THE ROLE OF O-LINKED N-ACETYLGLOUCOSAMINE MODIFICATION IN DIABETIC NEPHROPATHY

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Abstract

Increased O-linked β-N-acetylglucosamine glycosylation (O-GlcNAcylation) is a known contributor to diabetes; however its relevance in diabetic nephropathy is poorly elucidated. Here we studied the process and enzymes of O-GlcNAcylation with a special emphasis on Akt-endothelial nitric oxide synthase (eNOS) and heat shock protein 72 (HSP72) signaling. Since tubular injury is the prominent site of diabetic nephropathy the effect of hyperglycemia was first measured in proximal tubular (HK-2) cells cultured in high glucose. The in vivo O-GlcNAcylation and protein levels of O-GlcNAc transferase (OGT), O-GlcNAcase (OGA), pAkt/Akt, peNOS/eNOS and HSP72 were assessed in the kidney cortex of streptozotocin-induced diabetic rats. The effects of various renin-angiotensin-aldosterone system (RAAS)-inhibitors were also evaluated.

In proximal tubular cells hyperglycemia-induced OGT expression led to increased O-GlcNAcylation which was followed by a compensatory increase of OGA. In parallel peNOS and pAkt levels decreased, while HSP72 increased. In diabetic rats elevated O-GlcNAcylation was accompanied by decreased OGT and OGA. RAAS-inhibitors ameliorated diabetes-induced kidney damage; prevented the elevation of O-GlcNAcylation and the decrement of pAkt, peNOS and HSP72.

In conclusion hyperglycemia-induced elevation of O-GlcNAcylation contributes to the progression of DN via inhibition of Akt/eNOS phosphorylation and HSP72 induction. RAAS-blockers successfully inhibit this process suggesting a novel pathomechanism of their renoprotective action in the treatment of DN.

Keywords: diabetic nephropathy, O-GlcNAcylation, RAAS, eNOS, HSP72
Main text

Introduction

The increasing prevalence of diabetes mellitus is a major public health burden causing significant morbidity and mortality worldwide. Twenty percent of diabetic patients develop nephropathy (54); which is the leading cause of end stage renal failure in the adult population (10). However the molecular pathways critically involved in the development of diabetic nephropathy are still not fully understood.

Hexosamine biosynthesis pathway activity is increased in diabetes resulting in protein O-linked N-Acetylglucosamine modification (O-GlcNAcylation), which is a central contributor of glucose toxicity (8, 12, 20, 37). During the process of O-GlcNAcylation O-GlcNAc transferase (OGT) adds a single O-GlcNAc moiety to serine and/or threonine residues of various proteins (24, 25); while O-GlcNAcase (OGA) removes the modification (16, 21, 55). The two enzymes keep the rate of glycosylation and deglycosylation in a dynamic equilibrium and fine tune multiple cellular and metabolic processes in a glucose-dependent manner (37).

O-GlcNAcylation is one of the most common posttranslational modifications. In some proteins O-GlcNAcylation and phosphorylation competitively modify the same serine/threonine residues and thus have a reciprocal effect to each other (36, 58). This cross talk with phosphorylation is especially important in endothelial nitric oxide synthase (eNOS) activity. In endothelial cells hyperglycemia increased eNOS O-GlycNAcylation with an inverse relation to eNOS phosphorylation (19). The same has been observed in aortas of diabetic animals where O-GlcNAc modification of eNOS was increased parallel to decreased phosphorylation (7, 17).

The role of O-GlcNAc modification in cellular processes is very complex. While chronic accumulation of O-GlcNAc is associated with several diseases, in response to acute cellular
stress O-GlcNAc elevation contributes to cell survival (57). One of the key mediators of stress
response is heat shock protein 72 (HSP72) (23). HSP72 expression was increased by stress-
induced O-GlcNAcylation (30) and nitric oxide (NO) synthesis (42).

