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Augmented O-GlcNAc Signaling Attenuates Oxidative Stress and Calcium Overload in Cardiomyocytes

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Abstract

O-linked β -N-acetylglucosamine (O-GlcNAc) is an inducible, dynamically cycling and reversible post-translational modification of Ser/Thr residues of nucleocytoplasmic and mitochondrial proteins. We recently discovered that O-GlcNAcylation confers cytoprotection in the heart via attenuating the formation of mitochondrial permeability transition pore (mPTP) and the subsequent loss of mitochondrial membrane potential. Because Ca^{2+} overload and reactive oxygen species (ROS) generation are prominent features of post-ischemic injury and favor mPTP formation, we ascertained whether O-GlcNAcylation mitigates mPTP formation via its effects on Ca^{2+} overload and ROS generation. Subjecting neonatal rat cardiac myocytes (NRCMs, $n \geq 6$ /group) to hypoxia, or mice ($n \geq 4$ /group) to myocardial ischemia reduced O-GlcNAcylation, which later increased during reoxygenation/reperfusion. NRCMs ($n \geq 4$ /group) infected with an adenovirus carrying nothing (control), adenoviral O-GlcNAc transferase (adds O-GlcNAc to proteins, AdOGT), adenoviral O-GlcNAcase (removes O-GlcNAc to proteins, AdOGA), Vehicle, or PUGNAc (blocks OGA; increases O-GlcNAc levels), were subjected to hypoxia-reoxygenation or H_2O_2 and changes in Ca^{2+} levels (via Fluo-4AM and Rhod-2AM), ROS (via DCF), and mPTP formation (via calcein-MitoTracker Red colocalization) were assessed using time-lapse fluorescence microscopy. Both OGT and OGA overexpression did not significantly ($p > 0.05$) alter baseline Ca^{2+} or ROS levels. However, AdOGT significantly ($p < 0.05$) attenuated both hypoxia and oxidative stress-induced Ca^{2+} overload and ROS generation. Additionally, OGA inhibition mitigated both H_2O_2 -induced Ca^{2+} overload and ROS generation. Although AdOGA exacerbated both hypoxia and H_2O_2 -induced ROS generation, it had no effect on H_2O_2 -induced Ca^{2+} overload. We conclude that inhibition of Ca^{2+} overload and ROS generation (inducers of mPTP) might be one mechanism through which O-GlcNAcylation reduces ischemia/hypoxia-mediated mPTP formation.

Introduction

Ischemia-reperfusion injury is one of the major causes of morbidity and mortality in the western world. Calcium overload, oxidative stress, and the more recently implicated involvement of endoplasmic reticulum (ER) stress characterize pathologic components of ischemia-reperfusion injury. Additionally, ischemia-reperfusion injury also causes a wide variety of functional and structural changes to the mitochondria¹⁻⁵, including activation of the mitochondrial death pathway. The mitochondrial death pathway culminates with the formation of the mitochondrial permeability transition pore (mPTP), which represents a non-specific pore spanning both the outer and inner mitochondrial membranes that allows

molecules <1.5kDa to enter and exit the mitochondrial matrix. mPTP is activated by calcium overload and ROS, both of which are elevated in ischemia-reperfusion injury.

Manipulating the myocardium's response to ischemia-reperfusion is known to delay and/or reduce myocardial injury. Indeed, most cardioprotective interventions are known to mediate cytoprotection in part via attenuation of mPTP formation. Protein phosphorylation/dephosphorylation is one of the most studied biochemical aspects associated with these cardioprotective interventions. Interestingly, the novel post-translational sugar modification, O-linked β -N-acetylglucosamine (O-GlcNAc) has been shown in numerous studies and in different cell types to act as an inducible, cytoprotective stress response^{6, 7}. Our group⁸⁻¹² and others¹³⁻¹⁷ have shown that enhanced O-GlcNAcylation of proteins attenuates cardiomyocyte death and reduces infarct size in mice. Moreover, recent data from our laboratory reveal that O-GlcNAcylation is cardioprotective by attenuating mPTP formation^{8, 11, 12} and the activation of the maladaptive arm of the unfolded protein response⁹.

How O-GlcNAcylation mitigates mPTP formation is unknown. Therefore, we tested the hypothesis that O-GlcNAcylation reduces hypoxia-mediated mPTP formation via attenuating oxidative stress and Ca^{2+} overload in cardiomyocytes. We manipulated O-GlcNAc signaling and subjected myocytes to hypoxia-reoxygenation or oxidative stress to determine whether O-GlcNAcylation affected post-hypoxic or oxidative stress-induced ROS generation and Ca^{2+} overload. Our findings suggest that O-GlcNAcylation may attenuate mPTP formation by reducing Ca^{2+} overload and ROS generation.

Materials and Methods

Murine *in vivo* ischemia-reperfusion

Three month-old male C57BL6/J mice were subjected to *in vivo* coronary artery ischemia-reperfusion for assessment of O-GlcNAcylation levels according to a well-established protocol^{12, 18-39}. 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)-60 tubing connected to a mouse ventilator (Harvard Apparatus) and set the tidal volume and breathing rate set by standard allometric equations. The mice were supplemented with 100% oxygen via the ventilator side port. Body temperature was maintained between 36.5°C and 37.5°C using an electrically controlled rectal probe and a heat lamp. A left thoracotomy was performed using a thermal cautery, and the proximal left coronary artery was visualized with the aid of a dissecting microscope and completely occluded for 40 minutes with 7-0 silk suture mounted on a tapered needle (BV-1, Ethicon). After 40 minutes, the suture was removed and reperfusion was initiated and visually confirmed. The chest was closed in layers using 4-0 silk suture. The skin was closed using 4-0 nylon suture. Ketoprofen was given as analgesia prior to closing the chest. Upon recovery of spontaneous breathing, mice were removed from the ventilator, extubated and allowed to recover in a warm, clean cage supplemented with 100% oxygen.

Myocardial ischemic/non-ischemic zone determination

Following the 40 minute period of ligation of the left coronary artery in mice as described above, reperfusion was initiated. After zero, one, four, and twenty-four hours of reperfusion, mice were anesthetized with isoflurane. A tracheotomy was performed and mice were ventilated as described above. 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 coronary artery was re-ligated 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 non-ischemic zone. The heart was rapidly excised and serially sectioned along the long axis in 1-mm-thick sections. With the aid of a dissecting microscope, the non-ischemic and ischemic zones were separated and O-GlcNAcylation levels were assessed.

Neonatal rat cardiac myocyte isolation and culture

Neonatal rat cardiac myocytes (NRCMs) were isolated from 1-2 day old Sprague-Dawley rats and cultured according to an established protocol^{8, 9, 11, 40-43}. Cardiac myocytes in DMEM culture medium were plated on 35 mm glass bottom culture dishes at 75% density. During the first four days of culture, the medium contained the anti-mitotic, BrdU (0.1 mmol/L), to inhibit any potential fibroblast growth in addition to 5% fetal bovine serum, penicillin/streptomycin, and vitamin B₁₂. Twenty-four hours prior to experimentation, the medium was changed to serum-free DMEM. All experiments were performed on at least four separate NRCM cultures.

