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Glucose metabolism and metabolic flexibility in cultured skeletal muscle cells is related to exercise status in young male subjects

Running title: Enhanced glucose metabolism in myotubes from trained subjects

Jenny Lund[1*], Daniel S. Tangen[2¶], Håvard Wiig[2¶], Hans K. Stadheim[2¶], Siw A. Helle[1], Jesper B. Birk[3], Thorsten Ingemann-Hansen[4], Arild C. Rustan[1], G. Hege Thoresen[1,5], Jørgen F.P. Wojtaszewski[3], Eili T. Kase[1], Jørgen Jensen[2]

[1]*Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, Norway*

[2]*Department of Physical Performance, Norwegian School of Sport Sciences, Norway*

[3]*Section of Molecular Physiology, Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen, Denmark*

[4]*Department of Public Health – Sport Science, Aarhus University, Denmark*

[5]*Department of Pharmacology, Institute of Clinical Medicine, University of Oslo, Norway*

[¶]*Contributed equally.*

**Corresponding author:* Jenny Lund, Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, P.O. Box 1068 Blindern, 0316 Oslo, Norway. Tel.: +4722856544. E-mail: jenny.lund@farmasi.uio.no.

Author contributions: J.L., G.H.T., A.C.R., and J.J. conception and design of research; H.K.S. responsible for physical testing; T.I-H. responsible for taking skeletal muscle biopsies; H.W. responsible for immunohistochemistry; D.S.T. responsible for

immunoblotting on biopsies; J.B.B. and J.F.P.W. performed SDS-PAGE for myosin heavy chain identification; J.L., S.A.H. and E.T.K. performed experiments on myotubes; J.L., D.S.T., H.W., and H.K.S. analysed data; J.L., A.C.R., G.H.T., E.T.K., and J.J. interpreted results of experiments; J.L. prepared figures; J.L. and E.T.K. drafted manuscript; J.L., A.C.R., G.H.T., J.F.P.W., E.T.K., and J.J. edited and revised manuscript; all authors approved final version of manuscript.

Keywords: Training condition, myotubes, glucose, suppression, oxidation.

Abbreviations: BMI, body mass index; CA, cell-associated radioactivity; HF, heart frequency; HOMA-IR, homeostatic model assessment of insulin resistance; SMM, skeletal muscle mass; TAG, triacylglycerol; VO_{2max}, maximal oxygen uptake; WHR, waist-to-hip ratio.

Abstract

We hypothesized that skeletal muscles of healthy young people have a large variation in oxidative capacity and fibre-type composition, and aimed therefore to investigate glucose metabolism in biopsies and myotubes isolated from *musculus vastus lateralis* from healthy males with varying degrees of maximal oxygen uptake. Trained and intermediary trained subjects showed higher carbohydrate oxidation *in vivo*. Fibre-type distribution in biopsies and myotubes did not differ between groups. There was no correlation between fibre-type I expression in biopsies and myotubes. Myotubes from trained had higher deoxyglucose accumulation and fractional glucose oxidation (glucose oxidation relative to glucose uptake), and were also more sensitive to the suppressive action of acutely added oleic acid to the cells. Despite lack of correlation of fibre types between skeletal muscle biopsies and cultured cells, myotubes from trained subjects retained some of their phenotypes *in vitro* with respect to enhanced glucose metabolism and metabolic flexibility.

Introduction

Skeletal muscles of healthy young people have a large variation in oxidative capacity, fibre-type composition and insulin sensitivity (for review, see (Schiaffino and Reggiani, 2011)). Muscle fibre-type composition is mainly genetically determined (Schiaffino and Reggiani, 2011, Simoneau and Bouchard, 1995), whereas oxidative capacity, maximal oxygen uptake and metabolic health are very responsive to exercise training (reviewed by for example (Hood *et al.*, 2011, Strasser and Pesta, 2013, Jones and Carter, 2000)).

Muscle fibre types are defined by their expression of myosin heavy chain (MHC) isoforms, and many of the same transcription factors that regulate expression of type I MHC also regulate expression of oxidative enzymes (Schiaffino and Reggiani, 2011). Metabolic capacity in skeletal muscles during exercise varies greatly between untrained and trained subjects. Trained subjects have higher capacity for energy production, and particularly increased lipid oxidation on the expense of carbohydrate oxidation during exercise (Sidossis *et al.*, 1998, Melanson *et al.*, 2009).

Primary culture of differentiated human myotubes grown from satellite cells is a valuable tool to understand metabolic regulation. This *in vitro* system represents a model for intact skeletal muscle and has the most relevant genetic background for studying regulation of energy metabolism in human skeletal muscle (Aas *et al.*, 2013), thus allowing investigation of the innate characteristics of the donors from which they were established. Interestingly, we and others have previously shown that cultured muscle cells generated from type 2 diabetic subjects have reduced insulin-stimulated glucose uptake and glycogen synthesis compared to cells from non-diabetics (McIntyre *et al.*, 2004, Bakke *et al.*, 2015, Gaster *et al.*, 2002), and thus it is believed that the reduced insulin action results partly from genetic and partly from epigenetic changes (McIntyre *et al.*, 2004).

In addition, several studies have previously shown that prolonged training *in vivo* improves energy metabolism in skeletal muscle cells *in vitro* (Bourlier *et al.*, 2013, Lund *et al.*, 2017). Eight weeks of endurance training increased glucose oxidation, glycogen synthesis and glucose suppressibility in cultured myotubes established from obese donors. These improvements were associated with consistent changes in mRNA expression of *GLUT1*, *PDK4* and *PDHA1* (Bourlier *et al.*, 2013). Our group recently reported results on myotubes cultured from satellite cells isolated from biopsies from normal weight and overweight subjects participating in a 12-week combined endurance and strength training intervention, showing an increased exercise-induced fractional glucose oxidation in cultured myotubes from overweight subjects compared to normal weight subjects (Lund *et al.*, 2017).

In the present study we have studied myotubes derived from muscle biopsies obtained from healthy young men with different exercise status, *i.e.* different maximal oxygen uptake (VO_{2max}), hypothesizing that skeletal muscle from healthy young individuals will have a large variation in oxidative capacity and fibre-type composition. Our main aim was to explore possible differences in glucose metabolism in cultured myotubes established from donors with high and low VO_{2max}. We also wanted to compare the expression of selected genes and proteins involved in glucose metabolism in muscle biopsies with the expression pattern in cultured myotubes.

Materials and methods

Materials

Dulbecco´s modified Eagle medium (DMEM-Glutamax™) low glucose with sodium pyruvate, Dulbecco´s phosphate buffered saline (DPBS without Mg^{2+} and Ca^{2+}), foetal bovine serum (FBS), trypsin-EDTA, penicillin-streptomycin (10000 IE/ml), amphotericin B, Nunc™ Cell Culture Treated Flasks with Filter Caps, Nunc™ 96-MicroWell™ plates, Superfrost™ Plus Microscope Slides, TaqMan reverse transcription kit reagents, High-Capacity cDNA Reverse Transcription Kit, MicroAmp® Optical 96-well Reaction Plate, MicroAmp® Optical Adhesive Film, primers for TaqMan PCR, Power SYBR® Green PCR Master Mix, Goat anti-Mouse IgG (H+L) Alexa Fluor 488 secondary antibody (A-11001), Goat anti-Mouse IgG (H+L) Alexa Fluor 594 secondary antibody (A-11005), and SuperSignal™ West Dura Substrate were from ThermoFisher Scientific (Roskilde, Denmark). SkBM-kit (SkGM) and BioWhittaker® PBS were produced by Lonza (Wakersville, MD, USA). Ultroser G was from Pall Life Sciences (Cergy-Saint-Christophe, France), insulin (Actrapid® Penfill® 100 IE/ml) from Novo Nordisk (Bagsvaerd, Denmark) and VWR® Grade 703 Blotting Paper from VWR (Poole, UK). Bovine serum albumin (BSA, essentially fatty-acid free), L-carnitine, D-glucose, oleic acid (OA, 18:1, n-9), etomoxir, trypan blue (0.4%), HEPES, DMSO, L-glutamine, protease inhibitor, phosphatase II inhibitor, protease inhibitor cocktail P8340, benzamidine, DTT, and β-mercaptoethanol were from Sigma-Aldrich (St. Louis, MO, USA). D-[^{14}C(U)]glucose (2.9 mCi/mmol) and 2-[1-^{14}C]Deoxy-D-glucose (286.6 mCi/mmol) were from PerkinElmer NEN® (Boston, MA, USA). Bio-Rad Protein Assay Dye Reagent Concentrate, *DC* Protein Assay Reagents, Clarity™ Western ECL Substrate, Tris/glycine buffer, Tris/glycine/SDS buffer, SDS, Tween 20, bromophenol blue, Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (#170-6515) secondary antibody, and Mini-Protean® TGX™ gels were from Bio-Rad (Copenhagen, Denmark).

