The diabetic phenotype is preserved in myotubes established from type 2 diabetic subjects: a critical appraisal
Gaster, Michael

Published in:
APMIS

DOI:
10.1111/apm.12908

Publication date:
2019

Document version:
Accepted manuscript

Citation for published version (APA):

Go to publication entry in University of Southern Denmark's Research Portal

Terms of use
This work is brought to you by the University of Southern Denmark. Unless otherwise specified it has been shared according to the terms for self-archiving. If no other license is stated, these terms apply:

• You may download this work for personal use only.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying this open access version

If you believe that this document breaches copyright please contact us providing details and we will investigate your claim. Please direct all enquiries to puresupport@bib.sdu.dk

Download date: 15. Sep. 2023
Article Type: Review Article

The diabetic phenotype is preserved in myotubes established from type 2 diabetic subjects: A critical appraisal

Michael Gaster

Address for correspondence and reprint requests:

Michael Gaster, MD, PhD, DMsc
Laboratory for molecular physiology, Department of Pathology and Department of Endocrinology, Odense University Hospital, Denmark

Actual address
Lægerne Jernbanegade
Jernbanegade 4
7000 Fredericia
Tlf +45 23282164
Email: gasterpost@gmail.com

Abbreviated title: Myotube and primary insulin resistance
Summary

Cultured human myotubes offer a unique model to distinguish between primary and environmental factors in the etiology of insulin resistance in human skeletal muscle. The objective of this review is to summarise ours and other groups studies on insulin resistance in human myotubes established from lean, obese and T2D subjects. Overall, studies of human myotubes established from lean, obese and T2D subjects clearly show that part of the diabetic phenotype observed in vivo is preserved in diabetic myotubes. Diabetic myotubes express a primary coordinated impairment of lipid oxidation, oxidative phosphorylation and insulin-stimulated glucose metabolism. Currently, both the responsible molecular mechanisms as well as the extent to which these alterations depend on genetic and/or epigenetic alterations have yet to be identified. Based on the data, it is hypothesized that the impaired insulin-mediated glucose metabolism, impaired oxidative phosphorylation and reduced lipid oxidation observed in diabetic myotubes are caused by reduced peroxisome proliferator activated receptor gamma coactivator-1α (PCG-1α) expression.

Abbreviations:

**AMPK**: AMP-activated protein kinase, **ASM**: acid solube metabolites, **AMP**: adenosine-5-monophosphate, **ADP**: adenosine-5'-diphosphate, **AS160**: Akt substrate of 160 kDa, **ATP**: adenosine-5'-triphosphate, **CK**: creatinine kinase, **CS**: Citrate synthase, **CoA**: Coenzyme A, **DNA**: deoxyribonucleic acid, **ETC**: electron transport chain, **FCS**: foetal calf serum, **FDR**: First degree relatives, **FFA**: free fatty acid, **GLUT**: glucose transporter, **GS**: glycogen synthase, **GSK3**: glycogen synthase kinase 3, **GWA**: Genome wide association studies, **HS**: horse sera, **H₂O₂**: hydrogen peroxide, **IRS**: Insulin receptor substrate, **MRF**: myogenic regulatory factors, **MHC**: myosin heavy chain, **mtDNA**: mitochondrial DNA, **NAD**: Nicotinamide adenine dinucleotide, **NADPH**: reduced form of NADP, **NCAM**: neuronal cell adhesion molecule, **NRF-1**: Nuclear respiratory factor-1, **OXPHOS**: oxidative phosphorylation, **PA**: Palmitate, **PDHC**: pyruvate dehydrogenase complex, **PDK1**: phosphoinositide-dependent protein kinase-1, **PGC-1α**: peroxisome proliferator activated receptor gamma coactivator-1α, **PIK3**: phosphatidylinositol 3-kinase, **PKB**: protein kinase B, **PKC**: Protein kinase C, **PTM**: post-translational modifications, **RNA**: ribonucleic acid, **ROS**: reactive Oxygen Species, **Sc**: Satellite cell, **SDH**: succinate dehydrogenase, **T2D**: Type 2 diabetes, **TAG**: Triacylglycerol, **UG**: Ultrose G, **TCA**: tricarboxylic acids
1. Introduction

The constant increase in obesity in industrialized countries caused by a sedentary lifestyle and overly rich nutrition has resulted in an increase in the number of people diagnosed with diabetes worldwide (1-4). In the US alone, 23.6 million people have been estimated to suffer from diabetes. Of these, 17.9 million have been diagnosed, 90% of them with type 2 diabetes (T2D) (5). Furthermore, the increased prevalence in the U.S. has led the American Centers for Disease Control and Prevention to publically classify the disease as an epidemic. The worldwide picture shows the same trends, and today, T2D is seen as one of the main threats to human health in the western world (6). T2D is associated with increased morbidity and mortality rates, in particular, due to associated cardiovascular disease (3;7). Understanding of the pathophysiology and cellular mechanisms responsible for the development of T2D is necessary to prevent the disease. T2D is a complex disorder characterized by impaired insulin secretion (8) and insulin resistance in major metabolic tissues such as skeletal muscle, liver and adipose tissue, although skeletal muscle is the major site of insulin resistance (9;10). Insulin resistance in vivo is also seen during other pathological conditions, e.g., obesity (11;12) and polycystic ovary syndrome (PCOS) (13;14).

Insulin is an anabolic hormone that promotes a number of metabolic and mitogenic responses through a highly coordinated signalling network. In addition to being found in classically insulin-targeted tissues such as adipose, skeletal and liver tissues, insulin receptors can also appear in the central nervous system, heart and kidney (15;16). The intracellular action of insulin is mediated by modifications of the activity and/or subcellular localization of key regulatory proteins, kinases and phosphatases, which are regulated through their phosphorylation state (17;18). In physiological concentrations, insulin stimulates glucose uptake in human skeletal muscle in vivo by activating the insulin signalling cascade. Activation of this cascade results in glucose transport through insulin-mediation activation of the tyrosine kinase of the membrane-bound insulin receptor, which leads to autophosphorylation of the receptor. This effect increases tyrosine kinase activity and promotes signal transduction through the phosphorylation of insulin receptor substrate 1 (IRS1). IRS1 interacts with Src-homology-2 domains and activates phosphatidylinositol 3-kinase (PI3K), which catalyses the formation of PI(3,4,5)-P3 from PI(4,5)-P2 (19). The activation of phosphoinositide-dependent protein kinase-1 (PDK1), which occurs by increasing concentrations of PI(3,4,5)-P3, activates AKT2 (also named protein kinase B (PKB)) through phosphorylation. Further signal transduction is mediated through phosphorylation of the 160-kDa AKT substrate AS160 (also named TBC1D4). The final result is increased glucose uptake via translocation of glucose transporter 4 (GLUT4) to the cell surface (19;20), increased glycogen synthesis via AKT2-
mediated inhibition of glycogen synthase kinase 3 (GSK3) through phosphorylation and activation of glycogen synthase (GS) via dephosphorylation of serine residues at sites 2+2a and 3a+3b. Impaired insulin signalling is only evident in the metabolic effects of insulin; insulin signalling related to mitogenesis, growth and differentiation remains intact (21;22).

Insulin resistance in skeletal muscle in T2D in vivo is characterized by an impaired insulin response, specifically at the level of glucose uptake and its storage as glycogen (10;23-25). The most well described defect in the pathophysiology of T2D is reduced insulin-mediated glycogen synthesis in skeletal muscles (10;23). The impaired insulin response to GS in vivo seems to be caused by attenuated dephosphorylation of GS at site 2+2a, whereas the dephosphorylation of site 3+3a appears to be normal (26). Although findings vary across studies, defects in insulin signalling (IRS1, PI3K, AKT2 and AS160) are known in T2D, and these defects can explain impaired insulin activation of GLUT4 translocation and impaired insulin-mediated glucose uptake (21;26-28). The metabolic overload associated with T2D has been suggested to contribute to impaired insulin signalling. An increase in circulating free fatty acid (FFA) levels has been shown to decrease IRS1-associated PI3K activity and IRS1 tyrosine phosphorylation and to increase activation of protein kinase C (PKC) theta activity (29;30). Elevated levels of diacylglycerol, acyl-CoA, and ceramide, resulting from incomplete fatty acid oxidation and lipid overload, could activate PKC and lead to serine phosphorylation of IRS1 and consequently impaired insulin signalling (29;31;32). Mitochondrial dysfunction has been implicated in insulin resistance in skeletal muscle in T2D (33). Mitochondria express morphological abnormalities such as reduced size and fewer cristae. In addition, an altered distribution of mitochondria within the muscle fibres as a reduction of the number of sub-sarcolemmal mitochondria, are also observed (34;35)(36). Both the morphological abnormalities and the changes in the distribution and number of mitochondria decrease oxidative phosphorylation (OXPHOS), which is related to lower expression of the transcriptional peroxisome proliferator activated receptor gamma coactivator-1α (PGC-1α) (37;38). Metabolic inflexibility, or the reduced ability to switch between carbohydrate and fatty acid oxidation under insulin stimulation, has been described in T2D skeletal muscle by Kelley et al. (39).

It is generally accepted that both genetic and environmental factors contribute to insulin resistance and subsequently to the development of T2D, as highlighted by twin studies and epidemiological evidence (9). This assertion is supported by studies showing impaired insulin-stimulated glucose uptake, glycogen synthesis, and insulin signalling in glucose-tolerant first-degree relatives (FDRs) of patients with T2D (40). Despite some rare syndromes of monogenetic inheritance, for the vast majority of patients, the pattern of inheritance reflects varying penetrance or a polygenetic nature. Genome-wide association studies have identified approximately 65 common genetic variants,
typically with a low minor allele frequency (41-44). However, even the combination of these cannot explain more than 10-20% of the overall heritability of T2D (41). The identified alleles are located in genes important for β-cell function, stressing the importance of β-cell function during compensation for the hyperglycaemia that occurs secondary to insulin resistance. Increasing evidence indicates that the development of insulin resistance/T2D results from the interplay of genetic, epigenetic and environmental factors (41;42).

