Glucosamine inhibits angiotensin II-induced cytoplasmic Ca\(^{2+}\) elevation in neonatal cardiomyocytes via protein-associated O-linked N-acetylglucosamine

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ANG II is a vasoactive peptide that has a direct effect on cardiomyocytes and plays a critical role in the response of cardiomyocytes to increased hemodynamic stress. ANG II is a potent stimulator of cardiomyocyte hypertrophy; however, sustained exposure is also associated with increased apoptosis (12). The effect of ANG II on cardiomyocytes is mediated via the ANG II type 1 receptor (AT\(_1\)R) and the ANG II type 2 receptor. The AT\(_1\)R receptor has been shown to mediate many of the physiological actions of ANG II (44). AT\(_1\)R couples to the G\(_{\alpha}\) protein to stimulate PLC, generating two secondary messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\(_3\)) (4, 17, 39). The major target of DAG is PKC, whereas IP\(_3\) triggers an increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]). However, direct link between glucosamine or the HBP to [Ca\(^{2+}\)\(_i\)] homeostasis has yet to be established. The end product of the HBP, UDP-N-acetylglucosamine (UDP-GlcNAc), is the substrate for O-GlcNAcase that increases O-GlcNAc levels without changing UDP-GlcNAc concentrations, mimicked the effect of glucosamine on the ANG II-induced increase in [Ca\(^{2+}\)\(_i\)]. An inhibitor of O-GlcNAc transferase, alloxan, prevented the glucosamine-induced increase in O-GlcNAc but not the increase in UDP-GlcNAc; however, alloxan abrogated the inhibition of the ANG II-induced increase in [Ca\(^{2+}\)\(_i\)]. These data support the notion that changes in O-GlcNAc levels mediated via increased HBP flux may be involved in the regulation of [Ca\(^{2+}\)\(_i\)], homeostasis in the heart.

ANG II treatment leads to an increase in basal (i.e., diastolic) [Ca\(^{2+}\)\(_i\)], in both adult and neonatal cardiomyocytes (19, 43, 46). In addition to participating in excitation-contraction coupling (5), changes in [Ca\(^{2+}\)\(_i\)] also influence acute inotropic responses (29, 48a) as well as modulate a number of different signaling pathways regulating myocyte hypertrophy and apoptosis (1, 3, 15, 38). The intracellular targets of [Ca\(^{2+}\)\(_i\)] are numerous and include, e.g., CaM-regulated kinases (56), calcineurin (38, 41), PKC, MAPK (27), cytosolic phospholipase A2, and proteases (3, 33).

In most cell types, the IP\(_3\)-generated [Ca\(^{2+}\)\(_i\)] elevation is a consequence of an initial release from the endoplasmic reticulum followed by a subsequent influx of extracellular Ca\(^{2+}\) into the cytoplasm. This latter process is termed store-operated or capacitative Ca\(^{2+}\) entry (CCE) (35). Although CCE was first described in nonexcitable cells, studies have recently shown that CCE also coexists with L-type channels in smooth and skeletal muscle cells (25, 47). Furthermore, we have shown that CCE is present in neonatal and adult cardiomyocytes (15, 16) and that it appears to mediate the inotropic response of the intact heart to \(\alpha\)-adrenergic agonists such as phenylephrine (31).

We have found that hyperglycemia blunts the ANG II-induced hypertrophy in neonatal rat ventricular myocytes (NRVMs) (31, 32). Interestingly, the inhibition of ANG II-induced hypertrophy by hyperglycemia was blocked by aza-serine, which inhibits glucose entry into the hexosamine biosynthesis pathway (HBP). Furthermore, glucosamine, which enters cells via the glucose transporter system and selectively increases HBP flux, blocks CCE (48) and also inhibits ANG II-induced hypertrophy in NRVMs (15). However, a direct link between glucosamine or the HBP to [Ca\(^{2+}\)\(_i\)]; homeostasis has yet to be established. The end product of the HBP, UDP-N-acetylglucosamine (UDP-GlcNAc) is the substrate for O- and N-glycosylation of proteins (50, 51). The addition of O-linked N-acetylglucosamine (O-GlcNAc) to nuclear and cytoplasmic proteins, which is catalyzed by O-GlcNAc transferase (OGT), is a dynamic and abundant posttranslational modification that has increasingly been recognized as an important regulatory mechanism in signal transduction (14, 49, 52, 53) and that also may be especially important in mediating the cellular stress response (21, 55).

