Quantitative Protein Profiling in Heart Mitochondria from Diabetic Rats

Quantitative protein profiling based on in vitro stable isotope labeling, two-dimensional polyacrylamide gel electrophoresis, and mass spectrometry is an accurate and reliable approach to measure simultaneously the relative abundance of many individual proteins within two different samples. In the present study, it was used to define a set of alterations caused by diabetes in heart mitochondria from streptozotocin-treated rats. We demonstrated that the expression of proteins from the myocardial tricarboxylic acid cycle was not altered in diabetes. However, up-regulation of the fatty acid oxidation favored fatty acids over glucose as a source of acetyl CoA for the tricarboxylic acid cycle. Protein levels for several proteins involved in electron transport were modestly decreased. Whether this may depress overall ATP production remains to be established, since the protein level of ATP synthase seems to be unchanged. Other changes include down-regulation of protein levels for creatine kinase, voltage-dependent anion channel 1 (VDAC-1), HSP60, and Grp75. The mitochondria-associated level of albumin was decreased, while the level of catalase was substantially increased. All of the changes were evident as early as 1 week after streptozotocin administration. Taken together, these data point to a rapid and highly coordinated regulation of mitochondrial protein expression that occurs during the heart adaptation to diabetes.

Recent advances in quantitative protein profiling have made possible the relative quantification of individual proteins within two different samples (1–10). The approaches vary in details but all of them are based on labeling the proteins in two separate samples with isotopically normal and heavy reagents. A relative abundance of a protein in the original samples is determined by mass spectrometry as the ratio of signal intensities for the isotopically normal and heavy forms of a specifically labeled peptide. Several strategies of in vitro stable isotope labeling have been described previously (1–6). The most recent approach suggests an alkylation of cysteine residues in proteins with light (non-deuterated) and heavy (deuterated) forms of acrylamide (8, 9). In the present report, this method was used for quantifying protein expression in heart mitochondria from diabetic rats.

The studies on diabetic mitochondria generally suggest that diabetes causes dysfunctional mitochondria (11–14). However, little is known about the nature or extent of mitochondrial dysfunction in diabetes and the relationship between mitochondrial dysfunction and diabetic pathology has yet to be defined. The cardiac function is dependent on a constant rate of resynthesis of ATP by mitochondrial oxidative phosphorylation (OXPHOS).1 Fatty acids and glucose are normally major energy substrates in heart. Both pathways, glucose oxidation and fatty acid oxidation, merge in the mitochondria on the level of acetyl CoA production. Acetyl-CoA then enters the tricarboxylic acid cycle. The last step in ATP production is the entry of NADH and FADH2, derived from the tricarboxylic acid cycle, into the electron transport chain coupled with the mitochondrial ATP synthase. Insulin-dependent diabetes mellitus (IDDM) causes alterations in myocardial metabolism characterized by a decrease in glucose uptake and utilization and an increase in fatty acid oxidation (15–17). As a first step toward profiling the changes in diabetic mitochondria, we have analyzed the pattern of protein expression in heart mitochondria from streptozotocin (STZ)-treated rats. Our primary focus was on proteins involved in three major mitochondrial cycles associated with ATP production, namely on β-oxidation, tricarboxylic acid cycle, and OXPHOS. The diabetes-associated changes in expression levels of several other mitochondrial proteins were also estimated.

MATERIALS AND METHODS

Animals and Treatment—Male Harlan Sprague-Dawley rats (median body weight 250 g) were from Harlan Sprague-Dawley, Indianapolis, IN. Animals were maintained on standard rat chow and tap water ad libitum. STZ (Sigma) was dissolved in sterile 0.1 M citrate buffer (pH 4.5) and injected intraperitoneally at a dose of 60 mg/kg body weight. Control rats received the corresponding volume of citrate buffer. Blood glucose levels were monitored using Accu-Chek Advantage blood glucose monitor and test strips (Roche Applied Science). The STZ-treated rats that showed fasting blood glucose levels above 350 mg/dl, in addition to loss of weight and polyuria, were considered as insulin-dependent diabetes mellitus. Diabetic rats and age-matched control rats were sacrificed at 1 or 4 weeks after STZ injection.

