The significance of O-GlcNAcylation in diabetic nephropathy

PhD thesis

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Introduction

Diabetes mellitus (DM) and associated complications are a major public health concern, prevention and treatment are an enormous burden both to patients and society. According to WHO, currently 415 million people suffer from DM worlwide, moreover if the present growing tendency continues every tenth adult will be diabetic by 2040.

Among several complications of DM, diabetic nephropathy (DNP) develops in 30-40% of patients, and is the leading cause of end stage renal disease. While the number of patients increases worldwide as well as in Hungary, the exact patomechanism is not known and progression of the disease can lead to renal failure requiring renal replacement therapy. Currently among renin-angiotensin-aldosterone system (RAAS) blockers angiotensin converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB) are the first choice in treatment.

Beside the elevation of systemic and intraglomerular pressure and RAAS activation, direct glucotoxicity caused by persistent or not controlled hyperglycemia is also a major determining factor of the patomechanism of DNP.

It is known, that many alternative metabolic pathways are upregulated through excessive glucose levels leading to impairment mediated by glucotoxicity. Among these upregulation of the hexosamine pathway leads to increased O-linked ß-N-acetylglucosamin (O-GlcNAc) modification of proteins.

O-GlcNAcylation, which occurs in most proteins at serine and threonine residues and is similar to phosphorylation was discovered in the early 1980s. This dynamic and reversibe modification can compete with phosphorylation for the same site.

O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) mediate the addition and removal of O-GlcNAc, respectively.

Increasing evidence suggests that elevated O-GlcNAcylation is a prominent contributor to diabetic complications induced by glucotoxicity, however its relevance in DNP is poorly elucidated.

We hypothetize that O-GlcNAcylation plays a significant role in the pathomechanism and progression of DNP through the endothelial nitric oxide synthase (eNOS) system, Na-pump and heat shock signaling.

In DNP, the dysfunction of eNOS has been primarily emphasized in the renal vascular endothelium. eNOS is also present in proximal tubules and phosphorylation on Ser1177 catalyzed by phosphorylated Akt (pAkt) is essential for the activation of the enzyme. Preliminary experimental data suggests that the impairment of eNOS phosphorylation is in relationship with increased O-GlcNAcylation, which makes the enzyme dysfunctional.

In the kidney proximal tubules are especially sensitive to glucotoxycity, since they can not attenuate the extent of glucose transport appropriately in order to prevent the excessive intracellular glucose concentration. Sodium/potassium adenosin-triphosphatase (NKA) ensures the energetic background of glucose reabsorbtion in the proximal tubule.

We previously demonstrated in streptozotocin (STZ) induced DM rat model that renal NKA expression increases, but the enzyme is mislocated from the basal membrane to the cytoplasm thus becoming dysfunctional.

One of the main mediators of cellular stress-response, heat shock factor 72 (HSP72) is produced when cells are damaged. In DNP hyperglycemia and increased glomerular capillary pressure

induces HSP72, which is involved in the amelioration of tubular damage and the regeneration of kidney parenchyma through stabilizing the plasma membrane and repairing damaged or dysfunctional proteins. Previous studies supported that HSP72 stabilizes NKA in its physiological location, moreover repairs the damaged membrane protein. The complex role of O-GlcNAcyation in regulating cell function is demonstrated by the fact that O-GlcNAc modulation generated by cellular stress increases cell survival and HSP72 expression.

During my PhD-work we investigated the role of O-GlcNAcyation in the pathomechanism and therapy of DNP.

Aims

Our aims were to analyse the process and enzymes of O-GlcNAcylation in the pathomechanism of DNP, focusing on the Akt-eNOS pathway, Na-pump function, heat shock response and effect of RAAS-blockers as the first line clinical therapy. Our questions were as follows:

1. What is the extent of O-GlcNAcylation in an *in vivo* experimental model of DNP as well as *in vitro* in a proximal tubulular cell line after high glucose treatment?

2. How are OGT and OGA, the affecting enzymes of O-GlcNAcylation altered in these models?

3. How does hyperglycemia and high glucose treatment influence the amount of phosphorylated eNOS and Akt in the rat model of DNP and in proximal tubular cells?

4. What is the role of O-GlcNAcylation in altering the amount and function of NKA and HSP72 *in vivo* in DNP and *in vitro* in proximal tubular cells?

