O-GlcNAc signaling is essential for NFAT-mediated transcriptional reprogramming during cardiomyocyte hypertrophy

Hebert T. Facundo,1* Robert E. Brainard,1,2* Lewis J. Watson,1,2 Gladys A. Ngoh,1,2 Tariq Hamid,1 Sumanth D. Prabhu,1,2,3,4 and Steven P. Jones1,2,3

1Department of Medicine, Institute of Molecular Cardiology, 2Department of Physiology and Biophysics, 3Diabetes and Obesity Center, University of Louisville, and 4Veterans Administration Medical Center, Louisville, Kentucky

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Cardiac hypertrophy reflects a compensatory response to pressure or volume overload, sarcomeric abnormalities, or loss of contractile ability through partial death of the myocardium (6, 14). The development of pathological pressure overload-induced hypertrophy has deleterious consequences on the heart and often progresses to decompensation and overt heart failure, the leading cause of death in the industrialized world (30). The molecular mechanisms contributing to cardiac hypertrophy are common to several stressors (25), involve the activation of the nuclear factor of activated T-cells (NFAT), and include the fetal gene program (17). Intracellular Ca^{2+} participates as an obligate signaling molecule during hypertrophy after an increase in cardiac workload or in response to myocyte stretch (2). Elevated Ca^{2+} levels activate the phosphatase calcineurin culminating in NFAT dephosphorylation and nuclear translocation of NFAT (17). The final macromolecular response involves an increase in cell size and protein synthesis (25) and is accompanied by an eventual energetic defect (7, 14, 30).

Although several groups have investigated the metabolic defects accompanying cardiac hypertrophy, little attention has been given to the contribution of accessory pathways of glucose metabolism, such as the hexosamine biosynthetic pathway (see Refs. 20, 21 for review). The hexosamine biosynthetic pathway converts cellular glucose to uridine diphosphate-β-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc serves as the monosaccharide donor for the enzyme uridine diphospho-β-N-acetylglucosaminyltransferase (OGT), which catalyzes the addition of the sugar to serine/threonine residues on proteins. This sugar modification remains on the target protein until removed by the enzyme β-N-acetylglucosaminidase (OAG) (35). O-GlcNAcylation modulates the function of many target proteins (9). One remarkable and relevant example is the activation of the transcription factor NFAT in T-cells (8). In fact, the O-GlcNAcylation enzyme, OGT, may interact with NFAT and directly modify this protein and activate immune response genes in T-cells (8). Yet, there is no known relationship between NFAT activation and O-GlcNAc signaling during cardiac hypertrophy. Here, we demonstrate that O-GlcNAc signaling is essential for NFAT transcriptional activity during cardiac hypertrophy.

MATERIALS AND METHODS

Transverse aortic constriction surgery. The transverse aortic constriction (TAC) surgery was conducted in 3-mo-old, male C57BL/6J mice by constriction of the transverse aorta as described (1) and in accordance with the University of Louisville Animal Care and Use Committee. Briefly, C57BL/6J mice were anesthetized with ketamine (50 mg/kg, intraperitoneal) and pentobarbital (50 mg/kg, intraperitoneal), orally intubated with a polyethylene-60 tubing, and ventilated (Harvard Apparatus Rodent Ventilator, model 845) with oxygen supplementation. Mice were maintained under anesthesia with an isoflurane vaporizer (1%) supplemented with 100% oxygen. Tidal volumes and breathing rates were set based on standard allometric equations. The aorta was visualized through an intercostal incision. A 7-0 nylon suture was looped around the aorta between the brachiocephalic and left common carotid arteries. The suture was tied around a 27-gauge needle (put adjacent to the aorta) to constrict the aorta to a reproducible diameter. Then the needle was removed, leaving a discrete region of stenosis (TAC mice), and the chest was closed. Mice were extubated upon recovery of spontaneous breathing and were allowed to recover in warm, clean cages supplemented with oxygen. Analgesia (ketoprofen, 5 mg/kg, subcutaneous) was given before...
mice recovered from anesthesia (and by 24 and 48 h later). Sham age-matched mice were subjected to the same procedure except the suture was only passed underneath the aorta and not tied off. At the end of the study, TAC or sham-operated mice were euthanized, and the hearts were rapidly excised and weighed. The hearts were then immediately frozen in liquid nitrogen and stored at −80°C or perfused and fixed for immunohistochemical analysis.

