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Effects Of Hexosamine Biosynthesis On An In Vitro Model Of Cardiacischemia

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EFFECTS OF HEXOSAMINE BIOSYNTHESIS ON AN IN VITRO MODEL OF CARDIAC ISCHEMIA

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ABSTRACT

Increased levels of protein *O*-linked-N-acetylglucosamine (*O*-GlcNAc) have been correlated with increased cell survival following stress. Therefore the goal of this study was to determine whether enhanced flux through the hexosamine biosynthesis pathway (HBP), which leads to elevated levels of *O*-GlcNAc, improved the tolerance of isolated neonatal rat ventricular myocytes (NRVMs) to ischemia/reperfusion (I/R) injury. NRVMs were exposed to hypoxic medium for 4 hours, followed by oxygenated complete medium for 16 hours. Acute treatment with 5 mM glucosamine showed an increase in viability and decreased both necrosis and apoptosis following I/R. Glucosamine increased *O*-GlcNAc levels maximally at 2 hours and went down at 16 hours of reperfusion. Pretreatment with 30 mM glucose promoted survival and decreased apoptosis following I/R while pretreatment with azaserine, an inhibitor of GFAT, which regulates glucose entry into HBP, abrogated this protection. In addition, reperfusion in the absence of glucose reduced *O*-GlcNAc levels and resulted in lower viability. The improved viability seen with glucosamine treatment was also associated with attenuation of ischemia-induced NFAT activation; suggesting that increased *O*-GlcNAc levels may reduce cellular Ca^{2+} during ischemia and reperfusion. Overexpression of *O*-GlcNAc transferase (OGT) increased *O*-GlcNAc levels and survival whereas blocking *O*-GlcNAc

modification by OGT knockdown or pretreatment with alloxan, an inhibitor of OGT exacerbated cellular injury following I/R. Pretreatment with PUGNAc or NButGT, inhibitors of *O*-GlcNAcase was less protective against I/R, despite a 10 fold greater increase in overall *O*-GlcNAc levels. Interestingly, the protective effect of glucosamine was correlated with increasing levels of mitochondrial Bcl-2, protecting the loss of cytochrome C, which was also associated with attenuation of H_2O_2-induced loss of mitochondrial membrane potential. Therefore, the results from this study indicate at least two potential mechanisms contributing to the protection associated with elevated *O*-GlcNAc: 1) attenuation of cellular calcium and 2) accumulation of mitochondrial Bcl-2, consequently protecting in mitochondria function which in final reduced cardiomyocyte necrosis and apoptosis. Taken together, these data support the notion that elevating *O*-GlcNAc levels by increasing flux through OGT protects against ischemia-reperfusion injury in cardiomyocytes, suggesting that strategies designed to activate these pathways may represent novel interventions for inducing cardioprotection.

DEDICATION

I would like dedicate this dissertation to my mother, Ladda Champattanachai, who passed away while I was 11 years old and my father, Sompong Champattanachai, who has taken care of me ever since.

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LIST OF ABBREVIATION

AngII	angiotensin II
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	cytoplasmic free calcium
Ca^{2+} ATPase	xalcium dependent adenosine triphosphatases
CaMK	calcium/calmodulin dependent protein kinase
CCE	capacitative calcium entry
cTnI	cardiac troponin I
DON	6-diaz-*O*-norleucine
ER	endoplasmic reticulum
FBP	fructose 1,6 bisphosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAT	glutamine: fructose-6-phosphate amidotransferase
GlcN	glucosamine
HBP	hexosamine biosynthesis pathway
HSP	heat shock proteins
I/R	ischemia/reperfusion
IP_3	inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-triphosphate receptor
LDH	lactate dehydrogenase
MMP	mitochondrial membrane potential

LIST OF ABBREVIATION (Continued)

mMTP	mitochondrial membrane transition pore
NButGT	1,2-dideoxy-2'-methyl-alpha-d-glucopyranoso [2,1-d]-Delta2'-thiazoline
NFAT	nuclear factor of activated T cells
NRVMs	neonatal rat ventricular myocytes
O-GlcNAc	*O*-linked N-acetylglucosamine
OGA	*O*-GlcNAcase
OGT	*O*-GlcNAc transferase
PE	phenylephrine
PKC	protein kinase C
PUGNAc	*O*-(2-acetamido-2-deoxy-d-glucopyranosylidene) amino-N-phenylcarbamate
PVDF	polyvinylidene difluoride
ROS	reactive oxygen species
SERCA	Ca^{2+} adenosine triphosphatases
SOC	store-operated Ca^{2+} channel
SR	sarcoplasmic reticulum
Trp	transient receptor potential
UDP-GalNAc	UDP-*N*-acetylgalactosamine
UDP-GlcNAc	UDP-*N*-acetylglucosamine
UDP-HexNAc	UDP- *N*-acetylhexosamine

CHAPTER 1

INTRODUCTION

Cardiac Ischemia

Myocardial ischemia occurs when the supply of oxygen to the myocardium is inadequate for the oxygen demands of the tissue. Ischemic injury in the human heart can occur in many forms and may persist for only a few seconds or minutes (angina or angioplasty), for hours (cardiac surgery or infarction), or for years (chronic ischemic heart disease). Prolonged ischemia leads to an acute myocardial infarction (AMI) and consequently imperils cell viability and ultimately leads to cardiac dysfunction. The best way to treat ischemia is to minimize the ischemic time by restoring blood flow to the ischemic region as soon as possible, thereby minimizing irreversible injury and maximizeing the rate and extent of recovery.

It is paradoxical, however, that while reperfusion is needed to maximize recovery, reperfusion also hastens and may even exacerbate, the development of irreversible injury in tissue that may have been only reversibly injured in the moments before reperfusion; this phenomenon has been termed ‘reperfusion injury’ (11). The existence of lethal reperfusion injury as a separate entity is controversial, with some commentators suggesting that reperfusion exacerbates the cellular injury sustained during the ischemic period (79). Studies have demonstrated that reperfusion can exacerbate the necrotic component of cell death as evidenced by an extension in infarct size, following a fixed

period of ischemia (105, 164). However, other studies, indicate that the oxidative stress and abrupt metabolic changes that accompany reperfusion can initiate cellular injury in the absence of ischemia (59, 145).

The mechanism of reperfusion injury is complicated. In the last decade, extensive efforts have focused on the role of cytotoxic reactive oxygen species (ROS), complement activation, neutrophil adhesion, and the interaction between complement and neutrophils during myocardial reperfusion injury. One hypothesis is that myocardial stunning is a myofilament disease, caused by impaired calcium responsiveness of contractile machinery of myocytes, and that this effect results from both oxidative stress and calcium overload. Transient reversible ischemia followed by reperfusion results in increased production of ROS and calcium overload. Oxidative stress resulting from ROS generation can lead to a decrease in calcium responsiveness of myofilaments either directly by oxidative modification of contractile proteins (e.g., troponin I, α-actin) or indirectly by causing calcium overload (9). The cellular damage that results from reperfusion can be reversible or irreversible, depending on the length of the ischemic insult. One convincing means of demonstrating the existence of lethal reperfusion injury is to show that myocyte death can be modified by interventions administered at the time of reperfusion. Early interventions will profit more while late interventions less; multiple interventions acting at different levels will probably be more successful.

During past 50 years, reperfusion therapy has been developed and become a practical and effective treatment against the ischemic insult. However, despite the improvements in the management of patients with coronary heart disease, there is a growing need for the development of new treatments that can be administered

immediately before or at the time of revascularization to minimize irreversible tissue damage. Such treatment will both decrease acute mortality associated with infarction as well as reduce the incidence of the longer-term complications such as congestive heart failure.

Metabolic interventions and cardioprotection

Glucose and Glucose-Insulin-Potassium

There is a rich controversial literature surrounding the effectiveness of whether glucose and/or insulin and/or potassium (GIK) in protecting against damage following a variety of insults (36-38, 40, 128, 135, 136, 150). Several lines of evidence have been shown that hyperglycemia protects heart from ischemic injury in animal and cellular models. For instance, chronic hyperglycemia led to a reduction in hypoxia-induced apoptosis and necrosis in cardiomyocytes (128). In a swine model of hemorrhagic shock with 40% reduction in total blood volume, cardiac output in animals given 50% glucose upon resuscitation was more than double that seen in control pigs and 70% greater than iso-osmotic mannitol controls (135). In related contexts, GIK has also been shown to be effective in enhancing cardiac function following extensive burn injury (36), reversible cardiac failure following cardiac surgery (136), and myocardial infarction (4, 5).

Despite the fact that GIK therapy leads to a reduction in mortality that is greater than many accepted treatments, it has not been widely accepted. This is due in part to the complex nature of the treatment and the potential problems associated with the large fluid volumes required. Moreover, the data are often inconclusive and/or inconsistent due to a

wide range of mythological discrepancies (38). Nevertheless, on balance the evidence suggests that under well controlled conditions GIK treatment can lead to significantly reduced mortality rates following myocardial infarction. Thus, while data support the concept that GIK is protective in the treatment of ischemia/reperfusion, no mechanism has yet been solidly implicated.

Other metabolic interventions

In addition to high glucose and GIK treatment, numerous pharmacological interventions have been used to promote glucose oxidation (34, 84, 88, 96, 129). For example, dichloroacetate (DCA), an inhibitor for pyruvate dehydrogenase (PDH) kinase directly increases glucose oxidation and has been reported to improve functional recovery in studies of ischemia and reperfusion (84). Administration of L-carnitine, also increases carbohydrate metabolism and reduces ischemic myocardial injury (96). Moreover, additional regulatory sites suggested to potentially enhance glucose oxidation and function in postischemic myocardium include enhanced glucose transporter translocation to the sarcolemma, activation of acetyl-CoA carboxylase (88) and inhibition of malonyl-CoA decarboxylase (34). Alternatively glucose oxidation can be stimulated indirectly by inhibiting fatty acid oxidation. Trimetazidine and ranolazine, are two clinically effective anti-anginal agents that have reported to act by inhibiting mitochondrial ß-oxidation, thereby stimulating glucose oxidation and increasing cardiac efficiency (72, 87, 129). However, Wang et al., have shown that ranolazine had no effect on carbohydrate or fatty acid oxidation (152). They demonstrated that ischemic protection by ranolazine was not mediated by inhibition of fatty acid oxidation and conversely that inhibition of fatty acid

oxidation with CVT-4325, a new fatty acid oxidation inhibitor that reduced fatty acid oxidation up to approximately 7-fold with a concomitant increase in carbohydrate oxidation, was not associated with improved LV functional recovery (152). In addition, it has been shown that inhibition of fatty acid oxidation is implicated in apoptotic signaling pathway (101, 133). Therefore, long term inhibition of fatty acid oxidation may not be the best approach for enhancing cell survival.

Glutamine is another intervention that has been reported to have cardioprotecive effect for protection in cardiac ischemia (74, 75, 157). Moreover, elevating glutamine above the normal plasma level of 0.4 mM has been shown to enhance recovery from a variety of experimental and clinical challenges including bums, sepsis, and trauma (157). With respect to cardiac ischemia, glutamine provided remarkable protection either when administered prior to ischemia or when administered just prior to reperfusion in an isolated rat heart (74, 75). Glutamine has also been shown to be protective in an isolated cardiomyocyte model (157); however, the mechanism underlying the protection associated with glutamine in cardiomyocytes has yet to be identified. Recently, Liu et al., has been reported that, glutamine-induced protection of isolated rat heart from ischemia/reperfusion injury is mediated, at least in part, via the hexosamine biosynthesis pathway and increased protein *O*-GlcNAc levels (93).

Fructose 1,6 bisphosphate (FBP) is also another metabolic intermediate that has been shown to provide protection in the setting of myocardial ischemia and reperfusion in both experimental and clinical settings (35, 137). In a randomized placebo-controlled clinical trial, FBP administered during coronary artery bypass resulted in lower creatine kinase release and improved post-operative cardiac function (122). It has been reported

that FBP can be transported into the heart appeared to be mediated by a dicarboxylate carrier (56).

Each of the metabolic strategies discussed above have different proposed mechanisms of action, however they also all could have a potentially common target, namely the hexosamine biosynthesis pathway (HBP). Increased glucose entry into the cell, resulting from direct or indirect stimulation of glucose utilization either directly or indirectly, could also increase flux into the HBP. Glutamine is a necessary substrate for glutamine: fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme for glucose entry into the HBP. Finally, the presence of fructose-1,6- bisphosphatase (FDP) in the heart (56), it raises the intriguing possibility that FBP could be metabolized to FBP by FDP thereby increasing flux through the HBP. Thus, activation of the HBP may represent a common mechanism underlying the cardioprotection associated with increased glucose utilization, glutamine, and FBP treatment.

Hexosamine Biosynthesis Pathway

The Hexosamine Biosynthesis Pathway (HBP) is a relatively minor branch of the glycolytic pathway (Fig. 1). Approximately 2-4% of the glucose transported into the cell normally enters the HBP (104). In this pathway, fructose-6 phosphate is diverted from glycolysis to provide UDP-*N*-acetylglucosamine (UDP-GlcNAc) which is the major end product of this pathway. UDP-GlcNAc serves as sugar substrates for many reactions including sugar donor, multiple glycosylation reactions such as proteoglycan synthesis and the formation of *O*-linked glycoproteins or *O*-GlcNAcylation (15). Glutamine:

fructose-6-phosphate amidotransferase (GFAT) is the rate-limiting enzyme for entry into the HBP by converting fructose-6-phosphate and glutamine into glucosamine-6-phosphate. This enzyme can be inhibited with amidotransferase inhibitors such as azaserine (104). Glutamine, the donor of the amine group, has been shown to increase HBP flux (10, 124, 158). Glucosamine, although normally present at low levels in bodily fluids enters cells via the glucose transporters (143), and is phosphorylated to glucosamine-6-phosphate by hexokinase, thereby, bypassing GFAT and elevating UDP-GlcNAc levels.

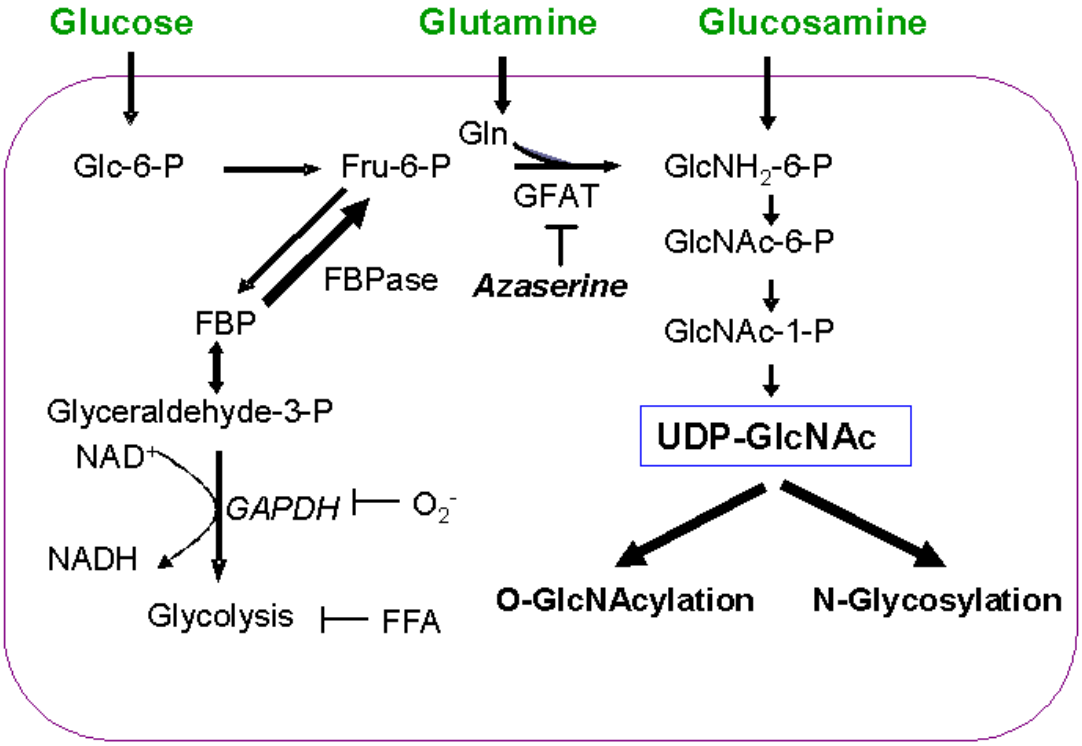

Figure 1: The Hexosamine Biosynthesis Pathway (HBP)

It has also been suggested that flux through the HBP is also increased by ROS generated as a result of hyperglycemia or stress as result of inhibition of glycolysis (33). GAPDH is the key enzyme controlling this entry point, and it has a reactive cysteine residue at position 149 that is necessary for enzymatic activity and particularly vulnerable to the effects of oxidants (144). Exposure of GAPDH to H_2O_2, ROS, or nitric oxide results in the rapid loss of enzymatic activity. Moreover, it has recently been suggested that ROS-induced inhibition of GAPDH leads to a redirection of glucose within cells such that diacylglycerol synthesis and flux through the HBP are increased (15). Because of this, GAPDH inhibition is proposed to be central to the development of many diabetic complications that arise either because of PKC activation by diacylglycerol or because of downstream consequences of increased flux through the HBP.

The role of the HBP in the development of insulin resistance was first described by Marshall et al. in rat adipocytes (104). Transgenic mice overexpressing GFAT in skeletal muscle and adipose tissue develop insulin resistance and hyperleptinaemia (26, 60). These data support the notion that increased flux through the hexosamine biosynthesis pathway plays an important role in the development of insulin resistance and glucose toxicity. Activation of the HBP has also been associated with glucose-induced transcriptional upregulation of TGFα (126), TGFβ (27), leptin (151), PAI-1 (33) and decreased phosphorylation of Akt and GSK3β (149).

Protein *O*-GlcNAc modification

O-GlcNAc transferase and O-GlcNAcase

UDP- *N*-acetylglucosamine (UDP-GlcNAc), the major end product of HBP is the obligatory substrate for transferring *O*-GlcNAc to proteins, a process termed *O*-GlcNAcylation (Fig. 2). This glycosylation is distinct from the well-characterized glycosylation cascades within the endoplasmic reticulum and Golgi, and utilizes completely different acceptor proteins. In all higher eukaryotes, *O*-GlcNAc is a common post-translational carbohydrate modification of nuclear and cytoplasmic proteins at side chain hydroxyl groups of serine or threonine residues (23, 58). *O*-GlcNAcylation is a reversible process which is regulated by two key-enzymes; uridine diphospho-N-acetylglucosamine: polypeptide β-N-acetylglucosaminyltransferase (*O*-GlcNAc transferase; OGT) -catalyzing the *O*-glycosylation and *N*-acetylglucosaminidase (O-GlcNAcase; OGA) -catalyzing the removal of sugar moiety from the proteins (58).

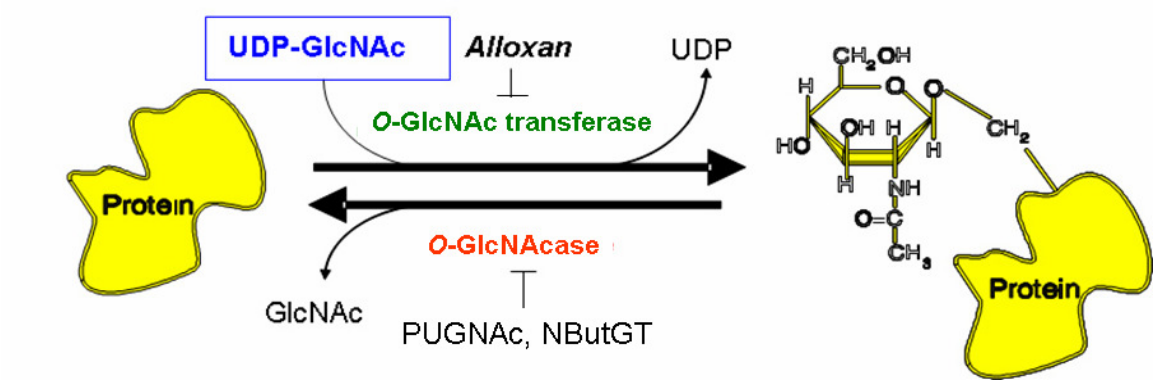

Figure 2: OGT and *O*-GlcNAcase regulate protein *O*-GlcNAc modification

Although, *O*-glycosylation is a highly dynamic posttranslational modification similar to phosphorylation, in contrast to the more than 500 kinases catalyzing phosphorylation (103), there is only one gene encoding OGT (81, 100), and a single gene encoding the complementary *O*-GlcNAcase (47). The enzymatic activity of OGT can be inhibited by alloxan (80) while *O*-GlcNAcase activity can be inhibited by *O*-(2-acetamid-*O*-2-deoxy-d-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc) and 1,2-dideoxy-2'-methyl-alpha-d-glucopyranoso[2,1-d]-Delta2'-thiazoline (NButGT) (102).

Both OGT and *O*-GlcNAcase are highly conserved from *Caenorhabditis elegans* to humans (47, 99). A single copy of the OGT gene is located on the X chromosome in humans and mice. It has been reported that OGT gene deletion in mice was embryonically lethal, demonstrating that OGT activity/O-glycosylation is vital for life (114, 130). In some tissues (e.g., liver, muscle and kidney) OGT is a hetero-trimer consisting of two 110 kDa and one 78 kDa subunits (53), whereas in other tissues it exists as a homo-trimer of three 110 kDa subunits (81). The structure of the 110 kDa subunit contains two main domains; an N-terminal domain containing multiple tetra-tricopeptide repeats (TPR), a catalytic domain in the C-terminus, and a nuclear localization sequence (81, 99). There is also a mitochondrial form of OGT (mOGT) primarily consisting of 103 kDa subunits, which contains a unique mitochondrial targeting sequence in the N-terminus (98). The TPR domains of OGT mediate protein-protein interactions (82). Because the transferase has a variety of binding partners and is itself post-translationally modified, OGT's substrate specificity, localization, and/or activity are likely to be regulated by signal transduction cascades. The longest form is a nucleocytoplasmic OGT (ncOGT) with 12 tetratricopeptide repeats (TPRs); a shorter form of OGT encodes a

mitochondrially sequestered enzyme with 9 TPRs and an N-terminal mitochondrion-targeting sequence (mOGT). An even shorter form of OGT (sOGT) contains only 2 TPRs (55). Furthermore, the activity of OGT is very sensitive to the intracellular concentration of UDP-GlcNAc (154) is readily altered in response to a diverse range of metabolic stimuli. In addition, acceptor protein specificity changes with hyperglycemia or glucosamine treatment (82, 150), which has profound implications with respect to cellular physiology.

O-GlcNAcase is a 106 kDa heterodimer complex containing a 54 kDa α and a 51 kDa β subunit (30) and the gene is localized to the 10q24 chromosomal location (153). O-GlcNAcase is distinct from lysosomal hydrolases, including hexosaminidase A and B and is specific for N-acetyl-β-D-glucosaminides, and shows no N-acetyl-β-D-galactosaminidase activity (30, 47, 153). The endogenous *O*-GlcNAcase appears to be in a complex with other proteins and is predominantly localized to the cytosol (153). It has also been shown that *O*-GlcNAcase has an intrinsic histone acetyl transferase (HAT) activity and a single point mutation abolished HAT activity (139). Interestingly, there is a caspase-3 cleavage site linking the *O*-GlcNAcase and HAT regions; however cleavage by caspase-3 does not alter the catalytic activity of *O*-GlcNAcase (139, 153).