Isolation of Heart Mitochondria—Mitochondria from individual rat hearts were isolated using differential centrifugation (18) in 5 mM Hepes-KOH (pH 7.4), 0.25 mM mannitol, 0.1 mM EDTA, 0.1% bovine serum albumin supplemented with protease and protease inhibitors: 1 mM 4-(2-aminoethyl)benzenesulfonfonyl fluoride, 0.5 mM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, 10 μM pepstatin A, 20 μM leupeptin, 20 μM lactacystin, and 20 μM MG-132. Purified mitochondria were further isolated using 30% Percoll, essentially as described in Ref. 18. Percoll-purified mitochondria were then sonicated

1 The abbreviations used are: OXPHOS, oxidative phosphorylation; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; STZ, streptozotocin; IDDM, insulin-dependent diabetes mellitus; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ACTH, adrenocorticotropin hormone; VDAC-1, voltage-dependent anion channel 1.
and centrifuged at 100,000 \times g for 60 min. The high speed supernatant fraction includes mitochondrial soluble proteins. The high speed pellet includes mainly mitochondrial membrane proteins. Total protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as the standard.

Labeling of Mitochondrial Proteins—The high speed supernatant and pellet fractions from Percoll-purified heart mitochondria from diabetic (1 week and 4 week of diabetes), and age-matched control rats were alkylated with acrylamide. 1 mg of total protein was dissolved in 1 ml of 2 M thiourea, 7 M urea, 4% CHAPS, 40 mM dithiothreitol, and incubated for 60 min at room temperature. The mitochondrial fractions from normal rats were then alkylated with 400 mM light (non-deuterated) acrylamide, while the mitochondrial fractions from diabetic rats were alkylated with 400 mM heavy (deuterated) acrylamide (Cambridge Isotope Laboratories, Woburn, MA) for 5 h at room temperature. The corresponding normal and diabetic fractions were then mixed in the 1:1 ratio.

Two-dimensional Gel Electrophoresis (Two-dimensional PAGE)—The first dimension was performed using the PROTEAN IEF cell (Bio-Rad). The 7-cm pH 3–10 immobilized pH gradient strips were rehydrated with 125 μl of a solution (2 M thiourea, 7 M urea, 4% CHAPS, 0.5% Triton X-100, 0.5% Biolytes 3–10, 1% dithiothreitol, and bromphenol blue) that contained 200 μg of the total protein. Isoelectric focusing was conducted at 250 V for 15 min, linearly increased over 2 h to a maximum of 4,000 V, and then run to accumulate a total of 20,000 V/h. For the second dimension, the immobilized pH gradient strips were equilibrated for 15 min in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and bromphenol blue. The strips were then embedded in 0.7% (w/v) agarose on the top of 10% acrylamide slab gels containing a 4% stacking gel.

Identification and Quantification of Mitochondrial Proteins—The proteins were identified by matrix-assisted laser desorption ionization/time-of-flight MS analysis (MALDI-TOF) of in-gel tryptic digest of selected Coomassie-stained spots. MALDI-TOF was performed using a Voyager-DE STR BioSpectrometry work station (PerSeptive Biosystems) operated in the delayed extraction and reflector mode with a matrix of α-cyano-4-hydroxy-cinnamic acid. Bradykinin fragment 1–7 (MH+ 757.3997), angiotensin II (MH+ 1046.5423), synthetic peptide PpR (MH+ 1533.8582), and ACTH fragment 18–39 (MH+ 2465.1989) were used for external calibration. All spectra were also calibrated internally on the trypsin peaks at 842.51 and 2211.0968. Peptide mass fingerprinting data were evaluated using the MASCOT software (www.matrixscience.com).

