmacological activators would enhance physical performance and even substitute for exercise. Indeed, administration of PPARδ agonists to adult mice resulted in an increase in myofiber numbers [163] and increase in oxidative capacity [163, 164] and PPARδ agonists are therefore proposed to act as exercise mimetics [165].

Physical activity plays an important role both in prevention and treatment of T2D [166-170], and as stated above, physical activity leads to major adaptations in skeletal muscle including altered gene expression and energy metabolism, but the individual contribution of these changes for improved physical health is unclear. It is important to remind that some exercise-mediated adaptations are reversible and thus the opposite effects are noted in response to inactivity [171].

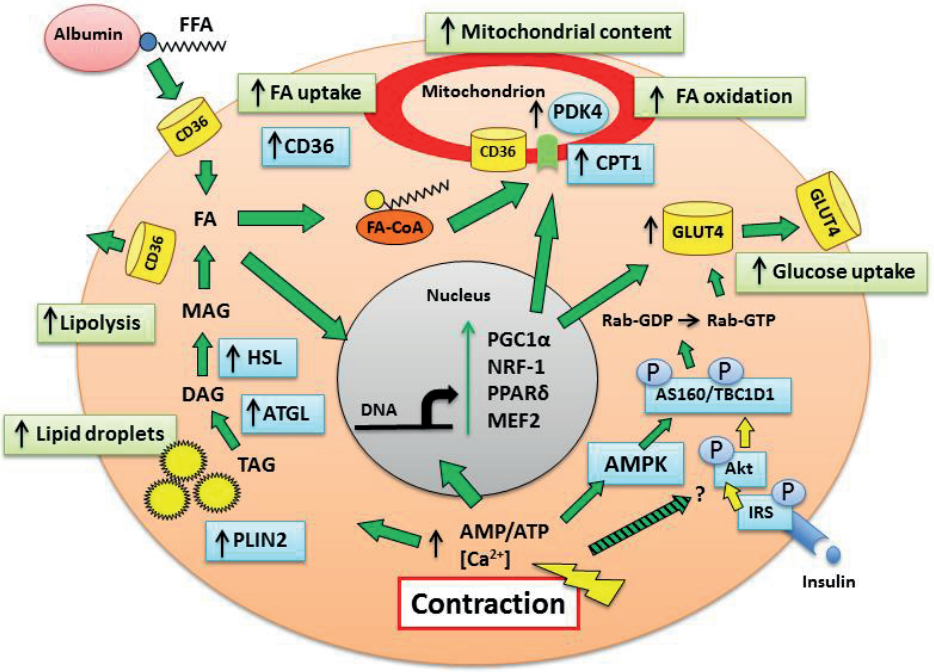

Figure 2. Proposed signalling pathways for contraction-stimulated effects on metabolism in skeletal muscle. Contraction leads to energy depletion (i.e., an elevated AMP/ATP ratio) and elevated intracellular [Ca^{2+}], which in turn leads to activation of AMP-activated protein kinase (AMPK) and calmodulin-dependent protein kinases (CaMK), respectively. Activated AMPK phosphorylates Akt substrate of 160 kDa (AS160, TBC1D4) and TBC1D1 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. Translocation of GLUT4 is also mediated through the canonical insulin-signalling pathway via activating of insulin receptor substrates (IRS) and leading to phosphorylation of Akt. Contraction also promotes GLUT4, carnitine palmitoyltransferase (CPT1) and pyruvate dehydrogenase kinase isozyme 4 (PDK4) expressions. Activated AMPK and/or CaMK promote relocation of fatty acid (FA) transporter (CD36) to the plasma membrane and the outer mitochondrial membrane to increase FA uptake and oxidation. Contraction also leads to increase in lipolysis of lipid droplets by activating adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). Prolonged influx of FAs and [Ca^{2+}] also activate peroxisome proliferator-activated receptor δ (PPARδ) and myocyte enhancer factor 2 (MEF2), respectively, and thereby their target genes. AMPK and/or CaMK increase expression of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) and nuclear respirator factor 1 (NRF-1), which then orchestrates the enhancement of mitochondrial biogenesis and function. Furthermore, PPARδ, MEF2 and PGC1α are all implicated in the oxidative fiber type program. Green arrows represent activation events, while yellow arrows represent processes probably not affected by contraction.

Skeletal muscle as a secretory organ

Skeletal muscle has been identified as a secretory organ that releases a diversity of biologically active proteins classified as myokines that can have autocrine, paracrine or endocrine functions (reviewed in [172]). Muscle contraction during exercise is a major stimulus of these endocrine functions, and the myokines are thought to mediate beneficial effects of exercise and may have a role in the protection against conditions associated with low-grade inflammation, such as T2D, obesity and metabolic syndrome [173-175]. Both interleukin 6 (IL-6) and IL-8 are produced in contracting muscles and released into plasma during the post-exercise period [176-178] when the insulin sensitivity is enhanced [179]. It seems that IL-6 works as an energy sensor and preserve fuel availability during exercise [180] by enhancing insulin-stimulated glucose disposal and FA metabolism [181, 182], whereas IL-8 has been suggested to be involved in angiogenesis in skeletal muscle [183]. Interestingly, while IL-6 released from skeletal muscle may promote insulin sensitivity [184], IL-6 secreted from adipose tissue may induce insulin resistance in skeletal muscle [185]. In fact, plasma IL-6 is chronically elevated in obese [186, 187] and T2D subjects [188], and is positively correlated with insulin resistance [189]. In addition, chronic exposure of C2C12 cells to IL-6 has been shown to impair insulin-stimulated glucose uptake [190]. On the other hand, regular exercise can lower basal plasma levels of IL-6 [191]. Thus, while it has become evident that contracting skeletal muscle releases myokines that may influence metabolism and function of skeletal muscle and other tissues and organs, the secretion of myokines from obese and/or diabetic muscle is yet to be fully clarified.

Insulin resistance, obesity and type 2 diabetes

The prevalence of T2D has increased worldwide during the last decades, and is projected to reach 592 million worldwide by the year 2035 [192]. It is established that a family history of T2D markedly increases the risk of the disease particularly in the first-degree relatives [193-195], however, genome-wide association studies revealed only 10% of the estimated heritability of T2D can be explained [196]. Lifestyle factors such as obesity, physical inactivity, and consumption of a high-fat diet are all strongly involved in development of T2D [166, 167, 171, 197].

Overweight and obesity are usually defined either by body mass index (BMI), waist circumference or waist-hip-ratio. BMI $\geq$ 25 kg/m^2 is considered overweight, BMI $\geq$ 30 kg/m^2 is considered obese, and BMI $\geq$ 40 kg/m^2 is considered morbidly or severely obese [198]. In most cases, obesity is caused by an imbalance between energy intake and energy expenditure, although genetics and chronic stress are contributors [199-201]. More recently, evidence has emerged that the gut microbiota also influences metabolic processes and contribute to low-grade inflammation and obesity [202-205]. Majority of subjects with T2D are classified as overweight or obese [206]. Moreover, visceral adipose tissue increases the risk of T2D, while subcutaneous adipose tissue decreases the risk [207]. Mechanism behind the reported association between obesity and T2D are multifactorial, and may involve increased FA release from visceral adipose tissue and raised level of plasma FFAs [208] which contribute to excess lipid accumulation in liver and skeletal muscle [209, 210]. Furthermore, visceral adipose tissue is also prone to inflammation and inflammatory cytokine production contributing to a chronic low-grade inflammation [211]. It has become more evident that adipose tissue secretes numerous bioactive peptides, collectively called adipokines, which are proposed to play an important role in the adipose tissue-skeletal muscle crosstalk [210, 212-214]. However, obesity is preventable and onset of T2D can be delayed or prevented by lifestyle interventions such as healthy diet, regular physical activity, weight loss and pharmacological treatments [166-169].

T2D is a metabolic disorder characterized by chronic hyperglycemia that affects the way the body utilizes energy. It is caused by a combination of factors, including defects in pancreatic β-cells and insulin secretion and insulin resistance, a condition in which the body's skeletal muscles, adipose and liver tissues do not respond effectively to insulin [215]. In skeletal muscle, insulin resistance is manifested as a decrease in glucose uptake and a decline in

muscle glycogen synthesis in response to insulin [209, 216]. In insulin-resistant subjects, the correlation between increased IMTG accumulation and insulin resistance has been firmly established [217-221]. Furthermore, decrease in IMTG storage after diet-induced weight loss is correlated with improvements in insulin sensitivity [222, 223]. However, despite the strong correlation of IMTG levels with insulin resistance, the exact mechanistic link between increased IMTG and reduced insulin sensitivity is unclear. The emerging idea is that increased IMTG turns to be deleterious when an increase in the supply of lipids to skeletal muscle is not balanced by an increase in the oxidative pathways, so that toxic intermediates, such as ceramides and DAG, accumulate in the cell and interfere with the insulin signaling [224, 225]. As the mitochondria are the main cellular sites devoted to FA oxidation, it has therefore proposed that impaired mitochondrial function leads to the accumulation of IMTG and lipid metabolites in skeletal muscles [195]. In fact, studies in humans have shown that T2D subjects exhibited alteration in mitochondrial morphology, as well as a decrease in the activity of the respiratory chain [226, 227]. Other studies showed a reduction in the expression of genes encoding key enzymes in oxidative mitochondrial metabolism such as PGC1α in diabetic subjects [228]. As also mentioned above, cultured myotubes established from subjects with T2D, as well as those from obese subjects, have reduced capacity to oxidize FAs compared to cells from lean subjects [61, 62]. Mitochondrial function was also lower in T2D subjects than BMI-matched control subjects despite of similar IMTG content, suggesting that impaired mitochondrial function may be a more important determinant of diabetes than IMTG levels [229, 230]. However, other observations argue against the hypothesis that mitochondrial dysfunction underlies the development of T2D or IMTG accumulation as the IMTG accumulation may precede the development of mitochondrial dysfunction and/or that insulin resistance arises when mitochondrial function is unaffected or even improved [231, 232]. Therefore, it is not clear whether mitochondrial dysfunction represent a cause or a consequence of T2D. More recently, studies in rats have shown that an increase in phosphorylation efficiency in skeletal muscle mitochondria may promote accumulation of IMTG and contribute to the development of high-fat induced insulin resistance in skeletal muscle as less substrates are need to be burned to obtain the same amount of ATP [233, 234].

Moreover, IMTG content is also reported to be similar in obese non-diabetic and obese diabetic subjects [235, 236]. Furthermore, in endurance-trained individuals, the IMTG content may be higher than in obese insulin-resistant subjects without affecting insulin

sensitivity or oxidative capacity, a phenomenon described as the athlete's paradox [235, 237, 238]. Similar to IMTG content in athletes, total muscle DAG content is also higher in athletes and correlated positively with insulin sensitivity [159]. Therefore, new theories to explain lipid-mediated muscular insulin resistance have emerged, which focus on abnormal lipid influx, storage, or TAG lipolysis and turnover rather than lipid storage *per se* [239]. Partitioning of more FAs toward TAG synthesis in skeletal muscle is correlated with increased insulin sensitivity [240], suggesting that a high expression levels of LD-associated proteins might be preferable. In fact, PLIN2 gene expression is lower in insulin-resistant obese subjects compared to obese controls [241], and higher PLIN2 protein content has been found in skeletal muscle of insulin-resistant subjects that have undergone weight loss or therapeutic treatments to increase muscle insulin sensitivity [242], suggesting that PLIN2 might play a role in decreasing intramuscular lipid toxicity by promoting lipid storage. Moreover, improvements in insulin sensitivity following either endurance [115] or resistance training [243] are linked to increase in the content of PLIN2 and PLIN5. On the other hand, similar muscular PLIN2 protein content is observed between obese non-diabetics and obese diabetic subjects, and more interestingly, it correlated negatively with insulin-stimulated glucose uptake [244]. Therefore, more insight into how PLIN2 regulates LD in skeletal muscle is needed.

AIMS OF PRESENT STUDIES

The overall aim of this thesis was to study the regulation of energy metabolism in human skeletal muscle cells related to obesity and T2D, focusing on the metabolic effects of PPARδ activation and *in vitro* exercise. To investigate the functional role of PLIN2 and LDs in skeletal muscle energy metabolism we also studied myotube cultures established from $PLIN2^{+/+}$ and $PLIN2^{-/-}$ mice. More specifically, the objectives of the present studies were:

1) To explore the effects of GW501516, a potent and selective PPARδ agonist, on global gene regulation, FA and glucose utilization, as well as lipid distribution in myotubes from lean non-diabetic subjects (**paper I**).

2) To investigate lipid accumulation, storage and turnover capacity, as well as oxidation and metabolic flexibility of myotubes from severely obese subjects with and without T2D (**paper II**).

3) To explore glucose and lipid metabolism and gene expression after electrical pulse stimulation (EPS), as an *in vitro* model of exercise, as well as lipid metabolism combined with PPARδ activation, in cultured myotubes established from lean non-diabetic subjects and severely obese subjects (**paper III**).

4) To explore lipid storage capacity and turnover, as well as lipid oxidation and glucose metabolism and muscle fiber type characteristics, in mice myotubes lacking PLIN2 (**paper IV**).

SUMMARY OF PAPERS

Paper I: PPARδ activation in human myotubes increases mitochondrial fatty acid oxidative capacity and reduces glucose utilization by a switch in substrate preference

The aim of this paper was to investigate the role of activation of peroxisome proliferator-activated receptor δ (PPARδ) on global gene expression and mitochondrial fuel utilization in myotubes from lean non-diabetic subjects. PPARδ is a well-established regulator of lipid metabolism in skeletal muscle, but effects on glucose utilization and fuel switching are less known. In addition, global gene analysis of PPARδ activation in human myotubes had not previously been reported.

Gene expression analysis using microarrays revealed that only 21 genes were significantly upregulated and 3 genes were significantly downregulated after 96 h of PPARδ activation with the potent and selective agonist, GW501516. Genes such as PDK4, ANGPTL4, CPT1A, PLIN2 and CD36 were increased after PPARδ activation. Pathway analysis showed upregulated mitochondrial FA oxidation, TCA-cycle and cholesterol biosynthesis. PPARδ activation increased oleic acid oxidation and mitochondrial oxidative capacity by 2-fold. Glucose uptake and oxidation were reduced by 25%, while total substrate oxidation was unaffected, suggesting a fuel switch from glucose to FA. Cholesterol biosynthesis was increased by 30%, but lipid biosynthesis, the number of lipid droplets, lipolysis, mitochondrial content, and insulin sensitivity were unaffected. The effects of PPARδ activation are summarized in **Figure 3**.

In conclusion, this study confirmed that the principal effect of PPARδ activation was to increase mitochondrial FA oxidative capacity. Moreover, results from the this study indicated that PPARδ activation reduced glucose utilization through a switch in mitochondrial substrate preference by upregulating PDK4 and genes involved in lipid metabolism and FA oxidation.

