

Are cultured human myotubes far from home?

Vigdis Aas¹, Siril Skaret Bakke², Yuan Z. Feng², Eili Tranheim Kase², Jørgen Jensen³, Sudip Bajpeyi⁵, G. Hege Thoresen^{2,4}, and Arild C. Rustan²

¹Institute of Pharmacy and Biomedical Laboratory Science, Faculty of Health Sciences, Oslo and Akershus University College of Applied Sciences, Oslo, Norway

²Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, Oslo, Norway

³Norwegian School of Sport Sciences, Oslo, Norway

⁴Department of Pharmacology, Institute of Clinical Medicine, Faculty of Medicine, University of Oslo and Oslo University Hospital, Oslo, Norway.

⁵University of Texas at El Paso, El Paso, TX, USA.

Introduction

Satellite cells can be isolated from skeletal muscle biopsies, activated to proliferating myoblast and differentiated into multinuclear myotubes in culture. These cell cultures represent an essential model system to intact human skeletal muscle, which can be modulated *ex vivo*. Advantages of this system include; having the most relevant genetic background to study human disease (as opposed to rodent cell cultures), the extracellular environment can be precisely controlled and the cells are not immortalized, thereby offering the possibility of studying innate characteristics of the donor. This review will focus on how human myotubes can be used as a tool to study metabolism in skeletal muscles, with a special attention to changes in muscle energy metabolism in obesity and type 2 diabetes. Limitations of this cell system and possible approaches to improve the current model will also be discussed.

Characteristics of human myotubes *in vitro*

Satellite cells were discovered in 1961 by electron microscopy (Mauro, 1961). The cells can be isolated from muscle samples by two principally different mechanisms: by enzymatic digestion (Yasin, et al., 1977) or by cell migration (Bonavaud, et al., 2002, Rosenblatt, et al., 1995). In adult skeletal muscle, satellite cells are in a quiescent state, but are activated in response to muscle growth or injury (Blau and Webster, 1981). Proliferation and differentiation of human muscle satellite cells can occur *in vitro*, where they form multinucleated myotubes (Blau and Webster, 1981). Over the past 30 years, these cells have been extensively used to study glucose and lipid metabolism. The myotubes display morphological, metabolic and biochemical similarities to adult skeletal muscle (Gaster, et al., 2001, Henry, et al., 1995, Thompson, et al., 1996), and thus offer a unique and powerful model to distinguish between genetic and environmental factors for instance in the etiology of insulin resistance. Examples of protocols for maintaining muscle cells in culture are given by Gaster et al. (Gaster, et al., 2001) and Henry et al. (Henry, et al., 1995).

Activated proliferating satellite cells, also denoted as myoblasts, express myogenic markers including MyoD (Hawke and Garry, 2001). After further proliferation, myoblasts either fuse with existing muscle fibers or fuse with other myoblasts and form multinucleated myotubes (Schultz and McCormick, 1994, Zammit, et al., 2006). The expression of key proteins for both glucose (Al-Khalili, et al., 2003) and lipid metabolism (Muoio, et al., 2002) increase during differentiation of myoblasts into myotubes. Compared to myoblasts, the protein expression pattern of myotubes exhibits a better resemblance to adult skeletal muscle, and myotubes are therefore preferred for experimental use (Berggren, et al., 2007).

Primary human myotubes are generally characterized by low mitochondrial oxidative capacity, and their fuel preference is for carbohydrates over lipids (Aas, et al., 2011). This may be due to predominant production of ATP by aerobic glycolysis rather than mitochondrial oxidative phosphorylation (OXPHOS) (Warburg effect) (Benard, et al., 2010), and is likely the result from a lack of proliferation of mitochondria and OXPHOS-activation in the absence of appropriate environmental signals *in vitro*. The cells are normally grown in energy rich media with excess glucose concentrations, and in addition the energy demand is low.

In addition to satellite cells, other progenitor cells also have a myogenic potential, such as pericyte-derived cells that can differentiate into myotubes *in vitro* (Dellavalle, et al., 2007). These myotube cultures differ from satellite cell cultures, and less is known about the metabolic characteristics of pericyte-derived myotubes.

Glucose and lipid metabolism

In addition to the glucose transporters GLUT1 and GLUT4, other hexose transporters as GLUT3, GLUT5 and GLUT12 are present in skeletal muscles (Stuart, et al., 2006). GLUT1 is thought to be involved mainly in basal glucose transport in both rodent (Hansen, et al., 1998) and human (Ciaraldi, et al., 2005) skeletal muscle. In response to insulin stimulation, GLUT4, but not GLUT1, is translocated to the cell surface of the skeletal muscle cells, resulting in an increase in glucose uptake (Ploug and Ralston, 1998). However, the ratio of GLUT1:GLUT4 is higher in human myotubes compared to adult skeletal muscle (Sarabia, et al., 1992), resulting in a lower insulin responsiveness of glucose transport (Al-Khalili, et al., 2003, Sarabia, et al., 1992). Insulin typically increases glucose uptake by 40-50 % in myotubes (Aas, et al., 2002, Al-Khalili, et al., 2003, McIntyre, et al., 2004, Montell, et al., 2001). Even though the effect of insulin is lower in myotubes than in intact skeletal muscle, the responsible molecular mechanisms of glucose transport remain the same (Al-Khalili, et al., 2003). Due to a decreased insulin responsiveness of human myotubes, insulin concentrations in the range of 0.1 – 1 μ M are often applied in studies of human myotubes (Aas, et al., 2004, Aguer, et al., 2011, Al-Khalili, et al., 2003, Bell, et al., 2010, Gaster, et al., 2004, Jackson, et al., 2000, Rune, et al., 2009a). This insulin concentration is markedly above the physiological concentration in the picomolar range. Insulin stimulation increases glycogen synthesis about 2-fold in human myotubes, which is similar to *in vivo* observations (Aas, et al., 2004, Al-Khalili, et al., 2003, Al-Khalili, et al., 2005). In intact muscle, glycogen content is linked to glucose uptake, and reduced glycogen content increases glucose uptake (Jensen, et al., 1997). Importantly, low glycogen content also increases insulin-stimulated glucose uptake (Jensen, et al., 1997), and reduced glycogen content after exercise is a major reason for the improved insulin sensitivity observed after exercise (Jensen, et al., 2011). In intact muscles, glycogen content also regulates glucose metabolism, and muscles with high

