Proteome Analysis Reveals Phosphorylation of ATP Synthase β-Subunit in Human Skeletal Muscle and Proteins with Potential Roles in Type 2 Diabetes*

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Insulin resistance in skeletal muscle is a hallmark feature of type 2 diabetes. An increasing number of enzymes and metabolic pathways have been implicated in the development of insulin resistance. However, the primary cellular cause of insulin resistance remains uncertain. Proteome analysis can quantitate a large number of proteins and their post-translational modifications simultaneously and is a powerful tool to study polygenic diseases like type 2 diabetes. Using this approach on human skeletal muscle biopsies, we have identified eight potential protein markers for type 2 diabetes in the fasting state. The observed changes in protein expression indicate increased cellular stress, e.g. up-regulation of two heat shock proteins, and perturbations in ATP (re)synthesis and mitochondrial metabolism, e.g. down-regulation of ATP synthase β-subunit and creatine kinase B, in skeletal muscle of patients with type 2 diabetes. Phosphorylation appears to play a key, potentially coordinating role for most of the proteins identified in this study. In particular, we demonstrated that the catalytic β-subunit of ATP synthase is phosphorylated in vivo and that the levels of a down-regulated ATP synthase β-subunit phosphoisoform in diabetic muscle correlated inversely with fasting plasma glucose levels. These data suggest a role for phosphorylation of ATP synthase β-subunit in the regulation of ATP synthesis and that alterations in the regulation of ATP synthesis and cellular stress proteins may contribute to the pathogenesis of type 2 diabetes.

Insulin resistance in skeletal muscle, defined as reduced insulin-stimulated glucose disposal, is a characteristic feature of type 2 diabetes mellitus (T2DM)1 and is believed to be largely accounted for by reduced non-oxidative glucose metabolism (1–3). Furthermore, insulin stimulation of glucose oxidation and suppression of lipid oxidation is significantly impaired in patients with T2DM (2, 3). Conversely, in the basal, fasting state increased glucose oxidation and reduced lipid oxidation is seen in skeletal muscle of insulin resistant subjects, whether caused by T2DM or obesity alone (4). These defects suggest an impaired capacity to switch between carbohydrate and fat as oxidative energy sources in insulin-resistant subjects. Together with reports of reduced oxidative enzyme activity and dysfunction of mitochondria in skeletal muscle of patients with T2DM (4–6) and the fact that mitochondrial DNA defects cause T2DM through impairment of oxidative phosphorylation (7, 8), these abnormalities in fuel metabolism have led to the hypotheses that perturbations in skeletal muscle mitochondrial metabolism (6, 9, 10) and defects in the signaling pathways of AMP-activated protein kinase (AMPK) are implicated in the pathogenesis of T2DM (11). That rates of fuel oxidation and mitochondrial function can affect glucose uptake and glycogen synthesis has been reported earlier (12, 13). In addition, both chronic activation of AMPK and induced expression of the transcriptional co-activator of peroxisome proliferator-activated receptor γ, PGC-1, result in improved mitochondrial biogenesis concomitant with increases in GLUT4 protein content in skeletal muscle (14–16). The reduction in oxidative enzyme capacity in patients with T2DM could be attributed to an increased proportion of glycolytic, type 2 muscle fibers (17). However, reduced oxidative enzyme capacity seems to be equally present in all muscle fiber types in patients with T2DM (5).

An increasing number of enzymes and metabolic pathways has been suggested to be involved in the development of skeletal muscle insulin resistance in T2DM. Therefore, there is a growing demand for techniques able to evaluate proteins from many signaling and metabolic pathways simultaneously. The recently introduced technique of proteome analysis, high resolution two-dimensional (2-D) gel electrophoresis followed by AMP-activated protein kinase; MS, mass spectrometry; %IOD, percentage integrated optical density; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; ATPsyn, ATP synthase β-subunit; CK-B, creatine kinase, brain isofrom; PGD1, phosphoglucomutase-1; HSP, heat shock protein; GRP78, 78-kDa glucose-regulated protein; α1(VI) collagen, α1 chain of type VI collagen; MRLC2, myosin regula-
tory light chain 2; ROS, reactive oxygen species; 2-D, two-dimensional; FFA, free fatty acids; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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Proteome Analysis of Skeletal Muscle in Type 2 Diabetes

**MATERIALS AND METHODS**

The study was approved by the Local Ethics Committee and was performed in accordance with the Helsinki Declaration.

