Increased O-GlcNAc levels during reperfusion lead to improved functional recovery and reduced calpain proteolysis

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Liu J, Marchase RB, Chatham JC. Increased O-GlcNAC levels during reperfusion lead to improved functional recovery and reduced calpain proteolysis. Am J Physiol Heart Circ Physiol 293: H1391–H1399, 2007. First published June 15 2007; doi:10.1152/ajpheart.00285.2007.—We have previously shown that preischemic treatment with glucosamine improved cardiac functional recovery following ischemia-reperfusion, and this was mediated, at least in part, via enhanced flux through the hexosamine biosynthesis pathway and subsequently elevated O-linked N-acetylglucosamine (O-GlcNAC) protein levels. However, preischemic treatment is typically impractical in a clinical setting; therefore, the goal of this study was to investigate whether increasing protein O-GlcNAC levels only during reperfusion also improved recovery. Isolated perfused rat hearts were subjected to 20 min of global, no-flow ischemia followed by 60 min of reperfusion. Administration of glucosamine (10 mM) or an inhibitor of O-GlcNACase, O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino- N-phenylcarbamate (PUGNAc; 200 μM), during the first 20 min of reperfusion significantly improved cardiac functional recovery and reduced troponin release during reperfusion compared with untreated control. Both interventions also significantly increased the levels of protein O-GlcNAC and ATP levels. We also found that both glucosamine and PUGNAc attenuated calpain-mediated proteolysis of α-fodrin as well as Ca2+/calmodulin-dependent protein kinase II during reperfusion. Thus two independent strategies for increasing protein O-GlcNAC levels in the heart during reperfusion significantly improved recovery, and this was correlated with attenuation of calcium-mediated proteolysis. These data provide further support for the concept that increasing cardiac O-GlcNAC levels may be a clinically relevant cardioprotective strategy and suggest that this protection could be due, at least in part, to inhibition of calcium-mediated stress responses.

hexosamine biosynthesis; protein O-glycosylation; ischemia-reperfusion

OPTIMIZING REPERFUSION CONDITIONS to minimize cardiac injury during global ischemia continues to be a major obstacle in the development of more effective therapies for cardiac disease (4). We have recently reported that preischemic treatment with glucosamine protects against ischemia-reperfusion (I/R) injury in both isolated rat hearts (8, 18) and neonatal rat ventricular myocytes (NRVM) (5), and this protection was associated with elevated levels of O-linked N-acetylglucosamine (O-GlcNAC) on nuclear and cytoplasmic proteins. The results from these studies were consistent with the report by Zachara et al. (42), who demonstrated that cells exposed to various stress stimuli were shown to have elevated O-GlcNAC levels, and this promoted cell survival. However, in addition to increasing O-GlcNAC levels, glucosamine also increases uridine diphos-

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fusión, and 3) to determine whether protection associated with increased O-GlcNAc levels was associated with attenuation of Ca^{2+}-induced stress responses such as calpain-mediated proteolysis.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Animals. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham 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). Nonfasted male Sprague-Dawley rats weighing 260–300 g were used throughout.

Isolated heart perfusion. Hearts were isolated and perfused as previously described (18, 26). Briefly, Sprague-Dawley rats were anesthetized (ketamine, 100 mg/kg ip) and decapitated, and hearts were rapidly excised and perfused in a modified Langendorff model with Krebs-Henseleit buffer equilibrated with 95% O2-5% CO2 (37°C, pH 7.4). The buffer contained (in mM) 118 NaCl, 4.8 KCl, 1.2 MgSO4, 1.25 CaCl2, 1.2 KH2PO4, 25 NaHCO3, and 5.5 glucose. Global ischemia was induced by stopping coronary flow for 20 min, followed by reperfusion at 75 mmHg for a further 60 min. At the end of each experiment, left ventricles were freeze-clamped in liquid nitrogen and stored at −80°C for further analysis.