Donkey Anti-Goat IgG (H+L) secondary antibody (#6420-05) was from SouthernBiotech (Birmingham, AL, USA). Glycerol, Tris-HCl and Goat Anti-Mouse IgG HRP Conjugate secondary antibody (#12-349) were from Merck Millipore (Darmstadt, Germany). 96-well Corning® CellBIND® tissue culture plates were from Corning Life-Sciences (Schiphol-Rijk, the Netherlands). Amersham™ Protran™ Premium 0.45 µm NC Nitrocellulose Blotting Membrane was from Amersham™ (GE Healthcare, Esbjerg, Denmark). Antibodies against human total and phosphorylated Akt at Ser473 (#9272 and #9271S, respectively), total and phosphorylated AMPKα at Thr172 (#2531 and #2532, respectively), total and phosphorylated TBC1D4 at Thr642 (#2670 and #4288, respectively), glycogen synthase (#3893), and β-actin (#4970S) were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against dystrophin (#ab15277) and human total OXPHOS (#ab110411) were from Abcam (Cambridge, UK). Antibody against HKII (#sc-6521) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cytostar-T® 96-well plate, OptiPhase Supermix, 96-well Isoplate®, Unifilter®-96 GF/B, and TopSeal®-A transparent film were from PerkinElmer (Shelton, CT, USA). RNeasy Mini Kit was from QIAGEN (Venlo, the Netherlands). Antibody against myosin heavy chain type 2 for immunohistochemistry was a gift from Professor S. Schiaffino (University of Padua, Padua, Italy), and the antibody against GLUT4 was a gift from David E. James (Garvan Institute of Medical Research, Darlinghurst, Sydney, Australia).

Ethics statement

The biopsies were obtained after informed written consent and approval by the Regional Committee for Medical and Health Research Ethics South East, Oslo, Norway (reference number: 2011/2207). The study adhered to the Declaration of Helsinki.

Donor characteristics and biopsy retrieval

A total of 24 healthy young (age 21-38 years) males with varying training status participated in the study. The subjects did not use any medications or smoked. The study group was divided in three groups based on VO_{2max}: untrained was defined as $VO_{2max} \leq 46$ ml/kg/min (7 participants), intermediary trained was defined as 46 ml/kg/min < $VO_{2max} \leq 60$ ml/kg/min and (11 participants) and trained was defined as $VO_{2max} > 60$ ml/kg/min (6 participants). All participants in the trained group performed both strength and endurance exercise, in average (± SEM) 2.7 (± 0.3) and 3.1 (± 0.7) workouts per week, respectively, 7.8 (± 0.7) hours weekly in total. The intermediary trained group performed in average 1 (± 0.4) strength workout and 1.5 (± 0.6) endurance workouts per week, 3.6 (± 1.4) hours weekly in total, whereas the untrained group practiced no systematic training (less than 1 hour per week). Donor characteristics for the three groups are presented in Table 2. A fasting blood sample was taken of each participant and analysed by Fürst Laboratories (Oslo, Norway). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as: fS-Insulin (pmol/l)×fS-glucose (mmol/l)/135. A small biopsy (100-200 mg) of *musculus vastus lateralis* of each participant was obtained under local anaesthesia with a Bergström cannula modified for suction (Bergström, 1975). Parts of the biopsy were immediately freeze dried while the rest was taken to isolation of satellite cells for culturing of myoblasts and subsequent differentiation into myotubes.

Measurement of VO_{2max}, HR_{max}, $lactate_{max}$, and fat oxidation *in vivo*

Measurement of VO_{2max} was performed on Monark Ergomedic 839E cycle (GIH, Stockholm, Sweden). Oxygen consumption and respiratory exchange ratio (RER) were measured with an Oxycon Pro metabolic system (Jaeger, Hochberg, Germany), and air was collected via a mouth V2-mask (Hans Rudolph Instruments, Shawnee, KS, USA) in combination with a nose

bracket as described (Stadheim *et al.*, 2015). Before the VO_{2max} test participants performed a test to evaluate glucose and fat oxidation. Participants started at 75 watts intensity and the load was increased by 15 watts every 5 min. The test was stopped once the participants reached: 1) 14-16 on Borgs scale (Borg, 1982), 2) lactate concentration > 3 mmol/l or 3) RER ≥ 1.00. After 5 min of cycling at 50 watt, VO_{2max} was tested. The load during the first min was the same load as last workload during the incremental test; workload thereafter increased by 15 watts every 30 sec until the participants no longer were able to maintain the required intensity despite strong encouragement. The mean of two subsequent 30 sec measurements were used as VO_{2max} once criteria for VO_{2max} were reached: a flattening in VO_2 with increasing load, RER ≥ 1.10, lactate concentration ≥ 7 mmol/l, or subjective exhaustion. Heart rate (HR) was measured during the test (Polar RS 800, Kempele, Finland), and maximal HR (HR_{max}) was defined. Lactate was measured in capillary blood using a YSI 1500 Sport Lactate Analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA) as described (Stadheim *et al.*, 2015). Oxidation rates were calculated from measured oxygen uptake and carbon dioxide (CO_2) output as described by Frayn (Frayn, 1983).

Immunohistochemistry of biopsies

Cross-sections (8 μm) of frozen muscle biopsies were cut using a CM3050 microtome at -20°C (Leica Microsystems GmbH, Wetzlar, Germany), mounted on Superfrost™ Plus microscope slides, air-dried, and stored at -80°C. The muscle sections were blocked for 30 min with 1% BSA, 0.05% PBS-T and 0.01% Tween 20 solution, before incubation with antibodies against myosin heavy chain type 2 (1:1000, stains both type IIa and IIx fibres) and dystrophin (1:500) for 2 h at 20°C, followed by incubation with appropriate secondary antibodies. After incubation the sections were washed for 2×10 min in 0.05% PBS-T. Digital pictures of the muscle sections were taken with a high-resolution Olympus DP72 camera

(Olympus Corp., Tokyo, Japan) through a Olympus BX61 microscope (Olympus Corp., Tokyo, Japan) with a X-cite 120PCQ fluorescence light source (EXFO Photonic Solutions Inc., Ontario, Canada), and analysed for fibre-type distribution and fibre cross-sectional area using TEMA software (CheckVision, Hadsund, Denmark). A mean of 401 fibres (range 103-928) were analysed on each cross-section.

Homogenization of biopsy samples

Parts of the muscle biopsies (~30 mg from each participant) were freeze dried and weighed. Samples were homogenized 1:120 in ice cold homogenization buffer (50 mmol/l HEPES, 150 mmol/l NaCl, 10 mmol/l $Na_4P_2O_7$, 30 mmol/l NaF, 1 mmol/l Na_3VO_4, 2.5 mmol/l benzamidine, 0.5 µl/2 mg/dw Protease Inhibitor Cocktail P-8340 in dH_2O) with a Retsch MR400 mixer mill (Retsch, Haan, Germany) for 3×30 sec with 5 sec break between each shaking. 50 µl crude homogenate was pipetted for measurements of glycogen content. Protein in homogenates was measured in a 96-well micro plate with Bio-Rad *DC* Protein Assay kit on FLUOstar Omega (IBMG, Ortenberg, Germany).

Glycogen measurements in the biopsies

Glycogen content was measured fluorometrically in an enzymatic reaction coupled to NADHP formation, as previously described (Jensen *et al.*, 2012, Passonneau and Lowry, 1993). 100 µl 1.8 mol/l HCl was added to 50 µl crude homogenate and glycogen was hydrolysed at 100°C for 3 h; samples were mixed every 30 min. Thereafter, samples were cooled to room temperature, spun at 1000×*g* for 2 sec, neutralized with 6 mol/l NaOH, and stored at -20°C. The reaction was performed in 96-well micro plates and fluorescence was measured before and after the reaction on FLUOstar Omega (IBMG, Ortenberg, Germany).

Immunoblotting of the biopsies

A mixture of 100 µl homogenate, 25 µl SDS buffer (200 mg/ml) and 5 µl DTT (5 mol/l) was incubated at room temperature for 60 min before quick centrifugation. Thereafter, 14 µl of each sample was applied on 4-15% Mini-Protean® TGX™ gels with Tris/glycine/SDS buffer and electrophoretically separated (200 V for 45 min). Membranes were soaked in methanol for 15 sec, dH_2O for 5 min and finally in Tris/glycine/SDS/methanol for 10 min. After electrophoresis the gels were equilibrated with the membranes in Tris/glycine/SDS/methanol buffer on a tilting table for 10 min. Thereafter, proteins were blotted to membranes (100 V for 30 min), and incubated with antibodies for hexokinase (HK) II (1:4000), Akt kinase (1:1500), glucose transporter (GLUT) 4 (1:200000), glycogen synthase (GS, 1:10000), and total OXPHOS (1:1000). Immunoreactive bands were visualized with Chemiluminescent Substrate SuperSignal WestDura on a Chemidoc™ MP System (Bio-Rad, Hercules, CA, USA) and quantified with Image Lab™ software (Bio-Rad, Hercules, CA, USA). All samples were derived at the same time and processed in parallel. Expression levels were normalized to a standard homogenate.

