Glucosamine protects neonatal cardiomyocytes from ischemia-reperfusion injury via increased protein-associated O-GlcNAc

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Submitted 7 April 2006; accepted in final form 30 July 2006

Champattanachai V, Marchase RR, Chatham JC. Glucosamine protects neonatal cardiomyocytes from ischemia-reperfusion injury via increased protein-associated O-GlcNAc. Am J Physiol Cell Physiol 292: C178–C187, 2007. First published August 9, 2006; doi:10.1152/ajpcell.00162.2006.—Increased levels of protein-associated O-linked N-acetylglucosamine (O-GlcNAc) have been shown to increase cell survival following stress. Therefore, the goal of this study was to determine whether in isolated neonatal rat ventricular myocytes (NRVMs) an increase in protein O-GlcNAcylation resulted in improved survival and viability following ischemia-reperfusion (I/R). NRVMs were exposed to 4 h of ischemia and 16 h of reperfusion, and cell viability, necrosis, apoptosis, and O-GlcNAc levels were assessed. Treatment of cells with glucosamine, hyperglycemia, or O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenylcarbamate (PUGNAc), an inhibitor of O-GlcNAcase, significantly increased O-GlcNAc levels and improved cell viability, as well as reducing both necrosis and apoptosis compared with untreated cells following I/R. Alloxan, an inhibitor of O-GlcNAc transferase, markedly reduced O-GlcNAc levels and exacerbated I/R injury. The improved survival with hyperglycemia was attenuated by azaserine, which inhibits glucose metabolism via the hexosamine biosynthesis pathway. Reperfusion in the absence of glucose reduced O-GlcNAc levels on reperfusion compared with normal glucose conditions and decreased cell viability. O-GlcNAc levels significantly correlated with cell viability during reperfusion. The effects of glucosamine and PUGNAc on cellular viability were associated with reduced calcineurin activation as measured by translocation of nuclear factor of activated T cells, suggesting that increased O-GlcNAc levels may attenuate I/R induced increase in cytosolic Ca2+. These data support the concept that activation of metabolic pathways leading to an increase in O-GlcNAc levels is an endogenous stress-activated response and that augmentation of this response improves cell survival. Thus strategies designed to activate these pathways may represent novel interventions for inducing cardioprotection.

The O-GlcNAc modification of proteins is unlike other glycosylation events in that it occurs through an enzyme-catalyzed reaction in the cytosol and the nucleus rather than in the Golgi apparatus or the endoplasmic reticulum. Posttranslational modification by O-GlcNAc is a dynamic and reversible process, regulated by the activities of two key enzymes, O-GlcNAc transferase (OGT) (18, 21) and N-acetylglucosaminidase (O-GlcNAcase) (11). In some cases, O-GlcNAcylation is reciprocal with phosphorylation (5, 7), and proteins that have been identified as being modified by O-GlcNAcylation include kinases, phosphatases, cytoskeletal proteins, nuclear hormone receptors, nuclear pore proteins, signal transduction molecules, and actin regulatory proteins (38). Furthermore, O-GlcNAcylation has also been reported to influence protein transcription and translation, nuclear targeting and transport, and protein degradation (7, 13, 29). Consequently, alterations in O-GlcNAc levels have important and wide-ranging effects on cell function.

Schaffer et al. (30) reported that hyperglycemia significantly reduced hypoxia-induced apoptosis and necrosis in isolated cardiomyocytes and showed that this was associated with decreased Ca2+ overload. Our laboratory (25) recently reported that increased levels of O-GlcNAc in cardiomyocytes decreased angiotensin II-mediated increase in cytosolic Ca2+. The facts that O-GlcNAc levels are increased by hyperglycemia and that elevated O-GlcNAc levels increase the tolerance of cells to stress raise the possibility that the protection associated with increased glucose utilization could be mediated, at least in part, via this pathway. Therefore, the goal of this study was to determine in isolated neonatal cardiomyocytes whether an increase in protein O-GlcNAcylation resulted in improved survival following ischemia-reperfusion and if so, whether protection conferred by hyperglycemia was also mediated via the same pathway.

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).

