Unique Hexosaminidase Reduces Metabolic Survival Signal and Sensitizes Cardiac Myocytes to Hypoxia/Reoxygenation Injury

Gladys A. Ngoh, Heberly T. Facundo, Tariq Hamid, Wolfgang Dillmann, Natasha E. Zachara, Steven P. Jones

Abstract—Metabolic signaling through the posttranslational linkage of N-acetylglucosamine (O-GlcNAc) to cellular proteins represents a unique signaling paradigm operative during lethal cellular stress and a pathway that we and others have recently shown to exert cytoprotective effects in vitro and in vivo. Accordingly, the present work addresses the contribution of the hexosaminidase responsible for removing O-GlcNAc (ie, O-GlcNAcase) from proteins. We used pharmacological inhibition, viral overexpression, and RNA interference of O-GlcNAcase in isolated cardiac myocytes to establish its role during acute hypoxia/reoxygenation. Elevated O-GlcNAcase expression significantly reduced O-GlcNAc levels and augmented posthypoxic cell death. Conversely, short interfering RNA directed against, or pharmacological inhibition of, O-GlcNAcase significantly augmented O-GlcNAc levels and reduced posthypoxic cell death. On the mechanistic front, we evaluated posthypoxic mitochondrial membrane potential and found that repression of O-GlcNAcase activity improves, whereas augmentation impairs, mitochondrial membrane potential recovery. Similar beneficial effects on posthypoxic calcium overload were also evident. Such changes were evident without significant alteration in expression of the major putative components of the mitochondrial permeability transition pore (ie, voltage-dependent anion channel, adenine nucleotide translocase, cyclophilin D). The present results provide definitive evidence that O-GlcNAcase antagonizes posthypoxic cardiac myocyte survival. Moreover, such results support a renewed approach to the contribution of metabolism and metabolic signaling to the determination of cell fate. (Circ Res. 2009;104:41-49.)

Key Words: O-GlcNAc ■ mitochondria ■ hypoxia ■ cell death ■ posttranslational modification

O-linked β-N-acetylglucosamine (O-GlcNAc) is a metabolic posttranslational modification of nucleocytoplasmic proteins. Following its discovery in 1984,1 numerous proteins have been identified as being O-GlcNAc–modified. Such targets are diverse and include transcription factors, RNA-binding proteins, cytoskeletal proteins, nuclear pore proteins, phosphatases, and kinases.2,3 Unlike traditional N-linked protein glycosylation, O-GlcNAcylation of proteins involves the addition of one GlcNAc molecule to the Ser/Thr amino acid residues with no further elongation into more complex oligosaccharides. The GlcNAc moiety is added to serine and threonine amino acid residues by O-GlcNAc transferase (OGT) and removed by O-GlcNAcase. O-GlcNAc is highly inducible, dynamic, and reversible, and the cycle of turnover of the sugar moiety exceeds the turnover of the protein itself.4 O-GlcNAc differs from phosphorylation in that the enzymes involved, OGT and O-GlcNAcase, are coded for by single genes, contrary to the numerous genes controlling protein phosphorylation/dephosphorylation.

Zachara and coworkers5,6 showed that O-GlcNAc levels change in response to stress and that augmentation of O-GlcNAc levels attenuated cell injury following lethal stress. We recently showed that enhanced O-GlcNAc levels attenuated injury following myocardial infarction, oxidative stress, and hypoxia.7,8 In the present study, we address the role of O-GlcNAcase in cardiac myocyte survival following hypoxic stress. Here, we evaluate whether manipulation of O-GlcNAcase to alter O-GlcNAc levels affects sensitivity to in vitro hypoxia/reoxygenation. Our findings definitively implicate O-GlcNAcase in the pathogenesis of posthypoxic cardiomyocyte death. In addition, this study emphasizes the significant contribution of metabolic signaling to posthypoxic damage.

Materials and Methods

Neonatal Rat Cardiac Myocyte Isolation and Culture
Neonatal rat cardiac myocytes (NRCMs) were isolated from 1- to 2-day-old Sprague–Dawley rats and cultured according to a well-
characterized protocol. The first 4 days of culture medium contained the antimitotic 5-bromodeoxyuridine (0.1 mmol/L) to inhibit fibroblast growth in addition to 5% FBS, penicillin/streptomycin, and vitamin B12. Twenty-four hours before experimentation, medium was changed to serum-free DMEM. All animals were used in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health. The experimental protocol for the present study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisville.

