Glucosamine protects neonatal cardiomyocytes from ischemia-reperfusion injury via increased protein O-GlcNAC and increased mitochondrial Bcl-2

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Champattanachai V, Marchase RB, Chatham JC. Glucosamine protects neonatal cardiomyocytes from ischemia-reperfusion injury via increased protein O-GlcNAC and increased mitochondrial Bcl-2. Am J Physiol Cell Physiol 294: C1509–C1520, 2008. First published March 26, 2008; doi:10.1152/ajpcell.00456.2007.—We have previously reported that glucosamine protected neonatal rat ventricular myocytes against ischemia-reperfusion (I/R) injury, and this was associated with an increase in protein O-linked-N-acetylglucosamine (O-GlcNAC) levels. However, the protective effect of glucosamine could be mediated via pathways other than O-GlcNAC formation; thus the initial goal of the present study was to determine whether increasing O-GlcNAC transferase (OGT) expression, which catalyzes the formation of O-GlcNAC, had a protective effect similar to that of glucosamine. To better understand the potential mechanism underlying O-GlcNAC-mediated cytoprotection, we examined whether increased O-GlcNAC levels altered the expression and translocation of members of the Bcl-2 protein family. Both glucosamine (5 mM) and OGT overexpression increased basal and I/R-induced O-GlcNAC levels, significantly decreased cellular injury, and attenuated loss of cytochrome c. Both interventions also attenuated the loss of mitochondrial membrane potential induced by H2O2 and were also associated with an increase in mitochondrial Bcl-2 levels but had no effect on Bad or Bax levels. Compared with glucosamine and OGT overexpression, NBuTG (100 μM), an inhibitor of O-GlcNAcase, had less protective against I/R and H2O2 and did not affect Bcl-2 expression, despite a 5- to 10-fold greater increase in overall O-GlcNAC levels. Reduced OGT expression resulted in lower basal O-GlcNAC levels, prevented the I/R-induced increase in O-GlcNAC and mitochondrial Bcl-2, and increased cellular injury. These results demonstrate that the protective effects of glucosamine are mediated via increased formation of O-GlcNAC and suggest that this is due, in part, to enhanced mitochondrial Bcl-2 translocation.

IN MAMMALIAN CELLS, A VARIETY of stress stimuli have been shown to increase the level of O-linked-N-acetylglucosamine (O-GlcNAc) on nuclear and cytoplasmic proteins (54). Inhibition of this response increased sensitivity to stress, whereas augmentation of the O-GlcNAc levels increased tolerance to the same stress stimuli and improved cell survival (54). We have previously reported that ischemic stress in isolated cardiomyocytes and the intact heart also increases O-GlcNAc levels; furthermore, treatment with glucosamine augments this increase in O-GlcNAc level, improving the tolerance to ischemic injury (9, 15, 32, 33). These data are consistent with the notion that, in the heart, an increase in O-GlcNAc protein modification is an endogenous stress response and that the protection seen with glucosamine is mediated via this same pathway.

However, in addition to increasing O-GlcNAc levels, glucosamine also increases UDP-GlcNAc levels and 6-phosphate levels and could potentially be metabolized to fructose-6-phosphate, thereby increasing glycolytic flux. Thus, in addition to increasing O-GlcNAc levels, the protection associated with glucosamine treatment in cardiomyocytes and the intact heart could be mediated via a number of other pathways. Support for the hypothesis that the protective effect of glucosamine is mediated by O-GlcNAc levels was provided by studies showing that increasing O-GlcNAc levels with PUGNAc, an inhibitor of O-GlcNAcase, had effects similar to those of glucosamine (9, 32). However, while the inhibition of O-GlcNAcase with PUGNAc is frequently used to increase cellular O-GlcNAc levels, it also inhibits other β-hexosaminidases (23, 34, 42) and thus will alter processing of glycoconjugates in addition to O-GlcNAc. We have also found that the pattern of O-GlcNAc-modified proteins is different in glucosamine- and PUGNAc-treated cardiomyocytes (9). This suggests that these two different methods for increasing cellular O-GlcNAc levels may not have equal effects on cell function, which may be a consequence of their actions on other pathways.

Importantly, the mechanisms underlying the protection associated with increased protein O-GlcNAc levels have yet to be determined. Zachara et al. (54) reported that increased survival seen with elevated O-GlcNAc levels was associated with increased expression of heat shock protein 70 (HSP70); in contrast, Sohn et al. (47) reported improved survival associated with increased O-GlcNAc levels without any change in HSP70 levels, which suggests that other mechanism(s) may also contribute to the protection associated with increased O-GlcNAc levels. We found that hyperglycemia-mediated protection of cardiomyocytes against ischemic injury also appeared to be mediated, at least in part, by increased O-GlcNAc levels (9). It has also been reported that hyperglycemia-induced protection against hypoxia-induced apoptosis and necrosis was associated with upregulation of the antiapoptotic factor Bcl-2 (45).

