O-Linked GlcNAc Modification of Cardiac Myofilament Proteins
A Novel Regulator of Myocardial Contractile Function

Genaro A. Ramirez-Correa,* Wenhai Jin,* Zihao Wang,* Xin Zhong, Wei Dong Gao, Wagner B. Dias, Cecilia Vecoli, Gerald W. Hart, Anne M. Murphy

In addition to O-phosphorylation, O-linked modifications of serine and threonine by β-N-acetyl-d-glucosamine (GlcNAc) may regulate muscle contractile function. This study assessed the potential role of O-GlcNAcylation in cardiac muscle contractile activation. To identify specific sites of O-GlcNAcylation in cardiac myofilament proteins, a recently developed methodology based on GalNAz-biotin labeling followed by dithiothreitol replacement and light chromatography/tandem mass spectrometry site mapping was adopted. Thirty-two O-GlcNAcylated peptides from cardiac myofilaments were identified on cardiac myosin heavy chain, actin, myosin light chains, and troponin I. To assess the potential physiological role of the GlcNAc, force–[Ca\(^{2+}\)] \textit{pCa}50, whereas maximal force (\(F_{\text{max}}\)) and Hill coefficient (\(n\)) were not modified. Using a pan-specific O-GlcNAc antibody, it was determined that acute exposure of myofilaments to GlcNAc induced a significant increase in actin O-GlcNAcylation. This study provides the first identification of O-GlcNAcylation sites in cardiac myofilament proteins and demonstrates their potential role in regulating myocardial contractile function.

Diabetes mellitus is a risk factor for the development of heart failure, and abnormal glucose metabolism may contribute directly to depressed cardiac function. Studies in humans and animal models of diabetes mellitus have demonstrated abnormal myofilament function and impaired excitation–contraction coupling, which may depress myocardial function. Posttranslational modifications of myofilament proteins regulate cardiac function and phosphorylation of myofilament proteins may result in functional abnormalities in heart failure. In addition to O-linked phosphorylation of serine (Ser) and threonine (Thr) residues of proteins, dynamic O-linked β-N-acetyl-d-glucosamine (O-GlcNAc) modifications can also regulate protein structure and function, and interplay between O-GlcNAcylation and O-phosphorylation may have an important role in cellular function. Although recent studies have suggested that O-linked modifications of Ser/Thr by GlcNAc could be involved in the regulation of myofilament Ca\(^{2+}\) activation properties in skeletal muscle, cardiac myofilament proteins have not been examined for this modification. Conversely, recent studies on isolated myocytes have directly associated diabetic cardiac dysfunction with increased levels of O-GlcNAcylation of cardiac proteins (>50kD), albeit the specific proteins and residues modified remained unknown. Those studies prompted us to verify the hypothesis that modification of cardiac myofilament proteins by O-GlcNAc could also regulate cardiac contractile function.

Materials and Methods

Mass Spectrometric Identification of O-GlcNAc–Modified Proteins

To label the specific sites (further details are in the online data supplement, available at http://circres.ahajournals.org), GlcNAc-modified peptides were labeled with GalNAz-biotin and enriched by avidin chromatography, and then dithiothreitol (DTT) was used to replace the GlcNAc-GalNAz-biotin by β-elimination and Michael addition (BEMAD) as previously described.

Isolated Skinned Fiber Studies

For skinned fiber studies, cardiac trabeculae were isolated and mounted as previously described.

Immunobots

Myofilament proteins were isolated as previously described, with minor modifications. To determine the global GlcNAc modifications of myofilament proteins, a pan-GlcNAc antibody (CTD 110.6, Covance) was used as previously described. To assess cardiac troponin I (cTnI) phosphorylation, a phospho-TnI (Ser23/Ser24) antibody (Cell Signaling, Danvers, Mass) was used as previously described.