Although glomeruli have been accepted as the primary site of diabetic kidney injury for
long; it is becoming evident that hyperglycemia-induced tubulointerstitial lesions are also
prominent components of nephropathy (2, 51). Therefore in this study the process of O-
GlcNAcylation and the Akt-endothelial nitric oxide synthase (eNOS) and heat shock protein
72 (HSP72) signaling were investigated both in proximal tubular cells and in diabetic rats.
The effect of renin-angiotensin-aldosterone system (RAAS)-inhibitors as the first line clinical
treatment of diabetic nephropathy was also evaluated.
Materials and Methods

Animals

6-week old male Wistar rats (weighing 180–200 g) were purchased from Toxi-Coop Toxicological Research Center (Dunakeszi, Hungary). The animals were kept under standard laboratory conditions with free access to standard chow and drinking water.

All experimental protocols were approved by the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University of Budapest, Hungary (TUKEB 380/2013).

In vivo model

All reagents were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary) unless stated otherwise.

Diabetes was induced in male Wistar rats by a single intraperitoneal injection of streptozotocin (STZ) (65 mg x bwkg\(^{-1}\)) dissolved in 0.1 M citrate buffer (pH 4.5). Blood glucose level was measured from tail vein after an overnight fast. Animals with ≥15 mmol/L blood glucose concentrations were considered to be diabetic.

Five weeks after the induction of diabetes rats were randomized into 4 groups (n=8/group). For a period of two weeks rats received once daily either vehicle (D), or Enalapril (D+Enalapril, 40 mg/bwkg/day), or Losartan (D+Losartan, 20 mg/bwkg/day), or selective aldosterone antagonist Eplerenone (D+Eplerenone 50 mg/bwkg/day) by oral gavage. The effective dose of each RAAS inhibitor was adopted from previous studies to avoid blood pressure changes while remaining effective in blocking the components of RAAS (13, 18, 50). Age-matched non-diabetic animals (n= 8/group) injected with citrate buffer, and gavaged with vehicle only served as controls.
At the end of the protocol anaesthetized rats were sacrificed. Blood and urinary samples were collected, kidneys were removed and weighted, and the cortical parts of the kidneys were separated with the naked eye and were immediately snap-frozen for further investigation.

In vitro model

Human proximal tubular cell line (HK-2) was cultured in DMEM containing 35 mM glucose (Gibco, Life Technologies Corporation) and supplemented with 10% FBS (Gibco, Life Technologies Corporation), 1% L-glutamine and 1% antibiotic-antimycotic solution (Sigma-Aldrich).

The cells were incubated in humidified 95% air/5% CO₂ at 37°C. Cells were plated (5x10⁵ cells/well) in 6-well plates (Sarstedt) for 24 hours in serum-free medium. Cells were kept under normal (C; 5.5 mM) or high glucose (HG; 35 mM) condition either for 24 (HG24) or 48 hours (HG48). HG cells were treated either with Enalapril (HG+Enalapril, 1µM), Losartan (HG+Losartan, 10µM) or Eplerenone (HG+Eplerenone 10µM) (n=12 well/group, 500 000 cells/well). All substances were purchased from Sigma-Aldrich. Cells were detached with 0.25% trypsin-EDTA (Gibco, Life Technologies Corporation) then lysed with the same buffer used for the kidney homogenates. In both time points untreated and mannitol (35 mM) treated cells were used as controls.

To determine cell viability cells were incubated with Thiazolyl-blue (MTT) for 4 h followed by lysis in DMSO/ethanol (1:1). The formation of water-insoluble formazan was determined by measuring optical density at 570 nm in Plate Chameleon V Fluorometer-Luminometer-Photom reader (Hidex, Turku, Finland).

Arterial blood pressure
Arterial blood pressure (systolic, diastolic, mean (MAP)) was measured on tail vein using CODA Standard monitor system (EMKA Technologies, Paris, France) which uses proprietary volume pressure recording, a clinically validated technology. While less accurate than telemetry this method was sufficient to confirm non-pressor drug dosages in our study (34).

**Metabolic and renal parameters**

Body and kidney weight were measured, kidney/body weight ratio and GFR were calculated. Serum metabolic (glucose, fructosamine, cholesterol, HDL-C, triglyceride) and renal functional (blood urea nitrogen (BUN), creatinine) parameters were determined with commercially available kits on Hitachi 912 chemistry analyzer (Roche Hitachi, Basel, Switzerland).

