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Cardiac hypertrophy drives PGC-1α suppression associated with enhanced O-glycosylation

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ABSTRACT

The peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) regulates metabolism and is essential for normal cardiac function. Its activity is suppressed during pressure overload induced cardiac hypertrophy and such suppression at least partially contributes to the associated morbidity. The O-linked β-*N*-acetylglucosamine post-translational modification (O-GlcNAc) of proteins is a glucose-derived metabolic signal. The relationship between O-GlcNAc, and PGC-1α activity in cardiac hypertrophy is unknown. We hypothesized that hypertrophy-induced suppression of PGC-1α was at least partially regulated by O-GlcNAc signaling. Treatment of neonatal rat cardiac myocytes with phenylephrine (an inducer of cardiomyocyte hypertrophy) significantly enhanced global O-GlcNAc signaling. Quantitative real-time PCR analysis revealed a down-regulation of PGC-1α with concomitant suppression of fatty acid oxidation/mitochondrial genes. Transverse aortic constriction in mice decreased the basal expression of PGC-1α and its downstream genes. Reduction of O-GlcNAc signaling alleviated suppression of PGC-1α and most of its downstream genes. Interestingly, augmentation of O-GlcNAc signaling with glucosamine or PUGNAC (a O-GlcNAcase inhibitor) reduced glucose starvation-induced PGC-1α upregulation even in the absence of hypertrophy. Finally, we found that PGC-1α itself is O-GlcNAcylated. Together, these results reveal the recruitment of O-GlcNAc signaling as a potentially novel regulator of PGC-1α activity during cardiac hypertrophy. Furthermore, O-GlcNAc signaling may mediate constitutive suppression of PGC-1α activity in the heart. Such findings illuminate new possibilities regarding the inter-regulation of O-GlcNAc signaling and also may have some implications for metabolic dysregulation during cardiac diseases.

1. Introduction

Cardiac hypertrophy reflects a compensatory response to pressure overload or volume overload [1], which can lead to heart failure, and represents a leading cause of morbidity and mortality in patients in the industrialized world. The normal heart preferentially utilizes fatty acids as an energetic substrate [2], while the hypertrophic heart shifts toward using a variety of substrates [3,4]. The Peroxisome Proliferator-Activated Receptor-coactivator 1α (PGC-1α) is an important regulator of metabolism in various organs. Its activity, and associated mitochondrial function [5,6], are reduced during cardiac hypertrophy and heart failure [7–9]. Despite evidence that suppression of PGC-1α [10] reduces cardiac energy reserve and PGC-1α knockout mice are more susceptible to cardiac hypertrophy and heart failure after transverse aortic constriction [8], the mechanism of PGC-1α suppression remains unknown.

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-*N*-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyl-transferase (OGT), which catalyzes the addition of the sugar to serine/threonine residues on proteins. The sugar remains on the protein until its removal by the enzyme β-*N*-acetylglucosaminidase (O-GlcNAcase) [11]. O-GlcNAc modification dynamically increases in response to cellular stress [12] and protects against acute myocardial infarction, oxidative stress, and hypoxia [13–19]. Conversely, the role of O-GlcNAc in the chronic setting may be quite different.

The enzyme OGT has been shown to be recruited to promoter regions

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and to mediate transcriptional modifications in association with the transcriptional corepressor mSin3A *via* histone deacetylases [20,21]. The O-GlcNAc modification on an SP1 derived peptide represses gene expression *via* an inhibition of protein-protein interactions [22]. Additionally, O-GlcNAc modification of RNA polymerase II in the same residues of phosphorylation induces a conformational change, blocks phosphorylation on these residues, and potentially regulates gene expression [23]. Clearly O-GlcNAc can regulate transcriptional (and translational) events related to gene expression. In this context, the nutrient sensor O-GlcNAc [24] is an attractive candidate to play a role in PGC-1α suppression during events of cardiac hypertrophy.

2. Materials and methods

2.1. 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. Briefly, The first four days of culture medium contained the antimitotic, BrdU (0.1 mmol/L), to inhibit fibroblast growth in addition to 5% fetal bovine serum, penicillin/streptomycin, and vitamin B_{12}. Each 60-mm dish was plated with 1×10^6 myocytes. Cells were maintained at 37 °C in the presence of 5% CO_2 in a humidified incubator. The NRCMs were cultured on DMEM containing 5 mmol/L D-glucose, 10% fetal bovine serum. Where indicated, cells were treated with 100 μmol/L phenylephrine (Phe) in serum-free DMEM plus insulin (10 μg/mL) for 48 h. Where indicated, NRCMs were pre-incubated with 40 μmol/L 6-diazo-5-oxonorleucine (DON – an inhibitor of glutamine: fructose-6-phosphate amidotransferase) for 24 h before any additional treatment/intervention.

2.2. Adenoviral infection

Adenoviral infection: Replication-deficient adenovirus was used to infect NRCMs as described elsewhere. CDNA encoding the human O-GlcNAcase gene was inserted into the E1 region of an adenoviral vector construct using methods described previously [25]. The recombinant vectors were expanded and purified using cesium chloride gradients, yielding adequate concentration (10^{10}–10^{11} plaque forming units - pfu/ml). Functional expression was confirmed by appropriate immunoblot analysis or real-time PCR. AdGFP was used as a control virus. Cells were infected with Ad-O-GlcNAcase or Ad-GFP at a multiplicity of infection (MOI) of 100 as used before [17,26]. Adenoviruses were delivered to the cells for 2 h, then the medium was changed to fresh DMEM. The cells were infected with adenovirus 24 h before treatment with phenylephrine at the indicated concentration.

