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Proteomic analysis of the mitochondria-enriched fraction from diabetic rat skeletal muscle

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ABSTRACT

Mitochondrial dysfunction in muscle has been implicated to play a causative role or being an indirect consequence of insulin resistance in type-2 diabetes. In order to investigate potential diabetes-related alterations in the mitochondrial proteome of muscle, we have carried out a mass spectrometry-based proteomic analysis of the gastrocnemius muscle from normal versus diabetic Goto-Kakizaki rats. A generally perturbed protein expression pattern was observed in the mitochondria-enriched fraction from diabetic muscle. Various mitochondrial markers, including NADH dehydrogenase, cytochrome b-c1 complex and isocitrate dehydrogenase were reduced in diabetic preparations. Isoforms of pyruvate dehydrogenase and ATP synthase exhibited differential changes in their abundance. The altered protein expression levels of these key metabolic enzymes might trigger a diabetes-dependent decrease in mitochondrial oxidative phosphorylation levels. The proteomic findings presented here support the idea that mitochondrial abnormalities are involved in the molecular pathogenesis of type-2 diabetes and may be crucial for the development of insulin resistance.

Keywords: Diabetes; DIGE; Goto-Kakizaki Rat; Mitochondria; Muscle Proteomics.

1. Introduction

Besides being involved in intermediary metabolism, protein transport, cell cycle progression, calcium signaling and regulation of apoptosis, mitochondria represent the primary site for energy generation via oxidative phosphorylation [1]. Altered functioning or changed expression levels of mitochondrial proteins play a key role during development, natural aging and in various pathologies [2, 3]. Mass spectrometry-based subproteomics suggests itself as an ideal analytical method to determine global changes in the mitochondrial protein complement [4-6]. Proteomic screening of mitochondrial preparations suggest the existence of approximately 1,500 individual mitochondrial protein species, whereby considerable tissue-specific differences exist within the mitochondrial proteome [4]. The recent difference in-gel electrophoretic analysis of the mitochondria-enriched fraction from aged rat skeletal muscle has revealed drastic changes in many metabolic enzymes involved in oxidative phosphorylation [7]. The natural functional decline of the aged neuromuscular system is clearly associated with a shift to more oxidative-aerobic metabolism in a slower twitching fibre population [8]. In analogy, we have used here a similar proteomic approach to determine whether type-2 diabetes is associated with significant alterations in the mitochondrial proteome from skeletal muscle. We have focused our two-dimensional gel electrophoretic study on the pH 4 to 7 range since most soluble mitochondrial proteins can be separated in this acidic to neutral environment.

Impaired glucose homeostasis and diabetic complications affect millions of patients worldwide [9]. Insulin resistance impinges especially on contractile fibres, making abnormal glucose handling in skeletal muscle tissues a key issue for treating side effects of type-2 diabetes [10]. The etiology of diabetes mellitus appears to be influenced by both environmental and genetic factors, whereby obesity and a sedentary lifestyle have an extremely negative influence on disease progression [11]. Many diabetics suffer from glucotoxic side

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effects and severely disturbed metabolic homeostasis [12]. Since muscle fibres are the most abundant cell type in the body and skeletal muscles represent the major organ for insulin-mediated whole body glucose disposal, insulin resistance in the musculature is one of the most striking pathophysiological features of type-2 diabetes [13]. In this respect, cellular dysfunction of muscle mitochondria is believed to play a crucial part in triggering insulin resistance [14-16]. It is not clear whether mitochondrial abnormalities are a secondary consequence of impaired hormonal signaling or the actual primary cause for a diminished glucose uptake into peripheral tissues [17]. However, independent of the exact initial role of mitochondria in diabetes, the long-term pathological consequences of disturbed mitochondrial bioenergetic functions are clearly a decline in crucial ATP-dependent cellular processes [16].