Therefore, the goal of the present study was to test the hypothesis that the impact of the HBP on the response of neonatal cardiomyocytes to the IP\(_3\)-generating agonist ANG II is mediated by an increase in protein O-GlcNAcylating...
alters the regulation of $[\text{Ca}^{2+}]_{i}$. We found that glucosamine increased HBP flux in isolated neonatal cardiomyocytes, resulting in increased O-GlcNAcylation in isolated neonatal cardiomyocytes, re-
Bethesda, MD). Images, we used IPLab version 3.6 software (Scanalytics, Rockville, MD) to directly influence $[\text{Ca}^{2+}]_{i}$, elevation and thapsigargin-evoked CCE. We also have demonstrated that independent of the HBP, up- or downregulation of protein O-GlcNAcylation directly influenced $[\text{Ca}^{2+}]_{i}$. These data demonstrate for the first time a direct link between protein O-GlcNAcylation and cardiomyocyte $\text{Ca}^{2+}$ homeostasis, which may represent a novel mechanism for the regulation of cardiomyocyte function under normal and stress conditions.

**MATERIALS AND METHODS**

Preparation of NRVMs. All animal experiments were approved by the University of Alabama Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication no. 85-23, 1996). Primary culture of NRVMs was obtained from 2- to 5-day-old neonatal Sprague-Dawley rats and cultured as described previously (8, 37). Briefly, after isolation using collagenase digestion, cells were resuspended in PBS and maintained at $37^\circ \text{C}$ in a 95% air-5% CO$_2$ atmosphere. The cell suspensions from each digestion step were combined and centrifuged at 500 g for 5 min. The pellets were resuspended in DMEM and medium-199 (M-199) at a 4:1 ratio; supplemented with 15% FBS, penicillin (100 U/ml), streptomycin (100 $\mu$g/ml), and arabinose C (10 $\mu$M); and preplated for 10 min on collagen-coated, 100-mm culture dishes to deplete fibroblasts. Finally, NRVMs were plated densely on six-well plates (2 x $10^6$ cells/well) or on four-chamber coverslips (0.3 x $10^6$ cells/chamber). After 24 h, the medium was replaced with serum-free DMEM-M-199 supplemented with 2% Nutridoma (Roche) and antibiotics. Primary cultures were maintained for 1 wk, with the medium replaced every second day.

Materials. All experiments were performed at $37^\circ \text{C}$ in HBSS supplemented with 1.2 mM CaCl$_2$ and 1.0 mM MgSO$_4$ unless otherwise indicated. To elicit an increase in $[\text{Ca}^{2+}]_{i}$, 1 $\mu$M ANG II (Sigma) or 1 $\mu$M thapsigargin (Molecular Probes) was added directly to the coverslip chambers during image acquisition. O-(2-acetamido-2-deoxy-$\beta$-D-glucopyranosylidine)-$\alpha$-D-mannoside (PUGNAc, 100 $\mu$M; Carbogen), an inhibitor of O-GlcNAcase (13), and alloxan (2.5 mM), an inhibitor of OTG (23), were added 45 min before ANG II or thapsigargin, whereas glucosamine (5 mM) was added 10 min before ANG II or thapsigargin.