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, 1996). Primary cultures of neonatal rat ventricular myocytes (NRVMs) were obtained from 2- to 3-day-old neonatal Sprague-Dawley rats and cultured as

INCREASING GLUCOSE Utilization has been long recognized as a method for increasing tolerance of the heart to ischemic injury, and the primary mechanisms attributed to this protection are increased glycolytic ATP production and reduced fatty acid oxidation (9, 33). However, increasing extracellular glucose levels also activates the hexosamine biosynthesis pathway and increases the level of O-linked N-acetylglucosamine (O-GlcNAc) on serine and threonine residues of cytoplasmic and nuclear proteins (37, 38). Interestingly, activation of this pathway has been associated with increased tolerance of cells to stress (40), and recent studies have suggested it may also be associated with ischemic cardioprotection (20).

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Glucosamine treatment decreases cardiomyocyte injury following ischemia and reperfusion. Glucosamine treatment had no effect on viability, necrosis, or apoptosis when present under normoxic incubation conditions for 20 h in time-control experiments (Fig. 1, A–C). However, at the end of reperfusion, glucosamine treatment resulted in a significant increase in survival and significantly decreased both necrosis and apoptosis (Fig. 1, A–C). Glucosamine treatment also significantly increased O-GlcNAc and UDP-HexNAc levels under both normoxic conditions and following ischemia-reperfusion (Fig. 1, D–F). Although ischemia-reperfusion alone appeared to increase O-GlcNAc levels in the control group, this did not reach statistical significance (1.00 ± 0.09 vs. 1.64 ± 0.33 arbitrary units (AU); P = 0.13). However, glucosamine treatment markedly augmented the response to ischemia-reperfusion, resulting in an approximately threefold increase compared with normoxia. Equal protein loading was confirmed by densitometric analysis of Coomassie blue staining of duplicate gels (Fig. 1, D, right), and this was consistent with Western blot analysis of β-actin levels (data not shown). In the control group, UDP-HexNAc levels were significantly increased at the end of ischemia compared with normoxic conditions (2.3 ± 0.2 vs. 3.0 ± 0.2 μmol/g; P < 0.05).

The protection against ischemic injury with glucosamine may be a consequence of increased energy production, since glucosamine can potentially be metabolized to fructose-6-phosphate via glucosamine-6-phosphate isomerase or deaminase (2, 41), thereby increasing glycolytic flux. Therefore, we
assessed ATP levels in glucosamine-treated and untreated cells at the end of ischemia and the end of reperfusion (Table 1). As anticipated, ATP levels were significantly reduced at the end of ischemia; however, at the end of 16 h of ischemia-reperfusion, ATP levels had returned to normoxic levels. Glucosamine treatment had no effect on ATP levels under any conditions. This was true regardless of whether ATP levels were normalized to micrograms of total protein or total numbers of cells per plate (data not shown). Thus the protection resulting from glucosamine treatment shown in Fig. 1 does not appear to be a consequence of increased glycolytic ATP production.

Table 1. ATP levels in NRVMs in untreated control and glucosamine-treated groups under conditions of normoxia, ischemia, and ischemia-reperfusion

<table>
<thead>
<tr>
<th>Condition</th>
<th>ATP, μmol/g protein</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Normoxia</td>
<td>27.8±2.6</td>
</tr>
<tr>
<td>Ischemia</td>
<td>9.4±0.8</td>
</tr>
<tr>
<td>I/R</td>
<td>34.4±4.3</td>
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Data are presented as means ± SE of 6 experiments [i.e., 3 separate neonatal rat ventricular myocyte (NRVM) isolations with each experiment performed in duplicate]. I/R, ischemia-reperfusion.

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**Time course of O-GlcNAc levels during ischemia and reperfusion.** In the experiment shown in Fig. 1, we demonstrated that glucosamine improved viability after 4 h of ischemia and 16 h of reperfusion. To evaluate the temporal relationship between increased O-GlcNAc levels and cell viability, we assessed O-GlcNAc levels and cell viability at different time points during ischemia-reperfusion in untreated and glucosamine-treated cells. In some additional experiments, we also examined the role of glucose in regulating O-GlcNAc levels by reperfusing without glucose in the medium.
During ischemia, O-GlcNAc levels increased ~45% in the control group ($P = 0.07$) and more than twofold in the glucosamine-treated group ($P < 0.05$) (Fig. 2, A and B). During the first hour of reperfusion, O-GlcNAc levels increased in both untreated and glucosamine-treated groups, but this response was markedly enhanced in the glucosamine-treated group (Fig. 2B). In contrast, in the absence of glucose there was no increase in O-GlcNAc levels on reperfusion (Fig. 2, A and B). In untreated cells, O-GlcNAc levels returned to baseline after 8 h of reperfusion and remained relatively constant for the remainder of the reperfusion period. However, in the glucosamine-treated group, O-GlcNAc levels remained elevated for ~8 h of reperfusion, returning close to baseline levels by 12 h. In the absence of glucose, there was a gradual decrease in O-GlcNAc levels throughout the reperfusion period. The ability to increase O-GlcNAc levels during the first few hours of reperfusion was associated with improved viability (Fig. 2C), and there was a significant correlation between O-GlcNAc levels and cell viability during both early (2 h) and late (16 h) reperfusion (Fig. 2D).

