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Noncanonical Glycosyltransferase Modulates Post-hypoxic Cardiac Myocyte Death and Mitochondrial Permeability Transition

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Abstract

O-linked β -N-acetylglucosamine (*O*-GlcNAc) is a dynamic, inducible, and reversible post-translational modification of nuclear and cytoplasmic proteins on Ser/ Thr amino acid residues. In addition to its putative role as a nutrient sensor, we have recently shown pharmacologic elevation of *O*-GlcNAc levels positively affected myocyte survival during oxidant stress. However, no rigorous assessment of the contribution of *O*-GlcNAc transferase has been performed, particularly in the post-hypoxic setting. Therefore, we hypothesized that pharmacological or genetic manipulation of *O*-GlcNAc transferase (OGT), the enzyme that adds *O*-GlcNAc to proteins, would affect cardiac myocyte survival following hypoxia/reoxygenation (H/R). Adenoviral overexpression of OGT (AdOGT) in cardiac myocytes augmented *O*-GlcNAc levels and reduced post-hypoxic damage. Conversely, pharmacologic inhibition of OGT significantly attenuated *O*-GlcNAc levels, exacerbated post-hypoxic cardiac myocyte death, and sensitized myocytes to mitochondrial membrane potential collapse. Both genetic deletion of OGT using a cre-lox approach and translational silencing via RNAi also resulted in significant reductions in OGT protein and *O*-GlcNAc levels, and, exacerbated post-hypoxic cardiac myocyte death. Inhibition of OGT reduced *O*-GlcNAc levels on voltage dependent anion channel (VDAC) in isolated mitochondria and sensitized to calcium-induced mitochondrial permeability transition pore (mPTP) formation, indicating mPTP may be an important target of *O*-GlcNAc signaling and confirming the aforementioned mitochondrial membrane potential results. These data demonstrate that OGT exerts pro-survival actions during hypoxia-reoxygenation in cardiac myocytes, particularly at the level of mitochondria.

Keywords

heart; hypoxia; mitochondria; post-translational modification

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Introduction

Understanding the complex metabolic changes that significantly affect acute myocardial ischemia remains a significant hurdle to our achievement of effective cardioprotective interventions. Entry of glucose into the cell is an essential step for metabolic homeostasis, which is significantly disturbed during myocardial ischemia. Although such a disturbance might affect glucose oxidation and ATP production, the focus of the present study involves an accessory pathway known as the hexosamine biosynthetic pathway (HBP). The HBP uses molecular glucose-6-phosphate to ultimately form uridine diphospho-N-acetylglucosamine (UDP-GlcNAc). While several cellular processes use UDP-GlcNAc as a sugar donor, most germane to the present study is the post-translational modification, *O*-GlcNAc. *O*-GlcNAc was first described by Torres and Hart [1] as an inducible and dynamically cycling post-translational modification in metazoans.

O-GlcNAc modification is distinct from other glycosylation processes because it occurs in the nucleus and cytosol instead of the Golgi apparatus and endoplasmic reticulum. Like phosphorylation, *O*-GlcNAc occurs on serine/threonine amino acid residues of nucleocytoplasmic proteins. On some proteins, *O*-GlcNAcylation actually may compete with phosphorylation [2–4], but it is unlikely that such reciprocity is universal and obligatory. UDP-GlcNAc levels are sensitive to extracellular glucose in studies of cell lines [5], thereby distinguishing global regulation of *O*-GlcNAc levels from phosphorylation.

Two enzymes are responsible for the presence of *O*-GlcNAc on proteins. *O*-GlcNAc transferase (OGT) catalyzes the addition of *O*-GlcNAc to proteins while β -N-acetylglucosaminidase (*O*-GlcNAcase) removes *O*-GlcNAc. Unlike the enzymes involved with the phosphorylation process coded for by several genes, enzymes of the *O*-GlcNAc modification process, OGT and *O*-GlcNAcase, are unique and are encoded by single genes each [6]. Such control is consistent with the idea that *O*-GlcNAc serves as a nutrient, metabolic, and/or stress sensor. Intracellular UDP-GlcNAc levels, protein-protein interactions [7], glycosylation, and phosphorylation [8] play roles in regulating OGT activity while *O*-GlcNAcase activity is regulated by protein-protein interactions and phosphorylation [9–11]. Cell death following deletion of OGT gene in embryonic cells by day five of embryogenesis emphasizes the importance of *O*-GlcNAc in the most elemental biological processes [6,12].

O-GlcNAc modified protein groups include nuclear pore proteins, transcription factors, phosphatases, kinases, adaptor proteins, RNA binding proteins, and cytoskeletal proteins [13,14]. *O*-GlcNAc modification plays important roles in processes like transcription, translation, protein degradation, nuclear targeting and transport, and cell cycle control [15] as well as in diseases [16–20]. Moreover, *O*-GlcNAc has been shown to act as a nutrient or metabolic sensor [21,22]. Zachara *et al* showed that *O*-GlcNAc levels increase in cell lines in response to various stressors [23]. Unfortunately, limited definitive evidence exists regarding the role of OGT in the heart. Accordingly, we sought to rigorously interrogate the contribution of OGT (add *O*-GlcNAc) to isolated cardiac myocyte survival by genetic, molecular, and pharmacologic gain- and loss-of-function approaches. The present data identify OGT as an essential enzyme in post-hypoxic cardiac myocytes and provide mechanistic insights for its activity at the mitochondrial level.

Materials and Methods

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 a well characterized protocol [24–28]. The first four days of culture medium contained the anti-mitotic, BrdU (0.1 mmol/L), to inhibit fibroblast growth in addition

to 5% fetal bovine serum, penicillin/streptomycin, and vitamin B₁₂. Twenty-four hours prior to experimentation, medium was changed to serum-free DMEM.

