Cardiac mitochondrial damage and biogenesis in a chronic model of type 1 diabetes


Despite the importance of diabetic cardiomyopathy, the disease or other known cardiac risk factors. That and numerous studies reporting similar findings (11, 19, 48) first proposed in 1972 (44) on the basis of postmortem findings of heart failure in diabetic patients free of coronary artery disease or other known cardiac risk factors. That and numerous following studies reporting similar findings (11, 19, 48) strongly support the existence of a specific cardiomyopathy with origins in diabetic cardiac muscle.

Despite the importance of diabetic cardiomyopathy, the pathogenesis of this complication is still not well characterized.

Among the postulated mechanisms, oxidative stress is a strong candidate. Excess reactive oxygen species (ROS) have been widely implicated in diabetes and its many complications (3, 33). The heart contains low levels of antioxidants, making it prone to oxidative damage (9). It has been shown that diabetic hearts display increased levels of lipid peroxidation (24, 38) and induction of antioxidant enzymes (24, 38, 56, 57), indicating the involvement of oxidative stress in this disease.

Mitochondria are the major source of ROS due to continuously generated superoxide, a by-product of electron transport (30). The significance of mitochondrial generated ROS in diabetes has been proposed by several laboratories (27, 37, 39, 45, 54). Brownlee’s laboratory has provided strong evidence that ROS from mitochondria activate other pathological pathways, such as protein kinase C, aldose reductase, and advanced glycation end products, all of which induce diabetic complications (13, 21, 37). The normalization of these changes by overexpression of manganese superoxide dismutase (37) clearly suggests that mitochondria act as a potent source of oxidative stress in diabetic complications. Because mitochondria appear to be the source of most ROS, it is not unexpected that mitochondria have been shown to be a primary target of damage in diabetes (16, 17, 40).

Proteomics is a powerful technology that provides an objective, large-scale analysis of proteins in normal or pathological conditions. It has been applied to the study of complex changes in cardiovascular disease because of its ability to examine hundreds to thousands of proteins simultaneously (2, 14, 34). Proteomics has recently been applied to cardiac mitochondria following short-term (1 or 4 wk after streptozotocin injection) diabetes (53), but it has not been used to examine changes in total cardiac protein expression or changes that occur in chronic diabetes. To address these questions, we used proteomic analysis to screen for changes in total heart proteins chronically diabetic for 18 wk. Our unbiased proteomic results point to mitochondria as the major target of diabetes-induced changes. This was confirmed by studies focused on the morphology and function of diabetic mitochondria. We further determined that diabetes or diabetes-induced damage stimulates cardiac mitochondrial biogenesis, which may ameliorate the metabolic effects of mitochondrial damage.

WEB ADDRESS: http://www.ajpendo.org

Address for reprint requests and other correspondence: P. N. Epstein, Dept. of Pediatrics, Univ. of Louisville, 570 S. Preston St., Suite 304, Louisville, KY 40202 (E-mail: paul.epstein@louisville.edu).

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EXPERIMENTAL PROCEDURES

**Animals.** We have previously described the development and maintenance of OVE26 diabetic mice (15, 32). All transgenic and nontransgenic animals were maintained on the inbred FVB background. A US Department of Agriculture-certified institutional animal care committee approved all animal procedures.

Western blotting. Protein was extracted from islets and hearts of 4- to 5-mo-old control and OVE26 mice. Protein concentrations were determined by bicinchoninic acid protein assay (Pierce, Rockford, IL). Protein (10 μg) was loaded per lane and separated by 18% homogeneous SDS-PAGE. Protein was transferred to PVDF membrane and probed with antibody against rabbit calmodulin (1:1,000; Zymed Laboratories, San Francisco, CA) at 4°C overnight. After a washing, the membrane was incubated with horseradish peroxidase-conjugated goat antibodies to rabbit immunoglobin G (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, and the final image was detected with enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ).