Culturing of human myotubes

Cells from the intermediary trained group were not used for the culturing experiments in the current study. Furthermore, only six of the seven donors in the untrained group gave rise to usable myotube cultures and were used in the cell experiments. Multinucleated human myotubes were established by activation and proliferation of satellite cells isolated from *musculus vastus lateralis* from six sedentary untrained young men and six well-trained young men (Table 2). Normally myotubes from each donor were seeded out on its own plate, but for the experiments behind the data in Figure 4A and Figure 6C a mixture of the six donors within the groups were used. The culturing was based on the method of Henry *et al.* (Henry

et al., 1995) and modified according to Gaster *et al*. (Gaster *et al.*, 2001a, Gaster *et al.*, 2001b). For proliferation of myoblasts a DMEM-Glutamax™ (5.5 mmol/l glucose) medium supplemented with 2% FBS and 2% Ultroser G was used. At approximately 80% confluence the medium was changed to DMEM-Glutamax™ (5.5 mmol/l glucose) supplemented with 2% FBS and 25 pmol/l insulin to initiate differentiation into multinucleated myotubes. The cells were allowed to differentiate for 7 days; the cells from both donor groups differentiated equally well as indicated by no change in mRNA or protein expressions of MHCI (*MYH7* on gene level) and MHCIIa (*MYH2* on gene level) in the myotubes (Figure 3E and 3G, respectively), and by visual examination in the microscope. During the culturing process the muscle cells were incubated in a humidified 5% CO_2 atmosphere at 37°C, and medium was changed every 2-3 days. Experiments were performed on cells from passage number two to four. For each experiment the passage number remained constant. Isolation of satellite cells from all biopsies was performed at the same location and by the same trained researchers.

RNA isolation and analysis of gene expression by qPCR

Total RNA was isolated using RNeasy Mini Kit according to supplier's protocol. RNA was reversely transcribed (25°C for 10 min, 37°C for 80 min and 85°C for 5 min) with a High-Capacity cDNA Reverse Transcription Kit and TaqMan Reverse Transcription Reagents using a 2720 Thermal Cycler (ThermoFisher Scientific, Roskilde, Denmark). Primers were designed using Primer Express® (ThermoFisher Scientific, Roskilde, Denmark). qPCR was performed using a StepOnePlus Real-Time PCR system (ThermoFisher Scientific, Roskilde, Denmark). Target genes were quantified in duplicates, carried out in a 25 µl reaction volume according to supplier's protocol. All assays were run for 44 cycles (95°C for 15 s followed by 60°C for 60 s). Expression levels were normalized to the average of the housekeeping gene acidic ribosomal phosphoprotein P0 (*RPLP0*). The housekeeping gene glyceraldehyde-3-

phosphate dehydrogenase (*GAPDH*) was also analysed; there were no differences between normalizing for *RPLP0* or *GAPDH*. The following forward and reverse primers were used at concentration of 30 µmol/l: *GAPDH*, *RPLP0*, cytochrome c-1 (*CYC1*), *GLUT1*, *GLUT4*, *MYH7*, *MYH2*, and pyruvate dehydrogenase kinase isoenzyme 4 (*PDK4*). The primers are presented in Table 1.

Table 1. Description of primers.

Gene	Acc.no.	Forward sequence	Reverse sequence
RPLP0	M17885	CCA TTC TAT CAT CAA CGG GTA CAA	AGC AAG TGG GAA GGT GTA ATC C
GAPDH	NM_002046	TGC ACC ACC ACC TGC TTA GC	GGC ATG GAC TGT GGT CAT GAG
CYC1	NM_001916	CTG CCA ACA ACG GAG CAT T	CGT GAG CAG GGA GAA GAC GTA
GLUT1	K03195	CAG CAG CCC TAA GGA TCT CTC A	CCG GCT CGG CTG ACA TC
GLUT4	M20747	GCT ACC TCT ACA TCA TCC AGA ATC TC	CCA GAA ACA TCG GCC CA
MYH2	NM_017534	AAG GTC GGC AAT GAG TAT GTC A	CAA CCA TCC ACA GGA ACA TCT TC
MYH7	NM_000257	CTC TGC ACA GGG AAA ATC TGA A	CCC CTG GAC TTT GTC TCA TT
PDK4	BC040239	TTT CCA GAA CCA ACC AAT TCA CA	TGC CCG CAT TGC ATT CTT A

Primers designed in primer express. Abbreviations: CYC1, cytochrome c-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter; MYH, myosin heavy chain; PDK4, pyruvate dehydrogenase kinase isozyme 4; RPLP0, acidic ribosomal phosphoprotein P0.

Immunoblotting of the myotubes

Myotubes were incubated with or without 100 µM oleic acid for 4 h or 100 nmol/l insulin for 15 min before cells were harvested in Laemmli buffer (0.5 mol/l Tris-HCl, 10% SDS, 20% glycerol, 10% β-mercaptoethanol, and 5% bromophenol blue). Proteins were electrophoretically separated on 4-20% Mini-Protean® TGX™ gels with Tris/glycine buffer (pH 8.3) followed by blotting to nitrocellulose membranes and incubation with antibodies for

total Akt kinase and Akt phosphorylated at Ser473 (1:1000), total AMP-activated protein kinase (AMPK) α and AMPKα phosphorylated at Thr172 (1:1000), total TBC1 domain family member 4 (TBC1D4, also known as Akt substrate of 160 kDa, AS160) and TBC1D4 phosphorylated at Thr642 (1:1000), MHCI (1:10000), MHCIIa (1:1000), OXPHOS (1:500), PDK4 (1:2500), and β-actin (1:5000). Immunoreactive bands were visualized with enhanced chemiluminescence on a Chemidoc XRS (Bio-Rad, Copenhagen, Denmark) and quantified with Image Lab (version 4.0) software (Bio-Rad, Copenhagen, Denmark). All samples were derived at the same time and processed in parallel. Expression levels were normalized to one sample used as loading control. Expressions of MHCI, MHCIIa, total OXPHOS, and PDK4 were further normalized to the endogenous control β-actin.

Myosin heavy chain determination by SDS-PAGE

Myotubes were harvested in Laemmli buffer (0.5 mol/l Tris-HCl, 10% SDS, 20% glycerol) and each sample was diluted to 2 mg/ml protein before immunoblotting. Muscle biopsy homogenates were likewise diluted in Laemmli buffer to the same concentration, after which all samples were further diluted in heavy Laemmli buffer, *i.e.* glycerol:Laemmli buffer (1:1), to a concentration of 0.3 mg/ml. Muscle biopsy homogenates were not available from the intermediary trained group. For SDS-PAGE on stain-free gels 4 µg were used as previously described (Albers *et al.*, 2015). Before calculation of relative expression of MHC bands it was corrected for tryptophan residues (ten in MHCI and nine in both MHCIIa and MHCIIx).

Glucose metabolism

Skeletal muscle cells were cultured on 96-well CellBIND® microplates. D-[^{14}C(U)]glucose (0.58 µCi/ml), 200 µmol/l, with or without 100 µmol/l oleic acid, were given during 4 h CO_2 trapping as previously described (Wensaas *et al.*, 2007). In brief, a 96-well UniFilter®-96

GF/B microplate was mounted on top of the CellBIND® plate and CO_2 production was measured in DPBS medium with 10 mmol/l HEPES and 1 mmol/l L-carnitine adjusted to pH 7.2-7.3. CO_2 production and cell-associated (CA) radioactivity were assessed using a PerkinElmer 2450 MicroBeta^2 scintillation counter (Waltham, MA, USA). The sum of $^{14}CO_2$ and remaining CA radioactivity was taken as a measurement of total cellular uptake of substrate: CO_2+CA. Protein levels in the lysate were measured with Bio-Rad protein assay using a PerkinElmer VICTOR™ *X*4 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA). Fractional complete oxidation was calculated as: $CO_2/(CO_2+CA)$. Suppression of [^{14}C]glucose oxidation by acutely added oleic acid was calculated as: [(1-(oxidation of [^{14}C]glucose at 100 µmol/l oleic acid / oxidation of [^{14}C]glucose with no oleic acid added)) × 100%].

Scintillation proximity assay (SPA)

Skeletal muscle cells were cultured and differentiated on 96-well CytoStar-T® plates. Measurement of [1-^{14}C]deoxy-D-glucose (2 µCi/ml, 7 µmol/l) accumulation by scintillation proximity assay (SPA) were performed in DMEM with 5.5 mmol/l glucose but without phenol red. Cells were incubated (5% CO_2 atmosphere at 37°C) for 8 h, with measurements at 0, 2, 4, 6, and 8 h on a PerkinElmer 2450 Microbeta^2 scintillation counter (Waltham, MA, USA). The radiolabelled deoxyglucose taken up and accumulated by adherent cells will be concentrated close to the scintillator embedded in the bottom of each well in the plate, and therefore provide a stronger signal than the radiolabel dissolved in the medium. Cells were harvested in 0.1 mmol/l NaOH and samples were stored at -20°C before analysis of protein content.

