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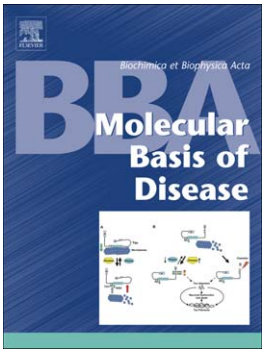

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Chronic hyperglycemia reduces substrate oxidation and impairs metabolic switching of human myotubes

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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 conclusions, chronic hyperglycemia reduced substrate oxidation in skeletal muscle cells and impaired metabolic switching. The effect is most likely due to an induced mitochondrial dysfunction.

Keywords:

Myotubes, Skeletal Muscle, Energy metabolism, Mitochondria

Abbreviations:

ACC; acetyl-CoA carboxylase, AMPK; AMP-activated protein kinase, ASM; acid soluble metabolites, B2M; beta-2 microglobulin, ChREBP; charbohydrate 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.

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 showed that the metabolic phenotype of the donor was maintained in myotube cultures after removal of *in vivo* neuroendocrine factors [4]. 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.

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 can not 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 micoplates 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 *M. 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-^{14}C]oleic acid was measured in the presence or absence of various compounds known to modify mitochondrial function; dinitrophenol (DNP 100 µmol/l) and carbonylcyanide-4-trifluoromethoxyphenylhydrazone (FCCP 0.6 mmol/l) (both mitochondrial uncouplers), and oligomycin (ATP synthase inhibitor, 1 µg/ml). From these experiments we calculated baseline (routine) oxidation (R) and uncoupled oxidation with FCCP (E), as well as baseline oxidation in the presence of

oligomycin (L) [27]. The net routine flux control ratio was calculated as netR=(R-L)/E. Protein content in each well was determined [28], and the data are presented as CO_2/mg protein, cell-associated substrate/mg protein or ASM/mg protein.

2.4. Lipid distribution

Myotubes were incubated with [1-^{14}C]oleic acid (18.5 kBq/ml, 0.2 mmol/l) for 4 h before they were harvested into ice-cold water, centrifuged (1000 g, 5 min), resuspended in distilled water and sonicated. Cell-associated lipids were extracted with chloroform:methanol and separated by thin layer chromatography as described by Folch et al. [29].

2.5. RNA isolation and analysis of gene expression by real-time RT-PCR

Human skeletal muscle cells were washed, trypsinized and pelleted before total RNA was isolated using Agilent Total RNA isolation kit (Agilent Technologies, Santa Clara, CA, USA) according to the supplier's protocol. Total RNA (0.1 µg/µl) was reversely transcribed with oligo primers using a PerkinElmer Thermal Cycler 9600 (25°C for 10 min, 37°C for 1 h, 99°C for 5 min) and a TaqMan reverse-transcription reagents kit (Applied Biosystems, Warrington, UK). Real-time PCR was performed using an ABI PRISM® 7000 Detection System (Applied Biosystems, Warrington, UK). DNA expression was determined by SYBR® Green, and primers were designed using Primer Express® (Applied Biosystems, Warrington, UK) (sequences in Table 1). Each target were quantified in triplicates and carried out in a 25 µl reaction volume according to the supplier's protocol. All assays were run for 40 cycles (95°C for 12 s followed by 60°C for 60 s). The transcription levels were normalized to the reference genes GAPDH and 36B4.

2.6. Microarray

Total RNA was prepared from primary myotubes from three donors using Agilent Total RNA isolation kit according to the supplier's protocol (Agilent Technologies). RNA was used individually and RNA integrity was checked on chip analysis (Agilent 2100 bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. RNA was judged as suitable for array hybridization only if samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number > 9.0). cRNA synthesis was performed using Illumina TotalPrep RNA Amplification (San Diego, CA, USA) according to

the supplier's protocol. Hybridization, washing, and scanning of Illumina Human-6 Express BeadChips version 3 arrays (> 48 000 probes) were according to standard Illumina protocols (San Diego, CA, USA). Data extraction and quality control was performed using BeadStudio version 3.1.3.0 (Illumina) and the Gene Expression module 3.2.7. Arrays were normalized using quantile normalization, and expression estimates were calculated by GC robust multiarray average background adjustment. Fold change (FC) was calculated as log2-transformed expression level after HG treatment divided by log2-transformed expression level after NG treatment (control), followed by identification of differentially expressed probe sets using Intensity-based moderated t-statistics [30]. P-values were corrected for multiple testing by using Benjamini and Hochberg's false discovery rate (FDR) method [31]. Probe sets that satisfied the criterion of FDR < 10 % (q-value < 0.1) and fold-change >1.2 or < -1.2, were considered to be significantly regulated. GEO accession number GSE19620.

2.7. Mitochondrial DNA copy measurement

DNA was isolated from whole cell lysates by PureGene DNA isolation kit (Qiagene, Germantown, MD, USA). Quantification of mitochondrial DNA (mtDNA) relative to nuclear DNA (nDNA) was done by real-time qPCR with TaqMan probes using a Stratagene 3000mxp. Primer and probe sequences were taken from He et al [32], Bogacka et al [33] and Bai et al [34], and confirmed with Primer3. Each target were quantified in triplicates and carried out in a 25 µl reaction volume (12.5 µl 2 x qPCR mix (dNTP, HotGoldStar DNA polymerase, $MgCl_2$ (5 mmol/l), ROX), 0.75 µl fwd primer (300 nmol/l), 0.75 µl rew primer (300 nmol/l), 0.5 µl probe (200 nmol/l), 5 µl template (10 ng), 5.5 µl H_2O). All assays were run for 95ºC in 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. The threshold cycle number (Ct) was used for calculating the relative copy number (Rc) by expressing differences in Ct of the mtDNA and nDNA qPCR: $Rc = 2^{\Delta Ct}$ [35-36].

2.8. Immunoblotting

Aliquots with 20 µg cell protein (total cell lysate prepared in Laemmli buffer) were separated by SDS-PAGE (10 % resolving gel) and transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with AMPK (1:1000), phospho-AMPK (1:1000), ACC (1:1000) and phospho-ACC (1:1000) antibodies (Cell Signaling Technology, Beverly, MA, US) over night. Immunoreactive bands were visualized with enhanced chemiluminescence

(ECL) (Amersham Biosciences, Buckinghamshire, UK), and quantified with Gel-Pro Analyzer (version 2.0) software.

2.9. Intracellular ATP and lactate concentrations

Intracellular concentration of ATP in NG and HG cells was measured with ATPlite 1step kit from PerkinElmer Life and Analytical Sciences (Shelton, CT, USA). Luminescence was detected with Victor^3 Wallac 1420 multilabel counter (PerkinElmer). ATP concentration was quantified according to a standard curve and given as μM ATP/mg cell protein. DNP (100 μmol/l) which was added as a control, reduced ATP concentration by 30 %.

Cell medium lactate concentration was measured with a radiometer ABL 715 blood gas analyzer (Diamond Diagnostics, Holliston, MA, USA). Medium from 6 well plates were harvested after 48 h and analyzed without dilution.

2.10. Protein synthesis

The cells were grown in CytoStar-T scintillating microplates and incubated with [^{14}C(U)]leucine (37 kBq/ml, 0.8 mmol/l) on day 8 after initiated differentiation. Incorporation of [^{14}C]leucine was followed for 10 h with scintillation proximity assay (SPA) technology. Uptake and accumulation of leucine in cells treated with hyperglycemia (20 mmol/l glucose for 4 days) were compared to normoglycemic control cells (5.5 mmol/l glucose).

2.11. Statistics

All data are presented as mean ± SEM. Statistical comparison between different treatments was performed by Student's t-test and linear mixed models (LMM) analysis (SPSS ver. 16.0). The parameter of interest was entered as the dependent variable and pretreatment (HG and NG) and acute treatments were entered as fixed variables. Differences were considered statistically significant at $p<0.05$. All experiments were performed with at least triplicate observations, and replicate experiments are performed on cells from different donors and given as n.

3. RESULTS

3.1. Effect of acute glucose on oleic acid oxidation

Simultaneous accumulation and oxidation of oleic acid (OA) was determined in human skeletal muscle cells (myotubes) as previously described [26]. The suppressive effect of acute addition (4 h during CO_2 trapping) of glucose on [1-^{14}C]OA oxidation was evident at 0.5 mmol/l glucose (Fig. 1), and at 5 mmol/l glucose OA oxidation was suppressed by 53 %. OA oxidation was not significantly suppressed by acute addition of 5 mmol/l deoxyglucose (DOG), 1.43 (± 0.83) nmol/mg protein of CO_2 was trapped in absence and 1.07 (± 0.55) nmol/mg protein in presence of 5 mmol/l DOG ($p=0.31$, $n=3$), indicating that the suppressive effect of glucose on OA oxidation is due to some downstream metabolites in the glycolytic pathway.

