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ORIGINAL ARTICLE

O-GlcNAcylation involvement in high glucose-induced cardiac hypertrophy via ERK1/2 and cyclin D2

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Abstract Continuous hyperglycemia is considered to be the most significant pathogenesis of diabetic cardiomyopathy, which manifests as cardiac hypertrophy and subsequent heart failure. O-GlcNAcylation has attracted attention as a post-translational protein modification in the past decade. The role of O-GlcNAcylation in high glucose-induced cardiomyocyte hypertrophy remains unclear. We studied the effect of O-GlcNAcylation on neonatal rat cardiomyocytes that were exposed to high glucose and myocardium in diabetic rats induced by streptozocin. High glucose (30 mM) incubation induced a greater than two-fold increase in cell size and increased hypertrophy marker gene expression accompanied by elevated O-GlcNAcylation protein levels. High glucose increased ERK1/2 but not p38 MAPK or JNK activity, and cyclin D2 expression was also increased. PUGNAc, an inhibitor of β-*N*-acetylglucosaminidase, enhanced O-GlcNAcylation and imitated the effects of high glucose. OGT siRNA and ERK1/2 inhibition with PD98059 treatment blunted the hypertrophic response and cyclin D2 upregulation. OGT inhibition also prevented ERK1/2 activation. We also observed concentric hypertrophy and similar changes of O-GlcNAcylation level, ERK1/2 activation and cyclin D2 expression in myocardium of diabetic rats induced by streptozocin. In conclusion, O-GlcNAcylation plays a role in high glucose-induced cardiac hypertrophy via ERK1/2 and cyclin D2.

Keywords Cardiomyocyte hypertrophy · O-GlcNAcylation · High glucose

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Introduction

Diabetic cardiomyopathy is defined as ventricular dysfunction occurring independently of a recognized cause, such as coronary artery disease or hypertension, which manifests as cardiac hypertrophy and subsequent heart failure (Murarka and Movahed 2010). Continuous hyperglycemia is considered to be the most significant pathogenesis; this condition induces reactive oxygen species (ROS) generation and activation of pathological pathways, including hexosamine pathway (HBP) flux (Rolo and Palmeira 2006).

Intracellular glucose is phosphorylated to form glucose-6-phosphate, which is further converted to fructose-6-phosphate, of which the majority is channeled to glycolysis. Less than 5 % of intracellular glucose is converted to glucosamine-6-phosphate (GlcN-6-P) by glutamine: fructose-6-phosphate amidotransferase (GFAT), which is rate limiting for hexosamine synthesis; this fraction then enters the HBP. The end product of the HBP, UDP-GlcNAc, is a substrate for uridine diphospho-*N*-acetylglucosamine: polypeptide β-*N*-acetylglucosamine transferase (OGT), which forms O-linked β-*N*-acetylglucosamine (O-GlcNAc)-modified proteins. O-GlcNAc removal is catalyzed by β-*N*-acetylglucosaminidase (OGA). Protein

O-GlcNAcylation cycles dynamically, similar to protein phosphorylation/dephosphorylation. O-GlcNAc-mediated post-translational protein modifications were first identified in 1984 (Torres and Hart 1984), and more than 1,000 proteins localized to the nucleus, cytoplasm and mitochondria have been reported to be modified by O-GlcNAc. O-GlcNAcylation was reported to regulate sp1 (Clark et al. 2003) and the voltage-dependent anion channel (Jones et al. 2008; Ngoh et al. 2008) in cardiomyocytes as well as Akt (Luo et al. 2008; Lima et al. 2009) and eNOS (Musicki et al. 2005; Lima et al. 2008) in aorta, through which it respectively impaired cardiac myocyte function, exerted pro-survival actions during hypoxia-reoxygenation in cardiomyocytes and reduced angiogenesis in endothelial cells. It is also reportedly associated with the cell cycle, as several cell cycle-related proteins were modified by O-GlcNAcylation, including histone H3 (Fong et al. 2012), E2F-1 associated pRB (Wells et al. 2011), vimentin (Slawson et al. 2008), c-Myc (Kamemura et al. 2002), YY1 (Hiromura et al. 2003), keratin 8 and 18 (Chou and Omary 1993), and Sec23 (Dudognon et al. 2004), etc.

The three mitogen-activated protein kinase (MAPK) subfamilies include extracellular signal-regulated kinase (ERK), c-jun NH2-terminal kinase (JNK) and p38 MAPK. ERK1/2 is particularly involved in growth-associated responses (Clerk and Sugden 2001), while JNK and p38 MAPK are both transducers of stress responses (Wang 2001) in cardiomyocyte hypertrophy.

D-type cyclins, especially cyclin D2, play essential roles in cardiac hypertrophy (Busk et al. 2005; Angelis et al. 2008). Cyclin D2 was induced in response to extracellular mitogenic signals via the Ras/MEK/ERK pathways in proliferating cells (Piatelli et al. 2002; Kayampilly and Menon 2012). There is reason to accept the association between ERK1/2 and cyclin D2 in cardiomyocytes.

We hypothesized that O-GlcNAcylation affected ERK1/2 and cyclin D2 since they were associated with growth and the cell cycle. Here, we demonstrate a connection among protein O-GlcNAcylation, ERK1/2 activation and cyclin D2 expression in neonatal rat cardiomyocytes (NRCM) exposed to high glucose and in heart of diabetic rat models, providing a link between high glucose-induced cardiac hypertrophy and O-GlcNAcylation.