In order to determine potential diabetes-related changes in the mitochondrial subproteome from skeletal muscle, we have performed here a mass spectrometry-based proteomic analysis of the gastrocnemius muscle from normal versus diabetic Goto-Kakizaki (GK) rats. The non-obese GK animal model of type-2 diabetes is a spontaneously diabetic rat that shows clear signs of faulty insulin signaling within 4 weeks after birth [18]. GK rats exhibit increased levels of blood glucose, but no major changes in the concentration of non-fasting plasma insulin [19]. Diabetic GK skeletal muscles show: (i) an inhibition of insulin receptor autophosphorylation [20], (ii) impaired activities of insulin signaling intermediates [21], (iii) a diminished recruitment of the glucose transporter GLUT4 [22], (iv) membrane cytoskeletal defects in the dystrophin-dystroglycan complex [23], (v) a reduced percentage of oxidative fibres [24], (vi) a generally perturbed protein expression pattern [25], and (vii) abnormal mitochondrial functioning [26].

The proteomic analysis of the mitochondria-enriched fraction from normal versus diabetic skeletal muscles presented here has revealed a reduced expression of several mitochondrial enzymes, including NADH dehydrogenase, cytochrome b-c1 complex and isocitrate dehydrogenase. The changed abundance of these metabolic proteins might play a central role in the well-established decrease of mitochondrial oxidative phosphorylation in diabetic fibres. Hence, the proteomic results shown in this report have demonstrated that the mitochondrial subproteome is altered in type-2 diabetes and suggest that the changed density of key mitochondrial enzymes might aid in the pathological development of insulin resistance.

2. Material and methods

2.1 *Materials*

For the gel electrophoretic separation of muscle proteins, materials and analytical-grade chemicals were purchased from Amersham Biosciences/GE Healthcare, Little Chalfont, Buckinghamshire, UK (Imobiline IPG dry-strips, ampholytes, acetonitrile, Destreak agent, iodoacetamide, CyDye DIGE fluor minimal dyes Cy3 and Cy5), Biorad Laboratories, Hemel-Hempstead, Hertfordshire, UK (Laemmli-type buffer system; protein molecular mass markers) and National Diagnostics, Atlanta, GA, USA (ultrapure Protogel acrylamide stock solution). Protease inhibitors were obtained from Roche Diagnostics (Mannheim, Germany). Sequencing grade-modified trypsin was from Promega, Madison, WI, USA. Nitrocellulose transfer stacks were from Invitrogen (Carlsbad, CA, USA) and chemiluminescence substrate was purchased from Roche Diagnostics (Manheim, Germany). Primary antibodies ab66484 to mitofilin and ab14730 to the beta-subunit of mitochondrial ATP synthase were obtained from Abcam Ltd. (Cambridge, UK). Peroxidase-conjugated secondary antibodies were obtained from Chemicon International (Temecula, CA, USA).All other chemicals used were of analytical grade and purchased from Sigma Chemical Company, Dorset, UK.

2.2 *Preparation of mitochondria-enriched fraction from skeletal muscle*

Gastrocnemius muscle tissue (3g wet weight) from 9-week old normal versus age-matched diabetic GK rats was cut into small pieces and homogenised in 20 ml of buffer (220mM mannitol, 70mM sucrose, 20mM Hepes, pH 7.4), supplemented with 1 Roche PIC complete mini tablet per 10 ml of solution [7]. The resulting mixture was centrifuged at 1,100*g* for 5 min at 4ºC to remove cellular debris. The supernatant was retained. The pellet was re-suspended in 10 ml of homogenisation buffer and re-centrifuged as described above. Supernatants from the above separation steps were pooled and centrifuged at 7,000*g* for 15 min. The mitochondrial fraction was obtained as a pellet after repeated centrifugation steps at 20,000*g* for 15 min. The final mitochondria-enriched pellet was re-suspended in 2D lysis buffer for DIGE analysis. Samples were adjusted to a pH-value of 8.5. The protein content of individual samples was determined by the Bradford assay system [27].

