Cardioprotection by N-Acetylglucosamine Linkage to Cellular Proteins

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Background—The modification of proteins with O-linked β-N-acetylglucosamine (O-GlcNAc) represents a key posttranslational modification that modulates cellular function. Previous data suggest that O-GlcNAc may act as an intracellular metabolic or stress sensor, linking glucose metabolism to cellular function. Considering this, we hypothesized that augmentation of O-GlcNAc levels represents an endogenously recruitable mechanism of cardioprotection.

Methods and Results—In mouse hearts subjected to in vivo ischemic preconditioning, O-GlcNAc levels were significantly elevated. Pharmacological augmentation of O-GlcNAc levels in vivo was sufficient to reduce myocardial infarct size. We investigated the influence of O-GlcNAc levels on cardiac injury at the cellular level. Lethal oxidant stress of cardiac myocytes produced a time-dependent loss of cellular O-GlcNAc levels. This pathological response was largely reversible by pharmacological augmentation of O-GlcNAc levels and was associated with improved cardiac myocyte survival. The diminution of O-GlcNAc levels occurred synchronously with the loss of mitochondrial membrane potential in isolated cardiac myocytes. Pharmacological enhancement of O-GlcNAc levels attenuated the loss of mitochondrial membrane potential. Proteomic analysis identified voltage-dependent anion channel as a potential target of O-GlcNAc modification. Mitochondria isolated from adult mouse hearts with elevated O-GlcNAc levels had more O-GlcNAc–modified voltage-dependent anion channel and were more resistant to calcium-induced swelling than cardiac mitochondria from vehicle mice.

Conclusions—O-GlcNAc signaling represents a unique endogenously recruitable mechanism of cardioprotection that may involve direct modification of mitochondrial proteins critical for survival such as voltage-dependent anion channel.

Key Words: infarction ■ ischemia ■ mitochondria ■ myocardial infarction ■ acetylglucosamine

The modification of nuclear and cytoplasmic proteins with monosaccharides of N-acetylglucosamine (O-GlcNAc) is an essential posttranslational modification of metazoans. The addition of O-GlcNAc to the protein backbone is analogous to protein phosphorylation. O-GlcNAc levels are dynamic and change in response to extracellular stimuli, morphogens, cell cycle, development, and cellular stress.1,2 The addition of O-GlcNAc to serine and threonine residues is catalyzed by uridine diphospho-N-acetylglucosamine:polypeptide β-N-acetylglucosaminyltransferase (OGT),3,4 whereas removal of O-GlcNAc is catalyzed by a β-hexosaminidase (O-GlcNAcase).5 Ultimate confirmation of the key role of O-GlcNAc in regulating processes within the cell is reflected by lethality consequent to deletion of OGT at the single-cell level.6,7

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Clinical Perspective p 1182

Up to 5% of glucose imported into the cell is converted to UDP-GlcNAc through the hexosamine biosynthetic pathway. UDP-GlcNAc serves as a donor for the synthesis of other sugar nucleotides, glycolipids, glycosylphosphatidylinositol anchors, N-linked glycosylation, Golgi-mediated O-linked glycosylation, and O-GlcNAc. Levels of O-GlcNAc within the cell are sensitive to changes in extracellular glucose concentrations.1,2 Accordingly, extensive efforts have focused on the idea that O-GlcNAc is a metabolic sensor or signal.8 Zachara and coworkers9 demonstrated recently that cell lines respond to various stressors by augmenting O-GlcNAc levels and that this may by a critical maneuver for cell survival. This finding suggests an endogenous autoproective mechanism that motivated us to investigate whether
transient cellular stress may trigger such a recruitable process in the myocardium. We initially addressed the cardioprotective process known as ischemic preconditioning, wherein brief periods of ischemia render the heart resistant to subsequent lethal ischemia. In this report we describe changes in O-GlcNAc levels during cardiac myocyte injury and specifically evaluate whether augmentation of O-GlcNAc levels is sufficient to confer cardioprotection in vitro and in vivo. Our findings implicate O-GlcNAc modification during ischemic preconditioning and represent the first reversible posttranslational modification other than phosphorylation to figure prominently in determining cardiac myocyte fate during lethal stress. Furthermore, our data indicate that at least 1 potential target for the O-GlcNAc modification is voltage-dependent anion channel (VDAC), a member of the mitochondrial permeability transition pore (mPTP), thereby providing unique insights into the cardioprotective mechanism.

**Methods**

See the online-only Data Supplement for complete experimental methods.

**Murine In Vivo Ischemia/Reperfusion and Infarct Size Determination**

Ligation of the left coronary artery and infarct size determination were performed as described previously.10–22

**Cardiac Myocyte Isolation, Culture, and Fluorescence**

Cardiac myocytes were isolated from 1- or 2-day-old Sprague-Dawley rats as described.23 Fluorescence-activated cell sorting and confocal microscopy were performed as described previously.20,24,25

**Identification of O-GlcNAc–Modified Proteins From Adult Mouse Hearts**

Cells were lysed in buffer containing 25 mmol/L HEPES, pH 7.0, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% NP-40, 0.1% SDS, 1% protease inhibitor cocktail (Sigma, St Louis, Mo), 1% phosphatase inhibitor cocktail (Pierce), and 1 μmol/L O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNac) (O-GlcNcase inhibitor). Proteins modified by O-linked GlcNAc were immunoprecipitated with anti-O-GlcNAc antibodies (Covance) with the use of a protein immunoprecipitation kit (Sigma). Proteins were released from the beads by boiling in Laemml buffer containing 50 mmol/L dithiothreitol and separated by 12% SDS-PAGE. Separated proteins were subjected to Western blot analysis with the use of anti-VDAC antibodies (Sigma).

**Adult Mouse Heart Mitochondrial Isolation and Swelling Assay**

Mitochondria were isolated from whole mouse hearts as described previously.26,27 The mitochondrial swelling assay was performed as described in previous reports.28

**Statistical Analyses**

Data were analyzed by unpaired t test or ANOVA with post hoc analysis (Bonferroni) with the use of StatView (SAS Institute) software. For box plots, data in the upper border indicate the 75th percentile, and data in the lower border indicate the 25th percentile; the median (50th percentile) is between the 2. For bar and line graphs, data are reported as mean±SEM. Differences were accepted as significant when P<0.05.

All authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**O-GlcNAc Levels After Ischemic Preconditioning In Vivo**

Mice were subjected to either acute or delayed ischemic preconditioning (or respective sham). At the appropriate time (acute, immediately; delayed, 24 hours later), the hearts were removed, total proteins were isolated as described in Methods, and samples were subjected to Western blotting with the O-GlcNAc–specific antibody (CTD110.6).29 As shown in Figure 1, cardiac O-GlcNAc levels were significantly augmented after acute (135±7%) or delayed (140±4%) ischemic preconditioning compared with the respective sham group (104±3% and 95±4%). The appearance of numerous immunopositive bands was expected and has been reported previously.9,29 The antibody (CTD110.6) used here is specific for the posttranslational modification (O-GlcNAc), not for a single protein.29

**Reduction of Myocardial Infarct Size by PUGNac In Vivo**

We intraperitoneally injected mice with 50 mg/kg of PUGNac (O-GlcNcase inhibitor28), 8 hours before surgery, to ascertain whether augmentation of O-GlcNAc levels is sufficient to reduce infarct size in vivo. Several mice not undergoing surgery were also euthanized to determine whether the dosing regimen of PUGNac was sufficient to increase cardiac O-GlcNAc levels. As shown in Figure 1D, such treatment significantly augmented myocardial O-GlcNAc levels. Additional mice were treated similarly and subjected to 40 minutes of left coronary artery ischemia and 24 hours of reperfusion. At the end of reperfusion, Evans blue dye and 2,3,5-triphenyltetrazolium chloride were used to define the area at risk and infarct size, respectively (Figure 1E). PUGNac significantly reduced infarct size compared with vehicle (Figure 1C). The ischemic preconditioning group is presented to give a better idea of the ability to observe protective effects in our model and demonstrate the endogenous capacity to augment O-GlcNAc levels and reduce infarct size.

**O-GlcNAc Levels in Cardiac Myocytes**

O-GlcNAc levels were assessed via Western blot analysis with the use of an O-GlcNAc–specific antibody in isolated cardiac myocytes after exposure to various durations of hydrogen peroxide in serum-free media (Figure 2). PUGNac was used to test the hypothesis that reversal of such decline in O-GlcNAc levels could attenuate the extent of cardiomyocyte death. As shown in Figure 2B, PUGNac significantly augmented O-GlcNAc levels in cardiac myocytes compared with vehicle in isolated cardiac myocytes. Additional myocytes were treated with vehicle or PUGNac and exposed to varying durations of hydrogen peroxide exposure. Cells were then harvested and examined for changes in O-GlcNAc levels. Myocytes treated with PUGNac showed markedly higher levels of O-GlcNAc throughout the time course compared with myocytes treated with vehicle. Furthermore, the decrement in O-GlcNAc levels was minimized and retarded in the PUGNac–treated preparations (Figure 2B).
The decrement in cellular O-GlcNAc levels may correspond to important catastrophic events culminating in cardiomyocyte death, as addressed below. We next tested the hypothesis that reversal of the decrement in O-GlcNAc levels could attenuate cell death.

**O-GlcNAc and Cardiomyocyte Survival**

Cardiac myocytes were treated with PUGNAc or vehicle, exposed to hydrogen peroxide (0.1 mmol/L), and coincubated with propidium iodide and annexin V to assess cell death (Figure 3). At the end of 150 minutes of hydrogen peroxide exposure, the percentage of propidium iodide–positive cells was significantly augmented in vehicle compared with PUGNAc and control groups. Cardiomyocyte death was significantly attenuated in cells treated with PUGNAc compared with cells treated with vehicle. Saponin permeabilization was used at the end of the experimental protocol to confirm equal numbers of cells per field (Figure 3B). Additional groups of myocytes were treated with vehicle or PUGNAc, then challenged with peroxide, and cells were harvested for total caspase activity after 16 hours (Figure 3E). This indicates evidence of persistent protective effects, potentially related to apoptosis.

**Preservation of Mitochondrial Membrane Potential**

To evaluate the mechanistic implications of the protective effects of enhanced O-GlcNAc protein modification, we focused on early events governing cell death. Specifically motivated by the importance of maintaining mitochondrial integrity to enhance cell survival, we ascertained whether mitochondrial membrane potential was affected by alterations in O-GlcNAc levels. Such an avenue is particularly attractive given the early decline in O-GlcNAc levels shown in Figure 2B and 2C, which mirrors the early loss of mitochondrial membrane potential we have reported previously.

Figure 2 clarifies that treatment of cardiac myocytes with the O-GlcNAcase inhibitor PUGNAc augments O-GlcNAc levels, and Figure 3 indicates that PUGNAc treatment attenuates
the extent of cardiomyocyte death. In Figure 4, we treated cardiomyocytes with PUGNAc or vehicle, loaded them with the mitochondrial membrane potential indicator tetramethylrhodamine ethyl ester (TMRE), and exposed the cells to oxidant stress (hydrogen peroxide). Vehicle cardiomyocytes experience a catastrophic loss of mitochondrial membrane potential after exposure to hydrogen peroxide for 1 hour. PUGNAc, which augments O-GlcNAc levels, significantly attenuated the loss of mitochondrial membrane potential according to confocal microscopy (Figure 4A and 4B) and flow cytometry (Figure 4C and 4D) in a dose-dependent manner. These data provide important mechanistic implications for the protective effect of O-GlcNAc, which will be discussed below.

**PUGNAc-Mediated Protection Is Glibenclamide Sensitive**

Glibenclamide blocks numerous forms of “preconditioning,” both ischemic and pharmacological. In Figure 5, we show that PUGNAc-mediated cytoprotection in neonatal rat cardiomyocytes is largely blocked by the K<sub>ATP</sub> channel antagonist glibenclamide. These data support the notion that some of the protective mechanisms required by augmented O-GlcNAc levels are shared with ischemic preconditioning (ie, K<sub>ATP</sub> activation). Although glibenclamide-sensitive pathways are apparently required for PUGNAc-mediated protection, it is unclear whether such pathways are the only mechanism of PUGNAc-mediated protection. To evaluate other potential changes that might occur after PUGNAc treatment, we performed Western blots for the cytoprotectant heat shock protein (HSP)70 in neonatal rat cardiomyocytes (Figure 5E). PUGNAc treatment significantly augmented HSP70 levels compared with vehicle treatment. Whether augmentation of HSP70 levels is instrumental for PUGNAc-mediated protection remains to be tested.

