Diabetes and the accompanying hyperglycemia impairs cardiomyocyte calcium cycling through increased nuclear O-GlcNAcylation*

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SUMMARY

Diabetic cardiomyopathy is characterized by impaired cardiac contractility leading to poor myocardial performance. We investigated the role that the hexosamine pathway, and especially altered nuclear $O$-GlcNAcylation, plays in the development of diabetic cardiomyopathy. Incubating neonatal rat cardiomyocytes in high glucose (25 mM) resulted in prolonged calcium transients when compared with myocytes incubated in normal glucose (5.5 mM), which is consistent with delayed myocardial relaxation. High glucose-treated myocytes also exhibited reduced sarcoendoplasmic reticulum Ca-ATPase 2a (SERCA2a) mRNA and protein expression, decreased SERCA2a promoter activity, and increased $O$-GlcNAcylation of nuclear proteins compared with myocytes treated with normal glucose. Exposure of myocytes to 8mM glucosamine or an adenovirus expressing $O$-GlcNAc transferase (OGT) resulted in prolonged calcium transient decays and significantly reduced SERCA2a protein levels, whereas treatment with an adenovirus encoding O-GlcNAcase (GCA) resulted in improved calcium transients and SERCA2a protein levels in myocytes exposed to high glucose. Effects of elevated glucose or altered $O$-GlcNAcylation were also observed on essential transcription factors involved in cardiomyocyte function. High glucose-treated myocytes (with or without OGT adenovirus) exhibited increased levels of $O$-GlcNAcylated Sp1 compared with control myocytes, whereas infecting high glucose-treated myocytes with GCA adenovirus reduced the degree of Sp1 GlcNAcylation. Treatment of myocytes with 25 mM glucose, 8 mM glucosamine, or OGT adenovirus also significantly reduced levels of MEF-2A protein compared with control myocytes, whereas infection with GCA adenovirus resulted in improved MEF2 expression. Our results suggest that the hexosamine pathway, and $O$-GlcNAcylation in particular, is important in impaired cardiac myocyte function and the development of diabetic cardiomyopathy.
INTRODUCTION

The leading cause of mortality among diabetic patients in the United States is heart disease. Despite the numerous effects diabetes exerts on the cardiovascular system, there is substantial evidence indicating that a diabetes-specific cardiomyopathy occurs in the absence of coronary artery disease or hypertension (1,2). Diabetic cardiomyopathy is characterized by impaired cardiac contractility and poor myocardial performance without an attendant vascular or valvular disease, and can lead to congestive heart failure. Studies in diabetic human patients and animal models have demonstrated the early development of diastolic dysfunction prior to the alteration of systolic function (3,4). Eventually, however, nearly all aspects of cardiac contractility appear to become impaired (5,6). Increasing evidence indicates that abnormalities in cardiac Ca\(^{2+}\) handling may be an important contributor to decreased contractile function in the diabetic heart. Ventricular contraction and relaxation are controlled largely by Ca\(^{2+}\) release from and uptake into the sarcoplasmic reticulum (SR\(^{4}\))(7). All aspects of SR function in the diabetic heart are depressed as documented by decreased SR Ca\(^{2+}\) uptake, reduced SR Ca\(^{2+}\) content, and impaired SR Ca\(^{2+}\) release (8-10). Impaired SR function appears to result from reduced activity and expression of the SR Ca\(^{2+}\) ATPase (SERCA2a)(5,11), which is responsible for sequestering Ca\(^{2+}\) and inducing diastolic relaxation. The underlying mechanisms leading to abnormal SR function and calcium flux in the diabetic heart are poorly understood.

Diabetic hyperglycemia results in a number of pathophysiological changes in the vascular system, but investigations of its role in diabetic cardiomyopathy are limited. Recently, studies exposing cardiac myocytes to elevated extracellular glucose resulted in impaired cardiomyocytes contractility and calcium flux (12) and increased \([\text{Ca}^{2+}]_i\) (13). The observation that the diastolic
dysfunction observed in myocytes exposed to elevated extracellular glucose could be duplicated by incubation of cardiomyocytes with glucosamine, a precursor to cellular N- and O-linked glycosylation, suggests that the mechanism may involve increased flux of glucose into the hexosamine pathway (12). Increased hexosamine flux is known to lead to insulin resistance in many tissues (14), and recent studies indicate that dynamic O-GlcNAcylation (the dynamic addition and removal of a single O-linked N-acetylglucosamine residue) may prove to be an important player in diabetes (15,16).

In this study, we investigated specifically whether the impaired myocardial calcium cycling observed in diabetic cardiomyopathy is linked to O-GlcNAcylation in a hyperglycemia-dependent manner. Using cultured neonatal rat cardiomyocytes, we demonstrate that elevated extracellular glucose impairs calcium cycling, that these changes appear specifically via increased cellular O-GlcNAcylation, and that the detrimental effect of increased cellular O-GlcNAcylation can be mitigated against through the use of adenovirally-transfected O-GlcNAcase protein.