2.3 *Fluorescent labelling of proteins*

Potential differences in the protein expression patterns of normal versus diabetic mitochondria were determined by difference in-gel electrophoresis (DIGE) using the CyDye DIGE fluor minimal dyes Cy3 and Cy5. DIGE dyes were reconstituted as a stock solution of 1mM in fresh dimethylformamide [9]. The stock solution was diluted to a working solution of 0.2mM prior to fluorescent labelling. Samples of 50 mg protein, representing each biological replicate, were minimally labelled with 200 pmols of Cy3 working solution. A pooled sample consisting of equal quantities of protein from all replicates used in the experiment were labelled at 200 pmol of Cy5 working solution to 50 mg of protein. The pooled fraction served as an internal standard. All samples were labelled at pH 8.5 with the appropriate amount of CyDye and after brief vortexing incubated on ice in the dark for 30 min. For the electrophoresis of individual gels, Cy3- and Cy5-labelled samples were mixed at an equal ratio. The

labeling reaction was terminated by the addition of 1µl of 10 mM lysine per 25 µg of muscle protein. Suspensions were briefly vortexed and then centrifuged at 12,000*g* for 10s and incubated on ice in the dark for 10 min. For gel electrophoretic separation, samples where loaded onto IPG strips with an equal volume of 2x sample buffer (7M urea, 2M thiourea, 65mM CHAPS, 2% ampholytes and 2% DTT) during rehydration [28].

2.4 *Two-dimensional gel electrophoresis*

The fluorescently labelled subproteomes from the mitochondria-enriched fraction of normal (n=4) versus diabetic (n=4) skeletal muscle tissues were separated in the first dimension by isoelectric focusing and in the second dimension by sodium dodecyl sulphate polyacrylamide gel electrophoresis, as previously optimized by our laboratory [25]. All biological replicates were analyzed individually. IPG strips were rehydrated in rehydration buffer (7M urea, 2M thiourea, 4% (w/v) CHAPS, 1.2% Destreak agent and 2% (v/v) pH 3-10 ampholytes) and 700 mg of protein sample for 12 hours. The first-dimension protein separation was conducted using the IPG strips on an Amersham IPGphor IEF system following the protocol previously described in detail [28]. First dimension strips were subjected to reduction and alkylation prior to second-dimension separation on 12.5% (w/v) slab gels. Gels were electrophoresed in an Amersham Ettan DALT-Twelve system [28].

2.5 *Image Acquisition and data analysis*

Fluorescently-labeled muscle proteins were visualised with the help of a Typhoon Trio variable mode imager from Amersham Biosciences/GE Healthcare (Little Chalfont, Bucks., UK). Two-dimensional gel images were analysed using Progensis SameSpots analysis software version 3.2.3 from Non Linear Dynamics (Newcastle upon Tyne, UK). All analytical DIGE gels were alligned to a reference gel. Following detection and filtering of spots, images were separated into groups (Normal rat muscle versus Goto-Kakizaki rat muscle) and analysed to determine significant changes in two-dimensional spot abundance. A hit list was generated of protein species that changed in density. An Anova score was included for each spot and any muscle proteins with an Anova score above 0.5 were excluded from further consideration. Any two-dimensional protein spot with a significant change in abundance was subsequently identified by mass spectrometry. Protein spots were excised from DIGE gels that had subsequently been stained with colloidal Coomassie Blue.