Mouse genotyping

At 6–8 weeks of age, mice were ear tagged and tail snips were taken. Total DNA was isolated from tail snips using the Qiagen DNeasy Tissue Kit. The DNA was stored at –20°C until PCR was performed. PCR was performed using the Taq PCR Core Kit from Qiagen. Mixes were created as follows: tube 1 contained 1 µl DNTP, 1 µl of 20 µmol/L Primer oIMR3203 (5'-CATCTCTCCAGCCCCACAACTG-3'), 1 µl of 20 µmol/L Primer oIMR3204 (5'-GACGAAGCAGGAGGGGAGAGCAC-3'), 10 µL Enzyme Q, and 7 µL water per sample. Tube 2 contained 5 µl 10X buffer, 0.5 µL Taq, and 14.5 µL water per sample. 20 µL of each tube were added to PCR tube containing 10 µL of purified DNA. PCR was performed at the following conditions: 1 cycle of 94°C for 3 min, 35 cycles of 94°C for 30 sec, 61°C for 1 min and 72°C for 1 min, 1 cycle of 72°C for 2 min then hold at 4°C ad infinitum. PCR samples were run on a 1.2% agarose gel with SYBR Safe stain (Invitrogen). Gels were visualized under UV light using a Fuji LAS-3000 imaging system. Once the line was taken to OGT-loxP flanked homozygosity, neonatal mouse cardiac myocytes were isolated and cultured as described below. OGT-loxP mice are commercially available from The Jackson Laboratory (Bar Harbor, ME).

Neonatal mouse cardiac myocyte isolation and culture

Neonatal mouse cardiac myocytes (NMCs) were isolated from 1–2 day old homozygous loxP-flanked OGT mice using a modified protocol for NRCM isolation. Mice were decapitated, hearts removed, rinsed and minced in calcium- and bicarbonate-free Hank's buffer with HEPES. The tissue fragments were digested by stepwise trypsin (2 mL) dissociation. The dissociated cells were mixed with 3mL FBS, and centrifuged at 180g at room temperature for 5 minutes. The pellet was resuspended in 6 mL of warm fortified DMEM containing to 5% fetal bovine serum, penicillin/streptomycin, and vitamin B₁₂, and centrifuged at 180g for 5 minutes. Pellet was then resuspended in 10 mL of warm fortified DMEM, and then preplated in 100mm dishes for one hour to allow fibroblast to adhere and enrich culture with myocytes. The nonadherent myocytes were then plated at a density of 0.6–1.0× 10⁶ cells/mL. BrdU (0.1mmol/L) was added to the medium the first four days of culture to inhibit fibroblast growth. The cells were maintained at 37°C in the presence of 5% CO₂ in a humidified incubator.

Gene transfer

NRCMs were infected with replication-deficient adenoviruses carrying OGT gene (AdOGT, 48 hours), or green fluorescent protein (AdGFP) as described previously [26]. The recombinant vector was expanded and purified using cesium chloride gradients, yielding adequate concentrations (10¹⁰–10¹¹ plaque forming units/milliliter). Doses used include 0, 20, and 100 multiplicity of infection (MOI) of AdOGT. NMCs were infected with replication-deficient adenovirus carrying the Cre recombinase gene (0 or 50 MOI AdCre) for 72 hours to remove the loxP-flanked OGT gene. Twenty-four hours prior to experimentation, medium was changed to serum-free DMEM. An initial aliquot of AdOGT[9] was subsequently expanded and purified, while AdCre and AdGFP were purchased from Vector Biolabs. Functional expression was confirmed by appropriate immunoblot analysis.

OGT inhibition

NRCMs were treated with [2H-1, 3-thiazine-6-carboxylic acid, 2-[(4-chlorophenyl) imino] tetrahydro-4-oxo-3- (2-tricyclo [3.3.1.1.3, 7] dec-1-ylethyl-)] (i.e. TT04: 0, 0.5, 1 µmol/L) or 3(2H)-bensoxazolecarboxylic acid, 5-chloro-2-oxo-phenyl ester (i.e. TT40: 0 and 10 µmol/L) dissolved in DMSO [29] to inhibit OGT prior to hypoxia/reoxygenation or two hours before

protein harvest for western blotting. TT04 and TT40 compete with UDP-GlcNAc for binding to OGT there by blocking the addition of GlcNAc to protein [29]. For comparison to the original identification of these compounds, 'TT04' is the same as 'Compound 4' in Ref 29, while 'TT40' represents 'Compound 5' in Ref 29. These compounds were purchased from TimTec, Inc. (Newark, DE).

RNA Interference

Cultured NRCMs were transfected with short interfering (si) RNA directed against OGT (siRNA ID # 173150; 5'-GCCUGACAAUACUGGUGUtt-3') or scrambled sequence fluorescently tagged as a non-silencing control (CyTM3-labeled negative control, Ambion). Myocytes were transfected with Ambion's transfection kit II system according to manufacturer's instructions. Thirty-six hours following transfection with 30 nmol/L siRNA, myocytes were subjected to H/R or whole cell lysates were harvested for assessment of OGT protein and O-GlcNAc levels as described below.

Reverse Transcription Real-Time PCR

Total RNA was extracted with Trizol reagent (Invitrogen). Total RNA (1 µg) was subjected to reverse transcriptase reaction to synthesize the cDNA using IScriptTM cDNA synthesis kit (BioRad). OGT sequences used were: ACTGTGTTTCGCAGTGACCTG (sense) and CACGAAGATAAGCTGCCACA (anti-sense). GAPDH sequences used were: TGATGACATCAAGAAGGTGGTGAAG (sense) and TCCTTGGAGGCCATGTGGGCCAT (anti-sense). The relative levels of OGT mRNA transcripts were quantified by real-time PCR using SYBR® Green (Applied Biosystems). The data generated were normalized to GAPDH threshold cycle (C_T) values by using the $\Delta\Delta C_T$ comparative method [30].