Cardiac function was monitored via a fluid-filled balloon inserted into the left ventricle through the mitral valve connected to a TDX-310 pressure transducer and analyzed with a heart performance analyzer (HPA-410; Micro-Med). End-diastolic pressure (EDP) was set to ~5 mmHg by adjusting the balloon volume, and coronary flow rate was adjusted to maintain 75 mmHg of perfusion pressure. Contractile parameters assessed included heart rate (HR), left ventricular developed pressure (LVDP), EDP, LV maximal rate of change in pressure (+dP/dt), LV minimum rate of change in pressure (~dP/dt), and rate-pressure product (RPP; RPP = HR × LVDP).

Experimental groups. Hearts were equilibrated for 30 min, followed by 20 min of global no-flow ischemia. After ischemia, hearts were randomly distributed to three experimental groups: I) control, untreated (n = 6); 2) glucosamine, 10 mM for first 20 min of reperfusion (n = 6); and 3) PUGNAc, 200 μM for first 20 min of reperfusion (n = 4). The total reperfusion time for each of the three groups was 60 min.

Western blot analysis. Protein extracts from LV tissue were obtained using a lysis buffer consisting of 0.5% NP-40, 150 mM NaCl, 10 mM Tris (pH 7.4), 10% glycerol, protease inhibitor cocktail, and 40 μM PUGNAc (inhibitor of O-GlcNAcase). Solubilized protein was suspended in Laemmli sample buffer and then separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Protein loading was assessed via Coomassie blue staining or α-tubulin immunostaining (18).

Blots were probed with anti-O-GlcNAc antibody CTD110.6 (7) (a kind gift from Mary Ann Accavitti, University of Alabama at Birmingham), anti-CaMKII (BD Biosciences, Franklin Lakes, NJ), and anti-fodrin antibodies (Chemicon, Temecula, CA). The immunoblots were developed with enhanced chemiluminescence (Pierce, Rockford, IL), and the signal was recorded on X-ray film. 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.

Measurement of AT P and UDP-GlcNAc levels. ATP and UDP-GlcNAc levels were determined using HPLC analysis of perchloric acid extracts of tissue samples as previously described (26). Briefly, neutralized acid extracts were loaded onto a SAX Partisil 10 anion-exchange column (250 × 4.6-mm Partisil SAX; Thermo) eluted with a gradient of ammonium dihydrogen phosphate from 15 (pH 2.8) to 1 mM (pH 3.7). The elution time for ATP was ~35 min, and that for UDP-GlcNAc was ~17 min. The concentrations of ATP and uridine diphosphate-N-acetylatedhexosamine (UDP-HexNAc) were determined using ultraviolet detection after calibration with appropriate standards. This method does not distinguish UDP-GlcNAc and uridine diphosphate-N-acetylatedglactosamine (UDP-GaINAc) (30); therefore, all results are presented as the sum of UDP-GlcNAc plus UDP-GaINAc (i.e., UDP-HexNAc). However, in the heart, the ratio of UDP-GlcNAc to UDP-GaINAc is ~3:1 (6).

Measurement of cardiac troponin I release in effluent. As a marker of tissue injury, cardiac troponin I (cTnI) concentration was determined in pooled coronary effluent at 30 and 60 min after reperfusion by using ELISA (cardiac troponin I ELISA kit; Life Diagnostics).

Data analysis. All data are means ± SE. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparisons test as appropriate. Statistically significant differences between groups were defined as P < 0.05 and are indicated in legends.

RESULTS

Postischemic treatment of glucosamine and PUGNAc improved functional recovery following I/R. In Fig. 1, A–C, typical LV pressure traces for the three groups are shown; there were no significant differences in LV function among the three groups before ischemia (Table 1). Consistent with our earlier study (17), in untreated time-controlled (110 min) normoxic perfusions, there was a small (<10%) decrease in all functional parameters over the duration of the experiment (data not shown).