O-GlcNAc and cell signaling

It has been shown that post-translational modification by *O*-GlcNAc is an important regulatory mechanism involved in signal transduction and protein-protein interactions in many area including insulin and diabetes, cell cycle regulation, and stress responses (54, 148, 154, 156). Over 600 proteins have been identified as being modified

by *O*-GlcNAcylation (58). Examples are of transcription factors, Sp1 (22, 49), p53 (41), NF-κB (69); insulin signaling and glucose metabolism, IRS1/2 (39), PI3K (39), GLUT4 (116), Akt (44), GSK3-β (100), glycogen synthase (117); and other signaling pathways such as PLC-β1 (77).

Post-translational modification by *O*-GlcNAc and *O*-phosphate can also occur at multiple sites on the same protein (97, 131). However, in some case, *O*-GlcNAcylation and *O*-phosphorylation can be reciprocal on some proteins (e.g., eNOS (32), estrogen receptor-β (19), C-terminal domain of RNA polymerase II (73), c-myc (21)). Thus, regulation of *O*-GlcNAc levels by OGT and O-GlcNAcase can be thought to be analogous to the regulation of phosphorylation by kinases and phosphatases; furthermore, alterations in *O*-GlcNAcylation could have direct effects on multiple phosphorylation cascades.

O-GlcNAc signaling and cardioprotection

Many publications on the effects of HBP and *O*-GlcNAc on cellular function are in the context of nutrient excess and the adverse effects of insulin resistance and diabetes. However, it has been shown that a wide variety of stress stimuli rapidly increased the levels of protein *O*-GlcNAc modification in mammalian cells, suggesting that acute activation of the pathways leading to *O*-GlcNAc formation was an endogenous stress response (162). Furthermore, decreasing or blocking this modification rendered cells more sensitive to stress and resulted in decreased cell survival, whereas increased levels of *O*-GlcNAc with PUGNAC, an inhibitor of *O*-GlcNAcase protected cells against the same stress (162). In addition, it has been shown that global OGT gene deletion is

embryonically lethal (114) and even cell specific OGT gene deletion resulted in cell death during embryogenesis (114, 130). Others reported that, in Chinese hamster ovary (CHO-K1) and Human hepatocarcioma (Hep3B), inhibition of GFAT decreased *O*-GlcNAc levels and reduced cell survival following heat stress (132).

Liu et al., reported that in the isolated perfused heart, glucosamine increased cardiac *O*-GlcNAc levels 3-fold and protected the heart against injury resulting from the calcium paradox and ischemia/reperfusion (95). Alloxan an inhibitor of OGT, blocked the protection associated with glucosamine and prevented the increase in *O*-GlcNAc levels (95). They also showed that glutamine an amino acid donor of glutamine:fructose6-phosphate amidotransferase (GFAT) augmented *O*-GlcNAc levels which is associated with improvement of heart functional recovery following ischemia/reperfusion (93). Pre-treatment with azaserine, an inhibitor of GFAT, completely reversed the protection seen with glutamine and prevented the increase in protein *O*-GlcNAc. Furthermore, administration of glucosamine or PUGNAc an inhibitor of *O*-GlcNAcase, during the early reperfusion significantly improved cardiac functional recovery and reduced troponin I release during reperfusion compared to untreated control (94). Both interventions also significantly increased the levels of protein *O*-GlcNAc and ATP levels. Interestingly, it also has been shown that treatment of glucosamine (159) or PUGNAc (167) improved cardiac function in vivo in a rat model of trauma-hemorrhage and this was associated with increased cardiac *O*-GlcNAc levels compared to untreated controls. These data support the notion that the cardioprotection of glucosamine, glutamine or PUGNAC is due, at least in part, to enhanced flux through the HBP and increased protein *O*-GlcNAc levels.

The specific mechanisms underlying the cardioprotection associated with increased *O*-GlcNAc levels remain to be determined. Zachara et al., (162) reported that increased survival seen with elevated *O*-GlcNAc levels was associated with at least in part via increased heat shock protein 70 (Hsp70) expression. Lim et al., (90) also demonstrated decreasing *O*-GlcNAc levels deteriorated thermal stability of Sp1 and *O*-GlcNAc modified Sp1 resisted thermal aggregation in vitro. They also showed that heat-induced elevation of Hsp70 was facilitated by Sp1 but blunted under low *O*-GlcNAc levels, suggesting that *O*-GlcNAc might upregulate the expression of heat shock protein 70 through thermoprotection of Sp1, which eventually enhanced cellular thermotolerance. In contrast Sohn et al. (132) reported improved survival associated with increased *O*-GlcNAc levels without any change in Hsp70 levels, which suggests that other mechanism(s) may also contribute to the protection associated with increased *O*-GlcNAc levels. Others also suggested that *O*-GlcNAc play an important role to regulate the several pathways in a manner consistent with stress tolerance. Yang et al., (160) showed that *O*-GlcNAcylation at Ser 149 stabilized p53 by blocking ubiquitin-dependent proteolysis, which suggests that *O*-GlcNAc may play a important role in protein stability. Zhang et al., (163) also reported that increased levels of *O*-GlcNAc result in the inhibition of the proteasome and this could be also contribute to increase cell survival.

It has been shown that glucosamine treatment was associated with attenuation of the ischemia-induced increase in p38 phosphorylation but also, paradoxically, increased p38 phosphorylation at the end of reperfusion, suggesting that glucosamine-induced cardioprotection may be mediated via the p38 MAPK pathway (43). On another hand, Liu et al., (93-95) proposed that in isolated perfused heart, increased *O*-GlcNAc levels

decreased calcium overload and this was correlated with attenuation of calcium-mediated proteolysis of structural proteins such as troponin I and α-fodrin. Furthermore, in neonatal cardiomyocytes, increased levels of *O*-GlcNAc also attenuated the increase in cytosolic Ca^{2+} suggests that protein could also be due to the inhibition of calcium influx into the cell (65, 110, 115). These data suggest that increasing cardiac *O*-GlcNAc levels may be a clinically relevant cardioprotective strategy and indicate that this protection could be due at least in part to inhibition of calcium-mediated stress responses.

Calcium homeostasis

Calcium regulation in heart

A critical component of both cell signaling and cell survival is the maintenance of the concentration of cytoplasmic free Ca^{2+} or $[Ca^{2+}]_i$. The major intracellular Ca^{2+} stores are in endoplasmic reticulum (ER) and mitochondria where cytoplasmic Ca^{2+} ions are interacted in tight complexes with cellular polysaccharides, lipids, and proteins such as calmodulin. In cardiomyocytes, both sarcolemmal (SL) and sarcoplasmic reticulum (SR) are considered to be the major organelles involved in the regulation of $[Ca^{2+}]_i$ on cardiac contractility and relaxation. In normal heart, the extracellular concentration of ionized Ca^{2+} is about 1.25 mM, whereas the intracellular concentration varies between 0.1 and 10 μM during diastole and systole, respectively. During an action potential, the SL membrane depolarization increases $[Ca^{2+}]_i$ via activation of SL voltage-gated L-type Ca^{2+} channels. A small amount of Ca^{2+} from the extracellular space triggers the release of a large amount of Ca^{2+} from the SR via Ca^{2+} release channels known as ryanodine

receptors (RyR). The transient increase in $[Ca^{2+}]_i$ ($Ca^{2+}{}_i$ transient) binds to troponin C and causes the activation of contractile myofilaments and therefore induces systolic contraction. During diastole, the level of Ca^{2+} is restored to the resting level via three mechanisms: (a) by pumping back cytosolic Ca^{2+} into the SR lumen by an energy-dependent mechanism, the SR Ca^{2+} pump ATPase (SERCA) (89, 91); (b) by the electrogenically neutral Na^+-Ca^{2+} exchanger cross SL membrane (8, 112), (c) by the SL Ca^{2+} pump ATPase against a concentration gradient. In addition to ryanodine receptors, Ca^{2+} is also released from SR via inositol triphosphate (IP_3) activated channels; however, but the contribution of this pathway of Ca^{2+} release to excitation-contraction coupling is unclear (147).

Calcium is perhaps the most critical modulator of cardiac physiology and pathology. In addition to the role $[Ca^{2+}]_i$ plays in the ongoing control of excitation-contraction coupling, agonists present in the extracellular milieu that alter the handling of $[Ca^{2+}]_i$ in cardiomyocytes can have profound effects on heart diseases such as cardiac hypertrophy (20), heart failure and diabetic cadiomyopathy (28). Abnormalities in regulating $[Ca^{2+}]_i$ have been shown to result in a high level of cytosolic Ca^{2+}, which then produces cellular toxicity. This phenomenon is known as intracellular Ca^{2+} overload (42) and is reported to occur in myocardial ischemia, ischemia/reperfusion injury, and catecholamine-induced cardiomyopathy (120). The molecular mechanisms are not fully understood, but recent studies suggest a major role for the Ca^{2+}-dependent cysteine protease, calpain implicated the contractile dysfunction during I/R injury, in part because of proteolysis of structural proteins and subsequent myocardium dysfunction (45, 46). While there are clearly a number of different calcium entry pathways that could

contribute to calcium overload, recent studies have shown a role for Capacitative Calcium Entry (CCE) in modulating calcium influx in cardiomyocytes (65, 115).

Capacitative Calcium Entry

Capacitative Calcium Entry or CCE (Fig. 3) refers to the Ca^{2+} influx pathway mediated by non-voltage-gated, store-operated Ca^{2+}channels (SOCs) and activated when endoplasmic (ER) or sarcoplasmic reticulum (SR) Ca^{2+} stores are depleted, usually by agonists leading to the activation of phospholipase C (PLC) and the generation of inositol 1,4,5 trisphosphate (IP_3) (13). CCE characterizes all non-excitable cells but erythrocytes (121), and has now been shown to co-exist with voltage-gated Ca^{2+} channels in smooth (140), skeletal (85), and cardiac (65, 115) muscle cells.

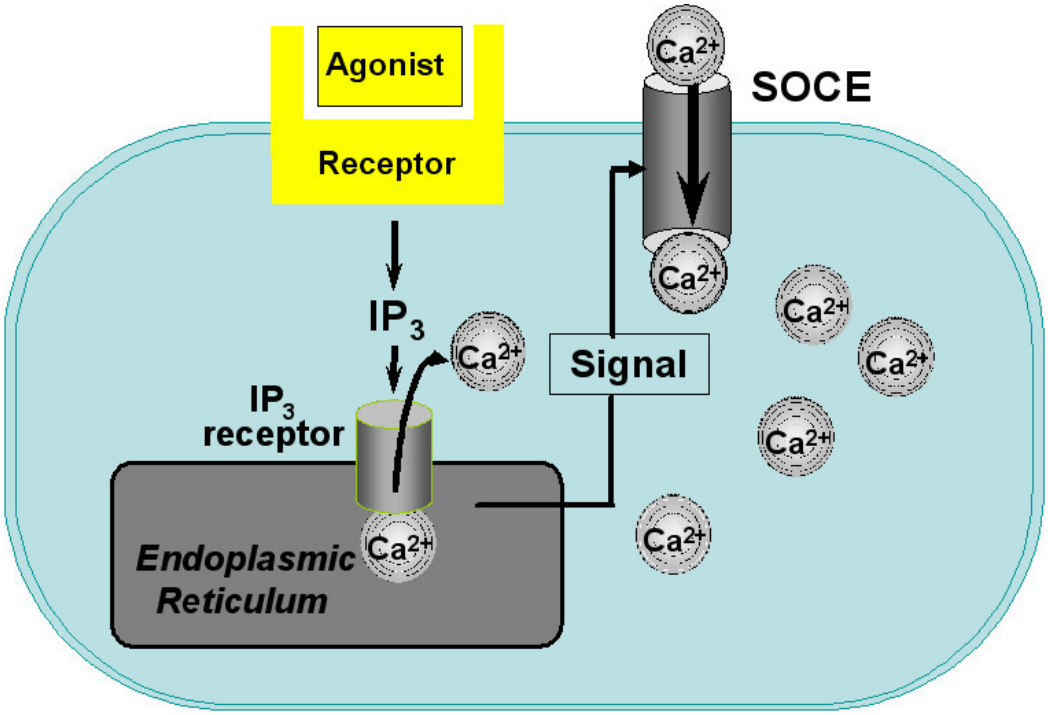

Figure 3: Capacitative Calcium Entry

There were two lines of circumstantial evidence supporting the presence of CCE in cardiomyocytes. First, the prime candidate genes for SOCs are members of the transient receptor potential (Trp) superfamily (63) mRNA transcripts for Trp 1 and Trp 4 have been detected in cardiac tissue from various species at high expression levels, and Trps 2, 3, 5, and 6 have been amplified from cardiac tissue by RT-PCR (48). Second, evidence supporting the existence of a voltage-independent calcium entry pathway in cardiomyocytes is plentiful (3, 76). Furthermore, it has been established that in both neonatal (65) and adult (66) cardiomyocytes that CCE is activated by IP_3-generating agonists such as angiotensin II (Angll) or phenylephrine (PE) and by other store-depleting strategies.

Capacitative Calcium Entry and Hexosamine Biosynthesis Pathway

Hunton et al., reported that glucosamine is an effective inhibitor of CCE in neonatal cardiomtocytes (65). Others have demonstrated that among the aberrancies associated with hyperglycemia is the inhibition of CCE (115, 123). Further, it has also determined that this inhibition is due, at least in part, to enhanced flux through the HBP (115). Further, Nagy et al. found that glucosamine blocked ANG II-induced $[Ca^{2+}]_i$ increase in neonatal cardiomyocytes and that this phenomenon was associated with a significant increase in UDP-GlcNAc and *O*-GlcNAc levels (110). On another hand, Clark et al., has reported that diabetes and the accompanying hyperglycemia impairs cardiomyocyte calcium cycling through increased nuclear *O*-GlcNAc (22). These investigations into CCE in cardiomyocytes has also suggested that this pathway may play an important role in the Ca^{2+} influx seen following ischemia resulting in $[Ca^{2+}]$ overload.

The existence of CCE in cardiomyocytes has yet to be universally accepted, however a link between the HBP and decreased calcium overload represents an intriguing and novel mechanism whereby increased myocardial glucose metabolism leads to increased protection against ischemic injury. IP_3-mediated store depletion and the subsequent increase in $[Ca^{2+}]_i$ that results from activation of CCE could be a primary contributor to the development of reperfusion-induced damage.

Cardiac apoptosis

Apoptotic pathways

Apoptosis is a highly regulated form of cell death, and contributes to the continuous decline of ventricular function in heart failure observed in a variety of cardiovascular diseases, including myocardial infarction and ischemia-reperfusion injury. (51). Apoptosis can be initiated by extracellular means via death receptors (extrinsic) or intracellular mechanisms via mitochondrial (intrinsic) stimuli, leading to the activation of caspases, a family of cysteine proteases, and subsequent cell death (51). The extrinsic pathway is induced through the activation of death receptors such as Fas and TNFR by their respective ligands. This causes recruitment of a death-inducing signaling complex and leads to activation of initiator caspase-8, which subsequently activates effector caspases (109). In contrast, in the intrinsic pathway, the mitochondria play a central role in the integration and execution of a wide variety of apoptotic signals, including loss of growth factors, hypoxia, oxidative stress, and DNA damage. The mitochondria provide the energy required for execution of the apoptotic program and release of proapoptotic

proteins such as cytochrome *c*, endonuclease G, and apoptosis-inducing factor. Release of cytochrome *c* leads to apoptotic protease-activating factor (Apaf-1)-mediated activation of initiator caspase-9, which in turn activates effector caspases (166). Thus the extrinsic and intrinsic pathways have different initiator caspases but converge at the level of the effector caspases. In addition, endonuclease G and apoptosis-inducing factor (AIF), apoptogenic factors which are released from the intermembrane space of the mitochondria into cytosol upon apoptotic stimuli are capable of inducing DNA fragmentation independent of caspases (6).

Bcl-2 family members

The apoptotic program is complex, involving both pro- and anti-apoptotic proteins, and apoptosis occurs when the equilibrium between these opposing factors is perturbed (127). One of these factors is intrinsic to the apoptotic pathway, Bcl-2 family proteins. This family is composed of pro and antiapoptotic proteins that share up to four conserved regions known as Bcl-2 homology (BH) domains (1). Anti-apoptotic members such as Bcl-2 and Bcl-XL contain all four subtypes of BH domains and promote cell survival by inhibiting the function of the pro-apoptotic Bcl-2 proteins. Anti-apoptotic Bcl-2 proteins have been reported to protect cells from many different apoptotic stimuli and are important for cell survival (108, 111, 146). The pro-apoptotic members can be separated into two structurally distinct subfamilies; 1) the “multidomain” proteins (Bax and Bak) share three BH regions and lack the BH4 domain. They are structurally similar to the anti-apoptotic proteins (1); 2) “BH3-only” proteins, which include Bnip3, Nix/Bnip3L, Bid, Noxa, Puma, and Bad, share only the BH3 domain and are structurally

diverse (64). Most of the Bcl-2 family members contain a transmembrane domain at their COOH terminus, which is important for their targeting to intracellular membranes. Anti- and pro-apoptotic Bcl-2 proteins can be found in the cytosol, endoplasmic reticulum, mitochondria, and nuclear envelope (92).

It is becoming evident that the Bcl-2 family proteins play a central role in regulating apoptosis in the cardiovascular system such as myocardial infarction, dilated cardiomyopathy, and ischemic heart disease (7, 86). Pro- and anti-apoptotic Bcl-2 proteins are expressed in the myocardium during development and in adult hearts (25). Anti-apoptotic Bcl-2 proteins have therapeutic potential for heart disease, since they have been shown to protect myocardial cells from various stresses. Bcl-2 has been shown to block p53-mediated apoptosis in cardiac myocytes (78), increase the calcium threshold for permeability transition pore opening in heart mitochondria (165), and inhibit hypoxia/reoxygenation induced apoptosis in isolated adult cardiac myocytes (71). Moreover, transgenic mice overexpressing Bcl-2 in the heart had fewer apoptotic cells, reduced infarct size, improved recovery of cardiac function after ischemia-reperfusion (14, 18, 68). The pro-apoptotic Bcl-2 proteins have been implicated in the pathogenesis of various cardiac diseases, including myocardial hypertrophy, myocardial infarction, and heart failure. For instance, chronic hypoxia, stretch, and chronic pressure overload caused significant apoptosis in rat hearts, which correlated with increased levels of Bax and decreased levels of Bcl-2 (24, 70). Moreover, Bax has been reported to be activated in cardiac cells in response to oxidative stress (52) and during ischemia (17). Capano and Crompton (17) showed that Bax translocation to the mitochondria during ischemia was dependent on AMP activated protein kinase and p38 MAPK in neonatal cardiac myocytes.

Mitochondrial damage was reduced and infarct size was decreased after ischemia-reperfusion in hearts from Bax deficient mice compared with wild-type animals, implicating Bax as a major player in ischemia-reperfusion injury (62).

Fiordaliso et al., (41) reported that incubation of adult cardiomyocytes in hyperglycemic conditions resulted in increased *O*-GlcNAc levels on the transcriptional regulator p53 leading to increased angiotensin II synthesis and increased apoptosis. They found that an AT1 receptor antagonist blocked hyperglycemia induced apoptosis, whereas inhibition of *O*-glycosylation with BAG (benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside) prevented the *O*-GlcNAc modification of p53 and blocked the synthesis of Ang II. However, since BAG was originally described as an inhibitor of mucin glycosylation in the Golgi (61), which is not catalyzed by OGT, it lacks specificity. In contrast, Schaffer *et al.*, (128) reported that hyperglycemia significantly reduced hypoxia induced apoptosis and necrosis in isolated cardiomyocytes and showed that glucose treatment also mediated an upregulation of the cardioprotective factor Bcl-2 but did not affect the cellular content of the pro-apoptotic factors Bax and Bad. These data suggest this protection may be mediated at least in part by increased HBP flux and *O*-GlcNAc levels which are associated with Bcl-2 upregulation. Although it is not clear how *O*-GlcNAc modification regulates apoptosis in cardiomyocytes, *O*-GlcNAc may play an important role via Bcl-2 family proteins and p53 activity and stability.

Hypothesis

The central hypothesis of this dissertation is to better understand the acute effects of glucosamine and *O*-GlcNAc modification on cardiomyocytes following ischemia/reperfusion injury and to elucidate the specific pathway(s) associated with glucosamine-induced ischemic protection. Preliminary studies presented in Dr. Marchase and Chatham Lab provide compelling evidence that acute activation of the hexosamine biosynthesis pathways (HBP) leads to protection against ischemic injury and calcium overload. This is in stark contrast to adverse effects usually associated with chronic activation of this pathway. To test this hypothesis, studies are performed in the isolated neonatal rat ventricular myocytes (NRVMs). Available inhibitors of *O*-GlcNAcylation are used to test the hypothesis of this protective effect. In addition, the consequences of manipulation and reduction of the gene responsible for *O*-GlcNAc addition, *O*-GlcNAc transfer (OGT) are studied. This approach can be more possibly done in the cellular models compared to with the intact heart model. The proposed experiments are designed to elucidate the mechanisms underlying the protection associated with glucosamine and increased *O*-GlcNAc modification. The successful outcome of this dissertation has the potential to identify a new area for the development of novel therapeutic agents and may present a novel strategy for inducing cardioprotection.

CHAPTER 2

GLUCOSAMINE PROTECTS NEOTATAL CARDIOMYOCYTES FROM ISCHEMI-REPERFUSION INJURY VIA INCREASINED PROTEIN-ASSOCIATED *O*-GlcNAc

by

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Abstract

Increased levels of protein *O*-linked-N-acetylglucosamine (*O*-GlcNAc) have been shown to increase cell survival following stress. Therefore, the goal of this study was to determine whether in isolated neonatal rat ventricular myocytes (NRVMs) an increase in protein *O*-GlcNAcylation resulted in improved survival and viability following ischemia/reperfusion (I/R). NRVMs were exposed to 4 hours ischemia and 16 hours of reperfusion and cell viability, necrosis, apoptosis and *O*-GlcNAc levels assessed. Treatment of cells with glucosamine, hyperglycemia or O-(2-acetamido-2-deoxy-dglucopyranosylidene) amino-N-phenylcarbamate (PUGNAc), an inhibitor of *O*-GlcNAcase, significantly increased *O*-GlcNAc levels and improved cell viability, as well as reducing both necrosis and apoptosis compared to untreated cells following I/R. Alloxan, an inhibitor of *O*-GlcNAc transferase, markedly reduced *O*-GlcNAc levels and exacerbated I/R injury. The improved survival with hyperglycemia was attenuated by azaserine, which inhibits glucose metabolism via the hexosamine biosynthesis pathway. Reperfusion in the absence of glucose reduced *O*-GlcNAc levels on reperfusion compared to normal glucose conditions and decreased cell viability. *O*-GlcNAc levels significantly correlated with cell viability during reperfusion. The effects of glucosamine and PUGNAc on cellular viability were associated with reduced calcineurin activation as measured by nuclear NFAT translocation suggesting that increased *O*-GlcNAc levels may attenuate I/R induced increase in cytosolic Ca^{2+}. These data support the concept that, activation of metabolic pathways leading to an increase *O*-GlcNAc levels is an endogenous stress activated response, and that augmentation of this response improves

cell survival. Thus, strategies designed to activate these pathways may represent novel interventions for inducing cardioprotection.

