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**PROTEOMICS AND MORPHOLOGY DEMONSTRATE MITOCHONDRIAL
CHANGES IN DIABETIC CARDIOMYOPATHY**

By

Xia Shen
B.S., P.R.China, 1999

A Thesis
Submitted to the faculty of the
Graduate School of University of Louisville
in Partial Fulfillment of the Requirements
for the degree of

Master of Science

Department of Pharmacology & Toxicology
University of Louisville
Louisville, Kentucky

August, 2002

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A Thesis Approved on

May 28, 2002

By the following Thesis Committee:

Thesis Director

DEDICATION

This thesis is dedicated to my family in China

My parents

Mr. Shangqi Shen & Mrs. Ninglan He

And

My sister

Ms. Jian Shen

For their endless support, love and faith for me

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I would like to thank my mentor, Dr. Paul N. Epstein, for his guidance and patience for the past three years. I would also like to thank the other committee members, Dr. David W. Hein, Dr. William M. Pierce, Dr. Jon Klein and Dr. Jon Eaton, for their valuable advice and suggestions on my research proposal. Also, I am very grateful to Dr. Edward C. Carlson, the professor at the Department of Anatomy and Cell Biology, University of North Dakota, for his expert assistance on cardiac morphology analysis. Finally, I would like to extend my thanks to all the members in our diabetic research group, as well as the faculty and staffs in the Department of Pharmacology & Toxicology, Department of Pediatrics at the University of Louisville, for their help and friendship. It is my honor and pleasure to be in this group.

ABSTRACT

Proteomics and morphology demonstrate mitochondrial changes in
diabetic cardiomyopathy

Xia Shen

August 9, 2002

Diabetic cardiomyopathy (DCM) is a common complication leading to accelerated cardiovascular failure in diabetic patients. Even though the exact mechanism(s) behind this disease still remain unclear, research from several laboratories including our own suggests that reactive oxygen species (ROS) play a very important role in the development of DCM.

In our lab, we have developed and characterized a diabetic transgenic mice line, designated OVE26, which clearly develops diabetic cardiomyopathy. Comparison of protein expression patterns in the hearts of 120-day-old control and diabetic mice reveals 22 proteins that are differentially expressed in OVE26 mice hearts, most of which are involved in energy metabolism. Surprisingly, 10 of 20 proteins that are identified by mass spectrometry are located in mitochondria, suggesting mitochondria as a critical target for ROS damage. The finding of the up-regulation of all 10 proteins also corresponds well with the concept that moderate oxidative stress can stimulate mitochondrial biosynthesis to compensate for the impaired cell functions. Study on mitochondrial morphology also shows abnormal cardiac structure and damage, further pointing to mitochondria as one of the major sites of ROS attack in diabetic cardiomyopathy.

In an effort to prevent the mitochondrial damage we saw in diabetic hearts, we have developed transgenic lines that overexpress the main mitochondrial antioxidant protein, MnSOD specifically in cardiomyocytes. Preliminary data from the transgenic mice that overexpress MnSOD in diabetic heart showed that this antioxidant enzyme was effective in protecting the morphology of the diabetic heart.

Our proteomic and morphologic analyses indicate that mitochondria are an important target of diabetic cardiomyopathy. Protection by overexpression of mitochondrial MnSOD indicates that ROS are involved in this damage.

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TABLE OF ABBREVIATIONS

2-D PAGE.....	Two-Dimensional Pholyacrylaminde Gel Electrophoresis
CHAPS.....	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DCM.....	Diabetic Cardiomyopathy
DTT.....	1,4-Dithio-DL-threitol
MnSOD.....	Manganese Superoxide Dismutase
MW.....	Molecular Weight
P/O.....	ATP/Oxygen consumption
PBS.....	Phosphate Buffered Saline
pI.....	Isoelectric Point
RCR.....	Respiratory Control Rate
ROS.....	Reactive Oxygen Species
SDS.....	Sodium Dodecyl Sulphate
TEM.....	Transmission Electron Microscopy

CHAPTER I

INTRODUCTION

Diabetes mellitus is now one of the main threats to human health in the 21st century (1). Up to the year 2000, 14.2 million people in United States had been diagnosed with diabetes, and 151 million people worldwide. And these numbers are set to rise to 17.5 and 220 million by 2010 (1;2). Prevention of diabetes and control of its complications are one of the greatest tasks for health scientist in the next decade.