Apoptosis, a genetically programmed form of cell death, contributes to myocyte cell loss in a variety of cardiac pathologies, including cardiac failure and those related to ischemia-reperfusion injury. The apoptotic program is complex, involving both pro- and antiapoptotic proteins, and apoptosis occurs when the equilibrium between these opposing factors is perturbed (44). The pro- and antiapoptotic members of the Bcl-2...
family are intrinsic to the apoptotic pathway; Bcl-2 and Bcl-xL protect cells from apoptosis, whereas Bax and Bad promote the response. It has been reported that increased Bcl-2 expression significantly limits cell death after acute myocardial infarction (55) or during cardiac posttransplant ischemia-reperfusion injury (20). Several lines of evidence have also shown that members of the Bcl-2 protein family are associated with the loss of apoptogenetic factors, including release of cytochrome c from the intermembrane space of the mitochondria into cytosol (1, 12, 28, 30). Many, if not all, apoptotic responses involve mitochondrial dysfunction and a loss of the mitochondrial membrane potential (MMP) (19). Thus antiapoptotic Bcl-2 may function in the mitochondrial membrane to prevent loss of cytochrome c and thus inhibit apoptosis.

Therefore the goals of the present study were to determine whether increased O-GlcNAc transferase (OGT) expression had effects similar to those of glucosamine treatment on the response to ischemic injury and acute oxidative stress. Studies were also performed with 1,2-dideoxy-2-methyl-α-D-glucopyranosonic[2,1-d]-β-thiazoline (NBuGT), an inhibitor of O-GlcNAcase, which has ~1,500-fold greater specificity for O-GlcNAcase over β-hexosaminidase than PUGNAc (34). To better understand the potential mechanism underlying O-GlcNAc-mediated cytoprotection, we examined whether increased O-GlcNAc levels altered the expression and translocation of members of the Bcl-2 protein family. Finally, we also examined the consequences of decreased OGT expression on the response to ischemic injury.

MATERIALS AND METHODS

Materials. Unless otherwise noted, except for glucosamine (Fluka), all chemicals were purchased from Sigma Chemical (St. Louis, MO). Culture medium products were purchased from GIBCO Invitrogen (Grand Island, NY). Adenovirus containing OGT was a kind gift from Dr. W. H. Dillmann (Department of Medicine, University of California, San Diego). NBuGT, an inhibitor of O-GlcNAcase, was a kind gift from Dr. David Vocadlo (Simon Fraser University, Burnaby, BC, Canada) (34). NBuGT has ~1,500-fold greater specificity for O-GlcNAcase over β-hexosaminidase than PUGNAc (34).

Neonatal rat ventricular myocyte primary cultures. 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, Revised 1996). Primary cultures of neonatal rat ventricular myocytes (NRVMs) were obtained from 2- to 3-day-old neonatal Sprague-Dawley rats and were cultured as described previously (9, 24). NRVMs were grown on collagen-coated plates in the culture growth medium containing 15% fetal bovine serum (FBS) on the first day. On the next day, medium was replaced, and cells were grown in the culture growth medium without FBS. Within 1–2 days of isolation, a confluent monolayer of spontaneously beating NRVMs had formed, and cells were used as described below.

Ischemia and reperfusion. Ischemia and reperfusion were induced as described previously (4, 5, 9). Briefly, following 2 days in culture, NRVMs were exposed to ischemia by the addition of a fresh Esumi buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (vol/vol) NP-40, 0.5% (wt/vol) sodium deoxycholate, 1 mM EDTA, and 0.1% SDS] containing 2% protease inhibitor cocktail (Sigma) on ice for 30 min. Lysed proteins were harvested and assayed for protein concentration using the Bio-Rad protein assay kit. Proteins (10 μg) were separated on 7.5% or 12% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). After being transferred, the blots were soaked in 100% methanol and dried completely under the hood. Dried blots were probed with the following antibodies: anti-O-GlcNAc antibody CTD110.6 (Covance), at 1:1,000, β-actin (Sigma) at 1:1,000, and GAPDH (Abcam) at 1:5,000. Blots and antibodies were incubated in 1 × PBS/casein blocker (Pierce) containing 0.01% Tween 20 for 2 h at room temperature. After being washed three times with PBS, the membrane was then incubated with an appropriate secondary antibody: goat anti-mouse IgG (Calbiochem) for anti-O-GlcNAc antibody CTD110.6, goat anti-rabbit IgG (Cell Signaling) at the same blocking buffer for 1 h at room temperature. After a further washing in PBS, the immunoblots were developed with enhanced chemiluminescence (Super Signal West Pico or Femto Maximum Sensitivity, Pierce), and immunoblots were developed with enhanced chemiluminescence (Super Signal West Pico or Femto Maximum Sensitivity, Pierce), and visualized was performed using the Bioimaging System of UV (Upland, CA). Densitometric analysis was performed on the entire lane of each sample using LabWorks analysis software (UVP), and the mean intensity was normalized to the control group.

