The role of O-glycosylation in diabetes and in diabetic cardiomyopathy and nephropathy.

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Summary:

Protein O-glycosylation and the hexosamine biosynthesis pathway are increasingly recognized as an important cell-signaling pathway. It has been proposed that O-glycosylation plays a role in the response to acute stress stimuli, and that increased O-glycosylation and hexosamine biosynthesis pathway flux is responsible at least in part for the development of diabetes and diabetic complications.

In our experiments we investigated the role of these pathways in mediating the response to acute ischemic stress in the heart. We have shown that activation of O-glycosylation occurs as an acute stress response and that enhanced O-glycosylation and increased hexosamine flux lead to protection against ischemia/ischemia-reperfusion injury in the heart and our experiments revealed the fact that this protective effect was associated with alteration in p38 MAPK phosphorylation.

As proposed by other studies O-glycosylation and the hexosamine pathway is associated with age related diseases. We found that with age O-glycosylation was increased in the heart, skeletal muscle, aorta and in the brain. Our results also suggested that the increase in O-glycosylation was a consequence of the increased glutamine: fructose 6-phosphate amidotransferase expression and increased flux through the hexosamine biosynthesis pathway.

In type-2 diabetic hearts hexosamine biosynthesis flux was also increased and O-glycosylation was elevated on high molecular weight proteins. This increase was associated with impaired excitation-contraction coupling, a hallmark of diabetic cardiomyopathy. We found that increasing O-glycosylation lead to changes in cardiac energy metabolism similar to those seen in diabetic hearts, namely increased fatty acid oxidation and decreased lactate utilization. We demonstrated that altered AMP-activated protein kinase and acetyl-CoA carboxylase activation was not involved in

the pathomechanism how enhanced O-glycosylation has changed energy metabolism.

We also examined the effect of O-glycosylation in the human diabetic kidney and found that O-glycosylation was increased both in the glomerular and tubular cells of diabetic individuals and this increase was associated with the presence of diabetic nephropathy.

Introduction:

Diabetes mellitus:

Type-2 diabetes mellitus is an increasing problem both in the developed and in the developing countries. According to the European Cardiovascular Disease Statistics Database the incidence of diabetes is increasing, it has been estimated that over 48 million adults had diabetes in Europe in 2005. The estimated prevalence of diabetes in Hungary was over 10% (11,2%).
(http://www.ehnheart.org/content/sectionintro.asp?level0=1457)

Type-2 diabetes is characterized by insulin resistance, hyperglycemia and dyslipidemia. Although the mechanisms underlying the development of diabetic complications is not completely understood, it has been proposed that there are four different mechanism mediating the adverse effect of chronic hyperglycemia; including increased flux through the polyol pathway [1]; increased formation of advanced glycation end-products (AGE) [1, 2]; increased protein kinase C (PKC) activation [1, 3] and increased flux through the hexosamine biosynthesis pathway (HBP) and/or increased levels of *O*-linked N-acetylglucosamine (*O*-GlcNAc) on proteins [1, 4-6].

Hexosamine Biosynthesis Pathway and its role in diabetes and the development of insulin resistance:

A small proportion of the uptaken glucose into the cells goes through the hexosamine biosynthesis pathway producing uridine 5'-diphospho N-acetylglucosamine (UDP-GlcNAc) (Figure 1).
It has been estimated that in cultured adipocytes HBP consumes 2-5 % of the glucose taken up by the cells [7]. The key regulatory enzyme of the pathway is the glutamine: fructose 6-phosphate amidotransferase (GFAT), converting fructose 6-phosphate to glucosamine 6-phosphate using glutamine as the amine donor [8]. Uridine 5'-diphospho N-acetylglucosamine is the endproduct of the pathway, serving

as a substrate for the addition of a single N-actetylglucosamine to serine or threonine residues of both nuclear and cytosolic proteins (O-glycosylation, O-GlcNAc). There are two isoforms of GFAT; GFAT1 is ubiquitously expressed, while GFAT2 is mainly expressed in the central nervous system [9]. GFAT has been shown to be regulated by the hexosamine products [8, 10]. Broschat et al. showed that both UDP-GlcNAc and glucosamine 6-phosphate ($GlcNH_2$-6-P) were potent inhibitors of human GFAT1 in vitro [10]. Combined treatment with insulin, glucose and glutamine decreased GFAT activity in adipocytes, while insulin or high glucose treatment alone or in combination failed to reduce the activity of GFAT [11]. Glucosamine, which enters the hexosamine biosynthesis pathway bypassing GFAT (Figure 1), also decreased the activity of the enzyme, suggesting the important role of the hexosamine products in GFAT regulation [11].

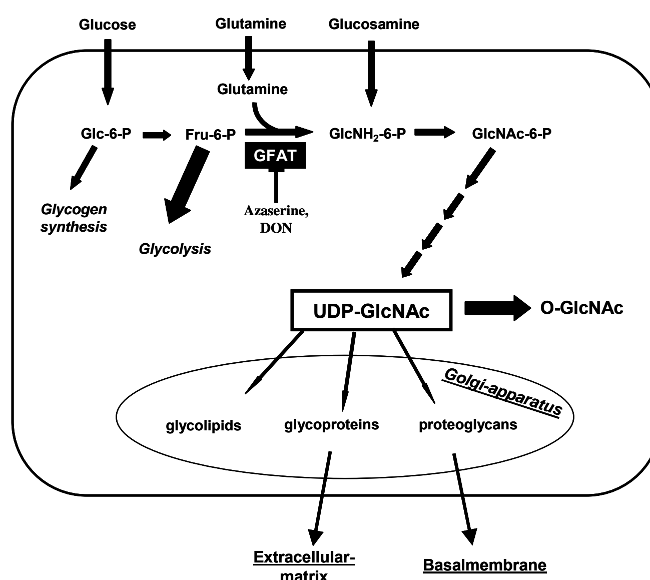


Figure 1.: *The Hexosamine Biosynthesis Pathway*

Muscle of GLUT1 overexpressing mice have increased GFAT activity without changes in GFAT mRNA levels, resulting in 2-3 fold increase of nucleotide-linked sugar concentrations and leading to insulin resistance [12]. While the glucose transporter-4 (GLUT4) overexpressing animals, with only a mild increase in basal glucose transport, do not develop insulin resistance or have elevated UDP-

hexosamine levels [12], suggesting that increased glucose transport can also enhance the flux through the pathway.

The role of the hexosamine biosynthesis pathway in the development of insulin resistance was first described by Marshall et al. in rat adipocytes [7]. Transgenic mice overexpressing GFAT in skeletal muscle and adipose tissue develop insulin resistance and hyperleptinaemia [13, 14]. In vivo glucosamine infusion also resulted in skeletal muscle insulin resistance [15-17]. These data suggests the notion that increased flux through the hexosamine biosynthesis pathway is playing an important role in the development of insulin resistance and glucose toxicity. It has been shown in type-1 diabetes - induced by streptozotocin (STZ) – rats that the hexosamine pathway flux was increased [5].

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. UDP-GlcNAc, the end-product of the HBP, serves as a substrate for O-glycosylation, which is thought to be the mediator of increased HBP flux leading to diabetes and insulin resistance.

Nuclear and cytoplasmic O-glycosylation and its relationship with diabetes and insulin resistance:

O-glycosylation is an ubiquitous post-translational modification of nuclear and cytoplasmic proteins present in all higher eukaryotes and is increasingly recognized as an important regulatory mechanism involved in signal transduction [18-21]. Unlike other glycosylation events this reaction occurs in the cytosol and in the nucleus rather than in the Golgi or the endoplasmic reticulum [22, 23], and is regulated by two key-enzymes; uridine diphospho-N-acetylglucosamine: polypeptide β-N-acetylglucosaminyltransferase (O-GlcNAc transferase; OGT) - catalyzing the addition of N-acetylglucosamine to the proteins [24, 25], and N-acetylglucosaminidase (O-

GlcNAcase, OGA) - catalyzing the removal of the sugar moiety from the proteins (Figure 2) [26].

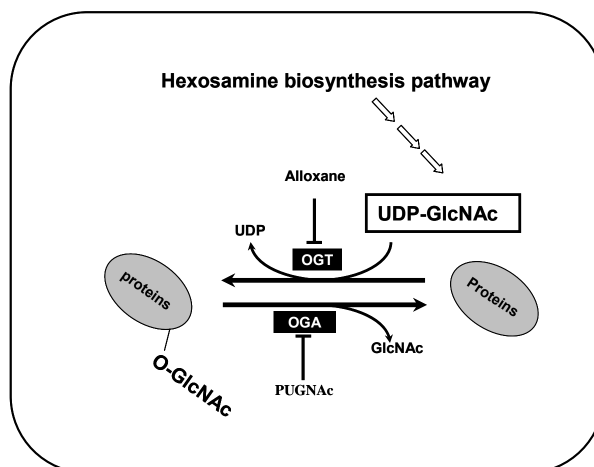


Figure 2.: *O-glycosylation*

O-glycosylation is highly dynamic posttranslational modification similar to phosphorylation, in that the processes, both O-glycosylation and deglycosylation are enzymatically controlled [27-30] [24-26]. However in contrast to the more than 500 kinases regulating phosphorylation [31], there is a single gene encoding OGT [32, 33]. All metazoans have a highly conserved OGT, C. elegans OGT shows 68% identity to the human form at amino acid sequence level [34]. A single copy of OGT gene is located on the X chromosome in humans and mice [35]. OGT is a trimer; in certain tissues (eg.: liver, muscle, kidney) it is a heterotrimer consisting of two 110 kDa and one 78 kDa subunit [24], while in other tissues it exists as a homotrimer of three 110 kDa subunits [32]. The 78 kDa subunit may result from an internal translation start site or from an alternate splicing [32]. Recently a mitochondrial form of OGT (mOGT) has been identified, which is mainly consisting of 103 kDa subunits [36, 37]. The N-terminus of the shorter splice variant contains a unique mitochondrial targeting information [37]. Love et al. demonstrated that the longer isoform localizes mainly in the nucleus, while the shorter isoform targets the mitochondria [37]. Although the mOGT is enzymatically active in vitro, there is only a few O-glycosylated proteins found in mitochondria [37].

The structure of the 110 kDa subunit is well known, it contains two main domains; an N-terminal domain containing multiple tetratricopeptide repeats (TPR) and a catalytic domain in the C-terminus, and also contains a nuclear localization sequence [32, 34]. The TPR domains mediate protein-protein interactions and at least 5 TPR domains are required for substrate binding [33].

Regulation of OGT activity is complex and not yet fully understood. The K_m value for UDP-GlcNAc of the OGT is one of the lowest (545 nM) among galactosyltransferases, which gives a competitive advantage over the UDP-GlcNAc transporters [24], which transport UDP-GlcNAc from the cytosol to the Golgi apparatus or to the endoplasmic reticulum. Kreppel and Hart showed that multimerization of the enzyme alter the affinity for UDP-GlcNAc [33]. OGT is also known to be a target of posttranslational modifications; tyrosin phosphorylation and O-glycosylation, although the role of these modifications is not clear [32]. Recently Whisenhunt and colleagues demonstrated that OGT directly associates with the O-GlcNAcase [38], the other enzyme regulating O-glycosyalation by removing the single N-acetylglucosamine moiety from the Ser/Thr residues of the modified proteins.

N-acetylglycosidase first characterized by Hart and colleagues, consists of a 106 kDa heterodimer complex containing a 54 KDa α and a 51 kDa β subunit [39]. However the native O-GlcNAcase activity corresponded with an approximately 600 kDA protein complex purified from cow brain, containing heat shock proteins (HSP110, HSC70) and intracellular signal transducers (DRP-2, calcineurin and amphiphysin) [40]. The O-GlcNAcase gene is localized on the chromosomal location 10q24 [40]. O-GlcNAcase is distinct from the lysosomal hydrolases, including hexosaminidase A and B, both of them having an acidic pH optimum and can be inhibited by both N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) [41]. The pH optimum of O-GlcNAcase is 6.4, below this pH it loses it`s activity, while at pH 6.0 or higher the activity is maintained, and the activity is not

inhibited by GalNAc [26, 39, 40]. The enzyme is specific for N-acetyl-β-D-glucosaminides, and shows no N-acetyl-β-D-galactosaminidase activity [26, 39, 40]. O-GlcNAcase seems to be responsible for the hexosaminidase C activity in higher eukaryotes [40]. 90% of the O-GlcNAcase activity is localized in the cytosol, and the remaining 10% is found in the nucleus, protein assays showed similar results [40]. The activity of the enzyme is variable in different tissues. In rats the spleen, kidney, liver, brain, and the lung have strong O-GlcNAcase activity, while the heart, uterus and spinal cord showed significantly less activity [39].

Toleman et al. identified splice variants from rats. In Goto-Kakizaki rat (which is a model of type-2 diabetes) an exon 8 sequence missing variant was expressed as protein of 90 kDa and had no O-GlcNAcase activity but the histone acetyltrasferase (HAT) activity was preserved [42]. In these animals decreased O-GlcNAcase activity may lead to increased O-GlcNAc levels and together with the increased OGT activity playing an important role in the development of diabetic complications.

O-glycosylation is becoming increasingly recognized as an important regulatory mechanism involved in signal transduction [27-30]. Wide range of proteins have been listed in recent reviews that are modified by O-glycosylation, including kinases, phosphatases, transcription factors, metabolic enzymes, chaperons, cytoskeletal proteins [20, 21, 43]. The role of O-glycosylation is extensive due to the wide range of protein groups known to be modified by OGT [19-21, 27, 43-45]. O-glycosylation has been shown to modify DNA binding [46], enzyme activity [47, 48], protein-protein interactions [49, 50], half-life of the proteins [50] and subcellular localization [51].

It has been shown that on some proteins O-glycosylation and phosphorylation are reciprocal [30] (e.g., eNOS [47], estrogen receptor-β [52], C-terminal domain of RNA polymerase II [53], c-myc [54]), since both post-translational modifications involve Ser/Thr residues. The study by Wells et al. demonstrated that OGT forms a

complex with protein phosphatase-1β (PP1β) and PP1γ [55], which further supports the notion that there is a dynamic interplay between O-GlcNAc and phosphorylation. However, this is not simply reciprocal relationship, since there can be multiple sites on the same protein which can be both O-glycosylated or phosphorylated.

Importantly, Shafi et al. showed that knock out of OGT in mice was embryonically lethal, and deletion of the OGT gene in embryonic fibroblasts was also lethal [35], suggesting that OGT activity/O-glycosylation is vital for life. Chromosomal proteins are known to be O-glycosylated [20, 21, 43], and several transcription factors and RNA polymerase II itself [53] are also a target for O-glycosylation. Sp1 an extensively investigated transcription factor, responsible for the transcription of housekeeping proteins, is modified by O-glycosylation [56]. A recent study showed that OGT and O-GlcNAcases form a complex accompanied by histone deacetylases, this complex acts as a transcription corepressor, regulating gene-expression [38].. That study, combined with the fact that increased flux through the HBP is associated with transcriptional upregulation of a number of key proteins, such as transforming growth factor (TGF)-α [57], TGFβ [58], leptin [59], plasminogen activator inhibitor-1 (PAI-1) [60] and that many transcription factors are O-glycosylated, provides strong evidence to suggest that O-GlcNAc is involved in the regulation of gene-expression [44].

Kudlow and colleagues have reported that reduction in the O-glycosylation of Sp1 increased Sp1 degradation and that could be inhibited by proteosome inhibitors [61]. In a recent study Zhang et al. demonstrated that OGT blocked Sp1 cleavage in vitro by inhibiting proteosome activity, for the inhibition of proteosomal function OGT catalytic activity was required [62].

Previously we have discussed the role of the hexosamine biosyntheis pathway in the development of diabetes and insulin resistance. It has been proposed that increased O-glycosylation causes insulin resistance and leads to the

development and progression of diabetes. Yki-Järvinen et al. showed that glucosamine infusion increased O-GlcNAc levels in rat and skeletal muscle and lead to insulin resistance [63]. Overexpression of OGT induced insulin resistance and hyperleptinaemia in vivo [64]. O-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenyl carbamate (PUGNAc), a potent inhibitor of O-GlcNAcase increased levels of O-GlcNAc levels in 3T3-L1 adipocytes and resulted in defects in insulin stimulated Akt and glycogen synthase kinase-3β (GSK3β) phosphorylation [65]. Patti et al. reported that glucosamine induced insulin resistance was associated with increased O-glycosylation of insulin receptor substrate (IRS)-1 and IRS-2 [17]. In a recent study Park et al. showed that PUGNAc treatment of primary rat adipocytes lead to decreased GLUT4 translocation, and insulin resistance [66]. They also showed that PUGNAc administration resulted in increased GLUT4, IRS-1 and Akt2 O-glycosylation, and this was accompanied by a reduction in insulin stimulated phosphorylation of IRS-1 and Akt [66]. Akimoto et al. reported that in Goto-Kakizaki rats (an animal model of type-2 diabetes) OGT expression and activity was increased compared to the non-diabetic Wistar rats, and they also found increased O-GlcNAc levels in the whole pancreas and pancreatic islets of Langerhans of the diabetic animals. They showed that increasing O-GlcNAc by PUGNAc administration decreased the glucose stimulated insulin secretion of the isolated islets [67]. These results suggest that O-glycosylation plays a crucial role in the development of insulin resistance and diabetes. Furthermore, it has been shown that increased levels of O-GlcNAc are associated not only with the development of diabetes but also with diabetic complications [68].

Diabetic cardiomyopathy:

Diabetic cardiomyopathy is defined as a ventricular dysfunction in absence of hypertension and/or coronary vascular disease [69]. Diabetes leads to diabetic cardiomyopathy in part by affecting function at the cardiomyocyte level [70]. Adult

cardiomyocytes isolated as little as 4-6 days following STZ induced diabetes exhibit slower relaxation and slower rates of Ca^{2+} transient decay [71] consistent with decreased sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity; however, SERCA expression was unchanged. Incubation of normal adult cardiomyocytes in hyperglycemic media induced similar alterations in excitation-contraction (EC) [72], which were not associated with changes in SERCA, or phospholamban expression or phosphorylation [73]. Since, hyperglycemia increases HBP flux and *O*-GlcNAc levels, it is possible that O-GlcNAc modification of Ca^{2+}-handling proteins may contribute to hyperglycemia-induced cardiomyocyte dysfunction. This is supported by the fact that glucosamine, which rapidly increases *O*-GlcNAc levels, had a similar effect on EC coupling as hyperglycemia [74].

A more direct link between increased *O*-GlcNAc levels and cardiomyocyte dysfunction was reported by Clark et al., [5], who showed that in neonatal cardiomyocytes increasing *O*-GlcNAc levels with hyperglycemia, glucosamine or increased OGT expression prolonged Ca^{2+} transient decays, whereas overexpression of *O*-GlcNAcase improved Ca^{2+} transients in cells exposed to hyperglycemia. In contrast to the effects of hyperglycemia in adult cardiomyocytes [73], hyperglycemia significantly decreased SERCA mRNA and protein expression in neonatal cardiomyocytes and this was prevented by increased *O*-GlcNAcase expression [5]. Increased *O*-GlcNAc levels on the transcription factor Sp1 were also observed. This may account for the decrease in SERCA expression since the SERCA promoter contains multiple Sp1 binding sites that are required for adequate expression. Sp1 expression levels were unaffected by increased *O*-GlcNAc levels; however, the expression levels of MEF-2, a transcription factor important for cardiomyocyte function and maturation, was depressed but it was not subject to O-GlcNAc modification itself. Subsequently, Hu et al., [4] showed that in hearts from STZ-induced diabetic mice there was an increase in OGT expression and *O*-GlcNAc levels, which was accompanied by cardiomyocyte dysfunction. Increased expression

of *O*-GlcNAcase significantly lowered cardiac *O*-GlcNAc levels and improved function at both the isolated cardiomyocyte and whole heart level, which was associated with increased SERCA expression relative to diabetic controls.

