

Cardiac O-GlcNAc signaling is increased in hypertrophy and heart failure

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¹Institute for Experimental Medical Research, Oslo University Hospital Ullevaal; ²Center for Heart Failure Research, University of Oslo; ³Bjørknes College; ⁴Department of Pharmacology, University of Oslo and Oslo University Hospital Rikshospitalet; ⁵Departments of ⁵Cardiology and ⁶Cardiothoracic Surgery, Oslo University Hospital Ullevaal; and ⁷Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway

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Lunde IG, Aronsen JM, Kvaløy H, Qvigstad E, Sjaastad I, Tønnessen T, Christensen G, Grønning-Wang LM, Carlson CR. Cardiac O-GlcNAc signaling is increased in hypertrophy and heart failure. *Physiol Genomics* 44: 162–172, 2012. First published November 29, 2011; doi:10.1152/physiolgenomics.00016.2011.— Reversible protein O-GlcNAc modification has emerged as an essential intracellular signaling system in several tissues, including cardiovascular pathophysiology related to diabetes and acute ischemic stress. We tested the hypothesis that cardiac O-GlcNAc signaling is altered in chronic cardiac hypertrophy and failure of different etiologies. Global protein O-GlcNAcylation and the main enzymes regulating O-GlcNAc, O-GlcNAc transferase (OGT), O-GlcNAcase (OGA), and glutamine-fructose-6-phosphate amidotransferase (GFAT) were measured by immunoblot and/or real-time RT-PCR analyses of left ventricular tissue from aortic stenosis (AS) patients and rat models of hypertension, myocardial infarction (MI), and aortic banding (AB), with and without failure. We show here that global O-GlcNAcylation was increased by 65% in AS patients, by 47% in hypertensive rats, by 81 and 58% post-AB, and 37 and 60% post-MI in hypertrophic and failing hearts, respectively ($P < 0.05$). Noticeably, protein O-GlcNAcylation patterns varied in hypertrophic vs. failing hearts, and the most extensive O-GlcNAcylation was observed on proteins of 20–100 kDa in size. OGT, OGA, and GFAT2 protein and/or mRNA levels were increased by pressure overload, while neither was regulated by myocardial infarction. Pharmacological inhibition of OGA decreased cardiac contractility in post-MI failing hearts, demonstrating a possible role of O-GlcNAcylation in development of chronic cardiac dysfunction. Our data support the novel concept that O-GlcNAc signaling is altered in various etiologies of cardiac hypertrophy and failure, including human aortic stenosis. This not only provides an exciting basis for discovery of new mechanisms underlying pathological cardiac remodeling but also implies protein O-GlcNAcylation as a possible new therapeutic target in heart failure.

O-GlcNAc transferase; O-GlcNAcase; aortic stenosis; phosphorylation; glycosylation

SUBSEQUENT TO THE DISCOVERY by Torres and Hart (41), O-GlcNAc signaling has been implicated in a diverse array of physiological and pathological functions in the cell (17). Although there is a growing recognition that global protein O-GlcNAc alterations are involved in cardiovascular pathophysiology in diabetes and acute ischemia (13, 27, 33, 35, 51), regulation in the heart is currently not well understood.

In hypertrophic and failing hearts, a shift toward a more glycolytic metabolism occurs (2, 10, 23, 37, 48). As it has been estimated from noncardiac cells that a small (<5%), but constant fraction of glucose is shunted into metabolic pathways

accessory to glycolysis, e.g., the hexosamine-biosynthetic pathway (HBP) (7, 32, 46), more substrate for O-GlcNAc signaling could be available in the failing heart. Thus, we hypothesized that in hypertrophied and failing hearts, numerous signaling proteins may be affected by O-GlcNAcylation.

In the HBP, fructose-6-phosphate is, through several enzymatic steps, converted into uridine diphosphate- β -*N*-acetylglucosamine (UDP-GlcNAc). The rate-limiting enzyme in this conversion is glutamine-fructose-6-phosphate amidotransferase (GFAT). UDP-GlcNAc from the HBP serves as the nucleotide donor sugar for the biosynthesis of proteoglycans and prototypical glycoproteins in the endoplasmic reticulum-Golgi (4), and for reversible, posttranslational modification of nuclear, cytoplasmic, and mitochondrial proteins by O-linked β -*N*-acetylglucosamine, known as O-GlcNAcylation (16–18). Modulation of protein O-GlcNAcylation is achieved by two evolutionary conserved enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). OGT deletion is embryonically lethal, suggesting O-GlcNAcylation to be essential for life (39), and to date, OGT has been found in all tissues examined (7). OGT and OGA antagonistically add and remove the GlcNAc via an O-linkage to serine/threonine residues of proteins in response to cellular signals, in a comparative fashion to O-phosphorylation by kinases and phosphatases.

The aim of our study was to determine if cardiac O-GlcNAcylation, OGT, OGA, and GFAT levels were altered in human heart disease and in animal models of the three most common etiologies of heart failure; i.e., hypertension, myocardial infarction (MI), and aortic constriction. We here show that O-GlcNAcylation of numerous cardiac proteins was increased in all conditions including human aortic stenosis (AS), providing novel insight into signaling in cardiac remodeling and thus representing an exciting basis for discovery of underlying mechanisms.

MATERIALS AND METHODS

An expanded methods section is available in the Supplementary Methods.¹

Human myocardial biopsies. The human myocardial biopsy protocol was approved by the local ethics committee and conforms to the Declaration of Helsinki. Informed written consent was obtained from each patient. Myocardial biopsies from the left ventricle (LV) were obtained from 12 patients (two females) with severe, symptomatic AS during elective aortic valve replacement. Myocardial biopsies from a nonischemic area of 12 patients (one female) with coronary artery disease were taken during coronary artery bypass operation, serving as

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controls. Biopsies were snap-frozen in liquid nitrogen and stored at -70°C .

Rat models and echocardiographic/hemodynamic evaluation. Experiments on rats were approved by the Norwegian National Animal Research Committee and conformed to the Guide for the Care and Use of Laboratory Animals [National Institutes of Health (NIH) publication no. 85-23, revised 1996, US]. Ligation of the left coronary artery (MI model) and aortic banding (AB) was performed on male Wistar rats as previously described (5, 40), while spontaneously hypertensive rats (SHR) were included at 23 wk of age. Sham-operated animals served as controls and underwent the same surgical procedure, except coronary artery ligation in MI Sham and without tightening the silk suture around the ascending aorta in AB Sham. Echocardiography was performed in SHR and 6 wk after MI and AB as previously described (40). Thereafter, left ventricular catheterization was performed by retrograde insertion of a 1.4 Fr Millar pressure transducer into the right common carotid artery and the LV in the MI and SHR models. The thorax was opened and the heart was excised and washed in saline to remove blood in the cardiac chambers. For molecular studies, the LV was dissected, washed in saline, and blotted dry, before being snap-frozen in liquid nitrogen and stored at -70°C . Selection into heart failure (HF) or hypertrophy (HT) groups was based on echocardiographic, hemodynamic, and postmortem analysis. In all surgical procedures performed and during echocardiography, a mixture of 67% N_2O , 28% O_2 , and 4% isoflurane in an anesthesia chamber was used for preoperative sedation. Perioperatively, a mixture of 69% N_2O , 29% O_2 , and 2% isoflurane was given by the endotracheal tube to maintain anesthesia. Buprenorphine were given as postoperative analgesia after coronary artery ligation and AB.