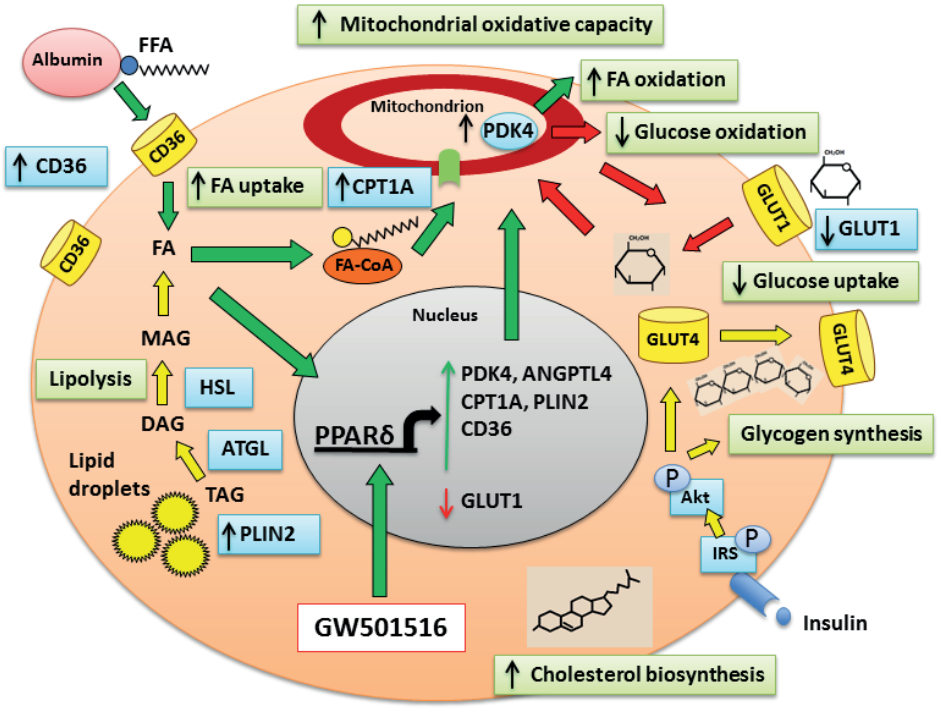

Figure 3: Effects of PPARδ activation in myotubes from lean non-diabetic subjects. Myotubes were treated for 96 h with 10 nM GW501516. Green arrows represent activation events, while red arrows represent inhibition processes. Yellow arrows indicate no effect of GW501516. Abbreviations: ANGPTL4, angiopoietin-like protein 4; ATGL, adipose triglyceride lipase; CD36, fatty acid transporter; CPT1A, carnitine palmitoyltransferase 1A; DAG, diacylglycerol; FA, fatty acid; FFA, free fatty acid; GLUT, glucose transporter; HSL, hormone-sensitive lipase; IRS, insulin receptor substrate; MAG, monoacylglycerol; PDK4, pyruvate dehydrogenase kinase isozyme 4; PLIN2, perilipin 2; PPARδ, peroxisome proliferator-activated receptor subtype delta; TAG, triacylglycerol.

Paper II: Myotubes from severely obese type 2 diabetic subjects accumulate less lipids and show higher lipolytic rate than myotubes from severely obese non-diabetic subjects

About 80% of patients with T2D are classified as overweight. However, only about 1/3 of severely obese subjects have T2D suggesting that several severely obese individuals may possess certain characteristics that protect them against developing T2D. Since skeletal muscle is the organ where insulin resistance is most pronounced, it was hypothesized that this apparent paradox could be related to fundamental differences in skeletal muscle lipid handling. Thus, the main focus of this study was to investigate lipid storage and turnover capacity, as well as oxidation and metabolic flexibility of myotubes from severely obese subjects (BMI $\geq$ 40 kg/m^2) with and without T2D.

Lower insulin sensitivity was observed in myotubes from severely obese subjects with T2D confirming that myotubes maintain their diabetic phenotype in culture. Lipolysis rate was higher, and oleic acid accumulation, TAG content, and FA adaptability were lower in myotubes from severely obese subjects with T2D compared to severely obese non-diabetic subjects. There were no differences in lipid distribution and mRNA and protein expression of the lipases HSL and ATGL, the lipase cofactor CGI-58, or the LD-associated proteins PLIN2 and PLIN3. In addition, glucose and FA oxidation, and the expression of oxidative phosphorylation (OXPHOS) proteins were also similar in cells from the two donor groups despite of lower mitochondrial staining. The findings in myotubes from severely obese diabetic subjects are summarized in **Figure 4**.

Thus, lower lipid accumulation and higher lipolysis without correspondingly increased FA oxidation and impaired metabolic flexibility, as observed in T2D myotubes, could contribute to accumulation of lipotoxic intermediates, which can interfere with insulin signalling. In conclusion, difference in intramyocellular lipid turnover might be fundamental in evolving T2D.

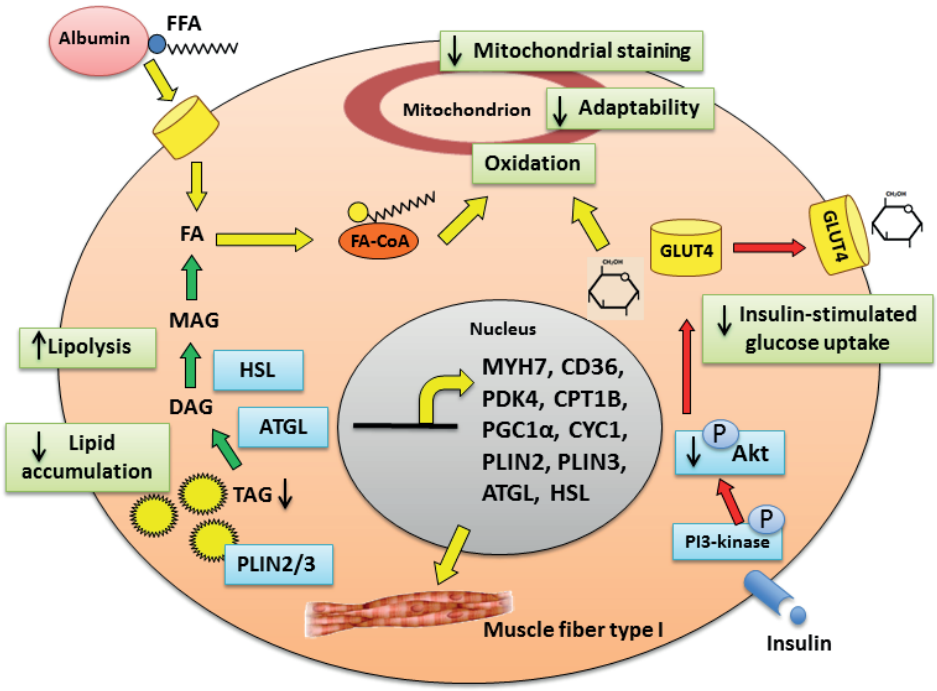

Figure 4: Energy metabolism and metabolic flexibility in myotubes from severely obese diabetic subjects when compared to myotubes from non-diabetics with similar BMI. Green arrows represent a higher event in diabetic than in non-diabetic myotubes, while red arrows represent a lower process. Yellow arrows indicate no differences between diabetic and non-diabetic myotubes. Adaptability is the ability to increase fatty acid oxidation with increasing fatty acid concentration. Abbreviations: ATGL, adipose triglyceride lipase; CD36, fatty acid transporter; CPT1B, carnitine palmitoyltransferase 1B; CYC1, cytochrome C-1; DAG, diacylglycerol; FA, fatty acid; FFA, free fatty acid; GLUT, glucose transporter; HSL, hormone-sensitive lipase; MAG, monoacylglycerol; MYH7, myosin heavy chain 7 (the gene that regulates protein expression enriched in type I fibers); PDK4, pyruvate dehydrogenase kinase isozyme 4; PGC1α, peroxisome proliferator-activated receptor gamma coactivator-1α; PI3-kinase, phosphatidylinositol 3-kinase; PLIN2/3, perilipin 2/3; TAG, triacylglycerol.

Paper III: Myotubes from lean and severely obese subjects with and without type 2 diabetes respond differently to an *in vitro* model of exercise

Exercise improves insulin sensitivity and oxidative capacity in skeletal muscles. However, the effect of exercise on substrate oxidation is less clear in obese and T2D subjects than in lean subjects. In this paper, we used an *in vitro* model of exercise (EPS) to explore whether there were differences in EPS response on insulin sensitivity, glucose and lipid metabolism and gene expression in cultured myotubes established from lean non-diabetic subjects and severely obese subjects (BMI $\geq$ 40 kg/m^2) with and without T2D. Moreover, we explored the combined effect of EPS together with GW501516, on lipid metabolism as well.

We observed an EPS-induced increase in insulin sensitivity, but no improvement in lipid oxidation in myotubes from severely obese subjects. Thus, the EPS-induced increases in insulin sensitivity and lipid oxidation were positively and negatively correlated to BMI of the subjects, respectively. Further, EPS enhanced oxidative capacity of glucose in myotubes from all subjects, while mitochondrial content were only increased in myotubes from lean subjects. FA oxidation was increased after GW501516 treatment in myotubes from all subjects, whereas combination of GW501516 treatment and EPS showed no additional effect on FA oxidation. Furthermore, EPS reduced mRNA expression of oxidative fiber-type marker (MYH7) in myotubes from diabetic subjects; however the protein expression of this marker was not significantly affected by EPS in neither of the donor groups. On the contrary, mRNA levels of IL-6 and IL-8 were unaffected by EPS in myotubes from diabetic subjects, while IL-6 mRNA expression was increased in myotubes from non-diabetic subjects. EPS-stimulated mRNA expression levels of MYH7, IL-6 and IL-8 correlated negatively with the subjects' HbA_{1c} and/or fasting plasma glucose, suggesting an effect linked to the diabetic phenotype. The effects after EPS or PPARδ activation are summarized in **Table 1**.

In conclusion, these data indicate that myotubes from various donor groups respond differently to EPS, and this effect also reflect the *in vivo* characteristics of the donor groups, suggesting that some individuals may inherently respond differently to exercise. Results suggest that pharmacological activation of PPARδ could be one way to increase FA oxidation in subjects that seem to be unresponsive to exercise-mediated effect on FA metabolism.

Table 1: Effects of EPS for 48 h or PPARδ activation for 96 h in myotubes established from lean, non-diabetics and severely obese subjects with and without type 2 diabetes (T2D). An increase or a decrease after treatment with either EPS or PPARδ activation is indicated with ↑ and ↓, respectively. (-) indicates no effect of EPS. Lipid oxidation and uptake were not further increased with PPARδ activation and EPS in combination. Abbreviations: EPS, electrical pulse stimulation; MYH7, myosin heavy chain 7 (the gene that regulates protein expression enriched in type I fibers) pAkt/Akt, phosphorylation of Akt/total Akt.

After EPS	Lean non-diabetic	Obese non-diabetic	Obese diabetic
pAkt/Akt	-	-	-
Glycogen synthesis	-	-	-
Insulin sensitivity	-	↑	↑
Glucose oxidation	↑	↑	↑
Lipid oxidation	↑	-	-
Mitochondrial content	↑	-	-
Lipid droplet number	-	-	-
Neutral lipid content	-	-	-
Lipid uptake	-	-	-
MYH7 (gene)	-	-	↓
Interleukin-6 (gene)	↑	↑	-
After PPARδ activation			
Lipid oxidation	↑	↑	↑
Lipid uptake	↑	↑	↑

Paper IV: A metabolic shift in energy metabolism from utilization of glucose towards fatty acids in myotubes lacking perilipin 2

LD-associated proteins are essential for the formation and stability of intracellular LDs. However, the roles of PLIN2, which is an abundant LD-associated protein in skeletal muscle, remain unclear. In this paper, by using cultured myotubes established from $PLIN2^{+/+}$ and $PLIN2^{-/-}$ mice we aimed to investigate the functional role of PLIN2 on lipid storage and turnover capacity, as well as on lipid oxidation and glucose metabolism. We further explored the role of PLIN2 on insulin-stimulated responses and muscle fiber type characteristics.

We observed reduced number of LDs, reduced incorporation of labeled oleic acid (OA) into TAG and DAG and increased T**A**G hydrolysis in $PLIN2^{-/-}$ myotubes. Absence of PLIN2 resulted in a metabolic shift in energy metabolism from utilization of glucose towards FAs. Accordingly, myotubes established from $PLIN2^{-/-}$ mice had higher OA oxidation and lower glycogen synthesis and glucose oxidation compared to $PLIN2^{+/+}$ cells. Ablation of PLIN2 also resulted in higher gene expressions of PDK4, a key enzyme important for switching fuel source from glucose to FA, PGC1α, known to stimulate expression of genes important for FA oxidation. We also found that loss of PLIN2 resulted in higher gene expression of the oxidative fiber type marker (MYH7) and lower expressions of the glycolytic fiber type markers (MYH1, -2 and -4). However, loss of PLIN2 had no impact on insulin-stimulated responses. Energy metabolism and lipid turnover in $PLIN2^{-/-}$ myotubes are summarized in **Figure 5**.

In conclusion, these results suggest that PLIN2 is essential for balancing the pool of skeletal muscle LDs to avoid an uncontrolled hydrolysis of the intracellular TAG pools and the metabolic consequences of an increased release of FAs from LDs.

