Biochimica et Biophysica Acta 1812 (2011) 94–105

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Chronic hyperglycemia reduces substrate oxidation and impairs metabolic switching of human myotubes

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ARTICLE INFO

Article history:
Received 3 July 2010
Received in revised form 14 September 2010
Accepted 28 September 2010
Available online 1 October 2010

Keywords:
Myotubes
Skeletal Muscle
Energy metabolism
Mitochondria

ABSTRACT

Skeletal muscle of insulin resistant individuals is characterized by lower fasting lipid oxidation and reduced ability to switch between lipid and glucose oxidation. The purpose of the present study was to examine if chronic hyperglycemia would impair metabolic switching of myotubes. Human myotubes were treated with or without chronic hyperglycemia (20 mmol/l glucose for 4 days), and metabolism of [^{14}C]oleic acid (OA) and [^{14}C]glucose was studied. Myotubes exposed to chronic hyperglycemia showed a significantly reduced OA uptake and oxidation to CO_2, whereas acid-soluble metabolites were increased compared to normoglycemic cells (5.5 mmol/l glucose). Glucose suppressibility, the ability of acute glucose (5 mmol/l) to suppress lipid oxidation, was 50% in normoglycemic cells and reduced to 21% by hyperglycemia. Adaptability, the capacity to increase lipid oxidation with increasing fatty acid availability, was not affected by hyperglycemia. Glucose uptake and oxidation were reduced by about 40% after hyperglycemia, and oxidation of glucose in presence of mitochondrial uncouplers showed that net and maximal oxidative capacities were significantly reduced. Hyperglycemia also abolished insulin-stimulated glucose uptake. Moreover, ATP concentration was reduced by 25% after hyperglycemia. However, none of the measured mitochondrial genes were downregulated nor was mitochondrial DNA content. Microarray and real-time RT-PCR showed that no genes were significantly regulated by chronic hyperglycemia. Addition of chronic lactate reduced both glucose and OA oxidation to the same extent as hyperglycemia. In conclusion, chronic hyperglycemia reduced substrate oxidation in skeletal muscle cells and impaired metabolic switching. The effect is most likely due to an induced mitochondrial dysfunction.

1. Introduction

Metabolic flexibility is the capacity of the body to switch between lipid oxidation in the fasting state to carbohydrate oxidation in the fed state and vice versa, i.e. the capacity to adjust fuel consumption according to availability [1]. Insulin resistance and type 2 diabetes (T2D) have been associated with impaired ability to switch between lipid and glucose oxidation [2,3], and it has been postulated that this impaired metabolic switching is an intrinsic property of skeletal muscle [4]. Ukropcova et al. [4] showed that the metabolic phenotype of the donor was maintained in myotube cultures after removal of *in vivo* neuroendocrine factors. However, metabolic flexibility *in vivo* has been shown to improve by weight loss [3], indicating that lifestyle factors can affect metabolic flexibility. We recently showed that treatment with n-3 fatty acids improved metabolic switching of myotubes [5]. Whether exposure to chronic hyperglycemia affects metabolic flexibility of skeletal muscle is at present unknown.

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ASM, acid soluble metabolites; B2M, beta-2 microglobulin; ChREBP, carbohydrate responsive element binding protein; CPT, carnitine palmitoyl transferase; CYC, cytochrome *c*; DAG, diacylglycerol; DGAT, acyl-CoA:1.2-diacylglycerol acyltransferase; DOG, deoxyglucose; DNP, dinitrophenol; ECM, extracellular matrix; ETS, electron transport system; FCCP, carbonylcyanide-4-trifluoromethoxyphenylhydrazone; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; HG, hyperglycemia; IMCL, intramyocellular lipids; LCA-CoA, long chain fatty acyl-CoA; LMM, linear mixed model; mtDNA, mitochondrial DNA; ND, NADH-ubiquinone oxidoreductase; NG, normoglycemia; OA, oleic acid; PDK, pyruvate dehydrogenase kinase; SCD, stearoyl-CoA desaturase; SPA, scintillation proximity assay; TAG, triacylglycerol; UCP, uncoupling protein

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doi:10.1016/j.bbadis.2010.09.014