**Gene Transfer**

NRCMs were infected with replication-deficient adenoviruses carrying O-GlcNAcase gene (AdO-GlcNAcase, 48 hours), or green fluorescent protein (AdGFP, Vector Bioslabs), as described previously. Doses used include 0 and 100 multiplicities of infection (moi) of AdO-GlcNAcase or AdGFP. Twenty-four hours before experimentation, medium was changed to serum-free DMEM. Functional expression was confirmed by immunoblot analysis.

**Enzyme Inhibition**

NRCMs were treated with O-(2-acetamido-2-deoxy-O-glucopyranosyl) amino-N-phenylcarbamate (i.e., PUGNAc; 200 μmol/L), an inhibitor of O-GlcNAcase, or vehicle (0.1% ethanol) overnight and subjected to hypoxia/reoxygenation or total protein isolated. Sample size is equal to 6 per group per treatment.

**RNA Interference**

Cultured NRCMs were transfected with 60 mmol/L short interfering (si)RNA directed against O-GlcNAcase (siRNA ID no. 190811; with sense strand 5'-GCAACUUAGAUCAUGACtt-3' and antisense strand 3'-GAUGAGAGUCAUAGUGCCG-5') or scrambled sequence as a nonsilencing control (Ambion). Myocytes were transfected with SIPORT NeoFX transfection reagent according to the instructions of the manufacturer (Ambion). Seventy-two hours following transfection, total protein was isolated and immunoblotted for O-GlcNAcase protein and O-GlcNAc levels. Similarly treated myocytes were subjected to hypoxia/reoxygenation and posthypoxic mRNA harvested to assess cell injury.

**Protein Expression**

Total cellular proteins were isolated from NRCMs as described previously. NRCMs were washed with PBS, harvested in ice-cold lysis buffer (containing 68 mmol/L sucrose, 200 mmol/L mannitol, 50 mmol/L KCl, 1 mmol/L EGTA, 1 mmol/L DTA, and 5 mmol/L HEPES) with freshly added 0.2 μmol/L DTT, 0.1% (vol/vol) protease inhibitor stock, 0.4% (vol/vol) NP-40, 0.4% (vol/vol) Triton-X, and posttranslational modification inhibitor stock. Extracts were sonicated and the resulting lysates centrifuged at 15 000g for 5 minutes at 4°C to remove cell debris. Forty micrograms of protein (according to Bradford assay) was applied to each lane of a 6% SDS-PAGE electrophoretically onto poly(vinylidene fluoride) membranes for cyclophilin D (CypD), adenine nucleotide translocase (ANT), and voltage-dependent anion channel (VDAC), whereas 50 (CypD), adenine nucleotide translocase (ANT), and voltage-dependent anion channel (VDAC), whereas 50

**In Vitro Hypoxia/Reoxygenation Injury in Cardiac Myocytes**

Cardiac myocytes were subjected to hypoxia using 1× Esumi lethal ischemic media, pH 6.2 (containing 117 mmol/L NaCl, 12 mmol/L KCl, 0.49 mmol/L MgCl₂, 4 mmol/L HEPES, 20 mmol/L sodium lactate, and 5.6 mmol/L L-glucose) by sealing the myocytes in humidified hypoxic chambers (Billups-Rothenberg Inc.), flushing each chamber with a gas mixture consisting of 5% CO₂ and 95% N₂ for 15 minutes, and incubating the hypoxic chamber in a modular incubator at 37°C for 3 hours. Following hypoxia, the media were changed to 1× Esumi control media (pH 7.4, 137 mmol/L NaCl, 3.8 mmol/L KCl, 0.9 mmol/L CaCl₂, 0.49 mmol/L MgCl₂, 4 mmol/L HEPES, and 5.6 mmol/L L-glucose) and culture dishes were reoxygenated for 1 or 6 hours in the modular incubator or on the fluorescent microscope during imaging, as appropriate. Similarly treated NRCMs were subjected to 4 or 9 hours of normoxia in 1× Esumi control media to serve as normoxic/aerobic controls.