Glycogen synthesis

Myotubes were exposed to DMEM supplemented with D-[^{14}C(U)]glucose (2 µCi/ml, 0.67 mmol/l) and 5.5 mmol/l glucose, in presence or absence of 100 nmol/l insulin for 3 h to measure glycogen synthesis. The cells were washed twice with PBS and harvested in 1 mol/l KOH. Protein content was determined with Pierce BCA Protein Assay Kit, before 20 mg/ml glycogen and more KOH (final concentration 4 mol/l) were added to the samples. Then, D-[^{14}C(U)]glucose incorporated into glycogen was measured as previously described (Hessvik *et al.*, 2010).

Presentation of data and statistics

Data are presented as means ± SEM in nmol/mg cell protein unless stated otherwise. The value *n* represents the number of different donors, each with at least duplicate observations. Statistical analyses were performed using GraphPad Prism 6 for Windows (GraphPad Software Inc., La Jolla, CA, USA) or SPSS version 22 (IBM® SPSS® Statistics for Macintosh, Armonk, NY, USA). Wilcoxon matched-pairs signed rank test was used within groups for the experiments where cells from each donor were cultured individually, whereas unpaired t test was used within groups for the experiments where a mixture of the six donors within the groups were used. Mann-Whitney test was used to evaluate effects between groups. Linear mixed-model analysis was used to compare differences between conditions with within-donor variation and simultaneously compare differences between groups with between-donor variation. The linear mixed-model analysis includes all observations in the statistical analyses and takes into account that not all observations are independent. Correlation studies were performed with Spearman´s test and are presented as Spearman´s correlation coefficient (r). A p-value < 0.05 was considered significant.

Results

In vivo and *ex vivo* metabolic traits

The study included 24 healthy young male participants, and they were separated into three groups based on VO_{2max}: untrained ($VO_{2max} \leq 46$ ml/kg/min, $n = 7$), intermediary trained (46 ml/kg/min < $VO_{2max} \leq 60$ ml/kg/min, $n = 11$) and trained ($VO_{2max} > 60$ ml/kg/min, $n = 6$). During the test of maximum exercise capacity we observed that trained subjects had significantly higher $workload_{max}$ and $lactate_{max}$ than untrained (Table 2). Subjects in the trained group also had significantly lower fasting serum C-peptide (fS-C-peptide) than the untrained group (Table 2). Subjects in the intermediary trained group also had significantly higher $lactate_{max}$ than untrained, in addition to significantly lower body mass index (BMI), fasting serum insulin (fS-insulin), HOMA-IR, fS-C-peptide, serum cholesterol (S-cholesterol), and serum low-density lipoproteins (S-LDL) than untrained (Table 2). Furthermore, trained subjects had significantly higher $workload_{max}$ than subjects in the intermediary trained group (Table 2).

Table 2. Clinical parameters.

	Untrained	Intermediary trained	Trained
n	7 (6[†])	11	6
Age, y	27.0 ± 2.4	26.8 ± 1.5	24.3 ± 0.7
VO_{2max}, ml/kg/min	41.3 ± 1.4	54.3 ± 0.9*	64.9 ± 1.7*[#]
HF_{max}, beats/min	190.4 ± 4.4	193.4 ± 2.3	192.5 ± 3.2
$Workload_{max}$, watt	262.5 ± 58.7[†]	299.1 ± 17.3	387.5 ± 14.1*[#]
$Lactate_{max}$, mmol/l	8.3 ± 0.6[†]	10.1 ± 0.3*	10.3 ± 0.4*
Body weight, kg	93.0 ± 7.4	75.0 ± 3.1	81.4 ± 2.9
BMI, kg/m^2	27.7 ± 2.1	22.6 ± 0.5*	23.9 ± 0.9
fS-Glucose, mmol/l	5.2 ± 0.2	4.9 ± 0.1	4.9 ± 0.2
fS-Insulin, pmol/l	108.7 ± 32.0	43.7 ± 5.1*	49.5 ± 10.6
HOMA-IR	4.2 ± 1.2	1.6 ± 0.2*	1.8 ± 0.4
fS-C-peptide, pmol/l	592.6 ± 103.0	354.1 ± 37.9*	314.3 ± 10.3*
fS-TAG, mmol/l	1.3 ± 0.2	0.9 ± 0.1	1.4 ± 0.3
S-Cholesterol, mmol/l	4.8 ± 0.4	4.0 ± 0.3*	4.6 ± 0.3
S-HDL, mmol/l	1.1 ± 0.1	1.3 ± 0.04	1.3 ± 0.1
S-LDL, mmol/l	3.0 ± 0.3	2.2 ± 0.2*	2.6 ± 0.3

Data are presented as means ± SEM, and n represents number of subjects. [†]Missing data from one participant. *$p < 0.05$ vs. untrained (Mann-Whitney test), [#]$p < 0.05$ vs. intermediary trained (Mann-Whitney test). BMI, body mass index; f, fasted samples; HDL, high-density lipoprotein; HF, heart frequency; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; S, serum; TAG, triacylglycerol; VO_{2max}, maximal oxygen uptake.

Oxidation of glucose and fat were calculated at exercise intensities from 50% to 80% during the incremental test (Figure 1). The highest rate of glucose oxidation was observed at 80% of maximal exercise intensity (Figure 1A), whereas the highest rate of fat oxidation was observed at 55% (Figure 1C). Overall capacity for glucose and fat oxidation increased significantly with increasing training status (Figure 1B and 1D, respectively).