**Renal histology**

Paraformaldehyde (10%-fixed), paraffin-embedded kidney sections were stained with Masson’s trichome. To determine tubulointestial fibrosis 10 areas of 200 x magnification, without glomeruli were randomly selected from each kidney cross-section with Panoramic Viewer (3DHISTECH Ltd.) and the stained interstitial area was measured. The number of pixels containing blue fibrotic tissue was divided by the total number of pixels in the area to obtain the percentage of tubulointerstitial fibrosis (14). Analysis was performed by a nephropathologist in a double blinded fashion with computer-assisted morphometry using Adobe Photoshop CS6 (Adobe Systems Corporation, USA) and Scion Image 1.49v (National Institutes of Health, USA) softwares.

**Western blot**
Kidney cortex samples were homogenized using lysis buffer containing 100 mM Tris (pH 7.4), 10 mM EGTA, protease and phosphatase inhibitors. For the measurement of O-GlcNAcylation the lysates also contained 40µM O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenyl-carbamate (PUGNAC, Sigma-Aldrich). PUGNAC is an effective and non-toxic inhibitor of OGA and hexosamine, and selectively alters the amount of O-GlcNAc without effecting OGT. Supernatants were centrifuged (10 min, 13000 rpm, 4°C) and protein concentrations were determined with a detergent-compatible protein assay kit (Bio-Rad, Budapest, Hungary). Appropriate amounts of proteins were loaded onto polyacrylamide precast gels and transferred to nitrocellulose membranes (Bio-Rad, Budapest, Hungary). The membranes were probed with specific antibodies: anti-O-GlcNAc (1:2000), anti-OGT (1:1000, Sigma-Aldrich), anti-OGA (MGEA-5, 1:1000, Proteintech), Akt (1:1000, Cell Signaling Technology), pAkt (1:1000, Cell Signaling Technology), eNOS (1:1000, Abcam), peNOS (1:1000, Cell Signaling Technology) and HSP72 (1:9000, Novus Biologicals). After repeated washing blots were incubated with the corresponding secondary antibodies: goat anti-mouse (1:2000, Santa Cruz Biotechnology), goat anti-rabbit (1:2000, 1:3000, 1:5000 and 1:6000 respectively, Cell Signaling Technology). Bands of interest were detected using enhanced chemiluminescence detection (ECL; GE Healthcare Life Sciences) and quantified by densitometry (Versadoc, Quantity One Analysis software; Bio-Rad) as integrated optical density (IOD) after subtraction of background. IOD was factored for Ponceau S red staining to correct for any variations in total protein loading and for internal control. Protein abundance was represented compared to controls.

**Fluorescent immunohistochemistry**

Frozen kidney sections were embedded in Shandon cryomatrix (Life Science Ltd.,) and cut to 10 µm slides with a cryostat. Samples were incubated for one hour with the specific
rabbit HSP72 (1:500) primary antibody. After repeated washing slides were incubated with the specific secondary anti-rabbit Alexa Fluor 568 (Invitrogen) conjugate and counterstained with Hoechst 33342 (Life Technologies) to visualize nuclei. Appropriate controls were performed omitting the primary antibody to assure specificity and to avoid autofluorescence. After drying sections were fixed with Vectashield Mounting Medium (Vector Labs). Coverslipped slides were analyzed with a Zeiss LSM 510 Meta confocal laser scanning microscope (Zeiss, Germany) with objectives of 200x and 630x magnification.

HK2 cells were cultured in tissue culture chambers (Sarstedt). After repeated washing the cells were fixed in 4% paraformaldehyde, washed again and permeabilized with Triton X-100 (Sigma Aldrich). Cells were incubated with the specific rabbit HSP72 (1:200) antibody. After repeated washing chambers were incubated with anti-rabbit Alexa Fluor 488 (Invitrogen) conjugate and counterstained with Hoechst 33342 (Life Technologies) to visualize nuclei. Appropriate controls were performed omitting the primary antibody to assure specificity and to avoid autofluorescence.