5. How do RAAS-blockers in monotherapy modulate the process and enzymes of O-GlcNAcylation in DNP?

Methods

In vitro model

In our *in vitro* experiments immortalised human kidney-2 (HK2) proximal tubular cells was cultured in normal (5 mM) and high glucose (HG, 35 mM) contained medium either for 24 (HG24) and 48 hours (HG48). HG cells were treated either with Enalapril (HG+Enalapril, 1μM), Losartan (HG+Losartan, 10μM) or Eplerenone (HG+Eplerenone 10μM). In both time points untreated and mannitol (35 mM) treated cells were used as controls.

Rat model of type 1 DM (T1DM) and experimental groups

All experiments were performed on mature male Wistar rats weighing 180-200 grams. T1DM was induced by a single intraperitoneal injection of STZ (65 mg/bwkg), dissolved in 0,1 M citrate buffer.

5 weeks after the induction of diabetes rats were randomized into 4 groups (n=8/group) and received the following for a period of two weeks:

1. Enalapril (40mg/bwkg/day)
2. Losartan (20mg/bwkg/day)
3. Selective aldosterone antagonist eplerenon (50mg/bwkg/day)
4. or isotonic saline as vehicle.

Age-matched non-diabetic animals served as controls.

Measurement of arterial blood pressure and pulse

Arterial blood pressure was measured using the tail-cuff method with a CODA Standard monitor system before and after RAAS treatment. Rats were anesthetized by inhalation of isoflurane.

Renal and metabolic parameters

Serum glucose, fructosamine, creatinine, blood urea nitrogen (BUN), cholesterol, HDL-C and trigliceride parameters were measured, creatinine was determined from collected urine, creatinine clearance was calculated.

Histology

Tubulointerstitial fibrosis was evaluated on Masson trichrome-stained kidney sections. Mesangial matrix expansion was measured on periodic acid-Schiff-stained sections. The localization of NKA and HSP72 were determined using fluorescent immunohistochemistry.

Measurement of protein levels

O-GlcNAcylation, OGT, OGA, peNOS, eNOS, pAkt, Akt, NKA and HSP72 proteins were measured with Western blot.

Statistical analysis

Results are presented as means ± SEM. Statistical analysis was performed using GraphPad Prism software (version 5.00). Multiple comparisons and possible interactions were evaluated by one-way ANOVA followed by Bonferroni's multiple comparison post hoc test. For non-parametrical data the Kruskal–Wallis ANOVA on ranks was used. P values <0,05 were considered significant.

Results and discussion

High glucose induces protein O-GlcNAcylation and isoform-specific expression of OGT and OGA in proximal tubular cells

In proximal tubular cells O-GlcNAcylation was elevated both after 24 and 48 hours of high glucose treatment, while did not show the same elevation in mannitol treated cells.

We are the first to show the two isoforms with various substrate-specificities and intracellular functions in proximal tubular cells: the full length nucleocytoplasmic (ncOGT, 110kDa), the mitochondrial (mOGT, 103 kDa) OGT, the long (OGA-L, 130 kDa) and short isoform of OGA (OGA-S, 75 kDa).

In HG24 cells ncOGT was increased, which is responsible for the synthesis, in HG48 cells was normalised. mOGT did not change in HG24 cells, but decreased in HG48 cells compared to controls.

OGA-L decreased below the level of controls in HG24 cells, while increased in HG48 suggesting a compensatory mechanism against increased O-GlcNAcylation. OGA-L is primarily responsible for the removal of the O-GlcNAc moiety due to its presence in the cytoplasm and higher enzyme activity. OGA-S was present in a much smaller amount without any difference between groups.

RAAS blockers only influenced the level of OGA-L in proximal tubular cells: the protein was increased after 24 hours of Enalapril and Losartan treatment.

eNOS phosphorylation is decreased by high glucose in proximal tubular cells

In high glucose treated proximal tubular cells eNOS was unchanged and peNOS was decreased suggesting eNOS phosphorylation was damaged in hyperglycemic conditions.

The phosphorylation of eNOS on Ser1177 is catalized by Akt, which is essential for the activation of the enzyme.

The level of phosphorylated Akt (pAkt) was increased in HG24 cells, while Akt remained unchanged. Conversely decreased peNOS levels suggest that increased pAkt is either not active or its function is inhibited. In HG48 the increase of Akt phosphorylation was diminished while total Akt was elevated.

High glucose increases NKA and HSP72 levels in proximal tubular cells

Protein levels of NKA in high glucose treated HK2 cells remained unchanged initally, but increased in HG48 cells

compared to controls. HSP72 protein levels rapidly increased in HG24 cells and returned to the level of controls by HG48.