Glycoprotein staining

Total cellular protein was isolated from NRCMs treated with either Vehicle, OGT inhibitor (1µmol/L TT04) or infected with adenovirus overexpressing OGT as described above. Following SDS-PAGE, gels were fixed by incubation in 50% methanol and 5% glacial acetic acid for two hours at 25°C. The fixed gels were then washed by gentle agitation three times with 3% glacial acetic acid solution for 10minutes, oxidized with periodic acid for one hour at room temperature, stained with PrO-Q emerald for one hour in the dark and imaged using a 488 laser and 520 BP 40 emission filter on TYPHOON 9400.

Enzymatic labeling of O-GlcNAc-modified proteins

O-GlcNAc modified proteins were labeled using Invitrogen's Click-iT enzymatic labeling kit according to manufacturer's instructions. Briefly, detergents were precipitated out of 100µL of 2mg/ml whole cell lysate (n=6/group) or 50µL of immunoprecipitated protein using the chloroform/methanol precipitation method by adding 600 µL of methanol, 150 µL of chloroform and 400 µL of distilled water and centrifuging at 14000g for five minutes at 4°C. The interface layer containing the protein precipitate was washed twice with methanol and centrifuged at 14000g for five minutes at 4°C. The resulting pellet was then covered with lint-free paper and allowed to dry for fifteen minutes in fume hood. The dried pellet was resuspended in 40 µL of 1% SDS and 20nM HEPES buffer pH 7.9, boiled at 90°C for five minutes, vortexed briefly and allowed to cool on ice for three minutes. 49 µL of distilled water, 80 µL of labeling buffer and 11 µL of MgCl₂ were added and mixture vortexed briefly. 10 µL of UDP-GalNAz (azide-modified UDP-N-Acetylglactosamine) and 7.5 µL of mutant β-1-4-galactosyltransferase were added. The mixture was vortex briefly and incubated 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, 50mM Tris, and pH 8 and . The azide-labeled proteins were tagged with a fluorescent dye, TAMRA by adding 100 μ L of 2x click-iT TAMRA reaction buffer, 10 μ L of distilled water, 10 μ L of CuSO₄, 10 μ L of Click-iT reaction buffer additive 1 and 20 μ L of Click-iT reaction buffer additive 2. The mixture was vortexed for five seconds after the addition of each component. The mixture was then rotated for one hour at 4°C for the conversion of the azide group to a stable triazole conjugate. 25mM DTT was added, incubated at 4°C for fifteen minutes to stop the reaction and 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 using a 532nm laser and 580 BP 30 emission filter on a TYPHOON 9400 imager. For the experiment in Figure 7D, the samples were first IP'd for VDAC (see below) prior to Click labeling.

SYPRO Ruby staining

Gels were stained for total protein using SYPRO ruby gel stain according to manufacturer's instructions. Briefly, gels were first washed three times using distilled water, fixed by incubation in 50% methanol and 5% glacial acetic acid for fifteen minutes at 25°C, and washed by gentle agitation three times with distilled water for ten minutes. The fixed gels were then rapid stained with SYPRO ruby for one hour in the dark by microwaving for and agitating for 30 seconds, microwaving for 30 seconds and agitating for five minutes and microwaving for 30 seconds and agitating for 23 minutes. The stained gels were then destained by gentle agitation in 7% glacial acetic acid and 10% methanol solution for 30 minutes, washed by gentle agitation three times with distilled water for 5 minutes, and imaged using a 488 laser and 610 BP 30 emission filter on TYPHOON 9400.

Cell death

Cell death was assessed for NRCM treated as mentioned above and subjected to hypoxia/reoxygenation by measuring post-hypoxic LDH release (expressed as LDH release relative total LDH in the cells) using the *in vitro* assay kit from SIGMA and staining with the fluorescent DNA-binding dyes Hoechst 33342 (5 μ g/mL) and propidium iodide, 5 μ g/mL for 30 minutes similar to previous reports [31]. The stained nuclei were then visualized at 20x on a Nikon-TE2000E2 fluorescent microscope.

Assessment of mitochondrial membrane potential

Using time-lapse fluorescence microscopy [24–28,32], detection of mitochondrial membrane potential changes was performed by following changes in tetramethylrhodamine methyl ester (TMRM) fluorescence. Cardiac myocytes were plated on 35 mm glass bottom culture dishes and loaded with 50 nmol/L TMRM prior to hypoxia-reoxygenation. Imaging was initiated at reoxygenation in isolated myocytes by exciting TMRM with an Xcite light source through a 568 nm bandpass filter and emission assessed through a 600 nm bandpass filter. Fluorescence intensity was monitored throughout the protocol every 90 seconds. All experimental groups were repeated in at least four separate isolations.