Neonatal rat cardiac myocyte isolation and culture. Neonatal rat cardiac myocytes (NRCMs) were isolated from 1- to 2-day-old Sprague-Dawley rats and cultured according to a well characterized protocol (11, 19, 23, 30). Briefly, the first 4 days of DMEM culture medium contained the antiangiogenic BrDU (0.1 mM) to inhibit fibroblast growth in addition to 5% fetal bovine serum, penicillin/streptomycin, and vitamin B12. Cells were maintained at 37°C in the presence of 5% CO2 in a humidified incubator. Where indicated, cells were treated with 100 μM phenylephrine (Phe) in serum-free DMEM plus insulin (10 μg/ml) for 48 h. Where indicated, NRCMs were pre-incubated with 40 μM 6-diaz-o-5-oxonorleucine (DON; an inhibitor of glutamine:fructose-6-phosphate amidotransferase) for 24 h before any additional reagents.

Adenoviral infection. Replication-deficient adenovirus was used to infect NRCMs as described previously (19, 23, 30). Functional expression was confirmed by appropriate immunoblot analysis or real-time PCR. Cells were infected with Ad-OGA, Ad-OGT, Ad-Null, or Ad-GFP at a multiplicity of infection (MOI) of 100 (19). Adenoviruses were delivered to the cells for 2 h (NRCMs) or 6 h (H9c2), and then the medium was changed to fresh DMEM. The cells were infected with adenovirus 24 h before treatment with phenylephrine at the indicated concentration.

NFAT-tagged GFP nuclear translocation. For the NFAT3-GFP nuclear translocation studies, the cells were infected with Ad-OGA, Ad-OGT, or Ad-null for 24 h. The cells were then exposed to NFAT-GFP expressing adenovirus (kindly donated by Dr. Jeffery Molkentin’s laboratory) incubated for 24 h, serum starved for 6 h, and then imaged. The cells were treated with DON for the same period of time as the adenoviruses. The effect of calcium treatment was imaged after 90 min of Ca2+ (4 mM) in DMEM lacking FBS. The NRCMs were grown in 35-mm glass-bottom dishes, and images were acquired with a Nikon A1 confocal at ×60/1.4 magnification Plan Apochromat oil immersion objective (Nikon, Japan). NFAT3-GFP nuclear translocation was analyzed with NIS elements software (Nikon Instruments).

Stretch procedures. H9c2 myoblasts (ATCC, catalog no. CRL1446) were grown in six-well flexible surface Flexcell culture plates coated with laminin. The myoblasts were then subjected to repetitive cycles of stretch and relaxation for 24 h (45 cycles/min) using a computer-driven, vacuum-operated instrument (Flexcell Stretch Unit FX-4000 Tension Plus, Flexcell International). The elongation in the diameter of the flexible surface was maintained at 10–12%. The temperature was maintained at 37°C in a 5% CO2 and 95% O2 atmosphere. Flexcell culture plates exposed to the same conditions but not submitted to stretch and relaxation procedures served as the control.

Heart and NRCM lysates. Following isolation and respective treatments, the NRCM cellular protein content was harvested using a cell scraper in buffer containing (in mM) 5 Hepes, 1 EDTA, 1 EGTA, 50 KCl, 200 mannitol, and 68 sucrose (pH 7.4 with KOH). DTT (1 mM), protease inhibitor (0.0001%), Triton X-100 (0.4%), NP-40 (0.4%), sodium orthovanadate (1 mM), sodium fluoride (1 mM), alloxan (OGT inhibitor, 1 mM), and O-(2-acetamido-2-deoxy-D-glucopyranosylidnamino)-N-phenylcarbamate (i.e., PUGNAc, which is an OGA inhibitor, 1 mM) were added to the buffer to avoid artificial O-GlcNAc addition or removal, respectively, to the proteins in vitro. Hearts were homogenized with buffer containing (in mM) 50 Tris-HCl (pH 7.4), 150 NaCl, 0.01 deoxycholic sodium salt, 1 EDTA, 1 sodium orthovanadate, 1 sodium fluoride, 0.001 PUGNAc, and 0.001 alloxan monohydrate. Protease inhibitor (556 μl/l; Sigma P8340) and NP-40 (1%) were freshly added to the buffer. Heart and NRCM lysates were sonicated twice at 4°C for 25 s each, with 30 min of time separating the sonications. After the second sonication, the lysates were centrifuged 15,000 g (NRCMs) or 12,500 g (hearts) at 4°C for 5 min. The protein content was determined and normalized using Bio-Rad protein assay (Bio-Rad), and bovine albumin served as a control. Heart and cell lysates were frozen in liquid nitrogen immediately and stored at −80°C until used.