Results and Discussion

Myofilament Proteins Are Modified by O-GlcNAc

With the enrichment and BEMAD experiments described, mass spectrometric (MS) data demonstrated that at baseline cardiac myofilament proteins are O-GlcNAcylated at the specific amino acid residues noted in Table I in the online data supplement. Thirty-two O-GlcNAcylated peptides from cardiac myofilaments were identified: 21 from cardiac myosin heavy chain, 6 from α-sarcomeric actin, 2 from myosin light chain 1, 1 from myosin light chain 2, and 1 from troponin I. As a control for specificity, in a parallel preparation treated extensively with β-N-acetyl-hexosaminidase to remove O-GlcNAc before enrichment, no modified sites were
Figure 1. Representative MS/MS fragmentation spectra. A, MS/MS spectrum of Ser54-modified peptide from actin. B, MS/MS spectrum of Ser150-modified peptide from cTnI.
detected. Typical tandem mass MS (MS/MS) spectra of O-GlcNAc–modified peptides from TnI and actin are shown in Figure 1A and 1B. The DTT modification on Ser54 of cardiac actin was confirmed by the observation of multiple matched ion pairs that contain the DTT mass (Figure 1A). We also identified O-GlcNAcylation at Ser150 of TnI (Figure 1B). Interestingly, Ser150 is also phosphorylated by p21-activated kinase 3, a modification that increases calcium sensitivity.17

This is the first report to define specific sites of O-linked GlcNAc of cardiac myofilament proteins. Although studies of skeletal muscle suggested GlcNAcylation of myosin heavy chain, actin, and myosin light chains, they did not define specific sites.11 Most of the newly identified O-GlcNAcylated sites in cardiac myofilaments were not previously described as phosphorylated, with the exception of cTnI Ser150 and MLC2 at Ser15. Interestingly, the O-GlcNAc targets in MLC1 at Thr93/Thr164 are different from phosphorylation sites at Thr69 and Ser200, previously found in pharmacological preconditioning.18

**GlcNAc-Desensitized Myofilaments to Calcium Without Altering Phosphorylation of TnI Ser23/24**

To characterize the effects of O-linked GlcNAc in cardiac muscle steady-state force-[Ca\(^{2+}\)]\(_{10^{-4}}\) relationships were studied in freshly skinned rat trabeculae. Baseline force-[Ca\(^{2+}\)]\(_{10^{-4}}\) relationships were established in individual trabeculae, thereafter the trabeculae were washed in relaxing solution and exposed to either 200 mmol/L Glycerol (n=5), 200 mmol/L GlcNAc (n=7) or 5 mmol/L GlcNAc (n=3) in a relaxing solution. After 1 hour incubation at room temperature, force-[Ca\(^{2+}\)]\(_{10^{-4}}\) relationships were determined. As shown in Figure 2A through 2C, a significant desensitization of the force-calcium relationship was observed in GlcNAc but not glycerol exposed trabeculae (pCa \(50 = 1.81 \pm 0.13\) mol/L for Control versus \(3.83 \pm 0.44\) mol/L for 200 mmol/L GlcNAc, n=7, \(P < 0.001\), pCa50 \(1.86 \pm 0.51\) mol/L for Control versus \(3.24 \pm 0.44\) mol/L for 5 mmol/L GlcNAc, n=3, \(P < 0.05\), whereas maximal force (\(F_{\text{max}}\)) and Hill coefficient (n) were not significantly modified. Notably, despite washing out GlcNAc and bathing for 1 hour with fresh relaxing solution, pCa50 did not return to basal levels (data not shown). These results indicate that the changes observed are not dependent on simple chemical artifacts or osmotic alterations reflected on myofilament lattice, the later usually sensitize rather than desensitize myofilaments.19 Brief (≈5 to 10 minutes) skinning in relaxing solution containing 1% Triton X-100 allows permeation of membranous system to calcium but preserves endogenous O-GlcNAc transferase activity. Skinned trabeculae showed immuno-reactivity to anti-O-GlcNAc transferase antibody and possessed metabolically active enzymes to incorporate \(^{3}\)H-GlcNAc into proteins of a wide range of

