Published in final edited form as:

J Mol Cell Cardiol. 2007 April ; 42(4): 884–895. doi:10.1016/j.yjmcc.2006.12.018.

Alterations in the Diabetic Myocardial Proteome Coupled With Increased Myocardial Oxidative Stress Underlies Diabetic Cardiomyopathy

Milton Hamblin^a, David B. Friedman^b, Salisha Hill^b, Richard M. Caprioli^b, Holly M. Smith^a, and Michael F. Hill^a

^aDepartment of Biomedical Sciences, Division of Cardiovascular Biology, Meharry Medical College, Nashville, TN 37208, USA

^bMass Spectrometry Research Center, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

Introduction

Diabetes mellitus is a syndrome that markedly potentiates the risk of heart failure (HF) in diabetic patients [1-3]. The increased incidence of HF is a major cause of morbidity and mortality for patients with diabetes [4]. The high frequency and poor prognosis of this common and serious comorbidity in diabetic patients has been linked in part to the presence of an underlying diabetic cardiomyopathy characterized by abnormal ventricular systole and diastole in the absence of such diabetes-associated risk factors as obesity, hypertension, hypercholesterolemia, and coronary artery disease [5-8]. Preclinical cardiac studies of diabetic patients have also demonstrated cardiac dysfunction in the absence of clinically detectable atherosclerosis and/or coronary artery disease [9]. Diabetic cardiomyopathy has been documented to progress to congestive heart failure (CHF) in both type 1 and 2 diabetic patients. However, approximately 30% of all type 1 diabetic patients suffer from this specific cardiomyopathy [10,11]. Despite these clinical observations, the pathophysiological mechanism(s) responsible for this diabetes-related cardiomyopathy remain poorly understood.

It has been reported that increased free-radical mediated oxidative stress is associated with diabetic complications in both humans and animals [4,12-16]. Reactive Oxygen Species (ROS) or Free Oxygen Radicals (FOR's) such as superoxide radical (O_2^-), hydroxyl radical ($OH^\cdot$), and hydrogen peroxide (H_2O_2), generated during oxygen metabolism, are controlled by various cellular defense mechanisms consisting of enzymatic [(superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSHPx)] and non-enzymatic (vitamin E, β -carotene, vitamin C) antioxidants [17].

However, during pathological conditions, the delicate balance between free-radical production and the protective antioxidant defense system may shift in favor of a relative increase in free-radical production and resultant ROS-induced tissue injury. Hyperglycemia can elevate levels

Address for correspondence: Michael F. Hill, Ph.D. Department of Medicine Division of Cardiovascular Medicine Vanderbilt University Medical Center 2220 Pierce Avenue Room # 383, Preston Research Building Nashville, TN 37232 Telephone: (615) 936-2412 Fax: (615) 936-1872 E-mail: E-mail: michael.f.hill@Vanderbilt.Edu.

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of FOR's or ROS by increasing mitochondrial superoxide anion production or by the process of glucose auto-oxidation [18,19]. Most of the available evidence implicating free-radical mediated oxidative stress in diabetic cardiomyopathy stems from reports evaluating the rate of lipid peroxidation [20-22]. However, much of this evidence lacks specificity given that most traditional methods previously used to assess lipid peroxidation are nonspecific, inaccurate, and unreliable [23,24]. In addition, investigations aimed at characterizing differences at the cardiac proteome level in hearts with diabetic cardiomyopathy and accompanying oxidative stress are lacking. Consequently, no definitive information as to the importance of this process *in vivo* in contributing to the functional alterations demonstrated in the hearts of diabetic patients as well as those of experimental animals is available.

Accordingly, the present investigation was designed to address these issues. Isoprostanes are members of a newly described family of prostaglandin isomers that are produced *in vivo* primarily from oxidative modification of polyunsaturated fatty acids via a free radical-catalyzed mechanism [25]. Of these, 8-isoprostane (8-*iso* PGF_{2α}) has recently been shown to be a specific and sensitive quantitative index of oxidative stress *in vivo* [26]. Therefore, to evaluate myocardial oxidative stress in diabetic cardiac complications, we measured myocardial levels of 8-*iso* PGF_{2α} along with myocardial reduced (GSH) and oxidized (GSSG) glutathione in relation to left ventricular (LV) hemodynamic function during diabetic cardiomyopathy using a rat streptozotocin (STZ) model of chronic type I diabetes. To identify cardiac protein changes that may accompany diabetic cardiomyopathy, we analyzed LV protein expression profiles from STZ-diabetic rats using a proteomics approach. The results demonstrate, for the first time, that myocardial levels of 8-*iso* PGF_{2α} and GSSG are increased in STZ-diabetic rats concomitant with reduced expression levels of proteins involved in antioxidant defense and apoptosis resistance. These findings support a potential link between oxidative stress damage and alterations at the cardiac proteome level which may play an important role in the pathogenesis of diabetic cardiomyopathy.

Methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Experimental Animals

Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee at Meharry Medical College.