Western blotting. The proteins harvested from NRCM or whole heart were submitted to electrophoresis in SDS-PAGE (4–10%) and transferred to nitrocellulose membrane. For the O-GlcNAc antibody samples, whole hearts were first precleared with sepharose G (GE Healthcare) to limit the interaction of the secondary antibody (antimouse) with endogenous immunoglobulins. The membrane blot was blocked (room temperature) using Tris-buffered saline pH 7.5 (TBS) containing nonfat milk (0.5%). After that, the blot was probed with primary antibody against O-GlcNAc: RL2 (1:1,000, Affinity Bioreagents) or CTD 110.6 (1:1,000, Covance), OGT (SQ-17, 1:2,000, Sigma-Aldrich), alpha-tubulin (1:2,000, Sigma-Aldrich) in TBS containing nonfat milk (1%). After overnight incubation at 4°C, the blot was washed in TBS containing Tween-20 (TBS-T; 0.1%). The blot was again blocked for 15 min in TBS-T plus nonfat milk (1%) and incubated with the horseradish peroxidase-labeled secondary antibody goat anti-mouse IgG-HRP (Santa Cruz Biotechnology), goat anti-mouse IgM-HRP (Santa Cruz Biotechnology), goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) in dilutions from 1:2,000 to 1:4,000, depending on the antibody, for 1 h. After washing four times with TBS-T, the blot was detected with an enhanced chemiluminescent detection system (Pierce). Densitometry was executed using nonsaturated chemiluminescent membranes exposed and quantified using Fuji LAS-3000 bio-imaging analyzer. To confirm the linear range of the signal, multiple exposures from every experiment were performed. Levels of proteins in each lane were normalized to loading protein content (tubulin) or to Ponceau stain and expressed as relative to control (set as 100%).

NFAT-luciferase assay. NRCMs were infected with adenovirus containing firefly luciferase under the transcriptional control of four repeats of the NFAT consensus sequence binding site gacaaa (NFAT-Luciferase, 10 MOI; Vector Biolabs). For an adenoviral loading control, we treated cells with adenovirus to overexpress the enzyme beta-galactosidase (Ad-beta-gal, 10 MOI; Vector Biolabs). All other adenoviruses when used in the luciferase experiment were added at 80 MOI. The control adenovirus Ad-null was added to apply the same virus load per condition. The cells were treated with phenylephrine for 6 h without serum and subsequently lysed for 20 min at room temperature using passive lysis buffer (Promega), followed by centrifugation at 2,500 g for 2 min to sediment the debris. A total of 20 μl of the cell lysate was mixed in 100 μl of the luciferase assay solution (Promega). The relative luminescence was measured with a single-tube multimode reader from Turner Biosystems.

β-Galactosidase assay. The normalization of the NFAT-Luciferase activity was performed by β-galactosidase assay in 10 μl of NRCMs lysate in 90 μl of buffer containing 2-nitrophenyl-β-D-galactopyranoside (1 mg/ml), 2-mercaptoethanol (50 mM), magnesium chloride (1 mM), and sodium phosphate (200 mM, pH 7.5). All were all purchased from Sigma (St. Louis, MO). The plate was covered and incubated for 30 min at 37°C, and absorbance at 405 nm was determined with a Thermo Scientific Multiscan Spectrum plate reader. β-Galactosidase activity was expressed as A405 U/mg total protein.

Subcellular fractionation assay. NRCMs were fractionated using Thermo Scientific Subcellular Protein Fractionation Kit, according to the manufacturer’s protocol.