3.2. Effect of chronic hyperglycemia on oleic acid metabolism

To examine whether chronic hyperglycemia affects the suppressive effect of glucose on OA metabolism, myotubes were pretreated with hyperglycemia (HG, 20 mmol/l glucose) for 4 days and OA (0-300 µmol/l) metabolism was measured in the absence and presence of acute glucose (5 mmol/l) (Fig. 2). As shown in Fig.1, acute glucose (5 mmol/l) suppressed OA-oxidation to CO_2 by 50 % in normoglycemic cells (Fig. 2A, $p<0.0005$ overall effect), whereas this suppressive effect of acute glucose was not significant in hyperglycemic cells (Fig. 2B). Chronic exposure to HG reduced complete oxidation of OA to CO_2 by 47 % compared to exposure to NG (Fig. 2A and B, $p<0.0005$, overall effect), indicating that oxidation was already suppressed after chronic HG exposure. Incomplete OA oxidation to acid-soluble metabolites (ASM) was however increased by 22 (± 8) % in HG cells compared to NG cells ($p=0.02$, data not shown). ASM consist mainly of tricarboxylic acid cycle metabolites and reflect incomplete oxidation of fatty acids. Acute glucose did not suppress ASM production, neither in NG nor HG cells (data not shown).

Cellular uptake of OA in NG cells, assessed as the sum of cell-associated and CO_2-trapped radioactivity, increased with increasing concentrations of OA (20-300 µmol/l) ($p<0.0005$) and in presence of glucose ($p<0.0005$) (Fig. 2C). HG cells on the other hand showed no significantly increased OA uptake with increasing OA concentrations ($p=0.12$) or in presence of glucose ($p=0.56$) (Fig. 2D), showing a reduced sensitivity towards acute glucose after chronic HG exposure. The overall cellular uptake of OA was also significantly reduced by 32 % in HG cells ($p=0.004$). However, myotubes exposed to HG showed 21 %

increased incorporation of OA into triacylglycerol compared to NG cells (p=0.02, data not shown), in accordance with previous findings [24]. The other lipid classes (free fatty acid, diacylglycerol, phospholipids, and cholesterol ester) were not significantly affected by HG pretreatment.

The fraction of OA that was oxidized (CO_2/cellular uptake) was independent of OA concentration, 4.7 (± 0.5) % in NG cells and 4.4 (± 0.5) % in HG cells in absence of glucose. Glucose reduced this fraction significantly in NG cells to 1.5 (± 0.2) % ($p<0.0005$), whereas there was no significant reduction (2.6 (± 1.0) % (p=0.17)) in HG cells. The observed effects of HG on metabolism, was not due to hypertonicity as previously shown [24].

3.3. Glucose suppressibility and adaptability in myotubes treated with chronic hyperglycemia

To investigate whether HG affected metabolic switching, the parameters suppressibility and adaptability were calculated according to previous definitions [5]. Glucose suppressibility, the ability of acutely added glucose to suppress lipid oxidation, was markedly reduced to 21 % in HG cells compared to 50 % in NG cells (Fig. 3A) (p=0.03). Adaptability, defined as the capacity to increase lipid oxidation upon exposure to increasing fatty acid concentrations, was present in both NG and HG cells (p=0.04 overall effect). The adaptability seemed to be reduced in cells pretreated with HG, although not significantly (Fig. 3B).

3.4. Effect of oleic acid on glucose metabolism in normoglycemic and hyperglycemic cells

The effect of hyperglycemia on glucose metabolism was also examined. Glucose uptake, assessed as the sum of cell-associated and CO_2-trapped radioactivity from [6-^{14}C]glucose, was reduced by exposure to hyperglycemia for 4 days (Fig. 4A), as reported before using a standard deoxyglucose uptake assay [24]. The overall glucose uptake in HG cells was 61 % of the amount measured in NG cells (p=0.03).

To evaluate the effect of fatty acids on glucose metabolism, oleic acid (OA) was added to the cell culture media during acute glucose uptake (4 h) in concentrations up to 300 µmol/l. Glucose uptake seemed to increase with increasing OA concentrations, although not significantly (p=0.06), and there was no difference between NG and HG cells (Fig. 4A).

Oxidation of glucose to CO_2 was reduced by HG to the same degree as glucose uptake, to 62 % of control cells (p=0.03, Fig. 4B). There was no significant effect of acute oleic acid addition on glucose oxidation. The fraction of glucose that was oxidized (CO_2/cellular uptake) was constant and not dependent on chronic hyperglycemia or concentration of oleic acid (data

not shown). The effect of hyperglycemia on glucose metabolism was confirmed with two other isotopes of glucose, [1-^{14}C]glucose (data not shown) and [^{14}C(U)]glucose (Fig. 5B).

3.5. Effect of chronic hyperglycemia on mitochondrial capacity

To determine the mitochondrial capacity of myotubes pretreated with NG or HG, complete oleic acid oxidation and glucose oxidation to CO_2 was measured in presence of the mitochondrial uncoupler 2.4-dinitrophenol (DNP). DNP (100 µmol/l) increased OA oxidation 1.5-fold in both NG and HG cells in absence of glucose (Fig. 5A). In accordance with the results in Fig. 2, glucose suppressed OA oxidation by about 50 % in NG cells, and 20 % in HG cells. There was no statistically significant difference between NG and HG in maximal fatty acid oxidation in presence of DNP. With DNP, acute glucose suppressed OA oxidation by 39 % in NG cells and by 24 % in HG cells. Additional experiments on OA oxidation were performed to further investigate mitochondrial energy production. By use of compounds modifying substrate oxidation and calculating net routine flux control ratio (netR) (see Experimental) we observed that 52 % of electron transport system (ETS) capacity was activated for ATP production in NG cells, whereas this was decreased to 41 % after HG treatment ($p=0.04$, $n=3$).

The maximal capacity to oxidize [^{14}C(U)]glucose was strongly compromised in HG cells (Fig. 5B) to only 47 % of the capacity seen in NG cells ($p<0.001$). In presence of DNP, the suppressive effect of OA on glucose oxidation (OA suppressibility) was also evident. OA suppressibility was significantly lower in HG compared to NG cells (23 % vs. 42 % in HG and NG cells, respectively, Fig. 5C).

3.6. Gene regulation by chronic hyperglycemia

To further elucidate the molecular mechanism behind the effects of hyperglycemia on glucose and lipid metabolism, we examined by real-time RT-PCR and microarray analysis, gene expression in NG and HG cells. Some of the essential genes involved in carbohydrate (ChREBP, PDK-4) and fatty acid metabolism (FAS, SCD-1, ACC) and fatty acid uptake (CD36) were examined by real-time RT-PCR (Fig. 6). HG did not affect the gene expression level of any of the examined genes. Neither were genes associated with mitochondrial function (CPT1, CYC1, UCP-2 and UCP-3) changed (Fig. 6). The gene 36B4 was used as reference gene, and similar results were obtained when gene expression were related to the reference gene glyceraldehyd-3-phosphate dehydrogenase (GAPDH) (data not shown). Microarray analysis of gene expression surprisingly showed that no genes were significantly

regulated by 4 days of chronic HG (data not shown, Illumina arrays with > 48 000 probes, GSE19620). However, it must be emphasized that cells from only 3 subjects were included in these experiments, and that might be too few to detect small changes.

3.7. Mitochondrial DNA, ATP and lactate concentration

Since mitochondrial capacity seemed to be reduced after chronic HG compared to NG, we wanted to examine the mitochondrial content, by measuring mitochondrial DNA, of the myotubes. Mitochondrial DNA relative to nuclear DNA was not altered by HG (Fig. 7A). However, cells exposed to HG contained 25 % less ATP than NG cells (Fig. 7B, p=0.03).

To examine whether the HG effect could be explained by an increased production of lactate, myotubes were pretreated with sodium lactate (1 or 10 mmol/l) for 1-4 days before glucose oxidation was measured (Fig. 8A). The effect of HG on glucose oxidation was mimicked by lactate, at 10 mmol/l lactate for 4 days glucose oxidation was reduced by 44 % (p=0.03). The effect of lactate on glucose uptake was similar to the effects on oxidation (Fig. 8B). Preincubation with sodium lactate (10 mmol/l) also reduced OA oxidation to the same extent as HG (data not shown). Addition of sodium lactate to the cell media did not change pH. For comparison the effect of lactic acid (0.01, 0.1 or 0.2 mmol/l) was examined on glucose oxidation and uptake, and a similar effect as of HG and lactate treatments was observed (Fig. 8C and D). In this case, pH of the culture medium was reduced to 6.8. The calculated concentration of non-dissociated lactic acid in the media was similar after addition of lactate and lactic acid. To exclude an effect of acidification, HCl was added in an amount that gave the same reduction in pH as lactic acid, but HCl had no effect on glucose oxidation (data not shown). Insulin-stimulated glucose uptake was abolished by HG (Fig. 9). Lactate seemed to reduce the insulin responses as well, although not significantly (p=0.14). However, the lactate concentration in the cell media was only slightly increased in cells exposed to HG for 4 days, 4.0 (± 0.6) mmol/l versus 3.7 (± 0.5) mmol/l in media from NG cells (n=5, p=0.08). The average pH value in the cell media was similar, 7.2 (± 0.2) in both HG and NG. This implied a specific effect of lactate independent of pH.