Introduction

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

The *O*-GlcNAc modification of proteins is unlike other glycosylation events in that it occurs through an enzyme-catalyzed reaction in the cytosol and the nucleus rather than in the Golgi apparatus or the endoplasmic reticulum. Post-translational modification by *O*-GlcNAc is a dynamic and reversible process, regulated by the activities of two key enzymes, *O*-GlcNAc transferase (OGT) (18, 21) and N-acetylgucosaminidase (*O*-GlcNAcase) (11). In some cases, *O*-GlcNAcylation is reciprocal with phosphorylation (5, 7) and proteins that have been identified as being modified by *O*-GlcNAcylation include kinases, phosphatases, cytoskeletal proteins, nuclear hormone receptors, nuclear pore proteins, signal transduction molecules and actin regulatory proteins (38). Furthermore,

O-GlcNAcylation has also been reported to influence protein transcription and translation, nuclear targeting and transport, and protein degradation (7, 13, 29). Consequently, alterations in O-GlcNAc levels have important and wide-ranging effects on cell function.

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

Exprimental procuders

Materials: Unless otherwise noted, except for glucosamine (Fluka) all chemicals were purchased from Sigma Chemical (St. Louis, MO). Culture medium products were purchased from GIBCO Invitrogen (Grand Island, NY).

Neonatal rat ventricular myocyte (NRVM) primary cultures: Animal experiments were approved by the University of Alabama Institutional Animal Care and Use Committee

and conformed to the Guide for the Care and Usage of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, 1996). Primary cultures of neonatal rat ventricular myocytes (NRVMs) were obtained from 2-3 days old neonatal Sprague Dawley rats and cultured as described previously (15). Within 1-2 days of isolation, a confluent monolayer of spontaneously beating NRVMs had formed and cells were used as described below.

Simulated ischemia and reperfusion: Ischemia and reperfusion was induced based on the method described by Brar et al. (3). Following 1-2 days in culture, NRVMs were exposed to ischemia by adding a fresh Esumi modified ischemic medium (137 mM NaCl, 12 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$.2H2O, 4 mM HEPES and 20 mM sodium lactate, pH 6.2) and then incubated in a chamber with an atmosphere of 95% argon and 5% CO_2 for 4 hours. Following 4 hours of ischemia, cells were returned to the maintenance growth medium (serum free 4:1 (v/v) Dulbecco's modified Eagle's medium (DMEM) / Medium 199 with Hanks salts (M199), supplemented with 2% Nutridoma and 1% penicillin/streptomycin) and then incubated in an atmosphere of 21% O_2 and 5% CO_2 for 16 hours. In control normoxia experiments, cells were incubated with fresh maintenance growth medium in an atmosphere of 21% O2 and 5% CO2 for 20 hours.

Measurement of cell viability, necrosis and apoptosis: Cell viability was measured by Trypan blue exclusion; 10 µl of cell suspension was mixed with 90 µl of 0.04% Trypan blue in PBS. A total of at least 200 cells were counted using haemocytometer.

Necrosis was assessed by determining the release of lactate dehydrogenase (LDH) in culture medium and LDH in remaining attached cells using an LDH assay kit (Sigma). The percent LDH release was calculated by the ratio of the released LDH into the media by the total LDH (release plus cellular content).

Apoptosis was assessed by using In Situ Cell Death Detection Kit (Roche). Permeabilized cells were exposed to the TUNEL reaction mixture and were counter stained with 0.1 mg/ml of propidium iodide solution. In each treatment, a total of at least 300 cells were counted through a 40x objective with excitation wavelength at 495 nm.

Measurement of UDP-GlcNAc and ATP: UDP-GlcNAc and ATP were analyzed in acid extracts by HPLC as described previously (26, 28). Nucleotide sugars were monitored by UV detector at 262 nm and known standard sugars were used for calibration. This method cannot separate UDP-GlcNAc from UDP-N-acetyl galactosamine (UDP-GalNAc) so the results are presented as the summary of UDP-GlcNAc and UDP-GalNAc (UDP-HexNAc) (28); however, in cardiomyocytes the ratio of UDP-GlcNAc-to-UDP-GalNAc is approximately 3:1 (6).

Measurement of O-GlcNAc levels: O-GlcNAc levels were determined by Western blot analysis using the anti-*O*-GlcNAc antibody CTD110.6 (Covance) as previously described (20). The specificity of CTD110.6 for *O*-GlcNAc has been previously reported by Comer et al., (8). Cells were lyzed with 1xRIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA and 0.1% SDS) containing 2% protease inhibitor cocktail (Sigma) on ice for 30 min. Lysed proteins were harvested

and assayed for protein concentration using the Bio-Rad protein assay kit. Proteins (10 μg) were separated on 7.5% SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Milipore). The blots were then soaked in 100% methanol, dried and then probed with the anti-*O*-GlcNAc antibody CTD110.6 (Covance) diluted 1:5000 times in casein blocking buffer (Pierce) containing 0.01% Tween 20 for 2 hours at room temperature. After washing three times with PBS, the membrane was then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgM (Calbiochem) in 1% casein/PBS with 0.01% Tween 20 for 1 h. After further washing in PBS, the immunoblots were developed with enhanced chemiluminescence (SuperSignal West Pico; Pierce) and visualization was performed using Bioimaging system of UVP (UVP, Inc., CA).

Previous studies reported that stress resulted in a global increase in *O*-GlcNAc levels (40). Therefore, densitometric analysis of CTD110.6 immunoblots was performed on the entire lane of each sample using LabWorks analysis software (UVP, Inc., CA) and the mean intensity normalized to the control group. Duplicate gels were stained by coomassie brilliant blue R-250 for assessment of equal protein loading.

Assessment of ischemia and ischemia/reperfusion on cytosolic calcium levels and NFAT translocation: A direct measure of Ca^{2+} levels at the end of ischemia was performed using the fluorescent Ca^{2+} indicator Fura 2-AM (Molecular Probes). NRVMs were loaded with Fura 2-AM as previously described and cytosolic calcium levels were determined in cells at the end of 4 hours using alternating 340- and 380-nm excitation wavelengths and emission at 510 nm (12).

We have previously shown (15) that a sustained increases in cytosolic calcium levels $[Ca^{2+}]_i$ resulted in the nuclear translocation of a nuclear factor of activated T cells (NFAT) transcription factor, which is mediated by activation of the Ca2+/CaM-dependent protein phosphatase calcineurin. Since calcineurin activation has been implicated in mediating cardiomyocyte apoptosis (24, 27), we examined NFAT translocation at the end of ischemia reperfusion as an indirect assessment of calcium influx and a surrogate marker for calcineurin activation. One day after cell isolation, NRVMs were infected with an adenovirus (100 moi) encoding enhanced green fluorescent protein linked to the nuclear localization region of NFAT (GFP-NFAT). The day after infection, cells were subjected to ischemia-reperfusion experiments as described above. This chimeric GFP-NFAT protein remains in the cytoplasm in resting cells and translocates into the nuclear when cytoplasmic calcium increases sufficiently to activate calcium dependent phophatase calcineurin. At the end of the experiment, cells were fixed and imaged through a 40x objective with excitation wavelength at 495 nm. 200 cells were counted for each condition.

Statistics: All data are presented as means±SEM. Unpaired T-tests, one-way and repeated measure ANOVA were used where appropriate followed by a Bonferroni's multiple comparison test using Prism 4.0c (GraphPad Software Inc., San Diego CA). Statistically significant differences between groups were defined as $P < 0.05$.

Results

Glucosamine treatment decreases cardiomyocyte injury following ischemia and reperfusion.

Glucosamine treatment had no effect on viability, necrosis or apoptosis when present under normoxic incubation conditions for 20 hours in time control experiments (Fig. 1A-C). However, at the end of reperfusion, glucosamine treatment resulted in a significant increase in survival and significantly decreased both necrosis and apoptosis (Fig. 1A-C). Glucosamine treatment also significantly increased *O*-GlcNAc levels and UDP-HexNAc levels under both normoxic conditions and following ischemia/reperfusion (Fig 1D-F). Although ischemia/reperfusion alone appeared to increase *O*-GlcNAc levels in the control group this did not reach statistical significance (1.00±0.09 Vs 1.64±0.33 AU; p=0.13). However, glucosamine treatment markedly augmented the response to ischemia/reperfusion, resulting in ~3-fold increase compared to normoxia. Equal protein loading was confirmed by densitometic analysis of Commassie blue staining of duplicate gels (Fig 1D right) and this was consistent with Western blot analysis of ß-actin levels (data not shown). In the control group, UDPHexNAc levels were significantly increased at the end of ischemia compared to normoxic conditions (2.3±0.2 Vs 3.0±0.2 $p<0.05$).

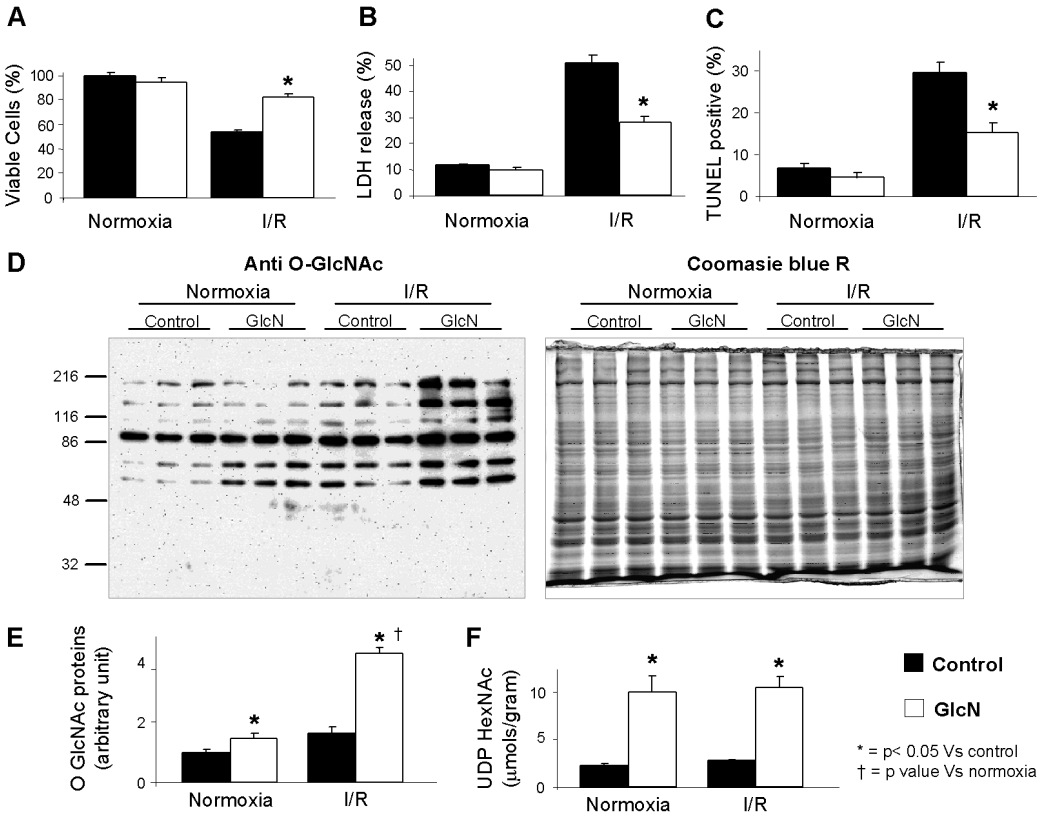

Figure 1: Glucosamine treatment decreases cardiomyocyte injury following ischemia and reperfusion mediated via *O*-GlcNAc modification. A) Cell viability assessed by Trypan blue exclusion; B) necrosis assess by LDH release as a % of total LDH; C) apoptosis assessed by % TUNEL positive cells; D) Representative CTD110.6 immunoblot of *O*-GlcNAc proteins and protein staining by coomassie blue; E) Mean intensity of all *O*-GlcNAc proteins determined by densitometric analysis normalized levels in normoxic control untreated cells; F) UDP-HexNAc levels analyzed by HPLC analysis. Control, untreated cells and with glucosamine treatment (5mM) were incubated under normoxic condition for 20 hours or subjected to 4 hours ischemia followed by 16 hours reperfusion. Data presented as mean ± SEM of six experiments (i.e., three separate NRVM isolations with each experiment performed in duplicate); * = p< 0.05 Vs control # p<0.05 Vs normoxia.

The protection against ischemic injury with glucosamine may be a consequence of increased energy production, since glucosamine can potentially be metabolized to fructose-6-phosphate via glucosamine-6-phosphate isomerase or deaminase (2, 41), thereby increasing glycolytic flux. Therefore, we assessed ATP levels in glucosamine treated and untreated cells at the end of ischemia and the end of reperfusion (Table 1). As anticipated ATP levels were significantly reduced at the end of ischemia; however, at the end of 16 hours of ischemia/reperfusion ATP levels had returned to normoxic levels.

Glucosamine treatment had no effect on ATP levels under any conditions. This was true regardless of whether ATP levels were normalized to μg of total protein or total number of cells per plate (data not shown). Thus the protection resulting from glucosamine treatment seen in Fig. 1 does not appear to be a consequence of increased glycolytic ATP production.

Table 1: ATP levels in NRVMs in untreated control and glucosamine treated groups under normoxia, ischemia and ischemia/reperfusion (I/R) conditions. Data presented as mean±SEM of six experiments (i.e., three separate NRVM isolations with each experiment performed in duplicate).

	ATP (μmols/gram protein)	
	Control	Glucosamine
Normoxia	27.8±2.6	25.9±3.5
Ischemia	9.4±0.8	11.1±0.9
I/R	34.4±4.3	25.6±3.3

Time course of O-GlcNAc levels during ischemia and reperfusion.

In Figure 1 we showed that glucosamine improved viability after 4 hours ischemia and 16 hours reperfusion. To evaluate the temporal relationship between increased *O*-GlcNAc levels and cell viability we assessed *O*-GlcNAc levels and cell viability at different time points during ischemia/reperfusion in untreated and glucosamine treated cells. In some additional experiments we also examined the role of glucose in regulating of *O*-GlcNAc levels by reperfusing without glucose in the media.

During ischemia, *O*-GlcNAc levels increased ~45% in the control group ($p=0.07$) and more than two fold the glucosamine treated group ($p<0.05$) (Fig 2A and B). During the first hour of reperfusion, *O*-GlcNAc levels increased in both untreated and glucosamine groups, but this response was markedly enhanced in the glucosamine treated group (Fig 2B). In contrast in absence of glucose there was no increase in *O*-GlcNAc levels on reperfusion (Fig 2A, B). In untreated cells *O*-GlcNAc levels returned to baseline after 8 hours of reperfusion and remained relatively constant for the remainder of the reperfusion period. However, in the glucosamine treated group *O*-GlcNAc levels remained elevated for approximately 8 hours of reperfusion returning close to baseline levels by 12 hours. In the absence of glucose, there was a gradual decrease in *O*-GlcNAc levels throughout the reperfusion period. The ability to increase *O*-GlcNAc levels during the first few hours of reperfusion was associated with improved viability (Fig 2C) and there was a significant correlation between *O*-GlcNAc levels and cell viability during both early (2hrs) and late reperfusion (16hrs) (Fig 2D).

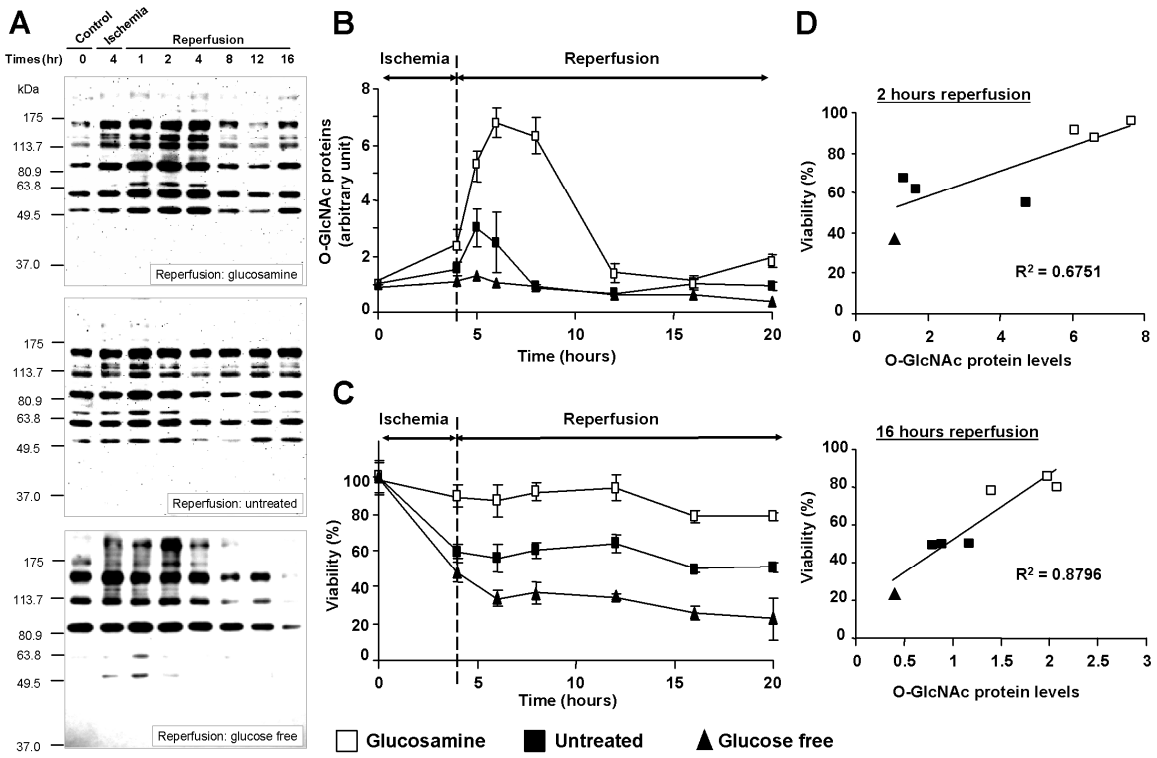

Figure 2: Time course of O-GlcNAc levels during ischemia and reperfusion. A) Representative CTD110.6 immunoblot of *O*-GlcNAc proteins; B) Mean intensity of all *O*-GlcNAc proteins determined by densitometric analysis normalized levels in normoxic control untreated cells; C) Viable cells assessed by Trypan blue exclusion during ischemia and reperfusion in control untreated cells, glucosamine treated (5 mM) cells and cells reperfused in the absence of glucose; D) Correlation of CTD density and viability after 2 and 16 hours of reperfusion. Data presented as mean±SEM of six independent experiments.

Hyperglycemia induced protection against ischemia/reperfusion mediated via HBP.

In Fig. 2 we showed that the absence of glucose during reperfusion decreased *O*-GlcNAc levels and this was associated with decreased cell survival, suggesting that glucose was required for the increase in *O*-GlcNAc in reperfusion. Since hyperglycemia increases flux through the HBP (22) and has also been shown to be protective (30) we asked whether hyperglycemia mediated protection may be due at least in part, by activation of this pathway. Hyperglycemia significantly increased cell viability and decreased NRVM apoptosis after ischemia and I/R (Fig 3A-B) and this was associated with an increase in *O*-GlcNAc levels of selected bands in the CTD110 immunoblot (Fig 3C and D). Treatment with azaserine, an inhibitor of GFAT (22) which regulates glucose entry into the HBP, blocked the protection seen with hyperglycemia and reversed the increase in *O*-GlcNAc levels. In contrast to glucosamine treatment, the increase in overall *O*-GlcNAc levels associated with hyperglycemia was less pronounced; this might be expected since glucosamine enters the HBP directly and essentially unregulated whereas glucose entry into the HBP is regulated by GFAT. Densitometric analysis of individual bands was performed (Fig 3D). Due to small sample size the apparent differences in *O*-GlcNAc levels following I/R in all bands and in Band No. 1 were not significant different. However, Bands 2, 4 and 5 all showed significant increase in the high glucose groups following I/R, which was significantly attenuated with azaserine treatment. Interestingly, hyperglycemia also increased the intensity of Bands 4 and 5 under normoxic conditions.

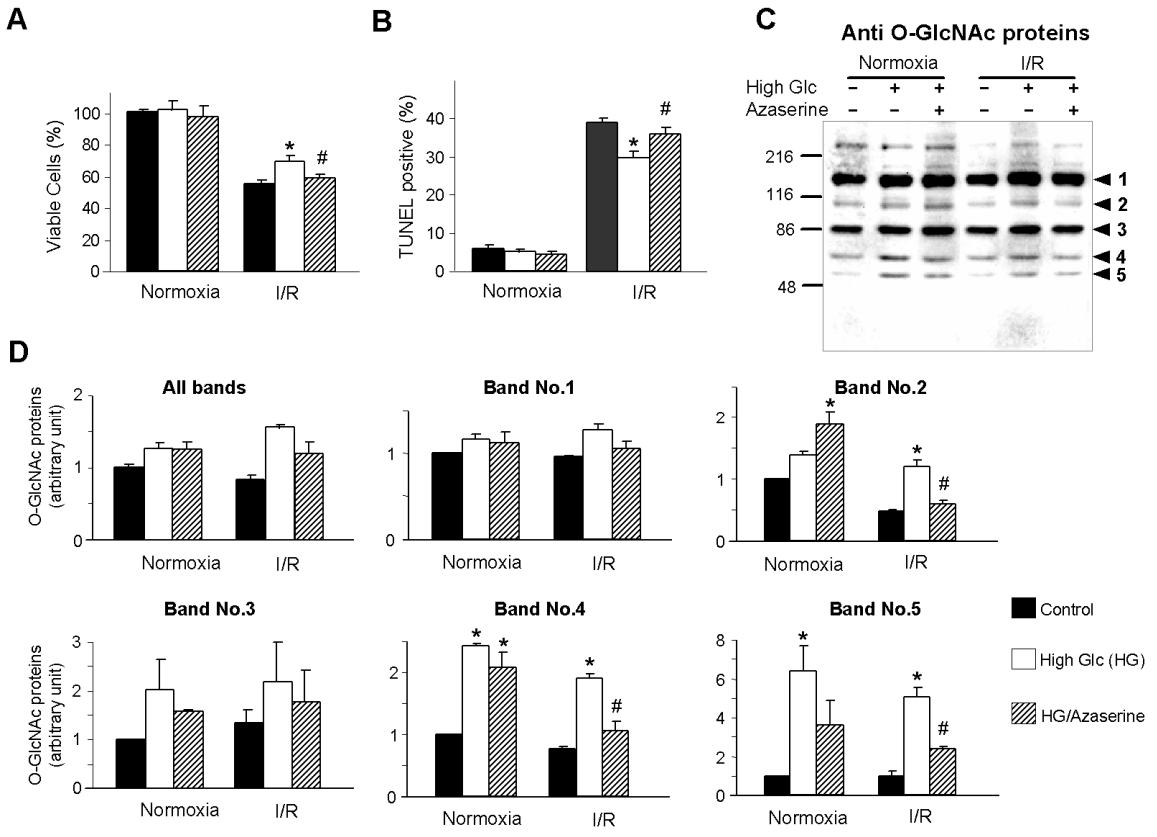

Figure 3: Hyperglycemia induced protection against ischemia/reperfusion mediated via HBP. A) Cell viability assessed by Trypan blue exclusion; B) apoptosis assessed by % TUNEL positive cells; C) Representative CTD110.6 immunoblot of *O*-GlcNAc proteins D) Mean intensity of *O*-GlcNAc proteins determined by densitometric analysis normalized levels in normoxic control untreated cells; for all bands, and bands 1-5 as indicated. Control untreated cells, cells incubated with high glucose (30mM Glucose) and high glucose plus the GFAT inhibitor azaserine (5 µM) were incubated under normoxic condition for 20 hours or subjected to 4 hours ischemia followed by 16 hours reperfusion. Data presented as mean±SEM of four independent experiments; *= $p< 0.05$ Vs control, # = $p<0.05$ Vs high glucose.

