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Reduced TCA flux in diabetic myotubes: A governing influence on the diabetic phenotype?

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ABSTRACT

The diabetic phenotype is complex, requiring elucidation of key initiating defects. It is unknown whether the reduced tricarboxylic acid cycle (TCA) flux in skeletal muscle of obese and obese type 2 diabetic (T2D) subjects is of primary origin. Acetate oxidation (measurement of TCA-flux) was significantly reduced in primary myotube cultures established from T2D versus lean subjects. Acetate oxidation was acutely stimulated by insulin and respiratory uncoupling. Inhibition of TCA flux in lean myotubes by malonate was followed by a measured decline in; acetate oxidation, complete palmitate oxidation, lipid uptake, glycogen synthesis, ATP content and increased glucose uptake, while glucose oxidation was unaffected. Acute TCA inhibition did not induce insulin resistance. Thus the reduced TCA cycle flux in T2D skeletal muscle may be of primary origin. The diabetic phenotype of increased basal glucose uptake and glucose oxidation, the reduced complete lipid oxidation and increased respiratory quotient, are likely to be adaptive responses to the reduced TCA cycle flux.

Introduction

There is increasing evidence for a link between insulin resistance and impaired mitochondrial oxidative phosphorylation (OXPHOS) in human skeletal muscle *in vivo*. Consistently, several microarray-based studies of skeletal muscle have reported a coordinated down-regulation of OXPHOS genes in patients with type 2 diabetes (T2D) and high-risk individuals [1,2]. Transcriptional profiling of myotubes established from T2D subjects did not show evidence for a primary defect in OXPHOS genes [3]. Less attention has been given to the tricarboxylic cycle, in which the acetyl group of acetyl coenzyme A (acetyl-CoA) is oxidised to two CO_2 and four pairs of electrons transferred to coenzymes, which are utilised by the respiratory chain. Insulin resistant offspring of T2D patients show a reduced mitochondrial TCA cycle flux [4]. Recently Schrauwen and Hesselink [5] confirmed this observation in exercising T2D patients by revisiting previously published data on acetate recovery factor (ARF), which is based on oxidation of acetate. The ARF expression reflects the degree of recovery of acetate by oxidation. Acetate is either oxidised to CO_2 or lost in an exchange reaction of the TCA cycle [6]. Previously, we showed that diabetic myotubes expressed a 14% reduction in basal activity of citrate synthase (CS) compared to lean myotubes, implying a mild TCA cycle impairment [7]. To date, it is unknown whether the TCA cycle flux is primarily reduced in skeletal muscle of obese and obese type 2 diabetic subjects or whether the reduction reflects an adaptive response. Nor is it known whether the TCA flux is sensitive to insulin stimulation. The TCA flux can be measured by determining ^{14}C-acetate oxidation to $^{14}CO_2$ [5]. Our current knowledge of substrate oxidation in skeletal muscle originates mainly from *in vivo* studies. The oxidative capacity of skeletal muscle is highly influenced by physical activity, ageing, hormonal status, and fibre type composition, rendering it difficult to determine the contribution of single factors to the alteration in oxidative metabolism. Cultured myotubes offer a unique model to distinguish between genetic and environmental factors in the aetiology of insulin resistance and T2D [8–11]. In the present study, we took advantage of this model to investigate acetate oxidation at baseline and during insulin stimulation in myotubes established from lean, obese and T2D subjects.

Materials and methods

Materials. Dulbecco's modified Eagle's medium, fetal calf serum (FCS), penicillin–streptomycin–amphotericin B, and trypsin–EDTA were obtained from Invitrogen (Invitrogen, Scotland, UK). Further reagents (and suppliers) were as follows: Ultroser G (Pall Biosepra, Cergy-Saint-Christophe, France). [1-^{14}C]-acetic acid,

Abbreviations: ARF, acetate recovery factor; CS, citrate synthase; OAA, oxaloacetate; OXPHOS, oxidative phosphorylation; PC, pyruvate carboxylase; TCA, tricarboxylic acid cycle; T2D, type 2 diabetes.

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[^{14}C(U)]-Glucose, [1-^{14}C]-palmitate, 2-[1-^{14}C]-deoxy-glucose and ATP kit (Perkin-Elmer, Boston, USA). Protein assay kit (BioRad, Copenhagen, DK). Sodium acetate, 2,4-Dinitrophenol, Sodium malonate, L-carnitine, and ECM-gel (Sigma Chemical Co., St. Louis, USA). Bovine serum albumin (BSA) (essentially fatty acid free) (Calbiochem, VWR, Roskilde, DK). Insulin Actrapid was from Novo Nordisk (Bagsvaerd, DK).

Human study subjects. Ten lean, ten obese control subjects, and ten obese T2D patients participated in the study (Table 1). Their clinical characteristics have been published [7,12]. Muscle biopsies were obtained from the *vastus lateralis* muscle by needle biopsy under local anesthesia. Diabetic patients were treated either with 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.

Cell culture. Cell cultures were established as previously described [13,14]. In brief, muscle tissue was minced, washed and dissociated for 60 min by three treatments with 0.05% trypsin–EDTA. The cells obtained were seeded for up-scaling on ECM-gel coated dishes after 30 min of preplating. Growth medium contained 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. 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.

Experimental design. 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) for 4 days. Differentiated myotubes were exposed to two different protocols: (I) Determination of acetate oxidation day 4 at an external acetate concentration of 0.1 mmol/l at baseline (5.5 mmol/l glucose and 25 pmol/l Insulin) and during acute insulin stimulation (5.5 mmol/l glucose and 1 μmol/l insulin) with/without respiratory uncoupling by 1.0 mmol/l 2,4-Dinitrophenol (DNP) [15]; (II) Myotubes were harvested day 4 at basal condition for determination of enzyme activities. (III) Acetate oxidation (Aox), glucose uptake (GT) and glucose oxidation (Gox), glycogen synthesis (GS), lipid uptake (Lup), and complete palmitate oxidation (Lox) were determined at baseline and during acute insulin stimulation with/without inhibition of the TCA cycle by 5.0 mmol/l malonate (competitive inhibitor of succinate dehydrogenase, [16]) in myotubes established from lean subjects.

