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

Ph.D theses
(Summary)

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Abbreviations:

ACC: acetyl-CoA carboxylase
AD: Alzheimer disease
AGE: advanced glycation endproduct
AMPK: AMP-activated protein kinase
DNP: diabetic nephropathy
EC: excitation-contraction coupling
EDP: end-diastolic pressure
ERK: extracellular-signal regulated kinase
FFA: free fatty acid
Fru-6-P: fructose-6-phosphate
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
HBP: hexosamine biosynthesis pathway
LFI: low flow ischemia
MAPK: mitogen activated protein kinase
OGA: N-acetylglycosidase, O-GlcNAc-ase
O-GlcNAc: O-linked N-acetylglucosamine
OGT: O-glucoronyl transferase, O-GlcNAc transferase
PKC: protein kinase C
TCA: tricarboxylic acid
TG: triglyceride
UDP-GlcNAc: Uridine diphospho-N-acetyl-glucosamine
ZDF: Zucker Diabetic Fatty

Introduction:

Diabetes mellitus:

Type-2 diabetes mellitus is an increasing problem both in the developed and in the developing countries. The incidence of diabetes is increasing, it has been estimated that the prevalence of diabetes in Hungary was over 10% (11,2%).

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; increased formation of advanced glycation end-products (AGE); increased protein kinase C (PKC) activation and increased flux through the hexosamine biosynthesis pathway (HBP) and/or increased levels of *O*-linked N-acetylglucosamine (*O*-GlcNAc) on proteins.

Hexosamine Biosynthesis Pathway:

A small proportion of the uptaken glucose (2-5%) into the cells goes through the hexosamine biosynthesis pathway producing uridine 5'-diphospho N-acetylglucosamine (UDP-GlcNAc) (Figure 1). The key regulatory enzyme is the glutamine: fructose 6-phosphate amidotransferase (GFAT). 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).

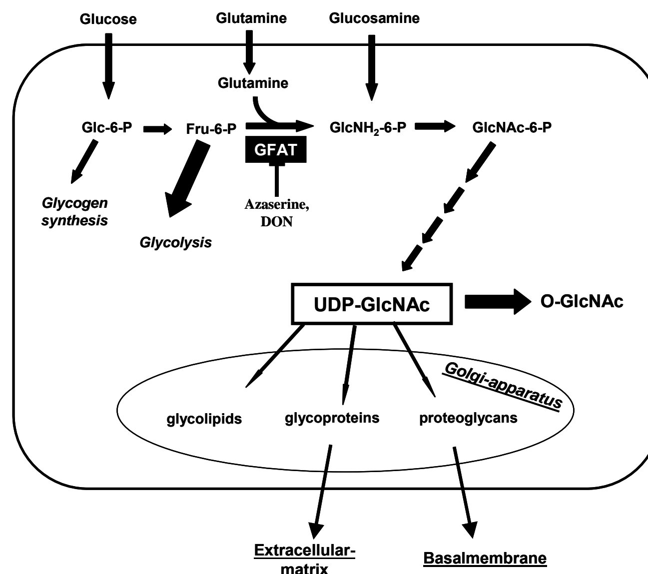

Figure 1.: *The Hexosamine Biosynthesis Pathway*

Nuclear and cytoplasmic O-glycosylation:

O-glycosylation is a post-translational modification of nuclear and cytoplasmic proteins and is increasingly recognized as an important regulatory mechanism involved in signal transduction. O-GlcNAc occurs in the cytosol and in the nucleus rather than in the Golgi or the endoplasmic reticulum, 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, and N-acetylglucosaminidase (O-GlcNAcase, OGA) - catalyzing the removal of the sugar moiety from the proteins.

Wide range of proteins have been identified to be O-glycosylated, including kinases, phosphatases, transcription factors, metabolic enzymes, chaperons, cytoskeletal proteins. O-glycosylation has been shown to modify DNA binding, enzyme activity, protein-protein interactions, half-life of the proteins and subcellular localization.

Several studies demonstrated that increasing the flux through the HBP leading to increased O-GlcNAc plays a crucial role in the development of insulin resistance and diabetes. It has been shown that increased levels of O-GlcNAc are also associated with diabetic complications.

Diabetic cardiomyopathy:

Diabetes leads to diabetic cardiomyopathy in part by affecting function at the cardiomyocyte level. In cell culture experiments and in animal models diabetes, high glucose or glucosamine treatment increased O-GlcNAc, and caused cardiomyocyte dysfunction by altering Ca^{2+} homeostasis.

Altered cardiac substrate utilization is another mechanism responsible for the development of diabetic cardiomyopathy. In diabetes carbohydrate utilization is decreased while palmitate oxidation is elevated in the heart. The effect of increased O-GlcNAc is a result – at least in part - of altered activity of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) - two of the key enzymes regulating substrate utiilization.