One of the leading causes of mortality in diabetics is heart failure (3). The Framingham Heart Study showed that the risk of heart failure was increased two fold among diabetic men and five fold among diabetic women (4). Heightened risks for heart failure are even more pronounced in African-Americans and certain Native Americans (3).

Nearly every physician is familiar with the concept that diabetic patients have increased cardiovascular morbidity (5). Prior to 1972, it had been attributed to only vascular diseases such as hypertension and coronary arteriosclerosis. In 1972, Rubler et al. first suggested a specific cardiomyopathy that exists independently of coronary artery disease and other known cardiac risk factors (6;7). The term “diabetic cardiomyopathy” was therefore proposed. This work had been subsequently followed by numerous studies

reporting similar findings (8;9), providing strong support for the existence of a specific diabetic cardiomyopathy with origins in diabetic cardiac muscles.

Diabetic cardiomyopathy is manifested by early diastolic dysfunction, i.e. reduced left ventricular end diastolic (LVED) volume and filling rate, followed later in the disease by abnormalities in systolic function (3). Biopsies of the diabetic heart also revealed histopathological abnormalities including interstitial fibrosis, myocyte hypertrophy and mitochondrial abnormalities (9;10). Patients with diabetic cardiomyopathy have a much worse prognosis following myocardial infarction than non-diabetics even when the infarct size is smaller (11). When diabetic cardiomyopathy presents with other cardiovascular diseases such as hypertension, they exacerbate one another, adding significantly to their overall morbidity and mortality (12).

Despite the obvious importance of diabetic cardiomyopathy, the underlying mechanisms of this complication at the cellular and molecular level are still not well characterized. Several mechanisms have been postulated, including free radical production, altered lipid content of membranes, as well as the abnormal carbohydrate metabolism and excessive fatty acid oxidation (3). Research from several laboratories including our own suggests that reactive oxygen species (ROS) play a very important role in the development of diabetic cardiomyopathy (13;14). But only limited data are available now and the pathogenesis of this disease is still unclear. In addition, little emphasis has been put on developing novel approaches to prevention and treatment of diabetic cardiomyopathy. We are expecting to make progress in characterizing the role of mitochondrial ROS and mitochondrial damage in this disease through this study, and also to shed some light on the potential application of antioxidant therapy.

In order to study diabetic cardiomyopathy, our laboratory has developed and characterized a diabetic transgenic mice line, designated OVE26 (15-18). These transgenic mice develop very early onset of diabetes due to the overexpression of the calcium binding protein calmodulin in pancreatic beta cells that induces pancreatic beta cell death and dysfunction. The exact mechanism of why elevated calmodulin was remarkably deleterious to β cells is still poorly understood, but it has been suggested that abnormal calcium homeostasis is harmful for both function and viability of β cells (16).

The OVE26 transgenic mice provide us an excellent model to study diabetic complications. Transgenic mice demonstrate elevated blood glucose levels within the first days of life and have blood glucose values over 450 mg/dl by 30 to 55 days of age. By the time when they are 120 days old, the typical blood glucose values are over 600 mg/dl. Surprisingly, they can survive at least a year without exogenous insulin therapy despite such high blood glucose levels. The specificity of the transgene and insulin promoter assures that calmodulin only induces the degeneration of pancreatic β cells. Since the diabetes is induced genetically, they require no administration of any beta cell toxin that might induce variability and nonspecific toxicity. Successive generations have continued to express their characteristic of very early onset of diabetes for more than 10 years now. They are readily maintained because they are fertile up to 4 months of age. The transgenic diabetic mice can be easily recognized by the development of obvious cataracts resulting from the co-injection and co-integration of the cataract-inducing GR19 gene (16;19). In summary, this OVE26 mice line is a very attractive animal model for study of diabetic complications because of its early onset, long life span, severe consistent diabetes and simple breeding and maintenance.