Mitochondrial fractionation. NRVMs (10 × 10⁶ cells) were seeded into 10-cm tissue culture dishes (Falcon) and mitochondria were prepared using a Mitochondrial/Cytosol Fractionation Kit (K256,
BioVision) with slight modifications. Briefly, NRVMs were washed with PBS and resuspended in 200 μl of 1 × Cytosol Extraction Buffer on ice for 10 min. Cells were homogenized and then centrifuged at 700 g for 10 min at 4°C, and the supernatant was centrifuged at 10,000 g for 30 min at 4°C. At the end of this second centrifugation, the supernatant (postmitochondria), consisting of the cytosolic/microsomal fraction, was collected for subsequent immunoblot analysis, and the pellet (lysed proteins) was resuspended in 50 μl of Mitochondrial Extraction Buffer. Lysed proteins were assayed for protein concentration using the Bio-Rad protein assay kit. Proteins (3–5 μg) were separated on 12% SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Millipore). Immunoblot analysis was performed as described above.

**Electroporation of small interfering RNA oligonucleotides.** Small interfering RNA (siRNA) oligonucleotides, OGT, and negative control (Silencer negative control 1) were purchased from Ambion (Austin, TX). The sense and antisense sequences of si-OGT were 5′-CACCUUGACCAUUUUCGUCUU-3′ and 5′-UCGGGUGGAAAGTGGTG-3′, respectively. Transfection of the siRNA oligos into neonatal cardiomyocytes was carried out according to the manufacturer’s instructions (Amaxa) with slight modification. Briefly, 1 day after cell isolation, attached cardiomyocytes (105 cells in a 10-cm culture dish) were detached by trypsin-EDTA solution (Sigma) and resuspended in the transfection reagent Nucleofector kit (Amaxa). A total of 2 μg of siRNAs per 2 × 106 cells were transfected using the Nucleofector II Device (Amaxa) according to the manufacturer’s instructions. After transfection, cells were grown in the culture medium containing 15% FBS, and on the next day, medium was replaced and cells were grown in the culture growth medium without FBS. Approximately 2 days following transfection, OGT levels were confirmed by Western blot analysis as described above, and cells were subjected to ischemia-reperfusion.

**Assessment of MMP.** Mitochondrial function was assessed by using JC-1 reagent, Mitochondrial Membrane Potential Detection Kit according to the manufacturer’s instructions (Stratagene). After isolation, NRVMs were seeded into a four-chambered coverglass (Lab-Tek) and pretreated with a unique fluorescent cationic dye, 5,5′,6,6′-tetrachloro-1,1,3,3-tetraethyl-3H-tetrazolium (WST-1, Sigma) commonly known as JC-1, for 15 min followed by washing with assay buffer to remove remaining reagent. The culture growth medium (500 μl) was added to the cells, which were then visualized through a ×20 objective with excitation wavelength at 623 nm to detect Texas Red dye and at 528 nm to detect rhodamine using an inverted fluorescence microscope (Olympus). In living cells, JC-1 exists as a monomer in the cytosol, which exhibits green fluorescence, and also accumulates as aggregates in the mitochondria, where it exhibits red fluorescence. In apoptotic and dead cells, JC-1 exists in the monomeric form only, staining the cytosol green. MMP was monitored before and after the addition of hydrogen peroxide (1 mM final concentration). A reduction in the ratio of red to green fluorescence indicated a fall in MMP. The ratio of red and green intensity of cells incorporating JC-1 dye was measured by using IPLab analysis software (version 3.6, BD Biosciences).

**Statistical analysis.** All data are presented as means ± SE. Unpaired t-tests and one-way and repeated-measures ANOVA were used where appropriate, followed by a Bonferroni’s multiple comparison test using Prism 4.0c (GraphPad Software, San Diego, CA). Statistically significant differences between groups were defined as P < 0.05.