**EXPERIMENTAL METHODS**

**Materials** Antibodies used in this study were: anti-Sp1 (07-124, Upstate Biotechnology, Lake Placid, NY), anti-MEF-2 (predominantly MEF-2a, sc-313, Santa Cruz Biotechnology, Santa Cruz, CA), anti-O-GlcNAc monoclonal (CTD 110.6, provided as a gift by Dr. Gerald Hart, Johns Hopkins University, Baltimore, MD) (17), and horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) and anti-mouse IgM (Sigma). Indo-1/AM and Pluronic were purchased from Molecular Probes (Eugene, OR). Pre-cast Tris/Glycine SDS gels and all electrophoresis supplies were from Bio-Rad (Hercules, CA). PUGNAc (O-(2-acetamido-2-
deoxy-D-glucopyranosylidene)amino-N-phenyl carbamate) was purchased from Toronto Research Chemicals (North York, Ontario, Canada). All other chemicals were purchased from Sigma unless otherwise noted.

**Cardiomyocyte Isolation and Culture.** Primary cultures of neonatal rat cardiomyocytes were prepared as described previously (18). Cells were plated onto gelatin-coated culture dishes or laminin-coated glass chamber slides. Plating medium consisted of 4.25:1 DMEM:M199, 10% horse serum, 5% fetal bovine serum, 1% penicillin/streptomycin/fungizone (PSF), and 5.5 mM D-glucose. Cells were allowed to adhere to the plates for 24 hours before changing to basic experimental culture medium (4.5:1 DMEM:M199, 2% fetal bovine serum, 1% PSF, and 100 μM insulin) and supplemented with glucose at either physiological (5.5 mM; normal glucose) or elevated (25 mM; high glucose) concentrations. For some experiments, media was supplemented with 8 mM glucosamine, 40 μM aminoguanidine, 0.5 μg/ml tunicamycin, 50 μM PUGNAc or an osmotic control (20 mM mannitol). In cases where cells were plated at exceptionally high density (5 x 10⁶ per 100 mm plate), the culture medium was changed daily until the cells were harvested.

**Construction of adenoviral vectors.** The rat cDNA corresponding to the UDP-N-acetylglucosamine:peptide N-acetylglucosaminyl transferase gene (O-GlcNAc transferase; OGT), which encodes the enzyme responsible for the O-linkage of single N-acetylglucosamine molecules to serine/threonine residues (19), and the human cDNA encoding the O-GlcNAcase gene (GCA), the enzyme responsible for removing O-linked N-acetylglucosamine residues (20), were both kindly provided by G. Hart. Each cDNA was inserted into the E1 region of an
adenoviral vector construct using previously described methods (21). Replication-deficient adenovirus particles containing the target gene or empty vector (SR-) were generated by in vivo recombination in 293 cells and single plaques were isolated and propagated to achieve high titer. Adenoviral particles were CsCl-purified and quantified by plaque titer assay. Myocytes were infected with a multiplicity of infection (MOI) of 25 for all viruses.

Measurement of \([Ca^{2+}]_i\) transients. Indo-1 facilitated measurement of \(Ca^{2+}\) transients has been described previously (22). Briefly, cells were plated (5 x 10⁴) onto glass chamber slides and incubated for 48-72 hours in experimental medium as above. Cells were rinsed twice with appropriate serum-free culture medium (5.5 or 25 mM glucose) then incubated with the same medium containing 10\(\mu\)M Indo-1/AM, 1 mg/ml bovine serum albumin, and 0.01% (wt/vol) Pluronic F-127 for 30 minutes in a 37°C incubator. Cells were rinsed and incubated for 20 min at RT in appropriate serum-free culture medium containing 1.8 mM CaCl₂ prior to making fluorescence measurements to allow for deesterification of the Indo-1/AM. Myocytes were stimulated to contract (0.3 HZ) using platinum electrodes, and Indo-1 ratios (405/484 nm) were collected at 20 Hz at room temperature using methods and equipment as described previously (23). Diastolic and systolic \(Ca^{2+}\) levels were defined as the resting ratio and maximal ratio per cycle, respectively. Transients were measured from at least 18 cells per slide per treatment. For data analysis, transients were aligned and averaged using the initial upstroke of each transient as a reference point, then normalized using the diastolic and systolic ratios as references.

Western Immunoblotting. To determine SERCA2a protein levels, myocytes (1 x 10⁶) were washed with phosphate buffered saline (PBS), then extracted with 0.2 ml lysis buffer (20 mM
Tris, pH 7.4, 20 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 0.5% Na-deoxycholate, 1 mM dithiothreitol). Lysates were incubated on ice for 30 min, and cellular debris was pelleted at 10,000 x g for 20 min at 4°C. Protein concentration in the supernatant was determined by BioRad protein reagent. 30 µg of protein diluted in extraction buffer and 4X Laemmli sample buffer were loaded without boiling onto pre-cast 4-20% Tris-glycine gels. Separated proteins were transferred to nylon membranes using a Bio-Rad Mini Trans-Blot apparatus and blocked overnight at 4°C. Blots were incubated with a rabbit polyclonal SERCA2a antibody(18) (1:5000) for 1 hour at RT, followed by a 1 hour incubation with a 1:5000 dilution of goat anti-rabbit IgG-HRP conjugated secondary antibody (Amersham). Bands were visualized by reacting with chemiluminescent substrate (NEN) and exposed to film. Protein bands were quantified from scanned images using Scion imaging software (Scion, Frederick, MD).