**Protein Modification by O-GlcNAc**

Numerous studies have documented an ever-growing list of proteins modified by O-GlcNAc. However, sparse information exists on specific cardiac proteins modified by
According to the immunoblotting data in Figures 1 and 2, it appears that several proteins are modified by \( \text{O-GlcNAc} \) in cardiac myocytes. In Figure 6, we attempted to specifically identify some of these as potential candidates for cardioprotection. Proteins isolated from cultured cardiac myocytes treated with vehicle or PUGNAc were harvested as described in the online-only Data Supplement. Two-dimensional gel electrophoresis revealed at least 13 spots on

Figure 3. Assessment of cell death in isolated cardiac myocytes \((n=3\) per group). Myocytes were treated with vehicle or PUGNAc before exposure to hydrogen peroxide for 150 minutes in the presence of the cell death indicators propidium iodide (PI) and annexin V. A, After 150 minutes of oxidant stress, most vehicle-treated myocytes become propidium iodide positive, a phenomenon inhibited by PUGNAc. Control cells were not challenged with peroxide; they are time controls. B, Representative confocal images of myocytes from panel A at the end of the experiment (pre-saponin) and after saponin permeabilization, indicating equivalent numbers of cells per field. C, During the time course of oxidant stress (hydrogen peroxide), annexin V positivity increases in vehicle-treated myocytes, an effect attenuated by PUGNAc. D, Representative confocal images of myocytes from panel C at the end of the experiment. E, In additional groups of myocytes, late caspase activity (16 hours after hydrogen peroxide) was reduced in PUGNAc-treated myocytes compared with vehicle \((n=3\) per group). \(^{*}P<0.05\) vs vehicle.
which the O-GlcNAc modification (as evidenced by CTD antibody positivity) was augmented after PUGNAc treatment. Analysis of these 13 spots (Table) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry revealed several proteins involved in metabolism and VDAC that piqued our interest because of its participation in the mPTP. Next, we performed an immunoprecipitation experiment to confirm that VDAC was modified by pulling down the O-GlcNAc–modified proteins and performing an immunoblot against VDAC. Indeed, the band corresponding to 30 kDa was also augmented in the PUGNAc sample compared with vehicle (Figure 6C).

Our focus on VDAC is based on its identity as a central element in formation of the mPTP, which represents a significant and proximal event in the commitment to cell death. Although this serves only as an initial foray into the likely numerous proteomic changes associated with O-GlcNAc in this system, this singular finding is intriguing and consistent with the in vitro evidence for O-GlcNAc–mediated cardioprotection. It is important to note that lower-molecular-weight proteins are not shown in the 1-dimensional gels earlier in this study because the Western blots were not optimized for lower-molecular-weight ranges. Nevertheless, the appearance of the lower-molecular-weight bands appeared after the saturation of some of the higher-molecular-weight bands (by which time the exposure was stopped for the analyses shown in the earlier figures).

On the basis of the preliminary findings in Figure 6A through 6C (and the Table), adult wild-type mice were treated with PUGNAc (50 mg/kg IP) or isovolumic vehicle (as in Figure 1D), and cardiac mitochondrial proteins were evaluated to ascertain whether such changes in VDAC modification suggested by the 2-dimensional gels (Figure 6A through 6C) also occurred in the adult myocardium. Fractionation of hearts from vehicle- and PUGNAc-treated mice yielded clean, largely intact mitochondria (Figure 6D). Further examination of the mitochondrial fraction revealed that more VDAC was O-GlcNAc modified after PUGNAc treatment compared with vehicle and that such change occurred without a difference in total VDAC (Figure 6E).

**O-GlcNAc Modification and mPTP Formation**

To test the potential link between O-GlcNAc modification and a functional biochemical assessment of mitochondrial...
function within the mechanistic context of cell survival, cardiac mitochondria isolated from PUGNAc- and vehicle-treated adult mouse hearts (see above) were subjected to calcium-induced mitochondrial swelling (Figure 7). Mitochondria isolated from vehicle- and PUGNAc-treated mice were intact (Figure 6D), were free of cellular debris (Figure 7A), and maintained stable calcium-free absorbance levels throughout the assay (Figure 7B). The formation of mPTP occurred in vehicle-treated mitochondria, as evidenced by the decrease in spectrophotometric absorbance (520 nm) after the addition of 0.1 mmol/L calcium chloride (Figure 7C). Conversely, cardiac mitochondria isolated from PUGNAc-treated mice revealed resistance to the induction of mPTP. These data provide a novel molecular link between the identity of an O-GlcNAc-modified protein and a potential direct mechanism of cytoprotection.

**Discussion**

Innumerable studies have focused on phosphorylation, the most widely described posttranslational modification in mammalian cells. The goal of this study was the primary establishment of the relationship between cardiac myocyte pathophysiology and the less studied posttranslational modification O-GlcNAc. The salient findings of the present study are as follows: (1) ischemic preconditioning enhances O-GlcNAc levels in vivo; (2) elevation of O-GlcNAc levels is sufficient to reduce infarct size after in vivo myocardial ischemia/reperfusion injury; (3) cardiac myocytes experience a decrement in O-GlcNAc levels during lethal cell injury; (4) the decrement in O-GlcNAc levels occurs early in cardiomyocyte injury in conjunction with the loss of mitochondrial membrane potential; (5) augmentation of O-GlcNAc levels attenuates the loss of mitochondrial membrane potential and cell death; (6) PUGNAc-mediated protection apparently
shares some mechanisms with ischemic preconditioning; (7) at least several proteins are O-GlcNAc modified; and (8) augmenting O-GlcNAc levels apparently reduces sensitivity to mPTP formation. The present study establishes a new mechanistic paradigm in experimental cardioprotection and provides a previously unrecognized potential mechanism of ischemic preconditioning.

