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Mitochondrial mass is inversely correlated to complete lipid oxidation in human myotubes

Michael Gaster *

Department of Endocrinology, Odense University Hospital, DK-5000 Odense, Denmark

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ABSTRACT

Exercise increases while physical inactivity decrease mitochondrial content and oxidative capacity of skeletal muscles *in vivo*. It is unknown whether mitochondrial mass and substrate oxidation are related in non-contracting skeletal muscle. Mitochondrial mass, ATP, ADP, AMP, glucose and lipid oxidation (complete and incomplete) were determined in non-contracting myotubes established from 10 lean, 10 obese and 10 subjects with type 2 diabetes precultured under normophysiological conditions. ATP, ADP, AMP, mitochondrial mass and energy charge were not different between groups. In diabetic myotubes, basal glucose oxidation and incomplete lipid oxidation were significantly increased while complete lipid oxidation was lower. Mitochondrial mass was not correlated to glucose oxidation or incomplete lipid oxidation in human myotubes but inversely correlated to complete lipid oxidation. Thus within a stable energetic background, an increased mitochondrial mass in human myotubes was not positive correlated to an increased substrate oxidation as expected from skeletal muscles *in vivo* but surprisingly with a reduced complete lipid oxidation.

1. Introduction

Mitochondria are the "powerhouse" of cells, generating energy through oxidation of various carbon substrates as glucose and lipids, in the tricarboxylic acid cycle (TCA), creating a trans membrane potential, powering the phosphorylation of ADP to ATP. The mitochondrial content and function are reduced in skeletal muscle *in vivo* with ageing, physical inactivity and metabolic diseases associated with insulin resistance, while exercising of skeletal muscle increases both the content and the oxidative capacity [1–6]. In contrast to *in vivo* we recently showed that the mitochondrial mass was not significantly different between myotubes established from lean, obese and T2D subjects [7,8] where the diabetic myotubes express an increased basal glucose oxidation and a reduced complete palmitate oxidation with a concomitant increased release of acid soluble metabolites [9–11]. Human myotubes in culture are non-contracting cells as they only seldom show spontaneous contractions [12]. Thus increasing mitochondrial mass seems *in vivo* to be positive correlated to an increased oxidative capacity, while this relationship is not as clear for human myotubes *in vitro*. The question arises whether mitochondrial mass in non-contracting human myotubes is related to its substrate oxidation. In the present study, we took advantage of our model of human myotubes to investigate whether the mitochondrial mass in myotubes established from lean, obese and T2D subjects correlates with their corresponding substrate oxidation of glucose and lipids. Moreover, the content of ATP, ADP, and AMP in human myotubes was determined and their energy charge calculated in order to estimate their energetic state.

2. Methods

2.1. Human study subjects

Ten lean, 10 obese control subjects, and 10 obese T2D patients participated in the study (Table 1) and their clinical characteristics have been published [13,14]. Muscle biopsies (200–300 mg) were obtained from the *vastus lateralis* muscle by needle biopsy under local anesthesia. Diabetic patients were treated with either diet alone or in combination with sulfonylurea, metformin or insulin withdrawn one week before the study. The patients suffered from no diabetic complications except for *simplex* retinopathy. The control subjects had normal glucose tolerance and no family history of diabetes. All subjects gave written informed consent, and the local ethics committee of Funen and Vejle County approved the study.

2.2. Materials

Dulbecco's modified Eagle's medium, fetal calf serum (FCS), penicillin–streptomycin–amphotericin B, and trypsin–EDTA were

* Fax: +45 65919653.
E-mail address: michael.gaster@ouh.regionsyddanmark.dk

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obtained from Invitrogen (Invitrogen, Scotland, UK). Ultroser G was purchased from Pall Biosepra (Cergy-Saint-Christophe, France). Protein assay kit was purchased from BioRad (Copenhagen, DK). Palmitic acid, L-carnitine, and ECM-gel were purchased from Sigma Chemical Co. (St. Louis, USA). Bovine serum albumin (BSA) (essentially FA free) was from Calbiochem (VWR, Roskilde, DK). Insulin Actrapid was from Novo Nordisk (Bagsvaerd, DK).

2.3. Cell culture

Cell cultures were established as previously described [12,15,16]. In brief, muscle tissue was minced, washed, and dissociated for 60 min by three treatments with 0.05% trypsin–EDTA. The cells harvested were pooled and FCS was added to stop trypsination. The cells obtained were seeded for up-scaling on ECM-gel coated dishes after 30 min of preplating. Cell cultures were established in DMEM medium supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1.25 μg/ml amphotericin B. After 24 h, cell debris and non-adherent cells were removed by change of growth medium to DMEM supplemented with 2% FCS, 2% Ultroser G, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1.25 μg/ml amphotericin B. Cells were subcultured twice before final seeding (4–6 weeks). At 75% confluence, the growth medium was replaced by basal medium (DMEM supplemented with 2% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml amphotericin B, and 25 pmol/l insulin) in order to induce differentiation. The cells were cultured in humidified 5% CO_2 atmosphere at 37 °C, and medium was changed every 2–3 days.