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**Figure 2.** GlcNAc desensitizes the myofilaments to calcium. Steady-state force-[Ca\(^{2+}\)]\(_{10^{-4}}\) relationship in skinned trabeculae, in control conditions, and after incubation with 200 mmol/L glycerol (n=5) (A) or GlcNAc (n=7) (B) or 5 mmol/L GlcNAc (n=3) (C). Note the rightward shift after exposure to 200 or 5 mmol/L GlcNAc. D, To quantify phospho-TnI, 3 trabeculae per group were pooled and immunoblotted for phospho-TnI (Ser23/Ser24) and then stripped and probed for sarcomeric α-actin. E, Intensity of phospho-TnI/sarcomeric α-actin bands was calculated by NIH ImageJ. There was no significant difference in the phosphorylation status of TnI between GlcNAc and glycerol samples. F, Immunoreactive bands to anti-O-GlcNAc transferase (anti-OGT) antibody on whole trabeculae crude protein extracts at the expected size of \(~105\) to \(110\) kDa. Lane 1, \(~20\) μg of trabecula 18; lane 2, \(~60\) μg of trabecula 19; lane 3, \(~20\) μg of trabecula 23; lane 4, empty; lane 5, \(~70\) ng of recombinant O-GlcNAc transferase (EG-OGT).
molecular weight extracted from freshly isolated trabeculae using UDP-[3H]-GlcNAc as substrate, as shown in supplemental Figure II, A and B. In support of this notion, other endogenous enzymatic activities, ie, pyruvate kinase, have been demonstrated in rat skeletal fibers skinned with Triton X-100 in comparable experimental conditions.20

It is well known that PKA phosphorylation of TnI Ser23/24 results in desensitization of the myofilaments to calcium.21 Therefore we sought to exclude the possibility that GlcNAc exposure altered calcium sensitivity by modifying phosphorylation levels at cTnI Ser23/Ser24. Using an antibody specific for phosphorylation at Ser23/Ser24 we concluded that GlcNAc exposure had no effects on phosphorylation at Ser23/Ser24 of TnI (Figure 2D). It is plausible that a balance between O-GlcNAcylation and O-Phosphorylation at cTnI Ser150 and MLC2 Ser15 could have an important role in regulating cardiac contractility; however that was not directly investigated in this work. Taken together, these results strongly suggest that excess of GlcNAc levels in myofilaments from cardiac trabeculae decreases cardiac myofilament sensitivity to calcium, through a mechanism that does not involve TnI phosphorylation at Ser23/Ser24.

Exposure to GlcNAc Alters Cardiac Myofilament O-GlcNAcylation Pattern

To further assess the effects of acute GlcNAc exposure, fresh cardiac myofilament preparations were examined using the pan-specific O-GlcNAc antibody CTD 110.6. We determined the pattern of myofilament GlcNAcylation at baseline (Figure 3A). Proteins of a wide range of molecular weight, from ≈15 to ≈250 kDa were detected (Figure 3A, lane 1). Next, the effects of preincubating myofilament preparations in vitro with 0.2 mol/L GlcNAc on GlcNAcylation pattern were studied (Figure 3A, lane 2). Interestingly, GlcNAc exposure was associated with an increase in actin GlcNAcylation levels (from 27.6±4.2% to 35.1±2.36%, n=4, P<0.05, Figure 3B). There were no significant differences in total signal level by densitometry after GlcNAc exposure. Treatment with PNGaseF (an enzyme that removes specifically N-linked glycans) did not significantly alter the staining pattern or content (data not shown), suggesting that most of the observed GlcNAcylation signal derived from O-linked GlcNAc.