Materials and methods

Neonatal rat cardiomyocyte isolation and culture

Cardiomyocytes were derived from 3 to 5-day-old Sprague–Dawley rats (from the Zhejiang University experimental animal center). Ventricular tissues were cut into small pieces and digested in PBS containing 0.1 % trypsin and 0.1 % type II collagenase for 5 min at 37 °C. Cell supernatants were collected, and three times the volume of DMEM cell culture medium (glucose concentration: 5.5 mM, GIBCO) containing 10 % (v/v) newborn calf serum was added to stop the digestion. The above steps were repeated until all of the tissues were completely digested. The cell suspension was then centrifuged, red blood cell lysis buffer was added (Beyotime, China), and the pellet was resuspended in DMEM. After a second centrifugation and resuspension, the cells were seeded in six-well plates at a density of 100,000-cells/cm^2 and were incubated at 37 °C in humidified air with 5 % (v/v) CO_2. Fibroblast numbers were reduced by pre-plating for 1 h using differential cell adhesion and 0.01 mM 5′-BrdU during the first 48 h. All of the animal procedures were performed according to *the Guide for the Care and Use of Laboratory Animals* (NIH, revised 1996), and approval was granted by the university ethics review board. The cardiomyocytes were treated with reagents 72 h after seeding (Yu et al. 2010). D-Glucose, mannitol, glucosamine and PUGNAc were purchased from Sigma-Aldrich (USA). PD98059 (MEK1/MEK2 inhibitor) was obtained from Merck (Darmstadt, Germany).

Rat model

All procedures were performed according to the Guide for the Care and Use of Laboratory Animals (NIH, revised 1996), and approval was granted by the university ethics review board. Twenty male Sprague–Dawley rats weighing 200–220 g (from the Zhejiang University experimental animal center) were randomly divided into a control group and a diabetes group ($N = 10$ for each group). Rats of the diabetes group were induced by a single intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA) at 60 mg/kg body weight in 0.1 mol/l citrate buffer, PH 4.5. The remaining rats received injection of similar volume of vehicle. Rats were considered to be diabetic if they had fasting plasma glucose concentrations of 16.7 mmol/l or greater 72 h later. Blood samples were taken from the tail vein, and glucose levels were measured with a glucometer (Johnson & Johnson Company, USA). All rats were fed normally for 4 weeks. At the end of 4 weeks, the rats were weighed, fasting plasma glucose was measured, and they were anesthetized with intraperitoneal injection of 10 % chloralic hydras (0.35 ml/100 g). After echocardiography had been performed, the rats were killed. Their hearts were rapidly excised and rinsed with cold saline. Every heart was dissected free from the atria, and the whole heart weights were determined. Then the ventricular tissue was cut into three pieces. One was immersed in 4 % formalin; two were frozen in liquid nitrogen and stored at −80 °C until analyzed.

Echocardiography

Short-axis view M-mode echocardiography was performed using a Hewlett-Packard 5500 instrument with a 14-MHz probe. An average of three measurements per each rat was used to assess intraventricular septum (IVS) and left ventricular posterior wall (LVPW) thickness as well as left ventricular internal dimensions at end diastole (LVIDd) and left ventricular internal dimensions at end systole (LVIDs). The left ventricular fractional shortening (FS) was determined by the equation: $FS = [(LVIDd - LVIDs)/LVIDd] \times 100\ \%$.

Histology

LV tissue fixed in 4 % formalin was embedded in paraffin. Sections (4 μm) were cut in the direction of the fibers and mounted on slides. After deparaffinization and rehydration, the sections were stained with hematoxylin-eosin (H&E) and observed at 100× magnification using an optical microscope (Eclipse 80i, Nikon, Japan).

RNA interference

Small interfering RNA (siRNA) oligonucleotides directed towards OGT (gene ID: 26295) and negative controls were purchased from GenePharma (Shanghai, China). The OGT siRNA sequence was 5′-CUGCUUGGAUAAGAUUAA UTT-3′. Forty-eight hours after seeding, NRCMs were transfected with siOGT and the negative control using HiPerFect transfection reagent (QIAGEN, Hilden, Germany) at a final concentration of 20 nM, according to the manufacturer's instructions. Expression was assessed by real-time PCR and Western blot 24 h after transfection.

Immunoblot analyses

Proteins were obtained from cell or tissue supernatants that had been lysed with RIPA lysis buffer containing 1 mM PMSF. Equal protein amounts were run on a 10 % Tris-glycine gradient gel, transferred to PVDF membranes and blocked with 5 % nonfat milk or BSA in Tris-buffered solution (TBS) for 1 h at room temperature (RT). Membranes were then incubated with primary antibodies at 4 °C for at least 6 h and with secondary antibodies at RT for 1 h. Membranes were washed three times with TBST (TBS containing 0.1 % Tween 20) for 10 min before and after the incubations. Finally, proteins were visualized using enhanced chemiluminescence reagent and exposed to Image Quant LAS-4000 (Fujifilm, Tokyo, Japan). Grayscale images were identified using image Multi-Gauge Software (Fujifilm, Tokyo, Japan). Antibodies including CTD110.6, cyclin D2, p-ERK42/44, goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were purchased from CST (Cell Signaling Technology, USA). OGT, t-ERK42/44 and β-actin antibodies as well as goat anti-mouse IgM-HRP were obtained from Santa Cruz (USA).

Real-time reverse transcription-PCR (real-time RT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and converted into cDNA with the PrimeScript RT-PCR kit (Takara, Dalian, China). Primer sequences (ordered from Invitrogen, Carlsbad, CA, USA) are shown in Table 1. Real-time RT-PCR was performed with SsoFast EvaGreen supermix with low ROX (Bio-Rad, Richmond, USA) in an ABI 7500 cycler (Applied Biosystems, Foster City, CA, USA). The conditions were 95 °C for 30 s (1 cycle) and 95 °C for 15 s, 60 °C for 45 s (40 cycles). All of the reactions were performed in triplicate. GAPDH was used as an internal control.