Our current knowledge of insulin resistance of skeletal muscle originates mainly from in vivo studies that combined assessments of whole-body insulin sensitivity and indirect calorimetry with muscle biopsies, allowing us to integrate information obtained from clinical characterization, whole-body metabolism and cellular events on the molecular, biochemical, and morphological level. Over the last three decades, research has focused especially on the identification and characterization of molecules involved in insulin signalling to understand the defects that result in impaired insulin stimulation in insulin resistance in vivo. Despite intensive study, the characteristics of primary insulin resistance and the underlying pathophysiological mechanism that causes inherited insulin resistance in skeletal muscle in T2D remained unclear.

Skeletal muscle expresses a high degree of plasticity, i.e., the capacity to adjust the size and functional properties of muscle fibres in response to endogenous and exogenous influences, rendering it difficult to determine the contribution of heritable factors to alterations of skeletal muscle metabolism in vivo. Studying the pathophysiology of insulin resistance of skeletal muscle in T2D requires a clearer model that will enable focus on the genetic component and/or the impact of environmental factors. Cultured human myotubes offer a unique model to distinguish between primary and environmental factors in the aetiology of diseases (45-52). Human myotubes are established from satellite cells (Sc’s), the stem cells of skeletal muscle. Sc’s can proliferate in culture and fuse to form human myotubes (multinucleated large cells) that express muscle characteristics (47;53-55). The possibility to control the extracellular environment, thereby removing the acquired confounding influences associated with the diabetic state, enables the elucidation of whether primary traits known from in vivo studies are present in the intermediary metabolism of myotubes established from subjects with T2D during normo-physiological conditions and acute insulin stimulation.

In a series of studies, we have characterized the intermediary metabolism during normo-physiological conditions and during insulin stimulation of myotubes established from lean, obese and T2D subjects. The objective of this review is to summarize our and other studies of primary insulin resistance in myotubes established from subjects with T2D. Emphasis is placed on primary traits found under basal conditions and during insulin stimulation in myotubes established from
T2D subjects cultured under normo-physiological conditions by us and other groups to clarify whether primary insulin resistance is present and if so, to determine its characteristics and investigate the underlying molecular mechanism.

2. **Human myotubes as a model for skeletal muscle**

Over the years, various models for human skeletal muscle tissue have been proposed, including cultures of human myotubes based on the stem cell of skeletal muscle, the Sc (53,56), and intact human muscle strips incubated ex vivo. Recently, due to the rapidly growing stem cell technology, other progenitor cells, such as pericyte-derived cells, which can differentiate into myotubes in vitro, have also been used (57). To study the inherited characteristics of T2D, investigators depend on the human genome and need to investigate human skeletal muscle from healthy and T2D subjects. Animal models are not suitable in this context.

Sc’s in culture have already been established over the course of many years (53,56,58), either from embryonic muscle tissue or from mature muscle tissue obtained through biopsies. Sc’s lie between the basal membrane and the sarcolemma of muscle fibres as quiescent cells (59) and can be isolated from muscle biopsy samples by enzymatic digestion (53,56) or by migration from muscle tissue explants (58). Isolated Sc’s can proliferate, fuse and differentiate into human myotubes and can be kept in culture for weeks. Sc’s were initially used in studies of muscle cell development and regeneration as well as in studies of inherited metabolic disorders with identified biochemical defects; however, they have also been used in studies of enzyme defects that affect multiple organ systems (please refer to review (60-62)). Later, human myotube cultures were used in metabolic and signal transduction studies (45-47,49,63-66). We and other groups use differentiated human myotubes to clarify whether pathophysiological findings in skeletal muscle in vivo, i.e., traits associated with insulin resistance in the skeletal muscle of T2D subjects, are of primary origin and/or are induced in response to a changed milieu (45-47,52,67-74). An alternative model to cultured human myotubes is isolated human muscle strips, which are obtained during surgical procedures where muscle fibre endings are fixed by a clamp (75). However, the preservation of membrane integrity/metabolism in this preparation has been questioned because human muscle fibres are extremely long, and muscle fibres cannot be obtained in toto. The lifetime of muscle strips is also short, hampering the ability to study whether traits of the intermediary metabolism are primary or secondary. As muscle tissue biopsies, isolated muscle strips are also strongly influenced by physical activity, ageing, hormonal status, and fibre type composition,
rendering it difficult to determine the contribution of genetically inherited factors. Pericyte-derived myogen cultures have yet to be established as a model for skeletal muscle.

The idea underlying the utilization of human myotubes is that cultured myotubes express only their genetic/epigenetic background when pre-cultured under normo-physiological conditions (5 mM glucose and 25 μM and express adaptive traits when pre-cultured under ‘inducing’ conditions. A primary (genetic/epigenetic) cause for a known in vivo pathophysiological abnormality will be evident if the abnormality is present under normo-physiological conditions in vitro. The model is based on the assumption that pre-conditioning in vivo is not a serious problem, i.e., that acquired defects, as a consequence of chronic hyperglycaemia, dyslipidemia and/or hyperinsulinemia, for example, will not be preserved after the myotubes are cultured under physiological conditions and allowed to replicate for weeks. As quiescent cells, Sc’s are partially protected against epigenetic alteration due to their low metabolism and because they are located in a specific environment that maintains their quiescent state (niche). Sc’s express very low mitochondrial oxidative phosphorylation, which protects them from DNA and protein damage via ROS production. Protein acetylation can affect both protein function and gene expression and depends on the presence of acetyl CoA. The low mitochondrial activity of Sc’s reduces the production of acetyl CoA. Although all of these processes are minimal in quiescent Sc’s, epigenomic alteration at the genomic scale cannot be completely excluded in Sc’s. Notably, when the host muscle fibre experiences epigenetic pressure, Sc’s are partially protected in their niche.

Although Sc’s may have experienced the same milieu as the muscle fibres from which they were derived, under normo-physiological conditions, they must build up an actively replicating myoblast population. Previous metabolic influences, i.e., post-translational modification of proteins, will decrease due to dilution through replication and through new synthesis. As a result, the new environment will influence the myoblasts in a different way than the old milieu. Moreover, the differentiation of myoblasts to myotubes induces an isoform shift of various proteins, shifting protein expression and metabolism in the direction of mature muscle fibres and further reducing the influence of the in vivo metabolic milieu. Thus, myotubes are suitable for studying genetic/epigenetic primary traits when they are evaluated under normo-physiological conditions. The presence of either primary or secondary traits allows their molecular biological backgrounds to be studied. Thus, the concept underlying the model is fundamentally valid and potentially revealing.

Proof of concept is provided by the fact that major single gene defects are expressed in the culture model, as shown for various primary metabolic myopathies, such as phosphorylase deficiency (61). Moreover, mutations are also carried over into the myotubes. Costford et al. (76) showed that
the naturally occurring R225W mutation in the γ-3 subunit of AMP-activated protein kinase (AMPK) and the resulting phenotype were retained in culture. A primary explanation cannot be excluded based on negative findings in human myotube cultures, as they may appear when the myotubes mature.

Isolated Sc’s can proliferate, fuse and differentiate into human myotubes and be kept in culture for weeks. On the ultra-structural level, differentiated myotubes display a basal membrane, cross and longitudinal striation, and sarcomere units, indicating that the contractile apparatus is present (54). Aneural cultured human myotubes express acetylcholine receptors, but the receptors cluster at motor nerve endplates only when the myotubes are innervated (53;77;78), and the myotubes rarely spontaneously contract (54). Innervation improves maturation of myotubes and induces contractions but at present, no reproducible models utilizing innervation have been established.

Human muscle tissue consists of mixed fibre types (79), and isolated Sc’s originate from all fibre types. Human Sc’s are not programmed to be either fast- or slow-twitch muscle fibres (80;81). Studies of the expression pattern of myosin heavy chain (MHC) isoforms in our cultures showed that myotubes established in accordance with our optimized culturing technique all express fast myosin. Some co-express neonatal or developmental myosin, and a few myotubes co-express slow MHC (54). Similar results were seen by Bonavaud et al. (81). Although this limitation of the model may be seen as a disadvantage, the model still allows the detection of primary defects in the insulin signalling cascade/insulin-stimulated metabolism as they are present in all fibre types, although to a lower extent in fast-twitch fibres compared to slow-twitch fibres. Several approaches have been developed to improve slow-twitch fibre expression in human myotubes, such as by utilizing a chemical cocktail (containing palmitic acid, forskolin and ionomycin) (82), electric pulse stimulation (83) or chronic electric stimulation. These approaches improve the oxidative capacity of myotube cultures, the fraction of slow fibres and insulin sensitivity. Maturation into only slow fibres, however, has not been obtained. Isolated Sc’s express some plasticity/multipotency, as human Sc’s can convert to more adipocyte-like cells in the presence of thiazolidinediones (65).

The functionality of skeletal muscle tissue in vivo is characterized by contractility, sensitivity to various stimuli, e.g., insulin, hypoxia and other hormones. The best-investigated stimulus in human myotubes is insulin stimulation. Human myotubes express most of the same proteins that characterize the intermediary metabolism (84;85) and all the essential components of the insulin signalling cascade (49;64;68;86-89) seen in vivo. However, some differences in isoforms may be present due to differences in the degree of maturation. Nearly all proteins from the major pathways in the intermediary metabolism have been identified by proteomic analysis of human myotubes set up in accordance with our protocol (84). Pathways that are insulin sensitive in vivo are also insulin
sensitive in vitro, including glucose uptake, glucose oxidation, glycogen synthesis, and lipid uptake and storage pathways (47;52;67;69;73;90;91).