To examine cellular O-GlcNAcylation and MEF-2a levels, nuclear extracts were prepared from neonatal cardiomyocytes (2 x 10⁶) using modifications of the procedures described by Dignam et al (24). Samples of each extract (25-50µg) were fractionated by SDS-PAGE on precast 4-20% Tris-glycine gels (BioRad) and transferred to nylon membranes. Membranes were blocked overnight, exposed to the O-GlcNAc-specific antibody 110.6 (17) (1:10,000) overnight at 4°C, then exposed for 1 hour to 1:5,000 goat anti-mouse IgM-HRP. Alternatively, membranes were exposed to anti-MEF-2 (1:200) for one hour at RT followed by a one hour exposure to 1:10,000 goat anti-rabbit IgG-HRP. Visualization and quantification was as described above.

Sp1 Immunoprecipitation. Nuclear extracts were made from neonatal cardiomyocytes (5 x 10⁶) using the procedures of Dignam et al. (24). Protein (50 µg) was diluted to 0.5 ml with binding
buffer (10 mM Tris pH 7.9, 2 mM MgCl₂, 0.15 mM NaCl, 1 mM dithiothreitol, 10% glycerol, and 1 mM PMSF) and incubated with 4 µg of anti-Sp1 antibody (Santa Cruz Biotech) on a rotator for two hours at 4°C, followed by overnight incubation with 20 µl of protein A/G plus agarose (Santa Cruz Biotech.). Immunoprecipitates were washed four times in RIPA buffer, resuspended in 40 µl of 1X sample buffer and boiled for 3 minutes, separated on 7.5% Tris-glycine gels and transferred to nylon membranes. Blots were incubated for 1 hour with the same antibody used to IP Sp1 (1:1000 in TBSTM) followed by exposure to a goat anti-rabbit IgG-HRP secondary antibody (1:20,000 in TBSTM). Visualization and quantification were as described above.

**Northern Blot Analysis.** Cellular RNA was extracted from cultured myocytes using the guanidine thiocyanate method (25) as described previously (5). The RNA was fractionated on a 1% agarose gel and transferred to a nylon membrane. A 1.6 kb fragment of the 5’ end of a cDNA of the rat SERCA2a gene was used to generate a ³²P-labeled probe (Multiprime DNA labeling system, Amersham). A probe for 28S rRNA was used as a loading control. Bands were visualized on radiographic film and the resulting images scanned and quantified using Scion imaging software.

**Transfections and SERCA2a Promoter Luciferase Assays.** DNA transfection of neonatal cardiomyocytes was performed using the calcium phosphate-DNA co-precipitation method (26). Cells (0.5 x 10⁶) were transfected overnight in appropriate culture medium (either NG- or HG-containing medium) with 50 ng of either a 0.6 kb fragment of the rat SERCA2a promoter (corresponding to the 600 bases immediately upstream of the transcriptional start codon) (27) or
a control plasmid (pGL3; Promega) and 3 µg of fill plasmid (pBS; Stratagene), then washed with PBS and changed into fresh culture medium. Cells to be treated with adeno-OGT or adeno-GCA were first infected with an MOI of 25 followed 24 hours later by transfection. Transfected cells were incubated for 48 hours, then washed with PBS and incubated for 10 min with gentle agitation at RT in 200 µl of harvest buffer (50 mM MES/Tris pH 7.8, 0.1% Triton X-100, and 1.3 mM dithiothreitol). Luciferase activity was measured using 100 µl of extract in a luminometer.

Animals and Determination of Cardiac Sugar Nucleotides. Male CB6F1 mice weighing 20-25 g (Charles River, Wilmington, MA) were made diabetic using methods described previously (5). Briefly, diabetes was induced with a single intraperitoneal injection of streptozocin (150 mg/kg in citrate saline, pH 4.5) to animals fasted overnight (16 hrs). Diabetic animals were sacrificed three weeks following injection. To determine the degree of diabetes, blood was drawn via direct cardiac puncture in CO2-euthanized animals and plasma glucose concentration was determined using the Sigma Glucose Kit (510-A).

