Excess protein O-GlcNAcylation and the progression of diabetic cardiomyopathy

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Fricovsky ES, Suarez J, Ihm S, Scott BT, Suarez-Ramirez JA, Banerjee I, Torres-Gonzalez M, Wang H, Ellrott I, Maya-Ramos L, Villarreal F, Dillmann WH. Excess protein O-GlcNAcylation and the progression of diabetic cardiomyopathy. Am J Physiol Regul Integr Comp Physiol 303: R689–R699, 2012. First published August 8, 2012; doi:10.1152/ajpregu.00548.2011.—We examined the role that enzymatic protein O-GlcNAcylation plays in the development of diabetic cardiomyopathy in a mouse model of Type 2 diabetes mellitus (DM2). Mice injected with low-dose streptozotocin and fed a high-fat diet developed mild hyperglycemia and obesity consistent with DM2. Studies were performed from 1 to 6 mo after initiating the DM2 protocol. After 1 mo, DM2 mice showed increased body weight, impaired fasting blood glucose, and hyperinsulinemia. Echocardiographic evaluation revealed left ventricular diastolic dysfunction by 2 mo and O-GlcNAcylation of several cardiac proteins and of nuclear transcription factor Sp1. By 4 mo, systolic dysfunction was observed and sarcoplasmic reticulum Ca2+ ATPase expression decreased by 50%. Fibrosis was not observed at any timepoint in DM2 mice. Levels of the rate-limiting enzyme of the hexosamine biosynthetic pathway, glutamine:fructose-6-phosphate amidotransferase (GFAT) were increased as early as 2 mo. Fatty acids, which are elevated in DM2 mice, can possibly be linked to excessive protein O-GlcNAcylation levels, as cultured cardiac myocytes in normal glucose treated with oleic acid showed increased O-GlcNAcylation and GFAT levels. These data indicate that the early onset of diastolic dysfunction followed by the loss of systolic function, in the absence of cardiac hypertrophy or fibrosis, is associated with increased cardiac protein O-GlcNAcylation and increased O-GlcNAcylation levels of key calcium-handling proteins. A link between excessive protein O-GlcNAcylation and cardiac dysfunction is further supported by results showing that reducing O-GlcNAcylation by O-GlcNAcase overexpression improved cardiac function in the diabetic mouse. In addition, fatty acids play a role in stimulating excess O-GlcNAcylation. The nature and time course of changes observed in cardiac function suggest that protein O-GlcNAcylation plays a mechanistic role in the triggering of diabetic cardiomyopathy in DM2.

Type 2 diabetes mellitus; high-fat diet; diabetic cardiomyopathy; protein glycosylation

THE PREVALENCE OF DIABETES mellitus (DM) is a growing health concern in the United States and in the developed world. It is estimated that by the year 2025, there will be more than 300 million diabetics worldwide (19). The rise in Type 2 diabetes mellitus (DM2) cases is largely attributed to increases in the incidence of obesity. Evidence demonstrates that DM can alter cardiac structure and function, even in the absence of hypertension or coronary artery disease (4). This condition is termed diabetic cardiomyopathy (DC). In humans, DC is characterized at early stages by diastolic left ventricular (LV) dysfunction and later progressing to LV systolic dysfunction, which is accompanied by cardiac hypertrophy and fibrosis (21). DC can ultimately progress to congestive heart failure (4). Diastolic dysfunction can be secondary to impaired calcium handling, muscle hypertrophy, altered titin levels, and/or changes in fibrillar collagen deposition (1, 6). However, the mechanisms that lead to the development of cardiac dysfunction in the setting of DC are incompletely understood.

The hexosamine biosynthetic pathway (HBP) is a major branch of the glycolytic pathway and normally utilizes 2–5% of glucose (22). Under high-glucose conditions, such as those seen with DM, flux through the HBP increases (23). The end product of the HBP is uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc serves as a substrate in glycoprotein synthesis, and its accumulation is an indicator of the amount of glucose flux through the pathway (22, 35). O-GlcNAcylation is a postranslational protein modification, whereby N-acetylglucosamine is enzymatically attached by an O-linkage to serine and threonine residues by N-acetylglucosamine transferase (OGT). O-GlcNAc residues are removed from proteins by O-GlcNAcase (GCA). Excessive protein O-GlcNAcylation has been implicated in the development of insulin resistance in DM2 (35). However, the role that excessive O-GlcNAcylation may play in the pathophysiology of DC is not well understood. We have previously demonstrated that reducing the excessive protein O-GlcNAcylation in cardiac myocytes obtained from Type 1 diabetic hearts treated with GCA-expressing adenovirus has beneficial effects on calcium handling and cardiac function (15). In addition, we previously reported that nuclear transcription factor Sp1, an important gene expression regulator of sarcoplasmic reticulum Ca2+ ATPase (SERCA2a), can be abnormally O-GlcNAcylated when cardiac myocytes are exposed to high glucose (8). Increased O-GlcNAcylation of Sp1 leads to decreased expression of the SERCA2a gene (Atp2a2), which can impair Ca2+ reuptake during diastole (8). In vivo evidence to support a role for increased O-GlcNAcylation of Sp1 as a mechanism to downregulate SERCA2a in DM2 is lacking. There have been reports that suggest fatty acids may play a role in stimulating protein O-

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GlcNAcylation via GFAT stimulation (34); however, this possibility also has not been examined in diabetic myocardium.

