Abstract—To examine whether excessive protein O-GlcNAcylation plays a role in the dysfunction of the diabetic heart, we delivered adenovirus expressing O-GlcNAcase (Adv-GCA) into the myocardium of STZ-induced diabetic mice. Our results indicated that excessive cellular O-GlcNAcylation exists in the diabetic heart, and that in vivo GCA overexpression reduces overall cellular O-GlcNAcylation. Myocytes isolated from diabetic hearts receiving Adv-GCA exhibited improved calcium transients with a significantly shortened $T_{\text{decay}}$ ($P<0.01$) and increased sarcoplasmic reticulum Ca$^{2+}$ load ($P<0.01$). These myocytes also demonstrated improved contractility including a significant increase in $+dL/dt$ and $-dL/dt$ and greater fractional shortening as measured by edge detection ($P<0.01$). In isolated perfused hearts, developed pressure and $-dP/dt$ were significantly improved in diabetic hearts receiving Adv-GCA ($P<0.05$). These hearts also exhibited a 40% increase in SERCA2a expression. Phospholamban protein expression was reduced 50%, but the phosphorylated form was increased 2-fold in the diabetic hearts receiving Adv-GCA. We conclude that excess O-GlcNAcylation in the diabetic heart contributes to cardiac dysfunction, and reducing this excess cellular O-GlcNAcylation has beneficial effects on calcium handling and diabetic cardiac function. (Circ Res. 2005;96:1006-1013.)

Key Words: O-GlcNAcase ■ adenovirus ■ gene therapy ■ diabetic cardiomyopathy ■ Ca$^{2+}$ handling

Diabetic cardiomyopathy is characterized by impaired cardiac function independent of cardiovascular disease. The functional changes of diabetic cardiomyopathy are indicated by an early diastolic dysfunction, followed by systolic dysfunction. Impaired sarcoplasmic reticulum calcium cycling, including decreased intracellular Ca$^{2+}$ concentration, reduced SR Ca$^{2+}$ content, decreased diastolic Ca$^{2+}$ uptake and systolic Ca$^{2+}$ release, and increased Ca$^{2+}$ leak, have been well documented; however, the underlying mechanism has not been fully elucidated.

O-GlcNAcylation (O-linked β-N-acetylglucosamine enzymatic glycosylation), which is defined as the covalent attachment of a single β-N-acetylglucosamine (O-GlcNAc) molecule to serine or threonine residues, is a posttranslational modification of intracellular proteins. This modification affects transcription, translation, nuclear transport, and cell signaling. Similar to phosphorylation, O-GlcNAc addition by O-GlcNAc transferase (OGT), or its removal by N-acetylglucosaminidase (O-GlcNAcase, GCA) is a highly dynamic and reversible process that is susceptible to perturbation under certain pathophysiological circumstances. Hyperglycemia, commonly seen in diabetes mellitus, increases glucose flux through the hexosamine pathway, resulting in increased production of UDP-GlcNAc (uridine diphosphatide GlcNAc, donor of O-GlcNAc), which serves as a substrate for O-GlcNAc modification. Excess O-linked glycosylation has been detected in the pancreas and corneas of diabetic rats as well as coronary endothelial cells and atherosclerotic plaques in diabetic patients. Furthermore, alteration of cellular O-GlcNAcylation levels in rat skeletal muscle or 3T3-L1 adipocytes has been linked to the development of insulin resistance in diabetes.

The potential role of O-GlcNAcylation in diabetic hearts has been implicated by several studies. Exposing cardiac myocytes to high glucose or elevated glucosamine (a precursor of O-GlcNAc) results in a reduction in contractility and calcium flux in cardiac myocytes. Exposing isolated perfused hearts to glucosamine blunts the positive inotropy by phenylephrine. Moreover, increasing cellular O-GlcNAcylation by infecting neonatal cardiac myocytes with adenovirus expressing OTG or incubating with GCA inhibitor (PUGNAc) significantly impairs the calcium transient, whereas reducing cellular O-GlcNAcylation by GCA reverses the calcium transient dysfunction induced by incubation with high glucose. We hypothesize that excess O-GlcNAcylation in the diabetic heart plays a significant role in cardiac dysfunction. Using in vivo adenovirus-mediated gene delivery to the heart, we overexpressed GCA in STZ-induced diabetic mice and evaluated the effects of reducing cellular O-GlcNAcylation on diabetic cardiac dysfunction.
Materials and Methods

Preparation of Diabetic Mice

NIH Swiss mice (25 g) were made diabetic by a single IP injection with freshly prepared streptozotocin (STZ) solution (200 mg/kg body weight in citrate saline, pH 4.2) after overnight fasting. The diabetic status was assessed by measuring urine glucose (>22 mmol/L = diabetic) 3 days after STZ injection and was confirmed by blood glucose measurement at the time of euthanasia.