**Proteomic analysis.** Global protein expression profiles in hearts of 4-mo-old OVE26 and FVB mice were evaluated according to proteomic methods described by Thongboonkerd et al. (51), with modification. In brief, frozen heart tissues taken from 120-day-old control and diabetic mice were homogenized in sample buffer containing 7 M urea, 1.9 M thiourea, 4% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 58 mM DTT, and 4.45% carrier ampholytes (pH 3–10). The mixture was shaken at 4°C for 1 h and centrifuged at 16,000 g for 10 min at 4°C. The supernatant containing the soluble proteins was removed, and protein concentration was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Protein (100 μg) was rehydrated overnight with immobilized pH gradient (IPG) strips (nonlinear pH 3–10; Amersham Biosciences, Fairfield, NJ), followed by isoelectric focusing with maximal 5,000 V and 80 μA for 24 h at 17°C using a two-dimensional PAGE running system (Genomic Solutions, Ann Arbor, MI). Then the sample was equilibrated and loaded onto 22 × 22-cm precast 10% homogeneous slab gels (Genomic Solutions) for second-dimension separation at maximal 500 V and 20,000 mW for 4–4.5 h at 4°C. The gel was removed after electrophoresis and fixed in 10% methanol and 7% acetic acid for 30 min followed by staining with SYPRO Ruby (Bio-Rad) overnight. The gel image after staining was taken by a high-resolution 12-bit camera with UV light box system (Genomic Solutions).

After proteins from each sample were separated and visualized on individual gels (n = 5 in each group), quantitative intensity analysis was performed using Investigator HT analyzer software (Genomic Solutions). Because we used proteomic analysis as a tool to screen changes in any protein without a specific initial assumption for changes in a set of multiple proteins, intensity values for each protein spot were analyzed individually using unpaired Student’s t-test (multiple comparisons were not performed because our objective was not to define a specific pattern for multiple proteins expressed in each sample or to determine differential expression among many different proteins). P ≤ 0.05 was considered significant. The protein spots that were significantly altered were subjected to in-gel tryptic digestion and identification by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using a Micromass Tof-Spec 2E instrument with a 337 mM N2 laser (29). Protein identification from tryptic fragment sizes was performed by peptide mass fingerprinting using the MASCOT search engine (http://www.matrixscience.com). The NCBI protein database was restricted to mammalian entries, and peptides were assumed to be monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Up to 1 missed trypsin cleavage and 150-ppm mass tolerance (error window) were allowed for matching peptide mass values. Probability-based MOWSE (MOlecular Weight SEarch) scores greater than 58 were considered significantly matched (P < 0.05).

**Morphology study.** After animals were anesthetized with ketamine (150 mg/kg ip) and xylazine (22.5 mg/kg ip), they were perfused from left ventricle with ~30 ml of wash-out solution containing propanic hydrochloride to prevent vasoconstriction and a 0.66% solution of paraformaldehyde to prefix the heart, followed by 100 ml of full-strength Karnovsky’s fixative (8). The heart was then removed and weighed. Ventricles were cut perpendicular to the long axis into rings of 1–2 mm wide. Left ventricular walls were then collected and trimmed into blocks. After storage in fixative overnight, the sections were postfixed in 1% osmium tetroxide for 1 h. They were dehydrated through a series of graded ethanol and embedded in LX112 (Ladd Research Industries, Burlington, VT). Semithin sections were cut at ~1 μm, mounted on glass slides, and stained with 1% azure II in 1% sodium borate. Random areas and areas with abnormal ultrastructure identified under the light microscope on semithin sections were taken for transmission electron microscope (TEM) study. Ultrathin sections were cut at ~80 μm thick on an LKB 8800 Ultratome III, stained with uranyl acetate and lead citrate, and observed under a Philips CM10 microscope operating at 60 kV.