Pang et al., [6] found that short-term diabetes blunted, the inotropic response of the heart to the α-adrenergic agonist phenylephrine and that this effect was partially abrogated by pre-treatment with azaserine, an inhibitor of the HBP [6]. In contrast, a brief pre-treatment of normal hearts with 5mM glucosamine, sufficient to increase *O*-GlcNAc levels approximately 3-fold [75] also attenuated the response to phenylephrine [6]; whereas the response to ß-adrenergic stimulation was unaffected. Kim et al., [76] demonstrated in C2C12 myoblasts that increased *O*-GlcNAc levels, blunted the bradykinin mediated increase in intracellular Ca^{2+}, which like phenylephrine is also mediated by phospholipase-C (PLC) activation. Importantly, they showed that PLC-ß1 was subject to *O*-GlcNAc modification and this modification was enhanced by glucosamine, PUGNAc or hyperglycemia. Consequently, they proposed that increased *O*-GlcNAc levels on PLC-ß1 decreased its activity, thereby attenuating the response to bradykinin. Since PLC activation is critical to G-protein coupled signaling, *O*-GlcNAc induced attenuation of PLC activity, could have broad implications for the development of diabetic cardiomyopathy.

Others propose that altered cardiac substrate utilization is another possible mechanism responsible for the development of diabetic cardiomyopathy [77, 78]. Chatham and colleagues demonstarted that in Zucker Diabetic Fatty (ZDF) rats (an animal model of type-2 diabetes) carbohydrate utilization was decreased while palmitate oxidation was elevated in the heart of the diabetic animals compared to he lean animals [77]. McClain and co-workers showed that incubation of 3T3L1 adipocytes with glucosamine for 48 hours increased palmitate oxidation [79], similar to the changes seen in the diabetic hearts [77]. They also showed that increased O-GlcNAc lead to changes in metabolism by altering the activity of AMP-activated

protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) [79], two of the key enzymes regulating substrate utiilization [80-82].

Diabetic nephropathy:

Recently due to the high prevalence of diabetes and due to the improved therapeutic strategies there has been a continous increase in the incidence end-stage renal failure among patients with diabetes [83].

The accumulation of the extracellular matrix proteins, like laminin, fibronectin or type-IV collagen [18, 20], is one of the major characteristic of the early diabetic nephropathy (DNP). It has been demonstrated that high glucose leads to increased expression and accumulation of fibronectin and laminin [18, 20]. However there is only a very little known about the role of hexosamine biosynthesis pathway and O-glycosylation in the development of diabetic nephropathy.

Kolm-Litty et al. showed that high-glucose caused a dose-dependent increase in the production of laminin and they demonstrated that it was dependent on the de-novo synthesis of TGF-β1 [84]. They also showed that both high-glucose and glucosamine increased the hexosamine products and enhanced the production of TGF-β and lead to a subsequent production of fibronectin in a dose dependent manner [85]. Inhibition of GFAT by azaserine or an antisense GFAT oligonucleotide prevented the effect of high-glucose, but it could not alter the effect of glucosamine [85]. Increased expression of GFAT even under normoglycemic conditions lead to increased expression of TGF-β and fibronectin in mesangial cells and increased the mRNA level of the TGF-β receptor [86, 87].

Another factor that plays an important role in the accumulation of matrix proteins is PAI-1, which inhibits tissue plasminogen activator and urokinase. Scholey and colleagues demonstrated in mesangial cells that hyperglycemia or overexpression of GFAT increased the activity of PAI-1 promoter in a dose-

dependent manner and they showed that DON - a GFAT inhibitor - blocked the effect of GFAT overexpression [87].

Nerlich et al. investigated the expression of GFAT - the key enzyme of HBP - in human renal tissue. They found that kidney tubular epithelial cells showed a positive staining using a GFAT antibody [88]. In the specimens of non-diabetic individuals GFAT protein expression was below the detection level. However in the glomeruli of individuals with diabetic nephropathy positive GFAT staining was seen focally in 7 out of 10 cases. Mesangial cells and glomerular epithelial cells showed GFAT positive staining [88].

Cellular hypertrophy both in the tubuli and in the glomeruli are characteristics of early diabetic nephropathy [89-91]. Slawson et al. showed in several mammalian cell lines, that increased O-GlcNAc results in growth defects by altering the cyclin dependent kinase activity [92]. Masson demonstrated in rat mesangial cells that glucosamine, known to increase flux through the HBP and increases O-glycosylation, induces cell cycle arrest and hypertrophy [93]. Hsieh et al. found that both high-glucose and glucosamine administration increased angiotensinogen gene expression and cellular hypertrophy in immortalized renal proximal tubular cells [94]. They demonstrated that azaserine blocked the stimulating effect of high glucose, but did not alter the effect of glucosamine [94], suggesting, that at least in part increased flux through the HBP is responsible for the activation of the renin-angiotensin system (RAS) in diabetes thus leading to accumulation of extracellular matrix proteins.

Cell survival:

It was recently shown that in mammalian cells a variety of different stress stimuli increased the level of *O*-linked-N-acetylglucosamine on nuclear and cytoplasmic proteins [95]. Inhibition of this response increased the sensitivity to stress whereas augmentation of the *O*-GlcNAc levels increased tolerance to the same stress stimuli and improved cell survival [95]. Sohn *et al.* reported that in

Chinese hamster ovarian cancer cells inhibition of GFAT decreased *O*-GlcNAc levels and reduced cell survival following heat stress [96]. Liu et al. reported that in the heart glucosamine afforded significant protection against injury resulting from both calcium paradox and zero-flow global ischemia [75].

O-glycosylation is thought to be involved in the pathogenesis of cancers. C-myc, an oncogene, has been shown to be O-glycosylated [54]. The mutation of c-myc on Thr58 is present in human lymphomas, this site is also a reciprocal O-glycosylation/phosphorylation site [54]. On the other hand p53, a tumor suppressor protein is also O-glycosylated [97]. However the role of O-glycosylation of these proteins in apoptosis and gene regulation is not clear [27].

Changes is O-GlcNAc regulation may also play an important role in the development of neurodegenerative disorders, such as Alzheimer’s disease (AD) [20, 27, 43]. There are O-GlcNAc modified proteins involved in the pathomechanism of AD: tau [98, 99], β-amyloid precursor [100], chlatrin assembly protein (AP) 3 [101] and AP 180 [102]. Furthermore the chromosomal localization of O-GlcNAcase has been identified as the locus of Alzheimer disease on the chromosome 10 [20, 27]. Decrease of AP3 and AP180 O-glycosylation has been described in patients with AD [101, 102]. It has been shown that tau can be both O-glycosylated and phosphorylated on the same site, while the tangled tau protein is hyperphosphorylated, suggesting that O-glycosylation may prevent tau aggregation [98, 99].

Zachara and Hart proposed that acute activation of the pathway results in protection, acting as an internal stress response, while chronic increase in O-glycosylation leads to the development of disease [21]. They also suggested that any disturbance in O-GlcNAc results in altered response to stress [21].

The role of the mitogen activated protein kinases (MAPK) in cardioprotection and its relationship with O-GlcNAc:

In the heart the response to external stress stimuli, including ischemia is mediated, in part, by a number of signaling pathways, including extracellular-signal regulated kinase (ERK) and p38 mitogen activated protein kinase (MAPK) [103]. The role of p38 in mediating the response of the heart to ischemic injury is somewhat controversial. In many studies ischemia increases p38 phosphorylation, and inhibition of p38 phosphorylation during sustained ischemia is reported to be cardioprotective [103-109]. However, in contrast, brief activation of p38 prior to ischemia has been shown to contribute to the protection associated with ischemic preconditioning, although this protective effect is critically dependent on both timing and duration of activation [110]. It is worth noting that p38 is frequently associated with activation of pro-apoptotic pathways such as Caspase-3, or p53 [111-113]. However, αB-crystallin and HSP-27 are anti-apoptotic, have been shown to play a role in ischemic protection [114-117] and are both downstream of p38 [118, 119]. Recently several studies have demonstrated that ERK activation, especially during reperfusion is important in mediating protection associated with insulin and ischemic preconditioning [104, 105, 110, 120]. A number of studies have also reported that Akt activation protects against ischemia/reperfusion injury in the heart [121-123]. Recently Hausenloy and Yellon [120] described the reperfusion injury salvage kinase (RISK) pathway in which activation of pro-survival kinases Akt and ERK1/2 particularly at the time of reperfusion contribute to ischemic protection.

Interestingly activation of HBP and increased *O*-GlcNAc levels have been reported to modify p38 phosphorylation and activity [48, 94, 124]. Kneass et al. showed that increasing *O*-GlcNAc levels in human neutrophils, increased basal and agonist stimulated phosphorylation of both p38 and ERK1/2 [125]. Other studies have also demonstrated that increased HBP and/or *O*-GlcNAc levels also affect Akt phosphorylation [65, 66]. Taken together, these studies suggest that key kinases

implicated in ischemic cardioprotection can be modulated by changes in HBP flux and *O*-GlcNAc levels.

Aims:

1. **The relationship between hexosamine biosynthesis pathway, O-glycosylation and stress or ageing - acute vs. chronic activation.**
    1.1. Investigate whether stress leads to increase in HBP flux and O-GlcNAc levels in the rat heart.
    1.2. Verify that acute increase in O-GlcNAc by glucosamine administration protects the heart against ischemia-reperfusion injury.
    1.3. Determine the effect of ageing on HBP and O-glycosylation.
2. **The role of the hexosamine biosynthesis pathway and O-glycosylation in diabetic cardiomyopathy in animal models.**
    2.1. Examine the effect of the development of type-2 diabetes on O-GlcNAc and HBP in the heart.
    2.2. Investigate the effect of the increased O-GlcNAc on excitation-contraction coupling in isolated cardiomyocytes.
    2.3. Determine whether increase in HBP and O-GlcNAc leads to changes in cardiac substrate utilization, similar to those seen in type-2 diabetic animals, and examine whether altered AMPK or ACC activation was responsible for the changes in cardiac metabolism.
3. **O-glycosylation and diabetic nephropathy.**
    3.1. Verify that O-GlcNAc is present in human renal biopsy specimens.
    3.2. Show differences in O-GlcNAc between samples taken from kidneys of diabetic and non-diabetic patients.

Methods and Results:

1.1. Effect of stress on HBP and O-GlcNAc in the heart.

Our goal was to investigate the effect of ischemia and ischemia/reperfusion on HBP and O-GlcNAc in cardiac tissue.

Methods:

Animals: Non-fasted, male Sprague-Dawley rats (Charles Rivers Laboratories) weighing 351±6 g were used.

Experimental groups: Hearts were divided into five perfusion groups: 1) Normoxia; 2) 5 minutes of low flow ischemia (LFI) (0.3mls/min); 3) 10 minutes of LFI; 4) 30 minutes of LFI and 5) 30 minutes of LFI and 60 min reperfusion. In the normoxic group hearts were perfused for a total of 60 minutes. In the LFI groups hearts were allowed to stabilize for 30 minutes then subjected to LFI for the indicated time period.

Isolated heart perfusions: Animals were anesthetized with intraperitoneal ketamine hydrochloride injection (100mg/kg, Lloyd Laboratories). Hearts were rapidly excised and perfused retrogradely, as previously described [126] and coronary flow was adjusted to maintain a constant perfusion pressure of 75 mmHg. The perfusion buffer consisted of a Krebs-Henseleit buffer containing in mmol/L: lactate 1.0, pyruvate 0.1, palmitate 0.32, glutamine 0.5 and 3% BSA (fatty acid free) (Serologicals Proteins Inc.) plus 50 μU/mL insulin (NovoNordisk). Cardiac function was monitored via a fluid-filled balloon placed into the left ventricle. End-diastolic pressure (EDP) was set to 5 mmHg by adjusting balloon volume. All hearts were paced at 320 beats/min rate during the whole experiment except the LFI period and the first five minutes of reperfusion. At the end of the experiment hearts were freeze-clamped with liquid nitrogen cooled tongs.

Western blots: Hearts were ground to a fine powder under liquid nitrogen and homogenized in T-PER (Pierce) containing 5% protease inhibitor cocktail, 40 μmol/L PUGNAc (Carbogen) and 1 mmol/L sodium-orthovanadate and 20 mmol/L sodium-fluoride on ice and centrifuged for 10 min at 15000g. The protein concentration of the supernatant was measured using Bio-Rad Protein Assay Kit. Whole heart lysates were separated on 10% SDS-PAGE and transferred to PVDF membrane (Pall). Equal loading of protein was confirmed by Sypro Ruby staining (Bio-Rad) on the membranes. Blots were probed with the appropriate antibody in casein blocking buffer. Anti-*O*-GlcNAc antibody: CTD110.6 (Covance). Blots were visualized with enhanced chemiluminescent assay (Pierce) and the signal was detected with UVP BioChemi System (UVP). Densitometry was quantified using Labworks analysis software (UVP).

HPLC: Approximately 50 mg of frozen tissue powder was homogenized in 1 mL ice-cold 0.3mol/L perchloric acid (PCA) and centrifuged for 15 min at 15000g at 4^oC. PCA was removed from the supernatant with 2 volumes of 1:4 trioctylamine:1,1,2-trichloro-trifluoroethan mixture. Samples were loaded on Partisil 10 SAX column (Beckman), nucleotide sugars were measured at 262 nm using 2 mL/min flow rate and linear salt and pH gradient from 5 mmol/L to 750 mmol/L $(NH_4)H_2PO_4$ and from pH 2.8 to 3.7 [127]. This method cannot separate UDP-GlcNAc from UDP-N-acetyl galactosamine (UDP-GalNAc) so the results are presented as the sum of UDP-GlcNAc and UDP-GalNAc (UDP-HexNAc); however in the heart the ratio of UDP-GlcNAc to UDP-GalNAc is approximately 3:1 [5].

Results:

As previously reported in this model [128, 129] , the onset of LFI resulted in a rapid decrease in contractile function and gradual increase in EDP (Figure 5 and 6). 5-min of ischemia significantly increased UDP-HexNAc concentrations, and longer

periods of ischemia (10 and 30 minutes) increased levels further (Figure 3). After 60 minutes reperfusion UDP-HexNAc levels returned to the normoxic levels.

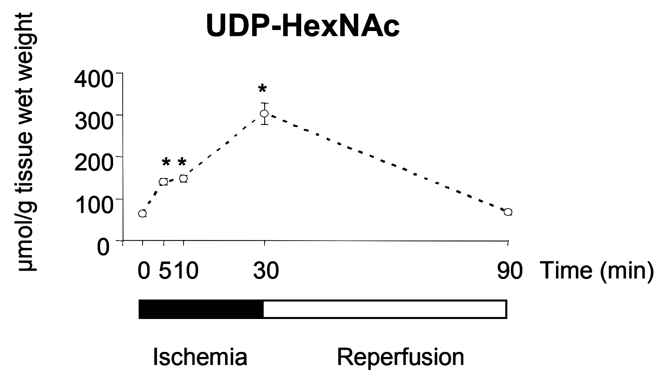


Figure 3.: *UDP-GlcNAc levels of the hearts under normoxic conditions and subjected to 5 min, 10 and 30 minutes of ischemia or 30 minutes of ischemia and 60 minutes reperfusion. *:p<0.05 vs. 0 min ischemia (One-Way ANOVA with Dunnet posthoc test)*

Protein O-GlcNAc levels increased in response to ischemia; after 10 minutes of ischemia O-GlcNAc levels were ~1.5-fold higher than normoxic levels. However, in contrast to UDP-HexNAc levels, O-GlcNAc levels declined between 10 and 30 min of ischemia and at the end of reperfusion they were significantly lower than normoxic levels (Figure 4).

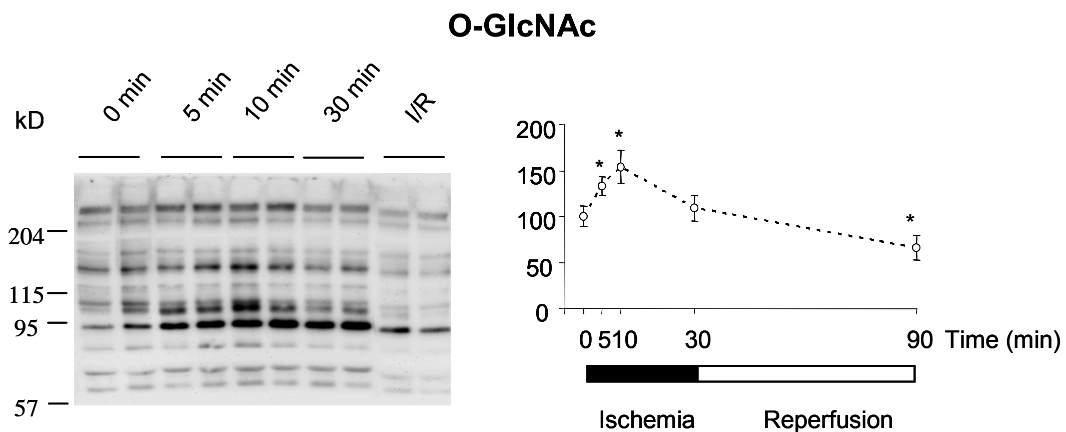


Figure 4.: *Effect of ischemia and reperfusion on O-GlcNAc. Left panel: representative CTD110.6 immunoblots form control hearts, right panel: CTD110.6 area densities relative to 0 min ischemia (n=4 in each group) *: p<0.05 vs. 0 min ischemia (One-Way ANOVA with Dunnet posthoc test)*

1.2. Acute increase in O-GlcNAc by GlcN administration protects the hearts form ischemia/reperfusion injury.

We investigated whether increasing O-GlcNAc levels by GlcN treatment protects the hearts from ischemia/reperfusion and examined the effect of GlcN on the stress signaling pathways, which could contribute to the cardioprotective effect of GlcN.

Methods:

Animals: Non-fasted, male Sprague-Dawley rats (Charles Rivers Laboratories) weighing 351±6 g were used.

Experimental groups: We used the hearts from the previous experiments as controls and perfused another set of animals as described in Chapter 1.1. with buffer containing 5 mmol/L GlcN throughout the whole experiment.

Western blots: Samples were prepared as described in Chapter 1.1. Anti-*O*-GlcNAc antibody, CTD110.6 (Covance), total and phospho (Thr180/Tyr182) p38 (Santa Cruz), total and phospho (Thr202/Tyr204) ERK1/2 (Cell Signaling) and total and phospho (Ser473) Akt (Cell Signaling) antibodies were used.

HPLC: The previously described HPLC protocol was used.