To rule out any potentially toxic effects of STZ on the heart, several STZ-injected mice were intensively treated with Ultraalente human insulin (Eli Lilly and Company) with 50 U/g per day subcutaneously. The insulin treatment commenced on the third day after STZ injection and lasted for 2 weeks. The cellular O-GlcNAcylation in these hearts was compared with that of the noninsulin-treated diabetic hearts. In addition, several 20-week-old male polygenic diabetic NONcNZO10/LJ mice (The Jackson Laboratory, Bar Harbor, Me)23,24 were used as a type II diabetic model to investigate the excess O-GlcNAcylation level in the diabetic heart independent of STZ administration. These mice developed hyperglycemia at 12 to 16 weeks. At the time of euthanasia, blood glucose levels were above 33.3 mmol/L. Animal procedures were performed in accordance with the guidelines established by the Committee on Animal Research at the University of California, San Diego.

In Vivo Adenoviral Gene Delivery

In vivo adenovirus gene delivery in diabetic mice was performed as previously described.7 Either adenovirus encoding human GCA (Adv-GCA) or adenovirus without encoding gene sequence (Adv-SR) was directly injected into the left ventricular wall (5 sites, 10 fL of 109 pfu/mL each). In the experiments involving individual myocyte studies, a green fluorescent protein expressing adenovirus (Adv-GFP) was coinjected to help identify either Adv-GCA– or Adv-SR–infected myocytes after myocytes isolation.7 Another group of normal age-matched NIH Swiss mice receiving Adv-SR was used as a control group in some series of experiments.

Measurement of Ca2+ Transients and Sarcoplasmic Reticulum Calcium Load

Single ventricular myocytes were enzymatically isolated and Ca2+ transients were measured as previously described.7,25 Only those myocytes infected with Adv-GCA or Adv-SR (as indicated from GFP fluorescence) were studied. Ca2+ transients were recorded from at least 20 cells per heart and for at least 3 hearts per treatment. Diastolic and systolic intracellular Ca2+ levels were inferred from the basal and maximal indo-1 ratio per cycle, respectively. Diastolic decay time (T decay) was calculated from the normalized Ca2+ transient curve.

Sarcoplasmic reticulum calcium load was measured as described by Shannon et al.26 In brief, cells were superfused with normal Tyrode solution (1 mmol/L Ca2+) and paced at 0.3 Hz to steady state. The solution was then rapidly switched to a Na+/free Tyrode solution with a rapid solution exchanger device. After 20 seconds, cells were rapidly exposed to 10 mmol/L caffeine (in Na+-free Tyrode solution). The difference between the basal and peak Ca2+ transient induced by caffeine was used as an index of sarcoplasmic reticulum Ca2+ load.

Measurement of Myocyte Contractility by Edge Detection

The contractile properties of single myocytes were measured using edge detection as described.27 Myocyte fractional shortening, maximal shortening rate (+dL/dt), and relengthening rate (−dL/dt) were analyzed with Felix32 software (Photon Technology International Inc.). Again, only infected myocytes as indicated by GFP signal were studied. Data were collected from at least 10 cells per heart and 3 hearts per treatment.

Measurement of Ventricular Function by Isolated Perfused Hearts

Diabetic mice were randomly divided into 2 groups, with each receiving either Adv-GCA or Adv-SR via in vivo adenovirus gene delivery. Five days after the procedure, hearts were isolated and Langendorff-perfused for functional analysis as previously described.27,28 The hearts were paced at 400 bpm, and the resulting pressure waves were analyzed for pressure derivatives [rate of contraction (+dP/dt), rate of relaxation (−dP/dt)] and developed pressure. Another set of normal mice served as a nondiabetic control group and underwent the same procedure. At the end of the experiment, hearts were frozen in liquid N2 for Western blot analysis.

