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The role of protein O-linked β -N-acetylglucosamine in mediating cardiac stress responses

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Abstract

The modification of serine and threonine residues of nuclear and cytoplasmic proteins by O-linked β -N-acetylglucosamine (O-GlcNAc) has emerged as a highly dynamic post-translational modification that plays a critical role in regulating numerous biological processes. Much of our understanding of the mechanisms underlying the role of O-GlcNAc on cellular function has been in the context of its adverse effects in mediating a range of chronic disease processes, including diabetes, cancer and neurodegenerative diseases. However, at the cellular level it has been shown that O-GlcNAc levels are increased in response to stress; augmentation of this response improved cell survival while attenuation decreased cell viability. Thus, it has become apparent that strategies that augment O-GlcNAc levels are pro-survival, whereas those that reduce O-GlcNAc levels decrease cell survival. There is a long history demonstrating the effectiveness of acute glucose-insulin-potassium (GIK) treatment and to a lesser extent glutamine in protecting against a range of stresses, including myocardial ischemia. A common feature of these approaches for metabolic cardioprotection is that they both have the potential to stimulate O-GlcNAc synthesis. Consequently, here we examine the links between metabolic cardioprotection with the ischemic cardioprotection associated with acute increases in O-GlcNAc levels. Some of the protective mechanisms associated with activation of O-GlcNAcylation appear to be transcriptionally mediated; however, there is also strong evidence to suggest that transcriptionally independent mechanisms also play a critical role. In this context we discuss the potential link between O-GlcNAcylation and cardiomyocyte calcium homeostasis including the role of non-voltage gated, capacitative calcium entry as a potential mechanism contributing to this protection.

Keywords

Hexosamine biosynthesis; protein O-glycosylation; O-GlcNAc transferase; capacitative calcium entry (CCE); STIM1; Orai1

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1) Introduction

The modification of serine and threonine residues of nuclear and cytoplasmic proteins by O-linked β -N-acetylglucosamine (O-GlcNAc) was first identified by Torres and Hart in 1984 [1]. It has emerged as a highly dynamic post-translational modification that plays a critical role in regulating numerous biological processes, such as nuclear transport, transcription and translation, cytoskeletal organization, signal transduction, proteasomal function and cell survival [2–13]. Much of our understanding regarding the consequences of altered O-GlcNAcylation is in the context of chronic diseases, including aging [14–16], cancer [17–19], neurodegenerative disorders [5,9,20,21] and diabetes and diabetic complications [22,23]. The metabolism of glucose via the hexosamine biosynthesis pathway (HBP) is essential for the synthesis of O-GlcNAc (Fig 1); consequently, there have been numerous reports on the role of sustained increases of O-GlcNAc in contributing to glucose toxicity and insulin resistance [22,23].

However, from an evolutionary perspective the pathways involved in O-GlcNAc synthesis are highly conserved [24] and gene deletion of O-GlcNAc transferase (OGT), which catalyzes the final step in O-GlcNAc synthesis, is embryonically lethal [25]. Thus, the presence of O-GlcNAc must provide some survival advantage to cells and organisms. This has been supported by a number of studies demonstrating that acute increases in O-GlcNAc synthesis improve tolerance to a variety of stress stimuli and increase cell survival. Similarly, while sustained hyperglycemia, such as that associated with diabetes, is clearly associated with adverse effects at the cellular and organismal level, acute increases in circulating glucose levels and cellular glucose utilization undoubtedly have survival benefit. For example, at the organismal level stress induced hyperglycemia is a classical characteristic of the “fight or flight” response, and inhibition of the hyperglycemic response to trauma leads to decreased survival [26–29]. There is also an extensive literature demonstrating that increasing glucose utilization protects against a wide range of injuries including trauma, shock, cardiac surgery and myocardial infarction [30–35].

The beneficial effects of hyperglycemia have typically been thought to be a result of facilitated mobilization of interstitial fluid reserves by increasing osmolarity or, particularly under ischemic or hypoxic conditions, as a fuel for vital organ systems by increasing glycolytic energy production [26,36–42]. However, as noted above, glucose is also essential in the synthesis of O-GlcNAc levels, raising the possibility that the beneficial effects of stress-induced hyperglycemia or metabolic interventions designed to improve glucose utilization are mediated through increases in O-GlcNAc synthesis (Fig 1). Therefore, the goal of this review is evaluate our current understanding regarding the role of O-GlcNAcylation in mediating the cardiac stress response. In addition, we will explore the potential link between O-GlcNAc and cardiomyocyte calcium homeostasis as a potential mechanism contributing to this protection.