**Muscle Samples**—Muscle samples were obtained from 10 patients with T2DM and 10 healthy age- and gender-matched control subjects. Five patients with T2DM were treated by diet alone, and five patients were treated by low doses of either sulfonylurea or metformin. These drugs were withdrawn 1 week prior to the study. The patients were all GAD65 antibody-negative and without signs of diabetic retinopathy, nephropathy, or macrovascular complications. Six of the patients had normal results on screening blood tests of hepatic and renal function. The control subjects had normal glucose tolerance and no family history of diabetes. Participants were instructed to avoid vigorous exercise for 48 h before the study, which was carried out after a 10-h overnight fast. Fasting blood samples were analyzed for glucose (Glucose Analyzer II, Beckman Instruments, Fullerton, CA), free fatty acids (FFA) (Wako Chemicals Gmbh, Neuss, Germany), insulin, and C-peptide (Wallac Oy, Turku, Finland). Percutaneous needle biopsies were obtained from the vastus lateralis muscle under local anesthesia using a biopsy pistol, and the muscle specimens (25 mg) were immediately blotted free of blood, fat, and connective tissue and frozen in liquid nitrogen.

**Sample Preparation**—The frozen muscle samples were homogenized for 25 min in 100 μl of ice-cold DNase/RNase buffer (20 mM Tris-HCl buffer, pH 7.5, containing 30 mM sodium chloride, 5 mM sodium chloride, 5 mM magnesium chloride, and 25 μg/mL RNase A, 25 μg/mL RNase I (Worthington, Freehold, NJ)). After homogenization, the samples were lyophilized overnight and then dissolved in 120 μl of lysis buffer (7 mM urea (ICN Biomedicals), 2 mM thiourea (Fluka), 2% CHAPS (Sigma), 0.4% dithiothreitol (Sigma), 0.5% Phospharylate 3–10, and 0.5% Phospharylate 6–11 (Amersham Biosciences) by shaking overnight. The homogenization was carried out in a buffer without kinase and phosphatase inhibitors (salts) to avoid destruction of the first dimensional gels. As this study was carried out at 0–4 °C, we assumed the activity of potential kinases and phosphatases to be very low and that the phosphorylation state of most proteins was stable. To confirm this, we have, after the present study, performed similar 2-D gels, in which the muscle specimens were solubilized directly in the lysis buffer for running the first dimensional gel. The pattern of the proteins on these 2-D gels and in particular the relative abundance of the different phosphoisoforms identified by MS showed no difference from the 2-D gels presented in this study.

**Protein Determination**—The protein concentration in the muscle samples was determined using the Bradford method, which was adopted for use with lysis buffer as described before (19).

**2-D Electrophoresis**—First dimension gel electrophoresis was performed on an IPG covering the pH range from 4 to 7 (Amersham Biosciences). Rehydration buffer for IPG 4–7 strips was identical with lysis buffer used for sample preparation, and the sample was applied by in-gel rehydration. 400 μg of protein were loaded on each gel. Focusing was performed on a Multiphor II at 20 °C using a voltage/time profile (C using a voltage/time profile and 300 Ci of [32P]orthophosphate (Amersham Biosciences) for 2.5 h. Phosphate groups in proteins of human myoblasts were labeled biosynthetically by incubating them in 300 μl of serum-free phosphate-free Dulbecco’s modified Eagle’s medium containing 0.2% bovine serum albumin for 2.5 h. Phosphate groups in proteins of human myoblasts were labeled biosynthetically by incubating them in 300 μl of serum-free phosphate-free Dulbecco’s modified Eagle’s medium containing 0.2% bovine serum albumin for 2.5 h. Immediately after labeling, medium was removed, and cells were lysed in 400 μl of lysis buffer as described above. Determination of 32Porthophosphate incorporation into myoblast proteins was performed using trichloroacetic acid precipitation as described (19). 2-D gel electrophoresis was run as described above loading 30 μl lysis buffer corresponding to 4 × 10^5 cpm on the gel. 32P-labeled proteins of myoblasts were visualized by exposing dried gels to phosphorimaging plates (AGFA).

**RESULTS**

**Quantitative Proteome Analysis**—Using computerized image analysis 489 spots in each gel image were matched and quantified using a Bio Image computer program (version 6.1; Bob Luton, Ann Arbor, MI). For comparison we have used 2-D gels from six subjects in the control group and nine subjects in the diabetes group (Table I). The remaining gels revealed that some protein degradation had occurred, and therefore these gels were excluded from the analysis. The expression of each protein was measured and expressed as its percentage integrated optical density (%IOD) (a percentage of the sum of all the pixel gray level values on and within the boundary of the spot in question compared with that of all detected spots). Images from each group were then matched, edited, and compared statistically. The average value of spot %IOD and S.D. were calculated for each protein in each group and then compared using the two-sided Student’s t test. Protein spots whose expression was found different between the two groups at the significance level of p < 0.05 were selected for further analysis. For correlation analysis Spearman’s rho was used.