To explore the relevance of this phenomenon in relevant pathophysiologic models, GlcNAcylation pattern was examined in similar fashion on cardiac myofilament preparations from ob/ob mice and streptozotocin treated rats and their respective controls. Interestingly, ob/ob mice and STZ rats myofilaments showed an increase in actin GlcNAcylation levels (36.4±7.8% to 53.2±2.1%, n=3 versus n=3 *P<0.05 for ob/ob mice and 42.7±2% to 50.9±3.2%, n=3 versus n=4 *P<0.05 for STZ rats, supplemental Figure I). Current evidence shows that acute enhancement of O-GlcNAcylation of specific myocardial protein targets may have cardioprotective effects22; however, it is also plausible that chronic increase of O-GlcNAcylation may impair cardiac function in diabetic cardiomyopathy.9,23 These data suggest that O-GlcNAcylation of actin can be increased by GlcNAc exposure in vitro and that this phenomenon is also present in vivo in relevant models of diabetes mellitus. O-GlcNAcylation of actin and/or other myofilament proteins may be responsible for the decreased submaximal force development observed in diabetes mellitus; however, increased ROS production and other factors may contribute.24 The O-GlcNAc antibody may not be sensitive enough to detect subtle changes in GlcNAcylation of other myofilament proteins.
In summary, this study has identified specific sites of O-GlcNAc modification of myofilament proteins and provides evidence that this posttranslational modifications can regulate myofilament function. This also raises the possibility that there could be a dynamic interaction between O-GlcNAc and O-phosphate modification of MLC2 and TnI as is well described for other proteins. Further studies of how O-GlcNAcylation of myofilament proteins regulates cardiac contractility may reveal novel and useful therapeutic targets in heart failure, especially in diabetic cardiomyopathy.

Acknowledgments
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Disclosures
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Key Words: O-GlcNAc | myofilaments | posttranslational modifications | cardiac contractility | diabetic cardiomyopathy
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SUPPLEMENT MATERIAL

SUPPLEMENTAL MATERIALS AND METHODS

Mass spectrometry identification of O-GlcNAc modified proteins

Myofilament proteins were isolated as previously described \(^1\) and dephosphorylated using Alkaline Phosphatase (Roche). After 1D SDS-PAGE gel electrophoresis, the bands of myofilament proteins were excised, pooled and trypsinized. The resulting peptides were sequentially treated with PNGaseF (New England Biolabs), to remove N-linked glycosylation, and again dephosphorylated with Alkaline Phosphatase (Roche) at 37 °C for 2 h. An azido-modified galactosamine (GalNAz) was attached to the O-GlcNAc moiety utilizing a mutant \(\beta\)-1, 4-galactosyltransferase (Gal-T1, Molecular Probes). A Biotin tag was then attached to the GalNAz and the modified peptides were enriched by avidin chromatography. To label the specific sites, DTT was used to replace the GlcNAc-GalNAz-Biotin by BEMAD (\(\beta\)-elimination and Michael addition). Details of this methodology have been recently reported by Wang Z \textit{et al}\(^2\). The modified peptides were subsequently identified by Finnigan LTQ mass spectrometer online coupled with a nano-LC. Experimental conditions: capillary column, 5 µm C18, 6 cm x 75 µm ID x 375 µm OD; flow rate, 300 nL/min; mobile phase: A, 0.1% formic acid; B, 80% acetonitrile in 0.1% FA, linearly gradient from 5% A to 100% B in 20 min. The MS conditions are: one full MS scan (350-1800 m/z) was followed by fragmentation and MS/MS scans of the
top eight most intensive ions with the following Dynamic Exclusion settings: repeat count 2, repeat duration at 30 s, exclusion duration at 60 s; spray voltage, 2.1 kV.

The subsequent MS data were searched by Mascot against the non redundant Swiss-Prot 50.4 Rattus database. The search parameters were: maximum allowed missed cleavages, 1; variable modifications, oxidation (Met), DTT modifications (Ser/Thr); peptide tolerance, ±1.5 Da, MS/MS tolerance, ±0.8 Da. Output peptides cutoff Mascot score, 32. All reported MS/MS from DTT modified peptides were manually checked. As a control for specificity, a parallel preparation was treated extensively, as per manufacturer instructions, with β-N-Acetyl-hexosaminidasef (NEB) to remove O-GlcNAc prior to enrichment, and did not demonstrate any modified sites.

