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

Jia Liu[1], **Richard B. Marchase**[1], and **John C. Chatham**[2]

[1] Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294

[2] Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294

Abstract

We have previously shown that pre-ischemic treatment with glucosamine improved cardiac functional recovery following ischemia/reperfusion (I/R) and this was mediated, at least in part, via enhanced flux through the hexosamine biosynthesis pathway (HBP) and subsequently elevated protein O-GlcNAc levels. However, pre-ischemic 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 global, no flow ischemia followed by 60 min of reperfusion. Administration of glucosamine (10mM) or an inhibitor of O-GlcNAcase O-(2-acetamido-2-deoxy-d-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc, 200μM), during the first 20 min of reperfusion significantly improved cardiac functional recovery and reduced troponin release during reperfusion compared to 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 Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) 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.

Keywords

hexosamine biosynthesis; protein O-glycosylation; ischemia/reperfusion; calpain; proteolysis

Introduction

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 pre-ischemic treatment with glucosamine protects against ischemia/reperfusion (I/R) injury in both isolated rat hearts (8,18) and neonatal rat ventricular myocytes (NRVMs) (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., who demonstrated that cells exposed to various stress stimuli were shown to have elevated

Address correspondence to: John C. Chatham, Department of Medicine, 684 MCLM Building, University of Alabama at Birmingham, Birmingham, AL 35294-0005, Tel: 205 934-0240, Fax: 205 934-0950, jchatham@uab.edu.

O-GlcNAc levels, and this promoted cell survival (42). However, in addition to increasing O-GlcNAc levels, glucosamine also increases UDP-GlcNAc levels, glucosamine-6-phosphate levels and could potentially be metabolized to fructose-6-phosphate thereby increasing glycolytic flux. Thus, the protection associated with glucosamine treatment could be mediated via a number of other pathways. Inhibition of O-GlcNAcase, which is responsible for the removal of O-GlcNAc from proteins, has been shown to rapidly increase O-GlcNAc levels in cell culture studies (35). We have previously shown that the O-GlcNAcase inhibitor O-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc) had similar effects to glucosamine in NRVMs (5,22); however, it is not know whether it would be effective in increasing O-GlcNAc levels in the intact heart and if so whether it would lead to protection against ischemic injury.

The mechanisms underlying the cardioprotection associated with increased protein O-GlcNAc levels have yet to be determined; however, we have previously shown in isolated cardiomyocytes that elevated O-GlcNAc levels are involved in the regulation of $[Ca^{2+}]_i$ homeostasis (22). We have also reported that activation of the HBP by glucosamine and elevated protein O-GlcNAc levels are associated with myocardial protection against the calcium paradox in isolated perfused rat hearts (18). Therefore, since cytosolic Ca^{2+} overload is known to mediate ischemia/reperfusion injury (13), cardioprotection associated with increased O-GlcNAc levels may be mediated by changes in $[Ca^{2+}]_i$ homeostasis. Elevated intracellular Ca^{2+} can activate numerous Ca^{2+}-regulated enzymes including protein kinases, protein phosphatases, phospholipases, NO synthases, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and the cysteine protease calpain (46). There is increasing evidence that calpain plays a major role in post-ischemic injury (15,33), in part because of proteolysis of structural proteins (9,10) including the cytoskeletal protein α-fodrin (34,40,41). Increased CaMKII activity has been implicated in contractile dysfunction following ischemia/reperfusion (21) and has also been associated with increased apoptosis/necrosis (38). Furthermore, Otani et al., recently suggested that CaMKII might be a target for calpain (25).

Many promising treatment strategies that are beneficial when used prior to ischemia prove to be ineffective when used only during reperfusion (4). Therefore, the goals of this study were 1) to determine whether increasing protein O-GlcNAc levels, only during reperfusion would be cardioprotective; 2) to compare the effectiveness of glucosamine and PUGNAc, in mediating functional recovery when administered during reperfusion 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 university of Alabama Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of laboratory Animals published by national Institute of Health (NIH Publication No. 85-23, 1996). Non-fasted, male Sprague-Dawley rats weighing 260–300g were used throughout.