**Protein Visualization and Protein Identification**—Proteins of interest were cut out from the gels and, after in-gel digestion, analyzed by mass spectrometry using a Bruker REFLEX matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (19, 20). The mass spectra obtained were internally calibrated using trypsin autodigestion peptides, and the masses were used to search the NCBI database using the ProFound, FindPept, and FindMod programs (www.proteometrics.com). Data base searches were performed using the following attributes with minor modification needed for each program: all species, no restrictions for molecular weight and protein pI, trypsin digest, one missed cleavage allowed, cysteines modified by acrylamide, and oxidation of methionines possible, mass tolerance 0.1 Da, and 0.4 NS 6.6 0.002

| Duration of diabetes | 46.1 ± 1.5 | 44.8 ± 1.5 | NS |
|----------------------|------------|------------|----|
| Body mass index (kg/m²) | 25.7 ± 1.2 | 33.3 ± 1.9 | 0.01 |
| Male/female          | 3/3        | 5/4        | NS |
| Cholesterol (mmol/l) | 5.1 ± 0.4  | 5.9 ± 0.4  | NS |
| HbA1c                | 5.3 ± 0.1  | 6.6 ± 0.4  | 0.02 |
| Glucose (mmol/l)     | 5.3 ± 0.1  | 8.5 ± 0.7  | 0.002 |
| FFA (mmol/l)         | 0.28 ± 0.04| 0.44 ± 0.08| NS |
| Insulin (pmol/l)     | 47 ± 11    | 93 ± 12    | 0.005 |
| C-peptide (pmol/l)   | 575 ± 120  | 1195 ± 93  | 0.001 |

**TABLE I**

**Fasting characteristics of study subjects**

Clinical characteristics of subjects whose 2-D gels were included in the computerized image analysis. Data represent means ± S.E. NS, not significant.
titated. Fifteen protein spots were expressed at statistically significant different levels in the two groups (Fig. 1). These potential protein markers of T2DM were excised from the 2-D gels for identification by MALDI-TOF-MS analysis. Eleven of these protein spots were positively identified: three metabolic enzymes, two heat shock proteins, and different isoforms of three structural proteins (Table II). Of the metabolic enzymes, ATP synthase β-subunit (ATPsynβ) and creatine kinase, brain isoform (CK-B) were significantly down-regulated, whereas phosphoglucomutase-1 (PGM-1) was significantly up-regulated in skeletal muscle of patients with T2DM. Heat shock protein (HSP) 90β and 78-kDa glucose-regulated protein (GRP78) were both significantly up-regulated in diabetic muscle. Of the structural proteins, four isoforms of α1 chain of type VI collagen (α1(VI) collagen) and one isoenzyme of myosin regulatory light chain 2 (MRLC2-A and MRLC2-B) were significantly up-regulated, whereas another isoenzyme of myosin regulatory light chain 2 (MRLC2-A) was significantly down-regulated in diabetic muscle.

In the diabetic group the levels of ATPsynβ (spot 180) and CK-B (spot 541) correlated negatively with fasting plasma glucose values (r = −0.75; p < 0.05) and (r = −0.82; p < 0.01), respectively (Fig. 2, A and B), whereas the levels of α1(VI) collagen (spot 11) correlated positively with fasting plasma glucose values (r = 0.67; p < 0.05). Furthermore, the levels of ATPsynβ (spot 180) correlated positively with the levels of CK-B (spot 541) (r = 0.71; p < 0.05) (Fig. 2C) and negatively with the levels of α1(VI) collagen (spot 11) (r = −0.72; p < 0.05) in the diabetic group. In contrast, in the control group the levels of ATPsynβ (spot 180) correlated negatively with fasting FFA values (r = −0.81; p < 0.05) rather than fasting plasma glucose values (Fig. 2D), and the levels of PGM-1 (spot 456) correlated positively with insulin (r = 0.95; p < 0.01). No other correlations among glucose, FFA, insulin, or C-peptide levels and the identified potential protein markers of T2DM were observed in the two groups. In particular, none of these protein markers correlated with body mass index either in the entire study population or within the study groups.

Identification of Protein Marker Isoforms—CK-B, HSP 90β, GRP78, PGM-1, and MRLC2 have been reported previously (22–26) to be phosphorylated. Several of the identified protein spots were located within a row of three-six protein spots with the same molecular weight indicating multiple post-translational modifications (possibly phosphorylation) of the same protein (Fig. 1). These protein spots were excised and subjected to MALDI-MS analysis. Two additional isoforms of HSP 90β, GRP78, PGM-1, α1(VI) collagen, MRLC2-A, and MRLC2-B and three additional isoforms of ATPsynβ were positively identified (Fig. 3). Calculation of sums of expression of the different isoforms of each protein marker showed that the sum of expression of HSP 90β, GRP78, α1(VI) collagen, and ATPsynβ were significantly up- or down-regulated in the same direction as the protein markers (Table III). This was also the case with the sum of expression of MRLC2-A and MRLC2-B, although these differences did not reach statistical significance (Table III). The relative abundance of the down-regulated ATPsynβ isoform (spot 180) was significantly reduced in muscle of patients with T2DM, as well (p = 0.03), indicating a role for post-translational modification of this protein. Interestingly, in the diabetic group the sum of expression of ATPsynβ and α1(VI) collagen correlated significantly with fasting plasma glucose values (r = −0.76 and r = 0.76; p < 0.05, respectively), and the sum of expression of ATPsynβ correlated significantly with the sum of expression of α1(VI) collagen (r = −0.87; p < 0.01).