Induction of Diabetes

Hyperglycemia was induced in male Sprague-Dawley rats (150 ± 10 g) by administering a single intraperitoneal (i.p.) injection of streptozotocin (STZ) (65 mg/kg body wt) prepared daily in citrate buffer pH 4.5 for maximal stability. The control group was injected with the vehicle only. To ensure that the animals were diabetic (D), urine analysis was performed 24-48 hours (h) after STZ injection by Chemstrip uGK (Roche Diagnostics, Indianapolis, IN). Rats with urine glucose values of >2000 mg/dL with polyuria 24-48 h after STZ injection were considered to be diabetic. Rats with urine glucose values of <2000 mg/dL 24-48 h after STZ injection were not considered to be diabetic and were excluded from further study. Animals were used at eight weeks after vehicle or STZ injection for different studies.

Cardiac Function

Rats were anesthetized with ketamine- xylazine (90 mg/kg:10mg/kg i.p.). The right carotid artery was cannulated with a Millar miniature catheter (SPC-320, 2F, Millar Instruments,

Houston, Texas) and advanced into the aorta to record arterial pressure. The aortic catheter was then advanced into the LV for recording of the following pressures: left ventricular end-diastolic pressure (LVEDP); left ventricular systolic pressure (LVSP); rate of pressure rise (+dP/dt); and rate of pressure decay (-dP/dt). After these assessments, the rats were killed, and the hearts were removed for further studies.

Measurement of Myocardial Oxidative Stress

8-iso PGF_{2α}—LV tissue was homogenized in 0.1 M phosphate, pH 7.4, containing 1 mM EDTA. An equal volume of 15% wt/vol KOH was added to the tissue homogenate and incubated for 60 minutes at 4°C. 2-4 volumes of ethanol containing 0.01% BHT was added to the sample and incubated for 5 minutes at 4°C and then centrifuged at 1,500 × g for 10 minutes to remove precipitated proteins. The supernatant was then decanted into a clean test tube and the ethanol was evaporated to < 10% v/v under a gentle stream of nitrogen. The samples were then reconstituted with EIA buffer, vortexed, and then analyzed for 8-iso PGF_{2α} using a commercially available kit (8-Isoprostane EIA Kit, Cayman Chemical Company, Ann Arbor, MI) and expressed as picomoles/g tissue.

Reduced and Oxidized Glutathione—LV tissue was homogenized in 5-10 ml of cold buffer (i.e., 50 mM MES, pH 6-7, containing 1 mM EDTA) per gram tissue and centrifuged at 10,000 × g for 15 minutes at 4°C. Reduced (GSH) and oxidized (GSSG) glutathione levels were examined in the supernatants using a commercially available kit (Glutathione Assay Kit, Cayman Chemical Company, Ann Arbor, MI). Exclusive measurement of GSSG was accomplished by derivatizing GSH with 2-vinylpyridine. Values were expressed as μM/mg tissue.

Proteomic Analysis

Sample Preparation—Myocardial tissue originating from the LV of control and diabetic rats at 8 weeks after vehicle or STZ injection were immediately frozen in liquid nitrogen and stored at -80°C until use. Frozen LV tissues taken from control and STZ-diabetic rats were homogenized in lysis buffer consisting of 7 M urea, 2 M thiourea, 30 mM Tris, 5 mM magnesium acetate, 4% CHAPS, and 58 mM DTT using a ratio of 1 g tissue:10 ml lysis buffer. The lysate was centrifuged at 12,000 × g for 1 h at 10°C. The pellet was discarded and the protein concentration of the supernatant was determined using a 2-D Quant protein assay kit (Amersham Biosciences/GE Healthcare, Piscataway, New Jersey).

Difference Gel Electrophoresis and Differential-Display Proteome Analysis—All equipment was manufactured by GE Healthcare/Amersham Biosciences (Piscataway, New Jersey) unless otherwise noted. Control and diabetic samples were prepared in replicate (n=5). 250 μg from each of the 10 samples were precipitated with methanol/chloroform [27,28] and resuspended in 40 μL labeling buffer (7M urea, 2M thiourea, 4% CHAPS, 30 mM Tris, 5 mM magnesium acetate). One-third of each sample (83 μg) was removed and pooled into a separate tube for the mixed-sample internal standard. The remaining 167 μg of each sample was separately labeled with 200 pmoles of either Cy3 or Cy5 NHS-ester minimal labeling reagents (2 μL of a 100 pmol/μL in NN di-methyl foramide) for 30 min on ice in the dark, followed by quenching with 2 μL of 10 mM lysine for 10 min on ice in the dark. The pooled sample mixture (830 μg total) was similarly labeled with 1,000 pmol of Cy2 and quenched with 10 μL of 10 mM lysine. Individual Cy3- and Cy5-labeled samples were combined with an equal portion of the Cy2-labeled internal standard for a total of approximately 500 μg on each gel. Each gel contained one control and one diabetic sample, but the Cy3 and Cy5 dyes were swapped to control for any dye-specific labeling artifacts.