2.4. Experimental

Human myotubes established from lean, obese, and T2D subjects were allowed to differentiate under physiological conditions of insulin (25 pmol/l) and glucose (5.5 mmol/l). All myotube cultures were used for analysis day eight after onset of differentiation. Myotubes established from lean, obese, and T2D subjects were exposed to (a) 0.6 mmol/l PA followed by determination of complete, incomplete and total basal PA oxidation and protein content; (b) 5.0 mmol/l glucose followed by determination of basal glucose oxidation and protein content. (C) Mitochondrial mass and (d) intramyotubelar concentrations of ATP/ADP/AMP and protein level, PA:BSA ratio was 2.5:1. Control and treated myotubes were exposed for equal amount of fatty acid free BSA.

2.4.1. Glucose oxidation

Cells were cultured on 12.5 cm^2 flasks and differentiated as described above. Cultures were exposed to DMEM with 0.24 mmol/l fat free albumin, 50 U/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml amphotericin B, D-[14C] glucose (2.0 μCi/ml) and supplemented with glucose and PA as indicated above with 25 pmol/l insulin under basal condition or 1 μmol/l insulin during insulin stimulation. Flasks were air-tightened with a rubber stopper. After 4 h, 300 μl phenyl ethylamine-methanol (1:1, v/v) were added with a syringe to a centre well containing a folded filter paper. Three hundred microliters 1 M perchloric acid was subsequently added to the cells through the stopper tops by means of a syringe. The flasks were placed for a minimum of 1 h at room temperature to trap labeled CO_2. Cell-free flasks (no cell controls) went through the same procedure to correct for unspecific CO_2 trapping.

2.4.2. FA oxidation

Cells were cultured on 12.5 cm^2 flasks, differentiated, and treated as described above. Myotubes were exposed to DMEM supplemented with 0.24 mmol/l FA free albumin (BSA), 0.5 mmol/l L-carnitine, 20 mmol/l HEPES, [1-^{14}C]palmitic acid (2.0 μCi/ml) and supplemented with glucose and PA as indicated above with 25 pmol/l insulin under basal condition or 1 μmol/l insulin during insulin stimulation. Flasks were air-tightened with stopper tops. After 4 h, 300 μl phenyl ethylamine-methanol (1:1, v/v) were added with a syringe to a centre well containing a folded filter paper. Three hundred microliters 1 mol/l perchloric acid were subsequently added to the cells through the stopper tops using a syringe. The flasks were placed for a minimum of 1 h at room temperature to trap labeled CO_2. Cell-free flasks (no-cell controls) went through the same procedure to correct for unspecific CO_2 trapping. Measurements of acid soluble metabolites (ASM) were performed as described by Skrede et al. [17]. Total oxidation from endogenous or extracellular PA was determined as the sum of ASM and complete PA oxidation.

2.5. Atp/adp/amp

Acid soluble metabolites were extracted with tricloroacetic acid. Extracts were neutralized in Tris buffer (0.1 mol/l) [12] and ATP, ADP and AMP determined on a VICTOR Plate Reader model 1420-050 (PerkinElmer, Turku, Finland) with the ATP monitoring Reagent from Bio-Orbit as described by Kristensen et al. [18]. Energy charge of myotubes were calculated as: ([ATP] + ½[ADP])/([ATP] + [ADP] + [AMP]).

2.6. Mitochondrial mass

For quantification of mitochondrial mass, we used MitoTracker Green Probe (Molecular Probes, Eugene, OR) which preferentially accumulates in mitochondria regardless of the mitochondrial membrane potential and gives an assessment of the mitochondrial mass. Myotubes were incubated at 37 °C for 30 min with 100 nM Mitotracker Green in PBS and subsequently washed with PBS. Fluorescence intensity was determined on a VICTOR plate reader model 1420-050 (PerkinElmer, Turku, Finland) with excitation and emission wavelength of 485 and 535, respectively as described previously [18]. Values were corrected for protein, and indicated as arbitrary units.

2.7. Statistical analysis

Data in text, tables, and figures are given as mean ± SE. Statistical analyses were performed with SPSS (version 17.0). ANOVA test were used to assess significant differences between groups. Pearson correlation coefficient was used for covariance analysis. $P \leqslant 0.05$ was considered to be significant.

3. Results and discussion

Cultured myotubes offer a unique model to distinguish the impact of single factors on the oxidative metabolism of skeletal muscle i.e. oxidative capacity or mitochondrial content. Human myotubes in culture are non-contracting cells. Spontaneously contractions are first seen after 8–12 days of differentiation [12]. The mitochondrial mass of myotubes established from lean, obese and T2D subjects were determined day eight after induction of differentiation. There were no significantly differences between groups (Fig. 1), or differences in ranges of mitochondrial mass in myotubes established from lean (36896–105561 arb. units (min, max)), Obese (29896–89908 arb. units (min, max)) and T2D (26900–92560 arb. units (min, max)). The intramyocellular content of ATP, ADP, and AMP in myotubes established from the three study groups were not significantly different (Fig. 2A, B) neither were the calculated energy charge (Fig. 2C). Mitochondrial masses were not correlated over all to ATP ($r = -0.13$, $p = 0.82$, $N = 30$), ADP ($r = 0.02$, $p = 0.93$, $N = 30$), AMP ($r = -0.04$, $p = 0.79$, $N = 30$)