O-GlcNAcylation plays an important role in cellular survival during ischemia and reperfusion injury.

To better understand the role of *O*-GlcNAc in mediating the protection, we compared the effects of glucosamine with alloxan an inhibitor of OGT that should block the formation of *O*-GlcNAc and with O-(2-acetamido-2-deoxy-d-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc) an inhibitor of which prevents cycling of *O*-GlcNAc from proteins thereby increasing *O*-GlcNAc levels independent of HBP flux (35). Consistent with Fig 1 glucosamine increased viability and decreased injury following ischemia/reperfusion (Fig. 4A-C). In contrast, alloxan markedly reduced viability and increased both necrosis and apoptosis; whereas the effect of PUGNAc on cell viability and LDH release was similar to glucosamine, although it did not reduce apoptosis compared to untreated controls. In the *O*-GlcNAc immunoblots (Fig 4D) it is clear that at the end of I/R *O*-GlcNAc was virtually absence in the alloxan group where with PUGNAc there was a dramatic increase; however the effect of glucosamine on *O*-GlcNAc levels was less marked that that seen in Fig 1. ANOVA of the mean densitometric data (Fig. 4E) clearly indicated a significant treatment effect ($p<0.05$); however this was due to the more that 10-fold increase in *O*-GlcNAc in the PUGNAc treated group. Although at the end of I/R there was a ~50% increase in *O*-GlcNAc in the glucosamine treated group, this did not reach statistical significance. We believe that the apparently attenuated response of *O*-GlcNAc to glucosamine treatment in these experiments is most likely a consequence of the short exposure time required because of the very high levels of *O*-GlcNAc seen with PUGNAc treatment. Under normoxic conditions alloxan had a small but significant effect on viability compared to controls

(100±0.7 Vs 91±3% p<0.05), but had no effect on necrosis or apoptosis. PUGNAc had no adverse effects on viability, necrosis or apoptosis.

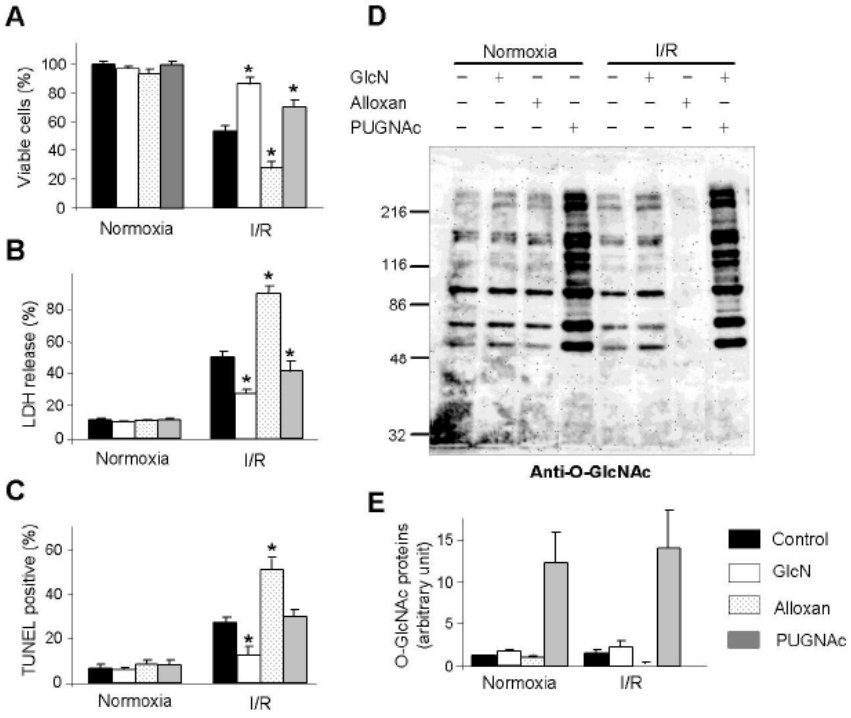

Figure 4: *O*-GlcNAcylation plays an important role in cellular survival during ischemia and reperfusion injury. A) Cell viability assessed by Trypan blue exclusion; B) necrosis assess by LDH release as a % of total LDH; C) apoptosis assessed by % TUNEL positive cells; D) Representative CTD110.6 immunoblot of *O*-GlcNAc proteins and E) mean intensity of all *O*-GlcNAc proteins determined by densitometric analysis normalized levels in normoxic control untreated cells. Control untreated cells, glucosamine treated (5mM), alloxan treated (1mM), and PUGNAc treated (100 µM) cells were incubated under normoxic condition for 20 hours or subjected to 4 hours simulated ischemia followed by 16 hours incubation under normoxic conditions. Data presented as mean±SEM of six independent experiments. * = p< 0.05 Vs control.

Increased O-GlcNAc attenuates calcium mediated nuclear translocation of NFAT during ischemia and ischemia/reperfusion.

Cytosolic Ca^{2+} levels were measured using Fura-2 in untreated and glucosamine treated NRVMs under normoxic conditions and at the end of 4 hours ischemia. In untreated cells cytosolic Ca^{2+} concentration increased from 110±12nM under normoxic conditions to 150±5 nM at the end ischemia ($p<0.05$). In glucosamine treated cells normoxic cytosolic Ca^{2+} levels were not significantly different than untreated cells (114±8nM); however, at the end of ischemia, cytosolic Ca^{2+} levels were significantly lower than untreated cells (106±7nM; $p<0.05$).

To determine whether the reduction in Ca^{2+} during ischemia in the glucosamine treated group might contribute to the reduction in injury seen in Figs 1 and 2 we assessed nuclear translocation of GFP-NFAT as an indicator of Ca^{2+}-induced calcineurin activation as previously described (15). Calcineurin has been implicated in mediating cardiomyocyte apoptosis (24, 27). As shown in Fig. 5 under normoxic conditions GFP-NFAT was restricted to the cytoplasm in >90% of the transfected cells; however, at the end of ischemia/reperfusion there was marked increase in nuclear translocation (36±2%) in untreated cells. Glucosamine treatment significantly reduced NFAT translocation compared to untreated controls (18±2%; $p<0.05$); whereas, alloxan markedly increased NFAT nuclear translocation (87±3%; $p<0.05$). Similar to glucosamine, PUGNAc treatment attenuated NFAT nuclear translocation compared to controls (13±1%; $p <0.05$). The impact of glucosamine, alloxan and PUGNAc on NFAT translocation, was remarkably similar to their effects on viability seen in Fig. 4A.

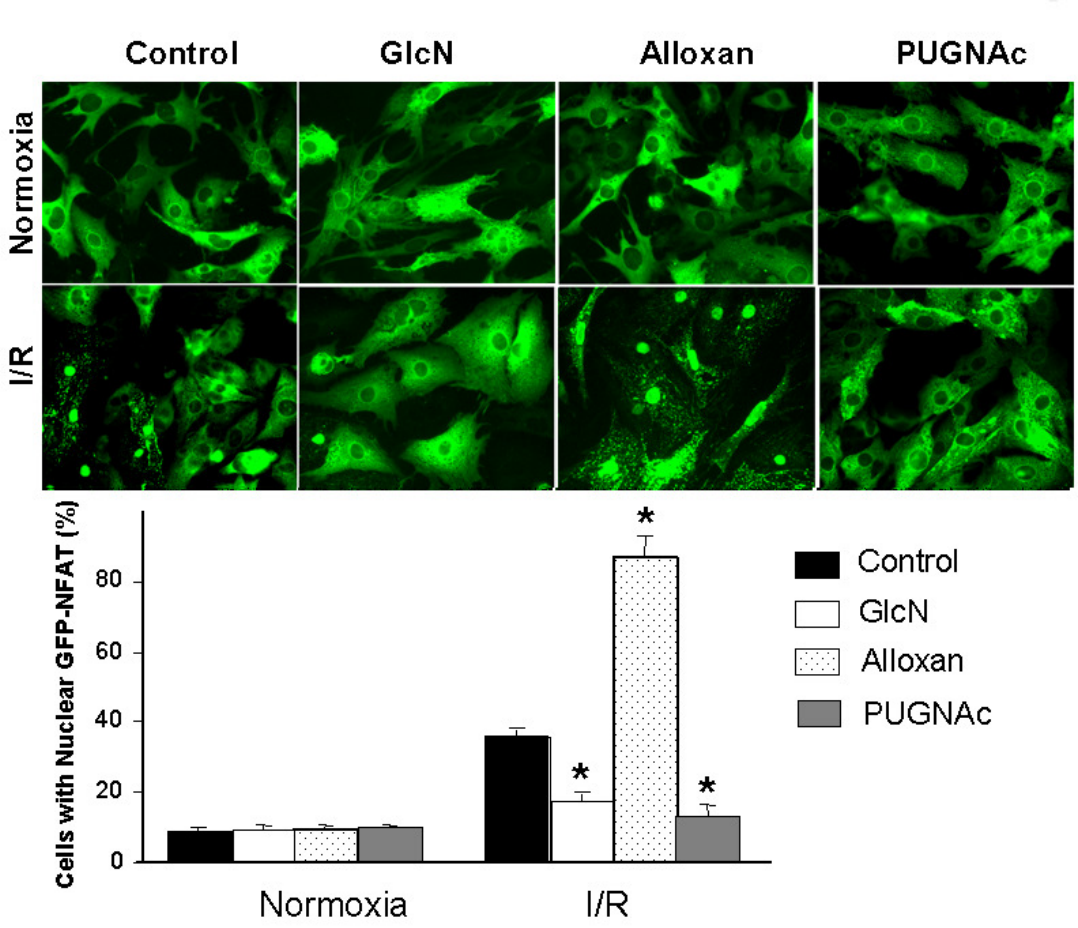

Figure 5: Increased *O*-GlcNAc attenuates calcium mediated nuclear translocation of NFAT during ischemia and ischemia/reperfusion. A) Representative images of NRVMs infected with GFP-NFAT adenovirus obtained at 40X with 495nm excitation wavelength; B) Percentages of cells with nuclear localization of GFP-NFAT. Control untreated cells, glucosamine treated (5mM), alloxan (1 mM), and PUGNAc treated (100 µM) cells were incubated under normoxic condition for 20 hours or subjected to 4 hours ischemia followed by 16 hours reperfusion under normoxic conditions (I/R). Data presented as mean±SEM of six independent experiments, 200 cells were counted for each observation. * = $p < 0.05$ Vs control.

Discussion

We show here, for the first time, that in isolated neonatal cardiomyocytes ischemia/reperfusion increased protein *O*-GlcNAc levels and that augmentation of this response was associated with increased viability, decreased necrosis and apoptosis following simulated ischemia/reperfusion. Conversely, attenuation of this response by reperfusion in the absence of glucose was associated with reduced cell viability. The protection associated with increased *O*-GlcNAc was independent of whether *O*-GlcNAc levels were elevated by increasing the flux through the HBP and OGT with glucosamine, hyperglycemia or by inhibition of *O*-GlcNAcase using PUGNAc. Further, we demonstrated that alloxan, a putative inhibitor of OGT, markedly reduced O-GlcNAc levels and increased cellular injury following ischemia/reperfusion. Taken together, these data provide compelling evidence that activation of pathways leading to increased protein *O*-GlcNAc levels is an endogenous stress response in cardiomyocytes and that alteration of this response influences survival following ischemia/reperfusion.

Zachara et al., (40) demonstrated that in COS-7 cells *O*-GlcNAc levels increased almost 2-fold within 3 hours following heat stress returning to baseline levels between 24-48 hours and they showed that this was due at least in part to an increase in OGT activity. Here we found that ischemia/reperfusion stress in untreated NRVMs increased *O*-GlcNAc levels even more quickly with a maximum level, almost 3-fold higher than baseline and that this was completely ablated when cells were reperfused in the absence of glucose (Fig 2B). This suggests that increased metabolism of glucose via the HBP is required for the increase in *O*-GlcNAc in response to ischemia and may also contribute to the increase in *O*-GlcNAc reported by Zachara et al. (40). This is supported by the fact

that increasing glucose concentrations also increased cell survival via a mechanism consistent with increased HBP flux and *O*-GlcNAc levels (Fig 3). Furthermore, the strong correlation between cell viability and *O*-GlcNAc levels during reperfusion provide additional support for the notion that the level of *O*-GlcNAc in cells is an important determinant of cell viability following a stress such as ischemia/reperfusion (Fig 2D).

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

Increasing glycolytic ATP production, especially during ischemia has been shown to decrease injury and improve recovery on reperfusion (9). Since, glucosamine could increase glycolytic flux by metabolism via glucosamine-6-phosphate isomerase or deaminase (2, 41), this may represent an alternative protective mechanism independent of alterations in O-GlcNAc levels. We therefore assessed ATP levels in control and glucosamine treated groups under normoxic conditions, at the end of ischemia and at the end of ischemia/reperfusion. If the addition of glucosamine increased glycolytic ATP synthesis we would have expected to see increased levels of ATP, especially at the end of

ischemia. However, although at the end of ischemia ATP levels were slightly increased and at the end of reperfusion they were decreased, these differences did not reach statistical significance (Table 1). Thus, these data suggest that the protection seen with glucosamine was not due to increased glycolytic ATP synthesis during ischemia. It is important to note that while maintenance of ATP levels either during ischemia or reperfusion, is often associated with improved functional recovery and decreased injury, this is not a prerequisite for ischemic protection. For example, although ischemic preconditioning significantly improves function recovery compared to control hearts following ischemia and reperfusion, ATP hydrolysis during ischemia was reported to be increased (16) and following reperfusion ATP levels were not increased in preconditioned group (4).

Stimulation of myocardial glucose utilization has repeatedly been shown to afford protection against ischemic injury (33); however, there is little consensus regarding the mechanism(s) underlying this protection. Here we found that hyperglycemia was protective against ischemia/reperfusion in NRVMs (Fig 3) and that this protection was associated with an increase in *O*-GlcNAc levels and that when glucose entry into the HBP was inhibited with azaserine, this attenuated both the protection and the increase in *O*-GlcNAc (Fig 3). Thus, while we cannot exclude changes in energy metabolism playing a role in hyperglycemic protection, these data suggest this protection may be mediated at least in part by increased HBP flux and *O*-GlcNAc levels. The fact that the increase in *O*-GlcNAc seen with hyperglycemia was less pronounced that that seen with glucosamine is not surprising, since glucosamine enters the HBP directly and essentially unregulated whereas glucose entry into the HBP is regulated by GFAT. However, it is noteworthy

that in the hyperglycemia experiments densitometric analysis showed differential response of individual bands in the CTD110 immunoblot, suggesting that the protection might be mediated via specific proteins rather than a global increase in O-GlcNAc levels.

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

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

We show here that alloxan a putative inhibitor of OGT (17) markedly reduced *O*-GlcNAc levels at the end of I/R reperfusion and this was associated with increased cellular injury. We also showed that the absence of glucose during reperfusion also attenuated the increase in *O*-GlcNAc levels and reduced cell viability. These data support the notion that metabolism of glucose via the HBP and the formation of *O*-GlcNAc is a normal stress response and inhibition of these pathways decreases the tolerance to ischemia/reperfusion stress. However, it is important to note that alloxan is a uracil analog and thus we cannot rule out potential non-specific effects of alloxan (34).

Nevertheless, in the absence of ischemia/reperfusion alloxan had minimal effects on viability and no effects on necrosis or apoptosis. Since ablation of the OGT gene is embryonically lethal (31), more specific demonstration of the role of OGT in mediating glucosamine cardioprotection will require the development of tissue-specific, conditional knockout mice or the use of siRNA approaches to reduce OGT expression in cardiomyocytes.

There was a significant correlation between *O*-GlcNAc levels and cell viability in untreated cells or when glucosamine was used to increase *O*-GlcNAc (Fig 2); however PUGNAc did not lead to greater protection despite the fact that *O*-GlcNAc levels were markedly higher that the glucosamine treated group (Fig 4). Indeed, if anything, PUGNAc appeared to be somewhat less protective than glucosamine. It has been reported that acceptor protein specificity changes with hyperglycemia or glucosamine treatment (19, 36) and we found that glucosamine treatment and PUGNAc lead to increased *O*-GlcNAc levels in different protein bands. Thus, the fact that PUGNAc was not as protective as glucosamine despite the higher overall level of *O*-GlcNAc may be due to different proteins being *O*-GlcNAcylated in response to the different interventions. Further, Zachara and Hart (39) proposed that high as well as low levels of *O*-GlcNAc might trigger apoptosis. Consequently, the dose and duration of PUGNAc treatment used here may have resulted in an excessive level of *O*-GlcNAc that was sustained for the entire reperfusion period. In contrast, while glucosamine treatment increased *O*-GlcNAc levels 7-8 fold during reperfusion (Fig 2), this was a transient response and at the end of reperfusion *O*-GlcNAc levels were only approximately 2-fold higher than the untreated group. This suggests that protection may be a result not only of increased *O*-GlcNAc

levels on specific proteins, but also on the duration of the increase in *O*-GlcNAc levels. Cleary further studies are warranted not only to identify the specific proteins involved in *O*-GlcNAc mediated protection, but also to better understand the dynamics of *O*-GlcNAcylation in response to stress.

In conclusion, we have shown that increasing *O*-GlcNAc levels with glucosamine, high glucose or PUGNAc are all associated with increased cell survival following ischemia and reperfusion. We also found that ischemia/reperfusion increased *O*-GlcNAc levels in untreated cells, which were inhibited by reperfusion in the absence of glucose and that this was associated with lower viability. Further, the improved cell survival in glucosamine and PUGNAc treated cells was associated with reduced cytosolic Ca^{2+} levels during ischemia and ischemia/reperfusion. These data support the concept that, activation of metabolic pathways leading to an increase *O*-GlcNAc levels is an endogenous stress activated response, and that augmentation of this response improves cell survival. Thus, strategies designed to activate these pathways may represent novel interventions for inducing cardioprotection.

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CHAPTER 3

GLUCOSAMINE PROTECTS NEONATAL CARDIOMYOCYTES FROM ISCHEMIA-REPERFUSION INJURY VIA INCREASED PROTEIN *O*-GlcNAc AND INCREASE MITOCHONDRIAL BCL-2

by

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Abstract

We have previously reported that glucosamine protected neonatal rat ventricular myocytes (NRVMs) against ischemia/reperfusion (I/R) injury, and this was associated to an increase in protein *O* linked-N-acetylglucosamine (*O*-GlcNAc) levels. However, the protective effect of glucosamine could be mediated via pathways other that *O*-GlcNAc formation; thus, the initial goal of this study was to determine whether increasing *O*-GlcNAc transferase (OGT) expression, which catalyzes the formation of O-GlcNAc, had similar protective effect as glucosamine. To better understand the potential mechanism underlying *O*-GlcNAc-mediated cytoprotection we examined whether increased *O*-GlcNAc levels altered the expression and translocation of members of the Bcl-2 protein family. Both glucosamine (5mM) and OGT overexpression increased basal and I/R induced *O*-GlcNAc levels, significantly decreased cellular injury, and attenuated loss of cytochrome C. Both interventions also attenuated the loss of mitochondrial membrane potential induced by H_2O_2 and were also associated with an increase in mitochondrial Bcl-2 levels, but had no effect on Bad on Bax levels. Compared to glucosamine and OGT overexpression, NButGT (100 μM), an inhibitor of *O*-GlcNAcase, was less protective against I/R and H_2O_2 and did not affect Bcl-2 expression, despite a 5 to 10 fold greater increase in overall *O*-GlcNAc levels. Decreased OGT expression resulted in lower basal *O*-GlcNAc levels, prevented the I/R induced increase in O-GlcNAc and mitochondrial Bcl-2 and increased cellular injury. These results demonstrate that the protective effects of glucosamine are mediated via increased formation of *O*-GlcNAc and suggest that this is due in part to enhanced mitochondrial Bcl-2 translocation.

Introduction

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

However, in addition to increasing *O*-GlcNAc levels, glucosamine also increases UDP-GlcNAc levels, glucosamine-6-phosphate levels and could potentially be metabolized to fructose-6-phosphate thereby increasing glycolytic flux. Thus, in addition to increasing *O*-GlcNAc levels, the protection associated with glucosamine treatment in cardiomyocytes and the intact heart could be mediated via a number of other pathways. Support for the hypothesis that the protective effect of glucosamine is mediated by *O*-GlcNAc levels, was provided by studies showing that increasing *O*-GlcNAc levels with PUGNAc an inhibitor of *O*-GlcNAcase had similar effects to glucosamine. However, while the inhibition of *O*-GlcNAcase with PUGNAc is frequently used to increase cellular *O*-GlcNAc levels, it also inhibits other ß-hexosamindases and thus will alter processing of glycoconjugates in addition to *O*-GlcNAc. We have also found that the pattern of *O*-GlcNAc modified proteins is different in glucosamine and PUGNAc treated

cardiomyocytes. This suggests that these two different methods for increasing cellular *O*-GlcNAc levels may not have equal effects on cell function, which may be a consequence of their actions on other pathways.

Importantly, the mechanisms underlying the protection associated with increased protein *O*-GlcNAc levels have yet to be determined. Zachara et al., reported that increased survival seen with elevated *O*-GlcNAc levels was associated with increased expression of Hsp70; in contrast Sohn et al., reported improved survival associated with increased *O*-GlcNAc levels without any change in Hsp70 levels, which suggests that other mechanism(s) may also contribute to the protection associated with increased *O*-GlcNAc levels. We found that hyperglycemia-mediated protection of cardiomyocytes against ischemic injury also appeared to be mediated, at least in part, by increased *O*-GlcNAc levels. It has also been reported that hyperglycemia-induced protection against hypoxic injury induced apoptosis and necrosis was associated with upregulation of the antiapoptotic factor Bcl-2.