2.3. Heart and NRCM lysates

Following isolation and respective treatments, the NRCM cellular protein content was harvested using a cell scraper in buffer containing in mmol/L: 5 Hepes, 1 EDTA, 1 EGTA, 50 KCl, 200 mannitol and 68 sucrose pH = 7.4 with KOH. The following reagents were freshly added to the buffer: 1 μmol/L DTT, 0.0001% protease inhibitor, 0.4% Triton X-100, 0.4% NP-40, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1 mmol/L alloxan (putative OGT inhibitor), and 1 μmol/L O-(2-Acetamido-2-deoxy-D-glucopyranosylidenamino) *N*-phenylcarbamate (PUGNAc – an O-GlcNAcase inhibitor) were added to the buffer in order to avoid O-GlcNAc addition or removal, respectively, to the proteins *in vitro*. Hearts were homogenized with buffer containing in mmol/L: 50 Tris-HCl (pH 7.4), 150 NaCl, 0.01 mmol/L deoxycholic sodium salt, 1.0 EDTA, 1.0 sodium orthovanadate, 1.0 sodium flouride, 0.001 PUGNAc, 0.001 alloxan monohydrate. Protease Inhibitor 556 μl/L (Sigma P8340) and 10% NP-40 were freshly added to the buffer. Heart and NRCM lysates were sonicated two times at 4 °C for 25 s each intercalated by 30 min period of time. After the second sonication, the lysates were centrifuged 15,000 ×*g* (NRCMs) 12,500 ×*g* (hearts) at 4 °C for 5 min. The protein content was determined and normalized using Biorad protein assay using bovine albumin as a control and used for SDS PAGE and western blot. The heart and cell lysates were frozen in liquid nitrogen immediately and stored at −80 °C until used.

2.4. Western blotting

The proteins harvested from NRCM or whole hearts were subjected to electrophoresis in SDS-PAGE (4–10%) and transferred to nitrocellulose membrane. For the O-GlcNAc antibody samples, whole hearts were first pre-cleared with sepharose G (GE Healthcare) to limit the interaction of the secondary antibody (anti-mouse) with endogenous immunoglobulins. The membrane blot was blocked (1 h, room temperature) using Tris-buffered saline pH 7.5 (TBS) containing nonfat milk 5%. After that the blot was probed with primary antibody against: O-GlcNAc RL2 (1:1000, Affinity Bioreagents) or CTD 110.6 (1:1000, Covance), OGT (SQ-17 - 1:2000, Sigma-aldrich), anti-GFP (1:2000, Santa Cruz Biotechnology) in TBS containing nonfat milk 1%. After overnight incubation in 4 °C the blot was washed in TBS containing 0.1% Tween-20 (TBS-T). 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 1:2000 to 1:4000 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 non-saturated 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 was 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%).

2.5. Reverse transcriptase PCR and real-time PCR

The total RNA from NRCMs or from hearts were extracted with Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA levels were quantified using the ratio of absorbance at 260 nm to 280 nm (A260/A280 ratio) with the NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). To verify the organic contaminants like phenol and other aromatic compounds (Trizol, for example) the total RNA was also verified the absorbance ratio 260 nm to 230 nm (A260/A230). We limited the use of RNA to samples with 260/230 ratio greater than 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 (BioRad, Herculus, CA). The relative levels of mRNA transcripts were quantified by real-time PCR using SYBR® Green (BioRad, Herculus, CA). The data generated was normalized to 18s ribosomal RNA or βactin threshold cycle (C_T) values by using the $\Delta\Delta C_T$ comparative method. The 18S ribosomal RNA (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 2% agarose gel. The absence of extra bands was also verified. All the primers were designed using Primer 3.0 software. The list of primers used for qRT-PCR is presented in Table Is (Supplement).

2.6. Genotyping, breeding and induction of transgenic OGT-floxed mice

Total DNA was isolated from tail snips using the Qiagen DNeasy Tissue Kit from 4 week-old mice. PCR was performed using the Taq PCR Core Kit from Qiagen. The reaction was conducted in the presence of 1 μM Primer OIMR3203 (catctctccagccccacaaactg), Primer OIMR3204 (gacgaagcaggaggggagagcac), 0.2 mM DNTPs, 0.025 units of Taq

polymerase/reaction. 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 held at 4 °C. PCR samples were electrophoresed on a 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 they were bred to the transgenic MerCreMer mouse line. To determine transgenic expression the same protocol as above was used with primers OIMR 3797 - ataccggagatcatgcaagc and OIMR 3798 - aggtggacctgatcatggag. Mice (adult mice 3 months old) were injected with 4-hydroxy-tamoxifen (Sigma-Aldrich - dissolved in 10% ethanol and 90% peanut oil) in single, daily doses for five days in order to induce the cardio-specific knockout of OGT gene. Five days after secession of the injections, mice were subjected to tissue harvesting ($n = 3$), mice MerCreMer negative that have been injected with tamoxifen served as controls ($n = 4$). Mice had been on a C57BL/6 background for at least five generations prior to use.

2.7. Transverse aortic constriction (TAC) surgery

The TAC surgery was conducted in 3-month-old, male C57BL/6J mice (Jax #000664) by constriction of the transverse aorta as described and in accordance with the University of Louisville Animal Care and Use Committee. All experiments were conducted conform to PHS guidelines. Briefly, C57BL/6J mice were anesthetized with tribromoethanol (375 mg/kg, intra-peritoneal), 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 consistent diameter. Then the needle was removed, leaving a discrete region of stenosis (TAC mice) and the chest was closed. Mice are extubated upon recovery of spontaneous breathing and allowed to recover in a warm clean cage supplemented with oxygen. Analgesia (ketoprofen, 5 mg/kg, sc) was given before mice recovered from anesthesia (and by 24 and 48 h later). Sham age-matched mice ($n = 4$) were subjected to the same procedure except the suture was only passed underneath the aorta and not tied off. TAC ($n = 3$) or Sham operated mice (n = 4) were sacrificed the hearts were rapidly excised and weighed. The hearts were then immediately frozen in liquid nitrogen and stored at