![FIG. 1. Scheme describing simultaneous identification and relative quantification of proteins. Quantification of a specific mitochondrial protein is shown. Normal and 4-week diabetic mitochondria from rat heart were labeled with light and heavy acrylamide, respectively. The samples were then mixed at 1:1 ratio, and proteins were separated using two-dimensional PAGE. A spot was randomly selected and identified as a dienoyl-CoA isomerase, chain A. The predicted cysteine-containing peptides from this protein were analyzed. It appears that a peak [M + H]+ (m/z 1420.62) corresponds to a tryptic peptide 185YCTQDAFFQV195 [M + H]+ (m/z 1349.52) modified with light acrylamide (+71.08 mass unit). A peak [M + H]+ (m/z 1423.65) corresponds to the same peptide modified with heavy acrylamide (+74.08 mass unit). Relative heights of these peaks give a relative abundance of dienoyl-CoA isomerase, chain A in the normal and 4-week diabetic mitochondria.](image1)

![FIG. 2. Two-dimensional PAGE patterns of mitochondrial proteins labeled with acrylamide. Normal and 4-week diabetic heart mitochondria were labeled with light and heavy acrylamide, respectively, and mixed at 1:1 ratio. The combined sample was then sonicated and centrifuged at 100,000 \times g for 60 min. Proteins in supernatant (A) and pellet (B) fractions were separated by two-dimensional PAGE and detected by Coomassie staining. The spots containing proteins that were subsequently identified and quantified are numbered. MW, molecular mass.](image2)
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TABLE I
Identification of proteins in Coomassie-stained gel spots numbered in Fig. 2.
The sixth column shows the MOWSE score from Mascot software at ca.expasy.org/sitemap.html. The number in parentheses is the score at which statistical significance (p < 0.05) occurred for that particular search. ubi, ubiquinone.

| Spot and protein | Mass | Peptide matches | Coverage | Score |
|-----------------|------|----------------|----------|-------|
|                 | kDa  |                | %        |       |
| β-Oxidation     |      |                |          |       |
| 1. Carnitine palmitoyltransferase II | 74.0 | 8 | 10 | 99 (64) |
| 2. Dienoyl-CoA isomerase, chain A | 36.1 | 8 | 29 | 133 (63) |
| 3. Acyl-CoA dehydrogenase, very long chain specific | 70.7 | 14 | 30 | 209 (64) |
| 4. Acyl-CoA dehydrogenase, long chain | 47.8 | 10 | 27 | 127 (63) |
| 5. Acyl-CoA dehydrogenase, short chain | 44.9 | 12 | 37 | 197 (59) |
| 6. 1,3-Dihydroxyacyl-CoA dehydrogenase, short chain | 34.4 | 4 | 16 | 63 (59) |
| 7. Electron transfer flavoprotein, α-chain | 34.9 | 5 | 13 | 78 (59) |
| Tricarboxylic acid cycle |      |                |          |       |
| 8. Pyruvate dehydrogenase (lipoamide) β | 38.9 | 9 | 37 | 152 (64) |
| 9. Acnotinase | 85.4 | 10 | 18 | 136 (63) |
| 10. Malate dehydrogenase | 35.6 | 4 | 19 | 65 (63) |
| 11. Dihydrolipoamide dehydrogenase | 54.2 | 5 | 13 | 79 (63) |
| 12. Fumarase | 54.4 | 11 | 24 | 153 (63) |
| 13. Isocitrate dehydrogenase 3 (NAD+/NADH) α | 39.6 | 10 | 36 | 161 (64) |
| 14. Succinyl-CoA ligase (ADP-forming), β-chain | 46.2 | 8 | 19 | 120 (64) |
| OXPHOS |      |                |          |       |
| 15. NADH dehydrogenase (ubi) 1 α subcomplex 5 | 13.4 | 4 | 39 | 66 (63) |
| 16. NADH dehydrogenase (ubi) flavoprotein 1 | 50.8 | 10 | 29 | 140 (63) |
| 17. NADH dehydrogenase (ubi) Fe-S protein 8 | 24.0 | 9 | 50 | 131 (63) |
| 18. NADH dehydrogenase (ubi) 24-kDa subunit | 26.5 | 9 | 44 | 123 (63) |
| 19. Cytochrome c-1 | 35.3 | 9 | 35 | 139 (63) |
| 20. Ubiquinol-cytochrome c reductase core protein 1 | 52.7 | 6 | 15 | 72 (64) |
| 21. ATP synthase, subunit d | 18.8 | 6 | 41 | 92 (63) |
| 22. ATP synthase, α-subunit | 55.2 | 17 | 44 | 226 (59) |
| Others |      |                |          |       |
| 23. Creatine kinase | 47.4 | 14 | 38 | 140 (63) |
| 24. Glutamate oxaloacetate transaminase 2 | 47.3 | 9 | 24 | 96 (59) |
| 25. VDAC-1 | 32.3 | 10 | 50 | 180 (64) |
| 26. HSP60 | 57.9 | 11 | 34 | 190 (63) |
| 27. Grp75 | 73.7 | 7 | 13 | 102 (59) |
| 28. Annexin II | 38.7 | 10 | 34 | 166 (64) |
| 29. Albumin | 68.7 | 15 | 29 | 135 (63) |
| 30. Catalase | 93.7 | 17 | 44 | 269 (59) |