Morphology of cells

Cardiomyocytes were digested at the end of treatments and re-plated on chamber slides at a density of 50,000-cells/cm^2 to measure the cell sizes; 24 h later, cells were fixed in 4 % paraformaldehyde for 30 min. F actin was stained with 5 μg/ml FITC-labeled phalloidin (Sigma-Aldrich, USA) for 45 min, and nuclei were stained with 1 μg/ml DAPI (Sigma-Aldrich) for 10 min at RT. Slides were washed with PBS three times for 5 min each between the two steps. Finally, the preparations were mounted on glass coverslips and observed at 400 × magnification using a confocal laser scanning microscope (LSM510 META, Carl Zeiss, Germany) with an Olympus 40×/0.8 NA water immersion objective. Cell size was analyzed using Image J software (National Institutes of Health, USA) and was expressed as arbitrary units. At least 50 cells from 10 representative fields of three experimental repeats were examined.

Table 1 Real-time RT-PCR primer sequences

Genes	Primers
ANF	Forward: 5′-CAACACAGATCTGATGGATTTCA-3′
	Reverse: 5′-CGCTTCATCGGTCTGCTC-3′
BNP	Forward: 5′-TTCCAAGATGGCACATAGTTCAA-3′
	Reverse: 5′-AGCCAGGAGGTCTTCCTAAAACA-3′
β-MHC	Forward: 5′-AAGTCCTCCCTCAAGCTCCTAAGT-3′
	Reverse: 5′-TTGCTTTGCCTTTGCCC-3′
OGT	Forward: 5′-CGTTTTCCAGCAGTAGGAGAGCCC-3′
	Reverse: 5′-TGGTCGGGTTTGTTGCCAGC-3′
GAPDH	Forward: 5′-CTACCCACGGCAAGTTCAAC-3′
	Reverse: 5′-CCAGTAGACTCCACGACATAC-3′

Statistical analysis

Statistical analysis was performed using SPSS 10.0 software for PC (SPSS Inc, Chicago, IL, USA). The results were presented as the mean ± SEM for at least three individual experiments. One-way ANOVA followed by the Newman-Keuls test was performed for multi-group comparisons. A P value of < 0.05 was considered to be statistically significant.

Results

High glucose and PUGNAc but not glucosamine increased O-GlcNAcylation

O-GlcNAcylation levels were assessed using CTD110.6 (anti-O-GlcNAc) antibodies in cardiomyocytes that had been treated with high glucose (30 mM) for different times to confirm the cellular actions of continuous high glucose levels. O-GlcNAcylation levels began to rise after 30 min and continued to trend upward until 72 h after treatment (Fig. 1a), at which time there was a significant increase in protein-O-GlcNAcylation levels. The results are normalized to β-actin.

Glucosamine and PUGNAc are common 'enhancers' of O-GlcNAcylation. Glucosamine bypasses GFAT to form glucosamine-6-phosphate, and PUGNAc inhibits OGA to increase O-GlcNAcylation levels independent of the HBP. We evaluated O-GlcNAcylation levels in cells that were treated for 72 h with the control (5.5 mM glucose), mannitol (24.5 mM, osmotic control), high glucose (30 mM), high glucose accompanied by OGT siRNA (siOGT) or PD98059 (20 μM), PUGNAc (100 μM) or glucosamine (5 mM). Compared with the control, high glucose and PUGNAc-treated cardiomyocytes had a greater than two- and eightfold increase in O-GlcNAcylation levels, respectively, and the high glucose effect was inhibited by siOGT treatment, as shown in Fig. 1b. Unexpectedly, there were no statistically significant differences between the control and the cells that had been incubated with glucosamine for 72 h. Furthermore, we detected that glucosamine induced a high level of O-GlcNAcylation between 6 and 24 h, which descreased with time (data not shown). It was also observed that negative control siRNA (siNEG) had little effect on O-GlcNAcylation levels elevated by high glucose (Fig. 1c).

OGT inhibition by siRNA was assessed at the mRNA and protein levels. As illustrated in Fig. 1d, siRNA treatment decreased OGT protein levels by about 70 % and mRNA levels by nearly 80 % after 24 h.

O-GlcNAcylation is implicated in ERK1/2 activation and cyclin D2 expression

Seventy-two hours of high glucose and PUGNAc treatment increased p-ERK1/2 levels, respectively. PD98059 and siOGT inhibited high glucose-mediated ERK1/2 activation, while siNEG has little effect on it (Fig. 2a, b). P38 and JNK were unchanged after 72 h of high glucose and PUGNAc treatment (data not shown).

We confirmed the relationship of cyclin D2 to high glucose, O-GlcNAcylation and ERK1/2 by assessing its protein expression in cells under different conditions. Similar to ERK1/2 activation, cyclin D2 expression increased after high glucose and PUGNAc treatments compared with the control. The high glucose-mediated increase in cyclin D2 expression was inhibited by siOGT or PD98059 treatment, while it wasn't by siNEG (Fig. 2a, c).

O-GlcNAcylation and ERK1/2 play roles in high glucose-induced cardiomyocyte hypertrophy

Cardiac hypertrophy is defined by increased heart size because of increased cardiomyocyte size. Cardiomyocytes replated at a lower density made it easy for measurement of cell size, yet the initial density was necessary for the cell junctions. High glucose increased cardiomyocyte size, which was reduced by siOGT or PD98059 treatment. There was no effect of siNEG. Myocytes that were treated with PUGNAc for 72 h also had enlarged cell size, analogous to high glucose treatment (Fig. 3a, b).

Hypertrophy marker genes such as atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC) were measured. Real-time RT-PCR analyses demonstrated an upregulation of hypertrophy marker genes in association with glucose-induced hypertrophy. Similarly, siOGT and PD98059 treatment significantly decreased the expression of these markers, but not siNEG. PUGNAc treatment yielded results similar to those of high glucose treatment (Fig. 3c).