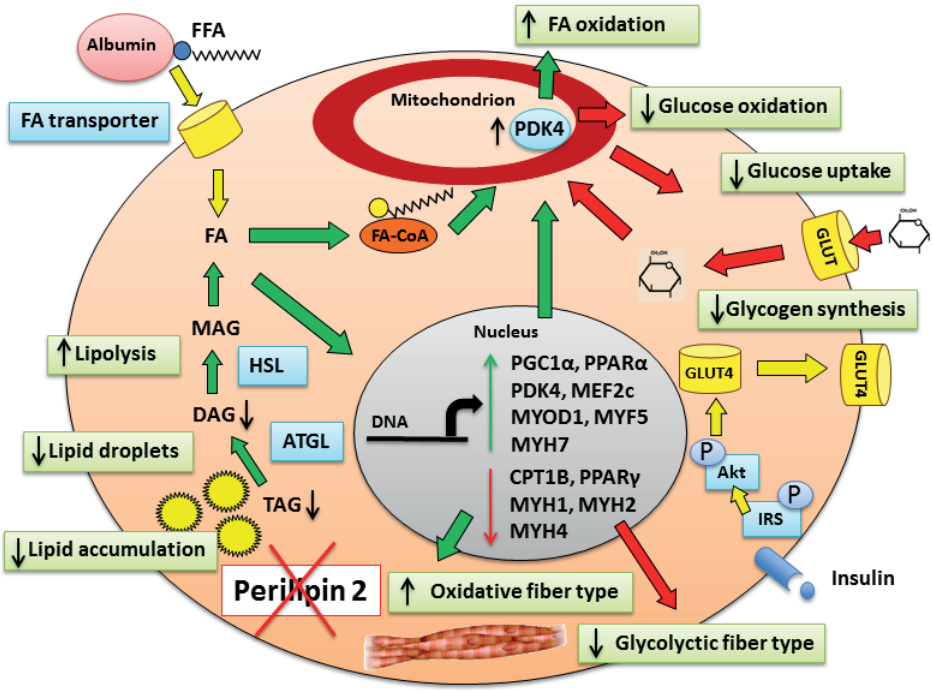

Figure 5: Energy metabolism and lipid turnover in myotubes lacking perilipin 2 ($PLIN2^{-/-}$) when compared to myotubes with a functional perilipin 2 ($PLIN2^{+/+}$). Green arrows represent a higher event in $PLIN2^{-/-}$ than $PLIN2^{+/+}$ myotubes, while red arrows represent a lower process. Yellow arrows indicate no differences between $PLIN2^{-/-}$ myotubes and $PLIN2^{+/+}$ myotubes. Abbreviations: ATGL, adipose triglyceride lipase; CPT1B, carnitine palmitoyltransferase 1B; DAG, diacylglycerol; FA, fatty acid; FFA, free fatty acid; GLUT, glucose transporter; HSL, hormone-sensitive lipase; IRS, insulin receptor substrate; MAG, monoacylglycerol; MEF2c, myocyte enhancer factor 2c; MYF5, myogenic factor 5; MYH, myosin heavy chain; MYOD1, myogenic differentiation protein 1; PDK4, pyruvate dehydrogenase kinase isozyme 4; PGC1α, peroxisome proliferator-activated receptor gamma coactivator-1α; PPAR, peroxisome proliferator-activated receptor; TAG, triacylglycerol.

METHODOLOGICAL CONSIDERATIONS

Cultured skeletal muscle cells as an *in vitro* model

Cultured human myotubes represent an *in vitro* model system for intact skeletal muscle, and have the most relevant genetic background for study of metabolic pathways and processes in human skeletal muscle (as opposed to rodent cell cultures) [245]. Moreover, human myotubes used here are not immortalized which allows investigation of the innate characteristic of the donors they were established from. Extracellular environment can be precisely monitored, thus making this system possible to study cellular mechanism and underlying signaling pathways under controlled conditions.

The human muscle cells used in this thesis were obtained from biopsies from *M. obliquus internus abdominis*. The lean non-diabetic donor biopsies were obtained from subjects donating a kidney at Oslo University Hospital, Norway (**papers I and III**) and the severely obese donor biopsies were obtained from subjects undergoing bariatric surgery at The Morbid Obesity Center, Vestfold Hospital Trust, Norway (**papers II and III**). The isolation of satellite cells from all biopsies was performed at the same location and by the same researchers. To be able to study the role of PLIN2 in skeletal muscle, muscle myoblast cultures were established from the hind leg containing *M. gastrocnemius* and *M. soleus* from PLIN2$^{+/+}$ and PLIN2$^{-/-}$ mice (**paper IV**). We disrupted the PLIN2 gene using standard homologous recombination in embryonic stem cells. The use of knockout mice enables us to study the role of one specific protein at a time; however, silencing of a gene may elicit compensatory cellular responses through up-regulation of genes sharing overlapping functions. However, as we showed **in paper IV**, complete loss of PLIN2 was not compensated by other related PLIN genes. Full knockout of the gene is superior compared to silencing with siRNA, as some expression of the target gene will be retained after silencing with siRNA. In comparing metabolic studies between different species, it is important to consider the differences existing between the species with regard to metabolic regulation (reviewed in [246]). For example, mouse has a basal metabolic rate that is ~7.5-fold greater than that of the human. Furthermore, it appears that glucose tolerance in mice is more closely related to hepatic rather than skeletal muscle insulin action.

Cultured human myotubes are generally characterized by low mitochondrial capacity and their fuel preference for carbohydrate over lipids [245]. The GLUT1:GLUT4 ratio is higher in cultured myotubes compared to adult skeletal muscle [247-249] resulting in lower insulin

responsiveness on glucose transport. Typically, insulin increases glucose uptake by 40-50 % in myotubes [247, 250]. However, despite the reduced insulin-responsiveness, the mechanisms involved in glucose uptake *in vivo* are conserved *in vitro* [247].

Mature human myotubes do not typically contract spontaneously [250], and to overcome this limitation, myotubes were stimulated with electrical pulses. As shown in **paper III** and also reported by others [251, 252], EPS can induce responses in cultured myotubes that are similar to those observed in exercising muscle *in vivo*, making this model important for clarifying the mechanisms for contraction-stimulated glucose uptake and exercise-induced adaptations in cellular energy metabolism.

Another aspect to consider when comparing myotubes obtained from different sources of muscle satellite cells is heterogeneity with respect to muscle fiber types. However, studies have shown that human satellite cells isolated from either fast or slow muscle fibers form myotubes *in vitro* which co-express both fast and slow fibers independently of the fiber type from which they originated [253]. Further, it has been reported that myotubes express fast fiber type regardless of donor muscles having mixed fiber type expression *in vivo* [254]. Furthermore, murine satellite cells isolated from various muscles are uniform regardless of muscle origin and the dominant muscle fiber type is the intermediate fiber type, MHCIIa [255]. In our cells, after 8 days of differentiation, we observed a significant amount of slow fiber type as well [245]. Taken together, these findings demonstrate that myotubes differ from donor muscle with respect to MHC expression. However, we have previously shown that cell content of MHCI is increased in electrically pulse stimulated myotubes [252], showing a plasticity potential of these cells.

Several characteristics of the *in vivo* phenotype are conserved in culture. For instance, the diabetic phenotype is conserved in myotubes [256, 257]. The ability of the skeletal muscle to switch between lipid and glucose oxidation appears to be an intrinsic characteristic, as it was retained *in vitro* [63, 258]. Further, altered lipid metabolism in skeletal muscle with diabetes and obesity [61, 62] is also retained in culture. The precise mechanisms by which myotubes are able to retain the *in vivo* characteristics are not known. However, a combination of genetic and epigenetic mechanisms are probably involved, and this has been reviewed in a recent paper [245]. For example, epigenetic regulation of skeletal muscle stem cells and skeletal muscle differentiation, exercise, diet and a family history of T2D have all been described to influence DNA methylation and/or histone modifications in human

skeletal muscle [90, 259], and these are traits that might follow the isolated satellite cells into their corresponding cultured myotubes.

Although the diabetic phenotype is stable in an uniform culture environment [260], it has also been shown that the ability of the myoblast to fuse and differentiate into myotubes and metabolic processes such as glucose uptake, glycogen synthesis, glucose and FA oxidation can gradually become impaired with increasing passage number [261]. For that reason, all the experiments performed in this thesis were on cells from passage numbers that exerted normal responses. Taken together, although some limitations, the cell model used in this thesis appears to be valuable to study skeletal muscle metabolism including conditions such as T2D, insulin resistance, obesity and EPS-induced responses.

Donor characteristics

Cultured myotubes used in the thesis were established from biopsies from adult donors of different ages (26-70 years). Both genders are included, although the majority of the donors were women (64%). The average age of the donors was 46 years. The lean non-diabetic donors used in the thesis had an average BMI of 24 kg/m^2, while the severely obese donors had average BMI of 43 kg/m^2, with lower HDL and higher TAG levels than the lean non-diabetic donors. Fasting plasma glucose and HbA_{1c} were higher in severely obese donors with T2D than in non-diabetic donors (**Table 2**). In addition to T2D, the donors may also possess other diseases or conditions such as hypertension.

Table 2: Donor characteristics for the three donor groups used in the thesis: lean non-diabetic (L-nD, n = 20), severely obese non-diabetic (SO-nD, n = 16) and severely obese with type 2 diabetes (SO-T2D, n = 14). Mean values are presented. HbA_{1c} and plasma insulin were not measured in the lean non-diabetic cohort. *Significantly different from L-nD. †Significantly different from the non-diabetic groups. #Significantly different from SO-nD. $P < 0.05$, two-tailed unpaired Student's t-test was used to compare two groups, whereas ANOVA with Bonferroni adjustment was used for multiple comparisons. BMI; body mass index, HbA_{1c}; glycosylated hemoglobin, HDL; high-density lipoprotein, LDL; low-density lipoprotein, TAG; triacylglycerol.

	Age years	BMI (kg/m^2)	Fasting glucose (mM)	HbA_{1c} (%)	Insulin (pM)	HDL (mM)	TAG (mM)	Total cholesterol (mM)
L-nD	48	24	5.4	-	-	1.5	1.0	5.1
SO-nD	41	44*	5.0	5.4	96	1.2*	1.6*	4.8
SO-T2D	49	41*	7.9†	7.1$^{\#}$	82	1.1*	2.0*	4.5

BMI as a measure of obesity is widely used because of its simplicity and validation in multiple epidemiologic studies. The main limitation of BMI is that it cannot differentiate body fat from lean mass and central (visceral) fat from peripheral (subcutaneous) fat. Therefore, athletes with enhanced body muscle mass may be misclassified as obese when using only BMI to diagnose obesity, whereas individuals with low lean body mass but high body fat content may still have a normal BMI. Furthermore, individuals with normal BMI (≤ 25 kg/m^2) and high body fat percentage also show a high degree of metabolic dysregulation (normal weight obesity) [198].

Gender is definitely a factor affecting energy metabolism *in vivo* as women are more insulin sensitive than men, despite higher IMTG levels [262-264], higher adipose mass and less skeletal muscle mass [265] compared to men. In addition, women have been reported to have higher expression of FA transport proteins [266], higher lipid turnover such as increased esterification of FAs and lipolytic activity [267, 268] and higher level of muscle fiber type 1 [68, 262] than men, while men have higher whole-body resting energy expenditure [269] than women. However, these differences do not seem to be maintained in cultured myotubes, as both glucose and palmitic acid metabolism were similar in myotubes from male and female donors [270, 271] indicating the importance of influence from sex hormones and other factors *in vivo*. Similarly, gene expression of several genes involved in glucose and lipid metabolism that been observed to be higher in muscle biopsies from females than men, the gene expression was similar in cultured myotubes from the same donors [270, 271]. In addition, the expression patterns of fiber type markers seem to be the same in myotubes from female or male donors [253]. Thus, in the studies included in this thesis, data obtained from donors of both genders were merged.

In vivo, age affects several metabolic processes in skeletal muscles such as impaired insulin sensitivity and increased obesity with increasing age [272-274]. Increasing age has also been associated with elevated decline in skeletal muscle mass [275] and increased proportions of fast muscle fibers [276], oxidative damaged to mitochondrial DNA [277-279], reduced mitochondrial content [280, 281] and function [272]. Additionally, the PPARδ content of skeletal muscles has been reported to decline with increasing age [282], whereas IMTG content increased with advancing age [281]. However, obesity and inactivity rather than age *per se* are suggested to be more important in age-related declines in insulin sensitivity [273]. Moreover, several of these age-related effects may be prevented by exercise [274, 283], and the ability to adapt to exercise with an increase in muscle mitochondria is maintained up to

at least 70 years [284, 285]. In this thesis, the majority of the donors used were under the age of 60 years, and experimental data from donors of all ages were merged.

Methods used to measure energy metabolism in cultured skeletal muscle cells

Metabolic processes in the thesis were described by combining functional studies using radiolabeled substrates with gene expression analysis using both real-time qPCR and microarray, as well as staining of LDs and mitochondria followed by live cell imaging. In this thesis, scintillation proximity assay (SPA) was used to study both real-time substrate accumulation and lipolysis. After 24 h of accumulation of radiolabeled oleic acid (OA), SPA was performed without OA present in the media where efflux of accumulated OA represented a measure of lipolysis. In addition, the radioactivity released to the culture media was also measured. The substrate oxidation assay [286] was used to study oxidation and metabolic flexibility. Microarray (used in **paper I)** is a valuable screening tool for gene expression, generating great amount of data, although relatively high costs and risk of false positive findings being the main drawbacks of this method. On the other hand, real-time qPCR is useful for investigation of a limited number of anticipated regulated genes and gene set enrichment analysis with focus on groups of genes that share common biological function, chromosomal location, or regulation [287]. Immunoblotting was also applied to assess the expression and phosphorylation of relevant proteins, thereby addressing changes in post-translational modifications. However, immunoblotting is highly dependent on the quality of the antibodies that are used, and is therefore a semi-quantitative method. Staining of LDs and mitochondria followed by live imaging is another method that was used in order to get a broader understanding of the cellular changes. The cells were incubated with fluorescent substances that are known to diffuse through the plasma membrane and bind to intracellular organelles. After excitation of the fluorophore, the emitted light can be quantified. In this thesis, muscle cells were labeled with Hoechst 33258, MitoTracker®Red FM and Bodipy 493/503. Hoechst 33258 binds to double stranded DNA and Bodipy 493/503 is a lipophilic dye that accumulates in non-polar neutral lipids and is rather specific for LDs [288]. The image acquisition and analysis was automated and the images were taken randomly, avoiding the potential bias from the operator. MitoTracker®Red FM is a fluorescent dye containing a mildly thiol chloromethyl moiety that accumulates within active mitochondria. However, there are some controversies regarding the MitoTracker®Red

FM dye's limitation as a specific marker, for instance its dependence on membrane potential and oxidative capacity [289], and that interpretations should be carried out with caution. In our lab, we have observed increased staining of MitoTracker®Red FM in parallel with higher OA oxidation [252], while we have also observed that MitoTracker®Red FM was unchanged despite increased oxidation [64, 290] as well also shown in **paper I**. Further, we have also treated cells with a mitochondrial uncoupler (FCCP), without effect on MitoTracker®Red FM staining (unpublished data). In support of our conclusion regarding use of MitoTracker®Red FM, we and others have shown that citrate synthase activity, which is another measure of mitochondrial content, had similar pattern as MitoTracker®Red FM in human myotubes [252, 291]. Thus, this dye seems to stain mitochondria independently of oxidative status in myotubes and therefore possibly independently of membrane potential, implying that, limited to these cells and our conditions, it is a good measure of mitochondrial content. More importantly, mitochondrial content should be assessed in more than one way, such as shown in **paper II and III** where we combined MitoTracker®Red FM staining with measuring expression levels of proteins involved in mitochondrial oxidative phosphorylation (OXPHOS)**.**

In the present thesis, several approaches were applied to explore energy metabolism in cultured skeletal muscle cells. In **paper I,** pharmacological activation of PPARδ with a selective and highly potent agonist, GW501516, was used to explore mitochondrial fuel utilization in myotubes. We have previously established an *in vitro* model of regular exercise of human myotubes by applying chronic, low-frequency electrical pulse stimulation (EPS, single, bipolar pulses of 2 ms, 30 V, 1 Hz continuously for 48 h) [252]. In **paper III**, we used this *in vitro* exercise model to explore EPS responses in myotubes from different donor groups. Further, we also explored the combined effect of EPS together with GW501516 on lipid metabolism.