Sugar nucleotides were extracted from fresh ventricles and quantified by HPLC as described by Weckbecker and Keppler (28). Hearts were homogenized in 3 vols of ice-cold 0.3 M HClO4 and debris pelleted at 10,000 x g for 15 min at 4°C. Two vols of 1:4 trioctylamine:1,1,2-trichlorotrifluoroethane (Freon) were added to the supernatant, followed by vortexing for 30 sec and centrifuging at 1000 x g for 5 min at 4°C. The aqueous phase was carefully transferred to a fresh tube and frozen at -80°C until assayed. Samples were diluted in 0.5 ml H2O and desalted on a Mono-Q column (flow rate 1 ml/min). The combined UDP-GlcNAc/GalNAc fraction was collected as a peak at 23 minutes and the sample was lyophilized.
overnight. Dried samples were subsequently dissolved in borate solution (0.22 M boric acid, 0.09 M disodium tetraborate, and 1.2 M glycerol, pH 6.85), and separated into distinct UDP-GlcNAc and UDP-GalNAc peaks on a Partisil SAX-10 column. Calibration of the peaks was performed by processing known amounts of UDP-GlcNAc and UDP-GalNAc using the same methods as above and comparing total area under the curve for the experimental vs. control peaks for each fraction.

Data Collection and Statistical Analysis. Experimental treatments were evaluated on myocyte cultures from at least three different isolations to control for variation among cultures. Data are presented as mean ± SEM. Statistical significance ($P < 0.05$) was evaluated using one-way ANOVA (SigmaStat, Chicago, IL). Post-hoc multiple comparisons for calcium transients were made using a Student-Newman-Keuls test.

RESULTS

Calcium transients. To determine whether elevated extracellular glucose and increased O-GlcNAcylation would exert an effect on intracellular calcium flux, Indo-1 calcium transients were determined in neonatal rat cardiomyocytes. Because the exact Ca$^{2+}$-binding dynamics of Indo-1 are uncertain, and given that Indo-1 has the potential to be compartmentalized intracellularly, we present data as the ratio of Indo-1 fluorescence observed at 405 and 485 nm, rather than as a calculated intracellular calcium concentration. To simplify comparisons, we also normalized transients to the diastolic (basal) and systolic (maximal) ratios. Figures 1A and 1C illustrate typical traces from these experiments. Treatment of myocytes with 5.5 mM or 25 mM glucose resulted in similar kinetics for achieving peak Indo-1 ratios during systole, but the
diastolic decay phase was significantly prolonged in myocytes exposed to high glucose. The
time to achieve a 50% decrease in the calcium transient (T_{1/2}) in normal and high glucose-treated
myocytes was 914±31 and 1149±24 msec, respectively, and represents a 26% slower T_{1/2} in the
latter group (P < 0.01; Fig 1B). Exposing myocytes to treatments designed to increase
nucleocytoplasmic GlcNAcylation further prolonged the T_{1/2} and suggested a trend, albeit not
significantly beyond high glucose treatment, toward slower systolic kinetics. Treatment of
myocytes with 5.5 mM glucose supplemented with either 8 mM glucosamine or 50 µM
PUGNAc increased T_{1/2} to 1320±61 and 1310±85 msec, respectively (Fig. 1B), which
corresponds to a 43% increase in diastolic calcium decay for both treatments relative to controls
(P < 0.01). Infection of myocytes with adeno-SR (control virus), adeno-GCA or adeno-OGT had
no significant effect on normal glucose-treated myocytes (data not shown), but adeno-OGT
infected cells exposed to 25 mM glucose exhibited prolonged T_{1/2} (1213±86) that was similar to
treatment with glucosamine (Fig. 1C/D). Infection of high glucose-treated myocytes with adeno-
GCA resulted in a reduced T_{1/2} that was similar to controls (Fig. 1D).

**SERCA2a mRNA, protein expression and promoter activity.** Northern blot analysis of mRNA
extracted from myocytes demonstrated a substantial decrease in SERCA2a mRNA with exposure
to 25 mM glucose compared with normal glucose-treated cells (-37±6%; P < 0.05; Fig. 2A). The
decrease in SERCA2a mRNA with high glucose treatment paralleled a similar decrease in
SERCA2a protein (-28±4%; P < 0.01; Fig. 2B). To examine the role of specific glycosylation
pathways, myocytes were incubated in 40 µM aminoguanidine (an inhibitor of non-enzymatic
glycosylation), 0.5 µg/ml tunicamycin (an inhibitor of N-linked glycosylation)(29), or 8 mM
glucosamine. Cells exposed to both 5.5 mM glucose and 8 mM glucosamine exhibited a
significant decrease (-25±3%; \( P < 0.01 \)) in SERCA2a protein compared with controls (Fig. 2B). Interestingly, SERCA2a protein levels in myocytes exposed to both 25 mM glucose and glucosamine were unchanged compared with myocytes exposed only to 5.5 mM glucose. This effect was observed reproducibly but we cannot explain it satisfactorily. Neither aminoguanidine nor tunicamycin appeared to influence SERCA2a protein levels regardless of glucose concentration (Fig. 2B), nor did we observe any osmotic effect when cells were treated with 5.5 mM glucose and a cell-impermeable osmotic carrier (20 mM mannitol) (data not shown).