Mitochondrial isolation

C57BL/6 mice were injected (intraperitoneal) with 10 mg/kg TT04 or Vehicle. After 18 hours, the mice were anesthetized with 100 mg/kg pentobarbital. Hearts were harvested, immediately rinsed with PBS, and placed in Kontes Dual homogenizer containing 4 mL sucrose buffer A (300 mmol/L sucrose, 10 mmol/L Tris-HCl, 2 mmol/L EGTA and 5 mg/mL BSA, pH 7.4) on ice. Hearts were homogenized using 12 strokes and the homogenate centrifuged at 2,000 $\times$ g for 2 minutes at 4°C to remove cell debris. The supernatant was further centrifuged at 10,000 $\times$ g for 5 minutes 4°C to sediment impure mitochondria. Mitochondria were washed twice with 1 mL of sucrose buffer A (minus BSA) [33] and purified by adding 19% Percoll and centrifuging

at 14,000×g for 10 minutes at 4° C. Mitochondrial pellet was washed twice with 0.5 mL of sucrose buffer B (300 mmol/L sucrose, 10 mmol/L Tris-HCl, pH 7.4)[34]. Purified mitochondrial pellet was resuspended in 0.5 mL of sucrose buffer B. Mitochondrial protein concentration was determined using BIO-RAD protein assay buffer. Adult mouse hearts were used to confirm the insights from the neonatal rat cardiomyocytes in the adult myocardium, and more importantly, because of the relatively high yield of intact mitochondria from the adult mouse heart.

CO-immunoprecipitation

CO-immunoprecipitation was performed with Protein G immunoprecipitation kit (Sigma) according to manufacturer's instructions on adult mouse cardiac mitochondrial proteins. Briefly, 5 µL of the anti-*O*-GlcNAc antibody (CTD110.6) or 10 µL of the anti-VDAC antibody and 245 µL of 1x IP buffer were added to 50 µL of sample and rotated in an end-to-end fashion at 4°C for 1 hour. 50 µL of washed Protein-G-sepharose beads was added to antibody-sample mixture and rotated in a head-to-tail fashion at 4°C overnight. The antibody-sample-bead mixture was then washed five times with 300 µL of 1x IP buffer and once with 0.1x IP buffer centrifuging at 12,000 × g at 4°C for 30 seconds between washes. 1x Sample buffer, reducing agent and 1x TBS was added and the mixture heated at 95°C for 5 minutes. Samples were then immunoblotted for VDAC using anti-VDAC (1:2000, Santa Cruz) or *O*-GlcNAc using RL2 (1:1000, Affinity BioReagents). Standard densitometry was performed and the value for the Vehicle group was set at 100%. All samples were normalized to their total VDAC levels (which were not different between the two groups).

Mitochondrial swelling

200 µL of mitochondria in sucrose buffer B (2 mg/mL protein concentration) was loaded on to 96-well plate and challenged with 100 µmol/L CaCl₂[35]. Absorbance was measured every 2 seconds for 600 measurements at 520 nm using a Thermo MultiSkan spectrophotometer. Sanglifehrin A (250 nmol/L) pretreatment of mitochondria was used to inhibit CaCl₂ induced mitochondrial swelling to confirm the mPTP dependence of calcium-induced swelling in the present system.

Statistical Analysis

Data were analyzed using one-way ANOVA, student's unpaired t-test, or Dunnett's t tests using SSPS software as required. Data are reported as mean ± standard error of the mean with differences between treatment groups accepted as significant when $p < 0.05$.

Results

OGT overexpression attenuates post-hypoxic cardiac myocyte death

Adenovirus carrying the OGT gene was used to study gain-of-function for *O*-GlcNAc transferase (AdOGT). Forty eight hours after infecting isolated neonatal cardiac myocytes (NRCMs) with AdOGT, total cellular proteins were harvested and OGT protein and *O*-GlcNAc levels assessed via western blot analysis. Infection of NRCMs with AdOGT dose dependently augmented OGT levels (Figure 1A). Assessment of the functional readout, *O*-GlcNAc, showed a dose dependent increase in *O*-GlcNAc levels with a significant ($p < 0.05$) increase observed at 100 MOI (145 ± 19% of control), but did not reach statistical significance at 20 MOI, compared to 0 MOI AdOGT (Figure 1B). Because a trend was obvious, two additional approaches were used. First, the same samples were also evaluated using another *O*-GlcNAc antibody (RL2), which showed a significant ($p < 0.05$) increase at 20 and 100 MOI compared to 0 MOI. Second, additional samples were prepared for evaluation using a non-immune technique (enzymatic labeling of *O*-GlcNAc with TAMRA fluorophore; i.e. click-chemistry

based approach). The enzymatic labeling technique also showed a significant augmentation of *O*-GlcNAc levels at 20 MOI ($132 \pm 12\%$) and 100 MOI ($155 \pm 9\%$) compared to 0 MOI AdOGT (Figure 1C). Immunoblots/gels for *O*-GlcNAc levels show multiple immunopositive bands because *O*-GlcNAc is a post-translational modification, not a single protein (Figure 1B & C). In addition to the significant increases in *O*-GlcNAc levels, there was no significant difference in total glycoprotein levels following OGT overexpression (Supplemental Figure IIIA). Equal protein loading was confirmed by densitometric analysis of Ponceau-stained membranes (Supplemental Figure V) or SYPRO ruby stained gels (Supplemental Figure IV-A).

To evaluate the effects of OGT overexpression on post-hypoxic cardiac myocyte survival, similarly-treated cardiac myocytes were subjected to hypoxia (three hours) and reoxygenation (one hour). Post-hypoxic media was harvested and assayed for LDH release and cells evaluated for PI positivity. Significant ($p < 0.05$) decreases in post-hypoxic LDH release were seen with 20 MOI ($60 \pm 12\%$ of control) and 100 MOI ($67 \pm 15\%$ of 0 MOI) compared with 0 MOI AdOGT treated NRCMs (Figure 2A). Also, we observed a significant reduction in PI positivity at 100 MOI ($72 \pm 19\%$ of 0 MOI) compared with 0 MOI ($100 \pm 14\%$) AdOGT treated NRCMs (Figure 2B).