**Statistical analysis**

Results are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism software (version 5.00; GraphPad Software, Inc., USA). 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

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

First the time-dependent effects of hyperglycemia-induced O-GlcNAcylation were investigated in proximal tubular cells. Both after 24 and 48 hours of high glucose treatment (HG24 and HG48) O-GlcNAcylation was elevated including the ~140 kDa molecular weight proteins representing eNOS. GlcNAcylation was not increased in untreated or mannitol treated cells (Figure 1. A-B) suggesting that the elevation is a direct consequence of hyperglycaemia, but not hyperosmolarity caused by increased glucose concentration.

OGT and OGA have different isoforms with various substrate-specificities and intracellular functions, however their renal distribution is yet unknown. We are the first to show the two isoforms in proximal tubular cells: the full length nucleocytoplasmic (ncOGT, 110kDa) and the mitochondrial (mOGT, 103 kDa) OGT. Here we demonstrated a time-dependent and isoform-specific alteration of OGT: in HG24 cells ncOGT was increased, while mOGT did not change. In HG48 cells both OGT isoforms decreased compared to HG24. The protein level of mOGT was even lower in HG48 than in controls (Figure 1. C-D).

Similarly the two distinct OGA isoforms also changed differently in time. The long isoform (OGA-L) which is found predominantly in the cytoplasm (11, 31) 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 (35). The short isoform (OGA-S) was present in a much smaller amount without any difference between the groups (Figure 1. E-F).
RAAS-blockers did not influence hyperglycemia-induced protein O-GlcNAcylation or OGT expression at any of the time points in proximal tubular cells. OGA-S was not affected either, while OGA-L was increased after 24-hours of Enalapril or Losartan treatment (supplemental figure).

The phosphorylation of eNOS and Akt is altered by high glucose in proximal tubular cells

The phosphorylation of eNOS on Ser1177 is essential for the activation of the enzyme (15). Although eNOS levels were unchanged in all groups, peNOS was decreased suggesting that high glucose impaired eNOS phosphorylation (Figure 2. A-C).

The level of pAkt, which phosphorylates eNOS on Ser1177 (15) was increased in HG24 cells, while total Akt levels remained unchanged. In HG48 the increase of Akt phosphorylation was diminished while total Akt was elevated (Figure 2. D-F).

High glucose increases HSP72 level and induces its translocation within proximal tubular cells

While HSP72 showed prominent perinuclear staining in control cells, the protein was detected throughout the whole cytoplasm after high glucose treatment (Figure 3. A-C). HSP72 protein level rapidly increased in HG24 cells, and returned to the control level by HG48. Mannitol-induced hyperosmolality had no effect on HSP72 (Figure 3. D).

Development of diabetic nephropathy in diabetic rats

Blood pressure, metabolic and renal parameters are summarized in Table 1. After 7 weeks of diabetes rats had significant weight loss, higher blood glucose, fructosamine and lipid levels reflecting to metabolic changes associated with the development of diabetes.
RAAS inhibitors did not influence the metabolic parameters with the exception of Losartan which reduced the level of cholesterol.

In diabetic rats serum creatinine and BUN increased, while GFR decreased. Here we confirmed our previous results (5) showing that these RAAS-blockers, even the aldosterone antagonist Eplerenone are renoprotective in monotherapy. The concentrations used here were enough to exert their renoprotective effects without altering blood pressure.

Parallel with functional impairment, histological examination also confirmed the development of diabetic nephropathy (Figure 4). Kidneys of untreated diabetic rats showed extensive tubulointerstitial fibrosis with tubular dilatation, epithelial atrophy and interstitial expansion, while all RAAS-blockers reduced renal structural damage by attenuating tubulointerstitial fibrosis.

The effect of RAAS-blockers on protein O-GlcNAcylation and O-GlcNAcylation enzymes in the diabetic kidney cortex

Increased protein O-GlcNAcylation was observed in kidney cortex homogenates of diabetic rats. All RAAS-blockers decreased protein O-GlcNAcylation (Fig. 5. A-B). We are the first to detect different OGT and OGA isoforms in the kidney. Renal ncOGT and mOGT were decreased in diabetic rats compared to controls, while RAAS-inhibitors had no effect on OGT levels (Figure 5. C-D).