There are numerous reports on the effect of environmental and dietary factors on development of intramuscular lipid accumulation and insulin resistance. Skeletal muscle of obese and insulin-resistant subjects is characterized by increased intramyocellular lipids (IMCL) and reduced mitochondrial oxidative capacity [6,7]. We have previously shown that myotubes from type 2 diabetic subjects have reduced fatty acid oxidation after culturing in a non-diabetic environment, implying a genetic defect [8]. Experiments in rats confirmed that there is an inherited connection between oxidative capacity and a diabetic phenotype, as rats bred to obtain low aerobic capacity were insulin resistant and displayed a diabetic metabolic profile [9]. These animals also expressed reduced level of several proteins involved in mitochondrial biogenesis, suggesting a genetic association between mitochondrial dysfunction and insulin resistance. Several studies have shown reduced mitochondrial content and/or mitochondrial dysfunction in skeletal muscle of T2D subjects [10–12]. There is substantial support for the hypothesis that reduced oxidative capacity might be the fundamental cause of IMCL accumulation and insulin resistance [11,13]. However, the possibility that mitochondrial dysfunction rather is the consequence of insulin resistance cannot be excluded.

In rat, chronic glucose infusion induced insulin resistance in skeletal muscle concurrent with increased triacylglycerol (TAG), malonyl-CoA and long-chain fatty acyl-CoA (LCA-CoA) levels in muscle [14]. Lipid intermediates, such as fatty acyl-CoA, ceramides and diacylglycerol, are linked to muscle insulin resistance both *in vivo* and in several *in vitro* models [15]. Glucose oversupply has also been associated with a range of metabolic changes, such as increased lactate production [16,17], and it has been shown that diabetic and obese patients have increased plasma lactate levels [18,19]. The role of lactate is not known, but it is most likely more complex than being a simple waste product of metabolism (reviewed in [20,21]). It has been hypothesized that lactate is a signaling molecule directing metabolic activity. In muscle, lactate inhibits 6-phosphofructo-1-kinase and consequently decreases glucose consumption [22].

Chronic hyperglycemia (HG) has been shown to contribute to insulin resistance in skeletal muscle [23]. The mechanism, however, by which HG induces insulin resistance is not clear. We have previously shown that treatment of human myotubes with chronic HG reduced acute glucose uptake and glycogen synthesis. This reduction accompanied accumulation of TAG in the cells, an increased *de novo* lipogenesis and increased acyl-CoA:1.2-diacylglycerol acyltransferase (DGAT) activity, whereas total cell content of glycogen was unchanged [24]. The effect of HG was maximal after 4 days and reversible, at least when it came to glycogen synthesis [24]. Exposure of 3T3-L1 adipocytes to HG has been found to induce insulin resistance and loss of mitochondrial membrane potential [25]. Moreover, mitochondria became smaller and more compact, whereas mitochondrial DNA was unaffected by HG [25], indicating that HG can induce mitochondrial dysfunction. Therefore, we hypothesized that exposure to chronic HG induces some kind of mitochondrial dysfunction leading to impaired metabolic switching of myotubes.