Others have shown that glucose transport activity is significantly enhanced after ischemic preconditioning.34 This could increase glucose flux and ultimately contribute to enlargement of the UDP-GlcNAc pool. Theoretically, increasing the size of the UDP-GlcNAc pool could increase O-GlcNAc levels (because UDP-GlcNAc is the sugar donor), as demonstrated previously in isolated cells9 and supported by the present findings from in vivo ischemic preconditioning. However, the approximate equivalence of PUGNAc and ischemic preconditioning in the extent of infarct size reduction does not conclusively demonstrate dependence of ischemic preconditioning on the O-GlcNAc modification, which is the subject of several ongoing investigations.

The current list of O-GlcNAc targets is sizable and growing, a fact not surprising when it is considered that O-GlcNAc is a posttranslational modification involving serine and threonine residues. As indicated by the present data, enhanced O-GlcNAc protein modification may act (at least partially) on the mitochondria to effect protection. Active pursuit of potential mitochondrial targets should continue, especially in light of the importance of mitochondria in cell survival. Additional emerging evidence suggests that O-GlcNAc protein modification of the proteasome could be responsible for the protective effects of enhanced O-GlcNAcylation.35 Marchase et al36 recently found that hyperglycemia, via enhanced flux through the hexosamine biosynthetic pathway, attenuated capacitative calcium entry in a manner similar to that of the in vitro model system. Thus, the present protective effects may at least partially

Figure 6. A, Two-dimensional immunoblotting for O-GlcNAc identified several proteins with enhanced immunoreactivity, which were subsequently submitted for mass spectrometry analysis. Several proteins displayed augmented O-GlcNAc modification after PUGNAc treatment, including VDAC (spot 6); n=2. See the Table for protein identities. pI indicates isoelectric point. B, Additional samples separated on narrower isoelectric point and molecular weight ranges to enhance the ability to identify the VDAC spot. C, Additional protein isolates were subjected to immunoprecipitation (IP) with an antibody against O-GlcNAc, then immunoblotted (IB) for VDAC. D, Adult mice were treated with vehicle (V; n=4) or PUGNAc (P; n=4), and cardiac mitochondria were isolated. The presence of cytochrome c oxidase, presence of subunit 4 (COX IV), and absence of tubulin in the mitochondrial fraction, whereas the converse was observed in the cytosolic fraction, indicated sound mitochondrial isolation. E, Mitochondrial protein isolates (n=4 per group) were immunoprecipitated (IP) with an anti-VDAC antibody, then immunoblotted (IB) with an anti-O-GlcNAc antibody (top). Additional aliquots were immunoprecipitated with an anti-O-GlcNAc antibody, and the precipitate was immunoblotted for VDAC (middle). Additional immunoblotting was performed on VDAC pulldown samples with an anti-VDAC antibody to confirm equivalent VDAC levels among the different samples (bottom).
be explained by attenuation of calcium overload, which is a known contributor to cardiomyocyte death. Subsequently, the same group found that elevating flux through the hexosamine biosynthetic pathway (thereby increasing O-GlcNAc levels) reduced cardiac myocyte damage in vitro.37,38 Such findings are consistent with those of the present study.

The formation of the mPTP is a harbinger of cell death.39–41 Although a consensus has not been reached on the obligatory presence of all putative members, it is widely accepted that VDAC (also known as porin), adenine nucleotide translocator, and cyclophilin-D constitute the core components of mPTP. Within the context of the present study, we are tempted to speculate that the O-GlcNAc modification of VDAC represents a heretofore unappreciated cardioprotection mechanism. Further, such modification may, at least in part, offer an explanation for the mechanism of cardioprotection.

The opening of the mPTP produces unregulated ingress and egress of molecules <1.5 kDa in the mitochondria. Such pathological disturbance uncouples electron transport from the production of ATP by destroying the electrochemical gradient across the inner mitochondrial membrane (ie, the mitochondrial membrane potential, indicated by TMRE) and releases proapoptotic proteins. Thus, the correlation of augmented levels of O-GlcNAc–modified VDAC with cytoprotection warrants attention as a potential mechanism. One might conjecture that the modification of VDAC by O-GlcNAc interferes with the formation of mPTP and thus protects the myocytes. Indeed, our swelling data from adult cardiac mitochondria support this precise notion, leading to our hypothetical scheme (Figure 1 in the online-only Data Supplement). At a minimum, these findings provide a molecular link between O-GlcNAc modification and cardioprotection, although definitive evaluation of such an exciting possibility and the complete characterization of O-GlcNAc–modified proteins in the heart will undoubtedly be the subject of numerous future studies. A recent report also suggests that mPTP can occur in murine embryonic fibroblasts in the genetic absence of VDAC,42 although this was not shown in cardiac mitochondria. Although this may be true in fibroblasts that have no VDAC, the present mt mitochondria all had abundant expression of VDAC, and the former report42 does not indicate that constitutive VDAC does not participate significantly in the formation of mPTP. Thus, it is difficult to make direct comparisons between the 2 studies. It is important to emphasize that many proteins are apparently O-GlcNAc modified and that VDAC is a likely mechanistic possibility; however, our data do not exclude the contribution of other protein targets in this system, such as adenine nucleotide translocator, cyclophilin-D, and other unidentified proteins. Indeed, it is likely that other O-GlcNAc–modified proteins contribute to this process, or the de novo production of cardioprotective proteins may also be involved (Figure 5).

Although the performance of in vivo myocardial ischemia/reperfusion studies lends credence to the idea that this could be a future therapeutic avenue in humans, the present data serve merely as proof of principle on which additional investigations will be based. The present in vivo infarct model has several obvious limitations. Healthy adult mice are likely unrepresentative of diseased human patient populations, and animal models with known risk factors should yield further insight into the potential clinical applicability of such findings.