Mitochondrial area and number estimations were accomplished by taking TEM micrographs from random areas. Areas with severely damaged mitochondria were eliminated from this process to avoid possible bias due to swollen mitochondria. Six to eight micrographs were taken for each heart. Three control and four diabetic animals were used. Mitochondrial areas on these micrographs were determined by manually blackening in mitochondrial outlines on photographs. These images were then scanned into Adobe Photoshop to determine the percentage of mitochondrial area over total cardiac area, excluding the areas of nucleus or blood vessels. Mitochondrial numbers on these TEM micrographs were counted manually by a blind observer and normalized to unit area.

**Mitochondrial isolation.** Fresh heart was immediately washed with prechilled isolation buffer containing 220 mM mannitol, 70 mM sucrose, and 5 mM MOPS, pH 7.4. The heart tissues were then minced and homogenized by a motor-driven Teflon pestle in the isolation buffer. The homogenate was centrifuged at 500 g for 10 min at 4°C, and supernatant was collected by passing it through cheesecloth. The filtrate was centrifuged at 5,000 g for 10 min at 4°C. The pellet was washed again with isolation buffer and gently resuspended in respiration buffer (225 mM mannitol, 70 mM sucrose, 10 mM KH2PO4, and 1 mM EGTA, pH 7.2). Protein concentration of the mitochondria preparation was determined by the Lowry method.

**Measurement of respiratory control ratio and mitochondrial reduced glutathione.** After isolation, mitochondria viability and function were evaluated by measuring oxygen consumption with a Clark-type oxygen electrode (model 1302; Strathkelvin Instruments, Glasgow, UK). The reaction was carried out in respiration buffer at 25°C with 80–100 μg of mitochondrial protein added. Pyruvate at 20 mM and 10 mM malate were also added as substrates. State 3 and state 4 respiration rates were measured in the presence or after depletion of 1 mM ADP. Oligomycin (1 μM) was also added for measurement of state 4 respirations. To examine mitochondrial integrity across all preparations, respiration was also measured in the presence or absence of 8 μM cytochrome c. Respiratory control ratio (RCR) was defined as the ratio of state 3 respiratory rate to state 4 rate. Isolation and assay of diabetic and control mitochondria were always carried out at the same time as matched pairs.

Mitochondrial (mt) reduced glutathione (mtGSH) level was measured by Bioxytech GSH/GSSH 412 kit (OxisResearch, Portland, OR) with modification. Twenty-microliter mitochondrial samples were vortexed in the same amount of 8.75% metaphosphoric acid and centrifuged at 2,000 g at 4°C for 10 min to extract mtGSH. Ten microliters of the supernatant were mixed with 700 μl of assay buffer containing 100 mM Na2PO4 and 4 mM EDTA, pH 7.5. Samples or standards were then mixed with 5,5'-dithiobis-(2-nitrobenzoic acid) and GSH reductase and incubated at room temperature for 5 min. The
Table 1. Probe and primer sequences for RT-PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Probe</th>
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<tr>
<td>ACA2</td>
<td>5'-CTCCGCTCCGTGTTCCAGAAA-3'</td>
</tr>
<tr>
<td>Acon 2</td>
<td>5'-AGCAATGAGCAGACGACTTGG-3'</td>
</tr>
<tr>
<td>Cyto B</td>
<td>5'-CCACCTCTCACTAATCATATATCTG-3'</td>
</tr>
<tr>
<td>COI</td>
<td>5'-GCGAAGATATCTTGGTGATGAGG-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-CTGGCTTGGGACGACAG-3'</td>
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</table>

AACA2, acetyl-CoA acyltransferase 2; Acon 2, aconitase 2; Cyto b, cytochrome b; COI, cytochrome c oxidase subunit I.

absorbance was read at 412 nm for 3 min after the addition of NADPH.

