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ARTICLE

Transcriptional profiling of myotubes from patients with type 2 diabetes: no evidence for a primary defect in oxidative phosphorylation genes

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Abstract
Aims/hypothesis Microarray-based studies of skeletal muscle from patients with type 2 diabetes and high-risk individuals have demonstrated that insulin resistance and reduced mitochondrial biogenesis co-exist early in the pathogenesis of type 2 diabetes independently of hyperglycaemia and obesity. It is unknown whether reduced mitochondrial biogenesis or other transcriptional alterations co-exist with impaired insulin responsiveness in primary human muscle cells from patients with type 2 diabetes.
Methods Using cDNA microarray technology and global pathway analysis with the Gene Map Annotator and Pathway Profiler (GenMapp 2.1) and Gene Set Enrichment Analysis (GSEA 2.0.1), we examined transcript levels in myotubes established from obese patients with type 2 diabetes and matched obese healthy participants, who had been extensively metabolically characterised both in vivo and in vitro. We have previously reported reduced basal lipid oxidation and impaired insulin-stimulated glycogen synthesis and glucose oxidation in these diabetic myotubes.
Results No single gene was differently expressed after correction for multiple testing, and no biological pathway was differently expressed using either method of global pathway analysis. In particular, we found no evidence for differential expression of genes involved in mitochondrial oxidative metabolism. Consistently, there was no difference in mRNA levels of genes known to mediate the transcriptional control of mitochondrial biogenesis (*PPARGC1A* and *NRF1*) or in mitochondrial mass between diabetic and control myotubes.
Conclusions/interpretation These results support the hypothesis that impaired mitochondrial biogenesis is not a primary defect in the sequence of events leading to insulin resistance and type 2 diabetes.

Keywords Cell culture · Genetics · Oxidative phosphorylation · Microarray · Skeletal muscle · Type 2 diabetes

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Abbreviations

FDR	false discovery rate
FWER	familywise error rate
GenMAPP	Gene Map Annotator and Pathway Profiler
GSEA	Gene Set Enrichment Analysis

NRF1 nuclear respiratory factor 1
OXPHOS oxidative phosphorylation
PGC-1α peroxisome proliferator-activated receptor γ coactivator 1α

Introduction

Insulin resistance in skeletal muscle is a hallmark of type 2 diabetes and is characterised by increased intramyocellular lipid content, and several abnormalities in the biological response to insulin, including decreased glucose transport, glucose oxidation and glycogen synthesis [1–4]. Based on twin studies and epidemiological evidence, it is generally accepted that both genetic and environmental factors contribute to insulin resistance and subsequent development of type 2 diabetes [5]. This is supported by studies showing impaired insulin-stimulated glucose uptake and glycogen synthesis in glucose-tolerant first-degree relatives of patients with type 2 diabetes [6] and in primary human muscle cells (myotubes) from patients with type 2 diabetes [7–9]. Consistently, impaired insulin activation of phosphatidylinositol 3-kinase and glycogen synthase has been demonstrated in muscle of patients with type 2 diabetes [2,10], their first-degree relatives [6] and diabetic myotubes [7, 8, 11, 12]. It is still uncertain whether most of the abnormalities observed in vivo represent adaptive responses at the cellular level or are the consequences of a genetic defect. Hence, the primary molecular mechanisms underlying insulin resistance in human skeletal muscle remain largely unknown.

There is increasing evidence for a link between insulin resistance and impaired mitochondrial oxidative phosphorylation (OXPHOS) in human skeletal muscle in vivo. Most studies suggest that mitochondrial dysfunction in type 2 diabetes is primarily due to a lower content of muscle mitochondria [13, 14], whereas a role for decreased functional capacity per mitochondrion needs to be further explored [15–17]. Consistently, several microarray-based studies of skeletal muscle have reported coordinated downregulation of OXPHOS genes (mitochondrial biogenesis) in patients with type 2 diabetes and high-risk individuals [18–21], and that reduced expression of the genes encoding transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and nuclear respiratory factor 1 (NRF1) could play a key role in these transcriptional changes [19–21]. Studies using magnetic resonance spectroscopy have shown lower rates of mitochondrial ATP production and substrate oxidation in lean, severely insulin-resistant first-degree relatives of patients with type 2 diabetes [22–23]. Thus, mitochondrial dysfunction and insulin resistance co-exist at an early point in the development of type 2 diabetes. However, it is clear that both insulin resistance and mitochondrial dysfunction in skeletal muscle are highly influenced by hyperglycaemia, physical activity, ageing, obesity and fibre type composition [13, 14, 17, 18, 24, 25]. Thus, it is difficult to determine the extent to which these abnormalities are primary or secondary to environmental factors and metabolic changes associated with diabetes and prediabetic states.