Functional studies in isolated ventricular muscle strips. In three rats fulfilling the criteria for inclusion in the post-MI heart failure group (MIHF), four LV muscle strips from each heart were harvested from the noninfarcted region and stimulated at 1 Hz as previously described (1). A similar set of muscle strips from three sham-operated rats served as control. Contraction-relaxation cycles (CRC) were recorded before and after addition of 200 μM PUGNAc (132489-69-1, Sigma) (31) in two of the four strips, while the other two served as time- and animal-matched controls. Blockers of α_1 -adrenoreceptors (1 μM prazosin, 19237-84-4), muscarinic cholinergic receptors (1 μM atropine, 51-55-8) and the β -adrenoreceptors (1 μM timolol, 26921-17-5) were present throughout the study (all purchased from Sigma). Contractility was measured as maximal development of force ($\text{dF}/\text{dt}_{\text{max}}$) during the CRC, and inotropic responses were measured as percentage alteration in $\text{dF}/\text{dt}_{\text{max}}$. The muscle strips were snap-frozen in liquid nitrogen and stored at -70°C . In a similar set of experiments, CRC were recorded before and after addition of Thiamet-G (13237, Cayman Chemical) in the dose range of 50 nM–50 μM .

Immunoblot analysis. LV lysates were made from frozen human biopsies and rat tissue by slightly different protocols. We added 40 mM glucosamine to the rat samples to provide excess substrate for OGA, while no OGA inhibitor was used for the human samples. SDS-PAGE and immunoblotting were performed essentially as described in the Criterion protocol (BIO-RAD). For immunodetection the following primary antibodies were used: anti-OGT (O6264, Sigma), anti-O-GlcNAc CTD110.6 (MMS-248R, Covance) anti-OGA (a kind gift from Dr. Sidney W. Whitehart, University of Kentucky, Lexington, KY), anti-GFAT2 (sc-134710; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-serine (61–8100, Invitrogen), and anti-phospho-threonine (9381, Cell Signaling Technology). For loading control, anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc-20357, Santa Cruz Biotechnology) was used. Blots were developed using ECL (Amersham/ GE Healthcare) and visualized on a Las-4000 mini (Fujifilm). Quantification of protein band density and processing of immunoblots were performed using ImageJ (National Institutes of Health) and Adobe Photoshop CS5. For total-O-GlcNAc quantification, the densities of all immunoreactive protein bands were merged.

Real-time RT-PCR analysis. Total RNA was isolated from frozen human biopsies and rat ventricles using the RNeasy Mini Kit (Qiagen Nordic). RNA quality was validated using the 2100 Bioanalyzer from Agilent Technologies, and samples with an RNA Integrity Number >7 were accepted for analysis. RNA quantity was determined using the Nanodrop ND-1000 Spectrophotometer (Nanodrop technologies, DE). Reverse transcription into cDNA was performed using the iScript cDNA Synthesis Kit (BIO-RAD). Predesigned TaqMan assays (Applied Biosystems) were used to determine gene expression levels of rat OGT (Rn00820779_m1), rat OGA (Rn00590870_m1), human OGT (Hs00269228_m1), and human OGA (Hs00201970_m1). For normalization, rat GAPDH (Rn01775763_g1) and human ribosomal protein L32 (Custom made for Rpl32 exon 23) (6) were used, as there were no differences in the expression of these genes between the groups (Suppl. Fig. S1, A–D). The results were detected on a 7900HT Fast Real Time PCR System, and the data analyzed using Sequence Detection Software 2.3 (Applied Biosystems).

Statistics. Data are expressed as group means \pm SE relative to control. Statistical differences were tested in GraphPad Prism 5 and were considered significant for $P < 0.05$. Human mRNA ($n = 9$) data were tested using an unpaired *t*-test, while human protein data were not tested statistically due to $n = 3$. Rat mRNA and protein data ($n = 6$) were tested using the nonparametric Mann-Whitney test or Kruskal-Wallis with Dunn's posttest, while contractility data were tested using a Mann-Whitney test.

RESULTS

Cardiac protein O-GlcNAc levels were increased in aortic stenosis patients. To investigate if protein O-GlcNAcylation was regulated in human heart disease, O-GlcNAc, OGT, OGA, and GFAT levels were measured by immunoblotting LV tissue from AS patients and controls. OGT and OGA gene expression was analyzed by real-time RT-PCR, and the clinical features of these patients are detailed in the Supplemental Methods. Protein O-GlcNAc levels were elevated in three out of three AS patients compared with control, on average $64.7 \pm 7.6\%$ higher (Fig. 1A, top, and D), with the most extensive O-GlcNAcylation observed in the area of 20–100 kDa (areas marked 2–5 in Fig. 1A and Table 1). There was little O-GlcNAcylation of proteins of <20 kDa in size, while there was some on proteins of 100–250 kDa in size. Interestingly, compared with immunoblotting detecting global threonine and serine phosphorylation, we detected relatively little phosphorylation of proteins >50 kDa in size (areas marked 1–3 in Fig. 1C and Table 1), while there was extensive phosphorylation of proteins of <50 kDa in size (areas marked 4–6 in Fig. 1C and Table 1). The level of nucleocytoplasmic OGT (ncOGT, 116 kDa) was elevated in 3 out of 3 AS patients compared with controls, on average $33.5 \pm 6.9\%$ higher in the AS group (Fig. 1A, middle, and E). Additionally, we observed two OGT-immunoreactive bands of ~ 85 and 75 kDa, interpreted as mitochondrial OGT (mOGT, 103 kDa) and short OGT (sOGT, 74.5 kDa) (7, 15), which on average were 13.2 ± 4.2 and $61.8 \pm 3.2\%$ higher in AS patients than controls, respectively ($n = 3$, Fig. 1E). Control immunoblotting with antibodies against proteins representing various cellular compartments confirmed that the LV lysates contained cytoplasmic, nuclear, and mitochondrial proteins, together with sarcolemmal and cytoskeletal proteins (Suppl. Fig. S2A). Consistent with an increased OGT protein level, gene expression of OGT was $68.1 \pm 21.8\%$ higher in AS than in controls ($P < 0.05$, $n = 9$, Fig. 1F). The level of ncOGA (130 kDa) was elevated in three out of three AS patients compared with controls, on average