**Isolated skinned fiber studies**

For skinned fiber studies thin right ventricular cardiac trabeculae were isolated and mounted as previously described. The trabeculae were skinned by 5 to 10 min incubation in 1% Triton X-100 on relaxing solution (80 mM KCl, 25 mM HEPES, 10 mM K$_2$EGTA, 15 mM creatine phosphate sodium salt (Na$_2$CrP), 5 Na$_2$ATP, 5.15 mM MgCl$_2$, and 0.5 mM leupeptin). The pH of the above solution was adjusted to 7.2 with KOH, and all experimental procedures were carried out at room temperature. Varied Ca$^{2+}$ concentrations [Ca$^{2+}$] were achieved by mixing the relaxing solution and activating solution (10 mM Ca$^{2+}$-EGTA, 80 mM KCl, 25 mM HEPES, 15 mM Na$_2$CrP, 5 mM Na$_2$ATP, 4.75 mM MgCl$_2$, and 0.5 mM leupeptin, pH 7.2) in various ratios. After reaching the highest calcium concentration trabeculae were washed in relaxing solution.
and incubated 1 hr in relaxing solution containing either 200 mM of Glycerol (Sigma), 200 mM or 5 mM GlcNAc (N-acetyl-D-glucosamine, Sigma). A new calcium activation protocol was carried out in the presence of 200 mM of Glycerol or GlcNAc and 5 mM GlcNAc. [Ca^{2+}] was calculated as previously described in detail. Skinned steady-state force-[Ca^{2+}] relationships were determined experimentally and fit to the Hill equation to yield Fmax (maximal Ca^{2+}-activated force), pCa50 (the [Ca^{2+}] required for 50% of maximal activation), and n (the Hill coefficient), as previously described. After the second relaxation phase skinned muscles were flash-frozen in liquid nitrogen and stored at -80°C for further Western Blot analyses.

**Animal models of Diabetes Mellitus**

Hearts derived from ob/ob (n=3, 29.27±1.27 g) and wild type mice (n=3, 19.36±2.35 g) are a kind gift from Dr. Natasha Zachara (Department of Biological Chemistry, Johns Hopkins School of Medicine). Before euthanasia with pentobarbital 50 mg/kg i.p., mice post-prandial glucose levels were 433.79±22.69 mg/dL for ob/ob mice and 256.32±44.16 mg/dL for wild type mice (p < 0.05), then hearts were harvested, flash frozen in liquid nitrogen and stored at -80 °C until further use.

To induce uncontrolled diabetes, Wistar rats of 200 to 225 g were exposed to a single i.p. injection of streptozotocin (STZ) (50 mg/kg). Two weeks after STZ injection hyperglycemia was confirmed and followed every two weeks up to eight weeks. On the day of euthanasia (Pentobarbital 80mg/kg i.p.), STZ rats glucose levels were 463.4±19.1 mg/dL whereas aged matched control rats glucose levels were 119±2.6
mg/dL (STZ $n=5$, Control $n=4$, $p<0.001$), then hearts were removed, flash frozen in liquid nitrogen and stored at -80 ºC until further use.

**Immunoblot Analyses**

**Immunoblotting for O-GlcNAc modification**

Myofilament proteins were isolated as previously described$^1$, with the following modifications: all solutions contained PhosStop (Roche) and for frozen hearts all solutions contained 20μM NAGT (NAG-thiazoline, 1,2-dideoxy-2'-methyl-alpha-d-glucopyranoso[2,1-d]-Delta2'-thiazoline)$^4$. For *in vitro* myofilament GlcNAcylation pattern determination, fresh myofilament preparations were incubated 1h at room temperature in relaxing solution alone or containing 200 mM GlcNAc. Samples were separated by 4-12 % NuPage Bis-Tris SDS gel electrophoresis (Invitrogen) and transferred onto nitrocellulose membranes (Invitrogen) at 30 v for 2.5 h at room temperature. To determine the global GlcNAc modifications of myofilament proteins, a pan-GlcNAc antibody (CTD 110.6, Covance) was diluted (1:5,000) in 3%BSA-TBST 0.05% and incubated O/N at 4°C$^5$, followed by 3 washes in TBST 0.05% and incubation for 1 h with anti-mouse IgG HRP-antibody (Santa Cruz) diluted (1:10,000) in 3 %BSA-TBST 0.05%. Membranes were developed by a chemo-luminescence method (WestPico, Pierce, Rockford IL) as per manufacturer instructions. Controls for specificity include pretreatment of the samples using PNGaseF at 5 U/μg (New England Biolabs) and incubation of the membrane with primary antibodies with or without 1M GlcNAc. Membranes incubated with CTD110.6 and 1M GlcNAc were devoid of signal. GlcNAc
signals were normalized to membrane total protein staining by Direct Blue 71 (DB71, Sigma)\textsuperscript{6}. Densitometry analysis was performed using NIH ImageJ software.