Gene transfer

NRCMs were infected for twenty-four hours with 100 MOI of replication-deficient adenoviruses carrying either the rat O-GlcNAc transferase gene (AdOGT), the rat O-GlcNAcase gene (AdOGA), or the green fluorescent protein (AdGFP; Vector Biolabs), as described previously^{8, 9, 11, 42}. Medium was changed to serum-free DMEM and myocytes were subjected to hypoxia-reoxygenation or challenged with H₂O₂. Sample size is equal to at least four per group per treatment.

Enzyme inhibition

NRCMs were treated overnight with *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene) amino-N-phenylcarbamate^{8, 9, 12, 44} (i.e., PUGNAc, 200 μmol/L) an inhibitor of OGA, or Vehicle (0.1% ethanol), prior to the H₂O₂ challenge or hypoxia-reoxygenation. Sample size is equal to at least four per group per treatment.

In vitro hypoxia-reoxygenation injury in cardiac myocytes

Untreated cardiac myocytes were subjected to six hours of hypoxia in Esumi Lethal Media in humidified hypoxic chambers (Billups-Rothenberg, Inc) as previously described^{9, 11, 45}. Following hypoxia, the media was changed to Esumi Control Media^{8, 11} and culture dishes were reoxygenated for zero, one, or six hours in the modular incubator. Normoxic/aerobic controls were achieved by subjecting NRCMs to six hours of normoxia and six hours of reoxygenation in Esumi control media. Total cellular protein was isolated and immunoblotted for O-GlcNAc modification. Sample size is equal to six per group per treatment.

Induction of oxidative stress

Oxidative stress was induced in cultured NRCMs by adding 100 μmol/L H₂O₂, mixing and immediately imaging.

Protein expression

Total cellular proteins were isolated from NRCMs and heart sections, as previously described. Fifty micrograms of protein (according to Bradford assay) were applied to each lane of a 10% SDS-PAGE gel that was then electroblotted onto a PVDF membrane and probed for O-GlcNAc. A solution of 5% (v/v) reagent-grade nonfat milk in Tris buffered saline was used for blocking, before incubating it in anti-O-GlcNAc antibody (1:1000 CTD 110.6; Covance) overnight at 4°C. The blot was then incubated for one hour with 0.05 μg/mL

of goat anti-mouse IgM HRP-conjugated secondary antibody and detected with an enhanced chemiluminescent detection system (Pierce).

Enzymatic labeling of O-GlcNAc-modified proteins

O-GlcNAc modified proteins were labeled using Invitrogen's Click-iT enzymatic labeling kit, as previously described¹¹. Briefly, detergents were precipitated out of 200 µg of whole cell lysate (n=6/group) using the chloroform/methanol precipitation method. The resulting pellets were then covered with lint-free paper and allowed to dry for 15 mins in fume hood. The dried pellets was resuspended in 40 µL of 1%, and allowed to cool on ice for 3 mins. O-GlcNAc-modified proteins were then labeled with UDP-GalNAz (azide-modified UDP-N-acetylgalactosamine) using mutant β-1-4-galactosyltransferase at 4°C overnight. The next day, the GalNAz-labeled O-GlcNAc-modified protein mixture was precipitated using the chloroform/methanol precipitation method and resuspended in 50 µL of buffer containing 1% SDS and 50 mmol/L Tris-HCl (pH 8). The azide-labeled proteins were tagged with a fluorescent dye, TAMRA-alkyne for one hour at 4°C for the conversion of the azide group to a stable triazole conjugate. 25mM DTT was added for 15 mins to stop the reaction and proteins were precipitated using the chloroform/methanol precipitation method. The dried-labeled protein sample was resuspended in SDS-PAGE buffer for electrophoresis. The gel was then imaged by excitation using a 532nm laser on a TYPHOON 9400 imager.

Fluorescence Microscopy

All fluorescent probes were purchased from Invitrogen. NRCMs were imaged in imaging/reoxygenation medium (DMEM with HEPES and minus phenol red and pyruvate). Images were captured using a Photometrics CoolSNAP ES camera attached to a Nikon-TE2000E2 fluorescence microscope with a T-PFS Perfect Focus Unit, all controlled with MetaMorph 6.3r2 software. The Perfect Focus System was used to prevent minute defocusing caused by changes over time during time-lapse imaging. Xcite 120 Fluor light source (level of 12%) was used and a Plan Apo 60xA/oil (NA=1.4) objective was used for magnification. Neutral density filter setting was set at ND4 and binning of 2x for all image acquisition. Images were captured every 90 seconds for 60 minutes. Exposure duration, excitation filters, and emission filters varied depending on the fluorescent dye, as mentioned below.

Assessment of reactive oxygen species (ROS) production

ROS levels were assessed in NRCMs using time-lapse fluorescence microscopy by following the changes in 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCF) fluorescence⁴². NRCMs were loaded with 2 µmol/L DCF for 30 minutes at 37°C and medium was changed to imaging/reoxygenation medium. For certain normoxic NRCM cultures, oxidative stress was induced and imaging was initiated immediately by excitation of DCF through 470/40 nm bandpass filter and emission through 522/40 nm bandpass filters. Exposure duration was set at 100 ms and all experimental groups were repeated in at least four separate isolations.

Assessment of calcium overload

Intracellular calcium overload was assessed in NRCMs using time-lapse fluorescence microscopy by following the changes in rhod-2^{8, 42, 46} and fluo-4 fluorescence⁴⁶. For the hypoxia-reoxygenation study, NRCMs were loaded with 2 µmol/L rhod-2AM or 1 µmol/L fluo-4AM prior to hypoxia and imaging was initiated during reoxygenation. For oxidative stress studies, NRCMs were loaded with 1 µmol/L rhod-2AM (used to assess mitochondrial calcium) or 1 µmol/L fluo-4AM (used to assess cytosolic calcium) for 30 minutes at 37°C and media was changed to imaging media. NRCMs were then treated with 100 µmol/L H₂O₂ and imaging was immediately initiated by sequentially exciting rhod-2 and fluo-4

through 560/28 nm and 470/40 nm bandpass filters respectively. Emission was sequentially assessed through 646/38 nm (for rhod-2) and 522/40 nm (for fluo-4) bandpass filters. Exposure duration was set at 100 ms for both rhod-2 and fluo-4. All experimental groups were repeated in at least five separate NRCM cultures.

Assessment of mitochondrial permeability transition pore (mPTP)

Formation of mPTP was assessed in NRCMs using time-lapse fluorescence microscopy by following the changes in calcein fluorescence using a modified cold and warm loading protocol from Lemaster's group⁴⁷. NRCMs were loaded with 0.5 $\mu\text{mol/L}$ calcein-AM for 180 minutes at 4°C. Next, NRCMs were loaded with 1 $\mu\text{mol/L}$ MitoTracker Red FM at 37°C for 60 minutes. NRCMs were then washed and oxidative stress induced with 50 or 100 $\mu\text{mol/L}$ H_2O_2 . Imaging was initiated immediately afterwards by excitation of calcein through 470/40 nm bandpass filter and emission through 522/40 nm bandpass filter. Excitation for MitoTracker Red was through a 560/28 nm bandpass filter while emission was through 646/38 nm bandpass filter. Exposure duration was set at 50 ms for calcein and 100 ms for MitoTracker Red. Mitochondrial calcein fluorescence was determined using MetaMorph software in regions where both MitoTracker Red and calcein fluorescence colocalized. Mitochondrial calcein localization was assessed by measuring the standard deviation (SD) of the intensity of all green fluorescent pixels within the same region of interest using MetaMorph software. Each given region of interest had several mitochondria, each undergoing mPTP at different times. The SD reflected how the calcein fluorescence intensity of each mitochondrion varied from the average fluorescent intensity for that region of interest. A region of interest was defined by drawing a circle ($43.61 \mu\text{m}^2$) enclosing several mitochondria. SD is high when fluorescence is punctate and low when fluorescence is diffuse. All experimental groups were repeated in at least five separate isolations.