Impaired glucose oxidation, reduced ATP concentrations and increased lactate production could be explained by a dysfunctional pyruvate dehydrogenase (PDH) complex. To test whether HG impaired PDH activity, glucose oxidation was measured after incubation with a new PDHK inhibitor, AZD7545 (10-100 nM for 2-4 days). The HG-induced reduction of glucose oxidation was not abolished by the PDHK inhibitor. Glucose oxidation was reduced by 22 % after chronic HG to 13.8 (± 0.9) nmol CO_2/mg protein in cells without

PDKH inhibition and by 23 % to 10.6 (± 1.8) nmol CO_2/mg protein in myotubes pretreated for 4 days with a combination of HG and 100 nM AZD7545 (n=3). The activity of the inhibitor was confirmed by an acute stimulatory effect on glucose oxidation. AZD7545 (100 nM for 4 h) increased glucose oxidation by 45 % in NG cells and by 35 % in HG cells.

3.8. Activation of AMPK

AMPK is known to be an important energy sensor in cells. The involvement of AMPK in HG-treated myotubes was therefore of interest. HG had no effect on either AMPK or ACC phosphorylation compared to NG control cells (data not shown). However, pretreatment with deoxyglucose (DOG) increased phosphorylation of both AMPK and ACC to the same extent in NG and HG cells, showing responses to acute energy depletion.

3.9. Effect of chronic hyperglycemia on protein synthesis

To explore the effect of HG on cellular viability and energy requirement, protein synthesis was assessed as [^{14}C(U)]leucine uptake and accumulation, measured by a scintillation proximity assay (SPA). Protein synthesis was about 2-fold increased after chronic HG (p=0.02 overall effect), indicating that hyperglycemia did not cause a reduced energy requirement (Fig. 10). Leucine uptake and accumulation was stimulated by insulin (100 nmol/l) in both NG and HG cells. After 10 h insulin-stimulated [^{14}C(U)]leucine accumulation was increased by 35.6 (± 7.4) nmol/mg protein in NG cells and by 24.7 (± 10.2) nmol/mg protein in HG cells, showing an impaired relative insulin response on protein synthesis in HG-treated cells (p=0.04, data not shown).

4. DISCUSSION

In the present study we showed that chronic exposure of differentiated human skeletal muscle cells (myotubes) to hyperglycemia (HG) significantly reduced both glucose and oleic acid oxidation and reduced the suppressive effect of acute glucose on oleic acid oxidation. Glucose oxidation in presence of a mitochondrial uncoupler (DNP) was also markedly reduced, and the same was cellular ATP concentration. Mitochondrial content, as measured by mitochondrial DNA, was unchanged and so was the expression level of several mitochondrial genes. Moreover, HG did not affect the expression level of any genes measured by real-time RT-PCR or microarray. Addition of lactate to control cells mimicked the effect of HG on inducing insulin resistance and reducing substrate oxidation. In addition, lactate concentration in cell media was slightly increased after hyperglycemia. These results imply that chronic hyperglycemia can induce impaired metabolic switching and reduce substrate oxidation in healthy myotubes without changing gene expression.

Metabolic switching of myotubes, characterized by the parameters suppressibility and adaptability, has been suggested to be of intrinsic origin and to correlate with *in vivo* characteristics [4]. However, metabolic switching can also be affected by extrinsic factors, as we recently showed that n-3 fatty acids improve metabolic switching of myotubes [5]. In the present study, glucose suppressibility (the suppressive effect of acute glucose on oleic acid oxidation) was significantly reduced by chronic HG. Adaptability (the capacity to increase fatty acid oxidation with increasing fatty acid availability) seemed to be reduced by HG exposure, although not significantly. This induced impairment of metabolic switching was not observed in the opposite situation; oleic acid suppressibility of glucose oxidation at baseline conditions. However, in presence of DNP, at maximal mitochondrial electron transport system (ETS) capacity, a suppressive effect of oleic acid on glucose oxidation was evident after both NG and chronic HG pretreatment of the cells, although significantly lower after HG. A possible explanation could be that glycolytic metabolism is preferred in these cells, and that a substrate competition was evident only at maximum flux through the ETS caused by DNP.

The acute glucose suppression of lipid oxidation has similarities with the switch from fatty acid to carbohydrate oxidation in the postprandial phase and at high intensity exercise. The mechanism behind this substrate switch is not known, but the suppressive effect on CO_2 but not on ASM indicates an effect downstream of fatty acid β-oxidation. The mechanism is probably due to a mitochondrial competition between carbohydrates and fatty acids. This

could be mediated by malonyl-CoA inhibiting CPT1 and thereby entry and oxidation of fatty acids in mitochondria [37-38]. Another explanation focus on the ETS, and is based on that catabolism of carbohydrates produces redox equivalents (NADH) feeding electrons into complex I, whereas catabolism of fatty acids produces redox equivalents feeding electrons into both complex I and II. Substrate competition at ETS level could possibly be explained by differences in relative activity of the complexes and in feedback control on PDH and β-oxidation by redox state [39]. Our results on substrate oxidation in presence of DNP showed that maximal oxidation capacity of glucose, but not OA, was compromised by HG, suggesting a defect at the complex I level or upstream. Measurements of mitochondrial function using OA as substrate further revealed that 21 % less of ETS capacity was activated for ATP production after HG treatment.

The present results indicate that chronic exposure of myotubes to high glucose concentration impairs mitochondrial function. Complete oxidation of glucose and oleic acid to carbondioxide was reduced. Incomplete oxidation of oleic acid, i.e. production of acid-soluble metabolites (ASM), was increased, as was oleic acid distribution into intracellular triacylglycerol (TAG), in accordance with previous findings [24]. The increased storage of oleic acid as TAG could partly explain why HG suppresses oleic acid oxidation. Decreased complete oxidation in combination with increased incomplete oxidation of fatty acids as observed after chronic HG exposure has also been reported to occur in myotubes from obese type 2 diabetic individuals [40]. Moreover, an imbalance between β-oxidation and TCA cycle or electron transport chain activity has been suggested to lead to increased incomplete fatty acid oxidation and accumulation of lipid-derived metabolites in the mitochondria, further resulting in mitochondrial stress and development of skeletal muscle insulin resistance [41-43]. Thus, the observed impaired substrate oxidation might be due to an affected step somewhere in the TCA cycle or electron transport chain machinery, leading to accumulation of lipid-derived metabolites in the mitochondria and possibly impaired metabolic switching.

Glycolytic products such as lactate and pyruvate might play a regulatory role in the mechanism behind metabolic switching. The effect of HG on glucose uptake and oxidation was mimicked by extracellular addition of lactate. The activity of CPT-1 is strongly pH-dependent, and could possibly be inhibited by lactate [44]. However, in the present study addition of lactate also inhibited glucose oxidation and this effect was rather specific since acidification by HCl did not decrease glucose oxidation. In addition, lactate did not change the pH of the cell culture medium. A specific effect of lactate is also supported by the lactic acid experiments. Recently, lactate was found to be the ligand for the orphan G-protein-

coupled receptor GPR81, and it was shown that lactate regulated adipocyte lipolysis through this receptor [45]. In muscle lactate is known to decrease glucose consumption and glycolytic flux [46]. Although lactate concentration was only slightly increased in cell media of myotubes exposed to chronic HG, it suggests that glucose oversupply pushes the glycolytic pathway. Intramyocellular lactate concentration was not measured, but could be increased to a greater extent. Possibly, recycling of lactate back into glucose could also explain the reduced ATP level, and there exist evidence that this futile cycle is active in myotubes [47]. An increased lactate production can be caused by increased lactate dehydrogenase activity, reduced activity of pyruvate dehydrogenase (PDH) or lack of acetyl CoA. PDH activity is controlled by PDH kinase, and if HG increased PDHK activity and thereby reduced PDH activity, an inhibitor of PDHK might then relieve the HG effect. The PDHK inhibitor, AZD7545, however showed no effect on the reduced glucose oxidation caused by HG. Changed PDH activity by any other mechanism can however not be excluded by these experiments. Whether the effect of HG is mediated by lactate is uncertain, and should be more thoroughly studied. Any effect of lactate or chronic HG on lipolysis in muscle is not known and should also be examined. The present finding of increased storage of oleic acid as triacylglycerol (TAG), together with previous observations of increased TAG content in myotubes treated with chronic hyperglycemia [24] could imply that lipolysis might be affected. In theory, glucose oversupply could also possibly increase cell glycogen content and thereby favor utilization of glucose derived from stored glycogen and suppress lipid oxidation. However, based on previous results we have no reasons to believe that glycogen content is increased after HG in absence of insulin [24].