Phosphorylation of ATP Synthase β-Subunit—The identifi-
Correlation of fasting plasma glucose levels with the expression of the down-regulated ATPsyn in vivo phosphorylated modification of ATP synthase.

The correlation of fasting plasma glucose levels with the expression of the down-regulated ATPsyn (Fig. 4) and that a putative non-modified variant was identified by MALDI-MS analyses (summed up in Table III).

**TABLE II**

| Spot no. | Protein | Database accession no. | Theoretical pl/mW | Sequence coverage | Matched peptides | Control group | Diabetic group | \( p \)  |
|----------|---------|------------------------|-------------------|------------------|-----------------|---------------|---------------|------|
| 180      | ATP synthase \( \beta \)-subunit | P06576              | 5.0/52            | 56/19            | 0.56 ± 0.05     | 0.41 ± 0.03   | 0.03           |
| 295      | Myosin regulatory light chain 2 (A) | P10916              | 4.9/19            | 58/9             | 1.26 ± 0.10     | 0.92 ± 0.10   | 0.03           |
| 541      | Creatine kinase B                  | P12277              | 5.3/43            | 42/12            | 0.32 ± 0.04     | 0.20 ± 0.02   | 0.04           |
| 10       | Collagen \( \alpha(VI) \) chain   | P12109              | 5.3/110           | 10/10            | 0.06 ± 0.01     | 0.09 ± 0.01   | 0.04           |
| 11       | Collagen \( \alpha(VI) \) chain   | P12109              | 5.3/110           | 5/5              | 0.04 ± 0.00     | 0.06 ± 0.01   | 0.02           |
| 407      | Collagen \( \alpha(VI) \) chain   | P12109              | 5.3/110           | 6/6              | 0.05 ± 0.01     | 0.08 ± 0.01   | 0.03           |
| 520      | Collagen \( \alpha(VI) \) chain   | P12109              | 5.3/110           | 6/5              | 0.06 ± 0.01     | 0.09 ± 0.01   | 0.02           |
| 51       | Heat shock protein 90\( \beta \)  | P08238              | 5.0/84            | 15/11            | 0.03 ± 0.01     | 0.05 ± 0.00   | 0.02           |
| 375      | Glucose-regulated protein 78       | P11021              | 4.9/78            | 28/12            | 0.06 ± 0.01     | 0.08 ± 0.01   | 0.02           |
| 445      | Myosin regulatory light chain 2 (B) | AAK55797           | 4.9/19            | 52/7             | 0.30 ± 0.08     | 0.69 ± 0.12   | 0.03           |
| 456      | Phosphoglucomutase 1               | P36871              | 6.2/62            | 25/12            | 0.14 ± 0.04     | 0.28 ± 0.04   | 0.03           |

**FIG. 2.** Relation among ATPsyn\( \beta \), CK-B, and plasma glucose in type 2 diabetes. Correlation of fasting plasma glucose levels with the expression of the down-regulated ATPsyn isoform and with the down-regulated CK-B isoform in skeletal muscle in type 2 diabetic subjects (T2DM) (A and B). C, correlation between the expression of the down-regulated ATPsyn isoform and with the down-regulated CK-B isoform in skeletal muscle in T2DM. D, correlation of fasting plasma FFA levels with the expression of the down-regulated ATPsyn isoform in control subjects.

**TABLE III**

| Protein                              | Control group | Diabetic group | \( p \)  |
|--------------------------------------|---------------|---------------|------|
| ATP synthase \( \beta \)-subunit      | 2.28 ± 0.16   | 1.86 ± 0.10*a |      |
| Myosin regulatory light chain 2 (A)   | 3.67 ± 0.21   | 3.16 ± 0.29   |      |
| Collagen \( \alpha(VI) \) chain      | 0.29 ± 0.04   | 0.43 ± 0.03*a |      |
| Heat shock protein 90\( \beta \)     | 0.09 ± 0.01   | 0.13 ± 0.01*a |      |
| Glucose-regulated protein 78          | 0.13 ± 0.01   | 0.17 ± 0.01*a |      |
| Myosin regulatory light chain 2 (B)   | 3.64 ± 0.21   | 4.77 ± 0.67   |      |

*a \( p < 0.05 \) vs. control.