Statistical analyses

Data were analyzed using one-way ANOVA and/or Dunnett's *t* tests using GraphPad Prism 4 software. Data are reported as mean $\pm$ standard error of the mean with differences between treatment groups accepted as significant when $p < 0.05$.

Results

O-GlcNAc signaling changes during myocardial ischemia-reperfusion

We and others have shown that O-GlcNAcylation of proteins serves as an endogenously recruitable stress response⁷. Moreover, studies from Chatham's group show that in isolated perfused rat hearts, simulated ischemia augments O-GlcNAcylation⁴⁸⁻⁵⁰. Because nothing is known about *in vivo* changes in O-GlcNAcylation during myocardial ischemia-reperfusion injury, we directly addressed this question. We used the exogenous enzymatic labeling of O-GlcNAcylated proteins (click chemistry technique) to assess changes in O-GlcNAcylation levels in the ischemic (area at risk, $n \geq 4/\text{group}$) and non-ischemic zones ($n \geq 4/\text{group}$) of mice subjected to 40 minutes of myocardial ischemia (MI) via left coronary artery ligation or sham surgery and reperused for zero (i.e. harvested after 40 minutes of ischemia without removing the ligature), one, four, or twenty-four hours. Levels of O-GlcNAcylated proteins in the ischemic zone were reduced after 40 minutes of myocardial ischemia (MI group) compared to the sham group (Figure 1B, $p < 0.05$, representative images shown in 1A). After one hour of reperfusion, O-GlcNAcylation levels in the ischemic zone were 11.6-fold higher than Sham and dropped to baseline levels after 4 hrs or 24 hrs of reperfusion (Figure 1B). Despite such changes in the ischemic zone of hearts from the MI group, there was no significant change in O-GlcNAcylated proteins in the non-ischemic (i.e. remote) zone from either MI or sham groups following ischemia or throughout reperfusion (Figure 1C). To correlate the level of O-GlcNAcylated proteins to the amount of damage suffered by the

myocardium plasma was harvested prior to sacrifice in all groups. Using an ELISA-based technique, plasma levels of cardiac troponin-I (cTnI, an intracellular protein released from cardiomyocytes following damage or death) were measured (Figure 1D).

O-GlcNAcylation levels are altered following hypoxia-reoxygenation

To characterize the changes in O-GlcNAcylation during hypoxia and reoxygenation, we isolated NRCMs and subjected them to hypoxia and reoxygenation for 0, 1, or 6 hours. Hypoxia significantly decreased O-GlcNAc levels compared to normoxia (Figure 2A, $p < 0.05$, representative images shown in B). Upon reoxygenation, we observed a time dependent increase in O-GlcNAc levels peaking after 6 hours (Figure 2A, representative image shown in 2B) and falling by 18 hours of reoxygenation (data not shown).

Hypoxia damaged cardiac myocytes as reflected by an increase in post-hypoxic LDH release compared to normoxia. Reoxygenation further exacerbated hypoxia-mediated myocyte injury, mirrored by the time dependent increase in post-hypoxic LDH release for hypoxic myocytes compared to normoxia (Figure 2C).

Manipulation of O-GlcNAc signaling alters oxidative stress

ROS are important contributors to ischemia-reperfusion injury and interventions that prevent a rise in ROS generation reduce myocardial ischemia-reperfusion injury. Several studies have shown that the addition of antioxidants or ROS scavengers delay the onset or attenuate ischemia-reperfusion injury⁵¹⁻⁵³. Therefore, we evaluated whether or not O-GlcNAc-mediated cardioprotection could be partially attributed to a reduction in oxidative stress-mediated ROS generation. NRCMs were treated with an adenovirus carrying the Null, OGT, or OGA genes, or, treated with vehicle or PUGNAc. NRCMs were then subjected to three hours of hypoxia or oxidative stress induced with 100 $\mu\text{mol/L}$ hydrogen peroxide (H_2O_2). A change in DCF fluorescence during reoxygenation (or immediately following H_2O_2 treatment) indicates changes in ROS levels. Hypoxia induced a significant ($p < 0.05$) increase in ROS generation in NRCMs, reflected by increased DCF fluorescence (Figure 3A). This effect was significantly ($p < 0.05$) attenuated by overexpression of OGT (Figure 3A-C) or exacerbated by OGA overexpression. Additionally, inhibition of OGA with PUGNAc significantly reduced post-hypoxic ROS production (Figure 3D-F).

Similarly, H_2O_2 treatment induced significant ROS generation, which was mitigated by OGT overexpression and worsened by OGA overexpression according to DCF fluorescence (Figure 4A-C). The protective effects of augmented O-GlcNAc signaling on ROS levels were also observed when O-GlcNAc signaling was increased via pharmacologic OGA inhibition using PUGNAc (Figure 4D-F). Despite the changes in ROS generation observed following hypoxia or peroxide treatment, there was no difference in ROS levels with either genetic (AdOGT or AdOGA) or pharmacologic (PUGNAc) manipulation of O-GlcNAc signaling during normoxia (data not shown).

Under normoxic conditions, basally generated ROS are efficiently detoxified by endogenous enzymatic antioxidants such as superoxide dismutase, glutathione peroxidase, and catalase^{54, 55}. However, during ischemia-reperfusion injury, there is excess ROS generation such that ROS generated exceeds the capacity of endogenous oxidant defense mechanisms to detoxify ROS and hence leading to deleterious ROS-mediated reactions. Accordingly, we ascertained whether altered O-GlcNAcylation affected baseline expression of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (Cat). qRT-PCR for Cat, GPX, SOD1 and SOD2 mRNA levels was performed on normoxic NRCMs treated with AdGFP, AdOGA, Vehicle or PUGNAc. OGT overexpression (AdOGT) did not significantly alter ($p > 0.05$) GPX, SOD1, SOD2, and Cat expression

(Figure 5A). Overexpression of OGA (AdOGA) did not significantly change GPX, SOD1 or SOD2 expression, though catalase was significantly reduced (Figure 5A, $p < 0.05$). Likewise, inhibition of OGA with PUGNAc significantly increased catalase mRNA levels without changing SOD2 (Figure 5B, $p < 0.05$).

O-GlcNAc signaling attenuates hypoxic/oxidative stress induced Ca^{2+} overload

Several studies have implicated Ca^{2+} overload as a key contributor to mitochondrial permeability transition leading to ischemia-reperfusion injury. Interventions that reduce or delay the rise in Ca^{2+} can block or delay myocardial death. In addition, inhibiting the rise in mitochondrial Ca^{2+} confers cardioprotection following acute ischemia^{56, 57}. Moreover, dysregulation of mitochondrial Ca^{2+} is known to induce mPTP formation^{58, 59}. Accordingly, we assessed whether O-GlcNAcylation affects Ca^{2+} overload following hypoxia or oxidative stress in NRCMs ($n \geq 6$ /group). OGT overexpression did not alter baseline cytosolic or mitochondrial Ca^{2+} levels (data not shown) but significantly attenuated hypoxia-induced cytosolic (Figure 6A-C) and mitochondrial (Figure 6D-F) Ca^{2+} overload according to fluo-4 and rhod-2 fluorescence, respectively. The protective effect of augmented O-GlcNAcylation on both post-hypoxic cytosolic and mitochondrial Ca^{2+} overload was absent during the first eleven minutes of reoxygenation (Figure 6C and 6F respectively). Although fluo-4 and rhod-2 are generally accepted as cytosolic and mitochondrial calcium indicators (respectively), this is not a point of emphasis for the present study. Our point is to show two different indicators of *intracellular* calcium overload.