To examine specifically the effect of increased \( O \)-GlcNAcylation on SERCA2a protein expression, we infected myocytes with adenovirus encoding either OGT or GCA at a (MOI) of 25. Treatment of myocytes with high glucose and adeno-OGT resulted in a 47±9% decrease in SERCA2a protein expression compared with control cells infected with an empty adenovirus (SR-; Fig. 2C), Importantly, infection of high glucose-exposed myocytes with adeno-GCA resulted in remarkably improved SERCA2a protein levels (Fig 2C).

The reduction in SERCA2a mRNA and protein expression observed following treatment of myocytes with high glucose prompted us to examine whether these effects were being exerted, at least in part, by interactions with the SERCA2a gene promoter. Cells were transfected with a 0.6kb fragment of the rat SERCA2a promoter inserted into the luciferase vector, pGL3 (Promega). Luciferase assays indicated that myocytes exposed to either 25 mM glucose or 5.5 mM glucose/8 mM glucosamine exhibited a significant reduction in SERCA2a promoter activity (28% and 33%, respectively; \( P < 0.01 \)) compared with normal glucose controls (Fig. 3). Overexpression of OGT had no effect on SERCA2a promoter activity in control cells (data not shown), but we measured considerably reduced promoter activity in high glucose-treated cells
infected with adeno-OGT (31%; $P < 0.01$; Fig. 3). Exposing myocytes to adeno-GCA and high glucose resulted in improved SERCA2a promoter activity, albeit activity did not return to control levels. Infection of myocytes with control virus had no effect on SERCA2a promoter activity relative to uninfected controls (data not shown).

**Effects of nuclear O-GlcNAcylation.** The effects of 25 mM glucose and adeno-OGT treatment on myocytes suggested that increased O-GlcNAcylation was affecting transcriptional activity in the cell. Because numerous transcription factors interact with the SERCA2a promoter and other elements of myocardial Ca$^{2+}$ cycling), we examined whether the overall level of O-GlcNAcylation was altered in nuclear extracts from cardiomyocytes. The monoclonal antibody 110.6 recognizes the O-linkage of a single N-acetylglucosamine molecule to serine/threonine residues (17) and thus provides a useful tool for examining the GlcNAcylation status of nuclear proteins. Our results revealed that overall levels of nuclear O-GlcNAcylation were substantially increased in myocytes exposed to 25 mM glucose, 5.5 mM glucose supplemented with either 8 mM glucosamine or 50 µM PUGNAc, or 25 mM glucose and infected with adeno-OGT (Figure 4). Infection of high glucose-treated cells with adeno-GCA resulted in a significant reduction in overall cellular O-GlcNAcylation (Fig. 4). No such changes in O-GlcNAcylation were observed when myocytes were incubated with control virus (data not shown).

**Effects on Sp1 and MEF2 expression and glycosylation.** The inhibitory effects of high glucose treatment and elevated nucleocytoplasmic O-GlcNAcylation on SERCA2a expression and promoter activity prompted us to examine a possible molecular mechanism linking increased glycosylation directly to alterations in SERCA2a expression. The transcription factor Sp1
contains sites that are modified specifically by O-GlcNAcylation, and the SERCA2a promoter contains several Sp1 recognition sequence sites. To investigate whether hyperglycemia could influence Sp1, we exposed myocytes to 5.5 mM glucose, 25 mM glucose, or high glucose and adeno-OGT and isolated Sp1 by immunoprecipitation. Our results indicate that levels of Sp1 protein were unchanged in myocytes exposed to either 25 mM glucose, with or without adeno-OGT, compared with normal glucose-treated cells (Fig. 5A). However, these treatments substantially increased levels of Sp1 O-GlcNAcylation (Fig. 5B). Incubation with control virus had no effect on Sp1 expression or glycosylation regardless of the glucose concentration of the media (data not shown).

Although the MEF-2 transcription factor is not known to be subject to O-GlcNAcylation, it is nevertheless important for the expression of a variety of essential proteins in cardiomyocytes, including SERCA2a (30). To examine whether elevated glucose could affect MEF-2 levels, we exposed myocytes to 5.5 mM or 25 mM glucose, and supplemented with each of the following: 8 mM glucosamine, adeno-OGT, or adeno-GCA. Results from immunoblots of nuclear extracts indicated that MEF2a expression was decreased considerably by treatments that increased overall levels of nuclear glycosylation (Fig 6). Specifically, treatment with 25 mM glucose, 5.5 mM glucose supplemented with 8 mM glucosamine, or 25 mM glucose and infected with adeno-OGT resulted in 49%, 52%, and 41% decreases in MEF-2a expression levels, respectively, compared with normal glucose controls. Infection of high glucose-exposed myocytes with adeno-GCA restored MEF-2a levels to near-normal levels. Infection with empty adenovirus had no effect regardless of glucose concentration (data not shown). We observed no evidence of O-GlcNAcylation of MEF-2a protein.
**Effects of STZ-induced diabetes on UDP-nucleotide levels.** To provide for a possible mechanism linking diabetic hyperglycemia to substrate flux through the hexosamine pathway, we examined concentrations of sugar nucleotides (UDP-GlcNAc and UDP-GalNAc) in hearts from animals made diabetic by injection with streptozocin (STZ) (Figure 7). STZ-injected animals exhibited severe hyperglycemia relative to controls ([blood glucose] = 969±40 vs. 238±20 mg/dL, respectively). Diabetic animals also exhibited elevated levels of both UDP-GlcNAc and UDP-GalNAc compared with control animals (1.36±0.03 and 0.51±0.01 nmol in diabetic animals vs. 0.88±0.04 and 0.28±0.03 nmol in controls, respectively).