Diabetic nephropathy:

The accumulation of the extracellular matrix (ECM) proteins, like laminin, fibronectin or type-IV collagen and increased cellular hypertrophy of the renal cells are the major characteristics of the early diabetic nephropathy (DNP). It has been shown that increased HBP flux leads to increased expression of ECM proteins. In human tissue Nehrlich et al. demonstrated that GFAT is present in the kidney and in

diabetes the expression of GFAT is increased. However there is only very little known about O-GlcNAc in the kidney. In rat mesangial cells glucosamine, known to increase flux through the HBP, increased O-glycosylation and induced cell cycle arrest and cellular hypertrophy.

Cell survival:

It was shown that stress stimuli increased the level of *O*-GlcNAc. Inhibition of this response increased the sensitivity to stress whereas augmentation of the *O*-GlcNAc levels increased stress tolerance and improved cell survival.

O-glycosylation is also thought to be involved in the pathogenesis of cancers and changes in O-GlcNAc regulation may also play an important role in the development of neurodegenerative disorders, such as Alzheimer's disease (AD).

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.

Mitogen activated protein kinases (MAPK) and cardioprotection:

In the heart the response to ischemia is mediated, in part, by extracellular-signal regulated kinase (ERK) and p38 mitogen activated protein kinase (MAPK) and Akt. The role of p38 in mediating the response of the heart to ischemic injury is controversial. Prolonged activation of p38 is detrimental. In contrast, brief activation of p38 prior to ischemia has been shown to be associated with ischemic preconditioning/protection. ERK activation is important in mediating protection associated with insulin and ischemic preconditioning. Akt has also been reported to protect against ischemia/reperfusion injury in the heart.

Activation of HBP and increased *O*-GlcNAc levels have been reported to modify MAPK phosphorylation and activity. 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. Therefore we perfused isolated rat hearts and subjected them to low flow ischemia (LFI) (0, 5, 10, 30 minutes) or to 30 minutes LFI and 60 minutes reperfusion. Protein O-GlcNAc was assessed by Western blot analysis and tissue UDP-GlcNAc by HPLC analysis.

The onset of LFI resulted in a rapid decrease in contractile function and gradual increase in end diastolic pressure (EDP). 5-min of ischemia significantly increased UDP-GlcNAc concentrations, and longer periods of ischemia (10 and 30 minutes) increased levels further. After 60 minutes reperfusion UDP-GlcNAc levels returned to the normoxic levels.

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-GlcNAc 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.

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. We used the hearts from the previous experiments as controls and perfused another set of animals with 5 mmol/L GlcN under the same conditions. Western blot analysis was used to evaluate protein expression, O-GlcNAc and phosphorylation. ATP and UDP-GlcNAc levels were determined by HPLC.

Before ischemia there was no significant difference in contractile function between control and glucosamine groups. At the end of LFI, EDP was significantly attenuated in the glucosamine group. After 30 minutes of LFI and 60 minutes reperfusion functional recovery was significantly higher in the glucosamine group.

Glucosamine increased UDP-GlcNAc levels almost 2-fold. Similar to the control group there was a significant increase in UDP-GlcNAc levels in response to ischemia.

Ischemia did not change O-GlcNAc levels in the glucosamine treated group. Glucosamine treatment significantly increased *O*-GlcNAc levels by at least 60% at all time points compared to the controls.

As expected ATP levels decreased in both control and glucosamine treated groups during ischemia. 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.

Glucosamine treatment had no effect on ERK1/2 or Akt phosphorylation compared to the control under any conditions. 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. In contrast at the end of reperfusion, p38 phosphorylation was increased in the glucosamine group.

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-GlcNAc levels between the different tissues of young adult and senescent animals. We harvested heart, aorta, skeletal muscle and brain from 5 and 24 month old Brown Norway rats. Serum glucose, insulin, lipid and free fatty acid levels were measured. Real time RT-PCR was used to measure GFAT, OGT and OGA mRNA levels. HPLC measurements were made to evaluate UDP-GlcNAc and Western blot to compare protein O-GlcNAc and OGT expression.

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. There were no changes in cholesterol, HDL or FFA levels with age.

O-GlcNAc was increased in the heart, aorta, brain and in the skeletal muscle of the senescent animals. In the heart, brain and skeletal muscle the pattern of O-GlcNAcylated proteins also changed with age.

With age OGA mRNA levels were significantly increased in the heart, whereas OGT mRNA levels were unchanged. There were no changes in OGT or O-GlcNAcase mRNA in brain or skeletal muscle with age. We found that OGT protein levels were significantly decreased in heart, aorta and brain, and unchanged in skeletal muscle.