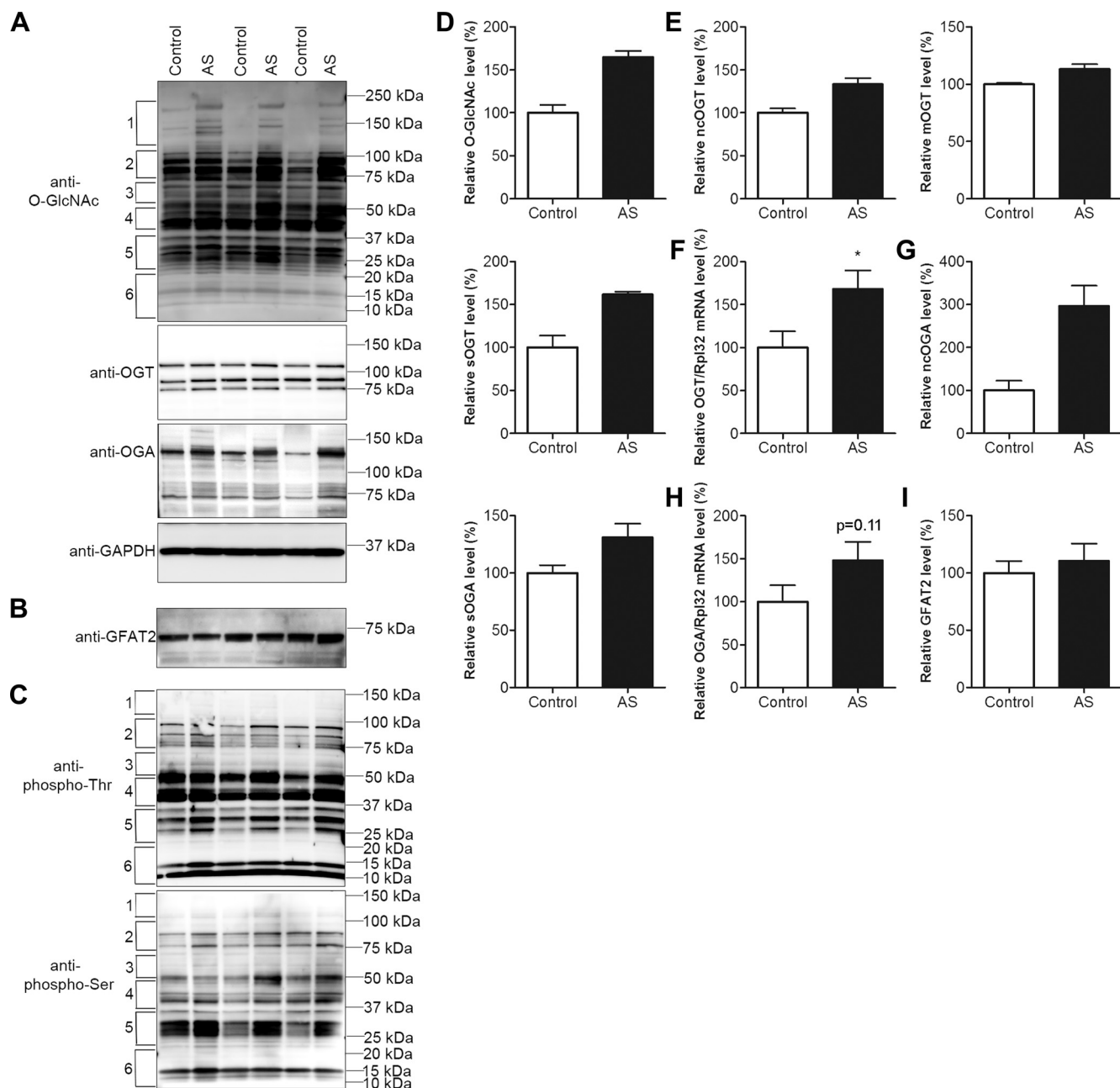


Fig. 1. Cardiac protein O-GlcNAc levels were increased in aortic stenosis patients. Immunoblots of O-GlcNAcylated proteins, O-GlcNAc transferase (OGT; ncOGT, nucleocytoplasmic; mOGT, mitochondrial; sOGT, short), O-GlcNAcase (OGA; ncOGA, nucleocytoplasmic; sOGA, short) (A), glutamine-fructose-6-phosphate amidotransferase 2 (GFAT2) (B), and proteins phosphorylated at threonine (Thr) and serine (Ser) residues (C) in left ventricular biopsies from patients with aortic stenosis (AS) and controls, $n = 3$. Average data of immunoblots in A and B (D, E, G, and I) and OGT and OGA gene expression ($n = 9$; F, H) are shown as means \pm SE. GAPDH was used for loading control for protein data, while ribosomal protein L32 (Rpl32) was used for mRNA normalization. Numbers 1–6 in blots in A and C denote areas of proteins of various molecular weights (kDa), also see Table 1. For patient details, see Suppl. Material. Differences in mRNA expression were tested using an unpaired t -test ($n = 9$); $*P < 0.05$ vs. control. For protein data, all samples are shown and no statistical testing was performed due to $n = 3$.

196.4 \pm 46.9% higher in AS patients, while there were tendencies toward higher sOGA protein (75 kDa, Ref. 7) (Fig. 1A, middle, and G) and OGA mRNA expression level in AS patients ($P = 0.11$, Fig. 1H). No differences were found in the protein level of GFAT2, the most abundantly expressed GFAT isoform in the heart (33) (Fig. 1, B and I).

Cardiac protein O-GlcNAc levels were enhanced by aortic constriction. Similarly, we investigated O-GlcNAc, OGT, OGA, and GFAT2 levels in a model of aortic constriction in

the LV of rats 6 wk post-AB and in sham-operated controls. Rats with hypertrophic (ABHT) and failing (ABHF) hearts had significantly increased LV weight (LVW) and posterior wall thickness in diastole (PWD). ABHF rats also had increased left atrial diameter (LAD) and lung weight (LW) vs. ABHT ($n = 6$, Fig. 2A and Suppl. Table S1). Protein O-GlcNAc levels were 80.6 \pm 13.1 and 58.2 \pm 10.0% higher in ABHT and ABHF than controls, respectively ($P < 0.01$, $P < 0.05$) (Fig. 2B, top, and E), with an extensive increase in O-GlcNAcylation of

Table 1. Extent of global serine/threonine O-GlcNAcylation compared with phosphorylation of human cardiac proteins

Molecular Weight Categories in Fig.1	Serine/Threonine O-GlcNAcylation	Threonine Phosphorylation	Serine Phosphorylation
1 >100 kDa	+	–	–
2 75–100 kDa	++	+	+
3 50–75 kDa	+	–	–
4 37–50 kDa	++	++	+
5 20–37 kDa	++	+	++
6 <20 kDa	–	++	+

Protein blots in Fig.1, A and C, were divided into 6 categories (1–6) based on molecular weight (kDa). The relative level of O-GlcNAcylation (CTD110.6 antibody) (A) and phospho-threonine and phospho-serine (C), within each blot was evaluated and categorized into high (++), medium (+), and low signal (–).

