NOVEL THERAPEUTIC OPTIONS IN DIABETIC KIDNEY DISEASE

PhD Thesis

Dóra Bianka Balogh

Doctoral School of Clinical Medicine
Semmelweis University

Supervisor: Andrea Fekete, MD, PhD

Official reviewers:
Gábor Kökény, MD, PhD
Ágnes Haris, MD, PhD

Head of the Final Examination Committee:
Miklós Kellermayer, MD, DSc

Members of the Final Examination Committee:
Nóra Hosszúfalusi, MD, PhD
Tibor Kovács, MD, PhD

Budapest, 2020

INTRODUCTION

Over the past few decades, diabetes mellitus has become one of the largest epidemics the world has faced, making it an enormous challenge for modern healthcare systems. Without urgent multi-sectoral strategies and actions, 700 million people are predicted to live with diabetes by 2045. Diabetic patients have an increased risk of developing life-threatening health complications resulting in reduced quality of life, increased mortality and higher medical care costs.

Diabetic kidney disease (DKD) is a major cause of chronic kidney disease (CKD), accounting for approximately 50% of cases in the United States and 30-40% of cases in Hungary. It typically develops after 10 years of diabetes in the case of type 1 diabetes (T1DM; ca. 30%), but may already be present at diagnosis of type 2 diabetes (T2DM; ca. 40%).

DKD is a complex and heterogeneous disease with numerous overlapping metabolic and hemodynamic pathways. Hyperglycemia affects most renal cell types; however, some cells are more vulnerable to high glucose concentrations than others. Glucose entry into proximal tubular cells is insulin independent; therefore, these cells are not capable of decreasing glucose transport adequately making them particularly susceptible to high glucose concentrations.

Prolonged hyperglycemia results in production of advanced glycation end products (AGE), activation of the hexosamine and polyol pathway, increased vasoactive renin-angiotensin-aldosterone system (RAAS) activity and hypoxia. All these glucose-induced general mechanisms change renal hemodynamics and promote renal inflammation and fibrosis.

In diabetes, high glucose concentration stimulates renal angiotensinogen and renin synthesis resulting in angiotensin II (Ang

II) increment. The high Ang II levels and overactivated RAAS lead to elevated blood pressure, mesangial cell contraction with decreased surface area for filtration, renal cell proliferation and hypertrophy, increased production of extracellular matrix (ECM) and induction of growth factors.

O-GlcNAcylation is the product of nutrient flux through the hexosamine biosynthetic pathway (HBP). Protein *O*-GlcNAcylation is a posttranslational modification regulating protein function in many cellular processes (e.g. signaling, transcription, cytoskeletal functions) and chronic upregulation of *O*-GlcNAc is a known contributor to in insulin resistance and glucose toxicity in diabetes. Moreover, *O*-GlcNAcylation has a major role in the regulation of renal fibrosis.

Tubular hypoxia is a major driver of DKD progression. Glomerular hyperfiltration and elevated glucose reabsorption through Na^+/glucose cotransporters (SGLT) enhance Na^+/K^+-ATPase activity resulting in increased O_2 consumption in diabetes. Cells harbor many mechanisms with which they withstand hypoxic challenges. Hypoxia-inducible factor (HIF)-1α activation is one of the most critical factors that triggers hypoxia adaptation. During hypoxia HIF-α stabilizes and translocates to the nucleus, where it dimerizes with HIF-β. The HIF complex then binds to hypoxia responsive elements in the promoter sequences of numerous genes involved in maintaining cellular and tissue O_2 homeostasis e.g. erythropoietin (EPO), vascular endothelial growth factor (VEGF) and glucose transporter-1 (GLUT1).

ACE inhibitors or ARBs are the preferred first-line medication regimen for blood pressure treatment in diabetic patients with hypertension and moderately increased urinary albumin excretion (UAE). However, these merely ameliorate renal impairment, but cannot prevent renal failure. Thus, novel therapies and early intervention directly targeting the diabetic kidney are of paramount importance.

The kidney bears a crucial role in the regulation of glucose homeostasis. Almost all filtered glucose (ca. 99%) is reabsorbed by SGLT and urine is essentially glucose free. Renal glucose filtration and the tubular glucose reabsorption are enhanced by three to four times in diabetes. If glomerular filtration rate (GFR) is normal, the renal transport maximum of glucose is reached when blood glucose levels exceed 11.1 mmol/L leading to a linear increase in glucosuria.