O-GlcNAcylation level, ERK1/2 activation and cyclin D2 expression were upregulated in hypertrophic ventricular myocardium of diabetic rat

The body weight (BW), blood glucose level (BG), heart weight (HW) and HW to BW ratio of all the rats are presented in Table 2, as well as IVS and LVPW thickness, and left ventricular FS. Diabetic rats had significantly lower body weight while high HW to BW ratios compared with control rats. The BG measured with a glucometer was 33.3 mmol/l even when it was greater. It was recorded as 33.3 mmol/l. The BG in the diabetes group was higher than in the control group. The IVS and LVPW were increased in the diabetes group compared to the control group, while the value of IVS/LVPW indicated concentric hypertrophy in diabetic rats. FS in diabetic rats did not decrease at 4 weeks after diabetes was induced. H&E staining of myocardium

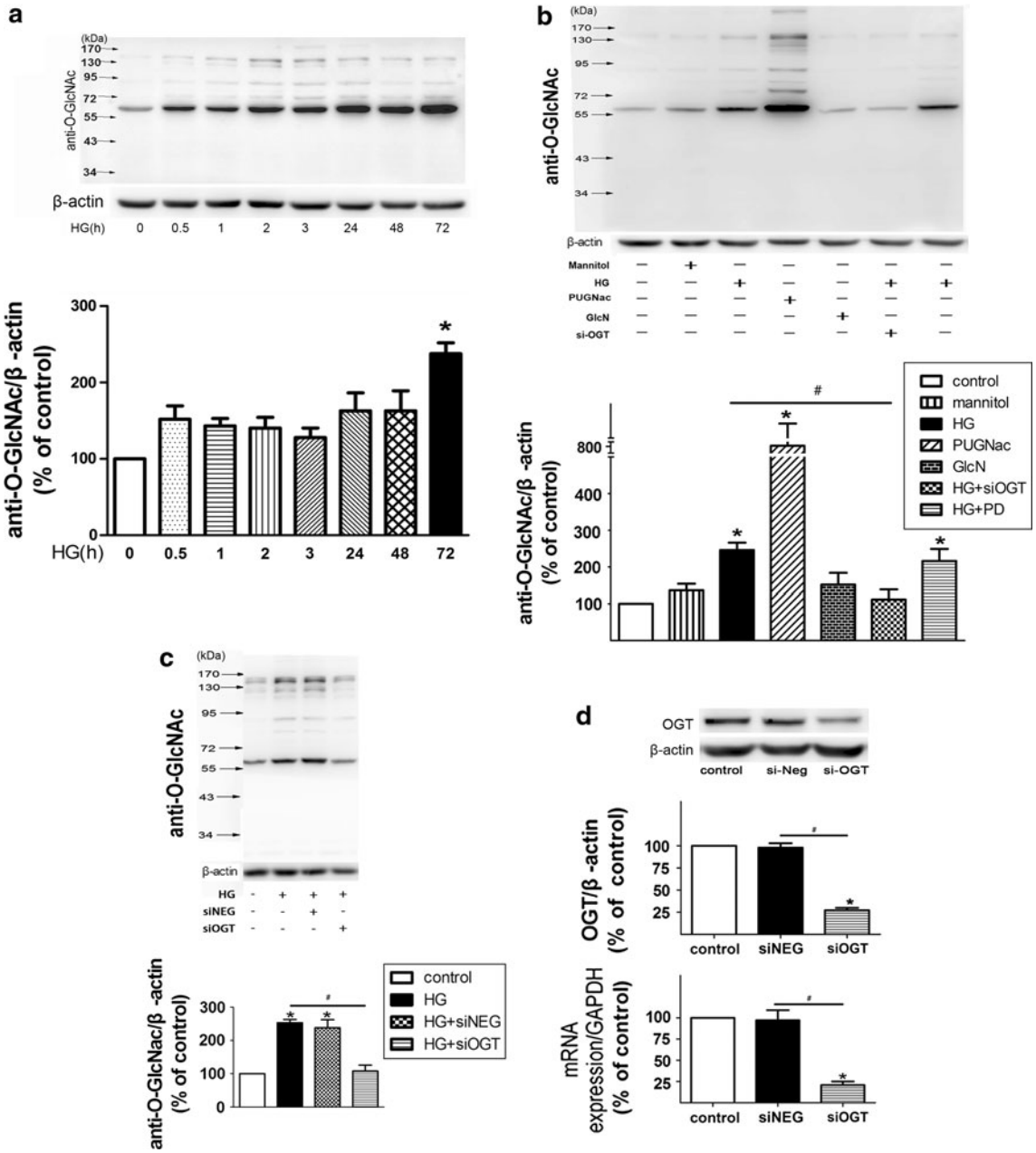

Fig. 1 O-GlcNAcylation levels were increased by high glucose in neonatal rat cardiomyocytes (NRCMs). **a** O-GlcNAcylation levels from high glucose-treated (HG, 30 mM) NRCMs for 0.5 h to 72 h were analyzed by Western blotting with CTD110.6. $N = 4$. **b** O-GlcNAcylation levels from NRCMs that were incubated for 72 h in control, mannitol (24.5 mM, osmotic control), high glucose (HG, 30 mM), high glucose and OGT siRNA treatment 24 h prior (HG + siOGT), high glucose and pre-treatment with PD98059 for 30 min (20 μM, HG + PD), PUGNAc (100 μM) and glucosamine (GlcN, 5 mM) were analyzed by Western blot with CTD110.6. Equal loading was assessed by immunoblot for β-actin. $N = 4$. **c** O-GlcNAcylation levels from NRCMs that were incubated for 72 h in control, high glucose (HG, 30 mM), high glucose and negative control siRNA (HG + siNEG) or OGT siRNA (HG + siOGT) treatment 24 h prior were analyzed by Western blot. Equal loading was assessed by immunoblot for β-actin. $N = 3$. **d** OGT protein expression for the control, negative control (siNeg) and siRNA (siOGT) were analyzed by Western blot 24 h after transfection. Equal loading was assessed with β-actin antibodies ($N = 6$). OGT mRNA expression in the control, negative control (siNeg) and siOGT was analyzed by real-time RT-PCR 24 h after transfection. The relative target gene expression was normalized to GAPDH ($N = 4$). Values are the mean ± SEM. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ between the indicated groups