**FIG. 3.** Isoforms of protein markers of type 2 diabetes. Enlarged regions of the silver-stained 2-D gel demonstrating the potential additional isoforms of HSP 90\( \beta \), GRP78, PGM-1, \( \alpha(VI) \) collagen, ATPsyn\( \beta \), MRLC2-A, and MRLC2-B. The asterisks indicate the protein markers (Table II), and the arrows indicate the additional isoforms positively identified by MALDI-MS analyses.

**DISCUSSION**

Using the methods of proteome analysis we have identified and characterized eight potential protein markers of T2DM in skeletal muscle in the fasting state. Although proteome analysis is somewhat limited by narrow dynamic ranges of silver staining and difficulties in MS identification of low abundant proteins recovered from fixed and or stained gels, it is the only technique that provides the possibility to quantitatively study global changes in expression profile of proteins, as well as certain post-translational protein modifications in a given cell or tissue. Because of the method of sample preparation used in this study the interpretation of the relative abundance of the specific protein marker phos-
The study of molecular processes underlying a complex disease such as T2DM. Combined 2-D gel and MS technology can be powerful tools for identifying markers and several additional isoforms demonstrates that regulation of mitochondrial function and glucose uptake activity despite reduced total levels of ATPsynβ is regulated by phosphorylation and that the expression of ATPsynβ might be altered in skeletal muscle of patients with T2DM. Because there is no report to date on the phosphorylation of human ATPsynβ it is uncertain how such post-translational modification interacts with the proposed binding-change model for ATP synthesis, in which conformational changes in the nucleotide-binding sites of the three β-subunits are coupled to the catalytic activity of F1-ATP synthase (27). That phosphorylation of human ATPsynβ may play a role for the catalytic activity of F1-ATP synthase is suggested by the recent observation that phosphoserine/phosphothreonine-binding 14–3–3 proteins were found to be associated with mitochondrial ATP synthase in plants in a phosphorylation-dependent manner through direct interaction with the β-subunit of F1-ATP synthase and that the activity of ATP synthase was reduced by recombinant 14–3–3 protein (28).

Consistent with the reduced expression of ATPsynβ in our study, several other studies argue for a lower ATP synthase activity in diabetic muscle. Thus, gene expression of several subunits from the other four complexes of the mitochondrial electron transport chain were found to be decreased in skeletal muscle of streptozotocin-diabetic mice (29), and during fasting conditions reduced activity of cytochrome c oxidase and citrate synthase activity, as well as reduced overall activity of the respiratory chain, have been demonstrated in skeletal muscle of patients with T2DM (4–6). Moreover, mutations in mtDNA can, via impaired oxidative phosphorylation, cause T2DM, with both muscle insulin resistance and impaired insulin secretion (7–8). Indeed, decreased ATP synthase activity and increased formation of reactive oxygen species (ROS) have been reported in hybrids constructed from mitochondria of patients with T2DM (9). However, whether the down-regulation of a specific ATPsynβ phosphoisoform is a mechanism to maintain normal activity despite reduced total levels of ATPsynβ or whether it reflects an altered activity of ATPsynβ per se warrants further studies. Endurance training up-regulates ATPsynβ protein levels in soleus muscle of rats (30). Thus, a sedentary lifestyle and reduced amounts of oxidative type 1 fibers in type 2 diabetic subjects (17) might play a role in the altered levels of ATPsynβ observed in our study. In control subjects we observed a negative correlation between the down-regulated ATPsynβ phosphoisoform and fasting plasma-free fatty acids. In contrast, in the patients with T2DM both this phosphoisoform and the total expression of ATPsynβ correlated inversely with fasting plasma glucose. These findings are consistent with a predominant reliance on lipid oxidation in the fasting state in muscle of lean non-diabetic subjects, which is changed to a poor reliance on lipid oxidation in muscle of patients with T2DM (4, 5). Recently, mRNA levels of the ATP synthase β-subunit, as well as of GLUT-4, were shown to be decreased 2–3-fold in skeletal muscle from patients with T2DM, in whom insulin treatment was withdrawn for 14 days (31), indicating that altered expression of ATPsynβ could be secondary either to hyperglycemia or to relative deprivation of the effects of insulin signaling (insulin resistance). Mitochondria are the intracellular sites for fuel oxidation and ATP production, and that mitochondrial dysfunction might play a key role for the impaired glucose metabolism observed in muscle of patients with T2DM is supported by the predominantly maternal transmission of T2DM in Caucasian populations (32) and by several recent studies demonstrating that regulation of mitochondrial function and glucose uptake seem to be tightly coupled; thus, both chronic activation of AMPK (14) and induced expression of PGC-1 (15, 16) result in improved mitochondrial biogenesis concomitant with increases in GLUT4 protein.