proteins of 20–75 kDa. Similar to in the human heart (see Fig. 1 and Table 1), phospho-threonine- and phospho-serine proteins were mostly seen in the 10–55 kDa range (Fig. 2D). The level of ncOGT was 38.8 ± 9.4 and $56.6 \pm 6.3\%$ higher in ABHT and ABHF than controls ($P < 0.05$, $P < 0.01$) (Fig. 2B, middle, and F), also reflected in significant increased OGT mRNA levels (Fig. 2G). NcOGA was 54.0 ± 10.2 and $55.9 \pm 7.0\%$ higher in ABHT and ABHF, respectively ($P < 0.01$), and there was a significant increase in OGA mRNA in the ABHF group (Fig. 2B, middle, and H and I). There were no differences in protein levels of O-GlcNAc, ncOGT, or ncOGA between ABHT and ABHF. Although control immunoblotting experiments revealed cytoplasmic, nuclear, mitochondrial, sarcolemmal, and cytoskeletal proteins in the LV lysate (Suppl. Fig. S2B), mOGT, sOGT, and sOGA hardly appeared in the rat samples (Fig. 2B, middle), suggesting that the immunoreactivity of the antibodies toward rat OGT and OGA isoenzymes was low. Thus, these were not quantified. The GFAT2 protein level was significantly increased by $47.8 \pm 3.9\%$ in ABHT compared with controls and ABHF (Fig. 2, C and J). Of note, LV protein lysates from rat were prepared with addition of 40 mM glucosamine to provide excess substrate for OGA, while human protein lysates were prepared without OGA inhibition. Despite this difference, O-GlcNAc profiles of comparable quality were obtained.

Cardiac protein O-GlcNAc levels were increased by hypertension. To investigate if O-GlcNAcylation was regulated in hypertension, another etiology of heart failure, O-GlcNAc, OGT, OGA, and GFAT2 were measured in LV tissue of hypertensive rats (SHR) and controls. SHR had significantly higher systolic blood pressure, and hypertrophy characterized by increased PWd and LVW ($n = 6$; Fig. 3A and Suppl. Table S2). Protein O-GlcNAc was $47.2 \pm 6.5\%$ higher in SHR than controls ($P < 0.01$; Fig. 3B, top, and E). There was an extensive increase in O-GlcNAc of proteins of ~50 kDa in size. Also in SHR/WKY rats, serine and threonine phosphorylation was mostly observed in low-molecular-weight proteins <50 kDa (Fig. 3D). The protein level of ncOGT was $38.7 \pm 2.3\%$ higher in SHR than controls ($P < 0.01$; Fig. 3B, middle, and F), and there was a significantly higher expression level of OGT mRNA ($P < 0.05$, Fig. 3G). There was no difference in ncOGA protein level (Fig. 3B, middle, and H); however, OGA mRNA level was $49.9 \pm 12.8\%$ higher in SHR ($P < 0.05$, Fig. 3I). GFAT2 protein level was $71.5 \pm 4.3\%$ higher in SHR than WKY ($P < 0.01$; Fig. 3, C and J).

Cardiac protein O-GlcNAc levels were increased by MI. Finally, we investigated if O-GlcNAc was regulated in MI, the most common etiology of HF. O-GlcNAc, OGT, OGA, and GFAT2 levels were measured in the LV 6 wk after coronary artery ligation and in sham-operated controls. Rats with hypertrophic hearts after MI (MIHT) had increased LVW, despite large anterolateral infarctions, and increased PWd, while rats with MIHF had increased end diastolic pressure, LAD, and LW ($n = 6$; Fig. 4A and Suppl. Table S3). O-GlcNAcylation was 36.7 ± 6.9 and $59.7 \pm 3.3\%$ higher in MIHT and MIHF than controls, respectively ($P < 0.01$ and $P < 0.001$), and significantly higher in MIHF than MIHT ($P < 0.05$) (Fig. 4B, top, and E). Specifically, there were extensive elevations in O-GlcNAcylation of proteins of 37–50 kDa in size and relatively little of proteins <20 kDa in size. Phospho-threonine and phospho-serine signals appeared strongest in the <50 kDa range (Fig. 4D). Despite the increased levels of O-GlcNAcylation, there were no significant differences in ncOGT, ncOGA, or GFAT2 protein nor OGT and OGA mRNA expression levels between controls, MIHT, or MIHF (Fig. 4B, middle; C; and F–J).

Cardiac contractility was reduced by OGA inhibition in post-MI failing hearts. To investigate if increased O-GlcNAcylation affects contractility in the diseased heart, we tested if the OGA inhibitor PUGNAc had effects of contraction-relaxation cycles in LV muscle strips ($n = 6$) from post-MI failing hearts (MIHF). In post-MI failing hearts, addition of PUGNAc induced a negative inotropic response in all muscles tested, with a rapid decline (1st phase) and a following increase (2nd phase) ending in a decline in contractility (3rd phase). The long-term decrease in contractility, measured ~55 min after addition of PUGNAc (3rd phase), was significantly larger than in control (13 ± 3 vs. $4 \pm 1\%$ reduction, $P < 0.05$) (Fig. 5, A and B). In sham-operated hearts, addition of 200 μ M PUGNAc induced a rapid decline in contractility (1st phase) and a following increase (2nd phase), but the long-term decrease in contractility (3rd phase) was not significantly different in PUGNAc-treated sham muscle strips compared with controls (8 ± 5 vs. $7 \pm 5\%$ reduction) (Fig. 5, C and D). A dose of 200 μ M was used, as this dose increased O-GlcNAc levels in the isolated, perfused rat heart and reduced injury following acute ischemic stress (31). Immunoblotting confirmed that 200 μ M of PUGNAc inhibited OGA in our muscle strips preparations as protein O-GlcNAc levels were $59 \pm 13\%$ higher in tissue treated with PUGNAc compared with controls ($P < 0.01$, Fig. 5E). Of notice, the OGA inhibitor Thiamet-G, which increased O-GlcNAc in the brain when administered to rats in vivo (50), did not affect contractility in a similar set of muscle strips in a dose range of 50 nM–50 μ M (data not shown), suggesting that PUGNAc was a more effective OGA inhibitor in muscle strips preparations such as used in our study.

DISCUSSION

We show here increased O-GlcNAcylation of numerous cardiac proteins in nonfailing, hypertrophic, and failing hearts of three common etiologies of chronic cardiac disease: in rat models of hypertension, MI and AB, and in patients with AS. Our results indicate dramatically altered O-GlcNAc signaling in chronic heart disease.