Protein-to-DNA ratio. Cells were washed with PBS, then 200 μl of perchloric acid (0.2 N) were added to each well. Plates were placed on a rocker for 5 min, after which cells were scraped and collected in 1-ml tubes. Samples were then centrifuged for 10 min at 10,000 g at
4°C. Samples were then incubated at 60°C with 30–40 μl of KOH for 20 min, and protein was analyzed using standard Bradford technique. DNA content was determined by using 1 mM Hoechst solution in Tris·NaCl. Diluted Hoechst solution (200 μl) was placed in each well on a 96-well plate along with 10–20 μl of cell homogenate. Fluorescence was measured at 350-nm excitation (slit 2.5) and 460-nm emission (slit 2.5) at 200 scan speed.

**Tritiated leucine assay.** The rate of protein synthesis in NRCMs was determined by [3H]leucine incorporation. Briefly, NRCMs were infected with vehicle + Ad-null, phenylephrine + Ad-null, or phenylephrine + Ad-OGA for 48 h. In the last 8 h, the cells were incubated with [3H]leucine (5 μCi/ml). After incubation, cells were rinsed with PBS, and protein was harvested in 10% TCA. The precipitate was resuspended in 0.5 N NaOH and then measured by scintillation. Values were normalized to DNA concentration measured by Hoechst fluorescence using salmon sperm DNA as a standard.

**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, chloroform/methanol was added to the sample to precipitate the detergents briefly, and allowed to cool on ice for 3 min. Labeling buffer and DTT (25 mM) was added followed by UDP-GalNAz (azide-modified UDP-N-acetylglalactosamine) and mutant β-1,4-galactosyltransferase. The mixture was then rotated for 1 h at 4°C for the conversion of the azide group to a stable triazole conjugate. DTT (25 mM) was added after the addition of each component. The mixture was vortexed for 5 s after the addition of each component. The mixture was then rotated for 1 h at 4°C for the conversion of the azide group to a stable triazole conjugate. DTT (25 mM) was added and incubated at 4°C for 15 min to stop the reaction. Precipitation was via the chloroform/methanol precipitation method. The dried-labeled protein sample was resuspended in SDS-PAGE buffer for electrophoresis.

**Reverse transcriptase PCR and real-time PCR.** The total RNA from NRCMs or from hearts was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA levels were quantified using the ratio of absorbance at 260–280 nm (A260/A280 ratio) with the NanoDrop 1000 Spectrophotometer (Thermo Scientific). To check for organic contaminants like phenol and other aromatic compounds (Trizol, for example), the total RNA was verified by the absorbance ratio of 260 to 230 nm (A260/A230). We limited the use of RNA samples to 260/230 ratio >1.8. Total RNA (1 μg) was then subjected to reverse transcriptase in a 20-μl final volume reaction for 30 min to synthesize the cDNA using IScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The relative levels of mRNA transcripts were quantified by real-time PCR using SYBR Green (Bio-Rad). The data generated was normalized to 18S ribosomal RNA (rRNA) or β-actin threshold cycle (Ct) values by using the ΔΔCt comparative method (13). rRNA was diluted 1:32 with nuclease-free water. In each well, the final concentration of all other primers was 0.1 μg/μl. The expected size of DNA for each primer was confirmed by electrophoresing the DNA product of the qRT-PCR in agarose gel (2%). The absence of extra bands was verified. All the primers were designed using Primer 3.0 software (25).

**Statistical analysis.** Results are shown as means ± SE. The statistical analysis (GraphPad 5.0) was conducted using Student’s t-test or one-way ANOVA followed by Newman-Keuls multiple comparison test when appropriate. Differences were considered statistically significant at P ≤ 0.05.

**RESULTS**

Hyper trophy reactivates fetal gene program concomitantly with O-GlcNAc signaling. Several signaling pathways are implicated in the pathogenesis of hypertrophy (5). Here, we used the TAC model to induce cardiac hypertrophy. After 1 wk, hypertrophy was indicated by significantly elevated heart weight/tibia length (HW/TL) and ANP/BNP mRNA levels (Fig. 1, A and B, respectively). To determine whether O-GlcNAc signaling is involved in the development of hypertrophy in vivo, we analyzed total O-GlcNAc levels using a non-immune technique (Click chemistry-based approach) in hearts from sham or TAC mice and found an enhancement of O-GlcNAc signaling 1 wk after TAC (Fig. 1, C and D). These results were confirmed by evaluating the O-GlcNAc content of cardiac lysates by Western blot analyses (not shown). Interestingly, we also found an upregulation in OGT protein in TAC mice compared with sham (Fig. 1, E and F).