**Immunoblotting for O-GlcNAc Transferase:**

To assess O-GlcNAc Transferase immunoreactivity in isolated cardiac trabeculae from physiology studies, three trabeculae snap frozen and maintained at -80 °C were thawed on ice cold relaxing solution, 1X PhosStop (Roche) and 20\(\mu\)M NAGT and sonicated. Myofilament preparations derived from ob/ob and wild type mice were prepared as above described. All samples were denatured at 70°C for 10 minutes in 1X NuPAGE LDS sample buffer (Invitrogen), 1X Reducing Agent (Invitrogen) and separated by 4-12 % NuPAGE Bis-Tris SDS gel electrophoresis (Invitrogen) and transferred onto nitrocellulose membranes (iBlot Dry Blotting System, Invitrogen) at 23 v for 11 minutes and blocked in 5%BSA-TBST 0.05% for 1hr at room temperature. To identify OGT in myofilaments preparations and whole trabeculae crude protein extracts, an anti-OGT antibody (AL-28, Hart’s Lab) was diluted (1:1,000) in 3%BSA-TBST 0.05%, 0.02% Sodium Azide and incubated O/N at 4°C, followed by 3 washes in TBST 0.05% and incubation for 1 h with anti-rabbit IgG HRP-antibody (Amershan GE) diluted (1:2,000) in 3 %BSA-TBST 0.05%. Membranes were developed by a chemoluminescence method (WestDura Femto, Pierce, Rockford IL) as per manufacturer instructions. Controls for specificity include 70 ng of recombinant O-GlcNAc Transferase (EC-OGT, Hart’s Lab) and a duplicate membrane with no primary antibody.
**Immunoblotting for TnI phosphorylation and actin:**

To assess cTnI phosphorylation in isolated cardiac trabeculae from Glycerol or GlcNAc group physiology studies, three trabeculae per group were thawed pooled in NuPAGE LDS Sample buffer (1X) and denatured at 70°C for 10 min. Protein samples were separated as above described. A phospho-TnI (Ser23/Ser24) antibody (Cell Signaling, Danvers, MA) was diluted (1:200) and incubated for 1 h at 25 °C, followed by incubation with anti-rabbit secondary HRP-antibody (1:10,000) (Upstate Biotechnology, Lake Placid, NY) for 1 h at 25 °C. Membrane was stripped for 2 h at 25 °C in Restore (Pierce, Rockford IL) and re-blotted with anti-sarcomeric alpha-actin (Sigma) as previously described 7. Radiographs were densitometrically analyzed with NIH ImageJ software, and the ratio of phospho-TnI to alpha-sarcomeric actin was calculated.