Quantitative real-time PCR for mtDNA. To quantitate mtDNA copy number per nuclear genome, well-conserved nuclear and mitochondrial genes were selected. Cytochrome b was used as a marker for mtDNA and β-actin for nuclear DNA (5). Cardiac DNA was extracted from frozen heart tissues of 4- to 5-mo-old diabetic and control hearts with a DNeasy tissue kit (QIAGEN, Valencia, CA). Total DNA concentration was determined using a PicoGreen DNA quantitation kit (Molecular Probes, Eugene, OR). Sequences for mouse cytochrome b and β-actin DNA were obtained from GenBank and input into Beacon Designer 2.0 (PREMIER Biosoft International, Palo Alto, CA) for Taqman probe design (for sequence of probe and primers, see Table 1). Primers and FAM-labeled TAMRA-quenched probes were ordered from Biosearch Technologies. Amplification and quantitation of mRNA were accomplished using a Brilliant Plus quantitative RT-PCR core reagent kit (Stratagene), ABS Taqman Universal PCR Master Mix (Applied Biosystems) and Matrix 4000 Real Time PCR system (Stratagene). The results were analyzed by the Mx4000 real-time quantitative detection software version 3.0 and normalized to β-actin mRNA level.

Statistical analysis. Two-tailed Student’s t-test was used for comparison between diabetic and control groups. P ≤ 0.05 was considered statistically significant.

RESULTS

Calmodulin Western blotting. We (32) have previously reported diabetic cardiomyopathy in OVE26 mice, a chronic model of type 1 diabetes. Diabetes in these transgenic mice is induced by overexpression of the calcium-binding protein calmodulin specifically in pancreatic β-cells, which dramatically reduces insulin secretion (15). OVE26 mice have elevated blood glucose values by 1 wk of age (15) and maintain blood glucose values >500 mg/dl after 6 wk of age (32). To exclude the effect of calmodulin overexpression on heart, we performed Western blotting on control and OVE26 cardiac protein. As demonstrated in Fig. 1, calmodulin expression levels in transgenic hearts are indistinguishable from those of control hearts. Additional experiments with another two pair of samples produced similar results (data not shown).

Proteomic analysis. In an effort to study diabetic cardiac complications at the molecular level, we performed proteomic analysis in 4-mo-old control and diabetic hearts (n = 5 per group). Approximately 300 protein spots were visualized on each gel, and protein spot patterns were essentially identical among the individuals, as determined by spot matching and analysis using Investigator HT analyzer software. Quantitative intensity analysis revealed differential expression of 22 protein spots between the two groups (indicated by arrows, Fig. 2). Of these significantly altered proteins, 20 were identified by MALDI-TOF mass spectrometry followed by peptide mass fingerprinting (Table 2). To our surprise, over one-half of these proteins (12 of 20) were from mitochondria, and all but one of these 12 proteins were upregulated in diabetic heart. This result.
OVE26/FVB: ratio of abundance in diabetic hearts divided by abundance in FVB hearts.

Significantly altered in 4-mo-old OVE26 diabetic hearts compared with control cardiac morphology in OVE26 mice. In the present study, we had previously observed the detrimental effects of diabetes on mitochondria, suggesting the hypothesis that mitochondria are the major target for diabetes-induced cardiac damage. To address this hypothesis, we focused our attention on morphological and functional markers.