The reduced mitochondrial capacity could not be explained by altered gene expression or by a decreased number of mitochondria. In fact, the protein synthesis experiments showed that the cells in energy surplus were more actively accumulating leucine, indicating high metabolic activity. This is in accordance with a previous study showing increased protein synthesis in hyperglycemic diabetic patients [48]. Resistance towards insulin-stimulated protein anabolism has also been reported in type 2 diabetics [49]. Insulin-stimulated protein synthesis was reduced in cells pretreated with HG confirming insulin resistance, which was also observed on glucose uptake. Why the muscle cells then turn down energy production (ATP) and increases energy storage is not known. Of course, other energy demanding processes than protein synthesis could be affected by HG, such as calcium recycling or other membrane-associated pumping activities.

Surprisingly, no genes were found to be significantly regulated by chronic HG. These results suggest that the metabolic effects of HG might be mediated by altered protein activities caused by some form of post-translational modification, e.g. phosphorylations, glycations or acetylations. Previously, we have reported that neither phosphorylation of PKB [24] nor ERK [50] is affected by chronic HG. Glucose infusion to rats has been shown to induce increased PKC activation [51] and to reduce phosphorylation and activation of AMPK, an important sensor of energy status and regulator of energy metabolism in cells [52]. In the present study, chronic HG did not change phosphorylation of AMPK or its substrate ACC. These data suggest that although energy uptake and oxidation is reduced, the cells are in an energy neutral state. Protein expression and activity of diacylglycerol kinase δ has been shown to be decreased in type 2 diabetic subjects and normalized upon correction of hyperglycemia [53]. This kinase is thought to be involved in the metabolic switch between lipid and glucose metabolism and might play a role in the metabolic inflexibility seen in type 2 diabetes. Other enzymatic changes and protein modifications by HG such as glycosylations or glycations can not be excluded and are currently under investigation.

In conclusion, chronic hyperglycemia impairs substrate oxidation in skeletal muscle cells and reduces the metabolic switching of the cells. This shows that hyperglycemia can induce a diabetic-resembling phenotype in former healthy cells. The effect is most likely due to an induced mitochondrial dysfunction or post-translational modifications.

5. ACKNOWLEDGEMENTS

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Figure legends

Figure 1. Suppression of oleic acid oxidation by acute glucose. Myotubes were incubated with [1-^{14}C]oleic acid (OA) (37 kBq/ml, 100 µmol/l) for 4 h in presence of 0, 0.1, 0.5, 1, 2 or 5 mmol/l glucose. CO_2 was trapped in a filter and counted by liquid scintillation as described in Experimental. Complete oleic acid oxidation to CO_2 is given as mean ± SEM (n=5).

Figure 2. Oleic acid metabolism in normoglycemic (NG) and hyperglycemic (HG) cells in absence and presence of acute glucose (5 mmol/l). Myotubes were pretreated with 5.5 mmol/l glucose (NG) or 20 mmol/l glucose (HG) for 4 days before they were incubated with [1-^{14}C]oleic acid (OA) (37 kBq/ml, 20-300 µmol/l) for 4 h in absence or presence of acute glucose (5 mmol/l). CO_2 (A, B) was trapped in a filter and counted by liquid scintillation as described in Figure 1. Uptake of OA was assessed as the sum of oxidized OA (trapped CO_2) and remaining cell-associated radioactivity (C, D). Data are given as mean ± SEM (n=3-5).

Figure 3. Suppressibility (A) and adaptability (B) of oleic acid (OA) oxidation in NG and HG cells. Suppressibility was calculated as [1-(OA oxidation (100 µmol/l) in presence of 5 mmol/l glucose/OA oxidation at 0 mmol/l glucose)]*100 %. Adaptability was calculated as fold increase in OA oxidation from 20 µmol/l OA, here 0 mmol/l and 5 mmol/l glucose are merged. These data were calculated from the results in Figure 2. $^{*}p<0.05$ vs. NG.

Figure 4. Glucose metabolism at increasing oleic acid concentrations in normoglycemic (NG) and hyperglycemic (HG) cells. Myotubes were pretreated with 5.5 mmol/l glucose (NG) or 20 mmol/l glucose (HG) for 4 days before incubation with [6-^{14}C]glucose (37 kBq/ml, 5 mmol/l) for 4 h in presence of 0-300 µmol/l oleic acid during CO_2 trapping. Glucose uptake assessed as the sum of cell-associated glucose and trapped CO_2 (A) and CO_2 production from glucose (B) were assessed as described in Experimetal. Data are given as mean ± SEM (n=3).

Figure 5. Oxidation of oleic acid and glucose in presence of the mitochondrial uncoupler 2.4-dinitrophenol (DNP). Myotubes were pretreated with 5.5 mmol/l glucose (NG) or 20 mmol/l glucose (HG) for 4 days before they were incubated with radiolabeled substrates in the absence or presence of DNP (100 µmol/l) for 4 h. (A) [1-^{14}C]oleic acid (OA) (37 kBq/ml, 100 µmol/l) in absence or presence of acute glucose (5 mmol/l) and (B) [^{14}C(U)]glucose (37 kBq/ml) in absence or presence of acute OA (100 µmol/l). (C) Oleic acid suppressibility of

glucose oxidation was calculated as [1-(glucose oxidation in presence of 100 µmol/l OA/glucose oxidation without OA added] *100 %. Data are given as mean (± SEM) (n=4). *p<0.05 vs. basal oxidation without acute glucose and without DNP, § p<0.05 vs. basal oxidation without acute OA and without DNP, $^{¤}$ p<0.05 vs. NG, $^{\#}$ p<0.05 vs. NG + DNP.

Figure 6. Gene expression in myotubes after pretreatment with hyperglycemia (HG) for 4 days relative to normoglycemic (NG) cells. Data are given as mean ± SEM (n=4). Expression of the fatty acid transporter CD36, fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD-1), acetyl CoA carboxylase (ACC), carbohydrate responsive element binding protein (ChREBP), cytochrome C (CYC1), carnitine palmitoyltransferase (CPT-1A, -1B), pyruvate dehydrogenase kinase (PDK-4) and uncoupling proteins (UCP-2, -3) were studied relative to the reference gene 36B4 by real-time PCR as described in Experimental.

Figure 7. Mitochondrial DNA (A) and ATP concentration (B) in human myotubes. The myotubes were grown in normoglycemic medium (NG) or in medium containing 20 mmol/l glucose (HG) for 4 days before mitochondrial DNA (mtDNA) relative to nuclear DNA (nDNA) was measured by real-time PCR, and ATP concentration was measured with an ATPlite 1step kit as described in Experimental. Data are given as mean (± SEM) (n=5-7). * p<0.05 vs. NG.

Figure 8. Effect of sodium lactate and lactic acid on glucose oxidation and uptake. Myotubes were preincubated with sodium lactate (1 and 10 mmol/l) (A, B) or lactic acid (10-200 µmol/l) (C, D) for 4 days, before CO_2 production from [^{14}C(U)]glucose (37 kBq/ml, 5 mmol/l) was measured for 4 h. Glucose uptake was assessed as the sum of cell-associated glucose and trapped CO_2. Data are given as mean (± SEM) (n=4). * p<0.05 vs. NG.

Figure 9. Insulin-stimulated glucose uptake in myotubes. Myotubes were preincubated with normoglycemia (5.5 mmol/l glucose, NG), hyperglycemia (20 mmol/l glucose, HG), sodium lactate (10 mmol/l) or lactic acid (0.01 mmol/l) for 4 days, before insulin-stimulated (100 nmol/l) glucose uptake was measured. CO_2 production from [^{14}C(U)]glucose (37 kBq/ml, 5 mmol/l) was measured for 4 h, and glucose uptake was assessed as the sum of cell-associated glucose and trapped CO_2. Insulin-stimulated glucose uptake is given as % of basal (glucose uptake in absence of insulin). Data are given as mean (± SEM) (n=4). * p<0.05 vs. NG.

Figure 10. Protein synthesis in normoglycemic (NG) and hyperglycemic (HG) myotubes. The cells were grown in CytoStar-T scintillating microplates and incubated with [^{14}C(U)]leucine (37 kBq/ml, 0.8 mmol/l) as described in Experimental. Incorporation of [^{14}C]leucine was followed for 10 h with scintillation proximity assay (SPA) technology. Data are given as mean ± SEM (n=3).