**Cell Death**

Cell death was assessed for NRCMs as previously described. Normoxic or posthypoxic LDH release was spectrophotometrically determined using a commercially available kit (Sigma) following hypoxia/reoxygenation, and the results were expressed as LDH release relative to total LDH in the cells and normalized to the appropriate controls (1 hour of reoxygenation data) or normoxic untreated control (for 6 hours of reoxygenation data). Similarly treated NRCMs were stained with the fluorescent DNA-binding dyes Hoechst 33342 (5 μg/mL) and propidium iodide (PI) (5 μg/mL) (Invitrogen) during the last 30 minutes of reoxygenation. The stained nuclei were then visualized using a ×20 objective on a Nikon-TE2000E2 fluorescence microscope, Xcite light source; 350/50 nm excitation and 470/40 nm emission filter for Hoechst and 560/40 nm excitation and 630/60 nm emission filter for PI. Four fields per treatment in triplicate were counted and data were expressed as percentage PI-positive nuclei/total nuclei. Because the nuclear stain Hoechst 33342 is membrane-permeable, it was used to determine total cells in each field and not as an index of apoptosis.

**Assessment of Mitochondrial Membrane Potential**

Using time-lapse fluorescence microscopy, detection of mitochondrial membrane potential changes was performed by following changes in tetramethylrhodamine methyl ester (TMRE) fluorescence treated with AdGFP, AdO-GlcNAcase, vehicle, PUGNAc, scrambled (Scr) RNAi or O-GlcNAcase RNAi as described previously.

**Assessment of Calcium Overload**

Calcium levels were assessed in NRCMs treated with AdGFP, AdO-GlcNAcase, vehicle, or PUGNAc and subjected to 3 hours of hypoxia using time-lapse fluorescent microscopy by following the changes in Rhod-2AM fluorescence. Cardiac myocytes were plated on 35 mm glass bottom culture dishes and loaded with 2 μmol/L Rhod-2AM before hypoxia/reoxygenation. Imaging was initiated at reoxygenation in isolated myocytes by exciting Rhod-2AM with an Xcite light source through a 546/11 nm bandpass filter and emission assessed through a 567/15 nm bandpass filter. Fluorescence intensity was monitored throughout the protocol every 90 seconds. All experimental groups were repeated in at least 4 separate isolations.
Results

O-GlcNAcase (AdO-GlcNAcase) Overexpression Exacerbates Posthypoxic Cardiac Myocyte Death

Forty-eight hours following AdO-GlcNAcase infection of isolated NRCMs (n=5 per group), total cellular proteins were isolated for O-GlcNAcase protein and O-GlcNAc levels (via western blot analysis). Adenoviral overexpression of O-GlcNAcase significantly (P<0.05) augmented O-GlcNAcase protein levels (Figure 1A). Such elevation corresponded with a significant (P<0.05) reduction in O-GlcNAc levels (48±7% of 0 moi AdO-GlcNAcase) (Figure 1B). Similar findings were seen with another O-GlcNAc antibody, RL2 (65±8% of 0 moi AdO-GlcNAcase; supplemental Figure V, A). Immunoblots for O-GlcNAc levels show multiple immunopositive bands because O-GlcNAc is a posttranslational modification, not a single protein (Figure 1C and supplemental Figure V, A). Previous studies have demonstrated similar findings in various cell lines and NRCMs. Equal protein loading was confirmed by densitometric analysis of Ponceau-stained membranes (see supplemental Figure VII).

To evaluate the effects of O-GlcNAcase overexpression on posthypoxic cardiac myocyte survival, similarly treated cardiac myocytes were subjected to hypoxia (3 hours) and reoxygenation (1 or 6 hours). Posthypoxic media was harvested for LDH release, and additional posthypoxic NRCMs were stained with PI to assess cell death. Myocytes infected with AdO-GlcNAcase were more sensitive to hypoxia-induced injury by the first hour of reoxygenation according to LDH release (150±23% of 0 moi AdO-GlcNAcase, P<0.05; supplemental Figure III, A) and PI positivity (17±2% versus 10±1% for AdGFP; supplemental Figure III, D) compared to 0 moi AdO-GlcNAcase or AdGFP, respectively.
To determine whether the detrimental effect of AdOGlcNAcase on posthypoxic cardiac myocytes was short-lived, similarly treated NRCMs were reoxygenated for 6 hours after 3 hours of hypoxia. The longer duration of reoxygenation (6 hours) still showed exacerbated cellular injury in AdOGlcNAcase NRCMs, according to LDH release (179 ± 14% versus 136 ± 8% for AdGFP, *P* < 0.05; Figure 1D) and PI positivity (34 ± 3% versus 24 ± 2% for AdGFP, *P* < 0.05; Figure 1E) compared to AdGFP.