**Reversal of O-GlcNAc signaling reverses hypertrophic transcriptional reprogramming.** The observation that pressure overload (TAC) induced O-GlcNAc signaling prompted us to evaluate the effect of reduced O-GlcNAc modification during cardiac hypertrophy. To better understand this process, we exposed NRCMs to the alpha adrenergic receptor agonist phenylephrine, which produces cellular hypertrophy (1, 26) through activation of NFAT signaling and consequent resumption of the fetal gene program (17). Similar to the in vivo hypertrophy model, we observed that O-GlcNAc signaling was upregulated by phenylephrine treatment of NRCMs (Fig. 2, A and C). Similarly, we found that the treatment of NRCMs with phenylephrine increased OGT protein (Fig. 2B) and significantly elevated ANP mRNA (Fig. 2D). Interestingly, exposing NRCMs to OGA adenovirus (Ad-OGA; 100 MOI) or inhibiting the rate-limiting enzyme (i.e., GFAT) of the hexosamine biosynthetic pathway with 6-diaz-o-5-oxo-L-norleucine (DON; 40 μM) significantly reversed the phenylephrine-induced increase in O-GlcNAc (Fig. 2, A and C) and minimized the induction of ANP during phenylephrine treatment (Fig. 2D). We also showed that O-GlcNAc is necessary for the increased protein production that accommodates hypertrophy (Fig. 2, E and F).

**Inhibition of O-GlcNAc signaling blocks NFAT activation during hypertrophy.** ANP mRNA expression is largely regulated by the activity of the hypertrophy transcription factor NFAT (17). To evaluate whether changes in O-GlcNAc signaling influenced NFAT activation during hypertrophy, we infected NRCMs with Ad-NFAT-luciferase reporter and exposed them to phenylephrine (Fig. 3C). Luciferase reporter activity was stimulated by phenylephrine and blunted by reversal of enhanced O-GlcNAc signaling using Ad-OGA (Fig. 3C). To confirm these findings, we used another adenoviral vector containing full-length NFATc3 fused to GFP (AdNFATc3-GFP), which indicates nuclear translocation of NFAT (i.e., another surrogate of activation). In Fig. 3, A and B, supraphysiological calcium (4 mM) induced nuclear translocation of NFAT within 90 min. In parallel experiments, we co-infected AdNFATc3-GFP NRCMs with Ad-OGA (or Ad-null) and found that reduction in O-GlcNAc signaling blocked translocation of NFAT to the nucleus. DON, an inhibitor of the hexosamine biosynthetic pathway, exerted similar effects.
Such experiments indicate that enhanced O-GlcNAc signaling was necessary for hypertrophic reprogramming but did not answer the question of sufficiency. Next, we augmented O-GlcNAc signaling (in the absence of high calcium) by adenoviral overexpression of OGT (Ad-OGT; Fig. 4, A and B). Here, we found augmented NFAT activation (according to the Ad-NFAT-luciferase driven gene reporter) and nuclear translocation of NFAT (Fig. 4C). We also confirmed that cyclosporin A (CsA) could inhibit the Ad-OGT-induced nuclear translocation of NFAT.