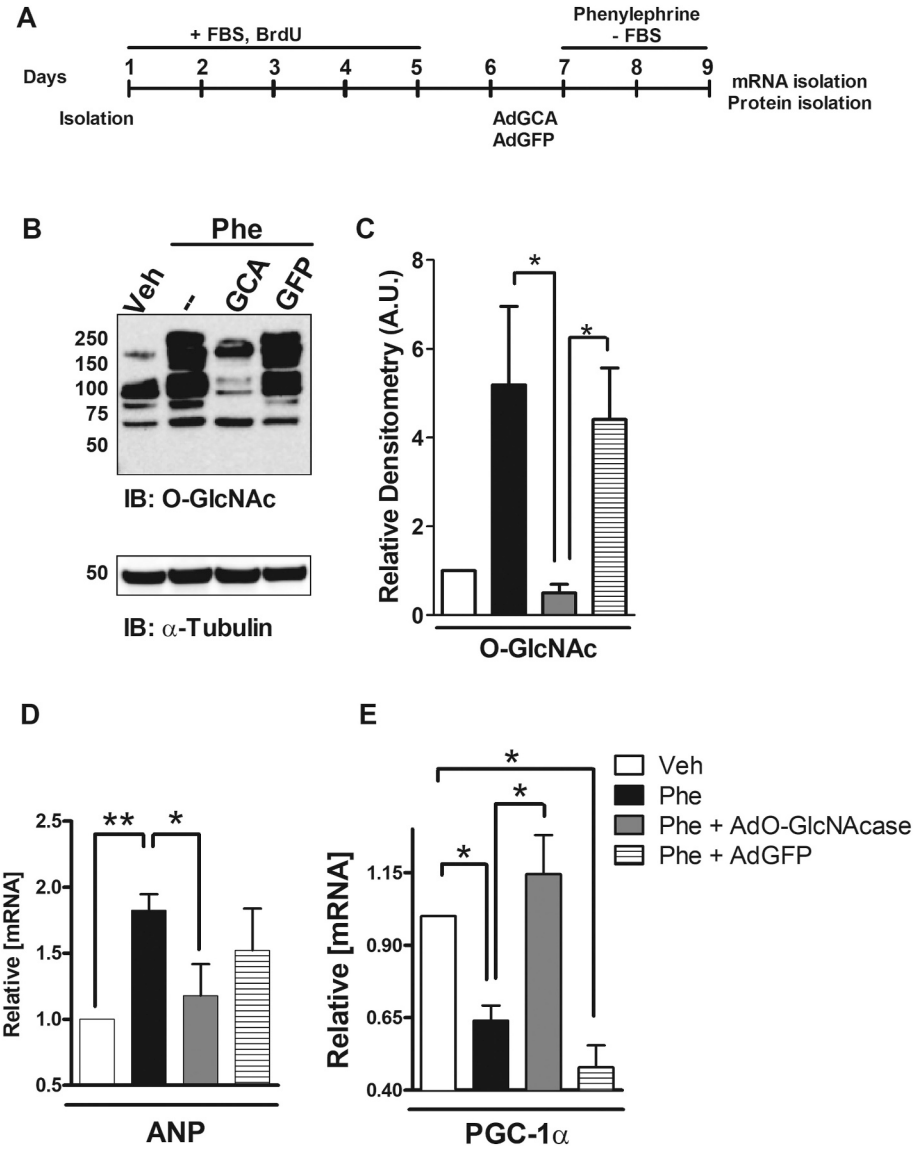

Fig. 1. PGC-1α repression in response to phenylephrine is reversible by O-GlcNAcase overexpression. A, Timeline indicating the *in vitro* experimental protocol. B, The effect of phenylephrine (Phe, 100 mM for 48 h) on total protein O-glycosylation. Cardiomyocytes were exposed to phenylephrine either alone or in the presence of ad-O-GlcNAcase (GCA - 100 MOI) or Ad-GFP (100 MOI) as indicated. The bottom panel represents tubulin as a loading control. C, Quantitative densitometry of O-GlcNAc levels relative to tubulin from panel B. D, quantitative real-time PCR analysis of ANP mRNA from NRCM treated with phenylephrine (Phe, 100 mM) for 48 h. E, quantitative real-time PCR analysis of PGC-1α mRNA. O-GlcNAcase (ad-O-GlcNAcase – 100 MOI) treatment reverses the repression of PGC-1α. mRNA levels are expressed relative to 18S ribosomal RNA. This figure demonstrates that viral overexpression (Ad-GFP) *per se* does not explain the effects of AdO-GlcNAcase in the present system. Results are expressed as means ± S.E. $N = 3$ at least, *, $P < 0.05$; **, $P < 0.01$.

−80 °C, or, perfused and fixed for immunohistochemical analysis.

2.8. Immunoprecipitation

Immunoprecipitation protocol was performed using lysates form NRCMs treated with PUGNAC (200 μM) or 2-[(4-Chlorophenyl)imino] tetrahydro-4-oxo-3-(2-tricyclo[3.3.1.13,7]dec-1-ylethyl)-2H-1,3-thiazine-6-carboxylic acid (TTO4–2.5 uM, 2 h) or vehicle of both drugs. Anti-PGC-1α (Santa Cruz Biotechnology) or anti-O-GlcNAc (CTD110.6 antibody) were applied to cells lysed with IP buffer contained in a Protein G Immunoprecipitation Kit (Sigma-aldrich IP50). The immunprecipitates were washed 3 times and separated with SDS-PAGE. Anti-PGC-1α (Santa Cruz Biotechnology) or anti-O-GlcNAc (CTD110.6 antibody) were used to identify PGC-1α directly of to detect whether PGC-1α was O-glycosylated.