RNase Protection Assay

RNase protection assays were performed as previously described.29 The human GCA (hGCA) probe spans residues 1035 to 1143 in the published human cDNA sequence (NM_012215), and the mouse GCA (mGCA) probe spans residues 881 to 1020 in the published mouse sequence (AF132214). The mouse OGT probe spans residues 57 to 215 in the published sequence (AF363030), which yields a 159-bp signal for full-length OGT nucleoeytoplasmic isofrom (nOGT) and a 150-bp signal for the OGT mitochondrial isofrom (mtOGT).

Western Immunoblotting

Cytosolic and nuclear fractions were prepared by differential centrifugation. Cardiac tissues were homogenized with a Polytron homogenizer in a buffer containing 30 mmol/L Tris, 300 mmol/L sucrose, 50 mmol/L GlcNAc, and protease inhibitor cocktail (1:1,000, Sigma). First centrifugation was performed at 1500g for 15 minutes at 4°C to spin down the crude nuclear fraction. The supernatant was spun at 8000g for 15 minutes at 4°C and subsequently for 1 hour at 160,000g at 4°C. The final supernatant represented the cytosolic fraction. Whole heart tissue homogenate was prepared with 0.2 mL of lysis buffer (in mmol/L: 20 Tris, pH 7.4, 20 NaCl, 0.1 EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1 dithiothreitol, 1 β-glycerophosphate, 10 Na-pyrophosphate, 50 NaF, 1 Na-ortho-vanadate, 50 GlcNAC, proteinase inhibitor cocktail).

Twenty micrograms of protein was mixed with Laemml sample buffer at room temperature for 10 minutes (samples for anti-O-GlcNAc antibody detection was heated at 70°C for 10 minutes) and then loaded onto 4% to 10% gradient Tris/glycine gels. Separated proteins were transferred to nitrocellular membranes and blocked overnight with 3% BSA at 4°C. Blots were incubated with a primary antibody (1:5000 CTD 110.6 antibody, a gift by Dr Gerald Hart, The Johns Hopkins University, Baltimore, Md; 1:1000 polyclonal SERCA2a antibody, Affinity Bioreagents, Inc; phospholamban an- tibody, phosphorylated phospholamban antibody, Upstate; α-actin antibody, Sigma; 1:1000 anti-OGT antibody, a gift by Dr John A. Hanover, National Institutes of Health, Boston, Mass) for 1 hour at room temperature, followed by a 1-hour incubation with a 1:5000 dilution of secondary antibody (anti-rabbit IgG-HRP conjugated, anti-rabbit IgG-HRP conjugated, anti-mouse IgM-HRP conjugated, anti-rabbit IgG-HRP conjugated; Sigma). Bands were visualized by reacting with chemiluminescent substrate (PerkinElmer Life Sciences) and exposed to film. Films were scanned and analyzed by Image-J software (NIH).

OGT Activity and GCA Activity Assay

The OGT activity assay was measured with the protein precipitated by 30% saturated ammonium sulfate from heart tissue extract as previously described,30,31 The peptide PGGTTPVSSANMM was used as a substrate. The OGT activity was expressed as dpm/μg protein.

Cytosolic fractions prepared as described above were used for GCA activity measurement,32 except that 1 mmol/L PMSF was added to the homogenization buffer but not GlcNAC and proteinase inhibitor cocktail; 50 mmol/L GalNAC was added to inhibit lysoso- mal hexosaminidases. The activity was expressed as units/mg.
Results

General Features of the Experimental Animals
All the diabetic mice used in this study had a blood glucose level >22 mmol/L at their time of euthanasia. As described previously, the diabetic mice studied here also had lower body weights (22.4 ± 1.1 versus 29.1 ± 0.5 g; P < 0.01) and higher blood glucose levels after STZ injection (40.6 ± 2.6 versus 9.5 ± 0.5 mmol/L; P < 0.05) than normal NIH Swiss mice. Diabetic mice were randomly divided into 2 groups: Dia+SR− group (mice receiving Adv-SR−) and Dia+GCA (mice receiving Adv-GCA). Before gene therapy, both body weights and blood glucose levels were comparable between these 2 groups (body weight: Dia+SR−, 22.2 ± 1.3 g versus Dia+GCA, 22.6 ± 1.0 g; blood glucose: Dia+SR−, 38.2 ± 2.1 mmol/L versus Dia+GCA, 43.0 ± 3.1 mmol/L; P > 0.05), which indicated that mice in these 2 groups had a similar severity of diabetes. After gene therapy, no significant changes were observed in body weight or blood glucose level in both groups (P > 0.05).