**Cardiac trabeculae UDP-[³H]-GlcNAc incorporation**

Freshly isolated cardiac trabeculae were mounted as previously described 3 and skinned by 5 to 10 min incubation in 1% Triton X-100 in relaxing solution (80 mM KCl, 25 mM HEPES, 10 mM K₂EGTA, 15 mM creatine phosphate sodium salt (Na₂CrP), 5 Na₂ATP, 5.15 mM MgCl₂, and 0.5 mM leupeptin), washed three times in relaxing solution (No Triton). Skinned fibers were separated into trabeculae per se (Trab) and ventricular wall attachment (VS), transferred to a 1.5 ml eppendorf tube containing; relaxing solution, 1X Complete Mini, EDTA-free proteinase inhibitors (Roche) and 20μM NAGT. Skinned fibers (Trab and VS) were homogenized by hand with a plastic pellet pestle (Thomas Scientific). Crude protein extracts were incubated over night with 3μCi
of UDP-[3H]-GlcNAc (50 Ci/mmol Uridine-diphospho-N-acetyl[6-3H]glucosamine, American Radiolabeled Chemicals, St. Louis, MO) at room temperature. Following that, crude protein extracts from trabeculae were sonicated, denatured in Laemmlli loading buffer at 95 ºC, separated by SDS-PAGE and stained with Coomassie G-250. Following de-staining the gel was treated with En3Hance (Perkin Elmer Life Science) and exposed to autoradiography film for 5 days at -80°C.

Statistical analysis

Data are expressed as mean ± standard error of the mean. Statistical analyses were performed using ANOVA and Student t-test, where appropriate, a p value of < 0.05 was considered to be statistically significant.

SUPPLEMENTAL FIGURE AND TABLE LEGENDS

Online Table I. Summary of O-GlcNAcylation sites in cardiac myofilament proteins. For MHC 21 GlcNAcylated peptides are shown, 6 for alpha cardiac Actin, 2 for MLC1, and 1 each for cardiac TnI and MLC2. See table for peptide ID and modified amino acid residue. Modified residues are denoted by an asterisk and corresponding residue symbol in blue color.
Online Figure I. Diabetes Mellitus alters cardiac myofilament O-GlcNAcylation pattern. All myofilament preparations were isolated in the presence of phosphatase and O-GlcNAcase inhibitors (1X PhosStop and 20μM NAGT, respectively). Densitometry analysis of the blots was performed using ImageJ NIH Software. A, GlcNacylation pattern of control wild type and ob/ob mice myofilaments with their respective densitometry plots (Top panels) and actin signals, as loading control (Bottom panels). B, GlcNacylation pattern of control rats and STZ rats myofilaments with their respective densitometry plots (Top panels) and actin signals, as loading control (Bottom panels). C, D, Show the quantification of the contribution of actin peak to total GlcNAcylation staining pattern, where actin peak is increased from 36.4±7.8% to 53.2±2.1% (n=3 vs n=3 *p<0.05) for ob/ob mice and actin peak increased from 42.7±2% to 50.9±3.2% (n=3 vs n=4 *p<0.05) for STZ induced diabetic rats.

Online Figure II. O-GlcNAc Transferase immunoreactivity in myofilaments and metabolic activity in skinned cardiac trabeculae. Myofilament preparations from ob/ob mice or whole trabeculae crude protein extracts (15 μg) were separated by SDS-PAGE gels. A, Shows immuno-reactive bands to Anti-OGT antibody on both myofilament preparations and whole trabeculae crude protein extracts at the expected size of ~105-110 kDa. Lane 1 and 2, control wild type mice myofilaments, lane 3 to 5 ob/ob mice myofilaments, lane 6 to 8, trabeculae crude proteins extracts, Lane 9 empty, lane 10 recombinant O-GlcNAc transferase (70 ng total). B, Shows that skinned trabeculae possess metabolically active enzymes to process UDP-[3H]-GlcNAc and incorporate [3H]-GlcNAc into a wide range of proteins.
extracted from both trabeculae per se (Trab) and ventricular side attachment (VS).
These data are highly suggestive that OGT is in close association with myofilaments
and that OGT is metabolically active in skinned cardiac trabeculae.

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phosphorylation impair ventricular relaxation-afterload and force-frequency
### Online Table I. Summary of O-GlcNAcylation sites in cardiac myofilament proteins.

<table>
<thead>
<tr>
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<th>Peptide ID</th>
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<td><strong>Actin, alpha cardiac, P68035</strong></td>
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Online Figure I. Diabetes Mellitus alters cardiac myofilament O-GlcNAcylation pattern.
Online Figure II. O-GlcNAc Transferase immunoreactivity in myofilaments and metabolic activity in skinned cardiac trabeculae.