2) Metabolic Cardioprotection

There is an extensive literature dating from the 1960s [43] surrounding the effectiveness of acute glucose-insulin-potassium (GIK) treatment in protecting against a wide range of injuries, including hypovolemic shock [34], burn trauma [33], septic shock [32], cardiac surgery [30, 31] and myocardial infarction [35]. GIK has been most widely used in the settings of cardiac surgery and myocardial infarction; in the latter it has been reported to reduce relative mortality by ~30% [35], which is greater than many widely used treatments such as aspirin, thrombolytics and angiotensin converting enzyme inhibitors [44–46]. More recently GIK therapy has been shown to improve outcomes when used during cardiac bypass surgery [47,48]. However, despite repeated studies showing benefit in a number of different pathological and surgical settings, GIK therapy has not gained widespread use, due at least in part to the disparate results

of the various studies [31,49]. In addition, the mechanisms underlying the beneficial effects of GIK therapy are uncertain since it is accompanied not only by high levels of insulin and increased glucose uptake but also lower serum lipid levels as well as increased circulating lactate [50].

While there are numerous systemic factors that may contribute to the cardioprotective effects of GIK, for the past 30 years or so, much of the focus regarding metabolic cardioprotection has been on increasing cardiac glucose metabolism, specifically stimulating glycolysis and glucose oxidation at the expense of fatty acid utilization. This is in part due to the fact that under anaerobic conditions, glycolysis is the sole source of ATP production, and many experimental studies demonstrating the potential benefit of increased glycolysis have utilized models of zero-flow ischemia. However, in humans and animals collateral flow during acute left anterior descending coronary artery occlusion can range from 10–40% [51,52]. In an isolated heart model of low flow ischemia oxidative metabolism contributes >95% of total ATP production, a significant proportion of which is from fatty acid oxidation [53]. Thus, under such conditions increasing glycolysis will yield little in the way of additional ATP production. An alternative rationale proposed for the benefit of increasing glucose metabolism at the expense of fatty acid metabolism is that glucose oxidation is more efficient than fatty acid oxidation with regard to oxidative ATP production. Theoretically, there is an approximately 12% decrease in oxygen required for ATP synthesis when shifting from 100% fatty acid oxidation to 100% glucose oxidation. However, such extreme metabolic shifts are unlikely to occur under physiological conditions, particularly when in the presence of other physiologically relevant fuels, glucose represents only 10–25% of total myocardial oxidative energy production [54]. Nevertheless, in experimental models [55] and patients [47,48], interventions that promote overall myocardial glucose metabolism result in significantly improved outcomes following myocardial ischemia.

While the predominant focus of metabolic cardioprotection has been on glucose, other metabolic interventions, in particular glutamine, have also been shown to be protective in a number of settings including ischemic injury [56,57] and trauma [58,59]. For example, Singleton *et al.* reported that glutamine administration increases the expression of HSP70 in gut epithelial cells and was protective against oxidant and heat injury [60]. Glutamine treatment also successfully reduced TNF- α and IL-1 β expression and led to an improved survival rate following endotoxemia. In addition the authors demonstrated that glutamine treatment prevented the activation of NF- κ B and stress kinase pathways [61]. A number of mechanisms have been postulated for the protection associated with glutamine treatment including increased energy production [56,57], attenuation of NF- κ B activation [61] and increased HSP70 expression [62].