Isolated heart perfusion

Hearts were isolated and perfused as previously described (18,26). Briefly, Sprague Dawley rats were anesthetized (Ketamine 100mg/kg i.p.), decapitated and hearts were rapidly

excised and perfused in a modified Langendorff model with Krebs-Henseleit buffer equilibrated with 95% O_2/5% CO_2 (37°C, pH 7.4). The buffer contained (in mmol/l): NaCl, 118; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 1.25; KH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 5.5. 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 TXD-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 perfusion pressure. Contractile parameters assessed included heart rate (HR), left ventricular developed pressure (LVDP), EDP, left ventricular maximal dP/dt (+dP/dt), left ventricular minimum dP/dt (−dP/dt), and rate-pressure product (RPP; RPP = HR * LVDP).

Experimental groups

Hearts were equilibrated for 30 min followed by 20 min global no-flow ischemia. After ischemia, hearts were randomly distributed to 3 experimental groups 1) control, untreated (n=6), 2) glucosamine, 10 mM for first 20 min of reperfusion (n=6), 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 left ventricular tissue were obtained using a lysis buffer consisting of 0.5% NP-40, 150 mM NaCl, 10mM Tris (PH 7.4), 10% glycerol, protease inhibitor cocktail and 40 μM PUGNAc (inhibitor of O-GlcNAcase). Solubilized protein was suspended in lamellae sample buffer, and then separated by SDS-PAGE and transferred to a polyvinylidenedifluoride (PVDF) membrane. Protein loading was assessed via Coomassie blue staining or α-tubulin immuno-staining (18).

Blots were probed with anti-O-GlcNAc antibody CTD110.6 (7) (a kind gift from Mary Ann Accavitti, UAB), anti-CaMKII (BD Biosciences, NJ), anti-fodrin (Chemicon, CA) antibodies. The immunoblots were developed with enhanced chemiluminescence (Pierce, IL) and the signal was recorded on X-ray film. Densitometry 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 ATP and UDP-GlcNAc levels

ATP and uridine diphosphate-N-acetyl-glucosamine (UDP-GlcNAc) levels were determined by 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 X 4.6 mm Partisil SAX; Thermo) eluted with gradient of ammonium dihydrogen phosphate from 15 (pH 2.8) to 1 mmol/L (pH3.7). The reaction time for ATP was ~ 35 min and ~17 min for UDP-GlcNAc. The concentrations of ATP and uridine diphosphate-N-acetylhexosamine (UDP-HexNAc) were determined by ultraviolet detection after calibration with appropriate standards. This method does not distinguish UDP-GlcNAc and uridine diphosphate-N-acetyl-galactosamine (UDP-GalNAc)(30); therefore all results are presented as the sum of UDP-GlcNAc plus UDP-GalNAc (i.e. UDP-HexNAc). However, in the heart the ratio of UDP-GlcNAc to UDP-GalNAc is approximately 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 minutes after reperfusion using ELISA (cardiac troponin I ELISA kit, Life Diagnostics, Inc.).

Data analysis

All data are presented as means ± SE. Statistical analysis was performed by 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 the legends to the figures.

Results

Post-ischemic treatment of glucosamine and PUGNAc improved functional recovery following ischemia/reperfusion

In Fig. 1A, B and C typical LV pressure traces for the three groups are shown; there were no significant differences in LV function between 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 to the untreated control group. There were no significant differences in coronary flow between groups either prior to ischemia or at the end of reperfusion (data not shown). ANOVA analysis 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 was significantly lower than untreated control group (Fig. 1F).

The effects of post-ischemic 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 to levels found in time-controlled normoxic perfusions (2.55±0.1 μmol/g wet wt). At the end of reperfusion ATP levels were approximately 2-fold higher in both glucosamine and PUGNAc groups compared to untreated control group (Fig. 2A).

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

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

Post-ischemic 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 Figs 4G and 4H, ischemia/reperfusion increased phospho/total CaMKII 9-fold compared to 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 4C, D) and not in levels of phosphorylation (Fig 4E,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 ischemia/reperfusion. The loss of CaMKII was not a consequence of non-specific protein loss, since these changes were not seen with α-tubulin, which was used as a protein loading control (Fig. 4A,B).

Otani et al., recently suggested that CaMKII might be a target for the Ca^{2+} sensitive cysteine protease calpain (25). This raised 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 which 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 ischemia-reperfusion compared to normoxic control hearts. Furthermore, post-ischemic 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 was not a consequence of non-specific proteolysis, since these changes were not seen with α-tubulin, which was used as a protein loading control.