Human myotubes exposed to 5.5 mM glucose oxidize 20 % of the glucose taken up and store 16 % of it as glycogen (92); the rest is found as intermediary metabolites (lactate, citrate, malate, etc.) as the de novo lipid synthesis rate is very low (93). The pentose phosphate shunt (PPS) accounts for 2 % of the glucose taken up (92). Acute insulin stimulation increased glucose uptake by up to 50 %, glucose oxidation by up to 45 %, and glycogen synthesis by up to 110 %, changing the overall partitioning in the direction of glucose storage (92). Human myotubes exposed to 0.6 mM PA and 5.5 mM glucose incorporated most of the FFAs taken up into phospholipids, triacylglycerols (TAGs) and, to a lesser extent, other lipid classes (FFAs, diacylglycerols, and cholesterol esters), while only 5 % of PA taken up was completely oxidized to CO$_2$; 10 % was found as acid-soluble metabolites (ASMs) (67;93). FFA oxidation was twice as high if glucose was not present and vice versa (94;95). Endogenous TAGs contributed to substrate oxidation in human myotubes to a minimal extent under these conditions (96). Acute insulin stimulation increases lipid uptake, and these lipids are mainly incorporated into TAGs (67). Myotubes also express high lipolytic activity (71). Inhibition of mitochondrial energy production by respiratory uncoupling or inhibition of ATP synthase increases glucose uptake and lactate production 3-fold, indicating that oxidative metabolism contributes considerably to energy production in myotubes (92).

GLUT1, GLUT3, GLUT4 and GLUT11 are expressed during human muscle tissue development, while only GLUT4 and GLUT11 are important in adult human muscle tissue (97-99). Basal glucose uptake seems to be performed only by GLUT4 in adult fast muscle fibres, as it is the only GLUT isoform expressed at a detectable level (97-99). In human myotubes, it seems that GLUT1 and GLUT3 are responsible for basal glucose transport, while GLUT4 is translocated to the cell surface during insulin stimulation, increasing glucose uptake as seen in vivo (100;101). GLUT4 expression is low in myotubes compared to in vivo (102), resulting in reduced insulin responsiveness, i.e., during insulin stimulation, a smaller lesser amount of GLUT4 is translocated in vitro than in vivo. During insulin stimulation, glucose uptake can be increased by several orders of magnitude in vivo, while glucose uptake in human myotubes can be increased up to 50 % over basal levels (47;52;69;88;92;101;103). Supra-physiological insulin concentrations (0.1-1 μM) are used to obtain maximal insulin stimulation because of the low insulin responsiveness of human myotubes. Insulin stimulation increases glycogen synthesis about twofold in human myotubes, similar to in vivo observations (47;52;88;92;101;104). In vivo, contraction-dependent glucose uptake is an important mechanism that is mediated though contraction-induced AMPK activation, which stimulates translocation of GLUT4 to the plasma membrane. Both direct stimulation of AMPK with AICAR (88)
and electrical stimulation of myotubes increase glucose uptake in vitro (83;105). The palmitate oxidation rate and the activity of citrate synthase (CS) are comparable between human myotubes and homogenates of the corresponding donor muscle (106;107). Thus, human myotubes in culture represent a suitable model to mimic skeletal muscle in the study of inherited traits, as they express an intermediary metabolism sensitive to insulin and contraction comparable to that observed in vivo.

Cell culturing has traditionally been regarded with scepticism. The usefulness of data generated from cultured myotubes and the putative translation to an in vivo condition are questioned. Importantly, human myotubes express the relevant human genes needed to clarify the presence of inherited components. Indeed, cultured muscle cells do not obtain all the features of adult skeletal muscle. As such, differences such as the example of GLUT expression described above may prevent full translation to mature in vivo muscle, but as long as the relevant trait under investigation is expressed, the trait is worth investigating. The extracellular environment can be precisely controlled and kept constant over time, which allows not only the importance of the genetic background to be studied but also the impact of various compensatory mechanisms. The culture environment lacks systemic homeostatic regulatory components from the nervous and endocrine systems, and this lack promotes a more constant cellular metabolism. Contraction-induced changes are not a major problem, as contractions are rarely seen in human myotubes. The muscle cell cultures are easily handled, and experimental designs allow many variables and replicates. Muscle cell culture tissue is suitable for almost the same analyses as muscle biopsy tissue, and often, the same assays and analysis can be directly applied to extracts from myotubes. In contrast to in vivo muscle biopsies, cultured myotubes allow studies of the living muscle cell, e.g., testing the effects of a particular hormone, substrate, drug or compound on different metabolic and signalling pathways, including time-course and dose-response analyses, and thereby allow the investigation of causal relationships and the interactions of various pathways. Myotube cultures facilitate study of the cellular mechanisms that regulate cellular physiology, something that has not been possible using extracts of skeletal muscle and because of the difficulty of manipulating specific variables in skeletal muscle in vivo.

3. **Sex, age and physical activity - study design**

The intermediary metabolism in skeletal muscle is associated with sex, age and physical activity. Sc donor differences related to these characteristics may hamper the detection of primary traits related to insulin resistance in established myotubes.
3.1 Sex

Sex differences in metabolism are well described (please see review (108)). The expression of several genes involved in glucose and lipid metabolism has been observed to be higher in skeletal muscle biopsies from female versus male donors, but these sex-related differences were absent from the corresponding established myotubes (81;109;110). We investigated the sex specificity of ATP flux, but our results revealed no significant differences between sexes (111). In addition, the expression patterns of fibre-type markers seem to be the same in myotubes established from male and female donors (81). Thus, sexual dimorphism in glucose and lipid metabolism in vivo seems likely to be adaptive to systemic factors rather than intrinsic. Although sex differences do not seem to be conserved in human myotube cultures, most studies are designed with sex matching between groups to avoid sexual dimorphism at the level of clinical characteristics.

3.2 Age

Increasing age is associated with reduced insulin sensitivity (112;113). In a study comparing myotubes established from young and middle-aged sedentary and exercise-trained men, Bunprajun et al. (114) described insulin resistance at the level of glucose transport in the aged group based on reduced/impaired translocation of GLUT4 to the plasma membrane during insulin stimulation. AKT phosphorylation, however, was intact. Moreover, basal glucose uptake tended to be lower in the aged group (114). In contrast, myotubes established from the aged trained men retained both the insulin-stimulated increase in glucose uptake and GLUT4 translocation to the plasma membrane (114). Thus, a sedentary lifestyle affects ageing-associated insulin resistance through impaired GLUT4 translocation. The number of Sc’s in normal human muscle decreases with increasing age (115), indicating that signs of senescence may appear in cultures established from older compared to younger tissue and impact results due to the increased number of doublings needed before the cell yield in culture is high enough for studies to be conducted. Notably, the proliferative capacity of Sc’s decreases with age during the first two decades and is remarkably stable thereafter (116). The minimal age difference between groups that results in age-dependent differences at the level of myotubes are at present unknown. With the exception of some of the first myotube studies, most studies have depended on age-matched Sc donor groups.
3.3 Exercise

A sedentary lifestyle is associated with the development of insulin resistance and T2D (117;117;118), but exercise/physical activity can partially counteract this development (114). Several studies have tried to clarify if exercise may imprint donor Sc's and whether established myotubes recapitulate these alterations. Berggren et al. (119) found that myotubes established from endurance-trained athletes express increased basal glucose uptake compared to sedentary individuals, while the fold-change in insulin-stimulated glucose uptake did not differ between the groups. Green et al. (120) compared myotubes established from highly active males with those from matched sedentary males and found that the effect of physical activity was retained in the trained cultures as partial protection against fatty acid-induced insulin resistance. Moreover, lifelong physical activity prevented aging-associated insulin resistance in myotubes by increasing glucose transporter expression (114) and was associated with increased expression of type 2a fibres. Slow myosin expression did not differ between cultures established from trained versus sedentary controls in the two studies (114;120). Bourlier et al. (121) studied myotubes established from obese subjects before and after 8 weeks of training and found enhanced glucose oxidation and glycogen synthesis with increased expression of GLUT1, PDK4 and PDHA1. In contrast, myogen differentiation was unaffected. Data from myotubes established after single bouts or short periods of exercise are currently not available to clarify whether the effects of an acute bout of training will be retained in established differentiated Sc's. Importantly, these studies show that training induces micro environmental changes in the Sc niche, leading to imprinting of Sc's that can be translated into established myotubes. The mechanisms responsible for the imprinting at the Sc level are currently unknown. Most myotube-studies are designed to match the training status of the Sc donors to avoid the confounding impact of training imprinting on the results.

3.4 Obesity

Most individuals with T2D are obese, and many obese individuals are insulin resistant. There is large variation of insulin resistance within obesity, but those with the highest degree of insulin resistance have the highest risk for cardiovascular disease and T2D (12). It is generally accepted that both genetic and environmental factors, e.g., excessive energy intake and low levels of physical activity, contribute to obesity (122-124). Several genetic variants that are important for hypothalamic function have been associated with obesity, indicating that regulating food intake
may be central to the pathogenesis of obesity (125-127). Vollenweider et al. (128) reported that myotubes established from obese subjects with impaired glucose tolerance express reduced insulin-mediated glucose uptake, which was associated with impaired activation of IRS-2-associated PI3K and PKC ζ/λ compared to that in lean controls. However, the study could not determine whether the alterations were genetic or epigenetic in origin. T2D is most commonly found in obese subjects. Obese individuals and obese T2D subjects may share many genetic and epigenetic traits, some of which may affect skeletal muscle metabolism. To identify primary traits related to insulin resistance in skeletal muscle in the most common form of T2D, study design will benefit from including both a lean and obese control group.