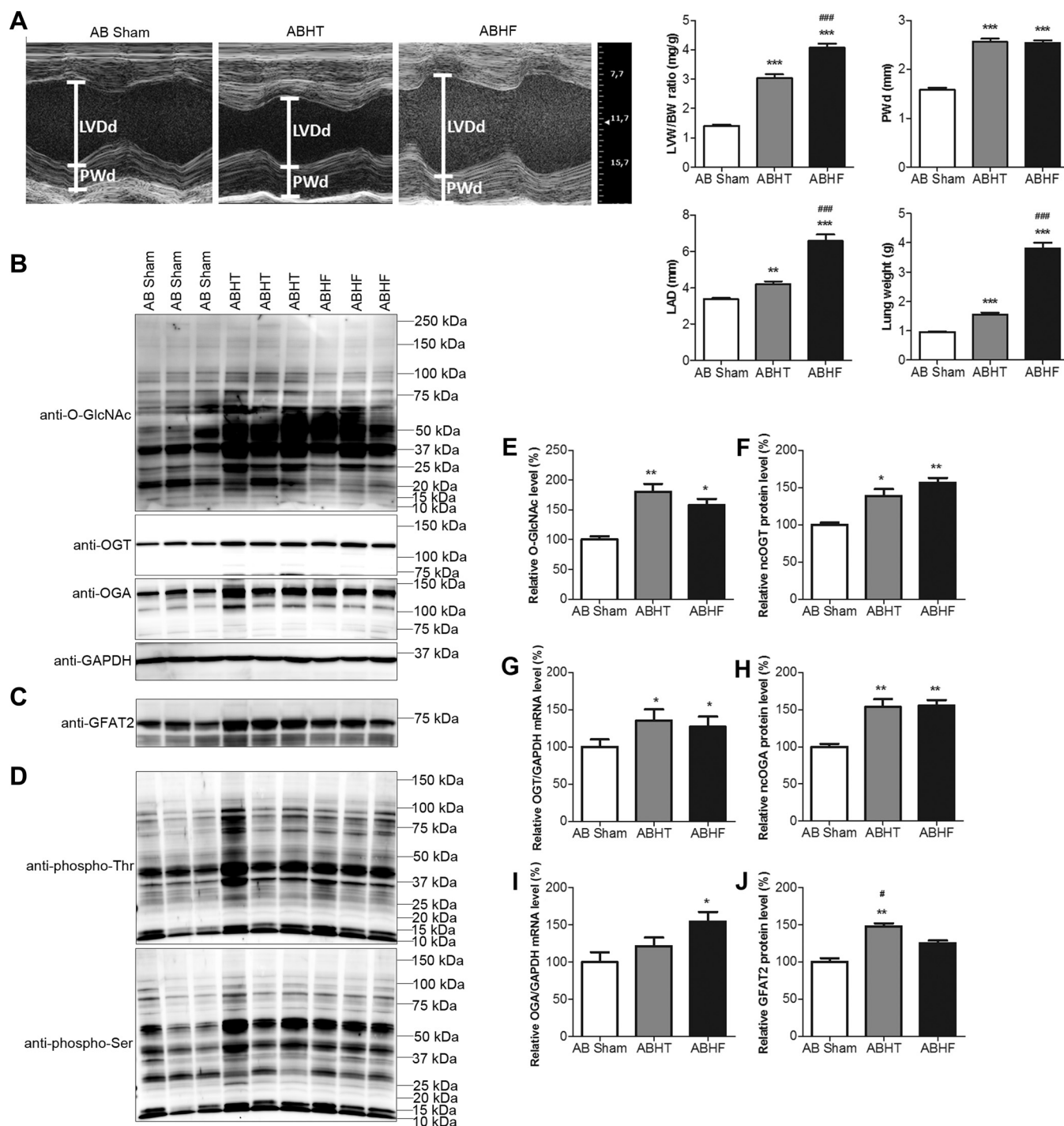


Fig. 2. Cardiac protein O-GlcNAc levels were enhanced by aortic banding. Representative echocardiographic images, left ventricular weight (LVW)-to-body weight (BW) ratio, posterior wall thickness in diastole (PWd), left atrial diameter (LAD), and lung weight in rats 6 wk post-aortic banding (AB), with either cardiac hypertrophy (ABHT) or cardiac failure (ABHF) (A). For details, see Suppl. Table S1. O-GlcNAc, OGT, OGA (B), GFAT2 (C), and proteins phosphorylated at Thr and Ser residues (D) in the LV shown as representative immunoblots. Average data of immunoblots in B and C (E, F, H, and J) and OGT and OGA gene expression (G, I) are shown as means \pm SE, $n = 6$ for all analyses. GAPDH was used for loading control and normalization in protein and mRNA analyses, respectively, and differences were tested using Kruskal-Wallis with a Dunn's posttest; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. AB Sham; # $P < 0.05$; ### $P < 0.001$ vs. ABHT.

The increase in cardiac O-GlcNAc in the HF etiologies studied likely reflects a common denominator: a glycolytic metabolism (2, 10, 23, 37, 48), providing increased glucose flux through the HBP and thus more substrate for O-GlcNAc signaling. Yet, although we observed a global increase encom-

passing numerous proteins, some individual proteins showed decreased levels of O-GlcNAc while others increased, varying in the hypertrophic vs. failing heart. In brain and spinal cord, proteomic analyses have identified nearly 1,000 O-GlcNAcylated proteins (12), and almost all functional classes