In Fig 6A, B it can be seen that there were 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

Even though 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 prior to 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). Here we report for the first time that increasing O-GlcNAc levels by two different mechanisms, namely by increasing synthesis with glucosamine or inhibition of 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 an increase cardiac O-GlcNAc levels and suggests that strategies for increasing O-GlcNAc levels on reperfusion is a feasible approach for reducing ischemia/reperfusion 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 in order to survive. They showed that modulation of the levels of O-GlcNAc resulted in altered tolerance to lethal levels of cellular stress including heat shock, osmotic, ethanolic, reductive, and oxidative stress. Consistent with this, we reported that pre-treatment with glucosamine significantly increased O-GlcNAc levels and this was associated with increased tolerance to ischemia/reperfusion 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. Here we showed that increasing protein O-GlcNAc levels during reperfusion by 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 ischemia/reperfusion has been linked to increase in cytosolic Ca^{2+} (32). Here, as well as with pre-treatment 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. While large increases in cytosolic Ca^{2+} can lead to necrosis, more subtle increases in cytosolic Ca^{2+} can also have adverse effects mediated via activation of Ca^{2+}-activated enzymes such as CaMKII. In vivo studies have implicated that CaMKII in cardiac hypertrophy and in heart failure (20,21,31,44,45). CaMKII has also bee 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). Here we found that both glucosamine and PUGNAc significantly attenuated the ischemia-induced increase in phospho-to-total CaMKII ratio (Fig 4). While 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 non-specific proteolysis, since these changes were not observed in α-tublin (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-kD fragment of α-fodrin was significantly increased during I/R compared to normoxic perfusion. Furthermore, similar to the CaMKII results we found that both glucosamine and PUGNAc blocked the degradation of α-fodrin 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 ischemia/reperfusion-induced calcium-mediated stress responses such as calpain activation. It is noteworthy that Otani and colleagues (24,25) have reported that both GLUT4 and AMPK are also targets for calpain-mediated proteolysis. Thus, attenuation of calpain activation could also improved cardiac energy metabolism following ischemia/reperfusion; 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 capacitive calcium entry (CCE) (27) and that increased O-GlcNAc levels blocked angiotensin-II induced $[Ca^{2+}]_i$ increase in NRVMs (22). Others have also shown altered Ca^{2+} handling under conditions of increased O-GlcNAc levels (6,14). Thus, taken together, the decrease in CaMKII and α-fodrin proteolysis seen with glucosamine and PUGNAc are consistent with the notion that increasing levels of O-GlcNAc attenuates the increase in cytosolic Ca^{2+} that occurs during reperfusion. However, we cannot rule out other possible mechanisms; for example, it has been shown that increased levels of O-GlcNAc results in inhibition of proteasome activity (43). However, it should be noted that while 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 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 trauma-hemorrhage (37), which is supportive of the idea that these same strategies might protect against myocardial ischemic injury in vivo. It should also 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 here 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 indication 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., recently described a new transgenic mouse model in which calpain activation can be monitored in vivo using FRET imaging techniques (1).

In conclusion, we have shown here 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 HBP 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 α-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.

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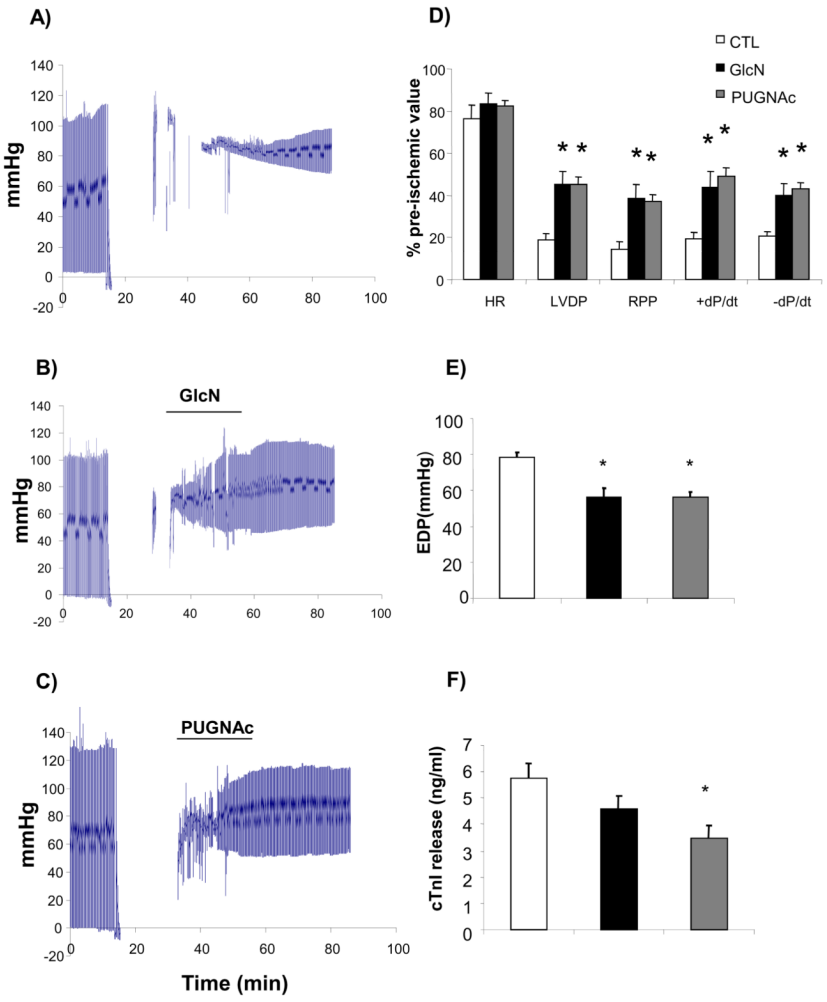