OGT inhibition exacerbates post-hypoxic cardiac myocyte injury

Loss-of-function for OGT was evaluated by incubating cultured NRCMs ($n=6/\text{group}$) with OGT inhibitors (TT04 or TT40) for two hours prior to protein harvest and immunoblotting for *O*-GlcNAc levels. The concentrations of TT04 (0, 0.5, and $1.0 \mu\text{mol/L}$) used were not toxic to the cells under normoxic conditions. Significant reductions ($74 \pm 9\%$, $65 \pm 7\%$, $p < 0.05$) in *O*-GlcNAc levels was observed at 0.5, and $1.0 \mu\text{mol/L}$ compared to $0 \mu\text{mol/L}$ TT04 (Figure 3A). We observed similar results with RL2, an additional *O*-GlcNAc antibody. A similar phenomenon was observed with a related OGT inhibitor, 3(2H)-benzoxazolecarboxylic acid, 5-chloro-2-oxo-phenyl ester (see Supplemental figure IA). There was no significant difference in glycoprotein levels follow OGT inhibition ($100 \pm 8\%$ of Vehicle, $p = \text{NS}$) compared with Vehicle ($100 \pm 4\%$) according to Pro-Q Emerald staining (see Supplemental Figure IIIB) demonstrating that the OGT inhibitor (TT04) does not alter other glycosylation processes. As with virtually all known compounds, no absolute and exclusive claims can be made regarding potential non-specific effects.

To evaluate the effects of OGT inhibition on cardiac myocyte survival post-hypoxia, cardiac myocytes ($n=6/\text{group}$) were treated with TT04, subjected to hypoxia-reoxygenation, and media harvested to measure LDH release. Results showed a dose dependent increase in post-hypoxic LDH release with a significant increase ($172 \pm 18\%$, $p < 0.05$) by $1 \mu\text{mol/L}$ TT04 compared with $0 \mu\text{mol/L}$ TT04 (Figure 3B). PI positivity was significantly more at $1 \mu\text{mol/L}$ TT04 ($132 \pm 10\%$, $p < 0.05$) compared with $0 \mu\text{mol/L}$ TT04 (Figure 3C). Another OGT inhibitor (TT40) significantly ($p < 0.05$) exacerbated post-hypoxic cell death (see Supplemental Figure I).

Knockdown of OGT sensitizes cardiac myocytes to post-hypoxic death

NRCMs ($n \geq 4/\text{group}$) were treated with OGT siRNA or scrambled control siRNA for 36 hours to knockdown OGT expression at the mRNA level. The transfection efficiency for the siRNA was estimated to be greater than 90% (Figure 4A). Real time PCR showed a significant decrease in OGT mRNA levels ($72 \pm 8\%$ of scrambled control) for OGT RNAi-treated cells compared with control RNAi (Figure 4B). Whole cell lysates immunoblotted for OGT and *O*-GlcNAc showed significant reductions in OGT levels ($49 \pm 8\%$ of control, $p < 0.05$) and *O*-GlcNAc levels ($64 \pm 5\%$ of control, $p < 0.05$) for OGT siRNA-treated NRCMs compared with control siRNA-treated NRCMs (Figure 4C & D). There was no difference in α -tubulin levels between OGT siRNA- or control siRNA-treated myocytes according to western blot

(Figure 4B). A similar decrement in *O*-GlcNAc levels was observed with RL2, another *O*-GlcNAc antibody (data not shown).

To investigate the effects of OGT gene knockdown on cardiac myocyte survival post-hypoxia, cardiac myocytes were treated with 30 nmol/L OGT siRNA or scrambled-control siRNA for 36 hours, subjected to hypoxia-reoxygenation, and media harvested to measure LDH release. There was a significant (120 $\pm$ 2% of control $p < 0.05$) augmentation in post-hypoxic LDH release in OGT siRNA-treated NRCMs compared to control siRNA-treated NRCMs (Figure 4E).

Deletion of OGT gene sensitizes cardiac myocytes to hypoxic injury

Neonatal mouse cardiac myocytes (NRCMs) carrying only loxP-flanked copies of the OGT gene were infected with adenovirus expressing Cre-recombinase for 72 hours. Whole cell lysates were immunoblotted for Cre-recombinase, OGT, and *O*-GlcNAc modification. NRCMs expressing Cre recombinase showed significant reductions in OGT (44 $\pm$ 16% of control) and *O*-GlcNAc levels (28 $\pm$ 5% of control, Figure 5C & 5D). To assess the effect of OGT deletion on cardiac myocyte survival following hypoxia, 72 hours post AdCre infection, NRCMs were subjected to three hours of hypoxia and one hour of reoxygenation and media harvested for LDH assay. A significant ($p < 0.05$) elevation in LDH release was observed at 50 MOI (122 $\pm$ 12% of control) compared with 0 MOI AdCre (Figure 5E).

Effect of OGT manipulation on mitochondrial membrane potential preservation

Because mitochondria are arbiters of cell fate and the present data support the role of OGT as a prosurvival enzyme, we assessed the effects of blocking endogenous OGT activity on post-hypoxic mitochondrial membrane potential. NRCMs were treated with either AdGFP (100 MOI, 48hrs), AdOGT (100 MOI 48hrs), TT04 (OGT inhibitor; prior to H/R, 1 μ mol/L) or equal volume of DMSO, and loaded with the mitochondrial membrane potential indicator, tetramethylrhodamine methylester (TMRM; 50 nmol/L). Cardiac myocytes were then subjected to three hours of hypoxia and one 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. Imaging was initiated at reoxygenation. For reference purposes, the normoxic group averaged between 190 and 200 AU. Thus, the mitochondria are not 'increasing' their fluorescence, more precisely, they are 'recovering'.