Cell damage was not significantly different among AdOGlcNAcase, AdGFP, or uninfected NRCMs under normoxia according to LDH release (109 ± 3% of control versus 93 ± 3% of control, *P* > 0.05; supplemental Figure I, A) and PI positivity (14 ± 1% for AdOGlcNAcase versus 13 ± 1% for AdGFP versus 12 ± 1% for control, *P* > 0.05; supplemental Figure II, A). Such results confirm that viral infection does not affect cell survival or O-GlcNAc levels in this system.

**O-GlcNAcase Inhibition Attenuates Posthypoxic Cardiac Myocyte Injury**

NRCMs (n=6 per group) were treated with PUGNAc (O-GlcNAcase inhibitor) overnight before protein harvest, then immunoblotted for O-GlcNAc levels. PUGNAc significantly increased O-GlcNAc levels (878 ± 215% of control, *P* < 0.05) compared to vehicle (Figure 2A and 2B). Supplemental Figure V, B, contains results with an additional O-GlcNAc antibody (RL2).

Additional NRCMs (n=6 per group) were treated with PUGNAc, subjected to hypoxia/reoxygenation, and media harvested to measure LDH release. Inhibition of O-GlcNAcase (with PUGNAc) significantly attenuated posthypoxic LDH release 70 ± 10% of control, *P* < 0.05; supplemental Figure III, B) and PI positivity (9 ± 1% versus 13 ± 1% for vehicle, *P* < 0.05; supplemental Figure III, E) at the end of the first hour of reoxygenation.

The protective effect of augmented O-GlcNAc levels (with PUGNAc) was still seen at 6 hours of reoxygenation with LDH release (133 ± 11% for PUGNAc, *P* < 0.05 versus 177 ± 14% for vehicle; Figure 2C) and PI positivity (21 ± 2% versus 36 ± 2% of vehicle; Figure 2D). PUGNAc or vehicle treatment did not significantly alter normoxic/aerobic cellular viability compared with untreated NRCMs under normoxia according to LDH release (P > 0.05, 90 ± 8% of control versus 104 ± 8% of control; supplemental Figure I, B) and PI

![Image](https://example.com/image.png)

**Figure 2.** NRCMs were subjected to pharmacological repression of O-GlcNAcase activity. A, Representative immunoblots for O-GlcNAc levels following PUGNAc treatment (n=6 per group) show a significant increase in O-GlcNAc levels compared to vehicle. Multiple bands occur because O-GlcNAc is a posttranslational modification. B, Densitometric analyses of O-GlcNAc Western blots show significantly elevated O-GlcNAc levels compared to vehicle. C, O-GlcNAcase inhibition with PUGNAc diminished posthypoxic injury in NRCMs (according to LDH release) compared to vehicle. D, O-GlcNAcase inhibition with PUGNAc reduced posthypoxic injury (per PI positivity) compared to vehicle. *P* < 0.05 vs vehicle.
Knockdown of O-GlcNAcase Reduces Posthypoxic Cardiac Myocyte Injury