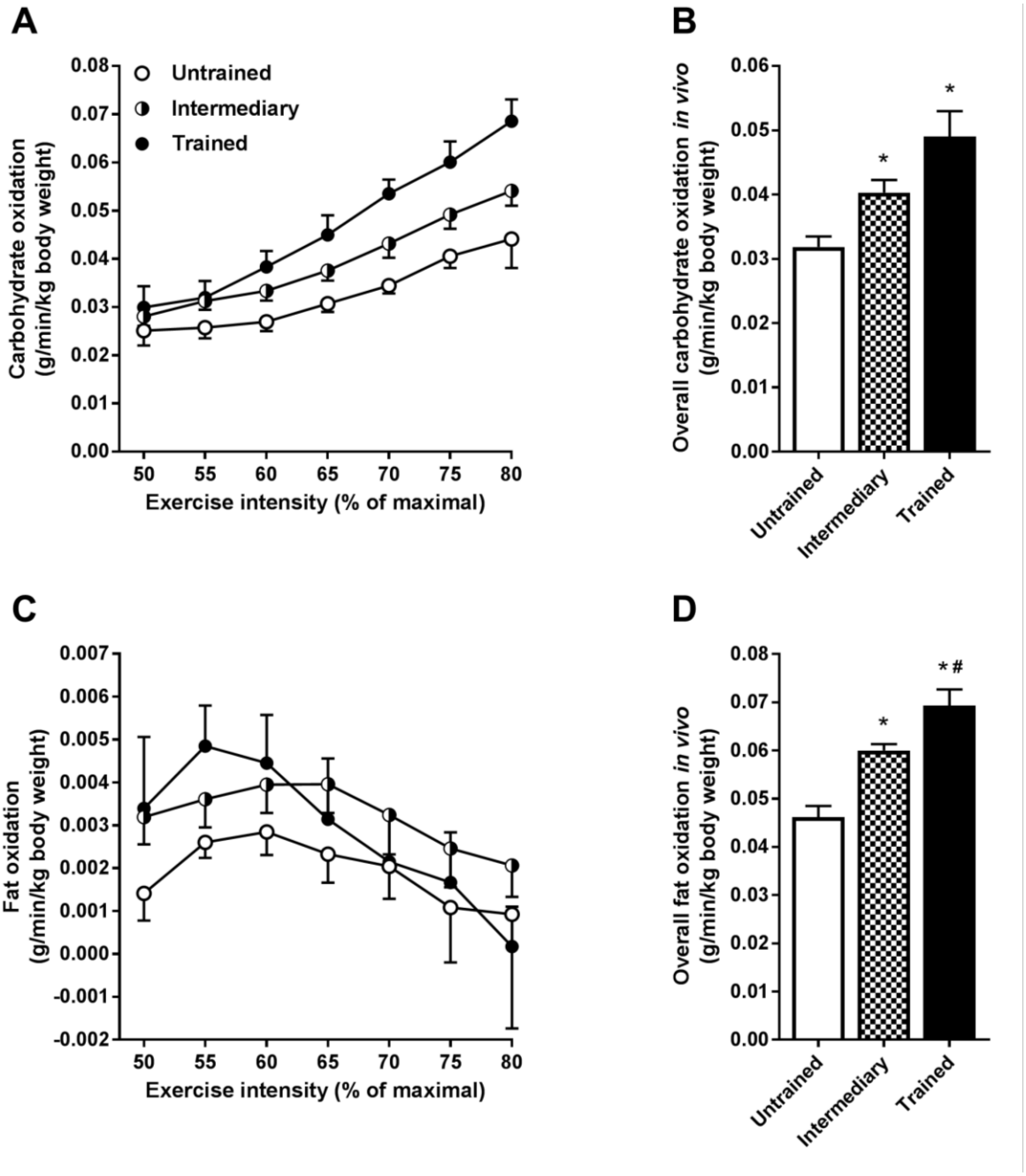

Figure 1. Carbohydrate and fat oxidation *in vivo*. Each participant completed an incremental test where oxygen uptake and CO_2 output were measured as described in "Materials and methods". Oxidation rates were calculated using Frayn's equations (Frayn 1983). (A) Carbohydrate oxidation *in vivo* at exercise intensities from 50% to 80%. (B) Overall carbohydrate oxidation *in vivo*. (C) Fat oxidation *in vivo* at exercise intensities from 50% to 80%. (D) Overall fat oxidation *in vivo*. Data are presented as means ± SEM in g/min/kg body weight (n = 7 in untrained group, n = 10 in intermediary trained group, and n = 6 in trained group). Statistically significant versus untrained (p<.05, linear mixed-model analysis, SPSS). [#]Statistically significant versus intermediary trained (p<.05, linear mixed-model analysis, SPSS).

Characteristics of the *musculus vastus lateralis* biopsies are shown in Figure 2. There were no differences between the three groups in muscle glycogen content (Figure 2A). Immunoblotting was performed on lysates from the biopsies, and no significant differences were observed for protein expressions of HKII, PKB/Akt, GLUT4, or mitochondrial OXPHOS proteins, detected with an antibody cocktail recognizing complex I subunit NDUFB8, complex II subunit, complex III subunit core 2, complex IV subunit II and ATP synthase subunit (Figure 2B).

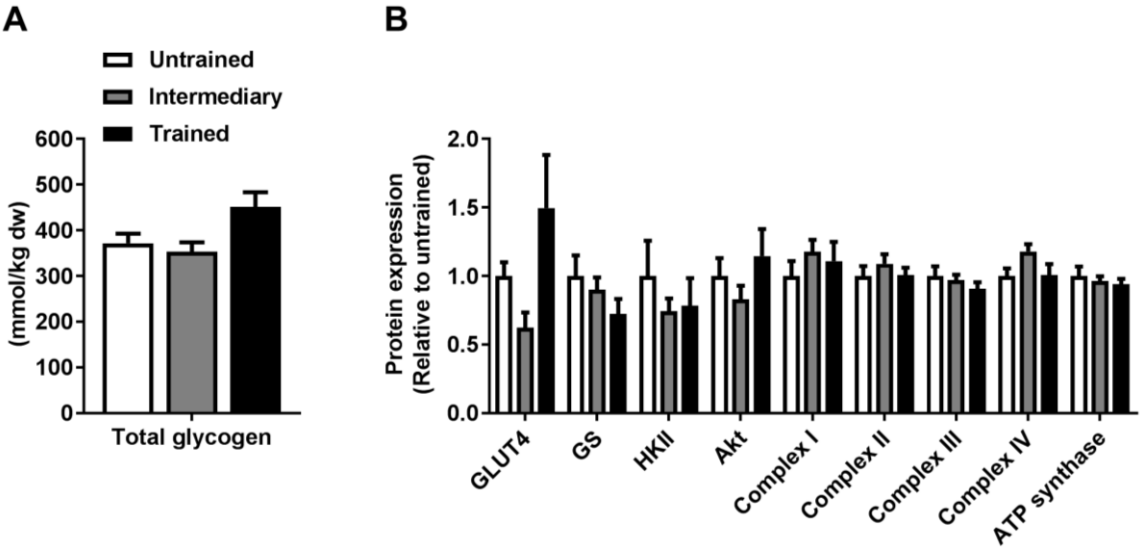

Figure 2. *Ex vivo* metabolic traits. (A) Glycogen content in muscle biopsies. Biopsy was taken from *musculus vastus lateralis* of each study participant and total glycogen content was measured as described in "Materials and methods". Values are presented as means ± SEM (n = 7 in untrained group, n = 10 in intermediary trained group, and n = 6 in trained group). (B) Expression of selected proteins in the biopsies. Biopsy was taken from *musculus vastus lateralis* of each study participant and immunoblotting was performed as described in "Materials and methods". Values are presented as means ± SEM (n = 7 in untrained group, n = 11 in intermediary trained group, and n = 6 in trained group) relative to untrained.

Fibre-type distribution in muscle biopsies and cultured myotubes

Immunohistochemistry (illustrated in Figure 3A) showed that there were no differences in distribution of fibre-type I (that is non-type II fibres) between biopsies from trained (range 41.2-66.4%), intermediary trained (range 26.8-68.1%) and untrained (range 27.2-55.3%)

subjects (Figure 3B). Assessment of fibre-type content in biopsies by SDS-PAGE in stain-free gels showed expression of MHCI, MHCIIa and MHCIIx (Figure 3C). Consistency between the two methods was shown in Figure 3D; percentage fibre-type I in the biopsies assessed by immunohistochemistry correlated positively with percentage fibre-type I in the biopsies when assessed by SDS-PAGE.

Satellite cells were isolated from muscle biopsies from the untrained and trained groups, and fibre-type expressions were also evaluated in the cells after proliferation and differentiation into myotubes (Figure 3E-H). There were no significant differences in mRNA expressions of *MYH7* or *MYH2* (Figure 3E) or protein expressions of MHCI or MHCIIa (Figure 3F and G) in the myotubes, and mRNA and protein expressions did not correlate with any of the fibre types (data not shown). The same isoforms of MHC was found in the cell lysates as in the biopsies when analysed on SDS-PAGE, however, in the cell lysates also other, unidentified bands were detected (Figure 3H). Percentage of fibre-type I in the biopsies did not correlate with protein expression of MHCI in the myotubes (r = -0.07, p = .82, n = 12 [combination of trained and untrained]).