The three sets of tripartite-labeled samples were separated by standard 2D gel electrophoresis using a manifold-equipped IPGphor first-dimension isoelectric focusing unit and 24 cm 4-7 immobilized pH gradient (IPG) strips, followed by second-dimension 12% SDS-PAGE homogenous on hand-cast gels that had one plate pre-silanized to ensure accurate robot picking in subsequent steps, using an Ettan DALT 12 unit according to the manufacturer's protocols. The Cy2 (mixed standard), Cy3 (sample X) and Cy5 (sample Y) components of each gel were individually imaged at 100 μ m resolution with mutually-exclusive excitation/emission wavelengths using a Typhoon 9400 Variable Mode Fluorescence Imager. A Sypro Ruby total protein post-stain (Invitrogen/Molecular Probes) was used to ensure accurate protein excision, as the low stoichiometry of Cy-dyes label only 1-3% of the total protein.

DeCyder software v6.5 was used for simultaneous comparison of abundance changes across all three sample pairs with statistical confidence and without interference from gel-to-gel variation [29,30]. Control:standard and Diabetic:standard normalized volume ratios were calculated for each resolved protein on every gel, and the internal standard signals were used to normalize and compare these ratios across the 5 DIGE gels to allow for the calculation of average abundance changes and Student's *t*-test *p*-values for the variance of these ratios for each protein-pair across all five independent gels.

Mass Spectrometry and Database Interrogation—Proteins of interest were robotically excised, digested into peptides in-gel with modified porcine trypsin protease (Trypsin Gold, Promega) and peptides applied to a stainless steel target using an integrated Spot Handling Workstation per the manufacturer's recommendations. Matrix assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) and data-dependant TOF/TOF tandem MS/MS was performed on a Voyager 4700 mass spectrometer (Applied Biosystems, Framingham, MA). The resulting peptide mass maps and the associated fragmentation spectra were collectively used to interrogate mammalian sequences present in the SWISS-PROT and NCBItr databases to generate statistically significant candidate identifications using GPS Explorer software (Applied Biosystems) running the MASCOT search algorithm (www.matrixscience.com). Searches were performed without constraining protein molecular weight or isoelectric point, with complete carbamidomethylation of cysteine, partial oxidation of methionine residues, and 1 missed cleavage also allowed in the search parameters. Molecular Weight Search (MOWSE) scores from database interrogation above 58 ($p < 0.05$), number of matched ions, number of matching ions with independent MS/MS matches, percent protein sequence coverage, and correlation of gel region with predicted MW and pI were collectively considered for each protein identification.

Protein Determination and Statistical Analysis—Protein concentration of LV tissue samples assessed for oxidative stress status were determined using the Modified Lowry Protein Assay Reagent Kit (Pierce Biotechnology). Protein concentration of LV tissue samples prepared for 2-dimensional difference gel electrophoresis was determined using a 2-D Quant Kit (Amersham Biosciences, Piscataway, New Jersey). Data were expressed as the mean $\pm$ SEM and were statistically evaluated using analysis of variance, followed by Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

General Observations

STZ-diabetic animals showed a significant decrease in body weight and heart weight compared with controls (Table 1). The results in Table 1 also show an increase in heart/body weight ratio in the STZ-diabetic animals compared with controls. The development of diabetes was confirmed by a marked elevation in the urine glucose level (>2000 mg/dl). We chose not to

measure blood glucose values as anesthetization is required and this process has been shown to be associated with an increased risk of mortality, particularly in rats that are chronically and severely diabetic, as was the case in the present study.

Ventricular Function

LV hemodynamic parameters were measured in control and diabetic groups for assessment of ventricular performance. Figure 1 shows LV pressure and LV dP/dt recordings obtained from a control and diabetic rat. The diabetic rat displays a lower LVSP and a higher LVEDP than the control rat (Figure 1A). In addition, compared to the control rat, the diabetic rat also displays a depressed LV dP/dt (Figure 1B).

Hemodynamic data are summarized in Figure 2. The 8-week diabetic animals exhibited a significantly decreased LVSP and a significantly increased LVEDP compared to control animals (Figures 2A + 2B, respectively). The 8-week diabetic animals also demonstrated a significantly lower LV peak positive and negative dP/dt (Figures 2C + 2D, respectively). These data demonstrate obvious diabetic cardiomyopathy in STZ-diabetic rats. In addition, diabetes in the rats was also characterized by significant reductions in MAP (Figure 2E) as well as HR (Figure 2F).

Myocardial Oxidative Stress

The amount of free-radical mediated oxidative stress was determined by evaluation of myocardial levels of 8-*iso* PGF_{2α}; these data are shown in Figure 3A. Myocardial 8-*iso* PGF_{2α} levels increased by 77% in the diabetic group versus the control group, indicating higher myocardial oxidative stress among the diabetic animals.

Myocardial reduced (GSH) and oxidized (GSSG) glutathione levels were also examined in addition to 8-*iso* PGF_{2α} to assess oxidative stress. Significantly increased levels of myocardial GSSG was observed in the diabetic group (+137%) compared to the control animals (Figure 3B). Myocardial levels of GSH were observed to decrease by 21% in the diabetic animals versus the control animals (Figure 3C). These data provide further evidence of increased myocardial oxidative stress among the diabetic animals.