**RESULTS**

Glucosamine reduces cellular injury during ischemia-reperfusion and increases O-GlcNAc levels. We have previously reported that, in NRVMs, glucosamine decreased both necrosis and apoptosis following ischemia-reperfusion and that the increase in O-GlcNAc levels was maximal at 2 h of reperfusion following 4 h of ischemia (9). Therefore, in the present study, we focused on the 2-h reperfusion time point to investigate the effects of ischemia-reperfusion on protein and cellular function. In Fig. 1A, we demonstrate that, consistent with our earlier study, glucosamine decreased cell injury following ischemia-reperfusion. Using two O-GlcNAc antibodies, which recognize different O-GlcNAc motifs (CTD110.6 and RL2), we confirm that ischemia-reperfusion alone significantly increases overall O-GlcNAc levels in the control, untreated group (Fig. 1B; CTD: 2.55 ± 0.47 vs. 1.07 ± 0.06, P < 0.05; RL2: 1.29 ± 0.02 vs. 0.97 ± 0.10, P < 0.05). Consistent with our earlier study, glucosamine treatment significantly augmented the response to ischemia-reperfusion compared with the untreated groups (CTD: 4.89 ± 0.37 vs. 2.55 ± 0.47 and RL2: 2.03 ± 0.43 vs. 1.29 ± 0.02). Equal protein loading was confirmed by densitometric analysis of β-actin levels.

The increase in O-GlcNAc in response to ischemia and glucosamine appears to be less robust when assessed with RL2 compared with CTD110.6. However, this is primarily due to the fact that, just below 77 kDa, there is a very intense band in the RL2 immunoblots that is relatively constant across the different groups. Above 77 kDa, the changes in intensity in the CTD110.6 and RL2 immunoblots are similar (data not shown).

**Glucosamine mediates translocation of mitochondrial Bcl-2.** In Fig. 2A, it can be seen that glucosamine had little or no effect on whole cell expression of Bcl-2, Bad, or Bax levels either under normoxic conditions or following ischemia-reperfusion. Since mitochondria play a critical role in the regulation of cell death, we examined expression levels of the same proteins in mitochondrial and postmitochondrial (cytosolic/microsomal) fractions. There was no effect of glucosamine or ischemia-reperfusion on levels of Bcl-2, Bad, or Bax in the postmitochondrial fraction; however, in the mitochondrial fraction, ischemia significantly increased Bcl-2 levels (Fig. 2C; 1.0 ± 0.0 vs. 1.91 ± 0.22, P < 0.05). Glucosamine treatment significantly increased mitochondrial Bcl-2 levels under normoxic conditions (1.0 ± 0.0 vs. 2.59 ± 0.54) and augmented the response to ischemia-reperfusion (1.0 ± 0.12 vs. 2.71 ± 0.11) (Fig. 2, B and C).

Following ischemia-reperfusion, mitochondrial Bad levels appeared to be reduced in the glucosamine-treated group (2.5 ± 1.4 vs. 1.5 ± 0.6); however, this difference was not significant, because of the high variance in the untreated group. In contrast to Bcl-2 and Bad, Bax was virtually undetectable in the mitochondrial fraction.

**The effect of increased OGT expression and O-GlcNAcase inhibition on response of NRVMs to ischemia-reperfusion.** Since glucosamine may affect cell function independent of increased O-GlcNAc formation, we asked whether increasing OGT expression levels would also afford protection against ischemia-reperfusion injury and, if so, whether this was also associated with increased mitochondrial Bcl-2. In Fig. 3A, it can be seen that transfection of NRVMs with the OGT adenovirus increased OGT expression levels four- to fivefold and this was associated with increased O-GlcNAc levels under normoxia and following ischemia-reperfusion. Unlike glucosamine treatment, ischemia-reperfusion did not increase O-GlcNAc levels further in the OGT-transfected group. Interestingly, however, the increase in O-GlcNAc achieved by OGT transfection was approximately four- to fivefold compared with nontransfected...
cells in both conditions, which was similar to the increase seen in the glucosamine-treated group following ischemia and reperfusion injury (Figs. 1 and 3B). Increased OGT expression significantly attenuated cell injury following ischemia-reperfusion, as indicated by reduced LDH release (Fig. 3B). Consistent with glucosamine treatment, increased OGT expression was also associated with increased mitochondrial Bcl-2 expression (Fig. 3C).

Inhibition of O-GlcNAcase, which is responsible for removal of O-GlcNAc from proteins, is another means by which cellular O-GlcNAc levels can be increased. We have previously shown that the O-GlcNAcase inhibitor PUGNAc increased O-GlcNAc levels to a much greater extent than glucosamine; however, it was less protective (9). While PUGNAc is typically used as an O-GlcNAcase inhibitor, it also inhibits lysosomal β-hexosaminidases, which could also affect the response of cardiomyocytes to ischemia-reperfusion (34). Therefore, we examined the effect of NButGT, a new O-GlcNAcase inhibitor, which shows ~1,500-fold greater specificity for O-GlcNAcase over β-hexosaminidase than PUGNAc (34). NButGT treatment increased O-GlcNAc levels more than 10-fold compared with untreated cells (Fig. 3A); however, while it decreased LDH release compared with untreated cells, this effect was significantly attenuated compared with increased OGT expression (Fig. 3B) and glucosamine treatment (Fig. 1A). Interestingly, NButGT treatment did not increase mitochondrial Bcl-2 expression and blocked the ischemia-reperfusion-induced increase in mitochondrial Bcl-2 seen in untreated cells (Fig. 3C).