NRCMs (n=6 per group) were treated with 60 nmol/L O-GlcNAcase RNAi or Scr RNAi for 72 hours to knockdown O-GlcNAcase expression. O-GlcNAcase knockdown significantly (P<0.05) reduced O-GlcNAcase protein levels compared to Scr RNAi, despite no change in α-tubulin levels (Figure 3A). O-GlcNAcase RNAi significantly (P<0.05) augmented O-GlcNAc levels (132±12% of control, P<0.05) compared to Scr RNAi (Figure 3B and 3C). Additional NRCMs were treated with O-GlcNAcase or Scr RNAi, subjected to hypoxia/reoxygenation, and media harvested to measure LDH release. O-GlcNAcase RNAi significantly (P<0.05) reduced posthypoxic LDH release after 1 hour (72±10% of Scr RNAi; supplemental Figure III, C) and 6 hours (130±4% for O-GlcNAcase RNAi versus 170±26% for Scr RNAi; Figure 3D) of reoxygenation compared to Scr RNAi. In addition, O-GlcNAcase RNAi significantly diminished PI positivity at 6 hours (22±2% for O-GlcNAcase RNAi versus 33±2% for Scr RNAi, P<0.05; Figure 3E) compared with Scr RNAi. O-GlcNAcase or Scr RNAi treatment did not cause significant cell damage compared with untreated NRCMs under normoxia/aerobic conditions (supplemental Figures I, C, and II, C).

Effect of O-GlcNAcase Manipulation on Mitochondrial Membrane Potential Preservation

Based on our recent findings linking O-GlcNAc–mediated cardioprotection to mitochondria,7,8 we assessed the effects of altered O-GlcNAcase activity on posthypoxic mitochondrial membrane potential. NRCMs were treated with either AdGFP...
(48 hours, 100 mos), AdO-GlcNAcase (48 hours, 100 mos), vehicle (overnight), PUGNAc (before hypoxia/reoxygenation, 200 μmol/L), Scr RNAi (72 hours, 60 nmol/L), or O-GlcNAcase RNAi (72 hours, 60 nmol/L) and loaded with the mitochondrial membrane potential indicator TMRM (50 nmol/L). Cardiac myocytes were then subjected to either 4 hours of normoxia, or, 3 hours of hypoxia and 1 hour of reoxygenation. During the reoxygenation period, myocytes were evaluated for changes in mitochondrial membrane potential using time-lapse fluorescence microscopy. Dissipation of mitochondrial membrane potential is reflected by a loss of TMRM fluorescence. AdGFP-infected NRCMs loaded with TMRM maintained mitochondrial membrane potential over time under normoxia and showed no significant difference in mitochondrial membrane potential over time (supplemental Figure IV). Hypoxia/reoxygenation induced mitochondrial membrane potential loss. O-GlcNAcase overexpression aggravated the loss of mitochondrial membrane potential on reoxygenation, shown by impaired recovery of TMRM fluorescence (Figure 4A and 4B). Conversely, inhibition of O-GlcNAcase (via PUGNAc or O-GlcNAcase RNAi) attenuated the loss of mitochondrial membrane potential over time under normoxia and showed no significant difference in mitochondrial membrane potential over time (supplemental Figure IV). Hypoxia/reoxygenation induced mitochondrial membrane potential loss. O-GlcNAcase overexpression aggravated the loss of mitochondrial membrane potential on reoxygenation, shown by impaired recovery of TMRM fluorescence (Figure 4A and 4B). Conversely, inhibition of O-GlcNAcase (via PUGNAc or O-GlcNAcase RNAi) attenuated the loss of mitochondrial membrane potential over time under normoxia and showed no significant difference in mitochondrial membrane potential over time (supplemental Figure IV). Hypoxia/reoxygenation induced mitochondrial membrane potential loss.

Effect of O-GlcNAcase Manipulation on mPTP Component Expression
To test the hypothesis that O-GlcNAc-mediated protection involved alteration in expression of the putative major components of mPTP, we treated cells with the various interventions and harvested proteins. Lysates were immunoblotted for the likely mPTP components VDAC, ANT, and CypD. None
of the interventions significantly altered the expression of
VDAC, ANT, or CypD (Figure 5).

Effect of O-GlcNAcase Manipulation on
Calcium Levels
Cardiac myocytes were treated with either AdGFP (48 hours, 100 mois), Ad-O-GlcNAcase (48 hours, 100 mois), vehicle (overnight), or PUGNAc (overnight, 200 μmol/L), loaded with the mitochondrial calcium indicator Rhod-2AM (2 μmol/L) and subjected to 3 hours of hypoxia and one of reoxygenation. Time-lapse fluorescence microscopy was initiated at the beginning of reoxygenation for changes in mitochondrial calcium levels. Augmentation of calcium levels is reflected by an increase in Rhod-2AM fluorescence. Hypoxia sensitized myocytes to calcium overload.