Augmented OGT expression significantly attenuated H_2O_2 -induced cytosolic (Figure 7A-C) and mitochondrial (Figure 8A-C) Ca^{2+} overload. Although OGA overexpression did not affect H_2O_2 -induced cytosolic (Figure 7A-C) or mitochondrial Ca^{2+} (Figure 8A-C), OGA inhibition with PUGNAc significantly reduced H_2O_2 -induced cytosolic (Figure 7D-F) and mitochondrial (Figure 8D-F) Ca^{2+} overload. To determine whether or not the lack of effect on H_2O_2 -induced cytosolic and mitochondrial Ca^{2+} overload observed with OGA overexpression was because of maximally induced Ca^{2+} overload, a lower concentration (50 $\mu\text{mol/L}$) of H_2O_2 was used to induce oxidative stress. There was no difference in either cytosolic (Figure 9A/C) or mitochondrial Ca^{2+} (Figure 9B/C) overload induced by H_2O_2 . Thus, the mechanism of exaggerated cell death in the context of elevated OGA activated may differ from loss of OGT activity (despite the fact that both treatments reduce O-GlcNAc levels).

O-GlcNAc signaling mitigates H_2O_2 induced mPTP formation

With mounting evidence suggesting that mPTP opening may be critical for the transition from reversible to irreversible myocardial ischemia-reperfusion injury², inhibition of mPTP formation likely represents an effective therapeutic approach in protecting the heart from reperfusion-induced damage. Indeed, direct and indirect inhibitors of mPTP opening have been shown to improve cardiac myocyte recovery after ischemia-reperfusion injury⁶⁰⁻⁶². Therefore, we investigated whether manipulation of O-GlcNAc signaling affected oxidative stress induced mPTP opening using calcein in NRCMs. Fluorescent calcein is concentrated in the mitochondrial matrix and can be used to detect mPTP opening⁶³, as reflected by change from punctate to diffuse calcein fluorescence upon H_2O_2 exposure, despite there being no change in MitoTracker red fluorescence (Figure 10A-C). H_2O_2 mediated mPTP opening was significantly delayed and attenuated by OGT overexpression (Figure 10A), while OGA overexpression accelerated and worsened mPTP formation (Figure 10A) compared to AdNull + H_2O_2 , though not significantly. Additionally, OGA inhibition (PUGNAc) significantly mitigated mPTP formation (Figure 10D-F) and CsA, a known mPTP blocker, significantly inhibited H_2O_2 -mediated mPTP opening (data not shown).

Discussion

The salient findings of the present study are as follows: 1. Ischemia/hypoxia reduced, while reperfusion/reoxygenation augmented O-GlcNAcylated proteins. 2. OGT overexpression reduced hypoxia and oxidative stress-induced cytosolic and mitochondrial Ca^{2+} overload. 3. Augmented O-GlcNAcylation (AdOGT and PUGNAc) attenuated hypoxia and oxidative stress-induced ROS production, while diminished O-GlcNAcylation (AdOGA) exacerbated it. 4. Enhanced O-GlcNAcylation suppressed mPTP opening in cardiac myocytes following oxidative stress, while reduced O-GlcNAcylation sensitized to it. These results demonstrate that a potential mechanism through which O-GlcNAcylation mitigates mPTP is by blocking Ca^{2+} overload and ROS generation.

In addition to its roles in signal transduction and in regulation of redox cell signaling, ROS generation contributes to myocardial ischemia-reperfusion injury⁶⁴. Enhanced O-GlcNAcylation of proteins (AdOGT and PUGNAc) mitigated both hypoxia- and oxidative stress-mediated ROS generation while AdOGA exacerbated hypoxia-induced ROS generation. One might conjecture that O-GlcNAcylation attenuates ROS generation by upregulating the expression and/or activity of the antioxidant enzymes catalase, glutathione peroxidase, and superoxide dismutase. Moreover, forkhead box O1 (FoxO1), a regulator of the transcription of the oxidative stress responsive enzymes catalase and MnSOD (SOD2), was shown recently to be O-GlcNAc modified⁶⁵. O-GlcNAcylation of FoxO1 activates FoxO1 and can in turn lead to activation of transcription of oxidative stress response enzymes Cat and MnSOD. Here, we show that neither augmented (AdOGT and PUGNAc) nor diminished (AdOGA) O-GlcNAcylation significantly altered baseline mRNA levels of superoxide dismutase or glutathione peroxidase. However, inhibition of OGA (PUGNAc) augmented, while overexpression of OGA, reduced baseline catalase mRNA. The alteration in Cat expression with OGA manipulation partially explains the exacerbated post-hypoxic ROS generation seen with OGA overexpression and reduced ROS generation with OGA inhibition. Likewise, OGA inhibition reduced oxidant stress-induced ROS production but OGA overexpression surprisingly did not seem to have a negative effect in the same system.

Another important finding in the present study is that enhanced O-GlcNAcylation attenuates post-hypoxic and oxidative stress mediated Ca^{2+} overload. These data support previous findings by our group showing that augmented O-GlcNAcylation, via inhibition of OGA (PUGNAc), mitigated hypoxia-induced calcium overload, while reduction of O-GlcNAcylation, via OGA overexpression, worsened hypoxia-induced Ca^{2+} overload. Additionally, Nagy *et al.* previously showed that enhanced O-GlcNAcylation with glucosamine treatment blocked angiotensin II-induced cytosolic Ca^{2+} overload in neonatal cardiomyocytes and that such beneficial effects were dependent on OGT⁶⁶.

How O-GlcNAcylation attenuates Ca^{2+} overload in acute stress is unknown. Because a primary source for the rise in intracellular Ca^{2+} during/following ischemia or hypoxia might be via reverse functioning NCX, it is possible that enhanced O-GlcNAcylation levels mitigate cytosolic Ca^{2+} overload via indirect effects on an upstream activator of NCX or direct effect on NCX. Other possible mechanisms may be enhanced SERCA2a activity and/or decreased phospholamban binding to SERCA2a. Such hypotheses remain to be tested but are certainly worthy of investigation.

Enhanced O-GlcNAcylation also attenuates mitochondrial Ca^{2+} overload. Ca^{2+} likely enters the mitochondria via the mitochondrial uniporter. Because there may exist a mitochondrial OGT isoform (mOGT), which is thought to be membrane bound, it is possible that mOGT interacts with the mitochondrial Ca^{2+} uniporter preventing Ca^{2+} uptake into the

mitochondria. Therefore, understanding how O-GlcNAcylation affects Ca^{2+} handling in the heart is critical in the determination of how O-GlcNAcylation modulates mPTP.