$\text{Ca}^{2+}$ imaging. NRVMs were plated densely on chambered glass coverslips and cultured for 3–7 days to encourage spontaneous beating. On the day of the experiment, NRVMs were washed in HBSS and loaded for 45 min at $37^\circ \text{C}$ with 3 $\mu$M fluo-3 AM (Molecular Probes) in HBSS containing 1% BSA (45). When the cells were pretreated with alloxan or PUGNAc, loading occurred during the treatment. After being loaded with fluo-3, NRVMs were washed three times with dye-free HBSS and then the buffer was replaced with fresh HBSS containing 1.2 mM CaCl$_2$ and 1.0 mM MgSO$_4$ and incubated for 10 min with or without glucosamine. Image acquisition was performed at $37^\circ \text{C}$ using an Olympus IX70 inverted microscope set at a $\times 100$ objective with excitation at 488 nm and emission at 524 nm. To visualize fast $\text{Ca}^{2+}$ events, 100-ms frames were captured for a total of 30 s of continuous recording. The fluorescence intensity was normalized to the initial, diastolic fluorescence; thus the normalized fluorescence is reported as F/F$_0$. To capture and process the fluorescent images, we used IPLab version 3.6 software (Scanalytics, Rockville, MD) and ImageJ version 1.29 software (National Institutes of Health, Bethesda, MD).

Immunoblotting with CTD110.6. NRVMs were washed in ice-cold PBS, scraped, and harvested in modified RIPA buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate) containing 5% protease inhibitor cocktail (Sigma) on ice for 30 min and centrifuged for 10 min at 14,000 g. Protein concentration from the supernatant was measured using a $D_6$$_{280}$ protein assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins were separated on a 7.5% SDS-PAGE gel (26) and transferred to a PVDF membrane (Millipore). Equal loading of proteins was confirmed by using Zn$^{2+}$ stain (Bio-Rad) on the gel before transfer. Blots were probed with CTD110.6, a mouse IgM MAb (1:5,000 dilution; Covance) that is highly specific for O-glycosylated proteins (21) with no cross reactivity to similar carbohydrate antigens (10) in casein blocking buffer, followed by horseradish peroxidase-conjugated goat anti-mouse IgM antibody (1:10,000 dilution; CN). To visualize the blots, Pico chemiluminescence substrate was used (Pierce), and the signal was detected with the Bio Chemi System (UVP, Upland, CA). Densitometric measurements were quantified using LabWorks analysis software (UVP).

Immunofluorescence microscopy. NRVMs were plated on coverslips as indicated above, and the cells were fixed in 3% formaldehyde-PBS for 45 min at room temperature, washed once in PBS, and preincubated with 0.5% Triton X-100-PBS for 2 min. The cells were rinsed in PBS, blocked in 5% BSA-PBS for 5 min, and then incubated with a 1:200 dilution of the anti-O-GlcNAc antibody CTD110.6 in 5% BSA-PBS for 30 min at $37^\circ \text{C}$. After being rinsed in PBS, the coverslips were incubated with a 1:200 dilution of secondary antibody Alexa Fluor 594-conjugated goat anti-mouse IgM (Molecular Probes) in 5% BSA-PBS for 30 min at $37^\circ \text{C}$. Finally, the coverslips were washed in PBS and mounted with a 9:1 ratio of glycerol-PBS. Image acquisition was performed with an Olympus IX70 inverted microscope using IPLab version 3.6 software.

HPLC. Approximately 1–2 x $10^6$ NRVMs were scraped in ice-cold PBS, and after centrifugation at 10,000 g for 10 s, the pellets were precipitated with ice-cold 0.3 M perchloric acid (PCA). PCA was extracted from the supernatant with 2 volumes of a trioctylamine-freon mixture at a 1:4 ratio (36). Samples were loaded onto an anion exchange HPLC column (Partisil 10 SAX; Beckman), and nucleotide sugars were detected at 262 nm using a 2 ml/min flow rate, a linear salt gradient from 5 to 750 mM (NH$_4$)$_2$HPO$_4$, and a pH gradient from 2.8 to 7.3. This method cannot fully separate UDP-GlcNAc from UDP-N-acetylgalactosamine (UDP-GalNAc) (36); consequently, the results are presented as the sum of UDP-GlcNAc and UDP-GalNAc and referred to as UDP-HexNAc. In cardiomyocytes, the ratio of UDP-GlcNAc to UDP-GalNAc is ~3:1 (9).