The purpose of this study was to investigate the role that excessive cardiac protein O-GlcNAcylation may play in the development of DC in a high-fat diet/streptozotocin (HFD/STZ) mouse model of DM2. Our results show that excessive O-GlcNAcylation of cardiac proteins occurs early in the development of DM2 and may explain the onset of cardiac dysfunction, whereas muscle tissue properties (cardiac fibrosis) remained unaltered. In addition, our results suggest a role for fatty acids in stimulating O-GlcNAcylation with GFAT induction.

**MATERIALS AND METHODS**

The animal protocol was approved by the University of California, San Diego (UCSD), Institutional Animal Care and Use Committee as outlined by the National Institutes of Health (NIH).

R690

**DIABETIC CARDIOMYOPATHY AND PROTEIN O-GlcNAcylation**

**Table 1. Morphometrics and blood chemistry profiles in normal and DM2 mice**

<table>
<thead>
<tr>
<th>Type</th>
<th>Normal BW, g</th>
<th>1 mo BW, g</th>
<th>2 mo BW, g</th>
<th>3 mo BW, g</th>
<th>6 mo BW, g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>210 ± 1.3</td>
<td>24.6 ± 1.6*</td>
<td>25.9 ± 0.25</td>
<td>29.9 ± 1.1*</td>
<td>31.2 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>0.13 ± 0.01</td>
<td>0.11 ± 0.01*</td>
<td>0.14 ± 0.01</td>
<td>0.14 ± 0.01*</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>ME/W, mg/g</td>
<td>5.2 ± 0.33</td>
<td>3.54 ± 0.63*</td>
<td>5.5 ± 0.1</td>
<td>4.8 ± 0.1*</td>
<td>5.11 ± 0.5</td>
</tr>
<tr>
<td>ME/W, mg/g</td>
<td>0.77 ± 0.01</td>
<td>0.60 ± 0.03*</td>
<td>0.77 ± 0.05</td>
<td>0.79 ± 0.05</td>
<td>0.79 ± 0.17</td>
</tr>
<tr>
<td>FPG, mg/dl</td>
<td>98 ± 7.0</td>
<td>144 ± 17.0*</td>
<td>89.1 ± 2.0</td>
<td>72 ± 10.4*</td>
<td>90 ± 2.0</td>
</tr>
<tr>
<td>Plasma Insulin, ng/ml</td>
<td>0.61 ± 0.03</td>
<td>2.08 ± 0.43*</td>
<td>0.35 ± 0.10</td>
<td>2.94 ± 0.70*</td>
<td>1.6 ± 0.85</td>
</tr>
<tr>
<td>PGC, mg/dl</td>
<td>177 ± 7.0</td>
<td>269 ± 30*</td>
<td>112.2 ± 6.0</td>
<td>208.9 ± 16*</td>
<td>133 ± 1.0</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. BW, body weight; DM2, diabetes mellitus 2; HW, heart weight; TL, tibia length; PGC, post glucose challenge. n = 6/group; *P < 0.05 vs. normal.

**Table 2. Echocardiographic evaluation of LV diastolic function**

<table>
<thead>
<tr>
<th>Type</th>
<th>Normal E vel., cm/s</th>
<th>1 mo E vel., cm/s</th>
<th>2 mo E vel., cm/s</th>
<th>3 mo E vel., cm/s</th>
<th>4 mo E vel., cm/s</th>
<th>6 mo E vel., cm/s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>931.3 ± 187</td>
<td>923.3 ± 125</td>
<td>767.9 ± 49</td>
<td>762.8 ± 66</td>
<td>1012 ± 214</td>
<td>887.9 ± 86</td>
</tr>
<tr>
<td></td>
<td>476.8 ± 77.7</td>
<td>482.0 ± 46.6</td>
<td>406.0 ± 37</td>
<td>456.3 ± 48</td>
<td>513 ± 123</td>
<td>541.4 ± 63</td>
</tr>
<tr>
<td></td>
<td>1.95 ± 0.09</td>
<td>1.93 ± 0.32</td>
<td>1.90 ± 0.21</td>
<td>1.68 ± 0.08*</td>
<td>1.98 ± 0.07</td>
<td>1.64 ± 0.07*</td>
</tr>
<tr>
<td></td>
<td>1.47 ± 1.0</td>
<td>1.41 ± 0.5</td>
<td>1.53 ± 0.8</td>
<td>1.85 ± 1.4*</td>
<td>1.58 ± 0.9</td>
<td>2.04 ± 0.5*</td>
</tr>
<tr>
<td></td>
<td>15.5 ± 2.0</td>
<td>15.5 ± 2.0</td>
<td>18.3 ± 2.2</td>
<td>22.9 ± 4.3*</td>
<td>16.5 ± 2.6</td>
<td>20.9 ± 1.6*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. E vel., late diastolic peak velocity; A vel., late distolic peak velocity; E/A ratio, ratio representing E vel. of the LV inflow over A vel.; IVRT, isovolumic relaxation time; DT, deceleration time. n = 6/group; *P < 0.05 vs. normal.
Table 3. Echocardiograph evaluation of cardiac structure and LV systolic function