Excessive O-GlcNAcylation of cellular proteins in STZ-induced diabetic hearts was directly confirmed by Western blot analysis with anti-O-GlcNAc antibody. As shown in Figure 1, more O-GlcNAcylated proteins were detected in nuclear fractions (indicated by arrows) isolated from diabetic hearts than from normal hearts. Similarly, more O-GlcNAcylated proteins were also detected in cytosolic fractions (indicated by arrows) isolated from diabetic hearts than from normal hearts. Excess O-GlcNAcylation of cellular protein in diabetic hearts was prevented by insulin treatment. Additionally, as shown in Figure 2, similar excess cellular O-GlcNAcylation was also observed in the cytosolic and nuclear fraction from the polygenic type II diabetic hearts.

In order to further understand excess O-GlcNAcylation in the diabetic heart, the expression levels and enzymatic activities of OGT and GCA were also determined. As shown in Figure 3, the mRNA expression level of both ncOGT and mitOGT isoform was upregulated ~20% to 30% in the diabetic heart. The protein expression levels of OGT were increased ~30% in the diabetic heart. However, the OGT activity was not significantly changed in the diabetic heart relative to that in the normal heart (110.52 ± 3.42 versus 96.51 ± 9.29 dpm/μg; P > 0.05). Additionally, the mRNA expression level of GCA was also increased approximately 30% in the diabetic heart. Similarly to the OGT enzymatic activity, the GCA activity was not increased in the diabetic heart (0.36 ± 0.03 versus 0.39 ± 0.04 U/mg; P > 0.05).

Confirming the Overexpression of GCA in Diabetic Hearts After Receiving In Vivo Adenoviral Gene Delivery
The overexpression of GCA mediated by adenovirus was confirmed by RNase protection assay with a human GCA (hGCA)–specific probe. As shown in Figure 4A, hGCA mRNA was well expressed in diabetic hearts 5 days after the Adv-GCA injection. The mRNA expression level of endogenous mouse GCA, detected by a second probe specific to mouse GCA sequence, was not affected by exogenous GCA overexpression. As shown in Figure 4B, the overall GCA activity was increased ~50% in diabetic hearts receiving Adv-GCA gene therapy.

The reduction of cellular O-GlcNAcylation in diabetic hearts after GCA overexpression was further evaluated by
Western blot using an O-GlcNAc antibody. Compared with diabetic hearts receiving Adv-SR−, O-GlcNAcylated proteins were less abundant in diabetic hearts receiving Adv-GCA treatment. As shown in Figure 5, a reduction in overall cellular O-GlcNAcylation was observed both in nuclear (indicated by arrows) and cytosolic fraction (indicated by arrows) from Dia−/H11001 GCA group in comparison with those from Dia−/H11001 SR− group. The overall reduction appeared more prominent in the nuclear fraction.

Effects of Overexpression of GCA on Ca2+ Transients in Diabetic Cardiac Myocytes
The effects of overexpression of GCA on Ca2+ transients were studied by directly coinjecting Adv-GCA and Adv-GFP into the hearts of diabetic mice 2 weeks after STZ induction. Cardiac myocytes were isolated after 5 days, and only GFP-positive cells were analyzed.

As shown in Figure 6A and 6B, cardiac myocytes from Dia−/H11001 SR− group (n=80) exhibited significantly lower systolic and diastolic calcium concentration, as indicated by lower basal and peak indo-1 ratios (Rdia and Rsys) compared with cells isolated from Nor−/H11001 SR− group (n=98) (Rdia: Dia−/H11001 SR−, 0.67±0.01 versus Nor−/H11001 SR−, 0.71±0.01; Rsys: Dia−/H11001 SR−, 0.78±0.01 versus Nor−/H11001 SR−, 0.86±0.01; P<0.01). In addition,
the diastolic $T_{\text{decay}}$ was prolonged ≈40% in myocytes from Dia+S−R− in comparison with that in normal myocytes from Nor+S−R− group (0.25±0.02 versus 0.15±0.01 seconds; $P<0.01$) (Figure 5C and 5D). These results indicate that calcium handling in diabetic cardiomyocytes was significantly impaired in STZ-induced diabetic hearts.