Morphological damage of cardiac mitochondria. We previously observed the detrimental effects of diabetes on cardiac morphology in OVE26 mice. In the present study, we carried out a more comprehensive analysis of mitochondrial damage (Fig. 3) based on the results from our initial proteomic screening. In typical micrograph sections of diabetic heart (Fig. 3B), mitochondria were intact and appeared to be increased in relative area and number, as confirmed by quantitative and statistical analyses (Fig. 3, E and F). In quantitative analysis, random sections were studied, except that regions with swollen or less dense mitochondria were excluded to reduce bias. Assuming that the observable 18% increase in cross-sectional area also occurred in the depth of the mitochondria, which cannot be measured by our method, the overall increase in mitochondrial volume would be ~28%, which matched well with the 30% increase in mitochondrial number we found. Approximately 4% of left ventricular area displayed much more severe mitochondrial damage (Fig. 3, C and D). In these areas, signs of mitochondrial damage ranged from swollen size and reduced density (Fig. 3D) to virtually hollow mitochondria with broken double membranes (Fig. 3C). Interestingly, in some of these areas with severely damaged mitochondria, the myofibrils appeared to be intact. These myofibrils had sarcomeres that were parallel and continuous with clear and regular Z-lines (Fig. 3D). In contrast to diabetic hearts, matched control animals showed normal myocardial ultrastructure, with continuous myofibrils and mitochondria with homogeneously dense matrix (Fig. 3A). Very severe mitochondrial damage was never seen in control hearts.

Impaired mitochondrial function. We further investigated whether mitochondrial function was altered in OVE26 hearts. Function was assessed by measuring the RCR and ADP-to-O2 consumption ratio (P/O) (42). After isolation, purified diabetic and control mitochondria appeared similar to one another by TEM analysis (data not shown). The comparison of RCR and P/O between control and diabetic cardiac mitochondria revealed a significant reduction of RCR in OVE26 mitochondria (Fig. 3A), even though P/O remained unchanged (data not shown). Further examination showed that the drop in RCR was

![Fig. 2. Representative 2-dimensional image of cardiac proteins with indication of modified proteins in OVE26 heart. Cardiac protein extract (100 µg) was separated on a 2-dimensional gel system and analyzed as described in experimental procedures.](http://ajpendo.physiology.org/)

Table 2. Diabetes-altered proteins identified by mass spectrometry

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<th>Spot</th>
<th>Protein</th>
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<th>pI</th>
<th>MW</th>
<th>OVE26/FVB</th>
<th>P Value</th>
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<td>85</td>
<td>1.9</td>
<td>0.02</td>
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<tr>
<td>27</td>
<td>Dihydropyrimidinase-related protein-2 (DRP-2)</td>
<td>gi1351260</td>
<td>6</td>
<td>63</td>
<td>1.5</td>
<td>0.01</td>
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<tr>
<td>43</td>
<td>Glucose regulated protein, 58 kDa</td>
<td>gi6679687</td>
<td>6</td>
<td>57</td>
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<td>65</td>
<td>ATP synthase, F1 complex, α</td>
<td>gi6680748</td>
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<td>60</td>
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<td>80</td>
<td>Enolase 3, β-muscle</td>
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<td>88</td>
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<td>92</td>
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<td>3-ketoacyl-CoA thiole, mitochondrial</td>
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<td>118</td>
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<td>199</td>
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<tr>
<td>211</td>
<td>Crystallin α2; ß-crystallin</td>
<td>gi6753530</td>
<td>6.8</td>
<td>20</td>
<td>0.8</td>
<td>0.03</td>
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Spot nos. correspond to nos. in Fig. 1. Identifier, protein gi no. in NCBI protein database; pI, isoelectric point; MW, molecular weight (mass, kDa). OVE26/FVB: ratio of abundance in diabetic hearts divided by abundance in FVB hearts. P value from t-test of OVE26 vs. FVB (n = 5). Rows highlighted in boldface indicate mitochondrial proteins.
due to lower state 3 respiration accompanied by no change in state 4 respiration (Fig. 4, B and C). The addition of 8 μM cytochrome c did not change RCR in preparations of control (9.50 ± 0.40 vs. 9.49 ± 0.26 in the presence of cytochrome c) or diabetic mitochondria (5.33 ± 0.53 vs. 5.38 ± 0.41 in the presence of cytochrome c). State 3 respiration rate was also not altered (data not shown).

Oxidative stress has been implicated in damage to diabetic mitochondria. We measured GSH as an indicator of mitochondrial oxidative stress and found the mtGSH level to be significantly reduced in older diabetic heart (Fig. 5).