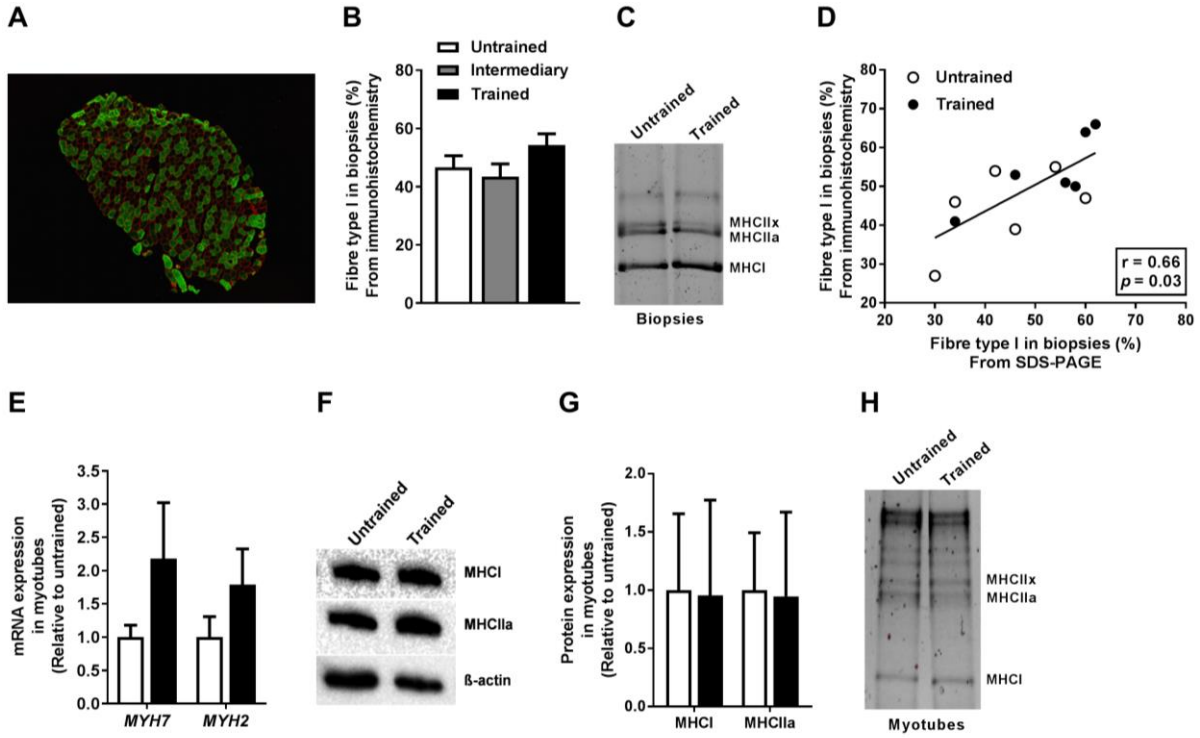

Figure 3. Fibre-type expressions *ex vivo* and *in vitro*. (A and B) Biopsy was taken from *musculus vastus lateralis* of each participant and cross-sections were cut, mounted on microscope slides and prepared for antibody-incubation against MHCII (staining both type IIa and IIx fibres green) and dystrophin 1 (staining red) and imaging. (A) One representative picture after immunohistochemistry. A mean of 401 fibres were analysed on each cross-section. (B) Quantified percentage of nontype II fibres, that is type I fibres based on the immunohistochemistry. Values are presented as means ± SEM (n = 7 in untrained group, n = 11 in intermediary trained group, and n = 6 in trained group). (C) Expression of MHC isoforms in biopsies was assessed on SDS-PAGE gels. All samples were derived at the same time and processed in parallel. One representative line for untrained and trained is shown. (D) Spearman's test of correlation between percentage fibre-type I *ex vivo* from immunohistochemistry and percentage fibre-type I as measured on SDS-PAGE gels (n = 12). Spearman's correlation coefficient, r = 0.66, and p = .03. (E) mRNA expression of *MYH* isoforms by qPCR. RNA was isolated and mRNA reversely transcribed before expressions of *MYH7* and *MYH2* were assessed by qPCR. Data are presented as means ± SEM (n = 6 in each group) relative to untrained. All values were corrected for the housekeeping control *RPLP0*. (F and G) MHCI and MHCIIa expressions by immunoblotting. Protein was isolated and expressions of MHCI and MHCIIa were assessed by immunoblotting. (F) One representative immunoblot. (G) Quantified immunoblots relative to untrained. All values were corrected for the housekeeping control β-actin, and are presented as means ± SEM (n = 6 in each group). (H) Protein was isolated from cultured myotubes and expression of MHC isoforms was assessed by SDS-PAGE on stain-free gels. All samples were derived at the same time and processed in parallel. One representative line for untrained and trained is shown.

Glucose metabolism in myotubes from trained and untrained subjects

Myotubes from trained subjects showed higher basal accumulation of deoxyglucose compared to myotubes from untrained subjects (Figure 4A). No differences in mRNA expressions of *GLUT1* or *GLUT4* were observed in myotubes from the two groups (Figure 4B). There were no differences in glycogen content in muscle biopsies (Figure 2A), and we did not observe a difference in glycogen synthesis in the myotubes from the two groups either (Figure 4C). Uptake (sum of oxidized CO_2 and CA radioactivity) and oxidation (CO_2) of glucose was statistically similar in the two groups (Figure 4D), whereas fractional glucose oxidation, *i.e.* oxidation related to glucose uptake was significantly higher in myotubes from trained subjects compared to untrained (Figure 4E).

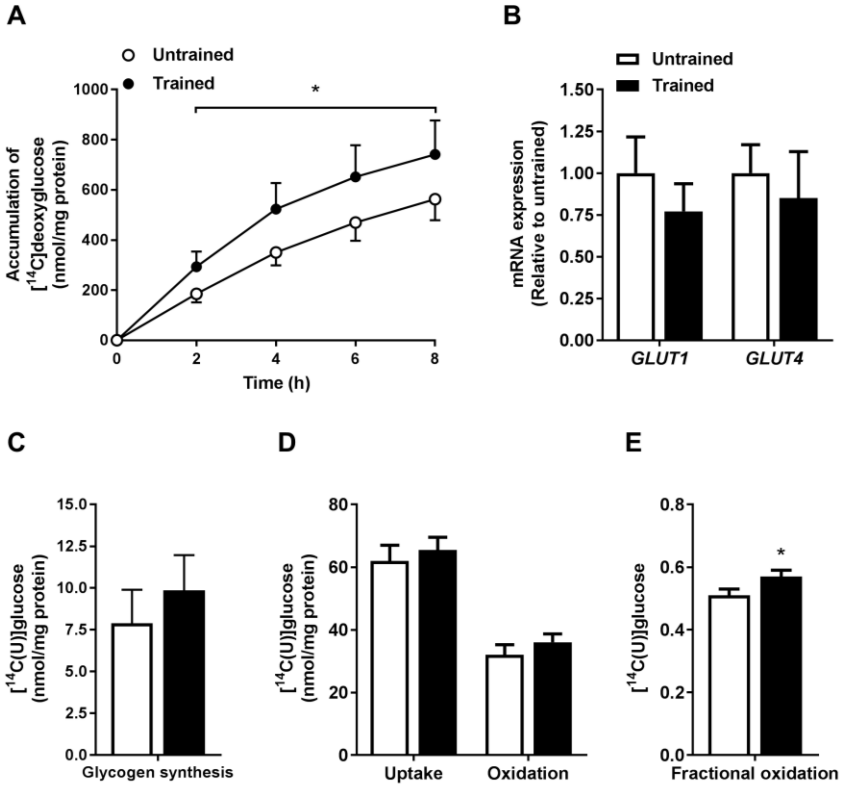

Figure 4. Glucose metabolism in myotubes. Satellite cells isolated from biopsies from *musculus vastus lateralis* from the untrained and trained subjects were cultured and differentiated into myotubes. (A) Basal accumulation of 5.5 mmol/l [^{14}C]deoxyglucose was measured at several time points over 8 h. Data are presented as means ± SEM (n = 6 in each group). Overall basal glucose accumulation statistically significant versus untrained (p<.05, linear mixed-model analysis, SPSS). (B) mRNA expressions of *GLUT1* and *GLUT4* by

qPCR. RNA was isolated and mRNA reversely transcribed before expressions of *GLUT1* and *GLUT4* were assessed by qPCR. Values are presented as means ± SEM (n = 6 in each group). (C) Overall glycogen synthesis in the myotubes. Glycogen synthesis was measured as incorporation of 6.2 mmol/l [^{14}C(U)]glucose into glycogen for 3 h. Values are presented as means ± SEM in nmol/mg protein (n = 6 in each group). (D) Uptake and oxidation of 200 µmol/l [^{14}C(U)]glucose. Oxidation (CO_2) and cell-associated (CA) radioactivity of [^{14}C(U)]glucose was determined after 4 h. The sum of CO_2 and CA radioactivity was considered total cellular uptake of glucose. Values are presented as means ± SEM (n = 6 in each group). (E) Fractional oxidation of 200 µmol/l [^{14}C(U)]glucose. Oxidation and cell-associated (CA) radioactivity were measured and fractional oxidation (CO_2/(CO_2+CA)) of [^{14}C(U)]glucose was determined. Values are presented as means ± SEM (n = 6 in each group). Statistically significant versus untrained (p<.05, Mann–Whitney test).

Insulin response in myotubes from trained and untrained subjects

To examine whether the difference in insulin sensitivity of the two groups *in vivo* (Table 2) was reflected *in vitro*, the response to 100 nmol/l insulin was assessed in myotubes by measurement of glycogen synthesis, Akt (Ser473) phosphorylation and TBC1D4 (Thr642) phosphorylation (Figure 5A-C). Significant insulin-induced effect was found for Akt phosphorylation (pAkt/total Akt ratio) only, not for glycogen synthesis or TBC1D4 phosphorylation (pTBC1D4/total TBC1D4 ratio). No differences between the two groups were observed (Figure 5).

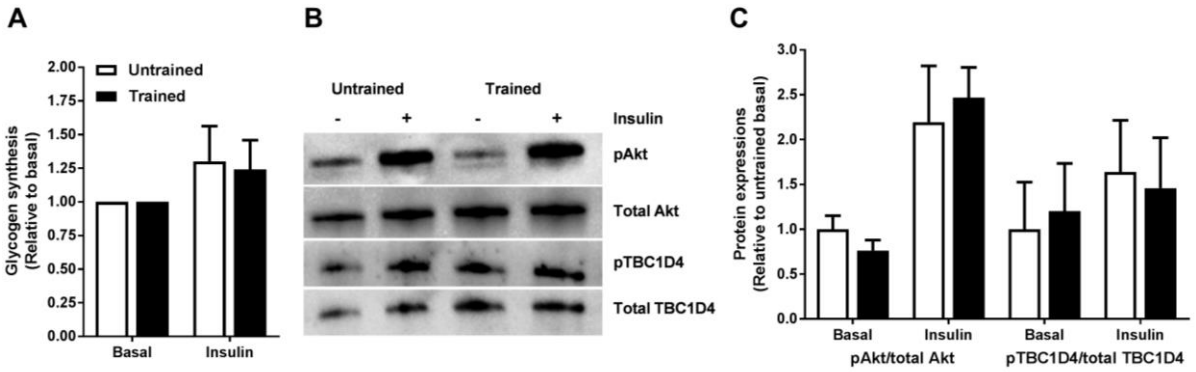

Figure 5. Effect of insulin on glycogen synthesis, Akt phosphorylation, and TBC1D4 phosphorylation in myotubes. Satellite cells isolated from biopsies from *musculus vastus lateralis* from the untrained and trained subjects were cultured and differentiated into myotubes. (A) Insulin-stimulated glycogen synthesis was measured as incorporation of 6.2 mmol/l [^{14}C(U)]glucose into glycogen in the presence or absence of 100 nmol/l insulin for 3

h. Values are presented as means ± SEM relative to basal (n = 6 in each group). (B and C) Akt phosphorylation and TBC1D4 phosphorylation by immunoblotting. Protein was isolated and total Akt, pAkt (Ser473), total TBC1D4, and pTBC1D4 (Thr642) expressions assessed by immunoblotting. (B) One representative immunoblot. (C) Quantified immunoblots relative to untrained basal. Values are presented as means ± SEM (n = 6 in each group).