glycogen content have reduced glycogen synthesis during insulin stimulation and channel more glucose into glycolysis (Jensen, et al., 2006). In myotubes, we did not observe increased glycogen content after incubation with high glucose although basal and insulin-stimulated glucose uptake decreased (Aas, et al., 2004). Although, the relationship between glycogen and glucose uptake has not yet been clearly established, cultured human muscle satellite cells may provide a suitable model for the study of glucose metabolism, both in the basal and insulin-stimulated state.

The capacity for long-chain fatty acid oxidation has been compared between homogenates of donor muscle biopsies and the derived skeletal muscle cell cultures (Jacobs, et al., 1987, Zuurveld, et al., 1985). The palmitate oxidation rate (Jacobs, et al., 1987, Zuurveld, et al., 1985) and the activity of citrate synthase (Jacobs, et al., 1987) were comparable between human myotubes and that measured in homogenates of the donor muscle (*m. quadriceps*). Based on their findings they concluded that cultured human muscle cells represent a suitable model for studies on mitochondrial oxidative metabolism of muscle.

Muscle contraction

Mature myotubes are quiescent and do not typically contract spontaneously. Muscle contraction stimulates translocation of GLUT4 and increases glucose uptake (Rose, et al., 2009). The mechanism for contraction-stimulated glucose uptake in myotube is not clear, but contraction activates AMP-activated protein kinase (AMPK), and AMPK activation stimulates glucose uptake. However, studies with AMPK knockdown mice showed that contraction could stimulate glucose uptake even in muscles where AMPK was kinase-dead (Maarbjerg, et al., 2009). Ca^{2+} /calmodulin dependent kinase also seems to be involved in contraction-stimulated glucose uptake, and a link between muscle glycogen content and contraction-stimulated glucose uptake has been suggested (Aslesen, et al., 2001). Recently, it has been shown that human myotubes respond to “exercise”, induced by electrical pulse stimulation, in a similar way as to exercising muscle *in vivo* (Lambernd, et al., 2012, Nikolic, et al., 2012a), and this model may be very important to clarify the mechanisms for contraction-stimulated glucose uptake. Chronic low-frequency stimulation (CLF) increases

glucose uptake and oxidation, oleic acid oxidation, mitochondrial content, AMPK activation, and interleukin-6 (IL-6) production (Lambernd, et al., 2012, Nikolic, et al., 2012a). In fact, insulin resistance induced by adipocyte-conditioned media, chemerin and monocyte chemoattractant protein-1 (MCP-1) was abrogated by electrical pulse stimulation (Lambernd, et al., 2012). All together, these observations imply that glucose metabolism, insulin signaling and responses to exercise are very similar in human myotubes as in skeletal muscle *in vivo*.

Fiber type comparison

Human muscle fibers express three isoforms of myosin heavy chain (MyHC): MyHC- β , MyHC-2A, and MyHC-2X (respective genes MYH7, MYH2, and MYH1) (Schiaffino and Reggiani, 2011). The fourth isoform MHC-2B (MYH4) is not detected in humans. The expression of the MyHC isoforms is associated with muscle fiber phenotype. The muscle fibers that express MyHC- β are characterized as slow, fatigue resistant and oxidative (type 1 muscle fibers), whereas the fibers that express MyHC-2A are fast oxidative (type 2A muscle fibers), and the MyHC-2X fibers are fast glycolytic (type 2X muscle fibers). Most human muscles consist of all three muscle fiber types in varying degrees, depending on the functional demands of the muscle (Harridge, et al., 1996), age and gender (Schiaffino and Reggiani, 2011). Studies performed in rodents have demonstrated that MyHC isoforms expressed in differentiated myotubes were identical to the MyHC expression pattern in the muscle fibers from which the satellite cells were isolated (Dusterhoft and Pette, 1993, Rosenblatt, et al., 1996). In human muscle cell cultures it has been found that all myotubes in 8-day differentiated cell cultures were immunoreactive for fast MyHC, regardless of donor muscles having mixed MyHC expression *in vivo* (Gaster, et al., 2001). In our hands, myotube cultures stained at day 8 after differentiation contained a noteworthy amount of slow muscle fibers as well (Fig. 1), and the cell content of MyHC- β proteins increased after chronic electrical pulse stimulation of the cells, showing plasticity potential (Nikolic, et al., 2012a). When satellite cells were isolated from pools of single human skeletal muscle fibers exclusively expressing either fast or slow MyHC isoform the isolated cells differentiated into myotubes that co-expressed both fast and slow MyHC isoforms (Bonavaud, et al., 2001). In addition to the fast and slow MyHC

isoforms present in adult skeletal muscle *in vivo*, several developmental MyHC isoforms are expressed in myotubes (Bonavaud, et al., 2001).