2.9. Statistical analysis

Results are shown as mean ± S.E. The statistical analysis (GraphPad 4.0) was conducted using 1-way ANOVA followed by Newman-Keuls Multiple Comparison Test for Figs. 1, 2, 4 or Student's *t*-test for Figs. 3, 4 and 6. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. O-GlcNAcylation suppresses PGC-1α-dependent gene program during hypertrophy

In order to test whether O-GlcNAc signaling is involved in hypertrophic suppression of PGC-1α we exposed NRCMs to the catecholamine phenylephrine (Fig. 1A), which produces cellular hypertrophy and suppression of PGC-1α and its dependent genes [8]. O-GlcNAc signaling was upregulated at 48 h of treatment with phenylephrine (Fig. 1B, C). Additionally, phenylephrine significantly elevated ANP mRNA (Fig. 1D) and significantly reduced PGC-1α (Fig. 1E). Strikingly exposing NRCMs to adenovirus overexpressing O-GlcNAcase (Ad-O-GlcNAcase – 100 MOI) significantly reversed the phenylephrine-induced increase in O-GlcNAc (Fig. 1B, C). Furthermore, overexpression of O-GlcNAcase minimized induction of ANP by phenylephrine (Fig. 2D). Such treatments (Ad O-GlcNAcase) largely negated hypertrophy-induced suppression of PGC-1α (Fig. 1E). Importantly, the control adenovirus expressing GFP had no apparent effect. Consistent with these observations we have previously shown that O-GlcNAcase also blocks increased hypertrophic protein synthesis, thereby suppressing the hypertrophic phenotype in NRCMs [27]. This result prompted us to analyze the efficacy of rescuing PGC-1α activity by reducing O-GlcNAc signaling.

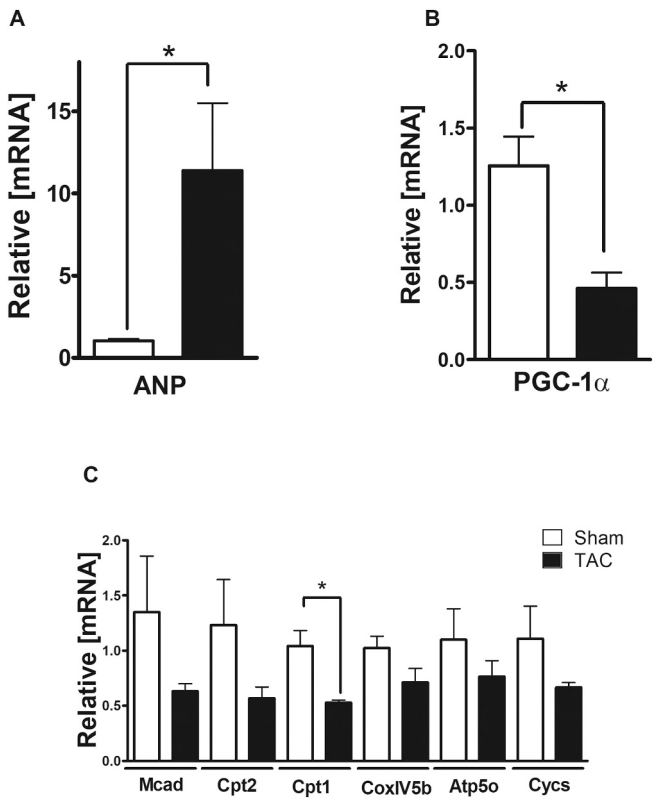

Fig. 3. Cardiac hypertrophy suppresses PGC-1α. Induction of hypertrophy after seven days of Transverse Aortic Constriction (TAC). A, Atrial natriuretic peptide mRNA levels after 7 days of TAC. B. Suppression of PGC-1α mRNA and its downstream genes fatty acid oxidation (Mcad, Cpt1, Cpt2) genes and mitochondrial genes (CoxIV5b, Atp5o) by transverse aortic constriction (TAC) for seven days compared to Sham (B). Results are expressed as means ± S.E., n≥3/group, *, $p < 0.05$.

In a set of parallel samples we show that similar to O-GlcNAcase

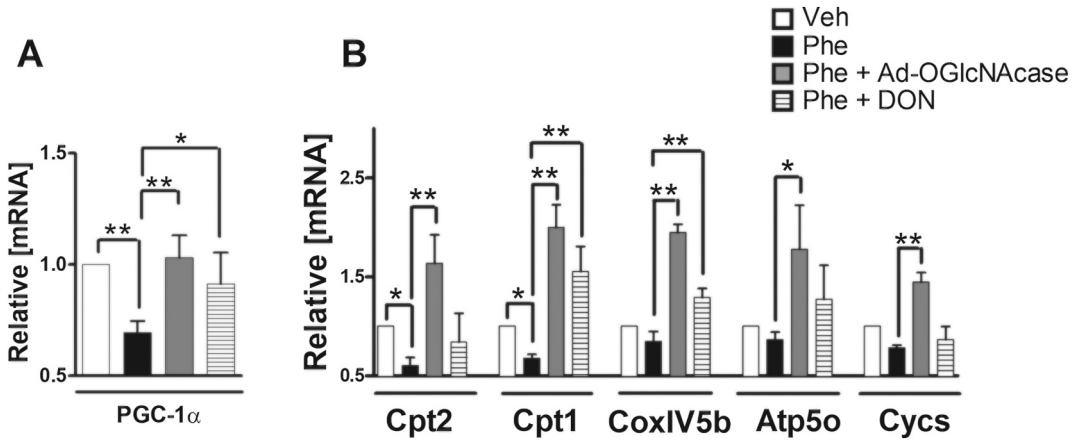

Fig. 2. Reduction of O-GlcNAc signaling alleviates hypertrophic suppression of PGC-1α downstream gene expression. A, Effect of Ad-O-GlcNAcase (AdGCA - 100 MOI) or the GFAT inhibitor 6-diazo-5-oxonorleucine (DON – 40 mmol/L) on mRNA levels of PGC-1α. Samples were treated with phenylephrine (Phe, 100 mmol/L for 48 h). B, Quantitative real-time PCR analysis of fatty acid metabolism (Cpt2, Cpt1) and mitochondrial genes (CoxIV5b, Atp5o, Cycs) mRNA expression from NRCM treated as indicated above. All mRNA levels are expressed relative to 18S ribosomal RNA. Results are expressed as means ± S.E., $n \geq 3$/group, *, $p < 0.05$; **, $p < 0.01$.