Figure 1.

Typical left ventricular pressure (LVP) tracing in hearts before and after 20 min global no flow ischemia in A) control B) glucosamine (10 mM) for first 20 min of reperfusion; C) PUGNAc (200 μM) for 20 min of reperfusion; D) Functional recovery of heart rate (HR), left ventricular developed pressure (LVDP), rate-pressure product (RPP) and positive and negative rates of pressure change (± dP/dt) following 20 minutes ischemia and 60 minutes reperfusion as a % of pre-ischemic values; E) End diastolic pressure (EDP) at the end of reperfusion; F) Total cardiac troponin I (cTnI) release during reperfusion in control (n=6), glucosamine (n=6) and PUGNAc (n=4) groups; *= $p<0.05$ compared to control group; one-way ANOVA with Bonfferoni's Comparison Test.

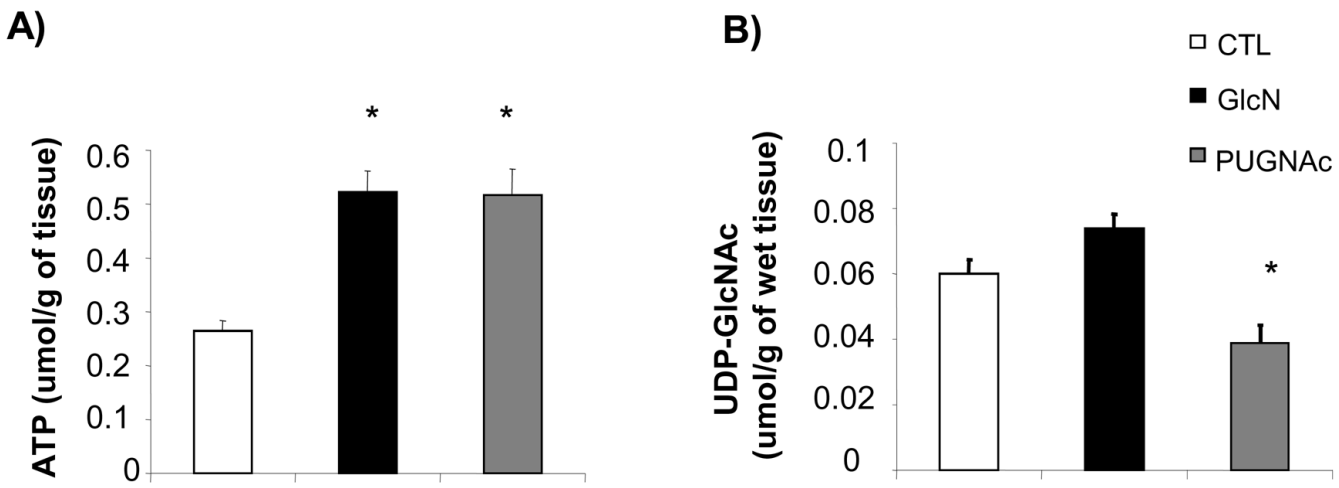

Figure 2.