PGM-1 is a glycolytic enzyme that plays a pivotal role in glycogen metabolism, catalyzing the interconversion of glucose-1-phosphate and glucose-6-phosphate. The activity of PGM-1 is increased by phosphorylation and is reduced in type 1 fibers of skeletal muscle and with endurance training (23, 33). The increased levels of the most acidic (phosphorylated) isoform observed in this study suggest that PGM-1 activity could be increased in skeletal muscle of patients with T2DM consistent with a reduced amount of type 1 fibers (17) and an increased glycolytic-to-oxidative ratio in the fasting state (4),...
as well as a sedentary lifestyle in such subjects. Together with the absence of correlation between the levels of PGM-1 and insulin in patients with T2DM, these observations raise the question of whether an increase in PGM-1 may play a role in the impaired glucose metabolism observed in patients with T2DM (1–3).

CK-B levels in skeletal muscle are much lower than in cardiac muscle (34) but are higher in type 1 muscle fibers and have been reported to increase with endurance training and to be positively correlated with oxidative enzyme capacity irrespective of muscle fiber type (35). As with ATPsynβ and PGM-1, the reduced levels of a CK-B isoform in skeletal muscle of patients with T2DM seen here could be explained by reduced amounts of type 1 fibers (37), reduced oxidative enzyme capacity (4, 5), and a sedentary lifestyle in such patients. In skeletal muscle of streptozotocin-diabetic mice a reduction in mRNA levels of CK-B was observed, together with decreased mRNA levels of several subunits in the electron transport chain (29). These data suggest a specific role for CK-B in mitochondrial fuel oxidation. We observed a negative correlation between CK-B and plasma glucose levels and a positive correlation between CK-B and the down-regulated ATPsynβ phosphoisoform in patients with T2DM, which may indicate that down-regulation of this ATPsynβ phosphoisoform actually reduces the catalytic activity of F$_\text{1}$-ATP synthase. Allowing resynthesis of ATP from phosphocreatine, creatine kinase plays a key role in the energy metabolism of heart and skeletal muscle, and the observed correlations support the hypothesis of a functional coupling of creatine kinase isoenzymes to mitochondrial energy production and glycolysis (36). Interestingly, the creatine kinase system is regulated by AMPK via phosphocreatine/creatine and ATP/AMP ratios (36, 37), and perturbations in the AMPK signaling system were suggested recently (11) as potential key players in the development of T2DM.

GRP78 and HSP 90 are stress-inducible proteins belonging to the group of heat shock proteins and function as molecular chaperones. The in vivo functions of both proteins are dependent on an inherent ATPase activity and phosphorylation (24, 25, 38, 39). Conditions giving rise to increased levels and activity of GRP78 are followed by a decreased phosphorylation of GRP78 (25), whereas phosphorylation of HSP 90 seems to be linked to its chaperoning function (24). We observed increased levels of GRP78 in patients with T2DM, and of the three isoforms identified only the most basic (non-phosphorylated) and most active isoform was significantly up-regulated. The increase in HSP 90β levels in patients with T2DM was caused mainly by an increase in an acidic (phosphorylated) isoform. What type of cellular stress induces HSP 90β and GRP78 in muscle of patients with T2DM is currently unknown. Both sustained hypoglycemia and hyperglycemia (40), as well as oxidative stress, lead to increases in GRP78 expression (41). Hyperglycemia increases ROS formation in cultured endothelial cells (42), and increased ROS formation and oxidative stress in heart muscle leads to increased mRNA levels of HSP 70 and HSP 90 (43). However, we observed no relationship between fasting plasma glucose levels and the levels of HSP 90β or GRP78 arguing against hyperglycemia as the direct cause of cellular stress. As mentioned above increased ROS formation and oxidative stress caused by mitochondrial dysfunction have been shown in hybrids constructed from mitochondria of patients with T2DM (9). Increased ROS formation and oxidative stress lead to increased amounts of incorrectly folded and denatured proteins, which induces the expression of cellular chaperones such as GRP78 and HSP 90β (44). Interestingly, both GRP78 and HSP 90 have been shown to play a role in the post-translational processing of certain mutant insulin receptors linking these molecular chaperones to severe insulin resistance (45, 46). In addition, HSP 90β has been shown to play an important role in maintaining the activity of protein kinase B (47), which seems to be involved in the regulation of insulin-mediated glucose transport and glycogen synthesis, two processes shown to be impaired in T2DM (1). Consistent with our data, mRNA expression of a heat shock protein (HSP 70) was found to be increased in skeletal muscle of patients with T2DM both before and after withdrawal of insulin treatment (31). Further studies are needed to establish what specific roles increased levels of certain stress proteins such as HSP 90β, GRP78, and HSP 70 play in the pathogenesis of insulin resistance in muscle of patients with T2DM.