**Hypertrophic NFAT activation requires O-GlcNAc signaling.** Both ANP and BNP promoters have binding sites for NFAT (17). We tested whether activation of NFAT by O-GlcNAc signaling during hypertrophy is of functional significance by focusing on the transcription of ANP and BNP (NFAT-dependent genes). We addressed this question by subjecting H9c2 cardiomyoblasts to stretch-induced hypertrophy (12). H9c2 cells express low levels of ANP and BNP under basal conditions, making them a suitable model to study the modulation of NFAT activation (at the level of ANP/BNP) by O-GlcNAc signaling, whereas ANP/BNP expression in NRMCs is relatively higher. Thus using H9c2 cells will address the potential influence of baseline elevations of ANP/BNP. As shown in Fig. 5, A and B, overexpression of OGT increased O-GlcNAc signaling, and, conversely, the overexpression of OGA reduced it. Stretching the H9c2 myoblasts for 24 h significantly augmented total O-GlcNAcylation, and such augmentation was sensitive to Ad-OGA. Both ANP and BNP mRNA were upregulated after stretch (Fig. 5, C and D). Strikingly, BNP mRNA was upregulated >100-fold during hypertrophy. Interestingly, the 1.8-kb BNP promoter has three binding sites for NFAT and is activated >100-fold in the presence of NFAT, GATA4, and calcineurin (17). In agreement with Fig. 4, Ad-OGT enhanced the levels of ANP and BNP in the absence of stretch. Conversely, Ad-OGT had no additive effects on these parameters when coupled with stretch (Fig. 5, A–D). Also, the control adenovirus (Ad-GFP) had no effect on any parameter analyzed (data not shown).

To determine whether reducing O-GlcNAc signaling during hypertrophy is sufficient to reduce ANP/BNP upregulation, we overexpressed OGA and submitted the cells to stretch for 24 h. Ad-OGA prevented the increase in O-GlcNAc signaling and also minimized the reactivation of the fetal gene program (according to qPCR for ANP and BNP; Fig. 5) during stretch. These results confirm that O-GlcNAc signaling is enhanced during hypertrophy and that reducing O-GlcNAc signaling could block NFAT function during hypertrophy.

We also found that hypertrophic O-GlcNAc signaling coincides with a dysregulation of GLUT1 and GLUT4 expression, the latter of which is regulated by PGC-1α. TAC (in vivo) or phenylephrine treatment in cardiomyocytes increased the ratio...
of GLUT1:GLUT4 due to elevated GLUT1 expression and reduced GLUT4 expression. Interestingly, Ad-OGA, which reversed the hypertrophic transcriptional program in NRCMs, also blocked the increase in GLUT1:GLUT4 ratio in cardiomyocytes (data not shown). These findings confirm the observation that myocardial hypertrophy involves dysregulation of basal glucose uptake and utilization (18), wherein insulin-independent glucose transport (GLUT1) is relatively more favored compared with insulin-dependent glucose transport (GLUT4).

DISCUSSION

We have established a new paradigm in which we show that O-GlcNAc levels not only control NFAT nuclear translocation but also NFAT activation. Following a hypertrophic stimulus, the phosphatase calcineurin is activated and dephosphorylates NFAT. Dephosphorylated NFAT translocates to the nucleus where it promotes the transcription of several hypertrophic genes, including ANP. This is a well established process, and several nuclear kinases, such as GSK and DYRK, phosphory-
late NFAT and promote its relocation back to the cytoplasm (10, 16). Here, we have established a novel paradigm in the regulation of cardiomyocyte hypertrophy whereby a metabolic signal (O-GlcNAc) represents an essential element of NFAT translocation to the nucleus. Moreover, augmentation of this signal, per se, is sufficient to induce NFAT activation in the absence of classical stimuli for hypertrophy.

O-GlcNAc signaling dynamically increases in response to cellular stress (36, 37) and protects against acute myocardial infarction, oxidative stress, and hypoxia (11, 19, 21, 22). Interestingly, activation of NFAT by calcineurin improves cardioprotection against ischemia/reperfusion injury both in vivo and in vitro (4). The concept of alterations in hexosamine signaling contributing to hypertrophy was first mentioned by Young et al. (34), in which they found elevation in UDP-GlcNAc levels following aortic banding in rats, although they were unable to draw specific conclusions related to changes in O-GlcNAc signaling or to demonstrate causality. Chatham’s group recently reported that protein O-GlcNAcylation may also be important in the diabetic heart (15). Nevertheless, their findings are consistent with the present results, both of which emphasize the importance of metabolism and metabolic signaling during hypertrophy and heart failure.

