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Original article

Non-canonical 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 that 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.
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1. 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

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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.

2. Materials and methods

2.1. 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 4 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 B_{12}. Twenty-four hours prior to experimentation, medium was changed to serum-free DMEM.

2.2. 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′-CATCTCTCCAGCCCCACAAACTG-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 10× buffer, 0.5 μL Taq, and 14.5 μL water per sample. 20 μL of each tube was 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 s, 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).

2.3. Neonatal mouse cardiac myocyte isolation and culture

Neonatal mouse cardiac myocytes (NMCMs) 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 3 mL FBS, and centrifuged at 180 ×g at room temperature for 5 min. The pellet was resuspended in 6 mL of warm fortified DMEM containing to 5% fetal bovine serum, penicillin/streptomycin, and vitamin B_{12}, and centrifuged at 180 ×g for 5 min. Pellet was then resuspended in 10 mL of warm fortified DMEM, and then preplated in 100 mm dishes for 1 h to allow fibroblast to adhere and enrich culture with myocytes. The non-adherent myocytes were then plated at a density of $0.6–1.0 \times 10^6$ cells/mL. BrdU (0.1 mmol/L) was added to the medium the first 4 days of culture to inhibit fibroblast growth. The cells were maintained at 37 °C in the presence of 5% CO_2 in a humidified incubator.

2.4. Gene transfer

NRCMs were infected with replication-deficient adenoviruses carrying OGT gene (AdOGT, 48 h), or green fluorescent protein (AdGFP) as described previously [26]. The recombinant vector was expanded and purified using cesium chloride gradients, yielding adequate concentrations (10^{10}–10^{11} plaque forming units/milliliter). Doses used include 0, 20, and 100 multiplicity of infection (MOI) of AdOGT. NMCMs were infected with replication-deficient adenovirus carrying the Cre-recombinase gene (0 or 50 MOI AdCre) for 72 h 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

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