The mPTP appears to be critical for the transition from reversible to irreversible myocardial ischemia-reperfusion injury². ROS and Ca^{2+} activate mPTP and both of these are elevated during myocardial ischemia and reperfusion. Numerous cardioprotective pathways work primarily by inhibition of mPTP formation directly or indirectly by preventing the conditions that promote mPTP formation. The present study demonstrates that augmented O-GlcNAcylation (AdOGT or PUGNAc) attenuates mPTP formation in NRCMs, while diminished O-GlcNAcylation sensitizes NRCMs to mPTP formation. These data support previous findings by our group showing that enhanced O-GlcNAcylation reduces cardiac mitochondria to mPTP formation, while diminished O-GlcNAcylation sensitizes them to mPTP formation. Based on the present data, it is possible that one way through which O-GlcNAcylation mitigates mPTP formation might be through indirect means by attenuation of Ca^{2+} overload and ROS generation.

In conclusion, it is abundantly clear that O-GlcNAcylation represents a significant mechanism in the heart's ability to successfully respond to a variety of stressors⁷. Future studies will identify calcium handling and mitochondrial proteins that are O-GlcNAcylated and attempt to elucidate how O-GlcNAcylation mitigates ROS and Ca^{2+} overload in acute myocardial ischemia.

References

1. Halestrap AP. Does the mitochondrial permeability transition have a role in preconditioning? *Circulation*. 2004; 110:e303. author reply e303. [PubMed: 15364824]
2. Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem J*. 1999; 341:233–249. [PubMed: 10393078]
3. Crow MT, Mani K, Nam YJ, Kitsis RN. The mitochondrial death pathway and cardiac myocyte apoptosis. *Circ Res*. 2004; 95:957–970. [PubMed: 15539639]
4. Di Lisa F, Menabo R, Canton M, Barile M, Bernardi P. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic nad⁺ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. *J Biol Chem*. 2001; 276:2571–2575. [PubMed: 11073947]
5. Wang G, Liem DA, Vondriska TM, Honda HM, Korge P, Pantaleon DM, Qiao X, Wang Y, Weiss JN, Ping P. Nitric oxide donors protect murine myocardium against infarction via modulation of mitochondrial permeability transition. *Am J Physiol Heart Circ Physiol*. 2005; 288:H1290–1295. [PubMed: 15528225]
6. Zachara NE, O'Donnell N, Cheung WD, Mercer JJ, Marth JD, Hart GW. Dynamic o-glcnae modification of nucleocytoplasmic proteins in response to stress: A survival response in mammalian cells. *J Biol Chem*. 2004; 279:30133–30142. [PubMed: 15138254]
7. Ngoh GA, Facundo HT, Zafir A, Jones SP. O-glcnae signaling in the cardiovascular system. *Circ Res*. 2010; 107:171–185. [PubMed: 20651294]
8. Ngoh GA, Facundo HT, Hamid T, Dillmann W, Zachara NE, Jones SP. Unique hexosaminidase reduces metabolic survival signal and sensitizes cardiac myocytes to hypoxia/reoxygenation injury. *Circ Res*. 2009; 104:41–49. [PubMed: 19023128]
9. Ngoh GA, Hamid T, Prabhu SD, Jones SP. O-glcnae signaling attenuates er stress-induced cardiomyocyte death. *Am J Physiol Heart Circ Physiol*. 2009; 297:H1711–1719. [PubMed: 19734355]
10. Ngoh GA, Jones SP. New insights into metabolic signaling and cell survival: The role of o-glcnae. *J Pharmacol Exp Ther*. 2008
11. Ngoh GA, Watson LJ, Facundo HT, Dillmann W, Jones SP. Non-canonical glycosyltransferase modulates post-hypoxic cardiac myocyte death and mitochondrial permeability transition. *Journal of Molecular and Cellular Cardiology*. 2008; 45:313–325. [PubMed: 18539296]

12. Jones SP, Zachara NE, Ngoh GA, Hill BG, Teshima Y, Bhatnagar A, Hart GW, Marban E. Cardioprotection by n-acetylglucosamine linkage to cellular proteins. *Circulation*. 2008; 117:1172–1182. [PubMed: 18285568]
13. Champattanachai V, Marchase RB, Chatham JC. Glucosamine protects neonatal cardiomyocytes from ischemia-reperfusion injury via increased protein-associated o-glcnaC. *Am J Physiol Cell Physiol*. 2007; 292:C178–187. [PubMed: 16899550]
14. Champattanachai V, Marchase RB, Chatham JC. Glucosamine protects neonatal cardiomyocytes from ischemia-reperfusion injury via increased protein o-glcnaC and increased mitochondrial bcl-2. *Am J Physiol Cell Physiol*. 2008; 294:C1509–1520. [PubMed: 18367586]
15. Yang S, Zou LY, Bounelis P, Chaudry I, Chatham JC, Marchase RB. Glucosamine administration during resuscitation improves organ function after trauma hemorrhage. *Shock*. 2006; 25:600–607. [PubMed: 16721268]
16. Zou L, Yang S, Champattanachai V, Hu S, Chaudry IH, Marchase RB, Chatham JC. Glucosamine improves cardiac function following trauma-hemorrhage by increased protein o-glcnaCylation and attenuation of nf- κ b signaling. *Am J Physiol Heart Circ Physiol*. 2009; 296:H515–523. [PubMed: 19098112]
17. Zou L, Yang S, Hu S, Chaudry IH, Marchase RB, Chatham JC. The protective effects of pugnac on cardiac function after trauma-hemorrhage are mediated via increased protein o-glcnaC levels. *Shock*. 2007; 27:402–408. [PubMed: 17414423]
18. Palazzo AJ, Jones SP, Girod WG, Anderson DC, Granger DN, Lefer DJ. Myocardial ischemia-reperfusion injury in cd18- and icam-1-deficient mice. *Am J Physiol*. 1998; 275:H2300–2307. [PubMed: 9843832]
19. Palazzo AJ, Jones SP, Anderson DC, Granger DN, Lefer DJ. Coronary endothelial p-selectin in pathogenesis of myocardial ischemia-reperfusion injury. *Am J Physiol*. 1998; 275:H1865–1872. [PubMed: 9815095]
20. Jones SP, Girod WG, Granger DN, Palazzo AJ, Lefer DJ. Reperfusion injury is not affected by blockade of p-selectin in the diabetic mouse heart. *Am J Physiol*. 1999; 277:H763–769. [PubMed: 10444504]
21. Jones SP, Girod WG, Palazzo AJ, Granger DN, Grisham MB, Jourdain'Heuil D, Huang PL, Lefer DJ. Myocardial ischemia-reperfusion injury is exacerbated in absence of endothelial cell nitric oxide synthase. *Am J Physiol*. 1999; 276:H1567–1573. [PubMed: 10330240]
22. Girod WG, Jones SP, Sieber N, Aw TY, Lefer DJ. Effects of hypercholesterolemia on myocardial ischemia-reperfusion injury in ldl receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. 1999; 19:2776–2781. [PubMed: 10559025]
23. Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Klevitsky R, Hewett TE, Jones SP, Lefer DJ, Peng CF, Kitsis RN, Molkentin JD. The mek1-erk1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. *Embo J*. 2000; 19:6341–6350. [PubMed: 11101507]
24. Jones SP, Trocha SD, Strange MB, Granger DN, Kevil CG, Bullard DC, Lefer DJ. Leukocyte and endothelial cell adhesion molecules in a chronic murine model of myocardial reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2000; 279:H2196–2201. [PubMed: 11045953]
25. Hoffmeyer MR, Scalia R, Ross CR, Jones SP, Lefer DJ. Pr-39, a potent neutrophil inhibitor, attenuates myocardial ischemia-reperfusion injury in mice. *Am J Physiol Heart Circ Physiol*. 2000; 279:H2824–2828. [PubMed: 11087237]
26. Hoffmeyer MR, Jones SP, Ross CR, Sharp B, Grisham MB, Laroux FS, Stalker TJ, Scalia R, Lefer DJ. Myocardial ischemia/reperfusion injury in nadph oxidase-deficient mice. *Circ Res*. 2000; 87:812–817. [PubMed: 11055986]
27. Jones SP, Trocha SD, Lefer DJ. Pretreatment with simvastatin attenuates myocardial dysfunction after ischemia and chronic reperfusion. *Arterioscler Thromb Vasc Biol*. 2001; 21:2059–2064. [PubMed: 11742885]
28. Condorelli G, Roncarati R, Ross J Jr, Pisani A, Stassi G, Todaro M, Trocha S, Drusco A, Gu Y, Russo MA, Frati G, Jones SP, Lefer DJ, Napoli C, Croce CM. Heart-targeted overexpression of caspase3 in mice increases infarct size and depresses cardiac function. *Proc Natl Acad Sci U S A*. 2001; 98:9977–9982. [PubMed: 11493678]