For quantifying the relative abundance of a protein, the peak height of the monoisotopic peak of the heavy (deuterated) acrylamide-labeled peptide divided by the peak height of the monoisotopic peak of the light (non-deuterated) acrylamide-labeled form of the peptide. Six MALDI-TOF mass spectra were acquired for each individual sample, and the results were averaged. All measurements were performed for n = 6 animals, which were handled individually. Data are presented as the mean ± S.E.

RESULTS

Principle of the Method—Fig. 1 shows a schematic presentation of the method of quantitative proteomics used in this report. It is based on the use of light (non-deuterated or d0) and heavy (deuterated or d6) acrylamide to alkylate cysteine residues in proteins before their separation by two-dimensional PAGE and subsequent analysis by MALDI-TOF MS. To estimate the relative amount of a specific protein in two separate samples, one sample is labeled with the light acrylamide and another sample is labeled with heavy acrylamide. Heavy acrylamide has 3 deuteriums instead of 3 protons and is 3 mass units heavier than light acrylamide. From the chemical point of view, there is no difference between heavy and light forms of acrylamide. This warrants equal efficiency of alkylation in separate samples and, after mixing, the co-migration of the same proteins from separate samples on two-dimensional PAGE (1, 7). The areas of interest were then in-gel digested with trypsin and identified using MALDI-TOF MS. The following quantitative profiling was accomplished by comparing a ratio between the intensities of the heavy and light mass components of the cysteine-containing peptide(s). An example in the Fig. 1 shows the cysteine-containing peptide, YCTQDAPFFQV, from dienoyl-CoA isomerase is represented by two isotopic envelopes that are separated by 3 mass units. The first envelope represents dienoyl-CoA isomerase from normal mitochondria. The second envelope represents dienoyl-CoA isomerase from 4-week diabetic mitochondria. Since mitochondrial samples were mixed 1:1, we concluded that the level of dienoyl-CoA isomerase was increased 2.5 times in the diabetic sample.