The beneficial effects of overexpressing GCA on Ca$^{2+}$ transients in diabetic cardiac myocytes are also shown in Figure 6. Compared with myocytes from the Dia+S−R− group, myocytes from the Dia+GCA group (n=104) had higher diastolic and systolic indo-1 ratio ($R_{\text{dia}}$, 0.71±0.01 versus 0.67±0.01; $R_{\text{syr}}$, 0.84±0.01 versus 0.78±0.01; $P<0.01$). The diastolic Ca$^{2+}$ $T_{\text{decay}}$ in diabetic myocytes from Dia+GCA group was significantly shorter than that in those from Dia+S−R− group (0.18±0.01 versus 0.25±0.02 seconds; $P<0.01$). All 3 parameters were altered toward a similar level as observed in myocytes from Nor+S−R− group ($P>0.05$).

To further evaluate the effects of Adv-GCA on calcium handling, sarcoplasmic reticulum Ca$^{2+}$ load was measured as caffeine-induced Ca$^{2+}$ release in these myocytes. Sarcoplasmic reticulum Ca$^{2+}$ load was ≈33% lower than normal in control diabetic myocytes from Dia+S−R− group (0.14±0.01 versus 0.21±0.01; $P<0.01$). However, sarcoplasmic reticulum Ca$^{2+}$ load was significantly increased 28% in cardiac myocytes from Dia+GCA group (0.18±0.02 versus 0.14±0.01; $P<0.05$).

We also examined the effects of Adv-GCA on Ca$^{2+}$ transient in normal myocytes. Our measurements indicated that $R_{\text{dia}}$ was higher ($P<0.05$) in myocytes isolated from normal hearts receiving Adv-GCA than those receiving Adv-S−R−. $R_{\text{syr}}$ and $T_{\text{decay}}$ were not changed in normal myocytes receiving Adv-GCA (data not shown).

### Effects of Overexpression of GCA on Contractile Function in Diabetic Cardiac Myocytes

To evaluate the effects of overexpression of GCA on contractile function, isolated cardiac myocytes were analyzed with edge-detection technique. As shown in the Table, cardiac myocytes from Dia+S−R− group manifested prominent contractile dysfunction, represented by a 48% reduction in fractional shortening ($P<0.01$) and a 53% reduction in +dL/dt ($P<0.01$) as well as 59% reduction in −dL/dt ($P<0.01$), compared with myocytes from Nor+S−R− group.

As shown in the Table, cardiac myocytes from Dia+GCA group manifested significantly improved contractile function. Compared with those from Dia+S−R− group, myocytes from Dia+GCA group had a 74% increase in fractional shortening ($P<0.01$), a 79% increase in +dL/dt ($P<0.01$), and an 85% increase in −dL/dt ($P<0.01$). Both fractional shortening and +dL/dt in myocytes from Dia+GCA group were recovered toward normal ($P>0.05$). However, there was still a 25% decrease in −dL/dt of these myocytes, compared with those from Nor+S−R− group ($P<0.05$).

### Effects of Overexpression of GCA on Contractile Function in the Intact Diabetic Heart

We reported previously an ≈30% decrease in contractile function in isolated perfused hearts with STZ-induced diabetes.4 Because the enzymatic isolation of individual myocytes can be a selective process, involving the survival of only the healthiest myocytes, we sought to confirm our observations by examining contractile function in the whole heart. In this study, STZ-induced diabetic hearts receiving Adv-S−R− (n=6) exhibited a 27% and a 21% reduction in −dP/dt and developed pressure (DP), respectively, in comparison with normal control hearts (n=9) (−dP/dt: 2296±120 versus 3138±259 mm Hg/S; DP: 90±5 versus 114±8 mm Hg; $P<0.05$). However, the diabetic hearts receiving Adv-GCA (n=7) had a significant 15% increase in −dP/dt and DP relative to the diabetic hearts receiving Adv-S−R− (−dP/dt: 2629±63 versus 2296±120 mm Hg/S; DP: 104±2 versus 90±5 mm Hg; $P<0.05$). There was no significant difference in +dP/dt either between normal and the diabetic hearts or between diabetic hearts receiving Adv-S−R− and diabetic hearts receiving Adv-GCA ($P>0.05$). These data demonstrated that overexpression of GCA in the diabetic heart had beneficial effects on its global contractility.