Previous studies in our laboratory have shown that OVE26 diabetic mice clearly exhibit cardiomyopathy characterized by significantly altered mRNA expression, severe morphological abnormalities, and reduced contractility under ischemic conditions (20). To better understand this disease on the molecular level, we decided to study changes in protein expression in an effort to reveal the mechanism of these deficits and some targets of ROS damage.

Proteomic analysis is chosen for this purpose because of the many advantages it offers over other methods for analysis of protein expression. Unlike Western blots which can examine the expression of only one protein at a time and which is limited by the availability of antibodies, proteomic analysis can examine the abundance of hundreds to thousands of proteins on one gel (21). What's more, proteomic analysis is able to detect some post-translational modifications. For example, it is possible to observe the different phosphorylation states of one protein by the presence of a series of protein spots with similar MW since the addition or the subtraction of phosphate groups will make detectable change on pI but not on molecular weight (22).

In the following study, we applied proteomic analysis to 4-month old FVB and OVE26 hearts to study the change of protein expressions in diabetic cardiomyopathy. Proteomic results combined with the observation from cardiac morphology strongly suggest that mitochondria are going through certain changes in this disease.

CHAPTER II

MATERIALS AND METHODS

Animals: We have previously described the development of OVE26 diabetic mice (Epstein, Overbeek, and Means 1989). Transgenic mice were recognized by the presence of small eyes caused by co-integration of the GR19 gene (Epstein, Overbeek, and Means 1989). All transgenic and non-transgenic mice were maintained on the inbred FVB background.

Whole heart protein extraction: Fresh heart taken from 4-month-old control and diabetic mice were immediately rinsed in pre-chilled PBS to wash out the blood. The heart was then frozen in liquid nitrogen and later transferred to -80°C freezer for storage. On the day before running the 2-D gel, the heart was powdered, weighed, and homogenized on ice in 600ml sample buffer/100mg heart tissue. The sample buffer contained 7 M urea, 1.9 M thiourea, 4% CHAPS, 58 mM DTT and 4.45% pH 3-10 carrier ampholytes. The mixture was shaken in cold room for one hour and centrifuged at 16,000g for 10 minutes at 4 degree. The supernatant containing the soluble proteins was removed and aliquoted to microtubes for further use. Protein concentration was determined using Bio-Rad protein assay based on Bradford's method (Bradford 1976).

2D-PAGE: Immobilized pH gradient (IPG) strips, nonlinear pH 3-10 (Amersham Pharmacia Biotech Inc.) were used for the first dimension. 100 ug of protein were

rehydrated overnight with the IPG strips in rehydration buffer containing 8 M urea, 2% CHAPS, 0.01 M DTT, 2% ampholytes and bromophenol blue. On the next morning, the sample was set up for isoelectric focusing (IEF) with maximal 5000 V and 80 μ A for 24 hours at 4°C. Then the sample was equilibrated with buffer containing 6 M urea, 130 mM DTT, 30% glycerol, 112 mM Tris base, 4% SDS, 0.002% bromophenol blue and acetic acid twice for 20 minutes, then with buffer containing 6 M urea, 135 mM iodoacetamide, 30% glycerol, 112 mM Tris base, 4% SDS, 0.002% bromophenol blue and acetic acid once for 10 minutes. For second dimension, the strips were loaded onto pre-cast 10% homogeneous, 20 x 20 cm slab gels (Genomic Solutions Inc.). Upper running buffer contained 0.2 M Tris base, 0.2 M tricine and 0.4% SDS. Lower running buffer contained 0.625 M Tris/acetate. The system was run with maximal 500 V and 20,000 mW for 4 to 4.5 hours at 4°C.