Various compounds (research tools) were used to modify lipid turnover from the IMTG pool in myotubes, such as an inhibitor of ACSL (triacsin C) [292], an inhibitor of HSL (CAY10499) [293] and an inhibitor of AGTL (Atglistatin) [294]. TAG resynthesis [45] and FA oxidation [290] are efficiently blocked by triacsin C, while lipolysis is increased [290]. Re-esterification was calculated as [triacsin C present - triacsin C absent] as previously described [290, 295, 296].

DISCUSSION AND CONCLUSIONS

In the present thesis, energy metabolism was investigated in cultured skeletal muscle cells established from different donor groups (lean non-diabetic, severely obese non-diabetic and severely obese type 2 diabetic) and mice ($PLIN2^{+/+}$ and $PLIN2^{-/-}$). In **paper I**, GW501516 was used to explore the effect of PPARδ activation on global gene expression and mitochondrial fuel utilization in myotubes from lean non-diabetic donors. In **paper II,** we explored differences in the metabolic processes in myotubes derived from severely obese subjects with and without T2D, whereas in **paper III** we investigated the effect of exercise-mimicking treatment by using EPS alone, or in combination with GW501516 on human myotubes from all three donor groups. Finally, in **paper IV** we investigated the functional role of PLIN2 on lipid turnover and energy metabolism as well as muscle fiber remodelling in myotubes from mice.

Oxidative capacity and metabolic flexibility

Cultured human myotubes are characterized by their low mitochondrial oxidative potential. Three of the papers (**I, III, IV**) presented in this thesis showed increased mitochondrial oxidative capacity for lipids, by using different experimental approaches. Impairments have been noted in skeletal muscle glucose and FA oxidation in obesity and T2D in the resting state [57, 62, 297]. It is therefore important to determine whether interventions, either exercise or pharmacological, can effectively reverse these impairments.

In skeletal muscle, PPARδ mRNA is expressed to a greater extent than PPARα, while PPARγ mRNA level is very low [65] as we also observed in cultured mice myotubes (**paper IV**). In **paper I**, we showed for the first time global gene analysis and gene set enrichment analysis of PPARδ activation in human myotubes from lean non-diabetic donors. Activation of PPARδ with GW501516 resulted in a robust activation of some essential genes involved in lipid metabolism and FA oxidation. In line with this, pathway analysis showed upregulated mitochondrial FA oxidation and TCA-cycle. Further, mitochondrial FA oxidative capacity of lipids was increased after PPARδ activation (**papers I and III**) in human myotubes from all donor groups, while lipid oxidation after EPS stimulation was only increased in myotubes from lean non-diabetic donors (**paper III**). Furthermore, uptake of FA was also increased after PPARδ activation in human myotubes from all donor groups

(**papers I and III**), but not in EPS-stimulated cells from neither lean nor obese donors (**paper III**). Although only shown in myotubes from lean non-diabetic donors, the increase in FA uptake and oxidation after PPARδ activation were accompanied by enhanced gene expressions of CD36 and CPT1A (**paper I**) without affecting mitochondrial content.

PPARδ agonist and exercise training are proposed to synergistically increase oxidative myofibers in adult mice [164]. However, factors important for FA oxidation such as CPT1B and PDK4 were not further induced with exercise compared to PPARδ activation alone [164], which is consistent with the findings that FA oxidation was not further increased when combining PPARδ activation and EPS (**paper III**). In comparison, mitochondrial content was increased after EPS-treatment (**paper III**) in lean non-diabetic donors, while it remained unchanged after PPARδ activation (**paper I**). This is in concordance with a previous report, which found that PPARδ increased FA oxidation without affecting mitochondrial gene expression and function [298]. In addition, neither mitochondrial biogenesis nor PGC1α have been shown to be increased after pharmacological activation of PPARδ [92, 101, 299-301]. Although it has been shown that exercise increase both mitochondrial content and intrinsic mitochondrial function in obese and insulin-resistant subjects [302], it has also been reported that exercise did not change key regulators of mitochondrial biogenesis in skeletal muscles in diabetic men after exercise [303]. Consistent with this, in our exercise model, myotubes from donors with higher BMI were less responsive to exercise-induced effects on FA oxidation and mitochondrial content than myotubes from lean donors (**paper III**). In contrast, increase in muscle *ex vivo* palmitic acid oxidation in obese subjects has been observed after *in vivo* exercise [304]. The discrepancy may to some extent be explained by differences between *in vivo* and *in vitro* exercise models. Without EPS, there was no difference in basal lipid content between myotubes in the three donor groups (**paper III**), excluding the possibility that the absence of EPS effect on FA oxidation is due to label dilution when using a labelled FA as a measurement of FA oxidation.

Taken together, after PPARδ activation, increased FA oxidation may partly due to an increase in FA uptake via CD36, an increase in flux of long-chain FAs across the mitochondrial membrane via CPT1A, and by switching the oxidation from glucose towards FA (**paper I**) without affecting mitochondrial content, while the EPS-induced effect on FA oxidation may due to an increase in mitochondrial content (**paper III**). Additionally, with no effect on FA oxidation after EPS, but increased FA oxidation after PPARδ activation (**paper**

III) in myotubes from obese donors may suggest that pharmacological activation of PPARδ could be one way to increase FA oxidation in subjects that seem to be unresponsive to exercise-mediated effect on FA metabolism.

Both glucose uptake and oxidation were decreased after PPARδ activation in human myotubes from all donor groups (**paper I and Figure 6** (unpublished data)), while glucose uptake assessed as glycogen synthesis remained unchanged after EPS stimulation (**paper III**).

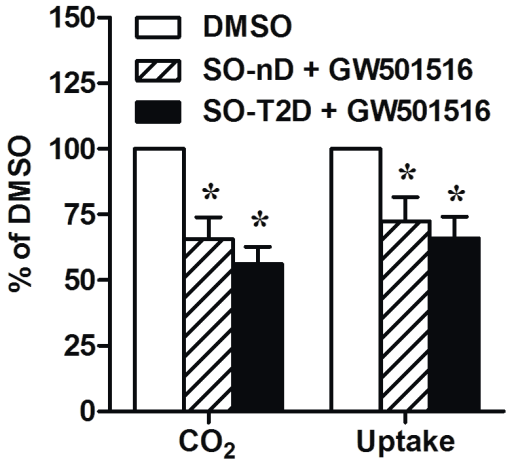

Figure 6: PPARδ activation reduced glucose oxidation and uptake. Myotubes established from severely obese non-diabetics (SO-nD) and severely obese type 2 diabetics (SO-T2D) were treated for 96 h with 10 nM GW501516 or 0.1% vehicle (DMSO). Glucose oxidation and uptake were measured in presence of [^{14}C]glucose (0.5 μCi/ml, 200 μM) for 4 h. Glucose uptake was assessed as the sum of oxidized glucose (trapped CO_2) and remaining cell-associated radioactivity (accumulated [^{14}C]glucose). Data are presented as mean ± SEM (n=5-7). *P < 0.05 versus DMSO.

Like PPARδ activation, ablation of PLIN2 also resulted in a metabolic shift in energy metabolism from utilization of glucose towards FAs (**paper IV**). Accordingly, a higher FA oxidation and lower glucose metabolism was observed in PLIN2$^{-/-}$ myotubes. In addition, transcription factors such as PPARα and PGC1α, known to facilitate FA oxidation [157, 305] were also higher expressed in PLIN2$^{-/-}$ myotubes, whereas PPARγ, which stimulates expression of genes promoting lipid storage [79], were lower (**paper IV**). Similar differences in PPARα expression have been observed in livers of PLIN2$^{+/+}$ and PLIN2$^{-/-}$ mice [306] whereas the oposite expression pattern is found in muscle electrophorated to

overexpress PLIN2 [307]. As stated above, PDC is positioned in such way that it plays one of the most central roles in regulation of glucose metabolism as well as fuel selection in skeletal muscle [54]. In fact, both PPARδ activation (**paper I**) and ablation of PLIN2 (**paper IV**) resulted in an increase in PDK4 expression. As PDK4 is known to inhibit PDC [54] and thereby reduces glucose oxidation, we confirmed in **paper I and IV** that PDK4 is an important enzyme for switching the fuel source from glucose to FA. In support, others have also shown that PDK4 is increased after PPARδ activation [96, 98, 102, 308]. Furthermore, PDK4 inhibition of glucose oxidation in response to elevated plasma FA availability has been observed during fasting [309, 310] or high-fat feeding [311]. Under these conditions, carbohydrate availability is low, so PDK4 contributes to glucose sparing by preventing its oxidation. Therefore, by regulating PDK4, PPARδ is suggested to play a role in utilization of FAs under physiological conditions. On the other hand, trained muscle fibers have been shown to use more glucose than untrained fibers [135, 312] with enhanced carbohydrate oxidation after exercise in obese subjects [313], and in subjects with T2D [314]. Consistent with this, we showed that EPS was able to increase oxidative capacity of glucose in human myotubes from all donor groups (**paper III**).

Adaptability of FA metabolism is shown to correlate positively with insulin sensitivity [63], and lower ability to increase FA oxidation with increasing FA concentration is observed in myotubes from obese type 2 diabetics [315]. In accordance, as we showed in **paper II**, obese diabetic myotubes had lower ability to increase FA oxidation with increased FA availability (i.e. lower adaptability). Additionally, reduced metabolic flexibility is also shown *in vivo* in insulin resistant muscle [60] implying that impairment in diabetic muscle mitochondria to exchange substrate in demand. In addition, we found lower mitochondrial staining in myotubes from obese donors with T2D compared to cells from obese non-diabetic donors, which is consistent with earlier study showing lower mitochondrial content in insulin resistant muscle [316]. This occurred despite no differences in FA and glucose oxidation between lean and obese myotubes (**paper III**) or differences in the OXPHOS protein expression or mRNA expression of CYC1, PGC1α CPT1B or PDK4 between the obese non-diabetic and obese diabetic myotubes (**paper II**). Although, FA oxidation at resting state is shown to be reduced in obese versus lean muscle [59, 62, 297, 317], other studies have also reported similar FA oxidation between myotubes from lean and obese diabetic subjects [236] and even increased oxidation in obese muscle compared to lean

[231]. The reason behind this discrepancy is unclear, but may be a result of different subgroups of obese populations studied and/or different methodology used.

Fast-to-slow transformations in skeletal muscles

An association between insulin sensitivity and the amount of oxidative type I fibers has been suggested, as a lower expression of type I fibers has been demonstrated in muscle biopsies from insulin resistant and T2D subjects when compared to healthy subjects [74, 318]. On the other hand, no differences in fiber type compositions were found between muscle biopsies from lean non-diabetic and obese diabetic subjects [316]. Furthermore, as discussed above, myotubes in culture differ from donor muscle as human satellite cells form myotubes *in vitro* independently of the fiber type from which they originated. Consistent with this, we showed in **paper II and III** that there was no difference in expression level of muscle fiber type I marker between the myotubes from lean non-diabetic and obese subjects (**paper III**), or between the myotubes from obese non-diabetic and diabetic subjects (**papers II and III**). After EPS, we observed reduced mRNA expression of muscle fiber type I marker (MYH7) in myotubes from diabetic subjects; however the protein expression of this marker was not significantly affected by EPS in neither the lean nor obese donor groups (**paper III**). We showed in **paper III** that metabolic adaptations such as EPS-induced increase in oxidative capacity and mitochondrial content can occur irrespective of an altered muscle fiber marker expression profile. Although the possibility of conversion from type II to type I muscle phenotype in adult humans has been debated [319, 320], this type of transition has been consistently shown in rodent with different approaches [78, 86, 92, 321, 322]. In **paper IV**, ablation of PLIN2 in mice resulted in myotubes that were more oxidative and less glycolytic. Furthermore, loss of PLIN2 resulted also in a higher gene expression of oxidative type I fiber marker (MYH7) and lower expressions of glycolytic fiber type markers (MYH1, -2, -4) together with higher gene expressions of PGC1α, MEF2c, and the myogenic markers (myoblast determination protein 1 (MYOD1) and myogenic factor 5 (MYF5)) (**paper IV**). In support of these results, MEF2 [323-325] and PGC1α [78] are shown to regulate fiber type switching from glycolytic type II to oxidative type I fibers. In fact, muscle-specific knockout of MEF2c resulted in decreased proportion of oxidative fibers, while overexpression of MEF2c increased proportion of oxidative fibers [77]. Furthermore, we have previously shown that PGC1α overexpression in human myotubes resulted in enhanced

lipid oxidative capacity and decreased gene expression of MYH2 [157]. MYOD and MYF5 are important transcription factors that activate many downstream genes to initiate muscle cell differentiation to multinucleated myotubes [326]. Additionally, MYOD is also proposed to be implicated in the fast-fiber formation [327]. However, other studies have shown that neither knocking out the MYOD gene in mice [328] nor increased expression of MYOD after exercise in rat were involved in fiber-type transitions [329]. How a lack of PLIN2 can influence skeletal muscle differentiation/fiber type remodeling remains unclear, but it is likely that increased efflux of FAs may contribute to the observed metabolic shift in energy metabolism from utilization of glucose towards FAs and to the reduced expression of glycolytic and increased expression of oxidative muscle fiber type markers in PLIN2$^{-/-}$ myotubes (**paper IV**).

Lipid storage and turnover

Lipid droplets (LDs) are dynamic organelles resulting from the balance between storage and breakdown of TAG by lipases to generate FAs available for oxidation in the mitochondria [48]. Exercise has been found to increase lipid storage in skeletal muscle [330-332], but as we showed in **paper III**, EPS-stimulation had no effect on LDs. We (**paper I**) and others [99, 333] have shown that PLIN2 gene expression was increased after PPARδ activation. However, as we showed in **paper I,** the increase in PLIN2 gene expression after PPARδ activation by GW501516 was not accompanied with an effect on number of LDs or on lipolysis, indicating that a 2-fold increase in mRNA level observed after PPARδ activation may not be accompanied by an increase in PLIN2 protein. Discrepancies between PLIN2 mRNA and protein levels exist due to a rapid proteasomal degradation of PLIN2 proteins that are not bond to LDs [334]. On the other hand, by using PLIN2$^{-/-}$ myotubes, we demonstrated that PLIN2 plays an essential role in skeletal muscle lipid storage and turnover (**paper IV**). Similar to the earlier studies based on partly loss of PLIN2 [307, 335], we showed that a complete loss of PLIN2 in myotubes (**paper IV**) generated cells with reduced number of LDs and with less accumulated TAG. We also showed that lack of PLIN2 did not affect FA uptake rate across the plasma membrane or the amount of FA incorporated into LDs, but solely increased degradation of the TAG deposited within LDs by interfering with lipolysis. In agreement, earlier studies have shown that overexpression of PLIN2 in embryonic kidney cells limited the interaction of lipases with LDs [336] and higher lipolysis

in hepatocytes with combined PLIN2 and PLIN3 knockdown [335]. As shown in **paper IV**, inhibitors against ATGL or HSL had only an effect on LD accumulation in PLIN2$^{-/-}$ myotubes, suggesting that ATGL and HSL gained more access to LDs or increased in activity in cells lacking PLIN2. Consistent with this, higher TAG accumulation and lower lipolysis in skeletal muscles has been observed in ATGL-KO mice [337] whereas HSL-KO mice contains higher levels of DAG. [338]. In line with a functional role of PLIN2 as TAG protector, PLIN2$^{-/-}$ myotubes exposed to OA accumulated less DAG (**paper IV**), a finding that contradicts the previous report of increased incorporation of palmitic acid (PA) into DAG in C2C12 cells knocked down for PLIN2 [307]. As a functional redundancy is shown among some PLINs [335], the incomplete removal of PLIN2 protein in PLIN2 knocked-down cells compared to complete removal of PLIN2$^{-/-}$ cells in **paper IV** likely contributes to these discrepancies. Additionally, the different types of labeled FAs used may also contribute as PA is accumulated to a lower extent into LDs than OA in myotubes [290].