OGA-L was downregulated in the diabetic kidneys, while O-GlcNAcylation was increased. In parallel OGA-S - which is found in the nucleus and is associated with lipid-droplets (11, 31) - was increased. Only Losartan ameliorated the reduction of OGA-L, while OGA-S was decreased by all RAAS-blockers (Figure 5. E-F).

RAAS-blockers rescued decreased eNOS phosphorylation in the diabetic kidney cortex
The level of phosphorylated eNOS, as well as peNOS/eNOS ratio 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 (Figure 6. A-C). However Akt was slightly elevated in diabetes, but neither pAkt nor pAkt/Akt ratio were altered by RAAS-blocker treatment (Figure 6. D-F).

**HSP72 protein level is decreased in the diabetic kidney cortex**

HSP72 was localized in the tubules as seen in the representative confocal images. Renal HSP72 protein level was lower in diabetes, RAAS-blockers tended to increase HSP72 (Figure 7.).
Discussion

OglcNAcylation is one of the most common, but least studied posttranslational modifications. While its relevance has been emphasized in the diabetic heart, OglcNAc modification has not been studied in the diabetic kidney yet. Here we showed that hyperglycemia induces O-GlcNAcylation, which contributes to the progression of diabetic nephropathy via the inhibition of Akt/eNOS phosphorylation and HSP72 induction. We also demonstrated that RAAS-blockers could ameliorate the detrimental effects of chronic accumulation of O-GlcNAc modified proteins.

However OGT and OGA, the isoforms of the O-GlcNAc cycling enzymes are different in subcellular localization and substrate specificity (11, 26); only a few studies discuss the effects of the two isoforms separately. Here we found that after hyperglycaemia ncOGT - which is responsible for O-GlcNAcylation in the nucleus and cytoplasm (39) - transiently increased, but on the long-run it was down-regulated. Similarly to ncOGT, the mitochondria-localized mOGT which also has catalytic activity (4) decreased as well, both in tubular cells and in the diabetic kidney cortex. These data suggest that in the case of chronic hyperglycaemia renal OGT decreases in a compensatory manner in order to protect tubular cells against over-O-GlcNAcylation. The same phenomenon was demonstrated in the red blood cells of diabetic human subjects (47).

In contrast to OGT, OGA appears to be less sensitive to nutritive changes and more susceptible to cellular O-GlcNAc levels. Here we found that after a transient decline, OGA-L quickly increases in proximal tubules, which could be an adaptive response to increased O-GlcNAcylation. This is in line with previous studies showing that OGA level is higher in the erythrocytes of pre-diabetic individuals suggesting that cells are trying to accommodate to hyperglycaemia by elevating OGA already at an early state of diabetes (47). On the other hand since OGA-L was here decreased in diabetic kidneys, it is also conceivable that instead
of a regulator, OGA is rather an O-GlcNAc-independent indicator of disease progression. Similar observations were made in pancreatic cancer (41).

OGA-S has lower enzymatic activity than OGA-L thus it makes less contribution to protein O-GlcNAcylation (35). In line with previous findings showing lower levels of OGA-S in HeLa cells, here we found that OGA-S is present in much smaller amount than OGA-L in the proximal tubules as well. On the contrary OGA-S was elevated in the suggesting that proximal epithelia are not the main source of renal OGA-S. Since lipid accumulation enhances OGA-S overexpression (33) we presume that increased OGA-S can also be a consequence of diabetes-induced enhanced lipid deposits. Higher cholesterol and triglycerid levels of diabetic rats correlated well with increased intracellular lipid deposition.

Activated RAAS and hexosamine biosynthesis pathways both play a significant role in the development of nephropathy. Angiotensin II has already been shown to increase O-GlcNAcylation in mesangial cells via activating the rate-limiting enzyme of hexosamine biosynthesis (29). This is also true the other way around; elevated O-GlcNAc modification stimulates ANG expression (28). We demonstrated that RAAS inhibitors successfully ameliorated diabetes-induced O-GlcNAcylation in the kidney primarily by increasing OGA. All these data suggest a bilateral activation of O-GlcNAcylation and intrarenal RAAS that generates a vicious circle contributing to a direct glucotoxic effect that can be inhibited by RAAS-blockers.