2.6 *Mass spectrometric identification of muscle proteins*

In order to identify individual muscle-associated protein species, peptide mixtures were analysed by mass spectrometry on a Model 6430 Ion Trap LC/MS apparatus from Agilent Technologies (Santa Clara, CA, USA). Excision, washing, destaining and treatment with trypsin were performed by a previously optimised method [7]. Following tryptic digestion, generated peptides were obtained by removing supernatants from digested gel plugs. Further recovery was achieved by adding 30% acetonitrile/0.2% trifluoricacid to the gel plugs for 10 min at 37 ^{0}C with gentle agitation. Peptide-containing supernatants were pooled and samples dried through vacuum centrifugation. Peptide mixtures were then re-suspended in 10µl mass spectrometry-grade water and 0.1% formic acid for identification by ion trap LC-MS analysis. Separation of peptides was performed with a nano flow Agilent 1200 series system, equipped with a Zorbax 300SB C18 5µm, 4 mm 40nl pre-column and a Zorbax 300SB C18 5µm, 43mm x 75µm analytical reversed- phase column using the HPLC-Chip technology. Mobile phases utilized were A: 0.1% formic acid, B: 50% acetonitrile and 0.1% formic acid. Samples (5µl) were loaded into the enrichment at a capillary flow rate set to 2 µl/min with a mix of A and B at a ratio 19:1. Tryptic peptides were eluted with a linear gradient of 10-90% solvent B over 2 µl/min with a constant nano pump flow of 0.6 ml/min. A 1 min post time of solvent A was used to remove sample carry over. The capillary voltage was set to 1700 V. The flow and the temperature of the drying gas were 4µl/ min and 300°C, respectively [7]. Database searches were carried out with Spectrum Mill Work Bench or Mascot MS/MS Ion search (Matrix Science, London, UK).

2.7 *Immunoblot analysis*

Standard one-dimensional immunoblotting was carried out in order to survey the mitochondrial isolation procedure and to validate expression changes in marker proteins as judged by DIGE analysis. Our optimized immunoblotting procedure for the immuno-decoration of muscle proteins has been previously described in detail [7]. Crude tissue extracts and the mitochondria-enriched fraction from normal versus diabetic skeletal muscle were electrophoretically separated with the help of a Mini-Protean-3 gel system (BioRad Laboratories, Hemel-Hempstead, Herts., UK). Gels were electrophoresed at 80V until the tracking dye ran off the end of the gel. For immunoblotting experiments, gels were transferred to an iblot transfer unit from Invitrogen (Carlsbad, CA, USA) for semi-dry blotting. Electrophoretic transfer was carried out for 6 min. Nitrocellulose membranes were blocked in a milk protein solution for 1h and then incubated overnight with gentle agitation with sufficiently diluted primary antibody, diluted in blocking solution containing 5% (w/v) fat-free milk powder in phosphate-buffered saline (0.9 % (w/v) NaCl, 50 mM sodium phosphate, pH 7.4). Following two washing steps with blocking solution for 10 min, sheets were incubated for 1h with secondary peroxidase-conjugated antibodies. Subsequently, nitrocellulose sheets were washed twice with blocking solution and then rinsed with phosphate-buffered saline. Immuno-decorated bands were visualized by the enhanced chemiluminescence method.

3. Results

Difference in-gel electrophoresis (DIGE) is a highly discriminating technique that can detect minute changes in the expression of soluble proteins. This makes DIGE analysis the

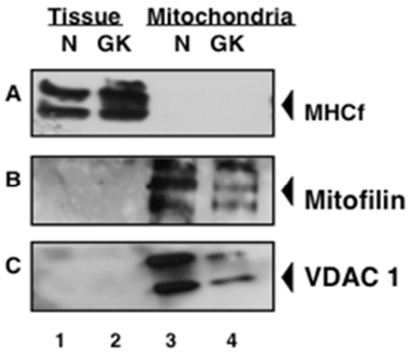

Figure 1. Immunoblot analysis of mitochondrial enrichment from normal and diabetic skeletal muscle. Shown are immunoblots of crude homogenates versus the mitochondria-enriched fraction from normal (N) versus Goto-Kakizaki (GK) rat muscle, labeled with antibodies to the fast MHCf isoform of myosin heavy cahin (A), the mitochondrial inner membrane marker mitofilin (B) and the mitochondrial outer membrane porin isoform VDAC1 of the voltage-dependent anion channel (C). Lanes 1 to 4 show tissue homogenates from normal and GK rat muscle and the mitochondrial fraction from normal and GK rat muscle, respectively.

