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Glutamine-induced protection of isolated rat heart from ischemia/reperfusion injury is mediated via the hexosamine biosynthesis pathway and increased protein O-GlcNAc levels

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Abstract

It has been shown that glutamine protects the heart from ischemia/reperfusion (I/R) injury; however, the mechanisms underlying this protection have not been identified. Glutamine: fructose-6-phosphate amidotransferase (GFAT) regulates the entry of glucose into the hexosamine biosynthesis pathway (HBP) and activation of this pathway has been shown to be cardioprotective. Glutamine is required for metabolism of glucose via GFAT; therefore, the goal of this study was to determine whether glutamine cardioprotection could be attributed to increased flux through the HBP and elevated levels of O-linked N-acetyl-glucosamine (O-GlcNAc) on proteins. Hearts from male rats were isolated and perfused with Krebs-Henseliet buffer containing 5mM glucose, and global, no-flow ischemia was induced for 20 minutes followed by 60 minutes of reperfusion. 30 minute pre-treatment with 2.5 mM glutamine significantly improved functional recovery (RPP: 15.6±5.7% Vs. 59.4±6.1%; p<0.05) and decreased cardiac Troponin I release (25.4±3.0 Vs. 4.7±1.9; p<0.05) during reperfusion. This protection was associated with a significant increase in the levels of protein O-GlcNAc and ATP. Pre-treatment with 80 μM azaserine, an inhibitor of GFAT, completely reversed the protection seen with glutamine and prevented the increase in protein O-GlcNAc. O-GlcNAc transferase (OGT) catalyzes the formation of O-GlcNAc, and inhibition of OGT with 5mM alloxan also reversed the protection associated with glutamine. These data support the hypothesis that in the ex vivo perfused heart glutamine cardioprotection is due, at least in part, to enhanced flux through the HBP and increased protein O-GlcNAc levels.

Keywords

Glutamine; hexosamine biosynthesis; protein O-GlcNAcylation; ischemia/reperfusion

INTRODUCTION

Despite significant advances in treatment, coronary heart disease still accounts for 1 in 5 deaths in the United States [1]; furthermore, deaths from congestive heart failure have also increased [2], in large part due to increased survival following myocardial infarction. Thus, despite improvements in the management of patients with coronary heart disease there is a growing need for the development of new treatments for use not only in response to an acute myocardial infarction, but also prior to surgery. Glutamine has been shown to preserve cardiomyocyte viability and enhance functional recovery from acute ischemia in isolated perfused rat heart [3,4]. In vivo administration of glutamine has also been shown to improve cardiac function

following ischemia/reperfusion [5]. However, the mechanisms underlying this protection have yet to be determined. Khogali et al., found that glutamine increased myocardial ATP/ADP and GSH/GSSG ratios following ischemia/reperfusion and suggested that the protection could be attributed to increased flux through the TCA cycle [3]. However, Wischmeyer et al., found that improved cardiomyocyte survival with glutamine treatment was associated with increased HSP72 expression [5].

Recently we reported that acutely increasing flux through the hexosamine biosynthesis pathway (HBP) with glucosamine significantly improved the tolerance of the isolated perfused heart to ischemia/reperfusion and appeared to be mediated by increased protein O-GlcNAc levels [6]. This was consistent with the report by Zachara et al., who showed that increased O-GlcNAc levels was an endogenous stress response and that augmentation of this response was cytoprotective [7]. Interestingly, glutamine:fructose-6-phosphate amidotransferase (GFAT) regulates the metabolism of glucose via the HBP and glutamine is the essential amino donor for the formation of glucosamine-6-phosphate, which is subsequently metabolized to UDP-N-acetylglucosamine (UDP-GlcNAc). Increasing glutamine concentrations dramatically increases UDP-GlcNAc, especially if glucose is also elevated [8]. Importantly, UDP-GlcNAc is the obligatory substrate for the transfer of O-GlcNAc to proteins by O-GlcNAc transferase (OGT) and flux through OGT is sensitive to changes in UDP-GlcNAc levels [9].

The importance of glutamine in regulating HBP flux combined with recent evidence demonstrating that increased HBP flux is cardioprotective, led us to postulate that the ischemic protection seen with glutamine was mediated via the same pathway. We report here that in the isolated rat heart, ischemic protection afforded by glutamine treatment was associated with a significant increase in the levels of UDP-GlcNAc and protein O-GlcNAc. Furthermore, inhibition of either GFAT or OGT blocked the glutamine-induced increase in O-GlcNAc and reversed the cardioprotection. These data provide further evidence supporting the role of the HBP and O-GlcNAc in mediating cardioprotection and provide new insights into the protective mechanisms associated with glutamine treatment.