3.5 Study cohorts and design

Currently, the utilization of human myotubes as a model for skeletal muscle requires careful matching of study groups with respect to age, gender, weight, BMI and physical activity to avoid introducing confounding factors in addition to the group differences that are under investigation. The clinical characterization includes metabolic profiling, including fasting insulin, glucose and lipid profiles, and verification of the presence of muscular insulin resistance using the hyperinsulinemic-euglycemic clamp technique (129). Initially, studies in the field compared myotubes established from obese individuals with those from obese patients with T2D. Currently, the majority of published studies are based on myotubes established from matched lean, obese and obese T2D subjects, taking into account the considerations discussed above. We conduct our studies on 3 different cohorts and compare myotubes established from T2D subjects with those from 1) a young lean control group (52), 2) a matched obese control group (67) and 3) age-matched lean and obese control groups (130). This design does not allow isolation the pure impact of T2D on skeletal muscle metabolism as it also takes the interaction of T2D and obesity into account. Including a lean group with T2D would enable factorial analysis, but this group of patients is hard to find and has not yet been included in this type of study.

4. **Myotube cultures - Methodological considerations**

To use human myotubes as a model for skeletal muscle tissue, the established cultures must be based on Sc’s, which differentiate into metabolically stable myotubes and express characteristics of skeletal muscle. Moreover, in the context of comparable studies, myogenicity has to be equal
between cultures to avoid confounding effects due to differences in culturing. Methodological variation arises at almost every stage of primary myotube culturing, especially in regard to initiation, proliferation and differentiation protocols. Considerations important for our protocol (54,55) for the establishment of human myotube cultures are reported below. Muscle biopsies for our studies were taken ad modum Bergstrøm (131) from the vastus lateralis muscle by needle biopsy. All our studies were done on myotubes that were allowed to differentiate for 8 days.

4.1 Culture principles

Sc’s can be isolated from muscle biopsy samples by enzymatic digestion (53,56) or by migration from muscle tissue explants (58). Both methods release cell types other than Sc’s, e.g., fibroblasts, smooth muscle cells and endothelial cells. Our studies are based on digestion cultures as the explant culture technique results in the outgrowth of more fibroblasts than Sc’s, a growth pattern that can only be minimally changed by modifying the cell culture method (132). Clonal cultures based on a single verified Sc can be established. However, the applicability of this technique is low due to the limited rate of doubling before senescence occurs, which reduces the number of analyses per clone. Myogenic purity after enzymatic digestion is initially high (>95 %) (47,133), but it decreases because the doubling time of Sc’s is higher than that of fibroblasts. Myogenic purity also decreases due to a reduction in the number of Sc’s as a result of their spontaneous differentiation into myotubes. The purity and differentiation degree of myotube cultures can be increased by favouring conditions for myogenic growth and differentiation (55,134). Different approaches have been employed to reduce the fibroblast content in inocula for cell culture, e.g., pre-plating, utilizing that fact that fibroblasts attach faster to uncoated dishes, fluorescence-activated cell sorting (135) or magnetic affinity cell sorting (136). A variety of usable combinations of media, supplements such as foetal calf serum (FCS), horse serum (HS), chick embryo extracts and recombinant growth factors as well as necessary coatings (gelatine, collagen, laminin, lysine) have been described for myotube cultures (61,134). Based on evaluation of the fractional Sc content (number of neuronal cell adhesion molecule (NCAM)-positive cells/total number of cells, to determine the number of Sc’s relative to the number of contaminating cells such as fibroblasts (137)) under various culture conditions, we could describe optimized Sc conditions based on pre-plating, Sc proliferation in the presence of the serum substitute Ultroser G (UG) and coating of the culturing surface with extracellular matrix gel (ECM) during growth; these conditions ensure high Sc content (82 %) at the 4th cell passage (55).
To identify the differentiation conditions that result in high survival of myotubes and a high grade of differentiation, Sc cultures were induced to differentiate in media supplemented with either 2% FCS, 2% HS or 10% HS (54). The differentiation process was evaluated through estimation of the maximal level of creatinine kinase (CK) and the presence of its mature isoenzymes, the fast myosin index (number of nuclei located in myotubes/total number of nuclei) and the detachment of myotubes during their exposure to the differentiation protocols described above (54). We recommend that Sc differentiation be induced by the removal of UG and the use of a low concentration of FCS, as these differentiation conditions resulted in the greatest CK expression and myotube survival. Based on studies of ATP, DNA, protein content and CK expression over time, we recommend that human myotubes be used on days 4 to 8 after the induction of differentiation, as only minor differentiation-related changes will occur in the myotubes during this period (54). Another frequently used protocol for maintaining myotubes in culture was provided by Henry et al. (47); according to this protocol, myotubes should be used on day 4 after the induction of differentiation. In general, independent of the protocol employed, established myotubes express a several fold increase in markers of skeletal muscle compared to myotubes on day 0 of differentiation after induction (54;88;138).

We studied the impact of increasing myoblast passage number on various metabolic and morphological characteristics of differentiated cultures (139). The capacity of myoblasts to fuse and differentiate into myotubes was reduced, and glucose uptake, storage and oxidation as well as fatty acid oxidation were gradually impaired as the passage number increased. High-passage cultures can be described as mononuclear myocytes rather than as multinuclear myotubes and exhibit a metabolism characterized by senescence. In parallel, the effects of insulin stimulation and the proliferation potential of myoblast were significantly reduced with increasing population doublings (140). The use of human myotubes after passage 5 should be carefully considered (139).

4.2 Culture studies - comparable myogenicity

When comparing myotube studies, it is important that the cultures are seeded with cells at the same passage, at the same density, at the same time and exposed to the same agents/reagents and protocols so that they reach the same myogenicity. Various qualities have been used to investigate the equality in myogenicity in myotubes established from T2D subjects and lean and obese control groups. This ensures control of the initial Sc purity of the seeded cells (NCAM and desmin expression) (47;88) and monitoring of myogen differentiation, as evaluated based on the
expression of myogenic regulatory factors (MRFs) (88), the fusion index (66), the expression of structural proteins (actin, myosin, troponin) (72) or measurement of CK activity and the expression of CK isoforms (88). The MRF family consists of the primary members, MyoD and myf-5, which are important in regard to commitment to the myogen lineage, and the secondary members, myogenin and MRF4, which are important for fusion and differentiation (141;142). Independent of the culture protocol and number of differentiation days, T2D and control myotube cultures handled side by side express comparable values of investigated parameters for Sc purity, proliferation and degree of differentiation (48;54;72;88;138;143). Differences in the magnitude of fold changes between various culture systems can be explained by variation in the protocol used and in the differentiation time for the establishment of myotubes. The most important observation is that cultures that are handled side by side under similar culture conditions, independent of donor group, exhibit equal myogenicity as evaluated by various parameters, indicating that myotubes established from lean, obese and T2D subjects express the same overall myogenicity and differentiation and are therefore suitable for comparison.

4.3 Reproducibility:

Comparing results from various metabolic assays conducted at three different days in myotubes established from the same donors in accordance to our protocols (73;92;144) shows that day-to-day variation is high, while within-day variation is small. Absolute rates of glucose uptake and glycogen synthesis can differ twofold between studies. A similar picture is found for data from other laboratories, e.g., glycogen synthesis by the Henry lab (47;48;145) and Walker lab (50;88) and glucose uptake by the Walker lab (88;146) and Krook lab (101;147); these data showed high day-to-day variation but small within-day variation. Although comparison studies from various labs have produced similar data using same-day cultures with high Sc densities that were established with nearly pure Sc inocula, that were cultured under similar optimized proliferation and differentiation protocols, that were handled side by side using the same protocols and agents/reagents and that were analysed simultaneously, such results are still sensitive to factors that may vary from experiment to experiment and that cannot be controlled between days, such as cell confluency or the degree of differentiation.

Thus, human myotubes are a suitable model for clarifying whether insulin resistance in skeletal muscle in vivo is at least partially primary in origin. Human myotubes express the right genes and insulin-sensitive intermediary metabolism and can be used for day-matched comparison studies if cultures for comparison are established, handled and analysed side by side under similar conditions/protocols.
5. **Insulin resistance is preserved in myotubes established from type 2 diabetic subjects**

Although initial studies focused on clarifying the extent to which T2D myotube cultures expressed insulin resistance based on the level of glucose uptake and glycogen storage and the insulin signalling cascade, such studies have since been extended to include various other aspects of the intermediary metabolism, i.e., primary alterations in substrate oxidation, mitochondrial function and lipid metabolism. Significant variation in methodology is seen, especially in regard to tracer methodology, insulin concentrations and durations, the number of study subjects and the assays used for the analysis of metabolites, enzyme kinetics, mRNA expression, protein expression and omics technologies. This variation is because muscle cell culture tissue is suitable for almost the same analyses as muscle biopsy tissue and other cell cultures, and the same assays and analyses can often be directly applied to myotubes with minor alterations, allowing the use of pre-existing technologies/methods across laboratories. The significant variation in methodology concerning myotube establishment as well as in the technologies and methods used to analyse various qualities make comparing results between various studies difficult, and such comparisons should be made with caution. The review below focuses on studies that investigated myotubes established from obese T2D subjects and controls under normo-physiological conditions and that were handled and analysed side by side to enable investigation the presence of primary traits in diabetic myotubes and comparison with results obtained in other studies. As differentiation time differs across studies, the number of days of differentiation is indicated.