In the heart mRNA levels of GFAT1 and GFAT2, were markedly increased with age; however, the increase in GFAT1 was not significant. Consistent with an increase in GFAT mRNA levels and the increase in O-GlcNAc, UDP-GlcNAc levels were significantly higher in hearts from 24-month old rats. In skeletal muscle and in the brain there were no changes in GFAT1, GFAT2 or UDP-GlcNAc levels between 5 and 24-month groups.

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. We perfused hearts from animals of 6 weeks or 22 weeks of age. Serum glucose, insulin and leptin levels were measured. Western blots and HPLC analysis were performed to asses O-GlcNAc, OGT and UDP-GlcNAc levels.

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.

There was no significant difference in UDP-GlcNAc levels between 6-week old Lean and ZDF groups. However, at 22 weeks of age UDP-GlcNAc levels were significantly increased in the ZDF group compared to the age-matched Lean group. The UDP-GlcNAc levels were also higher in the ZDF 22-week group compared to the 6-week ZDF group.

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 comparing the ZDF and Lean control groups. There was no difference in OGT expression between ZDF and Lean groups at either age.

Comparing O-GlcNAc levels in the 6 and 22 week groups we found that *O*-GlcNAc was significantly lower in both 22-week Lean and ZDF groups compared to their respective 6-week groups, which was associated with a significant reduction in OGT expression in both Lean and ZDF 22-week groups.

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. In all groups the anti-O-GlcNAc antibody, CTD110.6, identified 11 separate bands. In the Lean control group all of the bands showed significant decreases with age. In contrast, in the ZDF group the majority of the bands were unchanged between 6 and 22 weeks; however the intensity of 3 bands were significantly lower in the 22 week group. Interestingly, the intensity of high molecular weight band 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. Thus, it appears to be consistently increased in response to diabetes independent of age differences or leptin levels.

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. Isolated adult cardiomyocytes from 6 week and 22 week old ZDF rats were used. Mechanical properties and intracellular Ca^{2+} transients of ventricular myocytes were assessed simultaneously using a video-based detection system coupled to a fluorescent system.

At 6 weeks of age, myocyte contraction and relengthening was not significantly different between the ZDF and Lean control groups. 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 were consistent with the mechanical indices; at 6 weeks of age, there were no differences. However, at 22 weeks, peak Ca^{2+} was attenuated and cytosolic Ca^{2+} clearing was impaired in the diabetic ZDF group compared to the normoglycemic lean group.

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 and slower cytosolic Ca^{2+} clearing in the 22-week ZDF group compared to the 6-week ZDF group. This was consistent with the differences between the 22-week Lean and ZDF groups.

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. Therefore isolated rat hearts were perfused with buffers containing ^{13}C-labeled substrates with 0; 0.05; 0.1; 1.0; 5.0 or 10.0 mM GlcN. ^{13}C-isotopomer analyses were performed to determine the relative contribution of substrates to total acetyl-CoA entering the tricarboxylic acid (TCA) cycle. Western blots and HPLC analysis were performed to asses O-GlcNAc, ACC/phospho-ACC, AMPK/phospho-AMPK and UDP-GlcNAc, ATP levels.

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 function. We found that 60 minutes perfusion with even the lowest glucosamine concentration (i.e., 0.05 mM) resulted in elevated UDP-GlcNAc levels, and saw a further increase with higher GlcN concentrations, with no changes in the ATP content of the hearts. The changes in UDP-GlcNAc levels seen with the different GlcN concentrations correlated well with the changes seen in O-GlcNAc.

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.

Examining the effect of low (0.05, 0.1 mM) and high (5, 10 mM) GlcN on cardiac energy metabolism we found that at all concentrations glucosamine

significantly increased palmitate oxidation and decreased carbohydrate utilization compared to the control group. The maximal reponse on cardiac energy metabolism was seen with 0.1 mM GlcN. 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. GlcN did not change the glucose oxidation, or glycolytic lactate efflux in our experiments.

ACC and AMPK phosphorylation did not change with 0.1 mM GlcN compared to the control hearts, while the fatty acid oxidation was enhanced in the treted hearts.

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

We examined whether O-glycosylation is present in human kidney cells. Immunhistology was performed on percuteneous renal biopsy specimen with an anti-O-GlcNAc (CTD110.6 1:100) antibody.

We showed 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 and in the nuclei of the tubular epithelial cells. Furthermore the cytosol of the tubular epithelial cells showed positive CTD110.6 staining.

To verify that the antibody binding was specific we used 20 mmol/L N-acetylglucosamine to pre-adsorb the primary antibody. We found that all our immune-labeling was specific since 20 mmol/L N-acetylglucosamine completely blocked the immunostaining with the antibody specific for O-GlcNAc.

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. 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, and performed immunhistology with CTD110.6 antibody.