demonstrated disorderly arranged cardiomyocytes with loose interstitial tissue in diabetic rats (Fig. 4a). It was not reasonable to measure the myocytes size ignoring the effects of different angles in sections. Real-time RT-PCR analyses demonstrated an upregulation of hypertrophy marker genes in diabetic rats (Fig. 4b).

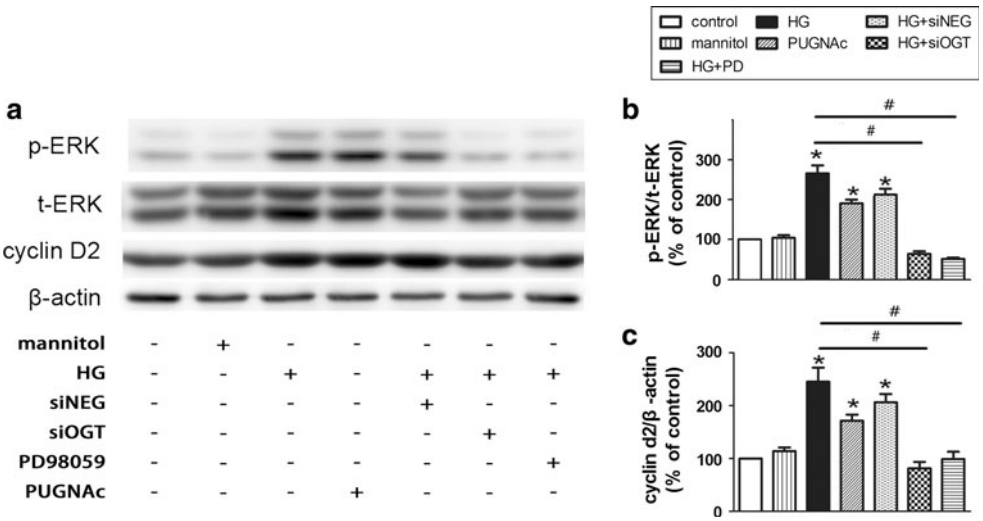

Fig. 2 ERK activation and cyclin D2 expression relative to O-GlcNAcylation increased after high glucose treatment in NRCMs. **a** p-ERK, t-ERK and cyclin D2 were analyzed by Western blot in NRCMs treated with blank, HG, HG + siNEG, HG + siOGT, HG + PD and PUGNAc. **b** p-ERK was normalized to t-ERK ($N = 5$). **c** Cyclin D2 was normalized to β-actin ($N = 4$). Values are the mean ± SEM. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ between the indicated groups

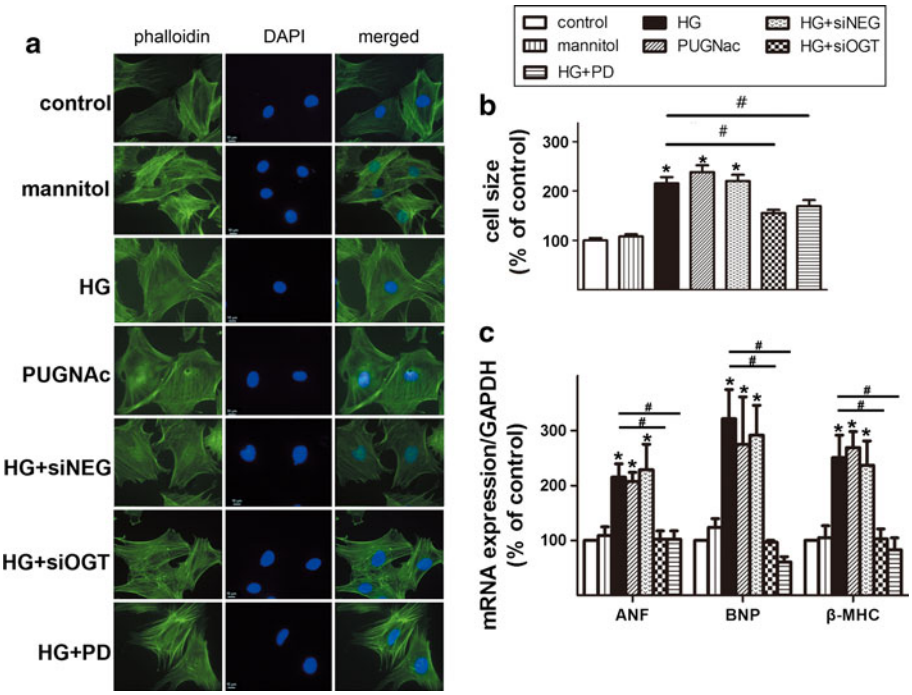

Fig. 3 O-GlcNAcylation, ERK1/2 and cyclin D2 were implicated in high glucose-induced cardiomyocyte hypertrophy. **a** Immunofluorescence was performed with FITC-labeled phalloidin and DAPI. Representative NRCM images for control, mannitol, HG, HG + siNEG, HG + siOGT, HG + PD and PUGNAc after 72 h are shown. F-actin (*green*), nuclear (*blue*) and merged images at ×400 magnification; *bar* 10 μm. **b** NRCM size was measured by directly tracing actin-stained cardiomyocytes in a merged image. Fifty cells were measured from ten representative fields of three experimental repeats in each group. $N = 50$. **c** ANF, BNP and β-MHC mRNA expressions in NRCMs with 72 h-treatment of control, mannitol, HG, HG + siNEG, HG + siOGT, HG + PD and PUGNAc were analyzed by real-time RT-PCR. Relative gene expression was normalized to GAPDH. $N = 3$. Values are the mean ± SEM; $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ between the indicated groups