Apoptosis, a genetically programmed form of cell death, contributes to myocyte cell loss in a variety of cardiac pathologies, including cardiac failure and those related to ischemia/reperfusion injury. The apoptotic program is complex, involving both pro- and anti-apoptotic proteins, and apoptosis occurs when the equilibrium between these opposing factors is perturbed. The pro- and anti-apoptotic members of the Bcl-2 family are intrinsic to the apoptotic pathway; Bcl-2 and Bcl-xL protect cells from apoptosis, whereas Bax and Bad promote the response. It has been reported that increased Bcl-2 expression is significantly limits cell death after acute myocardial infarction or during cardiac post-transplant ischemia/reperfusion injury. Several lines of evidence have also

shown that members of the Bcl-2 protein family are associated with the loss of apoptogentic factors, including release of cytochrome c from the intermembrane space of the mitochondria into cytosol. Many, if not all, apoptotic responses involve mitochondrial dysfunction and a loss of the mitochondrial membrane potential. Thus, anti-apoptotic Bcl-2 may function in the mitochondrial membrane to prevent loss of cytochrome c and thus inhibit apoptosis.

Therefore, the goals of this study were to determine, whether increased OGT expression had similar effects as glucosamine treatment on the response to ischemic injury and acute oxidative stress. Studies were also performed with NButGT, an inhibitor of *O*-GlcNAcase, which has ~1500-fold greater specificity for *O*-GlcNAcase over ß-hexosaminidase than PUGNAc. To better understand the potential mechanism underlying O-GlcNAc-mediated cytoprotection we examined whether increased *O*-GlcNAc levels altered the expression and translocation of members of the Bcl-2 protein family. Finally, we also examined the consequences of decreased OGT expression on the response to ischemic injury.

Experimental procuders

Materials: Unless otherwise noted, except for glucosamine (Fluka) all chemicals were purchased from Sigma Chemical (St. Louis, MO). Culture medium products were purchased from GIBCO Invitrogen (Grand Island, NY). Adenovirus containing *O*-GlcNAc transferase was a kind gift from Dr. W.H. Dillmann (Department of Medicine, University of California, San Diego). 1,2-dideoxy-2'-methyl-alpha-d-glucopyranoso[2,1-d]-Delta2'-thiazoline (NButGT), an inhibitor of *O*-GlcNAcase was a kind gift from Dr.

David Vocadlo (Simon Fraser University, Burnaby, BC, Canada). NButGT has ~1500-fold greater specificity for *O*-GlcNAcase over ß-hexosaminidase than PUGNAc.

Neonatal rat ventricular myocyte (NRVM) primary cultures: Animal experiments were approved by the University of Alabama Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Usage of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, 1996). Primary cultures of neonatal rat ventricular myocytes (NRVMs) were obtained from 2-3 days old neonatal Sprague Dawley rats and cultured as described previously. NRVMs were grown in collagen-coated plates in the culture growth medium containing 15% fetal bovine serum (FBS) on the first day. On the next day, medium was replaced and cells were grown in the culture growth medium without FBS. Within 1-2 days of isolation, a confluent monolayer of spontaneously beating NRVMs had formed and cells were used as described below.

Ischemia and reperfusion: Ischemia and reperfusion was induced as described previously. Briefly, following 2 days in culture, NRVMs were exposed to ischemia by adding a fresh Esumi modified ischemic medium (137 mM NaCl, 12 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2.2H_2O$, 4 mM HEPES and 20 mM sodium lactate, pH 6.2) and then incubated in the chamber atmosphere of 95% argon and 5% CO_2 for 4 hours. Following 4 hours of ischemia, cells were returned to the culture growth medium (serum free 4:1 (v/v) Dulbecco's modified Eagle's medium (DMEM) / Medium 199 with Hanks salts (M199), supplemented with 2% Nutridoma and 1% penicillin/streptomycin) and then incubated in

an incubator atmosphere of 5% CO_2 for 2 hours. In control normoxia experiments, cells were incubated with fresh culture growth medium in an incubator atmosphere of 5% CO_2 for 6 hours.

Cell injury in response to ischemia/reperfusion was determined as previously described. Necrosis was assessed by measuring the release of lactate dehydrogenase (LDH) in culture medium and LDH in remaining attached cells using an LDH assay kit (Sigma). NRVMs ($1x10^6$ cells) were seed into a multi 12 well-plate (Falcon). The percent LDH release was calculated by the ratio of the released LDH into the media by the total LDH (release plus cellular content) at the end of treatment. Apoptosis was determined by using In Situ Cell Death Detection Kit (Roche). NRVMs ($0.2\text{-}0.3x10^6$ cells) were seed into a 4-chambered cover glass (Lab-Tek). At the end of treatments, permeabilized cells were exposed to the TUNEL reaction mixture for 1.5 hour and were counter stained with 0.1 mg/ml of Hoechst 33258 (Invitrogen). In each treatment, a total of at least 200 cells were counted through a 40x objective with excitation wavelength at 528 nm.

Assessment of Protein O-GlcNAc and cytochrome C levels by Immunofluorescence: At the end of treatment, NRVMs were fixed with 3.7% Formaldehyde in PBS for 30 min at room temperature (RT). After washing cells with 3 times PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min on ice. Permeabilized cells were exposed to the with O-GlcNAc antibody, CTD 110.6 (Covance) at 1:50 and cytochrome C (556432, BD Pharmingen) in 3%FBS/PBS for 1 hour at room temperature. After washing cells with 3 times of PBS, cells were incubated with secondary antibodies; Alexa-Flour(R) 594 goat anti-mouse IgM (Invitrogen) at 1:200 for CTD 110.6 and Alexa-Flour(R) 488 goat

anti-mouse IgG (Invitrogen) at 1:200 for cytochrome C in 3%FBS/PBS for 1 hour at RT. After washing cells with PBS, cells were stained with 0.1 mg/ml of Hoechst 33258 (Invitrogen). Cells were visualized through a 20x objective with excitation wavelength at 528 nm for cytochrome C staining, 623 nm for *O*-GlcNAc staining, and 456 nm for Hoechst nuclear staining using an inverted fluorescent microscope (Olympus).

Immunoblot analysis: At the end of treatment, cells were lyzed with 1xRIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA and 0.1% SDS) containing 2% protease inhibitor cocktail (Sigma) on ice for 30 min. Lysed proteins were harvested and assayed for protein concentration using the Bio-Rad protein assay kit. Proteins (10 μg) were separated on 7.5% or 12% SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Milipore). After transferring, the blotted were soaked in 100% methanol and dried completely under the hood. Dried blots were probed with the following antibodies; anti-*O*-GlcNAc antibody CTD110.6 (Covance) at 1:2000, RL2 (Affinity Bioreagents) at 1:2000, OGT (Sigma) at 1:1000, β-actin (sigma) at 1:20000, Bcl-2 (Santa Cruz) at 1:200, Bax (Santa Cruz) at 1:200, Bad (Cell signaling) at 1:1000, COX4 (Abcam) at 1:20000, and GAPDH (Abcam) at 1:5000. Blots and antibodies were incubated in 1x PBS/casein blocker (Pierce) containing 0.01% Tween 20 for 2 hours at room temperature. After washing three times with PBS, the membrane was then incubated with an appropriate secondary antibody; goat anti-mouse IgM (Calbiochem) for anti-O-GlcNAc CTD110.6, goat anti-mouse IgG, or goat anti-rabbit IgG (Santa Cruz) at the same blocking buffer for 1 hour at room temperature. After further washing in PBS, the immunoblots were developed with

enhanced chemiluminescence (Super Signal West Pico or Femto Maximum Sensitivity; Pierce) and visualization was performed using Bioimaging system of UVP (UVP, Inc., CA). Densitometric analysis was performed on the entire lane of each sample using LabWorks analysis software (UVP, Inc., CA) and the mean intensity normalized to the control group.

Mitochondrial fractionation: NRVMs ($10x10^6$ cells) were seeded into a 10 cm tissue culture dishes (Falcon) and mitochondria were prepared using a Mitochondrial/Cytosol Fractionation Kit (K256, BioVision) with slight modifications. Briefly, NRVMs were washed with PBS and resuspended in 200 µl of 1x Cytosol Extraction Buffer on ice for 10 minutes. Cells were homogenized and then centrifuged at 700x g for 10 minutes at 4^oC and the supernatant centrifuged at 10,000x g for 30 minutes at 4^oC. At the end of this second centrifugation, the supernatant (post-mitochondria), consisting of the cytosolic/microsomal fraction was collected for subsequent immunoblot analysis and the pellet (mitochondria) was resuspended in 50 µl of Mitochondrial Extraction Buffer. Lysed proteins were assayed for protein concentration using the Bio-Rad protein assay kit. Proteins (3-5 µg) were separated on 12% SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Milipore). Immunoblot analysis was performed as described above.

Electroporation of siRNA oligonucleotides: siRNA oligonucleotides, *O*-GlcNAc transferase (OGT) and negative control (Silencer® negative control 1) were purchased from Ambion Inc. (Austin, TX). The sense and antisense sequences of si-OGT were 5'-

CCCUUGACCCAAUUUUCUtt-3' and 5'-AGAAAUUUGGGUCAAGGGtg-3', respectively. Transfection of the siRNA oligos into neonatal cardiomyocytes was carried out as described according to the manufacturer's instructions (Amaxa Inc.) with slight modification. Briefly, one day after cell isolation, attached cardiomyocytes (10^7 cells in a 10cm culture dish) were detached by trypsin-EDTA solution (Sigma) and resuspended in the transfection reagent Nucleofector kit (Amaxa Inc.). A total of 2 μg of siRNAs per 2 x 10^6 cells were transfected using the Nucleofector II Device (Amaxa Inc.) according to the manufacturer's instructions. After transfection, cells were grown in the culture medium containing 15% FBS and on the next day, medium was replaced and cells were grown in the culture growth medium without FBS. Approximately 2 days following transfection OGT levels were confirmed by western blotting as described above and cells were subject to ischemia/reperfusion.

Assessment of mitochondrial membrane potential: Mitochondrial function was assessed by using JC-1 reagent, Mitochondrial Membrane Potential detection Kit as described according to the manufacturer's instructions (Stratagene). After isolation NRVMs were seeded into a 4 chambered coverglass (Lab-Tek) and pre-treated with a unique fluorescent cationic dye, 5,5',6,6'-terachloro-1,1,3,3'-tetraethyl-0-benzamidazolocarbocyanin iodide, commonly known as JC-1 for 15 minutes followed by washing with assay buffer to remove remaining reagent. 500 μl of the culture growth medium was added to the cells, which were then visualized through a 20x objective with excitation wavelength at 623 nm to detect Texas Red dye and at 528 nm to detect rhodamine using an inverted fluorescent microscope (Olympus). In living cells, JC-1

exists as a monomer in the cytosol, which exhibits green fluorescence, and also accumulates as aggregates in the mitochondria, where it exhibits red fluorescence. In apoptotic and dead cells, JC-1 exists in the monomeric form only, staining the cytosol green. Mitochondrial membrane potential (MMP) was monitored before and addition of hydrogen peroxide (1mM final concentration). A reduction in the ratio of red to green fluorescence indicated a fall in MMP. The ratio of red and green intensity of cells incorporating JC-1 dye was measured by using IPLab version3.6 analysis software (BD Biosciences).

Statistics: All data are presented as means±SEM. Unpaired T-tests, one-way and repeated measure ANOVA were used where appropriate followed by a Bonferroni's multiple comparison test using Prism 4.0c (GraphPad Software Inc., San Diego CA). Statistically significant differences between groups were defined as $P < 0.05$.

Results

Glucosamine reduces cellular injury during ischemia/reperfusion and increases O-GlcNAc levels.

We have previously reported that in NRVMs glucosamine decreased both necrosis and apoptosis following ischemia/reperfusion and that the increase in *O*-GlcNAc levels was maximal at 2 hours of reperfusion following 4 hours of ischemia. Therefore, in this study we focused on the 2 hr reperfusion time point to investigate the effects of ischemia/reperfusion on protein and cellular function. In Fig. 1A, we demonstrate that,

consistent with our earlier study, glucosamine decreased cell injury following ischemia/reperfusion. Using two *O*-GlcNAc antibodies, which recognize different *O*-GlcNAc motifs (CTD110.6 and RL2) we confirm that ischemia/reperfusion alone significantly increases overall *O*-GlcNAc levels in the control, untreated group (Fig. 1B) (CTD: 2.55±0.47 Vs 1.07±0.06, $P<0.05$) and (RL2: 1.29±0.02 Vs 0.97±0.10, $P<0.05$). Consistent with our earlier study, glucosamine treatment markedly augmented the response to ischemia/reperfusion significantly compared to the untreated groups (CTD: 4.89±0.37 Vs 2.55±0.47 and RL2: 2.03±0.43 Vs 1.29±0.02). Equal protein loading was confirmed by densitometric analysis of β-actin levels.

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

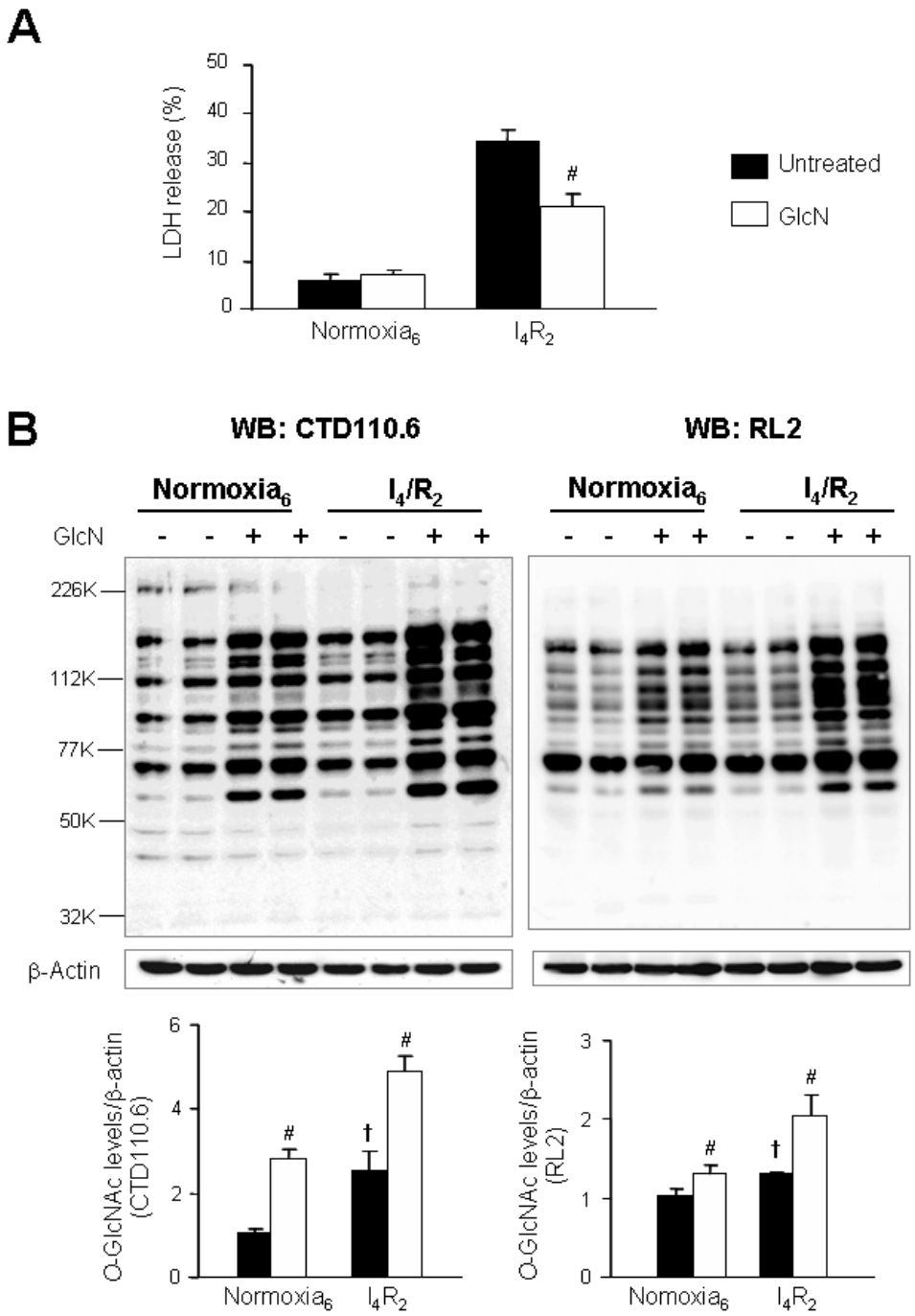

Figure 1: Increasing global *O*-GlcNAc protein levels by glucosamine (GlcN) reduced cellular injury during ischemia and reperfusion injury. A) Cell injury assessed by determining lactate dehydrogenase (LDH) release as a percentage of total LDH; B) Representative CTD110.6 and RL2 immunoblot of *O*-linked N-acetylglucosamine (*O*-

GlcNAc) proteins and mean intensity of all *O*-GlcNAc proteins determined by densitometric analysis. Equal protein loading was normalized by densitometric analysis of β-actin levels. *O*-GlcNAc levels are normalized to normoxic control untreated cells. Control untreated cells and glucosamine (5 mM)-treated cells were incubated under normoxic condition for 6 h or subjected to 4 h of ischemia followed by 2 h of reperfusion (I_4/R_2). Data are presented as means±SEM of more than 3 experiments. # =P< 0.05 Vs untreated, † P< 0.05 Vs normoxia.

Glucosamine mediates translocation of mitochondrial Bcl-2.

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

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

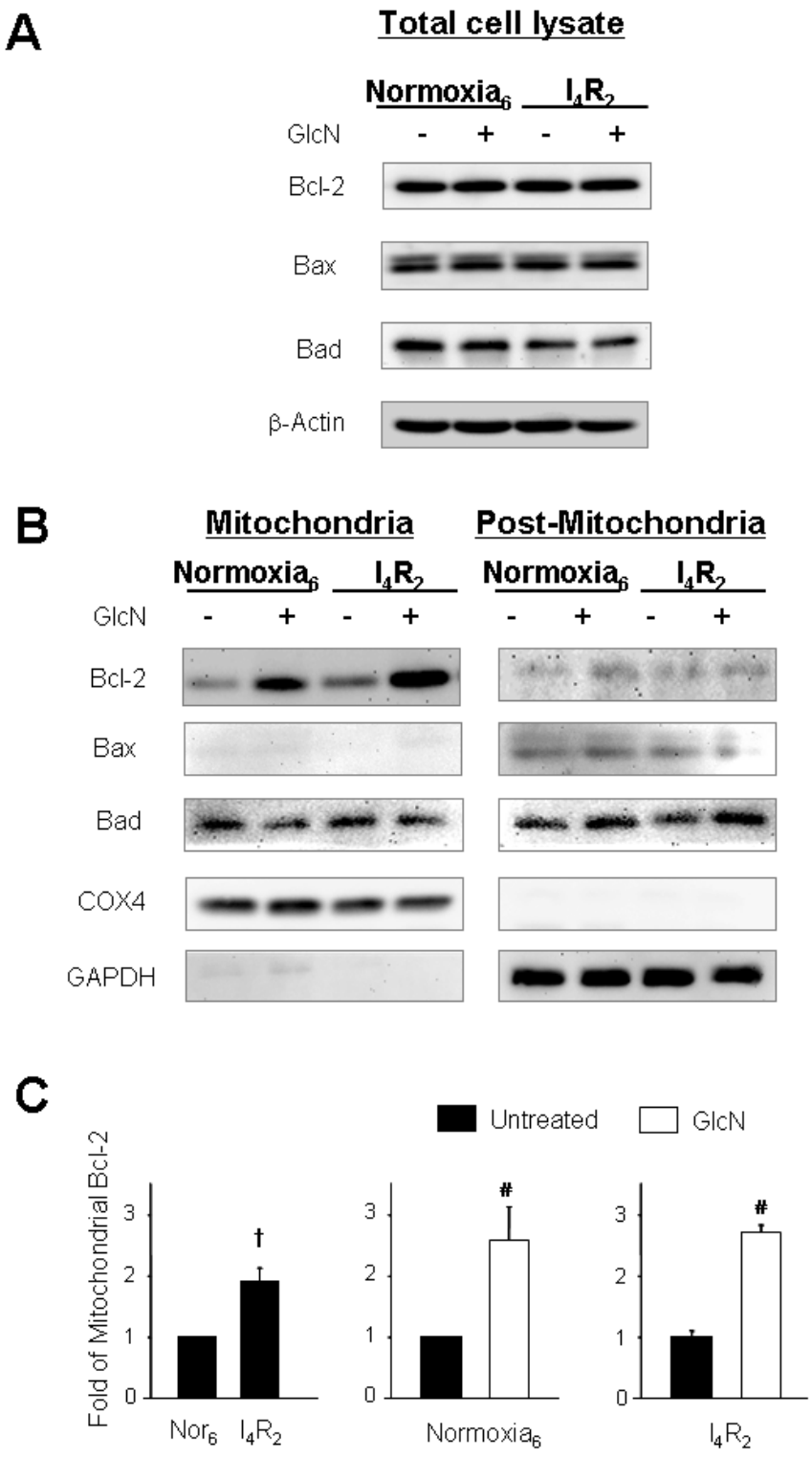

Figure 2: Glucosamine had no effect on Bcl-2 family proteins, Bcl-2, Bax, and Bad, but enhanced mitochondrial Bcl-2 translocation. A) Representative Bcl-2, Bax, and Bad

immunoblots of the whole cell lysate of NRVMs. Equal protein loading was confirmed by β-actin levels; B) Representative Bcl-2, Bax, and Bad of the mitochondrial and post-mitochondrial (cytosolic/microsomal) fraction of NRVMs. Purity of each fraction was assessed by GAPDH and COX4, respectively; C) Mean intensity of mitochondrial Bcl-2 determined by densitometric analysis. Levels are normalized to normoxic control untreated cells. Control untreated cells and glucosamine (5 mM)-treated cells were incubated under normoxic condition for 6 h or subjected to 4 h of ischemia followed by 2 h of reperfusion (I_4/R_2). Data are presented as means±SEM of more than 3 experiments. # $P< 0.05$ Vs untreated, † $P< 0.05$ Vs normoxia.

The effect of increased OGT expression and O-GlcNAcase inhibition on response of NRVMs to ischemia/reperfusion.

Since glucosamine may affect cell function independent of increased *O*-GlcNAc formation, we asked whether increasing OGT expression levels would also afford protection against ischemia/reperfusion injury and if so whether this also associated with increased mitochondrial Bcl-2. In Fig 3A it can be seen that transfection of NRVMs with the OGT adenovirus increased OGT expression levels 4-5 fold and this was associated with increased *O*-GlcNAc levels under normoxia and following ischemia/reperfusion. Unlike glucosamine treatment ischemia/reperfusion did not increase *O*-GlcNAc levels further in the OGT transfected group. Interestingly, however, the increase in *O*-GlcNAc achieved by OGT transfection was ~4-5 fold compared to non-transfected cells in both conditions, which was similar to the increase seen in the glucosamine treated group

following ischemia/reperfusion (Figs 1A, 3B). Increased OGT expression significantly attenuated cell injury following ischemia/reperfusion as indicated by reduced LDH release (Fig 3B). Consistent with glucosamine treatment, increased OGT expression was also associated with increased mitochondrial Bcl-2 expression (Fig 3C).