29. Jones SP, Trocha SD, Lefer DJ. Cardioprotective actions of endogenous il-10 are independent of inos. *Am J Physiol Heart Circ Physiol*. 2001; 281:H48–52. [PubMed: 11406467]
30. Scalia R, Gooszen ME, Jones SP, Hoffmeyer M, Rimmer DM 3rd, Trocha SD, Huang PL, Smith MB, Lefer AM, Lefer DJ. Simvastatin exerts both anti-inflammatory and cardioprotective effects in apolipoprotein e-deficient mice. *Circulation*. 2001; 103:2598–2603. [PubMed: 11382730]
31. Jones SP, Girod WG, Marotti KR, Aw TY, Lefer DJ. Acute exposure to a high cholesterol diet attenuates myocardial ischemia-reperfusion injury in cholesteryl ester transfer protein mice. *Coron Artery Dis*. 2001; 12:37–44. [PubMed: 11211164]
32. Lefer DJ, Scalia R, Jones SP, Sharp BR, Hoffmeyer MR, Farvid AR, Gibson MF, Lefer AM. Hmg-coa reductase inhibition protects the diabetic myocardium from ischemia-reperfusion injury. *Faseb J*. 2001; 15:1454–1456. [PubMed: 11387255]
33. Jones SP, Gibson MF, Rimmer DM 3rd, Gibson TM, Sharp BR, Lefer DJ. Direct vascular and cardioprotective effects of rosuvastatin, a new hmg-coa reductase inhibitor. *J Am Coll Cardiol*. 2002; 40:1172–1178. [PubMed: 12354446]
34. Sharp BR, Jones SP, Rimmer DM, Lefer DJ. Differential response to myocardial reperfusion injury in enos-deficient mice. *Am J Physiol Heart Circ Physiol*. 2002; 282:H2422–2426. [PubMed: 12003854]
35. Jones SP, Hoffmeyer MR, Sharp BR, Ho YS, Lefer DJ. Role of intracellular antioxidant enzymes after in vivo myocardial ischemia and reperfusion. *Am J Physiol Heart Circ Physiol*. 2003; 284:H277–282. [PubMed: 12485820]
36. Yang J, Jones SP, Suhara T, Greer JJ, Ware PD, Nguyen NP, Perlman H, Nelson DP, Lefer DJ, Walsh K. Endothelial cell overexpression of fas ligand attenuates ischemia-reperfusion injury in the heart. *J Biol Chem*. 2003; 278:15185–15191. [PubMed: 12576484]
37. Jones SP, Greer JJ, van Haperen R, Duncker DJ, de Crom R, Lefer DJ. Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice. *Proc Natl Acad Sci U S A*. 2003; 100:4891–4896. [PubMed: 12676984]
38. Jones SP, Greer JJ, Kakkar AK, Ware PD, Turnage RH, Hicks M, Van Haperen R, De Crom R, Kawashima S, Yokoyama M, Lefer DJ. Endothelial nitric oxide synthase overexpression attenuates myocardial reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2004; 286:H276–282. [PubMed: 12969888]
39. Jones SP, Greer JJ, Ware PD, Yang J, Walsh K, Lefer DJ. Deficiency of inos does not attenuate severe congestive heart failure in mice. *Am J Physiol Heart Circ Physiol*. 2005; 288:H365–370. [PubMed: 15319210]
40. Teshima Y, Akao M, Li RA, Chong TH, Baumgartner WA, Johnston MV, Marban E. Mitochondrial atp-sensitive potassium channel activation protects cerebellar granule neurons from apoptosis induced by oxidative stress. *Stroke*. 2003 In Press.
41. Akao M, Ohler A, O'Rourke B, Marban E. Mitochondrial atp-sensitive potassium channels inhibit apoptosis induced by oxidative stress in cardiac cells. *Circ Res*. 2001; 88:1267–1275. [PubMed: 11420303]
42. Teshima Y, Akao M, Jones SP, Marban E. Uncoupling protein-2 overexpression inhibits mitochondrial death pathway in cardiomyocytes. *Circ Res*. 2003; 93:192–200. [PubMed: 12855674]
43. Jones SP, Teshima Y, Akao M, Marban E. Simvastatin attenuates oxidant-induced mitochondrial dysfunction in cardiac myocytes. *Circ Res*. 2003; 93:697–699. [PubMed: 14512440]
44. Haltiwanger RS, Grove K, Philipsberg GA. Modulation of o-linked n-acetylglucosamine levels on nuclear and cytoplasmic proteins in vivo using the peptide o-glcnac-beta-n-acetylglucosaminidase inhibitor o-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-n-phenylcarbamate. *J Biol Chem*. 1998; 273:3611–3617. [PubMed: 9452489]
45. Kelly BD, Hackett SF, Hirota K, Oshima Y, Cai Z, Berg-Dixon S, Rowan A, Yan Z, Campochiaro PA, Semenza GL. Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. *Circ Res*. 2003; 93:1074–1081. [PubMed: 14576200]