O-GlcNAcase overexpression (Ad-O-GlcNAcase) exacerbated the hypoxia-induced calcium overload on reoxygenation reflected by a significant increase in Rhod-2AM fluorescence (Figure 6A and 6B), whereas O-GlcNAcase inhibition (PUGNAc) attenuated hypoxia-induced calcium overload compared to vehicle (Figure 6C and 6D).

Discussion
O-GlcNAc signaling has emerged as an integral element in the armamentarium of the cell against lethal stress. Previous work from this laboratory and others suggests that pharmacologically elevated O-GlcNAc levels are protective in cell models following hypoxia/oxidative stress and, in vivo, following myocardial ischemia/reperfusion. However, such studies were never able to achieve the standard of genetic gain- and loss-of-function insights on O-GlcNAc, as are available in the present work. Here, overexpression of O-GlcNAcase reduces O-GlcNAc levels, exacerbates the loss of mitochondrial membrane potential following hypoxia, and sensitizes myocytes to posthypoxic cell death. The present results also demonstrate that inhibition of O-GlcNAcase (via siRNA or PUGNAc) augments O-GlcNAc levels, enhances the recovery of mitochondrial membrane potential following hypoxia, and attenuates posthypoxic cell death.

Recently, Taylor et al identified a unique response of cells to O-GlcNAc levels, namely that O-GlcNAc is not a simple “readout” of glucose availability. These results were further confirmed by Cheung et al in Neuro-2a cells. Indeed, such insights are consistent with ongoing work from this laboratory that O-GlcNAc signaling represents a stress responsive program, not simply a fuel gauge. Such insights are particularly true in light of the data of Taylor et al and Cheung and Hart indicating that complete removal of glucose significantly augments O-GlcNAc levels. Because the hexosamine biosynthetic pathway produces the monosaccharide donor for the O-GlcNAcylation of proteins, several groups have manipulated this pathway as a means of better understanding the role of O-GlcNAc modification of proteins and have shown that enhanced hexosamine biosynthetic pathway flux is cytoprotective following acute stress.

Zachara at al first showed that enhanced O-GlcNAc levels improved cell survival following stress. Since then, we and others have reported on the protective role of enhanced O-GlcNAc levels in different systems. In the heart, Champanathanachai et al showed that inhibition of O-GlcNAcase with PUGNAc in NRCMs improved cardiac myocyte viability following hypoxia/reoxygenation. In several studies of the isolated perfused heart, Liu and colleagues showed that enhanced O-GlcNAc levels protected the heart against injury resulting from calcium paradox and hypoxia/
reoxygenation.20–22 We have also shown that pretreatment of mice with PUGNAc (an O-GlcNAcase inhibitor) reduced infarct size in vivo and that ischemic preconditioning augmented O-GlcNAc levels.7 Moreover, we recently showed that augmenting O-GlcNAc levels by overexpressing OGT attenuated posthypoxic injury, whereas inhibition of OGT (pharmacologically or genetically) in cardiomyocytes exacerbated posthypoxic injury at the mitochondrial level. Such findings are supported by the study by Champattanachai et al19 showing that overexpression of OGT attenuated loss of mitochondrial membrane potential induced by H2O2 and increased mitochondrial Bcl-2. Here, we show that manipulation of O-GlcNAcase to alter O-GlcNAc levels significantly affects cardiac myocyte survival following hypoxia, although we found no evidence for differences in apoptosis at 6 hours reoxygenation (see supplemental Figure VII).

From a molecular vantage point, we have identified VDAC,7,8 a putative member of the mitochondrial permeability transition pore, to be O-GlcNAc modified and also showed that enhanced O-GlcNAc levels attenuated calcium-induced mitochondrial permeability transition pore (mPTP) formation in adult cardiac mitochondria. The present study supports mitochondrial involvement as a potential mechanism in O-GlcNAc-mediated cardioprotection in that following hypoxia, reduction of O-GlcNAc levels (by O-GlcNAcase overexpression) diminished the recovery of mitochondrial membrane potential, whereas augmented O-GlcNAc levels using PUGNAc enhanced the recovery of mitochondrial membrane potential during reoxygenation. The calcium overload data further support potential involvement of mitochondria in O-GlcNAc signaling induced alterations in cell survival. Yet, there were no significant changes in total protein expression of the prominent, although putative, constituents of the mPTP. Whether the physical alteration of mPTP components by O-GlcNAc explains the protective effects observed remains the subject of ongoing investigation.