**DISCUSSION**

Diabetes mellitus leads to accelerated heart failure, which is manifested in part by early diastolic dysfunction (31). The reduction in myocardial performance in diabetic cardiomyopathy is associated with disruption of normal cardiomyocytes calcium flux (32), and has been linked to reduced sequestration of calcium into the SR as a result of reduced SERCA2a activity and expression (5,11). Because diabetes is a multifactorial disease, the underlying mechanism for these alterations in cardiomyocyte calcium homeostasis has been unclear. In this study we demonstrate that exposure of cells to high extracellular glucose concentrations ([Glc]o) can lead to impaired diastolic calcium sequestering, and this appears to occur through a high [Glc]o-induced reduction in SERCA2a expression. Furthermore, we provide for a possible mechanism for these perturbations in contractile function and calcium homeostasis with the observations that increased O-GlcNAcylation is sufficient to alter SERCA2a expression, at least in part through effects on nuclear transcription factors.
Treatment of adult rat ventricular cardiomyocytes with elevated [Glc]₀ has been shown recently to result in prolonged action potential duration, impaired diastolic calcium uptake, and poor contractile performance (12). These effects are similar to impaired cardiomyocyte function observed in myocytes isolated from diabetic animals (10). We support and extend these observations by demonstrating that neonatal rat cardiomyocytes exposed to elevated [Glc]₀ exhibit prolonged calcium transients, and that the observed diastolic impairment in calcium flux can be linked to reduced expression of the SERCA2a. SR calcium release and subsequent re-uptake serve as the primary determinants of myocardial systolic contraction and diastolic relaxation, respectively. The rate of calcium sequestration into the SR is controlled by SERCA2a, which also affects total SR calcium storage (33). A reduction in the expression of SERCA2a would likely result in a decreased rate of calcium reuptake and lead to prolonged calcium transients. The results of our experiments agree with observations we reported recently demonstrating that hearts from STZ-induced diabetic mice express reduced levels of SERCA2a protein, and that the contractile deficits observed in these animals could be rescued in transgenic mice expressing SERCA2a and made diabetic (5).

Diabetic hyperglycemia can exert detrimental effects on the myocardium via several mechanisms, but increased attention is being focused on the hypothesis that increased glucose flux through the hexosamine biosynthetic pathway plays an important role in the pathogenesis of diabetes (14,34-37). Under normal conditions approximately 2-3% of glucose entering the cardiomyocyte is shunted to the hexosamine pathway (14). Upon initial examination, it may seem counterintuitive that excess glucose may be available for hexosamine biosynthesis despite the relative glucose insensitivity of diabetic tissues. However, despite either reduced insulin levels or insulin resistance, elevated [Glc]₀ may still enter the myocyte via the glucose
transporter Glut1, which is less insulin sensitive than Glut4 (38). Furthermore, low or absent insulin levels in the diabetic milieu will impair glucose flux through glycolysis and glycogen synthesis, potentially shunting glucose to alternative metabolic pathways such as the hexosamine pathway, or for the non-enzymatic glycation of cellular proteins. Although measurements of glucose flux rates into hexosamine biosynthesis has not been measured in cardiomyocytes from diabetic animals, our results clearly demonstrate that hearts from diabetic mice exhibit elevated concentrations of UDP-GlcNAc, an important common substrate for both N- and O-linked glycosylation. Indeed, dysfunctional calcium cycling in diabetic hearts may be predicated, in part, on an increase in UDP-GlcNAc levels, as suggested by our observation that adeno-OGT infection had no effect on myocytes incubated in normal glucose (i.e., OGT activity is limited by substrate availability). The observed decrease in SERCA2a expression we observed in neonatal cardiomyocytes exposed to elevated [Glc]o does not appear to occur as a result of increased non-enzymatic glycation or N-linked glycosylation. This latter result is in direct contrast to the apparent amelioration of contractile performance and calcium homeostasis observed in adult rat myocytes exposed to elevated [Glc]o and treated with tunicamycin (12). We are uncertain why this difference exists. Instead, our results suggest that the abnormalities in cardiomyocyte calcium flux result from enhanced O-GlcNAcylation of cellular proteins, and in particular an increase in the O-linkage of N-acetylglucosamine molecules on target proteins.