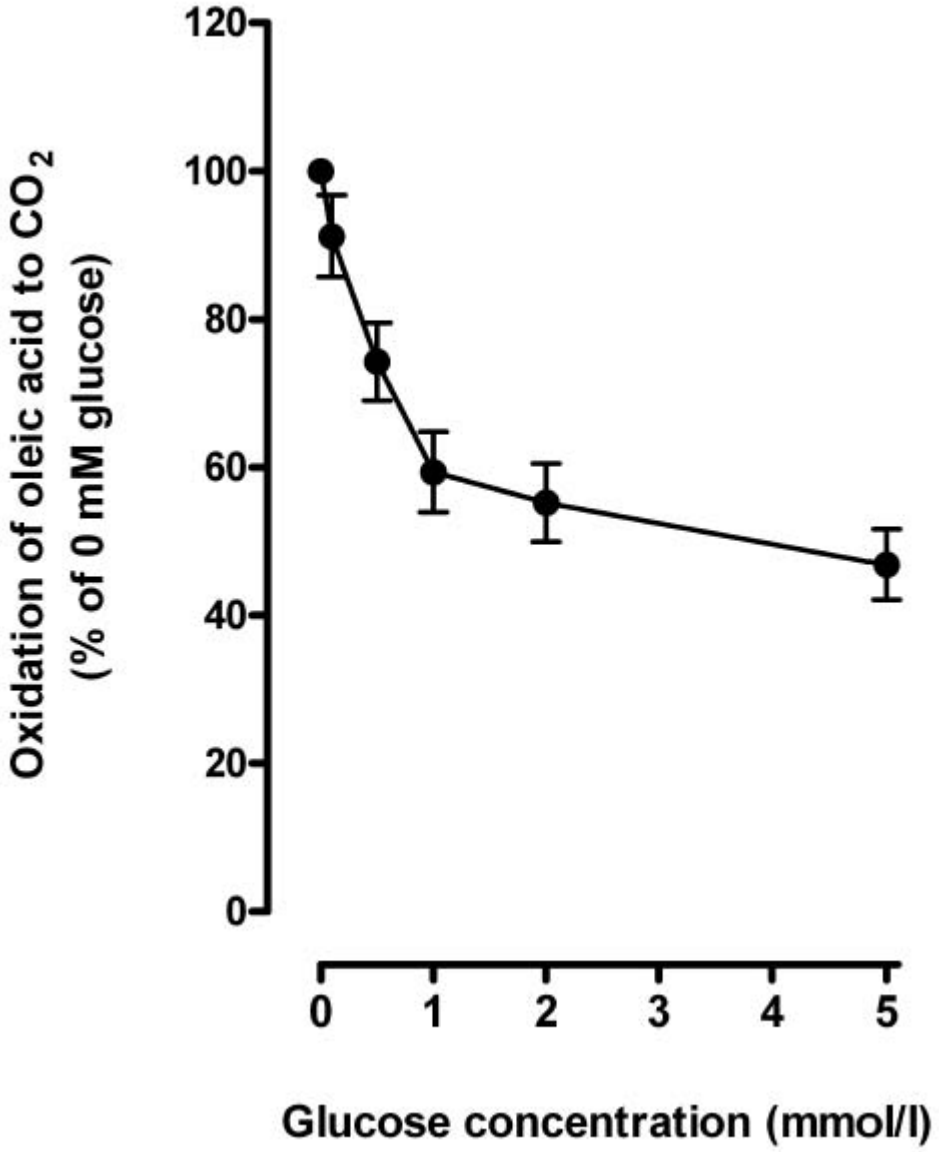


Figure 1

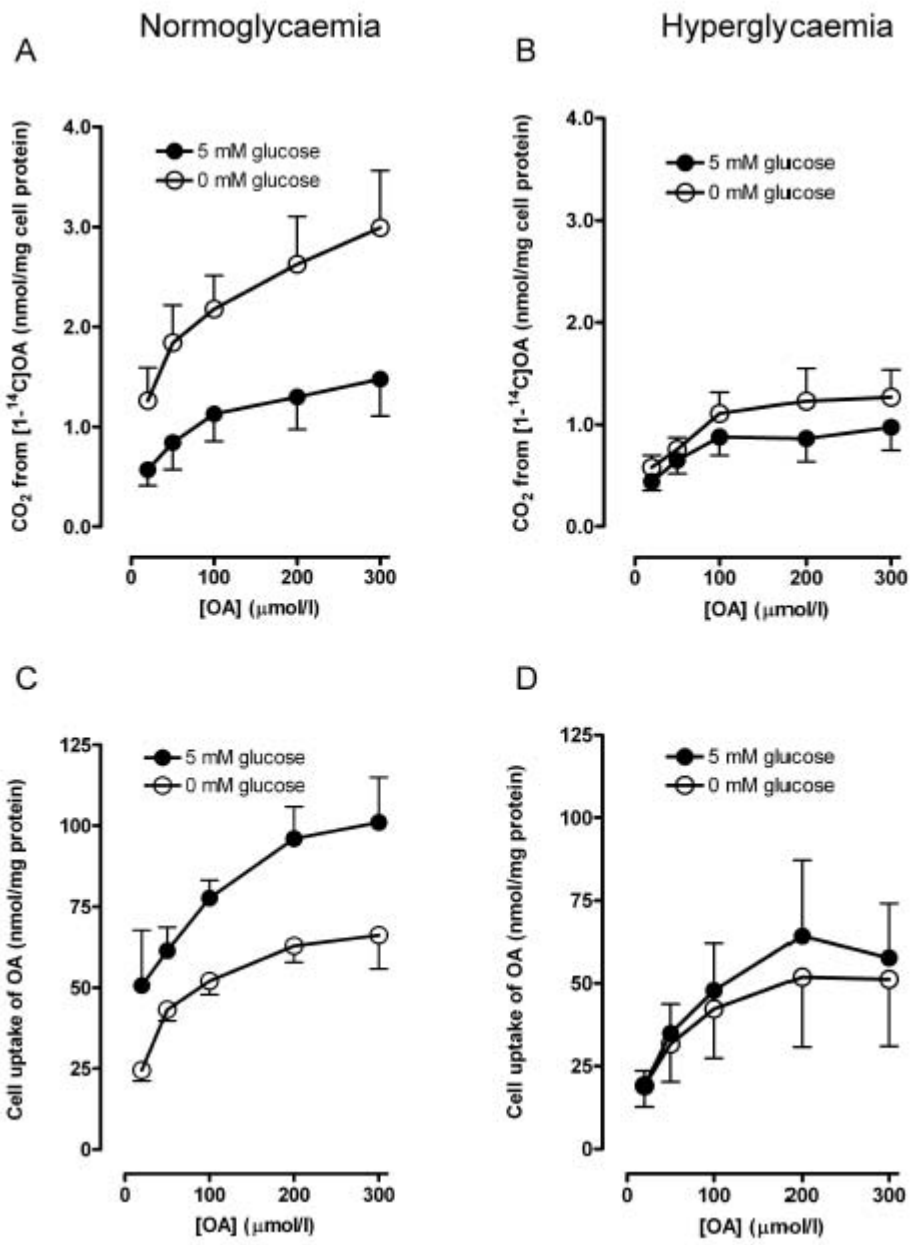
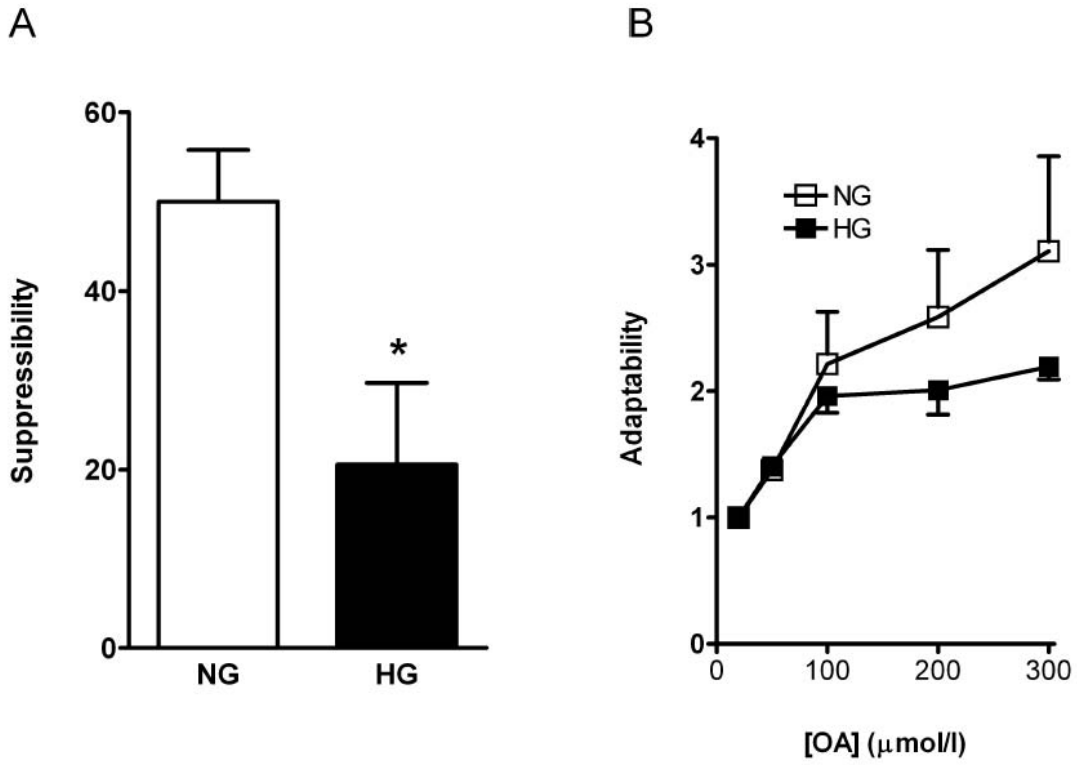