High levels of IMTG have been shown to correlate with insulin resistance [218-221]. On the other hand, insulin resistance has been shown to occur independently of changes in IMTG content, thus dissociating IMTG concentrations from insulin resistance [339]. Furthermore, increased storage of neutral lipids together with improved glucose metabolism and metabolic switching of the cells has been observed in studies with cultured human myotubes [64, 291, 340], suggesting that enhancing the partitioning of excess FAs toward storage is considered to be beneficial in preventing insulin resistance by limiting the accumulation of lipotoxic intermediates ([240, 341, 342] and reviewed in [239]). In accordance, as we showed in **paper II**, diabetic myotubes had lower lipid accumulation and FA incorporation into TAG and higher lipolysis without correspondingly increased FA oxidation compared to non-diabetic myotubes with similar BMI. Moreover, a higher DAG level in muscle has also been associated with obesity and insulin resistance [224, 225, 240, 267], whereas strategies that resulted in improved insulin sensitivity, on the other hand, did not consistently reduce DAG levels [124, 343, 344]. In accordance to this, we did not observe any differences in DAG content between obese diabetic myotubes and non-diabetic myotubes with similar BMI (**paper II**), confirming that it is not total DAG *per se* that is involved in insulin resistance. Furthermore, higher PLIN2 expression level in muscle has been shown to be associated with improved insulin sensitivity [241, 242]. On the other hand, PLIN2 loss-of-function studies in liver or animal models showed inconsistent results on insulin sensitivity [335, 345-347]. As we showed in **paper IV** and also shown by others [307], complete or

partly removal of PLIN2 expression in skeletal muscle cells, respectively, had no impact on insulin-stimulated responses, suggesting that enhanced FA oxidation in PLIN2$^{-/-}$ cells act as a compensatory mechanism to handle a higher availability of FAs released from LDs. Furthermore, consistent with others [236], we observed neither differences in ATGL, HSL, PLIN2, and PLIN3 protein expression nor phosphorylation of HSL at serine 660 in the obese myotubes compared to diabetic myotubes with similar BMI despite functional changes in lipolysis (**paper II**). Thus, conflicting results were observed, suggesting complexity of the process. In fact, regulation of lipase activity is influenced by multiple steps, including phosphorylation of lipases, cofactors and PLIN proteins and complex movement of these different partners between the lipid droplets and the cytosol [48]. Further, increased expression of ATGL by PA in myotubes did not increase lipolysis, implying that content and activity are not directly linked [290].

Effects on insulin sensitivity

Decreased insulin-stimulated glucose uptake into skeletal muscles is one of the hallmarks of T2D, and **in paper II**, we showed that myotubes from severely obese donors with T2D maintain their diabetic phenotype in culture. As stated above, it has been hypothesized that insulin resistance develops due to lower lipid accumulation and higher lipolysis without increased FA oxidation (**paper II**), processes which may lead to higher accumulation of lipotoxic intermediates that could interfere with insulin signalling. Consequently, much focus has been on the possibility of increasing lipid utilization with different approaches to avoid ectopic lipid accumulation in muscle [348-350]. However, as we showed in **paper III**, EPS improved insulin sensitivity in insulin-resistant myotubes from obese diabetic subjects without having impact on lipid oxidation or lipid accumulation. Furthermore, neither PPARδ activation (**paper I**) nor ablation of PLIN2 (**paper IV**) affected insulin sensitivity measured as an effect on insulin-stimulated responses despite of improvement in lipid oxidative capacity. Similarly, others have shown that PPARδ activation had no effect on insulin-stimulated Akt phosphorylation [101]. Thus, conflicting results were observed, suggesting that insulin resistance in skeletal muscle is not solely dependent on increased lipid accumulation. Although a complete loss of PLIN2 in myotubes did not affect insulin-stimulated responses, it generated cells with reduced expression of total Akt protein that may contribute to the reduced glucose metabolism observed in PLIN2$^{-/-}$ cells. However, the three different Akt isoforms (Akt1-3) are shown to have distinct roles, where Akt2 is specifically involved in the maintenance of glucose homeostasis [351, 352], and therefore remains to clarify which isoform(s) of the Akt proteins is reduced in PLIN2$^{-/-}$ cells.

Of particular interest, the observed effect on insulin sensitivity in terms of improved insulin-stimulated responses after EPS was more evident in myotubes from obese donors compared to lean donors (**paper III**), which is consistent with other studies [353, 354] reporting greater exercise-induced changes in insulin sensitivity in subjects who were more insulin-resistant at baseline, and more evident in obese men compared to lean men [354]. This suggests that interventions to increase physical activity may be particularly effective at improving insulin sensitivity in population groups who are more insulin resistant or have an increased predisposition to insulin resistance. In addition, EPS alone had no effect on Akt phosphorylation in neither of the human donor groups (**paper III**), supporting the hypothesis of distinct pathways of exercise- and insulin-induced GLUT4 translocation [139, 355-357]. Importantly, repeated contractions with EPS were able to restore insulin-

stimulated Akt phosphorylation in insulin resistant myotubes (**paper III**), suggesting that exercise triggers an additional effect on the insulin cascade for enhancing the glucose uptake [358, 359]. In agreement, we have previously shown that EPS improved the reduced insulin-stimulated glucose uptake observed in human myotubes after preincubation with 20 mM glucose (hyperglycemia) [250]. Similarly, others have shown that EPS rescued the reduced Akt phosphorylation observed in human myotubes after incubation with adipocyte-conditioned medium [251]. Lack of EPS-induced increase in insulin sensitivity in myotubes from lean non-diabetic subjects could be explained by maximal responsiveness to insulin in those cells *per se* and limited capacity to respond further.

In the present thesis, we have shown that EPS increased IL-6 mRNA expression in both lean and obese non-diabetic myotubes, whereas IL-6 mRNA expression was not affected in myotubes from obese diabetic subjects (**paper III**). In support, increase in IL-6 mRNA expression has been detected in muscle during exercise and in the post-exercise period in both lean and obese subjects [176, 360], whereas no effect on IL-6 mRNA levels were found in muscle biopsies from obese diabetic subjects [361] or in plasma from diabetic mice after *in vivo* exercise [362], suggesting an inherent abnormal IL-6 response in insulin-resistant skeletal muscle, also observed by others [363, 364]. Despite vast research on myokines the past decade, more research is needed in order to clarify whether there is an altered myokine response in skeletal muscles from subjects with disturbed metabolic status.

Final considerations

It should be noted that the myotubes used in this thesis were from donors with different age, both genders and different physical activity level, and this might have affected the observed results. For instance, plasma TAG values and BMI were higher and HDL lower in obese donors compared to lean donors (**Table 2**). Interestingly, we also observed that plasma TAG values correlated positively and HDL correlated negatively with the EPS-induced increase in insulin sensitivity reported in **paper III** (unpublished data), while BMI of the donors correlated negatively with the EPS-induced increase in lipid oxidation (**paper III**). In addition, we also observed that fasting plasma glucose levels (**paper III**) and age (unpublished data) of the donors correlated negatively with the EPS-induced increase in IL-6 mRNA expression. Thus, responses in the myotubes may reflect the *in vivo* characteristics of the donor and might be influenced by the genetics or epigenetics of the donors.

Conclusively, oxidative capacity of the cells was increased with indications of a shift from glucose utilization towards FA after PPARδ activation in human myotubes established from different donor groups (**papers I and III**) and in mice myotubes lacking PLIN2 (**paper IV**), while the EPS responses reflected the *in vivo* characteristics of the donors (**paper III).** We also conclude that PLIN2 is essential for balancing the pool of skeletal muscle LDs to avoid an uncontrolled hydrolysis of the intracellular TAG pool (**paper IV**), while increase of PLIN2 gene expression after PPARδ activation was not accompanied with an effect on the number of LDs or on lipolysis (**paper I**). Moreover, data presented suggest that PLIN2 itself or its effect on lipid storage capacity may affect muscle fiber type remodelling (**paper IV**). Data presented also suggest a beneficial role of increased capacity for intramyocellular lipid accumulation, as this improves metabolic flexibility, which is associated with higher insulin sensitivity and increased glucose metabolism in human myotubes (**paper II**). On the contrary, EPS was able to improve insulin sensitivity and glucose oxidation in insulin-resistant myotubes without having impact on lipid oxidation or lipid accumulation (**paper III**). Finally, neither increase in oxidative capacity of lipids after PPARδ activation (**paper I**) nor ablation of PLIN2 (**paper IV**) had any impact on insulin-stimulated responses in skeletal muscle cells.

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RESEARCH ARTICLE

Myotubes from Severely Obese Type 2 Diabetic Subjects Accumulate Less Lipids and Show Higher Lipolytic Rate than Myotubes from Severely Obese Non-Diabetic Subjects

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Abstract

About 80% of patients with type 2 diabetes are classified as overweight. However, only about 1/3 of severely obese subjects have type 2 diabetes. This indicates that several severely obese individuals may possess certain characteristics that protect them against type 2 diabetes. We therefore hypothesized that this apparent paradox could be related to fundamental differences in skeletal muscle lipid handling. Energy metabolism and metabolic flexibility were examined in human myotubes derived from severely obese subjects without (BMI 44±7 kg/m^2) and with type 2 diabetes (BMI 43±6 kg/m^2). Lower insulin sensitivity was observed in myotubes from severely obese subjects with type 2 diabetes. Lipolysis rate was higher, and oleic acid accumulation, triacylglycerol content, and fatty acid adaptability were lower in myotubes from severely obese subjects with type 2 diabetes compared to severely obese non-diabetic subjects. There were no differences in lipid distribution and mRNA and protein expression of the lipases HSL and ATGL, the lipase cofactor CGI-58, or the lipid droplet proteins PLIN2 and PLIN3. Glucose and oleic acid oxidation were also similar in cells from the two groups. In conclusion, myotubes established from severely obese donors with established type 2 diabetes had lower ability for lipid accumulation and higher lipolysis rate than myotubes from severely obese donors without diabetes. This indicates that a difference in intramyocellular lipid turnover might be fundamental in evolving type 2 diabetes.

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Introduction

Overweight and obesity are strongly associated with insulin resistance and type 2 diabetes, and the majority of subjects with type 2 diabetes are classified as overweight or obese [1]. However, a study at the Morbid Obesity Center in Norway revealed that only 31% of the severely obese patients enrolled for screening (BMI $>$ 35 kg/m^2) had type 2 diabetes [2]. Many organs are involved in obesity-related type 2 diabetes, but skeletal muscle is one of the organs where insulin resistance is most prominent.

Skeletal muscle uses both fat and carbohydrate as fuel, and fat predominates during fasting. Metabolic flexibility is defined as the muscle's ability to change between predominantly fatty acid oxidation in the fasting state and carbohydrate oxidation in the fed state (reviewed in [3]). This flexibility is reduced in insulin resistance and type 2 diabetes *in vivo* [4]. Treatments that have positive effects on muscle metabolism, such as omega-3 fatty acids, have been observed to increase both lipid accumulation and metabolic flexibility [5]. Interestingly, it has been observed that muscle cells isolated from overweight/obese patients with or without type 2 diabetes maintain these characteristics when grown in culture [6–8]. Studies performed on cultured skeletal muscle cells (myotubes) from severely obese subjects are few [9–13], but the main findings from these studies are that myotubes from the severely obese subjects have a reduced complete fatty acid oxidation compared to cells from lean subjects [10, 11, 13], in addition to a reduced mitochondrial content [11]. Other studies on myotubes from obese subjects with diabetes (BMI$\geq$30 kg/m^2) have shown reduced lipid oxidation associated with obesity and type 2 diabetes [14–16]. This reduced oxidation in obese/diabetic muscle has been attributed to impaired mitochondrial capacity [17] or lower mitochondrial content [18]. There are also reports on unaltered and/or increased fatty acid oxidation in human skeletal muscle of obese or insulin resistant individuals [19–21]. Increased fatty acid uptake [11] and partitioning of lipids towards storage rather than oxidation was observed in myotubes from severely obese donors compared to lean donors [10, 11]. Skeletal muscle store fat as triacylglycerols (TAG) in lipid droplets (LDs) and lipid droplet-binding proteins (perilipins) coat and regulate lipid droplet biogenesis and turnover, while adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) are enzymes involved in lipolysis [22–24]. Increased intramyocellular lipid storage and/or levels of intermediates in fatty acid metabolism have been shown to correlate with decreased insulin sensitivity (reviewed in [25]). However, insulin signaling was also improved in presence of increased lipid accumulation in human myotubes [26], and treatment of myotubes with metformin has been shown to increase lipid accumulation in cells from lean individuals [8]. Finally, athletes that are highly insulin sensitive have a higher content of lipid in skeletal muscle than both overweight sedentary and overweight type 2 diabetic individuals [27]. Based on these findings, new theories have emerged that focus on abnormal lipid storage or TAG lipolysis rather than lipid storage *per se* (reviewed in [25]).

Myotubes in culture are known to maintain many phenotypic characteristics of the donor. We wanted to study lipid storage and turnover capacity, as well as oxidation and metabolic flexibility of myotubes from severely obese with and without type 2 diabetes, to see whether lipid handling is inherently different between those who develop type 2 diabetes and those who don't.