Cultured human myotubes display the morphological, metabolic and biochemical properties of adult skeletal muscle [26] and offer a unique model to distinguish between genetic and environmental factors in the aetiology of insulin resistance and type 2 diabetes. We and others have reported a number of potential intrinsic defects in myotubes established from patients with type 2 diabetes, including lower basal palmitate oxidation and impaired insulin-stimulated glycogen synthesis and glucose oxidation [9, 27]. Whether these abnormalities co-exist with impaired mitochondrial biogenesis in diabetic myotubes is at present unknown.

To identify potential changes in the transcriptional profile associated with impaired insulin-responsiveness in diabetic myotubes, we compared microarray data from myotubes established and harvested under normoglycaemic and normoinsulinaemic conditions from a cohort of obese patients with type 2 diabetes and obese non-diabetic participants who had been extensively metabolically characterised in vivo and in vitro.

Methods

Materials DMEM, FCS, penicillin–streptomycin–amphotericin B and trypsin were obtained from Life Technologies (Paisley, UK). Ultroser G was purchased from Pall Biosepra (Cergy-Saint-Christophe, France). Uridine 5′-diphosphate-[^{14}C]glucose (10.63 GBq mol^{-1}) was obtained from DuPont NEN (Boston, MA, USA). A protein assay kit was purchased from Bio-Rad (Copenhagen, Denmark). Glycogen, pepstatin, leupeptin, phenylmethylsulfonyl fluoride and ECM gel were purchased from Sigma Chemical (St Louis, MO, USA). Insulin Actrapid was from Novo Nordisk (Bagsvaerd, Denmark).

Study participants Ten obese patients with type 2 diabetes and ten healthy control participants, matched according to age and BMI, participated in the study (Table 1). Only sedentary male participants were recruited. Patients with type 2 diabetes were either treated by diet alone or diet in combination with sulfonylurea or metformin, which were withdrawn 1 week prior to the study. The patients were all negative for glutamic acid decarboxylase antibody and

Table 1 In vivo and in vitro characteristics

Characteristic	Control participants (n=10)	Diabetic participants (n=10)
In vivo characteristics		
Age (years)	51.1±2.0	50.4±1.6
BMI (kg m^{-2})	29.6±0.9	31.1±1.1
HbA_{1c} (%)	5.1±0.1	6.9±0.5**
Fasting plasma triacylglycerol (mmol l^{-1})	1.4±0.2	3.4±0.8*
Fasting plasma glucose (mmol l^{-1})	5.5±0.1	10.3±1.1**
Fasting serum insulin (pmol l^{-1})	40±5	69±9*
Fasting serum NEFA (mmol l^{-1})	0.49±0.06	0.47±0.05
Glucose disposal rate, basal (mmol min^{-1} m^{-2})	0.41±0.02	0.49±0.02
Glucose disposal rate, insulin (mmol min^{-1} m^{-2})	1.61±0.14	1.00±0.15**
Glucose oxidation, basal (mmol min^{-1} m^{-2})[a]	0.24±0.02	0.29±0.05
Glucose oxidation, insulin (mmol min^{-1} m^{-2})[a]	0.58±0.02	0.43±0.05*
Lipid oxidation, basal (nmol min^{-1} m^{-2})[a]	56±2	58±5
Lipid oxidation, insulin (nmol min^{-1} m^{-2})[a]	27±3	43±4*
Non-oxidative glucose disposal, basal (mmol min^{-1} m^{-2})[a]	0.15±0.02	0.20±0.04
Non-oxidative glucose disposal, insulin (mmol min^{-1} m^{-2})[a]	1.09±0.12	0.66±0.13*
In vitro characteristics (myotubes)		
Glucose oxidation, basal (pmol [mg protein]$^{-1}$ min^{-1})	382±42	429±41
Glucose oxidation, insulin (pmol [mg protein]$^{-1}$ min^{-1})	531±46	423±38*
Lipid oxidation, basal (pmol [mg protein]$^{-1}$ min^{-1})	24.4±1.5	18.1±1.9*
Lipid oxidation, insulin (pmol [mg protein]$^{-1}$ min^{-1})	24.5±2.4	18.0±2.1*
Glycogen synthesis, basal (pmol [mg protein]$^{-1}$ min^{-1})	68±12	50±8
Glycogen synthesis, insulin (pmol [mg protein]$^{-1}$ min^{-1})	141±22	87±12*