Figure 2



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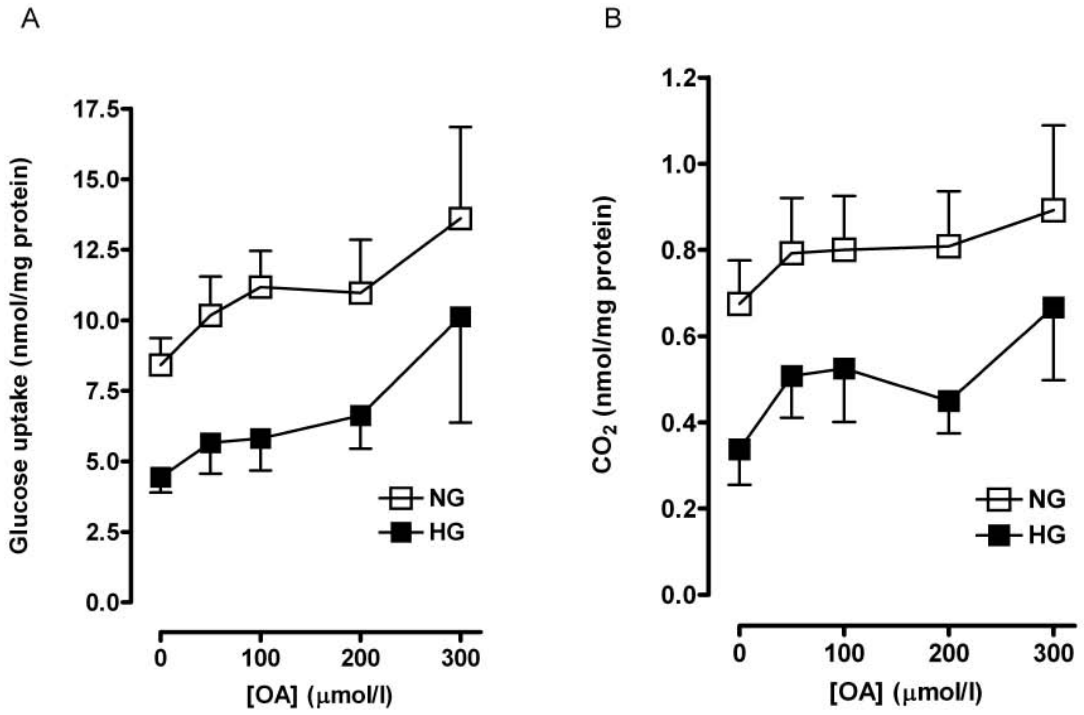

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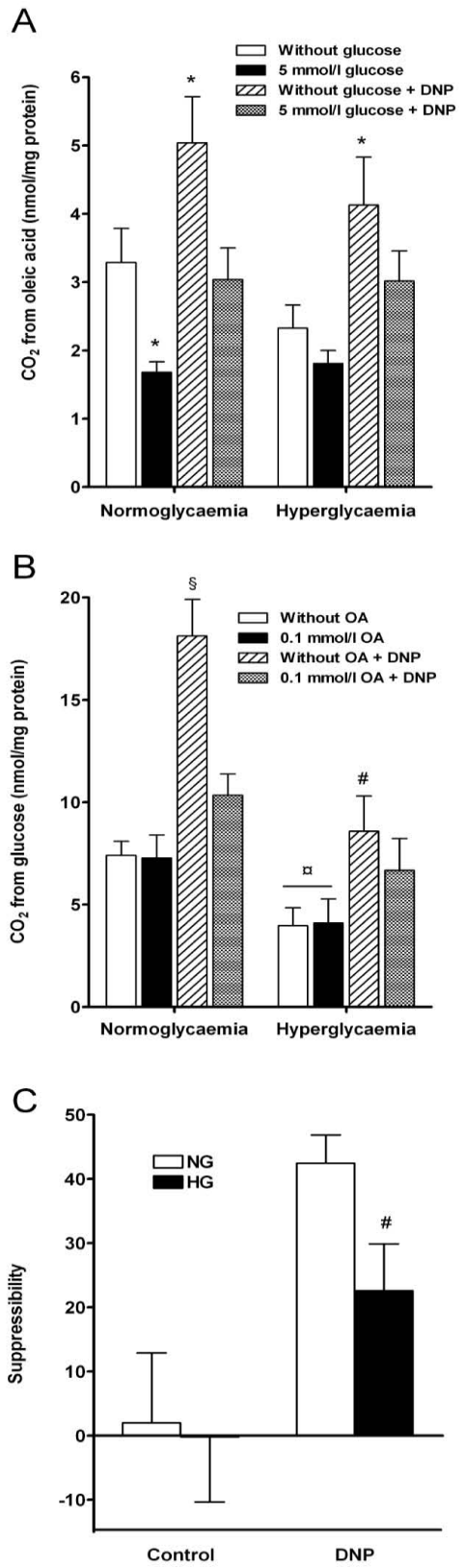

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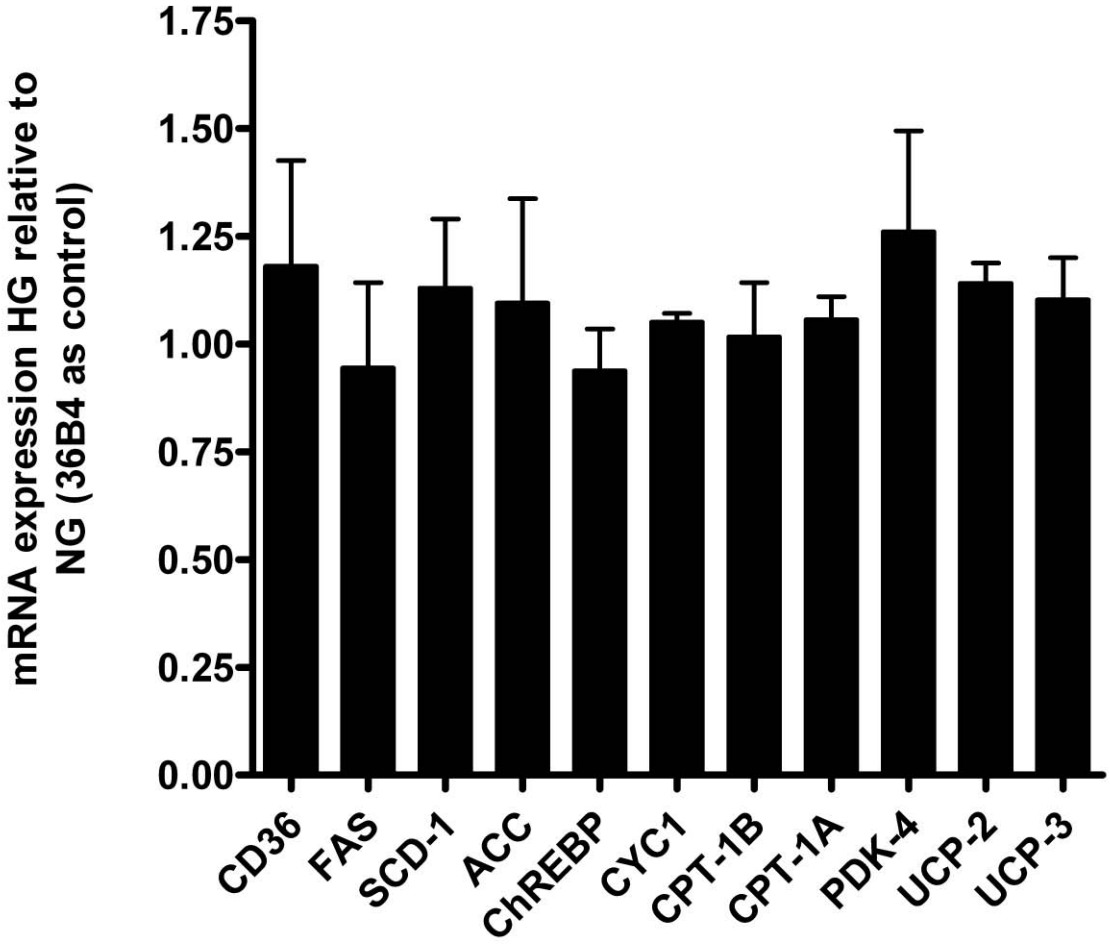