Proteomics of Diabetic Cardiomyopathy

In an effort to gain insight into the molecular mechanisms underlying the pathogenesis of diabetic cardiomyopathy, we performed a comparative analysis of the cardiac protein expression profiles of control and diabetic rats following long-term (8 weeks after STZ injection) diabetes using two-dimensional difference gel electrophoresis and mass spectrometry (DIGE/MS). Each DIGE gel contained a Cy3- and Cy5-labeled extract from individual samples, which was co-resolved with a Cy2-labeled pooled-sample internal standard that was present on every gel. This allowed for each resolved protein to be quantitatively measured relative to the cognate signal emanating from the standard within each gel (no gel-to-gel variation), as well as using each Cy2 signal to normalize and compare these ratios between the replicate samples across 5 DIGE gels (Methods). A total protein post-stain of a representative gel is shown in Figure 4, with numbers depicting points of excision corresponding to line entries for identified proteins in Table 2. Approximately 1000 protein spots were visualized on each gel and protein spot patterns were essentially identical among the animals, as determined by spot matching and analysis using DeCyder software. A comparison of left ventricles from STZ-diabetic rats (n=5 individual animals) with those from controls (n=5 individual animals) revealed differential expression of 42 protein spots between the 2 groups (each protein form indicated by number, Figure 4) that were identified by mass spectrometry and database interrogation as described in the Methods. Many of the 42 identified proteins were related isoforms, and collectively represented 24 different proteins (Table 2).

Interestingly, half of these proteins (12 out of 24) were localized to the mitochondria, and all but four of these 12 proteins were up-regulated in the myocardium of STZ-diabetic rats (Table 2). These results indicate that cardiac mitochondrial function may be modified during type I diabetic cardiomyopathy.

Diabetes-Specific Alterations in Metabolic Proteins

Major differences in protein expression were found related to metabolic pathways in the hearts of STZ rats with chronic diabetes. Proteins involved in fatty acid metabolism were observed to be up-regulated in STZ-diabetic hearts. Two isoforms of acyl coenzyme A thioester hydrolase (#24, 25) increased in average volume ratio by 123%-155% ($p<0.00003$) in STZ-diabetic hearts compared to control hearts while the levels of six resolved isoforms of acyl CoA dehydrogenase, long chain specific (#26-31) increased by 47%-109% ($p<0.0012$) in STZ-diabetic hearts compared to control hearts (Table 2), both of which are involved in fatty acid oxidation [31]. Glycolytic proteins were observed to be down-regulated in STZ-diabetic hearts as compared to control hearts. For example, pyruvate dehydrogenase protein $\times$ component (#14) was significantly reduced by 50% ($p=0.00017$) in the hearts of STZ-diabetic rats compared to the hearts of control rats (Table 2). Furthermore, two isoforms of the tricarboxylic acid (TCA) cycle protein 2-oxoglutarate dehydrogenase (#16, 17) were observed to decrease by 28%-31% ($p<0.00069$) in STZ-diabetic hearts as compared to control hearts (Table 2).

Fatty acid-binding protein, heart (H-FABP) and its related isoforms (#40, 42) were up-regulated by 64%-90% ($p<0.00036$, Table 2). In addition to acquiring mass spectral information for peptides accounting for 75% of the protein sequence for H-FABP, tandem mass spectrometry was consistent with tryptophan oxidation at two regions of this protein (Figure 5). The masses of these modified peptides was consistent with mono- and di-oxidation of tryptophan and subsequent conversion to kynurenine. 3-hydroxykynurenine (3-HK), a metabolic intermediate of the kynurenine pathway, has been shown to be an endogenous oxidative stress generator.

Diabetes-Specific Differences in Proteins Responsible for Myocardial Contractility

Striking changes in the expression levels of protein species associated with myocardial contractility and energy production were seen in STZ-diabetic rats. α -myosin heavy chain (MHC) and its proteolytic forms (#2, 3, 5, 15) were observed to be down-regulated by 55%-81% ($p<0.00071$) while the expression levels of β -MHC and its proteolytic forms (#7, 11, 18, 20) were found to increase by 61%-75% ($p<0.00036$, Table 2). Since α -myosin exhibits higher actin-activated ATPase than does β -myosin, these changes in protein expression among the cardiac MHC isoforms are consistent with the decreased contractility exhibited in rats with STZ-induced diabetes. In addition, two isoforms of creatine kinase (#21, 22) were reduced by 58%-66% in STZ-diabetic hearts compared to control hearts ($p<0.00009$, Table 2), which would reduce the level of available creatine phosphate, an important energy source for heart muscle.

Diabetes-Induced Changes in LV Proteins That Function to Protect Against Oxidative Stress

Alterations in the expression of a group of proteins that function to protect against oxidative stress was also observed in the LV of STZ-diabetic rats. Compared to control hearts, the protein level of aldehyde dehydrogenase (ALDH-2) (#13) significantly decreased by 34% ($p=0.00026$) while the protein level for 3-mercaptopyruvate sulfurtransferase (MST) (#34) was significantly reduced by 37% ($p=0.00097$) in rat hearts with STZ-induced diabetes (Table 2). Since both proteins are involved in antioxidant defense, the observed decrease in abundance of these cardiac proteins may play a role in the increased myocardial oxidative stress associated with diabetic cardiomyopathy.