The table shows characteristics of type 2 diabetic and control participants in the basal and insulin-stimulated states as reported previously [2, 9, 27]. Data are mean±SEM

*p<0.05 and **p<0.01 vs control

[a]Based on indirect calorimetry in eight control and nine diabetic participants

without signs of diabetic retinopathy, nephropathy, neuropathy or macrovascular complications. The control participants had normal glucose tolerance and no family history of diabetes. All participants had normal results of screening blood tests of hepatic and renal function. Informed consent was obtained from all participants before participation. The study was approved by the local ethics committee and was performed in accordance with the Declaration of Helsinki II.

In vivo characteristics Total glucose disposal rate and rates of glucose and lipid oxidation were assessed by euglycaemic–hyperinsulinaemic clamp studies (equilibration for 2 h followed by insulin infusion for 4 h, 40 mU min^{-1} m^{-2}) combined with indirect calorimetry as described in detail previously [2, 28]. Non-oxidative glucose disposal was calculated as the difference between the rates of total glucose disposal and glucose oxidation. Plasma glucose, serum insulin, NEFA and triacylglycerol were measured as described previously [29]. Skeletal muscle biopsies were obtained in the basal steady-state period of the clamp from the vastus lateralis muscle using a modified Bergström needle with suction under local anaesthesia.

Cell culture Cell cultures were established as described previously [8, 26]. In brief, muscle tissue was minced, washed and dissociated for 60 min by three treatments with 0.05% trypsin–EDTA. The harvested cells were pooled and FCS was added to stop trypsinisation. The cells obtained were seeded for upscaling on ECM gel-coated dishes after 30 min of preplating. Cell cultures were established in DMEM medium supplemented with 10% FCS, 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 1.25 μg ml^{-1} 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^{-1} penicillin, 50 μg ml^{-1} streptomycin and 1.25 μg ml^{-1} 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^{-1} penicillin, 50 μg ml^{-1} streptomycin, 1.25 μg ml^{-1} amphotericin B and 25 pmol l^{-1} insulin) in order to induce differentiation. The cells were cultured in a humidified 5% CO_2 atmosphere at 37°C, and medium was changed every 2–3 days. Human myotubes established from controls and participants with type 2 diabetes were allowed to differentiate under physiological

conditions of insulin (25 pmol l^{-1}) and glucose (5.5 mmol l^{-1}) for 8 days. All myotube cultures were used for analysis on day 8 after onset of differentiation.

In vitro characteristics Results of glycogen synthesis, glucose oxidation and lipid oxidation under basal and insulin-stimulated conditions in myotubes established from the diabetic and control participants included in this study have been reported previously [9, 27]. In brief, differentiated myotubes were exposed to either radiolabelled palmitic acid (0.6 mmol l^{-1}) or glucose (5.5 mmol l^{-1}), and either 25 pmol l^{-1} (basal) or 1 µmol l^{-1} insulin in serum-free DMEM for 4 h [9, 27]. Refer to these papers for further details.

Sample preparation Total RNA was prepared from muscle cell cultures using RNeasy (Qiagen, Hilden, Germany) and microarray analysis was performed using Human Genome U95Av2 GeneChips (Affymetrix, Santa Clara, CA, USA). Total RNA isolated from cultured human myotubes was reverse-transcribed using the SuperScript Choice system for cDNA synthesis (Life Technologies) according to the protocol recommended by Affymetrix (GeneChip Expression Analysis: Technical Manual [2001], p. 2.1.14–2.1.16). The sequence of the oligonucleotide used for priming was 5′-GGCCAGTGAATTGTAATACGACTCACTATAGG GAGGCGG-$(T)_{24}$-3′ (Genset Oligo, Paris, France) as recommended by Affymetrix. Double-stranded cDNA was cleaned by phenol–chloroform extraction and the aqueous phase was removed by centrifugation through Phase-lock Gel (Eppendorf, Hamburg, Germany). In vitro transcription was performed on 1 µg of cDNA using the Enzo BioArray high-yield RNA transcript labelling kit (Enzo Diagnostics, Farmindale, NY, USA) following the manufacturer's protocol. The cRNA was cleaned using RNAeasy clean-up columns (Qiagen). To improve recovery from the columns, the elution water was spun into the matrix at 27 *g* and then left for 1 min prior to the standard 8,000 *g* centrifugation recommended by Qiagen. This low-speed wetting step gave us nearly double the yield of eluted RNA. The cRNA was fragmented by heating in 1× fragmentation buffer (40 mmol l^{-1} Tris–acetate, pH 8.1, 100 mmol l^{-1} KOAc, 30 mmol l^{-1} MgOAc) as recommended by Affymetrix. Ten micrograms of fragmented cRNA was hybridised to a U95Av2 GeneChip (Affymetrix) using the manufacturer's standard procedure (45°C, 16 h). Washing and staining were performed in a Fluidics Station 400 (Affymetrix) using the protocol EukGE-WS2v4 and scanned in an Affymetrix GeneChip 2500 scanner.