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 250–300g were used throughout.

Isolated heart perfusion

Hearts were isolated and perfused as previously described [6,10]. Briefly, rats were anesthetized (Ketamine 100mg/kg i.p.), decapitated and hearts 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 mmol/l): NaCl, 118; KCl, 4.8; MgSO₄, 1.2; CaCl₂, 1.25; KH₂PO₄, 1.2; NaHCO₃, 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, stored in –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 hemodynamics 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

After 15 min equilibration, hearts were randomly distributed to 4 experimental groups 1) control (n=7), 2) glutamine, 2.5 mM added 30 min before the start of ischemia (n=7), 3) glutamine plus azaserine, an inhibitor of GFAT, 80 μ M (n=7); azaserine was added 15 min prior to addition of glutamine and was present throughout the experiment, and 4) glutamine plus alloxan, an inhibitor of OGT, 5 mM (n=4); alloxan was added 15 min prior to addition of glutamine and was present throughout the experiment. Previous studies have shown that the maximum protective effect of glutamine was observed at 2.5mM [3]. The concentration of azaserine used here was chosen based on preliminary experiments to determine the minimum concentration required to reverse the protection seen with glutamine. We have previously shown that 5mM alloxan blocks the protection associated with increased O-GlcNAc levels resulting from glucosamine treatment [6].

To determine whether azaserine or alloxan alone had adverse effects on the isolated perfused hearts, two additional series of experiments were performed. In one series hearts were perfused under normoxic conditions in the absence or presence of azaserine (80 μ M) or alloxan (5mM) for the same duration as the ischemia/reperfusion experiments. In another series of experiments hearts were subject to ischemia/reperfusion protocol with either azaserine (80 μ M) or alloxan (5mM) in the absence of glutamine.

To determine whether the effect of azaserine was specific for glutamine-mediated protection, an additional series of experiments were performed to assess the effect of azaserine on the ischemic protection observed with glucosamine [6], which also increases O-GlcNAc levels, but bypasses GFAT.

Assessment of O-GlcNAc levels

Protein O-GlcNAc levels were determined as previously described [6] via immunoblotting with the anti-O-GlcNAc antibody CTD110.6 [11] (a kind gift from Mary Ann Accavitti, UAB). Briefly, 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 μ M PUGNAc (inhibitor of O-GlcNAcase). 50–100 μ g solubilized protein was suspended in laemmli sample buffer, and then separated by 7.5% SDS-PAGE and transferred to a polyvinylidenedifluoride (PVDF) memberane. Protein loading was assessed via Sypro ruby blot staining or coomassie blue staining as previously described [6].

Blots were incubated with a 1:5000 dilution of CTD110.6 in 1% casein, phosphate-buffered saline (Pierce) with 0.01% Tween 20 overnight at 4°C and then washed three times in phosphate-buffered saline. The membranes were then incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgM (calbiochem) in 1% casein, phosphate-buffered saline with 0.01% Tween 20 for 1 h at room temperature. After further washing in phosphate-buffered saline the immunoblots were developed with enhanced chemiluminescence (Pierce) 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 UDP-GlcNAc and ATP levels

UDP-GlcNAc and ATP levels were determined by HPLC analysis of perchloric acid extract tissue samples as previously described [6,10]. Briefly, neutralized acid extracts were loaded onto a SAX Partisil 10 anion-exchange column (250 X 4.6 mm) eluted with gradient of ammonium dihydrogen phosphate from 15 (PH 2.8) to 1 mmol/L (PH3.7). The concentrations of ATP and uridine diphosphate-N-acetylhexosamine (UDP-HexNAc) were determined by ultraviolet detection after calibration with appropriate standards. This method is unable to separate uridine diphosphate-N-acetyl-glucosamine (UDP-GlcNAc) and uridine diphosphate-N-acetyl-galactosamine (UDP-GalNAc); therefore all results are presented as the sum of UDP-GlcNAc plus UDP-GalNAc (i.e. UDP-HexNAc)[12]. However, in the heart the ratio of UDP-GlcNAc to UDP-GalNAc is approximately 3:1 [13].

Measurement of cardiac troponin I release in effluent

As a marker of tissue injury, cardiac troponin I (cTnI) concentration was determined in coronary effluent collected 5 min prior to ischemia and pooled coronary effluent at 5, 30 and 60 minutes of reperfusion using ELISA (cardiac troponin I ELISA kit, Life Diagnostics, Inc.).