Mitochondrial biogenesis in diabetic heart. When we were isolating cardiac mitochondria for RCR measurement, we noted a nonsignificant trend (P = 0.07) for greater protein content in isolated OVE26 mitochondria (Fig. 6A), which yielded 30% more protein than isolated FVB mitochondria. To determine whether this was due to greater mitochondrial mass in OVE26 hearts, we performed quantitative RT-PCR of mi-
tochondrial and nuclear DNA. Cytochrome b was used as a marker for mtDNA and /H9252-actin for nuclear DNA. Diabetic mitochondria had 50% more DNA compared with normal mitochondria, as shown in Fig. 6B.

The mRNA level of Tfam, which plays a key role in the regulation of mtDNA replication, was also increased by 50% in OVE26 heart (Fig. 7A), consistent with the elevation of mtDNA. We further tested these samples to determine whether the mRNA for two of the mitochondrial proteins identified in our proteomic analysis was elevated. As shown in Fig. 7B, mRNA levels of Acon 2 and ACAA2 were elevated by 24 and 26%, respectively, in OVE26 compared with FVB hearts. These two proteins were chosen because they had relatively large increases in protein expression in diabetic hearts. We also measured mRNA levels of two mitochondrial encoded proteins, cytochrome b in electron transport chain complex II and cytochrome c oxidase subunit I in complex IV, both of which had significantly increased levels in OVE26 hearts (Fig. 7C).

**DISCUSSION**

The present study investigated the status of mitochondria in mouse heart following over 100 days of type 1 diabetes. Damage to mitochondria was indicated by ultrastructural changes, impaired function and reduced GSH. Damage was accompanied by indicators of mitochondrial biogenesis, including increases in 11 specific mitochondrial proteins, elevation of mRNA for the mitochondrial regulatory protein Tfam, and increased total mitochondrial DNA, area, and number. These results indicate that mitochondria are a primary focus of diabetic damage to the heart and that diabetic hearts might respond to this damage by augmenting mitochondrial biogenesis. Cardiac changes in the transgenic OVE26 model were secondary to diabetes, as there was no increase in calmodulin expression in transgenic heart.

To our knowledge, this is the first time that proteomic analysis has been used to evaluate diabetes-induced changes in whole heart protein expression. We found that 60% of the proteins altered by diabetes were localized to mitochondria. This is a striking finding in view of the fact that only 1–2% of cellular proteins are mitochondrial. All but one of the altered mitochondrial proteins were upregulated, and all were encoded by nuclear DNA. The absence of mitochondrial encoded proteins was likely due to the fact that there are relatively few proteins encoded by mitochondrial DNA, and they are difficult to solubilize by standard extraction buffers for two-dimensional PAGE because they are tightly embedded in the inner mitochondrial membrane (36). Evaluation of mRNA levels for two of the increased mitochondrial proteins demonstrated a
25% increase in mRNA. Although significant, this was appreciably less than the ~80% increase in protein abundance, implying that posttranscriptional steps must also contribute to the increase in protein level.

Our proteomic study produced results consistent with the known increase in fatty acid utilization of diabetic hearts (1, 49, 50). Four altered proteins (3-ketoacyl-CoA thiolase; acyl-CoA thioester hydrolase; enoyl-CoA hydratase 1; electron transfer flavoprotein β-subunit) are involved in fatty acid oxidation (25), and all of them were upregulated 1.7- to 2.4-fold. Similar findings of increased mRNA and protein levels of enzymes involved in fatty acid β-oxidation have been reported in diabetic skeletal and cardiac muscle by Yechoor et al. (59) and Turko and Murad (53). The fact that plasma triglycerides are elevated in OVE26 mice (32) may also have contributed to the increase in these fatty acid utilization proteins.