Despite the fact that myotubes can be of different types, it has been a question of heated debate whether muscle satellite cells are of multiple origin or not, and conflicting results have been reported in the literature depending on the markers used to identify cell type differences, the species used to obtain cells and the culturing conditions (for review (Schultz and McCormick, 1994)). Satellite cells may be heterogenous, and it is not known if there are distinct populations of fast twitch (type 2) and slow twitch (type 1) cells. However, when grown in culture, human satellite cells mature to myotubes that express MYH1, MYH2, and MYH7 (Nikolic, et al., 2012a). In accordance with our observations in human myotubes, murine satellite cells isolated from different muscles were uniform regardless of muscle origin, and the dominant muscle fiber type both in primary murine myoblast and in the murine muscle cell lines was the intermediate MyHC-2A (LaFramboise, et al., 2003). It has been suggested that MyHC-2X is the default form of the protein in human skeletal muscle (Harridge, 2007). An important additional observation is that the expression pattern of MyHC seems to be the same in myotubes from male and female donors (Bonavaud, et al., 2001). Taken together these findings demonstrate that myotubes differ from donor muscle with respect to MyHC expression.

Retention of metabolic characteristics of the muscle cell donor

Even though cultured human skeletal muscle cells differ from their donor in MyHC expression, some metabolic properties of the donor muscle were maintained in the derived cell cultures. Several studies have shown that the diabetic phenotype is conserved in myotubes established from type 2 diabetic (T2D) subjects (Gaster, et al., 2002, Henry, et al., 1996, Thompson, et al., 1996), and several potential intrinsic deficiencies in myotubes from individuals with T2D have been reported (Gaster, et al., 2002, Gaster, et al., 2004, Henry, et al., 1995, Thompson, et al., 1996). For instance, it has been observed primary defects in glucose metabolism such as decreased glucose transport and glycogen synthase activity in myotubes from diabetic individuals (Gaster, et al., 2002, Henry, et al., 1995, Henry, et al.,

1996, McIntyre, et al., 2004), decreased insulin-stimulated citrate synthase activities (Ortenblad, et al., 2005) and impaired glucose oxidation (Wensaas, et al., 2009). With respect to lipid metabolism, decreases in both complete palmitate oxidation and fatty acid beta-oxidation have been observed in myotubes from individuals with T2D when compared to control cells from obese individuals who were normoglycemic (Cha, et al., 2005, Gaster, et al., 2004, Wensaas, et al., 2009). It has also been demonstrated that there is an increased formation of fatty acid beta-oxidation products in diabetic cells when chronically exposed to fatty acids (Wensaas, et al., 2009), and a reduced tricarboxylic acid (TCA) cycle flux when compared to myotubes established from obese subjects (Gaster, 2009). Additionally, myotubes from individuals with T2D show increased lipogenesis and lipid accumulation when chronically treated with a liver X receptor (LXR) agonist (Kase, et al., 2005) or in the presence of the polyunsaturated fatty acid eicosapentaenoic acid (Wensaas, et al., 2009) when compared to lean and/or obese control cells. It should be noted that the metabolic differences between myotubes from lean donors and myotubes from obese T2D donors are very striking, but metabolic differences in myotubes from obese donors and myotubes from obese T2D donors are not always as obvious (Gaster, 2007a).

In myotubes established from young healthy subjects, the capacity to increase lipid oxidation in response to increased fatty acid availability *in vitro* (adaptability) was positively related to insulin sensitivity *in vivo* (Ukropcova, et al., 2005). In a recent study, we observed that intracellular lipid oxidation and adaptability were reduced in myotubes established from T2D individuals (Corpeleijn, et al., 2010). The variation in intracellular lipid oxidation *in vitro* was significantly related to the *in vivo* fasting respiratory quotient of the subjects (Corpeleijn, et al., 2010). This suggests that skeletal muscle fatty acid handling is intrinsically impaired in T2D individuals (Gaster, et al., 2004, Wensaas, et al., 2009).

Metabolic switching, representing dynamic changes in fatty acid oxidation in skeletal muscle cells, is also correlated with clinical phenotypes of cell donors. Myotubes retain the characteristics of the donor, which indicates that metabolic switching is an intrinsic characteristic of skeletal muscle (Gaster, 2007b, Ukropcova, et al., 2005). This suggests that defects in metabolic switching might be one of the primary events in the development of obesity and insulin resistance. We have also found that lipid content in myotubes was

strongly associated with *in vivo* maximal oxidative capacity, insulin sensitivity, physical activity level and the proportion of type 1 muscle fibers (Bajpeyi, S., unpublished data).

Insulin-stimulated activation of Akt (PKB) is impaired in skeletal muscle of T2D individuals *in vivo* (Krook, et al., 1998). This defect seems to be maintained in cultures as well (Cozzone, et al., 2008). However, insulin-stimulated phosphorylation of Akt is not reduced in muscles from 72 h fasted subjects although severe insulin resistance occurs (Vendelbo, et al., 2012). Other components in the insulin-signaling cascade, such as activation of Akt substrate of 160 kDa (AS160), are also impaired in muscles from type 2 diabetic subjects (Karlsson, et al., 2005). Interestingly, AS160 phosphorylation was also reduced after 72 h fasting, and AS160 may be a better intracellular signaling molecule reflecting insulin-stimulated glucose uptake (Vendelbo, et al., 2012). After inducing insulin resistance by palmitic acid in myotubes, phosphorylation of AS160, as well as other signaling proteins, was correspondingly reduced (Bikman, et al., 2010). These are only a few examples of reported signaling defects in myotubes from obese and insulin resistant subject showing that results from myotubes reflect the *in vivo* situation.

Gender is definitely a factor affecting energy metabolism *in vivo*, however, this difference does not seem to be maintained in myotube cultures (Rune, et al., 2009b, Salehzadeh, et al., 2011). Both glucose and palmitic acid metabolism were similar in myotubes from male and female donors. Gene (mRNA) expression of several genes involved in glucose and lipid metabolism was observed to be higher in skeletal muscle biopsies from female vs male donors, but the expression was similar in cultured myotubes from the same donors (Rune, et al., 2009b, Salehzadeh, et al., 2011).