The addition of either glucosamine or PUGNAc during the first 20 min of reperfusion significantly improved the recovery of LVDP, RPP, +dP/dt, and −dP/dt (Fig. 1D) and attenuated the increase in EDP (Fig. 1E) compared with the untreated control group. There were no significant differences in coronary flow among groups either before ischemia or at the end of reperfusion (data not shown). ANOVA indicated a significant treatment effect on cTnI release determined in pooled coronary effluent samples collected at 30 and 60 min of reperfusion; however, the post hoc analysis indicated that only the PUGNAc group had a significantly lower value than the untreated control group (Fig. 1F).

Effects of postischemic treatments of glucosamine and PUGNAc on ATP, UDP-GlcNAc, and O-GlcNAc. In all groups at the end of reperfusion, ATP levels were markedly reduced compared with levels found in time-controlled normoxic perfusions (2.55 ± 0.1 μmol/g wet wt). At the end of reperfusion, ATP levels were approximately twofold higher in both glucosamine and PUGNAc groups compared with the untreated control group (Fig. 2A).

In all groups, at the end of reperfusion, UDP-GlcNAc levels were significantly reduced compared with levels found in time-controlled normoxic perfusions (0.11 ± 0.01 μmol·min⁻¹·g wet wt⁻¹). UDP-GlcNAc levels were increased ~20% in the glucosamine group compared with untreated controls at the end of reperfusion (Fig. 2B); however, this did not reach statistical significance according to ANOVA. Surprisingly, in the PUGNAc group, UDP-GlcNAc levels were significantly lower than in untreated controls.

At the end of reperfusion, protein O-GlcNAc levels were ~1.5-fold higher in the glucosamine group compared with controls, and PUGNAc increased protein O-GlcNAc almost 2.5-fold compared with controls (Fig. 3). We have previously shown that glucosamine treatment significantly increased
O-GlcNAc levels compared with untreated normoxic control perfused hearts (17).

Postischemic treatments of glucosamine and PUGNAc attenuate CaMKII and α-fodrin proteolysis. To determine whether the protection seen with glucosamine and PUGNAc was associated with decreased CaMKII phosphorylation, we determined the tissue levels of total and phospho-CaMKII at the end of reperfusion (Fig. 4). As shown in Fig. 4, G and H, I/R increased phospho/total CaMKII ninefold compared with normoxic controls, and this was markedly attenuated by both glucosamine and PUGNAc. However, surprisingly, these changes in phospho/total CaMKII were due primarily to alterations in total CaMKII levels (Fig. 4, C and D) and not in levels of phosphorylation (Fig. 4, E and F). Thus the primary effect of both glucosamine and PUGNAc was to attenuate the loss of CaMKII that occurred in untreated control hearts subjected to I/R. The loss of CaMKII was not a consequence of nonspecific protein loss, since these changes were not seen with α-tubulin, which was used as a protein loading control (Fig. 4, A and B).

Table 1. Cardiac baseline function before ischemia in control, untreated hearts and hearts subsequently treated with glucosamine and PUGNAc

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Heart Rate, beats/min</th>
<th>LVDP, mmHg</th>
<th>RPP, mmHg/min \times 10^{-3}</th>
<th>+dP/dt, mmHg/s \times 10^{-3}</th>
<th>−dP/dt, mmHg/s \times 10^{-3}</th>
</tr>
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<tbody>
<tr>
<td>Untreated</td>
<td>7</td>
<td>303 ± 12</td>
<td>117 ± 6</td>
<td>35 ± 1</td>
<td>3.8 ± 0.3</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>7</td>
<td>331 ± 19</td>
<td>125 ± 5</td>
<td>41 ± 2</td>
<td>4.2 ± 0.4</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>PUGNAc</td>
<td>4</td>
<td>319 ± 18</td>
<td>120 ± 4</td>
<td>38 ± 2</td>
<td>3.8 ± 0.2</td>
<td>2.4 ± 0.1</td>
</tr>
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</table>