Other Cardiac Protein Changes Associated With Diabetic Cardiomyopathy

In addition to the effects on mitochondrial, myofibrillar, contractile, and antioxidant proteins, diabetic cardiomyopathy was found to be associated with several other changes in myocardial protein expression (Table 2). Among these, the marked reduction in expression levels of gelsolin, which decreased by 48% ($p=0.00078$) (Table 2) in the LV of STZ-diabetic animals, might be of functional relevance since its up-regulation in aging hearts is reported to confer apoptosis resistance [32].

Discussion

Although diabetes has been well documented to be associated with increased oxidative stress [20-22], these studies suffer from major limitations due to the inaccuracy intrinsic to previously available indices of oxidant stress *in vivo* (e.g. malondialdehyde, lipid hydroperoxides) [23, 33]. Furthermore, little is known about the relation between oxidative stress and changes at the cardiac proteome level. The present study provides significant new information that is relevant to diabetic cardiac complications. The salient results can be summarized as follows: (1) in STZ rats with type I diabetic cardiomyopathy, LV levels of 8-*iso* PGF_{2α} and GSSG are significantly increased, a reflection of increased oxidative stress; (2) proteomic analysis of the LV of STZ-diabetic rats revealed marked alterations in the expression of 24 different cardiac proteins concomitant with this increased myocardial oxidative stress; (3) 50% of the altered proteins identified were localized to the mitochondria, with the majority increasing in abundance; and (4) in addition to proteomic alterations in mitochondrial pathways, we also observed down-regulation of antioxidant and anti-apoptotic proteins not noted previously to be altered in the diabetic heart. Taken together, these results provide specific quantitative evidence consistent with increased myocardial oxidative stress *in vivo* in the setting of diabetic cardiomyopathy and that this damage coincides with and may stimulate alterations at the cardiac proteome level. To our knowledge, this is the first study to perform proteomic analysis on LV proteins following long-term STZ-induced diabetes in rats. This is also the first study to reveal diabetes-state specific protein patterns in the heart of STZ-diabetic rats and is a first step to demonstrating possible associations with diabetic cardiomyopathy.

Our results strengthen the hypothesis that myocardial oxidative stress is increased in diabetic cardiomyopathy. In the present study, we found myocardial levels of 8-*iso* PGF_{2α} to be increased in diabetic rats 8 weeks after STZ injection. To our knowledge, it has not previously been shown that 8-*iso* PGF_{2α} is increased in the LV of STZ-treated rats with chronic type I diabetes. The increased formation of myocardial 8-*iso* PGF_{2α} among the STZ-diabetic animals was associated with significant depressions in LVSP, +dP/dt, and -dP/dt and concomitant increases in LVEDP. These hemodynamic alterations in STZ-diabetic rats demonstrate abnormal LV systolic and diastolic function that is the hallmark of diabetic cardiomyopathy. Interventional experiments involving antioxidant therapy were not carried out and therefore these findings do not allow for a causative relationship to be established. Nonetheless, the observed increases in myocardial 8-*iso* PGF_{2α} levels in STZ rats with type I diabetic cardiomyopathy provides strong evidence for increased oxidation in diabetic hearts.

Having established that STZ-induced diabetic cardiomyopathy is accompanied by an increase in myocardial levels of 8-*iso* PGF_{2α} as well as GSSG, we used proteomic analysis to investigate changes in expression of LV total proteins. Our unbiased proteomic results are consistent with the known increase in fatty acid utilization of diabetic hearts. Two altered enzymes (acyl coenzyme A thioester hydrolase and acyl CoA dehydrogenase, long chain specific) are involved in fatty acid oxidation [31], and both of them were up-regulated. Similar findings of increased protein levels of enzymes involved in fatty acid β-oxidation as a consequence of decreased glucose uptake and utilization has been reported previously in type I diabetic cardiac muscle [34,35].

A key finding of the current investigation was the significant decline in the myocardial abundance of aldehyde dehydrogenase (ALDH-2) and 3-mercaptopyruvate sulfurtransferase (MST) antioxidant enzymes. Prior studies utilizing proteomic techniques in diabetic hearts have not identified differences in the cardiac protein levels of these specific antioxidant enzymes. Notably, recent studies have shown that ALDH-2 and MST may be susceptible to oxidative stress-based inactivation [36-38]. Hence, in view of our present results, it is tempting to speculate that the significant decrease in myocardial protein abundance observed for ALDH-2 and MST in rats with STZ-induced diabetic cardiomyopathy may be due in part to oxidative modifications resulting from direct free-radical attack. In that regard, a marked increase in plasma protein oxidation has recently been reported in young type I diabetic patients with and without microvascular complications [39]. Since ALDH-2 and MST function as antioxidants, it is plausible that these proteins are more prone to free radical oxidation as a consequence of diabetes-induced oxidative stress. Further studies on these potential targets of protein oxidation in the setting of diabetic cardiomyopathy are certainly warranted.