<table>
<thead>
<tr>
<th>Type</th>
<th>1 mo</th>
<th>2 mo</th>
<th>3 mo</th>
<th>4 mo</th>
<th>6 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>DM2</td>
<td>Normal</td>
<td>DM2</td>
<td>Normal</td>
</tr>
<tr>
<td>IVST, mm</td>
<td>0.54 ± 0.02</td>
<td>0.57 ± 0.06</td>
<td>0.57 ± 0.03</td>
<td>0.66 ± 0.05*</td>
<td>0.61 ± 0.06</td>
</tr>
<tr>
<td>PWT, mm</td>
<td>0.60 ± 0.04</td>
<td>0.62 ± 0.06</td>
<td>0.74 ± 0.08</td>
<td>0.81 ± 0.05</td>
<td>0.76 ± 0.16</td>
</tr>
<tr>
<td>LVDD, mm</td>
<td>3.26 ± 0.23</td>
<td>3.21 ± 0.10</td>
<td>3.44 ± 0.25</td>
<td>3.55 ± 0.08</td>
<td>3.57 ± 0.08</td>
</tr>
<tr>
<td>LVSD, mm</td>
<td>1.69 ± 0.23</td>
<td>1.73 ± 0.17</td>
<td>2.00 ± 0.30</td>
<td>2.02 ± 0.13</td>
<td>1.89 ± 0.11</td>
</tr>
<tr>
<td>FS, %</td>
<td>48.3 ± 5.9</td>
<td>45.9 ± 4.6</td>
<td>42.6 ± 5.4</td>
<td>43.2 ± 3.7</td>
<td>46.1 ± 1.4</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. IVST, interventricular septum wall thickness; PWT, posterior wall thickness; LVDD, left ventricle diastolic dimension; FS, fractional shortening. n = 6/group; *P < 0.05 vs. normal.

Wheat germ agglutinin (WGA). To detect the O-GlcNAcylated forms of SERCA2a and PLN. Briefly, 120 μg of myocardial protein in 120 μl of lysis buffer were used in each reaction. Then 30 μl of A/G beads (Santa Cruz Biotechnology) were added and incubated for 2 h and 4°C. After incubation, the beads were spun down at 5,000 rpm for 5 min, and the supernatant was collected and placed in a new microcentrifuge tube and added 30 μl of agarose-bound WGA and then incubated overnight. After incubation, the beads were spun down at 2,000 rpm for 5 min. The pellet-containing beads and pull-down proteins were washed three times with lysis buffer, and then mixed with 20 μl of 2× sample buffer, boiled for 5 min, and analyzed by Western blot.

HPLC analysis of HBP substrates and products. Hearts from mice 1 and 3 mo posttreatment were flash-frozen in liquid N2. Heart tissue was homogenized in 0.5 ml of 0.4 N perchloric acid. Homogenate was centrifuged, and 120 μl of 0.4 N perchloric acid. Homogenate was centrifuged, and 120 μl of 0.4 N perchloric acid. Homogenate was centrifuged, and 120 μl of 2.2 M KHCO₃ was added to the supernatant, left on ice for 15 min, and centrifuged again. Fructose-6-phosphate (F-6-P), UDP-N-acetylgalactosamine (UDP-GalNAc), and UDP-GlcNAc levels in heart homogenates were analyzed by the UCSD glyobiology core laboratory using a Dionex DX-600 BioLC equipped with a GP-50 quaternary gradient pump, AS-50 autosampler, LC30 chromatography enclosure, AD 25 variable-wavelength UV detector, an ED50-pulsed amperometric/conductivity detector, and a Jasco 1520 fluorescence detector, a column heater, an Eldex dual-channel postcolumn addition pump, and a heated postcolumn reactor is primarily used for the analysis of glycosaminoglycan-derived disaccharides enzymatically generated by the action of lyases. Detection is based on absorbance at 232 nm and on postcolumn fluorescent derivatization using 2-cyanoacetamide.