Data analysis. Data are presented as means ± SE throughout. Comparisons were performed using Student’s t-test, and statistically significant differences between groups were defined as $P < 0.05$.

RESULTS

ANG II-induced $[\text{Ca}^{2+}]_{i}$ elevation in NRVMs is inhibited by glucosamine. Cultured NRVMs beat spontaneously; the addition of 1 $\mu$M ANG II increased the frequency of these spontaneous contractions and also provoked a rapid elevation in baseline $[\text{Ca}^{2+}]_{i}$ (i.e., diastolic), which was sustained throughout the subsequent recording period (Fig. 1A). In contrast to the effect on diastolic $[\text{Ca}^{2+}]_{i}$, ANG II did not significantly increase the observed peak $[\text{Ca}^{2+}]_{i}$ (i.e., systolic). The increase in baseline $[\text{Ca}^{2+}]_{i}$ observed with the addition of ANG II was not observed in the absence of extracellular Ca$^{2+}$ (data not shown). These effects of ANG II on NRVMs are consistent with the findings of earlier studies in which investigators examined the effect of ANG II on cardiomyocyte function (19, 20, 43, 46).

Preincubation of NRVMs for 10 min with 5 mM glucosamine almost completely blocked the ANG II-induced increase in diastolic $[\text{Ca}^{2+}]_{i}$ level (Fig. 1A); however, glu-
cosamine did not block the ANG II-induced increase in beating frequency and did not attenuate the peak \([\text{Ca}^{2+}]_i\) levels. Addition of the O-GlcNAcase inhibitor PUGNAc, which increases protein-O-GlcNAc levels (13), had an effect similar to that of glucosamine, almost completely abolishing the effect of ANG II on baseline \([\text{Ca}^{2+}]_i\) but without causing a significant effect on beating frequency. Pretreatment with alloxan, an inhibitor of OGT, for 45 min partially reversed the effect of glucosamine, whereas alloxan alone did not inhibit the ANG II-induced increase in basal \([\text{Ca}^{2+}]_i\). The effects of glucosamine, PUGNAc, and alloxan on the ANG II-mediated increase in basal \([\text{Ca}^{2+}]_i\) and the frequency of the spontaneous contractions averaged from 25 cells from at least five separate experiments are shown in Fig. 1, B and C.

**HBP inhibits thapsigargin-induced \([\text{Ca}^{2+}]_i\) elevation.** We previously showed that the \([\text{Ca}^{2+}]_i\) increase caused by ANG II in NRVMs is mediated at least in part via CCE (15, 16). Thapsigargin is an inhibitor of SR \(\text{Ca}^{2+}\)-ATPase (SERCA) that prevents uptake of \(\text{Ca}^{2+}\) into the SR and also leads to activation of CCE in cardiomyocytes (15, 16). Therefore, we examined the effect of glucosamine and PUGNAc on thapsigargin-mediated increase in \([\text{Ca}^{2+}]_i\). The addition of 1 \(\mu\)M thapsigargin increased diastolic \([\text{Ca}^{2+}]_i\), after ANG II relative to controls calculated from area under the curves. Data are means ± SE from at least 5 independent experiments; \(n = 25\) cells. \#P < 0.05 vs. control; **P < 0.05 vs. glucosamine. C: change in spontaneous beating frequency after ANG II stimulation relative to controls. Data are means ± SE from at least 5 independent experiments.

**Glucosamine increases HBP flux and protein O-GlcNAc.** Glucosamine enters cells via the glucose transporter system and selectively increases HBP flux, which should increase both UDP-GlcNAc and protein O-GlcNAc. The mean basal level of UDP-HexNAc in controls was 14.5 ± 1.1 nmol/mg of protein, and this level was increased significantly by glucosamine treatment (Fig. 3). However, PUGNAc and alloxan treatment had no significant effects on UDP-HexNAc levels.