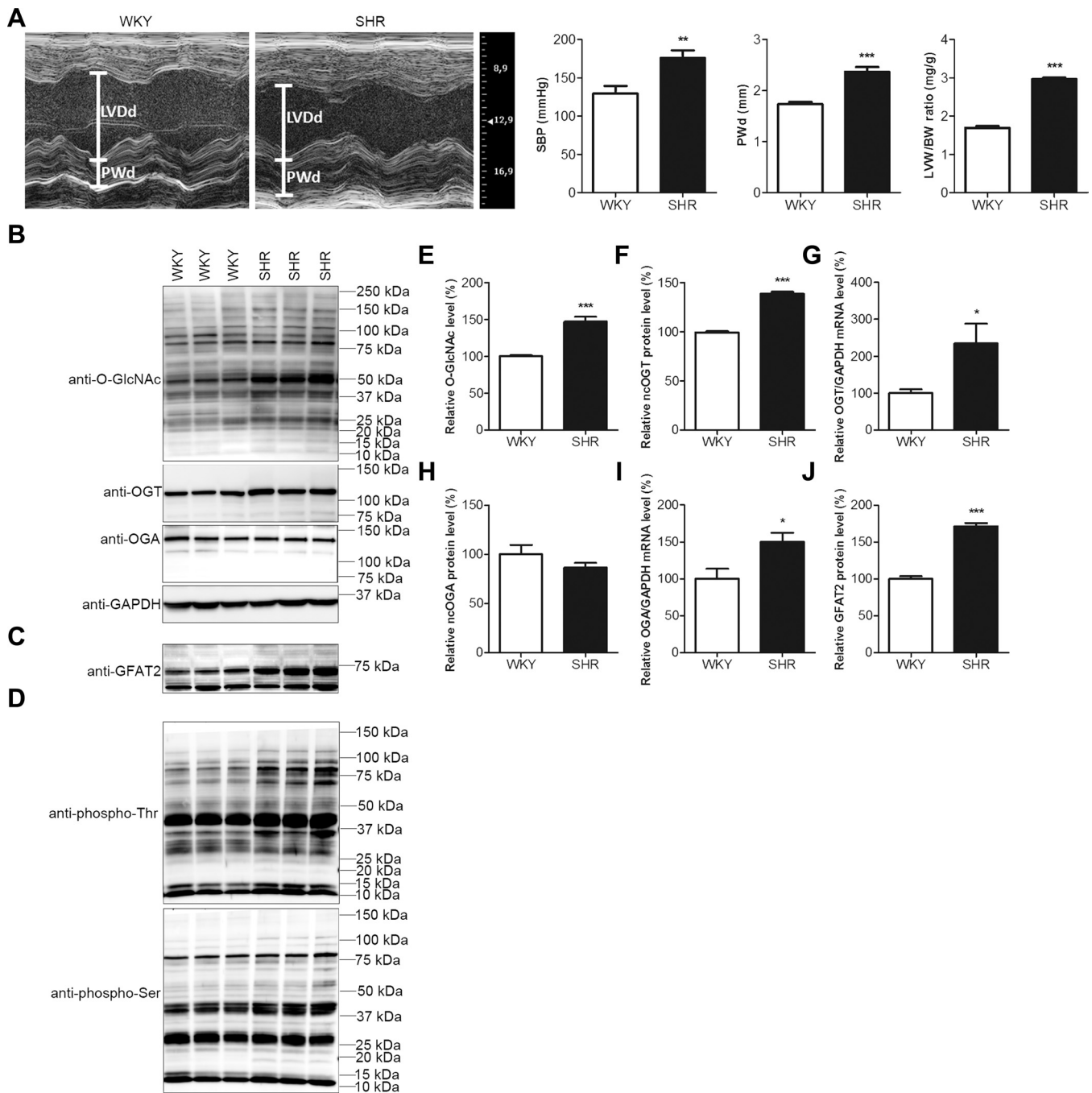


Fig. 3. Cardiac protein O-GlcNAc levels were increased by hypertension. Representative echocardiographic images, systolic blood pressure (SBP), PwD, and LVW/BW ratio in SHR and controls (WKY) (A). For details, see Suppl. Table S2. Protein O-GlcNAc, OGT, OGA (B), GFAT2 (C), and proteins phosphorylated at Thr and Ser residues (D) in the LV shown as representative immunoblots. Average data of immunoblots in B and C (E, F, H, and J) and OGT and OGA gene expression (G, I) are shown as means \pm SE, $n = 6$ for all analyses. GAPDH was used for loading control and normalization in protein and mRNA analyses, respectively. Differences were tested using a Mann-Whitney test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. WKY.

of proteins have been found to be subjected to O-GlcNAcylation (9, 17, 52). Recently, several key proteins involved in cardiac failure, such as PLN (47), myosin heavy and light chains, and troponin I (36) were found to be O-GlcNAcylated. Despite this, a consensus sequence for O-GlcNAcylation has not yet been identified (33), and thus cardiac targets and the mechanisms in which OGT/OGA modifies specific proteins at a specific time, remain unclear.

Although we did not investigate changes in O-GlcNAcylation of individual proteins in our study, some specific banding patterns were apparent in the immunoblots. For instance, a prominent increase of cardiac O-GlcNAcylation at ~ 50 kDa was observed in all four etiologies of cardiac dysfunction, which has also been reported for diabetic mice (21). While O-GlcNAc data for proteins < 50 kDa are rarely reported, Fülöp et al. (14) reported the most prominent O-GlcNAc elevations in

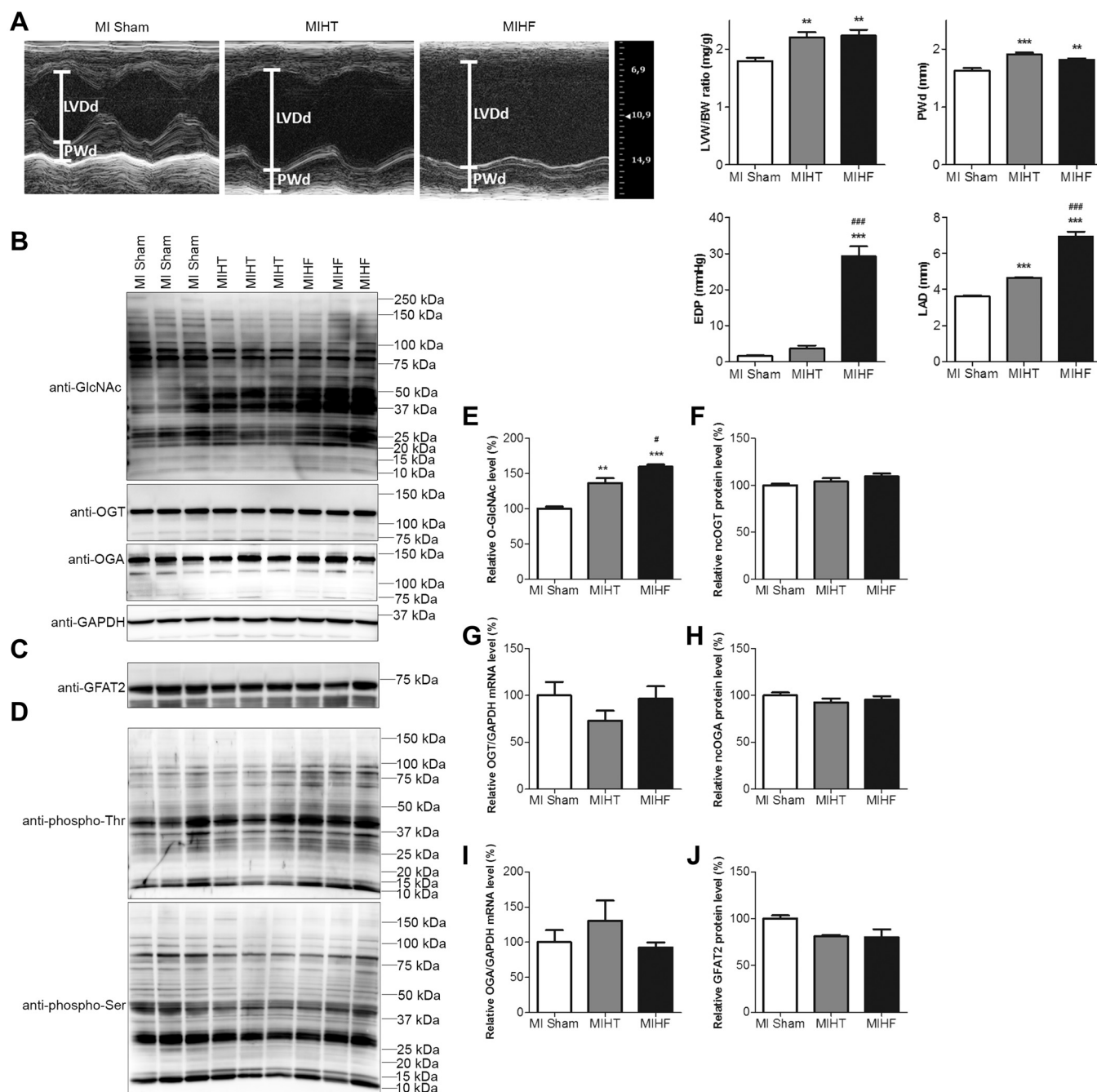


Fig. 4. Cardiac protein O-GlcNAc levels were increased by myocardial infarction (MI). Representative echocardiographic images, LVW/BW ratio, PW/d, end diastolic pressure (EDP), and LAD in rats 6 wk post-MI, with either cardiac hypertrophy (MIHT) or cardiac failure (MIHF) (A). For details, see Suppl. Table S3. O-GlcNAc proteins, OGT, OGA (B), GFAT2 (C), and proteins phosphorylated at Thr and Ser residues (D) in the LV shown as representative immunoblots. Average data of immunoblots in B and C (E, F, H, and J) and OGT and OGA gene expression (G, I) are shown as means \pm SE, $n = 6$ for all analyses. GAPDH was used for loading control and normalization in protein and mRNA analyses, respectively. Differences were tested using Kruskal-Wallis with a Dunn's *posttest*; ^{*} $P < 0.01$; ^{***} $P < 0.001$ vs. MI Sham, [#] $P < 0.05$; ^{###} $P < 0.001$ vs. MIHT.