Values are means ± SE of cardiac baseline functions measured before ischemia in control (untreated) hearts and hearts treated with 10 mM glucosamine or 200 μM O-(2-acetamido-2-deoxy-β-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc). LVDP, left ventricular developed pressure; RPP, rate-pressure product; ±dP/dt, rate of pressure development and relaxation.
Otani et al. (25) recently suggested that CaMKII might be a target for the Ca\(^{2+}\)-sensitive cysteine protease calpain. This raises the possibility that the loss of total CaMKII seen here might be a consequence of calpain-mediated proteolysis and that the protection seen with glucosamine and PUGNAc could be due to attenuation of calpain activation. Therefore, we examined α-fodrin, a cytoskeletal protein that is well established to be degraded by calpain following I/R (34, 40, 41). As shown in Fig. 5, consistent with previous reports (34, 40, 41), the 145/150-kDa cleavage fragment of α-fodrin was significantly increased (~3-fold) following I/R compared with normoxic control hearts. Furthermore, postischemic treatment with both glucosamine and PUGNAc significantly reduced the 145/150-kDa fragment levels, similar to their effects on CaMKII. As with the results in Fig. 4, the changes in α-fodrin were not a consequence of nonspecific proteolysis, since these changes were not seen with α-tubulin, which was used as a protein loading control.

In Fig. 6, A and B, there are significant linear correlations between the levels of 145/150-kDa fragment of α-fodrin with both RPP and EDP at the end of reperfusion. This suggests a strong association between the improvement in cardiac function resulting from glucosamine and PUGNAc treatment and the attenuation of α-fodrin proteolysis.

**Correlations between O-GlcNAc levels with cTnI release and functional recovery.** Although both glucosamine and PUGNAc treatment improved functional recovery, cTnI release was significantly reduced only in the PUGNAc group (Fig. 1); however, the increase in O-GlcNAc in the PUGNAc group was also markedly higher than that in the glucosamine group (Fig. 3). Therefore, we examined the relationship between cTnI release and O-GlcNAc levels and found that there was a significant correlation, with higher O-GlcNAc levels associated with reduced cTnI release (Fig. 6C).

**DISCUSSION**

We have previously shown in both the isolated perfused heart and in isolated cardiomyocytes that increasing protein O-GlcNAc levels before ischemia improved functional recovery and cell viability when given before ischemia (5, 18). However, treatments shown to be effective when administered before ischemia are frequently ineffective when administered during reperfusion (4). In this study, we report for the first time that increasing O-GlcNAc levels via two different mechanisms, namely, by increasing synthesis with glucosamine or inhibiting O-GlcNAcase with PUGNAc, only during early reperfusion significantly improved functional recovery and attenuated tissue injury assessed by cTnI release. Furthermore, both interventions attenuated the loss of CaMKII and α-fodrin cleavage consistent with a decrease in calpain-mediated proteolysis. These results, combined with our earlier report (18) support the notion that the protection resulting from glucosamine treatment is mediated via increased cardiac O-GlcNAc levels and suggests that strategies for increasing O-GlcNAc levels on reperfusion is a feasible approach for reducing I/R injury. These data also suggest that this protection might be due, at least in part, to inhibition of calcium-mediated proteolysis.

Zachara et al. (42) recently suggested that protein O-GlcNAc may be a unique signaling mechanism by which cells detect and respond to stress to survive. They showed that modulation of the levels of O-GlcNAc resulted in altered tolerance to lethal stress including heat shock, osmotic, ethanolic, reductive, and oxidative stress. Consistent with this, we reported that pretreatment with glucosamine significantly increased O-GlcNAc levels and that this was associated with increased tolerance to I/R injury (5, 8, 18). These studies suggested that the protection seen with glucosamine was mediated via the increase in O-GlcNAc. However, glucosamine increased levels of UDP-GlcNAc, a substrate for both N- and O-glycosylation, and in addition, glucosamine can potentially be metabolized via glycolysis; therefore, we could not entirely rule out other possible mechanisms contributing to glucosamine-induced ischemic protection. In the present study we showed that increasing protein O-GlcNAc levels during reperfusion via two independent mechanisms, either by increasing flux through the hexosamine pathway with glucosamine or by inhibiting O-GlcNAcase with PUGNAc, significantly improved cardiac functional recovery. This supports the concept that the previously reported protection seen with glucosamine (5, 8, 18) was indeed mediated by increased O-GlcNAc levels and also demonstrates that significant protection can be obtained even when administered only at reperfusion.