To assess the effect of glucosamine and PUGNAc on protein O-GlcNAc levels, NRVMs were labeled with CTD110.6 MAb

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**Fig. 1.** A: neonatal rat ventricular myocytes (NRVMs) were pretreated with control (HBSS), 5 mM glucosamine for 10 min, 2.5 mM alloxan for 45 min, 2.5 mM alloxan for 45 min with glucosamine (5 mM) added for 10 min, and 100 \(\mu\)M O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenyl-carbamate (PUGNAc) for 45 min. Cells were loaded with fluo-3 AM for 45 min during incubation with PUGNAc, alloxan, or HBSS. Baseline images represent NRVMs in the diastolic phase at the start of recording. The second set of images was captured in diastolic phase ~15–20 s after stimulation with 1 \(\mu\)M ANG II. The color scale at bottom of left column indicates the increasing fluorescence intensity from left (blue) to right (white) relative to intracellular \([\text{Ca}^{2+}]_i\) concentration ([Ca\(^{2+}\)]). The right column shows representative \([\text{Ca}^{2+}]_i\) fluxes in a single cell (time of ANG II addition is indicated by arrows). Frames selected for diastolic phase imaging (**) are shown at left. The 100-ms time frames were recorded, and data are expressed as average fluorescence of the cell at each time point divided by initial diastolic fluorescence. B: changes in baseline \([\text{Ca}^{2+}]_i\) after ANG II relative to controls calculated from area under the curves. Data are means ± SE from at least 5 independent experiments; \(n = 25\) cells. \*P < 0.05 vs. control; **P < 0.05 vs. glucosamine. C: change in spontaneous beating frequency after ANG II stimulation relative to controls. Data are means ± SE from at least 5 independent experiments.

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specific against protein O-GlcNAc, under conditions analogous to the Ca\(^{2+}\) imaging data (Fig. 4). The abundance of cytoplasmic O-GlcNAc was noticeable, and the labeling patterns displayed a punctuate, granulated structure rather than a homogeneous distribution. The density of the CTD110.6-labeled granules was highest at the perinuclear region, most likely the location of the SR. In contrast, the nuclei were almost completely excluded from labeling. This finding was somewhat unexpected, because other cell types have exhibited strong positive nuclear CTD110.6 staining (18, 24, 54).

The changes in O-GlcNAc levels also were assessed using immunoblot analysis of cell extracts with CTD110.6 (Fig. 5A), and the resulting banding patterns were similar to those published in previous reports (21, 22, 54). Using this method, we found that glucosamine treatment led to a modest but significant increase in intensity of overall staining that was blocked by alloxan (Fig. 5B). Consistent with the immunofluorescence data, CTD110.6 staining was increased much more after PUGNAc treatment than after glucosamine treatment. The intensities of five selected bands from the Western blot analysis shown in Fig. 5A are summarized in Fig. 5C. Although was observed in the glucosamine group, PUGNAc markedly increased the overall level of CTD110.6 fluorescence.

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Fig. 2. A: representative [Ca\(^{2+}\)]\(_i\) traces from single cells before and after the addition of 1 \(\mu\)M thapsigargin (arrows). NRVMs were pretreated with control (HBSS), 5 mM glucosamine for 10 min, 2.5 mM alloxan for 45 min, 2.5 mM alloxan for 45 min with glucosamine (5 mM) added for 10 min, and 100 \(\mu\)M PUGNAc for 45 min. B: changes in baseline [Ca\(^{2+}\)]\(_i\), after thapsigargin relative to controls calculated from area under the curves. Data are means ± SE from at least 5 independent experiments; \(n \geq 25\) cells. *\(P < 0.05\) vs. control; **\(P < 0.05\) vs. glucosamine. C: changes in spontaneous beating frequency after thapsigargin relative to controls. Data are means ± SE from at least 5 independent experiments. Each bar represents the average of at least 5 individual experiments.
PUGNAc had a similar effect on all bands, the effect of glucosamine was more variable; for example, the increase in band 5 was ~2.5-fold, whereas only a 25% increase in the intensity of band 3 was observed. This specificity of glucosamine for bands of a particular molecular weight may explain why the increase in overall CTD110.6 immunofluorescence was not significant (Fig. 4). As anticipated, alloxan treatment prevented the overall increase in CTD110.6 staining due to glucosamine treatment; however, this phenomenon was more pronounced for some protein bands than for others.