Thus, while both glutamine and glucose have been shown to be cardioprotective, there is little consensus regarding the mechanisms of either intervention. Increased glucose entry into the cell, resulting from direct or indirect stimulation of glucose utilization, can lead to increased flux into the HBP [63,64]. Furthermore, glutamine is a necessary substrate for glutamine: fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme for entry into the HBP (Fig 1); consequently, elevated glutamine levels have been shown to significantly increase UDP-N-acetylglucosamine (UDP-GlcNAc) levels [65]. OGT has a very high affinity for UDP-GlcNAc, with a K_m of 545nM at low micromolar UDP-GlcNAc concentrations [66]; it has also been reported that OGT has three distinct K_m values for UDP-GlcNAc (6, 35, 217 μ M) and that multimerization of the enzyme altered the affinity for UDP-GlcNAc [67]. This high affinity for UDP-GlcNAc not only provides OGT with a competitive advantage over nucleotide transporters in the endoplasmic reticulum and Golgi, which compete for cytoplasmic UDP-GlcNAc, but also makes it highly sensitive to changes in UDP-GlcNAc concentrations [67]. It should also be noted that insulin stimulates tyrosine phosphorylation and catalytic activity

of OGT [68]. Thus, a common feature of these approaches for metabolic cardioprotection is that they both have the potential to either increase UDP-GlcNAc levels by either by activating HBP or by stimulating OGT activity, thus augmenting O-GlcNAc synthesis (Fig 1).

At the cellular level hyperglycemia-mediated protection against ischemia/reperfusion injury has been shown to be dependent on flux through the HBP and was associated with increased O-GlcNAc levels [69]. We have also recently shown in the intact heart that glutamine-induced ischemic protection was dependent on flux through GFAT and the subsequent increase in O-GlcNAc [70]. It is noteworthy that the protection associated with both glutamine and O-GlcNAc have been linked to increased HSP70 expression [60,71] and attenuation of stress-induced inflammatory responses [61]. Thus, activation of the HBP leading to increased protein O-GlcNAcylation may represent a common mechanism underlying the cardioprotection associated with increased glucose utilization, insulin and glutamine (Fig 1).

3) Hexosamine biosynthesis, O-GlcNAcylation and Cardioprotection

As discussed above and illustrated in Fig 1, both high glucose and glutamine are potential activators of the HBP and at least part of their cardioprotective mechanism can be attributed to increased O-GlcNAc synthesis. At the cellular level O-GlcNAc levels are dramatically increased in response to multiple experimental stress stimuli [69,71,72] and when this response is prevented cell viability is reduced. Since OGT catalytic activity is highly sensitive to changes in UDP-GlcNAc concentrations [66,67], an acute increase in UDP-GlcNAc concentrations should be an effective means for increasing O-GlcNAc levels. Glucosamine is transported into cells via the glucose transporter system and is phosphorylated to glucosamine-6-phosphate by hexokinase, bypassing GFAT (Fig 1), thereby leading to a rapid increase in UDP-GlcNAc levels [73]. To determine whether glucosamine is cytoprotective, we used an isolated perfused rat heart model, which involves the rapid removal of the heart from the animal, cannulation of the aorta followed by perfusion with Krebs-Henseleit buffer. A fluid filled balloon placed into the left ventricle and connected to a pressure transducer permits the assessment of contractile function. Using this model, we found that a 10min perfusion with 5mM glucosamine resulted in a marked (2–3-fold) increase in O-GlcNAc levels in the heart, with no effect on cardiac function [74]. The first stress we used to test whether glucosamine was cardioprotective, was the so-called “calcium paradox” protocol [75]. In this protocol hearts are perfused for a brief period with calcium free buffer (~10min) leading to complete cessation of contractile activity; paradoxically, when the heart is subsequently reperfused with normal Ca^{2+} buffer, contractile activity is not restored, rather there is rapid and massive Ca^{2+} overload, resulting in severe tissue injury. As noted above treatment with glucosamine had no effect on normal contractile function and it did not prevent the decline in function on removal of Ca^{2+} . However, when reperfused with normal Ca^{2+} buffer, the addition of glucosamine almost completely prevented the tissue injury seen in untreated hearts and significantly improved contractile function [74].

There are many similarities between the Ca^{2+} -overload seen with the calcium paradox, compared to that which occurs following myocardial ischemia and reperfusion; therefore, we subsequently examined whether glucosamine would protect against ischemic injury. Similar to the results with the calcium paradox protocol, 10min pre-treatment of the isolated perfused heart with 5mM glucosamine, prior to ischemia and reperfusion, resulted in significantly improved functional recovery compared to untreated hearts [74]. We also found that alloxan, which inhibits OGT [76], prevented the increase in O-GlcNAc and reversed the protection afforded by glucosamine treatment [74]. Alloxan has significant limitations as an inhibitor of OGT [77] and glucosamine increases glucosamine-6-phosphate and UDP-GlcNAc levels as well as O-GlcNAc levels; nevertheless, this was the first report that supported the notion that increasing cardiac O-GlcNAc levels could be an effective cardioprotective strategy.