The present data demonstrate a previously unrecognized endogenously recruitable mechanism of cardioprotection involving enhancement of the posttranslational modification O-GlcNAc. As our understanding of this area matures, we can elucidate the identity of specific protein targets of O-GlcNAc and develop a more integrated understanding of this process. This area of study may yield viable therapeutic options to combat postischemic myocardial injury, and, in a broader sense, we can begin to establish the biological role of O-GlcNAc in both the healthy and diseased myocardium.43

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**Table.** Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Identification of Proteins With Positive Immunoreactivity With Anti-O-GlcNAc Antibodies*

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*See 2-dimensional immunoblots in Figure 6A.
PUGNAc-treated mice. (C), an effect largely reduced in cardiac mitochondria from mice (n/H11005/H11003). Challenge with 0.1 mmol/L calcium chloride significantly reduced absorbance (520 nm) in the vehicle mitochondria (B). A, Gross transmitted light evaluation of Percoll-purified cardiac mitochondria from vehicle- and PUGNAc-treated hearts (respectively) demonstrating structures of ~1 μm in diameter, with no apparent cellular debris (image size 100 × 75 m). Cardiac mitochondria from PUGNAc-treated (PUG) or vehicle-treated (Veh) mice (n=3 per group) were indistinguishable without calcium chloride (B). Challenge with 0.1 mmol/L calcium chloride significantly reduced absorbance (520 nm) in the vehicle mitochondria (C), an effect largely reduced in cardiac mitochondria from PUGNAc-treated mice.

**Figure 7.** Augmenting O-GlcNAc levels reduces calcium-induced mPTP activation in adult cardiac mitochondria. A, Gross transmitted light evaluation of Percoll-purified cardiac mitochondria from vehicle- and PUGNAc-treated hearts (respectively) demonstrating structures of ~1 μm in diameter, with no apparent cellular debris (image size 100 × 75 μm). Cardiac mitochondria from PUGNAc-treated (PUG) or vehicle-treated (Veh) mice (n=3 per group) were indistinguishable without calcium chloride (B). Challenge with 0.1 mmol/L calcium chloride significantly reduced absorbance (520 nm) in the vehicle mitochondria (C), an effect largely reduced in cardiac mitochondria from PUGNAc-treated mice.

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**Disclosures**
Under a licensing agreement between Covance Research Products and Johns Hopkins University, Dr Hart receives a share of royalty received by the university on sales of the CTD110.6 antibody. The terms of this arrangement are being managed by Johns Hopkins University in accordance with its conflict-of-interest policies. The other authors report no conflicts of interest.

**References**
Despite decades of intensive effort, our understanding of the mechanisms of myocardial cell injury and survival remains limited. In this report, we take a unique approach to the problem and illustrate a potentially new paradigm of ischemic cardiobiology. The present study identifies one of the first enzymatically reversible posttranslational modifications, other than phosphorylation, to figure prominently in myocardial ischemia/reperfusion injury in vivo. Previous data suggest that the posttranslational modification O-linked β-N-acetylglucosamine (O-GlcNAc) may act as an intracellular metabolic or stress sensor, linking glucose metabolism to cellular function. Considering this, we hypothesized that augmentation of O-GlcNAc levels represents an endogenously recruitable mechanism of cardioprotection. From a mechanistic vantage point, O-GlcNAc levels waned synchronously with events related to mitochondrial permeability transition pore formation.

Proteomic analysis identified several potential targets of O-GlcNAc modification, including a putative mitochondrial permeability transition pore member, voltage-dependent anion channel. Thus, O-GlcNAc signaling represents a unique endogenously recruitable mechanism of cardioprotection in vivo that may involve direct modification of mitochondrial proteins critical for survival (eg, voltage-dependent anion channel). Identification of all of the potential targets of O-GlcNAc modification in the heart, particularly in the human myocardium, and the question of whether augmentation of O-GlcNAc levels during ischemic preconditioning is necessary for the infarct-sparing effects of preconditioning will be the focus of future studies.
Cardioprotection by N-Acetylglucosamine Linkage to Cellular Proteins
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Methods

Cardiac Myocyte Isolation and Culture

Cardiac myocytes were isolated from 1- or 2-day-old Sprague-Dawley rats (Zivic-Miller Laboratories, Inc, Pittsburgh, PA) and cultured in modified DMEM media as previously described \(^1\). Daily media changes included 5% fetal bovine serum, penicillin/streptomycin, and vitamin B12 supplementation. During the first four days of culture, bromo-deoxyuridine (BrdU) is added to inhibit the growth of any potential contaminating non-myocytes. One day prior to experimentation, the media is changed to the supplemented DMEM without serum. Cardiac myocyte cultures were incubated with 0.2 mmol/L \(O-(2\text{-acetamido-2-deoxy-D-glucopyranosylidene})\text{-amino-N-phenylcarbamate (PUGNAc)}\), an inhibitor of \(O\text{-GlcNAcase}\) \(^2\), for 8 hours prior to 0.1 mmol/L hydrogen peroxide challenge unless otherwise indicated. This duration of PUGNAc exposure is required because of its poor cellular permeability. The sample size is four per group unless indicated otherwise. All animals were used in accordance with institutional, local, and federal regulations.

Flow Cytometric Analysis

Mitochondrial membrane potential (\(\Delta \varphi_m\)) was assessed by tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) using a fluorescence activated cell scanning (FACS) as previously described \(^3\text{-}^5\). TMRE (100 nmol/L) was loaded for 30 minutes in the dark at 37°C. Cardiac myocytes were subjected to flow cytometry after 60 minutes exposure to (0.1 mmol/L) hydrogen peroxide and fluorescence was monitored in the FL-2 channel of a FACScalibur (Becton Dickinson) flow cytometer. The parameters for assessment of TMRE fluorescence were not changed during the course of this study. However, positive and negative control myocytes were scanned at the beginning of each experiment to ascertain the
consistent validity of the experimental settings. Cell suspensions were filtered prior to analysis to avoid any clumps of cells. The myocytes were selected by a gate drawn at the beginning of each session which was designed to further exclude cellular debris and cells attached to one another. At least 20,000 myocytes were scanned in each sample.