**Attenuation of cytochrome c release by glucosamine and increased OGT expression.** It has been reported that the balance of anti- and proapoptotic Bcl-2 family proteins that reside in the outer mitochondrial membrane plays an important role in the regulation of mitochondria-mediated apopto-
One characteristic of mitochondria-mediated apoptosis is the release of cytochrome c from the mitochondria into the cytosol. However, using cell fractionation and immunoblotting techniques, we observed very low cytosolic cytochrome c levels following ischemia-reperfusion. Since there is significant loss of cytosolic proteins in this model of ischemia-reperfusion, as indicated by LDH release (Fig. 1A), the low levels of cytosolic cytochrome c could be due to loss of the protein from the cell. Indeed, we found appreciable levels of cytochrome c in the media following ischemia-reperfusion, which was attenuated by 50% in the glucosamine-treated group (data not shown).

Therefore we used immunofluorescence to look at the relationship between O-GlcNAc and cytochrome c levels (Fig. 4A). Consistent with immunoblot analysis of O-GlcNAc proteins (Fig. 1B and 3B), ischemia-reperfusion increased global O-GlcNAc levels in the untreated cells. Glucosamine, OGT overexpression, and NButGT all increased O-GlcNAc levels under normoxic conditions as well as following ischemia-reperfusion, and the increase in O-GlcNAc levels was clearly much greater in the NButGT-treated cells compared with the other groups. Following ischemia-reperfusion, there was a marked loss of cytochrome c staining in untreated cells, which was significantly attenuated by glucosamine, increased OGT expression, and NButGT (Fig. 4B). However, consistent with LDH release (Fig. 3B), despite the marked increase in O-GlcNAc levels, the effect of NButGT in attenuating cytochrome c release was significantly less than either glucosamine or increased OGT expression.

Increasing O-GlcNAc levels attenuates hydrogen peroxide-induced loss of MMP. Release of cytochrome c from the mitochondria is usually associated with opening of the mitochondrial membrane transition pore (mMTP), which is a critical step in mitochondria-mediated apoptosis (29). Opening of the mMTP is characterized by loss of MMP (29); therefore, we asked whether increasing O-GlcNAc levels attenuated the loss of MMP in response to transient exposure to hydrogen peroxide. Cardiomyocytes were pretreated with the fluorescent cationic dye, JC-1, which exhibits a red fluorescence in healthy cells with intact MMP. We used real-time immunofluorescence technique to monitor the MMP of individual cardiomyocytes before and after H$_2$O$_2$ treatment.

As shown in Fig. 5, in untreated cardiomyocytes, H$_2$O$_2$ resulted in a rapid decrease of the ratio of red to green fluorescence intensity, indicating loss of MMP. However, glucosamine, increased OGT expression, and NButGT all significantly attenuated the loss of MMP compared with untreated groups. This was apparent within 5 min of H$_2$O$_2$ treatment and was sustained for at least 20 min (Fig. 5B). Interestingly, after 20 min of H$_2$O$_2$ treatment, glucosamine and increased OGT expression were more effective in preventing the loss of MMP than NButGT-treated cells.

Decreased OGT expression decreases cardiomyocyte survival following ischemia-reperfusion. Zachara et al. (54) showed that OGT deletion using Cre-recombinase techniques in mouse embryonic fibroblasts significantly decreased survival following heat stress. Therefore, we asked whether decreased OGT expression in cardiomyocytes would also attenuate survival following ischemia-reperfusion injury. Since global OGT gene deletion is embryonically lethal (41) and even cell-specific OGT gene deletion resulted in cell death during embryogenesis (41, 46), we used siRNA approach to decrease OGT expression in NRVMs.