Considerable evidence indicates that hyperglycemia and elevated [Glu]o can affect gene expression in cardiac and non-cardiac tissue (39-41). Furthermore, it is known that dynamic O-GlcNAcylation modifies the activity and function of RNA polymerases, cytoskeletal proteins, and transcription factors (42,43). Recent reports demonstrate that the transcription factor Sp1 is subject to dynamic O-GlcNAcylation, and that this modification results in decreased
transcriptional activity of the protein (44,45). Given that the SERCA2a promoter contains multiple Sp1 binding sites which are important for adequate gene expression (46), we were intrigued by the possible nexus of these cellular mechanisms. Our results provide clear evidence that the effects of hyperglycemia on cardiac function, and SERCA2a expression in particular, may be transmitted through effects on the transcription factor Sp1. The significant increase in Sp1-specific O-GlcNAcylation provides for a molecular mechanism that, for the first time, links hyperglycemia, increased hexosamine flux, and the transcriptional regulation of SERCA2a expression, with cardiomyocyte dysfunction in the diabetic context. This linkage between hyperglycemia, hexosamine flux, and transcriptional control is further strengthened by our observations that treatments designed to increase cellular O-GlcNAcylation also exert powerful effects on the expression of MEF-2a. Although MEF-2 transcription factors are known to be important for both cardiomyocyte maturation and function (47), the exact molecular mechanism linking changes in MEF-2a expression to either cardiomyocyte calcium handling or contraction is not clear. Previous studies in this lab have demonstrated that overexpressing MEF-2 alone is insufficient to exert any effect on SERCA2a expression using reporter constructs (30). Nevertheless, we are intrigued by the interaction between hyperglycemia and MEF-2a expression, and we intend to study this further.

Finally, this study demonstrates for the first time that the removal of O-GlcNAc residues is sufficient, in most cases, to normalize cardiomyocyte function despite exposure to hyperglycemia. Virally-transmitted O-GlcNAcase was sufficient to improve diastolic calcium handling and to elevate SERCA2a levels and promoter activity to near normal levels in cells exposed to conditions that otherwise result in increased cellular O-GlcNAcylation and a subsequent reduction in function. Not surprisingly, a recent report by Parker et al. (16)
demonstrated similar results in improving insulin sensitivity in adipocytes. The utilization of a virally-encoded O-GlcNAcase gene to counteract the detrimental effects of elevated extracellular glucose may provide for a potential gene therapy approach to address cardiac dysfunction in diabetic patients.

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Footnote:

4 Abbreviations used: SR, Sarcoendoplasmic reticulum; SERCA2a, Sarcoendoplasmic reticulum Ca\[^{2+}\] ATPase; Sp1, specificity protein 1; MEF2, myocytes enhancer factor; PUGNAc, O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenyl carbamate; NG, normal (5.5 mM) glucose; HG, high (25 mM) glucose; OGT, UDP-N-acetylglucosamine:peptide N-acetylglycosaminyl transferase (O-GlcNAc transferase); GCA, O-GlcNAcase; NCM, neonatal rat cardiomyocytes; GlcN, glucosamine
FIGURE LEGENDS

Figure 1. Calcium transients in neonatal cardiomyocytes. (A and C) Intracellular Ca\textsuperscript{2+} transients in neonatal cardiomyocytes measured using Indo-1 fluorescence as described in “Experimental Procedures”. Cells (0.5 x 10\textsuperscript{6}/plate) were incubated for 72 hours in either (A) 5.5 mM glucose (NG, solid line) or 25 mM glucose (HG, dotted line), normal glucose supplemented with 8 mM glucosamine (GlcN, dashed line), or normal glucose treated with 50 µM PUGNAc (dashed and dotted line), or (C) normal glucose (solid line), high glucose (dotted line), and high glucose infected with either adenovirus encoding \textit{O}-GlcNAc transferase (OGT, dashed and dotted line) or \textit{O}-GlcNAcase (GCA, dashed line). Cells were loaded with Indo-1/AM and Indo-1 ratios were normalized relative to the baseline and the maximum of each trace set to 100%. Data represents the mean of 18 myocytes for each treatment, and experiments were performed using cultured cells from three separate isolations. (B and D) Graphical representation of the time to 50% decay of the calcium transient (T\textsubscript{1/2}) based on the data presented in 1A (B) or 1C (D). An * indicates a statistically significant (P<0.05) difference compared with myocytes cultured in 5.5 mM glucose.

Figure 2. Northern and western blots of neonatal cardiomyocytes. (A) Results of a northern blot for SERCA2a mRNA in neonatal cardiomyocytes cultured for 72 hours with 5.5 mM (NG) or 25 mM (HG) glucose. Total RNA (15µg) isolated from cultured myocytes was resolved using a denaturing agarose gel, transferred to a nylon membrane and probed with a full length \textsuperscript{32}P-labeled probe corresponding to rat SERCA2a as described in “Experimental Procedures”. (B) Western immunoblot analysis of SERCA2a protein levels from neonatal cardiomyocytes exposed to 5.5 mM or 25 mM glucose in the presence and absence of 40 µM aminoguanidine.
(AG), 0.5 µg/ml tunicamycin (Tun), or 8 mM glucosamine (GlcN, 5.5 mM glucose group only). Crude lysates (30µg) were resolved using 4-20% gradient SDS-PAGE gels, transferred to nylon membranes, and immunoblotted with a SERCA2a polyclonal antibody as described in “Experimental Procedures”. The apparent increase in SERCA2a in cells treated with 25 mM glucose and aminoguanidine is not statistically significant from controls with repeated trials. (C) SERCA2a protein levels based on Western immunoblots from adenovirus-infected neonatal cardiomyocytes. Cells were cultured in either NG or HG glucose and infected at an MOI of 25 with adeno-OGT, adeno-GCA, or empty virus (SR-).