It was subsequently demonstrated that the tolerance of neonatal cardiomyocytes to hypoxia and reoxygenation could also be improved by increasing O-GlcNAc levels with glucosamine or high glucose treatment [69]. The protection observed with high glucose was prevented by inhibiting GFAT, as was the associated increase in O-GlcNAc, demonstrating the role of the HBP in mediating this response. Conversely, alloxan treatment significantly increased apoptosis and necrosis, and decreased cell viability, supporting a role for OGT in mediating cell survival [69]. An alternative approach for increasing O-GlcNAc levels independent of the HBP is to prevent removal of O-GlcNAc from proteins by inhibiting O-GlcNAcase (Fig 1). PUGNAc [O-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-N-phenylcarbamate] is an O-GlcNAc analog that is a potent competitive inhibitor of O-GlcNAcase and has been widely used to increase O-GlcNAc levels [78,79]. We found that PUGNAc treatment had a similar effect on improving cardiomyocyte viability and reducing necrosis as glucosamine treatment [69]. Since glucosamine and PUGNAc increase O-GlcNAc levels by entirely different mechanisms, these data provided further evidence that augmentation of cardiomyocyte O-GlcNAc levels would increase the tolerance of the heart to acute stress.

In these initial studies, either in the isolated perfused heart [74] or in cardiomyocytes [69], glucosamine and/or PUGNAc were administered shortly before or at the beginning of the ischemic or hypoxic period. However, numerous promising cardioprotective treatment strategies that have been shown to be beneficial when used prior to ischemia, prove to be ineffective when used only during reperfusion [80]. Therefore, using the same isolated perfused rat heart model described above, we examined the effectiveness of both glucosamine and PUGNAc in mediating functional recovery when they were administered only during reperfusion [81]. Representative typical left ventricular (LV) pressure traces recorded from a fluid filled balloon placed in the left ventricle of the perfused heart and connected to a pressure transducer are shown in Fig. 2A–C. In all groups prior to ischemia the developed pressure was ≥ 100 mmHg; ischemia was induced by stopping flow of perfusate to the whole heart, which is indicated by absence of any pressure recording. After 20 min of ischemia, flow to the heart was restored and this is reflected by the gradual recovery of developed pressure. At the time of reperfusion hearts received either no treatment or were treated with glucosamine (10 mM) or PUGNAc (200 μ M) for the first 20 min of reperfusion. As can be seen from the LV pressure recordings (Fig 2A–C) the addition of either glucosamine or PUGNAc during the first 20 min of reperfusion substantially improved recovery of LV developed pressure; this is reflected in the averaged data in Figs. 2D and 2E. The improvement in function in the treatment groups was associated with a significant decrease in tissue injury as indicated by lower cardiac troponin I release during reperfusion (Fig 2F) [81]. This study demonstrated that acutely increasing O-GlcNAcylation at the time of reperfusion, either by increasing either synthesis or inhibiting degradation, may be a valuable therapeutic strategy for the treatment of ischemic heart disease. One limitation of these studies is that they were performed in an ex-vivo preparation; however, administration of both glucosamine and PUGNAc in vivo improved outcomes in a rodent model of hemorrhagic shock, which included greater cardiac output and contractility [82,83], demonstrating that these interventions are effective in vivo. Subsequently, PUGNAc was shown to decrease infarct size in a mouse model of myocardial ischemia/reperfusion [84], providing further support for the utility of these approaches in vivo.