Increasing evidence suggests that in contrast to chronic effects, acute elevation of O-GlcNAcylation stimulates pro-survival signaling pathways (9). Increased O-GlcNAc levels promote the translocation of heat shock factor and the expression of HSP72 – which is a prominent cytoprotective chaperon in the kidney (57). Here we found that HSP72 was promptly increased after high glucose treatment in proximal tubules, but decreased after chronic hyperglycaemia (in the diabetic kidneys). This suggest that heat shock response is
impaired in chronic hyperglycaemia resulting in diminished HSP synthesis contributing to the progression of diabetic nephropathy (1, 45, 46).

It became evident in the past decades that the development and/or progression of diabetic nephropathy is associated with alterations in eNOS, however its importance has been primarily emphasized in the renal vascular endothelium. It is also known that a bidirectional link exists between HSPs and nitric oxide (NO); NO has direct effects on heat-shock factor-mediated HSP expression (56), while HSP70 increases eNOS (53). We are the first to demonstrate that hyperglycemia inhibits eNOS phosphorylation in proximal tubular cells. In parallel lower eNOS phosphorylation was detected in diabetic kidney cortex as well. Since it is well known that O-GlcNAcylation interacts with protein phosphorylation (27), we presume that hyperglycemia-induced elevation of O-GlcNAc plays a role in decreased phosphorylation of eNOS in diabetes.

pAkt is a key mediator of eNOS activation via phosphorylating eNOS on Ser1177 (15).

It is well established that Akt is also O-GlcNAcylated, which decreases Akt phosphorylation (22, 40). Therefore we hypothesize that lower Akt phosphorylation in diabetic rats could be a result of increased O-GlcNAcylation. Furthermore it is also conceivable that the O-GlcNAc modification of Akt inhibits phosphorylation thus the activation of eNOS. Therefore our data might in a way explain the findings of various other studies showing that Akt/eNOS phosphorylation is depressed in the diabetic kidney (32, 52).

In summary here we highlighted the significance of increased protein O-GlcNAcylation in the pathomechanism of diabetic nephropathy. We showed a time-dependent and isoform-specific expression pattern of O-GlcNAcylation enzymes OGT and OGA under hyperglycemia. As a possible consequence of increased O-GlcNAcylation we also demonstrated the impairment of Akt/eNOS signaling and HSP72 expression in diabetes.
Finally we concluded that RAAS-blockers successfully ameliorate protein O-GlcNAcylation in diabetes suggesting a new therapeutic potential of these agents in the treatment of DN.
Acknowledgements

The authors are very grateful to Maria Bernath for her excellent technical assistance.

Grants

This study was founded by grants of OTKA PD105361-K112629-K108688-K116928-NN114607. It was also supported by MTA-SE Lendület Research Grant (LP-008/2016).

Disclosures

All authors declare no conflict of interest.
References


**Figure captions**

**Figure 1. Protein O-GlcNAcylation and the levels of OGT and OGA isoforms in proximal tubular cells after high glucose treatment**

HK2 proximal tubular cells were treated with high glucose (35 mM) for 24 (HG 24) or for 48 hours (HG 48).

A: Representative immunoblots of protein O-GlcNAcylation. Samples might be from different gels but were derived at the same time and processed in parallel.

B: Densitometric quantification of the blots are shown after normalisation to control.

Representative immunoblots of nucleocytoplasmic (C: ncOGT, 110 kDa), mitochondrial OGT (D: mOGT, 103 kDa), the long (E: L-OGA, 130 kDa) and the short (F: OGA-S, 75 kDa) isoform of OGA.

The graphs represent the relative protein levels of C: ncOGT, D: mOGT, E: OGA-L; F: OGA-S in control, HG24 and HG48 cells.