Following electroporation and transfection with siRNA, cell viability, measured by Trypan blue exclusion, was ~80–90%. Two days after transfection, O-GlcNAc and OGT levels were significantly attenuated in the si-OGT cells compared with
si-negative oligonucleotide (si-Neg)-transfected controls (Fig. 6A); furthermore, the ischemia-induced increase in O-GlcNAc was completely blocked in the si-OGT cells. The decrease in OGT and O-GlcNAc was associated with a significant increase in LDH release following ischemia-reperfusion (Fig. 6B). At the end of ischemia-reperfusion, there was little difference in total Bcl-2 expression between si-OGT and si-Neg groups. However, in si-OGT cells, ischemia-reperfusion significantly decreased Bcl-2 levels compared with si-Neg cells (Fig. 6C). These results are consistent with the notion that decreased flux through OGT attenuated the ischemia-reperfusion induced increase in mitochondrial Bcl-2 levels.

In Fig. 7A, cytochrome c levels are shown in si-Neg and si-OGT cells under normoxic conditions and following ischemia-reperfusion. Under normoxic conditions, there was no difference between the two groups; however, compared with si-Neg cells, there was much greater loss of cytochrome c following ischemia-reperfusion in si-OGT cells. Following ischemia-reperfusion, the decrease in cytochrome c levels in the si-Neg cells appears to be attenuated compared with that seen in untreated cells (Fig. 4A). Comparison of immunofluorescence images between different sets of experiments must be made with caution; however, it is possible that the process of electroporation used to transfect cells with the siRNA induces some sort of protection against ischemia-reperfusion stress, akin to a preconditioning response. Nevertheless, the relevant comparison here is between si-Neg and si-OGT groups, both of which have undergone the same electroporation treatment. It is evident that the si-OGT cells have markedly lower cytochrome c levels following ischemia-reperfusion than si-Neg cells, and this was associated with greater number of TUNEL-positive cells following reperfusion of 16 h (Fig. 7B).
DISCUSSION

There is increasing appreciation of the role of O-linked GlcNAc modification of proteins in the regulation of cell function (22, 52). While much of the focus has been on the role of increased O-GlcNAc in mediating the adverse effects of diabetes (2, 8, 37), there is a growing body of data indicating that activation of pathways leading to O-GlcNAc formation is an endogenous stress response and that augmentation of this response improves tolerance to stress (31, 47, 54). We have previously demonstrated that glucosamine is remarkably protective against ischemic injury at the cellular (9), organ (15, 32, 33), and organismal level (40, 51) and that this protection is associated with increased O-GlcNAc levels. However, the protection associated with glucosamine treatment could be mediated via pathways other than O-GlcNAc, such as gangliosides (36) or cell surface N-glycans (48). Although we have observed effects similar to those of glucosamine by increasing O-GlcNAc levels via inhibition of O-GlcNAcase with PUGNAc (32, 56), we have also observed some differences, in which PUGNAc appears to be less effective than glucosamine in attenuating cell death (9).

We show here for the first time that the effect of glucosamine is mimicked by increased OGT expression and that both interventions increase the tolerance of NRVMs to injury induced by either ischemia-reperfusion or H2O2. Both glucosamine and OGT overexpression augmented the ischemia-reperfusion-induced increase in mitochondrial Bcl-2. The marked similarity between glucosamine treatment and increased OGT expression demonstrates that the protective effect of glucosamine is mediated primarily via increased flux through OGT. Conversely, we found that decreasing OGT expression decreased tolerance to ischemia-reperfusion injury and blunted the ischemia-reperfusion-induced increase in mitochondrial Bcl-2. We also found that, while inhibition of O-GlcNAcase with NBuGT resulted in a much greater increase in O-GlcNAc levels than glucosamine or OGT overexpression, it was signif-
significantly less protective than either. This demonstrates that the similar observations previously reported with PUGNAc (9) were most likely not due to nonspecific effects of PUGNAc, but rather suggest that there may be some threshold for $\textit{O}$$\text{-GlcNAc}$ in increasing cell survival, beyond which the detrimental effects of excessive $\textit{O}$$\text{-GlcNAc}$ levels outweigh the prosurvival mechanisms.

While it is becoming increasingly apparent that elevation of $\textit{O}$$\text{-GlcNAc}$ levels improves the tolerance of cells to stress, the specific mechanisms underlying this protective response have not been fully defined. Zachara et al. (54) demonstrated that $\textit{O}$$\text{-GlcNAc}$-mediated tolerance to heat stress was due in part to increased transcription of heat shock proteins. Our studies have suggested that the protection associated with glucosamine treatment may be due at least in part to attenuation of calcium-mediated stress responses, such as calpain activation (9, 32). We have also shown that glucosamine treatment significantly attenuated cardiomyocyte apoptosis (9). Given the importance of the Bcl-2 family of proteins in the regulation of apoptosis (11, 21), we examined the effect of increasing $\textit{O}$$\text{-GlcNAc}$ levels on Bcl-2, Bad, and Bax. We found that there was no effect of either glucosamine or OGT overexpression on whole cell levels of Bcl-2, Bad, or Bax; however, both interventions specifically increased mitochondrial Bcl-2 levels. Surprisingly, this was observed under normoxic conditions as well as following ischemia-reperfusion. Ischemia-reperfusion increased mitochondrial Bcl-2 in untreated cells, and this response was augmented by glucosamine and OGT overexpression.