**DISCUSSION**

We previously reported that the increase in [Ca\(^{2+}\)], induced by ANG II occurs at least in part by CCE, both in NRVMs and in adult cardiomyocytes (15, 16). We also previously showed that the physiological responses to ANG II, such as cardiomyocyte hypertrophy, were attenuated by hyperglycemia and that this response could be mimicked by glucosamine, suggesting that this effect is mediated by the HBP (31, 32). In the present study, we have demonstrated for the first time that the attenuation of both ANG II- and thapsigargin-induced increases in [Ca\(^{2+}\)], by glucosamine not only is mediated by increased flux through the HBP but also is dependent on the subsequent formation of O-GlcNAc. Furthermore, we have demonstrated that PUGNAc, which increases protein O-GlcNAc levels independently of flux through the HBP, also attenuated ANG II- and thapsigargin-induced increase in [Ca\(^{2+}\)]. These results suggest that protein O-GlcNAc levels play a role in regulating Ca\(^{2+}\) homeostasis in NRVMs, which may have important implications for the understanding of the role of hyperglycemia in modulating cardiomyocyte function.

O-GlcNAcylation is increasingly recognized as an important and widespread posttranslational modification. The number of identified proteins capable of posttranslational O-glycosylation is quickly growing, including a wide range of proteins, such as NF-κB, annexin, endothelial nitric oxide synthase, αB-crystallin, OGt, α-tubulin, c-myc, and heat shock protein 70 (50, 51). Increased levels of O-glycosylation have been implicated in a range of cellular processes, including the development of insulin resistance in muscle (2), hyperglycemia-induced apoptosis (28), and impaired excitation-contraction coupling (9). Recently, it also was shown that increased protein O-GlcNAcylation occurs in a range of different cells in response to stress, suggesting that activation of this pathway may be a component of an endogenous cell survival pathway (55).

Previously, we showed that hyperglycemia blocks ANG II-elicted CCE and blunts its hypertrophic effects in both NRVMs and adult cardiomyocytes (16, 32). The impact of hyperglycemia on ANG II-mediated effects was mimicked by glucosamine, which increased HBP flux and was reversed by azaserine, an inhibitor of glucosamine: fructose-6-phosphate amidotransferase, which regulates glucose entry into the HBP (32).

We also previously showed that in the intact heart, the positive inotropic response to phenylephrine, another IP\(_3\)-generating agonist, was inversely correlated to UDP-GlcNAc concentrations after either short-term in vivo hyperglycemia or acute glucosamine treatment (31). Taken together, these data suggest that the physiological responses of cardiomyocytes to IP\(_3\)-generating agonists such as ANG II are blunted in response to increased HBP flux and that this could be due to increased levels of protein O-GlcNAc. However, a cause-and-effect relationship had not been established.

In the present study, we used increases in baseline [Ca\(^{2+}\)] to measure acute ANG II stimulation of NRVMs. A similar response was reported in several other studies, in which researchers used primary rat cardiomyocytes (19, 20, 43, 46). We have shown not only that the effect of ANG II on baseline [Ca\(^{2+}\)], was blunted by glucosamine but also that this event was mimicked by PUGNAc. In other cell systems, PUGNAc increases protein O-GlcNAc levels independently of HBP flux by inhibiting O-GlcNAcase thereby preventing the recycling of O-GlcNAc (13). Our results in NRVMs are entirely consistent with these findings, because we found that PUGNAc markedly increased levels of O-GlcNAc but had no significant effect on UDP-HexNAc levels. Further support for the role of increased O-GlcNAc levels in blocking the ANG II-induced rise in baseline [Ca\(^{2+}\)], comes from the fact that alloxan, an inhibitor of OGT, prevented the effect of glucosamine treatment. Furthermore, alloxan significantly reduced the glucosamine-induced increase in O-GlcNAc (Fig. 5B) but had no effect on UDP-HexNAc levels, which remained significantly increased compared with controls. These data support the notion that the attenuation of ANG II-induced elevation of baseline [Ca\(^{2+}\)], in NRVMs by glucosamine is due at least in part to increased levels of O-GlcNAc and suggest that cardiomyocyte Ca\(^{2+}\) homeostasis may be regulated by O-GlcNAcylation.