A) ATP and B) UDP-GlcNAc levels at the end of reperfusion in control (n=6), glucosamine (n=6) and PUGNAc (n=4) groups; *= $p<0.05$ compared to I/R CTL group; one-way ANOVA with Bonfferoni's Multiple Comparison Test.

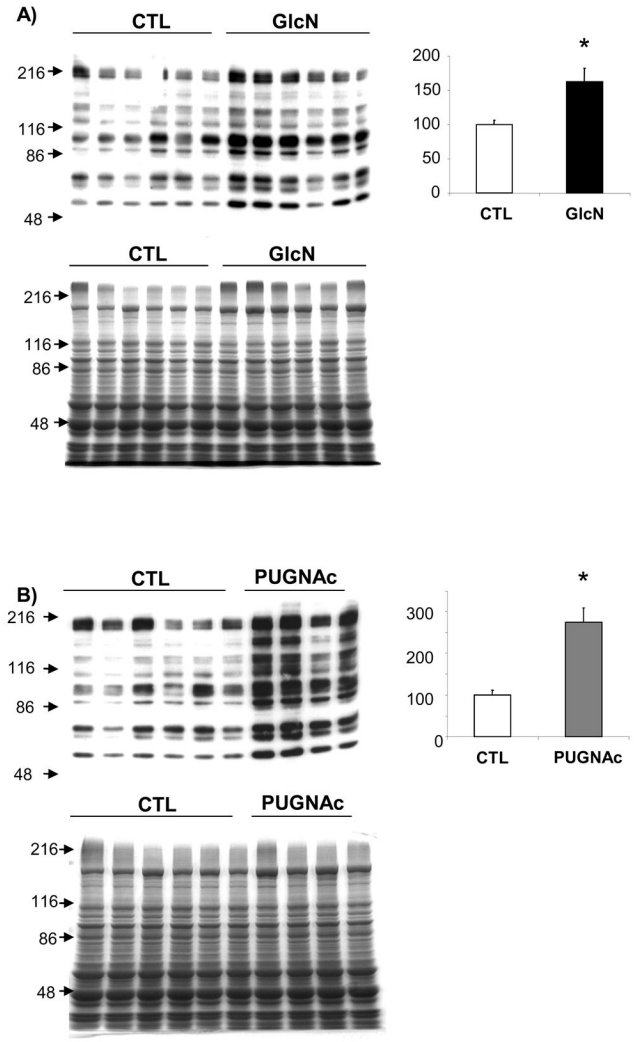

Figure 3.

Comparison of cardiac protein O-GlcNAc levels at the end of reperfusion in A) control (n=6) and glucosamine (n=6) treated groups and B) control (n=6) and PUGNAc (n=4) treated groups. In the upper panels are CTD110 immunoblots of solubilized proteins (left) and the mean intensities quantified by densitometric analysis of the immunoblots normalized to the mean intensities of control group (right). In the lower panels are the coomassie blue staining showing uniform protein loading of samples between groups. *= $p<0.05$ compared with control group; unpaired Students T-Test.