We confirmed the relationship of O-GlcNAcylation, ERK1/2 and cyclin D2 in vivo. Compared to control rats, O-GlcNAcylation level, ERK1/2 activation and cyclin D2 expression were all upregulated in diabetic rats at 4 weeks, consistent with the cell study (Fig. 5).

Discussion

Increased O-GlcNAcylated protein levels were reported in diabetic animal hearts and isolated high glucose-treated cardiomyocytes (Hu et al. 2009; Marsh et al. 2011).

Cardiac O-GlcNAc protein levels were significantly higher in rats receiving diets high in saturated fat and sugar, suggesting that a 'Western' diet may contribute to the adverse effects of metabolic syndrome and diabetes by an O-GlcNAc-mediated process (Medford et al. 2012). It was reported that glucosamine (Zou et al. 2009) and PUGNAc treatment (Ngoh et al. 2011) increased cardiomyocyte protein O-GlcNAcylation levels. The effects of glucosamine and PUGNAc on cultured cardiomyocytes were commonly assessed for short periods (no more than 6 h) in previous studies. Although glucosamine increased O-GlcNAcylation levels after a short time by forming the first product of the HBP and bypassing the GFAT, the actions of glucosamine were different from those of glucose as the rates of their uptake and utilization through the HBP were different from each other. Marshall revealed that the dynamic and early actions of glucose differed from those of glucosamine on hexosamine flux and GlcN-6-P and UDP-GlcNAc generation (Marshall et al. 2004). The long-term actions of the glucose and glucosamine might differ because glucose utilization through alternate pathways may modify the ultimate response of the HBP, and glucosamine-mediated massive accumulation of GlcN-6-P overwhelming the biosynthetic capacity of the HBP may alter cellular function through allosteric regulation of various enzymes such as hexokinase and glycogen synthase (Ciaraldi et al. 1999; Virkamaki and Yki-Jarvinen 1999). We found that high glucose maintained increased O-GlcNAcylation in cardiomyocytes for at least 72 h, but glucosamine did not. Thus, glucosamine was not a good glucose effector in these cells.

There is evidence that increased HBP flux and protein O-GlcNAcylation levels contribute to diabetic complications in many tissues (Laczy et al. 2009). Protein O-GlcNAcylation is associated with cardiomyocyte hypertrophy.

Table 2 General characteristics and cardiac structure and function of rats in the control group and diabetes group

	CON	DM
General characteristics		
BW (g)	395.5 ± 8.21	237.1 ± 13.41*
BG (mmol/l)	6.64 ± 0.19	32.5 ± 0.48*
HW/BW (%)	3.26 ± 0.08	4.58 ± 0.03*
Cardiac structure and function		
IVS (mm)	45.76 ± 4.47	52.22 ± 1.24*
LVPW (mm)	37.30 ± 1.69	49.87 ± 1.39*
IVS/LVPW	1.22 ± 0.08	1.05 ± 0.12
FS (%)	61 ± 3	60 ± 5

The body weight (BW), blood glucose (BG), heart weight to body weight ratio and the heart intraventricular septum thickness (IVS), left ventricular posterior wall thickness (LVPW), IVS to LVPW ratio and fractional shortening (FS) of all rats in different groups are presented as the mean ± SEM; n = 10 for each group