These observations suggest that the characteristics of the *in vivo* phenotype are to a certain extent carried over to the myotubes *in vitro*. Exactly how myotubes are able to retain the *in vivo* characteristics is not known, however a combination of genetic and epigenetic mechanisms are probably involved. Epigenetic regulation of skeletal muscle stem cells and skeletal muscle differentiation are well known (see e.g. (Bharathy, et al., 2012, Sousa-Victor, et al., 2011)). Exercise (Barres, et al., 2012, Nitert, et al., 2012), diet (Jacobsen, et al., 2012) and family history of type 2 diabetes (Nitert, et al., 2012) are all described to influence DNA methylation in human skeletal muscle, traits that may follow the isolated satellite cells into

cultured myotubes. Thus, acute exercise was found to induce hypomethylation of promoters of several metabolic genes (Barres, et al., 2012), and a 6-month exercise intervention induced decreased methylation of genes involved in e.g. retinol metabolism, calcium-signaling pathway, starch and sucrose metabolism, and the insulin-signaling pathway (Nitert, et al., 2012). Short-term (5 days) high-fat overfeeding introduced widespread DNA-methylation changes, changes that were only partly reversed after 6-8 weeks (Jacobsen, et al., 2012).

Mutations are of course carried over from the *in vivo* to the *in vitro* situation. One example is the naturally occurring R225W mutation in the skeletal muscle-specific γ_3 subunit of the cellular energy sensor AMPK. This mutation, identified in humans in 2007 (Costford, et al., 2007), was shown to result in increased muscle glycogen and decreased intramuscular triacylglycerol in R225W carriers and was linked to increased AMPK activity in R225W cultured myotubes. Further studies have shown that the fuel storage phenotype is maintained in the R225W myotubes, and that R225W myotubes also have increased mitochondrial content, oxidative capacity and glucose uptake; the latter of which is consistent with the fact that R225W carriers show a trend for increased skeletal muscle glucose uptake *in vivo*, as measured by [¹⁸F]-fluorodeoxyglucose positron emission tomography (Crawford, et al., 2010). The maintenance of the R225W carrier phenotype in R225W myotubes is another example of the donor-myotube similarities, which make primary human myotube culture systems an extremely useful tool for metabolic research.

Limitations of the myotube model

Cultured myotubes lack of course the *in vivo* microenvironment and the communication with other cells through direct contact and bioactive substances. Indeed, co-cultures with adipocytes may overcome some of these limitations (Trayhurn, et al., 2011). It is also important to take notice of that glucose uptake *in vivo* is regulated by delivery, transport and metabolism (Rose and Richter, 2005), whereas cultured myotubes only allow studies of transport and metabolism. In these studies, another limitation to the myotube model is that compared to *in vivo*, the insulin-stimulated glucose uptake in primary human myotubes is

relatively low (about 1.5-fold increased) (Aas, et al., 2004, Al-Khalili, et al., 2003, McIntyre, et al., 2004, Montell, et al., 2001), possibly caused by a low expression of the insulin-responsive glucose transporter GLUT4 (Al-Khalili, et al., 2003, Sarabia, et al., 1992). *In vivo*, GLUT4 is more expressed in type 1 oxidative muscle fibers than type 2 fibers (Daugaard, et al., 2000, Gaster, et al., 2000, Stuart, et al., 2006), and insulin sensitivity correlates with the proportion of slow twitch oxidative fibers in the muscle (Lillioja, et al., 1987). Insulin-stimulated glucose uptake is generally greater in slow twitch-enriched muscles than fast-twitch muscles (Zierath and Hawley, 2004). For example, white gastrocnemius in rat containing mostly type 2 fibers shows a 7-fold response to insulin, whereas glucose uptake increased 11-fold in red gastrocnemius (James, et al., 1985). Therefore ways to change the phenotype to a more oxidative one would improve the insulin sensitivity. It is also important to note that denervation of skeletal muscles reduces GLUT4 expression and causes insulin resistance (Jensen, et al., 2009). Indeed, innervation of human myotubes is possible *in vitro* by co-culturing with rat motor neurons, and such innervation increases GLUT4 expression (Chowdhury, et al., 2005). However, more convenient methodologies are required.

There are also clear differences in gene expression between muscle biopsies and myotubes. A direct comparison of mRNA expression between skeletal muscle biopsy and cultures from human myotube show that most genes we studied were expressed higher in biopsies than in cells (Fig. 2). The only exception was the lipid droplet protein PLIN3. This could imply less active cells, possibly not completely matured to muscle fibers or simply an indication of more quiescent cells. Indeed, muscles strips from human adult differentiated muscles can be used to study glucose uptake and GLUT4 translocation *in vitro* (Lund, et al., 1997). This methodology has provided important information, but the muscle strips also have their limitations. It is not possible to manipulate gene transcription and the strips have limited viability.

Adult stem cells are known to have a finite replication potential. Human myotubes from myoblast cultures undergoing senescence exhibit defects in glucose and lipid metabolism (Nehlin, et al., 2011). In that study muscle biopsy-derived human satellite cells were grown at different passages and differentiated to human myotubes in culture to analyze the functional state of various carbohydrate and lipid metabolic pathways. A number of cellular

functions were altered as the proliferative potential of myoblasts decreased with increasing passage number. The capacity of myoblasts to fuse and differentiate into myotubes was reduced, and metabolic processes in myotubes such as glucose uptake, glycogen synthesis, glucose oxidation and fatty acid β -oxidation became gradually impaired. Late-passage, non-proliferating myoblasts cultures showed strong senescence-associated β -galactosidase activity, indicating that the observed metabolic defects accompany the induction of a senescent state (Nehlin, et al., 2011).