Data analysis Microarray normalisation and calculation of expression measures were performed using Robust Multi-array Average [30], implemented in the statistical package R provided by Bioconductor (Seattle, WA, USA) [31]. The expression values were log 2 transformed to obtain a normal distribution across arrays and samples. Before analysing the expression data we removed Affymetrix control probes, resulting in a total of 12,558 probes.

Supervised analysis The Comparative Marker Selection suite in the GenePattern analysis environment (http://www.broad.mit.edu/genepattern) was used to find genes that could discriminate between diabetic and control myotubes. A total of 1,000 permutations were performed for the estimation of nominal *p* values, which were then corrected for multiple hypothesis testing using the false discovery rate (FDR) and the familywise error rate (FWER). FDR <0.01 and FWER <0.05 were considered significant in our study.

Global pathway analysis Gene Set Enrichment Analysis (GSEA) and Gene Map Annotator Profiler (GenMAPP 2.1) were used to evaluate the contributions of gene pathways to the transcriptional differentiation of samples. In GSEA, all genes were ranked by signal-to-noise ratio. A total of 513 gene sets were applied using the gene set browser module (MSigDB) in the GSEA software. This included 456 gene sets defined by Curated (Collection), Generic (Organism), Gene symbol (CHIP) and contributed by Biocarta, GenMAPP, SIGNALING Transduction KE, SIGNALINGAlliance, SigmaAldrich, GO and GEArray (CONTRIBUTOR). In addition, 57 gene sets defined by Curated, Human, Gene symbol and contributed by the Broad Institute and Vamsi Mootha were included. An enrichment score was generated for each gene set, and statistical significance was estimated using phenotype-based permutations. In this analysis, 2,000 permutations were used to obtain the nominal *p* values, and FDR <0.25 and FWER <0.05 was considered significant after correction for multiple hypothesis testing.

The MAPPFinder 2.1 tool in GenMAPP 2.1 was used to determine whether any gene sets or pathways were significantly up- or downregulated in the diabetic vs control myotubes. We used all contributed human pathways available in the MAPP archives. A fold change of >1.05 or <−1.05 and $p<0.05$ (unadjusted) were used as the criteria for gene expression changes between diabetic and control myotubes. The statistical rating of the relative gene expression activity was provided by the *z* score. The *z* score was based on the number of genes (*N*) linked to local MAPPs and the number of these genes (*R*) meeting the criteria for change in expression. A pathway with a *z* score >2.0 was considered significantly regulated. A non-parametric statistic, based on 2,000 permutations, was applied to access the statistical significance of the *z* score (permute P). To account for multiple hypothesis testing, the FWER was calculated for each pathway (www.GenMAPP.org). Under-

scoring between words in the text and Tables 2 and 3 indicates names of gene sets in GenMAPP GSEA.