Inhibition of *O*-GlcNAcase, which is responsible for removing *O*-GlcNAc from proteins, is another means by which cellular *O*-GlcNAc levels can be increased. We have previously shown that the *O*-GlcNAcase inhibitor PUGNAc increased *O*-GlcNAc levels to a much greater extent than glucosamine; however, it was less protective. While PUGNAc is typically used as an *O*-GlcNAcase inhibitor it also inhibits lysosomal ß-hexosaminidases, which could also affect the response of cardiomyocytes to ischemia/reperfusion. Therefore, we examined the effect of NButGT a new *O*-GlcNAcase inhibitor, which shows ~1500-fold greater specificity for *O*-GlcNAcase over ß-hexosaminidase than PUGNAc. NButGT treatment increased *O*-GlcNAc levels more than 10-fold compared to untreated cells (Fig 3A); however, while it decreased LDH release compared to untreated cells, this effect was significantly attenuated compared to increased OGT expression (Fig 3B) and glucosamine treatment (Fig 1A). Interestingly, NButGT treatment did not increase mitochondrial Bcl-2 expression and blocked the ischemia/reperfusion induced increase in mitochondrial Bcl-2 seen in untreated cells (Fig 3C).

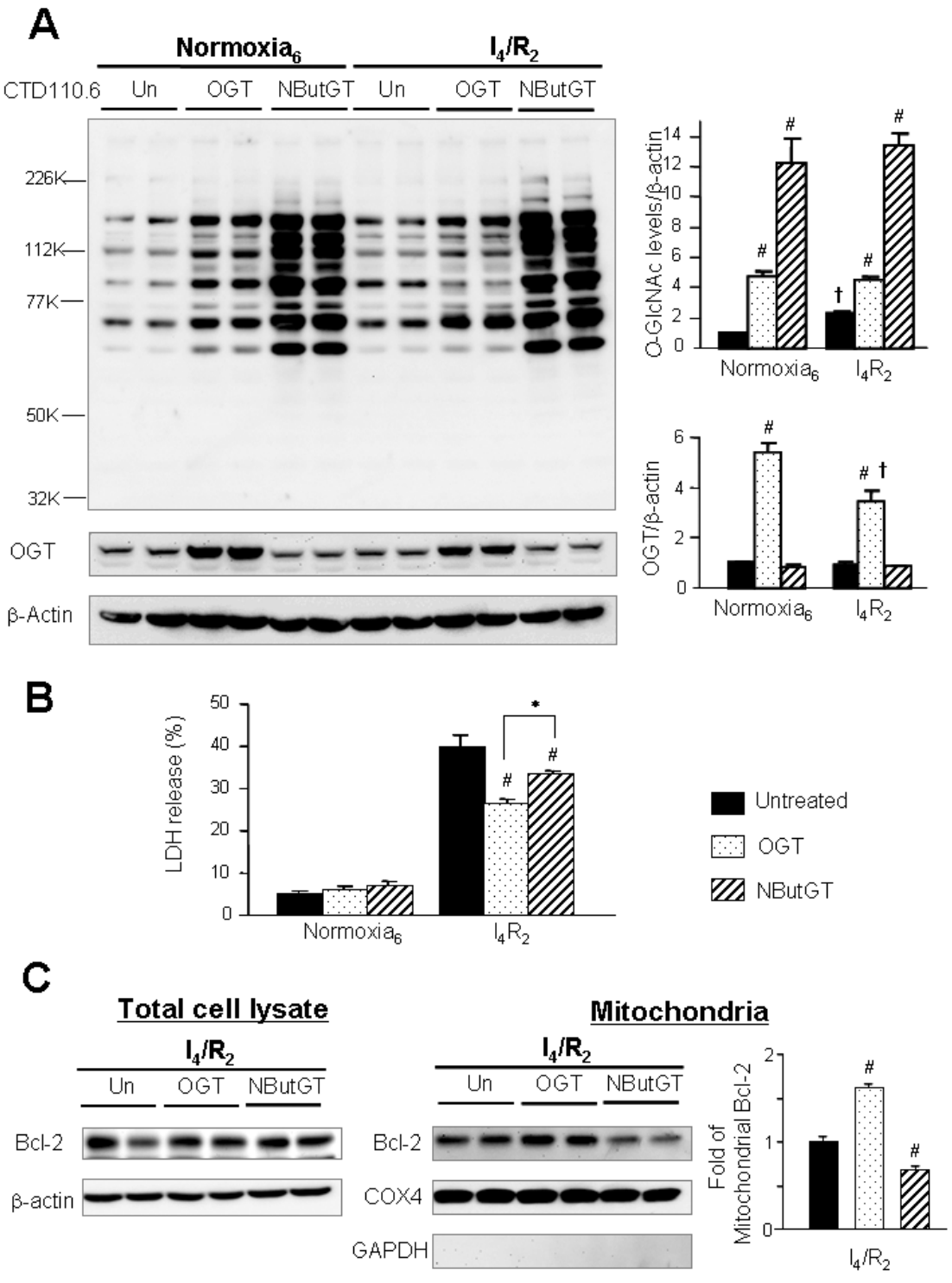

Figure 3: Increasing *O*-GlcNAc transferase (OGT) and blocking *O*-GlcNAcase by NButGT had different effects on *O*-GlcNAc protein modification, cellular survival and

Bcl-2 translocation. A) Representative immunoblotting CTD110.6 and OGT and mean intensity of all *O*-GlcNAc proteins and OGT of the total whole cell lysate determined by densitometric analysis. Equal protein loading was normalized by densitometric analysis of β-actin levels. Proteins are normalized to normoxic control untreated cells; B) Cell injury assessed by determining lactate dehydrogenase (LDH) release as a percentage of total LDH; C) Representative immunoblotting of Bcl-2 expression levels in total cell lysate and mitochondrial fractions and mean densitometric analysis of mitochondrial Bcl-2. Purity of mitochondrial fraction was assessed by COX4 and GAPDH. Control untreated cells, *O*-GlcNAc transferase (OGT) adenovirus infected cells, NButGT (100 μM)-treated cells were incubated under normoxic condition for 6 h or subjected to 4 h of ischemia followed by 2 h of reperfusion (I_4/R_2). Data are presented as means±SEM of more than 3 experiments. # =P< 0.05 Vs untreated, † P< 0.05 Vs normoxia, * P< 0.05 Vs OGT overexpression.

Attenuation of cytochrome C release by glucosamine and increased OGT expression.

It has been reported that the balance of anti- and pro- apoptotic Bcl-2 family proteins that reside in the outer mitochondrial membrane play an important role in regulating mitochondria-mediated apoptosis. One characteristic of mitochondria-mediated apoptosis is the release of cytochrome C from the mitochondria into the cytosol. However, using cell fractionation and immunoblotting techniques, we observed very low cytosolic cytochrome C levels following ischemia/reperfusion. Since there is significant loss of cytosolic proteins in this model of ischemia/reperfusion, as indicated by LDH release (Fig 1A), the low levels of cytosolic cytochrome C could be due to loss of the

protein from the cell. Indeed, we found appreciable levels of cytochrome C in the media following ischemia/reperfusion, which was attenuated by 50% in the glucosamine treated group (data not shown).

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

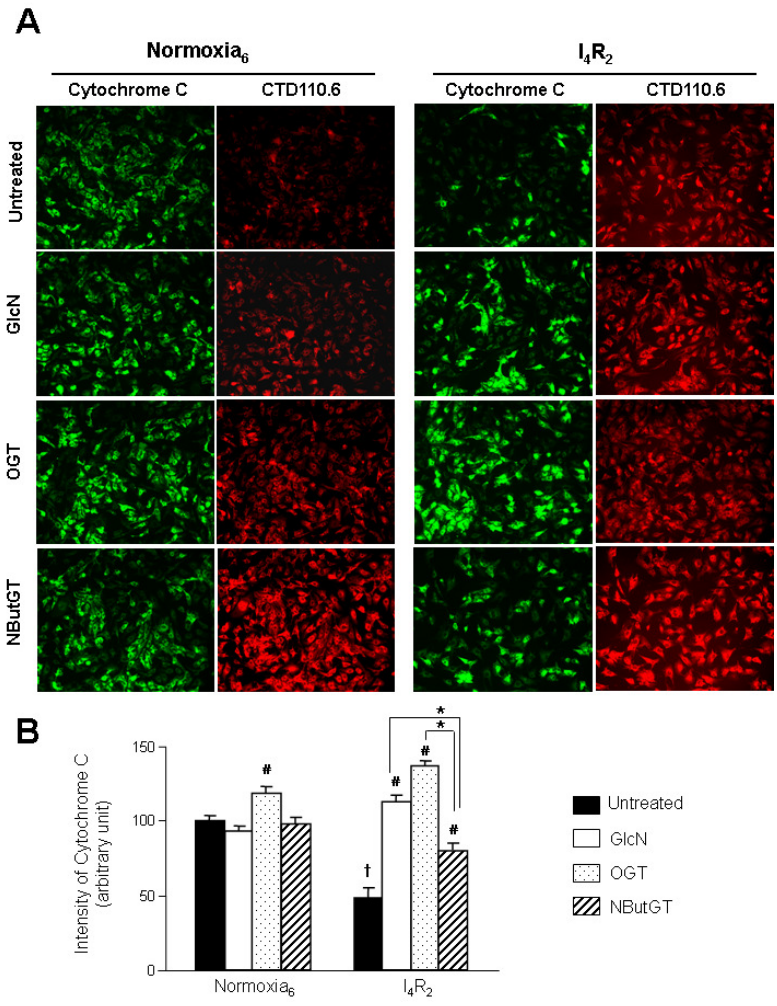

Figure 4: Increasing global *O*-GlcNAc proteins protected the loss of cytochrome C. A) Representative immunofluorescence of cytochrome C and *O*-GlcNAc proteins (CTD 110.6) under normoxia and following ischemia/reperfusion; B) Mean intensity of cytochrome C determined by IPLab analysis software was normalized with the control untreated cells. Data are presented as means±SEM of 150-200 cells. Control untreated cells, glucosamine (5 mM)-treated cells, *O*-GlcNAc transferase (OGT) adenovirus infected cells, NButGT (100 µM)-treated cells were incubated under normoxic condition for 6 h or subjected to 4 h of ischemia followed by 2 h of reperfusion (I_4/R_2). # = $P< 0.05$ Vs untreated, * $P< 0.05$ Vs NButGT.

Increasing O-GlcNAc levels attenuates loss of hydrogen peroxide-induced loss of mitochondrial membrane potential.

Release of cytochrome C from the mitochondria is usually associated with opening of the mitochondrial membrane transition pore (mMTP), which is a critical step in mitochondria-mediated apoptosis. Opening of the mMTP is characterized by loss of mitochondrial membrane potential; therefore, we asked whether increasing *O*-GlcNAc levels attenuated the loss of mitochondrial membrane potential in response to transient exposure to hydrogen peroxide (H_2O_2). Cardiomyocytes were pre-treated with the fluorescent cationic dye, JC-1, which exhibits a red fluorescence in healthy cells with intact mitochondrial membrane potential. We used real-time immunofluorescence technique to monitor the mitochondrial membrane potential (MMP) of individual cardiomyocytes before and after H_2O_2 treatment.

As shown in Fig. 5 in untreated cardiomyocytes, H_2O_2 resulted in a rapid decrease of the ratio of red to green fluorescence intensity, indicating loss of mitochondrial membrane potential. However, glucosamine, increased OGT expression and NButGT all significantly attenuated the loss of mitochondrial membrane potential compared to untreated groups. This was apparent within 5 min of H_2O_2 treatment and was sustained for at least 20 mins (Fig 5B). Interestingly, after 20 minutes of H_2O_2 treatment, glucosamine and increased OGT expression were more effective in preventing the loss of mitochondrial membrane potential than NButGT treated cells.

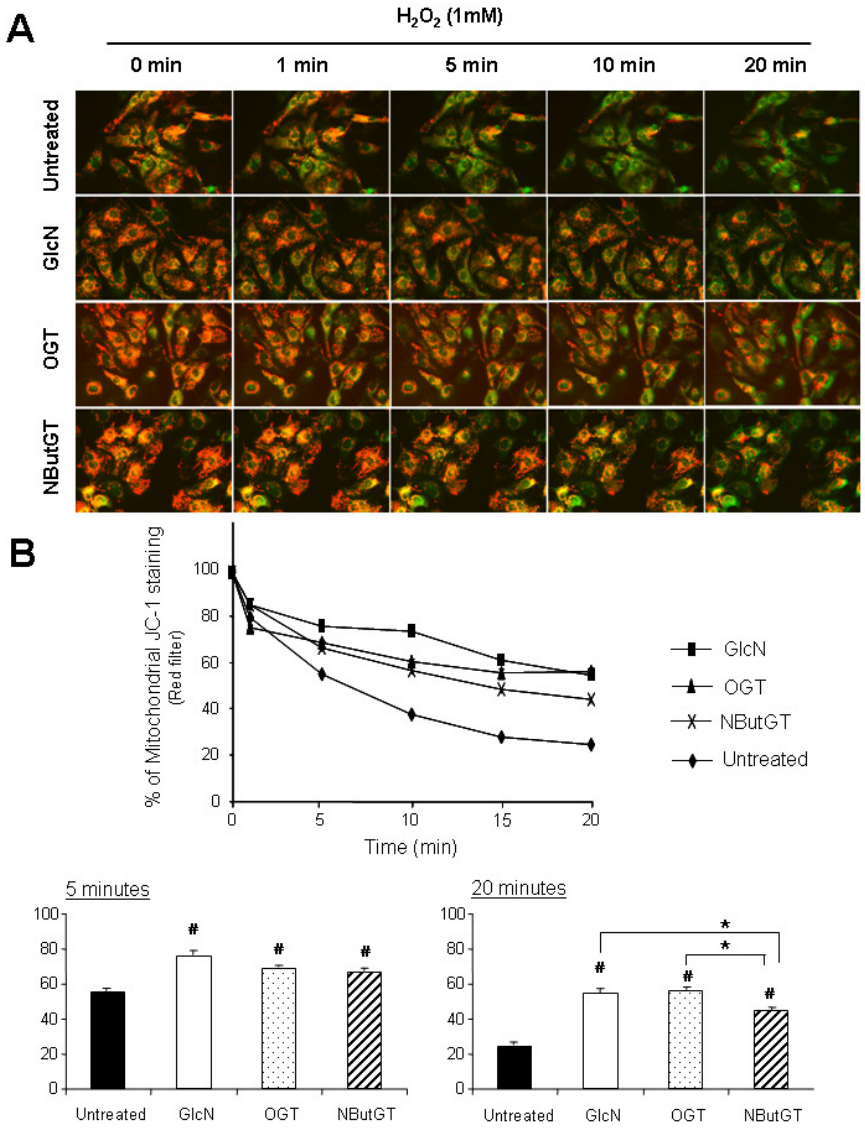

Figure 5: Increased *O*-GlcNAc levels attenuated the loss of mitochondrial membrane potential (MMP). A) Representative fluorescent merged images with red and green filters of JC-1 staining NRVMs treated with hydrogen peroxide at 1 mM from 0 to 20 mins; B) Mean intensity of mitochondrial JC-1 staining (Red filter) determined by IPLab analysis software and normalized with cells at starting time. Data are presented as means±SEM of 30 cells. Control untreated cells, glucosamine (5 mM)-1 hour pretreated cells, *O*-GlcNAc transferase (OGT) adenovirus infected cells, NButGT (100 μM)-1 hour

pretreated cells were incorporated with JC-1 to monitor MMP. # = P< 0.05 Vs untreated, * P< 0.05 Vs NButGT.

Decreased OGT expression decreases cardiomyocyte survival following ischemia/reperfusion.

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

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

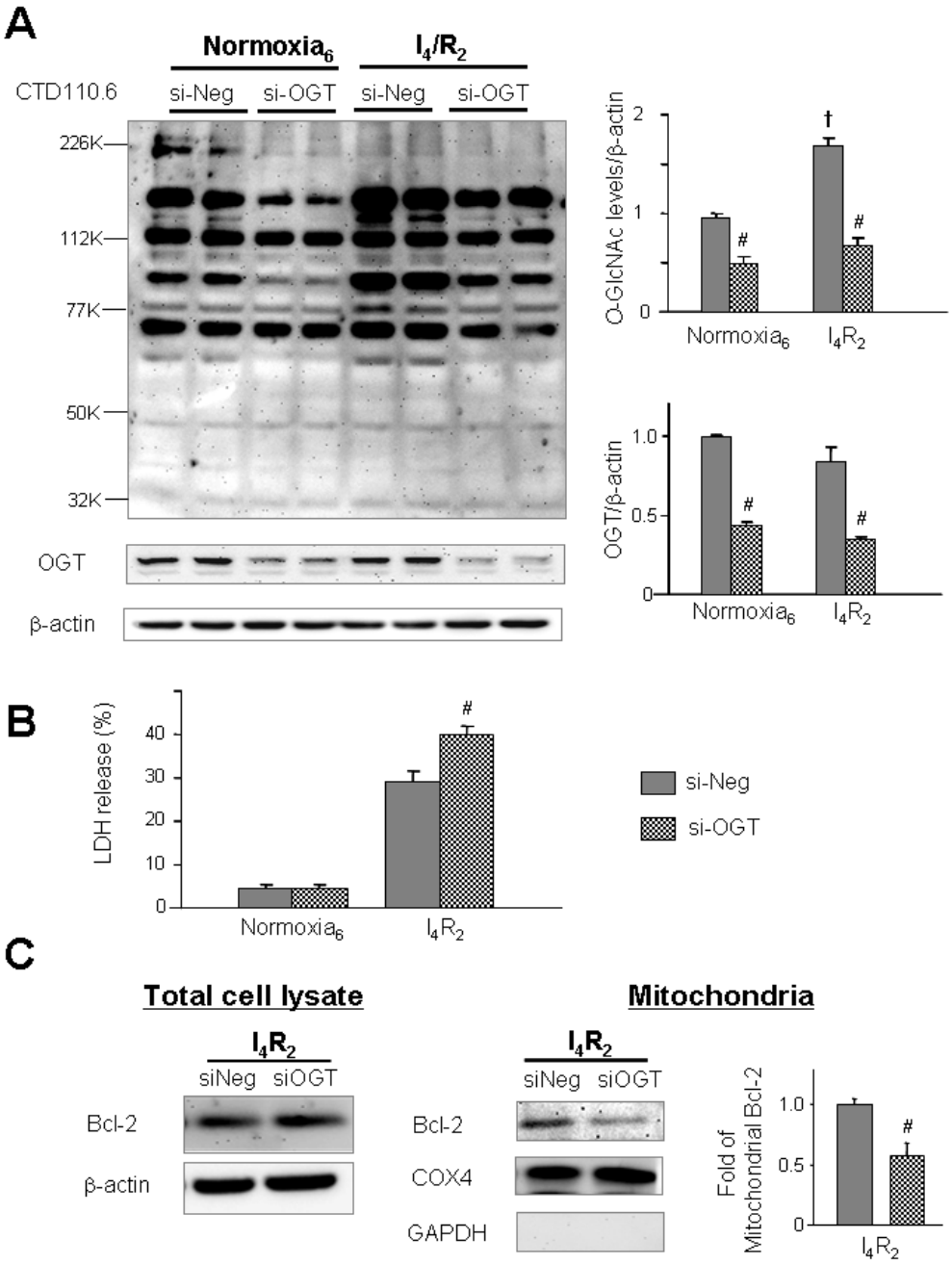

Figure 6: *O*-GlcNAc modification is essential for cellular survival and Bcl-2 translocation. A) Representative immunoblotting CTD110.6 and OGT and mean intensity of all *O*-GlcNAc proteins and OGT of the total whole cell lysate determined by densitometric analysis. Equal protein loading was normalized by densitometric analysis

of β-actin levels. Proteins are normalized to normoxic control untreated cells; B) Cell injury assessed by determining lactate dehydrogenase (LDH) release as a percentage of total LDH; C) Representative immunoblotting of Bcl-2 expression levels in total cell lysate and mitochondrial fractions and mean densitometric analysis of mitochondrial Bcl-2. Purity of mitochondrial fraction was assessed by COX4 and GAPDH. Control, si-Negative oligonuleotides transfected cells (si-Neg) and si-OGT oligonucleotides transfected cells (si-OGT) cells were incubated under normoxic condition for 6 h or subjected to 4 h of ischemia followed by 2 h of reperfusion (I_4/R_2). Data are presented as means±SEM of more than 3 experiments. # =P< 0.05 Vs untreated, † P< 0.05 Vs normoxia.

In Fig 7A it can be seen that in siOGT cells there was much greater loss of cytochrome C following ischemia/reperfusion, and this was associated with greater number of TUNEL positive cells (Fig 7B).

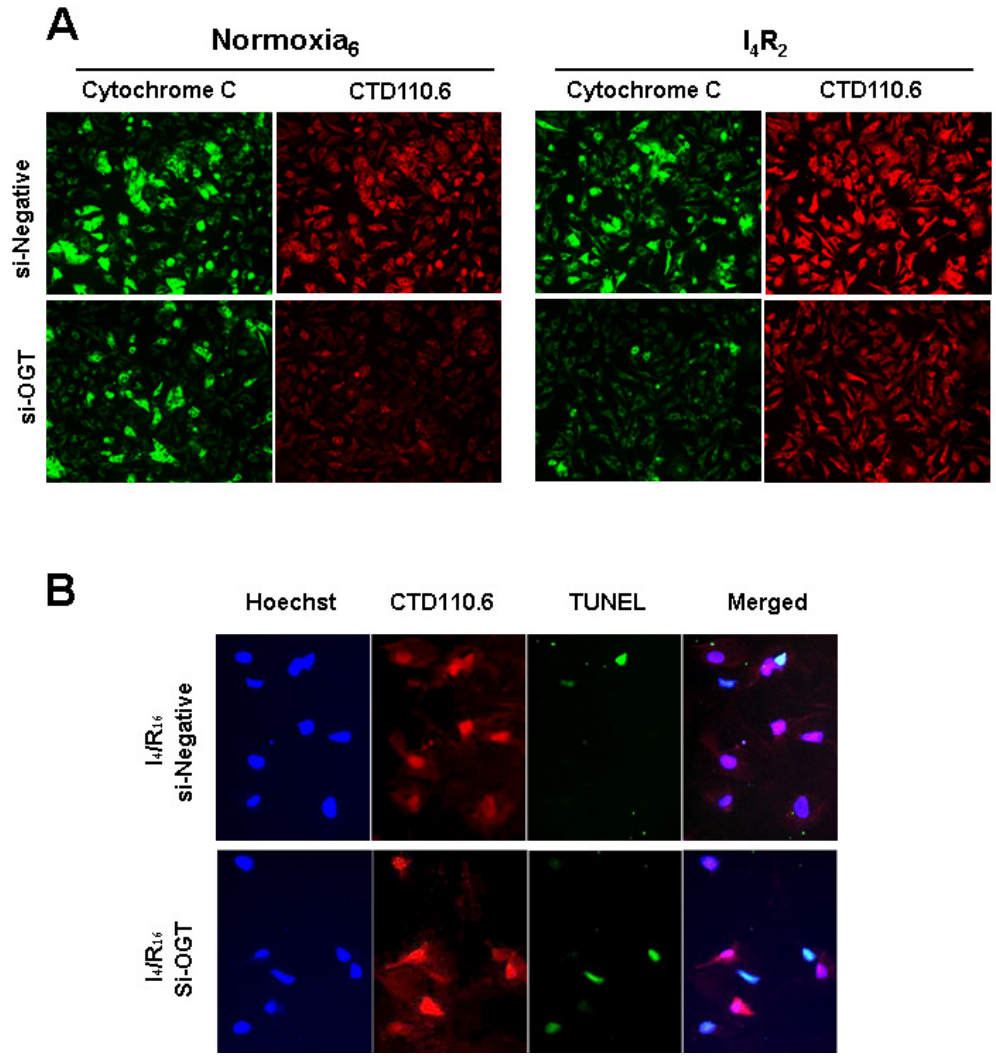

Figure 7: Decreasing global *O*-GlcNAc proteins exacerbated the loss of cytochrome C and apoptosis. A) Representative immunofluorescence of cytochrome C and *O*-GlcNAc proteins (CTD 110.6) under normoxia and following ischemia/reperfusion; B) Representative immunofluorescent of apoptosis (TUNEL), *O*-GlcNAc proteins (CTD 110.6), and nuclear staining (Hoechst). Control, si-Negative oligonuleotides transfected cells (si-Neg) and si-OGT oligonucleotides transfected cells (si-OGT) cells were incubated under normoxic condition for 6 h or subjected to 4 h of ischemia followed by 2 h or 16 h of reperfusion.