SYPRO Ruby staining: The gel was fixed in 10% methanol and 7% acetic acid for 30 minutes. The fixation solution was removed and 500 ml of SYPRO Ruby stain (Bio-Rad Laboratory, Hercules, CA) was added to each gel and incubated at room temperature for 18 hours.

Visualization: The gel images after staining were taken by a high-resolution 12-bit camera with UV light box system (Genomic Solutions Inc.) 1 and 3 seconds exposure time were used to scan the gels. The images were converted to TIFF files before the image analysis.

Image analysis: The protein expression patterns in control and diabetic heart were compared using Investigator HT analyzer software (Genomic Solutions Inc.). First a virtual reference gel was created by combining all of the detectable spots into one image.

This image was then used to determine the existence and differential expression of proteins in each group. Background of the gel was subtracted using “Average on the boundary” and the spot intensity was normalized by using “total volume ratio x total spot area” to minimize the intensity variability among the gels. A t-test was used to compare the protein intensities in control and diabetic hearts, and $p \leq 0.05$ was considered significant.

Protein spot picking and in-gel tryptic digestion: The protein spots were excised with a clean scalpel into 1 mm cube and transferred to clean 1.5 ml microtubes. The gel pieces were washed and sequentially dehydrated and rehydrated by the methods used described by Thongboonkerd, et al. (Thongboonkerd et al 2002). Then the gel tubes were chopped into smaller pieces and incubated with 20 ng/ul modified trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate at 37°C overnight in shaking incubator.

MALDI-TOF mass spectrometry: The samples were desalted and spotted onto the 96-well MALDI target plate by the method described by Thongboonkerd, et al. (Thongboonkerd, Luengpailin, Cao, Pierce, Cai, Klein, and Doyle 2002). Mass spectral data were obtained using a Micromass ToF-Spec 2E instrument with a 337 mN₂ laser.

Data analysis: Protein identification from tryptic fragment sizes was performed by peptide mass fingerprinting using the MASCOT search engine (<http://www.matrixscience.com>). NCBI nr and Swiss (ExPASy) protein databases were used for this purpose and MOWSE scores greater than 58 were considered statistically significant ($p \leq 0.05$).

Heart morphology: Cardiac ultrastructure was examined by transmission electron microscopy (TEM) in hearts of control and transgenic mice at the age of 4-5 month.

Hearts from anesthetized animals were first perfused with an approximately 30 ml of wash-out solution containing procaine hydrochloride to prevent vasoconstriction and a relatively weak solution of paraformaldehyde to pre-fix the heart, followed by the full-strength Karnovsky's fixative 100 ml for TEM observation. The heart was then removed and weighed. Left ventricular wall and interventricular septum were collected for conventional TEM studies. Fixed tissue was sent to Dr. Carlson at the University of North Dakota for preparation of EM micrographs. To further quantify the morphological changes in different groups of hearts, EM micrographs from the hearts of FVB, OVE26, OVE26-MnSOD and MnSOD mice were scored by 2-3 blinded observers for overall morphology, regularity and continuity of myofibrils, as well as mitochondrial structure and distribution. Heart weights from different groups were also compared as another parameter for normal heart features.

Construction of MnSOD transgene: The construction of MnSOD transgene, designated MySOD is shown in Figure 4. All the enzymes used in this process were ordered from New England BioLabs, Beverly, MA. The alpha myosin heavy chain promoter was obtained from another transgene MyADH (Liang et al 1999). In our laboratory, we have produced over 50 transgenic lines using this promoter, and all the transgenes have been very active and cardiac specific. MyADH was digested by restriction enzymes Sal I and Hind III to remove the ADH fragment. The remaining 9 Kb fragment contained the α myosin heavy chain promoter, vector for transformation and the polyadenylation region of the rat insulin II gene. The 830 bp MnSOD fragment containing complete coding sequences for human manganese SOD was removed from transgene InSOD by EcoRI digestion, followed by gel separation and purification. The 9