Data analysis

All data are presented as means $\pm$ SE. Statistical analysis was performed by either an unpaired t-tests or one-way analysis of variance (ANOVA) followed by Dunnett'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

Inhibition of HBP and OGT reverses protection associated with glutamine treatment

Cardiac function prior to ischemia in all four groups is summarized in Table 1. Although LVDP was significantly lower in the glutamine+azaserine group compared to untreated controls, heart rate, RPP and $\pm$ dP/dt were not significantly different between any of the groups. In time-control normoxic perfusions, there were no significant differences in any functional parameters after 105 min perfusion with either azaserine or alloxan compared to untreated control hearts (Table 2). Although RPP in both alloxan and azaserine groups was slightly depressed, this did not reach statistical significance (ANOVA $p = 0.053$). Over the time course of perfusion the decrease in RPP was $14 \pm 3\%$ in the azaserine group, $3 \pm 3\%$ in the alloxan group and $9 \pm 1\%$ in the untreated control group (NS). Thus, the data in Tables 1 and 2 suggest that both azaserine and alloxan may have a small negative inotropic effect. However, the fact that under normoxic perfusion conditions neither drug resulted in a decline in function over time that was significantly greater than that seen in untreated controls, suggests in these experiments, azaserine and alloxan do not adversely affect the heart.

Thirty min pre-treatment with 2.5 mM glutamine significantly improved functional recovery following ischemia/reperfusion (Fig. 1A,B). Pretreatment of hearts 15 min before glutamine with 80 μ M azaserine, an amidotransferase inhibitor that we have previously shown decreases flux through the HBP in perfused heart [10], significantly attenuated the protection associated with glutamine (Fig. 1A,B). Similarly 5mM alloxan, a putative inhibitor of OGT [14], also completely blocked the protective effects seen with glutamine following ischemia/reperfusion (Fig. 1A,B). There was no significant effect of any treatment on the development of contracture during ischemia as indicated by EDP at the end of ischemia (Fig 1C) or on time of onset of contracture, time to peak contracture or peak contracture (data not shown). However, at the end of reperfusion EDP was significantly lower in the glutamine treated group compared to control and this was reversed by both azaserine and alloxan (Fig 1D).

As an index of the degree of tissue injury we measured cTnI levels in the pooled samples of the coronary effluent at 5, 30 and 60 min of reperfusion by ELISA. Consistent with a reduction in tissue injury, glutamine reduced cTnI release compared to controls and this was reversed by azaserine (Fig 1E). The addition of alloxan increased cTnI release more than 5-fold compared to glutamine; however, this did not reach statistical significance, possibly due to the smaller sample size in this group.

The effect of glutamine on UDP-GlcNAc, O-GlcNAc and ATP levels

At the end of reperfusion UDP-GlcNAc levels were increased by ~35% in the glutamine group compared to untreated controls (Fig 2A); however, this did not reach statistical significance ($P=0.077$, ANOVA). It should be noted that, given the experimental design, this study is under powered to conclude that there is no effect of glutamine on UDP-GlcNAc levels.

At the end of reperfusion, ATP levels were markedly depressed in all groups compared to normoxic control levels ($2.6 \pm 0.1 \mu\text{mol}/\text{min}/\text{g}$ wet wt). Glutamine treatment increased ATP levels approximately 3-fold compared to untreated control group and both azaserine and alloxan reversed this effect (Fig. 2B). Under normoxic, time-control perfusion conditions azaserine and alloxan had no effect on ATP levels (data not shown). In a separate series of studies we found that the addition of up to 5mM glutamine did not change cardiac ATP levels during normoxic perfusion (unpublished data).

At the end of reperfusion O-GlcNAc levels were increased almost 40% in the glutamine group compared to controls (Fig 3A). The addition of azaserine attenuated the O-GlcNAc levels seen with glutamine treatment by ~30% (Fig 3B) and alloxan by ~50% (Fig 3C).

Azaserine and alloxan alone do not exacerbate ischemic injury or reduce O-GlcNAc levels

To determine whether azaserine or alloxan alone had any adverse effects on the response to ischemia, hearts perfused with the same concentrations of azaserine or alloxan as above but in the absence of glutamine were subjected to the same ischemia/reperfusion protocol. As shown in Fig 4A neither azaserine nor alloxan alone exacerbated functional recovery compared to untreated control hearts. There was also no effect of either drug alone on O-GlcNAc levels either following ischemia/reperfusion (Fig 4B) or in normoxic time-control perfusions (Fig 4C). During normoxic perfusion up to 5mM glutamine had no effect on O-GlcNAc levels in the heart (data not shown).