Discussion

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

We show here for the first time that the effect of glucosamine is mimicked by increased OGT expression and that both interventions increase the tolerance of NRVMs to injury induced by either ischemia/reperfusion or H_2O_2. Both glucosamine and OGT overexpression augmented the ischemia/reperfusion-induced increase in mitochondrial Bcl-2. The marked similarity between glucosamine treatment and increased OGT expression, demonstrates that the protective effect of glucosamine is mediated primarily via increased flux through OGT. Conversely, we found that decreasing OGT expression decreased tolerance to ischemia/reperfusion injury and blunted the ischemia/reperfusion-induced increase in mitochondrial Bcl-2. We also found that while inhibition of *O*-

GlcNAcase with NButGT resulted in a much greater increase in *O*-GlcNAc levels than glucosamine or OGT overexpression, it was significantly less protective and than either. This demonstrates that the similar observations previous reported with PUGNAc were most likely not due to non-specific effects of PUGNAc, but rather suggests that there may be some threshold for *O*-GlcNAc in increasing cell survival, beyond which the detrimental effects of excessive *O*-GlcNAc levels outweigh the pro-survival mechanisms.

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

Modulation of *O*-GlcNAc levels has been shown to modify sub-cellular localization of proteins and since there was no change in total Bcl-2, the increase in

mitochondrial Bcl-2 in the glucosamine and OGT overexpression groups presumably reflects its redistribution from other cellular compartments such as the endoplasmic reticulum, and nuclear envelope . We attempted to use immunofluorescence techniques to monitor Bcl-2 localization; however, all Bcl-2 antibodies we tested were ineffective (Santa Cruz, Abcam, and BioVision). Based on the prediction of potential *O*-GlcNAcylation sites by YinOYang 1.2 (http://www.cbu.dtu.dk/services/yinoyang), there are at least 4 potential sites for *O*-GlcNAc modification of rat Bcl-2 including Thr_{65}, $Ther_{69}$, Ser_{70}, and Ser_{84}. However, our attempts to determine whether the redistribution of Bcl-2 in these experiments could be due to *O*-GlcNAc modification of Bcl-2 were unsuccessful.

Despite the evidence demonstrating that anti-apoptotic Bcl-2 proteins may have therapeutic potential, the mechanism(s) by which they protect cells remains unclear. Bcl-2 has been shown to block p53-mediated apoptosis in cardiac myocytes and overexpression of Bcl-2 suppressed both p53-dependent and p53-independent activation of the intrinsic death pathway. Transgenic mice overexpressing Bcl-2 decreased apoptosis, reduced infract size and improved cardiac function after ischemia/reperfusion. It has been suggested that Bcl-2 inhibits opening of mMTP, possibly by direct interaction with voltage-dependant anion channel (VDAC), thereby preventing the release of death factors from mitochondria. Consistent with the notion that Bcl-2 protection is mediated via attenuation of mMTP opening, both glucosamine and OGT overexpression attenuated loss of cytochrome C release following ischemia/reperfusion (Fig. 4) and slowed the H_2O_2-induced loss of mitochondrial membrane potential (Fig. 5). Thus, these data suggest that an increase in mitochondrial Bcl-2 may be an important contributing factor

to the cellular protection associated with increased *O*-GlcNAc levels. Clearly, however, further studies are needed to delineate the mechanism(s) by which *O*-GlcNAc levels modulate the cellular distribution of Bcl-2.

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

O-GlcNAc levels can be increased not only by increasing the rate of synthesis, but also by decreasing the rate of removal via inhibition of *O*-GlcNAcase. PUGNAc is a widely used inhibitor of *O*-GlcNAcase, and we have previously reported that both glucosamine and PUGNAc are protective against ischemic injury. However, despite the fact that PUGNAc resulted in markedly greater *O*-GlcNAc levels than glucosamine, it was somewhat less protective. Therefore, here we used a new *O*-GlcNAcase inhibitor, NButGT, which has ~1500-fold greater specificity for *O*-GlcNAcase over ß-hexosaminidase than PUGNAc. NButGT increased *O*-GlcNAc levels 5-10 fold more than OGT overexpression; however, while compared to untreated cells NButGT attenuated

necrosis following ischemia/reperfusion, this was effect was significantly less than that seen with OGT overexpression (Fig. 3B). The difference between NButGT treatment and both OGT overexpression and glucosamine expression was particularly apparent with regard to the loss of cytochrome C following ischemia/reperfusion (Fig 4). It is also noteworthy that in contrast to OGT overexpression and glucosamine treatment, NButGT had no effect on mitochondrial Bcl-2 levels, under either normoxia or following ischemia/reperfusion.

These observations are rather contradictory to the notion that increasing protein *O*-GlcNAc levels is cytoprotective, since NButGT was clearly less protective than either glucosamine or OGT overexpression despite ~10-fold higher *O*-GlcNAc levels. This could point to differences resulting from increasing *O*-GlcNAc levels via new synthesis versus inhibiting the removal. For example, proteins, which under steady state conditions contain *O*-GlcNAc that is constantly cycling between on and off states, will show an enhancement of *O*-GlcNAc levels with *O*-GlcNAcase inhibition. In contrast, some proteins may become *O*-GlcNAcylated only when UDP-GlcNAc or OGT levels increase. Our results could be explained if some proteins important to mediating the protective response, including the increase in mitochondrial Bcl-2 levels, were in the latter class. The importance of OGT and *O*-GlcNAc in mediating cardiomyocyte survival was further supported by the fact that decreasing OGT expression by ~50%, using siRNA not only attenuated basal *O*-GlcNAc levels, but also prevented the increase induced by ischemia (Fig 6A, 7A). This was associated with an increase in necrosis (Fig 6B), greater loss of cytochrome C (Fig 7A) and increased apoptosis (Fig 7B) in OGT siRNA group. In contrast to OGT overexpression and glucosamine treatment, reduced OGT expression

resulted in an increase in whole cell Bcl-2 levels, thereby complicating the interpretation of the Bcl-2 data. Nevertheless, the increase in cardiomyocyte injury in the OGT siRNA group was associated with attenuation of mitochondrial Bcl-2 following ischemia/reperfusion, which is in contrast to the increase seen in control si-Neg cells.

In conclusion we have shown that in NRVMs the glucosamine-mediated protection against both ischemia/reperfusion and H_2O_2 is mimicked by OGT overexpression, and that decreasing OGT expression significantly increased sensitivity of NRVMs to ischemia/reperfusion injury. These results provide strong evidence to support the notion that the protective effects of glucosamine seen at the cellular, organ and whole animal level are mediated via increased formation of *O*-GlcNAc. We have also demonstrated that one potential mechanism contributing to this protection is an increase in mitochondrial Bcl-2 levels, which was also associated with attenuation of H_2O_2-induced loss of mitochondrial membrane potential. However, further studies are clearly warranted to better understand the mechanism by which increased *O*-GlcNAc levels modulate the cellular distribution of Bcl-2 and whether this is a consequence of *O*-GlcNAc modification of Bcl-2 itself.

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CHAPTER 4

CONCLUSIONS

Conclusion

The goal of this study was to test the hypothesis whether elevated flux through the hexosamine biosynthesis pathway (HBP) by glucosamine and increased levels of protein-associated *O*-GlcNAc were protective against ischemia/reperfusion injury in isolated cardiomyocyte models. While much of the focus has been on the role of increased *O*-GlcNAc in mediating the adverse effects of diabetes (2, 16, 106), several line of evidence indicates that activation of pathways leading to *O*-GlcNAc formation is an endogenous stress response and that augmentation of this response improves tolerance to stress (90, 132, 162). In addition, studies from the Marchase-Chatham Lab have demonstrated glucosamine is remarkably protective against ischemic injury at the organ (43, 94, 95), and in vivo (113, 159) level and that this protection was associated with increased *O*-GlcNAc levels. In this study, for the first time, it was shown that, in isolated neonatal cardiomyocytes (NRVMs), ischemia/reperfusion increased protein *O*-GlcNAc levels and that augmentation of this response by glucosamine was associated with increased viability, decreased necrosis and apoptosis following simulated ischemia/reperfusion. Furthermore, our results also demonstrated that the increase in *O*-GlcNAc formation was glucose dependant, demonstrating that during reperfusion glucose is metabolized not only via energy producing pathways but also via the hexosamine biosynthesis pathway.

Therefore, taken together this evidence supports the concept that activation of metabolic pathways leading to an increase in *O*-GlcNAc levels may represent novel interventions for improvement of cellular survival.

To better understand the role of *O*-GlcNAcylation in mediating cardioprotection, alternation of enzyme activities responsible to this modification were examined. *O*-GlcNAc transferase (OGT) catalyzes *O*-GlcNAcylation by adding a single UDP-GlcNAc to nuclear and cytoplasmic proteins at side chain hydroxyl groups of serine or threonine residues (23). The enzymatic activity of OGT can be inhibited by alloxan (80) and we found that in NRVMs, treatment of alloxan completely blocked *O*-GlcNAcylation following ischemia/reperfusion. This was associated with exacerbation of necrosis and apoptosis, further demonstrating that *O*-GlcNAcylation plays an important role in mediating the response to stress. However, it was surprising that under normoxic condition, alloxan had minimal effects on viability and no effects on necrosis or apoptosis. Alloxan is an uracil analog and conseqeuently, we cannot rule out potential non-specific effects of alloxan (138), particularly since the effective dose used in this study was in milimolar range. Therefore, a more specific demonstration of the role of OGT in mediating glucosamine cardioprotection was required.

Ablation of the OGT gene is embryonically lethal (130), therefore rather than using a conventional knockout approach we used an siRNA approaches to reduce OGT expression in cardiomyocytes. In NRVMs we were able to decrease OGT expression by ~50%, using OGT siRNA. This not only attenuated basal *O*-GlcNAc levels, but also prevented the increase in *O*-GlcNAc induced by ischemia/reperfusion and was associated with an increase in necrosis. In contrast, increasing the levels of OGT expression

increased *O*-GlcNAc levels and increased the tolerance of NRVMs to ischemia/reperfusion injury. These data together provide strong evidence that OGT and *O*-GlcNAc modification is vital for stress response and perturbation of this process exacerbates cellular injury whereas an increase of OGT levels is protective.

In addition to OGT, *O*-GlcNAcase (OGA) plays a key role in controlling *O*-GlcNAcylation by catalyzing removal of *O*-GlcNAc from proteins. Its activity can be inhibited by *O*-(2-acetamid-*O*-2-deoxy-d-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc), and this leads to an increase in protein *O*-GlcNAc levels. In this study, treatment of PUGNAc dramatically increased overall O-GlcNAc levels under both normoxic and ischemia/reperfusion conditions. Surprisingly, however, PUGNAc did not lead to greater protection than glucosamine, despite the fact that *O*-GlcNAc levels were markedly higher that the glucosamine treated group. Indeed, if anything, PUGNAc appeared to be somewhat less protective than glucosamine. Glucosamine and PUGNAc treatment lead to increased *O*-GlcNAc levels in different intensity and protein bands; thus, the fact that PUGNAc was not as protective as glucosamine despite the higher overall level of *O*-GlcNAc may be due to different proteins being *O*-GlcNAcylated in response to the different interventions. It should also be noted that while PUGNAc is typically used as an *O*-GlcNAcase inhibitor, it also inhibits lysosomal ß-hexosaminidases, which may also alter the response of cardiomyocytes to ischemia/reperfusion. A new *O*-GlcNAcase inhibitor was recently described, 1,2-dideoxy-2'-methyl-alpha-d-glucopyranoso[2,1-d]-Delta2'-thiazoline (NButGT), which has ~1500-fold greater specificity for *O*-GlcNAcase over ß-hexosaminidase than PUGNAc (102). Here we found that similar to PUGNAc, NButGT treated cells increased *O*-GlcNAc levels 10 fold more

than untreated cells. However, while NButGT attenuated necrosis following ischemia/reperfusion, this effect was significantly less than that seen with glucosamine. These observations are rather contradictory to the notion that increasing protein *O*-GlcNAc levels is cytoprotective, since PUGNAc and NButGT was clearly less protective than glucosamine despite markedly higher *O*-GlcNAc levels. This could point to differences resulting from selective modification. For example, proteins that at steady state contains *O*-GlcNAc that is constantly cycling between on and off states, will show an enhancement of *O*-GlcNAc levels with *O*-GlcNAcase inhibition. In contrast, some proteins may become *O*-GlcNAcylated only when UDP-GlcNAc or OGT levels increase. Furthermore, Zachara and Hart (161) proposed that high as well as low levels of *O*-GlcNAc might trigger apoptosis. Consequently, the dose and duration of PUGNAc or NButGT treatment used here may have resulted in an excessive level of *O*-GlcNAc that was sustained for the entire reperfusion period, thereby resulting in less protection.

Stimulation of myocardial glucose utilization has repeatedly been shown to afford protection against ischemic injury (134). In this context, Schaffer *et al.*, (128) reported that hyperglycemia significantly reduced hypoxia-induced apoptosis and necrosis in isolated cardiomyocytes. However, there is little consensus regarding the mechanism(s) underlying this protection. The fact that *O*-GlcNAc levels are increased by hyperglycemia and that elevated *O*-GlcNAc levels increase the tolerance of cells to stress, raises the possibility that the protection associated with increased glucose utilization, could be mediated, at least in part, via this pathway. Here, this study demonstrated that in NRVMs, hyperglycemia was protective against ischemia/reperfusion and that this protection was associated with an increase in *O*-

GlcNAc levels in specific protein bands. Hyperglycemic protection was inhibited with azaserine, an inhibitor of GFAT, which blocks glucose entry into the HBP, and was associated the decrease in *O*-GlcNAc levels. The fact that the increase in *O*-GlcNAc seen with hyperglycemia was less pronounced that seen with glucosamine is not surprising, since glucosamine enters the HBP directly and essentially unregulated whereas glucose entry into the HBP is regulated by GFAT. It is noteworthy that in the hyperglycemia experiments western blotting analysis showed differential response of individual *O*-GlcNAc protein bands, suggesting that the protection might be mediated via specific proteins rather than a global increase in *O*-GlcNAc levels. In addition, this study also demonstrated that the absence of glucose during reperfusion attenuated the increase in *O*-GlcNAc levels and reduced cell viability. All together, these data support the notion that metabolism of glucose via the HBP and the formation of *O*-GlcNAc is a normal stress response and inhibition of these pathways decreases the tolerance to ischemia/reperfusion stress.

It has been reported that in NRVMs, glucosamine inhibited angiotensin II induced and calcineurin-mediated NFAT translocation (65) and have subsequently shown that this was due to attenuation of Ca^{2+} influx (110). Since calcineurin activation has been implicated in mediating cardiomyocyte apoptosis (107), protection resulting from increased *O*-GlcNAc levels might also be associated with reduced calcineurin activation. This study showed that at the end of ischemia/reperfusion there was marked increase in nuclear localization of GFP-NFAT consistent with an increase in cytosolic Ca^{2+} and treatment of glucosamine remarkably decreased this response. In addition, PUGNAc treated cells significantly attenuated the nuclear translocation of GFP-NFAT whereas

alloxan treated cells significantly increased GFP-NFAT nuclear localization. Furthermore, the effects of the different interventions on GFP-NFAT nuclear localization are remarkably similar to their impact on cell viability. Glucosamine also attenuated an increase of cytosolic calcium concentration compared to untreated cells following ischemia alone. In a similar context, Liu et al., reported that in the whole heart glucosamine markedly reduced tissue injury due to severe calcium overload induced by the calcium paradox (95). Thus, the data shown here combined with earlier study (95) suggests that the protection associated with increased *O*-GlcNAc levels may be due, at least in part, to inhibition of Ca^{2+} entry into NRVMs and subsequent attenuation of Ca^{2+} mediated necrosis and apoptosis.

It is becoming increasingly apparent that elevating *O*-GlcNAc levels improves the tolerance of cells to stress; however, the specific mechanisms underlying this protective response have not been fully defined. Zachara et al., demonstrated that in COS-7 cells, *O*-GlcNAc-mediated tolerance to heat stress was due in part to increased transcription of heat shock proteins such as HSP70 (162). However, in NRVMs we found no increase in HSP70 expression in response to glucosamine treatment following ischemia/reperfusion; which is consistent with a study by Sohn et al., (132) who showed that improved survival associated with increased *O*-GlcNAc levels without any change in HSP70 levels, which suggests that other mechanism(s) may also contribute to the protection associated with increased *O*-GlcNAc levels.

As mentioned earlier, glucosamine treatment significantly attenuated cardiomyocyte apoptosis following ischemia/reperfusion injury. One important factor regulating apoptosis is the Bcl-2 family of proteins. Schaffer *et al.*, (128) reported that in

NRVMs, hyperglycemia reduced significantly hypoxia induced apoptosis which was mediated an upregulation of the cardioprotective factor Bcl-2 but did not affect the cellular content of the proapoptotic factors Bax and Bad. Increasing, HBP flux and *O*-GlcNAc levels by hyperglycemia, may at least in part, mediate this protective response. Therefore, we speculated that that glucosamine may also protect NRVMs against ischemia/reperfusion injury via regulation of the Bcl-2 family of proteins. We found that, glucosamine had no or little effect on whole cell expression levels of Bcl-2, Bad or Bax; however, glucosamine specifically increased mitochondrial Bcl-2 levels both under normoxic conditions as well as following ischemia/reperfusion. Importantly, we also found that overexpression of OGT also increased mitochondrial Bcl-2 levels.

It has been suggested that Bcl-2 inhibits opening of mMTP, possibly by direct interaction with voltage-dependant anion channel (VDAC) (141, 142) thereby preventing the release of death factors from mitochondria. Consistent with the notion that Bcl-2 protection is mediated via attenuation of mMTP opening, both glucosamine and OGT overexpression attenuated loss of cytochrome C release following ischemia/reperfusion. Surprisingly, NButGT, an inhibitor of *O*-GlcNAcase had no effect on mitochondrial Bcl-2 expression under either normoxia or following ischemia/reperfusion and this was correlated with less protection of the loss of cytochrome C release following I/R. Although NButGT resulted in a much greater increase in *O*-GlcNAc levels than glucosamine and OGT overexpression, it was less protective. This suggests that *O*-GlcNAc cycling is an important for maximum protection. A large increase in levels of *O*-GlcNAc modification alone may not represent a good protective indicator.

Since there was no change in total Bcl-2 expression, the increase in mitochondrial Bcl-2 in the glucosamine and OGT overexpression groups presumably reflects its redistribution from other cellular compartments such as the endoplasmic reticulum, and nuclear envelope (50). However, all techniques to monitor this distribution in this study were ineffective. In addition, attempts to determine whether the redistribution of Bcl-2 in these experiments could be due to *O*-GlcNAc modification of Bcl-2 were unsuccessful although based on the prediction of potential *O*-GlcNAcylation sites, there are at least 4 potential sites for *O*-GlcNAc modification of rat Bcl-2. Further studies are needed to delineate the mechanism(s) by which *O*-GlcNAc levels modulate the cellular distribution of Bcl-2 and whether this is a consequence of *O*-GlcNAc modification of Bcl-2 itself.

In some preliminary studies, we used ultracentrifugation to examine other sub-cellular compartments amd we found that Bcl-2 was distributed in cytosolic and microsomal (ER/SR) fractions (Fig 1). GAPDH (cytosolic marker), COX4 (mitochondrial marker), and IP3R (microsomal ER/SR marker) were used to check the purity of each fraction. Consistent with early studies (Chapter 2, figure 2), glucosamine increased mitochondrial Bcl-2 levels. Interestingly, following ultracentrifugation of the post-mitochondrial fraction, we found that treatment of glucosamine increased microsomal Bcl-2 and decreased cytosolic Bcl-2 levels under normoxic and ischemia/reperfusion compared to the untreated groups. These data suggest that glucosamine results in an translocation of Bcl-2 from the cytosol to both the mitochondrial and SR/ER compartments and that this may be an important contributing factor to the cellular protection associated with increased *O*-GlcNAc levels.

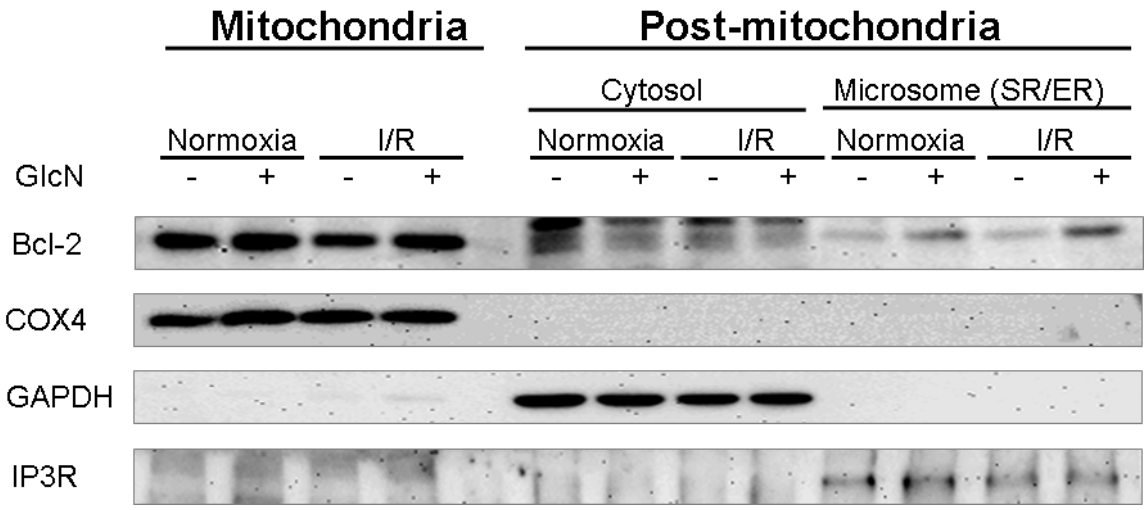


Figure 1: Glucosamine increased mitochondrial and microsomal Bcl-2 levels. Representative Bcl-2, COX4, GAPDH, and IP3R immunoblots of the sub-cellelar fractions. NRVMs untreated cells and glucosamine (5 mM)-treated cells were incubated under normoxic condition for 6 h or subjected to 4 h of ischemia followed by 2 h of reperfusion (I_4/R_2). Cells were resuspended in Cytosol Extraction Buffer (BioVision), then homogenized and centrifuged at 700x g for 10 minutes at 4^{o}C to separate cell debris and liquid supernatant. The supernatant was second centrifuged at 10,000x g for 30 minutes at 4^{o}C to separate the pellet (mitochondria) and the supernatant consisting of the cytosolic/microsomal (post-mitochondria). The post-mitochondria supernatant was further centrifuged at 100,000x g for 1 hour at 4^{o}C to separate the microsomal pellet and cytosolic supernatant. Proportional proteins were separated on 10% SDS-polyacrylamide gel electrophoresis and immunobloted onto a PVDF membrane.