method of choice for comparative biochemical studies of distinct protein complements [29]. Although two-dimensional gel electrophoresis underestimates the presence of certain classes of proteins, such as integral proteins and components with a very high molecular mass, the highly sensitive DIGE technique is ideal for the study of soluble proteins in the molecular range of 10 kDa to 200 kDa. We have applied here the DIGE method for the comparative analysis of the soluble mitochondrial subproteome from normal versus diabetic skeletal muscle tissue. As illustrated in Figure 1, the subcellular fractionation method applied here clearly resulted in the enrichment of mitochondrial markers. While the fast myosin heavy chain isoform MHCf drastically decreased in abundance during the isolation of the mitochondrial fraction (Figure 1A), marker proteins of the inner and outer mitochondrial membrane, mitofilin and the porin isoform VDAC1, increased in their density (Figure 1B, C). Thus, this differential centrifugation protocol removed the most abundant type of contractile muscle proteins from the mitochondrial preparation.

Following fluorescent labeling with CyDyes and the high-resolution two-dimensional gel electrophoretic separation of the mitochondria-enriched fraction, as shown in Figure 2, densitometric scanning revealed a changed abundance in 24 distinct protein spots. A reduced expression was found for 18 muscle-associated proteins and an increased density for 6 proteins in the mitochondria-enriched fraction. The first dimensional position of certain muscle-associated proteins, such as phosphoglycerate kinase, diacylglycerol kinase and phosphofructokinase, did not agree with the theoretical p*I*-values of these enzymes. However, the recent large-scale analysis of the aged mitochondrial proteome from rat skeletal muscle has shown that the mitochondrial organelle contains over a thousand different protein species, whereby many proteins are represented by a large number of distinct two-dimensional spots in analytical gels [7]. Thus, a specific type of enzyme exists in multiple sub-species and this explains why theoretical pI-values do not always perfectly agree with actual p*I*-values following two-dimensional gel electrophoresis. It is actually one of the great advantages of proteomic technologies that they can differentiate between differently charged or sized sub-species of individual proteins.

The DIGE master gel shown in Figure 3 outlines the position of the 24 2D-spots with a changed abundance in diabetic preparations. Mass spectrometry identified the proteins with a decreased abundance as the glycolytic enzyme enolase (spots 1 and 7), the molecular chaperones Hsp60 (spot 3) and Hsp72 (spot 8), muscle glycogen phosphorylase (spot 4), the mitochondrial enzyme NADH dehydrogenase (spots 5 and 12), subunit-1 of the cytochrome b-c1 complex (spot 6), phosphoglycerate kinase (spot 9) muscle creatine kinase (spot 10), diacylglycerol kinase (spot 11), isocitrate dehydrogenase (spot 13), myosin binding protein H (spot 14), the mitochondrial Tu translation elongation factor (spot 16), the 78 kDa glucose-regulated protein (spot 17) and the fast MLC1-f isoform of myosin light chain (spot 18). Interestingly, differential changes of individual 2D spots representing pyruvate dehydrogenase (spots 15 and 22) and ATP synthase (spots 2, 19 and 24) were observed. Increased proteins were identified as triose phosphate isomerase (spot 20), phosphofructokinase (spot 21) and pyruvate kinase (spot 23). Table 1 correlates spot numbers on the DIGE master gel with the proteomic information on individual protein species as determined by mass spectrometry. The table lists the protein name, accession number, predicted isoelectric point, predicted molecular mass, number of matched peptides, Mascot score, the percent sequence coverage and fold-change. The main subcellular