Total RNA isolation and RT-PCR analysis Total RNA was isolated from human myotubes using a single-step method with Trizol (Invitrogen, Tastrup, Denmark) according to the manufacturer's instructions. The integrity and purity of total RNA were verified spectrophotometrically and by gel electrophoresis on 0.8% SeaKem agarose (BMA, Hellerup, Denmark). cDNA was synthesised from 5 μg of total RNA using a Revertaid H Minus First Strand cDNA Synthesis Kit (Fermentas, Copenhagen, Denmark) according to the manufacturer's instructions. RT-PCR was performed using the iCycler IQ detection system (Bio-Rad, Herlev, Denmark) by using SYBR Green I as a double-strand DNA-specific binding dye. Thermocycling was performed in a final volume of 20 μl containing 3 μl of cDNA sample (diluted 1:20), 20 pmol of each primer and 2× iQ SYBR Green Supermix (Bio-Rad). The following human specific primers were used: *NRF1* forward, 5′-AACAAAATTGGGCCACGTTACA-3′; *NRF1* reverse, 5′-TCTGGACCAGGCCATTAGCA-3′; *PPARGC1A* forward, 5′-GCTTTCTGGGTGGACTCAAGT-3′; *PPARGC1A* reverse, 5′-TCTAGTGTCTCTGTGAGGACTG-3′. Quantifications of each target gene and β-actin mRNA were performed in separate tubes. Gene expression levels for each target gene were calculated using the comparative C_t method formula ($1/[2\Delta C_t]$), where ΔC_t is the difference between C_t target and C_t reference after normalisation to β-actin mRNA (PerkinElmer User Bulletin No. 2). Data were analysed using optical system software version 3.1 (Bio-Rad) and Microsoft Excel 2000 to generate relative expression values.

Mitochondrial mass For the quantification of mitochondrial mass, we used MitoTracker Green Probe (Molecular Probes, Eugene, OR, USA), 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 200 nmol l^{-1} MitoTracker Green in DMEM and subsequently washed with PBS. Fluorescence intensity was determined on a Victor plate reader model 1420-050 (PerkinElmer, Turku, Finland) with excitation and emission wavelengths of 485 and 535 nm, respectively. Values were corrected for protein and expressed as arbitrary units.

Results

In vivo and in vitro characteristics As reported previously [2], the diabetic patients had increased fasting levels of HbA_{1c}, plasma glucose, serum insulin and plasma triacyl-

Table 2 The ten most up- and downregulated gene sets analysed with GSEA

Name	Size	ES	NES	NOM *p* value	FDR *q* value	FWER *p* value
Downregulated in diabetic myotubes						
Cholesterol_biosynthesis	17	−0.73	−1.54	0.05	1.00	0.87
Rac1pathway	38	−0.48	−1.49	0.03	1.00	0.96
Pitx2pathway	21	−0.57	−1.47	0.07	1.00	0.97
Erk5pathway	24	−0.69	−1.45	0.07	1.00	0.98
Inositol_phosphate_metabolism	33	−0.49	−1.43	0.06	1.00	0.99
Calcium_regulation_in_cardiac_cells	180	−0.34	−1.41	0.01	1.00	1.00
Ctla4pathway	27	−0.56	−1.38	0.13	1.00	1.00
Mitochondrial_fatty_acid_oxidation	15	−0.57	−1.37	0.11	1.00	1.00
P53hypoxiapathway	38	−0.50	−1.37	0.06	1.00	1.00
Carm_Erpathway	36	−0.46	−1.33	0.14	1.00	1.00
Upregulated in diabetic myotubes						
Phenylalanine_metabolism	22	0.71	1.84	<0.001	0.12	0.08
Tyrosine_metabolism	36	0.76	1.79	<0.001	0.14	0.16
Prostaglandin_and_leukotriene_metabolism	31	0.72	1.66	<0.01	0.46	0.52
Electron_transporter_activity	121	0.48	1.65	<0.01	0.40	0.56
DNA_replication_reactome	57	0.52	1.59	0.03	0.64	0.77
Glycerolipid_metabolism	44	0.64	1.57	0.01	0.63	0.81
Eicosanoid_synthesis	21	0.77	1.52	0.02	0.83	0.91
Tryptophan_metabolism	72	0.44	1.52	0.02	0.78	0.93
St_interferon_gamma_pathway	16	0.76	1.50	0.03	0.77	0.95
Butanoate_metabolism	27	0.48	1.50	<0.01	0.70	0.95

All genes on the chip were ranked by difference in expression between diabetic and control myotubes using the *t* test
ES, enrichment score; NES, enrichment score normalised for differences in gene set size; NOM, nominal

glycerol, whereas plasma NEFA levels were similar (Table 1). The insulin-stimulated glucose disposal rate was 38% lower in diabetic patients than in controls, and this was primarily accounted for by a 40% reduction in non-oxidative glucose disposal, but also by a 25% decrease in glucose oxidation. Moreover, the ability of insulin to suppress lipid oxidation during the clamp was significantly impaired in diabetic patients.