5.1 **Myotubes established from T2D subjects at baseline**

The first step in glucose utilization by muscle fibres is glucose uptake through the plasma membrane, a process that is mediated by a family of glucose transporters (please refer to the following review (97)). Human myoblasts mainly express GLUT1 and minimally express GLUT4, while myotubes express an increased level of GLUT4 (102). By utilizing a photolabelling technique, Al-khalili et al. (101) demonstrated that GLUT4 is the predominant insulin-stimulated glucose transporter in human myotubes. Henry et al. (47;66) showed that on day 4 after the induction of differentiation, basal glucose uptake in diabetic myotubes established from middle-aged subjects...
was reduced compared to that in myotubes established from controls who were 8 years younger. Correspondingly, significantly reduced GLUT1 expression but similar GLUT4 content was observed in diabetic myotubes compared to controls (47;66); the low GLUT1 level explains the reduced basal glucose uptake. Consistently, using a similar setup, we (52) found that basal glucose uptake was reduced in day 8 diabetic myotubes. Age differences between study groups may explain the reduced glucose uptake in these studies (114). Basal glucose uptake was not different between diabetic and matched controls in day 7 myotubes, as shown by McIntyre et al. (88). Utilizing day 8 cultures, we showed that basal glucose uptake is increased in diabetic myotubes compared to age-matched lean myotubes (73;92); however, GLUT1 expression did not differ between the diabetic and control myotubes (84;148;149).

The taken-up glucose is phosphorylated to glucose-6-phosphate (G6P) by hexokinase (HX) and can be metabolized through glycolysis to pyruvate, enter the PPS, or be converted to glycogen, the primary storage form of glucose in skeletal muscles. The rate-limiting enzyme is GS. We found that the level of free intracellular glucose in day 8 diabetic myotubes was significantly lower than that in control myotubes, whereas the G6P content indicated that glucose uptake was not limited by glucose accumulation (52). HX expression in myotubes was not different between groups (130). Nikoulina et al. (48) reported that basal glycogen synthesis tended to be lower in day 4 cultures of diabetic myotubes compared to controls. GS mRNA and protein expression as well as the fractional velocity, $FV_{0.1}$, were also significantly reduced in diabetic myotubes, while the basal $KM_{0.1}$ was increased (48). There were no significant differences in total GS activity between cultures. McIntyre et al. (88) showed in day 7 cultures that basal glycogen synthesis was similar in control and diabetic myotubes, whereas GS protein expression was significantly reduced in diabetic myotubes. Basal glycogen synthesis in day 8 diabetic and control cultures did not significantly differ between age-matched lean, obese and T2D (69;92). The $FV_{0.1}$ and $A_{0.5}$ under basal conditions did not differ between day 8 diabetic and younger control myotubes (52). We determined the G6P-independent GS activity ratio (68), which measures the phosphorylation state of GS. Basal GS ratios were close to zero, indicating full phosphorylation of GS under basal conditions in both control and diabetic cultures (68). Our finding of comparable intracellular G6P concentrations in diabetic and control cultures (52) suggests that glycogen synthesis in diabetic and control myotubes are allosterically activated to the same extent.

The glycolytic pathway converts glucose to pyruvate, which is further reduced to lactate by lactate dehydrogenase (LDH) under aerobic conditions. Pyruvate is decarboxylated to acetyl-coenzyme A (acetyl-CoA) in the mitochondria by pyruvate dehydrogenase (PDH) and further oxidized into CO$_2$ through the tricarboxylic acid (TCA) cycle. LDH expression in myotubes did not differ between
lean, obese and T2D (70); however, lactate production was increased in day 8 diabetic myotubes compared to lean myotubes, indicating greater dependency on anaerobic glycolysis in diabetic myotubes (92).

The increased basal glucose oxidation in day 8 diabetic myotubes (92;94) can be explained by an increase in incomplete glucose oxidation due to pyruvate conversion to citrate and cataplerotic loss (95). The TCA system can only operate as long as the cataplerosis is compensated by a similar degree of anaplerosis to replenish the TCA pool; otherwise, the TCA flux will decrease. Anaplerosis is achieved through the carboxylation of pyruvate to oxaloacetate (OA); this reaction is catalysed by pyruvate decarboxylase (PC). The presence of PC in skeletal muscle has been a subject of debate, but we demonstrated that PC is expressed in myotubes as well as in skeletal muscle (150). Moreover, we measured basal PC activity and could not find differences between myotubes established from T2D subjects and controls (70). Thus, basal glucose metabolism in diabetic myotubes seems comparable with that in controls, with the exception of increased glucose uptake, increased incomplete glucose oxidation and increased lactate production, suggesting that diabetic myotubes may exhibit increased glycolysis.

Extracellular FFAs cross the plasma membrane either by diffusion or through various transporters, such as CD36/FAT, FATP-1 and FABPpm (151). Intracellular FFAs are activated by acyl-CoA synthases to long-chain acyl-CoA and can be further esterified with glycerol to TAGs, the primary storage form of FFAs. They can also be incorporated into other complex lipids (phospholipids, cholesterol esters) or oxidized in the mitochondria. Long-chain fatty acyl-CoA derivatives cannot be transported into the matrix directly but must first be converted to acylcarnitine by carnitine acyltransferases. Complete PA oxidation tended to occur (92;94;152) and was significantly reduced (67;71) in day 8 diabetic myotubes pre-cultured under normo-physiological conditions compared to controls. This reduction was not followed by increased release of acid-soluble metabolites (67;92;153). Kiitzman et al. (154) reported that beta-oxidation did not differ between lean control and obese T2D myotubes. Two cardinal principles seems to govern the reduced lipid oxidation in obese and diabetic myotubes; first, impaired coupling between endogenous lipids and mitochondria in obese and obese diabetic myotubes and second, a mismatch between beta-oxidation and the TCA cycle in obese diabetic myotubes (71). Moreover, we previously reported that neither lipid uptake into myotubes (67;73) nor the mRNA expression of CD36 and CPT1 (149) differed between diabetic and control myotubes, suggesting that the reduced lipid oxidation in diabetic myotubes could not be explained by impaired FFA availability to the mitochondria. Consistently, the level and distribution of acyl-CoA and TAG accumulation on day 8 did not significantly differ between myotubes established from lean, obese and T2D subjects (73;74).
Wilmsen et al. (90) found no differences in total palmitate uptake between day 4 cultures of myotubes established from obese individuals with and without T2D under basal conditions. Corpeleijn et al. (155) found that lipid accumulation measured as cell-associated radioactivity and the number of lipid droplets (LDs) per nucleus did not significantly differ between day 7 myotubes established from lean controls and obese individuals with T2D. The average LD size and density did not differ between groups (155). Finally, under basal conditions, neither the total amount of long-chain acyl-CoAs nor the amount of individual LCA-CoA species is increased in myotubes established from T2D subjects (74). Recently, we determined basal carnitine and acylcarnitine levels in myotubes established from lean, obese and T2D subjects and could not find differences in acylcarnitine levels between the groups. This observation was true for most acylcarnitines, excluding c16 and c18:1, the levels of which seemed to be decreased compared to those in lean myotubes (156). Thus, basal lipid metabolism in diabetic myotubes seems comparable with that in controls except for reduced complete lipid oxidation, which accounts for only a small part of the total lipid turnover. The primary reduced lipid oxidation is an important defect, as it can lead to an increase in levels of circulating FFAs and their derivatives, diacylglycerol, acyl-CoA, and ceramide, which could activate PKC and lead to serine phosphorylation of IRS1 and consequently impaired insulin signalling (29-32).

Mitochondria are the “powerhouse” of cells, generating energy through the oxidation of various carbon substrates, such as glucose and lipids, though the TCA cycle, which creates a transmembrane potential that powers the phosphorylation of ADP to ATP. Mitochondrial mass and content (mitochondrial copy number) were not significantly lower in day 5 myotubes compared to control myotubes (157). Corpeleijn et al. (155) reported no significant differences in mitochondrial mass between lean- and T2D-established myotubes (day 7). Kitzmann et al. (154) compared mitochondrial content and network characteristics between lean and obese T2D myotubes (day 8) and could not find significant differences in CS, HAD, NRF1, or CPT1 mRNA expression, in CS activity, or in the expression of the non-glycosylated protein component of mitochondria (Ab2). A comparable spaghetti-like network was also revealed though fluorescence staining for Ab2. To investigate the possibility of reduced mitochondrial content in day 8 diabetic myotubes, we measured mitochondrial number based on three different parameters: (1) mitochondrial yield, (2) CCCP uncoupled respiration, and (3) NADH respiration of the electron transport chain in permeabilised mitochondria. We could not identify differences between myotubes established from lean, obese and T2D subjects (130). Moreover, we found no significant differences in mitochondrial mass in myotubes established from lean, obese and obese T2D subjects (day 8) (148;152;152;158). TCA cycle flux is reduced in diabetic myotubes (70;159), and this reduction cannot be attributed to a single TCA defect, as shown by us (159). In relation to this, we previously
showed that day 8 diabetic myotubes exhibited a 14% reduction in basal CS activity compared to lean myotubes, indicating mild TCA cycle impairment (130). TCA cycle intermediate replenishment improved the TCA flux but did not improve lipid oxidation in myotubes established from T2D subjects, raising the question of whether passive replenishment of TCA intermediates can improve substrate oxidation (160). In contrast, respiratory uncoupling increased substrate oxidation twofold and reduced incomplete lipid oxidation, suggesting that stimulation of energy consumption, i.e., through physical activity, is the most profitable way to improve substrate oxidation (70; 92). ATP and OXPHOS content was not significantly lower in day 5 diabetic myotubes compared to control myotubes (157). We found that both ATP (111; 158) and ROS (161) production are reduced in mitochondria isolated from diabetic myotubes compared to controls, while the ROS/ATP ratio was comparable between groups (161), indicating that diabetic mitochondria produce less energy with the same mitochondrial mass compared to controls. Based on these observations, ROS production does not seem to be part of the pathogenesis of primary insulin resistance. Neither cellular AMP, ADP, and ATP levels nor the energy charge differ between myotubes established from lean, obese and T2D subjects (152). Hjelle et al. (157) showed that day 5 diabetic myotubes express a reduced mitochondrial oxygen consumption rate (OCR) compared to control myotubes established from younger lean subjects. The above findings related to diabetic myotube energy metabolism can be summarized as follows: diabetic myotubes primarily express impaired oxidative phosphorylation with concomitant enhanced glycolysis.