Quantification of Changes in Protein Levels in Heart Mitochondria Caused by Diabetes—To improve a resolution of two-dimensional PAGE, the mitochondria were first separated in two fractions: (i) the high speed supernatant fraction which includes mitochondrial soluble proteins and (ii) the high-speed pellet, which includes mainly mitochondrial membrane proteins. These two fractions were then analyzed separately. The representative Coomassie-stained two-dimensional gel patterns are shown in the Fig. 2. Our primary focus was on proteins that belong to β-oxidation, tricarboxylic acid cycle, or to OXPHOS. We have identified many proteins involved in these three major mitochondrial pathways. However, it should be noted that the quantitative method used in this study has some apparent limitations. It is not applicable to proteins without cysteine residues. The other problem is if the peptide overlaps with another ion, then quantification would be impossible. For these reasons we failed to quantify some of the identified proteins. Only those proteins for which a reliable quantification was achieved are numbered in the Fig. 2. The identification of these proteins is summarized in the Table I. For all of these identifications, the MOWSE score from Mascot software (ca.expasy.org/sitemap.html) search was higher than the score at which statistical significance (p < 0.05) occurred for that particular search. Identified proteins also corresponded well to the expected molecular mass and pI values.
Fig. 3 shows some examples of quantification when the level of a protein remains unchanged between control and 4-week diabetic samples. Quantification for these three proteins was made based on peptides containing two cysteine residues. This results in a 6-mass unit shift and a good resolution of light and heavy isotopic envelopes. Reliable quantification was possible even for a large peptide from aconitase with 5 peaks in the isotopic envelope. Fig. 4 shows some examples of quantification when the level of a protein was changed during the progression of diabetes. These examples include a gradual increase or a gradual decrease of the relative amount of a protein over the period of 1 and 4 weeks of diabetes. The results for all proteins numbered in Fig. 2 are summarized in Table II.

**DISCUSSION**

The quantitative method for protein profiling used in the present report has some intrinsic limitations. It is not applicable to proteins that do not have cysteine residues. The other limitation to be mentioned is if a peptide overlaps with another ion, then quantification would be impossible. For these reasons we were not able to quantify some proteins of interest, such as the β-subunit of ATP synthase (no cysteine residues) or several proteins involved in electron transport chain (3 mass unit shift was not enough to resolve big cysteine-containing peptides). Nevertheless, we found this method to be useful and reliable for quantification of many other selected proteins and applied it to profiling of heart mitochondrial proteins during the development of IDDM in rats.

Our primary focus was on mitochondrial pathways involved in ATP production, namely on β-oxidation of fatty acids, tricarboxylic acid cycle, and OXPHOS. Changes in protein levels for these three pathways are discussed in detail below.

**β-Oxidation**—The main source of energy for heart muscle is fatty acid oxidation, with the balance coming from the oxidation of pyruvate derived from glycolysis and lactate uptake. Diabetes increases level of circulating fatty acids and decreases glucose uptake and utilization. Our direct measurements show that various mitochondrial proteins involved in fatty acid oxidation are up-regulated in IDDM. The levels of carnitine palmitoyltransferase II (regenerates long chain acyl-CoA in the mitochondrial matrix), three different acyl-CoA dehydrogenases (the rate-limiting enzymes that catalyze the first dehydrogenation of fatty acids), dienoyl-CoA isomerase (catalyzes hydration followed the first dehydrogenation), and 3-hydroxyacyl-CoA dehydrogenase (catalyzes the second dehydrogenation) were gradually increased (Table II). The expression of electron transfer flavoprotein, a necessary electron acceptor for many mitochondrial dehydrogenases, was also increased. Our proteomic findings for heart muscle from rat model of IDDM concur well with a published study that is based on quantita-
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All measurements were performed for \( n = 6 \) individual animals. Data are presented as the mean ± S.E. ubi, ubiquinone.