Strategies to improve the human myotube model

Several parameters of the cell culture model can be improved using different approaches, and we have in this review focused on some ways of improving oxidative capacity (Fig. 3). The muscle phenotype can be changed; it has been long known that muscle contractile activity impacts the muscle phenotype, and that the phenotype can change when the motor nerve activity is altered (Vrbova, 1963). In humans, the muscle fiber type composition varies among individuals depending on age, gender, genetic background, and activity level. The gastrocnemius of sprint athletes, for instance, is dominated by fast twitch type 2 fibers and that of top endurance athletes by slow twitch type 1 fibers (Costill, et al., 1976). The transition from slow to fast twitch muscle fiber occurs after denervation and by disuse of the muscles, such as after spinal cord injury (Andersen, et al., 1996). Increasing age has also been associated with an increased proportion of fast fibers (D'Antona, et al., 2003). However, this is controversial, since loss of type 2 fibers with age has also been shown (Lexell, 1995), as well as changes in biochemical parameters without changes in fiber type composition (He, et al., 2001). Type 2 diabetes is also associated with an increased proportion of fast, glycolytic type 2 fibers (Marin, et al., 1994, Oberbach, et al., 2006). The transition from fast to slow fiber is more difficult to achieve in humans than the other way round. The experimental model of chronic low-frequency stimulation (CLF) in animals has shown such transitions in rabbits and rats (for review see (Pette and Vrbova, 1999)). The plasticity of skeletal muscle in general has been thoroughly reviewed by others (Fluck and Hoppeler, 2003, Gundersen, 2010, Schiaffino and Reggiani, 2011).

Since myotubes in culture seem to be rather glycolytic, strategies to improve oxidative capacity, i.e. induce fiber type switching from fast type 2 to slow type 1 fibers would improve the model. The mechanisms involved in fiber type switching are not known in detail, but it is clear that gene expression must be changed in some way. Prolonged increase in intracellular calcium, activation of the calcineurin pathway and dephosphorylation of the transcription factor nuclear factor of activated T-cells (NFAT) has been shown to initiate the slow fiber type program (Chin, et al., 1998). Slow fiber type genes were also shown to be upregulated by the extracellular signal-regulated kinases (ERK) pathway in rats (Murgia, et al., 2000) and by peroxisome-proliferator-activated receptor-gamma co-activator-1 alpha (PGC-1 α) in mice (Lin, et al., 2002). PGC-1 β , on the other hand, is involved in fast twitch (type 2X) fiber transition (Arany, et al., 2007). In human skeletal muscle *in vivo* there seems to be a correlation between the expression of the peroxisome proliferator-activated receptors (PPAR) α and δ and proportion of type 1 fibers (Kramer, et al., 2006, Russell, et al., 2003). Overexpression of a constitutive active PPAR δ in adult rat muscles induced slow muscle properties (Lunde, et al., 2007). PGC-1 α overexpression in primary human myotubes promoted an increased lipid oxidative capacity, increased the expression of genes involved in regulation of mitochondrial function and biogenesis, and decreased the expression of a fast fiber type gene marker (MyHC-2A) when compared to cells infected with an empty retrovirus-vector (Nikolic, et al., 2012b). We also observed that PGC-1 α overexpression increased expression of several genes which were markedly down-regulated during the process of cell isolation and culturing when compared to skeletal muscle *in vivo*. After treatment of PGC-1 α transfected cells with the selective PPAR δ agonist GW501516, palmitate oxidation was further increased. GW501516 also increased mRNA levels for carnitine palmitoyltransferase-1 (CPT-1). Additionally, a dose-dependent increase in oleate oxidation to CO₂ was observed in myotubes pretreated for three days with this compound during differentiation of the cells (Wensaas, et al., 2007). The PPAR δ agonist was also shown to increase basal and insulin-mediated glucose transport and lead to phosphorylation of AMPK and p38 mitogen-activated protein kinase (MAPK) in cultured human myotubes (Kramer, et al., 2007).

We have recently shown that chronic low-frequency electrical pulse stimulation of human myotubes increases glucose and fatty acid oxidation and mitochondrial content (Nikolic, et

al., 2012a). Protein content of MyHC- β increased, indicating a fiber type switch. Another recent study by Lambernd *et al.* showed that insulin stimulated glucose uptake was increased and insulin resistance could be prevented by chronic electrical pulse stimulation (Lambernd, et al., 2012). In murine C2C12 cells exposed to electrical pulse stimulation (40 V/60 mm, 24 ms, 1 Hz), there was a decrease in type 2 fibers and an increase in type 1 fibers. In addition, ERK1/2, ERK5 and c-Jun N-terminal kinase (JNK) were activated and glucose uptake was improved (Nedachi, et al., 2008). GLUT4 mRNA expression was unaltered, but GLUT4 recycling was increased, both under basal and insulin-stimulated conditions (Nedachi, et al., 2008). In animal studies, elevation in PGC-1 α is associated with fiber-type switch from glycolytic, fast-twitch type 2B/2X to oxidative, slow-twitch type 2A and 1 muscle fibers (Handschin, et al., 2007, Lin, et al., 2002). Expression of PGC-1 α was also increased in primary rat skeletal muscle cells after electric stimulation for 90 min a day for 5 days (Silveira, et al., 2006), as well as in C2C12 cells stimulated for 90 min a day for 4 days or after CLF for 24 h (Burch, et al., 2010). In this latter study, expression of several genes involved in oxidative phosphorylation, glucose and fatty acid metabolism increased concomitantly, although not followed by a clear fiber type switch since both MyHC- β and MyHC-2X increased (Burch, et al., 2010). All these studies indicate that oxidative capacity can be improved by electrical pulse stimulation (exercise mimetic) of the myotubes.