proteins of sizes at ~ 100 kDa, 140 kDa, and >200 kDa in the diabetic rat heart. In contrast, we detected the strongest O-GlcNAc immunoreactivity in the 20–100 kDa area in our rat and human hearts using the CTD110.6 antibody. Clearly, these comparisons are very superficial and antibody-dependent, and more sophisticated methods such as those employed to identify O-GlcNAcylated proteins in brain tissue (24, 25, 30, 38) are warranted to identify the individual proteins being O-GlcNAc-modified in the diseased heart.

Control of O-GlcNAc through OGT and OGA gene regulation remains elusive, as little is known about the promoters (15). Interestingly, the increased O-GlcNAc observed here in pressure overload, i.e., human AS, chronic hypertension, and AB in rats, was associated with increased nCOGT protein and OGT mRNA expression, suggesting OGT level as one regulatory mechanism of cardiac protein O-GlcNAcylation. Yet OGA mRNA and protein were also upregulated by pressure overload, suggesting additional regulation. Despite enhanced

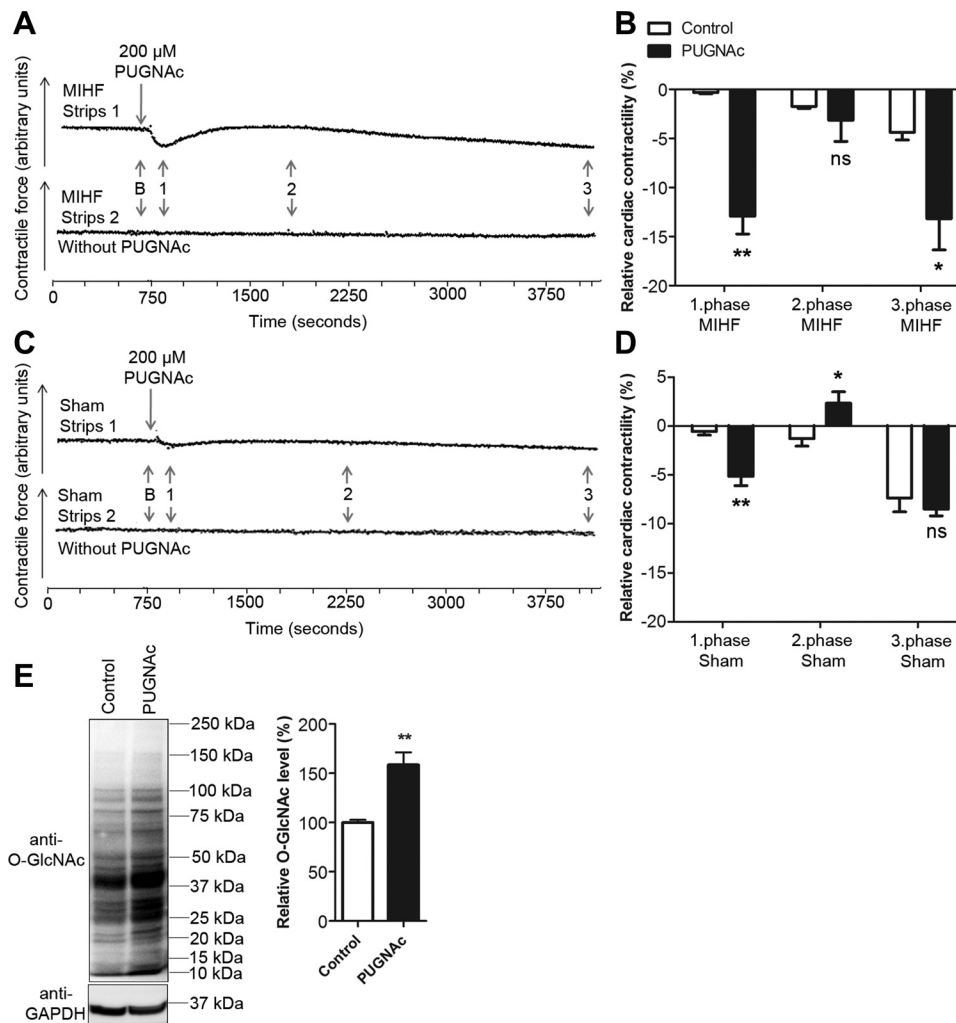


Fig. 5. Cardiac contractility was reduced by OGA inhibition in post-MI failing hearts. Representative tracings of contractility in LV muscle strips from post-MI failing rat hearts (MIHF, $n = 3$) with (strips 1, $n = 6$) and without (strips 2, $n = 6$) addition of 200 μ M of the OGA inhibitor PUGNAc (A). Contractility was measured as maximal development of force during contraction-relaxation cycles (CRCs). Basal ("B") CRCs were obtained in stimulated and control strips, before CRCs from the 1st phase, 2nd phase, and 3rd phase were analyzed as marked ("1," "2," and "3", respectively). Representative tracings of contractility in a similar set of LV muscle strips from sham-operated rats ($n = 3$) (C). Relative contractility (%) is shown as means \pm SE (B, D). O-GlcNAc proteins in muscle strips with and without PUGNAc shown as a representative immunoblot and as means \pm SE (E). GAPDH was used for loading control. Differences were tested using a Mann-Whitney test; ns, nonsignificant; * $P < 0.05$; ** $P < 0.01$.

protein O-GlcNAc levels in MI, OGT and OGA levels were not regulated, suggesting other mechanisms such as UDP-GlcNAc concentration, glycosylation, phosphorylation, protein interactions, or compartmentalization (7, 18) play a role in regulating O-GlcNAc in this etiology.