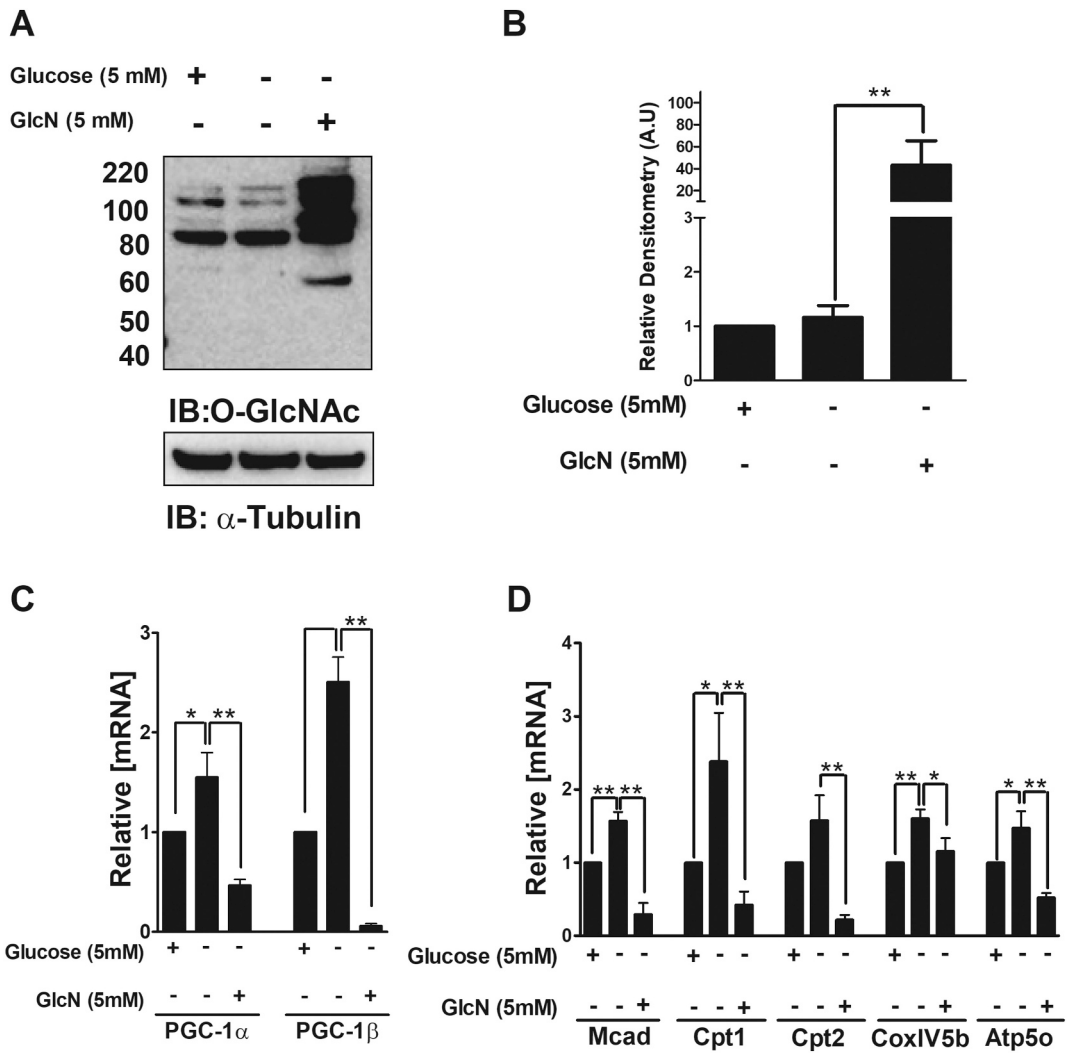

Fig. 4. O-GlcNAc constitutively suppresses PGC-1 gene expression. A, Immunoblot (IB) analysis of the effect of glucose deprivation (0 mmol/L) or glucosamine (GlcN – 5 mmol/L) for 24 h on total O-GlcNAc levels. Panel B represents the densitometric analysis of O-GlcNAc levels relative to tubulin. C, PGC-1α, and PGC-1b gene expression. D, fatty acid metabolism (Mcad), fatty acid transport (Cpt2, Cpt1), and mitochondrial genes (CoxIV5b, Atp5o) are elevated in the absence of glucose, which is reversed by elevating O-GlcNAc levels. NRCMs were treated for 24 h under euglycemic conditions (5 mmol/L), aglycemia (0 mmol/L), or in the presence of glucosamine (5 mmol/L) but no glucose. Results are expressed as means ± S.E. $n \geq 3$/group, *, $p < 0.05$; **, $p < 0.01$.

adenovirus, treating NRCMs with 6-diazo-5-oxo-L-norleucine (DON; 40 μmol/L), an inhibitor of the rate-limiting enzyme of the hexosamine biosynthetic pathway (GFAT) rescued PGC-1α (Fig. 2A) levels and activity toward its dependent genes (Fig. 2B), including fatty acid oxidation genes (*cpt1* and *cpt2*), with a slight decrease in mitochondrial genes (*CoxIV5b*, *Atp5o*, *Cycs*). We have published and characterized that transverse aortic constriction in mice increases O-glycosylation [26]. Here we show that this procedure suppresses PGC-1α levels as shown in Fig. 3A and by others [8] TAC also suppressed fatty acid oxidation genes (mcad, cpt1 and cpt2), with a slight reduction in mitochondrial genes (CoxIV5b, Atp5o, Cycs - Fig. 3B). Importantly, we show that TAC increases total levels of O-glycosylation (Fig. S1). Therefore, hypertrophic induction of O-GlcNAc signaling with a concomitant decline in PGC-1α mRNA expression supports the hypothesis that the two events are related.