One limitation of these studies is that unlike PUGNAc, which is a high-affinity inhibitor of O-GlcNAcase and is effective at fairly low concentrations, relatively high concentrations of alloxan were required to block the effect of glucosamine. Thus, while alloxan is known to inhibit OGT (23), given the relatively high concentrations needed, we cannot exclude the possibility that alloxan may have some effects on NRVMs independent of its effects on OGT. Unfortunately, alloxan is the only known inhibitor of OGT (23), and because ablation of the OGT gene is embryonically lethal (42), a more specific demonstration of the role of OGT in regulating cardiomyocyte [Ca\(^{2+}\)], would require the development of tissue-specific conditional knockout mice or the use of small interfering RNA approaches to reduce OGT expression in cardiomyocytes.

Despite the potential limitations of alloxan, it should be noted that incubation of NRVMs for 20 h with a slightly higher (3 mM) concentration of alloxan had no adverse effects on cell...
viability (7). Thus, because the experiments described herein were much shorter than previous studies, it is unlikely that the effects of alloxan are due to nonspecific toxicity. Furthermore, we also found that alloxan alone did not prevent the ANG II- or thapsigargin-induced increase in basal [Ca^{2+}] (Figs. 1 and 2). One limitation is that we did not determine whether alloxan had any impact on the effects of PUGNAc. However, alloxan does not block the PUGNAc-induced increase in O-GlcNAc levels (data not shown); consequently, we think it is unlikely that alloxan would inhibit the effects of PUGNAc shown in the present study. Nevertheless, we cannot entirely rule out the possibility that glucosamine and PUGNAc may act via different mechanisms to block the increase in basal [Ca^{2+}],

The effects of ANG II on cardiomyocytes are mediated via the formation of DAG and IP_{3} (4, 17, 39); however, the mechanisms leading to the positive inotropic effect of ANG II are still unclear. IP_{3} acts on specific receptors on the SR, and inhibition of these receptors has been shown to block the increase in basal [Ca^{2+}], induced by IP_{3}-generating agonists (57) but not their positive inotropic effects (39). It has been suggested that both the Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchangers may play a role in the positive inotropic effects of ANG II (11, 39). We found that glucosamine and PUGNAc abolished the increase in basal [Ca^{2+}] elicited by both ANG II and thapsigargin; however, neither intervention significantly affected peak Ca^{2+} levels or the ANG II-induced increase in spontane-

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**Fig. 4.** Immunofluorescence microscopic images of NRVMs using the anti-O-GlcNAc antibody CTD110.6. NRVMs were pretreated with control (HBSS), 5 mM glucosamine for 10 min, 2.5 mM alloxan for 45 min, 2.5 mM alloxan for 45 min with glucosamine (5 mM) added for 10 min, and 100 μM PUGNAc for 45 min. NRVMs show abundant cytoplasmic O-GlcNAc, which could be increased by glucosamine or by PUGNAc. Bar graph at bottom shows average fluorescence intensity of cells normalized to control levels. Each bar represents the average of at least 15 cells. *P < 0.05 vs. control.
ous beating frequency. We previously showed that thapsigargin increased basal \([Ca^{2+}]_i\) levels in NRVMs via a CCE-mediated pathway that was independent of both L-type \(Ca^{2+}\) channels and the Na\(^+/\)Ca\(^{2+}\) exchanger (15, 16) and that ANG II also increased basal \([Ca^{2+}]_i\) levels via a similar mechanism (15, 16). Therefore, these new data, combined with our findings published in earlier reports (15, 16), suggest that the effect of O-GlcNAcylation on \([Ca^{2+}]_i\) homeostasis is most likely due to the inhibition of \(Ca^{2+}\) entry via CCE rather than to decreased flux through L-type \(Ca^{2+}\) channels or the Na\(^+/\)Ca\(^{2+}\) exchanger. However, we cannot rule out the possibility that increased levels of O-GlcNAc might also affect \([Ca^{2+}]_i\) homeostasis mediated via the SR IP\(_3\) receptor or the Na\(^+/\)H\(^+\) exchanger.