Materials and Methods

Materials

Dulbecco's modified Eagles medium (DMEM-Glutamax) low glucose with sodium pyruvate, DMEM without phenol red, heat-inactivated FCS, penicillin-streptomycin and amphotericin B

were from Gibco Invitrogen (Gibco, Life Technologies, Paisley, UK). SkGM-bulletkit was from Lonza (Wakersville, MD, USA). Ultroser G was from PALL (St-Germain-en-Laye, France), insulin (Actrapid) from NovoNordisk (Bagsvaerd, Denmark), BSA, L-carnitine, oleic acid (OA, 18:1, n-9), and triacsin C from Sigma (St.Louis, MO, US). [1–^{14}C]oleic acid (2.15 GBq), [9,10-^{3}H]triolein (37 MBq) and D-[^{14}C-(U)]glucose (107 MBq or 185 MBq) were from PerkinElmer NEN (Boston, MA, US), 2-[^{3}H] deoxy-D-glucose (370 GBq) from American Radiolabeled Chemicals Inc. (St.Louis, MO, US). 96-well Scintiplate was from Perkin Elmer (Boston, MA, US), Corning CellBIND tissue culture plates and flasks from Corning Life-Sciences (Schiphol-Rijk, The Netherlands), glass bottom 6-well plates from MatTek (Ashland, MA, US), and Biocoat 25cm^2 cell flask from BD Biosciences (Franklin Lakes, NJ, US). MitoTrackerRed FM, Hoechst 33258, primers for TaqMan qPCR from Molecular Probes, Invitrogen (Carlsbad, CA, US). RNeasy minikit was from Qiagen (Venlo, The Netherlands), SYBR green from Applied Biosystems (Warrington, UK) and Roche Diagnostics (Mannheim, Germany). TaqMan reverse transcription kit reagents MicroAmp Optical Reaction Plate and High-Capacity cDNA revers transcription kit were from Applied Biosystems (Warrington, UK), Agilent Total RNA isolation kit from Agilent Technologies (Santa Clara, CA, US) and TRIzol Reagent from Invitrogen Dynal AS (Carlsbad CA, US). Immun-Star WesternC kit, Mini-Protean TGX gels were from BioRad (Copenhagen, Denmark). SPE column was from Macherey-Nagel (Düren, Germany).

Donor characteristics

Muscle biopsies were obtained from subjects undergoing bariatric surgery at The Morbid Obesity Center at Vestfold Hospital Trust, Norway. Biopsies were obtained after informed written consent and approval by the Regional Committee for Medical and Health Research Ethics, Oslo, Norway (approvement S-09078d). Blood samples were taken the day before biopsy retrieval or earlier (range 1 month to 2 years, median 1.5 years), due to practical reasons. The diagnosis of type 2 diabetes was based on fasting plasma glucose $\geq$7.0 mM, $HbA_{1c} \geq 6.5\%$ and/or the use of one or more antidiabetic drug. Of the 14 donors in the type 2 diabetic group, 6 were on metformin monotherapy, 2 on metformin and glimepiride, 1 on metformin in combination with rosiglitazone, 1 on pioglitazone monotherapy, 2 on insulin and 2 were untreated. The donors did not receive medication on the day of surgery.

Cell culturing

Satellite cells were isolated from *M. obliquus internus abdominis* and cultured as previously described [28]. Experiments were performed after 7–8 days of differentiation and 100 μM OA was added the last 24h of differentiation period. Protein concentration in each sample was determined [29], and the results were standardized according to this value for each well. Not all donors were included in each set of experiment, but 5–8 donors in each group were used, and these were carefully matched with respect to age and BMI.

Deoxyglucose uptake

Myotubes were preincubated for 1h in serum-free DMEM-Glutamax (5.5 mM glucose) ± 100 nM insulin at 37°C before addition of [^{3}H]deoxyglucose (37 kBq, 0.1 μM) in presence or absence of 100 nM insulin. Deoxyglucose uptake was measured for 1 h as previously described [30].

Scintillation proximity assay

Scintillation proximity assay (SPA) was performed as previously described [31] with [1–^{14}C] oleic acid (OA) (18.5 kBq, 100 μM) in medium without phenol red. Briefly, [^{14}C]OA taken up and accumulated by adherent cells will be concentrated close to the scintillator embedded in the plastic bottom of each well (Scintiplate, Perkin Elmer) and provide a stronger signal than the dissolved in the medium alone [32]. Lipid accumulation was monitored up to 24h with liquid scintillation. Thereafter, the cells were washed twice with DPBS with 0.5% BSA, and incubated in DPBS without radioactivity and liquid scintillation measurements were monitored up to 3h. The decline in [^{14}C]OA present in the cells in presence of triacsin C (total lipolysis, 10 μM) [33] was determined, before the remaining cell-associated (CA) radioactivity was assessed. The decline in [^{14}C]OA represent radioactivity released by the cells and provide a measure of lipolysis. Triacsin C inhibits long-chain fatty acyl-CoA synthetase and will therefore inhibit, among other pathways, re-esterification.

Lipid distribution

Myotubes were incubated with 100 μM OA (18.5 kBq, 100 μM) for 24 h. Myotubes were then washed twice with PBS and harvested with two additions of 125 μl distilled water. Cellular lipids were extracted as described earlier [5]. Briefly, homogenized cell fractions were extracted, lipids were separated by thin layer chromatography, and radioactivity was quantified by liquid scintillation. The amount of neutral lipids was related to total protein concentrations.

Determination of total TAG content

Total TAG content in the cells were measured as previously described [34]. Briefly, lipids were extracted in dichloromethane:methanol:water (2.5:2.5:2.1 (v/v/v)) in presence of internal standards. Neutral lipids were separated over a SPE column (glass Chromabond pure silica, 200 mg), and neutral lipids were eluted with chloroform:methanol (9:1 (v/v)). The organic phase was evaporated to dryness and dissolved in 20 μl of ethyl acetate. 1 μl of the lipid extract was analyzed by gas-liquid chromatography.

TAG hydrolase activity assay

TAG hydrolase activity was measured on cell lysates as previously described [35]. [9,10-^{3}H] triolein was emulsified with phospholipids by sonication and used to determine TAG hydrolase activity [5].

Live imaging

Myotubes were cultured on 6-well glass bottom plates coated with ECM gel. The cells were incubated with Hoechst 33258 to stain nuclei, Bodipy 493/503 to stain neutral lipids and LDs and MitoTrackerRed FM to stain mitochondria as previously described [5]. Images were randomly taken in 25–36 positions per well. After gating out aggregates and dead cells, each parameter was determined from about 240 images per donor group (average of 38±4 nuclei per image).

Substrate oxidation assay

Myotubes were cultured on 96-well CellBIND microplates. Substrates, [1–^{14}C]OA (18.5 or 37 kBq, 5 or 100 μM) or D-[^{14}C(U)]glucose (37 or 21.5 kBq, 111 or 200 μM/l) were given during 4h CO_2 trapping with or without 5 mM glucose or 100 μM/l OA present. A 96-well UniFilter-96 GF/B microplate was mounted on top of the CellBIND plate and CO_2 production was

measured in DPBS medium with 10 mM HEPES and 1 mM L-carnitine for 4h, as previously described [32]. The sum of $^{14}CO_2$ and remaining CA radioactivity reflects total cell uptake. Incomplete fatty acid oxidation, assessed as acid soluble metabolites (ASMs), was measured as described [31].

Metabolic parameters

Suppresibility is the ability of the cells to decrease OA oxidation by acutely added glucose, adaptability is the ability to increase OA oxidation with increasing OA concentration and substrate-regulated flexibility is the ability to increase OA oxidation while changing from "fed" (low fatty acid, high glucose) to "fasted" (high fatty acid, no glucose added) condition [5].

RNA isolation and mRNA expression

Total RNA from cells was isolated by Agilent Total RNA isolation kit according to the supplier's protocol. Total RNA from muscle biopsies was isolated using TRIzol and a clean-up procedure was performed using RNeasy kit. RNA was reverse-transcribed with oligo primers using a heat block (25°C for 10 min, 37°C for 1 h and 99°C for 5 min) or a PerkinElmer Thermal Cycler 9600 and qPCR was performed using an ABI PRISMT 7000 Detection System or a LightCycler 480 (Roche Diagnostic, Mannheim, Germany). Forward and reverse primers used at concentration of 30 μM are presented in S1 Table. The transcription levels were normalized to the average of housekeeping genes *GAPDH* and *RPLP0*.

Immunoblotting

Total cell lysates prepared either in Laemmli or RIPA buffer containing 10 μl/ml protease inhibitor, 10 μl/ml phosphatase I inhibitor and 10 μl/ml phosphatase II inhibitor were electrophoretically separated, blotted to nitrocellulose membrane and incubated with antibodies recognizing human total and phosphorylated Akt (Ser473), total HSL (#4107), Ser660 HSL (#4126) and ATGL (#2138, all from Cell Signaling Technology, Beverley, MA, US), comparative gene identification 58 (CGI-58) (#H00051099-M01, Abnova, Tapei, China), PLIN2 (#PA5-25042) and PLIN3 (#PA5-20272, Thermo Scientific, France), myosin slow muscle fiber (MAB1628, Millipore Billerica, MA, US), total OXPHOS WB antibody cocktail (#110411, Abcam), alpha-tubulin rabbit (#2144), GAPDH (#5174) and β-actin (#4970, all from Cell Signaling Technology). Immunoreactive bands were visualized with enhanced chemiluminescence (Chemidoc XRS, BioRad) and quantified with Gel-Pro Analyzer (version 2.0) software. Antibodies against GAPDH, alpha-tubulin or β-actin were used to normalize the protein-antibody signal.

Presentation of data and statistics

Two-tailed unpaired Student's t-tests were performed to determine the difference between the donor groups (GraphPad Prism 5.0, GraphPad Software Inc., San Diego, CA, US). For correlation studies, Spearman correlation analysis was performed and Spearman coefficient, ρ, defines the strength of the correlation (GraphPad Prism). Linear mixed model analysis (LMM, SPSS 20.0.0.1, IBM SPSS Inc., Chicago, IL, US) was used to compare the donors in time-course fatty acid accumulation and lipolysis experiments (SPA). 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. $P < 0.05$ was considered statistically significant.

Table 1. Donor characteristics for the donor group severely obese non-diabetics (nD) and severely obese with type 2 diabetes (T2D).

	Age (yrs)	BMI (kg/m^2)	Fasting Glucose (mmol/l)	HbA_{1c} (%)	Insulin (pmol/l)	LDL (mmol/l)	HDL (mmol/l)	Cholesterol (mmol/l)	TG (mmol/l)	*n*	Male
nD	40 ± 7	44 ± 7	5.0 ± 0.5	5.4 ± 0.5	97 ± 51	2.8 ± 0.6	1.2 ± 0.2	4.7 ± 0.8	1.6 ± 0.5	15	4
T2D	49[a] ± 10	43 ± 6	7.6[a] ± 1.5	7.1[a] ± 1.6	78 ± 48	2.4 ± 1.0	1.1 ± 0.3	4.4 ± 1.3	1.8 ± 0.7	14	5

Mean ± SD are presented. [a]Significantly different from nD. BMI; body mass index, HbA_{1c}; glycosylated hemoglobin, HDL; high-density lipoprotein, LDL; low-density lipoprotein, *n*; number, TG; triacylglycerol.

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Results

Cell and donor characteristics

Selected characteristics of the muscle biopsy donors are presented in Table 1. Severely obese without type 2 diabetes and severely obese with type 2 diabetes differed in fasting plasma glucose and in HbA_{1c}. There were no differences in plasma insulin, LDL, HDL, cholesterol, or triacylglycerol (data not shown). The severely obese subjects with type 2 diabetes were on average 9 years older than the severely obese subjects with normal glucose tolerance. However, the difference in age could not explain the metabolic differences between the groups, assessed by multivariate analysis, and the duration of obesity in the two groups was not statistically different. Physical activity level (self-reported) was also similar in the two groups (data not shown). Myotube cultures established from the two groups of donors revealed no difference in protein or mRNA expression of the marker for muscle fiber type I (MYH7, slow fiber type), determined by immunoblotting and qPCR. There were no differences in mRNA expression of myosin heavy chains in the biopsy samples either (data not shown).

Myotubes *in vitro* maintain their *in vivo* phenotype

To confirm that the myotubes maintained their diabetic phenotype in culture, insulin-stimulated glucose uptake and Akt phosphorylation (Ser473) were measured. Insulin-stimulated phosphorylation of Akt tended to be lower in myotubes from severely obese donors with type 2 diabetes compared to severely obese donors with normal glucose tolerance (Fig. 1A, $p = 0.11$). Insulin-stimulated glucose uptake was abolished in myotubes from type 2 diabetics compared to cells from non-diabetics (Fig. 1B, $p = 0.01$), implying a conserved insulin resistance in cultured cells.

Reduced lipid accumulation in type 2 diabetic myotubes

To evaluate lipid turnover in the cells, accumulation of oleic acid (OA), distribution of lipids and TAG hydrolase activity were determined. Without pre-incubation with OA, there were no differences in lipid droplet number (LD) or total neutral lipid content in myotubes from severely obese donors with or without T2D (Fig. 2A, $p = 0.65$, $p = 0.81$). Cellular accumulation of [^{14}C]OA was significantly lower in myotubes from type 2 diabetic subjects than in myotubes from non-diabetics (Fig. 2B, $p = 0.035$). TAG measured after incubation of [^{14}C]OA for 24 h tended to be lower in myotubes from type 2 diabetic subjects than in myotubes from non-diabetics (Fig. 2C, $p = 0.058$) with no differences in free fatty acid (FFA, $p = 0.22$), diacylglycerol (DAG, $p = 0.97$), cholesteryl ester (CE, $p = 0.59$) or phospholipids (PL, $p = 0.10$). In line with this, total cell content of TAG measured after incubation of OA for 24 h was 50% lower in myotubes from type 2 diabetic subjects than in myotubes from non-diabetics (Fig. 2D, $p = 0.01$).

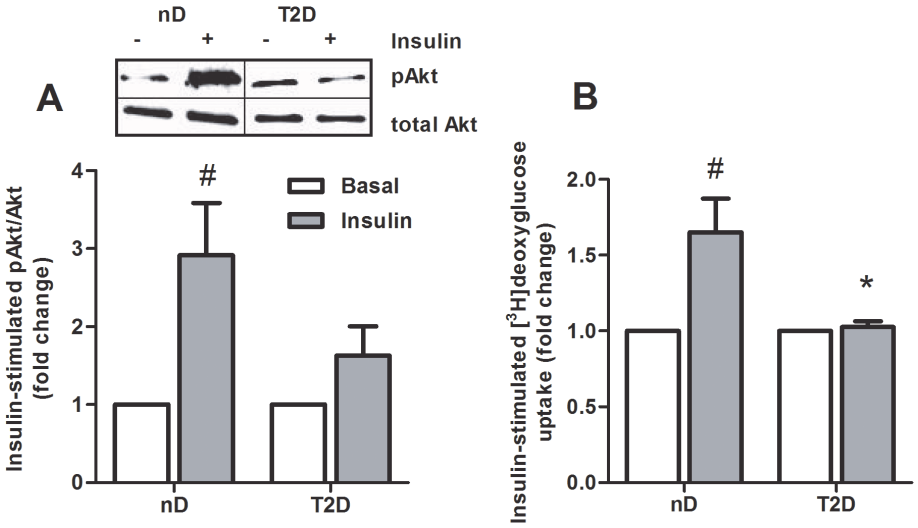

Fig 1. Decreased insulin-stimulated glucose uptake and Akt phosphorylation in myotubes from severely obese donors with type 2 diabetes. (*A*) Myotubes from severely obese non-diabetic donors (nD) and severely obese donors with type 2 diabetes (T2D) were incubated for 15 min with or without 100 nM insulin, before immunoblotting analysis with antibodies against phosho-Akt (Ser473) and total-Akt were performed. Data are shown as ratio phosho-Akt/total Akt and related to unstimulated cells (n = 4–5). Immunoblotting from one representative experiment. (*B*) Glucose uptake was measured by [^{3}H] deoxyglucose with or without 100 nM insulin for 1 h. Basal glucose uptake was 251 ± 68 nmol/mg protein (nD) and 203 ± 34 nmol/mg protein (T2D). Data are presented as mean ± SEM normalized to unstimulated cells, n = 7–8, $^{\#}p < 0.05$ versus basal, $^{*}p < 0.05$ versus nD.