Modulation of $\textit{O}$$\text{-GlcNAc}$ levels has been shown to modify subcellular localization of proteins (3, 17), and since there was no change in total Bcl-2, the increase in mitochondrial Bcl-2 in the glucosamine and OGT overexpression groups presumably reflects its redistribution from other cellular compartments, such as the endoplasmic reticulum and nuclear envelope (18, 43). We attempted to use immunofluorescence techniques to monitor Bcl-2 localization; however, all Bcl-2 antibodies we

![Fig. 5. Increased $\textit{O}$$\text{-GlcNAc}$ levels attenuated the loss of mitochondrial membrane potential (MMP).](image-url)
tested were ineffective (Santa Cruz, Abcam, and BioVision). On the basis of the prediction of potential O-GlcNAcylation sites by YinOYang software (version 1.2; http://www.cbs.dtu.dk/services/YinOYang), there are at least four potential sites for O-GlcNAc modification of rat Bcl-2, including Thr65, Thr69, Ser70, and Ser84. However, our attempts to determine whether the redistribution of Bcl-2 in these experiments could be due to O-GlcNAc modification of Bcl-2 were unsuccessful.

Despite the evidence demonstrating that antiapoptotic Bcl-2 proteins may have therapeutic potential, the mechanism(s) by which they protect cells remains unclear. Bcl-2 has been shown to block p53-mediated apoptosis in cardiac myocytes (27), and overexpression of Bcl-2 suppressed both p53-dependent and p53-independent activation of the intrinsic death pathway (35). Transgenic mice overexpressing Bcl-2 decreased apoptosis, reduced infarct size, and improved cardiac function after ischemia-reperfusion (7, 10, 25). It has been suggested that Bcl-2 inhibits opening of mMTP, possibly by direct interaction with voltage-dependent anion channel (49, 50), thereby preventing the release of death factors from mitochondria. Consistent with the notion that Bcl-2 protection is mediated via attenuation of mMTP opening, both glucosamine and OGT overexpression attenuated loss of cytochrome c release following ischemia-reperfusion (Fig. 4) and slowed the H2O2-induced loss of MMP (Fig. 5). Thus these data suggest that an increase in mitochondrial Bcl-2 may be an important contributing factor to the cellular protection associated with increased O-GlcNAc levels. Clearly, however, further studies are needed to delineate the mechanism(s) by which O-GlcNAc levels modulate the cellular distribution of Bcl-2.

We focused here on mitochondrial Bcl-2, because of the extensive data demonstrating its role in attenuating mitochondria-mediated apoptosis (38); however, Bcl-2 is also associated with other subcellular compartments such as the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) and nuclear envelope (18, 43), and it has been suggested that Bcl-2 localized to
the ER/SR may play a role in the attenuation of apoptosis, possibly by mediation of ER/SR calcium homeostasis (6, 13). There are also other antiapoptotic members of the Bcl-2 family of proteins, such as Bcl-XL and Bcl-w that are associated with the mitochondria (26), which were not examined in the present study and could also contribute to the protection seen with increased O-GlcNAc levels. However, it should be noted that, while there is considerable evidence demonstrating that mitochondrial Bcl-2 plays an important role in the mediation of apoptosis, the precise mechanisms by which this occurs are still not well defined (38).

O-GlcNAc levels can be elevated not only by increasing the rate of synthesis, but also by decreasing the rate of removal via inhibition of O-GlcNAcase. PUGNAc is a widely used inhibitor of O-GlcNAcase, and we have previously reported that both glucosamine and PUGNAc are protective against ischemic injury (9, 32). However, despite the fact that PUGNAc resulted in markedly greater O-GlcNAc levels than glucosamine, it was somewhat less protective (9). Therefore, here we used a new O-GlcNAcase inhibitor, NBuGT, which has ~1,500-fold greater specificity for O-GlcNAcase over β-hexosaminidase than PUGNAc (34). NBuGT increased O-GlcNAc levels 5- to 10-fold more than OGT overexpression; however, while compared with untreated cells NBuGT attenuated necrosis following ischemia-reperfusion, this effect was significantly less than that seen with OGT overexpression (Fig. 3B). The difference between NBuGT treatment and both OGT overexpression and glucosamine expression was particularly apparent with regard to the loss of cytochrome c following ischemia-reperfusion (Fig. 4). It is also noteworthy that, in contrast with OGT overexpression and glucosamine treatment, NBuGT had no effect on mitochondrial Bcl-2 levels, under either normoxia or following ischemia-reperfusion.