CON normal control rats, *DM* diabetic rats

* $P < 0.05$ versus control

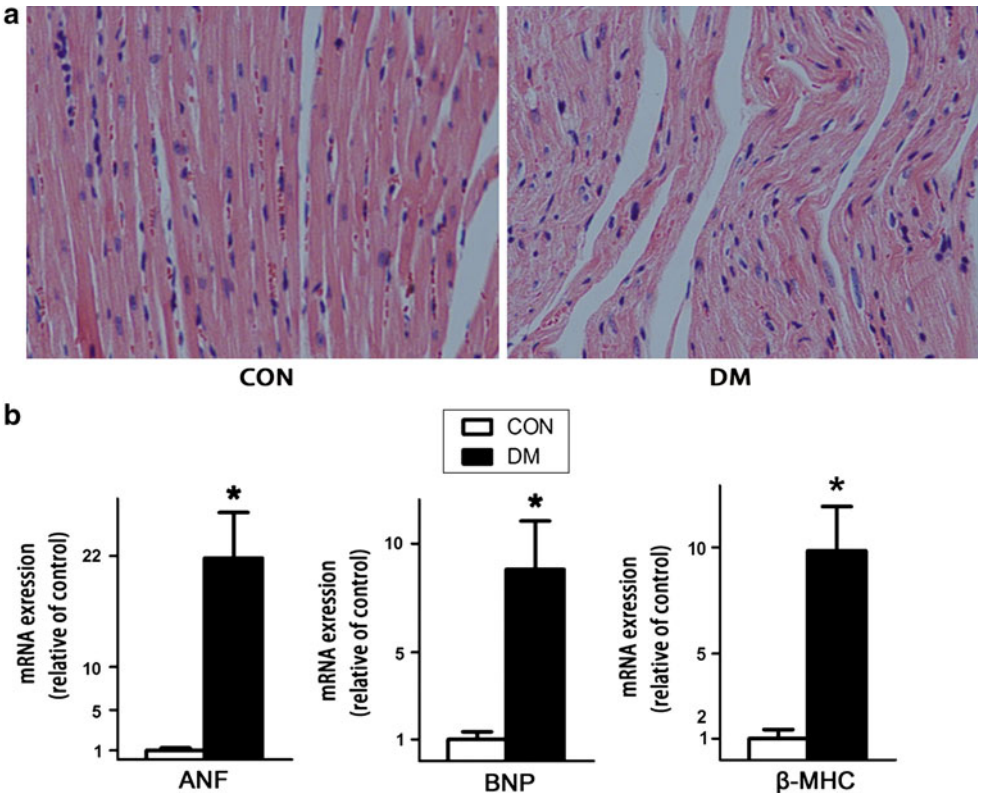

Fig. 4 Diabetic rats induced by STZ manifested cardiac hypertrophy 4 weeks later. **a** H&E staining of myocardium demonstrated pathological changes of cardiomyocytes. Myocardium of control rats were arranged neatly and closely, while in diabetic rats it was shown disordered arranged cardiomyocytes with loose interstitial tissue. The images were at ×100 magnification. **b** ANF, BNP and β-MHC mRNA expression in myocardial tissues of control and diabetic rats was analyzed by real-time RT-PCR. Relative gene expression was normalized to GAPDH. $N = 10$ for each group. Values are the mean ± SEM; *$P < 0.05$ vs. normal control. (*CON* normal control rats, *DM* diabetic rats)

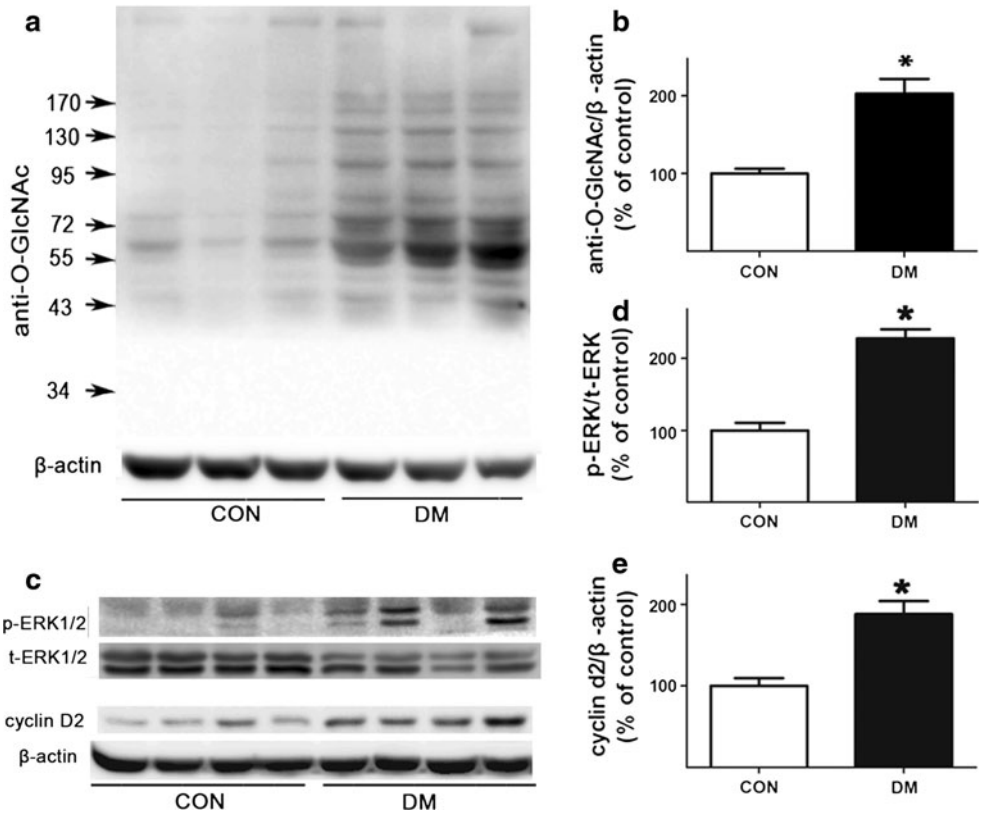

Fig. 5 O-GlcNAcylation level, ERK activation and cyclin D2 expression were upregulated in diabetic rats. **a**, **b** O-GlcNAcylation levels from myocardium of control and diabetic rats were analyzed by Western blot with CTD110.6. Equal loading was assessed by immunoblot for β-actin. $N = 10$ for each group. **c**, **d**, **e** p-ERK, t-ERK and cyclin D2 were analyzed by Western blot in myocardium of control and diabetic rats. p-ERK was normalized to t-ERK, and cyclin D2 was normalized to β-actin. $N = 10$ for each group. Values are the mean ± SEM. $^*P < 0.05$ vs. control. (*CON* normal control rats, *DM* diabetic rats)

O-GlcNAcylation of global cardiac proteins was increased in aortic stenosis patients and in rat models of hypertension, myocardial infarction and aortic banding (Lunde et al. 2012). O-GlcNAcylation also played a critical role in nuclear factor of activated T-cells activation during hypertrophy (Facundo et al. 2012). We determined that both high glucose and PUGNAc treatment increased cardiomyocyte cell size along with increased O-GlcNAcylation levels. OGT inhibition reversed the effect of high glucose, which supported the idea that O-GlcNAcylation played a role in hypertrophy.