It has also been shown in adult rat muscle that PPAR δ expression is influenced by muscle contractile activity and induces slow muscle fiber properties after somatic gene transfer (Lunde, et al., 2007). Thus, strategies to enhance PPAR δ activity, by either agonist treatment or by gene transfer (not yet shown in human myotubes), may increase the oxidative potential of myotubes and promote fiber type switching. However, also other PPARs may be targets for increased oxidative capacity. Muoio *et al.* demonstrated that PPAR α protein expression was induced during myocyte differentiation (Muoio, et al., 2002), and that its activation stimulated lipid oxidation (Djouadi, et al., 2005, Muoio, et al., 2002) and decreased triacylglycerol accumulation (Muoio, et al., 2002) in primary human myotubes. Treatment with the PPAR γ agonist troglitazone reversed impaired fatty acid metabolism in skeletal muscle cells from individuals with T2D (Cha, et al., 2005), whereas all the thiazolidinediones (troglitazone, rosiglitazone and pioglitazone) normalized the impaired fatty acid uptake (Wilmsen, et al., 2003).

Other pharmacological approaches, such as treatment of differentiated myotubes with the pan-PPAR agonist tetracylthioacetic acid (TTA) improved complete palmitate oxidation in diabetic myotubes, opposed increased lipid accumulation and increased glucose oxidation (Wensaas, et al., 2009). The activation of cAMP/PKA and calcium signaling pathways is known to promote mitochondrial biogenesis and increase lipid oxidation capacity *in vivo* (Baar, et al., 2002, Hood, 2001). We hypothesized that activation of these signaling pathways in human primary myotubes would induce functional changes in lipid and glucose metabolism. Activation of cAMP/PKA and calcium signaling pathways by PFI-treatment (palmitic acid, forskolin and ionomycin) increased lipid oxidation, storage and lipolysis, as well as improved insulin responsiveness in human primary myotubes (Sparks, et al., 2011).

Activation of LXR in myotubes by an LXR agonist (T0901317) increased glucose uptake and oxidation, but promoted also lipogenesis and increased lipid storage (Kase, et al., 2007, Kase, et al., 2005). This was further enhanced in myotubes established from T2D donors. However, the increased lipid storage in T2D myotubes did not impair insulin-stimulated glucose metabolism during the four-day treatment of the cells with an LXR agonist (Kase, et al., 2007, Kase, et al., 2005). A new strategy is to grow the myoblasts in nutrient limited culturing media to increase the pressure for metabolic remodeling. The OXPHOS vs. glycolytic capacity will possibly be changed to increase the ATP yield (Vander Heiden, et al., 2009). It is well known that cancer cells in particular, but also other cells, become highly glycolytic when grown in high glucose concentrations. Glucose inhibits oxidative phosphorylation, a phenomenon known as the Crabtree effect (Diaz-Ruiz, et al., 2011, Ibsen, 1961). This effect can possibly be avoided by growing cells in media with other nutrients, such as amino acids, lipids or lactate. A problem with this approach is that proliferating cells deficient in ATP will undergo apoptosis or cell cycle arrest. However, replacing glucose with galactose during differentiation of myoblasts to myotubes did increase the basal mitochondrial oxygen consumption rate (Aguer, et al., 2011). Mitochondrial biogenesis was not increased, but some mitochondrial enzymes were increased, as well as the AMPK activity (Aguer, et al., 2011). We have recently confirmed increased oxidative capacity in myotubes grown and differentiated in galactose-containing media, assessed as increased oxidation of glucose and oleic acid (Kase, et al., 2013). We also showed that suppression of oleic acid oxidation by glucose was improved. Preliminary data indicate that removal of glucose (no glucose DMEM

with 10 % FCS) and replacement of glucose with lactate (5 mM) could have similar effects on oxidative capacity (unpublished observations).

Like AMPK, the sirtuins (e.g. silent mating type information regulation 2 homologue 1, SIRT1) are also regarded as fuel-sensing enzymes. AMPK and SIRT1 have a long-standing partnership where they can regulate each other (Ruderman, et al., 2010). Although the effect is less than the effect from PPAR δ activation, treatment of human myotubes for 4 days with the AMPK-activator 5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide (AICAR) or the SIRT-activator SRT1720 led to about 60 % increased oleic acid oxidation (Fig. 4).

The antidiabetic drug metformin, which is an activator of AMPK, has been shown to increase glucose uptake (Sarabia, et al., 1992) as well as insulin-stimulated glycogen synthesis in human myotubes (Al-Khalili, et al., 2005). However, any stimulatory effect of metformin on oxidative capacity has, to our knowledge, not been shown. Neither have we been able to increase substrate oxidation by metformin (10-100 nM for 4-8 days). Glucose uptake increased significantly by about 25 % (\pm 3) and oleic acid uptake by 19 % (\pm 3), whereas oxidation of the same substrates did not increase equally (unpublished data).

In vitro, muscle cells fuse to multinucleated fibers that are randomly arranged, not like the parallel fibers in intact muscles. Different attempts to affect myotube organization *in vitro* have been tried, such as culturing the cells on micro-patterned glass (Yamamoto, et al., 2008), in three-dimensional gels or in flow chambers. Co-cultures with adipocytes or neurons are other approaches to mimic the *in vivo* situation more closely. Sophistication of the culturing technique will possibly improve the muscle cell system.

Conclusions

To summarize, human myotubes seem to be a valuable model for the study of glucose and lipid metabolism. There are some limitations in differentiation status (fiber type) of the cells and energy metabolism that can be improved by proper treatment such as electrical pulse

stimulation to mimic exercise. *In vitro* vs. *in vivo* translatability should be further studied since epigenetic mechanisms may be translated to cultured myotubes.

Figure legends

Figure 1

A. Myotubes were fixed, permeabilized and stained for myosin heavy chain 1 (mouse anti-slow muscle myosin monoclonal antibody), lipid droplets (bodipy), and nuclei (DAPI). Red represents fiber type 1, turquoise represents nuclei, and green represents lipids. B. Human muscle biopsy samples were frozen, sectioned and stained for myosin heavy chain 1 (MAB 1628, Millipore, MA, USA), and lipids (Bodipy 493/503, Invitrogen Molecular Probes). Red represents fiber type 1, and green represents lipids.