| Spot and protein | Relative change in diabetes |
|------------------|-----------------------------|
|                  | 1 Week                      | 4 Weeks                     |
| **β-Oxidation**  |                             |                             |
| 1. Carnitine palmitoyltransferase II | 1.43 ± 0.21 | 1.58 ± 0.19 |
| 2. Denvoy-CoA isomerase, chain A   | 1.34 ± 0.14 | 2.47 ± 0.26 |
| 3. Acyl-CoA dehydrogenase, very long chain-specific | 1.31 ± 0.12 | 1.75 ± 0.21 |
| 4. Acyl-CoA dehydrogenase, long chain | 1.45 ± 0.15 | 1.89 ± 0.21 |
| 5. Acyl-CoA dehydrogenase, short chain | 1.49 ± 0.14 | 1.56 ± 0.15 |
| 6. 1,3-Hydroxyacyl-CoA dehydrogenase, short chain | 1.51 ± 0.14 | 1.74 ± 0.25 |
| 7. Electron transfer flavoprotein, α-chain | 1.30 ± 0.12 | 1.55 ± 0.12 |
| **Tricarboxylic acid cycle** |                             |                             |
| 8. Pyruvate dehydrogenase (lipoyamide) β | 1.15 ± 0.10 | 0.98 ± 0.12 |
| 9. Aconitase | 1.08 ± 0.12 | 1.08 ± 0.12 |
| 10. Malate dehydrogenase | 1.00 ± 0.09 | 0.97 ± 0.09 |
| 11. Dihydropyruvate dehydrogenase | 0.87 ± 0.12 | 0.94 ± 0.12 |
| 12. Fumarase | 1.04 ± 0.14 | 1.07 ± 0.14 |
| 13. Isocitrate dehydrogenase 3 (NAD+) α | 1.04 ± 0.14 | 1.04 ± 0.14 |
| 14. Succinyl-CoA ligase (ADP-forming), β-chain | 1.06 ± 0.14 | 0.95 ± 0.13 |
| **OXPHOS**       |                             |                             |
| 15. NADH dehydrogenase (ubi) 1 α subcomplex 5 | 0.71 ± 0.10 | 0.75 ± 0.10 |
| 16. NADH dehydrogenase (ubi) flavoprotein 1 | 0.68 ± 0.09 | 0.93 ± 0.09 |
| 17. NADH dehydrogenase (ubi) Fe-S protein 8 | 0.72 ± 0.10 | 0.87 ± 0.10 |
| 18. NADH dehydrogenase (ubi) 24-kDa subunit | 0.75 ± 0.10 | 0.77 ± 0.12 |
| 19. Cytochrome c-1 | 0.54 ± 0.07 | 0.54 ± 0.07 |
| 20. Ubiquinol-cytochrome c reductase core protein 1 | 0.93 ± 0.13 | 0.97 ± 0.13 |
| 21. ATP synthase, subunit d | 1.08 ± 0.12 | 1.12 ± 0.15 |
| 22. ATP synthase, α-subunit | 1.02 ± 0.13 | 1.02 ± 0.13 |
| **Others**       |                             |                             |
| 23. Creatine kinase | 0.77 ± 0.11 | 0.54 ± 0.08 |
| 24. Glutamate oxaloacetate transaminase 2 | 1.07 ± 0.13 | 1.07 ± 0.13 |
| 25. VDAC-1 | 0.62 ± 0.09 | 0.94 ± 0.11 |
| 26. HSP60 | 0.68 ± 0.10 | 0.89 ± 0.12 |
| 27. Grp75 | 0.74 ± 0.11 | 0.78 ± 0.11 |
| 28. Annexin II | 1.00 ± 0.12 | 0.93 ± 0.12 |
| 29. Albumin | 0.58 ± 0.08 | 0.58 ± 0.08 |
| 30. Catalase | 2.56 ± 0.36 | 6.17 ± 0.92 |

tive measurement of mRNA levels in skeletal muscle from a mouse model of IDDM (19). Yechoor et al. (19) demonstrated that the mRNA levels for many proteins involved in β-oxidation of fatty acids were 1.2–1.9 times increased in the skeletal muscle from STZ-treated mice.

**Tricarboxylic Acid Cycle—β-Oxidation** produces acetyl-CoA for the tricarboxylic acid cycle. It appears that in contrast to β-oxidation, the levels of tricarboxylic acid cycle proteins were not altered in heart from diabetic rats (Table II). At least for seven proteins quantified, we did not observe a difference between diabetic and normal groups at both time points, 1 and 4 weeks, of IDDM development. Our direct proteomic measurements support earlier biochemical measurements of tricarboxylic acid cycle enzyme activities in myocardium of diabetic rats (20).