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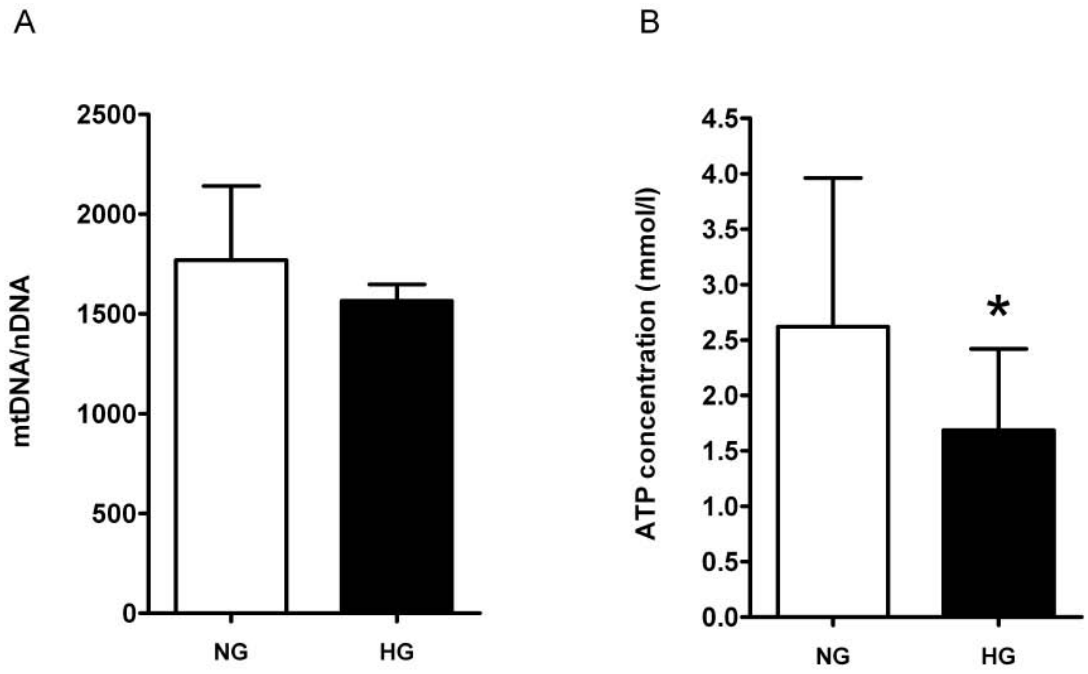