Echocardiogram. Echo was used to examine the time course of changes in cardiac structure and function. Under light anesthesia (1% isoflurane), transthoracic two-dimensional-targeted M-mode and
pulsed Doppler echocardiography was performed using a 40-MHz probe and Visual Sonics echo machine (Vevo 770 System; Visual Sonics, Toronto, Ontario, Canada). Measurements were recorded serially at 1 to 6 mo posttreatment. End-diastolic interventricular septal thickness (IVST) and posterior wall thickness (PWT), LV end-systolic dimension (LVSD), and LV end-diastolic dimension (LVDD) were measured. LV fractional shortening (FS%) was calculated as (LVDD-LVSD)/LVDD × 100. Two-dimensional guided pulsed-wave Doppler images were obtained from the apical four-chamber view. E/A ratio representing the early diastolic peak velocity (E velocity) of the LV inflow over the late diastolic peak velocity (A velocity), deceleration time (DT) and the LV isovolumic relaxation time (IVRT) were measured as an index of the LV diastolic function. All values were derived from three measurements, and mean values were used.

Histology. After thoracotomy, hearts from mice 3 and 6 mo posttreatment were excised and cross-sectioned at the center of the heart. The apical half of the heart was used for cryostat sectioning. The basal half was used for wax embedding. Sections were stained with hematoxylin and eosin, and Mason’s trichrome (for collagen quantification) by the UCSD Histology Core Laboratory.

Papillary muscle stretch. To examine cardiac tissue mechanical properties, papillary muscles isolated from mice 3 and 6 mo posttreatment were mounted horizontally with one end to a fixed hook and the other end attached to an isometric force transducer, as previously detailed (26). Starting from slack length, initial muscle length (L0) was established by stretching the muscle to the point at which tension just began to register on the gauge. L0 and midsection dimensions were measured with a microscale ruler. Cross-sectional area was calculated from the measurements assuming an elliptical shape. The muscle was slowly stretched 30% beyond L0 for three preconditioning runs. The final force gauge measurements were obtained by stretching the muscle 30% beyond L0 at a rate of ~3 mm/min, allowing 10 s between each stretch for recovery. Fiber stress and strains were derived from the measurements taken.

Hydroxyproline assay. This assay was adapted from previously reported methods and used to quantify collagen content (20). Briefly, hearts from 3 and 6 mo posttreatment DM2 and age-matched control mice were dried in glass containers for 48 h at 100°C. Acid hydrolysis was completed by adding 2 ml of 6 N HCl to each sample and was incubated under anoxic conditions for 24 h at 100°C. Vials were opened, and acid was evaporated for 24 h at 100°C. Each sample was reconstituted with 2 ml of sterile PBS, seeded, and incubated for 1 h in a 60°C water bath. Each sample was centrifuged twice for 10 min at 10,000 rpm, and the supernatants were analyzed for hydroxyproline. 4-Hydroxy-L-proline standards (0–10 μg/ml) were created from a 5 mg/ml hydroxyproline stock solution. All of the standards and sample dilutions (1:4) were created using PBS in a final volume of 1 ml. Samples were incubated with 0.5 ml of chloramine-T solution [50 mM chlorine-T, 30% (v/v) ethylene glycol monomethyl ether, 50% (v/v)], hydroxyproline buffer [0.26 M citric acid, 1.46 M sodium acetate, 0.85 M sodium hydroxide, 1.2% (v/v) glacial acetic acid], distilled H2O for the remaining volume for 20 min at room temperature, followed by 0.5 ml of 3.15 M perchloric acid for 5 min at room temperature. Samples were mixed well after each addition. p-dimethylamino-benzaldehyde solution (1.34 M p-dimethylaminobenzaldehyde dissolved in ethylene glycol monomethyl ether) was added to each (0.5 ml) and incubated for 20 min at 60°C for color development. The standards and samples were read in a 96-well plate at 557 nm on a spectrophotometer (μQuant, Biotek Instruments).

Isolation and culture of neonatal rat cardiac myocytes in oleic acid. To examine the role of fatty acids in stimulation of protein O-GlcNAcylation, primary cultures of neonatal rat cardiomyocytes were prepared as described previously (14). Cells (3 × 10^5 per 10-cm plate) were plated onto gelatin-coated culture dishes. Plating medium consisted of 4.25:1 DMEM: M199, 10% horse serum, 5% FBS, 1% penicillin/streptomycin/fungizone, and 5.5 mmol D-glucose. Cells were allowed to adhere to the plates for at least 24 h before different treatments. Cells were cultured in maintenance medium (4.5:1 DMEM: M199, 2% FBS, 1% penicillin/streptomycin/fungizone) supplemented with normal glucose (5.5 mM plus 25 mM mannitol), normal glucose (5.5 mM plus 25 mM mannitol) plus oleic acid (400 μM), high glucose (30 mM), or high glucose plus oleic acid (400 μM) (16, 17), referred to as control and oleic acid, respectively. The culture medium was changed daily for 3 days until the cells were harvested.