Data are presented as mean ± SEM; n = 12 well/group, 500000 cell/well. **p< 0.01; ***p<0.001 vs. Control, respectively; ###p<0.001 vs. Mannitol; $p<0.05, $$p<0.01, $$$p<0.001 vs. HG24, respectively. NS: non significant.

**Figure 2. Phosphorylated and total eNOS and Akt levels in proximal tubular cells after high glucose treatment**

Representative immunoblots of phosphorylated eNOS (Ser1177) (A: peNOS; 140 kDa), eNOS (B: 140 kDa), phosphorylated Akt (Ser473) (D: pAkt; 60 kDa) and Akt (E: 60 kDa).

The graphs represent the relative protein levels of A: peNOS, B: eNOS, C: peNOS/eNOS ratio, D: pAkt, E: Akt and F: pAkt/Akt ratio in control, HG24 and HG48 cells.

Data are presented as mean ± SEM; n = 12 well/group, 500 000 cell/well. *p< 0.05; **p< 0.01 vs. Control, respectively; $p<0.05 vs. HG24. NS: non significant.

**Figure 3. Confocal images and HSP72 protein levels in proximal tubular cells after high glucose treatment**

HK2 proximal tubular cells were treated with high glucose (35 mM) for 24 (HG24) or for 48 hours (HG48). Mannitol (35mM) treated cells served as controls.

Representative images of immunofluorescent staining in HK2 cells for heat shock protein 72 (HSP72, green) in control (A) and HG24 (B) cells (630x magnification, scale bar 50 μm).

Nuclei are stained blue with Hoechst 33342. C: The graphs represent the corrected total cell fluorescence (CTCF) of control and HG24 cells.
D: Top panel: representative immunoblots of HSP72 levels. The graphs represent the relative protein level of HSP72 in control, mannitol treated, HG24 and HG48 cells. Samples might be from different gels but were derived at the same time and processed in parallel.

Data are presented as mean ± SEM **p<0.01 vs. Control; ##p<0.01 vs. Mannitol; $$p<0.01$$ vs. HG24.

**Figure 4. Histopathology of kidney sections of control, diabetic and treated diabetic rats**

Representative Masson trichrome stained kidney sections (200x magnification; scale bar -100 μm) of non-diabetic control (A), untreated diabetic (B), Enalapril (C), Losartan (D), Eplerenone (E) treated diabetic rats (n = 8/group). F: Tubulointerstitial fibrosis (Vv) was estimated by the ratio of Masson stained interstitial area/tubulointerstitial area. Fibrotic tubulointerstitial tissue was defined by evaluation of Masson trichrome-positive and glomerulus-free areas in the tubulointerstitium. Diabetes-induced tubulointerstitial fibrosis was ameliorated by all RAAS inhibitors. Data are presented as mean ± SEM ***p<0.001 vs. Control; $$$p<0.001$$ vs. Diabetes.

**Figure 5. Protein O-GlcNAcylation and protein levels of OGT and OGA isoforms in the kidney of control, diabetic and treated diabetic rats**

A: Representative immunoblot of protein O-GlcNAcylation. Samples might be from different gels but were derived at the same time and processed in parallel.

B: O-GlcNAcylated protein levels in kidney homogenates of control, diabetic and RAAS-treated diabetic rats after normalization to control.

Representative immunoblots of C: ncOGT, D: mOGT, E: OGA-L and F: OGA-S. The graphs represent the levels of ncOGT (C), mOGT (D), OGA-L (E) and OGA-S (F) in kidney homogenates of control, diabetic and RAAS-treated diabetic rats.

Data are presented as mean ± SEM; n = 8/group. **p<0.01; ***p<0.001$$ vs. Control respectively, $p<0.05; $$$p<0.001$$ vs. Diabetes respectively. NS: non significant.

**Figure 6. Protein levels of phosphorylated eNOS and Akt in the kidney of control, diabetic and treated diabetic rats**

Representative immunoblots of phosphorylated eNOS (A), eNOS (B), phosphorylated Akt (D) and Akt (E) levels. Samples might be from different gels but were derived at the same time and processed in parallel.
The graphs represent the relative protein levels of peNOS (A), eNOS (B), peNOS/eNOS ratio (C), pAkt (D), Akt (E) and pAkt/Akt ratio (F) in kidney homogenates of control, diabetic and treated diabetic rats.