3.2. *O-GlcNAc signaling can suppress PGC-1α activity in the absence of hypertrophy*

Hypertrophic induction of O-GlcNAc signaling and PGC-1α suppression raises the question of whether O-GlcNAc signaling regulates PGC-1α in the absence of hypertrophy. Accordingly, we subjected NRCMs to glucose deprivation (*in vitro* 'fasting'), which is a known method to induce PGC-1α and reflects the importance of this coactivator in the maintenance of energy metabolism [28]. To test the ability of augmented O-GlcNAc signaling to suppress PGC-1α induction, we treated 'fasted' cells with hexosamine biosynthetic pathway donor glucosamine. Glucosamine significantly elevated O-GlcNAc signaling in glucose-starved cells (Fig. 4A, B). Quantitative real-time PCR analysis revealed up-regulation of PGC-1α and β (another PGC isoform) mRNA levels (Fig. 4C) and several of its target genes (Fig. 4D) in cardiomyocytes 24 h after glucose deprivation. Moreover, glucosamine treatment eliminated the induction of PGC-1α (Fig. 4C) with clear suppression of fatty acid metabolic (*Mcad*), fatty acid transport (*Cpt2*, *Cpt1*), and mitochondrial genes (*CoxIV5b*, *Atp5o*), as shown in Fig. 4D. One limitation is the artificial nature of PGC induction, however, this approach is used to generate a robust elevation in PGC-1α levels in the absence of hypertrophy. Therefore, we also treated cells with O-(2-acetamido-2-deoxy-D-glucopyranoslidene) amino-*N*-phenylcarbamate (PUGNAc - an O-GlcNAcase inhibitor) and found similar results (Fig. S2).

3.3. PGC-1α is a target for O-glycosylation

Others have shown that PGC-1α is O-glycosylated by an approach using anti-flag antibodies in cells overexpressing Flag- PGC-1α [29]. Here we use a more direct approach by immunoprecipitating PGC-1α and blotting for O-GlcNAc or immunoprecipitating all O-glycosylated proteins and blotting for PGC-1α. Using this technique we successfully detected O-glycosylated PGC-1α at basal levels. Interestingly, this modification was upregulated by treating NRCMs with PUGNAc. Additionally, 2-[(4-Chlorophenyl)imino]tetrahydro-4-oxo-3-(2-tricyclo [3.3.1.13,7]dec-1-ylethyl)-2H-1,3-thiazine-6-carboxylic acid (TTO4, an OGT inhibitor) treatment decreased the levels of O-glycosylated PGC-1α (Fig. 5A, B). Immunoprecipitating samples with the anti-O-GlcNAc antibody, CTD110., 6, and blotting for PGC-1α also reveal a similar pattern (Fig. 5C), confirming that PGC-1α is by itself a target for O-GlcNAc signaling.

3.4. Cardiomyocyte O-GlcNAc transferase deficiency relieves basal suppression of PGC-1α

Based on our evidence that O-GlcNAcylation can suppress PGC-1α levels (Figs. 1, 2), we evaluated whether endogenous *in vivo* O-GlcNAc signaling exerted a level of tonic, negative inhibition of PGC-1α by generating cardiomyocyte specific *Ogt* knockout mice (referred to as cmOGT KO). Previously we have shown that cmOGTKO does not induce apoptosis, or any acute morphological or functional changes in non-pathologic cardiac tissue. Additionally, this cmOGTKO induces a sustained loss of OGT protein in cardiomyocytes seen by western blot or by immunofluorescent sections stained for OGT [30]. Here, we show that the loss of cmOGT was confirmed at the mRNA levels, importantly the levels of O-GlcNAcase was maintained (Fig. 6A). In accordance with our hypothesis, cmOGT KO hearts had higher levels of PGC-1α, PGC-1β (Fig. 6B), and, fatty acid oxidation genes (*mcad*, *cpt1 and cpt2*), and mitochondrial genes (*CoxIV5b*, *Atp5o*), as indicated in Fig. 6C.

Altogether these results suggest that O-glycosylation modifies and contributes (partially or not) to PGC-1α suppression during pathological cardiac hypertrophy.

4. Discussion

In this study, we revealed an intricate relationship between the transcriptional coactivator PGC-1α and O-GlcNAc signaling, particularly in the context of cardiomyocyte hypertrophy. The suppression of PGC-1α, and several of its downstream genes in hypertrophy, apparently requires O-GlcNAc signaling. Indeed, the enzyme that catalyzes the addition of O-GlcNAc on serine or threonine in proteins has been shown to mediate repression at certain promoter regions [21]. Although work from our laboratory has clearly demonstrated the acute cardioprotective benefits of O-GlcNAc signaling [16,19,31], the present study provides surprising insights into the implications of chronic alterations in O-GlcNAc signaling. Although the focus of the present work was the mechanism of PGC-1α suppression during cardiac hypertrophy, the implications for other metabolic disturbances such as diabetes are clear.

Cardiac hypertrophy involves PGC-1α suppression [8] and a shift in substrate utilization from fatty acid to glucose oxidation [4]. Here, we demonstrate that the suppression of PGC-1α was sensitive to inhibition of hexosamine biosynthesis flux and O-GlcNAc signaling (*in vitro*) *via* O-GlcNAcase overexpression. Although *in vitro* hypertrophy did not suppress electron transport genes at the time evaluated, the reduction in O-GlcNAc seems to alter the balance between the repressive and the inductive stimulus (favoring the inductive) making the cells less susceptible to the hypertrophic stimulus. One could argue that the treatment of cells with adenoviral O-GlcNAcase could exacerbate the transcription of these genes due to the known histone acetyltransferase C-terminal domain on this protein [32]. In view of O-GlcNAcase histone acetyltransferase C-terminal domain to promote gene expression, we also tested the inhibitor of the hexosamine biosynthetic pathway DON that interferes only with O-glycosylation and similarly to O-GlcNAcase enhanced PGC-1α transcription during hypertrophic stimulus. O-GlcNAc signaling is not sufficient to promote hypertrophy *per se*, but is sufficient