Myotubes established from diabetic and control participants were inspected visually under phase-contrast microscopy, and did not differ in appearance. As reported previously [9, 27], diabetic myotubes showed reduced insulin-mediated glucose oxidation (20%) and glycogen synthesis (38%), whereas lipid oxidation, measured as production of CO_2 in response to palmitate exposure, was decreased by 26% under both basal and insulin-stimulated conditions (Table 1).

Quality control of microarrays To confirm the quality of the microarray data we made a box plot of the expression values after normalisation. The box plot showed that median expression values for all arrays were on the same level and were evenly distributed after normalisation.

Supervised analysis Using the Comparative Marker Selection suite in GenePattern, we observed that none of 12,558 probe sets could discriminate between diabetic and control myotubes when corrected for multiple hypothesis testing using either FDR <0.01 or FWER <0.05 (data not shown).

Global pathway analysis Using phenotype-based permutations on the 513 gene sets included in the GSEA analysis, no pathways were downregulated, whereas two gene sets, phenylalanine_metabolism and tyrosine_metabolism, were upregulated in diabetic vs control myotubes using FDR <0.25 as the criterion. However, when applying the more stringent FWER <0.05 as the criterion, no gene sets were differently regulated between diabetic and control myotubes (Table 2). The gene sets electron_transport_chain, oxidative_phosphorylation, and Mootha_voxphos, were not differentially regulated (all FDR >0.9, and FWER=1.00).

Table 3 The ten most up- and downregulated gene sets analysed with MAPPFinder

MAPP name	Changed (n)[a]	Measured (n)[b]	On MAPP (n)[c]	Changed (%)[d]	z score	Permuted p value	FWER p value
Downregulated in diabetic myotubes							
Integrin-mediated_cell_adhesion	7	87	99	8.0	2.9	<0.01	0.59
Fatty_acid_omega_oxidation	2	15	15	13.3	2.4	0.08	0.87
Focal_adhesion	10	169	187	5.9	2.4	0.02	0.87
Nucleotide_metabolism	2	16	17	12.5	2.3	0.08	0.91
Pentose_phosphate_pathway	1	5	7	20.0	2.3	0.15	0.94
RNA_transcription_reactome	3	32	40	9.4	2.2	0.05	0.95
Nuclear_receptors_in_lipid_metabolism_and_toxicity	3	32	33	9.4	2.2	0.05	0.95
S1P_signaling	2	20	25	10.0	1.9	0.12	0.99
Heme_biosynthesis	1	9	9	11.1	1.5	0.24	1.00
MAPK_signaling_pathway	7	145	162	4.8	1.4	0.21	1.00
Upregulated in diabetic myotubes							
Smooth_muscle_contraction	11	138	156	8.0	3.3	<0.001	0.41
Triacylglyceride_synthesis	3	18	24	16.7	3.3	0.02	0.44
Irinotecan_pathway	2	12	12	16.7	2.7	0.06	0.73
Oxidative_stress	3	24	28	12.5	2.6	0.03	0.74
Calcium_regulation_in_cardiac_cells	9	127	149	7.1	2.6	0.01	0.74
Fatty_acid_omega_oxidation	2	15	15	13.3	2.3	0.08	0.90
Biogenic_amine_synthesis	2	15	15	13.3	2.3	0.08	0.90
Prostaglandin_synthesis_regulation	3	30	31	10.0	2.2	0.07	0.91
Synthesis_and_degradation_of_ketone_bodies	1	5	5	20.0	2.2	0.17	0.94
Small_ligand_GPCRs	2	17	18	11.8	2.0	0.10	0.97

A fold change >1.05 or less than −1.05 and a p value <0.05 (unadjusted) were used as the criteria for gene expression changes between diabetic and control myotubes. Among the 2,544 genes linked to local MAPPS, R=74 and R=80 genes met the criteria for up- and downregulation, respectively (see "Methods")

[a]Number of genes changed

[b]Number of genes measured on the chip

[c]Number of genes on the MAPP

[d]Number changed divided by number measured

Using the MAPPFinder tool in GenMAPP on human pathways, significant *z* scores (FDR <0.01) were observed for downregulation of integrin_mediated_cell_adhesion and upregulation of smooth_muscle_contraction in diabetic vs control myotubes. However, when controlling for multiple hypothesis testing using FWER <0.05, no pathways were significantly regulated (Table 3). The Electron_transport_chain showed FDR >0.38 and FWER=1.00 in both analyses. In fact, only one of the 85 genes measured met the criteria for up- and downregulation, respectively.