46. Teshima Y, Akao M, Jones SP, Marban E. Cariporide (hoe642), a selective Na^{+} - H^{+} exchange inhibitor, inhibits the mitochondrial death pathway. *Circulation*. 2003; 108:2275–2281. [PubMed: 14568900]
47. Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, Herman B. The mitochondrial permeability transition in cell death: A common mechanism in necrosis, apoptosis and autophagy. *Biochim Biophys Acta*. 1998; 1366:177–196. [PubMed: 9714796]
48. Fulop N, Marchase RB, Chatham JC. Role of protein o-linked n-acetyl-glucosamine in mediating cell function and survival in the cardiovascular system. *Cardiovasc Res*. 2007; 73:288–297. [PubMed: 16970929]
49. Liu J, Marchase RB, Chatham JC. Glutamine-induced protection of isolated rat heart from ischemia/reperfusion injury is mediated via the hexosamine biosynthesis pathway and increased protein o-glcna levels. *J Mol Cell Cardiol*. 2007; 42:177–185. [PubMed: 17069847]
50. Liu J, Marchase RB, Chatham JC. Increased o-glcna levels during reperfusion lead to improved functional recovery and reduced calpain proteolysis. *Am J Physiol Heart Circ Physiol*. 2007; 293:H1391–1399. [PubMed: 17573462]
51. Chi L, Tamura Y, Hoff PT, Macha M, Gallagher KP, Schork MA, Lucchesi BR. Effect of superoxide dismutase on myocardial infarct size in the canine heart after 6 hours of regional ischemia and reperfusion: A demonstration of myocardial salvage. *Circ Res*. 1989; 64:665–675. [PubMed: 2702730]
52. Kilgore KS, Friedrichs GS, Johnson CR, Schasteen CS, Riley DP, Weiss RH, Ryan U, Lucchesi BR. Protective effects of the sod-mimetic sc-52608 against ischemia/reperfusion damage in the rabbit isolated heart. *J Mol Cell Cardiol*. 1994; 26:995–1006. [PubMed: 7799454]
53. Ambrosio G, Becker LC, Hutchins GM, Weisman HF, Weisfeldt ML. Reduction in experimental infarct size by recombinant human superoxide dismutase: Insights into the pathophysiology of reperfusion injury. *Circulation*. 1986; 74:1424–1433. [PubMed: 3779923]
54. McCord JM. The evolution of free radicals and oxidative stress. *Am J Med*. 2000; 108:652–659. [PubMed: 10856414]
55. Lucchesi BR, Werns SW, Fantone JC. The role of the neutrophil and free radicals in ischemic myocardial injury. *J Mol Cell Card*. 1989; 21:1241–1251.
56. Murata M, Akao M, O'Rourke B, Marban E. Mitochondrial atp-sensitive potassium channels attenuate matrix Ca^{2+} overload during simulated ischemia and reperfusion: Possible mechanism of cardioprotection. *Circ Res*. 2001; 89:891–898. [PubMed: 11701616]
57. Giffiths EJ, Halestrap AP. Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. *Biochem J*. 1995; 307:93–98. [PubMed: 7717999]
58. Hunter DR, Haworth RA. The Ca^{2+} -induced membrane transition in mitochondria. Iii. Transitional Ca^{2+} release. *Arch Biochem Biophys*. 1979; 195:468–477. [PubMed: 112926]
59. Crompton M, Costi A, Hayat L. Evidence for the presence of a reversible Ca^{2+} -dependent pore activated by oxidative stress in heart mitochondria. *Biochem J*. 1987; 245:915–918. [PubMed: 3117053]
60. Halestrap AP. The mitochondrial permeability transition pore in reperfusion injury and cardioprotection. *Cardiovasc J S Afr*. 2004; 15:S5.
61. Halestrap AP. Calcium, mitochondria and reperfusion injury: A pore way to die. *Biochem Soc Trans*. 2006; 34:232–237. [PubMed: 16545083]
62. Di Lisa F. Mitochondrial contribution in the progression of cardiac ischemic injury. *IUBMB Life*. 2001; 52:255–261. [PubMed: 11798040]
63. Bernardi P, Scorrano L, Colonna R, Petronilli V, Di Lisa F. Mitochondria and cell death. Mechanistic aspects and methodological issues. *Eur J Biochem*. 1999; 264:687–701. [PubMed: 10491114]
64. Lefer DJ, Granger DN. Oxidative stress and cardiac disease. *Am J Med*. 2000; 109:315–323. [PubMed: 10996583]
65. Housley MP, Udeshi ND, Rodgers JT, Shabanowitz J, Puigserver P, Hunt DF, Hart GW. A pgc-1alpha-o-glcna transferase complex regulates foxo transcription factor activity in response to glucose. *J Biol Chem*. 2009; 284:5148–5157. [PubMed: 19103600]

66. Nagy T, Champattanachai V, Marchase RB, Chatham JC. Glucosamine inhibits angiotensin ii-induced cytoplasmic ca^{2+} elevation in neonatal cardiomyocytes via protein-associated o-linked n-acetylglucosamine. *Am J Physiol Cell Physiol.* 2006; 290:C57–65. [PubMed: 16107505]

Figure 1. Effects of ischemia-reperfusion injury on level of O-GlcNAcylation. Adult C57BL6 (n=4-6 per group) mice were subjected to 40 minutes of ischemia and reperfusion for the indicated time (in hours). **A)** Representative TAMRA-GlcNAc gels for ischemic zone for time points with significant changes in O-GlcNAcylation. Ischemia induced significant decrement, while reperfusion for one hour augmented O-GlcNAcylation. **B)** Densitometric quantification of TAMRA-GlcNAc gels expressed as percent of sham for ischemic zones show significant reduction in O-GlcNAcylation following 40 minutes of ischemia (zero hour of reperfusion), augmented O-GlcNAcylation by 1 hour of reperfusion and no change in O-GlcNAcylation by four and twenty four hours of reperfusion compared to Sham. **C)** There was no significant difference in O-GlcNAcylation in the Non-ischemic zones of MI hearts compared with sham hearts at all reperfusion time points. **D)** Ischemia induced troponin I (cTnI) release. *p<0.05 vs. Sham

Figure 2. Effects of hypoxia-reoxygenation on O-GlcNAc levels. Myocytes were subjected to hypoxia for three hours and reoxygenated for zero, one or six hours. O-GlcNAcylation levels were assessed on whole cell lysates or cell injury assessed by measuring post-hypoxic LDH release in the media. **A)** Representative immunoblots of time course of O-GlcNAcylation levels in cardiac myocytes. **B)** Densitometric quantification of O-GlcNAc immunoblots expressed as percent of normoxic control. **C)** Hypoxia induced cell injury according to post-hypoxic LDH release.

Figure 3.

Evaluation of ROS production in NRCMs treated with AdNull, AdOGT, AdOGA, Vehicle or PUGNac using DCF (n=6/group). Hypoxia induced ROS production. AdOGT significantly attenuated, while AdOGA exacerbated, hypoxia-induced ROS production. OGA inhibition with PUGNac also reduced post-hypoxic ROS generation. **A.** Representative montage showing DCF fluorescence. **B.** Bar graph showing the change in DCF fluorescent intensity between 1 and 61 minutes. **C.** Quantitative changes in mean DCF fluorescence in NRCMs exposed to AdNull, AdOGT, or AdOGA using time-lapse fluorescent microscopy. **D.** Representative montage showing DCF fluorescence for Vehicle vs. PUGNac. **E.** Bar graph showing the change in DCF fluorescent intensity between 1 and 61 minutes for Vehicle vs. PUGNac. **F.** Quantitative changes in mean DCF fluorescence in NRCMs exposed to Vehicle or PUGNac using time-lapse fluorescent microscopy. *p<0.05 vs. AdNull or Vehicle, #p<0.05 vs. AdNull + Hypoxia or Vehicle + hypoxia.

Figure 4.