Statistical analysis. Results are expressed as means ± SE. Comparisons between means were analyzed, as appropriate, by Student’s t-test or one-way ANOVA followed by Bonferroni t-test. A value of P < 0.05 was considered statistically significant.

RESULTS

Morphometrics and blood chemistry. At 1 to 6 mo posttreatment, DM2 mice had significantly increased body weight. The standards and samples were read in a 96-well plate at 557 nm on a spectrophotometer (μQuant, Biotek Instruments).

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RESULTS

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A

1 month

Normal

DM2

SERCA2a

Actin

2 months

Normal

DM2

SERCA2a

Actin

4 months

Normal

DM2

SERCA2a

Actin

B

SERCA2a/Actin

1 month

Normal

0

1

2

3

4

DM2

SERCA2a/Actin

2 months

Normal

0

1

2

3

4

DM2

SERCA2a/Actin

4 months

Normal

0

1

2

3

4

DM2

SERCA2a/Actin

Fig. 2. Changes in sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA2a) levels with the development of DM2. A: immunoblots on cardiac lysates from mice 1, 2, and 4 mo after induction of DM2, and from age-matched controls (normal). B: graphical representation of normalized protein levels. n = 4/group, *P < 0.05 vs. normal.
Heart weight-body weight ratio was significantly lower in DM2 mice due to increased BW. Overall, there was no change in heart weight-tibia length ratio (HW/TL), except at 1 mo, when hearts were significantly smaller; thus cardiac hypertrophy was not found in the DM2 mice. FBG, plasma insulin, and PGC values were significantly increased compared with normal mice (Table 1). Plasma free fatty acids at 1 mo posttreatment (normal 164 ± 21 μM vs. DM2 260 ± 20 μM) were significantly elevated and persisted at 6 mo (normal 168 ± 4.0 vs. DM2 339 ± 7.0 μM, P < 0.05).

Cardiac function. Echocardiography demonstrated that at 1 mo posttreatment, DM2 mice had similar LV dimensions, and similar diastolic and systolic function (Tables 2 and 3) compared with normal mice. DM2 mice had significant increases in IVST at 2, 3, and 6 mo posttreatment, but no change in PWT (Table 3). LV diastolic function in DM2 mice, expressed as the E/A ratio, was significantly decreased at 2 and 3 mo. Doppler-derived DT and LV IVRT were significantly increased at 2, 4, and 6 mo. At 4 mo, DM2 mice develop enlarged LVDD and LVSD and decreased FS% of 25% (Table 3).

Cardiac structure and morphology. At 3 and 6 mo posttreatment, papillary muscle length-tension relationships (stress kPa) in DM2 showed no significant changes compared with normal (Fig. 1A). Hydroxyproline assay results demonstrated no significant difference in collagen content between DM2 and normal hearts (Fig. 1B). Histology of DM2 heart sections did not show evidence of changes in collagen area fraction compared with normal (Fig. 1C).

Expression of cardiac calcium handling proteins. SERCA2a protein levels were significantly decreased by 50% at 4 mo (Fig. 2). By 4 mo, the p-PLN/PLN ratio is significantly decreased by 70% because of the fall in p-PLN and the rise in PLN (Fig. 3).

Cardiac protein O-GlcNAcylation and HBP flux. Western blot analysis of DM2 hearts demonstrated significant increases in overall cardiac protein O-GlcNAcylation levels at 2 and 4 mo posttreatment (Fig. 4). HPLC measurements at 1 mo of F6P were significantly elevated in DM2 hearts (control 0.000087 ± 0.000022 nM/mg vs. DM2 0.00021 ± 0.00002 nmol/mg); however, no changes in UDP-N-acetylgalactosamine (UDP-GalNAc) and UDP-N-acetylglucosamine (UDP-GlcNAc) were observed (Fig. 5). At 3 mo, DM2 hearts had significantly increased levels of F6P (control 0.000075 ± 0.000009 nmol/mg vs. DM2 0.00013 ± 0.000009 nmol/mg), UDP-GalNAc (control 0.000009 ± 0.0000009 nmol/mg vs. DM2 0.0000131 ± 0.0000002 nmol/mg), UDP-GlcNAc (control 0.000009 ± 0.0000009 nmol/mg vs. DM2 0.0000131 ± 0.0000002 nmol/mg),

Fig. 3. Changes in phospholamban (PLN) and phospho-PLN (p-PLN) levels with the development of DM2. A: immunoblots on cardiac lysates from mice 1, 2, and 4 mo after induction of DM2, and from age-matched controls (normal). B and C: graphical representation of normalized protein levels PLN and p-PLN. D: ratio of p-PLN over PLN. n = 4/group, *P < 0.05, **P < 0.001 vs. normal.
and UDP-GlcNAc (control 0.000033 ± 0.000004 nmol/mg vs.
DM2 0.0000475 ± 0.0000006 nmol/mg) (Fig. 5).