Hyperglycemia-induced protection against ischemia-reperfusion mediated via hexosamine biosynthesis pathway. In the experiment shown in Fig. 2, we demonstrated that the absence of glucose during reperfusion decreased O-GlcNAc levels and that this was associated with decreased cell survival,
suggesting that glucose was required for the increase in O-GlcNAc in reperfusion. Since hyperglycemia increases flux through the hexosamine biosynthesis pathway (HBP) (22) and also has been shown to be protective (30), we asked whether hyperglycemia-mediated protection may be due, at least in part, to activation of this pathway. Hyperglycemia significantly increased cell viability and decreased NRVM apoptosis after ischemia and ischemia-reperfusion (Fig. 3, A and B), and this was associated with an increase in O-GlcNAc levels of selected bands in the CTD110 immunoblot (Fig. 3, C and D). Treatment with azaserine, an inhibitor of glutamine:fructose-6-phosphate amidotransferase (GFAT) (22), which regulates glucose entry into the HBP, blocked the protection seen with hyperglycemia and reversed the increase in O-GlcNAc levels. In contrast to glucosamine treatment, the increase in overall O-GlcNAc levels associated with hyperglycemia was less pronounced; this might be expected, since glucosamine enters the HBP directly and essentially unregulated, whereas glucose entry into the HBP is regulated by GFAT. Densitometric analysis of individual bands was performed (Fig. 3D). Because of small sample size, the apparent differences in O-GlcNAc levels following ischemia-reperfusion in all bands and in band 1 were not significantly different. However, bands 2, 4, and 5 all showed a significant increase in the high-glucose groups following ischemia-reperfusion, which was significantly attenuated with azaserine treatment. Interestingly, hyperglycemia also increased the intensity of bands 4 and 5 under normoxic conditions.

**O-GlcNAcylation plays an important role in cellular survival during ischemia and reperfusion injury.** To better understand the role of O-GlcNAc in mediating the protection, we compared the effects of glucosamine with alloxan, an inhibitor of OGT that should block the formation of O-GlcNAc, and with O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-

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**Fig. 3.** A: cell viability assessed using Trypan blue exclusion. B: apoptosis assessed as a percentage of TUNEL-positive cells. C: representative CTD110.6 immunoblot of O-GlcNAc proteins. D: mean intensity of O-GlcNAc proteins determined by densitometric analysis with levels normalized to normoxic control untreated cells for all bands and bands 1–5 as indicated. Control untreated cells, cells treated with high glucose (30 mM Glc), and cells treated with high glucose (HG) plus the glutamine:fructose-6-phosphate amidotransferase (GFAT) inhibitor azaserine (5 μM) were incubated under normoxic conditions for 20 h or subjected to 4 h of ischemia followed by 16 h of reperfusion. Data are presented as means ± SE of 4 independent experiments. *P < 0.05 vs. control. #P < 0.05 vs. high glucose.
N-phenylcarbamate (PUGNAC), an inhibitor that prevents cycling of O-GlcNAc from proteins, thereby increasing O-GlcNAc levels independently of HBP flux (35). Consistent with data in Fig. 1, glucosamine increased viability and decreased injury following ischemia-reperfusion (Fig. 4, A–C). In contrast, alloxan markedly reduced viability and increased both necrosis and apoptosis; whereas the effect of PUGNAC on cell viability and LDH release was similar to that of glucosamine, although it did not reduce apoptosis compared with untreated controls. In the O-GlcNAc immunoblots (Fig. 4D), it is clear that at the end of ischemia-reperfusion, O-GlcNAc was virtually absent in the alloxan group, whereas with PUGNAC, there was a dramatic increase; however, the effect of glucosamine on O-GlcNAc levels was less marked than that shown in Fig. 1. ANOVA of the mean densitometric data (Fig. 4E) clearly indicated a significant treatment effect \( P < 0.05 \); however, this was due to the more than 10-fold increase in O-GlcNAc in the PUGNAC-treated group. Although at the end of ischemia-reperfusion there was a \( \sim 50\% \) increase in O-GlcNAc in the glucosamine-treated group, this did not reach statistical significance. We believe that the apparently attenuated response of O-GlcNAc to glucosamine treatment in these experiments is most likely a consequence of the short exposure time required for visualizing the immunoblot due to the very high levels of O-GlcNAc seen with PUGNAC treatment.