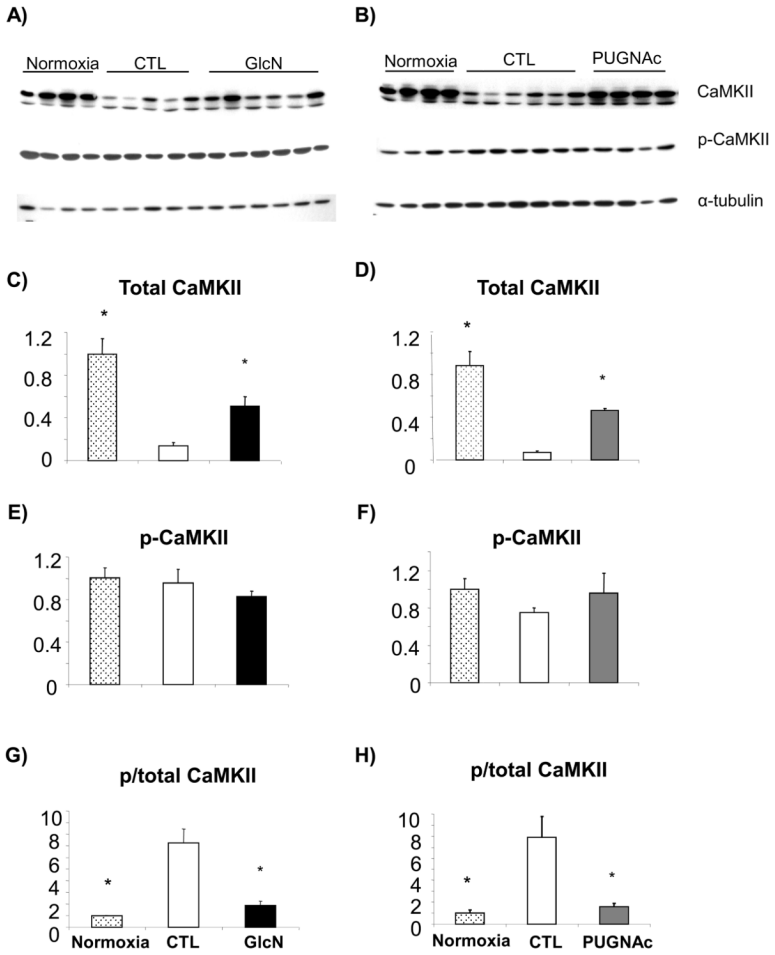

Figure 4.

Western blot analysis of total and phosphorylated CaMKII and α-tubulin as a protein loading control in A) normoxic perfused hearts (n=4); untreated control (CTL) (n=5) and glucosamine (GlcN) (n=6) treated groups after ischemia/reperfusion and B) normoxic perfused hearts (n=4); untreated control (CTL) and (n=6) and PUGNAc (n=4) treated groups after ischemia/reperfusion; C) and D) are the mean intensities of total CaMKII; E) and F) are the mean intensities of phosphorylated CaMKII quantified by densitometric analysis of the immunoblots normalized to α-tubulin and presented relative to normoxic control; G) and H) are the ratios of phosphorylated to total CaMKII. *= $p<0.05$ compared with I/R control group; one-way ANOVA with Bonfferoni's Multiple Comparison Test.

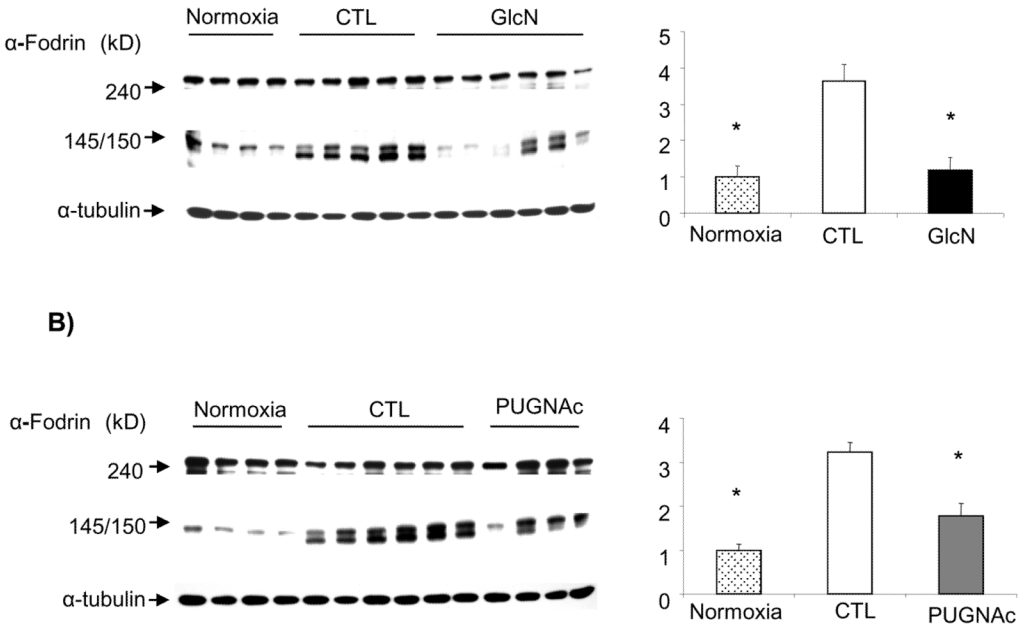

Figure 5.