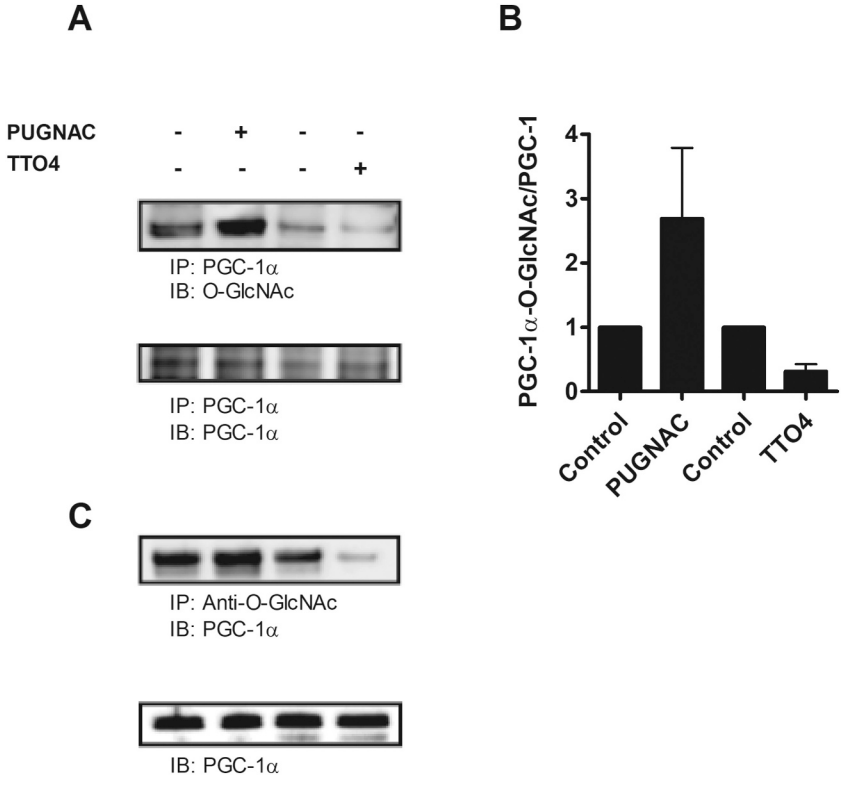

Fig. 5. PGC-1α is a target for O-GlcNAc modification. A, Immunoblot of O-GlcNAc from immunoprecipitation of PGC-1α from NRCMs treated with PUGNAC and TTO4 or vehicle. The bottom panel shows PGC-1α input levels. B, relative densitometry of O-glycosylated PGC-1α normalize for total PGC-1α immunoprecipitated. C, samples were immunoprecipitated using anti-O-GlcNAc antibody and immunoblotted for PGC-1α. The bottom panel shows total PGC-1α input levels. NRCMs were exposed to PUGNAC (200 uM, 16 h) or TTO4 (2.5 uM, 2 h) as indicated. $n \geq 3$/group.

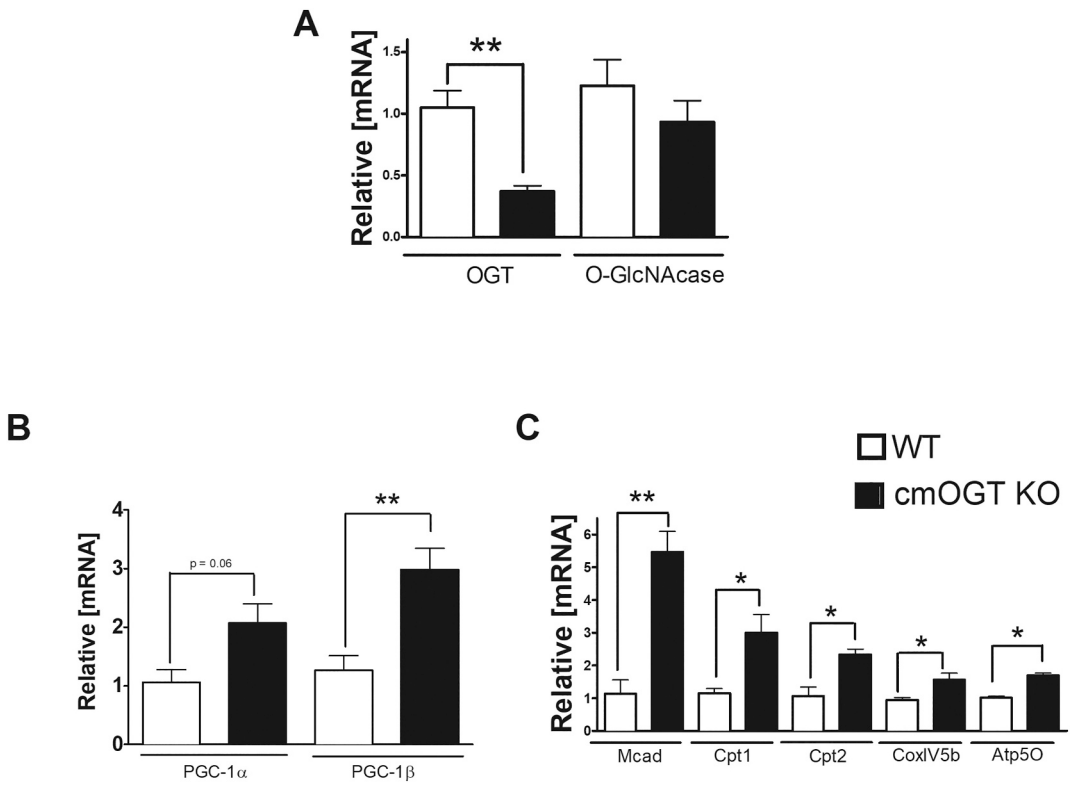

Fig. 6. Cardiomyocyte-specific O-GlcNAc transferase deletion induces PGC-1. Cardiomyocyte-specific OGT knockout (cmOGT KO) mice were created using the well-described 'MerCreMer' inducible mouse. A, cmOGT knockout reduced OGT mRNA expression in the heart, but not GCA mRNA. B, Deletion of cardiomyocyte OGT induced PGC-1α and PGC-1b mRNA and its downstream genes (panel C). Data are presented as mean ± S.E., $n \geq 3$/group. *, $p < 0.05$; **, $p < 0.01$.