### Effects of Overexpression of GCA on Sarcoplasmic Reticulum Ca$^{2+}$ Regulating Protein

To understand the mechanism of the beneficial effects of GCA overexpression on the diabetic heart, protein expression levels of SERCA2a, phospholamban (PLB), and phosphorylated PLB (p-PLB) were examined. As shown in Figure 7, the expression levels of SERCA, PLB, and p-PLB were all significantly reduced in diabetic hearts. Compared with the expression in Dia+S−R− group, SERCA2a expression was increased ≈40%, whereas PLB protein expression was reduced ≈50% in hearts from Dia+GCA group. Furthermore, the percentage of p-PLB from PLB was increased ≈2-fold in diabetic hearts from Dia+GCA group in comparison with Dia+S−R− group.

### Discussion

Our investigation demonstrates for the first time that excess protein O-GlcNAcylation occurs in vivo in the diabetic heart,
overexpression of GCA has been used successfully in our laboratory to reduce O-GlcNAcylation in high glucose- or glucosamine-treated neonatal cardiac myocytes.\textsuperscript{22} Although no significant GCA enzymatic change is detected in the diabetic heart, the present study demonstrates that overexpressing O-GlcNAcase in vivo is able to increase O-GlcNAcase activity \(\sim 50\%\) and is effective at reducing cellular O-GlcNAcylation levels in the heart. A reduction in cellular O-GlcNAcylation was observed both in cytosolic and nuclear fractions, suggesting that GCA overexpression may have diverse effects on cellular function.

The most interesting finding of our study is that overexpression of GCA has a beneficial effect on cardiac function in diabetes. The results presented here demonstrate that overexpression of GCA could dramatically improve calcium cycling in diabetic myocytes by enhancing the Ca\(^{2+}\) transient and sarcoplasmic reticulum Ca\(^{2+}\) loading. In the STZ-induced diabetic heart, decreased SERCA expression level and phosphorylation of PLB explain the cardiac dysfunction observed in these diabetic hearts. The alterations in sarcoplasmic reticulum protein expression observed after GCA overexpression further provide an explanation for these changes in calcium cycling. The higher SERCA\(_{2a}\) expression and lower PLB expression in diabetic hearts after Adv-GCA delivery increased the SERCA\(_{2a}/\)PLB ratio and SERCA\(_{2a}\) activity, which mirrors the observed normalization of calcium transients and increases in sarcoplasmic reticulum calcium load. The improvement in calcium cycling can certainly contribute to the enhancement of contractility in diabetic myocytes overexpressing GCA, and further facilitates the global functional improvement observed in the diabetic heart. However, unlike the restoration of contractile function observed in diabetic hearts treated with insulin,\textsuperscript{37,38} contractile function increased only 15\% in isolated perfused hearts overexpressing GCA. Other factors unrelated to O-GlcNAcylation, such as nonenzymatic glycation of SERCA\(_{2a}\),\textsuperscript{39} may also play a role in diabetic cardiac dysfunction.

There is compelling evidence indicating that O-GlcNAcylation involves regulation of gene transcription, protein synthesis, and degradation.\textsuperscript{10} A series of studies have established a role of O-GlcNAc in suppressing transcription through modifying RNA polymerase II,\textsuperscript{40} Sp1,\textsuperscript{41,42} and histone deacetylase.\textsuperscript{43} We have previously shown that SERCA\(_{2a}\) promoter activity negatively corresponds with the amount of O-GlcNAcylation on Sp1 in cardiac myocytes.\textsuperscript{22} Therefore, it is likely that the increased SERCA\(_{2a}\) expression observed in diabetic hearts receiving Adv-GCA results from a decrease in Sp1 O-GlcNAcylation via GCA overexpression. However, another series of studies suggest a positive role of O-GlcNAc in upregulating transcriptional events, including those specifically responsive to glucose.\textsuperscript{44,45} Additionally, O-GlcNAc modification has been demonstrated to inhibit the proteasome\textsuperscript{46–48} and stabilize certain proteins. We postulate that different mechanisms may contribute to the down regulation of PLB observed in diabetic heart receiving Adv-GCA delivery.