The fact that both glucosamine and PUGNAc afforded similar levels of cardioprotection and yet increased O-GlcNAc levels via different mechanisms provided strong support for the fundamental concept that the protection was mediated via activation of O-GlcNAcylation. Nevertheless, the effects of glucosamine could be mediated via pathways other than O-GlcNAc such as ganglioside [85] or cell surface N-glycan [86] synthesis. PUGNAc also inhibits other β -hexosaminidases [87–89], which may alter processing of glycoconjugates in addition to O-GlcNAc. However, in isolated cardiomyocytes both glucosamine and OGT overexpression increased O-GlcNAc levels and significantly decreased ischemia/reperfusion induced injury,

and attenuated the loss of mitochondrial cytochrome C [90]. Both interventions also attenuated the loss of mitochondrial membrane potential induced by oxidative stress [90]. Conversely, decreased OGT expression attenuated the ischemia-induced increase in O-GlcNAc, and this was associated with increased necrosis, greater loss of cytochrome C and increased apoptosis following ischemia/reperfusion [90]. Jones and colleagues have also shown by altering the levels of OGT and O-GlcNAcase expression that the tolerance of cardiomyocytes to hypoxia/reperfusion injury is clearly mediated via O-GlcNAcylation [91,92]. Taken together these studies demonstrate that strategies that augment O-GlcNAc levels are clearly pro-survival, whereas those that decrease O-GlcNAc levels reduce cell survival.

A number of different mechanisms have been proposed to account for the protection associated with increased O-GlcNAc levels, such as increased synthesis of HSP40 and HSP70 [71], both of which are also known to be subject to O-GlcNAc modification. The activity of the transcription factor Sp1 also increases when modified by O-GlcNAc [93,94] and this may contribute to the increased synthesis of heat shock proteins [95] as well as pro-survival Bcl-2 family members [96]. Increased O-GlcNAc levels as a result of glucosamine treatment resulted in increased p38 phosphorylation at the end of reperfusion [97]. While p38 activation is frequently considered to be pro-apoptotic, it can also lead to activation of pro-survival pathways through downstream effectors, such as α B-crystallin and HSP27. Both α B-crystallin and HSP27 have been shown to play a role in ischemic protection [98–102] and are targets for O-GlcNAc modification [9,103,104]. Increased levels of O-GlcNAc have also been reported to inhibit protein degradation [4,13], most likely due to inhibition of the proteasome [105], which may also contribute to enhanced cell survival.

It is noteworthy however, that in the isolated perfused heart, cardioprotection is conferred after a very brief (5 min) exposure to glucosamine [74,97]; this suggests that O-GlcNAc-related protection does not depend on de novo protein synthesis. In addition to protecting against ischemia/reperfusion injury, increasing O-GlcNAc synthesis with glucosamine also resulted in remarkable protection against calcium overload induced by the calcium paradox protocol [74]. These data suggest that transcriptionally independent mechanisms, involving attenuation of calcium overload, may contribute to O-GlcNAc mediated cardioprotection. In isolated cardiomyocytes increased O-GlcNAc levels were associated with a decrease in calcium influx as a result of ischemia/reperfusion [69]. One consequence of minimizing cytoplasmic calcium levels is to prevent activation of the protease calpain, and increasing O-GlcNAc levels with either glucosamine or PUGNAc, attenuated the ischemia/reperfusion-induced increase in calpain-mediated proteolysis [81]. Thus, one mechanism of protection associated with increased O-GlcNAc levels could be attenuation of the calcium overload that often accompanies stresses such as ischemia/reperfusion [106,107]. Consequently, while acute activation of pathways leading to O-GlcNAc formation may confer protection via increased transcription of pro-survival factors including heat shock proteins, it is also likely that there are also transcriptionally independent mechanisms that include modulation of stress-activated kinase pathways, post-translational modification of pro-survival factors as well as attenuation of calcium-mediated stress responses.

4) Protein O-GlcNAcylation and cardiomyocyte calcium homeostasis

A critical component of both cell signaling and cell survival is the maintenance of the concentration of cytoplasmic free Ca^{2+} ($[\text{Ca}^{2+}]_i$). When $[\text{Ca}^{2+}]_i$ is excessive, Ca^{2+} overload and cell death rapidly ensue. As cells evolved mechanisms for surviving during stress, a primary adaptation might be expected to have been a mechanism to stifle Ca^{2+} overload. While there may well be multiple mechanisms responsible for the influx leading to this overload, data we and others have gathered, suggest that at least under certain conditions capacitative calcium entry (CCE) is an important mediator of stress induced Ca^{2+} overload. CCE refers to a Ca^{2+}