SGLT2 inhibitors (SGLT2i) have recently been approved as new generation antidiabetics with a unique mechanism of action. SGLT2i act by inhibiting glucose reabsorption in renal proximal tubules, thereby lowering plasma glucose levels in an insulin-independent manner. SGLT2i have been routinely used in T2DM for years, but only dapagliflozin (DAPA) and sotagliflozin have been approved in T1DM to-date. Multicenter clinical trials EMPA-REG, CANVAS and DAPA-TIMI 58 indicate that SGLT2i considerably hinder the progression of DKD. Comparison studies suggest that SGLT2i are more renoprotective than other antidiabetics (e.g. GLP analogues) with similar glucose lowering effect. These results were substantiated by another study in which reduced albuminuria was independent of changes in HbA_{1c}, blood pressure or body weight in DAPA-treated T2DM patients. Thus, renoprotection may arise not only because of lower glucose levels, but due to other mechanisms of SGLT2i, such as inhibition of TGF anti-inflammatory or anti-fibrotic effects.

Considering the importance of SGLT2i as novel antidiabetics, it is crucial to characterize their renoporotective mechanisms especially in light of data suggesting that the beneficial effects of DAPA are beyond its blood glucose lowering properties.

OBJECTIVES

1. To determine the antifibrotic and renoprotective effects of various RAASi in monotherapy in experimental model of T1DM

2. To investigate the safety and efficacy of DAPA and DAPA combined with ARB losartan in T1DM

3. To analyze the renoprotective and antifibrotic effects of DAPA in both *in vivo* and *in vitro*

4. To determine the effects of DAPA and DAPA combined with losartan on protein *O*-GlcNAcylation and hypoxia in human proximal tubular cells

METHODS

Study approval
All experiments were conducted in accordance with guidelines of the Committee on the Care and Use of Laboratory Animals of the Semmelweis University Budapest, Hungary (PEI/001/1731-9-2015).

Animals and experimental design

Experiments were performed on eight-week old male Wistar rats (*Rattus norvegicus*). Rats were housed in plastic cages under a 12-hour light/dark cycle at constant temperature (24±2 °C) with *ad libitum* access to standard rodent chow and tap water.

Diabetes was induced with a single intraperitoneal injection of 65 mg/bwkg streptozotocin (STZ) dissolved in 0.1 M citrate buffer (pH 4.5). Blood glucose levels were measured three times from tail vein. Rats with a peripheral blood glucose value above 15 mmol/L 72 hours after the STZ injection were enrolled in the study. Two different experimental protocols were used.

Protocol I
After five weeks of diabetes rats were randomized into five groups (n=7-8/group) and were treated daily by oral gavage for 2 weeks as follows:

1. isotonic saline as vehicle (D)
2. enalapril (D+ ENA; 40 mg/bwkg/day)
3. losartan (D + LOS; 20 mg/bwkg/day)
4. spironolactone (D + SPI; 50 mg/bwkg/day)
5. eplerenone (D + EPL; 50 mg/bwkg/day).

Doses were adopted from our previous studies in line with literary data where effective blockade of ACE, AT1R or aldosterone activity was achieved without changes in systemic blood pressure.

Protocol II
Rats were randomly divided into three groups immediately after the onset of diabetes (n=6 in D and n=7 in treatment groups) and were treated *per os* as follows:

1. isotonic saline as vehicle (D)
2. DAPA (D + DAPA; 1 mg/bwkg/day for six weeks)
3. DAPA + losartan (D + DAPA + LOS; 1 mg/bwkg/day DAPA for six weeks + 20 mg/bwkg/day losartan in the last three weeks of the protocol)

In both protocols, age-matched controls received the equivalent volume of citrate buffer without STZ once, and the same amount of saline by oral gavage daily at the same time as the diabetic animals.

Cell cultures and experimental design

All *in vitro* experiments were performed on human proximal tubular epithelial cell line (HK-2).

Hyperglycemia model
The effect of high glucose was tested on HK-2 cells cultured in DMEM containing 5.5 mM glucose and treated with high glucose (HG; final cc. 35 mM) or high mannitol (final cc. 35 mM) for 24 hours. HG cells were treated with 10 μM DAPA (HG + DAPA) or 10 μM DAPA combined with 10 μM LOS (HG + DAPA + LOS) for 24 hours. Cells in 5.5 mM glucose conditions served as controls and mannitol treated cells were used for testing hyperosmolarity *per se*.

Hypoxia model
Hypoxia was induced in bold line stage top CO_2/O_2 incubator by keeping the cells in 1% O_2 for 2 hours. HK-2 cells cultured in medium containing 25 mM glucose were treated as follows: 10 μM DAPA (H + DAPA) or 10 μM DAPA combined with 10 μM LOS (H + DAPA

+ LOS) for 24 hours before harvest. Cells were harvested at the end of hypoxia. Cells cultured under normoxic conditions served as controls.

Metabolic and renal parameters
Serum (glucose, fructose, cholesterol, GOT, GPT, creatinine, BUN) and urinary parameters (creatinine clearance, albumin excretion, glucosuria) were determined. Urinary kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) levels were measured.