Under normoxic conditions alloxan had a small but significant effect on viability compared with controls \( (100 \pm 0.7 \text{ vs. } 91 \pm 3\% ; P < 0.05) \) but had no effect on necrosis or apoptosis. PUGNAC had no adverse effects on viability, necrosis, or apoptosis.

Increased O-GlcNAc attenuates Ca\(^{2+}\)-mediated nuclear translocation of NFAT during ischemia and ischemia-reperfusion. Cytosolic Ca\(^{2+}\) levels were measured using fura-2 in untreated and glucosamine-treated NRVMs under normoxic conditions and at the end of 4 h of ischemia. In untreated cells, cytosolic Ca\(^{2+}\) levels increased from 110 \(\pm\) 12 nM under normoxic conditions to 150 \(\pm\) 5 nM at the end of ischemia \( (P < 0.05) \). In glucosamine-treated cells, normoxic cytosolic Ca\(^{2+}\) levels were not significantly different from those in untreated cells \( (114 \pm 8 \text{ nM}) \); however, at the end of ischemia, cytosolic Ca\(^{2+}\) levels were significantly lower than in untreated cells \( (106 \pm 7 \text{ nM}; P < 0.05) \).

To determine whether the reduction in Ca\(^{2+}\) during ischemia in the glucosamine-treated group might contribute to the reduction in injury shown in Figs. 1 and 2, we assessed nuclear translocation of GFP-NFAT as an indicator of Ca\(^{2+}\)-induced calcineurin activation as previously described (15). Calcineurin has been implicated in mediating cardiomyocyte apoptosis (24, 27). As shown in Fig. 5, under normoxic conditions GFP-NFAT was restricted to the cytoplasm in >90% of the trans-

![Fig. 4](http://ajpcell.physiology.org/)

**Fig. 4.** A: cell viability assessed using Trypan blue exclusion. B: necrosis assessed by determining LDH release as a percentage of total LDH. C: apoptosis assessed as a percentage of TUNEL-positive cells. D: representative CTD110.6 immunoblot of O-GlcNAc proteins. E: mean intensity of all O-GlcNAc proteins determined by densitometric analysis with levels normalized to normoxic control untreated cells. Control untreated cells, glucosamine (5 mM)-treated, alloxan (1 mM)-treated, and O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenylcarbamate (PUGNAC; 100 \(\mu\text{M}\))-treated cells were incubated under normoxic conditions for 20 h or subjected to 4 h of simulated ischemia followed by 16 h of incubation under normoxic conditions. Data are presented as means \( \pm \) SE of 6 independent experiments. \( *P < 0.05 \) vs. control.
effect on cell viability. The augmented response influences survival following ischemia-reperfusion. Together, these data provide compelling evidence that activation of pathways leading to increased protein O-GlcNAc levels is an endogenous stress response in cardiomyocytes and that alteration of this response influences survival following ischemia-reperfusion.

Zachara et al. (40) demonstrated that in COS-7 cells, O-GlcNAc levels increased almost twofold within 3 h following heat stress, returning to baseline levels between 24–48 h, and they showed that this was due, at least in part, to an increase in OGT activity. In the present study, we found that ischemia-reperfusion stress in untreated NRVMs increased O-GlcNAc levels even more quickly with a maximum level, almost threefold higher than baseline, and that this was completely ablated when cells were reperfused in the absence of glucose (Fig. 2B). This finding suggests that increased metabolism of glucose via the HBP is required for the increase in O-GlcNAc in response to ischemia and also may contribute to the increase in O-GlcNAc reported by Zachara et al. (40). This is supported by the fact that increasing glucose concentrations also increased cell survival via a mechanism consistent with increased HBP flux and O-GlcNAc levels (Fig. 3). Furthermore, the strong correlation between cell viability and O-GlcNAc levels during reperfusion provides additional support for the notion that the level of O-GlcNAc in cells is an important determinant of cell viability following a stress such as ischemia-reperfusion (Fig. 2D).