Azaserine does not block glucosamine protection

To determine whether azaserine has an adverse effect on the hexosamine biosynthesis pathway, independent of inhibition of GFAT we assessed whether azaserine treatment attenuated protection associated with glucosamine treatment. We have previously shown that glucosamine, which is metabolized directly to glucosamine-6-phosphate bypassing GFAT, protects hearts from ischemia/reperfusion and that this protection was also due at least in part increased flux through the HBP and protein O-GlcNAc [6]. Consistent with our previous report, glucosamine treatment significantly improved cardiac recovery following ischemia/reperfusion (Fig 5). Azaserine treatment did not attenuate the protection seen with glucosamine.

DISCUSSION

Increasing glutamine levels above the normal plasma level of 0.4 mM has been shown to enhance recovery from a variety of experimental and clinical challenges including burns, sepsis, trauma, hyperthermia and myocardial ischemia [3–5,15–17]. However, the mechanism underlying the protection afforded by glutamine is unclear. We demonstrate here for the first time that ischemic protection resulting from glutamine treatment is mediated, at least in part,

via increased flux through the HBP resulting in elevated levels of O-GlcNAc on nucleocytoplasmic proteins. These results are consistent with recent reports by us [6,18] and others [7,19] demonstrating that increased O-GlcNAc levels are protective against a wide range of stress stimuli, including ischemia/reperfusion.

Khogali and colleagues showed that 2.5mM glutamine elicited maximal protection in response to 20 min low flow ischemia (~12% of baseline flow) and reperfusion, which was associated with improved bioenergetic status and reduced lactate accumulation and attenuation of glutamate depletion [3]. They proposed that the effect of glutamine was mediated via maintenance of TCA cycle activity due to conversion of glutamine to glutamate and subsequent formation of α -ketoglutarate. Subsequently, they reported that glutamine treatment increased both the ATP/ADP and GSH/GSSG ratios and suggested that the enhanced glutathione redox state may also contribute to the cardioprotective effect of glutamine [4]. Alternatively, Wischemeyer et al., showed that that glutamine mediated cellular protection is associated with enhanced expression of heat shock protein-70 (HSP-70) mediated via heat shock transcription factor-1 [17,20]. Also, Evans et al., reported that glutamine inhibited cytokine-induced apoptosis via the pyrimidine synthesis pathway [21].

In addition to its metabolism to glutamate, glutamine is also essential for the activity of GFAT, which regulates the metabolism of glucose via the HBP and is responsible for the conversion of fructose-6-phosphate to glucosamine-6-phosphate. We have recently demonstrated that glucosamine, which is converted to glucosamine-6-phosphate via hexokinase, thus bypassing GFAT and increasing HBP flux, provided significant protection against ischemia/reperfusion injury in the isolated perfused rat heart [6]. One consequence of glucosamine treatment was an increase in protein O-GlcNAc levels in the heart and treatment with alloxan, an inhibitor of OGT, blocked both the increase in O-GlcNAc and the protection [6]. These results were consistent with the report by Zachara et al, who proposed that activation of pathways leading to increased levels of O-GlcNAc was an endogenous stress-activated response and that augmentation of this response improved cell survival [7]. Here we show that, similar to glucosamine, glutamine treatment significantly improved functional recovery and decreased tissue injury, and this was associated with an increase in O-GlcNAc levels. This protection was reversed by both inhibition of GFAT with azaserine and of OGT with alloxan, and in both cases this also blocked the increase in O-GlcNAc. These data support the notion that the protection afforded by glutamine treatment is mediated via increased flux through the HBP and OGT.