Although ALDH-2 deficiency has been documented to contribute to the risk of diabetes [40], the risks have been mainly attributed to the association with alcohol consumption and the increase in the acetaldehyde concentration [36]. In conjunction with a previous study demonstrating that ALDH-2-deficient cell lines are highly vulnerable to exogenous 4-hydroxy-2-nonenal (HNE), an aldehyde derivative generated from free-radical mediated peroxidation of fatty acids [36], the present results suggest that reduced ALDH-2 may serve to amplify myocardial injury due to oxidative stress in diabetic hearts and thereby contribute to the pathogenesis of diabetic cardiac complications by this alternative pathway. Thus, decreased ALDH-2 in the diabetic heart may represent a heretofore unrecognized mechanism for the perpetuation of diabetes-induced LV dysfunction.

The precise mechanism (s) whereby MST and ALDH-2 function as antioxidants remains incompletely understood. However, it has been reported that MST can both oxidize thioredoxin, a key intermediate in cellular redox reactions, and react with peroxides and that this may be a physiologically significant mechanism for combating oxidative challenges [41, 42]. Recently, it has been reported that overexpression of ALDH-2 confers lung epithelial cell resistance to hyperoxia-induced cell death in part by reducing intracellular and mitochondrial-derived ROS production [43]. Another mechanism by which ALDH-2 may function as an antioxidant involves lipid peroxidation-derived aldehydes (LP-DA). By virtue of their increased chemical stability, LP-DA diffuse greater distances compared with their precursor ROS and behave as secondary toxic messengers that can propagate and amplify oxidative injury [44]. ALDH-2 has been documented to be the key enzyme for detoxifying the various aldehydes, including 4-HNE, a by-product of free-radical mediated lipid peroxidation [45]. Because 4-HNE reacts preferentially with sulfhydryl (SH)-containing molecules, the mitochondrial inner membrane is easily adducted with 4-HNE [46,47] which alters the electron transport chain and may increase free-radical production. Despite these observations, considerable work will be necessary to unravel the molecular and biochemical basis of MST and ALDH-2 antioxidant properties.

Previous studies have demonstrated that diabetes causes an increase in cardiac apoptosis [48, 49], which may cause loss of contractile myocytes, compensatory hypertrophy, and interstitial fibrosis [50]. This maladaptive cardiac remodeling associated with diabetes increases cardiac muscle stiffness and may contribute to the impaired diastolic function that accompanies diabetic cardiomyopathy. Recently, it has been shown that increased levels of gelsolin, a Ca^{+2} dependent actin-regulatory protein [51], in heart tissue of old rats, may be partly responsible for age-related apoptosis resistance [32]. In fact, prior studies conducted in non-diabetic hearts have reported an increased sensitivity to apoptosis-induction by gelsolin down-regulation [32]. To the best of our knowledge, this is the first study to document a reduction

in the protein expression levels of gelsolin in the diabetic heart. On the basis of these findings, it is conceivable to hypothesize that lower amounts of gelsolin contributes to cardiac apoptosis and thus to the development of diastolic dysfunction observed in the STZ-diabetic rats in the present study. Although we did not assess the contribution of myocardial apoptosis to LV function in the present study, the recognition that diabetes is associated with a loss of cardiac gelsolin raises the possibility that reductions in this protein may have deleterious consequences on the structure and function of the diabetic myocardium.

Our proteomic analysis of type I diabetic hearts did not reveal changes in the expression of myocyte Ca^{+2} -handling proteins (eg. phospholamban, SERCA2) that have been reported to occur using other techniques. This may be explained by the fact that phospholamban is too small (5.8 kDa) to be resolved in our gel system. Although SERCA2 certainly is predicted to resolve in our gel conditions, it may not have been detected because either it is not expressed at high enough levels for us to detect in the context of whole tissue extracts, or it was not changing significantly in this experiment. Other approaches may have detected these changes, but we elected to use the chosen approach to provide a global picture of proteomic changes in a way that enabled a high number of biological replicates and low technical noise.

For H-FABP, 13 peptides analyzed by mass spectrometry and database searching accounted for 75% of the predicted amino acid sequence. Intriguingly, 4 additional peptides that did not readily match the predicted H-FABP sequence were subsequently found to be highly related to the predicted sequence (Figure 5). The peptide fragmentation pattern resulting from tandem TOF/TOF mass spectrometry was consistent with these tryptophan-containing peptides (containing W8 and W97) being oxidized and subsequently converted to kynurenine. Kynurenine, a product of tryptophan degradation, is an immediate precursor of 3-hydroxykynurenine (3-HK) which has been shown to produce superoxide radical and hydrogen peroxide during its oxidation reactions [52,53]. 3-HK has also been demonstrated to auto-oxidize readily to generate ROS [53]. No evidence was found either by DIGE or MS for isoforms of H-FABP that did not contain this modification (although MW/pI shifts are not expected). Future experimentation focused on mass spectrometry will be required to determine whether this modification is a direct result of diabetes or a constant component of H-FABP.

Although possible confounding effects of STZ cannot be excluded, the half-life of the drug is ~15 minutes, and it is cleared from the organs within 4 hours [54]. Since the present study was conducted at such a late period after STZ treatment (8 weeks post-STZ injection), the results reported herein are likely related to the development of diabetes.