In isolated cardiomyocytes we found that increased O-GlcNAc levels attenuated both angiotensin II-induced and ischemia-induced increase in cytosolic Ca\(^{2+}\) levels (5, 22). In the isolated perfused heart, increased EDP following I/R has been linked to increase in cytosolic Ca\(^{2+}\) (32). In the present study, as well as with pretreatment protocols (18), we found that the improved functional recovery associated with increased O-GlcNAc levels was due primarily to lower EDP (Fig. 1E), suggesting that the protection may be mediated, at least in part, by decreased Ca\(^{2+}\) influx on reperfusion. Although large increases in cytosolic Ca\(^{2+}\) can lead to necrosis, more subtle increases in cytosolic Ca\(^{2+}\) also can have adverse
effects mediated via activation of Ca\(^{2+}\)-activated enzymes such as CaMKII. In vivo studies have implicated CaMKII in cardiac hypertrophy and heart failure (20, 21, 31, 44, 45). CaMKII also has been reported to play a role in mediating the beneficial effects of ischemic preconditioning on heart function by modifying the phosphorylation of sarcoplasmic reticulum proteins (23). Moreover, protection associated with ischemic preconditioning was attenuated by inhibition of CaMKII (2). In the present study we found that both glucosamine and PUGNAc significantly attenuated the ischemia-induced increase in phospho-to-total CaMKII ratio (Fig. 4). Although this is consistent with attenuation of Ca\(^{2+}\) activation, interpretation is confounded by the fact that the changes in the phospho-to-total CaMKII ratio were primarily due to alterations in the levels of total CaMKII, rather than changes in phospho-CaMKII.

The marked loss of CaMKII in untreated control hearts was not a result of nonspecific proteolysis, since these changes were not observed in α-tubulin (Fig. 4). In light of reports suggesting that CaMKII might be a substrate of the Ca\(^{2+}\)-activated protease calpain (12, 25), we examined the effects of glucosamine and PUGNAc on the cleavage of α-fodrin, a well-characterized target for calpain (41). Consistent with these reports, we found that the cleaved 145/150-kDa fragment of α-fodrin was significantly increased during I/R compared with normoxic perfusion. Furthermore, similar to the CaMKII results, we found that both glucosamine and PUGNAc blocked the degradation of α-fodrin.

Fig. 3. Comparison of cardiac protein O-linked N-acetylglucosamine (O-GlcNAc) levels at the end of reperfusion in control (n = 6) and glucosamine (n = 6)-treated groups (A) and control (n = 6) and PUGNAc (n = 4)-treated groups (B). The top blots are CTD110 immunoblots of solubilized proteins (left), and the mean intensities quantified by densitometric analysis of the immunoblots normalized to the mean intensities of the control group are shown at right. The bottom blots are the Coomassie blue staining showing uniform protein loading of samples between groups. *P < 0.05 compared with control group; unpaired Student’s t-test.
following I/R (Fig. 5). Interestingly, there was also a significant correlation between the amount of α-fodrin cleavage with both end-reperfusion RPP and EDP (Fig. 6), suggesting a strong association between decreased proteolysis and improved recovery of function. These results further support the notion that both glucosamine and PUGNAc attenuate I/R-induced calcium-mediated stress responses such as calpain activation. It is noteworthy that Otani and
colleagues (24, 25) have reported that both the glucose transporter GLUT4 and AMP-activated protein kinase (AMPK) are also targets for calpain-mediated proteolysis. Thus, attenuation of calpain activation could also improve cardiac energy metabolism following I/R; however, further experiments are required to determined whether this contributes to the improved recovery seen here.