We found no difference in the localization of O-GlcNAc staining in the glomerular cells between the diabetic and non-diabetic samples. We found no difference in the nuclear staining of the tubular epithelial cells either. 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.

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. 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. 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.

In the intact heart we found that after 5-10 min of ischemia UDP-GlcNAc levels were increased. Since flux through OGT is very sensitive to UDP-GlcNAc levels, the increase in UDP-GlcNAc could explain the increase in *O*-GlcNAc levels; however, we cannot rule out an increase in OGT activity. The increase in UDP-GlcNAc 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.

Interestingly even though UDP-GlcNAc continued to increase during ischemia, O-GlcNAc levels had decreased after 30 min ischemia. The dissociation between UDP-GlcNAc and O-GlcNAc, suggests that OGT activity might be inhibited after prolonged ischemia, which leads to the further decline in O-GlcNAc levels seen during reperfusion.

Enhancing O-GlcNAc lead to increased tolerance to stress in the isolated heart. Glucosamine increased normoxic levels of both UDP-GlcNAc and O-GlcNAc, prevented the decrease in O-GlcNAc seen at the end of reperfusion and improved functional recovery following ischemia reperfusion. Champattanachai has shown that that inhibition of nucleocytoplasmic O-glycosylation with alloxan resulted in loss of protection of glucosamine treatment, which supports the notion that the cardioprotective effect of glucosamine is due to the increase in nucleocytoplasmic O-glycosylation.

Activation of the Akt and MAPK pathway, particularly p38 and ERK1/2 MAPK, has been implicated in ischemic cardioprotection. Kneass and Marchase showed in cell culture system that agonist stimulation of the MAPK pathway was enhanced in

response to increased O-GlcNAc levels. We found that glucosamine significantly attenuated the ischemia-induced increase in p38 phosphorylation, this is consistent with reports that inhibition of p38 during sustained ischemia is protective. Surprisingly, at the end of reperfusion p38 phosphorylation was increased in the glucosamine treated group. Activation of p38 can be pro-apoptotic, mediated via Caspase-3 or p53; 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 to play a role in ischemic protection. We found that glucosamine had no effect on Akt or ERK1/2 phosphorylation under any perfusion 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. 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-GlcNAc concentration, consistent with increased HBP activity. 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.

We found a significant decrease in OGT expression in all tissues examined, except skeletal muscle. This is at odds with the increase in O-GlcNAc levels; however, other factors such as decreased O-GlcNAcase activity could contribute to an increase in O-GlcNAc. It should also 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. Unfortunately, due to the lack of a commercially available O-GlcNAcase antibody, we could not determine O-GlcNAcase protein levels in our experiments.

It has been shown, that O-GlcNAc is also playing an important role in the development of neurodegenerative disorders, such as Alzheimer’s disease. There

are O-GlcNAc modified proteins involved in the pathophysiological mechanisms underlying the development of Alzheimer's including tau, β-amyloid precursor, chlatrin assembly protein 3 and 180. Therefore we can conclude that altered O-glycosylation may contribute to the development of AD with age.

Another interesting observation was that the senescent animals were insulin resistant. Increased HBP flux and increased levels of O-GlcNAc are associated with insulin resistance and diabetes. Previous studies have demonstrated a link between increased *O*-GlcNAc levels and contractile dysfunction following a prolonged period of type-1 diabetes. We showed that at the age of 22 weeks - 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. 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. An increase in HBP flux seen with type-2 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.

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. We also found a decrease in overall O-GlcNAc and in OGT expression with age, comparing the 6 and 22-week Lean groups. 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.

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 the diabetic animals on proteins in the high molecular weight range (i.e., >205kD). 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 of high molecular weight proteins – such as titin, dystrophin, myosin, acetyl-CoA carboxylase 2 may play a role in the development of impaired cardiomyocyte function associated with diabetes.

It has been proposed that altered energy metabolism is associated with impaired function in the diabetic hearts. Wang et al. showed that in the heart of ZDF rats cardiac energy metabolism was altered compared to the non-diabetic lean littermates. In a recent study McClain`s group demonstrated that long term activation of the HBP increased O-GlcNAc and lead to increased palmitate oxidation in adipocytes.

GlcN increases the flux through the HBP, by bypassing GFAT - the rate-limiting enzyme of the pathway. 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 or cardiac function.

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 in type-2 diabetes the decrease in carbohydrate oxidation was mainly due to the decreased lactate oxidation, since glucose oxidation was unchanged. We have seen similar changes in carbohydrate oxidation; lactate and pyruvate oxidation decreased and glucose oxidation was unchanged.

AMPK plays a crucial role in maintaining cellular energy and metabolic homeostasis. 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. We also 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.

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 and causes cellular hypertrophy in mesangial cells and tubular epithelial cells. 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.

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.

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. Thus 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.

<u>Theses:</u>

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.

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