The insulin signalling cascade under basal conditions has been studied in human myotubes, but insulin receptor protein expression and activity have not been compared between diabetic and control myotubes. Insulin receptor mRNA levels did not differ between diabetic and control myotubes (162). The protein expression of IRS-1, PI3K subunit p85 (regulatory) and PI3K subunit p110 (catalytic) did not differ between lean, obese and diabetic myotubes (86). GSK3α and GSK3β activity and protein expression did not differ between day 8 diabetic and control myotubes (68). Under basal conditions, diabetic myotubes do not seem to express defects related to the insulin signalling cascade.

To identify, quantify and validate changes in protein abundance between day 8 myotubes obtained from lean, obese and T2D subjects, we conducted a quantitative proteomic study (84). Despite a clear diabetic phenotype in the diabetic myotubes, only twelve proteins were differentially expressed between the three different groups; however, these proteins were not known to be related to altered oxidative metabolism nor reduced insulin-mediated glucose metabolism. Myosin-2 (MHCIIa), myosin-3 (developmental) and myosin-8 (neonatal) are up-regulated in diabetic myotubes, but this up-regulation is not followed by a concomitant up-regulation of myosin-7 (MHC1)
in lean myotubes (84). Proteins from all the major pathways known to be important in T2D, including the TCA cycle, glucose and lipid oxidation, OXPHOS, the glycolytic pathway, and lipid and glycogen metabolism, were well characterized. None of these enzymes were found to be regulated at the level of protein expression or degradation, suggesting that an explanation for the diabetic phenotype may not be found at the protein expression level. Protein profiling of human myotubes established from obese and matched obese T2D subjects by Al-Khalili et al. (162) identified 47 proteins whose expression differed between the groups; however, the analysed diabetic myotubes expressed no primary insulin resistance. Therefore, this group difference might be explained by other inherited traits. Transcriptional profiling of myotubes established from obese and matched obese T2D subjects with a clear diabetic phenotype done by us (148) revealed no evidence of a primary defect in the main pathways or in single genes. In particular, we found no evidence for differential expression of genes involved in mitochondrial oxidative metabolism. Consistently, we found no difference in mRNA levels of proteins known to mediate the transcriptional control of mitochondrial biogenesis (PGC-1α and NRF1) or in mitochondrial mass between diabetic and control myotubes. These array and proteomic data indicate that there are no detectable intrinsic isolated or coordinated defects in the expression of mRNA and proteins under basal conditions that can explain the impaired mitochondrial oxidative metabolism and increased glucose turnover present in myotubes established from obese T2D subjects.

5.2 Myotubes established from T2D subjects during acute insulin stimulation

Henry et al. (47;66) showed that glucose uptake during acute insulin stimulation on day 4 after the induction of differentiation in diabetic myotubes established from middle-aged subjects was reduced compared to that in myotubes established from controls who were 8 years younger. McIntyre et al. (88) reported that insulin-stimulated glucose uptake was significantly reduced compared to that in controls. Utilizing day 8 cultures, we showed that insulin-mediated glucose uptake (fold change) in diabetic myotubes was significantly reduced compared to that in myotubes established from lean individuals (73;92) but not that in myotubes established from obese controls (69;72;92).

Cozonne et al. (87) described impaired insulin-mediated glycogen synthesis in day 4 myotubes established from T2D subjects compared to lean controls. Nikoulina et al. (48) found that day 4 diabetic myotubes exhibited significantly impaired insulin-stimulated glycogen synthesis compared to matched controls. The fractional velocity $F_{V0,1}$ was significantly lower in diabetic myotubes compared to controls but was insulin sensitive (46). The reduced GS activity in diabetic myotubes
during acute insulin stimulation can be explained by the fact that insulin stimulation is not followed by increased sensitivity to its substrate and its allosteric regulator G6P, as seen in control myotubes (46;48). In our study (52), acute insulin stimulation increased $F_{v_{1.1}}$ in day 8 diabetic and control myotubes, but the most significant increase occurred in control cultures. This observation can be explained by a substantial increase in sensitivity for G6P during acute insulin stimulation in control myotubes but not in diabetic myotubes. McIntyre et al. (88) showed in day 7 cultures that acute insulin stimulation of glycogen synthesis is significantly impaired in diabetic myotubes. Moreover, they also studied the dephosphorylation of GS site 3a+b under acute insulin stimulation and could not describe significant differences in the degree of dephosphorylation between control and diabetic myotubes. We studied insulin-stimulated glycogen synthesis in diabetic and control myotubes (day 8) and found that acute insulin-mediated glycogen synthesis was significantly reduced in diabetic compared to lean control myotubes (69;73;92). We determined the G6P-independent GS activity ratio (68), which reflects the phosphorylation state of GS, during acute insulin stimulation of myotubes established from T2D subjects and controls. Acute stimulation with a physiological insulin concentration significantly increased the GS activity ratio in the control myotubes. In contrast, the ratio did not significantly change in the diabetic myotubes, indicating that despite acute insulin stimulation, GS is still highly phosphorylated in diabetic myotubes (68).

Insulin-stimulated glucose oxidation in diabetic myotubes is impaired compared to that in myotubes established from lean individuals (69;73;92;94). Wilmsen et al. (90) found no differences in total palmitate uptake between day 4 diabetic and control myotubes during acute insulin stimulation. We also showed that insulin-stimulated lipid uptake and storage did not significantly differ between diabetic and control myotubes in day 8 cultures (73;93). Thus, diabetic myotubes exhibit impaired insulin-mediated glucose uptake, oxidation and storage compared to controls. In contrast, insulin-mediated lipid metabolism does not seem to be affected in diabetic myotubes.

Nikoulina et al. (86) showed that insulin-resistant myotubes established from individuals with T2D display impaired insulin stimulation of PI3K based on a decreased association of PI3K with IRS1. In contrast, AKT phosphorylation appeared normal. In day 15 myotubes established from T2D subjects, Bouzakri et al. (64) described defective insulin stimulation of PI3K activity and decreased association of PI3K with IRS1 but unaltered signalling through IRS2. Cozonne et al. (87) described impaired insulin-stimulated IRS1-associated PI3K activity and glycogen synthesis in day 4 myotubes established from T2D subjects compared to controls, while neither the phosphorylation nor protein expression of PDK1 differed. Moreover, insulin-stimulated activation of all AKT isoforms was impaired in diabetic myotubes, with impaired AKT2 Ser(473) phosphorylation and altered AKT1-Thr(308) phosphorylation. McIntyre et al. (88) found that PKB expression and
phosphorylation in response to insulin stimulation were comparable in day 7 diabetic and control cultures. We reported that GSK3α activity is reduced to the same extent in both day 8 control and diabetic cultures during acute insulin stimulation (68). GSK3β activity and protein expression did not differ between control and diabetic cultures (68). AMPK is a sensor of the AMP/ATP ratio in cells (163). AMPK promotes energy production by stimulating catabolic processes, e.g., glucose uptake and glycolysis, and inhibiting anabolic processes, e.g., lipid and glucose storage. The expression of AMPK and ACCβ, the basal activity of AMPKα1 and AMPKα2, Thr172 phosphorylation of AMPK and Ser221 phosphorylation of ACCβ in day 6 myotubes established from lean, obese and obese T2D subjects did not differ (164). Stimulation by 2 mM AICAR significantly stimulated palmitate oxidation, AMPKα2, Thr172 phosphorylation of AMPK and Ser221 phosphorylation of ACCβ to the same extent and level in all 3 groups (164). McIntyre et al. (88) could not find differences in AICAR (2 mM)-stimulated glucose uptake between day 7 diabetic and control myotube cultures. Thus, diabetic myotubes seem to express a primary defect in insulin signalling compared to control myotubes, whereas AMPK stimulation seems to be comparable between diabetic and control myotubes.

Analysis of correlation between the in vivo phenotype of the donor metabolism and the responses noted in the subsequent cultures is seldom seen in the literature. Few examples are present in the literature. Henry et al. (47) described a positive association between the in vivo glucose disposal rate during hyperinsulinemic-euglycemic clamp and insulin-stimulated 2-deoxy-glucose uptake in human myotubes (r = 0.65), and Corpeleijn et al. (155) showed an inverse association between total lipid oxidation in vitro and the fasting whole-body respiratory quotient (r = -0.66). The correlations are at best modest, which can be explained by the fact that the comparison variables are too different. Adult skeletal muscle tissue consists of highly differentiated fibres, the characteristics of which depend on genetic, epigenetic and environmental influences, especially its contractility and innervation. In contrast, myotubes are immature multi-nucleated cells that primarily express genetic and epigenetic traits that are mainly related to cell proliferation and differentiation.

In addition to studies of primary insulin resistance in diabetic myotubes, various aspects of insulin resistance induced by hyperinsulinemia (48;52;68;165), hyperglycaemia (48;66;73;165-168), hyperlipidemia (67;73;93;155) and other agents (92;144;169) have been investigated in human myotubes. Please refer to the relevant literature for further reading, as induced insulin resistance in skeletal muscle/myotubes is beyond the scope of the present review.