Both azaserine and alloxan had a small negative inotropic effect (Tables 1 and 2), however, in time-control, normoxic perfusion experiments neither treatment caused a decline in function that was significantly greater than that seen in untreated control hearts. Furthermore, in the absence of glutamine, neither treatment exacerbated ischemic injury compared to untreated controls (Fig 4A). There was also no effect of azaserine or alloxan alone on O-GlcNAc levels, either following ischemia/reperfusion or under normoxic conditions (Fig. 4B,C). These data suggest, therefore, that their effects on blocking the protection seen with glutamine and the attenuation of the increase in O-GlcNAc seen with glutamine is unlikely to be a consequence of non-specific toxicity. The fact that azaserine did not block the protection associated with glucosamine also suggests that its abrogation of glutamine-induced protection is not a consequence of effects independent of its inhibition of GFAT. However, it should be noted that while alloxan has been shown to inhibit OGT [14], it is reported to have other potential cytotoxic effects including oxidation of free thiol groups [22]. Unfortunately, alloxan is the only known inhibitor of OGT [14] and since ablation of the OGT gene is embryonically lethal [23], more specific demonstration of the role of OGT in mediating cardioprotection require the development of either tissue-specific, conditional knockout mice or more specific high affinity

inhibitors of OGT. Cardiac specific alterations of GFAT expression would also be valuable in helping to elucidate the role of this pathway in mediating cardioprotection.

The specific mechanisms underlying the cardioprotection associated with glutamine-induced activation of the HBP remain to be elucidated. However, because similar to glucosamine treatment [6], protection with glutamine was conferred relatively rapidly, it is unlikely to be due to *de novo* protein synthesis and more likely a direct consequence of post-translational modification of proteins with *O*-GlcNAc. Although, specific proteins involved in mediating this protection have yet to be identified, increased HBP flux and *O*-GlcNAcylation have been reported to modulate the insulin-signaling pathway, MAPK pathway, and PKC [24–27], each of which pathways has been implicated in mediating ischemic cardioprotection [28]. Alternatively, protection could also be due in part to decreased Ca^{2+} influx on reperfusion, which would be consistent with improved recovery of EDP and with the fact that increased levels of *O*-GlcNAc attenuated agonist induced increase in cytosolic Ca^{2+} in cardiomyocytes [29]. Others have also shown altered Ca^{2+} handling under conditions of increased *O*-GlcNAc levels [13,30].

As mentioned above due to the relatively short time course of these experiments, the protection is unlikely to be a consequence of increase HSP-70 expression as reported by Morrison et al., [20]. However, the fact that increased HSP-70 expression mediated via HSF-1 have been implicated in cytoprotection seen with both glutamine treatment [20] and increased *O*-GlcNAc levels [7], raises the possibility that the longer term protection seen with glutamine may [5, 15–17] also be mediated via the same pathways. Thus, therapeutic strategies designed to increase *O*-GlcNAc levels in the heart may afford protection both over the short term by post-translational modification of specific proteins as well over the long term by increased expression of proteins such as HSP-70.

It is important to note that these experiments were performed in an ex vivo isolated heart preparation; consequently, these observations cannot be readily extrapolated to the in vivo environment. Furthermore, we examined only a single period of 20 min ischemia and while this insult lead to only 20% recovery of function on reperfusion in untreated control hearts and significant cTnI release, it is possible that protection seen here with glutamine might not be observed following a more severe injury. Furthermore, the duration of reperfusion was only 60 min and while this is fairly common in ex vivo studies, the evolution of irreversible tissue injury following ischemia/reperfusion in vivo typically takes several hours. Nevertheless, Wischemeyer et al., have shown that that glutamine mediated cellular protection does occur in vivo [17,20] and we have shown that increasing *O*-GlcNAc levels in vivo with glucosamine is associated with improved cardiac function following trauma-hemorrhage and resuscitation [31].

In conclusion, we have shown that in the isolated perfused heart ischemic protection afforded by pre-treatment with glutamine is mediated, at least in part, by increased flux through the HBP leading to increased *O*-GlcNAc levels on nucleocytoplasmic proteins. These results provide further support for the notion that pathways leading to the formation of *O*-GlcNAc are endogenous stress activated pathways and that augmentation of this response may represent a novel strategy for inducing cardioprotection.