Although the function of Bcl-2 proteins including Bcl-2 itself is best characterized at the mitochondria, Bcl-2 is also associated with other intracellular membranes such as the ER/SR and nuclear envelope (50, 125). It has been suggested that Bcl-2 localized to the ER/SR may play a role in attenuating apoptosis possibly by mediating ER/SR calcium homeostasis (12, 31); however, the mechanism by which this occurs is controversial and not well defined (12). For example, several groups have reported that Bcl-2 increases the ER Ca^{2+} content and/or prevents ER Ca^{2+} release during apoptosis (29, 67). In contrast, Rizzuto and co-workers demonstrated that overexpression of Bcl-2 reduces the steady state levels of $[Ca^{2+}]_{ER}$ by increasing the permeability of the ER membrane to Ca^{2+} (118, 119). Therefore, according to our results, although the mechanisms are not well defined, augmentation of *O*-GlcNAc levels may be an important contributing factor to the cellular protection through increasing levels of mitochondrial and SR/ER Bcl-2. Thus, by localizing to both ER and mitochondria, Bcl-2 might prevent apoptotic crosstalk between the two compartments by lowering the amount of free $[Ca^{2+}]_{ER}$ and by increasing the tolerance of the mitochondria to high Ca^{2+} loads.

It has been suggested that release of cytochrome C from the mitochondria is usually associated with opening of the mitochondrial membrane transition pore (mMTP), which is a critical step in mitochondrially-mediated apoptosis (83). Opening of the mMTP is characterized by loss of mitochondrial membrane potential; therefore, an experiment was set to test whether increasing *O*-GlcNAc levels attenuated the loss of mitochondrial membrane potential in response to transient exposure to hydrogen peroxide. The results showed that increasing *O*-GlcNAc levels by glucosamine, OGT overexpression, and NButGT delayed the loss of mitochondrial membrane potential after

transient exposure to hydrogen peroxide. Interestingly, after 20 minutes of H_2O_2 treatment, glucosamine and increased OGT expression were more effective in preventing the loss of mitochondrial membrane potential than NButGT treated cells. Again, this suggests that *O*-GlcNAc cycling is important for maximum protection.

The potential contradiction between the beneficial effects of increased *O*-GlcNAcylation and the adverse effects referred to insulin resistance and diabetes could be that the protection is observed in response to acute increases in *O*-GlcNAc levels, whereas adverse effects are seen in response to chronic activation. Thus, if increased *O*-GlcNAcylation is an acute survival response, the development of pathophysiology may be a consequence of chronic activation as seen in metabolic disease. Furthermore, Zachara and Hart (161) suggested that an excessive elevation of *O*-GlcNAc may induce apoptosis; consequently, stress induced increase in *O*-GlcNAc on top of an already elevated level of *O*-GlcNAc may lead to cell death.

In conclusion, this dissertation has shown for the first time that in neonatal cardiomyocytes (NRVMs), ischemia/reperfusion increased *O*-GlcNAc protein levels, and augmentation of this modification by glucosamine, or PUGNAc, or NButGT, or hyperglycemia, or overexpression of OGT, is protective against ischemia/reperfusion injury. Elevation of *O*-GlcNAc levels also increase the tolerance of NRVMs to injury induced by H_2O_2. The maximum protection is required by increasing specific *O*-GlcNAc protein modification via *O*-GlcNAc transferase (OGT) rather than overall increasing via inhibiting *O*-GlcNAcase (OGA). In addition, blocking an increase of *O*-GlcNAc levels markedly exacerbated cellular injury following ischemia/reperfusion. These findings support the concept that, activation of metabolic pathways leading to an increase in *O*-

GlcNAc levels is an endogenous stress activated response, and that augmentation of this response improves cell survival. Although the specific mechanism regarding protection is still incompletely understood, this study demonstrates that at least two potential pathways contributing to this protection; attenuation of cytosolic calcium and accumulation of mitochondrial Bcl-2, consequently protecting in mitochondria function which in final reduced cardiomyocyte necrosis and apoptosis.

Future studies

O-GlcNAcylation was first discovered in late 1980's by Gerald W. Hart (57). This modification constitutes an abundant and dynamic reversible form of glycosylation for numerous cytoplasmic and nuclear proteins. More than 500 *O*-GlcNAc proteins have been identified to date: they belong to various classes of proteins including cytoskeletal components, hormone receptors, transcriptional factors, kinases, signaling molecules, nuclear pore proteins, vital proteins, suggesting that *O*-GlcNAc may be implicated in several key cellular systems (58). A number of techniques and tools have been developed for the detection and study of *O*-GlcNAc (155). These protocols include galactosyltransferase labeling, immunoblotting, using mass spectrometry based on beta-elimination followed by Michael addition with dithiothreitol, and chemoenzymatic labeling, enrichment, and detection.

The specific proteins affected by *O*-GlcNAcylation, that might be mediating the protection against ischemia/reperfusion injury remain to be identified. Proteomic approach by using 2D IEF/SDS-PAGE and Blue Native Gel are powerful techniques to

clarify and identify what those proteins are. Furthermore, cell fractionation also would be an interesting technique to separate and enrich proteins in subcellular particles for studying *O*-GlcNAc protein distribution in cells.

In preliminary studies, we found that mitochondrial proteins may be possibly potential targets for *O*-GlcNAc modification. Using serial centrifugation, in NRVMs, it was clear that there are *O*-GlcNAc proteins in the mitochondrial enriched fractions and their protein patterns are different from those in the post-mitochondrial (cytosol/microsome) enriched fractions and the total whole cell lyzates (Fig 2). GAPDH (cytosolic marker), COX4 (mitochondrial marker), and Histone H1 (nuclear marker) were used to check the purity of each fraction. Importantly, we also found that ischemia/reperfusion alone increased *O*-GlcNAc levels in both mitochondrial and post-mitochondrial fractions compared to normoxic conditions. Treatment of glucosamine clearly increased *O*-GlcNAc protein levels under normoxic conditions and augmented the response to ischemia/reperfusion in both mitochondrial and post-mitochondrial fractions. These data suggest therefore that several mitochondrial proteins are likely to be modified by *O*-GlcNAc and glucosamine augments this modification in mitochondrial fractions.

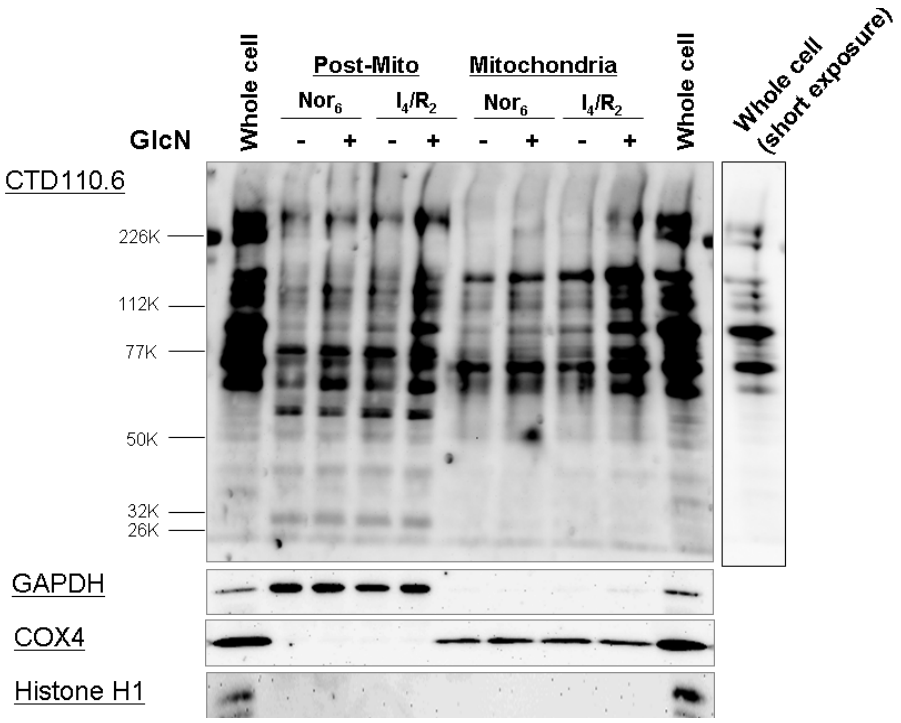
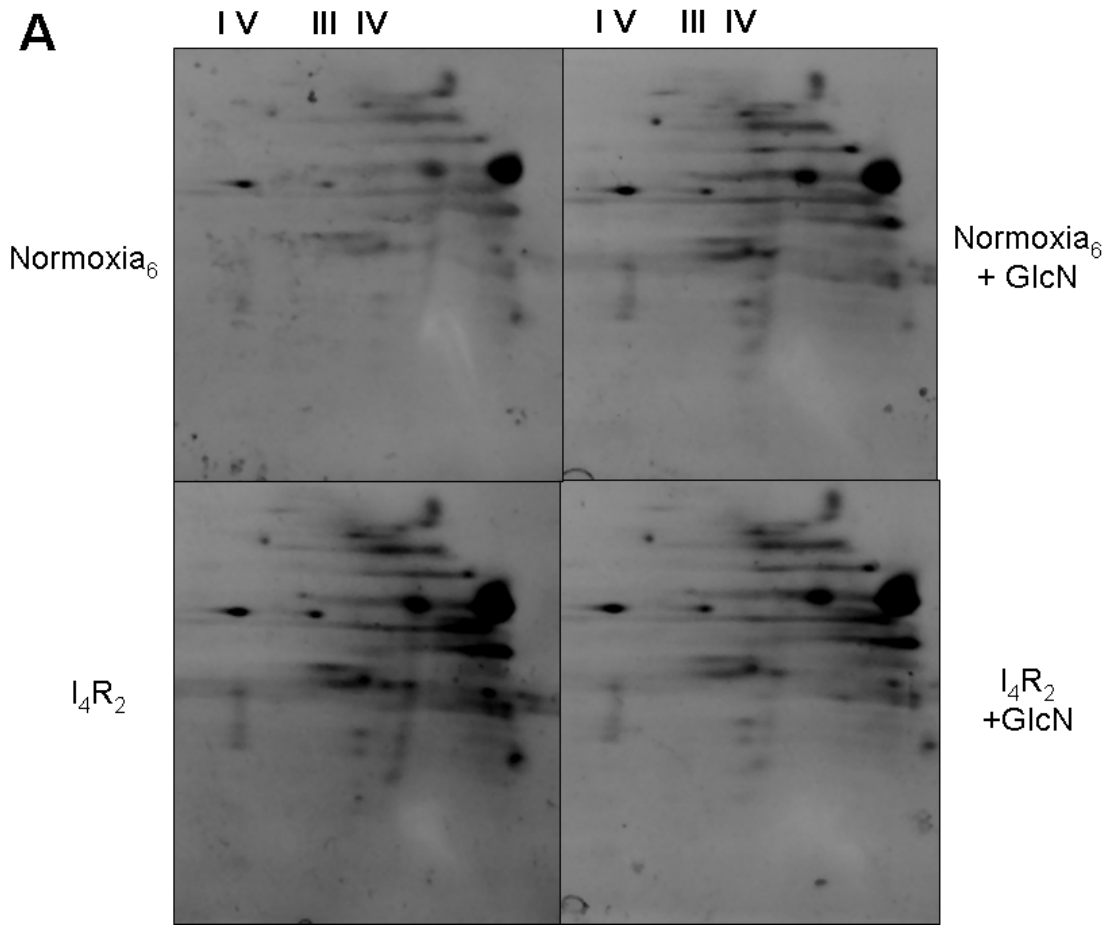


Figure 2: Glucosamine augmented *O*-GlcNAc levels in mitochondria and post-mitochondrial (cytosol/microsome) fractions under normoxic and ischemia/reperfusion in NRVMs. Representative CTD110.6 immunoblot of *O*-GlcNAc proteins. Purity of each fraction was assessed by GAPDH, COX4, and Histone H1 respectively. Control untreated cells and glucosamine (5 mM)-treated cells were incubated under normoxic condition for 6 h or subjected to 4 h of ischemia followed by 2 h of reperfusion (I_4/R_2). NRVMs were resuspended in Cytosol Extraction Buffer (BioVision), then homogenized and centrifuged at 700x g for 10 minutes at 4^{o}C to separate cell debris and liquid supernatant. The supernatant was second centrifuged at 10,000x g for 30 minutes at 4^{o}C to separate the pellet (mitochondria) and the supernatant consisting of the cytosolic/microsomal (post-mitochondria). Whole cells were lyzed in RAPA lysis buffer. Proteins (3 μg) were separated on 8% SDS-polyacrylamide gel electrophoresis and immunobloted onto a PVDF membrane.

To better identify *O*-GlcNAc mitochondrial proteins, we used 2D Blue native (BN) gels which are a powerful technique to separate membrane-soluble respiratory chain (RC) complexes. Figure 3 showed 2D Blue native gels of glucosamine treated and untreated NRVMs under normoxia and ischemia/reperfusion. We found that many proteins are modified by *O*-GlcNAc in untreated cells under normoxic condition. Ischemia/reperfusion alone increased *O*-GlcNAc protein levels compared to normoxic condition. Interestingly, treatment of glucosamine clearly increased *O*-GlcNAc protein levels under normoxic conditions and augmented the response to ischemia/reperfusion compared to untreated groups (Fig 3B). These results of 2D BN gels are consistent with those of 1D SDS-PAGE (Fig 2).



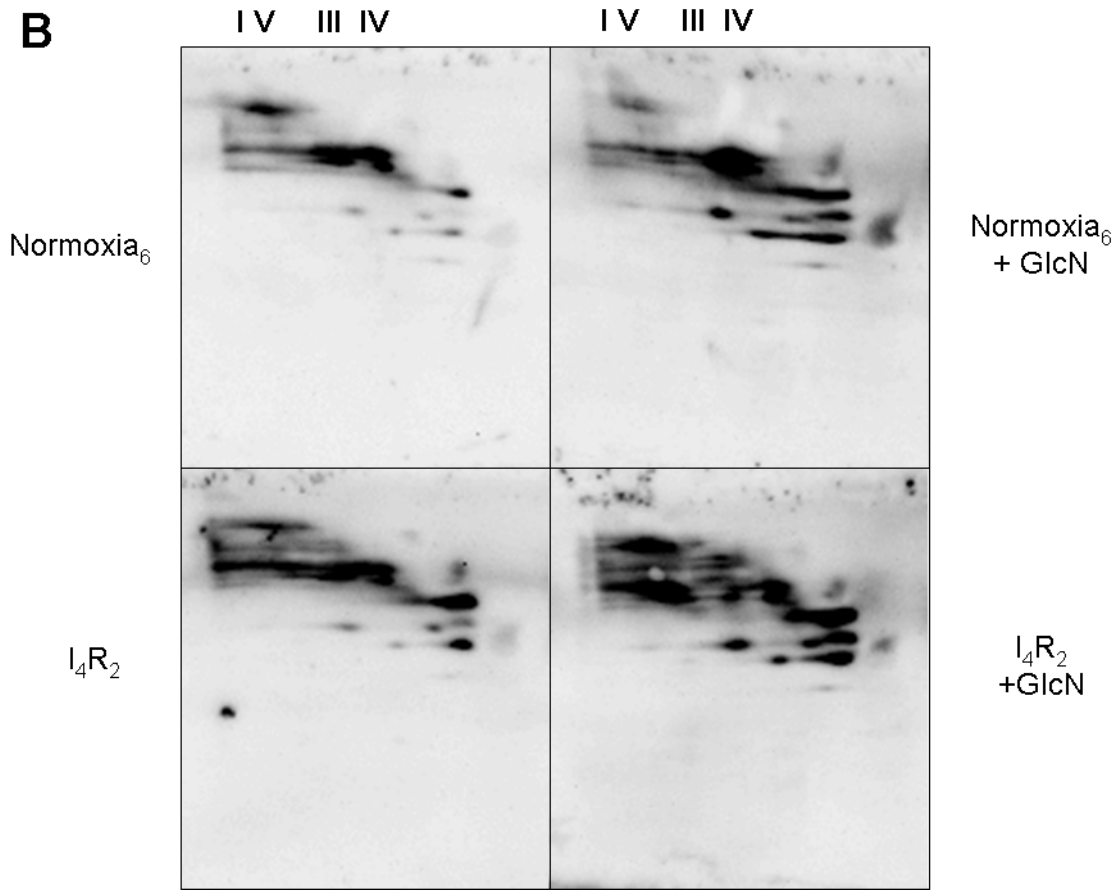


Figure 3: 2D Blue native gels of glucosamine treated and untreated NRVMs under normoxia and ischemia/reperfusion. A) Representative fluorescent protein staining gels by sypro ruby. B) Representative CTD110.6 immunoblot of *O*-GlcNAc proteins. Control untreated cells and glucosamine (5 mM)-treated cells were incubated under normoxic condition for 6 h or subjected to 4 h of ischemia followed by 2 h of reperfusion (I_4/R_2). Proteins (50 μg) were separated in the 1st native gradient gels to keep intact protein complexes and followed with the 2nd denaturing gradient gels. I, V, III, and IV are labels of respiratory chain complexes, respectively.

According to these results, mitochondrial proteins are likely to be an important target of *O*-GlcNAc modification and treatment of glucosamine results in an increase of this protein modification. These proteins may play an important role in mitochondrial function and perhaps improve cardiomyocyte survival following ischemia/reperfusion injury. Clearly an important goal for future studies is to identify the mitochondrial proteins that are modified by *O*-GlcNAc. Several high sensitivity techniques for protein identification are available including mass spectrometry, MOLDI-Q and MOLDI-TOF and could used for such studies.

Another important and as yet unresolved question is how mitochondrial proteins become O-GlcNAcylated. This could occur in two ways: (1) *O*-GlcNAc modification occurs in cytosol and the modified protein(s) is transferred into mitochondria or (2) *O*-GlcNacylation occurs within the mitochondria. It has been reported that, in addition to nucleocytoplasmic OGT (ncOGT), there is a shorter form of OGT, which encodes an N-terminal mitochondrion-targeting sequence (mOGT) (55, 98). Love et al. found that mOGT was tightly associated with the mitochondrial inner membrane (98); however, in contrast to our findings, they were unable to demonstrate any *O*-GlcNAcylated proteins in the mitochondrial fraction. One explanation for this is that they monitored *O*-GlcNAc proteins with the RL-2 antibody, which is recognizes *O*-GlcNAc proteins, mostly in nuclear pore complex proteins. If mOGT is functioning, however, it is still not clear how UDP-GlcNAc or equivalent substrate(s) transports into mitochondria in order to synthesize the *O*-GlcNAc formation or even whether hexosamine biosynthesis pathway (HBP) exists in the mitochondrial compartment.

In summary, we have shown for the first time, in NRVMs mitochondrial proteins are modified by *O*-GlcNAc and glucosamine augmented this protein modification. Clearly further studies are warranted not only to identify the specific proteins involved in *O*-GlcNAc mediated protection, but also to determine how these proteins are affected by ischemic stress and how changes in the levels of *O*-GlcNAc alters the response to stress.

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APPENDIX A

IACUC PROTOCOL NUMBER

Admission to Candidacy
Research Compliance Verification Form

Instructions
Complete this form, including all applicable information and the signatures of the student, Chair of the student's committee, and Graduate Program Director. For application forms and details regarding compliance issues, please contact the Institutional Review Board (IRB) (http://www.uab.edu/irb or 934-3789), the Institutional Animal Care and Use Committee (IACUC) (http://www.uab.edu/iacuc or 934-7692), and the Office of the Conflict of Interest Review Board (CIRB) (http://www.uab.edu/uabra/cirb or 975-9691).

Human Subjects
The University of Alabama at Birmingham defines a human subject as not only a living human being, but also human tissue, blood samples, pathology or diagnostic specimens, study of medical records, observation of public behavior, and all questionnaires or surveys.

Does the research proposed by the student involve human subjects? ____ Yes (continue below) X No
This research is:

Pending _____ Approved _____ Date _____

IRB Protocol No. _____
(Please attach copy of IRB approval form)

Animal Subjects
The University of Alabama at Birmingham defines a laboratory animal as any vertebrate animal (e.g., traditional laboratory animals, farm animals, wildlife, and aquatic animals) and specific invertebrate animal used in research, teaching, or testing.

Does the research proposed by the student involve animal subjects? X Yes (continue below) ____ No
This research is:

Pending _____ Approved YES Date 12/1/2004

IACUC Protocol No. 041207048
(Please attach copy of IACUC approval form)

Conflict of Interest Disclosure is required for all sponsored research.

Have you disclosed to the Office of the CIRB? Yes ✓ Date: 1/21/04 No _____

Has the project been released pending funding? Yes ✓ Date: 1/21/04 No _____

The chair of the committee and the student agree that no research will be initiated until an application is submitted for review and approved by the appropriate review boards. It is the responsibility of the chair of the committee and the student to comply with federal and UAB regulations associated with this research.

V. Chu	Cell Biology	Feb 25, 2005
Student's Signature	Dept.	Date
[signature]	Medicine/Cell Bio	02/25/2005
Chair of Committee Signature	Dept.	Date
_____	_____	_____
Graduate Program Director	Dept.	Date

Revised 10/9/2004

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

NOTICE OF APPROVAL

DATE: December 1, 2004

TO: Richard Marchase, Ph.D.
MCLM-690 0005
FAX: 975-2533

FROM: Suzanne M. Michalek, Ph.D., Vice Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Cytoplasmic Glycosylation and Hypovolemic Stress
Sponsor: NIH
Animal Project Number: 041207048

On December 1, 2004, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Pigs	B	25
Rats	C	200
Rats	B	500
Rats	A	25

Animal use is scheduled for review one year from December 2004. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 041207048 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

MEMORANDUM

DATE: December 1, 2004

TO: Richard Marchase, Ph.D.
MCLM-690 0005
FAX: 975-2533

FROM: Suzanne M. Michalek, Ph.D., Vice Chair
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on December 1, 2004.

Title of Application: Cytoplasmic Glycosylation and Hypovolemic Stress

Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW) (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

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