Conclusions

We have established a specific proteomic pattern in cardiac tissue that is indicative of diabetic cardiomyopathy presenting with higher oxidative stress. These changes in myocardial protein expression may serve as possible biomarkers for a 'diabetic milieu' and/or diabetic cardiac damage. These results offer new insight into understanding the cellular and molecular mechanisms that underlie cardiac dysfunction in diabetic cardiomyopathy and suggest that oxidative stress and concurrent proteome changes in diabetic hearts may proceed simultaneously and perhaps synergistically to predispose diabetic patients to cardiac complications. Data obtained in experimental animals must obviously be extrapolated to the clinical arena with caution. Nevertheless, the findings from the present study show that diabetes-induced proteomic changes in combination with attendant oxidative stress may be useful to predict the therapeutic response to established and novel antioxidant therapies for the prophylaxis of diabetic cardiomyopathy and subsequent HF in patients.

Acknowledgements

This study was supported by National Institutes of Health grants NIH NCRR G12 RR03032-17 and NIH/NIGMS 2 S06 GM08037-32 (M.F.H.) and by NIH/NIDDK predoctoral fellowship award F31 DK061284 (M. H.). We also acknowledge support for Proteomics through the Vanderbilt Academic Venture Capitol Fund. We thank Corbin W. Whitwell for technical assistance for DIGE experimentation.

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Figure 1.

Representative left ventricular (LV) hemodynamic recordings obtained from a control and diabetic rat (8 weeks post-STZ): **(A)** LV pressure; **(B)** dP/dt, first derivative of pressure.

Figure 2.

Summarized hemodynamic data from control (n=5) and STZ-diabetic (n=5) groups of rats. (A) LVSP, left ventricular systolic pressure; (B) LVEDP, left ventricular end-diastolic pressure; (C) +dP/dt, maximal rate of ventricular pressure increase; (D) -dP/dt, maximal rate of ventricular pressure decay; (E) MAP, mean arterial pressure; (F) HR, heart rate.

*Significantly different (p<0.05) vs. control group.

MYOCARDIAL CONTENT

Figure 3.

Myocardial oxidative stress in control and diabetic rats 8 weeks after STZ injection. Myocardial oxidative stress was determined by measuring the myocardial levels of 8-iso PGF_{2α} (A), oxidized (GSSG) glutathione (B), and reduced (GSH) glutathione (C). Data are mean ± SEM from 5 rats, with each assay done in duplicate. *P<0.05 vs. control group.

Figure 4.

(A) Representative 2D gel image of total protein stain (Sypro Ruby) from the 5-gel coordinated DIGE experiment. A total of 500 μ g of protein was loaded onto each DIGE gel. Altered proteins are indicated by numbers which correspond to line entries for identified proteins listed in Table 2. Examples of quantitative abundance changes are shown for H-FABP acidic isoform (B) and basic isoform (C), aldehyde dehydrogenase (D), and 3-mercaptopyruvate sulfurtransferase (E), with average fold-changes and supporting p -values calculated as described in the Methods.

A **B** **Figure 5.**

Representative mass spectra used in the identification and characterization of H-FAPB. **(A)** matrix-assisted laser desorption/ionization, time-of-flight (MALDI-TOF) mass spectrum of peptides derived from in-gel digestion as described in the Methods. Peptide ions matching H-FAPB (#40 Table 2) are labeled with mass accuracy < 20 ppm using trypsin autolytic peptides ($m/z=842.51$ and 2211.10) for internal calibration. **(B)** TOF/TOF tandem mass spectrum of the fragment ions of the peptide ion at $m/z=1040.51$, which is consistent with the displayed N-terminal H-FAPB sequence containing N-terminal acetylation (+42-A) and oxidized tryptophan converted to kynurenine (+4-W). Fragment ions with the charge retained on the N-terminus (b-ions, numbered left-to-right), and with charge retained on the C-terminus (y-ions,

numbered right-to-left) are indicated. Additional TOF/TOF spectra on peptide ions at m/z =1068.50, 1210.57, and 1238.57 were similarly consistent with H-FABP peptides containing tryptophan in various oxidized states (data not shown).

Table 1

Body Weight, Heart Weight, and Ratio of Heart/Body Weight at Eight Weeks After Streptozotocin or Vehicle Injection in Rats

Parameter	Control (n=5)	Diabetes (n=5)
Body weight (g)	431 ± 9.7	230 ± 11.5 [*]
Heart weight (g)	1.1 ± 0.03	0.77 ± 0.04 [*]
Heart/body weight (mg/g)	2.8 ± 0.07	3.4 ± 0.09 [*]

Heart weight represents the weight of both ventricles. Data are means ± SEM.

^{*} P<0.05 vs. control.