Zachara et al. (40) found that increased O-GlcNAc levels were associated with faster induction of heat shock protein 70 (HSP70) expression, suggesting a possible mechanism underlying the cytoprotection resulting from increased flux through OGT. However, we found no increase in HSP70 expression in response to glucosamine treatment in NRVMs (data not shown), which is consistent with a study by Sohn et al. (32), who showed that overexpression of OGT increased O-GlcNAc levels and cell survival in Chinese hamster ovary cells within 60 min without any change in HSP70 expression during this time frame. The dissociation between changes in cell survival and HSP70 expression suggests that other mechanism(s) operating over a shorter time frame also may contribute to the protection associated with increased O-GlcNAc levels.

Increasing glycolytic ATP production, especially during ischemia, has been shown to decrease injury and improve recovery on reperfusion (9). Since glucosamine could increase glycolytic flux by metabolism via glucosamine-6-phosphate
isomerase or deaminase (2, 41), this might represent an alternative protective mechanism independent of alterations in O-GlcNAc levels. We therefore assessed ATP levels in control and glucosamine-treated groups under normoxic conditions, at the end of ischemia, and at the end of ischemia-reperfusion. If the addition of glucosamine increased glycolytic ATP synthesis, we would have expected to see increased levels of ATP, especially at the end of ischemia. However, although ATP levels were slightly increased at the end of ischemia and decreased at the end of reperfusion, these differences did not reach statistical significance (Table 1). Thus these data suggest that the protection seen with glucosamine was not due to increased glycolytic ATP synthesis during ischemia. It is important to note that although maintenance of ATP levels during either ischemia or reperfusion is often associated with improved functional recovery and decreased injury, this is not a prerequisite for ischemic protection. For example, although ischemic preconditioning significantly improves function recovery compared with control hearts following ischemia and reperfusion, ATP hydrolysis during ischemia has been reported to be increased (16), and following reperfusion, ATP levels were not increased in a preconditioned group (4).

Stimulation of myocardial glucose utilization repeatedly has been shown to afford protection against ischemic injury (33); however, there is little consensus regarding the mechanism(s) underlying this protection. In the present study we found that hyperglycemia was protective against ischemia-reperfusion in NRVMs (Fig. 3), that this protection was associated with an increase in O-GlcNAc levels, and that when glucose entry into the HBP was inhibited with azaserine, both the protection and the increase in O-GlcNAc were attenuated (Fig. 3). Thus, although we cannot exclude changes in energy metabolism playing a role in hyperglycemic protection, these data suggest this protection may be mediated, at least in part, by increased HBP flux and O-GlcNAc levels. The fact that the increase in O-GlcNAc seen with hyperglycemia was less pronounced than that seen with glucosamine is not surprising, since glucosamine enters the HBP directly and essentially unregulated, whereas glucose entry into the HBP is regulated by GFAT. However, it is noteworthy that in the hyperglycemia experiments, densitometric analysis showed differential response of individual bands in the CTD110 immunoblot, suggesting that the protection might be mediated via specific proteins rather than a global increase in O-GlcNAc levels.

Work in our laboratory has previously shown that glucosamine inhibited angiotensin II induced, calcineurin-mediated NFAT translocation (15) and have subsequent studies shown that this was due to attenuation of Ca\(^{2+}\) influx (25). Since calcineurin activation has been implicated in mediating cardiomyocyte apoptosis (24, 27), we asked whether protection resulting from increased O-GlcNAc levels may also be associated with reduced calcineurin activation. We found that at the end of ischemia-reperfusion, there was marked increase in nuclear localization of GFP-NFAT consistent with an increase in cytosolic Ca\(^{2+}\) (Fig. 5). Importantly, both glucosamine and PUGNAc significantly attenuated the nuclear translocation of GFP-NFAT, whereas alloxan significantly increased GFP-NFAT nuclear localization. Furthermore, the effects of the different interventions on GFP-NFAT nuclear localization are remarkably similar to their impacts on cell viability (Fig. 4). It should be noted that these experiments do not address whether glucosamine attenuates severe Ca\(^{2+}\) overload that is also associated with ischemia-reperfusion injury. However, our laboratory has recently reported that in the whole heart, glucosamine markedly reduced tissue injury due to severe Ca\(^{2+}\) overload induced by the Ca\(^{2+}\) paradox (20). Thus the data shown in the present study combined with our group’s earlier study (20) suggests that the protection associated with increased O-GlcNAc levels may be due, at least in part, to inhibition of Ca\(^{2+}\) entry into NRVMs and subsequent attenuation of Ca\(^{2+}\)-mediated necrosis and apoptosis.