Overexpression of OGT (AdOGT) attenuated the loss of mitochondrial membrane potential compared to AdGFP as shown by increased TMRM fluorescence and thus protected myocytes from cell death following hypoxia (Figure 6A). Inhibition of OGT (via TT04) exacerbated the loss of mitochondrial membrane potential compared to H/R alone as shown by a further reduction in TMRM fluorescence and thus sensitized myocytes to mitochondrial damage (Figure 6B). Such findings support a role for OGT at the level of the mitochondrial permeability transition pore.

VDAC is a mitochondrial target of *O*-GlcNAc

Mitochondria play a central role in necrotic and apoptotic cell death with an important event being the formation of the mitochondrial permeability transition pore (mPTP). With recent findings identifying VDAC[28] as a target of *O*-GlcNAc modification, we assessed VDAC levels following OGT inhibition as a possible mechanistic link between *O*-GlcNAc signaling and the mitochondria. Adult cardiac mitochondria from TT04-treated mice contained reduced *O*-GlcNAc-modified VDAC compared with those from Vehicle-treated mice (Figure 7A&B) according to immunoblotting. Yet, there was no significant difference in total VDAC levels between cardiac mitochondria from TT04- and Vehicle-treated mice (Figure 7C). Moreover,

an immune independent technique further confirmed the co-IP findings showing reduction in *O*-GlcNAc modification of VDAC with OGT inhibition (Figure 7D). Again, VDAC is not the only *O*-GlcNAc modified protein, and, TT04 reduces *O*-GlcNAc levels on many proteins in the adult mouse heart (supplemental Figure VI).

Effects of OGT inhibition on calcium induced mitochondrial swelling

Because a hallmark of mPTP is the massive swelling of the mitochondria, we assessed sensitivity to calcium-induced mitochondrial swelling following OGT inhibition. Exposure of isolated mitochondria to supra-physiological concentrations of Ca^{2+} permeabilizes the mitochondrial inner membrane to small solutes (<1.5 kDa)[36]. Cardiac mitochondria from TT04- or Vehicle-treated mice were challenged with 100 $\mu\text{mol/L}$ CaCl_2 and the change in absorbance measured at 520 nm every two seconds. The mPTP inhibitor, Sanglifehrin A, significantly inhibited calcium-induced swelling in mitochondria from Vehicle-treated mice (Supplemental Figure II-B) showing that the swelling was mPTP dependent. Similar results were observed with mitochondria from TT04-treated mice (data not shown). Adult cardiac mitochondria from TT04-treated mice were significantly ($178 \pm 10\%$ of Vehicle) more sensitive to CaCl_2 induced swelling compared with Vehicle (Figure 7E; Supplemental Figure II-E). The rate of swelling in Supplemental Figure II-C was calculated as a simple change in absorbance with respect to time and normalized to the Vehicle group. Such functional data complement the biochemical data in Figure 7 (panels A and B).

Discussion

The present study determined the role of *O*-GlcNAc transferase (OGT) in post-hypoxic cardiac myocyte survival. The data show pharmacologic or genetic manipulation of OGT affects cardiac myocyte survival following hypoxia, indicating OGT is a critical survival protein in differentiated cardiac cells. Augmenting OGT levels favored *O*-GlcNAc modification and reduced cardiac myocyte death following hypoxia. Conversely, OGT inhibition (using siRNA, cre-lox, or pharmacologically with TT04) reduced *O*-GlcNAc levels, promoted loss of mitochondrial membrane potential, and exacerbated post-hypoxic cardiac myocyte death. Furthermore, OGT inhibition sensitizes mitochondria to mPTP formation (according calcium-induced swelling). Such findings demonstrate a unique role for the *O*-linked glycosyltransferase, OGT, as an endogenous defender of cell survival in cardiac myocytes.

This is the first direct demonstration that OGT, via alterations in *O*-GlcNAc levels, may act at the mitochondrial level by altering sensitivity to loss of mitochondrial membrane potential and mPTP formation. This provides a potential mechanism through which *O*-GlcNAc protects post-hypoxic cardiac myocytes. Specifically, the present data demonstrate that inhibition of OGT reduces *O*-GlcNAc levels, particularly but not exclusively, on VDAC on the outer mitochondrial membrane. As a putative member of the mitochondrial permeability transition pore (mPTP), such a reduction in the *O*-GlcNAc modification of VDAC implies a potential mechanism linking *O*-GlcNAc signaling with cell death. In congruence with the present findings, we recently demonstrated that pharmacologic augmentation of *O*-GlcNAc signaling elevated the levels of *O*-GlcNAc-modified VDAC and reduced mitochondrial swelling[28]. It is plausible that events favoring *O*-GlcNAcylation of VDAC may prevent the formation of the mPTP, preserve mitochondrial membrane potential, and hence prevent mitochondrial-mediated cell death, while events (or risk factors) that impair *O*-GlcNAc signaling may have contrary and deleterious effects.

With findings from the present study, and that of other groups focused on manipulation of the hexosamine biosynthetic pathway (HBP) [37–41], it is evident that *O*-GlcNAc signaling is protective following acute stress. The present pro-survival effect of OGT through enhanced *O*-GlcNAc modification complements previous data from Champathanachi *et al* in which

hyperglycemia or treatment of cells with glucosamine to increase flux via HBP augmented *O*-GlcNAcylation of proteins and was associated with increased cellular viability[37]. In addition, seminal work from Zachara *et al* [23] showed that reducing OGT and *O*-GlcNAc levels in Cos7 cells resulted in less tolerance to stress, while augmenting OGT and *O*-GlcNAc levels resulted in more stress tolerance. Marchase's group also showed that glucosamine treatment, associated with elevated HBP flux and protein *O*-GlcNAc levels, attenuated severe calcium overload induced by the calcium paradox[38], and inhibits angiotensin II induced elevated cytosolic calcium overload in isolated cardiomyocytes *in vitro*[39]. Because calcium overload has been shown to be a likely contributor to cardiac myocyte death following ischemia, attenuation of calcium overload by *O*-GlcNAc modification may in part explain the protective effect of *O*-GlcNAc presently observed following enhanced OGT levels, thereby providing a strong correlation between *O*-GlcNAc levels and mechanisms of cell survival.