**OXPHOS—OXPHOS** consists of five large enzyme complexes (Complexes I–V) that couple oxidation to phosphorylation in the inner mitochondrial membrane and provide most of the ATP produced. Biochemical studies suggest that this process may be impaired in the diabetic heart (11–14). Our study shows that the protein level for four subunits of Complex I was decreased by 20–25%. A 46% decrease was found for cytochrome c-1 from Complex III. These changes are relatively modest, but statistically reproducible for a group of proteins involved in OXPHOS, consistent with published biochemical studies (11–14) and seem to be a part of the overall program of adaptation in diabetic heart. A study based on mRNA measurements (19) provides an additional support to our proteomic observations. Yechoor et al. (19) demonstrated that several subunits from Complexes I–IV were 20–30% down-regulated in the skeletal muscle from STZ-treated mice. We also quantified the protein level for two subunits from ATP synthase (Complex V) and found them unchanged.

Taken together our direct measurements of protein levels in diabetic heart mitochondria show that adaptation of heart metabolism to IDDM includes up-regulation of fatty acid β-oxidation. This changed only the source of acetyl-CoA for the TCA cycle. At the same time, it seems that the efficiency of the TCA cycle to pump NADH and FADH₂ into the electron transport chain remains unchanged. It is expected that some down-regulations on the level of Complex I and Complex III would be accompanied by an increase in reactive oxygen species. Whether this affects the overall production of ATP is not clear, since Complex V was presumably unchanged. In brief, the primary difference in mitochondrial metabolism of diabetic heart appears to be an increased consumption of fatty acids. Whether production of ATP was compromised remains to be established. We also want to underline that our discussion is based on the direct measurements of protein levels in diabetic mitochondria rather than protein activities, and we cannot rule out the additional potentially negative effects of oxidative stress, which may cause modification of protein activities involved in ATP synthesis.

**Others—**Several proteins involved in other mitochondrial pathways were also quantified. Creatine kinase level was obviously decreased that would decrease the level of available creatine phosphate, an important energy source for heart muscle at high workloads. Similar 50% decrease of muscle creatine kinase from STZ-treated rats was reported based on mRNA measurements (16). The protein levels of glutamate oxaloacetate transaminase 2 (mitochondrial amino acid synthesis) and annexin II (maintaining Ca²⁺ homeostasis) were unchanged.
Voltage-dependent anion channel 1 was the only one example in the present study when the protein level dropped 1 week after STZ administration and then recovered later. Whether it is relevant to heart adaptation in diabetes warrant further studies. Two mitochondrial chaperones, HSP60 and Grp75, were moderately decreased. Cardioprotective function of molecular chaperones is well established (21). If diabetes causes a decline in mitochondrial chaperone levels, it may adversely affect mitochondrial refolding and contribute to mitochondrial dysfunction seen with diabetes.

The protein level of albumin was 42% decreased in diabetic mitochondria. For catalase it was an apparent time course of changes, 2.6-fold increase after 1 week and 6.2-fold increase after 4 weeks of diabetes. Albumin and catalase fall into a category of nonmitochondrial proteins, which are always found in mitochondria. The association of nonmitochondrial proteins with mitochondria may or may not be biologically relevant (22). However, control and diabetic mitochondria were purified in our experiments side-by-side, and since the protein levels for albumin and catalase were changed during the diabetes development, it is not possible to interpret their presence in mitochondria as just contaminants. In terms of function, both proteins may be involved in antioxidant defense and their association with diabetic mitochondria could be relevant to the increased oxidative stress in diabetes.

In summary, for the first time a quantitative proteomic approach was used in assessing the diabetes-associated changes in heart mitochondria. In addition to the previous biochemical and mRNA studies, we found several lines of proteomic evidence that mitochondrial function in diabetes may be modified as a result of simultaneously altered expression of multiple genes.

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