Data are presented as mean ± SEM; n = 8/group *p < 0.05; ***p < 0.001 vs. Control respectively, $p<0.05; $$p<0.01; $$$p<0.001 vs. Diabetes, respectively. NS: non significant.

**Figure 7. Confocal images and HSP72 protein levels of control, diabetic and treated diabetic rats**

Representative images of immunofluorescent staining of kidney sections for heat shock protein 72 (HSP72, red) in non-diabetic control (A), untreated diabetic (B) and Enalapril (C), Losartan (D), Eplerenone (E) treated diabetic rats (630 x magnification). Nuclei are stained blue with Hoechst 33342. F: Top panel: representative immunoblots of HSP72 levels detected by Western blot. The graphs represent the relative protein level of HSP72 in kidney homogenates of control, diabetic and treated diabetic rats.

Data are presented as mean ± SEM **p<0.01 vs. Control. NS: non significant.
Table 1

Table 1. Blood pressure, metabolic and renal parameters of control, diabetic and RAAS-treated diabetic rats

<table>
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<th>Parameters</th>
<th>Control</th>
<th>Diabetic (D)</th>
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<th>D+Losartan</th>
<th>D+Eplerenone</th>
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<td>Mean arterial blood pressure (Hgmm)</td>
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<td>291 ± 13.3***</td>
<td>263 ± 12.6***</td>
<td>284 ± 12.5***</td>
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<td>∆ weight gain</td>
<td>223±11.5</td>
<td>66.4±11.0***</td>
<td>90.0±15.7***</td>
<td>71.4±11.0***</td>
<td>95.5±12.1***</td>
</tr>
<tr>
<td>Serum glucose (mmol/L)</td>
<td>12.7 ± 0.59</td>
<td>47.3 ± 3.98***</td>
<td>35.3 ± 3.23*</td>
<td>43.8 ± 2.45***</td>
<td>34.8 ± 1.82</td>
</tr>
<tr>
<td>Fructosamine (µmol/L)</td>
<td>150±2.09</td>
<td>268±6.86***</td>
<td>250±5.09**</td>
<td>249±7.45*</td>
<td>229±6.85</td>
</tr>
<tr>
<td>Serum creatinine (µmol/L)</td>
<td>52.1 ± 3.90</td>
<td>70.5 ± 4.65*</td>
<td>66.3 ± 3.41</td>
<td>65.5 ± 2.90</td>
<td>62.2 ± 2.29</td>
</tr>
<tr>
<td>Urea nitrogen (mmol/L)</td>
<td>8.75 ± 0.20</td>
<td>26.3 ± 2.80***</td>
<td>17.5 ± 2.10*</td>
<td>18.1 ± 1.84*</td>
<td>20.2 ± 0.82**</td>
</tr>
<tr>
<td>GFR (mL/min/100g)</td>
<td>3.28 ± 0.10</td>
<td>1.37 ± 0.09*</td>
<td>1.95 ± 0.39</td>
<td>1.66 ± 0.43</td>
<td>2.00 ± 0.24</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>1.69 ± 0.04</td>
<td>2.35 ± 0.05**</td>
<td>1.80 ± 0.12</td>
<td>1.54 ± 0.05***</td>
<td>1.88 ± 0.08</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>2.21 ± 0.10</td>
<td>1.55 ± 0.18*</td>
<td>1.77 ± 0.20</td>
<td>1.87 ± 0.07</td>
<td>1.32 ± 0.08**</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.42 ± 0.03</td>
<td>1.90 ± 0.50**</td>
<td>1.49 ± 0.45*</td>
<td>1.31 ± 0.37</td>
<td>1.51 ± 0.33*</td>
</tr>
</tbody>
</table>

Values of blood pressure, metabolic and renal parameters are presented as mean ± SEM, n = 8/group. *p<0.05; **p<0.01; ***p<0.001 vs. Control, respectively, §§§p<0.001 vs. Diabetes, respectively.