The specific proteins affected by O-GlcNAcylation that might be mediating the protection against ischemia-reperfusion injury remain to be identified. However, our previous studies have shown that the effects of glucosamine on cardiomyocyte Ca\(^{2+}\) homeostasis were specific for a capacitative Ca\(^{2+}\) entry (CCE) pathway rather than other sarcolemmal Ca\(^{2+}\) channels (15). The transient receptor potential (TRP) channel protein family are prime candidates for mediating CCE (10, 23), and analysis of the protein sequences for both TRP1 and TRP4 indicates that both proteins contain serine residues with a potentially high affinity for O-GlcNAc modification (www.cbs.dtu.dk/services/YinOYang/). Alternatively, kinases such as Akt, ERK1/2, p38, and PKC that have been shown to play a role in mediating ischemic cardioprotection (1, 14) also have been shown to be targets for O-GlcNAcylation or for their activity to be modulated by changes in O-GlcNAc levels (38). Clearly, further studies are warranted not only to identify cardiac proteins that are targets for O-GlcNAcylation but also to determine how these proteins are affected by ischemic stress and how changes in the levels of O-GlcNAc alter the response to stress.

We have shown in the present study that alloxan, a putative inhibitor of OGT (17), markedly reduced O-GlcNAc levels at the end of ischemia-reperfusion, and this was associated with increased cellular injury. We also have shown that the absence of glucose during reperfusion also attenuated the increase in O-GlcNAc levels and reduced cell viability. These data support the notion that metabolism of glucose via the HBP and the formation of O-GlcNAc is a normal stress response and that inhibition of these pathways decreases the tolerance to ischemia-reperfusion stress. However, it is important to note that alloxan is a uracil analog, and thus we cannot rule out potential nonspecific effects of alloxan (34). Nevertheless, in the absence of ischemia-reperfusion, alloxan had minimal effects on viability and no effects on necrosis or apoptosis. Since ablation of the OGT gene is embryonically lethal (31), more specific demonstration of the role of OGT in mediating glucosamine cardioprotection will require the development of tissue-specific, conditional knockout mice or the use of small interfering RNA approaches to reduce OGT expression in cardiomyocytes.

There was a significant correlation between O-GlcNAc levels and cell viability in untreated cells or when glucosamine was used to increase O-GlcNAc (Fig. 2); however, PUGNAc did not lead to greater protection despite the fact that O-GlcNAc levels were markedly higher that in the glucosamine-treated group (Fig. 4). Indeed, if anything, PUGNAc appeared to be somewhat less protective than glucosamine. It has been reported that acceptor protein specificity changes with hyperglycemia or glucosamine treatment (19, 36), and we found that glucosamine and PUGNAc treatment led to increased O-GlcNAc levels in different protein bands. Thus the fact that
PUGNAc was not as protective as glucosamine despite the higher overall level of O-GlcNAc. These data support the concept that augmentation of this response improves cell survival. Thus protection may be a result not only of the effect of increased O-GlcNAc levels on specific proteins but also the duration of the increase in O-GlcNAc levels. Clearly, further studies are warranted not only to identify the specific proteins involved in O-GlcNAc-mediated protection but also to better understand the dynamics of O-GlcNAcylation in response to stress.

In conclusion, we have shown that increasing O-GlcNAc levels with glucosamine, high glucose, or PUGNAc are all associated with increased cell survival following ischemia and reperfusion. We also found that ischemia-reperfusion increased O-GlcNAc levels in untreated cells, which was inhibited by reperfusion in the absence of glucose, and that this was associated with lower viability. Furthermore, the improved cell survival in glucosamine- and PUGNAc-treated cells was associated with reduced cytosolic Ca\(^{2+}\) levels during ischemia and ischemia-reperfusion. This data support the concept that activation of metabolic pathways leading to an increase in O-GlcNAc was sustained for the entire reperfusion period. In contrast, although glucosamine treatment increased O-GlcNAc levels seven-to-eightfold during reperfusion (Fig. 2), this was a transient response, and at the end of reperfusion O-GlcNAc levels were only approximately twofold higher than in the untreated group. This suggests that protection may be a result not only of the effect of increased O-GlcNAc levels on specific proteins but also the duration of the increase in O-GlcNAc levels.