(at least *in vitro*) to suppress PGC-1α mRNA expression. It is important to note that PGC-a activity is not only determined by its mRNA expression levels but by the protein post-translational modifications such as phosphorylation, acetylation [33], and, as shown here (and by others) by O-glycosylation [29].

During the hypertrophic stimulus triggered by TAC or phenylephrine, the cell suffers an extensive change in transcription, translation, and even metabolism with several adaptive or maladaptive reactions occurring simultaneously. O-GlcNAcylation of proteins was found here to be upregulated during cardiac hypertrophy. So, is O-GlcNAc signaling, in the absence of hypertrophy, sufficient to repress PGC-1α and its downstream genes? PGC-1α has proven to be an important regulator of mitochondrial biogenesis and respiration as well as fatty acid oxidation [5,6]. Therefore, the levels of this protein must be controlled to prevent perturbations in cellular energetics [34]. Here, we show that treating NRCMs with glucosamine increases intracellular levels of O-GlcNAc and blunts the levels of PGC-1α and its downstream genes. The absence of PGC-1α in cardiac tissues leads to metabolic defects [34], exacerbates the maladaptive effects of hypertrophy [10], and makes cardiomyocytes more susceptible to apoptosis [9]. Indeed, heart failure induces a PGC-1α downregulation in human subjects [35]. Although we did not conduct experiments in order to determine the substrate utilization on these cells, we predict fatty acid oxidation is reduced due to downregulation of MCAD, CPT1 and CPT2. A controversial point is that modest PGC-1α overexpression in adult heart did not prevent the maladaptive effects of pressure overload in mice [36]. This mild overexpression was, indeed, detrimental. In our model we did not overexpress PGC-1α directly, but its levels were suppressed in mice subjected to TAC surgery or cardiomyocytes subjected to phenylephrine. Previously, our group found that OGT is not absolutely required for the development of cardiac hypertrophy during pressure overload. Indeed, OGT knockout in cardiomyocytes seems to exacerbate ventricular dysfunction (*via* suppression of GATA-4 expression), with no effects on fibrosis [37]. Surprisingly, the absence of OGT is not sufficient to upregulate PGC-1α during cardiac hypertrophy *in vivo*. In terms of mechanistic explanation, it is possible that the upregulation of PGC1a by the absence of OGT *in vivo* may be overcomed (during cardiac hypertrophy) by other repressing signals that are not yet known [37]. A lost threshold of cardiac PGC-1α protein might trigger additional alterations that are important to sustain cardiac contractile function.

Evidence from the Hart laboratory indicates that PGC-1α interacts with OGT in a complex with FOXO transcription factor, which they show to be O-GlcNAcylated [29]. Although protein levels of PGC-1α were not evaluated here, we clearly show regulation of PGC-1α transcript levels by O-GlcNAc signaling. The constitutive relationship between OGT and PGC-1α is highlighted by the fact that cardiac-specific knockout of OGT is sufficient to augment PGC-1α and its dependent genes. Unfortunately, this procedure leads to progressive and gradual cardiomyopathy demonstrating that OGT is essential for the maintenance of normal cardiomyocyte physiology [38]. Additionally, it is widely recognized that OGT and O-glycosylation of proteins is a cardioprotective signal. To further the knowledge of the relationship between OGT and PGC-1α we demonstrated that PGC-1α is O-glycosylated by a direct approach rather than using recombinant FLAG proteins as used before [29]. PGC-1α has been shown to regulate the activity of its own promoter by binding the myocyte enhancer factor 2 (MEF2) [29]. Therefore, it is possible that an interaction with OGT and direct O-GlcNAcylation of PGC-1α could inhibit the activity of this coactivator on its own promoter, consequently providing an additional mechanism of regulating PGC-1α transcription. MEF2 plays a role on transcription through binding coactivators proteins such as CBP, p300 and PGC [39], which is the result of phosphorylation events that release MEF2 from an inhibitory complex that includes histone deacetylases and mSin3. Interestingly, mSin3A has been shown be part of a repressive complex with OGT and histone deacetylase [21]. Additionally, O-glycosylation of PGC-1α weakens its interaction with PPARgamma [40]. Similar studies still need to be

conducted in cardiomyocytes during cardiac hypertrophy or with PPARalpha, the isoform highly expressed in cardiomyocytes. It is important to consider that OGT could regulate constitutive suppression of PGC-1α and its absence could induce PGC-1α transcription by reduction in O-GlcNAc signaling.

In summary, this study provides novel insights into mechanisms of metabolic reprogramming in the hypertrophic heart, with important implications for homeostatic metabolic regulation. Such mechanisms could provide a partial explanation for how PGC-1α dependent transcription is suppressed during the development of hypertrophy and heart failure. The PGC-1α levels and activity is maintained, upregulated, or repressed by intricate regulatory circuitry. This circuitry is controlled by transcriptional and post-translational modification mechanisms (for review see [33]). This circuitry might involve O-glycosylation (as shown here), phosphorylation, ubiquitination, methylation, and acetylation. O-glycosylation may target several proteins, including regulators of PGC-1α expression or activity or the protein PGC-1α at residue Serine 333 as seen before [29]. In addition, this work provides potential insights into metabolic dysregulation that occurs in diabetic tissues. Many of the critical targets of O-GlcNAc signaling remain elusive, but the significance of O-GlcNAc signaling in primary disease is becoming unquestionable. Future studies are necessary to dissect whether PGC-1α protein levels and O-glycosylated levels are still up or even down-regulated during compensated and decompensated cardiac hypertrophy progress. This will bring new and valid insights on the mechanisms of ventricular dysfunction during cardiac hypertrophy. As the influence of metabolic signaling in the form of O-GlcNAc remains unclear, this warrants more studies to investigate the possibility that novel therapeutics might arise from such discoveries.

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CRediT authorship contribution statement

Robert E. Brainard: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Heberty T. Facundo:** Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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