Figure 2

The myotube to muscle tissue mRNA expression ratio of selected genes.

Total RNA was isolated, cDNA was synthesized, and real-time qPCR was performed.

Expression of the selected genes were quantified according to the average of two housekeeping genes (GAPDH and 36B4), and the average ratio of expression in myotubes to muscle tissue from the same donor is given \pm SEM (n=4-18). GAPDH = glyceraldehyde 3-phosphate dehydrogenase, 36B4 = acidic ribosomal phosphoprotein P0, PGC-1 = peroxisome proliferator-activated receptor γ coactivator-1, PPAR = peroxisome proliferator-activated receptor, ERR = estrogen-related receptor, GABPA = GA binding protein transcription factor, alpha subunit 60 kDa, MCAD = medium-chain acyl-CoA dehydrogenase, CPT1b = carnitine palmitoyltransferase 1b, PDK4 = pyruvate dehydrogenase kinase 4, COXIV = cytochrome c oxidase IV, CD36 = cluster of differentiation 36/fatty acid translocase, PLIN = perilipin, MYH = myosin heavy chain, ATGL = adipose triglyceride lipase.

Figure 3

Suggested strategies to improve oxidative capacity of myotubes and some mechanisms involved. Muscle fiber type switch and improved oxidative capacity might be achieved by chronic low-frequency stimulation (CLF). Stimulation of AMP-dependent kinase (AMPK) by 5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide (AICAR) and GW501516, via

activation peroxisome proliferator-activated receptor (PPAR) δ , increase fatty acid oxidation. Other nuclear receptors, such as the other PPARs and liver X receptor (LXR), may improve myotube metabolism, and metformin increases energy uptake. PFI-treatment (palmitate, forskolin, ionomycin) improves lipid metabolism and insulin sensitivity. The activator of SIRT (silent mating type information regulation 2 homolog) SRT1720 increases lipid oxidation probably via PPAR gamma coactivator 1 α (PGC-1 α). Incubation of myotubes with certain nutrients such as galactose, fatty acids and lactate can increase oxidative energy metabolism.

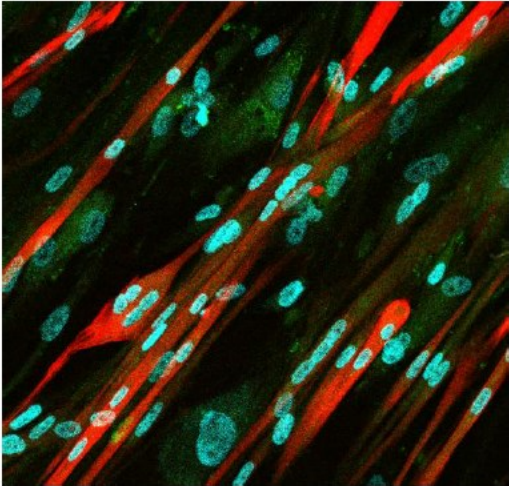
Figure 4

Oleic acid oxidation in human myotubes after pretreatment with the PPAR δ agonist GW501516, the AMPK-activator AICAR and the SIRT-activator SRT1720. Myotubes were pretreated with GW501516, AICAR or SRT1720 for 24 h, before 100 μ M 14 C-labeled oleic acid was added and CO₂ was trapped for 4 h. Average amount of CO₂ \pm SEM is given (n=10).

*Significantly increased from control (p<0.05).

Figure 1

A)



B)