The contradiction between the beneficial and adverse effects of O-GlcNAcylation has been described. Glucosamine protected cardiomyocytes from angiotensin II treatment (Nagy et al. 2006), ischemia-reperfusion injury (Champattanachai et al. 2008), trauma-hemorrhage (Zou et al. 2009) and LPS-induced damage (Gong and Jing 2011). It was suggested that this protection occurred because the acute rise in O-GlcNAcylation increased mitochondrial tolerance to oxidative stress (Darley-Usmar et al. 2012). That acute O-GlcNAc formation may confer protection via increased transcription of pro-survival factors such as heat shock proteins was also considered (Zachara et al. 2004; Lim and Chang 2006) and Bcl-2 family members(Dong et al. 1999), and transcriptionally independent mechanisms including modulation of stress-activated kinase pathways (Fulop et al. 2007), post-translational modification of pro-survival factors (Roquemore et al. 1996; Whelan and Hart 2003) as well as attenuation of calcium-mediated stress responses (Liu et al. 2006, 2007). Either hyperglycemia in diabetic individuals or high glucose-induced cardiomyocyte hypertrophy caused chronic multiple pathophysiological changes, including increased oxidative stress. O-GlcNAcylation was increased not only by glucose, but also by ROS (Du et al. 2000), which mediated the impairment of mitochondrial function and generated adverse effects.

O-GlcNAcylation is associated with the cell cycle. Slawson et al. (2005) demonstrated that global O-GlcNAc protein modification is regulated in a cell cycle-dependent manner, and this dynamic regulation is essential for cell cycle progression, which altered mitotic phosphorylation and cyclin expression, including cyclin D. It was also found the O-GlcNAc modification may play important roles in both the developmental and cell division processes of oocytes (Slawson et al. 2002). Lefebvre found that O-GlcNAc glycosylation promoted interactions between the proteins required for the G2/M transition and progesterone-induced maturation in oocytes (Lefebvre et al. 2004). And insulin-induced chondrocyte differentiation was associated with an increase in O-GlcNAcylated proteins (Andres-Bergos et al. 2012).

The important role of MAPK activation in diabetic complications was evidenced (Tomlinson 1999; Xin et al.

2004). It was less controversial that ERK1/2, p38 and JNK were activated between 5 and 30 min after high glucose treatment in cardiomyocytes (Singh et al. 2012). The effects of long-term (more than 48 or 72 h) high glucose treatment are controversial (Chen et al. 2007; Tsai et al. 2012). The Ras/Raf/MEK/ERK cascade is critically involved in the pathogenesis of cardiac hypertrophy. Studies on cardiac myocyte size as well as gene expression profiling demonstrated that the ERK1/2 cascade contributed to prohypertrophic agonist-induced hypertrophic gene expression (Clerk et al. 2006; Kennedy et al. 2006). Overexpression of MEK1 in vivo and in cultured myocytes promoted a physiological hypertrophy response associated with augmented cardiac function and partial apoptosis resistance, suggesting that ERK1/2 activity promoted a compensated form of hypertrophy (Bueno et al. 2000). MEK/ERK was considered to be particularly involved in growth-associated responses and compensated hypertrophy, while JNK and p38 MAPK were both defined as stress activated, such as in pressure or volume overload, and are possibly implicated in decompensated stages (LaMorte et al. 1994; Wang 2001). ERK1/2 but not JNK or p38 MAPK was activated in myocytes, indicating that the 72-h high glucose-induced hypertrophy might be compensated.

Cell-cycle regulatory proteins play a key role in cardiac hypertrophy (Bicknell et al. 2003; Ahuja et al. 2007). D-type cyclins are critical regulators of cardiac hypertrophy (Busk and Hinrichsen 2003). Ang II could upregulate cyclin D2 and promoted hypertrophy in the heart in vivo and in cardiomyocytes (Yang et al. 2011). Cyclin D2 protein levels were upregulated in the left ventricles and cardiomyocytes of rats with pressure overload at the beginning stages of hypertrophy (Li et al. 1998). Cardiac hypertrophy was blocked in cyclin D2-null mice (Zhong et al. 2006) and was inhibited after blocking cyclin D2 transcription with differentiating inducing factor-1 in vivo and in vitro. In the latter case, the hypertrophy was restored by cyclin D2 overexpression (Busk et al. 2002). Cardiac hypertrophy was accompanied by substantial increases in protein synthesis and transcription of tRNA genes by RNA polymerase III (Pol-III). Phosphorylated Rb via increased cyclin D2–CDK4/6 activity relieved TF IIIb and induced TF IIIb–TF IIIc–Pol III transcriptional unit formation (Angelis et al. 2008). Cyclin D2 is induced via the Ras/MEK/ERK signaling pathway in proliferating cells, whereas the connection of these pathways in cardiomyocytes is yet to be confirmed.

Our study demonstrated elevated O-GlcNAcylation levels, activated ERK1/2 and increased cyclin D2 expression in high glucose-induced hypertrophic cardiomyocytes and myocardium of diabetic rats. The hypertrophy and cyclin D2 upregulation were blunted by inhibiting O-GlcNAcylation or ERK1/2. PUGNAc treatment and high glucose had similar effects. Based on these results, we deduced that O-GlcNAcylation-mediated high glucose-induced cardiomyocyte hypertrophy via ERK1/2 and cyclin D2 and that ERK1/2 regulated cyclin D2 expression in cardiomyocytes.

There are multiple effects of glucose because of its various metabolic pathways. The increase in the HBP and O-GlcNAcylation plays a partial role in compensated states. In diabetic cardiomyopathy progression, O-GlcNAcylation induces a growth response that involves the ERK1/2 cascade and cyclin D2. Thus, O-GlcNAcylation might function in compensated stages for the selective ERK1/2 activation. Rat models also demonstrated concentric cardiac hypertrophy in our study. PUGNAc produced a fourfold O-GlcNAcylation level compared to high glucose and a similar level of effect on cardiomyocytes, which indicated the O-GlcNAcylation-mediated effect did not depend on the level of increase.

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