The mechanisms by which increased O-GlcNAc levels attenuate calpain-mediated proteolysis are not yet clear. However, these data are consistent with our observations in isolated cardiomyocytes that the protection seen with both glucosamine and PUGNAc was associated with reduced cytoplasmic calcium levels and attenuated activation of calcineurin (5). We have also reported that increasing hexosamine metabolites inhibited capacitative calcium entry (CCE) (27) and that increased O-GlcNAc levels blocked angiotensin II-induced [Ca²⁺], increase in NRVM (22). Others have also shown altered Ca²⁺ handling under conditions of increased O-GlcNAc levels (6, 14). Thus, together, the decreases in CaMKII and α-fodrin proteolysis seen with glucosamine and PUGNAc are consistent with the notion that increasing levels of O-GlcNAc attenuate the increase in cytosolic Ca²⁺ that occurs during reperfusion. However, we cannot rule out other possible mechanisms; for example, it has been shown that increased levels of O-GlcNAc result in inhibition of proteasome activity (43). However, it should be noted that although some studies have shown that inhibiting proteasome function reduces ischemic injury (16, 29), others have reported that this exacerbates ischemic injury (28). Consequently, at this time there is insufficient evidence to ascribe the cardioprotection associated with increased O-GlcNAc to proteasome inhibition; however, further studies on this subject are clearly warranted.

The observation that protection was seen with treatment during reperfusion is of potential clinical relevance; however, because these studies were performed in an ex vivo preparation, the results cannot be directly extrapolated to the in vivo environment. Nevertheless, we have shown that increasing O-GlcNAc levels in vivo with either glucosamine or PUGNAc is associated with improved organ function following traumahemorrhage (37), which is supportive of the idea that these same strategies might protect against myocardial ischemic injury in vivo. It also should be noted that in these experiments, we did not measure infarct size, which is commonly used to evaluate the effectiveness of cardioprotective strategies. Clearly, further studies looking at the effectiveness of glucosamine and PUGNAc in reducing infarct size would be valuable. We did, however, demonstrate that PUGNAc markedly reduced cTnI release, which has been shown to be a sensitive measure of tissue injury in the isolated perfused heart (3) and has been associated with decreased infarct size both in vitro (36) and in vivo (11, 39). The fact that glucosamine did not significantly reduce cTnI release is contrary to our earlier study (18). In that study, glucosamine was present both before ischemia and throughout reperfusion, whereas in this study it was present only during the first 20 min of reperfusion. Thus the lack of effect of glucosamine on cTnI release in the glucosamine group could be due to the duration and timing of treatment. Nevertheless, despite the short duration of treatment with glucosamine and PUGNAc in this study, there was significant correlation between cTnI release and O-GlcNAc levels at the end of reperfusion, which further supports a link between O-GlcNAc levels and attenuation of tissue injury.

Another limitation of these studies is that we only have an indirect measure of calpain activation; although it has been well established that α-fodrin cleavage is a result of calpain-mediated proteolysis (41). Future studies using the fluorescent calcium indicator rhod-2, as recently described by MacGowan et al. (19) would also provide more direct insight into the
relationship between protein O-GlcNAc levels and cytosolic Ca\(^{2+}\) in the intact heart. In addition, Bartoli et al. (1) recently described a new transgenic mouse model in which calpain activation can be monitored in vivo using FRET (fluorescence resonance energy transfer) imaging techniques.

In conclusion, we have shown that increasing cardiac protein O-GlcNAc levels in the isolated perfused heart only during reperfusion, significantly improved cardiac function and decreased tissue injury. The fact that increasing O-GlcNAc levels by either increasing flux through the hexosamine biosynthesis pathway with glucosamine or inhibiting O-GlcNAcase with PUGNAc had similar protective effects provides strong support that this protection was mediated via the increase in O-GlcNAc. We also found that this protection was associated with attenuation of the loss of CaMKII and reduced proteolysis of \(\alpha\)- fodrin, consistent with a decrease in calpain-mediated proteolysis. This raises the possibility that the protection seen with these interventions might be mediated at least in part by decreasing calcium influx into the cell during reperfusion.

GRANTS

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