The function of both collagen type VI and MRLC2 in skeletal muscle is only partially understood, and the potential implications of the observed changes in expression of these structural proteins are therefore unclear. However, mutations in the MRLC2 gene have been shown to cause cardiac and skeletal muscle myopathy with ragged red fiber histology similar to mitochondrial myopathy (48). This relationship between MRLC2 mutations and their energy source (mitochondria) suggests that the observed changes might be secondary to perturbations in skeletal muscle mitochondrial metabolism. In vitro, hyperglycemia up-regulates type VI collagen, and up-regulation of this collagen has been demonstrated in type 2 diabetes in myocardium and nerves (49, 50). The observed association of type VI collagen levels to both plasma glucose and ATPsynβ levels in diabetic muscle suggests that increased (oxidative) stress, whether caused by hyperglycemia or mitochondrial dysfunction (9), could be responsible for the accumulation of modified long-lived proteins such as type VI collagen.

In summary, using proteome analysis we have identified eight potential protein markers of T2DM in skeletal muscle in the fasting state. The observed changes in expression of these protein markers could all be linked to increased cellular stress and perturbations in skeletal muscle mitochondrial metabolism. Two proteins important for ATP synthesis, CK-B and ATPsynβ, were reduced in diabetic muscle and correlated inversely with plasma glucose levels. However, whether these changes are secondary to hyperglycemia, hyperinsulinemia, an altered fiber type composition in muscle, or whether they are indicators of a more primary defect in T2DM remains to be elucidated. Phosphorylation appears to play a key, potentially coordinating role for most of the proteins identified in this study. In particular, we demonstrated that the catalytic β-subunit of F$_\text{1}$-ATP synthase is phosphorylated in vitro, which may contribute substantially to the understanding of the regulation of ATP synthase. These data suggest a role for phosphorylation of ATPsynβ in the regulation of ATP synthesis and indicate that alterations in the regulation of ATP synthesis and cellular stress proteins might contribute to the pathogenesis of T2DM.