Table 2
Identification of Myocardial Protein Changes Induced By Diabetes

Protein Position	Protein(s) Identified	Identifier	pI	MW (kDa)	Fold Change	P Value	MOWSE ¹
1	Gelsolin	gil51854227	5.7	86	-1.92	0.00078	45
2	α -myosin heavy chain (MHC)	gil127741	5.3	220	-5.34	0.000003	128
3	α -myosin heavy chain (MHC)	gil127741	5.3	220	-5.33	0.00004	
4	Hypothetical protein	gil66910599	5.8	59	2.13	0.00054	83
5	α -myosin heavy chain (MHC)	gil127741	5.3	220	-2.53	0.00071	
6	Grp75 stress-70 protein	gil5584140	6.0	74	1.41	0.00043	442
7	β -myosin heavy chain (MHC)	gil127748	5.6	224	1.61	0.00036	145
8	Dihydropyridyl dehydrogenase	gil6014973	6.6	50	1.99	0.000001	78
9	Dihydropyridyl dehydrogenase	gil6014973	6.6	50	1.39	0.00044	
10	Dihydropyridyl dehydrogenase	gil6014973	6.6	50	1.11	0.13	
11	β -myosin heavy chain (MHC)	gil127748	5.6	224	1.75	0.00024	
12	α -1-antitrypsin	gil112889	5.6	44	2.09	0.00083	383
13	Aldehyde dehydrogenase	gil3121992	5.8	55	-1.52	0.00026	455
14	Pyruvate dehydrogenase protein $\times$ component	gil60688224	5.8	48	-1.99	0.00017	97
15	α -myosin heavy chain (MHC)	gil127741	5.3	220	-2.23	0.00004	
16	Dihydropyridyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase	gil62512126	5.9	41	-1.39	0.00069	351
17	Dihydropyridyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase	gil62512126	5.9	41	-1.46	0.00023	
18	β -myosin heavy chain (MHC)	gil127748	5.6	224	1.75	0.00001	
19	α -enolase	gil56757324	6.2	47	1.65	0.00064	194
20	β -myosin heavy chain (MHC)	gil127748	5.6	224	1.61	0.00029	
21	Creatine kinase- B type	gil125296	5.3	43	-2.98	0.00009	269
22	Creatine kinase- B type	gil125296	5.3	43	-2.38	0.00001	
23	Pyruvate dehydrogenase (lipoamide) kinase Isozyme 4, mitochondrial precursor	gil12585291	6.5	47	3.06	0.0000004	45
24	Acyl coenzyme A thioester hydrolase	gil6166586	6.3	45	2.55	0.00002	328
25	Acyl coenzyme A thioester hydrolase	gil6166586	6.3	45	2.23	0.00003	
26	Acyl-CoA dehydrogenase, long-chain specific	gil113016	6.7	46	2.09	0.000003	120
27	Acyl-CoA dehydrogenase, long-chain specific	gil113016	6.7	46	1.84	0.00031	
28	Acyl-CoA dehydrogenase, long-chain specific	gil113016	6.7	46	1.50	0.00005	

Protein Position	Protein(s) Identified	Identifier	pI	MW (kDa)	Fold Change	P Value	MOWSE ¹
29	Acyl-CoA dehydrogenase, long-chain specific	gi 113016	6.7	46	1.75	0.00003	
30	Acyl-CoA dehydrogenase, long-chain specific	gi 113016	6.7	46	1.47	0.0012	
31	Acyl-CoA dehydrogenase, long-chain specific	gi 113016	6.7	46	2.04	0.00002	
32	Suelg2_predicted protein (succinyl-CoA ligase)	gi 59808474	5.4	43	1.55	0.00037	186
33	Ndufa 10 protein	gi 62531217	6.0	37	-1.56	0.00028	245
34	3-mercaptopyruvate sulfurtransferase	gi 3122930	5.9	33	-1.60	0.00097	155
35	Delta3,5-delta2,4-dienoyl-CoA isomerase	gi 6015047	6.6	33	2.06	0.00020	391
36	Delta3,5-delta2,4-dienoyl-CoA isomerase	gi 6015047	6.6	33	2.32	0.00000003	
37	Apolipoprotein A-I precursor	gi 113997	5.5	27	3.02	0.00003	198
38	ATP synthase D chain	gi 1352051	6.2	19	2.47	0.00002	71
39	Heat-shock protein β -6	gi 6016271	6.0	18	-1.71	0.00017	139
40	Fatty acid-binding protein, heart	gi 119804	5.9	15	1.64	0.00036	574
41	Fatty acid-binding protein, adipocyte	gi 2494405	6.8	15	2.44	0.00011	68
42	Fatty acid-binding protein, heart	gi 119804	5.9	15	1.90	0.00008	

Protein position numbers refer to the annotated gel shown in Figure 4. Identifier = protein gi no. in NCBI protein database; pI = isoelectric point; MW = molecular weight (mass, kDa). Reported pI and MW values are predicted from genomic sequences and may not necessarily reflect migration of mature proteins. Several proteins are expressed as multiple isoforms with similar pI and MW on the gel. Isoforms listed in this table had comparable mass spectral signatures. Fold Change = fold change in abundance in STZ-diabetic hearts relative to control hearts. Fold change and associated probability scores (P value) were calculated from Student's *t*-test of STZ-diabetic hearts (n=5) versus control hearts (n=5) using DeCyder v6.5 software.

¹MOWSE = (Molecular Weight Search) scores as described in the Methods. Rows highlighted in bold indicate mitochondrial proteins.