Laser Scanning Confocal Microscopy

Mitochondrial membrane potential ($\Delta \Psi_m$) was assessed in neonatal cardiomyocytes. Myocytes were loaded with 100 nmol/L TMRE for 30 minutes at 37°C as described previously. The media was then changed to a phenol red-free DMEM and the myocytes were imaged every 2 minutes through bandpass excitation (568 ± 20 nm) and emission (600 ± 20 nm) filters. The 35 mm glass-bottomed dishes were maintained at 37°C via an external servo-controlled heating unit fitted to the microscope stage. Reference probes were maintained in the heating block and in the media of each sample to confirm a constant temperature. The power position of the laser was held constant (at 10 o'clock position) and never changed during the course of these studies. Prior to contact with the dish the laser intensity was attenuated using the highest neutral density filter setting. The cells were imaged with a 40x oil immersion objective. The camera exposure duration was 100 msec and the binning was set at 2x. After the images were captured, offline analysis allowed the normalization of the fluorescence intensities to background levels (which were similar among all groups). Ten regions of interest (ROI) were drawn in mitochondrial-rich areas (for TMRE experiments) in each experiment using the PerkinElmer confocal software. The fluorescence in each ROI was recorded within the PerkinElmer confocal software analysis module. The data from the ten ROI were averaged for each timepoint in each experiment.
For the Annexin V/propidium iodide studies, the cells were treated with Vehicle or PUGNAc and the dyes were loaded according to the manufacturer’s instructions (Roche). Hydrogen peroxide was then added and images were captured every 5 minutes through a 20x lens. The remainder of the settings was the same as described above with the addition of the green channel bandpass filtered images (Ex: 488 nm, Em: 525 nm). For late determination of caspase activity, cells were treated and challenged as described above, but were not harvested until 16 hours after peroxide exposure. Caspase activity was enzymatically determined using a commercially available system (Roche).

RNA Interference

Cultured cardiac myocytes were transfected with short interfering (si) RNAs directed against OGT or against luciferase (LUC) that served as a non-silencing control. Myocytes were transfected with Qiagen’s Transmessanger system according to the manufacturer’s instructions. Thirty-six hours following transfection with 50 nmol/L siRNA, myocytes were subjected to flow cytometry as described above or harvested for determination of protein levels as described below.

Extraction of proteins from cells

Cells were washed with ice-cold PBS, harvested and extracted with 1% (v/v) NP-40 in 15 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1 mmol/L PMSF, 2 μmol/L PUGNAc, 5 mmol/L KF, 0.5 mmol/L sodium orthovanadate, 5 mmol/L β-glycerophosphate, 2 mmol/L EDTA, PIC1, PIC2. Extracts were separated by non-continuous reducing SDS-PAGE on Tris-Glycine Gels (Criterion, BIORAD). Proteins transferred to nitrocellulose and blocked with 3% (w/v) BSA in Tris buffered saline 0.05% v/v Tween-20 (TBST) were detected with anti-O-
GlcNAc antibody (CTD 110.6; Covance)\(^6\), anti-OGT (AL28 from GWH’s lab)\(^7\), anti-tubulin (Sigma), or anti-actin (Sigma). Recombinant OGT used as a positive control was produced as reported previously\(^8\).

**Identification of O-GlcNAc-modified Proteins**

Protein from Vehicle and PUGNAc-treated samples was precipitated with 10% trichloroacetic acid (v/v) and centrifuged at 14,055 xg for 15 minutes at 4°C. The protein pellets were washed with acetone and dried under a gentle stream of nitrogen gas. Two-dimensional electrophoresis (2DE) sample buffer (20 mmol/L Tris, pH 6.8, containing 8 mol/L urea, 2% CHAPS, 1 mmol/L EDTA, and 50 mmol/L DTT) was then added and proteins were allowed to solubilize for 2 hours at room temperature with gentle vortexing. Samples were again centrifuged at 14,055 xg for 15 minutes at 4°C and the protein concentration in the supernatant was assayed by a modified method of Bradford using bovine serum albumin in 8 mol/L urea as a standard. DTT was added to a final concentration of 50 mmol/L, ampholytes were added to 1%, and 40 μg of protein from the Vehicle and PUGNAc-treated samples was loaded for passive rehydration (12 hours incubation) onto 3-10 NL and 7-10 IPG strips (Biorad). The proteins were isoelectrically focused for 26000 V/hrs at 25°C and strips were then equilibrated with base rehydration buffer containing DTT and iodoacetamide, respectively. IPG strips were then loaded on a 12% polyacrylamide gel and electrophoresed for 90 minutes at 140 V for protein separation in the second dimension. Proteins were then transferred to PVDF membranes by electroblotting overnight at 30 mA. Parallel gels were silver-stained using the EMBL silver-staining protocol. For western analysis, O-GlcNAc-modified proteins were probed using 4 μg/ml anti-O-GlcNAc monoclonal antibodies.
(CTD110.6) followed by 0.1 μg/ml goat anti-mouse secondary antibodies. Membranes were developed using ECL Plus reagents and analyzed with a Typhoon 9400 Variable Mode Imager (Amersham Biosciences).

To obtain peptides for MS analysis, protein spots of interest were excised from parallel silver-stained gels and digested with trypsin using a modified version of the method described by Jenson, et al. Briefly, the excised gel pieces were incubated for 15 min in 100 mmol/L NH₄HCO₃ and 50% acetonitrile and dried by vacuum centrifugation. Proteins were then reduced by incubation with 20 mmol/L DTT at 56°C for 45 min, followed by alkylation with 65 mmol/L iodoacetamide in the dark at room temperature for 30 min. After alkylation, gel pieces were incubated for 15 min in 50 mmol/L NH₄HCO₃ and 50% acetonitrile and dried by vacuum centrifugation. Proteins were hydrolyzed by incubation in 20 ng of modified trypsin (Promega) per ml at 37°C overnight. Trypsin-generated peptides were applied on stainless steel targets by thin-film spotting using α-cyanohydroxycinnamic acid (Aldrich) as a matrix. Mass spectral data were obtained with a TOF-Spec 2E instrument (Micromass) and a 337 nm N₂ laser at 20 to 35% power in the reflector mode. Spectral data were obtained by averaging 10 spectra, each of which was the composite of 10 laser firings. Mass axis calibrations were accomplished by using peaks from tryptic auto-hydrolysis. Peptide masses obtained by MALDI-TOF/MS analysis were analyzed with Protein Probe software (MassLynx) to identify intact proteins. Additionally, the National Center for Biotechnology Information (NCBI) database was also used for protein identification. Peptides displaying masses consistent with O-GlcNAc modification (monoisotopic mass = 203.079) were ascribed as potentially modified protein fragments.