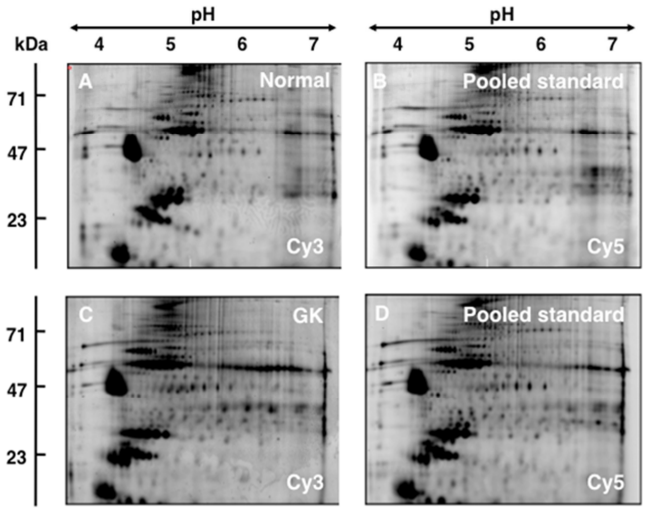

Figure 2. Comparative two-dimensional gel electrophoretic analysis of normal versus diabetic rat skeletal muscle. Shown is the 2D-DIGE analysis of the mitochondria-enriched fraction, using a pH 4-7 range in the first dimension. Individual panels represent Cy3-labelled gels of mitochondrial protein fractions from normal (A) and diabetic GK (C) skeletal muscle, as well as Cy5-labelled gels containing pooled standards (B, D). The pH-values of the first dimension gel system and molecular mass standards (in kDa) of the second dimension are indicated on the top and on the left of the panels, respectively.

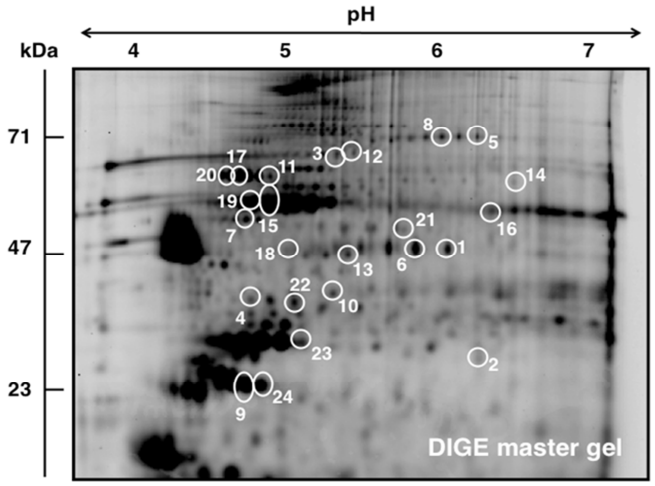

Figure 3. Two-dimensional DIGE reference gel of diabetic rat skeletal muscle. Shown is a DIGE reference gel of the mitochondria-enriched fraction from rat skeletal muscle, used for the mass spectrometric identification of proteins with a differential expression profile. The pH-values of the first dimension gel system and molecular mass standards (in kDa) of the second dimension are indicated on the top and on the left of the panel, respectively. Identified muscle proteins are marked by circles and are numbered 1 to 24. See Table 1 for a detailed listing of proteins that exhibited a diabetes-associated change in their abundance.

localizations of the identified proteins are predicted to be mitochondria (ATP synthase, NADH dehydrogenase, cytochrome b-c1 complex, isocitrate dehydrogenase, pyruvate dehydrogenase, F1-ATPase, 78kDa glucose-regulated protein, creatine kinase, Tu translation elongation factor, Hsp60), the cytosol (enolase, Hsp72, glycogen phosphorylase, phosphoglycerate kinase, diacylglycerol kinase, triose phosphate isomerase, phosphofructokinase, pyruvate kinase) and the contractile apparatus (myosin binding protein H, myosin light chain MLC-1f).

In order to verify DIGE-determined changes in marker proteins, immunoblotting was carried out. As shown in Figure 4, the molecular chaperone Hsp60 was clearly found to be reduced in GK preparations (Figure 4A), which agrees with the proteomic finding of a reduction in spot no. 3 (Figure 3; Table 1). Since the DIGE analysis of mitochondrial ATP synthase had revealed differential effects of diabetes on different sub-species of this mitochondrial enzyme, it was of interest to evaluate its molecular fate by immunoblotting. As illustrated in Figure 4B, the beta-subunit of ATP synthase was found to be increased in abundance. This agrees with the proteomic identification of increased spot no. 24 (Figure 3; Table 1). The statistical evaluation of the comparative immunoblot analysis of Hsp60 and mitochondrial ATP synthase is shown in Figure 4C, D and demonstrates the significance of the altered expression changes of these two proteins.