5.3 Insulin resistance in vitro and its characteristics
The central question of this review is whether part of the insulin resistance seen in T2D skeletal muscle is primary in nature. Studies by us and by other groups have clearly shown that diabetic myotubes, independent of differentiation day, express a primary reduced insulin-mediated glycogen synthesis \((48;69;86-88;92)\) - the most well described characteristic of muscular insulin resistance in vivo \((10;23;170-172)\). Correspondingly, these cultures also express insulin resistance both in glucose uptake on days 4, 7 and 8 \((47;66;73;88;92)\) and in glucose oxidation on day 8 \((73;92;94)\); these characteristics of insulin resistance are also seen in vivo \((170-172)\). In vitro recapitulation of in vivo characteristics of insulin resistance indicate that these physiological traits are primary in origin and clearly demonstrate that part of the insulin resistance seen in skeletal muscle in vivo is genetically/epigenetically determined.

To better understand the mechanism of primary insulin resistance in diabetic myotubes, various studies have been done. The supplementary research studies of interest for this review utilized myotubes that display primary insulin resistance at the level of glucose uptake and storage in order to link in vitro observations to the main finding of insulin resistance in skeletal muscle in vivo.

Henry et al. \((47;66)\) reported that day 4 diabetic myotubes expressed both a primary impairment of basal and insulin-mediated glucose uptake, which was explained by reduced GLUT1 protein expression in diabetic myotubes. In contrast, GLUT4 expression was comparable between diabetic and control myotubes.

Nicoulina et al. \((45;48)\) showed that day 4 diabetic myotubes expressed impaired insulin-stimulated glycogen synthesis but normal basal glycogen synthesis. GS protein and mRNA was also reduced. The reduced GS activity was explained by increased insulin-stimulated \(K_{m0.1}\) and an increased insulin-stimulated activity constant \(A_{0.5}\) for G6P.

The diabetic myotubes (day 4) described by Nicoulina et al. \((86)\) exhibited impaired stimulation of GS simultaneous with impaired insulin-stimulated IRS1-associated PI3K activity. In contrast, the protein expression of IRS1, p85, p110, and AKT and the phosphorylation of AKT were normal.

Cozonne et al. \((87)\) described impaired insulin-mediated glycogen synthesis in day 4 myotubes established from T2D subjects compared to that in those established from lean controls. In addition, impaired insulin-stimulated IRS1-associated PI3K activity and phosphorylation of AKT isoforms was observed in diabetic myotubes, while neither the phosphorylation nor the protein expression of PDK1 differed between the lean control and diabetic myotubes.

McIntyre et al. \((88)\) reported that diabetic myotubes (day 7) expressed impaired insulin-stimulated glucose uptake and storage. Although GS protein expression was significantly reduced compared
to that in controls, basal glycogen synthesis was normal. PKB protein expression and phosphorylation did not differ compared to controls on days 3 and 7. Normal dephosphorylation at GS site 3 during insulin stimulation was observed.

We (Gaster et al.) (68;70;111;148;152;158;159;161) found that day 8 diabetic myotubes express impaired insulin-stimulated glucose uptake, glycogen synthesis and glucose oxidation; impaired complete lipid oxidation; and reduced mitochondrial oxidative phosphorylation. In addition to the hyperphosphorylation of GS despite normal GSK3 activity and the reduced sensitivity of GS to the allosteric activator G6P under insulin stimulation, the primary abnormalities that occur at the transcriptional and protein level are unknown. Mitochondrial TCA flux, ATP and ROS production, and GS activity were lower in diabetic myotubes compared to control myotubes. Furthermore, mitochondrial mass and the expression of markers for mitochondrial biogenesis (PCG-1α and NRF1) did not differ between diabetic and control myotubes. MHCIIa, myosin-3 (developmental) and myosin-8 (neonatal) were up-regulated in diabetic myotubes, but this effect was not accompanied by up-regulation of MHCII in lean myotubes.

Although primary insulin resistance, as reflected in glucose uptake and storage, seems to be conserved in culture independent of the number of days of differentiation, surprisingly few mechanistic observations have been reported. The question thus is can these alterations explain the present primary insulin resistance in diabetic myotubes? Basal glucose uptake is reduced in day 4 diabetic myotubes (47;66), likely due to reduced GLUT1 expression. The age difference between study groups may explain the reduced glucose uptake observed in this study (114). Al-khalili et al. (101) demonstrated that GLUT4 is the predominant insulin-stimulated glucose transporter in human myotubes, but GLUT4 expression does not differ between diabetic and control myotubes (47). Acute insulin stimulation doubles glucose transport in both diabetic and control myotubes. The reduced insulin-mediated glucose uptake in observed in these studies results from reduced basal glucose uptake in diabetic myotubes. Thus, these diabetic cultures do not seem to be insulin resistant. GS protein expression has been reported to be reduced in day 4 (45;48) and 7 (88) diabetic myotubes, but basal glycogen synthesis did not differ between diabetic and control myotubes, suggesting that GS protein expression is not the limiting factor for glycogen synthesis. Nicoulinna et al. (86) reported that day 4 diabetic myotubes exhibit impaired insulin stimulation of GS concurrently with impaired insulin stimulation of IRS1-associated PI3K activity. In contrast, AKT protein expression and phosphorylation were normal. Paradoxically, AKT is unaffected by upstream alterations in PI3K, indicating that impaired PI3K function cannot be the mechanism responsible for the impaired insulin-mediated GS stimulation. Decreased GS activity in diabetic myotubes is not a direct effect of defective signalling through the classic insulin signalling
pathway that ends with PKB and GSK-3, as suggested by Cozonne et al. (87). In vivo studies have shown that impaired insulin activation of GS seems to be caused by hyperphosphorylation at site 2+2a, whereas the inhibition of GSK3 and the dephosphorylation of GS site 3a+3b seems normal (26); these observations are in line with the above studies of myotubes (68;88). Although the classic insulin signalling cascade has been found to be altered in various studies, these alterations have not been shown to result in primary insulin resistance in diabetic myotubes. Notably, other primary conditions may co-exist with primary insulin resistance and influence the cultures, potentially biasing observations in diabetic myotubes. Based on these considerations, primary insulin resistance may be associated with impaired mitochondrial oxidation and impaired GS hyperphosphorylation. Currently, no studies have compared mitochondrial oxidation or ATP production between day 4 diabetic myotubes with known insulin resistance and control myotubes. Hjelle et al. (157) measured the OCR in day 0 myoblasts (pre-differentiation) and day 4 myotubes originating from diabetics and controls and showed that both the diabetic myoblasts and the diabetic myotubes expressed a reduced OCR compared to the controls. These observations suggest that the primary reduction in mitochondrial oxidative phosphorylation and primary insulin resistance found in diabetic myotubes on days 4 and 8 are related.

In vitro recapitulation of the characteristics of insulin resistance observed in vivo indicates that these physiological traits are primary in origin and clearly demonstrates that part of the insulin resistance seen in skeletal muscle in vivo is genetically/epigenetically determined. Our and other studies clearly show that the in vivo phenotypic changes, i.e., impaired insulin-stimulated glucose uptake, storage and oxidation; reduced complete lipid oxidation; and impaired mitochondrial oxidative phosphorylation, are partially inherited. Currently, the molecular background and pathophysiological processes responsible for the primary diabetic phenotype in diabetic myotubes are unknown.

6. An explanation of the concurrent impairment of insulin-stimulated glucose metabolism, complete lipid oxidation and oxidative phosphorylation in day 8 diabetic myotubes

To elucidate the pathophysiological mechanism of the diabetic phenotype, analysis of single genes, proteins and their function is particularly effective under conditions where alterations are large or have a strong impact on cellular metabolism and the corresponding biological variation is small. In contrast, the diabetic phenotype in human myotubes consists of multiple modest
alterations in various pathways; such alterations may involve changes in the expression or activity of one or more members of the pathways. Currently, the mechanism responsible for the coordinated impairment of both oxidative metabolism and insulin sensitivity in diabetic myotubes as consistent alterations at the transcriptional and/or protein level is unknown (84;148;162).

One strategy to elucidate the mechanism of primary insulin resistance in diabetic myotubes is to use siRNA-mediated knock down of regulatory components of the affected pathways in diabetic myotubes and myotubes established from healthy lean subjects to search for similarities. However, this strategy would be like searching for a needle in a haystack and would not necessarily explain primary insulin resistance, as the procedure itself would induce a major alteration that is not seen in primary insulin resistance.

An alternative analytical approach is to combine relevant differences between controls and diabetics and compare this overall picture of differences with known biological patterns with the goal of generating a hypothesis to explain the differences. Consistently, there is increasing evidence of a link between insulin resistance and impaired mitochondrial OXPHOS in human skeletal muscle in vivo. Several microarray-based studies of skeletal muscle reported coordinated down-regulation of OXPHOS genes in patients with T2D as well as in high-risk individuals and that reduced expression of the transcriptional coactivator (PGC-1α) could play a key role for these transcriptional changes (37;38;173). Moreover, a common polymorphism of the PGC-1α gene (Gly482Ser), which is associated with reduced PGC-1α activity, has been linked to an increased risk of T2D (174;175).

The coordinated impairment of insulin resistance and oxidative phosphorylation in diabetic skeletal muscle both in vivo and in vitro indicate that the same biological processes/mechanisms may be responsible/present. The coordinated impairment further suggests that the alterations are primary in origin, in accordance with our myotube model. Impaired expression of PGC-1α seems to explain the inherited alterations of reduced mitochondrial oxidative phosphorylation and concomitant insulin resistance in diabetic myotubes.