influx pathway mediated by non-voltage-gated, store-operated channels (SOCs). Typically CCE is 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) [108]. CCE characterizes all non-excitabile cells except erythrocytes [109], and has been shown to co-exist with the voltage-gated Ca^{2+} channels in smooth [110] and skeletal muscle cells [111–115]. The presence of CCE in the heart is under explored; however, evidence supporting the existence of a voltage-independent calcium entry pathway in cardiomyocytes is plentiful although not integrated [116–122]. Furthermore, since 2002 there has been a gradual accretion of data from us [123–125] and more recently other investigators [126,127] clearly demonstrating the presence of CCE in cardiomyocytes.

The development of links between the HBP, CCE and attenuation of cellular damage due to calcium overload has evolved over the last decade, beginning with studies of CCE in response to IP_3 -generating agonists in excitable cells. In 1995, Rivera et al. [128] were among the first groups to describe a CCE pathway in smooth muscle cells. Of particular relevance, in that study, a marked diminution of CCE following exposure to hyperglycemia was observed; however, at that time the specific metabolic pathway responsible was not described. Subsequently, Vemuri and Marchase [129] showed that the addition of glucosamine to the J774 macrophage cell line resulted in inhibition of CCE without affecting ATP levels. As the primary pathway of glucosamine metabolism is via the HBP, this study provided the first indication that CCE may be regulated by HBP activity and thus by extension O-GlcNAc levels.

While there is broad acceptance of CCE as an important mediator of function of non-excitabile cells, as noted above its role in regulating cardiomyocyte function is less well established. However, in 2002, Hunton et al. [123] demonstrated for the first time that CCE was an important attribute in neonatal cardiomyocytes, demonstrating an increase in $[\text{Ca}^{2+}]_i$ that was dependent on ER store depletion and negated by agents shown to inhibit CCE in other cells, such as SKF96365 but not by inhibitors of voltage-gated channels. Agonists that generate IP_3 , such as phenylephrine and angiotensin II, which result in release of calcium from sarcoplasmic reticulum also caused $[\text{Ca}^{2+}]_i$ to increase via a CCE-like pathway. In addition, CCE inhibitors also negated the nuclear localization of NFAT, a transcription factor critical in regulating cardiomyocyte hypertrophy that occurs in response to IP_3 -generating agonists. Significantly, all of these responses were rapidly and reversibly inhibited by glucosamine [123] and hyperglycemia [125]; furthermore, the inhibition of CCE-mediated responses by hyperglycemia was blocked by inhibition of the HBP [125]. Nagy et al. subsequently demonstrated that the effects of glucosamine on CCE in cardiomyocytes were mediated via increased O-GlcNAc levels [130]. Additionally PUGNAc, which increases O-GlcNAc independently of the HBP by inhibiting O-GlcNAcase, was found to mimic the effects of glucosamine and attenuate CCE [130]. Further studies demonstrated that CCE was present in adult cardiomyocytes and this could also be inhibited by glucosamine [124]. In the isolated perfused heart the inotropic effects of the IP_3 -generating agonists phenylephrine and angiotensin II were also blunted by glucosamine as well as the independent inhibitor of CCE, SKF96365 [131]. Taken together these studies demonstrated not only a role for CCE in mediating cardiomyocyte function, but also for the regulation of CCE by the HBP and O-GlcNAcylation.

Our observations that activation of the HBP and increased O-GlcNAc levels attenuates CCE in cardiomyocytes, protects against Ca^{2+} overload induced by the calcium paradox, and blunts ischemia/reperfusion induced activation of Ca^{2+} -mediated proteases has led us to postulate that CCE may be a major factor contributing to the Ca^{2+} influx seen following ischemia or Ca^{2+} deprivation that contributes to $[\text{Ca}^{2+}]_i$ overload. This is supported by several reports demonstrating that in arterial myocytes hypoxia leads to store operated depletion and activates

CCE [132–134]; however, it remains to be determined whether this also occurs in cardiomyocytes. As a corollary therefore, we propose that one mechanism of protection associated with increased O-GlcNAc levels is attenuation of calcium overload via inhibition of CCE.