Our study also demonstrated an increased phosphorylated PLB in diabetic heart overexpressing GCA. This provides another explanation for enhanced SERCA\(_{2a}\) function after GCA gene delivery, as it has been shown that the phosphor-

---

**Figure 7.** Alterations of protein expression in diabetic hearts receiving Adv-GCA gene delivery. Whole cell extract was made from normal heart or diabetic hearts receiving either Adv-SR\(_{−}\) (Dia\(_{−}\)SR\(_{−}\)) or Adv-GCA (Dia\(_{+}\)GCA). SERCA and PLB expression level (pentamer form) were adjusted by actin and p-PLB level was adjusted by PLB. Top, Representative Western blot image. Bottom, Relative expression level was represented as mean±SE. *\(P<0.05\), compared with normal; # \(P<0.05\), compared with Dia\(_{+}\)SR\(_{−}\).

and that a reduction of excessive O-GlcNAc modification by overexpressing an adenovirus-encoded O-GlcNAcase enzyme in the heart has beneficial effects on cardiac function in STZ-induced diabetes.

We observed, through protein analyses, that diabetic hearts exhibited excessive O-GlcNAcylation in both type I and type II diabetes. This is similar to what has been observed in other tissues in diabetic animals and diabetic patients.\textsuperscript{17,31} Although both mRNA and protein expression levels of OGT were upregulated in the STZ-induced diabetic heart, the enzymatic activity of OGT did not increase with protein amount. This finding is in agreement with previous observations,\textsuperscript{31,33} indicating that additional factors may regulate the activity of OGT.\textsuperscript{34} Additionally, unlike the increase in OGT activity observed in the pancreas from STZ-induced diabetic rats, there was no significant change in enzymatic activity in hearts from STZ-induced diabetic mice. Thus, it is suggested that OGT activity is modulated differentially in different types of tissue. Our previous studies have shown that intracellular UDP-GlcNAc levels are significantly elevated in the diabetic heart when compared with the normal heart.\textsuperscript{25} It is likely that excess substrate supply, derived from the enhanced hyperglycemia-driven hexosamine pathway flux, rather than altered enzymatic activity, leads to increased protein O-GlcNAc modification in the STZ-induced diabetic heart. O-GlcNAcase has been characterized as an enzyme specific for O-GlcNAc removal,\textsuperscript{35,36} and the adenovirus-mediated...
ylation of PLB promotes oligomerization of PLB and decreases its inhibitory effects on SERCA2a. Reciprocal phosphorylation and O-GlcNAcylation occurring at the same amino acid site have been identified in several proteins, including Tau and DNA polymerase II. Moreover, recently, we determined that PLB could be immunoprecipitated with anti-O-GlcNAc antibody (CTD 110.6) from cardiac myocytes cultured with high glucosamine and adenosine to express OGT (data not shown). Therefore, we are currently investigating whether PLB phosphorylation is directly regulated by reciprocal PLB O-GlcNAcylation, or whether PLB phosphorylation was indirectly regulated by O-GlcNAcylation through altering protein kinase A or protein phosphatase activity.

In summary, our study demonstrates that excessive cellular O-GlcNAcylation exists in the diabetic heart, and that reducing excess O-GlcNAcylation by overexpressing GCA has beneficial effects on calcium handling and diabetic cardiac function. Overexpressing GCA mediated by adenosine may provide a therapeutic means to ameliorate diabetic cardiomyopathy.

Acknowledgments

This research was supported by the NIH grants R01 HL 66917 and R01 HL 52946.

References

6. Lagadic-Gossmann D, Buckler KJ, Le Prigent K, Feuvray D. Altered amino acid site have been identified in several proteins, including Tau and RNA polymerase II. Moreover, reciprocal phosphorylation or protein phosphatase activity.


Adenovirus-Mediated Overexpression of O-GlcNAcase Improves Contractile Function in the Diabetic Heart
Ying Hu, Darrell Belke, Jorge Suarez, Eric Swanson, Raymond Clark, Masahiko Hoshijima and Wolfgang H. Dillmann

Circ Res. 2005;96:1006-1013; originally published online April 7, 2005;
doi: 10.1161/01.RES.0000165478.06813.58

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/96/9/1006

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/