Western blot analysis of α-fodrin with α-tubulin as a protein loading control in A) normoxic perfused hearts (n=4); untreated control (CTL) (n=5) and glucosamine (GlcN) (n=6) treated groups after ischemia/reperfusion and B) untreated control (CTL) and (n=6) and PUGNAc (n=4) treated groups after ischemia/reperfusion. On the left are α-fodrin immunoblots and on the right are the mean intensities quantified by densitometric analysis of the 145/150 kD fragment; data are normalized to the mean intensities of normoxic control group. *= $p<0.05$ compared with I/R control group; one-way ANOVA with Bonfferoni's Multiple Comparison Test.

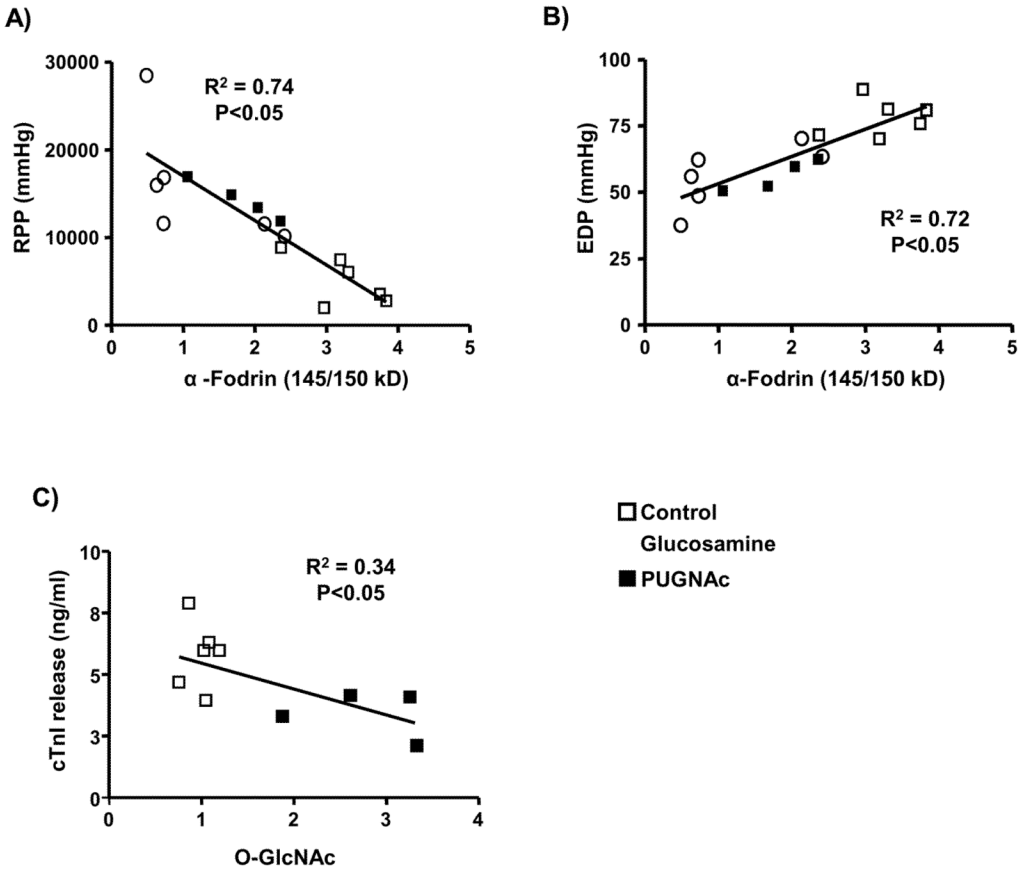

Figure 6.

Correlations between A) RPP and B) EDP and the 145/150 kDa α-fodrin fragment and between C) cTnI release and O-GlcNAc levels at the end of reperfusion. Data are presented for all control, glucosamine and PUGNAc groups.

Table 1

Cardiac baseline function prior to ischemia in control, untreated hearts; hearts treated with 10 mM glucosamine; heart treated with 200 μM PUGNAc groups. Data presented as Mean±SEM

	Heart rate	LVDP	RPP	+dP/dt	−dP/dt
Untreated (n=7)	303±12	117±6	35±1	3.8±0.3	2.4±0.1
Glucosamine (n=7)	331±19	125±5	41±2	4.2±0.4	2.6±0.1
PUGNAc (n=4)	319±18	120±4	38±2	3.8±0.2	2.4±0.1

LVDP = left ventricular developed pressure, mmHg; RPP = rate pressure product, mmHg/min × 10^{-3}; ±dP/dt = rate of pressure development and relaxation, mmHg/sec * 10^{-3}.