One of the limitations underlying the investigations in the regulation of CCE has been the lack of molecular mechanisms or channel proteins responsible for mediating this process; however, there is now growing consensus that STIM1 and Orai1 are essential for activation of CCE [115,135,136]. STIM1 contains 2 protein-protein interacting domains separated by a single membrane-spanning segment; the N terminal region is within the lumen of the ER/SR and contains a SAM protein interaction domain; between the N-terminal sequence and SAM region there is EF hand Ca^{2+} -binding domain that serves as the sensor of Ca^{2+} within the ER or SR (Fig 3A)[137]. In the cytoplasm there are two coiled-coil domains and closer to the C-terminal region there is a proline-serine rich domain, which contains putative phosphorylation sites [136]. Significantly, this region is not only a prime site for phosphorylation but also has the characteristics of preferred acceptor sites recognized by OGT for the addition of O-GlcNAc [138] (Fig 3A). The prevailing view is that in response to a decrease in the intra-ER Ca^{2+} levels, Ca^{2+} is released from the EF-hand of STIM1, which first triggers STIM1 oligomerization in the ER membrane. This followed by translocation of STIM1 to the plasma membrane and subsequent interaction with Orai1, leading to activation of CCE (Fig 3B) [136,137]. This is a simplified description as it does not include the potential roles of STIM2, Orai2 and Orai3; however, of relevance to our discussion, deletion of the serine-proline rich region of STIM1 prevented the ability of STIM1 to activate Ca^{2+} influx [136]. Therefore, we hypothesize that an increase in cellular O-GlcNAc levels leads to increased O-GlcNAcylation of the serine-proline rich region of STIM1, which either inhibits the initial oligomerization of STIM1, prevents translocation or blocks the interaction of STIM1 with Orai1 (Fig 3A,B). Indeed, it has been shown that O-GlcNAcylation of certain proteins inhibits or disrupts their interaction with specific binding partners [139–142]. Thus, we postulate that O-GlcNAcylation of STIM1 prevents the formation of channels required for CCE, resulting in the diminution of calcium influx seen in response to IP_3 -generating agonists as well as in response to stresses such as ischemia or the calcium paradox that otherwise results in calcium overload and cell damage.

5) Conclusion

While O-GlcNAc modification of serine and threonine residues of nuclear and cytoplasmic proteins was first identified in 1984 [1], it was only in 2004 that the potential beneficial role of O-GlcNAc in mediating cell survival was first clearly recognized [3]. Since that time, there is a rapidly emerging consensus that acute activation of O-GlcNAc synthesis by the addition of glucosamine or inhibition of O-GlcNAc degradation with O-GlcNAcase inhibitors affords remarkable ischemic cardioprotection. At the cellular level gain and loss of function studies in neonatal cardiomyocytes provide further support that OGT and O-GlcNAcase play critical role in regulating the response to acute ischemic and oxidative stress [90–92]; however, the specific mechanisms underlying the protection associated with increased O-GlcNAc levels remains elusive. At the transcriptional level, protection appears to be due at least in part by increased levels of heat shock proteins [71]. On the other hand, the fact that ischemia/reperfusion injury is attenuated even with very brief pre-treatment protocols, as well as interventions at the time of reperfusion, strongly suggests that transcriptionally independent mechanisms play a critical role. Potential mechanisms include activation of pro-survival kinase pathways [97], increased O-GlcNAcylation of small heat shock proteins or preventing loss of mitochondrial membrane potential [84,90]. However, our data suggests that an additional mechanism is attenuation of Ca^{2+} overload, which plays a key role in the evolution of ischemia/reperfusion injury. There are a number of Ca^{2+} entry pathways that have been implicated in Ca^{2+} overload in the heart; however, given the presence of CCE in cardiomyocytes and the fact that increased O-GlcNAc

levels attenuate CCE-mediated Ca^{2+} entry, we propose that inhibition of CCE is an important mechanism underlying the acute cardioprotection associated with O-GlcNAcylation. The recent identification of key proteins responsible for mediating CCE, namely STIM1 and Orai1, provide a foundation for testing this hypothesis.

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Figure 1.