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Although there was no difference in TAG hydrolase (TAGH) activity (Fig. 2D, p = 0.48), the ratio DAG/TAG tended to be higher in the myotubes from type 2 diabetic subjects (27%, p = 0.086).

Higher lipolysis rate in type 2 diabetic myotubes

To evaluate whether increased lipolysis rate could explain the lower accumulation and cell content of TAG in type 2 diabetic myotubes, lipolysis rate with triacsin C (total lipolysis) was measured. Indeed, lipolysis in presence of triacsin C was significantly higher in type 2 diabetic myotubes than in non-diabetic myotubes (Fig. 3A, p = 0.046) and total lipolysis rate related to cell-associated OA correlated positively with fasting plasma glucose levels of the subjects (Fig. 3B). However, there were no differences in mRNA or protein expression of the lipases HSL and ATGL and the lipase cofactor CGI-58, or the lipid droplet proteins PLIN2 and PLIN3 between the two groups (Fig. 3C, D). The fatty acid transporter CD36 was also equally expressed (Fig. 3C). These genes were also measured in presence of OA, but this revealed no further differences between the groups (data not shown). No differences were observed in biopsy samples either (S2 Table). Phosphorylation of HSL at serine 660, which is associated with activation of the lipase, was neither different between myotubes from the two groups (Fig. 3D).

Reduced mitochondrial staining but unchanged substrate oxidation

About 40% lower mitochondrial staining, measured as MitoTrackerRed intensity, was observed in type 2 diabetic compared to non-diabetic myotubes (Fig. 4A, B, p = 0.03). Glucose and OA oxidation (Fig. 4C, p = 0.5, p = 0.93, respectively) were not statistically different between the

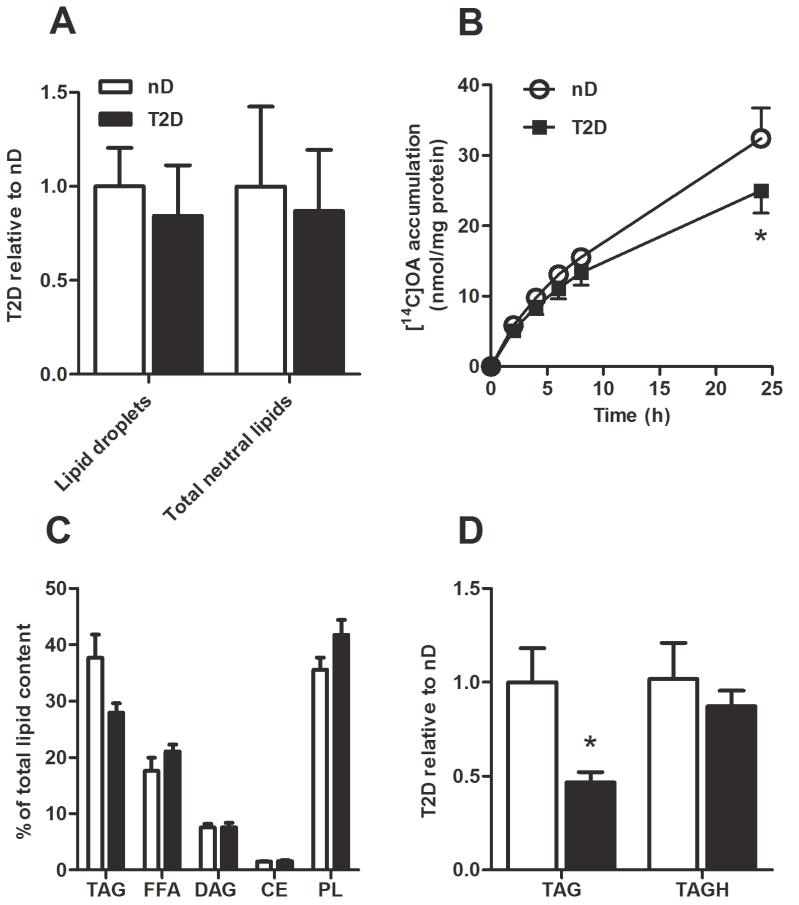

Fig 2. Lower lipid accumulation in myotubes from severely obese donors with type 2 diabetes. (*A*) Live imaging of lipid droplets and total neutral lipid content in myotubes from severely obese non-diabetic donors (nD) and severely obese donors with type 2 diabetes (T2D). The cells were incubated for 15 min with Hoechst 33258 to stain nuclei and Bodipy 493/503 to stain neutral lipids, $n = 5$. (*B*) [^{14}C]OA accumulation over 0–24 h. $n = 5$–6. (*C*) Lipid distribution measured as incorporation of [^{14}C]OA into TAG, FFA, DAG, CE and PL. Data are presented as % of total lipids in the cell, $n = 5$. (*D*) Total cell content of TAG and TAG hydrolase (TAGH) activity in non-diabetic and type 2 diabetic myotubes after 24 h incubation with 100 μM OA. Total cell content of TAG was 1.4±0.2 nmol/mg protein (T2D) and 3.0±0.6 nmol/mg protein (nD). TAGH was 3.7±0.3 nmol mg protein^{-1} h^{-1} (T2D) and 4.1±0.8 nmol mg protein^{-1} h^{-1} (nD) Data are presented relative to mean values of non-diabetics, $n = 6$–7. *$p<0.05$ versus non-diabetic. CE, cholesteryl ester; DAG, diacylglycerol; FFA, free fatty acid; OA, oleic acid; PL, phospholipid; TAG, triacylglycerol.

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groups, neither were OA and glucose uptake nor incomplete OA oxidation (ASMs) (S1 Fig.). Further, expressions of the two OXPHOS proteins, ATP synthase unit and Complex I subunit NDUFB8, did not differ significantly between the groups either ($p = 0.058$ and $p = 0.14$, respectively, Fig. 4D, E). We were not able to detect Complex II subunit 30 kDa, Complex III subunit Core 2 and Complex IV subunit 2 of the OXPHOS protein complex. Challenging the cells with

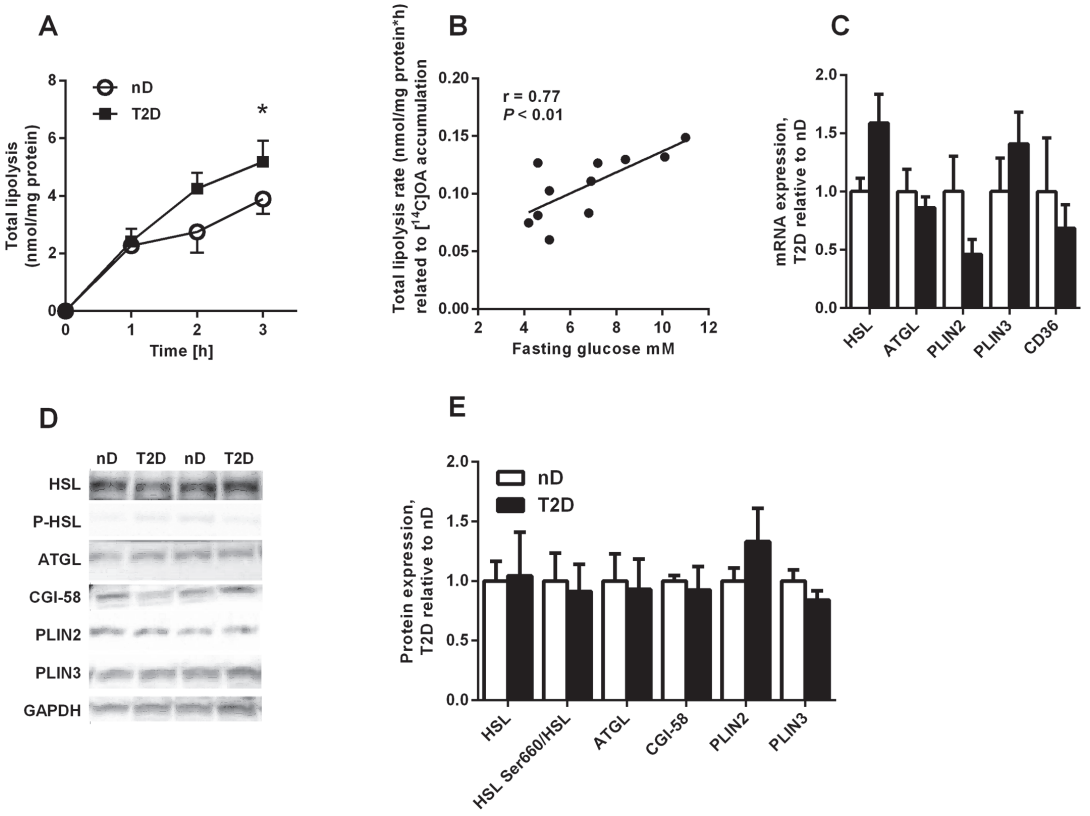

Fig 3. Higher lipolysis rate in myotubes from severely obese donors with type 2 diabetes. (*A*) Total lipolysis (with triacsin C) after 24 h incubation with [^{14}C]OA in non-diabetic (nD) and type 2 diabetic myotubes (T2D) $n = 5–6$. (*B*) Fasting plasma glucose levels correlated positively with total lipolysis rate, $n = 11$ (C) mRNA expression of hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), perilipin 2 (PLIN2), perilipin 3 (PLIN3) and the fatty acid transporter CD36. Data are presented relative to mean values of non-diabetics, $n = 6–10$. (*D-E*) Protein expression of HSL, ATGL, comparative gene identification 58 (CGI-58), PLIN2 and PLIN3 and phosphorylation of HSL at serine 660 (HSL Ser660) after 24h incubation with 100 μM OA. (D) Two representative blots are shown. Data are presented relative to mean values of non-diabetics, $n = 5$.

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the mitochondrial uncoupler FCCP (carbonyl cyanide 4-trifluoromethoxy phenylhydrazone, 1 μM) or higher OA concentrations (600 μM) revealed no further difference between groups (data not shown). Moreover, there were no differences between the groups in mRNA expression of essential genes in energy metabolism, e.g. carnitine palmitoyltransferase 1B (*CPT1B*), pyruvate dehydrogenase kinase isozyme 4 (*PDK4*), peroxisome proliferator-activated receptor gamma coactivator-1 alpha *(PPARGC1A*) and cytochrome C-1 (*CYC1*) (data not shown).

Metabolic flexibility

The metabolic flexibility parameters were determined as described before [5], and myotubes derived from type 2 diabetics showed an about 30% lower adaptability for OA oxidation than myotubes from non-diabetics, whilst the substrate-regulated flexibility and suppressibility parameters were not significantly different (Fig. 5, p = 0.02, p = 0.26, p = 0.45, respectively).

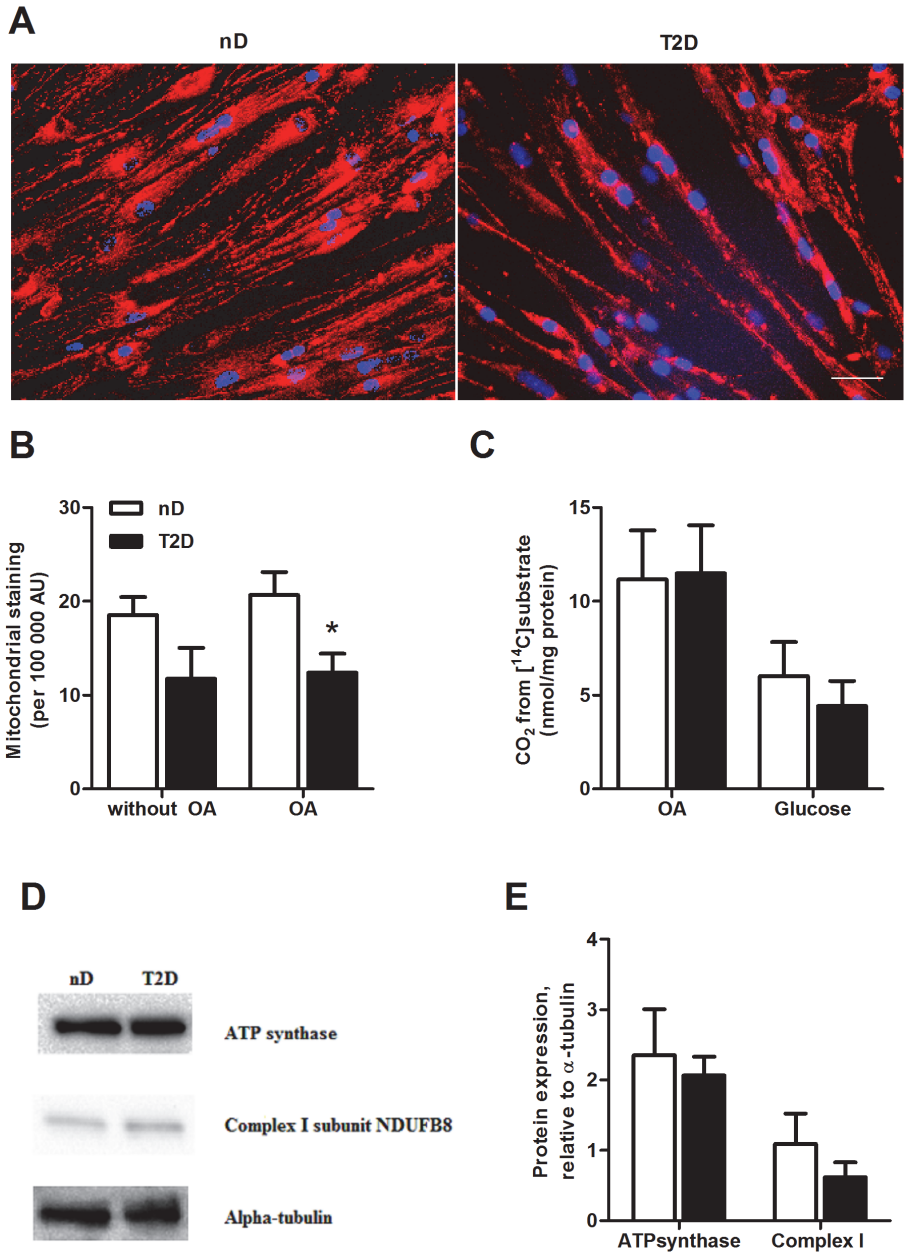

Fig 4. Lower mitochondrial staining of myotubes from severely obese donors with type 2 diabetes. Myotubes from severely obese non-diabetic donors (nD) and severely obese donors with type 2 diabetes (T2D) were stained with MitoTrackerRed (mitochondria) and Hoechst 33258 (nuclei). (*A*) Representative images after 24h incubation with 100 μM oleic acid (OA). Blue is nuclei and red is mitochondria. Magnification is 1:20, scale bar represents

50 μm. (*B*) Mitochondrial staining related to number of nuclei per 100 0000 AU. Myotubes were incubated with or without 100 μM oleic acid (OA). Mean±SEM are presented related to number of nuclei, $n = 5$, *$p<0.05$ versus non-diabetics. *(C)* OA oxidation or glucose oxidation were measured in presence of [^{14}C]OA (18.5 kBq, 100 μM) or [^{14}C]glucose (18.5 kBq, 200 μM) for 4h. Data are presented as mean±SEM, $n = 7$. AU, arbitrary units. *(D-E)* Protein expressions of ATP synthase subunit and Complex I subunit NDUFB8. Protein samples were harvested and analyzed as described in Materials and Methods, and expression levels were normalized to alpha-tubulin. *(D)* Representative Western blots from one experiment. *(E)* Protein expressions of ATP synthase subunit and Complex I subunit NDUFB8 related to alpha-tubulin. Data are presented as mean±SEM, $n = 5–8$.