Although the present study focuses on the acute effects of altered *O*-GlcNAc signaling, other studies interrogated the role of chronic elevation of *O*-GlcNAc levels and its possible role in diabetes. Several groups have shown that prolonged elevation of *O*-GlcNAc levels attenuates insulin signaling[17,22], though this issue is far from reconciled [42]. Hu *et al* showed that altered *O*-GlcNAcylation in diabetic hearts contributes to cardiac dysfunction and calcium handling [43]. Such efforts support the emerging notion that *O*-GlcNAc signaling participates in the pathogenesis of diabetes. Thus, acute and chronic effects of altered *O*-GlcNAc levels may have significantly different implications for health and disease. Despite the important insights provided in the aforementioned previous studies, significant additional investigations of *O*-GlcNAc signaling are necessary to fully appreciate its function in diabetes, especially in cardiac myocytes.

Studies show that augmenting glucose flux improves ischemic tolerance of the heart, potentially through an elevation in glycolytic ATP production and reduction of fatty acid oxidation[44,45]. The present data involve alterations in a glucose-derived metabolic signal, *O*-GlcNAc, leading one to question whether its manipulation would affect ATP levels. Such questions might be justified because of the present data indicating VDAC as an *O*-GlcNAc target and our recent work identifying metabolic (e.g. glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, pyruvate kinase) and mitochondrial proteins (including VDAC) as being *O*-GlcNAc modified [28]. If alteration in *O*-GlcNAc modification of these enzymes affects their function, then it might be reasonable to expect changes in cellular ATP levels. Moreover, one of VDAC's physiological functions is to facilitate ATP transport across the outer mitochondrial membrane. However, because the HBP normally uses less than 5% cellular glucose, minimal changes in glycolytic ATP (via fructose-6-phosphate) should occur, assuming *O*-GlcNAc modification of such enzymes would not affect their activity. In agreement with this notion, Champattanachai *et al* found that glucosamine treatment in cardiac myocytes did not significantly affect ATP levels[37]. We also found that inhibition of OGT (via TT04) did not affect ATP levels in cardiac myocytes [0 μ M TT04 (26.3 ± 15.3 nmol/L/mg protein) vs. 2.5 μ M TT04 dose (26.3 ± 11.9 nmol/L/mg)]. Future studies specifically designed to decipher the metabolic consequences of altered *O*-GlcNAc signaling will undoubtedly shed light on this largely untested aspect of metabolic control.

Prior to the present study, specific investigations of either of the enzymes controlling the *O*-GlcNAc modification have been conspicuously limited in the literature. Such deficiencies reflect more of a dearth of experimental tools than a lack of effort. Nevertheless, such limitations had previously hampered our ability to decipher the exact contribution of OGT to cardiac physiology. However, the present study has made significant progress in this regard. One of the few tools previously used to evaluate OGT activity, alloxan, has been used by several groups to inhibit OGT[9,46], but was recently shown to also inhibit *O*-GlcNAcase, which removes *O*-GlcNAc from proteins[47]. Whether the inhibitory effects of alloxan are exerted

on OGT and/or *O*-GlcNAcase remains unknown. Because of conflicting effects of alloxan on *O*-GlcNAc levels, the present design used two different inhibitors (TT04 & TT40) [29], RNA interference, and excision of the OGT gene (cre-lox), to delineate the loss-of-function effects of OGT. Thus, such a multi-faceted approach should allay concerns about off-target effects. Although one limitation of the present study is that it involves young, healthy, isolated myocytes, such a reductionist approach was necessary to definitively identify the role of OGT in cardiac myocytes, *per se*, and maintain the ability to elevate and reduce OGT activity. Such manipulations are currently not possible *in vivo*, particularly without off-target effects. Nevertheless, one must remain cautious about conclusions drawn from the pharmacologic OGT inhibitors (TT04 & TT40) in the present work because of their limited *in vivo* characterization. In addition, the presently used OGT inhibitors induced a greater increase in myocyte damage than the knockdown or knockout approaches. Although it is difficult to make such direct comparisons, such differences are important to note for future studies. It is also possible that the differences in *O*-GlcNAc levels at the end of hypoxia differed from the pre-hypoxic values we present.

Future work will focus on the full complement of proteins modified by *O*-GlcNAc, particularly in the mitochondria, and identify their relative contributions to cardiac health and disease. It is becoming increasingly evident that numerous post-translational modifications are critical for the maintenance of health and the development of disease. The present study lays the groundwork for understanding OGT's role in more complex cardiovascular phenotypes, such as diabetes, hypertension, obesity, and other pathologies that predispose patients to heart disease. Because of its direct link to glucose metabolism and involvement with acute cellular stress, *O*-glycosylation has emerged as an important post-translational modification that deserves extensive attention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Assessment of the effects of OGT overexpression (using AdOGT) on NRCM survival following hypoxia. Myocytes were infected with AdOGT 48 hours prior to protein harvest or hypoxia-reoxygenation. **A)** Summary densitometric analysis (n=3/group) and immunoblot of OGT (using SQ17) show a dose dependent increase in OGT protein with increasing MOI of AdOGT. A significant (p<0.05) increase is seen at 20 and 100 MOI compared with 0 MOI AdOGT. **B)** Densitometric analysis (n=6/group) and representative immunoblot of the functional readout of OGT, O-GlcNAc. A dose dependent increase in O-GlcNAc levels (n=6/group) is seen with the RL2 antibody, while a trend is evident with the CTD antibody. As expected, multiple immunopositive bands appear because the O-GlcNAc modification occurs on numerous proteins throughout the cell. **C)** OGT overexpression (n=6/group) dose-dependently augmented O-GlcNAc levels according to O-GlcNAc-TAMRA. *p<0.05 vs. 0 MOI AdOGT.