O-GlcNAcase structurally has both hexosaminidase and histone acetyltransferase domains and is functionally involved with the removal of O-GlcNAc from proteins and acetylation of free histones.28–30 Because O-GlcNAcase has been shown to be cleaved by caspase 3,30 an executioner caspase in apoptosis, into N-terminal hexosaminidase domain and C-terminal HAT domain, we hypothesize that cleavage of O-GlcNAcase might result in the loss of internal regulation of its hexosaminidase activity, thereby increasing the rate of removal of O-GlcNAc from proteins and hence the severity of posthypoxic injury. However, significant additional efforts should be directed toward understanding the molecular regulation of O-GlcNAc signaling.

The reductionist approach used in the present study allows clear focus on the role of O-GlcNAcase in hypoxia/reoxygenation injury. It is becoming increasingly evident that O-GlcNAc signaling exerts its influence based on context and such potentially differing effects deserve investigation.31,32 Ongoing pursuits will continue to identify the protein targets and attempt to elucidate how O-GlcNAc signaling might be altered in diabetes and aging, in contrast to acute events such as myocardial ischemia. Clearly, O-GlcNAc signaling war-
rants such continued and intensive attention in the cardiovascular system.

Acknowledgments
We acknowledge the expert technical assistance of Linda Harrison (University of Louisville).

Sources of Funding
This work is supported by NIH grant R01 083320, American Heart Association National Center Scientist Development Grant 0535270N, and Kentucky Science and Engineering Foundation grant KSEF-1677-RDE-011 (to S.P.J.). G.A.N. is an American Heart Association Predoctoral Fellow, Great Rivers Affiliate (0815502D). H.T.F. is an American Heart Association Postdoctoral Fellow, Great Rivers Affiliate (0825643D).

Disclosures
None.

References
Unique Hexosaminidase Reduces Metabolic Survival Signal and Sensitizes Cardiac Myocytes to Hypoxia/Reoxygenation Injury
Gladys A. Ngoh, Heberty T. Facundo, Tariq Hamid, Wolfgang Dillmann, Natasha E. Zachara and Steven P. Jones

Circ Res. 2009;104:41-49; originally published online November 20, 2008; doi: 10.1161/CIRCRESAHA.108.189431

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 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/104/1/41

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/11/26/CIRCRESAHA.108.189431.DC1

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/
SUPPLEMENT MATERIAL

Supplemental Figure Legends

Supplemental Figure I. NRCMs were infected with AdGFP (100 MOI, 48 hours), AdO-GlcNAcase (100 MOI, 48 hours), Vehicle (overnight), PUGNAc (200 µmol/L, overnight), Scr RNAi (60nmol/L, 72 hours), or O-GlcNAcase RNAi (60nmol/L, 72 hours), subjected to 3hrs of normoxia and reoxygenated for 6 hrs. Cell damage was assessed by measuring LDH release in the normoxic and reoxygenated media. O-GlcNAcase or GFP overexpression (A), Vehicle or PUGNAc treatment (B), and, Scrambled RNAi or O-GlcNAcase RNAi transfection (C) did not significantly (p>0.05) alter cellular viability compared to untreated NRCMs.

Supplemental Figure II. NRCMs were infected with AdGFP (100 MOI, 48 hours), AdO-GlcNAcase (100 MOI, 48 hours), Vehicle (overnight), PUGNAc (200 µmol/L, overnight), Scr RNAi (60nmol/L, 72 hours), or O-GlcNAcase RNAi (60nmol/L, 72 hours), subjected to 3hrs of normoxia and reoxygenated for 6 hrs. Cell damage was assessed by counting propidium iodide (PI) positive nuclei at the end of 6hrs of reoxygenation. There was no significant (p>0.05) difference in % PI positivity for O-GlcNAcase or GFP overexpression (A), Vehicle or PUGNAc treatment (B), and, Scrambled RNAi or O-GlcNAcase RNAi transfection (C) compared to untreated NRCMs.