For immunoprecipitation analysis, cells were lysed in buffer containing 25 mmol/L HEPES, pH 7.0, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% NP-40, 0.1% SDS, 1% mammalian
protease inhibitor cocktail (Sigma), 1% phosphatase inhibitor cocktail (Pierce), and 1 \( \mu \text{mol/L} \) PUGNAc (O-GlcNAcase inhibitor). Proteins modified by O-linked GlcNAc were immunoprecipitated with anti-O-GlcNAc antibodies (Covance) using a protein immunoprecipitation kit (Sigma). Briefly, 200 \( \mu \text{g} \) cell lysate protein was incubated with 5 \( \mu \text{l} \) anti-O-GlcNAc antibody overnight at 4\( ^\circ \)C. Protein G sepharose beads were then incubated with the antigen:antibody complexes for two hours. Proteins were released from the beads by boiling in Laemmlli buffer containing 50 mmol/L DTT and separated by 12% SDS-PAGE. Separated proteins were subjected to western analysis using anti-VDAC antibodies (Sigma).

**Extraction of proteins from tissues**

Tissues were cooled in liquid N\(_2\) and homogenized in a mortar and pestle. Ground tissue was defrosted on ice in 15 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1 mmol/L PMSF, 1 mmol/L DTT, 2 \( \mu \text{mol/L} \) PUGNAc, 5 mmol/L KF, 0.5 mmol/L orthovanadate, 5 mmol/L \( \beta \)-glycerophosphate, 2 mmol/L EDTA, PIC1, PIC2 (4 mL/g). Samples were homogenized using a polytron (5000-25000 rpm) on setting 4, 2x20s on ice. Debris was pelleted at 20,000xg, 30min, 4\( ^\circ \)C. Extracts were separated by non-continuous reducing SDS-PAGE on Tris-Glycine Gels (Criterion, BIORAD). Proteins transferred to nitrocellulose and blocked with 3% (w/v) BSA in TBST were detected with anti-O-GlcNAc (CTD 110.6; Covance) \(^6\), anti-OGT (AL28) \(^7\), anti-O-GlcNAcase, anti-tubulin (Sigma), or anti-actin antibodies (Sigma).

**Densitometry**
Densitometry was performed using non-saturated chemiluminescent exposed films and quantitated using MacBAS bio-imaging analyzer (version 2.5, Fuji Photo Film Co). Typically, multiple exposures from the same experiment were used to confirm that the signal was within the linear range. Levels of \( \text{O-GlcNAc} \) in the entire lane were normalized to the appropriate control (actin or tubulin), and then expressed as a percentage of control (set at 100%). In all instances, data is averaged from independent experiments.

**Mitochondrial isolation and swelling assay**

Mitochondria were isolated from whole mouse hearts similar to methods previously described\(^1^1\),\(^1^2\). Wild-type mice treated with PUGNAc (50 mg/kg) or vehicle overnight were anesthetized with 127 mg/kg pentobarbital intraperitoneally, hearts harvested, and immediately rinsed with PBS. Hearts were homogenized in 4 ml of Buffer A (300 mmol/L sucrose, 10 mmol/L Tris HCl, 2 mmol/L EGTA and 5 mg/ml BSA, pH 7.4) using an ice-cold Kontes glass homogenizer. The homogenate was centrifuged at 2,000 xg for 2 minutes at 4 \(^\circ\)C. The resulting supernatant was centrifuged at 10,000 xg for 5 minutes at 4 \(^\circ\)C. The pellet was rinsed twice with 1 ml of Buffer A (without BSA) and resuspended in 1 ml of Buffer A (without BSA). Mitochondria in Buffer A were suspended in 19% (v/v) Percoll and centrifuged at 14,000 xg for ten minutes at 4 \(^\circ\)C. The resultant purified mitochondrial fraction was then resuspended in 0.5 mL of Buffer B (300 mmol/L sucrose and 10 mmol/L Tris HCl). An aliquot of 100\( \mu \)L of mitochondrial stock was used for protein concentration determination according to the Bradford method.

The mitochondrial swelling assay was performed similar to previous reports\(^1^3\). Briefly, cardiac mitochondria from PUGNAc or Vehicle treated mice in buffer B with a protein concentration of 2 mg/ml were loaded on a 96-well plate and allowed to warm to room temperature. CaCl\(_2\) (100 \(\mu\)mol/L) was used to induce mitochondrial swelling and the change
in absorbance measured spectrophotometrically at 520 nm. A decrease in absorbance indicated an increase in swelling (i.e. mitochondrial permeability transition pore opening).

**Murine in vivo ischemia-reperfusion**

Three- to four-month-old male SV129 mice (mean body weight 25 +/- 1 g) were randomized to treatment groups. Ligation of the left coronary artery was performed similar to methods described previously \(^5, 14-25\). Briefly, mice were anesthetized with intraperitoneal injections of ketamine hydrochloride (50 mg/kg) and sodium pentobarbital (50 mg/kg). The animals were then attached to a surgical board with their ventral side up. The mice were orally intubated with polyethylene (PE)-90 tubing loosely connected to PE-240 tubing and then connected to a model 683 rodent ventilator (Harvard Apparatus). The tidal volume was set at 2.2 mL and the respiratory rate was set at 120 breaths/min. The mice were supplemented with 100% oxygen via the ventilator side port. A median sternotomy was performed using an electric cautery, and the proximal left main coronary artery was visualized and completely occluded for 40 minutes with 7-0 silk suture mounted on a tapered needle (BV-1, Ethicon).

**Myocardial infarct size determination**

At 24 h of reperfusion, the mice were anesthetized as described previously, intubated, and connected to a rodent ventilator. A catheter (PE-10 tubing) was placed in the common carotid artery to allow for Evans blue dye injection. A median sternotomy was performed, and the left main coronary artery was religated in the same location as before. Evans blue dye (1.2 ml of a 2% solution) was injected into the carotid artery catheter into the heart to delineate the ischemic zone from the nonischemic zone. The heart was rapidly excised and serially sectioned along the long axis in 1-mm-thick sections, which were then incubated in
1.0% 2,3,5-triphenyltetrazolium chloride for five minutes at 37°C to demarcate the viable and nonviable myocardium within the risk zone. Each of the five 1-mm-thick myocardial slices was weighed. A blinded observer assessed the areas of infarct, risk, and nonischemic zone using computer-assisted planimetry (Image J, version 1.38x). All of the procedures for area at risk (AAR) and infarct size determination have been previously described ²⁰⁻²².