Evaluating the results from both GSEA and GenMAPP, there were no pathways that were consistently down- or upregulated in diabetic vs control myotubes. In both analyses, we found no evidence for differential regulation of the pathways representing OXPHOS.

RT-PCR and mitochondrial mass To validate our results, we examined gene expression of *PPARGC1A* and *NRF1*, which are known to be involved in the transcriptional control of mitochondrial biogenesis. There was no difference in mRNA levels of *PGC-1α* (p=0.89) or *NRF1* (p=0.26) between diabetic and control myotubes (Fig. 1a, b). Moreover, in a subset of the diabetic (n=5) and control myotubes (n=6), we could not detect any difference in mitochondrial mass (p=0.32) (Fig. 1c).

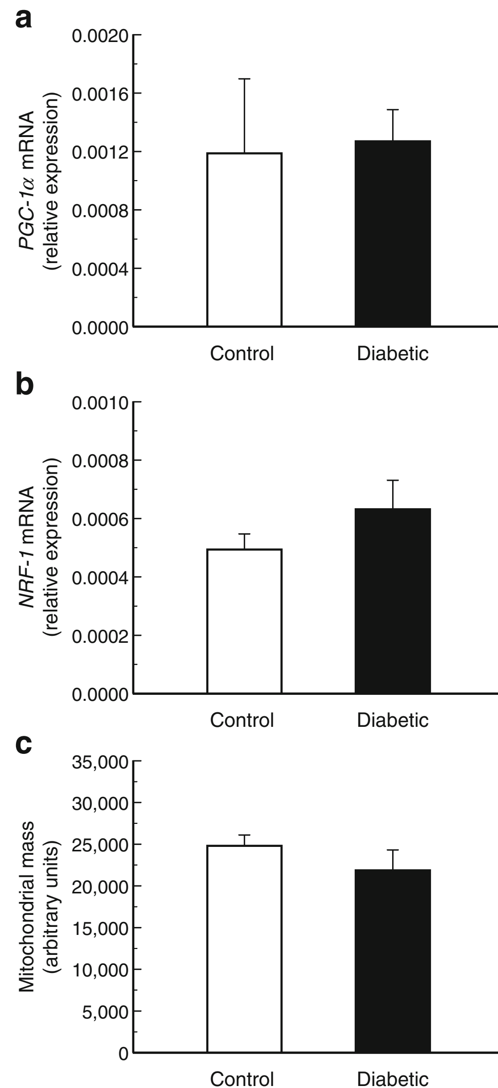

Fig. 1 *PPARGC1A* (translated into PGC-1α) (**a**) and *NRF1* (**b**) mRNA levels determined by quantitative real-time PCR in myotubes established from control participants (n=10) and patients with type 2 diabetes (n=10), expressed relative to β-actin. **c** Mitochondrial mass was determined by MitoTracker Green fluorescence in myotubes from a subset of the control (n=6) and diabetic participants (n=5). All data are mean±SEM. There were no significant differences between control and diabetic participants

Discussion

Cultured human myotubes represent a well-characterised in vitro model system of skeletal muscle in which the extracellular environment can be controlled precisely and kept constant over time [26]. In the present study, we took advantage of this model to compare the transcript levels of muscle genes required for basal homeostasis in myotubes established from obese patients with type 2 diabetes and matched healthy control participants. As in studies of muscle transcript levels in patients with type 2 diabetes in vivo [18–20], we could not find any single gene that was significantly regulated after correction for multiple hypothesis testing in diabetic myotubes. To search for gene sets enriched in diabetic vs control myotubes, we used two different methods for global pathway analysis, GenMAPP and GSEA, which are well-established tools used to identify novel biological pathways of interest in the pathogenesis of complex disorders such as insulin resistance [19–21]. In contrast to recent in vivo studies [18–20], we could not find evidence for differential gene expression in any biological pathways in diabetic vs control myotubes, and, in particular, genes and pathways representing mitochondrial OXPHOS displayed almost no variability. Consistently, we observed no difference in mRNA levels of *PGC-1α* and *NRF1*, which are known to mediate the transcriptional control of mitochondrial biogenesis, or in mitochondrial mass between diabetic and control myotubes. These data indicate that under basal conditions there are no intrinsic (inborn) errors in the factors controlling transcriptional regulation and mitochondrial biogenesis in diabetic muscle.