A schematic of the hexosamine biosynthesis pathway and protein O-GlcNAcylation adapted from Laczy et al. *Am. J. Physiol. – Heart Circ Physiol* 296: H13-H28, 2009 [143]. Glucose imported into the cells is rapidly phosphorylated to glucose-6-phosphate and converted to fructose-6-phosphate, which is subsequently metabolized to glucosamine-6-phosphate by L-glutamine-D-fructose 6-phosphate amidotransferase (GFAT) ultimately leading to the synthesis of uridine-diphosphate-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is the obligatory substrate for OGT (uridine-diphospho-N-acetylglucosamine: polypeptide β -N-acetylglucosaminyltransferase), which catalyzes the formation of O-linked β -N-acetylglucosamine (O-GlcNAc) on serine and threonine residues of proteins. β -N-

acetylglucosaminidase (O-GlcNAcase) catalyzes the removal of O-GlcNAc from the proteins. Glucose entry into the HBP can be attenuated by inhibition of GFAT by the glutamine analogs 6-diazo-5-oxo-L-norleucine (DON) and O-diazoacetyl-L-serine (Azaserine). The level of O-GlcNAc on proteins can be blocked by inhibiting OGT with the uridine analog Alloxan or with TTO4 (2[(4-chlorophenyl)imino] tetrahydro-4-oxo-3 (2-tricyclo[3.3.1.1^{3,7}]dec-1-ylethel)); whereas O-GlcNAcylation of proteins can be rapidly increased by inhibiting O-GlcNAcase with PUGNAc (O-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-N-phenylcarbamate) or with NAG-thiazoline (1,2 dideoxy-2-methyl-D-glucopyranoso(2,1-d)-2-thiazoline). As described in the text several different interventions that have been shown to be cardioprotective, such as increasing levels of glucose, glutamine, insulin and most recently glucosamine all have the potential to increase flux through the HBP and thus augment protein O-GlcNAc levels.

Figure 2.

Left ventricular (LV) pressure recording from isolated perfused rat hearts before and after 20 min global no flow ischemia in A) untreated, control B) glucosamine (10 mM) for first 20 min of reperfusion; C) PUGNAC (200 μ M). The LV pressure traces were recorded via a fluid filled balloon placed in the left ventricle of the perfused heart and connected to a pressure transducer. The onset of ischemia is indicated by absence of any pressure recording and reperfusion is reflected by the gradual recovery of developed pressure. D) Functional recovery of heart rate (HR), left ventricular developed pressure (LVDP), rate-pressure product (RPP) and positive and negative rates of pressure change ($\pm$ dP/dt) following 20 minutes ischemia and 60 minutes reperfusion as a % of pre-ischemic values; E) End diastolic pressure (EDP) at the end of reperfusion; F) Total cardiac troponin I (cTnI) release during reperfusion in control (n=6),

glucosamine (n=6) and PUGNAc (n=4) groups; *= p<0.05 compared to control group; one-way ANOVA with Bonferroni's Comparison Test. (From: Liu et al., Am J Physiol Heart Circ Physiol 293: H1391-H1399, 2007 [81]).

Figure 3.

A) STIM1 contains 2 protein-protein interacting domains separated by a single membrane-spanning segment; the N-terminal region is within the lumen of the ER/SR and contains a SAM (S) protein interaction domain; between the N-terminal sequence and SAM region there is EF-hand Ca^{2+} -binding domain, which acts as the sensor of Ca^{2+} within the ER/SR. In the cytoplasm STIM1 contains two coiled-coil domains and closer to the C-terminal region there is a proline-serine rich domain (P), which contains putative phosphorylation sites and is also has the characteristics of preferred acceptor sites recognized by OGT for the addition of O-GlcNAc. (Adapted from Cahalan, *Nature Cell Biology* 11: 669–677, 2009 [137]). B) In response to a decrease in the intra-ER Ca^{2+} levels, Ca^{2+} is released from the EF-hand of STIM1, triggering

STIM1 oligomerization followed by translocation of STIM1 to the plasma membrane and interaction with Orai1, leading to activation of CCE [136,137]. We hypothesize that an increase in O-GlcNAcylation of the serine-proline rich region of STIM1 either inhibits the initial oligomerization of STIM1, prevents translocation or blocks the interaction of STIM1 with Orai1 thereby attenuating CCE (Adapted from Liou et al., *PNAS*, 104: 9301–9306,2007 [144]).