2. Experimental

2.1. Materials

L-Glutamine, penicillin/streptomycin (10,000 IE/10 mg/ml), HEPES, amphotericin B, L-carnitine, sodium L-lactate, dinitrophenol (DNP), carbonylcyanide-4-trifluoromethoxyphenylhydrazone (FCCP), oligomycin and extracellular matrix (ECM) gel were from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium low glucose (DMEM), DMEM-Glutamax, foetal calf serum (FCS), Dulbecco's Phosphate Buffered Saline (DPBS) and trypsin/EDTA (0.05%) were from Gibco/Invitrogen (Grand Island, NY, USA). Ultroser G was from Pall Corporation (St-Germain-en-Laye Cedex, France) and lactic acid was from Apotekproduksjon AS (Oslo, Norway). D-[6-^{14}C]glucose (56 mCi/mmol–2.1 GBq/mmol), D-[1-^{14}C]glucose (54 mCi/mmol–2.0 GBq/mmol), D-[^{14}C(U)]glucose (10 mCi/mmol–0.37 GBq/mmol), [1-^{14}C]oleic acid (53 mCi/mmol–2.0 GBq/mmol) and L-[^{14}C(U)]leucine (318 mCi/mmol–11.766 GBq/mmol) were provided by either by American Radiolabeled Chemicals Inc. (St. Louis, MO, USA) or by NEN Radiochemicals, PerkinElmer (Boston, MA, USA). Insulin Actrapid® was from Novo Nordisk (Bagsvaerd, Denmark). The antibodies against AMPK (#2532), phosphorylated AMPK (Thr172, #2531), ACC (#3662) and phosphorylated ACC (Ser79, #3661) were from Cell Signaling Technology (Beverly, MA, US). The pyruvate dehydrogenase kinase (PDHK) inhibitor AZD7545 was a kind gift from CVGI Research Area, AstraZeneca (Alderley Park, UK). Corning® CellBIND® microplates were from Corning B.V. Life Sciences (Schipol-Rijk, The Netherlands), 96-well UNIFILTER® microplate from Whatman (Middlesex, UK), and 96-well Wallac Isoplates, TopSeal for 96-well microplates and the scintillation liquid Optiphase Supermix was from PerkinElmer (Waltham, MA, USA). The ATPlite 1 step kit was from PerkinElmer Life and Analytical Sciences (Shelton, CT, USA). Bio-Rad Protein Assay Dye Reagent was from Bio-Rad Laboratories (NY, USA). Thin layer chromatography (TLC) plates (Silica gel) were from Merck (Darmstadt, Germany). The primers for CD36, FAS, SCD-1, ACC, ChREBP, PDK-4, CYC1, CPT-1A, CPT-1B, UCP-2 and UCP-3 and the housekeeping genes GAPDH and 36B4 were provided by Invitrogen (Carlsbad, CA, USA). SYBR green and TaqMan reverse transcription kit reagents were obtained from Applied Biosystems (Warrington, UK). Agilent Total RNA isolation kit was purchased from Agilent Technologies (Santa Clara, CA, USA). Primers and probes for ND1 and B2M and qPCR MasterMix Plus Low ROX were from Eurogentec (Seraing, Belgium). Puregene DNA isolation kit was from Gentra Systems, Qiagen (Germantown, MD, USA).

2.2. Human skeletal muscle cell cultures

Satellite cells were isolated from the *Musculus obliquus internus abdominis* of 8 healthy donors, age 39.9 (±2.9) years, body mass index 23.5 (±1.4) kg/m^2, fasting glucose 5.3 (±0.2) mmol/l, insulin, plasma lipids and blood pressure within normal range and no family history of diabetes. The biopsies were obtained with informed consent and approval by the National Committee for Research Ethics, Oslo, Norway. The cells were cultured in DMEM-Glutamax (5.5 mmol/l glucose) with 2% FCS, 2% Ultroser G, penicillin/streptomycin (P/S) and amphotericin B until 70–80% confluence. Myoblast differentiation to myotubes was then induced by changing medium to DMEM-Glutamax (5.5 mmol/l glucose) with 2% FCS, 34 pmol/l insulin, P/S and amphotericin B. Experiments were performed after 8 days of differentiation, and pre-incubation with hyperglycemia (20 mmol/l glucose) was started after 4 days.

2.3. Substrate oxidation assay

The muscle cells were cultured on 96-well CellBIND® microplates as described above. Growth medium was completely removed before addition of substrates. Substrate, [1-^{14}C]glucose (37 kBq/ml), [6-^{14}C]glucose (37 kBq/ml), [^{14}C(U)]glucose (37 kBq/ml) or [1-^{14}C]oleic acid (37 kBq/ml), was given in DPBS with 10 mmol/l HEPES and 1 mmol/l L-carnitine (only added with oleic acid). Oleic acid was bound to BSA at a ratio of 2.5/1. A 96-well UNIFILTER® microplate was mounted on top of the CellBIND® plate as described before [26], and the cells were incubated at 37 °C for 4 h. The CO_2 trapped in the filter was counted by liquid scintillation in a MicroBeta™ Trilux scintillation counter (PerkinElmer). The remaining cell-associated radioactivity was also assessed by liquid scintillation, and the sum of CO_2 and cell-associated radioactivity was considered as total substrate uptake. Oxidation of [1-^{14}C]oleic acid to acid-soluble metabolites (ASM) was measured by acidic precipitation of the incubation media. CO_2 production from [1-