These observations are rather contradictory to the notion that increasing protein O-GlcNAc levels is cytoprotective, since NBuGT was clearly less protective than either glucosamine or OGT overexpression despite ~10-fold higher O-GlcNAc levels. This could point to differences resulting from increasing O-GlcNAc levels via new synthesis versus inhibiting the removal. For example, proteins, which under steady-state conditions contain O-GlcNAc that is constantly cycling between on and off states, will show an enhancement of O-GlcNAc levels with O-GlcNAcase inhibition. In contrast, some proteins may become O-GlcNAcylated only when UDP-GlcNAc or OGT levels increase. Thus our results could be explained if some proteins important to mediating the protective response, including the increase in mitochondrial Bcl-2 levels, were in the latter class. If this is the case, it is possible that the combination of glucosamine plus NBuGT could increase mitochondrial Bcl-2 levels and thus provide greater protection than NBuGT alone. It should also be noted that OGT is predominantly localized to nucleus, whereas O-GlcNAcase is found primarily in the cytosol (14); consequently, glucosamine and NBuGT may lead to a different spectrum of O-GlcNAcylated proteins in different cellular compartments, which could also account for their different levels of protection. However, it is also apparent with regard to the loss of cytochrome c following ischemia-reperfusion (Fig. 4).
possible that there is a threshold effect with cellular O-GlcNAc levels, such that beyond a certain level, increased O-GlcNAc levels will increase cell death. Indeed, it has been proposed that high as well as low levels of O-GlcNAc may trigger apoptosis (53). If this is the case, then a combination of glucosamine and NBuGT may lead to further loss of protection compared with either treatment alone.

The importance of OGT and O-GlcNAc in the mediation of cardiomyocyte survival was further supported by the fact that decreasing OGT expression by ~50%, using siRNA, not only attenuated basal O-GlcNAc levels, but also prevented the increase induced by ischemia (Fig. 6A and 7A). This was associated with an increase in necrosis (Fig. 6B), greater loss of cytochrome c (Fig. 7A), and increased apoptosis (Fig. 7B) in the OGT siRNA group. In contrast to OGT overexpression and glucosamine treatment, reduced OGT expression resulted in an increase in whole cell Bcl-2 levels, thereby complicating the interpretation of the Bcl-2 data. Nevertheless, the increase in cardiomyocyte injury in the OGT siRNA group was associated with attenuation of mitochondrial Bcl-2 following ischemia-reperfusion, which is in contrast with the increase seen in control si-Neg cells.

An alternative explanation for the improved survival associated with glucosamine and OGT overexpression could be that increased O-GlcNAc levels lead to decreased energy consumption as seen with β-blockers or calcium antagonists or by increasing energy production by stimulating glycolysis. In the present study, we did not assess cardiomyocyte contractility or energy metabolism; however, in an earlier study using the same NRVM model, we found that glucosamine had no effect on ATP levels under normocind conditions, at the end of ischemia, or the end of reperfusion (9). We have also shown that neither glucosamine nor PUGNac affected the beating rate of neonatal cardiomyocytes (39), suggesting that increasing O-GlcNAc formation or inhibiting O-GlcNAc removal does not block Ca²⁺-handling pathways associated with contractility. In the intact perfused heart, we have not observed any negative inotropic or chronotropic effect of glucosamine, and the rate of ATP loss during ischemia was not reduced by glucosamine treatment even though functional recovery was improved (15). Preliminary studies on the effect of glucosamine on cardiac metabolism demonstrate that, at least under normocind conditions, glucosamine has no effect on glycolytic flux (16). Thus, while we cannot entirely rule out an effect of increasing O-GlcNAc on improving cardiac energy metabolism, our data to date suggest this is not the case.

In conclusion, we have shown that in NRVMs, the glucosamine-mediated protection against both ischemia-reperfusion and H₂O₂ is mimicked by OGT overexpression, and that decreasing OGT expression significantly increased sensitivity of NRVMs to ischemia-reperfusion injury. These results provide strong evidence to support the notion that the protective effects of glucosamine seen at the cellular, organ, and whole animal level are mediated via increased formation of O-GlcNAc. We have also demonstrated that one potential mechanism contributing to this protection is an increase in mitochondrial Bcl-2 levels, which was also associated with attenuation of H₂O₂-induced loss of mitochondrial membrane potential. However, further studies are clearly warranted to better understand the mechanism by which increased O-GlcNAc levels modulate the cellular distribution of Bcl-2 and whether this is a consequence of O-GlcNAc modification of Bcl-2 itself.

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REFERENCES