Results:

Before ischemia there was no significant difference in contractile function between control and glucosamine groups; however baseline coronary flow was significantly increased in the glucosamine group (Table 1). At the end of LFI, EDP was significantly attenuated in the glucosamine group (Control: 43.1 ± 3.1 vs. GlcN: 23.8 ± 3.0 mmHg; $p<0.05$) (Figure 5). After 30 minutes of LFI and 60 minutes reperfusion functional recovery was significantly higher in the glucosamine group

Table 1: *Baseline function of control and 5 mmol/L GlcN perfused hearts of Sprague Dawley rats, *: p< 0,05 vs Control*

	Control (n=17)	**GlcN (n=17)**
LVDP (mmHg)	100.6 ± 2.8	108.1 ± 3.4
Heart rate (beats/min)	322 ± 0.2	321 ± 0.3
RPP (mmHg/min)	32250 ± 830	34337 ± 1074
+dp/dt (mmHg/s)	4498 ± 172	4436 ± 209
-dp/dt (mmHg/s)	2436 ± 94	2457 ± 116
EDP (mmHg)	5.9 ± 0.2	6.0 ± 0.3
Coronary flow (mL/min)	13.6 ± 0.7	17.3 ± 0.9*

compared to control (Figure 6); however, coronary flow was decreased by the same proportion in both groups compared to preischemic values (Figure 6). Interestingly, while four out of five control hearts (80%) fibrillated during early reperfusion none of the glucosamine treated hearts fibrillated (0%; p<0.05; chi-squared test).

End Diastolic Pressure

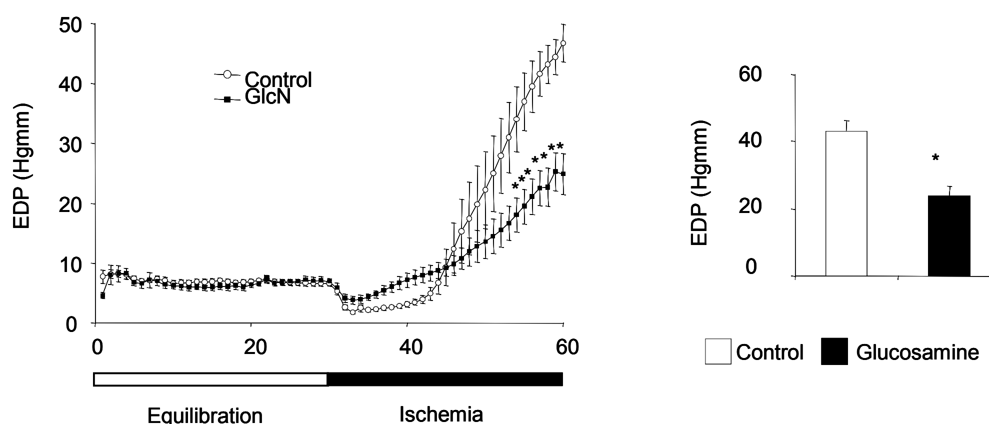


Figure 5.: *Left panel: Trace of end diastolic pressure (n=11 in each group), *: p<0.05 vs. control (Two-Way ANOVA with Bonferroni posthoc test); Right panel: End diastolic pressure at the end of 30 minutes ischemia (n=11 in each group), *: p<0.05 vs. control (Student`s t-test)*

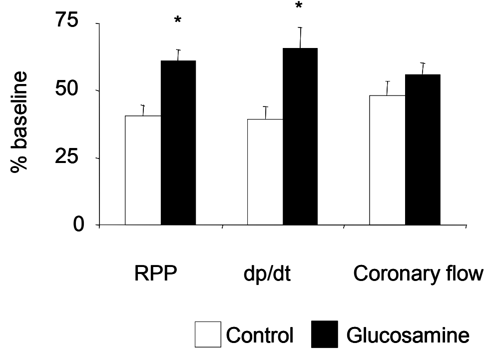


Figure 6.: *Percentage of recovery of function compared to baseline values (n=5 in each group), *: p<0.05 vs. control (Student`s t-test). (RPP: rate pressure product (heart rate x left ventricular developed pressure; dp/dt: maximal change in left ventricular pressure over time)*

Under normoxic conditions glucosamine increased UDP-HexNAc levels almost 2-fold (72 ± 7 Vs 126 ± 7 nmoles/g tissue wet weight, p<0.05; (Figure 7). Similar to the control group there was a significant increase in UDP-HexNAc levels in response to ischemia; however after 30 minutes ischemia there was no significant difference in UDP-HexNAc levels between control and glucosamine treated groups (Figure 7).

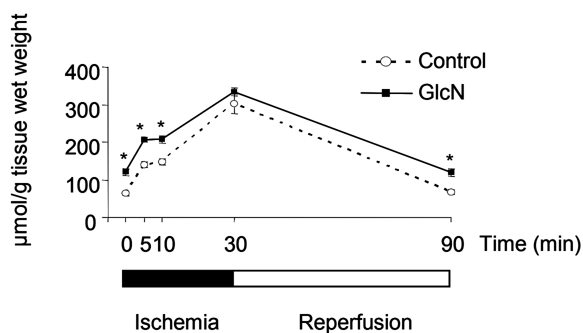


Figure 7.: *UDP-HexNAc levels (n=4 in each group), *: p<0.05 vs. control (Two-Way ANOVA with Bonferroni posthoc test)*

At the end of reperfusion UDP-HexNAc levels in the glucosamine treated group also decreased compared to end ischemia but were still elevated compared to control group. In contrast to the control group ischemia did not change O-GlcNAc levels in the glucosamine treated group; however at the end of reperfusion O-GlcNAc levels were significantly increased compared to normoxia (Figure 8). In Figure 9 comparisons of O-GlcNAc levels between control and glucosamine treated groups

are shown at each time point. It can be seen that glucosamine treatment significantly increased *O*-GlcNAc levels by at least 60% at all time points.

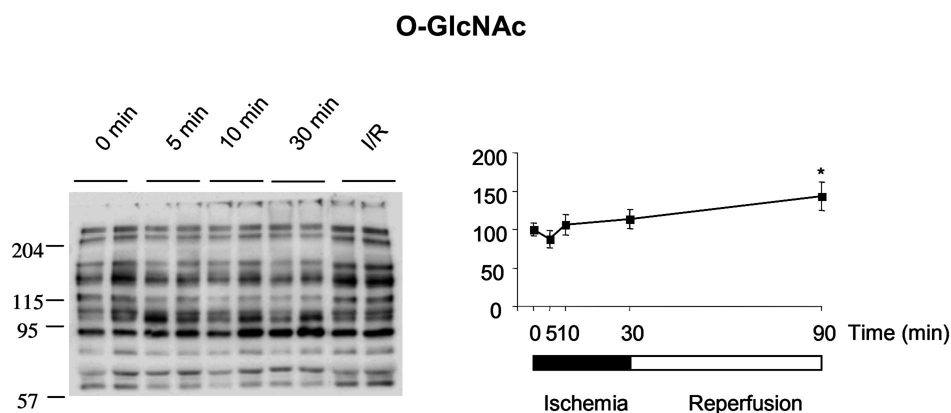


Figure 8.: *Effect of ischemia and reperfusion on O-GlcNAc. Top panel representative CTD110.6 immunoblots form glucosamine treated hearts, bottom panel: CTD110.6 area densities relative to 0 min ischemia (n=4 in each group); *: p<0.05 vs 0 min (One-Way ANOVA with Dunnet posthoc test)*

As expected ATP levels decreased in both control and glucosamine treated groups during ischemia and remained lower than normoxic perfusion at the end of ischemia/reperfusion (Figure 10). However, despite the attenuation of ischemic contracture and the improved functional recovery at the end of reperfusion, glucosamine treatment did not attenuate ATP loss during ischemia and did not increase ATP levels during reperfusion.

To identify a possible signaling pathway responsible for the protection seen with GlcN we examined changes in ERK, Akt and p38 phosphorylation in our samples. Comparisons of total and phosphorylation levels of ERK, Akt and p38 in control and glucosamine treated groups at each time point are shown in Figures 11, 12 and 13 respectively. The time course of changes in phosphorylation in the two groups normalized to normoxic conditions are summarized in Figure 14. Glucosamine treatment had no effect on ERK1/2 or Akt phosphorylation compared to the control under any conditions (Figure 11, 12). Consistent with other studies [120] there was a marked decrease in the ratio of phospho- to total-ERK1/2 during ischemia and a significant increase after reperfusion compared to normoxic

conditions in both groups (Figure 14A). Akt phosphorylation increased following 5 minutes ischemia; however after longer period of ischemia Akt phosphorylation

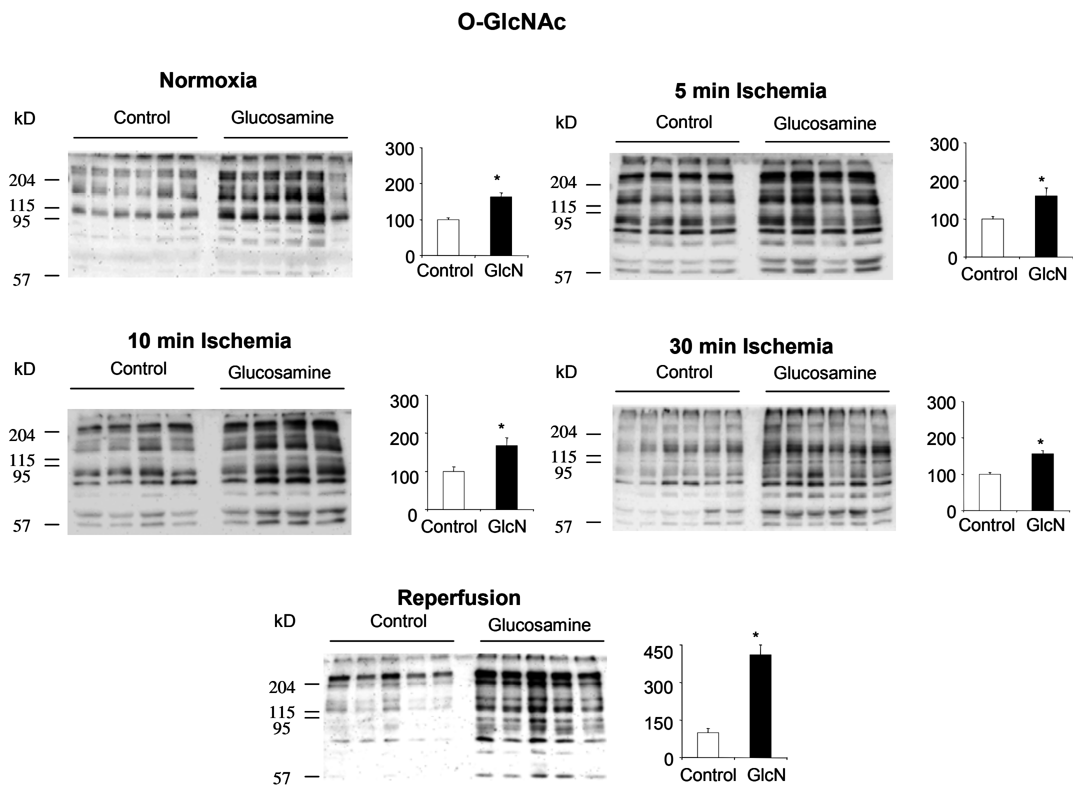


Figure 9.: *Effect of glucosamine on protein O-GlcNAc under normoxic conditions; after 5 minutes, 10 minutes and 30 minutes ischemia and following ischemia/reperfusion (I/R), left panels CTD110.6 immunoblots, right panels total area densities normalized to control group; *:p<0.05 vs. control (Student`s t-test)*

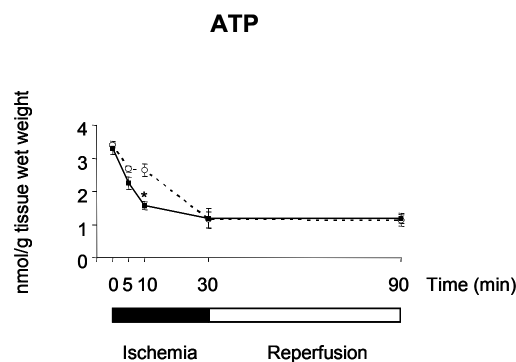


Figure 10.: *Effect of ischemia and ischemia/reperfusion on cardiac ATP levels in the control and GlcN treated hearts. *: p<0.05 vs. control (Two-Way ANOVA with Bonferroni posthoc test)*

markedly decreased (Figure 14B) similar to that seen with ERK (Figure 14A). At the end of reperfusion Akt phosphorylation returned to normoxic levels. The changes in

Akt phosphorylation after 30 minutes ischemia and reperfusion are consistent with previous studies [130].

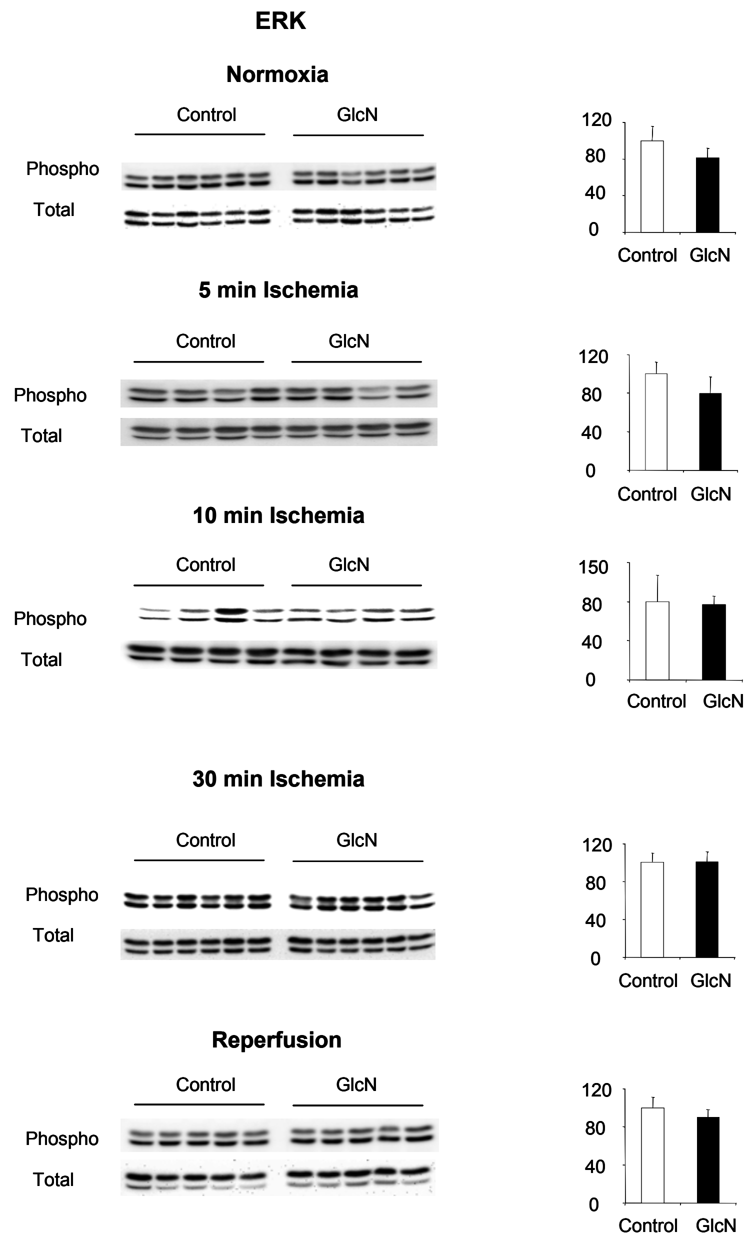


Figure 11.: *Effect of glucosamine on ERK1/2 phosphorylation, under normoxic conditions, and following 5 minutes, 10 minutes and 30 minutes ischemia or ischemia/reperfusion (I/R), left panels phopho- and total-ERK1/2 immunoblots, right panels phosphor/total area densities normalized to control group; *: p<0.05 vs. control (Student`s t-test)*

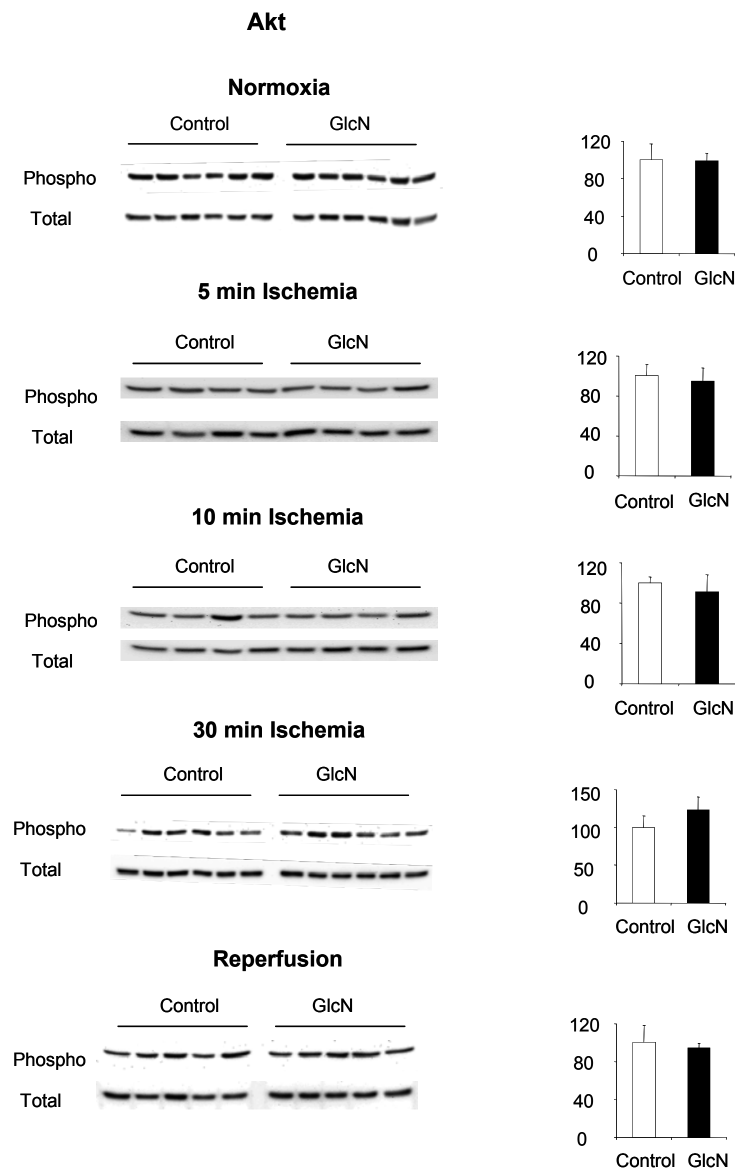


Figure 12.: *Effect of glucosamine on Akt phosphorylation, under normoxic conditions, and following 5 minutes, 10 minutes and 30 minutes ischemia or ischemia/reperfusion (I/R), left panels phopho- and total-Akt immunoblots, right panels phosphor/total area densities normalized to control group; *: p<0.05 vs. control (Student`s t-test)*