Figure 3. Luciferase assay for SERCA2a promoter activity. Neonatal cardiomyocytes were cultured in either 5.5 mM (NG) or 25 mM (HG) glucose media, and chemically-transfected with a 0.6 kb fragment of the rat SERCA2a promoter in pGL3 as described in “Experimental Procedures”. To determine the effects of altering cellular glycosylation specifically, cells were further treated with 8 mM glucosamine (NG/GlcN) or adenoviruses encoding either O-GlcNAc transferase (HG/OGT) or O-GlcNAcase (HG/GCA) for 48 hours prior to chemical transfection with the SERCA2a promoter construct glycosylation. Crude cell lysates were incubated with luciferase substrate and relative light units were measured on a luminometer. An (*) indicates a significant difference ($P<0.05$) existed between an experimental group and the control (NG).

Figure 4. Western immunoblot illustrating effects of various treatments on nuclear O-glycosylation in cultured neonatal cardiomyocytes. Cells were cultured for 72 hours in 5.5 mM glucose (NG) or 25 mM glucose (HG) and supplemented with one of the following: 8 mM glucosamine (NG/GlcN), 50 µM PUGNAc, or adenovirus encoding either OGT or GCA.
Adenoviruses were applied at an MOI = 25. Nuclear extracts (50 µg) were prepared according to the methods outlines in “Experimental Procedures”, resolved on 4-20% gradient SDS-tricine gels, transferred to nylon membranes and immunoblotted with a monoclonal anti-\(O\)-GlcNAc antibody 110.6 (kindly provided by G. Hart).

Figure 5. Sp1 immunoprecipitation from cultured neonatal cardiomyocytes. Cells were cultured for 72 hours in experimental medium (NG, HG, or HG with either adeno-OGT or adeno-GCA). Sp1 was immunoprecipitated from nuclear extracts (50µg) as described in “Experimental Procedures”, resolved electrophoretically on 7.5% SDS-PAGE gels, transferred to nylon membranes and immunoblotted with polyclonal antibodies to either Sp1 (A) or \(O\)-glycosylated residues (B)(anti-\(O\)-GlcNAc antibody 110.6).

Figure 6. MEF-2 protein levels from cultured rat neonatal cardiomyocytes. Cells were incubated in either 5.5 mM glucose (NG) or 25 mM glucose (HG) with or without the following: 8 mM glucosamine, adeno-OGT or adeno-GCA (MOI = 25). Nuclear extracts (50µg) were prepared as described in “Experimental Procedures”, resolved on 10% SDS-PAGE gels, transferred to nylon membranes and immunoblotted with anti-MEF2 antibody (Santa Cruz Biotech).

Figure 7. Blood glucose and UDP-nucleotide concentrations in control and diabetic mice. Mice (20-25g) were injected intraperitoneally with streptozocin (150 mg/kg) and subsequently developed diabetes over a three week period. Blood was drawn via direct cardiac puncture and plasma [glucose] was subsequently determined using a Sigma Glucose kit. Sugar nucleotides
were extracted immediately from hearts and quantitated using HPLC as described in “Experimental Procedures”. UDP-GlcNAc and UDP-GalNAc were collected in a single fraction via HPLC using a Mono-Q column, then separated into individual fractions using a Partisil SAX-10 column. Fractions were quantified by comparing total peak area with results from known concentrations of each sugar nucleotide.
Figure 1

A

Time to 50% decay (sec)

B

Time (sec)

% Maximum Indo-1 ratio

C

Time (sec)

% Maximum Indo-1 ratio

D

Time (sec)

% Maximum Indo-1 ratio
Figure 3
| NG  | HG  | NG/P | NG/GlcN | HG/OGT | HG/SR- | HG/GCA |
|-----|-----|------|---------|--------|--------|--------|
|     |     |      |         |        |        |        |
Figure 5

|       | NG | HG | HG/SR- | HG/OGT | HG/GCA |
|-------|----|----|--------|--------|--------|
| **A** |    |    |        |        |        |
| **B** |    |    |        |        |        |
Figure 6

NG  NGG  HG  HG/OGT  HG/GCA
Figure 7

A

Blood glucose (mg/dL)

0
200
400
600
800
1000
1200

control

STZ injected

B

UDP-nucleotides (nmol/g tissue wet weight)

0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

UDP-GlcNAc

UDP-GalNAc

control

STZ injected
Diabetes and the accompanying hyperglycemia impairs cardiomyocyte calcium cycling through increased nuclear O-GlcNAcylation

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