Expression of HBP proteins. Levels of the fructose-6-phosphate amidotransferase (GFAT) protein, the rate-limiting enzyme in the HBP, were increased at 2 and 4 mo posttreatment by 48% and 57%, respectively (Fig. 6). However, mRNA and protein levels of other key HBP enzymes, such as OGT and GCA, did not change in diabetic mice during the duration of this study (data not shown).

Excess O-GlcNAcylation of cardiac calcium-handling proteins. SERCA2a was found to be O-GlcNAcylated, but DM2 did not further increase basal O-GlcNAcylation levels (Fig. 7). However, O-GlcNAcylation of p-PLN was found to be significantly increased in DM2 hearts by 20% at 4 mo posttreatment. O-GlcNAcylation of nuclear transcription factor Sp1 was also significantly increased in DM2 hearts as early as 2 mo, while Sp1 protein levels remained unchanged (Fig. 8).

Cultured cardiomyocytes in oleic acid and protein O-GlcNAcylation. GFAT protein levels were significantly increased by 48% in cardiomyocytes cultured in normal glucose plus oleic acid compared with normal glucose (Fig. 9A). Subsequently, GFAT levels in high glucose (89%) and high glucose plus oleic acid (126%) were also significantly higher than normal glucose (Fig. 9B). Neonatal cardiomyocytes cultured in normal glucose (5.5 mM) plus oleic acid (400 μM) for 3 days demonstrated a three-fold increase in overall protein O-GlcNAcylation compared with control cells cultured in normal glucose (Fig. 9C, D).

GCA expression improves cardiac function in DM2. Results presented up to now indicate a relationship between excessive protein O-GlcNAcylation and progression of cardiac dysfunction in DM2; however, a direct link cannot be established. Therefore, we sought to investigate whether expressing GCA, the enzyme that removes O-GlcNAc residues from proteins, results in cardiac function improvement in DM2. We used inducible, cardiac-specific GCA transgenic mice characterized in Fig. 10. A robust GCA expression in cardiac myocytes can be obtained by 2 wk of doxycycline treatment (Fig. 10A). Four month-diabetic GCA transgenic mice showed improved FS% by 30%, as assessed by echocardiography after GCA induction (Fig. 10B). GCA expression was induced 2 mo prior to echo. GCA expression in nondiabetic mice had no effect on FS% (WT; 40.38 ± 1.8 vs. normal-GCA; 43.70 ± 2.3).

DISCUSSION

The principle and unique findings of this study are that over the course of 6 mo, a mouse model of DM2 demonstrated 1) the early onset of diastolic dysfunction followed by the loss of systolic function in the absence of cardiac hypertrophy or fibrosis, 2) increased early cardiac protein O-GlcNAcylation mediated by increased HP pathway flux, 3) increase in O-GlcNAcylation levels of key calcium handling proteins, 4) restoration of cardiac function after removal of excess O-GlcNAcylation, and 5) a potential role for fatty acids in stimulating excess O-GlcNAcylation. The nature and time course of changes observed in cardiac protein O-GlcNAcylation suggest that the HBP plays a mechanistic role in the triggering of DC in DM2.

Prior studies on the cardiac effects of DM have been performed using nongenetic and genetic models of diabetes, and these studies have focused on different time points (7, 31). In humans, DC develops slowly over time, and thus, there is a need to replicate the long-term course of the disease in animals to characterize the nature of the structural and functional changes that take place in...
can be combined with toxic stimuli to decrease glycemia, and hypertension (9). HFD-induced insulin resistance is highly susceptible to diet-induced DM2, developing central adiposity, hyperglycemia, and hypertension (9, 27, 28, 36). The C57BL/6J inbred mouse model is highly susceptible to diet-induced DM2, developing central adiposity, hyperglycemia, and hypertension (9, 27, 28). Thus, to replicate more advanced degrees of DM2, the HFD/STZ model of DM2, an important component of altered protein regulation may be the result of increased O-GlcNAcylation. In agreement with our results, excessive protein O-GlcNAcylation has been recognized as a factor in the development of DM (35). The role of O-GlcNAcylation of cardiac proteins in the development of DC is incompletely studied. We observed excessive overall protein O-GlcNAcylation in the hearts of DM2 mice. Excessive O-GlcNAcylation preceded all other alterations seen in more advanced stages of the disease in patients (13). The causes underlying changes in diastolic function can be identified by examining known determinants, as discussed below. Several studies have examined changes in cardiac structure and function in rodents with DM (18, 24, 29). Thus, to replicate more advanced degrees of DM2, the HFD/STZ model of DM2, an important component of altered protein regulation may be the result of increased O-GlcNAcylation in the natural course of DM2-induced DC in a shorter time course.