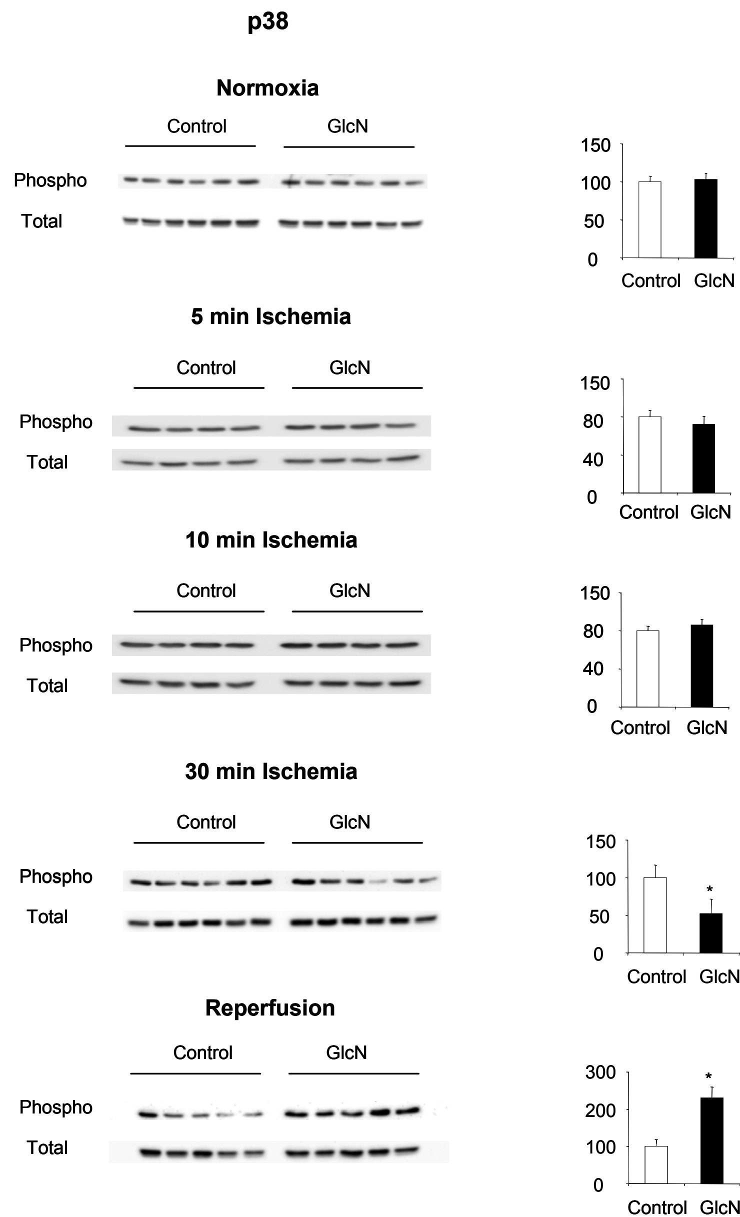
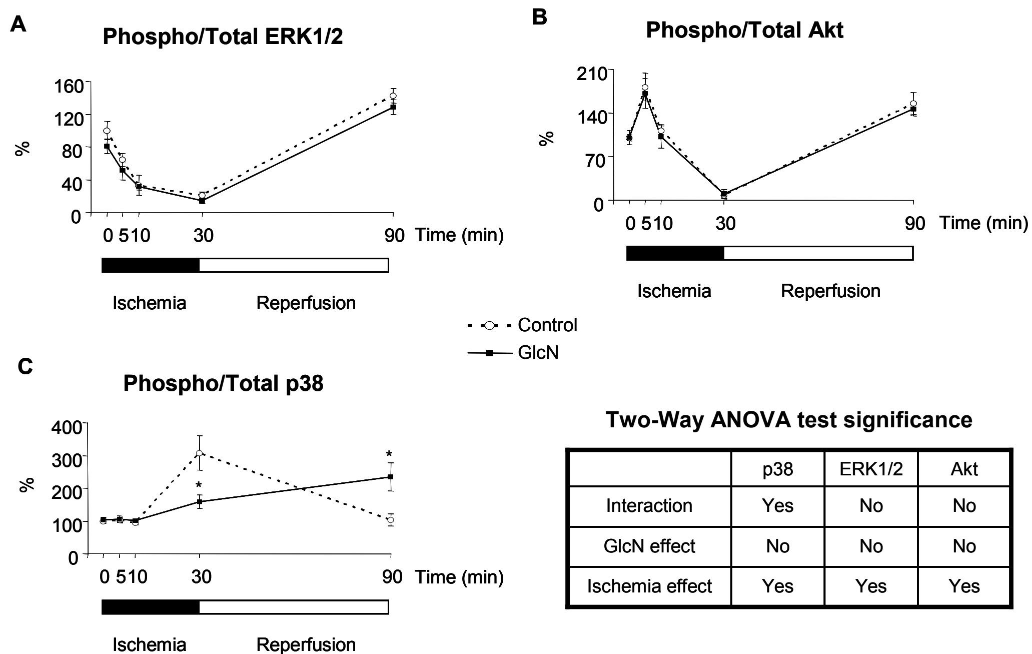


Figure 13.: *Effect of glucosamine on p38 phosphorylation, under normoxic conditions, and following 5 minutes, 10 minutes and 30 minutes ischemia or ischemia/reperfusion (I/R), left panels phopho- and total-p38 immunoblots, right panels phosphor/total area densities normalized to control group; *: p<0.05 vs. control (Student`s t-test)*

Under normoxic conditions and after 5 and 10 min of ischemia p38 phosphorylation was unchanged by glucosamine treatment; however at the end of ischemia p38 phosphorylation was significantly attenuated in the glucosamine group (Figure 13). In contrast at the end of reperfusion, p38 phosphorylation was increased in the glucosamine group (Figure 13). In Figure 14C it can be seen that consistent with previous reports ischemia increased phospho-p38 levels by more than 3-fold in control group compared to normoxic levels [131]. However, in the glucosamine group ischemia increased phospho-p38 by ~50%, consistent with the 30min data in Figure 13. At the end of reperfusion phospho-p38 levels returned to normoxic levels in the control group, whereas in the glucosamine group phospho-p38 levels were significantly elevated compared to both ischemic and normoxic conditions (Figure 14C).



	p38	ERK1/2	Akt
Interaction	Yes	No	No
GlcN effect	No	No	No
Ischemia effect	Yes	Yes	Yes

Figure 14.: *Effect of ischemia/reperfusion and glucosamine on signaling pathways.* ***A:*** *ERK1/2 phosphorylation;* ***B:*** *Akt phosphorylation;* ***C:*** *p38 phosphorylation; *: $p<0.05$ (Two-Way ANOVA with Bonferroni posthoc test) (n=6 in 0 min, 30 groups, n=5 in 90 min group and n=4 in 5 min and 10 min groups)*

1.3. Effect of ageing on the hexosamine biosynthesis pathway and O-glycosylation.

We examined whether there were differences in O-GlcNAc and/or UDP-HexNAc levels between the different tissues of young adult and senescent animals.

Methods:

Animals: Non-fasted, 5 and 24 month old, male Brown Norway rats (National Institute of Aging, NIH) were used.

Analysis of serum metabolites: Blood samples were collected immediately after decapitation from all animals. Serum glucose, insulin, lipid and free fatty acid levels were measured as previously described [128].

Real time RT-PCR analysis: RNA was extracted from tissues using TRIzol (Invitrogen Corp, Carlsbad, CA), treated with DNA-ase I to remove genomic DNA, purified using an RNA purification kit (Invitrogen), and reverse transcribed to cDNA using the SYBR Green RT-PCR kit (Applied Biosystems, Foster City, CA) and specific primers as described in Table 2.

Table 2: *Specific primers for GAPDH, GFAT1, GFAT2, OGT and O-GlcNAcase (OGA)*

Gene	Primer Sense Sequence	Primer Antisense Sequence	PCR Product, bp
GAPDH	TGGTCCAGGGTTTCTTACT	ATTCTTCCACCTTTGATGC	151
GFAT1	GCCAGCGACTTCTTGGATAG	AAGACCCATCAGGGTGTCAG	99
GFAT2	CCACAGCGTTCAGACAAAGA	CTCAAACTCGTAGCCCTTGC	108
OGT	ACAGCTCTTCGTCTGTGTCC	AGCAAACTCTGGGAAGACCT	134
OGA	AGCCAACTATGTTGCCATCC	AGTCATCACCACGTCCTTCC	82

cDNA was amplified by PCR in the iCycler for 40 cycles, and relative RNA levels were calculated using the iCycler software and a standard equation (Applied

Biosystems) and normalized using GAPDH mRNA. Results were standardized to the mean value of the 5 month-old group.

Immunoblot analysis: Hearts, abdominal aorta, liver, brain and skeletal muscle (m. gluteus) were freshly isolated and ground to a fine powder under liquid nitrogen. Protein extract was prepared as described in Chapter 1.1. Anti-*O*-GlcNAc antibody, CTD110.6 (a kind gift of Mary-Ann Accavitti), anti-OGT (SQ-17, Sigma) antibodies were used.

HPLC analysis: The previously described (Chapter 1.1.) HPLC protocol was used.

Results:

Body weight, heart weight, serum glucose, insulin, lipids and free fatty acid FFA) levels in the 5 and 24- month old animals are summarized in Table 3. Body weight, heart weight and serum insulin levels were significantly increased in the 24-month group. There was no difference in the heart weight: bodyweight ratio and although triglyceride levels were increased by ~45% this difference was not significant (p=0.068). There were no changes in cholesterol, HDL or FFA levels with age.

Table 3: *Body-, heart weight and serum parameters of fed 5 and 24 month old Brown-Norway rats. *: p<0.05 vs 5 month group. HDL = high density lipoproteins; TG = triglycerides; FFA = free fatty acids.*

	Body wt (g)	Heart/body wt (mg/g)	Glucose (mM)	Cholesterol (mM)	HDL (mM)	TG (mg/dL)	FFA (mEq/L)	Insulin (ng/mL)
5 month	333.8±4.7	2.7±0.1	9.4±0.4	2.25±0.23	0.84±0.02	1.1±0.2	0.17±0.02	0.51±0.04
24 month	507.6±20.7*	3.2±0.7	7.5±0.1*	2.86±0.11	0.85±0.03	1.6±0.1	0.18±0.06	2.08±0.36*

Anti-O-GlcNAc immunoblots from heart, aorta, brain and skeletal muscle from 5 and 24-month groups are shown in Figures 15 A, D and 16 A, D. In the heart overall O-GlcNAc levels were increased ~2-fold in the 24-month group (Figure 15A, B); the pattern of O-GlcNAcylated proteins was also altered, with several protein

bands appearing below 50 kD molecular weight (Figure 15A). In the abdominal aorta overall O-GlcNAc levels were increased ~50% in the 24-month group (Figure 15D, E); however there were no marked changes in the pattern of O-GlcNAc modified proteins.

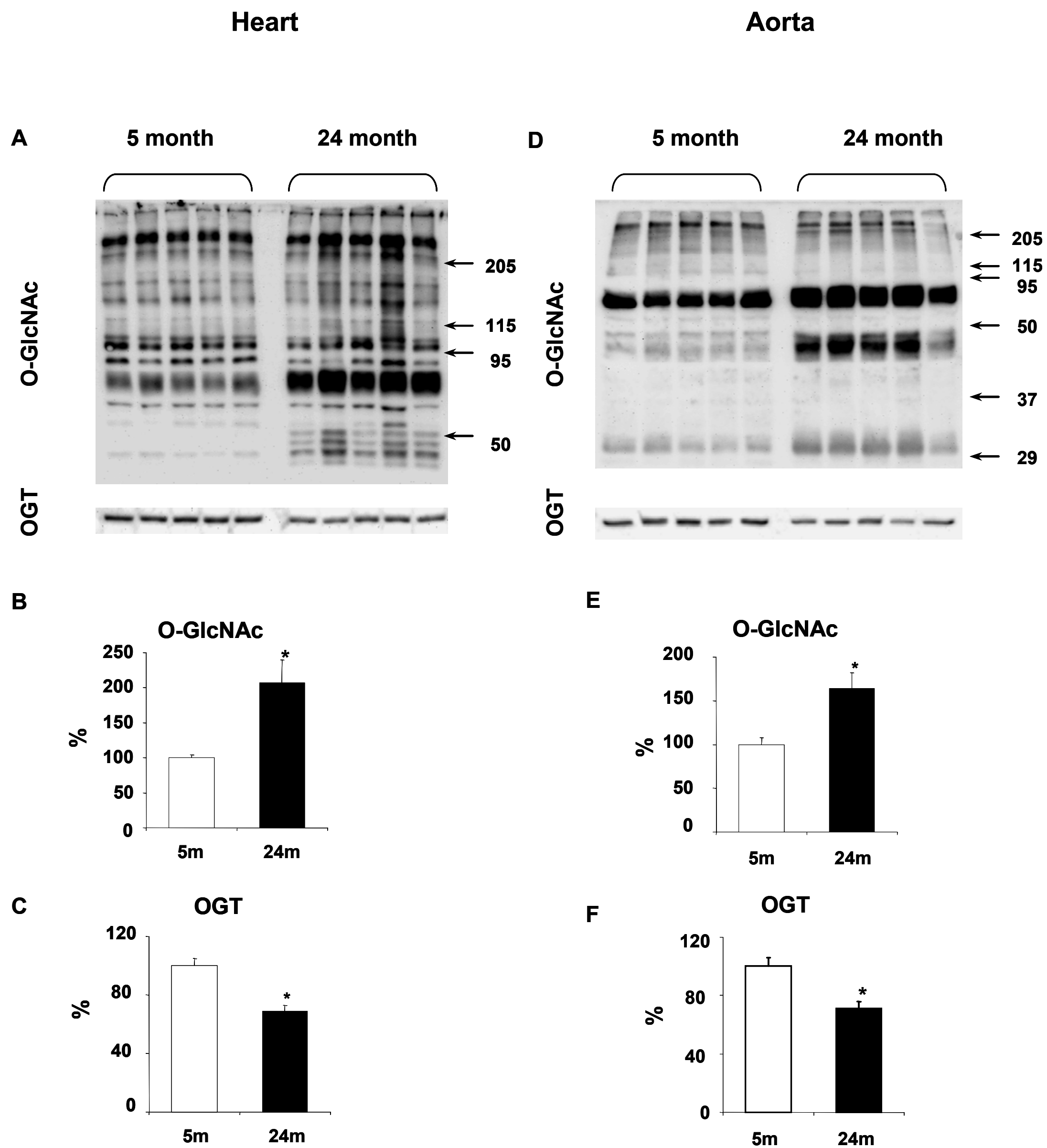


Figure 15.: *Anti O-GlcNAc and anti OGT Western blot results from hearts (**A,B,C**) and aortae (**D,E,F**) of 5- and 24-month old Brown-Norway rats. (**A,D**), representative Western blots; (**B,E**), mean area densities of anti O-GlcNAc blots; and (**C,F**), mean area densities of anti OGT blots. *: p<0.05 vs. control.*

In the brain O-GlcNAc levels increased ~30% in the 24-month group compared to the 5-month group (Figure 16 A, B) and we observed the appearance of a new O-GlcNAc band at around 80kD as indicated by a red arrow in Figure 16 A. In skeletal

muscle there was also a significant increase in overall O-GlcNAc (Figure 16 D, E) and we also found an approx. 50 kD band appearing in the 24-month group (Figure 16 D).

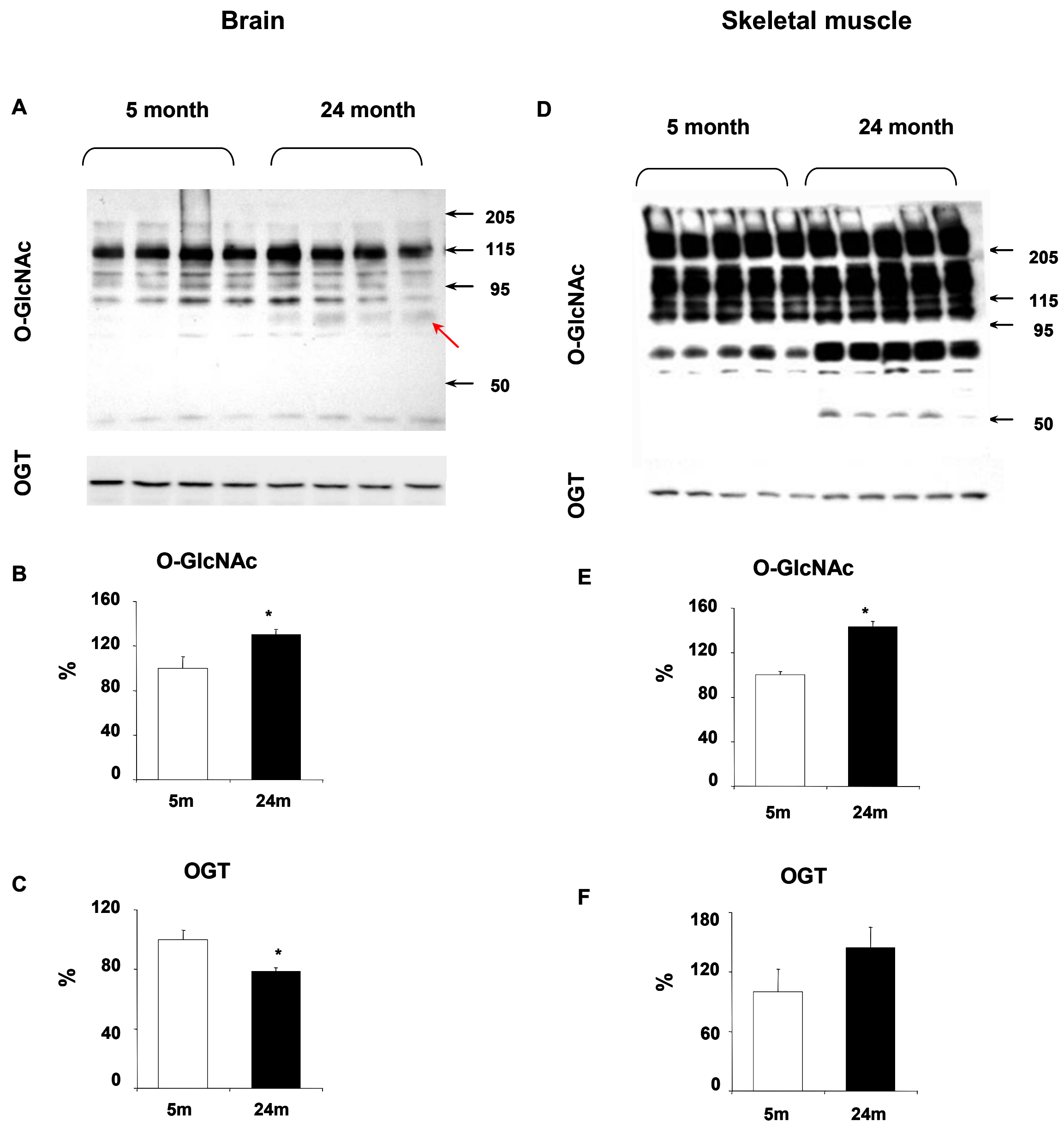


Figure 16.: *Anti O-GlcNAc and anti OGT Western blot results from brains (**A,B,C**) and skeletal muscles (**D,E,F**) of 5- and 24-month old Brown-Norway rats. (**A,D**), representative Western blots; (**B,E**), mean area densities of anti O-GlcNAc blots; and (**C,F**), mean area densities of anti OGT blots. *: p<0.05 vs. control.*

To better understand the factors contributing to the increase in O-GlcNAc levels we examined OGT and O-GlcNAc mRNA levels in heart, brain and skeletal muscle (Table 4). We found that with age O-GlcNAcase mRNA levels were significantly

increased in the heart in the 24-month group, whereas OGT mRNA levels were unchanged (Table 4). There were no changes in OGT or O-GlcNAcase mRNA in brain or skeletal muscle with age. There is no readily available O-GlcNAcase antibody therefore we were only able to determine OGT protein levels. We found that OGT protein levels were significantly decreased in heart, aorta and brain, and unchanged in skeletal muscle (Figures 15A, C, D, F and 16A, C, D, F).