In immunofluorescent stained sections HSP72 showed prominent perinuclear staining in control cells, however after high glucose treatment the protein was detected throughout the cytoplasm.

Development of diabetic nephropathy in T1DM rats

7 weeks after the development of STZ-induced T1DM rats had significant weight loss, serum creatinine and BUN levels were elevated, creatinine clearance was decreased, fructosamin and lipid parameters were elevated. After using the nonpressor doses of RAAS-inhibitors blood pressure remained unchanged in all groups. Histological examination also confirmed kidney damage in diabetic animals: in the Masson-stained sections the kidneys showed extensive tubulointerstitial fibrosis, while in PAS-stained sections mesangial matrix expansion was represented.

RAAS-blockers ameliorated renal functional parameters and reduced structural damage by attenuating tubulointerstitial fibrosis and mesangial matrix expansion, Losartan decreased serum cholesterol.

RAAS-blockers decrease protein O-GlcNAcylation in diabetic kidneys

We showed increased protein O-GlcNAcylation of T1DM rats compared to controls, which was decreased by all RAAS-blockers.

We detected the decrease of ncOGT and mOGT levels in diabetic kidneys, which was not influenced by RAAS-blockers. Similarly, OGA-L was downregulated in the diabetic kidney, while OGA-S, which is found in the nucleus and is associated with lipid-droplets was increased. Among RAAS-blockers, Losartan ameliorated the decrease of OGA-L, while OGA-S was decreased by all of the treatments.

RAAS-blockers rescue eNOS phosphorylation in the diabetic kidney

The levels of phosphorylated eNOS, as well as peNOS/eNOS ratios were decreased in diabetic rats. Treatment with RAAS inhibitors, especially with Losartan and Eplerenone increased peNOS levels. Elevated total eNOS levels were mitigated by RAAS-blockers. Akt was slightly elevated in diabetes, but neither pAkt nor pAkt/Akt ratio were altered by RAAS-blocker treatment.

HSP72 protein level is decreased in the diabetic kidney

Renal HSP72 protein was decreased in diabetes but showed an increasing tendency after RAAS-blocker treatment. Immunofluorescent staining revealed that HSP72 was localized in the tubules.

NKA protein level is increased and localisation is changed in the diabetic kidney

In T1DM NKA was increased and mislocated from the basal membrane to the cytoplasm, thus becoming dysfunctional. The

protein levels were decreased by RAAS-treatments except Enalapril.

Thesis

1. We confirmed that O-GlcNAcylation was increased after high glucose treatment in proximal tubular cells and in DNP.

2. We were the first to detect the different isoforms and time-dependent changes of OGT and OGA. After the impact of excess glucose in proximal tubular cells, ncOGT and then with compensatorial effects OGA-L were increased, which affected O-GlcNAcylation in the cells. During continued hyperglycemia the enzyme levels were decreased, which can be a damage-response to accumulated O-GlcNAcylation.

3. Phosphorylated eNOS was decreased, while eNOS remained unchanged in proximal tubular cells after high glucose treatment and in diabetic kidneys. These data suggest that eNOS phosphorylation was inhibited, which can be a result of increased O-GlcNAcylation.

4. After high glucose treatment NKA and HSP72 protein levels were increased and HSP72 appeared in the whole cytoplasm instead of its perinuclear localisation. In DNP the function of NKA was damaged and it translocated to the cytoplasm. HSP72 co-localized with NKA, but its expression decreased, which lead to vulnerability against glucotoxycity.

5. In DNP increased O-GlcNAcylation was attenuated by RAAS-inhibitors, presumably through enhancing OGA-L. RAAS-blockers increased peNOS and HSP72, which could contribute to their renoprotective effect in DNP.

Publication list

Publications related to the theme of the Ph.D. thesis

Gellai R, Hodrea J, Lenart L, Hosszu A, Köszegi S, Balogh D, Ver A, Banki NF, Fülöp N, Molnar A, Wagner L, Vannay A, Szabo J A, Fekete A: The role of O-linked N-acetylglucosamine modification in diabetic nephropathy. American Journal of Physiology: Renal physiology (2016) IF: 3,390

NF Bánki, S Kőszegi, L Wagner, L Lénárt, D Varga, R Gellai, J Hodrea, Á Vér, AJ Szabó, T Tulassay, A Fekete: Új terápiás támpontok a diabéteszes nefropátia kezelésében: a renin-angiotenzin-aldoszteron rendszer és a Na/K ATPáz szerepe. Gyermekgyógyászat (2013) 64: 70-74 2

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Other publications

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