4. Discussion

Abnormal mitochondrial functioning has been implicated to play a central role in the molecular pathogenesis of insulin resistance and contractile weakness in diabetic skeletal muscle tissues [17]. The proteomic DIGE analysis of the mitochondria-enriched fraction presented here confirms a disturbed protein expression pattern in the mitochondrial subproteome from GK gastrocnemius muscle. Our previous proteomic survey of crude total extracts from diabetic GK muscle had identified moderate differential expression patterns in 21 protein species. The diabetic phenotype seems to be associated with a generally altered composition of the muscle protein complement, affecting especially glucose, fatty acid, nucleotide and amino acid metabolism, as well as the contractile apparatus, the cellular stress response, the antioxidant defense system and detoxification mechanisms [25]. With respect to changes in mitochondria, a reduction in NADH dehydrogenase, cytochrome b-c1 complex and isocitrate dehydrogenase agrees with the previously reported decreased oxidative phosphorylation in type-2 diabetes [14-16].

The lower concentration of the molecular chaperones Hsp60 and Hsp72 indicates an impaired cellular stress response in GK muscle tissue, which might weaken the defense mechanisms of metabolically challenged skeletal muscles. Reduced levels of muscle glycogen phosphorylase could have a negative effect on the proper utilization of stored glycogen and a lower concentration of creatine kinase may negatively affect the creatine phosphate shuttle. Both reductions in key metabolic components probably worsen the bioenergetic status of diabetic fibres and explain the contractile weakness in certain skeletal muscles from patients with type-2 diabetes [30, 31]. Interestingly, glycolytic marker enzymes were differentially affected in GK muscle. The increased density of triose phosphate isomerase, phosphofructokinase and pyruvate kinase would suggest a higher glycolytic flux rate in tissues with a reduced mitochondrial content. Since phosphofructokinase and pyruvate kinase represent rate-limiting steps of glycolysis, this alteration might be interpreted as a glycolytic

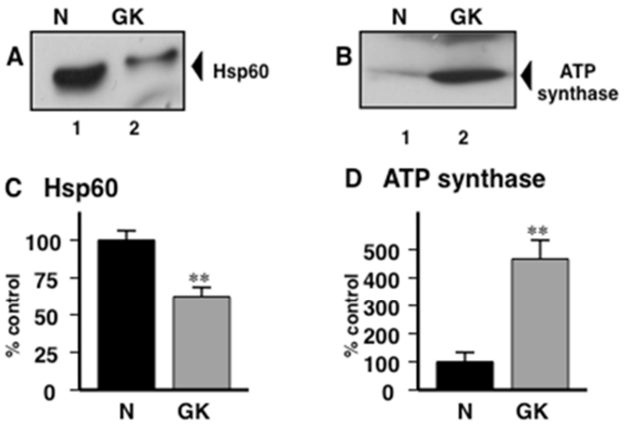

Figure 4. Immunoblot analysis of altered marker proteins in diabetic skeletal muscle. Shown are immunoblots of normal (lane 1) versus diabetic Goto-Kakizaki (lane 2) mitochondria-enriched preparations from skeletal muscle. Immuno-decoration was carried out with antibodies to the molecular chaperone Hsp60 (A, C) and the mitochondrial enzyme ATP synthase (B, D). The comparative blotting was statistically evaluated using an unpaired Student's t-test (n=5; **p<0.01).

Table 1. List of identified proteins that exhibit a changed abundance in mitochondria-enriched fraction from diabetic skeletal muscle.