The decrease in OGT protein levels and the increase in O-GlcNAcase mRNA described above is more consistent with a decrease in overall O-GlcNAc levels, rather than the increase seen in the 24-month group. Therefore, we examined GFAT1 and GFAT2 mRNA levels and UDP-GlcNAc levels in heart, brain and skeletal muscle to determine whether increased HBP activity could account for the increase in O-GlcNAc.

Table 4: *OGT and OGA mRNA levels determined by real-time RT-PCR of the 5 and 24 month old heart, skeletal muscle and brain of Brown-Norway rats. *: $p<0.05$ vs 5 month group*

	OGT		**OGA**	
	5 month	**24 month**	**5 month**	**24 month**
Heart	100±7	112±35	100±11	148±16*
Skeletal muscle	100±17	97±20	100±10	131±24
Brain	100±6	107±11	100±6	105±9

In the heart mRNA levels of GFAT1 and GFAT2, were markedly increased in the 24-month group compared to the 5 month old (Figure 17A); however, the increase in GFAT1 was not significant (p=0.14). Unfortunately, because there is no readily available antibody against GFAT we were not able to determine whether there were also changes in GFAT protein levels. However, consistent with an increase in GFAT mRNA levels and the increase in O-GlcNAc, UDP-HexNAc levels were significantly higher in hearts from 24-month old rats (Fig. 17A). In skeletal muscle

there was no change in GFAT1 mRNA; however, there was ~2-fold increase in GFAT2 mRNA (p=0.22) (Figure 17C).

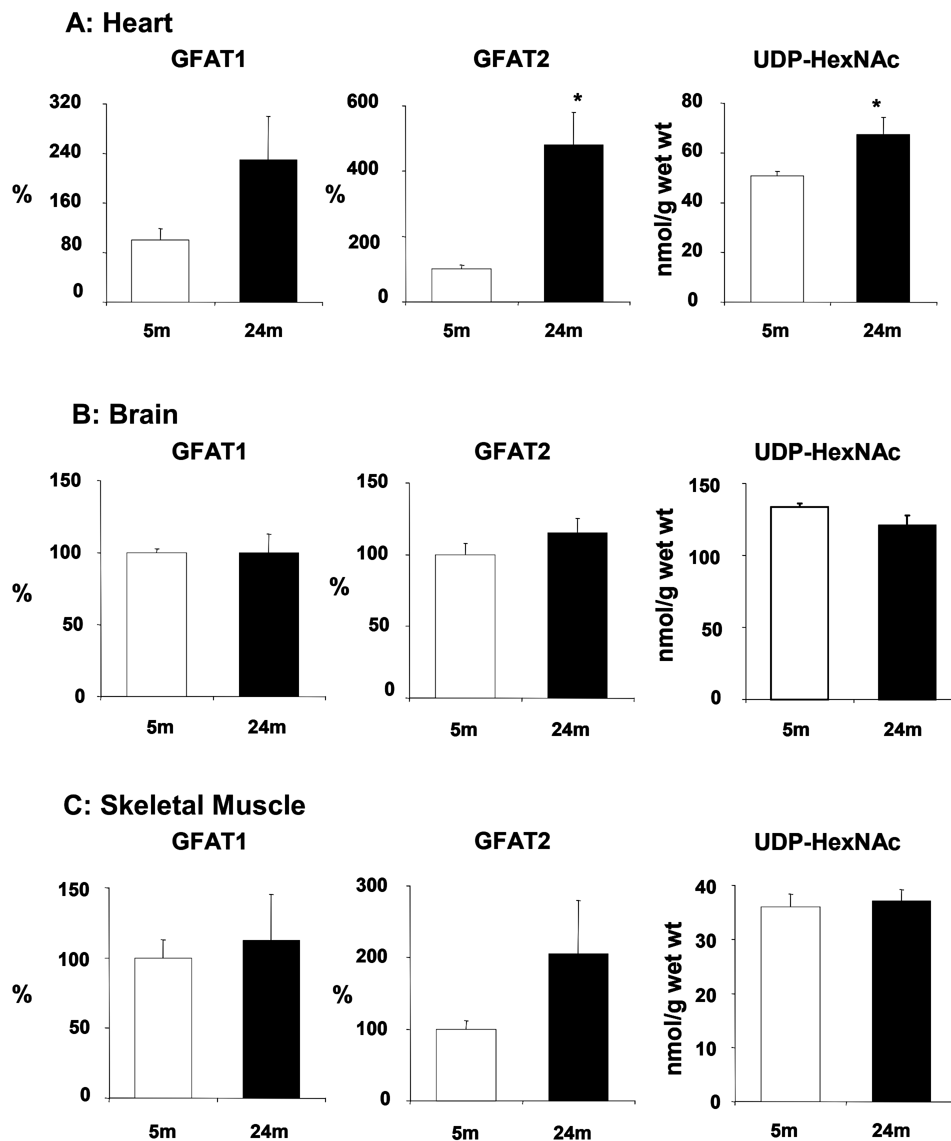


Figure 17: *GFAT1 mRNA levels, GFAT2 mRNA levels as % of 5-month group and UDP-GlcNAc levels in A: heart; B: brain and C: skeletal muscle isolated from 5 and 24 month old Brown-Norway rats, *: p<0.05 vs. 5 month old.*

Skeletal muscle UDP-HexNAc levels were not significantly different between 5 and 24-month groups. In the brain there were no changes in GFAT1, GFAT2 or UDP-GlcNAc levels between 5 and 24-month groups (Figure 17B).

2.1. Effect of type-2 diabetes mellitus on HBP and cardiac O-GlcNAc levels.

In this study we have investigated the effect of the development of type-2 diabetes on the hexosamine biosynthesis pathway in the heart and on cardiac O-glycosylation in Zucker Diabetic Fatty (ZDF) rats.

Methods:

Animals: As a model of Type-2 diabetes, non-fasted, male Zucker diabetic fatty rats (ZDF; Gmi-fa/fa, Charles Rivers Laboratories) were used with age matched lean littermates (?/+) as Controls. Animals were maintained on Purina 5008 diet as recommended by the supplier and sacrificed at 6 weeks or 22 weeks of age. To clarify that the differences between the 6 and 22 week old animals are not strain specific we used 6 and 22 week old non-fasted, male Spargue Dawley rats (Charles Rivers Laboratories). All hearts were excised and perfused as described in Chapter 1.1.

Analysis of serum metabolites: Blood samples were collected immediately after decapitation from all animals. Serum glucose, insulin and leptin levels were measured as previously described [128].

Western blots and HPLC: Tissue was ground to a fine powder under liquid nitrogen. For tissue preparation for Western blots and for the HPLC measurements we have used the protocols described in Chapters 1.1. and 1.3. For the Western blots we used calsequestrin antibody (Abcam) as an internal control.

Results:

Body weight, serum glucose, insulin and leptin levels in the 6 and 22 week old Lean and ZDF rats are summarized in Table 5. Consistent with our earlier studies [77, 128] the 6 week old ZDF group was both insulin and leptin resistant but normoglycemic; whereas, by 22 weeks of age insulin levels were lower and they had

become markedly hyperglycemic. Although leptin levels increased significantly in the 22 week Lean group compared to the 6-week group, they were still markedly lower than the levels in the 22-week old ZDF group.

Table 5: *Characteristics of 6 week and 22 week old Lean and Obese ZDF rats. *: $p<0.05$ vs. age matched Lean, †: $p<0.05$ vs. 6 weeks*

	6 weeks		22 weeks	
	Lean (n=5)	**ZDF (n=5)**	**Lean (n=6)**	**ZDF (n=6)**
Body weight (g)	148±2	150±0	400±9†	406±11†
Blood glucose (mmol/L)	8.8±0.2	9.5±0.5	10.3±0.2†	38.7±1.2*,†
Serum insulin (nmol/L)	0.7±0.1	2.1±0.0*	0.5 ± 0.1†	0.4±0.1†
Leptin (ng/mL)	2.2±0.4	19.2±0.5*	7.7±0.7†	16.2±1.7*†

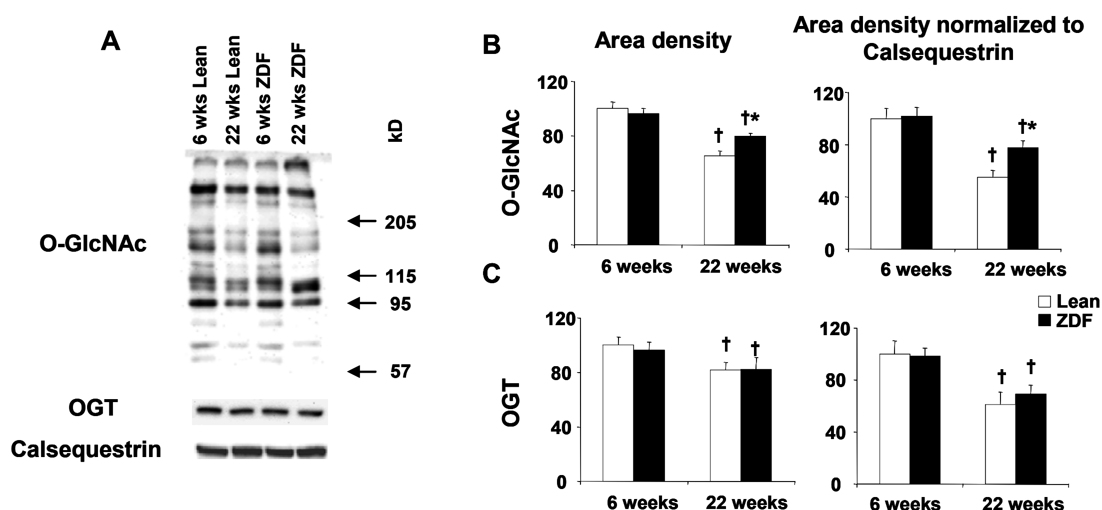


Figure 18. A: *Representative CTD110.6, OGT and Calsequestrin immunoblots from lean and ZDF groups;* ***B:*** *Area densities of CTD110.6 normalized to 6 week lean group (left) or to calsequestrin (right);* ***C:*** *CTD110.6 and Calsequestrin immunoblots for all samples from 22-week Lean and ZDF groups (right) and absolute area densities of 11 separate bands normalized to Calsequestrin (left);* ***D:*** *Area densities OGT relative to 6 week lean group (left) or to calsequestrin (right). *: $p < 0.05$ Vs. age matched Lean group; †: $p <0.05$ Vs. respective 6 week group (One-way ANOVA with Bonferroni post-hoc test). (n=5 in the 6 weeks old groups, and n=6 in the 22 weeks old groups)*

There was no significant difference in UDP-GlcNAc levels between 6-week old Lean and ZDF groups (86±6 and 87±5 nmol/g wet weight respectively). However, at 22 weeks of age UDP-GlcNAc levels were significantly increased in the

ZDF group compared to the age-matched Lean group (103±6 Vs 88±3 nmol/g wet weight p<0.05). The UDP-GlcNAc levels were also higher in the ZDF 22-week group compared to the 6-week ZDF group (p<0.05).

Consistent with the UDP-GlcNAc levels there was no difference in *O*-GlcNAc levels between 6 week old Lean and ZDF groups, whereas there was a significant increase in *O*-GlcNAc at 22-weeks of age between the ZDF and Lean control groups (Figure 18 A, B). The difference in O-GlcNAc levels between the ZDF and Lean 22-week groups was significant also when normalized to calsequestrin as a protein loading control. In all groups the anti-O-GlcNAc antibody, CTD110.6, identified 11 separate bands all of them greater than 57 kDa. Densitometric analysis of the individual bands in the O-GlcNAc immunoblots from 22-week Lean and ZDF groups (Figure 19) revealed that the overall increase in O-GlcNAc levels was primarily due to increased O-GlcNAc on proteins in only 3 bands. There was no difference in OGT expression between ZDF and Lean groups at either age (Figure 18A, C).

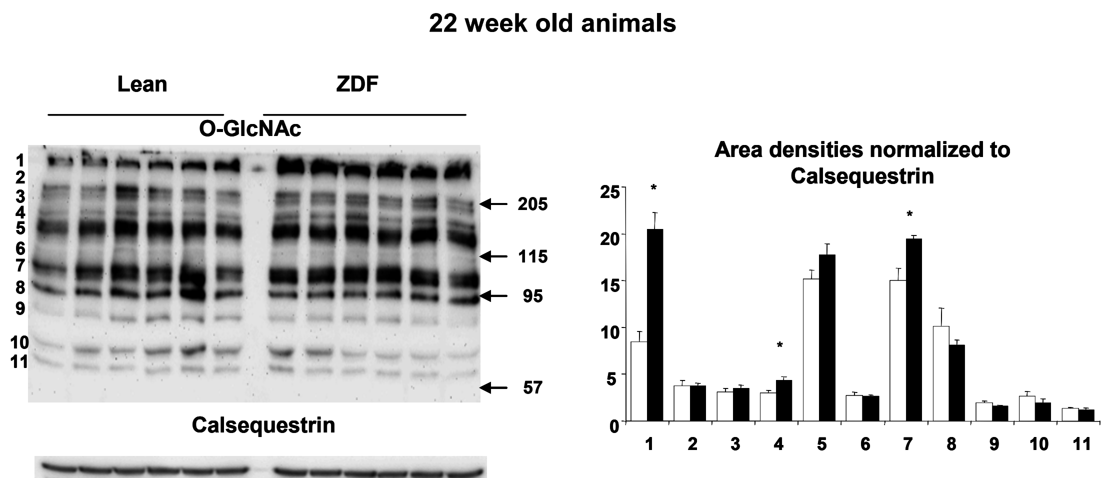


Figure 19.: *CTD110 and Calsequestrin immunoblots and absolute area densities of 11 separate bands normalized to Calsequestrin from the immunoblots from 22-week old lean and obese groups. *: p <0.05 vs. Lean group (Student's T-test).*

Since we saw similar functional alterations when comparing 22-week Lean and ZDF groups and 6 and 22-week ZDF groups, we also compared the O-GlcNAc levels in the 6 and 22 week groups. Surprisingly, we found that *O*-GlcNAc levels were significantly lower in both 22-week Lean and ZDF groups compared to their

respective 6-week groups (Figure 18A, B), which was associated with a significant reduction in OGT expression in both Lean and ZDF 22-week groups (Figure 18A, C).

To determine whether the age difference in O-GlcNAc and OGT expression levels were strain specific we also examined hearts from 6 weeks and 22 weeks old Sprague-Dawley rats. Similar to the Lean and ZDF groups there was a significant age-related decrease in both O-GlcNAc and OGT levels (Figure 20). Again consistent with the Lean and ZDF groups there was no difference in UDP-GlcNAc levels between the 6 and 22 week groups (82.3±4.3 vs. 83.6±5.0 nmol/g tissue wet weight, respectively).

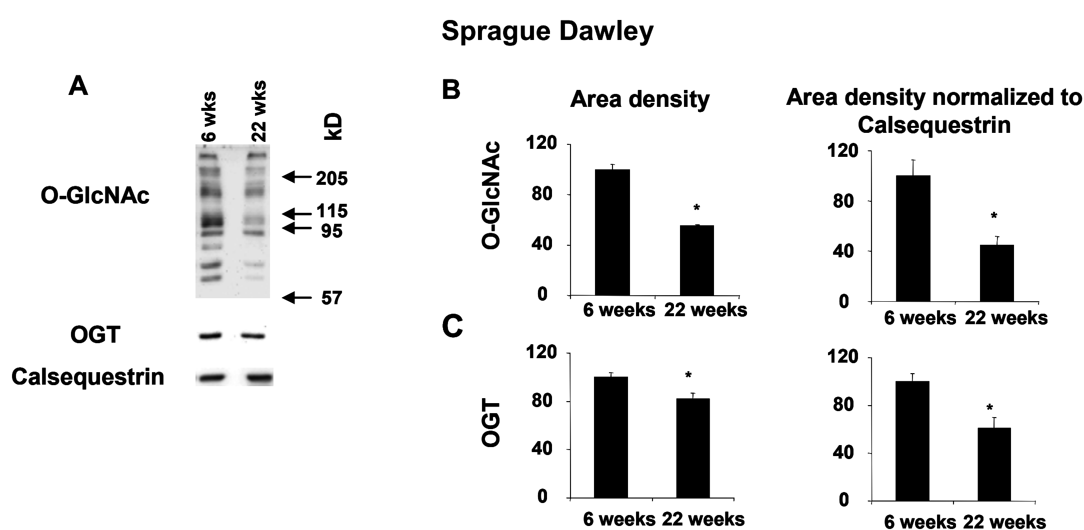


*Figure 20. A: Representative CTD110.6, OGT and Calsequestrin immunoblots from 6 and 22 week Sprague Dawley groups; B: Area densities of CTD110.6 and OGT relative to 6 week group; C: Area densities of CTD110.6 and OGT relative to 6 week group following normalization to Calsequestrin levels. *: p <0.05 Vs. 6 week group (Student's T-test). (n=5)*

To better understand the combination of age and diabetes on protein O-GlcNAc levels we evaluated the change in intensity of specific protein bands with age in Lean and ZDF groups (Figure 21A, B). The area densities of all 11 bands were measured and normalized to Calsequestrin levels. In the Lean control group all of the bands except #1 showed significant decreases with age (Figure 21A); the magnitude of this decrease ranged from 43-68% (Figure 21A). In contrast, in the ZDF group the majority of the bands were unchanged between 6 and 22 weeks (Figure 21B); however bands #5, #6 and #11 were significantly lower in the 22 week group.

Interestingly, the intensity of band #1 was significantly increased by ~3 fold in the 22-week ZDF group compared to the 6-week ZDF group. It is noteworthy that this same band was markedly increased in the 22-week ZDF compared to the 22-week Lean (Figure 19). Thus, O-GlcNAc levels of band #1, appears to be consistently increased in response to diabetes independent of age differences or leptin levels.