Although we provided new insights regarding the emerging role of protein O-GlcNAcylation in hypertrophy, the role of NFAT/calcineurin signaling has been well established as a regulator of hypertrophic growth. Genetic disruption/inhibition of NFAT inhibits cardiomyocyte hypertrophy, albeit to varying

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**Fig. 3.** O-GlcNAcylation is essential for calcium-activated NFATc3 activation and nuclear translocation. A: NRCMs expressing NFATc3-GFP protein were co-infected with Ad-OGA or Ad-null in the presence or absence of the calcium. The HBP inhibitor DON was given to the cells 24 h before treatment with calcium. Ad-null was added to samples not containing any adenovirus treatment to serve as a control. DAPI was used to mark the nucleus. B: quantification data of NFATc3-GFP translocation. C: the effect of phenylephrine on Ad-NFAT luciferase reporter activity. Tubulin served as a loading control. The Ad-null was added to samples not containing any adenovirus treatment to serve as a control. Results were normalized to beta-galactosidase activity. Values are expressed as means ± SE, n ≥ 3 per group. **Significant difference (P < 0.01).**
extents with different NFAT isoforms (31, 33). Moreover, targeted inhibition of calcineurin can exert similar results in terms of limiting hypertrophy (17, 23, 27, 28). More recently, novel, indirect approaches indicate that hypertrophic signaling via calcineurin/NFAT can be indirectly regulated by miRNA (3). With continued investigation, the present findings can be extended into more detailed molecular mechanistic insights.

Despite the novelty of this study, there are several limitations. The use of NRCM (or H9c2) may not mimic all of the changes observed in the intact adult heart. This is why we confirmed that O-GlcNAc signaling was induced during TAC in vivo. More importantly, we needed to use the NRCM system to transfer genes of interest and induce metabolic re-programming during phenylephrine treatment; this is currently not feasible in multi-day studies of isolated adult cardiac myocytes. Furthermore, had we performed these studies in vivo, we would lack the cardiomyocyte-specific insights gathered from the present data. Nevertheless, we are developing strategies to translate the present findings to in vivo systems in future studies, which represents an important question. This is particularly important given our recent findings that protein O-GlcNAcylation during infarct-induced heart failure appears to be important for the endogenous, although insufficient, compensatory response during heart failure (32).

In summary, this study provides novel insights into mechanisms of metabolic reprogramming in the hypertrophic heart (summarized in Fig. 6). In addition, this work provides potential insights into metabolic dysregulation that could be extended to studies of diabetic tissues since O-GlcNAc is produced in the hexosamine biosynthetic pathway (one accessory pathway of glycolysis). Undoubtedly, the interaction of O-GlcNAc signaling with NFAT translocation/activation should spark additional studies designed to identify the specific signaling modules and site-specific modification of O-GlcNAc in the hypertrophic cardiomyocyte. Many of the critical targets of O-GlcNAc remain elusive, but the significance of O-GlcNAc signaling in primary disease is becoming unquestionable. As our understanding of the influence of metabolic signaling in the form of O-GlcNAc continues to grow, the likelihood escalates that novel therapeutics might arise from such discoveries.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: H.T.F., R.E.B., L.J.W., G.A.N., and T.H. performed experiments; H.T.F., R.E.B., L.J.W., G.A.N., and T.H. analyzed data; H.T.F., R.E.B., G.A.N., T.H., S.D.P., and S.P.J. interpreted results of experiments; H.T.F. and R.E.B. prepared figures; H.T.F. and S.P.J. drafted the manuscript; R.E.B. and S.P.J. edited and revised the manuscript; R.E.B. and S.P.J. approved the final version of the manuscript; S.P.J. conception and design of research.

Fig. 5. O-GlcNAc mediates the fetal gene program reactivation during stretch-induced hypertrophy. A: representative immunoblotting showing the effect of stretch (24 h) on total protein O-GlcNAcylation and OGT levels in H9c2 cells. The stretch was conducted, as described in MATERIALS AND METHODS, alone or in the presence of Ad-OGA (100 MOI), Ad-OGT (100 MOI), or Ad-GFP (100 MOI). Tubulin serves as a loading control. B: quantification of blot in Fig. 5A. Mechanical stretch induces hypertrophy according to ANP (C) and BNP (D) levels. The mRNA levels are expressed relative to 18s ribosomal RNA. Results are expressed as means ± SE, n ≥ 4 per group. *Significant difference (P < 0.05).

Fig. 6. Hypothetical scheme of the interplay between protein O-GlcNAcylation and hypertrophic reprogramming.
REFERENCES