DM2 animals developed hyperglycemia and hyperinsulinemia comparable to levels observed in patients with uncontrolled DM2 (10a). Increases in body weight were observed, but no difference in heart weight or HW/TL were seen except that at 1 mo, DM2 hearts were significantly lower in weight, possibly as a result of the initial STZ injection. This difference was not observed at further time points. Thus, this animal model of DM2 does not develop cardiac hypertrophy until 6 mo into the pathology, which has been described as a prominent feature in human DC patients (10). We did not find changes in myocardial stiffness or any significant increases in myocardial fibrosis. This observation contrasts with reports from humans in which myocardial fibrosis appears to occur early with DM (32) and can be attributed to specific features of this experimental model.

In spite of the absence of structural changes, cardiac dysfunction was observed. The analysis of cardiac morphometry by echocardiography indicated modest increases in wall thickness and LV chamber dimensions. The assessment of diastolic function denoted the development of abnormalities in the E/A ratio, DT, and IVRT as early as 2 mo into DM2. These changes were essentially sustained until the end of the study. Systolic dysfunction became evident in DM2 mice by 4 mo posttreatment. Thus, diastolic dysfunction occurred fairly early into the pathology and recapitulates alterations observed in humans with early-stage diabetes (11). The subsequent loss of systolic function also parallels alterations seen in more advanced stages of the disease in patients (13). The causes underlying changes in diastolic function can be identified by examining known determinants, as discussed below. Several studies have examined changes in cardiac structure and function in rodents with DM (18, 24, 29). There appears to be a consensus that diastolic dysfunction develops relatively early and evolves over time into systolic dysfunction (25). Interestingly, echocardiography in rodents with DM does not appear to consistently report the development of a true hypertrophic response, in particular, when values are normalized to tibia length (18). This is in agreement with our results.

Cardiac dysfunction in DM has been partially explained by alterations of calcium-handling proteins. Previous studies performed in our laboratory using rodents with Type 1 DM demonstrated that SERCA2a protein levels decrease upon development of the disease (29). Other investigators using DM2 rodent models have reported decreased SERCA2a activity, together with alterations in contractile performance, with no changes in SERCA2a protein levels (3, 33, 37). Decreased SERCA2a activity has been attributed to increased PLN and/or decreased p-PLN.

In the present study, we examined in a systematic fashion the time course of changes in levels of SERCA2a and PLN. SERCA2a protein levels decreased at 4 mo into DM2. These changes were associated with a decrease in the ratio of p-PLN/PLN. Since diastolic dysfunction was observed already at 2 mo, a possible cause may relate to a decrease in the ratio of p-PLN/PLN and not to SERCA2a levels (3, 33, 37). These results suggested that other mechanisms may also be involved in the diastolic dysfunction observed here. Interestingly, systolic dysfunction correlated in time with significant decreases in SERCA2a levels. A novel finding in this work is that PLN can be O-GlcNAcylated, and the level of PLN O-GlcNAcylation is increased in DM2 mice. We found an inverse relationship between p-PLN and O-GlcNAcylated PLN in the DM2 mice. It has been documented that crosstalk between these two protein modifications can regulate the biological activity of some proteins (39). Thus, it is possible that O-GlcNAcylation may interfere with the PLN phosphorylation process, resulting in more nonphosphorylated PLN, which, in turn, could contribute to decreased systolic function. A recent report demonstrated that PLN can be O-GlcNAcylated at a known phosphorylation site, which supports our hypothesis (38).

Excessive protein O-GlcNAcylation has been recognized as a factor in the development of DM (35). The role of O-GlcNAcylation of cardiac proteins in the development of DC is incompletely studied. We observed excessive overall protein O-GlcNAcylation in the hearts of DM2 mice. Excessive O-GlcNAcylation was apparent early after the start of treatment at 2 mo. It is interesting that excessive O-GlcNAcylation preceded all other alterations found in the present study. Using the RL2 antibody to detect O-GlcNAcylation of multiple proteins, we observed increases at 2 and 4 mo into DM2, indicating that in this model of DM2, an important component of altered protein regulation may be the result of increased O-GlcNAcylation. In agreement with this idea, we found that Sp1 was excessively O-
GlcNAcylated. Sp1 is a known substrate for protein O-GlcNAcylation, and modification at serine and threonine residues by O-GlcNAc results in diminished transcriptional activity, thus decreasing SERCA2a mRNA levels. This paradigm was demonstrated by us in previous work (8) and may explain the reduced levels of SERCA2a observed in this DM2 model of DC. However, in the present study, Sp1 was O-GlcNAcylated early, at 2 mo after induction of diabetes, indicating that in vivo, other transcrip-

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**Fig. 6.** Changes in glutamine:fructose-6-phosphate amidotransferase (GFAT) with the development of DM2. A: immunoblots on cardiac lysates from mice 1, 2, and 4 mo after induction of DM2 and from age-matched controls (normal). B: graphical representation of GFAT levels normalized to actin. n = 4/group, *P < 0.05 vs. normal.