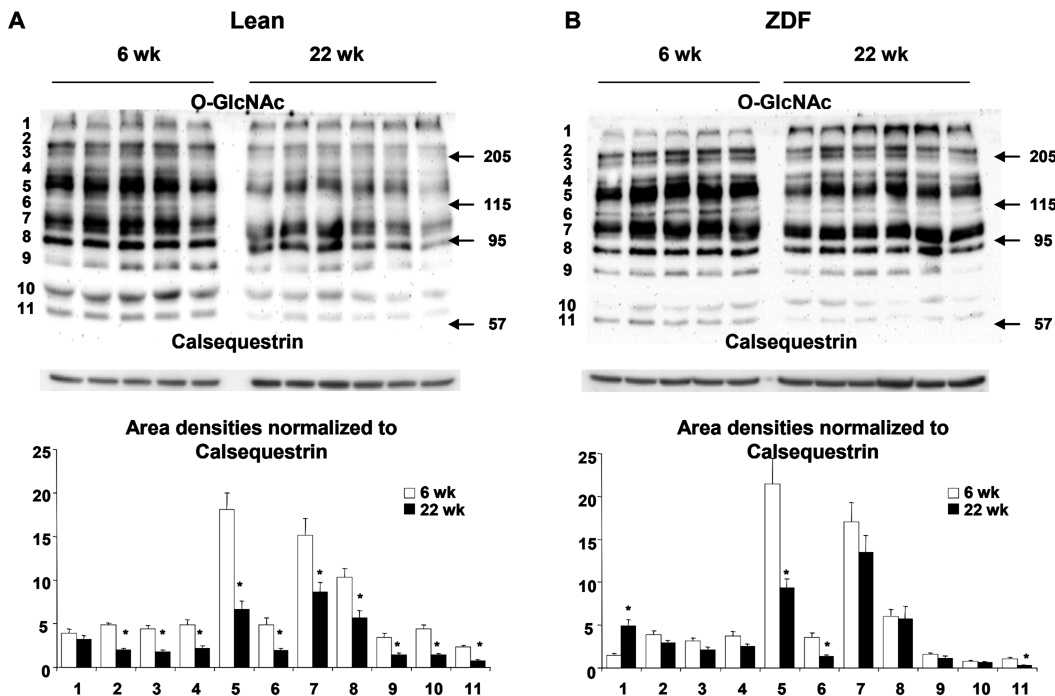


Figure 21.: *CTD110 and Calsequestrin immunoblots (Top panels) and absolute area densities of 11 separate bands normalized to Calsequestrin from the immunoblots (Bottom panels) from 6 and 22-week A: Lean and B: ZDF groups. *: p <0.05 Vs. 6 week old group (Student's T-test).*

2.2. Effect of the development of type-2 diabetes mellitus on excitation-contraction coupling in isolated adult cardiomyocytes.

Our goal was to verify that type-2 diabetes has similar effect on EC coupling compared to those seen with type-1 diabetes or GlcN treatment, described previously [71, 74].

Methods:

Animals: As a model of Type-2 diabetes, non-fasted, male Zucker diabetic fatty rats (ZDF; Gmi-fa/fa, Charles Rivers Laboratories) as described in Chapter 2.1.

Ventricular myocyte isolation, myocyte mechanics and intracellular Ca^{2+} transients: Ventricular myocytes were isolated by collagenase digestion as previously described [132]. Myocytes were allowed to attach on laminin (10 μg/ml) coated glass coverslips and maintained in a Tyrode's buffer (see below) at 37 ^{0}C in incubator and used within 6 hours of isolation. Mechanical properties and intracellular Ca^{2+} transients of ventricular myocytes were assessed simultaneously using a video-based detection system designed to measure dynamic changes in sarcomere lengths coupled to a fluorescent system (IonOptix Co., Milton, MA) as described previously [133]. Briefly, myocytes were electrically stimulated at 0.5 Hz and mechanical properties recorded at a sampling rate of 240 Hz. Ca^{2+} transients were recorded using fluo-4/AM (Molecular Probes Inc. Eugene, OR) while sampling at 1000 Hz.

As previously reported [132], the indices used to describe isotonic shortening and relengthening are include peak fractional shortening (PS; peak shortening amplitude normalized to resting sarcomere length), time to peak twitch (TPT; measured between 10% and 90% above baseline), and area under the shortening (contraction) phase normalized to peak shortening amplitude (A_C/PK) (Figure 22A). The indices for isotonic relengthening were time to relengthening (TR; measured between 10% and 90% below peak) and area under the relengthening (relaxation)

phase normalized to peak amplitude (A_R/PK). The indices used to describe Ca^{2+} transients and are summarized in Figure 23A. The total area under the Ca^{2+} transient normalized to peak fluorescence (A_T/PK) was used instead separating systole and diastole. The ratio of peak fluorescence (F) to basal fluorescence (F_0) was used as an index of the change in cytosolic Ca^{2+}, during contraction since this is independent of the intracellular concentration of the fluoroprobe [133].

All indices of shortening/relengthening and Ca^{2+} transients were determined in the same myocyte after averaging ~10 steady-state twitches for each myocyte. Myocytes were chosen randomly from those meeting the inclusion criteria of sharp, regular striations with visible basal fluorescence, and the ability to achieve steady-state twitches and Ca^{2+} transients during electrical stimulation. Steady-state was defined as consistent peak amplitudes during the recording period for each cell.

Results:

Cardiomyocyte mechanics are summarized in Figure 22B-D and Table 6. At 6 weeks of age, myocyte contraction (i.e., PS, A_C/PK, TPT and Max+dL/dt) was not significantly different between the ZDF and Lean control groups. Myocyte relengthening, (A_R/PK, TR and Max-dL/dt) were also similar in the ZDF and Lean control groups. The lack of impaired contraction and relaxation in the 6-week ZDF group was somewhat surprising since insulin resistance has been shown to adversely affect cardiomyocyte EC-coupling [134, 135]. However, at 22 weeks of age both contraction and relaxation were significantly impaired in the hyperglycemic ZDF group compared to Controls. The time course of the Ca^{2+} transients (Figure 23; Table 7) were consistent with the mechanical indices; for example, at 6 weeks of age, there were no differences between ZDF and Lean controls in peak Ca^{2+} (Figure 23B), A_T/PK (Figure 23C), TPT or TR (Table 7). However, at 22 weeks, peak Ca^{2+} was attenuated (Figure 23B) and cytosolic Ca^{2+} clearing was impaired (Figure 23C) in the diabetic ZDF group compared to the normoglycemic lean group. The

attenuation in peak Ca^{2+} did not, result in predictable changes in sarcomere shortening (Figure 22 and Table 6).

***Table 6**: Mechanical indices for 6 and 22 week old Lean and ZDF rats. SL = sarcomere length, TPT = time to peak twitch (10% above baseline, 90% to peak), TR = time to relaxation (90% from peak, 10% to baseline), Max ± dL/dt = maximum length change, *: p<0.05 vs. age matched Lean, †: p<0.05 vs. 6 weeks*

Twitch	n	SL (μm)	TPT (ms)	TR (ms)	Max +dL/dt (μm^{-4}/ms)	Max -dL/dt (μm^{-4}/ms)
6 wk Lean	30	2.02 ± 0.01	42 ± 2	146 ± 12	26 ± 3	-20 ± 3
6 wk ZDF	42	2.03 ± 0.01	45 ± 2	125 ± 7	29 ± 3	-21 ± 2
22 wk Lean	60	1.87 ± 0.01*†	49 ± 1†	186 ± 12	23 ± 2	-15 ± 1
22 wk ZDF	59	2.00 ± 0.01	55 ± 1*†	314 ± 23*†	18 ± 2†	-12 ± 1†

To assess the effects of hyperglycemia, independent of the effects of leptin, we also compared cardiomyocyte contraction and relaxation between 6 and 22 week old ZDF groups. We found significantly impaired contraction and relaxation (Figure 22D, Table 6) and slower cytosolic Ca^{2+} clearing in the 22-week ZDF group (Figure 23C, Table 7) compared to the 6-week ZDF group. This was consistent with the differences between the 22-week Lean and ZDF groups. However, peak Ca^{2+} (F/F_0) was significantly increased 22-week ZDF compared to the 6-week group, which was in contrast to the reduction in peak Ca^{2+} in the 22-week ZDF compared to the age-matched lean group. It should be noted that there were also age-related changes involving contraction in myocytes from the control group (A_C/PK and TPT); however, the impact of diabetes in the ZDF group exceeded these age-dependent effects. Similar to the ZDF groups F/F_0 was also higher in 22-week Lean groups compared to

the 6-week group; however this was not associated with significant changes in contraction.

Table 7: *Ca^{2+} transient indices for 6 and 22 week old Lean and ZDF rats. TPT = time to peak twitch (10% above baseline, 90% to peak), TR = time to relaxation (90% from peak, 10% to baseline), n = number of cells from 3 animals/group/age, *: p<0.05 vs. age matched Lean, †:p<0.05 vs. 6 weeks*

Ca^{2+} transients	n	TPT (ms)	TR (ms)
6 wk Lean	30	13 ± 1	234 ± 16
6 wk ZDF	42	17 ± 2	212 ± 8
22 wk Lean	60	53 ± 1†	256 ± 9
22 wk ZDF	59	53 ± 1†	331 ± 15*†

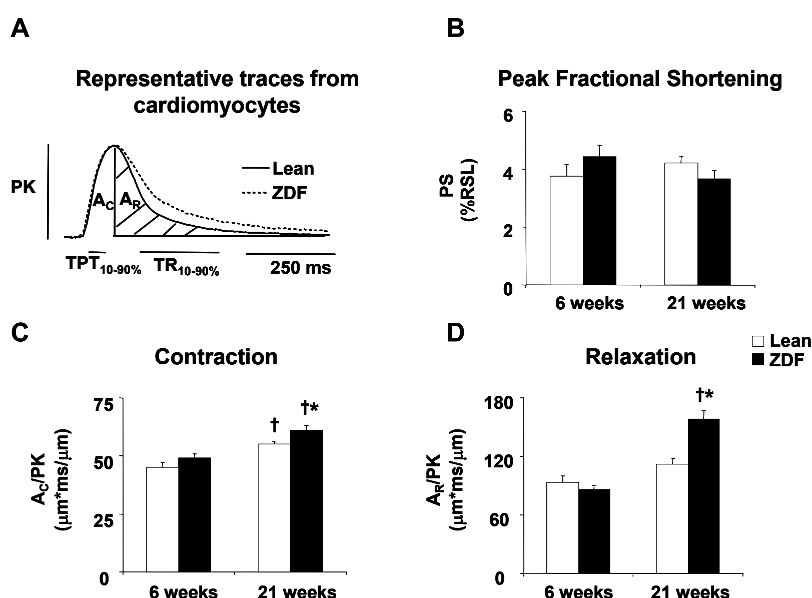


Figure 22. A: *Representative mechanical traces of myocytes from a 22-week old lean and ZDF rat. Most of the indices used to describe mechanical properties are illustrated (see methods for details);* ***B:*** *Peak fractional shortening (maximum sarcomere shortening normalized to resting sarcomere length);* ***C:*** *Area under the contractile phase (normalized to peak shortening) and* ***D:*** *area under the relaxation phase (normalized to peak shortening) of myocytes from 6 and 22 week old lean and ZDF groups. *: p<0.05 vs. age matched Lean, †:p<0.05 vs. 6 weeks*

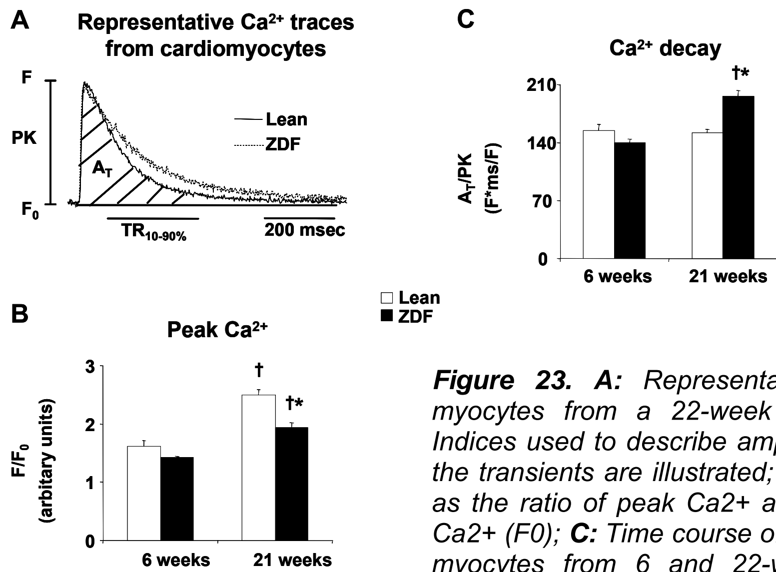


Figure 23. A: *Representative Ca2+ transients of myocytes from a 22-week old lean and ZDF rat. Indices used to describe amplitude and time course of the transients are illustrated;* ***B:*** *Peak Ca2+ expressed as the ratio of peak Ca2+ amplitude (F) and baseline Ca2+ (F0);* ***C:*** *Time course of Ca2+ transient (AT/PK of myocytes from 6 and 22-week old lean and ZDF groups. *: p<0.05 vs. age matched Lean, †: p<0.05 vs. 6 weeks*

2.3. Effect of acute increase in HBP flux and O-GlcNAc on cardiac substrate utilization.

Our goal was to show that GlcN alters cardiac energy metabolism by increasing HBP flux and O-GlcNAc.

Methods:

Animals: Non-fasted, 300-350 g Sprague Dawley male rats (Charles Rivers Laboratories) were used. Hearts were excised and perfused as previously described in Chapter 1.1.

Materials: ^{13}C-labeled substrates were obtained from Cambridge Isotope Laboratories.

Experimental groups: Hearts were divided into six groups and perfused for 60 minutes under normoxic conditions with perfusion buffer containing 1) 0 mM GlcN (n=8) 2) 0.05 mM GlcN (n=5) 3) 0.1 mM GlcN (n=8) 4) 1.0 mM GlcN (n=4) 5) 5.0 mM GlcN (n=8) and 6) 10.0 mM GlcN (n=7).

^{13}C-isotopomer analyses: Hearts were perfused with [U-^{13}C]palmitate, [3-^{13}C]lactate and [2-^{13}C] pyruvate for the final 40 minutes of the protocol at which time hearts were freeze-clamped, acid extracted and ^{13}C-NMR spectra collected as previously described [77, 136]. As previously described ^{13}C-NMR isotopomer analyses of heart extracts were performed to determine the relative contribution of substrates to total acetyl-CoA entering the tricarboxylic acid (TCA) cycle [77, 136].

Western blots: Hearts were freeze clamped and ground to a fine tissue powder under liquid nitrogen. Tissue powder was homogenized in the appropriate lysis buffer as previously described for O-GlcNAc (Chapter 1.1.), for phospho- and total-AMPK and for phospho- and total-ACC [137]. Whole heart lysates were separated on SDS-PAGE and transferred to PVDF membrane (Pall). Blots were visualized with enhanced chemiluminescent assay (Pierce) and the signal was detected with UVP

BioChemi System (UVP). Densitometry was quantified using Labworks analysis software (UVP).

HPLC: HPLC protocol described in Chapter 1.1. was used.

Results:

Perfusion of the hearts with 0.05, 0.1, 1.0, 5.0 or 10.0 mM GlcN for 60 minutes had no effect on rate pressure product, +dp/dt or coronary flow compared to the control hearts, perfused with perfusion buffer containing 0 mM GlcN (Figure 24).

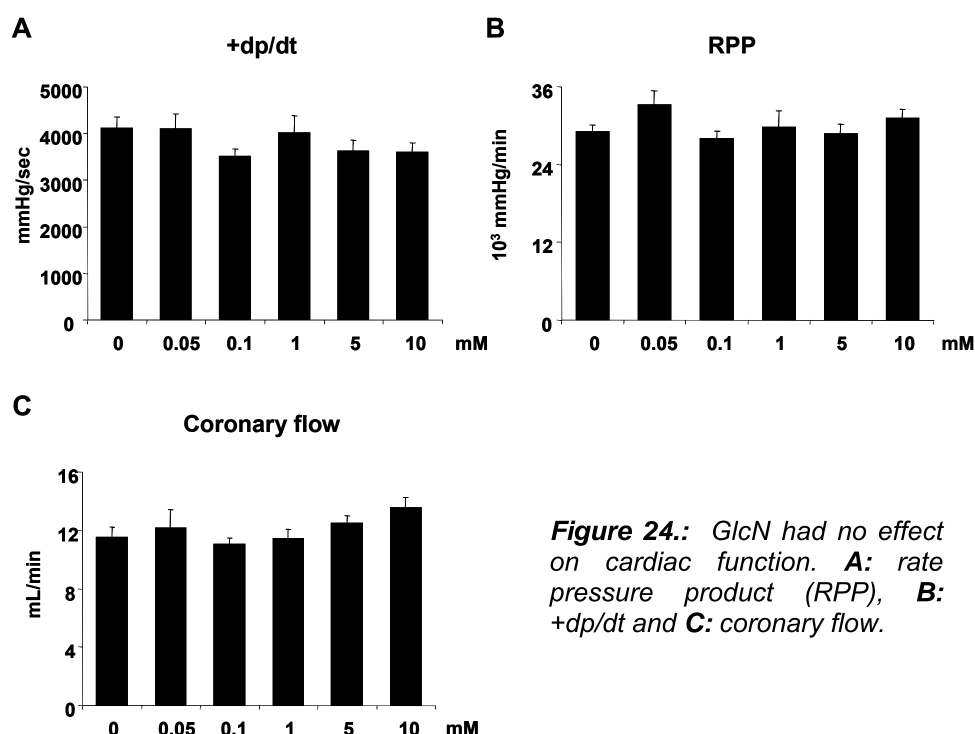


Figure 24.: *GlcN had no effect on cardiac function.* ***A:*** *rate pressure product (RPP),* ***B:*** *+dp/dt and* ***C:*** *coronary flow.*

We examined the effect of the different GlcN concentrations on the hexosamine biosynthesis pathway. We found that 60 minutes perfusion with even the lowest glucosamine concentration (i.e., 0.05 mM) resulted in elevated UDP-GlcNAc levels, which is the end-product of the HBP, suggesting an increased flux through the pathway. We saw a further increase with higher GlcN concentrations (Figure 25C), with no changes in the ATP content of the hearts (Figure 25D). The changes in UDP-

HexNAc levels seen with the different GlcN concentrations correlated well with the changes seen in O-GlcNAc (Figure 25A, B).