Supplemental Figure III. NRCMs were infected with AdGFP(100 MOI, 48 hours), AdO-GlcNAcase (0 or 100 MOI, 48 hours), Vehicle (overnight), PUGNAc (200 µmol/L, overnight), Scr RNAi (60nmol/L, 72 hours), or O-GlcNAcase RNAi (60nmol/L, 72 hours), subjected to 3hrs of hypoxia and 1 hour reoxygenation. Cell death was assessed by measuring LDH release in the hypoxic/reoxygenation media and counting propidium iodide (PI) positive nuclei.
at the end of 1hrs of reoxygenation.  **A)** O-GlcNAcase overexpression significantly augmented (p<0.05) LDH release compared to 0 MOI AdO-GlcNAcase. *p<0.05 vs. 0 MOI AdO-GlcNAcase  **B)** O-GlcNAcase inhibition with PUGNAc minimized post-hypoxic cardiac myocyte injury according to LDH release. *p<0.05 vs. Vehicle.  **C)** NRCMs (n=6/group) treated with O-GlcNAcase RNAi were less sensitive to hypoxia-induced injury (according to LDH release).  **D)** O-GlcNAcase overexpression exacerbated post-hypoxic injury mirrored by augmented % PI positivity (n=4/group). *p<0.05 vs. 100 MOI AdGFP  **E)** O-GlcNAcase inhibition with PUGNAc reduced post-hypoxic injury (according to PI positivity. *p<0.05 vs. Vehicle.

**Supplemental Figure IV.** NRCMs (n>/=4/group) were infected with AdGFP (48 hours) and subjected to 4 hours of normoxia.  **A)** Quantification of average fluorescent intensity of mitochondrial-rich regions during the last hour of normoxia showed no significant change in mitochondrial membrane potential.  **B)** Representative montage of time-lapse imaging during the last hour of normoxia (i.e. reoxygenation). This represents the normoxic/aerobic control for Figure 3.

**Supplemental Figure V.** NRCMs were infected with nothing, AdGFP, AdO-GlcNAcase (48 hours), Vehicle or PUGNAc (200 µmol/L), and whole cell lysates immunoblotted for O-GlcNAc using RL2.  **A)** Densitometric analysis and representative immunoblots for O-GlcNAc levels following AdO-GlcNAcase infection (n=6/group) showed significant reduction in O-GlcNAc levels with 100 MOI AdO-GlcNAcase compared with 0 MOI.  **B)** O-GlcNAcase inhibition (with PUGNAc) significantly (p< 0.05) augmented O-GlcNAc levels compared with Vehicle. Multiple bands occur because O-GlcNAc is a post-translational modification.
**Supplemental Figure VI.** Because several immunoblots were from cropped images, we elected to show the original immunoblots to confirm the data were derived from single membranes containing both the control and treatment groups of interest. **A)** Original image for O-GlcNAcase immunoblot shown in Figure 1A. **B)** Original image for O-GlcNAc immunoblots shown in Figure 2A. **C)** Original image for O-GlcNAc immunoblots shown in Supplemental Figure VB.

**Supplemental Figure VII.** Ponceau staining to confirm equal protein loading.

**Supplemental Figure VIII.** Assessment of apoptosis after 6 hours of post-hypoxic reoxygenation in NRCMs subjected to the various treatments. Caspase activity did not significantly differ between groups at 6 hours of reoxygenation (n>=5/group).
Supplemental Figure I

A) Normoxia/Reoxygenation LDH

B) LDH Release (% of Control)

C) LDH Release (% of Control)
Supplemental Figure III

A) LDH Release (% of Control)

B) LDH Release (% of Vehicle)

C) % PI Positivity

D) % PI Positivity

- A) 
  - Control
  - AdO-GlcNAcase

- B) 
  - Vehicle
  - PUGNAc

- C) 
  - Scr
  - O-GlcNAcase

- D) 
  - Vehicle
  - PUGNAc
Supplemental Figure V

(A) O-GlcNAc Levels (% of Vehicle)

(B) O-GlcNAc Levels (% of Vehicle)

IB: O-GlcNAc
Figure 1A

<table>
<thead>
<tr>
<th>AdO-GlcNAcase (MOI)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2A

<table>
<thead>
<tr>
<th>PUGNAc (μM)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Supplemental Figure VI

B) PUGNAc (μM)

C) PUGNAc (μM)