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References

1. Theroux P, Willerson JT, Armstrong PW. Progress in the treatment of acute coronary syndromes: a 50-year perspective (1950–2000). *Circulation* 2000;102:IV2–IV13. [PubMed: 11080126]
2. Heart Disease and Stroke Statistics: 2005 Update. American Heart Association; 2005.
3. Khogali SE, Harper AA, Lyall JA, Rennie MJ. Effects of L-glutamine on post-ischaemic cardiac function: protection and rescue. *J Mol Cell Cardiol* 1998;30:819–827. [PubMed: 9602431]
4. Khogali SE, Pringle SD, Weryk BV, Rennie MJ. Is glutamine beneficial in ischemic heart disease? *Nutrition* 2002;18:123–126. [PubMed: 11844641]
5. Wischmeyer PE, Jayakar D, Williams U, Singleton KD, Riehm J, Bacha EA, Jeevanandam V, Christians U, Serkova N. Single dose of glutamine enhances myocardial tissue metabolism, glutathione content, and improves myocardial function after ischemia-reperfusion injury. *JPEN J Parenter Enteral Nutr* 2003;27:396–403. [PubMed: 14621120]
6. Liu J, Pang Y, Chang T, Bounelis P, Chatham JC, Marchase RB. Increased hexosamine biosynthesis and protein O-GlcNAc levels associated with myocardial protection against calcium paradox and ischemia. *J Mol Cell Cardiol* 2006;40:303–312. [PubMed: 16337959]
7. Zachara NE, O'Donnell N, Cheung WD, Mercer JJ, Marth JD, Hart GW. Dynamic O-GlcNAc modification of nucleocytoplasmic proteins in response to stress. A survival response of mammalian cells. *J Biol Chem* 2004;279:30133–30142. [PubMed: 15138254]
8. Wu G, Haynes TE, Li H, Yan W, Meininger CJ. Glutamine metabolism to glucosamine is necessary for glutamine inhibition of endothelial nitric oxide synthesis. *Biochem J* 2001;353:245–252. [PubMed: 11139387]
9. Love DC, Hanover JA. The hexosamine signaling pathway: deciphering the "O-GlcNAc code". *Sci STKE* 2005;2005:re13. [PubMed: 16317114]
10. Pang Y, Bounelis P, Chatham JC, Marchase RB. The hexosamine pathway is responsible for the inhibition by diabetes of phenylephrine-induced inotropy. *Diabetes* 2004;53:1074–1081. [PubMed: 15047624]
11. Comer FI, Vosseller K, Wells L, Accavitti MA, Hart GW. Characterization of a mouse monoclonal antibody specific for O-linked N-acetylglucosamine. *Anal Biochem* 2001;293:169–77. [PubMed: 11399029]
12. Robinson KA, Weinstein ML, Lindenmayer GE, Buse MG. Effects of diabetes and hyperglycemia on the hexosamine synthesis pathway in rat muscle and liver. *Diabetes* 1995;44:1438–46. [PubMed: 7589852]
13. Clark RJ, McDonough PM, Swanson E, Trost SU, Suzuki M, Fukuda M, Dillmann WH. Diabetes and the accompanying hyperglycemia impairs cardiomyocyte cycling through increased nuclear O-GlcNAcylation. *J Biol Chem* 2003;278:44230–44237. [PubMed: 12941958]
14. Konrad RJ, Zhang F, Hale JE, Knierman MD, Becker GW, Kudlow JE. Alloxan is an inhibitor of the enzyme O-linked N-acetylglucosamine transferase. *Biochem Biophys Res Commun* 2002;293:207–212. [PubMed: 12054585]
15. Wischmeyer PE. Clinical applications of L-glutamine: past, present, and future. *Nutr Clin Pract* 2003;18:377–85. [PubMed: 16215069]
16. Wischmeyer PE, Vanden Hoek TL, Li C, Shao Z, Ren H, Riehm J, Becker LB. Glutamine preserves cardiomyocyte viability and enhances recovery of contractile function after ischemia-reperfusion injury. *JPEN J Parenter Enteral Nutr* 2003;27:116–22. [PubMed: 12665167]
17. Singleton KD, Wischmeyer PE. Oral glutamine enhances heat shock protein expression and improves survival following hyperthermia. *Shock* 2006;25:295–9. [PubMed: 16552363]
18. Yang S, Zou L, Bounelis PIC, Chatham JC, Marchase RB. Glucosamine administration during resuscitation improves organ function following trauma-hemorrhage. *Shock*. 2006In Press.
19. Sohn KC, Lee KY, Park JE, Do SI. OGT functions as a catalytic chaperone under heat stress response: a unique defense role of OGT in hyperthermia. *Biochem Biophys Res Commun* 2004;322:1045–1051. [PubMed: 15336570]
20. Morrison AL, Singleton KD, Dinges M, Odoms K, Wong HR, Wischmeyer PE. Glutamine's Protection Against Cellular Injury is Dependent on Heat Shock Factor-1. *Am J Physiol Cell Physiol*. 2006