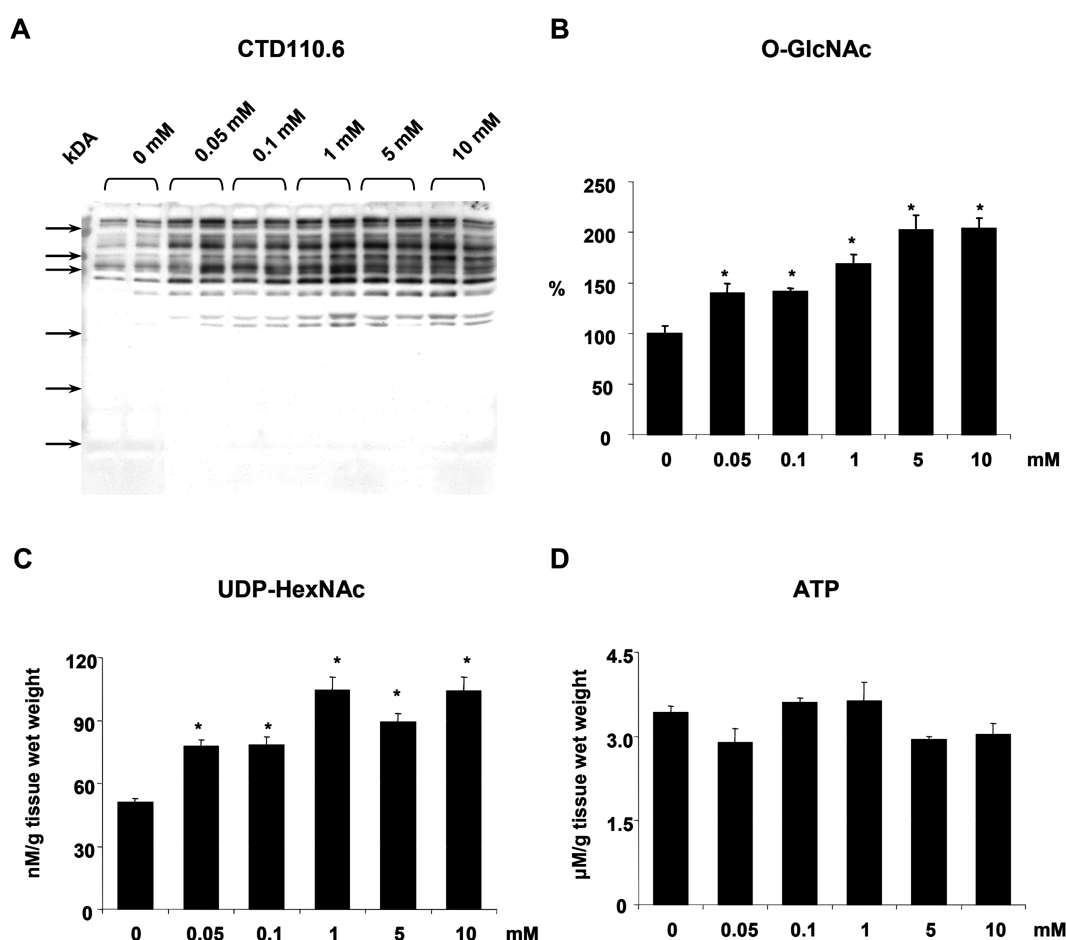


Figure 25.: *GlcN caused a dose dependent increase in O-GlcNAc (**A, B**), due to the increased levels of UDP-HexNAc (**C**), without altering cardiac ATP content (**D**). *: p<0,05 vs 0 mM, one-way ANOVA with Dunnet`s posthoc test*

Perfusion with low concentrations of GlcN (0.05 and 0.1 mM) for 60 minutes lead to significantly increased O-GlcNAc compared to the control hearts. Perfusion with 1 mM GlcN further enhanced O-glycosylation in the isolated perfused hearts. O-GlcNAc increased even more with the 5 mM GlcN however we did not see additional increase with the 10 mM GlcN compared to the 5 mM hearts (Figure 25A, B).

Examining the effect of low (0.05, 0.1 mM) and high (5, 10 mM) GlcN on cardiac energy metabolism by using ^{13}C-glutamate isotopomer analyses we found that at all concentrations glucosamine significantly increased palmitate oxidation and decreased carbohydrate utilization compared to the control group (Figure 26A). The

maximal reponse on cardiac energy metabolism was seen with 0.1 mM GlcN (Figure 26A). The decrease in carbohydrate oxidation was mainly due to the decreased lactate oxidation and with the 0.1, 5 and 10 mM GlcN in part due to the decreaed pyruvate oxidation (Figure 26B). GlcN did not change the glucose oxidation (Figure 26B), or glycolytic lactate efflux in our experiments (Figure 27B). As expected the lactate uptake was decreased with the 0.1 mM GlcN compared to the 0 mM (Figure 27A).

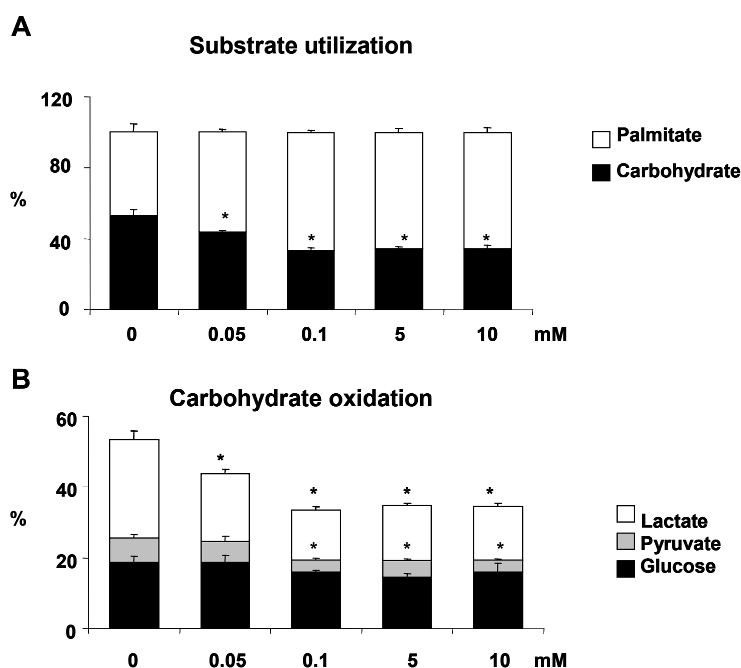


Figure 26. A: *60 minutes treatment with 0.05 mM GlcN increased palmitate oxidation and decreased carbohydrate utilization. 0.1 mM GlcN further increased fatty acid utilization, but we have not seen further increase with the higher GlcN concentrations.* ***B:*** *The overall decrease in carbohydrate oxidation was a result of decreased lactate (0.05, 0.1, 5, 10 mM) and decreased pyruvate (0.1, 5, 10 mM) oxidation compared to the 0 mM. Glucose oxidation was unchanged. (*: p<0,05 vs 0 mM, one-way ANOVA with Dunnet`s posthoc test)*

McClain and colleages previously demonstrated that chronic increase in O-GlcNAc resulted in increased AMPK activity and increased ACC phosphorylation leading to increased fatty acid oxidation. Therefore we also examined the effect of a short term GlcN treatment on AMPK and ACC phosphorylation in the rat heart. As shown in Figure 28 we found no difference with the 0.1 mM GlcN compared to the control hearts, while the fatty acid oxidation was enhanced in the treted hearts (Figure 26A).

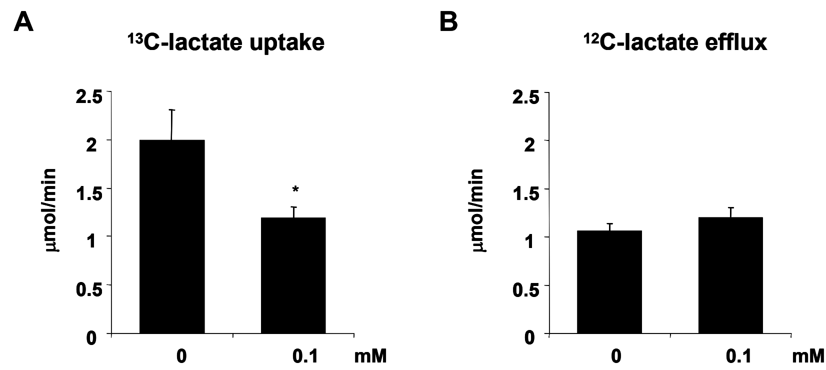


Figure 27.: *0.1 mM GlcN significantly decreased 13C-lactate uptake (**A**), but did not alter 12C-lactate (glycolytic) efflux (**B**). (*: p<0,05 vs 0 mM, Student's T-test)*

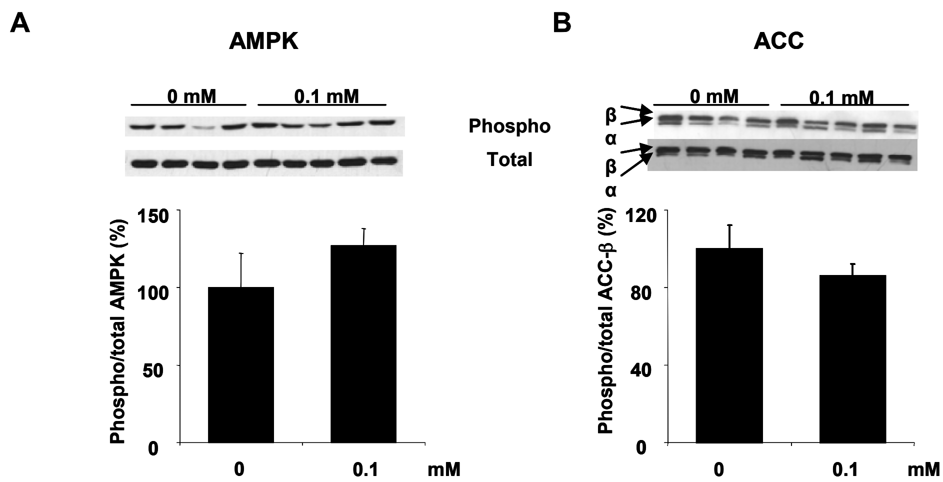


Figure 28.: *There was no change in AMPK (**A**) or ACC (**B**) phosphoryltaion in the heart after 60 minutes perfusion with 0.1 mM GlcN.*

3.1. The presence of O-glycosylation in human renal biopsy specimens.

We examined whether O-glycosylation is present in human kidney cells.

Methods:

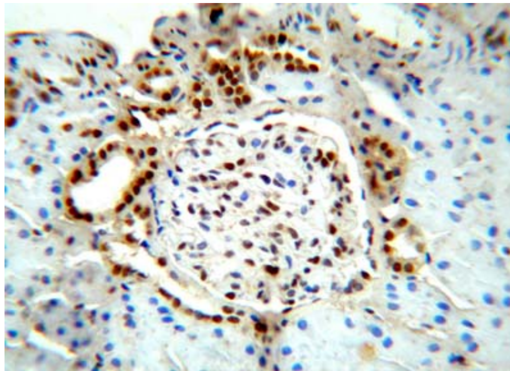
Immunhistology: Percuteneous renal biopsy was performed previously to diagnose kidney disease. Biopsy specimen was fixed in formaldehyde and embedded in paraffin. Remnant 3 μm sections were used. After endogen and background blocking slides were incubated in the primary antibody solution (CTD110.6 1:100) for overnight at 4 ^{o}C following the appropriate HRP labeled secondary antibody solution. We have used DAB (DAKO) as a chromogen and UltraVision DAKO for detection. To verify that the antibody binding was specific we used 20 mmol/L N-acetylglucosamine in the primary antibody solution to block the binding of the primary antibody to the antigens. We used haemtoxylin for nuclear staining.

Results:

We show here for the first time that O-glycosylation is present in the cells of the human kidney. We found O-GlcNAc positive staining in the nuclei of the podocytes in the glomeruli (Figure 29) and we also found that O-GlcNAc is present in the nuclei of the tubular epithelial cells (Figure 30). Furthermore the cytosol of the tubular epithelial cells showed positive CTD110.6 staining in our samples (Figure 30).

To verify that our immune labeling was specific we blocked the specific binding of the primary antibody using 20 mmol/L N-acetylglucosamine. We found that all our immune-labeling was specific and the 20 mmol/L N-acetylglucosamine completely blocked the immunostaining with the antibody specific for O-GlcNAc (Figure 31).

Control glomerulus



Diabetic glomerulus

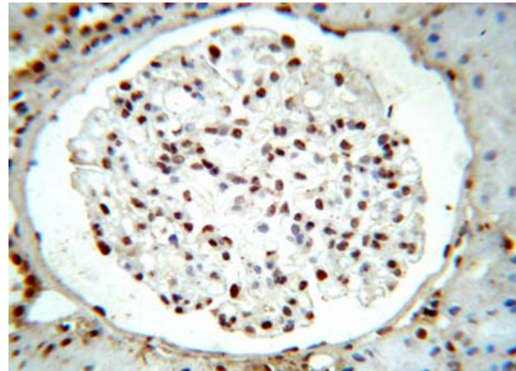
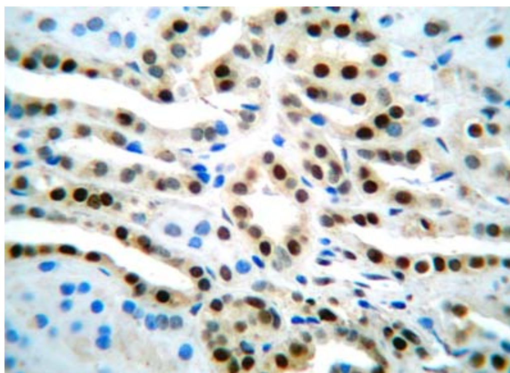


Figure 29.: *CTD110.6 immunostaining of human renal biopsy specimens. Representative pictures of a diabetic and a non-diabetic glomerulus.*

Control tubuli



Diabetic tubuli

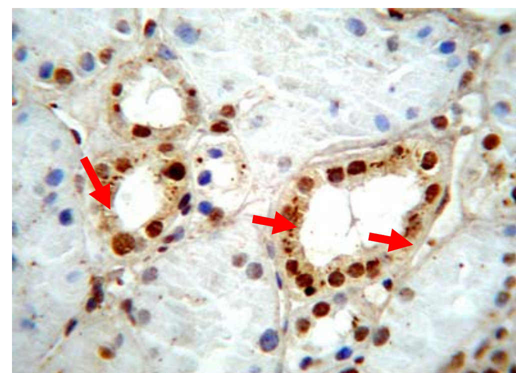


Figure 30.: *CTD110.6 immunostaining of human renal biopsy specimens. Representative pictures of diabetic and non-diabetic tubuli. The red arrows indicate the granular cytoplasmic staining of the diabetic samples.*

CTD110.6 + 20 mmol/L N-acetylglucosamine

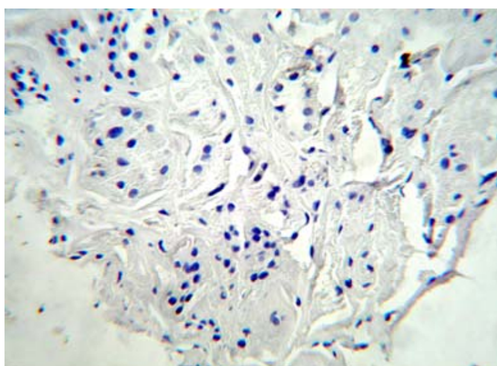


Figure 31.: *Specific blockade of CTD110.6 immunostaining using 20 mmol/L N-acetylglucosamine*

3.2. O-glycosylation in diabetic nephropathy.

We hypothesized that O-GlcNAc is increased in the kidney of the individuals with diabetic nephropathy, therefore we compared renal biopsy specimens of patients with diabetic nephropathy (diagnosed previously, n=2) with the biopsy specimens of 2 non-diabetic individual.

Methods:

Immunhistology: Percutaneous renal biopsy was performed previously to diagnose kidney disease. We used renal biopsy specimens of patients with previously diagnosed diabetic nephropathy (n=2) and as controls 2 specimens from individuals without diabetes, with previously diagnosed thin basal membrane syndrome. The protocol described in Chapter 3.1. was used to perform immunhistology.

Results:

We found no difference in the localization of O-GlcNAc staining in the glomerular cells between the diabetic and non-diabetic samples (Figure 29). Unfortunately our protocol was not able to show subtle differences in intensity.

Comparing 2 diabetic vs. 2 non-diabetic renal biopsies we found no difference in the nuclear staining of the tubular epithelial cells either (Figure 30). However in contrast with the absence of O-GlcNAc in the cytosol of the non-diabetic samples we found an intense, granular anti O-GlcNAc staining in the cytosol of the tubular epithelial cells of the diabetic individuals (Figure 30), suggesting a higher glucose reuptake in the proximal tubular cells and a consequent increase in HBP flux and O-GlcNAc.

However more detailed experiments are needed to provide stronger evidence of the role of O-GlcNAc in diabetic nephropathy.

Discussion:

There is increasing recognition that *O*-GlcNAc modification of serine and threonine residues on cytosolic and nuclear proteins is an important regulatory mechanism involved in signal transduction [18-21, 138]. In cell culture systems previous studies have demonstrated that O-GlcNAc levels are increased in response to stress and that augmentation of this response increased tolerance to stress [95, 96], suggesting that activation of this pathway maybe a component of endogenous cell survival pathway. We showed that short ischemic stress alone increased the flux through the HBP and lead to increased O-glycosylation in the isolated rat heart. Surprisingly, however, after longer periods of ischemia, O-GlcNAc level decreased and after 60 min of reperfusion O-GlcNAc levels further declined.

Zachara et al., [95] reported that *O*-GlcNAc levels increased in response to stress possibly as a consequence of an increase in OGT activity. Here in the intact heart we found that after 5-10 min of ischemia both UDP-HexNAc and *O*-GlcNAc level were significantly increased. Since flux through OGT is very sensitive to UDP-GlcNAc levels [29, 33], the increase in UDP-HexNAc could explain the increase in *O*-GlcNAc levels seen here; however, we cannot rule out an increase in OGT activity. The increase in UDP-HexNAc during ischemia could be due to either an increase in flux through the hexosamine pathway and/or a consequence of decreased utilization of UDP-GlcNAc via other pathways. Sohn et al., showed that inhibition of GFAT, which regulates the entry of glucose into the hexosamine biosynthesis pathway, prevented the stress induced increase in *O*-GlcNAc, and decreased hyperthermal tolerance. This would be consistent with increased flux through the hexosamine biosynthesis pathway also increasing UDP-GlcNAc. However, UDP-GlcNAc is also required for multiple N-glycosylation reactions that are involved in protein synthesis; since ischemia is known to inhibit protein synthesis [139] it is possible that this could also contribute to the increase in UDP-GlcNAc seen here.

Interestingly even though UDP-HexNAc continued to increase during ischemia, O-GlcNAc levels had decreased after 30 min ischemia. The dissociation between UDP-HexNAc and O-GlcNAc, suggests that OGT activity might be inhibited after prolonged ischemia. If so this could also contribute to the further decline in O-GlcNAc levels seen during reperfusion. Regulation of OGT activity is complex and poorly understood; however, OGT is known to be subject to both phosphorylation and O-GlcNAc modification and increased phosphorylation is believed to increase activity [140]. Clearly more studies are needed to understand the regulation of the hexosamine biosynthesis pathway and *O*-GlcNAcylation in response to ischemia.

Our findings were consistent with previous studies showing that enhancing O-GlcNAc leads to increased tolerance to stress both in cell culture systems [95, 96, 141] and in the isolated heart [75]. Glucosamine increased normoxic levels of both UDP-HexNAc and O-GlcNAc and improved functional recovery following ischemia reperfusion. Interestingly, while the addition of glucosamine increased O-GlcNAc levels at all time points relative to controls, ischemia did not result in a further increase; however, glucosamine did prevent the decrease in O-GlcNAc seen at the end of ischemia and during reperfusion. Champattanachai has shown that that inhibition of nucleocytoplasmic O-glycosylation with alloxan, an OGT inhibitor, resulted in loss of protection of glucosamine treatment in isolated cardiomyocytes [141], which supports the notion that the cardioprotective effect of glucosamine is due to the increase in nucleocytoplasmic O-glycosylation.

Activation of the MAPK pathway, particularly p38 and ERK1/2 MAPK, has been implicated in ischemic cardioprotection [103-110, 120]. Kneass and Marchase showed in another cell system that agonist stimulation of the MAPK pathway was enhanced in response to increased O-GlcNAc levels [125]; therefore, we examined the effect of glucosamine on the response of p38 and ERK1/2 MAPK to ischemia/reperfusion. We found that glucosamine significantly attenuated the

ischemia-induced increase in p38 phosphorylation, which this is consistent with reports that inhibition of p38 during sustained ischemia is protective [103-109]. Surprisingly, at the end of reperfusion p38 phosphorylation was increased in the glucosamine treated group. There is relatively little information regarding the impact of reperfusion on p38 phosphorylation or on whether modulation of p38 activation during reperfusion impacts recovery. However, it has been shown that brief activation of p38 may contribute to cardioprotection seen with ischemic preconditioning. Activation of p38 can be pro-apoptotic, mediated via Caspase-3 or p53 [109, 111-113]; however, p38 has also been reported to activate pro-survival pathways. For example, αB-crystallin and HSP-27 are downstream of p38 and both have been shown not only to play a role in ischemic protection but also to subject to *O*-GlcNAc modification [30, 43, 142, 143].