Figure 7

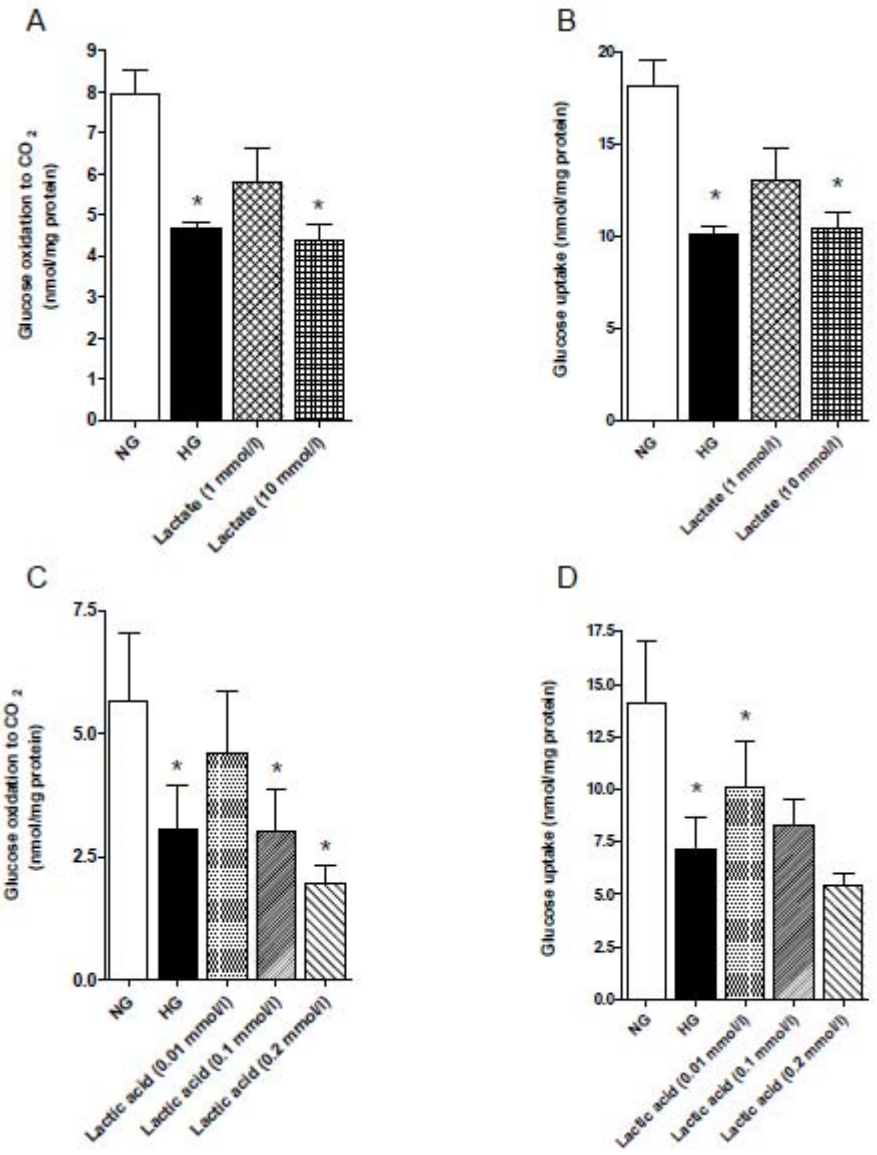

Figure8

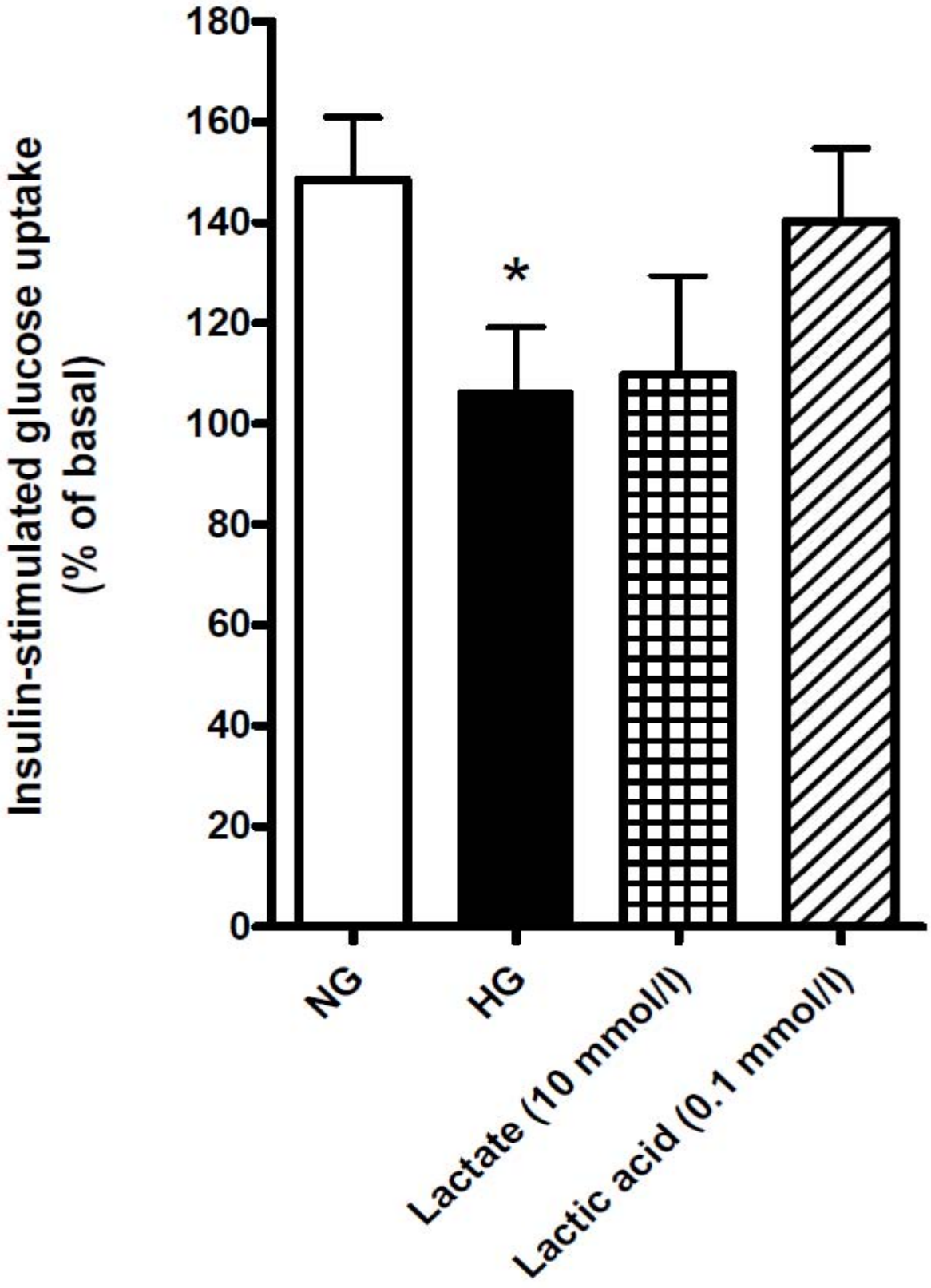

Figure 9

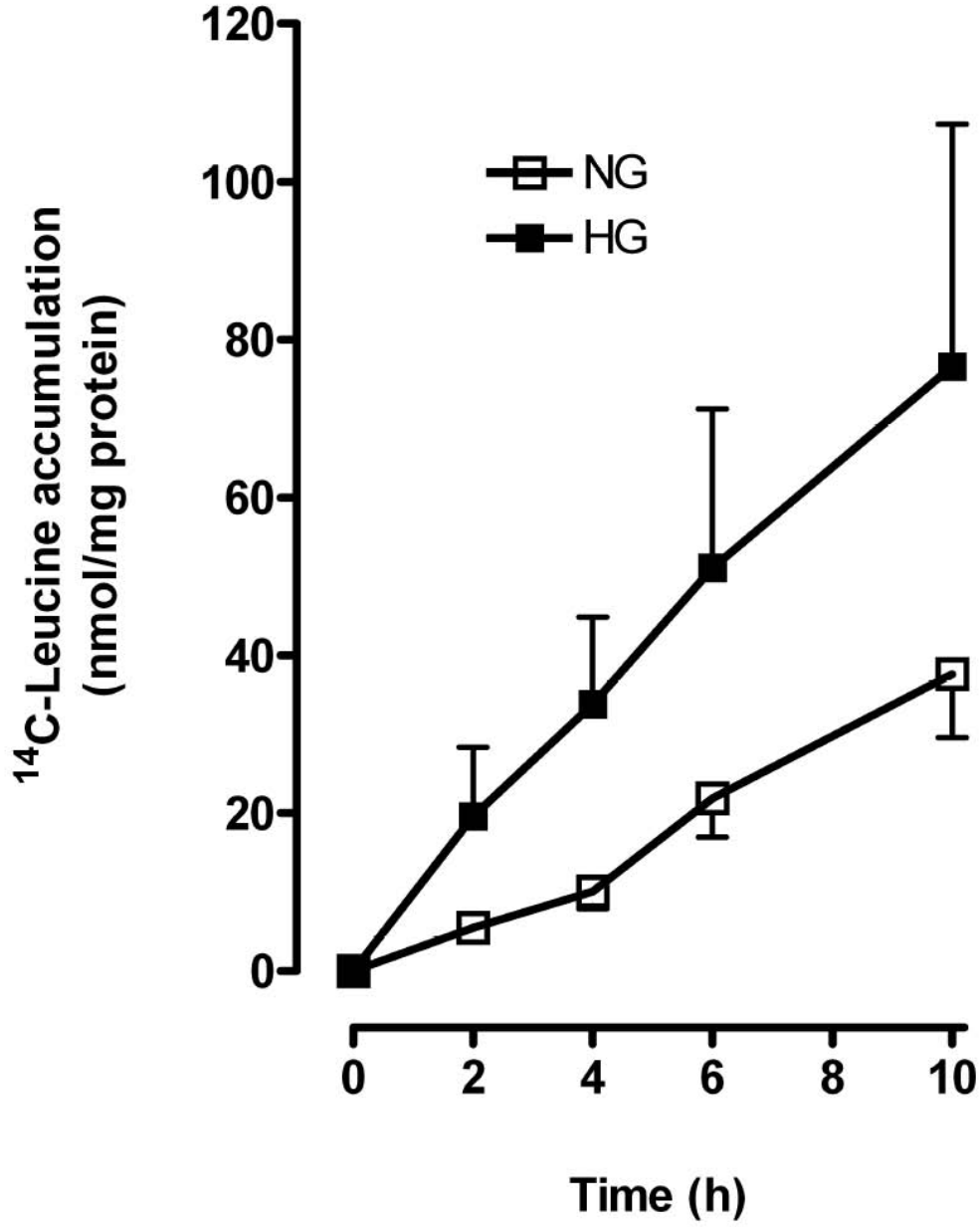

Figure 10

Table 1
Primer sequences

Gene	Acc.no.	Primer
GAPDH	NM_002046	Forward: TGCACCACCAACTGCTTAGC Reverse: GGCATGGACTGTGGTCATGAG
36B4	M17885	Forward: CCATTCTATCATCAACGGGTACAA Reverse: AGCAAGTGGGAAGGTGTAATCC
CD36	L06850	Forward: AGTCACTGCGACATGATTAATGGT Reverse: CTGCAATACCTGGCTTTTCTCAA
FAS	U26644	Forward: GAACTCCTTGGCGGAAGAGA Reverse: GTTCTGAGAAAGGTCGAATTTGC
SCD-1	AB032261	Forward: CTCCCCTGCCACACTGATG Reverse: GAGCGCTTGGCTTCTCATG
ACC1	U10822	Forward: AGAATTCACCCAGCAAAATAAAGCTA Reverse: CTCCGATCCACCTCATAGTTGAC
ChREBP	NM_032951	Forward: CGGCATTGAGCTCCTCAATC Reverse: GCAGAAGACAGCTGAGTACATCCTTA
CYC1	NM_001916	Forward: CTGCCAACAACGGAGCATT Reverse: CGTGAGCAGGGAGAAGACGTA
CPT-1B	NM_004377	Forward: GAGGCCTCAATGACCAGAATGT Reverse: GTGGACTCGCTGGTACAGGAA
CPT-1A	NM_001876	Forward: TGCTTTACAGGCGCAAACTG Reverse: TGGAATCGTGGATCCCAAA
PDK-4	BC040239	Forward: TTTCCAGACCAACCAATTCACA Reverse: TGCCCGCATTGCATTCTTA
UCP-2	AF019409.1	Forward: CCTGCGGCTCGGACACATA Reverse: GGGGCACCTTTAATCAGCAACA
UCP-3	AF050113	Forward: AGGACCTTTGCCCAACATCATG Reverse: AGTCCAGCAGCTTCTCCTTGAG
ND1		Forward: CCCTAAAACCCGCCACATCT Reverse: GAGCGATGGTGAGAGCTAAGGT
B2M		Forward: TGCTGTCTCCATGTTTGATGTATCT Reverse: TCTCTGCTCCCCACCTCTAAGT

ND1 probe: FAM-CCATCACCCTCTACATCACCGCC-BHQ
B2M probe: HEX-TTGCTCCACAGGTAGCTCTAGGAGG-BHQ

Research highlights of the manuscript ”Chronic hyperglycemia reduces substrate oxidation and impairs metabolic switching of human myotubes”;

- Chronic hyperglycemia (HG, 20 mmol/l glucose for 4 days) reduced both glucose oxidation and oleic acid oxidation by 39 % and 47 %, respectively.
- Glucose suppressibility of oleic acid oxidation was significantly reduced after HG, from 50 % in control cells to 21 % after HG.
- Incomplete oxidation of oleic acid was increased by 22 % after HG.
- The maximal mitochondrial capacity was reduced after HG without any changes in mitochondrial content or mitochondrial gene expression. Assessed by micro array, no genes at all were significantly regulated by HG after 4 days.
- The electron transport system capacity activated for ATP production was significantly lowered by HG, and the myotube content of ATP was also reduced.
- These effects of HG were not followed by activation of AMPK.
- However, the effect of HG could be mimicked by addition of lactate.
- The HG treated cells were not in a state of low energy requirement, since protein synthesis in fact was increased after HG.

In conclusion, chronic hyperglycemia reduces substrate oxidation and impairs metabolic switching. The effect seems to be mediated by some mitochondrial defect without changes in mitochondrial gene expression or mitochondrial content.