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**Fig. 7.** Influence of DM2 on O-GlcNAcylation of SERCA2a and PLN. A: immunoblots on cardiac lysates for O-GlcNAcylated SERCA2a and O-GlcNAcylated PLN were analyzed at the times shown. B and C: graphs represent levels of O-GlcNAcylated proteins. n = 4/group. *P < 0.05 vs. normal.
tion factors are involved in the regulation of SERCA2a expression.

Excessive O-GlcNAcylation has been observed in Type 1 and Type 2 DM; however, the mechanisms that lead to increased O-GlcNAc modification may be different. It is well recognized that elevated glucose levels increase the availability of substrates for the HBP in the setting of DM. An ultimate consequence of high levels of HBP activity is excessive protein O-GlcNAcylation (5, 12). We examined the level of the HBP substrates at 1 and 3 mo into DM2. Significant increases were noted at 3 mo of DM2, providing evidence to suggest an enhanced flux through the HBP in this DM2 model. However, in many cases, DM2 coincides with only mild hyperglycemia, as observed in this study. In spite of this, increased O-GlcNAc levels were observed as early as 2 mo posttreatment in DM2 mice. GFAT, the rate-limiting enzyme of the HBP, increased in parallel with O-GlcNAc levels. We found increased GFAT protein levels with no changes in the levels of OGT or GCA. Increase in GFAT expression could be a result of the high-fat diet in this model. Measurements of free fatty acids in plasma of DM2 mice were significantly increased at 1 mo and remained elevated at 6 mo. It has been shown that fatty acids can induce GFAT in other models (23). Therefore, we explored whether increased GFAT protein levels could be induced by fatty acids in the cardiac myocyte and offer a possible explanation for increases in protein O-GlcNAcylation. We determined whether fatty acids were able to increase protein O-GlcNAcylation in cardiac myocytes by exposing cells to oleic acid, while maintaining glucose levels at normal. Oleic acid increased O-GlcNAcylation levels in cardiac myocytes in the absence of hyperglycemia associated with an increase in GFAT protein levels, thus demonstrating the ability of fatty acids to enhance the activity of this pathway. These effects were similar to the effects produced by high glucose. These in vitro results are, therefore, in agreement with the findings from DM2 mouse hearts observed in this study.

Results in this work strongly suggest that excessive protein O-GlcNAcylation is involved with the development and progression of cardiac dysfunction in DM2. Support for this idea was obtained by expressing O-GlcNAcase in DM2 mice, which resulted in improvement of cardiac performance. GCA expression in normal mice had no effect on cardiac performance during this study. However, whether chronic GCA expression could have deleterious effects in the heart is unknown.

Perspectives and Significance

We studied a mouse model of Type 2 diabetes, which developed cardiac diastolic and systolic dysfunction over the course of 4 mo as DC develops. Excessive protein O-GlcNAcylation appears as an early sign of cardiac abnormality. Functional changes are related to changes in the levels of calcium-handling proteins.
and to PLN and Sp1 O-GlcNAcylation. The increased levels of fatty acids observed with DM2 may play a critical role in triggering excessive O-GlcNAcylation in the Type 2 diabetic heart. Furthermore, beneficial effects on cardiac function were obtained by facilitating the removal of O-GlcNAc residues from proteins by overexpressing GCA. Our findings support the idea of cardiac protein O-GlcNAcylation as a therapeutic target in DC.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


Fig. 9. Protein O-GlcNAcylation and glutamine:fructose-6-phosphate amidotransferase (GFAT) levels in neonatal cardiac myocytes cultured for 3 days in normal glucose (NG) (5.5 mM plus 25 mM mannitol) and NG plus 400 μM oleic acid (OA) or high (30 mM) glucose (HG) and HG plus OA. A and B: GFAT protein levels in whole cell lysates. Graphical representations are of GFAT levels normalized to actin levels. C: protein O-GlcNAcylation level of whole cell lysates using the RL2 anti-O-GlcNAc antibody. D: graphical representation of O-GlcNAc levels normalized to actin levels. n = 3/group, *P < 0.05, **P < 0.001 vs. NG.

Fig. 10. Beneficial effects of O-GlcNAcase (GCA) expression on cardiac function in DM2 mice. A: Western blot demonstrating increased GCA protein levels in transgenic mice after 7 days of induction by doxycycline (Dox). B: fractional shortening (FS%) obtained by echocardiography showing improvement in DM2 mice after GCA induction. *P < 0.05, n = at least 4 animals per group.
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