Assessment of effects of manipulation of O-GlcNAc signaling on H₂O₂-induced ROS generation. NRCMs treated with AdNull, AdOGT, AdOGA, Vehicle, or PUGNAc were subjected to oxidative stress (with H₂O₂) and ROS levels measured using DCF (n=4 per group). AdOGT significantly attenuated, while AdOGA exacerbated, H₂O₂-induced ROS production. OGA inhibition with PUGNAc also reduced H₂O₂-induced ROS production. **A.** Representative montage showing DCF fluorescence for AdNull, AdOGT and AdOGA treated NRCMs. **B.** Bar graph showing the change in DCF fluorescent intensity between 1 and 61 minutes for AdNull, AdOGT and AdOGA treated NRCMs. **C.** Quantitative changes in mean DCF fluorescence in NRCMs exposed to AdNull, AdOGT, or AdOGA using time-lapse fluorescent microscopy. **D.** Representative montage showing DCF fluorescence for Vehicle vs. PUGNAc. **E.** Bar graph showing the change in DCF fluorescent intensity between 1 and 61 minutes for Vehicle vs. PUGNAc. **F.** Quantitative changes in mean DCF fluorescence in NRCMs exposed to Vehicle or PUGNAc using time-lapse fluorescent microscopy. *p<0.05 vs. AdNull or Vehicle, #p<0.05 vs. AdNull + H₂O₂ or Vehicle + H₂O₂.

Figure 5. Evaluation of anti-oxidant enzymes by qRT-PCR in NRCMs treated with AdGFP, AdOGT, AdOGA, Vehicle, or PUGNAc. **A.** No change in mRNA levels of the anti-oxidant enzymes SOD1, SOD2, GPX or Cat occurred with either AdGFP or AdOGT. AdOGA did not affect SOD1, SOD2 or GPX but did significantly reduce Cat. **B.** Inhibition of OGA with PUGNAc did not affect SOD2 levels but did increase Cat mRNA. * $p < 0.05$ vs. AdGFP or Vehicle.

Figure 6. Evaluation of calcium overload in NRCMs treated with AdNull or AdOGT and subjected to hypoxia/reoxygenation. AdOGT significantly attenuated cytosolic and mitochondrial Ca^{2+} overload. **A.** Representative montage showing fluo-4 fluorescence for AdNull and AdOGT treated NRCMs. **B.** Bar graph showing the change in fluo-4 fluorescent intensity between 1 and 61 minutes for AdNull and AdOGT. **C.** Time-lapse graph of mean fluo-4 fluorescence in NRCMs treated with AdNull or AdOGT and exposed to hypoxia/reoxygenation. **D.** Representative montage showing rhod-2 fluorescence for AdNull or AdOGT treated NRCMs. **E.** Bar graph showing the change in rhod-2 fluorescent intensity between 1 and 61 minutes for AdNull or AdOGT treated NRCMs. **F.** Quantitative changes in mean DCF fluorescence in NRCMs exposed to AdNull or AdOGT using time-lapse fluorescent microscopy. * $p < 0.05$ vs. AdNull, # $p < 0.05$ vs. AdNull + hypoxia/reoxygenation.

Figure 7. Evaluation of calcium overload in NRCMs treated with AdNull, AdOGT, AdOGA, Vehicle, or PUGNac and subjected oxidative stress using fluo-4 (n=6 per group). AdOGT significantly attenuated, while, AdOGA did not affect H₂O₂-induced cytosolic Ca²⁺ overload. OGA inhibition with PUGNac also reduced H₂O₂-induced cytosolic Ca²⁺ overload. **A.** Representative montage showing fluo-4 fluorescence for AdNull, AdOGT, and AdOGA treated NRCMs. **B.** Bar graph showing the change in fluo-4 fluorescent intensity between 1 and 61 minutes for AdNull, AdOGT and AdOGA treated NRCMs. **C.** Time-lapse graph of mean fluo-4 fluorescence in NRCMs treated with AdNull, AdOGT, or AdOGA and exposed to H₂O₂. **D.** Representative montage showing fluo-4 fluorescence for Vehicle vs. PUGNac. **E.** Bar graph showing the change in fluo-4 fluorescent intensity between 1 and 61 minutes for Vehicle vs. PUGNac – treated NRCMs. **F.** Quantitative changes in mean DCF fluorescence in NRCMs exposed to Vehicle or PUGNac using time-lapse fluorescent microscopy. *p<0.05 vs. AdNull or Vehicle, #p<0.05 vs. AdNull + H₂O₂ or Vehicle + H₂O₂.

Figure 8. Evaluation of mitochondrial calcium overload in NRCMs treated with AdNull, AdOGT, AdOGA, Vehicle, or PUGNac and subjected oxidative stress using rhod-2 (n=6 per group). AdOGT significantly attenuated, while, AdOGA did not affect H₂O₂-induced mitochondrial Ca²⁺ overload. OGA inhibition with PUGNac also reduced H₂O₂-induced mitochondrial Ca²⁺ overload. **A.** Representative montage showing rhod-2 fluorescence for AdNull, AdOGT, and AdOGA treated NRCMs. **B.** Bar graph showing the change in rhod-2 fluorescent intensity between 1 and 61 minutes for AdNull, AdOGT and AdOGA treated NRCMs. **C)** Time-lapse graph of mean rhod-2 fluorescence in NRCMs treated with AdNull, AdOGT, or AdOGA and exposed to H₂O₂. **D.** Representative montage showing rhod-2 fluorescence for Vehicle vs. PUGNac. **E.** Bar graph showing changes in rhod-2 fluorescent intensity between 1 and 61 minutes for Vehicle vs. PUGNac –treated NRCMs. **F.** Quantitative changes in mean DCF fluorescence in NRCMs exposed to Vehicle or PUGNac using time-lapse fluorescent microscopy. *p<0.05 vs. AdNull or Vehicle, #p<0.05 vs. AdNull + H₂O₂ or Vehicle + H₂O₂.

Figure 9. Assessment of peroxide induced calcium overload in NRCMs exposed to a low (50 μ mol/L) concentration of H₂O₂. **A.** Cytosolic calcium levels as shown by fluo-4 fluorescence. **B.** Mitochondrial calcium levels as shown by rhod-2 fluorescence. **C.** Change in fluorescence in both fluo-4 and rhod-2.

Figure 10.

Assessment of sensitivity to formation of mitochondrial permeability transition pore (mPTP) in NRCMs treated with AdNull, AdOGT, AdOGA, Vehicle, or PUGNac prior to H_2O_2 treatment using calcein and MitoTracker Red. Loss of calcein fluorescence was used to indicate mPTP ($n \geq 6$ per group). H_2O_2 treatment induced mPTP opening, which was attenuated by OGT overexpression or exacerbated by OGA overexpression according to: **A.** Representative montages, **B.** Quantitative bar graph showing the change in calcein fluorescent intensity SD between 1 and 61 minutes, and **C.** Graph showing time dependent changes in mean calcein fluorescence intensity standard deviation in NRCMs treated with AdNull, AdNull + H_2O_2 , AdOGT + H_2O_2 , and AdOGA + H_2O_2 . OGA inhibition with PUGNac also mitigated mPTP formation according to: **D.** Representative montages of calcein fluorescence, **E.** Quantitative bar graph showing the change in calcein fluorescent intensity SD between 1 and 61 minutes, and **F.** Graph showing time dependent changes in mean calcein fluorescence intensity standard deviation in NRCMs treated with Vehicle, Vehicle + H_2O_2 , and PUGNac + H_2O_2 . * $p < 0.05$ vs. AdNull or Vehicle or # $p < 0.05$ vs. AdNull + H_2O_2 or Vehicle + H_2O_2 .