Kb fragment from MyADH and the MnSOD fragment were then blunt-ended and ligated by T4 DNA ligase to accomplish the construction. The resulting DNA solution was then incubated with Hind III to digest and eliminate the self-ligated product of MyADH fragment. This solution was then used to transform XL1-Blue competent cells for amplification. Correct construction and orientation was verified by sequencing the regions across the ligation. Finally, the transgene was microinjected into mouse embryo to produce MnSOD transgenic mice.

SOD enzyme activity assay: SOD activity was determined by measuring the inhibition of pyrogallol autoxidation (Marklund and Marklund 1974). Heart samples were homogenized in 1ml/25mg tissue in sample assay buffer containing 50 mM Tri-cacodylic acid (pH 8.2) with 1 mM diethylenetriaminepentaacetic acid (DTPA). The homogenate was incubated on ice for 30 minutes to solubilize SOD from tissue followed by centrifugation at 16,000g for 30 minutes at 4°C. The supernatant (2-4 ul) was mixed with 200 ul assay buffer in 96-well micro plates in triplicate. The reaction was initiated by adding 25ul of 2 mM pyrogallol in 10 mM HCl, and the increase in absorbance at 405 nm was followed for 2 minutes by SPECTRA Fluor Plus plate reader (Tecan U.S. Inc., Durham, NC). SOD activity was assessed as the degree of inhibition of the pyrogallol autoxidation rate. An inhibition of 50% by SOD standard was defined as 1 unit SOD. Sample protein concentration was measured by BCA method (Smith et al 1985). The final results were reported as SOD Units/mg of protein.

CHAPTER III

RESULTS

Mice cardiac protein expression: The pattern of protein expression in mice heart by two-dimensional PAGE was consistent and identical for over 80% of all protein spots in 10 control and diabetic heart samples. Hundreds of proteins were visualized on the gel and over two hundred spots were detected by gel image analysis software. Figure 1 is a typical two-dimensional gel image showing the cardiac protein expression pattern in mice.

Altered protein expression in diabetic heart: In our proteomic analysis comparing five control hearts to five OVE26 hearts, twenty-two proteins are found significantly altered in abundance, most of which have increased abundance in diabetic hearts. The positions of these proteins are shown in Figure 1 by red arrowheads. Twenty of them gave signal strong enough to be identified by the mass spectrometry (Table 1).

Among these twenty differentially expressed proteins, more than half of them are involved in energy metabolism, such as aconitase and isocitrate dehydrogenase for TCA cycle, enolase and aldolase for glycolysis, as well as 3-ketoacyl-CoA thiolase for fatty acid beta-oxidation. Some proteins are known to be induced by stress, such as prohibitin and crystallin. There are also some proteins with unknown functions such as HSPC326. Very surprisingly, 10 of these 20 proteins are in the mitochondria, and all of them are

Figure 1. Typical image of cardiac proteins.

100ug protein extract from heart was run in two-dimension gel system and stained with SYPRO ruby fluorescent stain. Gel images were then photographed with a video camera and analyzed by HT analyzer. 22 proteins were significantly altered in OVE26 diabetic hearts compared to the control hearts, as spots indicated by arrows (n=5 each group, $P \leq 0.05$ by Students t-test).