Protein O-GlcNAc level is also regulated by HBP activity, which is controlled by the rate-limiting enzyme GFAT. An increased flux through the HBP has previously been shown in the pressure-overloaded rat heart (49). Moreover, UDP-GlcNAc, the donor sugar for O-GlcNAc modification, and GFAT2 expression levels were increased by $\sim 40\%$ 7 wk post-AB. In accordance with this, we found increased cardiac GFAT2 levels following AB and in hypertensive rats, indicative of higher HBP activity following pressure overload. In contrast, the GFAT2 level was not significantly altered following MI, suggesting that HBP activity could not contribute to an explanation for the elevation of protein O-GlcNAcylation observed post-MI or that HBP activity is regulated in another way than by GFAT level. As it seemed that the O-GlcNAc-modulating enzymes, i.e., OGT, OGA, and GFAT2, were regulated in tandem, i.e., all upregulated by pressure overload and none regulated by MI, it is tempting to speculate that it is the potential for O-GlcNAc signaling rather than the actual protein O-GlcNAcylation that is controlled in the heart when levels of

these enzymes are altered. Indeed, this was also recently commented on by Belke (3), who found a downregulation of all three enzymes in murine hearts with physiological hypertrophy.

Functionally, the consequences of increased cardiac O-GlcNAcylation may be of importance in cardiac failure development. O-GlcNAc has been linked to cell survival (35, 39), and many forms of cellular stress induce O-GlcNAc and alter the activity, expression, and targeting of OGT (9, 53). In the heart, numerous studies have shown that during acute ischemic and hypoxic stress, O-GlcNAc mediates cardioprotection (8, 13, 22, 27, 33–35, 51, 54). Severe injury such as trauma-hemorrhagic shock induces O-GlcNAc in multiple tissues including the heart, and increasing O-GlcNAc levels during resuscitation has been shown to improve functional recovery of the heart and to reduce stress-mediated inflammation in rodent models (9, 56). Recently, the role of O-GlcNAcylation in post-MI failure was addressed by deletion of OGT in the adult mouse heart (45). These mice showed exacerbated dysfunction after MI, indicating a cardioprotective effect of protein O-GlcNAcylation following MI. Thus, these studies indicate that short-term O-GlcNAcylation is cardioprotective against stresses such as ischemia and hypovolemic shock.

Interestingly, cardiac O-GlcNAcylation was recently also assessed in a model of physiological hypertrophy (3). A ~50% increase in heart mass and an improved contractile performance was correlated to a significant decrease in global protein O-GlcNAcylation in swim-exercised mice. This suggests that our finding of increased cardiac O-GlcNAcylation in the chronically diseased heart, the opposite of what was found in physiologically hypertrophied hearts, is part of a pathological mechanism. Furthermore, our finding of increased O-GlcNAcylation in nonfailing hypertrophic ventricles suggests that increased O-GlcNAcylation is a cellular mechanism preceding development of failure.

In the long term, cardiac O-GlcNAcylation has been shown to have detrimental effects. Several studies have shown that chronically increased O-GlcNAcylation contributes to the cardiovascular dysfunction in diabetes (7, 33). For instance, increased O-GlcNAcylation has been shown to induce vascular dysfunction (28–30), a common complication in diabetic patients and a maladaptive shift in cardiac metabolism toward fatty acid oxidation (26). In cardiomyocytes from diabetic mice, reducing protein O-GlcNAc levels by OGA overexpression improves the contractile function (21), while hyperglycemia, mimicking diabetes by increasing O-GlcNAc levels, impairs cardiomyocyte calcium cycling (11). Furthermore, cardiomyocytes from hyperglycemic, diabetic rats exhibited a significantly impaired relaxation that was associated with increased O-GlcNAc levels (14), indicating that increased O-GlcNAcylation of cardiac proteins contributes to the cardiovascular dysfunction of the diabetic heart. We observed increased cardiac O-GlcNAc levels in chronically hypertrophied and failing hearts. Interestingly, we found that pharmacological inhibition of OGA in post-MI failing hearts, increasing O-GlcNAc, decreased cardiac contractility, suggesting a possible role for chronic O-GlcNAcylation in development of cardiac dysfunction. As several proteins such as PLN (47), actin, myosin heavy chain, myosin light chain, and troponin I (36) are known to be O-GlcNAcyated, it seems reasonable that the response observed in the failing LV muscle strips could be related to altered activity of several of these key regulatory proteins in the contractile apparatus. Thus, we speculate that the increased O-GlcNAcylation observed in our chronically diseased hearts is part of a pathological mechanism promoting cardiac dysfunction.

Another interesting aspect of our findings is interaction with protein phosphorylation. O-GlcNAc cycles on serine/threonine residues of proteins with a timescale, distribution, and abundance similar to phosphorylation, and there is extensive cross talk with the pathways and mechanisms regulated by phosphorylation cascades in response to cellular stimuli (7, 19, 20, 33, 42–44, 55). In fact, OGT and OGA themselves are activated by both O-GlcNAcylation and phosphorylation, and in many cases, OGT, OGA, kinases, and phosphatases are found in the same complex (19, 55). To date, all of the known O-GlcNAcyated proteins are also phospho-proteins, and the specific sites for modification are often the same. When phosphorylation of 700 sites was determined after increasing O-GlcNAcylation with an OGA inhibitor, phosphorylation of virtually all actively cycling sites was either increased or decreased (42), illustrating both competitive and noncompetitive regulation. Since we observed a relatively high number of proteins being O-GlcNAcyated in the heart, cross talk with phosphorylation is

also likely to be important in cardiac remodeling and failure. When we compared global cardiac O-GlcNAc levels to serine/threonine phosphorylation, we found relatively more O-GlcNAcylation on proteins of high molecular weight and phosphorylation on low, which may suggest competitive modification of many proteins in the heart as well. Furthermore, results from studies in which serine/threonine residues have been mutated have been accepted as evidence for the importance of specific kinases without considering O-GlcNAcylation. For instance, O-GlcNAcylation of serine 16 in PLN was recently identified (47). Thus, the biological effects observed after serine 16 mutation that were concluded to result from O-phosphorylation by protein kinase A might actually reflect O-GlcNAcylation. In view of this, serine/threonine posttranslational modifications might need re-evaluation to take into account O-GlcNAcylation of the specific residues.

In conclusion, we report major alterations in the posttranslational modification of O-GlcNAcylation of numerous cardiac proteins in chronic hypertrophy and failure, both in humans with AS and in rat models of increased afterload and MI. We show that O-GlcNAcylation was globally increased in all conditions, providing novel insight and thus representing an exciting basis for discovery of new mechanisms in cardiac remodeling. Although the consequences of increased O-GlcNAc remains speculative, new therapies for cardiac failure may in the future be based on increased knowledge about this essential signaling system.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: I.G.L., J.M.A., E.Q., I.S., T.T., G.C., L.M.G.-W., and C.R.C. conception and design of research; I.G.L., J.M.A., H.K., E.Q., I.S., T.T., and C.R.C. performed experiments; I.G.L., J.M.A., H.K., E.Q., L.M.G.-W., and C.R.C. analyzed data; I.G.L., J.M.A., H.K., E.Q., I.S., T.T., G.C., L.M.G.-W., and C.R.C. interpreted results of experiments; I.G.L. and E.Q. prepared figures; I.G.L. and J.M.A. drafted manuscript; I.G.L., J.M.A., E.Q., I.S., T.T., G.C., L.M.G.-W., and C.R.C. edited and revised manuscript; I.G.L., H.K., E.Q., I.S., T.T., G.C., L.M.G.-W., and C.R.C. approved final version of manuscript.

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