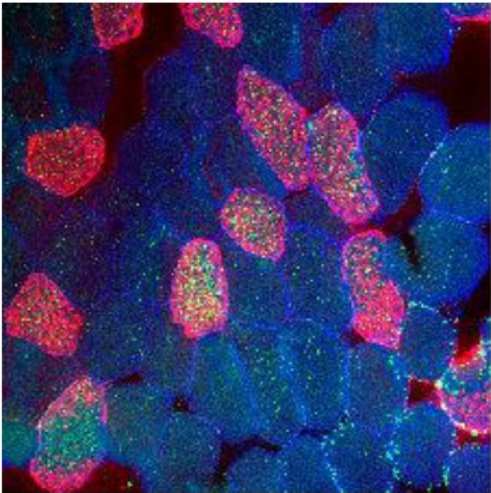


Figure 2

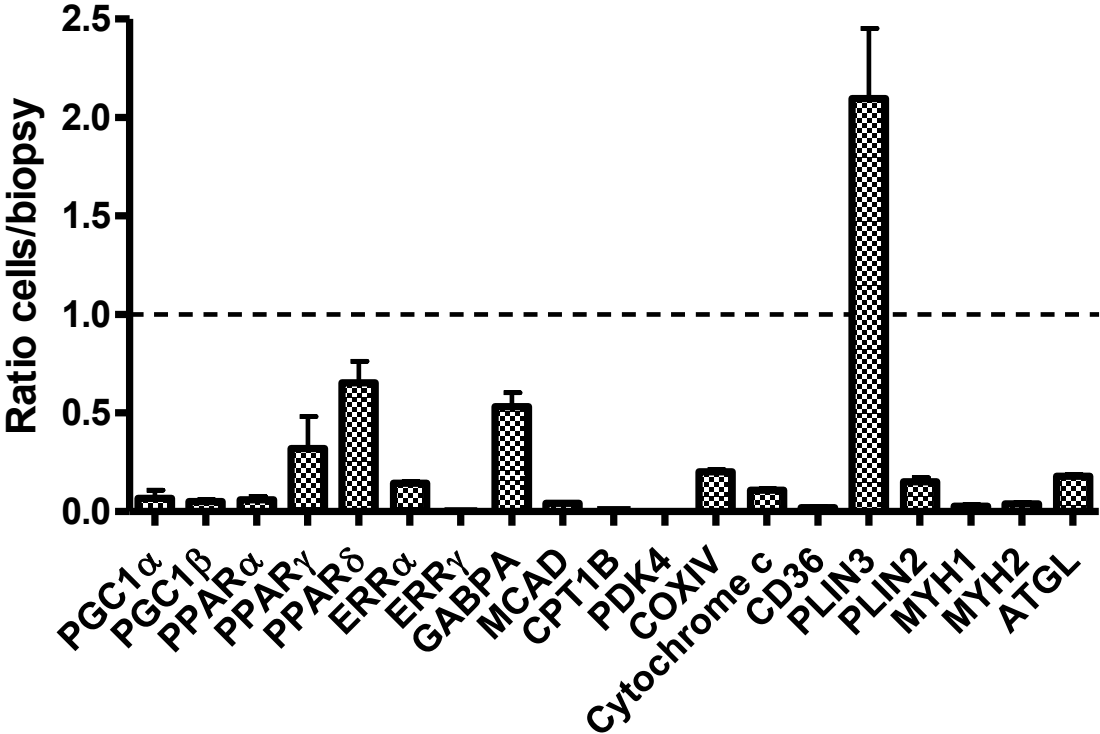


Figure 3

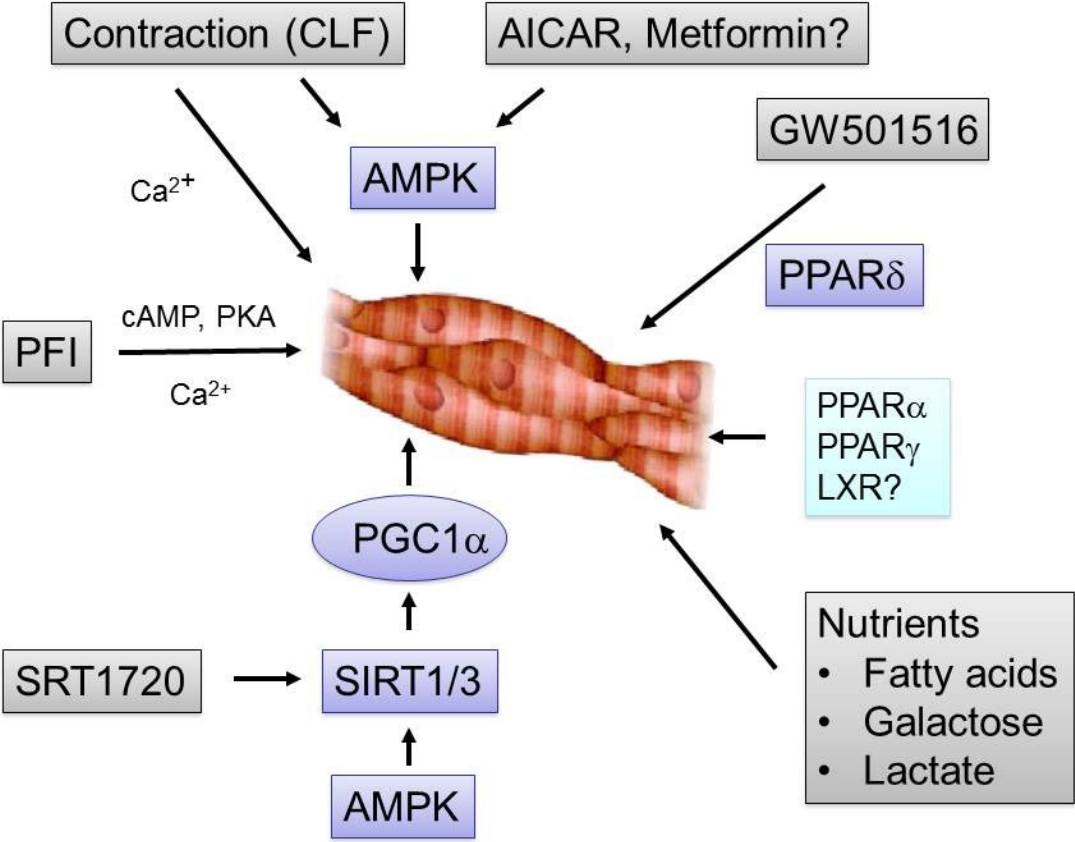
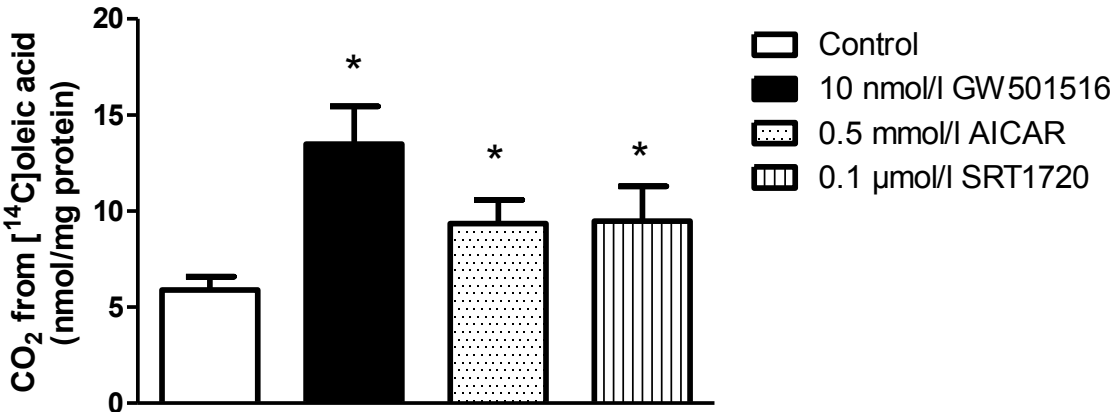


Figure 4



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