Table 1

Proteins identified by mass spectrometry that altered their abundances in OVE26 hearts

Spot	Protein	pI	Mw	OVE26/FVB	P value
2	Aconitase 2, mitochondria	8.1	85	1.9	0.02
27	Dihydropyrimidinase related protein-2 (DRP-2)	6.0	63	1.5	0.01
43	Glucose regulated protein, 58 kDa;	6.0	57	1.6	0.01
65	ATP synthase, F1 complex, alpha	9.3	60	1.3	0.01
80	Enolase 3, beta muscle	6.7	47	0.5	0.01
86	Ornithine aminotransferase	6.2	49	1.6	0.01
88	NADP+-specific isocitrate dehydrogenase	9.2	51	1.8	0.02
92	Translation elongation factor EF-Tu precursor	6.8	50	0.8	0.05
103	GTP-specific succinyl-CoA synthetase beta subunit	5.9	44	1.5	0.01
105	3-ketoacyl-CoA thiolase, mitochondrial	8.4	42	2.3	0.01
108	Acyl coenzyme A thioester hydrolase, precursor	6.9	50	2.4	0.01
118	Aldolase 1, A isoform	8.8	40	1.8	0.02
123	Glutamate oxaloacetate transaminase 2	9.4	48	1.5	0.03
159	HSPC326	5.5	36	1.4	0.01
162	Enoyl coenzyme A hydratase 1	7.9	36	1.7	0.01
163	Prohibitin	5.6	30	1.3	0.03
179	(homolog to electron transfer flavoprotein beta subunit)	8.6	27	2.3	0.04
193	Proteosome (prosome, macropain) activator subunit 3	5.8	31	1.5	0.02
199	(homolog to ATP synthase D chain, mitochondrial)	5.5	19	1.4	0.01
211	Crystallin, alpha 2; alpha B-crystallin	6.8	20	0.8	0.03

Spots correspond to the numbers on Figure 1. pI: isoelectric point. MW: molecular weight (KD). OVE26/FVB: the ratio of abundance in diabetic hearts divided by abundance in FVB hearts. P value: from t-test of OVE26 vs. FVB.

Rows highlighted indicate mitochondrial proteins.

up-regulated in diabetes. It has been reported that within a certain threshold, elevated ROS or reduced ATP production can induce a type of stress response to compensate impaired mitochondrial function by stimulating mitochondrial biosynthesis and/or increasing the expression of the genes required for energy metabolism (Fosslien 2001; Heddi et al 1999; Lee and Wei 2000). The fact that so many mitochondrial proteins are altered in abundance in diabetic heart also suggests that mitochondria might be a crucial target for ROS attack in diabetic cardiomyopathy.

Mitochondrial morphology in OVE26 heart: In order to further investigate the possible mitochondrial damage in diabetic cardiomyopathy, transmission electron micrographs (TEM) of left ventricular myocardium were taken in FVB and OVE26 mice to look into the mitochondrial morphology at different ages. Figure 2 shows the cardiac ultrastructure at 2, 5 and 11 months of age in control (A,C,E) and diabetic (B,D,F) heart. The data clearly indicate the development of cardiomyopathy in our transgenic mice and the progressive damage to mitochondria over the time course. By the age of two months, OVE26 hearts began to show disarrangement of myofilaments and disruption of sarcomeres (Figure 2, panel A and B) while the mitochondria still kept normal structure and appearance. At 5 months, mitochondria abnormalities such as broken membranes and cristae as well as rarified matrices became evident (panel C and D). By eleven months of age, damages to both myofibrils and mitochondria were exacerbated, myelin figures become prominent and the overall cytoarchitecture was disrupted (panel E and F).

A closer look at the cardiac mitochondria at 300-day-old diabetic mice (Figure 3) confirmed the damage we see in Figure 2. In some area, mitochondria are swollen or in irregular shape, some of them are even broken. Instead of the homogeneous dense matrix

Figure 2. Development of cardiomyopathy illustrated in representative TEMS of left ventricular myocardium in FVB (A,C,E) and OVE26 (B,D,F) mice at 2 months (A,B) 5 months, (C,D) and 11 months (E,F).