REFERENCES

1. Beck-Nielsen, H. (1998) J. Basic Clin. Physiol. Pharmacol. 9, 255–279
2. Eriksson, J., Franssila-Kallunki, A., Ekström, A., Saloranta, C., Widen, E., Schalin, C., and Groop, L. (1989) J. Physiol. 406, 19–31
3. Damaso, P., Hothier-Nielsen, O., and Beck-Nielsen, H. (1991) Diabetes 36, 831–835
4. Kelley, D. E., and Mandarino, L. J. (2000) J. Clin. Invest. 106, 2944–2950
5. Gehb, S., Shosfner, J. M., Kost, D., Kaufmann, A., and Wallace, D. (1996) J. Biol. Chem. 271, 13823–13828
6. Wallace, D. C. (1999) Science 283, 1482–1488
7. Anderson, C. M. (1999) Drug. Dev. Res. 46, 67–79
8. Antonetti, D. A., Reynet, C., and Kahn, C. R. (1995) J. Clin. Invest. 95, 1383–1388
11. Winder, W. W., and Hardie, D. G. (1999) Am. J. Physiol. Endocrinol. Metab. 277, E1–E10
12. Bashan, N., Burdett, E., Guma, A., Sargeant, R., Tumiati, L., Liu, Z., and Klip, A. (1995) Am. J. Physiol. 269, C430–C440
13. Katz, A., and Westerblad, H. (1995) Biochim. Biophys. Acta 1244, 229–332
14. Winder, W. W., Holmes, B. F., Rubink, D. S., Jensen, E. B., Chen, M., and Hollosy, J. O. (2000) J. Appl. Physiol. 88, 2219–2226
15. Michael, L. F., Wu, Z., Cheatham, R. B., Puigserver, P., Adelman, G., Lehman, J. J., Kelly, D. P., and Spiegelman, B. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3820–3825
16. Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Ley, A., Cinti, S., Lowell, B., Scarpulla, R. C., and Spiegelman, B. M. (1999) Cell 99, 115–124
17. Gaster, M., Staeahr, P., Beck-Nielsen, H., Schrøder, H. D., and Handberg, A. (2000) Diabetes 49, 1324–1329
18. Fey, S. J., and Larsen, P. M. (2001) Curr. Opin. Chem. Biol. 5, 26–33
19. Fey, S. J., Nawrocki, A., Larsen, M. R., Gorg, A., Roepstorff, P., Skews, G. N., Williams, R., and Larsen, P. M. (1997) Electrophoresis 18, 1361–1372
20. Jensen, O. N., Larsen, M. R., and Roepstorff, P. (1998) Proteins 2, 74–89
21. Hurel, S. J., Rochford, J. J., Northwick, A. C., Wells, A. M., Vandenheede, J. R., Turnbull, D. M., and Yeaman, S. J. (1996) Biochem. J. 320, 871–877
22. Hemmer, W., Skarli, M., Perriard, J. C., and Wallimann, T. (1993) FEBS Lett. 327, 35–40
23. Joshi, J. G., and Zimmermann, A. (1988) Toxicology 48, 21–29
24. Zhao, Y., Gilmore, R., Leone, G., Coffey, M. C., Weber, B., and Lee, P. W. K. (2001) J. Biol. Chem. 276, 32822–32827
25. Hendershot, L. M., Ting, J., and Lee, A. S. (1988) Mol. Cell. Biol. 8, 4250–4256
26. Manning, D. R., and Stull, S. (1982) Am. J. Physiol. 242, C234–C241
27. Menz, R. I., Walker, J. E., and Leslie, A. G. W. (2001) Cell 106, 331–341
28. Bunney, T. D., van Walraven, H. S., and de Boer, A. H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4249–4254
29. Vechoor, V. K., Patti, M., Sacccone, R., and Kahn, C. R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 10587–10592
30. González, B., Hernando, R., and Manso, R. (2000) Pfuiigers Arch. 440, 42–49
31. Sreekumar, R., Halvatsiotis, P., Schminke, J. C., and Nair, K. S. (2002) Diabetes 51, 1913–1920
32. Thomas, F., Blakan, B., Vauzelle-Krevsdan, F., and Papoz L. (1994) Diabetes 43, 63–67
33. Hickson, R. C., Heusner, W. W., and Van Huss, W. D. (1976) J. Appl. Physiol. 40, 868–871
34. Fredericks, S., Merton, G. K., Leina, M. D., Heining, P., Carter, N. D., and Holf, D. W. (2001) Clin. Chim. Acta 304, 65–74
35. Apple, F. S., and Tesch, P. A. (1989) J. Appl. Physiol. 66, 2717–2720
36. Wallmann, T., Dolder, M., Schlatter, T., Deder, M., Hornemann, T., O’Gorman, R., Rock, A., and Brindel, D. (1998) Biochemistry 8, 229–234
37. Ponticos, M., Lu, Q. L., Morgan, J. E., Handie, D. G., Partridge, T. A., and Carling, D. (1998) EMBO J. 17, 1688–1699
38. Panareto, B., Prodomou, C., Roe, S. M., O’Brien, R., Laidbury, J. E., Piper, P. W., and Pearl, L. H. (1998) EMBO J. 17, 4829–4836
39. Kassenbrock, C. K., and Kelly, R. B. (1998) EMBO J. 17, 1461–1467
40. Mote, P. L., Tillman, J. B., and Spindler, S. R. (1998) Mech. Ageing Dev. 104, 149–158
41. Chen, K. D., Lai, M. T., Cho, J. H., Chen, L. Y., and Lai, Y. K. (2000) J. Cell. Biochem. 76, 585–595
42. Nishikawa, T., Edelstein, D., Du, X. L., Yamagashi, S., Matsumura, T., Kaneda, Y., Yorek, M. A., Beebe, D. Oates, P. J., Hamnes, H. P. et al. (2000) Nature 404, 787–790
43. Nishizawa, J., Nakai, A., Matsuda, K., Komeda, M., Ban, T., and Nagata, K. (2000) Circulation 99, 934–941
44. Lenny, N., and Green, M. (1991) J. Biol. Chem. 266, 20552–20557
45. Sawa, T., Imamura, T., Haratou, T., Sasaoka, T., Ishiki, M., Takada, Y., Takada, Y., Morooka, H., Ishihara, H., Usui, I., and Kobayashi, M. (1996) Biochim. Biophys. Res. Commun. 218, 449–453
46. Imamura, T., Haratou, T., Takada, Y., Usui, I., Iwata, M., Ishihara, H., Ishiki, M., Ishihashi, E. U., Ueno, E., Sasaoka, T., and Kobayashi, M. (1998) J. Biol. Chem. 273, 11183–11188
47. Sato, S., Fujita, N., and Tsuruo, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10832–10837
48. Poetter, K., Jiang, H., Hassanazadeh, S., Master, S. R., Chang, A., Dalakas, M. C., Raymond, L., Sellers, J. R., Funanagapazir, L., and Epstein, N. D. (1996) J. Biol. Chem. 271, 641–649
49. Muona, P., jaakola, S., Zhang, R. Z., Pan, T. C., Pelliniemi, L., isteli, L., chu, M. L., llottf, J., and pelletier, J. (1993) Am. J. Pathol. 142, 1586–1597
50. Spiro, M. J., and Crowley, T. J. (1993) Diabetologia 36, 83–98
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