It should be noted that we used a fairly high (1 \(\mu\)M) concentration of ANG II, which is outside the normal physiological range and raises the possibility that the effects of ANG II that we observed might be more pharmacological than physiological. The 1 \(\mu\)M concentration of ANG II was used in the present study because in a previous study we found that stimulation of cardiomyocyte hypertrophy with 1 \(\mu\)M ANG II was inhibited by glucosamine (15). Furthermore, the use of ANG II in the micromolar range in cardiomyocyte studies is fairly common (39, 43, 46). Although we have not examined the effects of lower ANG II concentrations in our studies, previously published reports have demonstrated similar effects of ANG II in the 10–100 nM range on cardiomyocyte \(Ca^{2+}\) homeostasis (20, 46). Consequently, we think it likely that alterations in O-GlcNAc levels also affect the response of cardiomyocytes to changes in ANG II concentration in the physiological range; however, this hypothesis remains to be proved.

As shown in Fig. 5, glucosamine and PUGNAc lead to increased O-GlcNAc levels in several different protein bands. The specific proteins affected by O-GlcNAc that might mediate the effects of ANG II have yet to be identified. However, the transient receptor potential channel (TRPC) protein family is the prime candidate for the long-sought CCE channel proteins and thus may be involved in the O-GlcNAc-mediated effects on \(Ca^{2+}\) homeostasis. Analysis of the protein sequence for TRPC1 suggests a high-affinity site for O-GlcNAc close to the NH\(_2\)-terminal region (for further information, see the Center for Biological Sequence Analysis web site, available at: http://www.cbs.dtu.dk/services/YinOYang/).

In conclusion, we have shown that increasing levels of O-GlcNAc by either increasing flux through the HBP with glucosamine or inhibiting O-GlcNAcase with PUGNAc inhibits the increase in basal \([Ca^{2+}]_i\), in NRVMs induced by either ANG II or thapsigargin. Alloxan, an inhibitor of OGT, prevented the increase in O-GlcNAc induced by glucosamine but not the increase in UDP-HexNAc. Alloxan also blocked the effect of glucosamine on ANG II- and thapsigargin-mediated changes in \([Ca^{2+}]_i\). These data, in combination with data published in our earlier reports (15, 16), provide strong evi-
idence for the regulation of \( [\text{Ca}^{2+}]_i \) in cardiomyocytes by O-GlcNAcylation. This finding has important implications for the regulation of cardiomyocyte function because changes in \( \text{Ca}^{2+} \) homeostasis play a central role in the cardiomyocyte stress response, initiating numerous signaling cascades that can lead to apoptosis, hypertrophy, arrhythmia, or cell death (3, 27, 30, 33, 38, 41, 56). Increased flux through the HBP also has been implicated in complications associated with diabetes (6); thus alterations in \( \text{Ca}^{2+} \) homeostasis mediated by increased O-GlcNAc levels may contribute to the deleterious effects of prolonged hyperglycemia (9). It is also possible that regulation of cardiomyocyte \( [\text{Ca}^{2+}]_i \) by protein O-GlcNAcylation may contribute to the cytoprotection associated with increased levels of protein O-GlcNAc (55).

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DISCLOSURES

R. B. Marchase and J. C. Chatham have a patent pending that relates to the work presented in this article.

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