Despite the power and advantages that proteomic analysis provides, there are also limitations to our current study. Two-dimensional electrophoresis is capable of visualizing over a thousand proteins on a single two-dimensional gel (26), but we visualized only 300 protein spots on our gel of solubilized proteins, which seemed to simplify the complexity of the whole heart proteins. This was limited by the staining method we were using. In this study, we employed SYPRO Ruby staining, because its dynamic range is more linear than silver staining, because its dynamic range is more linear than silver staining. We utilized only 100 μg of total protein for each gel, and this limited the total number of proteins visualized. We could increase the amount of total protein for each gel, but major abundance proteins such as actin, tubulin, myosin, tropomyosin, etc., could be “oversaturated” by this fluorescence staining or could “overshadow” adjacent proteins during the scanning process. One could also argue that to get a more complete profile of total heart proteins we could have used narrow-range overlapping gradient strips (pH 3–6, 5–8, 7–10) or prefractionated samples. However, those procedures are quite laborious and repetitive, so as a tool for identifying major protein changes under diabetic condition, we used IPG strips of pH 3–10 range and total cardiac extract for the present study. Therefore, in the current study, the primary value of proteomics was to provide an initial screening tool.

The proteomics results focused our attention on mitochondria. Detailed ultrastructural analysis revealed discrete areas, comprising ~4% of total left ventricular area, with severe mitochondrial damage. Mitochondria seemed to be the primary target in these regions, because many of the cardiomyocytes appeared normal except for their mitochondria (Fig. 3D). The damage was not likely to be artifactual, as such severe mitochondrial damage was not seen in identically prepared control hearts and damaged mitochondria were seen adjacent to apparently normal myofibers. Mitochondrial structural damage has been reported in hearts of different diabetic animal models (17, 20). Fitzl et al. (17) identified mitochondria with decreased cristae density and degenerative intramitochondrial areas in heart of 6-mo-old streptozotocin-treated Wistar rats, consistent with our morphological findings.

Outside of focal regions of extreme damage, most diabetic mitochondria appeared comparatively normal. However, assays of respiration in isolated mitochondria indicated that this was not the case. As reported in streptozotocin and other models of diabetes (6, 16, 29, 40, 52), mitochondria from OVE26 hearts exhibited a significant reduction in RCR (36%), despite no apparent change in P/O. It did not appear that the impaired RCR of OVE26 mitochondria was due to damage sustained during isolation, since they appeared similar to FVB mitochondria by TEM analysis, and the addition of cytochrome c to OVE26 mitochondria did not alter their decreased RCR or state 3 respiration. Unchanged P/O suggests that most diabetic mitochondria retain normal coupling of oxidation and ADP phosphorylation for the substrate pyruvate. The reduction in RCR implies depressed respiratory chain activity and is consistent with reduced state 3 respiration found in our and other diabetic animal models. We do not know at this stage whether this was due to an overall reduction in electron transport or to inhibition of just one susceptible complex in the chain. Decreased pyruvate dehydrogenase activity (28) can also lower RCR when pyruvate is used as substrate. However, lower RCR has also been reported in diabetic mitochondria when glutamate, which does not utilize pyruvate dehydrogenase, is used as substrate (40, 52). This suggests that impaired electron transport is involved in the impairment of mitochondrial function caused by diabetes.

The cause of structural and functional damage to mitochondria in diabetes has not yet been established. Mitochondria are a major source of ROS in diabetes (7, 37). Oxidative stress has been widely implicated in development of diabetic complications, and we have previously reported (32) that an antioxidant transgene reduces damage to diabetic mitochondria. In the present study, we found that the GSH content of OVE26 cardiac mitochondria was reduced by 33%. This is in accord
with the recent results of Santos et al. (46), who reported a 21.5% reduction in GSH of cardiac mitochondria from the diabetic Goto-Kakizaki rat. GSH acts as a scavenger of free radicals and is oxidized by glutathione peroxidase during detoxification of hydrogen peroxide and lipid peroxides. The decrease in GSH content indicates that diabetic mitochondria are subject to oxidative stress and are less protected from damage than nondiabetic mitochondria.