**Evaluation of blood pressure, heart rate, and left ventricular function:**

In order to assess the closed-chest hemodynamic status, mice were anesthetized with ketamine (50 mg/kg, intraperitoneal) and pentobarbital (50 mg/kg, intraperitoneal) and supplemented with oxygen via a nasal cone. A fluid-filled polyethylene catheter was inserted into the right common carotid artery, similar to methods described previously ²⁶, for the acquisition of mean arterial blood pressure (MABP). In vivo transthoracic echocardiography of the left ventricle using a 15 MHz linear array transducer (15L8s) interfaced with a Sequoia C512 (Acuson) was performed as described previously ²⁷, ²⁸. All data were calculated from 10 independent cardiac cycles per experiment. Short axis views at the midpapillary level yielded left ventricular (LV) end diastolic diameter (EDD), end systolic diameter (ESD), heart rate (HR), and fractional shortening (%FS).
Supplemental Results

Evaluation of blood pressure, heart rate, and left ventricular function:

One potential explanation for the cardioprotective effects may be that PUGNAC favorably affects cardiovascular hemodynamics, thereby reducing oxygen demand and by extension, infarct size. Accordingly, we measured blood pressure in mice treated with Vehicle (n=3) or PUGNAC (n=3) and found no significant differences in mean arterial blood pressure (80 ± 3 mmHg and 78 ± 2 mmHg, respectively). Furthermore, we evaluated LV end diastolic diameter (3.33 ± 0.03 mm and 3.34 ± 0.24, respectively), LV end systolic diameter (2.30 ± 0.06 mm and 2.30 ± 0.20 mm, respectively), LV fractional shortening (30.1 ± 2.4% and 31.0 ± 2.6%, respectively), and heart rate (360 ± 23 bpm and 421 ± 33 bpm, respectively), and found no differences between the two groups. Similarly, PUGNAC did not alter blood glucose levels in the mice (143 ± 18 mg/dL) compared to Vehicle treatment (147 ± 20 mg/dL). Such data indicate that there were no gross reductions in myocardial work or circulating glucose levels that would explain the salubrious effects of PUGNAC during myocardial ischemia. Collectively, these findings provide a clinically-relevant counterpart to our more detailed and mechanistic in vitro studies.
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Disclosure

Under a licensing agreement between Covance Research Products and The Johns Hopkins University, Dr. Hart receives a share of royalty received by the University on sales of the CTD 110.6 antibody. The terms of this arrangement are being managed by The Johns Hopkins University in accordance with its conflict of interest policies.
Supplemental Figure Legends

**Figure S1.** Scheme of theoretical mechanism. Noxious cellular insults result in the formation of the mitochondrial permeability transition pore (mPTP). The present data indicate O-GlcNAc modification occurs on at least VDAC, a component of the mPTP. Such modification may preserve mitochondrial integrity and provide potential mechanistic insight into this emerging area of cardiovascular biology. VDAC is one of many potential targets of O-GlcNAc modification in the heart that may relate to the cardioprotective mechanism reported here.

**Figure S2.** RNAi silencing of OGT, the gene responsible for the addition of O-GlcNAc, in isolated cardiac myocytes. A) Transmitted light (top) and green fluorescence (bottom) of myocytes transfected with fluorescein-tagged short interfering (si)RNA indicating 50-70% transfection efficiency. B) Myocyte cultures were treated with control siRNA directed against luciferase (luc) or OGT. Cells were harvested and the protein isolates were subjected to immunoblot analysis of OGT. Recombinant OGT protein is shown in the first lane for reference as a positive control. This representative immunoblot demonstrates that siRNA directed against OGT reduced OGT protein levels. C) Myocytes were transfected with control (LUC) or OGT siRNA, loaded with the mitochondrial membrane potential indicator TMRE, challenged with peroxide, and subjected to flow cytometry. Knockdown of OGT sensitized cardiac myocytes to oxidant stress compared to control siRNA. D) Myocyte cultures were treated with the control (LUC) siRNA or the OGT siRNA, with or without the O-GlcNAcase inhibitor, PUGNAc, and evaluated for O-GlcNAc levels by western blot. In addition to diminishing OGT expression, siRNA directed against OGT also attenuated O-
GlcNAc levels. E) Representative histograms of TMRE fluorescence indicate that the protective effect observed with PUGNAc was largely reversible by OGT siRNA. All groups of myocytes were loaded with TMRE, challenged with peroxide for one hour, and subjected to flow cytometry. A peroxide alone group (solid grey) is shown for reference. The protective effect of PUGNAc was largely reduced by siRNA-mediated knockdown of OGT.

**Figure S3.** The phenomenon of ischemic preconditioning typically involves the elevated production of nitric oxide. Here, we attempted to identify whether *in vivo* PUGNAc treatment significantly alters nitric oxide production according to tissue NOx determination. PUGNAc did not significantly alter NOx levels in the hearts of adult mice compared to the Vehicle treatment. Such findings do not rule out the possibility that NO signaling is involved, but do not support the idea of augmentation of NO levels as the mechanism of cardioprotection following *in vivo* PUGNAc administration. (n=5/group) p = NS.
References


11. Halestrap AP. The regulation of the oxidation of fatty acids and other substrates in rat heart mitochondria by changes in the matrix volume induced by osmotic strength, valinomycin, and Ca2+. *Biochem J.* 1987;244:159-164.


Following oxidant stress/ischemia/hypoxia/calcium overload, the mitochondrion will form the mPTP, ultimately leading to cell death.

The O-GlcNAc modification (G) may directly interfere with the pathologic participation of VDAC in the formation of the mPTP, thereby preserving mitochondrial function and avoiding cell death.
H_2O_2 alone
H_2O_2 + siRNA^{luc} + PUGNAc
H_2O_2 + siRNA^{OGT} + PUGNAc

A

B

Cardiac Myocytes

OGT  siRNA^{luc}  siRNA^{OGT}

IB: O-GlcNAc transferase (OGT)

C

TMRE Intensity

Cell Count

Depolarized

Normal

10^0

10^1

10^2

10^3

10^4

siRNA sequence:

5'

AAG UUU GAG CCC AAA UCA UGC

3'
Cardiac NOx (nmol/mg protein)

Vehicle: 5

PUGNAc: 5