FVB mice showed normal myocardial ultrastructure at all ages (A,C,E) . Myofibrils are comprised of regular and continuous sarcomeres that demarcated by Z-lines (arrowhead). Myofibrils are separated by straight rows of mitochondria (m) with homogeneously dense matrix. In contrast, myocardium from matched OVE26 mice (B,D,F) showed severely disrupted myofibrils in all ages. Mitochondria (m) damage was not detectable in two-month-old heart (B), but by five months (D), mitochondria were loosely packed and highly disorganized. Focal areas showed mitochondria with broken membranes and cristae and their matrices appear mottled and regionally electron lucent. This damage was even more evident at 11 months (F) where mitochondria were devoid of normal cristae structure and swollen with mottled matrix. Numerous myelin figures suggested cellular damage. The magnification for all figures are X6,400.

Figure 3. Higher magnification (X28,000) transmission electron micrographs showing cardiac mitochondrial damage in 300-day-old OVE26 mice.

M: mitochondria; Mf: myofilament; My: myelin figures; Arrowhead: Z-line.

we see in the same age control heart, OVE26 mitochondria matrix was rarified and irregularly distributed.

The mitochondrial damage we observed in diabetic heart EM photographs combined with the proteomics results strongly suggest that mitochondrial structure and probably function are impaired in diabetic cardiomyopathy, and this further leads to the compensatory induction of the proteins involved in energy metabolism.

MnSOD protection: In an effort to prevent the abnormalities we saw in diabetic cardiomyopathy, especially the damage to mitochondria, we have developed transgenic lines that overexpress the major mitochondrial antioxidant enzyme, manganese superoxide dismutase (MnSOD), specifically in heart. The transgene shown in Figure 4 is constructed as described in methods.

Characterization of these MnSOD transgenic lines is still in progress. Initial study done in 3 of the 5 transgenic lines shows the SOD activities in the heart were 30-60 fold higher compared to FVB mice (Figure 5). The increased SOD protein expression was also confirmed by two-dimension PAGE (Figure 6).

We have just obtained data from the first OVE26-MnSOD mice. While this data is very preliminary and from only one OVE26-MnSOD mouse it clearly suggests that MnSOD protected the heart from diabetes-induced damage to overall structure, cardiomyofibils, as well as mitochondria. As shown in Figure 7, OVE26-MnSOD diabetic hearts were protected from the damage seen in diabetic OVE26 hearts.

Figure 4. Construction of MySOD transgene.

α MHC promoter: murine alpha myosin heavy chain promoter; ADH: Alcohol dehydrogenase; Poly A: polyadenylation region of rat insulin II gene; MnSOD: complete coding sequence for human MnSOD.

Figure 5. Enzyme assay shows increased SOD activity in 3 lines of MnSOD transgenic mice.

Activities are shown as the fold increase in transgenic mice vs. control mice. Data were obtained from the mean of 2-5 mice in each of four groups. Assay will be repeated later with more animals to confirm these results.

Figure 6. Comparison of 2-D gel images from control (A) and MnSOD (B) transgenic mice showing strong expression of SOD protein (dark circle).

Figure 7. Overexpression of MnSOD in heart prevents diabetic heart from morphology damage.

Representative transmission electron micrographs of left ventricle in FVB (A), OVE26 (B), and OVE26-MnSOD (C) mice at 5 months of age. M: mitochondria; arrowhead: z-lines.

CHAPTER IV

DISCUSSION

Cardiovascular disease is the major cause of morbidity and mortality from diabetes mellitus. In this paper, we studied the changes of protein expression in diabetic cardiomyopathy through our diabetic animal model – OVE26 transgenic mice. It is the first time for researchers to study systematically the protein expression in this disease. Proteomic analysis revealed over 20 proteins with altered abundance in a 4-month-old diabetic heart, most of which are involved in energy metabolism. We also noticed an unexpected high proportion of mitochondrial proteins that were altered. The fact that all of them are upregulated suggests that this compartment might be undergoing cellular damage in the diabetic state and a certain compensatory response is induced. Results from morphological study of cardiac mitochondria also showed progressive damage to its ultrastructure and appearance, further confirming our suspicions.