Substrate oxidation. Glucose, palmitate and acetate oxidation was determined by a 96 multi-well tracer technique as previous described [11]. Substrate oxidation was monitored by incubating myotubes with [1-^{14}C]-acetate (1.0 μCi/ml) in a final concentration of acetate of 0.1 mmol/l, [1-^{14}C]-Palmitate (2.0 μCi/ml) in a final concentration of 0.4 mmol/l palmitate and [^{14}C(U)]-glucose (2.0 μCi/ml) in a final concentration of 5.0 mmol/l glucose with subsequent capture of liberated ^{14}CO2 for 4 h at 37 °C. Trapped radioactivity was determined on a Microbeta counter (Perkin–Elmer, Finland).

Glucose and lipid uptake. Glucose uptake was measured by capturing 2-[1-^{14}C]-deoxy-glucose and lipid uptake was measured as the incorporation of [1-^{14}C]-Palmitate (2.0 μCi/ml) as previously described [10,12]. Radioactivity was determined on a Microbeta counter (Perkin-Elmer, Finland).

Glycogen synthesis. Glycogen synthesis was measured as previous described [10] in 96 well plates. Radioactivity was measured with a Microbeta counter (Perkin-Elmer, Finland).

ATP. ATP was determined as previous described [15] in 96 well plates and determined by Luminescence on a Microbeta counter (Perkin-Elmer, Finland).

Enzymes. The activity of the following enzymes was determined in accordance to Lowry et al. [17]: Phosphofructo kinase (PFK, EC 2.7.1.11), Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, EC 1.2.1.12), Phosphoglycerate-kinase (PGK, EC 2.7.2.3), Pyruvate kinase (PK, EC 2.7.1.40), Lactate-dehydrogenase (LDH, EC 1.1.1.27), Pyruvate-carboxylase (PC, EC 6.4.1.1).

Statistical analysis. The data in the text, tables, and figures are given as mean ± SEM. The statistical analyses were performed with INSTAT 2.01 (GraphPad, USA). $p \leqslant 0.05$ was considered to be significant.

Table 1
Clinical characteristics of the study subject.

	Control, lean	Control, obese	T2D
N	10	10	10
Age (years)	51 ± 1	49 ± 1	50 ± 1
Weight (kg)	71.6 ± 3.0	105.5 ± 6.4*	102.2 ± 4.1*
BMI (kg/m^2)	24.2 ± 0.5	33.7 ± 1.4*	33.5 ± 1.1*
Fasting plasma glucose (mM)	5.7 ± 0.1	5.7 ± 0.2	10.0 ± 0.7#
Fasting serum insulin (pM)	24.3 ± 5.7	52.7 ± 5.0*	94.6 ± 10.1#
Glucose infusion rate (mg/min)	383.3 ± 20.4	257.9 ± 28.3*	117.8 ± 18.6#
HbA_{1c} (%)	5.5 ± 0.1	5.4 ± 0.1	7.7 ± 0.5#
Fasting total cholesterol (mM)	5.29 ± 0.22	5.43 ± 0.41	5.42 ± 0.37
Fasting LDL cholesterol (mM)	2.94 ± 0.22	3.33 ± 0. 33	3.20 ± 0.27
Fasting HDL cholesterol (mM)	1.85 ± 0.15	1.48 ± 0.15	1.36 ± 0.03*
Fasting plasma triglyceride (mM)	1.12 ± 0.16	1.35 ± 0.18	1.93 ± 0.40

Data are means ± SE.
* Significant different from the lean controls ($p < 0.05$).
# Significant different from the lean and obese controls ($p < 0.05$).

Results and discussion

In the present study we measured acetate oxidation in myotubes established from lean, obese and obese T2D subjects (Fig. 1A). Diabetic myotubes express a significantly lower acetate oxidation (30%) compared to myotubes established from lean subjects, both at basal state and during acute insulin stimulation ($p < 0.05$). The insulin induced increment in acetate oxidation (fold change) tended to be lower in diabetic myotubes but did not reach significance between groups (Fig. 1B). The oxidation of acetate during exercise is dependent on the metabolic rate [18]. In line we found that acetate oxidation was significantly increased (foldchange) ($p < 0.05$) in myotubes exposed to respiratory uncoupling by the ionophor DNP (Fig. 1C), without significantly differences between groups ($p > 0.05$). A reduced TCA cycle flux has been shown in insulin resistant offspring of T2D patients [4] and exercising T2D patients *in vivo* [5]. Thus, the reduced TCA flux *in vivo* could be primary in origin. A primary reduced TCA cycle flux is a new characteristic of the diabetic phenotype in myotubes established from T2D subjects, which comprises an increased basal and insulin mediated glucose uptake, reduced to normal basal glycogen synthesis and reduced insulin mediated glycogen synthesis, increased basal glucose oxidation and insulin stimulated glucose oxidation compared to myotubes established from lean subjects [10,12,15,19]. Lipid uptake and incorporation was not primarily effected while lipid oxidation was reduced based on a mismatch between the TCA flux and beta-oxidation in diabetic myotubes [9,11,12,15]. Insulin resistance has been described for glucose uptake, glucose oxidation and glucose storage [8,10,12,15,19]. The question arises whether a reduced TCA flux may explain some of the other characteristic of the diabetic phenotype, as the TCA is integrating glucose and lipid