Suppression of glucose oxidation by oleic acid in myotubes from trained and untrained subjects

Myotubes from trained subjects showed significantly higher suppression of glucose oxidation by oleic acid (Figure 6A). Overall the highest rate of carbohydrate oxidation *in vivo* correlated positively with oleic acid-induced suppression of glucose oxidation *in vitro* (r = 0.61, p = .04, n = 12 [combination of trained and untrained], Figure 6B). Etomoxir, which inhibits fatty acid oxidation by targeting the mitochondrial carnitine palmitoyltransferase 1 (CPT1, reviewed in (Rupp *et al.*, 2002)) reversed the oleic acid-induced suppression of fractional glucose oxidation and quenched the difference in glucose oxidation between the two donor groups (Figure 6C). There were no differences in mRNA expression of *CPT1A* and *CPT1B* (data not shown), and neither mRNA nor protein expression of PDK4, a key enzyme in regulating switching of fuel between glucose and fatty acids (Badin *et al.*, 2012), were significantly differently expressed (Figures 6D-6F,). mRNA expression of the mitochondrial gene *CYC1* as well as expression of proteins involved in oxidative phosphorylation, complex III and ATP synthase, were also similar between myotubes from the two groups (Figures 6D-6F). AMPK phosphorylation after oleic acid stimulation did not differ between the groups (Figures 6G and 6H).

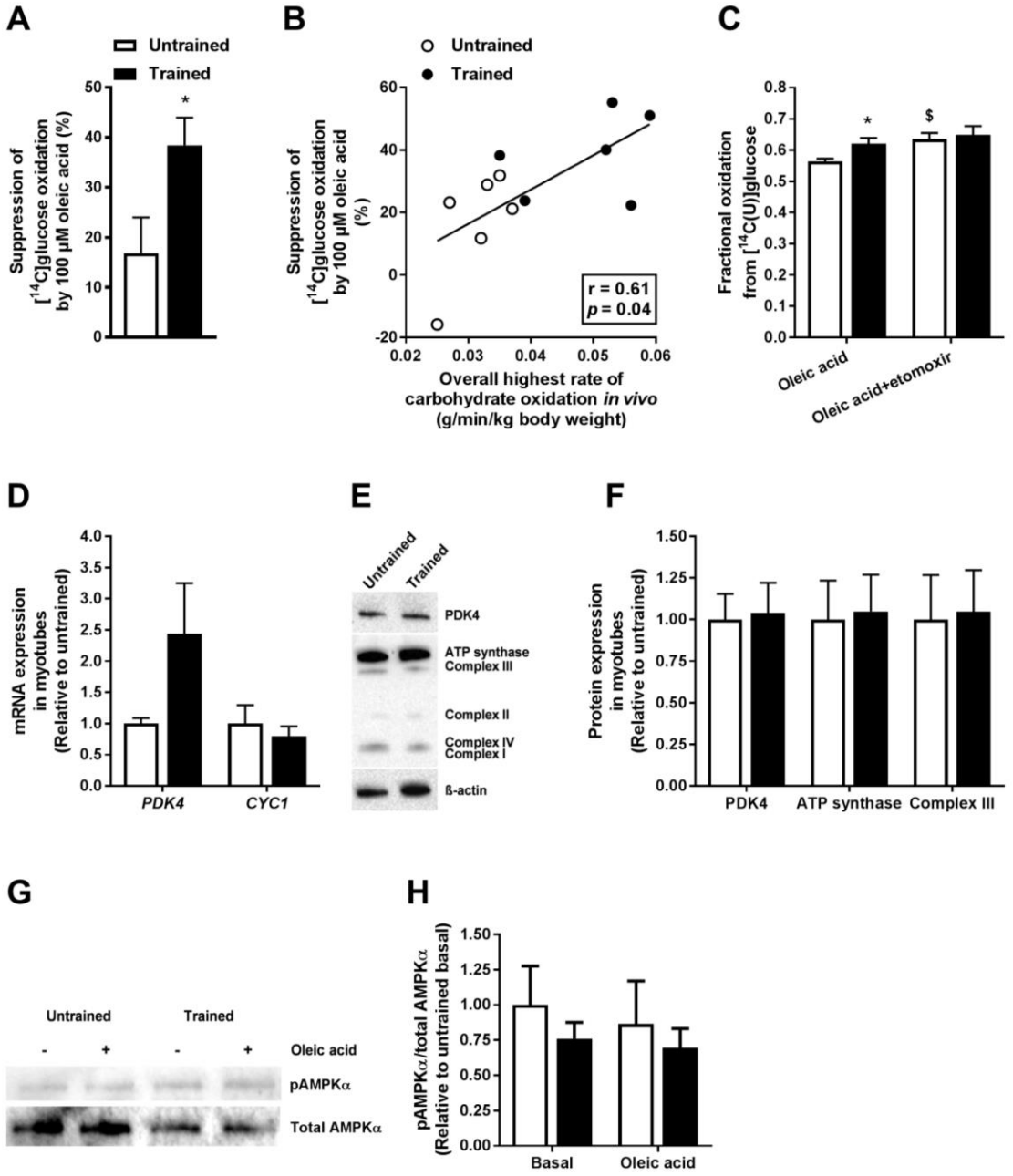

Figure 6. Oleic acid-induced suppression and effect of etomoxir on glucose oxidation in myotubes. Satellite cells isolated from biopsies from *musculus vastus lateralis* from the untrained and trained subjects were cultured and differentiated into myotubes. Glucose oxidation was examined after 4 h using 200 µmol/l [^{14}C(U)]glucose, where some parallels were treated with 100 µmol/l oleic acid and some parallels were treated with a combination of 100 µmol/l oleic acid and 10 µmol/l etomoxir (a CPT1-inhibitor that reduces fatty acid oxidation). Oxidation (CO_2) and fractional oxidation (CO_2/(CO_2+CA)) of [^{14}C(U)]glucose were determined. Values are presented as means ± SEM (n = 6 in each group). (A) Suppression of [^{14}C(U)]glucose oxidation by oleic acid, calculated as [(1-(oxidation of [^{14}C]glucose at 100 µmol/l oleic acid/oxidation of [^{14}C]glucose with no oleic acid added)) × 100%]. Statistically significant versus untrained (p<.05, Mann–Whitney test). (B) Spearman's test of correlation between overall highest rate of carbohydrate oxidation *in vivo* and suppression of glucose oxidation by oleic acid *in vitro* (n = 12). Spearman's correlation

coefficient, r = 0.61, and p = .04. (C) Effect of treatment with 100 µmol/l oleic acid and a combination of 100 µmol/l oleic acid and 10 µmol/l etomoxir on fractional glucose oxidation. Statistically significant versus untrained (p<.05, unpaired t test). $^{\$}$Statistically significant versus oleic acid (p<.05, unpaired t test). (D) mRNA expression of *PDK4* and *CYC1* by qPCR. RNA was isolated and mRNA reversely transcribed before expressions of *PDK4* and *CYC1* were assessed by qPCR. Values are presented as means ± SEM (n = 6 in each group). (E and F) Expression of PDK4 and OXPHOS complexes by immunoblotting. Protein was isolated and expression assessed by immunoblotting. (E) One representative immunoblot. (F) Quantified immunoblots relative to untrained. Values are presented as means ± SEM (n = 6 in each group). All samples were derived at the same time and processed in parallel. (G and H) AMPKα phosphorylation by immunoblotting in presence or absence of 100 µmol/l oleic acid. Protein was isolated and total AMPKα and pAMPKα (Thr172) expressions assessed by immunoblotting. (G) One representative immunoblot. (H) Quantified immunoblots relative to untrained basal. Values are presented as means ± SEM (n = 6 in each group). All samples were derived at the same time and processed in parallel.

Discussion

The main findings in the present study were that myotubes isolated from trained males with high VO_{2max} compared to sedentary males with low VO_{2max} showed a higher glucose metabolism, that is higher basal accumulation of deoxyglucose and higher fractional glucose oxidation compared to cells from untrained subjects. Furthermore, cells from trained subjects showed higher fatty acid-induced suppression of glucose oxidation than cells from untrained subjects, indicating that myotubes from trained subjects more efficiently can switch from carbohydrates to lipids as energy source when fatty acids are available.