21. Evans ME, Jones DP, Ziegler TR. Glutamine inhibits cytokine-induced apoptosis in human colonic epithelial cells via the pyrimidine pathway. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G388–96. [PubMed: 15878985]
22. Tiedge M, Richter T, Lenzen S. Importance of cysteine residues for the stability and catalytic activity of human pancreatic beta cell glucokinase. *Arch Biochem Biophys* 2000;375:251–60. [PubMed: 10700381]
23. Shafi R, Iyer SP, Ellies LG, O'Donnell N, Marek KW, Chui D, Hart GW, Marth JD. The O-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. *Proc Natl Acad Sci U S A* 2000;97:5735–9. [PubMed: 10801981]
24. Wells L, Vosseller K, Hart GW. Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc. *Science* 2001;291:2376–2378. [PubMed: 11269319]
25. Hanover JA. Glycan-dependent signaling: O-linked N-acetylglucosamine. *FASEB J* 2001;15:1865–1876. [PubMed: 11532966]
26. Whelan SA, Hart GW. Proteomic approaches to analyze the dynamic relationships between nucleocytoplasmic protein glycosylation and phosphorylation. *Circ Res* 2003;93:1047–1058. [PubMed: 14645135]
27. Vosseller K, Sakabe K, Wells L, Hart GW. Diverse regulation of protein function by O-GlcNAc: a nuclear and cytoplasmic carbohydrate post-translational modification. *Curr Opin Chem Biol* 2002;6:851–857. [PubMed: 12470741]
28. Murphy E. Primary and secondary signaling pathways in early preconditioning that converge on the mitochondria to produce cardioprotection. *Circ Res* 2004;94:7–16. [PubMed: 14715531]
29. Nagy T, Champattanachai V, Marchase RB, Chatham JC. Glucosamine inhibits angiotensin II induced cytoplasmic Ca²⁺ elevation in neonatal cardiomyocytes via protein-associated O-GlcNAc. *Am J Physiol Cell Physiol* 2006;290:C57–65. [PubMed: 16107505]
30. Hu Y, Belke D, Suarez J, Swanson E, Clark R, Hoshijima M, Dillmann WH. Adenovirus-mediated overexpression of O-GlcNAcase improves contractile function in the diabetic heart. *Circ Res* 2005;96:1006–1013. [PubMed: 15817886]
31. Yang S, Zou LY, Bounelis P, Chaudry I, Chatham JC, Marchase RB. Glucosamine administration during resuscitation improves organ function after trauma hemorrhage. *Shock* 2006;25:600–607. [PubMed: 16721268]

Figure 1.

A) Typical left ventricular pressure (LVP) in hearts before and after 20 min global zero flow ischemia in control, untreated heart; heart treated with 2.5 mM glutamine 30 min prior to ischemia; heart treated with 80 μ M azaserine 15 minutes before glutamine treatment; and heart treated with 5 mM alloxan 15 minutes before glutamine treatment; B) Functional recovery of heart rate (HR), left ventricular developed pressure (LVDP), rate-pressure product (RPP) and positive and negative rates of pressure change ($\pm$ dP/dt) following 20 minutes ischemia and 60 minutes reperfusion as a % of pre-ischemic values; C) End diastolic pressure (EDP) at the end of ischemia; D) EDP at the end of reperfusion; E) Total cardiac troponin I (cTnI) release during reperfusion in control hearts (n=7), hearts perfused with 2.5 mM glutamine (n=7), 2.5 mM

glutamine + 80 μ M azaserine (n=7), and 2.5 mM glutamine + 5 mM alloxan (n=4). * = $p < 0.05$ compared to glutamine group; ANOVA with Dunnett's Multiple Comparison Test.

Figure 2.

A) UDP-GlcNAc levels and B) ATP levels at the end of reperfusion in control hearts (n=7), hearts perfused with 2.5 mM glutamine (n=7), 2.5 mM glutamine + 80 μM azaserine (n=7), and 2.5 mM glutamine + 5 mM alloxan (n=4). * = $p < 0.05$ compared to glutamine group; ANOVA with Dunnett's Multiple Comparison Test.

Figure 3.

Comparison of protein O-GlcNAc levels at the end of reperfusion in A) control hearts (n=7) and hearts perfused with 2.5 mM glutamine (n=7); B) hearts perfused with 2.5 mM glutamine and 2.5 mM glutamine + 80 μ M azaserine (n=7), C) hearts perfused with 2.5 mM glutamine and 2.5 mM glutamine + 5 mM alloxan (n=4). On the left are CTD110 immunoblots of solubilized proteins from left ventricles of hearts and on the right are the mean intensities quantified by densitometric analysis of the immunoblots; in A) data are normalized to the mean intensities of control group in C) and D) data are normalized to the mean intensities of glutamine group; *= p<0.05, (unpaired Students T-Test).