PGC-1α mRNA expression in myotubes from obese and obese T2D subjects did not significantly differ despite the clear diabetic phenotype of the latter (148). Transcriptional profiling of diabetic myotubes and controls provided no evidence of a primary defect in OXPHOS genes (148). However, primary myotubes represent a cell system with a low maturation grade in which growth rate and differentiation are the most important cellular processes present. As a result, the expression of PGC-1α and other transcription factors is low compared to that of other induced genes. Differences, e.g., PGC-1α expression between diabetic and control myotubes, are thus
even smaller and therefore hard to detect. The PGC-1α content in human myotubes established from lean and obese individuals with/without T2D has not yet been compared. As the diabetic phenotype is more distinguishable between lean control and obese diabetic myotubes, comparing PGC-1α expression levels between these two groups may answer the question as to whether PGC-1α expression is decreased in diabetic myotubes.

Given our assumption that lean myotubes express a higher PGC-1α level than diabetic myotubes and that reduced PGC-1α expression may explain the diabetic phenotype in diabetes myotubes, the PGC-1α effect in lean myotubes could be explained as follows, if diabetic myotubes are considered as the controls.: decreased basal glucose uptake; reduced glucose oxidation with unaffected glycogen synthesis; increased complete lipid oxidation; increased mitochondrial ATP and ROS production; increased TCA flux; reduced expression of MHCIIa and of neonatal and developmental myosin; increased insulin sensitivity of glucose uptake, oxidation and storage; and finally, unaffected lipid uptake and storage.

Overexpression of PGC-1α in lean myotubes was studied by Nicolic et al. (176). Myotubes overexpressing PGC-1α exhibited decreased basal and insulin-stimulated glucose uptake; unaffected basal glycogen synthesis; increased lipid oxidation; increased mRNA and protein expression of cytochrome C, COX IV (both respiratory chain), oestrogen-related receptor α (ERRα) and mitochondrial transcription factor A (mtTFA) (both regulating mitochondrial biogenesis); unaffected NRF1 expression; significantly decreased MHCIIa mRNA expression; unaffected MHCI mRNA expression; increased GLUT4 and PDK4 mRNA expression but unaffected GLUT1 mRNA expression; and unaffected insulin-stimulated glycogen synthesis and lipid uptake.

Comparing the effect of increased PGC-1α expression in lean myotubes with the alterations of the diabetic phenotype in diabetic myotubes reveals the similarity of the two observations and strongly suggests that the increased PGC-1α level can explain most of the basal differences between lean and diabetic myotubes, i.e., increased lipid oxidation, reduced glucose uptake, unaffected glycogen synthesis and lipid uptake, and reduced MHCIIa expression. Although PGC-1α overexpression is followed by increased lipid oxidation, the present data do not verify that mitochondrial ATP production is increased, as the increased lipid oxidation could also be explained by increased PDK4 mRNA, which would result in PDH inhibition and switch oxidation towards lipids. Glucose uptake and glycogen synthesis in PGC-1α-overexpressing myotubes do not seem to exhibit increased insulin sensitivity, possibly because the overexpression time may be too short to increase GLUT4 protein expression by increasing GLUT4 mRNA and for increased expression of mitochondrial proteins to increase ATP production through an increase in OXPHOS transcripts. Less differentiated myotubes depend on AMPK and glycolysis as an energy source. Increased

This article is protected by copyright. All rights reserved
mitochondrial ATP production will reduce basal AMPK-dependent glucose uptake. GS site 2 can be phosphorylated by AMPK (177), with constitutive phosphorylation of site 2α. Increased mitochondrial ATP production may reduce phosphorylation of GS site 2+2a and increase GS sensitivity to insulin stimulation. Thus, in human myotubes, increased expression of PGC-1α may increase insulin-mediated glucose and glycogen synthesis by increasing GLUT4 expression and mitochondrial ATP production.

PGC-1α drives the formation of slow-twitch muscle fibres in transgenic animals (178). Compared with that in control subjects, the fraction of slow muscle fibres in patients with T2D and their FDRs (179) has been reported to be significantly lower in some studies (180-183) but not all (184;185). Proteomic profiling by Giebelstein et al. (186) showed increased glycolytic protein expression and reduced mitochondrial protein expression in diabetic individuals without changes in markers of fibre type. Slow fibres are more oxidative and insulin sensitive than fast fibres (184;187-189). Our proteomic-based observation that fast myosin (MHCIIa) expression is up-regulated in diabetic myotubes (84) is consistent with the idea that primary insulin resistance in myotubes may also be associated with loss of slow fibres, although we did not confirm a concomitant increase in slow myosin expression in lean myotubes. Currently, it is unknown to what extent fibres co-express developmental and neonatal myosin represent more than a transitional stage in the development of mature muscle fibres (190).

PGC-1α operates through a broad range of transcription factors that are involved in a wide variety of biological responses, including mitochondrial biogenesis, glucose-fatty-acid metabolism, and fibre type switching. Currently, we do not know if the diabetic phenotype in diabetic myotubes can be solely explained by a primary reduction of PGC-1α expression or a combination of the reduction with other transcription factor alterations. No other regulatory protein has the same characteristics as PGC-1α, making PGC-1α the only probable explanatory factor for the whole picture of impaired lipid oxidation, impaired mitochondrial oxidative phosphorylation and insulin resistance in diabetic myotubes at the functional-pathways level.

Thus, based on the above finding, it is hypothesized that the impaired insulin-mediated glucose metabolism and impaired oxidative phosphorylation and reduced complete lipid oxidation in diabetic myotubes is caused by reduced PGC-1α expression. This hypothesis allows a simple explanation of the phenotypic findings observed in diabetic myotubes compared to controls, i.e., the simultaneous impairment of insulin-mediated glucose metabolism and mitochondrial oxidative phosphorylation and complete lipid oxidation, and enables these findings to be linked to the same findings in skeletal muscle in vivo. Further studies are needed to verify this hypothesis. Most importantly, whether PGC-1α expression in diabetic myotubes is reduced compared to that in lean
myotubes must be investigated, possibly using a model system of more mature myotubes. Additional PGC-1α transgene experiments in diabetic myotubes should be conducted to clarify if increasing PGC-1α expression improves insulin resistance, lipid oxidation and oxidative phosphorylation. Moreover, siRNA-mediated PGC-1α knock down in lean myotubes could be used to investigate whether this manipulation induces the diabetic phenotype and to identify the underlying mechanism. Although the myotube model has made it possible to identify the presence of primary insulin resistance, impaired oxidative phosphorylation and reduced lipid oxidation in diabetic myotubes, the observed alterations do not allow further examination of the extent to which the primary findings, at the functional-pathway level, depend on genetic or epigenetic mechanisms due to the lack of information about differences at the molecular biological level. Based on the available data, it is hypothesized that the impaired insulin-mediated glucose metabolism and oxidative phosphorylation in diabetic myotubes is caused by reduced PGC-1α expression. Therefore, we need a model system that requires more energy, specifically energy supplied from oxidative metabolism. Improved maturation of established myotubes may give a clearer picture of differences between diabetic and control myotubes at the molecular level and allow testing of this hypothesis. Such a model would also enable further investigation into the impact of epigenetic and genetic factors in primary insulin resistance.

7. Conclusion and further perspectives

Overall, studies of human myotubes established from lean, obese and T2D subjects clearly show that part of the diabetic phenotype observed in vivo is preserved in diabetic myotubes. Diabetic myotubes express a primary coordinated impairment of lipid oxidation, oxidative phosphorylation and insulin-stimulated glucose metabolism. Currently, both the responsible molecular mechanisms as well as the extent to which these alterations depend on genetic and/or epigenetic alterations have yet to be identified. Based on the data, it is hypothesized that the impaired insulin-mediated glucose metabolism, impaired oxidative phosphorylation and reduced lipid oxidation observed in diabetic myotubes are caused by reduced PGC-1α expression. The immaturity of the model system, small differences between diabetic and control myotubes, and small sample sizes all reduce the ability to detect significant differences. New culture protocols are required that enable the achievement of differentiation states that are closer to in vivo skeletal muscle, especially specific muscle fibre types. Gou et al. (191) described an improved culturing technique that allows further maturation of myotubes through the utilization of a second differentiation media that
contains various trophic factors later in the differentiation process. Currently, neither the fibre type
distribution nor a characterization of the intermediary metabolism in myotubes established from
diabetic or control subjects in accordance with this protocol has been published. Ultimately,
innervation is the key to making a myotube more like a muscle fibre, such as through co-culturing
with rat spinal cord. In addition to the huge task of establishing the number of cultures necessary
for metabolic studies, and in addition to day-to-day variation, differences between same-day
cultures are to be expected due to the high dependence of differentiation on innervation. Highly
differentiated myotubes would allow further investigation into the mechanisms underlying primary
insulin resistance, primary reduced lipid oxidation and primary reduced oxidative phosphorylation
in myotubes established from subjects with T2D and would facilitate research into whether the
responsible mechanism is purely genetic and/or epigenetic based on a clearer expression of
differences at the molecular level. In particular, highly differentiated cultures would enable testing
of whether reduced PGC-1α expression is at least partially responsible for primary insulin
resistance, impaired lipid oxidation and impaired oxidative phosphorylation in myotubes.
Identification of the primary molecular mechanisms will allow to manipulate the mechanism and its
regulation and possibly lead to the development of new treatments for muscular insulin resistance,
T2D and its serious complications.

7. Acknowledgment

The Danish Medical Research Council, the Novo Nordisk Foundation, the Danish Diabetes
Association, Lægeforeningens Forskningsfond (Mimi and Victor Larsens Fund), Aage and Johanne
Louis-Hansens Foundation, Schested-Hansens Foundation, Eva and Hans A. Holm mindelegat,
Niels and Desire Ydes Foundation, Clinical research Institute and the Free Research Funds of
Odense University Hospital are thanked for financial support.

8. References