Individual variations can be detected in the fibre-type composition of human skeletal muscles (for review, see (Schiaffino and Reggiani, 2011)). Analyses of muscle biopsies from monozygotic and dizygotic twins indicate that almost 50% of this variance is associated with genetic factors (Simoneau and Bouchard, 1995). However, competitive endurance-trained athletes tend to have a higher percentage of type I fibres, whereas sprinters tend to have a higher percentage of type II fibres (Gollnick *et al.*, 1972). In the present study, fibre-type distribution varied substantially in all three groups and there were no differences in fibre-type composition between muscle biopsies from trained, intermediary trained and untrained subjects. This may reflect that these subjects represented a heterogeneous group with respect to what kind of exercise they performed. In the present study, fibre-type distribution by immunohistochemistry and expression of myosin heavy chain was investigated on stain-free gels, and the two methods for determination of fibre-type distribution correlated. To the best of our knowledge, this is the first investigation of quantitative comparison between myosin heavy chain expression in myotubes and skeletal muscle biopsies. The three isoforms expressed in human skeletal muscle biopsies (MHCI, MHCIIa and MHCIIx) were expressed in the myotubes, but in addition at least three other bands were visible. This may possibly represent developmental forms of myosin as previously described by others (see for example

(Bonavaud *et al.*, 2001, Gaster *et al.*, 2001b)), highlighting the more limited ability of cultured myotubes to fully differentiate to a similar degree as skeletal muscles *in vivo*. In this study we did not do any attempt to determine which isoforms of myosin heavy chains that were present, however, in a study using cultured myotubes derived from other donors ((Feng *et al.*, 2014), microarray data reported to Gene Expression Omnibus, GSE40789), we found substantial mRNA expression of both the embryonic myosin *MYH3* and the neonatal myosin *MYH8* (developmental myosins, reviewed in (Schiaffino *et al.*, 2015)). A purpose of the present study was to investigate whether expression of MHC *in vivo* was maintained in myotubes; however, we did not observe any correlation between MHC expression in muscles and differentiated myotubes. Taken together, these findings demonstrate that myotubes differ from donor muscle with respect to MHC expression. Thus, our data suggest that the differentiation of satellite cells to muscle fibres is not predetermined.

We observed an overall higher carbohydrate oxidation in trained subjects *in vivo* compared to untrained subjects, whereas fat oxidation was higher in both trained and intermediary trained subjects *in vivo* compared to untrained subjects. Myotubes from trained subjects showed higher basal accumulation of deoxyglucose, but no differences in mRNA expression of *GLUT1* or *GLUT4*. Furthermore, there was no difference in basal glycogen synthesis between the two groups and insulin did not significantly stimulate glycogen synthesis. We have previously seen 30-100% increase in glycogen synthesis during insulin stimulation (Aas *et al.*, 2004, Feng *et al.*, 2014). The reason for this variation is not obvious, but may be related to different donor groups used in the various studies. We did not observe any differences in glycogen content between biopsies from the three groups in this study. Insulin increased Akt Ser473 phosphorylation as expected, but phosphorylation of TBC1D4 at Thr642 did not increase during insulin stimulation. Myotubes from trained subjects also

showed higher oxidation of glucose, reflecting the higher carbohydrate oxidation *in vivo* in these subjects.

Skeletal muscle uses both carbohydrates and fatty acids as fuel (Henriksson, 1995, Kelley *et al.*, 1990), and trained subjects have higher activities of mitochondrial enzymes for oxidation of both glucose and fatty acids (Spina *et al.*, 1996, Holloszy, 1974, Russell *et al.*, 2014). Furthermore, Phielix *et al.* observed higher mitochondrial capacity as measured by respirometry in muscle biopsies from endurance-trained athletes compared to untrained young subjects (Phielix *et al.*, 2012). In the present study we were unable to detect higher expression in the biopsies of any of the selected proteins involved in oxidation of glucose or fatty acids, which was surprising. However, it is worth to note that the trained subjects were selected according to VO_{2max} rather than amount or type of training. The trained subjects were mainly physical education students and trained as much strength as endurance, in average 2.7 and 3.1 workouts per week, respectively. Endurance-trained muscles have in particular higher capacity to oxidize lipids, whereas glucose oxidation is lower at the same absolute workload (Melanson *et al.*, 2009, Sidossis *et al.*, 1998). Ukropcova *et al.* was amongst the first to describe that metabolic flexibility was correlated with clinical phenotypes of the cells' donors, thus showing that the myotubes retained, at least to a certain extent, the characteristics of the donor, suggesting that defects in metabolic switching might be one of the main events in development of obesity and insulin resistance (Ukropcova *et al.*, 2005). Furthermore, metabolic flexibility has previously been described as the ability to adapt fuel oxidation by fuel availability and exercise intensity (Galgani *et al.*, 2008). To study metabolic flexibility *in vitro*, we incubated the myotubes with radiolabelled glucose with and without unlabelled oleic acid. We observed that myotubes from trained subjects were in fact more flexible, with higher suppression of glucose oxidation by acutely added oleic acid, and that the difference was quenched when inhibiting fatty acid oxidation by etomoxir (Rupp *et al.*,

2002). In the study of Bourlier *et al*. on myotubes from middle-aged, obese subjects after 8 weeks of exercise (Bourlier *et al.*, 2013), flexibility was enhanced in that the inhibition of palmitate oxidation by glucose was more effective after exercise. They partly explained this by an upregulation of *PDK4*, but this mechanism was not confirmed in the present study with myotubes from young healthy men. We did not observe any changes in mitochondria-related genes or proteins, neither in biopsies nor myotubes, to explain these differences, in line with Lund *et al.* (Lund *et al.*, 2017). However, the differences we found regarding glucose metabolism in the two donor groups were relatively small and possible changes in mRNA and protein may be difficult to detect. Boyle *et al.* hypothesized that human skeletal muscle cell cultures from obese, insulin resistant subjects would display inability to appropriately regulate mitochondrial respiration in response to lipid pre-incubation (Boyle *et al.*, 2012). They concluded that muscle cell cultures from obese humans inherently lacked metabolic flexibility in response to lipid exposure through an inability to increase mitochondrial respiration in presence of lipid substrate and perhaps also by an inability to induce mitochondrial proliferation (Boyle *et al.*, 2012). Kitzmann *et al.* compared myotubes from type 2 diabetics with myotubes from healthy subjects (Kitzmann *et al.*, 2011) and observed that the mitochondrial gene expression, content and network were similar in myotubes from the two groups. Further, basal palmitate β-oxidation was not affected in myotubes from the type 2 diabetics, whereas after pre-treatment with palmitate, type 2 diabetic myotubes but not healthy myotubes, showed an inability to increase the palmitate β-oxidation, indicating a mitochondrial dysfunction in the myotubes from the type 2 diabetics (Kitzmann *et al.*, 2011). Acetyl-CoA carboxylase (ACC) phosphorylation tended to be increased, although not statistically significant, after palmitate pre-treatment in healthy myotubes but not in type 2 diabetic myotubes, which may explain the inability of the type 2 diabetic myotubes to increase palmitate β-oxidation (Kitzmann *et al.*, 2011). Furthermore, treatment with the

AMPK activators AICAR and metformin did not decrease lipid content or affected phosphorylation of ACC or AMPK in the type 2 diabetic myotubes, but AICAR increased AMPK phosphorylation in healthy myotubes (Kitzmann *et al.*, 2011). Interestingly, both metformin and mitochondrial inhibition by antimycin altered the lipid content in the healthy myotubes (Kitzmann *et al.*, 2011). Recently, Fan *et al.* showed in mice myotubes that activation of PPARδ was sufficient to drive a higher fat oxidation and less glucose oxidation without any changes in muscle glycogen content, fibre-type expression or mitochondrial complex activities (Fan *et al.*, 2017).

The strength of the study is the possibility to investigate protein expression, *e.g.* MHC expressions, in both biopsies and myotubes established from the same biopsies. The weaknesses are the limited numbers and heterogeneity of participants in each group. The inclusion criteria for the untrained group were exercising less than 1 hour weekly the last years and $VO_{2max} \leq 46$ ml/kg/min. These were young untrained individuals and some of them had exercised on a regular basis previously (several years earlier), and we may thus not eliminate possible epigenetic marks from the periods where they were more physically active. For the trained group, subjects were athletes in different sports and trained both strength and endurance. With more homogeneous participants in each group possible differences might have been more clearly shown between the groups, both in biopsies and in cultured cells.

Conclusions

This study showed that despite the lack of correlation of MHC expressions between skeletal muscle biopsies and differentiated cultured skeletal muscle cells, myotubes retained some of their phenotypic traits *in vitro* with respect to higher glucose metabolism. For the first time we show that myotubes from trained subjects were more flexible when it came to exploit the

fuel source available, thus fatty acids inhibited glucose oxidation more effectively in myotubes from trained subjects, possibly explained by altered access for fatty acids to mitochondria.

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Declaration of interest

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