Accumulating evidence suggests that reactive oxygen species (ROS) play a very important role in the development of diabetic cardiomyopathy, and mitochondria are the main source of ROS in the cell under most conditions. Excess ROS had been shown to be widely involved in diabetes and its many complications (Baynes 1991). Whether the superoxide produced by the mitochondria is upregulated in diabetes is still unclear.

However, aside from the well-known fact that mitochondria are the main intracellular source of oxidative stress, they are also the major target of ROS that are continually generated as the by-products from the respiratory chain under normal physiological conditions (Lee and Wei 2000). It has been suggested that a decline in mitochondrial function plays a critical role in aging and other degenerative diseases by producing excess ROS due to defects in the electron transport chain (Lee and Wei 2000; Wallace 1999).

Mitochondrial damage and dysfunction has been widely reported in diabetes and cardiomyopathy. Several laboratories including our own have found severe mitochondrial structural damage in diabetic heart electronmicrographs (Giacomelli and Wiener 1979). Evidence for mitochondrial dysfunction include depressed state 3 respiration and ATPase activity, decreased mitochondrial calcium uptake, as well as defective oxidative phosphorylation (OXPHOS) (Pierce and Dhalla 1985). Decreased pyruvate dehydrogenase activity and reduced activities of Complex I and Complex V have also been reported in the heart of STZ-induced diabetic animal models (Flarsheim et al 1996; Tomita et al 1996). Moreover, mitochondrial DNA (mtDNA) mutations have also been implicated in diabetes (Flarsheim, Grupp, and Matlib 1996; Graff et al 1999). Some characteristics of mitochondria such as a lack of protective histones for mtDNA and absence of an efficient repair system render mtDNA vulnerable to oxidative insult by ROS and prone to mutations (Lee and Wei 2000). Mutations in mtDNA can in turn deteriorate OXPHOS and reduce mitochondrial energy production (Esposito et al 1999). Because heart exclusively relies on mitochondrial ATP synthesis for contraction and ion transport, it makes sense that defective energy production would preferentially affect the

heart (Wallace 2000). In diabetic heart, long-term exposure to ROS might result in defective oxidative phosphorylation and bioenergy production, leading further to the development of cardiomyopathy. Our finding of upregulation of a significant number of mitochondrial proteins in diabetic cardiomyopathy is well in accordance with the above theory.

Another possible explanation for the elevated percentage of mitochondrial proteins was that the mitochondrial mass was increased in 120-day-old diabetic mice. In morphological study, we did see swollen cardiac mitochondria with increased mass in the mice of 11 months age (Figure 2F). However, this increase was not so distinct in 5-month-old diabetic heart (Figure 2D), and we consider it to be a minor factor that counted for the high proportion of mitochondrial proteins in 4-month-old diabetic hearts as we saw in proteomic results.

From the severe damage we saw in mitochondrial morphology, it is reasonable to postulate that mitochondrial function might be affected by ROS attack as well. Our next study is to investigate mitochondrial integrity and activity under the diabetic state. Initial data from Shirong Zheng, a postdoctoral fellow in our laboratory, shows that in OVE26 cardiac mitochondria, even though P/O remains the same as control, RCR is significantly decreased (data not shown), suggesting impaired control of mitochondrial metabolism and possibly reduced rate of ATP synthesis. We will also measure the activities of four complexes in mitochondrial electron transport chain to study the effect of oxidative stress on mitochondrial functions.

In support of our hypothesis that ROS induced mitochondrial damage in diabetic cardiomyopathy, initial results from MnSOD transgenic mice has shown that

overexpression of MnSOD in diabetic heart successfully blocked diabetes-induced damage in heart morphology. Since MnSOD only protects against ROS, this result suggests that ROS causes the morphology damage in diabetic hearts. We are planning to further study the protective effect of MnSOD in diabetic heart from protein expression, ROS production, mitochondrial function, as well as cardiomyocyte contractility. These studies will be a part of our continued efforts to find an effective antioxidant regimen for the prevention or treatment of diabetic complications.

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