Figure 2.

A) OGT overexpression dose-dependently improves post-hypoxic cardiac myocyte survival. LDH release was spectrophotometrically determined from the media following hypoxia-reoxygenation (n=6/group). **B)** OGT overexpression attenuates post-hypoxic injury according to propidium iodide staining. *p<0.05 vs. 0 MOI AdOGT or 100 MOI AdGFP.

Figure 3.

Evaluation of effects of OGT inhibition on cardiac myocyte survival post-hypoxia. NRCMs were treated with the OGT inhibitor, TT04, prior to protein harvest or H/R (n=6/group). **A)** Densitometric analysis and representative immunoblots for *O*-GlcNAc levels following TT04 treatment show a dose dependent decrease in *O*-GlcNAc levels. Multiple bands occur because *O*-GlcNAc is a post-translational modification occurring on a plethora of intracellular proteins. **B)** OGT inhibition dose-dependently exacerbates post-hypoxic cardiac myocyte survival. LDH release was spectrophotometrically determined from the media following hypoxia-reoxygenation. **C)** OGT inhibition via TT04 worsens post-hypoxic injury reflected by the significant increase in PI positivity release. *p<0.05 vs. 0 μmol/L TT04.

Figure 4.

Effects of OGT gene knockdown on post-hypoxic NRCM survival (n=6/group). **A)** NRCMs treated with fluorescently tagged scrambled (Scr) control RNAi showing a transfection efficiency of more than 90%. **B)** Knockdown of OGT significantly reduces OGT mRNA levels according to qRT-PCR in OGT siRNA-treated compared with scrambled control siRNA-treated NRCMs. **C)** Knockdown of OGT significantly reduces OGT protein levels according to immunoblotting in OGT siRNA-treated compared with scrambled control siRNA-treated NRCMs. **D)** Whole cell lysates from OGT siRNA-treated NRCMs showed a significant decrease in O-GlcNAc levels compared with those from scrambled control siRNA-treated NRCMs according to western blotting. **E)** NRCMs treated with OGT siRNA were more

sensitive to hypoxia-induced injury (according to LDH release) compared with NRCMs treated with scrambled control siRNA. * $p < 0.05$ vs Scr siRNA.

Figure 5. Effects of OGT deletion on survival following hypoxia in cardiac myocytes. NCMs were infected with AdCre 72 hours prior to protein harvest or H/R. **A)** Representative genotyping PCR results showing loxP-flanked or WT OGT gene. Cardiac myocytes from homozygous litters were exposed to AdCre or no virus. There was a significant ($p < 0.05$) increase in Cre-recombinase expression (panel **B**) and a significant ($p < 0.05$) decrease in OGT levels (panel **C**) with 50 MOI compared with 0 MOI AdCre ($n = 4/\text{group}$). **D)** O-GlcNAc levels were markedly decreased at 50 MOI compared with 0 MOI AdCre ($n = 4/\text{group}$). **E)** LDH release was significantly ($p < 0.05$) elevated at 50 MOI compared with 0 MOI AdCre following hypoxia-reoxygenation ($n = 4/\text{group}$). * $p < 0.05$ vs. 0 MOI AdCre

Figure 6.

Assessment of sensitivity to loss of mitochondrial membrane potential in NRCMs following inhibition of OGT (TT04) during hypoxia. TMRM fluorescence was used to indicate mitochondrial membrane potential (n=6/group) beginning at post-hypoxic reoxygenation and continuing through the first hour of reoxygenation. **A)** In post-hypoxic cardiomyocytes, OGT overexpression improved the recovery of mitochondrial membrane potential compared to Hypoxia + AdGFP. Quantification of the change in relative fluorescent intensity (left) and representative montages (right) are shown. **B)** In post-hypoxic myocytes, OGT inhibition (via TT04) repressed the recovery of mitochondrial membrane potential compared to Hypoxia + Vehicle. Quantification of the change in relative fluorescent intensity (left) and representative montages (right) are shown.

Figure 7. OGT inhibition (via TT04) reduces *O*-GlcNAc levels on at least one mitochondrial protein (VDAC). **A–C)** Co-immunoprecipitation for *O*-GlcNAc-modified-VDAC showed a reduction in *O*-GlcNAc-modified-VDAC in adult cardiac mitochondria from TT04-treated mice compared with Vehicle-treated mice, despite no difference in total VDAC levels from both groups (n=4/group; *p<0.05 vs. Vehicle). **D)** Immunoprecipitation for VDAC and enzymatic labeling of *O*-GlcNAc-modified proteins confirmed the reduction in *O*-GlcNAc-modified-VDAC in cardiac mitochondria from TT04-treated compared with Vehicle-treated mice. There was no difference in protein loading according to SYPRO ruby staining. **E)** Sensitivity to mPTP formation according to calcium-induced mitochondrial swelling. Adult mouse cardiac mitochondria were subjected to time control or challenged with 100 μmol/L CaCl₂. Change in absorbance (at 520 nm) was measured over time. Cardiac mitochondria from TT04-treated mice were significantly more sensitive to calcium-induced swelling compared with those from Vehicle-treated mice (n=4/group). p< 0.05 vs. Vehicle.