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Discussion

Disturbances in skeletal muscle lipid metabolism are clearly associated with insulin resistance and type 2 diabetes. In the present study, we wanted to examine lipid handling in myotubes

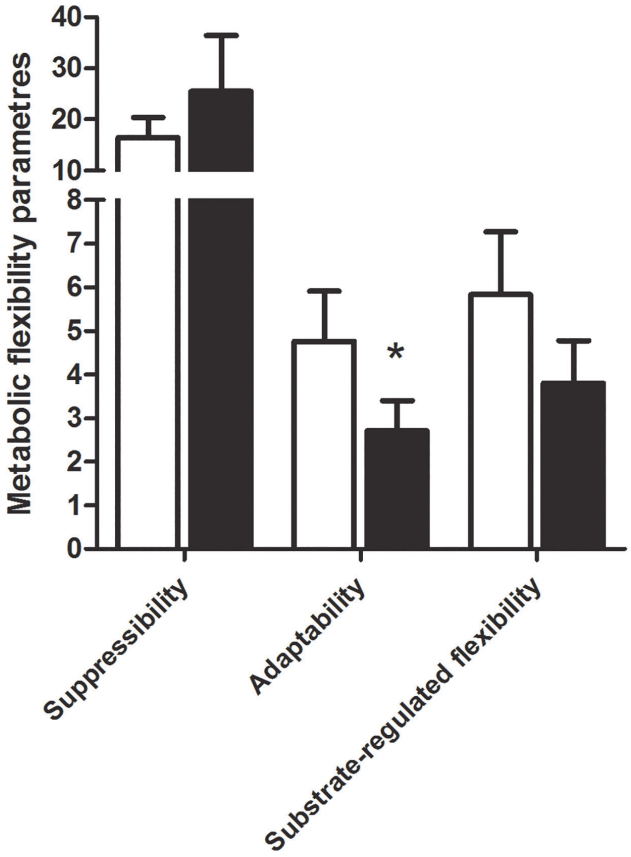

Fig 5. Metabolic flexibility parameters in myotubes derived from severely obese non-diabetic donors (nD) and severely obese donors with type 2 diabetes (T2D). Calculations of metabolic parameters are performed as described [5]. Suppressibility is (1- oxidation of 100 μM OA at 5 mM glucose/oxidation of 100 μM OA at no glucose added)*100%, adaptability is oxidation of 100 μM OA/oxidation of 5 μM OA, and substrate-regulated flexibility is oxidation of 100 μM OA without glucose added/oxidation of 5 μM OA at 5 mM glucose. Linear mixed model was performed for adaptability and suppressibility, while unpaired two-tailed t-test was performed for substrate-regulated flexibility, *$p<0.05$ versus non-diabetics, $n = 7–8$.

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isolated from severely obese that have developed type 2 diabetes and severely obese with normal glucose tolerance to see whether there are fundamental differences. We observed that myotubes established from severely obese type 2 diabetic donors had higher lipolysis rate and were less able to accumulate fatty acids than myotubes from severely obese with normal glucose tolerance. In line with this, TAG content was lower in myotubes from type 2 diabetic donors but there were no significant differences in lipid distribution into FFA, DAG, CE or PL. There were no differences in fatty acid and glucose oxidation either. Although the diabetic cells had lower mitochondrial content as assessed by Mitotracker, expression levels of the OXPHOS proteins did not differ significantly between the groups. Further, diabetic cells showed less ability to increase fatty acid oxidation with increased oleic acid availability (adaptability). We also confirmed that myotubes derived from severely obese donors with T2D had lower insulin sensitivity compared to cells from non-diabetic donors, a trait apparently conserved from the *in vivo* situation.

There are numerous studies showing an association between insulin resistance and increased lipid accumulation in skeletal muscle of type 2 diabetic subjects [25, 36]. However, there are also indications that this association is not solely due to ectopic lipid accumulation, but rather caused by dysregulation of lipolysis and/or lipid turnover [25, 27, 37]. Lower lipid accumulation and higher lipolysis without correspondingly increased fatty acid oxidation, as observed in type 2 diabetic myotubes in this study, could contribute to accumulation of lipotoxic intermediates, which are reported to interfere with insulin signaling [38–40], (reviewed in [25]). Although we could not observe any differences in DAG content, the ratio of DAG/TAG tended to be higher in type 2 diabetic myotubes. In correspondence with this, higher DAG level in muscle has been associated with obesity and insulin resistance in both rats and humans [39, 41], while muscle ceramides are also elevated in lean or obese insulin resistant humans and rats [41]. Although TLC is a sensitive method, we cannot exclude that small differences that we were not able to detect, in for instance DAG or other lipid intermediates could mediate important signaling effects.

We observed lower lipid accumulation and higher lipolysis rate in myotubes from severely obese type 2 diabetic donors without any difference between cells from the two groups with respect to protein and mRNA expression levels of CD36, PLINs, ATGL, HSL and CGI-58. Reduced protein expression of the lipase HSL has previously been shown in obese insulin-resistant subjects [24, 42], and increased protein levels of ATGL in both obese subjects with type 2 diabetes [22] and obese non-diabetic subjects [24]. However, no apparent changes in lipases and PLIN proteins were observed in the present study despite functional changes in lipolysis. The regulation of lipase activity is a complex process regulated at multiple steps, including phosphorylation of lipases and PLIN proteins and complex movement of these different partners between the lipid droplets and the cytosol [43]. HSL activity is probably mostly regulated by phosphorylation [43]. However, we were not able to detect any differences in HSL660 phosphorylation in myotubes from the two donor groups, but other phosphorylation sites, not studied here, may be involved. Recently, we observed that increased expression of ATGL by palmitic acid in myotubes, did not increase lipolysis, implying that content and activity are not directly linked [31]. ATGL can also be phosphorylated, although its activity appears mainly controlled by the co-activator CGI-58 [43]. Exactly how lipolysis rate is regulation by PLINs is not known, but PLIN2 has been found co-localized with HSL after epinephrine stimulation, and PLIN5 is suggested to channel fatty acids to the mitochondria after lipolysis [43]. In accordance with a recent observation in myotubes from obese individuals with or without type 2 diabetes [44], we could not detect any change in expressions of PLIN2 and PLIN3, and further investigation on how PLINs and lipases interact is needed.

TAG content of type 2 diabetic myotubes was about 50% of the content in non-diabetic controls after 24 h incubation with OA. This difference can possibly be explained by increased lipolytic rate, combined with less lipid accumulation. However, there might also be differences in TAG synthesis, for instance DGAT activity or in lipid uptake. A complete picture is difficult to interpret. CD36 expression was similar as well as fatty acid uptake, assessed as cell associated OA after 4 h (data not shown), and Sparks et al. showed very recently that DGAT-activity did not differ between myotubes from obese males with T2D and BMI and age matched controls [44]. Altogether, we have documented disturbed lipid handling, but still the explanation is puzzling.

In addition to lipid turnover, metabolic flexibility was also impaired in myotubes derived from type 2 diabetic donors, assessed as reduced adaptability for fatty acid oxidation. In support, lower ability to increase oxidation with increasing fatty acid concentration in myotubes from overweight/obese type 2 diabetics [8] and reduced metabolic flexibility *in vivo* in insulin resistant muscle [4] compared to healthy lean donors have been reported. The combination of decreased adaptability and higher lipolysis rate might be unfavorable and likely contribute to an abnormal intracellular lipid profile. Interestingly, we found lower mitochondrial staining in myotubes from severely obese with type 2 diabetes compared to cells from severely obese non-diabetic donors, in accordance with earlier studies showing lower mitochondrial content in insulin resistant muscle [17, 45]. This occurred despite no differences in fatty acid oxidation, nor in the OXPHOS protein expression or mRNA expression of *CYC1*, *PPARGC1A*, *CPT1B* or *PDK4* in myotubes from severely obese donors with type 2 diabetes compared to cells from severely obese non-diabetic donors. The focus in recent years has been directed towards a mitochondrial deficiency/dysfunction in relation to development of type 2 diabetes, and several studies conclude that the observed reduced mitochondrial function in type 2 diabetes is due to, and secondary to, a lower mitochondrial content in muscle (reviewed in [46]). Although one might expect that oxidation and mitochondrial content are positively associated, an inverse correlation between mitochondrial content and degree of complete lipid oxidation has been observed in myotubes [47].

Increasing age is related to elevated insulin resistance and so is increasing obesity related to aging. The subjects with type 2 diabetes in this study were in average older than the non-diabetic subjects, however, the duration of obesity did not differ and age *per se* could not explain the metabolic differences. It is also hypothesized that obesity and inactivity rather than age *per se* is the primary determinant of age-related declines in insulin sensitivity [48, 49].

Altogether, our results show that there are retained differences in cells cultured from subjects with and without diabetes. This is in accordance with many previous observations [6, 14, 44]. Actually, how these differences are conserved from the *in vivo* to the *in vitro* situation is not known. Most studies have failed to explain this by genetic polymorphism, but a possibility, yet undetected, is that epigenetic changes could accumulate *in vivo* and be transmitted to in the cultured cells, causing a diabetic phenotype.

In conclusion, myotubes established from severely obese subjects with established type 2 diabetes had lower ability for lipid accumulation and higher lipolysis rate than myotubes from severely obese donors without diabetes. Our hypothesis is that by storing more lipids as triacylglycerol, the formation of lipotoxic intermediates that could interfere with insulin signaling may be prevented. Higher lipolysis not followed by a higher fatty acid oxidation, in combination with lower adaptability and lower mitochondrial content could contribute to a diabetic metabolic profile.

Supporting Information

S1 Fig. Oleic acid and glucose uptake and acid soluble metabolites (ASM) in myotubes derived from severely obese non-diabetics (nD) and severely obese diabetics (T2D). (*A*)

Cellular uptake of oleic acid and glucose, assessed as the sum of cell associated and CO_2-trapped radioactivity after 4 h, $n = 7$. (*B*) Incomplete fatty acid oxidation, detected as ASM, were assessed after incubation of 100 μM or 600 μM oleic acid (OA) for 24 h, $n = 5$. Data are presented as mean ± SEM.
(TIF)

S1 Table. Primer sequences used in real time RT-PCR.
(PDF)

S2 Table. mRNA expression in biopsies for the donor groups severely obese non-diabetics (nD) and severely obese with type 2 diabetes (T2D). Data are presented as fold change to the average of two housekeeping genes (*GAPDH* and *RPLP0*). Mean ± SEM are presented for $n = 7$ donors per group.
(PDF)

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Author Contributions

Conceived and designed the experiments: SSB YZF NN ETK CM ACR GHT VA. Performed the experiments: SSB YZF NN ETK CM CS LD MOL VA. Analyzed the data: SSB YZF NN ETK CM LD MOL ACR GHT VA. Contributed reagents/materials/analysis tools: RS BMS JH. Wrote the paper: SSB YZF NN ETK CM CS LD MOL RS BMS ACR JH GHT VA.

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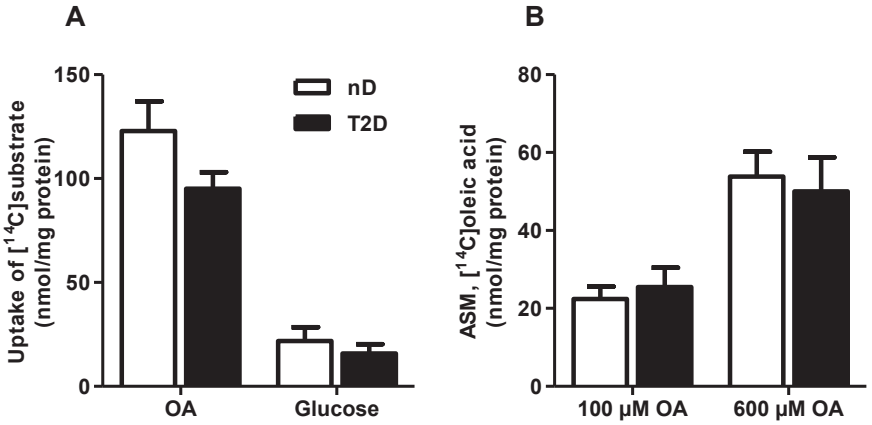
Table S2. mRNA expression in biopsies for the donor groups severely obese non-diabetics (nD) and severely obese with type 2 diabetes (T2D).

	CD36	PLIN2	PLIN3	ATGL	PDK4	CPT1B
nD	2.43±0.28	1.62±0.15	0.37±0.04	1.76±0.10	2.21±0.50	2.40±0.30
T2D	2.70±0.63	1.63±0.28	0.48±0.05	1.90±0.27	2.43±0.42	2.59±0.31

Data are presented as fold change to the average of two housekeeping genes (*GAPDH* and *RPLP0*). Mean ± SEM are presented for n =7 donors per group.

S1 Fig.



(*A*) Cellular uptake of oleic acid and glucose, assessed as the sum of cell associated and CO_2-trapped radioactivity after 4 h, $n = 7$. (*B*) Incomplete fatty acid oxidation, detected as ASM, were assessed after incubation of 100 μM or 600 μM oleic acid (OA) for 24 h, $n = 5$. Data are presented as mean ± SEM.

III

IV