Spot no.	Protein name	Accession no.	Isoelectric point (p*I*)	Molecular mass (kDa)	Matched peptides	ANOVA (p value)	Mascot score	Coverage (%)	Fold change
1	Enolase	gi|126723393|	7.08	47,332	17	0.009	314	44	-4.5
2	Mitochondrial H-ATP synthase	gi|220904|	5.78	18,828	3	0.021	74	16	-3.3
3	Heat shock protein Hsp60	gi|56383|	5.11	61,101	5	0.043	100	12	-3.2
4	Muscle glycogen phosphorylase	gi|158138498|	6.65	97,749	10	0.032	112	13	-3.1
5	NADH dehydrogenase, FeS protein 8	gi|157821497|	5.87	24,419	6	0.031	56	25	-2.9
6	Cytochrome b-c1 complex, subunit 1	gi|51948476|	5.57	53,511	9	0.004	241	19	-2.7
7	Enolase	gi|126723393|	7.08	47,332	17	0.002	206	44	-2.6
8	Heat shock protein Hsp72	gi|347019|	5.43	71,116	12	0.011	113	17	-2.6
9	Phosphoglycerate kinase	gi|40254752|	8.02	44,916	9	0.003	96	29	-2.5
10	Muscle creatine kinase	gi|6978661|	6.57	43,224	7	0.015	111	17	-2.4
11	Diacylglycerol kinase, delta	gi|149037714|	8.09	126,993	2	0.008	50	1	-2.3
12	NADH dehydrogenase, FeS protein 1	gi|53850628|	5.65	80,348	2	0.032	44	4	-2.2
13	Isocitrate dehydrogenase	gi|16758446|	6.47	40,052	7	0.049	198	22	-2.2
14	Myosin binding protein H	gi|38303941|	6	53,044	7	0.048	177	23	-2.1
15	Pyruvate dehydrogenase	gi|56090293|	6.2	39,305	11	0.031	294	34	-2.1
16	Tu translation elongation factor, mitochondrial	gi|149067905|	7.65	44,023	2	0.020	74	10	-2
17	78kDa glucose-regulated protein	gi|25742763|	5.07	72,476	2	0.024	60	4	-2
18	Myosin light chain MLC-1f	gi|205485|	4.99	20,795	15	0.003	200	67	-2
19	F1-ATPase, beta subunit	gi|203033|	5.07	38,747	4	0.009	187	12	2
20	Triose phosphate isomerase	gi|38512111|	7.07	27,223	10	0.006	152	59	2.1
21	Phosphofructokinase	gi|62825891|	8.07	86,159	5	0.040	89	7	2.2
22	Pyruvate dehydrogenase	gi|56090293|	6.2	39,305	3	0.044	37	10	2.6
23	Pyruvate kinase	gi|16757994|	6.63	58,303	13	0.013	283	30	2.7
24	ATP synthase, mitochondrial F1 complex	gi|149029719|	6.43	23,434	8	0.004	94	53	3.1

shift that has previously been described in obese skeletal muscles [32]. On the other hand, the enzymes enolase and phosphoglycerate kinase are reduced in GK tissue, which might be associated with the multi-functionality of many glycolytic elements [33]. The enzyme diacylglycerol kinase catalyzes the conversion of diacylglycerol to phosphatidic acid and uses ATP as a source of the phosphate. Thus reduced ATP levels in diabetic muscle tissues due to a reduced content of functional mitochondria could affect this enzyme. Altered levels of the fast MLC1-f isoform of myosin light chain and myosin binding protein H indicate a certain degree of re-modeling of the contractile apparatus. However, since this subproteomic analysis was carried out with a mitochondria-enriched fraction, it is difficult to interpret changes in regulatory elements of the acto-myosin filaments. Compensatory mechanisms to counter-act the loss of mitochondrial functioning appear to be an increase in certain isoforms of pyruvate dehydrogenase and ATP synthase [17].

In conclusion, the novel candidate proteins with a changed expression level in the mitochondria-enriched fraction, as shown in this proteomic survey of the GK rat model of non-obeses type-2 diabetes, should be helpful for complementing the biomarker signature of diabetes mellitus [34-38]. Changes in the functioning and/or density of mitochondrial enzymes

may be useful for the identification of new therapeutic targets, the development of better diagnostic criteria, the improved monitoring of disease progression and the biomedical evaluation of experimental treatment regimes.

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