Figure 4.

A) Functional recovery of heart rate (HR), left ventricular developed pressure (LVDP), rate-pressure product (RPP) and positive and negative rates of pressure change ($\pm$ dP/dt) following 20 minutes ischemia and 60 minutes reperfusion as a % of pre-ischemic values in untreated control hearts; hearts treated with 80 μ M azaserine (n= 4) or 5mM alloxan (n=4). Note that the control data are the same as that shown in Fig 1 and are shown here for comparison purposes only; B) Comparison of protein O-GlcNAc levels at the end of reperfusion in control hearts (n=7) and hearts treated with 80 μ M azaserine (n= 4) or 5mM alloxan (n=4); C) Comparison of protein O-GlcNAc levels following time-control normoxic perfusions in untreated control hearts (n=5) and hearts treated with 80 μ M azaserine (n= 4) or 5mM alloxan (n=4).

Figure 5.

A) Functional recovery of heart rate (HR), left ventricular developed pressure (LVDP), rate-pressure product (RPP) and positive and negative rates of pressure change ($\pm$ dP/dt) following 20 minutes ischemia and 60 minutes reperfusion as a % of pre-ischemic values in control hearts (CTL) (n=4), hearts perfused with 10 mM glucosamine (n=5), and hearts perfused with 10 mM glucosamine + 80 μ M azaserine (n=4). B) End diastolic pressure (EDP) at the end of reperfusion. # = $p < 0.05$ compared to control; ANOVA with Dunnett's Multiple Comparison Test.

Table 1

Cardiac function immediately prior to ischemia in control, untreated hearts; heart treated with 2.5 mM glutamine for 30 min; heart treated with 80 μ M azaserine 15 minutes before glutamine treatment; and heart treated with 5 mM alloxan 15 minutes before glutamine treatment baseline. Data presented as Mean $\pm$ SEM.

	HR	LVDP	RPP	-dP/dt	+dP/dt
Control (n=7)	269 $\pm$ 21	109 $\pm$ 6	29.0 $\pm$ 1.2	4.3 $\pm$ 0.2	2.3 $\pm$ 0.1
Glutamine (n=7)	284 $\pm$ 15	94 $\pm$ 2	26.9 $\pm$ 1.9	4.1 $\pm$ 0.3	2.1 $\pm$ 0.1
Glutamine + Azaserine (n=7)	274 $\pm$ 13	88 $\pm$ 5*	24.2 $\pm$ 2.0	3.2 $\pm$ 0.3	1.8 $\pm$ 0.1
Glutamine + Alloxan (n=4)	262 $\pm$ 10	94 $\pm$ 6	24.5 $\pm$ 2.2	3.8 $\pm$ 0.4	1.9 $\pm$ 0.2

HR = heart rate; LVDP = left ventricular developed pressure, mmHg; RPP = rate pressure product, mmHg/min $\times 10^{-3}$; $\pm$ dP/dt = rate of pressure development and relaxation, mmHg/sec $\times 10^{-3}$.

* $p < 0.05$ vs control; ANOVA with Dunnett's Multiple Comparison Test.

Table 2

Cardiac function after 120 min of normoxic perfusion in control, untreated hearts and hearts perfused with 80 μ M azaserine or 5 mM alloxan. Azaserine and alloxan were added after 15 min perfusion and were present for the remaining 105 min. Data presented as Mean $\pm$ SEM.

	HR	LVDP	RPP	- dP/dt	+dP/dt
Control (n=5)	314 $\pm$ 16	97 $\pm$ 4	30.2 $\pm$ 1.1	3.5 $\pm$ 0.2	2.0 $\pm$ 0.1
Azaserine (n=4)	282 $\pm$ 10	85 $\pm$ 6	24.2 $\pm$ 3.0	3.5 $\pm$ 0.2	1.7 $\pm$ 0.2
Alloxan (n=4)	273 $\pm$ 6	90 $\pm$ 2	24.7 $\pm$ 1.0	3.3 $\pm$ 0.4	1.7 $\pm$ 0.2

HR = heart rate; LVDP = left ventricular developed pressure, mmHg; RPP = rate pressure product, mmHg/min $\times 10^{-3}$; $\pm$ dP/dt = rate of pressure development and relaxation, mmHg/sec $\times 10^{-3}$.

* $p < 0.05$ vs control; ANOVA with Dunnett's Multiple Comparison Test.