Our finding by proteomic analysis that over 11 mitochondrial proteins were significantly more abundant in OVE26 heart suggested that diabetes might induce mitochondrial biogenesis. To test this hypothesis, we measured four additional parameters of mitochondrial content: surface area, number, protein, and DNA. All of these measures were elevated in mitochondria of 5-mo-old OVE26 heart by 18–50%, strongly indicating that mitochondrial biogenesis had occurred. Diabetes has been shown to increase mitochondrial protein yield from kidney and heart (40, 43). Dysfunctional mitochondria bring about synthesis of mitochondrial components in patients with inherited mitochondrial defects (22) and in cultured cells (18, 30) with impaired mitochondria. Nevertheless, this is the first time that mitochondrial biogenesis was shown in diabetic heart. Exercise training and cold exposure also induce mitochondrial biogenesis (23, 58). In each instance, mitochondrial biogenesis occurred in tissues with damaged mitochondria or increased energy demand. Although we do not have evidence for increased energy demand in the present study, it is clear from functional and structural measures that OVE26 hearts have damaged mitochondria. The fact that diabetes induced mitochondrial damage and several indicators of mitochondrial biogenesis raises the possibility that mitochondrial biogenesis may be a compensatory response to reduced mitochondrial function in diabetic hearts. Additional studies are needed to understand the underlying mechanism(s) for mitochondrial biogenesis. The expression of thermogenic coactivator peroxisome proliferator-activated receptor-γ coactivator 1 (58) or activation of AMP kinase (4, 60) might both play a role in this process. In addition, increased utilization of fatty acid in diabetic heart (1, 49) might also contribute to the observed mitochondrial biogenesis.

Mitochondrial biogenesis requires coordinate control of nuclear and mitochondrial genes encoding mitochondrial proteins. mtDNA encodes only 13 proteins, whereas the vast majority of the mitochondrial proteins are derived from nuclear DNA. Tfam plays a critical role in nuclear-mitochondrial interactions, and we found it to be upregulated in our diabetic heart. This nuclear encoded transcription factor is one of the most important proteins for control of mtDNA replication and transcription (10, 41). The significance of this protein for mtDNA maintenance in heart was clearly demonstrated in cardiac specific Tfam knockout mice. Absence of Tfam caused a threefold reduction in heart mtDNA, leading to lethal dilated cardiomyopathy (55). In our study, we found that the level of Tfam mRNA was increased by 50% in OVE26 hearts, similar to the increase found in mtDNA and protein. Levels of mRNA for several mitochondrial proteins, encoded by nuclear DNA or mtDNA, were also all elevated in diabetic hearts. The known function of Tfam and our results in diabetic hearts suggest that Tfam may mediate the coordinate elevation of mtDNA and protein induced by diabetes. Tfam has been shown to be increased in several pathologies that produce mitochondrial damage (12, 31, 35, 47). Human aging produces mitochondrial damage and a large increase in Tfam (31). We suggest that Tfam induction may coordinate the compensatory mitochondrial biogenesis in diabetic heart.

In summary, we found clear evidence of mitochondrial damage in type 1 diabetic hearts, indicating that mitochondria are a major target of injury. Signs of increased mitochondrial mass indicated that biogenesis might be induced to compensate
for impaired mitochondria. Further study is needed to establish whether the damage to mitochondria causes or exacerbates diabetic cardiomyopathy. Reduced GSH content suggested the involvement of oxidative stress. If oxidative stress is the main cause of mitochondrial damage, then enhanced mitochondrial antioxidant defenses should prevent this change. We have recently produced transgenic mice with cardiac-specific overexpression of manganese superoxide dismutase and are testing this hypothesis in diabetic mice.

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