A number of studies have provided support for the hypothesis of a link between mitochondrial dysfunction and insulin resistance in human skeletal muscle [13–24, 32], and that these abnormalities co-exist at an early point in the development of type 2 diabetes [21–23]. The lack of

transcriptional alterations in diabetic myotubes in our study, however, suggests that the downregulation of mitochondrial OXPHOS genes observed in insulin-resistant skeletal muscle in vivo is caused by differences in the magnitude of or response to factors outside the muscle cell, such as neuronal regulation, blood flow and circulating levels of substrates, cytokines and hormones. This idea is supported by several recent studies, which have indicated that both absolute and relative insulinopenia (insulin resistance), hyperglycaemia and circulating levels of NEFA are possible factors contributing to impaired mitochondrial biogenesis in vivo [14, 17, 18, 32–34]. Accordingly, none of the present human studies have been able to prove a cause-and-effect relationship between mitochondrial dysfunction and insulin resistance in vivo [13–24]. Further studies in human myotubes are warranted to study the effects of these factors separately, e.g. whether an abnormal transcriptional response to palmitate exposure could contribute to the impaired lipid oxidation observed in diabetic myotubes [27].

In vivo, skeletal muscle takes up and stores the most of the glucose during insulin stimulation, and the response to this hormonal factor is reduced in patients with type 2 diabetes [2]. This insulin-resistant phenotype can also be found in diabetic myotubes [7–9], indicating that at least insulin resistance could be a primary defect. It is likely that the maintenance of mitochondria in vivo involves a normal adaptation to the repeated bursts of physiological hyper-insulinaemia elicited by food intake. Recent microarray-based studies of skeletal muscle from healthy humans have shown that most genes upregulated in response to acute insulin infusion are involved in transcriptional and translational regulation as well as a number of genes involved in mitochondrial processes such as lipid oxidation, the citric acid cycle and OXPHOS [35–36]. Hence, an intrinsic defect in the adaptation to insulin in muscle may affect gene transcription and mitochondrial biogenesis. In support of this idea, the ability of insulin to stimulate ATP production and OXPHOS gene expression in skeletal muscle is impaired in patients with type 2 diabetes and their first-degree relatives [37–39]. Our study cannot exclude the possibility that a primary defect in insulin action on the muscle cell causes mitochondrial dysfunction, and that if myotubes were studied under conditions similar to those observed in vivo we would find reduced mitochondrial biogenesis. On the other hand, it is known that muscle cells in culture are quite different from skeletal muscle regarding their oxidative capacities, mitochondrial content and subcellular distribution of mitochondria. We have shown previously that insulin may regulate different pools of genes in skeletal muscle in vivo and cultured muscle cells in vitro [40–41]. Using diabetic and control myotubes stimulated with supraphysiological insulin levels (1 μmol l^{-1}), none of the 144 genes that responded differentially to insulin in diabetic vs control myotubes were OXPHOS genes [41]. These data argue against impaired insulin-mediated transcriptional regulation of OXPHOS genes in diabetic myotubes.

In another study from our group, we found no difference in maximal ADP-stimulated respiration between myotubes established from lean, obese and type 2 diabetic participants [42]. Together with the lack of abnormalities in mRNA levels of *PGC-1α* and *NRF1* and mitochondrial mass in the present study, these data indicate that an insulin-resistant phenotype is present in a human muscle cell model of type 2 diabetes without alterations in the transcriptional levels of OXPHOS genes, mitochondrial content and function. Although most studies of human skeletal muscle in vivo have reported the co-existence of impaired insulin sensitivity and reduced expression of OXPHOS genes and mitochondrial content/function [13–24], there is so far no clear evidence for a mechanistic link or even strong correlation between these parameters. In fact, two recent studies have provided evidence for disassociation between mitochondrial dysfunction and insulin resistance in skeletal muscle of mice and humans [43, 44].

In summary, we tested the hypothesis that alterations in the transcriptional profile contribute to the diabetic phenotype of myotubes established from patients with type 2 diabetes. Despite clear defects in insulin-stimulated glucose metabolism both in vivo and in vitro, and an impaired ability to oxidise lipids during palmitate exposure in vitro, we did not demonstrate the existence of differential transcript levels of muscle genes between diabetic and control myotubes. In particular, there were no changes in genes and pathways representing the mitochondrial OXPHOS. This is in contrast to most in vivo data, and indicates that, at least under basal conditions, no major primary defect in gene transcription or mitochondrial biogenesis precedes or co-exists with the abnormalities in insulin-stimulated glucose metabolism.

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