While we saw the decrease in ERK1/2 phosphorylation during ischemia and an increase on reperfusion as recently reported by Hausenloy [120], we found that glucosamine had no effect on ERK1/2 phosphorylation under any perfusion conditions. Akt has also been shown to mediate the effect of both ischemic pre- and postconditioning [120, 144, 145]. We saw a brief increase in Akt phosphorylation after 5 minutes of ischemia; and a decrease in Akt phosphorylation at the end of ischemia and an increase during reperfusion. Despite the importance of Akt as a mediator of cardioprotection, we found no difference in the phosphorylation state of Akt between the control and the glucosamine group under any of the conditions. Thus, these data suggest that glucosamine cardioprotection cannot be attributed to activation of either ERK1/2 or Akt pro-survival pathways. These data provide further support for the idea that acute increase in O-GlcNAc provides cardioprotection and it is associated with an altered response of p38 MAPK to ischemia/reperfusion.

Although we have shown that acute changes in O-glycosylation are protective, chronic changes in O-GlcNAc levels have been implicated in the

development of age-related diseases. Rex-Mathes et al. showed aged related changes in O-GlcNAc in the mouse brain between 3 and 13 months of age [146]. Therefore we examined whether HBP flux and O-GlcNAc change with age.

We found that in senescent rats compared to young adult rats, O-GlcNAc levels in heart, aorta, brain and skeletal muscle were significantly increased. However, paradoxically the protein levels of OGT, which catalyzes O-GlcNAc synthesis were significantly decreased with senescence. The increased O-GlcNAc levels in the heart were associated with increased levels of GFAT2 mRNA and increased UDP-HexNAc concentration, consistent with increased HBP activity. No such changes were seen in brain or skeletal muscle.

As discussed above the regulation of O-GlcNAc is complex and poorly understood. It is known that O-GlcNAc is dependent on UDP-GlcNAc, the end-product of HBP. Flux through the HBP is regulated in part by GFAT and we found here that in the heart GFAT2 mRNA levels were increased ~3 fold and this was also associated with a significant increase in UDP-GlcNAc levels in the heart. Thus, in the heart the increase in O-GlcNAc levels in the 24-month group, could be attributed at least in part by increased HBP flux. On the other hand there were no significant changes in either GFAT mRNA or UDP-GlcNAc levels in either brain or skeletal muscle, despite the fact that O-GlcNAc levels were significantly increased in the 24-month group. However, it should be noted that whereas, in the heart O-GlcNAc levels increased ~2-fold, in brain and skeletal muscle the increase was only ~30-40%.

Although the effect of age on GFAT mRNA and UDP-GlcNAc levels were variable between tissues, we found a significant decrease in OGT expression levels in all tissues examined, except skeletal muscle. This is clearly at odds with the increase in O-GlcNAc levels seen in the same tissues; however, other factors such as decreased O-GlcNAcase activity could contribute to an increase in O-GlcNAc. On the other hand, it should be noted that we saw an increase in O-GlcNAcase mRNA in

the heart; however, changes in mRNA levels do not necessarily reflect changes at the protein level, as found here with OGT, where mRNA levels were unchanged but protein levels were decreased. Unfortunately, due to the lack of a commercially available O-GlcNAcase antibody, we could not determine O-GlcNAcase protein levels in our experiments.

Several nervous system specific cytoskeletal proteins have been shown to be O-glycosylated, including neurofilaments and synapsin [147, 148]. It has been shown, that O-GlcNAc is also playing an important role in the development of neurodegenerative disorders, such as Alzheimer's disease [20, 27, 43]. There are O-GlcNAc modified proteins involved in the pathophysiological mechanisms underlying the development of Alzheimer's including tau [98, 99], β-amyloid precursor [100], AP 3 [101] and AP 180 [102]. Furthermore the chromosomal localization of O-GlcNAcase has been identified as the locus of Alzheimer disease on the chromosome 10 [20, 27]. Therefore we can conclude that altered O-glycosylation may contribute to the development of AD with age.

The potential consequences of increased O-GlcNAc in aorta is somewhat less clear; however, increased HBP flux and O-GlcNAc levels have been associated with impaired eNOS activity [47], increased PAI-1 expression [149] and TGF-ß1 expression [60, 85, 87]. Thus, elevated O-GlcNAc levels in the 24-month old group, could contribute to the development of impaired vascular reactivity and increased incidence of atherosclerosis commonly associated with aging [150].

In the heart increased HBP flux and O-GlcNAc levels have been associated with impaired function, particularly in the context of diabetes. For example, Pang et al., found that a brief period of streptozotocin induced diabetes and acute glucosamine treatment blunted the inotropic response of the isolated perfused heart to phenylephrine, [6]. Clarke et al. demonstrated that in neonatal cardiomyocytes increasing protein *O*-GlcNAc levels either by hyperglycemia or glucosamine treatment resulted in impaired sarcoplasmic reticulum (SR) function [5].

Subsequently, Hu et al. showed that increased O-GlcNAcase expression improved whole heart function in diabetic mice [4].

Another interesting observation was that the senescent animals were insulin resistant, since the insulin levels in the 24 months old rats were increased by 4-fold. Increased HBP flux and increased levels of O-GlcNAc are associated with insulin resistance and diabetes [7, 15-21]. Previous studies have demonstrated a link between increased *O*-GlcNAc levels and contractile dysfunction following a prolonged period of type-1 diabetes [4, 5]. However there is very little information about the relationship between EC-coupling and *O*-GlcNAc levels in a model of type-2 diabetes. Therefore we examined 6 week old - insulin resistant - and 22 week old - type-2 diabetic - ZDF rats and age matched lean controls.

We showed that there are no differences in any of the indices of contraction or relaxation between 6-week old insulin resistant ZDF rats and Lean controls; however, by 22 weeks of age - when ZDF animals are hyperglycemic - cardiomyocytes from ZDF rats exhibited significantly impaired relaxation and slowed cytosolic Ca^{2+} removal compared to both age-matched Lean control and 6 week old normoglycemic ZDF groups. In the 22-week groups the impaired relaxation was associated with a significant increase in overall *O*-GlcNAc levels in the ZDF group compared to the Lean group. This increase in *O*-GlcNAc was associated with elevated UDP-GlcNAc levels with no change in OGT expression suggesting that increased HBP flux was a primary mediator of the increased *O*-GlcNAc levels.

Although we observed similar impaired relaxation and increased UDP-GlcNAc levels in the diabetic 22-week old ZDF group compared with the 6-week, normoglycemic ZDF group, paradoxically we saw a significant decrease in overall *O*-GlcNAc levels as well as a decrease in OGT expression. However, when comparing either 22-week ZDF and Lean groups or 6- and 22-week ZDF groups, we found that independent of age and leptin levels, hyperglycemia was associated with increased *O*-GlcNAc levels.

In the 22 week old diabetic ZDF group, UDP-GlcNAc levels were significantly increased compared to their age-matched lean counterparts, which would suggest that increased flux through the HBP was a contributing factor in the increase in O-GlcNAc levels in this model. An increase in HBP flux seen with diabetes may be a result not only of elevated plasma glucose levels, but also to the redirection of the glucose fluxes due to increased circulating lipids and decreased activity of pyruvate dehydrogenase, both hallmarks of diabetes. It has been shown in skeletal muscle cells that elevated fatty acid concentration increased UDP-GlcNAc and expression of GFAT [151], which regulates the entry of glucose into the HBP.

Surprising, despite the marked hyperglycemia in the 22-week group and increased in cardiac UDP-GlcNAc concentrations, there was a significant decrease in *overall* O-GlcNAc compared to the 6-week group, which was associated with reduced OGT expression. Even though there was an overall decrease in O-GlcNAc levels between 6 and 22-week ZDF groups, there was a significant increase in O-GlcNAc levels in proteins in the high molecular weight range (i.e., >205kD). Interestingly in the study examining the effect of ageing in the senescent hearts an O-GlcNAc positive band appeared below the molecular weight of 50 kDa, which suggests that the pathomechanism of the functional impairment seen in diabetic animals may be different from those seen with age.

Proteins in the same molecular weight range also showed increased O-GlcNAc levels in the 22-week ZDF group compared to age-matched Lean group. It is tempting to suggest therefore, that increased O-GlcNAc levels in this group of proteins may play a role in the development of impaired cardiomyocyte function associated with diabetes. Cardiomyocytes contain many high molecular weight proteins including cytoskeletal and contractile proteins such as titin, dystrophin, myosin, as well as proteins that may play a critical role in regulating the changes in metabolism seen in response to diabetes, such as acetyl-CoA carboxylase 2.

It has been proposed that altered energy metabolism is associated with impaired function in the diabetic hearts [78]. We have shown increased O-GlcNAc in hearts from type-2 diabetic ZDF rats. Wang et al. showed that in the heart of ZDF rats cardiac energy metabolism was changed and it was associated with contractile dysfunction compared to the non-diabetic lean littermates [77]. In a recent study McClain and his group demonstrated that long term activation of the HBP increased O-GlcNAc and lead to increased palmitate oxidation in adipocytes [79]. These data suggests that in the heart increased O-GlcNAc may lead to changes in energy utilization similar to those seen in diabetic animals. Therefore we examined the effect of GlcN on cardiac energy metabolism.

GlcN increases the flux through the HBP, by bypassing GFAT - the rate-limiting enzyme of the pathway (Figure 1). We showed that short term – 60 minutes – perfusion with GlcN leads to a dose dependent increase in UDP-GlcNAc and O-GlcNAc levels. As expected within such a short time glucosamine had no effect on ATP levels in the heart at any of the examined concentrations and we did not see changes in cardiac function either.

Short-term perfusion with as little as 0.05 mM GlcN decreased carbohydrate oxidation and caused an increase in fatty acid utilization. Using 0.1 mM GlcN further increased the changes seen in substrate utilization, but increasing GlcN concentrations to 1-10 mM concentrations had no additional effect. Wang et al. showed that type-2 diabetes increased fatty acid oxidation and decreased carbohydrate utilization, the decrease in carbohydrate oxidation was mainly due to the decreased lactate oxidation, since glucose oxidation was unchanged [77]. We have seen similar changes in carbohydrate oxidation; lactate and pyruvate oxidation decreased and glucose oxidation was unchanged. Since in our perfusate both glucose and glucosamine were unlabeled, if glucosamine had been metabolized via glycolysis and the TCA cycle, this would have been seen as an increase in unlabeled glycolytic lactate efflux and glucose oxidation. However, this was not seen in these

experiments, which strongly suggests that the effect of glucosamine on the heart is not due to its metabolism via glycolysis and the TCA cycle..

AMPK plays a crucial role in maintaining cellular energy and metabolic homeostasis [80-82]. Luo et al. demonstrated that chronically enhancing HBP flux with GlcN increased the activity of AMPK and lead to increased phosphorylation of ACC, thus increasing fatty acid oxidation [79]. Transfecting the cells with dominantly negative AMPK blocked the effect of GlcN. They showed that AMPK was recognized by wheat germ agglutinin - a lectin binding O-glycosylated proteins - and the removal of O-GlcNAc by hexosaminidase reduced AMPK activity [79]. This would suggest that AMPK is a target for O-glycosylation; however, using a specific anti-O-GlcNAc antibody they were unable to detect O-GlcNAc modification of AMPK. It is possible that the antibody used was not sensitive in detecting proteins without multiple O-GlcNAc moieties. We examined the phosphorylation levels of AMPK and ACC and found no difference between the 0.1 mM GlcN treated and control groups. The discrepancy described here can be explained by the differences of acute vs. chronic stimulation of the HBP. It is also known that HBP activation has a tissue specific effect on AMPK regulation [152], therefore the paradox can be explained by the difference between the adipose tissue and the heart.

In conclusion we can postulate that in the hearts of type-2 diabetic animals the flux through the HBP and O-glycosylation was increased and it was associated with impaired excitation-contraction coupling. Furthermore increasing O-GlcNAc leads to changes in cardiac energy metabolism, similar to the changes in the diabetic heart. Taken together these results strongly suggest that altered regulation of protein O-glycosylation plays a critical role in the development of diabetic cardiomyopathy.

Increased HBP flux and O-glycosylation also plays an important role in the development of diabetic nephropathy. It has been shown that increased HBP flux leads to accumulation of extracellular matrix proteins in a TGF-β dependent manner

[84-86]. Another important mediator if PAI-1, which is also known to be activated by enhanced HBP [87].

Most of the studies investigating the role of HBP and O-GlcNAc in diabetic nephropathy have been performed in cell culture. Nerlich et al. demonstrated that in human autopsy samples the key enzyme of the HBP is present in kidney tubular epithelial cells and they found that the glomeruli of diabetic subjects showed anti-GFAT positive staining in the mesangial- and in the glomerular epithelial cells [88].

Enhanced cell growth and hypertrophy is another possible mechanism how elevated glucose levels lead to the development of diabetic nephropathy. Slawson et al., demonstrated that increased O-GlcNAc causes perturbations in cell cycle in a cyclin dependent kinase dependent manner [92]. It has also been shown that in mesangial cells [89, 93] and in tubular epithelial cells [94] activation of the hexosamine biosynthesis pathway stimulates cell hypertrophy.

Most of the literature provides evidence, that HBP is playing a role in the development of diabetic nephropathy, however there is no direct evidence, that O-GlcNAc is present in the kidney cells, or it is elevated in diabetes. Therefore we examined whether CTD 110.6 - an O-GlcNAc specific antibody – recognizes cells in human kidney samples. Shafi et al. showed that knock out of OGT in mice was embryonically lethal, and deletion of the OGT gene in embryonic fibroblasts was also lethal [35], suggesting that OGT activity/O-glycosylation is vital for life, thus we expected that human kidney cells will show anti-O-GlcNAc staining.

We showed here for the first time that glomerular and tubular cells of human kidney biopsy specimen show anti-O-GlcNAc positive staining. We found that CTD 110.6 showed a granular staining in the cytosol of the tubular epithelial cells. Proximal tubular epithelial cells reabsorb glucose from the filtrate. Diabetic patients have glucosuria, therefore tubular epithelial cells absorb more glucose and have elevated intracellular glucose levels. Higher intracellular glucose increases the flux through HBP and provide excessive substrate – UDP-GlcNAc – for OGT and leads to

increased O-GlcNAc formation. As discussed above increased HBP flux and increased O-GlcNAc is one potential mechanism, how diabetic nephropathy is evolved.

Finally we may conclude that O-GlcNAc is playing an important role in cell signaling pathways. Acute activation of the pathway is cytoprotective, while chronic activation of the pathway is involved in aging related diseases and diabetes, diabetic complications.

Theses:

1. Activation of pathways leading to protein O-glycosylation is an internal stress response and augmentation of these pathways leads to enhanced tolerance to stress in the rat heart.

   - In the isolated rat heart ischemic stress alone increased flux through the hexosamine biosynthesis pathway and increased O-glycosylation

   - Increasing O-glycosylation by glucosamine administration resulted in enhanced tolerance to ischemia/reperfusion injury in the isolated rat heart, and it was associated with altered p38 MAPK activation.

2. In the rat brain, skeletal muscle, aorta and heart O-glycosylation was increased with age. The increase in O-glycosylation was not a consequence of the alterations seen in the expression of the regulating enzymes (O-GlcNAcase and/or O-GlcNAc transferase), but it was associated with increased expression of glutamine: fructose 6-phosphate amidotransferase and increased hexosamine biosynthesis flux seen in the heart of aged rats.

3. Increased O-glycosylation in the heart as a result of diabetes was associated with changes in excitation-contraction coupling and substrate utilization.

   - The development of type-2 diabetes resulted in impaired cardiomyocyte excitation-contraction coupling and this was associated with increased hexosamine biosynthesis pathway flux and a consequent increase in O-glycosylation, especially on high molecular weight proteins.

   - Increasing O-glycosylation in the isolated rat heart by glucosamine administration lead to decreased carbohydrate and increased fatty acid utilization. These changes were similar to those seen in the heart of type-2 diabetic rats.

- The changes of substrate utilization were not associated with altered AMP-activated protein kinase and/or acetyl-CoA carboxylase activation in the rat heart.

4. O-glycosylation was present in the human kidney and increased O-glycosylation was associated with diabetic nephropathy.

- O-glycosylation was present in the glomerular and tubular cells of kidney biopsy specimen of both diabetic and non-diabetic human individuals.

- Diabetic nephropathy was associated with increased O-glycosylation in glomerular and tubular cells of human biopsy specimens.

Abbreviations:

A_C/PK: area under the contraction phase normalized to peak shortening amplitude
ACC: acetyl-CoA carboxylase
AD: Alzheimer disease
AGE: advanced glycation endproduct
AMPK: AMP-activated protein kinase
AP: chlatrin assembly protein
A_R/PK: area under the relaxation phase normalized to peak amplitude
A_T/PK: area under the Ca^{2+} transient normalized to peak fluorescence
DNP: diabetic nephropathy
EC: excitation-contraction coupling
EDP: end-diastolic pressure
eNOS: endothelial nitrogen oxide synthase
ERK: extracellular-signal regulated kinase
F: peak fluorescence
F_0: basal fluorescence
FFA: free fatty acid
Fru-6-P: fructose-6-phosphate
GalNAc: N-acetylgalactosamine
GFAT: Glutamine: fructose-6-phosphate amidotransferase
Glc-6-P: glucose-6-phosphate
GlcN: glucosamine
GlcNAc: N-acetylglucosamine
GlcNAc-6-P: N-acetylglucosamine-6-phosphate
$GlcNH_2$-6-P: glucosamine-6-phosphate
GLUT: Glucose transporter
GSK: glycogen synthase kinase
HAT: histone acetyltransferase
HBP: hexosamine biosynthesis pathway
HDL: high density lipoprotein
I/R: ischemia-reperfusion
IRS: insulin receptor substrate
LFI: low flow ischemia
LVDP: left ventricular developed pressure
MAPK: mitogen activated protein kinase

OGA: N-acetylglycosidase, O-GlcNAc-ase

O-GlcNAc: O-linked N-acetylglucosamine

OGT: O-glucoronyl transferase, O-GlcNAc transferase

PAI-1: plasminogen activator inhibitor-1

PCA: perchloric acid

PKC: protein kinase C

PLC: phospholipase-C

PP: protein phosphatase

PS: peak shortening

PUGNAc: O-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenyl carbamate

RAS: renin-angiotensin system

RISK: reperfusion injury salvage kinase

RPP: rate pressure product

SERCA: sarcoplasmic reticulum Ca^{2+}-ATPase

SL: sarcomer length

SR: sarcoplasmic reticulum

STZ: streptozotocin

TCA: tricarboxylic acid

TG: triglyceride

TGF: transforming growth factor

TPT: time to peak twitch

TR: time to relengthening

UDP-GalNAc: Uridine diphospho-N-acetyl-galactosamine

UDP-GlcNAc: Uridine diphospho-N-acetyl-glucosamine

UDP-HexNAc: Uridine diphospho-N-acetyl-hexosamine

ZDF: Zucker Diabetic Fatty

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