Renal histology and immunocytochemistry
Mesangial matrix expansion was evaluated on periodic acid-Schiff stained kidney sections. Masson's trichrome staining was used to evaluate tubulointerstitial fibrosis and Picrosirius red staining to evaluate collagen accumulation. Fibronectin accumulation was determined with fluorescent immunohistochemistry. Changes in *O*-GlcNAc and HIF-1α in the proximal tubules were detected with immunocytochemistry.

Measurement of biomarkers of ECM formation and degradation
The biomarkers collagen III formation (rPRO-C3), MMP-9-mediated degradation of type III collagen (uC3M) and type IV collagen (TUM) were measured in rat urine samples.

Quantitative RT-PCR
Tgfb1, Ctgf, Pdgfb, Fn1, Havcr1, Lcn2, Rn18S, TGFB1, CTGF, PDGFB, HIF1A, GAPDH and *RN18S* mRNA expressions were determined. Target gene expressions were normalized against *Rn18S* or *RN18S* mRNA or *GAPDH* housekeeping genes.

Western blot
Western blot was used to measure the following proteins: α-SMA, CTGF, EPO, HIF-1α, OGA, *O*-GlcNAc, OGT, PDGF, VEGF-A. Bands of interest were factored for Ponceau S staining to correct for

variations in total protein loading. Each blot was normalized to an internal control so that bands on separate blots could be compared.

Statistical analysis

Data are expressed as means±standard deviations (SD) or means±95% confidence intervals. Statistical analysis was performed using GraphPad Prism software. Multiple comparisons and interactions were evaluated by one-way ANOVA followed by Holm-Sidak *post hoc* test. For non-parametrical data, the Kruskal-Wallis ANOVA on ranks followed by with Dunn correction was used. *P* values of <0.05 were considered significant.

RESULTS

RAASi improves renal function and ameliorates tubulointerstitial fibrosis in diabetes

Our aim was to investigate the effect of RAAS blockade regardless of their antihypertensive properties. Mean arterial pressure remained unchanged in all groups confirming that the examined effects of RAASi are independent of their antihypertensive properties. Rats had significant weight loss, elevated blood glucose, fructosamine and lipid levels confirming the development of T1DM. RAASi did not alter any of these parameters.

Development of DKD was confirmed by the decline of renal function. Creatinine clearance decreased, while serum creatinine, BUN and UAE elevated in diabetic rats. ENA, SPI and EPL improved creatinine clearance. BUN and UAE were significantly reduced by all treatments. The quantity of tubulointerstitial fibrotic tissue increased in diabetic rats. All of the RAASi decreased tubulointerstitial fibrosis.

DAPA prevents diabetes-induced metabolic decline and slows the progression of renal impairment

As expected, DAPA markedly improved all the metabolic parameters that was elevated due to diabetes. By the end of the experiment, blood glucose levels were 47% lower in DAPA *vs.* diabetic group. In parallel, urinary glucose excretion was enhanced in DAPA-treated groups. Our results confirm the efficacy of DAPA in T1DM experimental rat model.

DAPA markedly improved creatinine clearance, serum creatinine, BUN and albumin excretion. Urinary and renal KIM-1 and NGAL were elevated in the diabetic group, while DAPA decreased their levels by more than 50% indicating milder tubular damage. Histologic

changes were consistent with functional decline. Evaluation of PAS-stained sections revealed massive hypertrophy, mesangial matrix expansion and basal membrane thickening in the glomeruli of diabetic rat kidneys. DAPA minimized mesangial matrix expansion and ameliorated structural damage.

Renal fibrogenesis is alleviated by DAPA

rPRO-C3, uC3M, and TUM are novel urinary biomarkers of ECM remodeling, which are promising in early diagnosis and prognosis of renal fibrosis. In our experiment, urinary rPRO-C3, uC3M and TUM were elevated in diabetic rats. DAPA treatment decreased rPRO-C3 and TUM levels. Beside the novel markers, we investigated the renal mRNA expressions of profibrotic growth factors, *Tgfb1, Pdgfb* and *Ctgf*, which were upregulated in diabetic rats. DAPA decreased *Pdgfb* and *Ctgf* to control levels, while surprisingly it h ad no effect on *Tgfb1* expression.

Diabetes-induced myofibroblast marker α-SMA increment was minimized by DAPA. Extensive tubulointerstitial fibrosis and dilated tubules were observed in diabetic kidneys. DAPA reduced the amount of renal fibrotic tissue. Weak collagen staining was detected in glomeruli and around blood vessels in control kidneys. Extensive fibrotic tissue accumulation was observed in diabetic kidneys as shown by collagen deposition in the interstitium. Diabetes-induced collagen deposition was lower in the DAPA group compared to the diabetic group. Considerable fibronectin-positive staining was detected in the glomeruli and to a lesser extent in the tubulointerstitium of diabetic kidneys which was attenuated by both treatments. In parallel with histology, renal fibronectin mRNA expression increased in diabetes and was decreased by 50% in DAPA-treated rats.

Correlation analysis was performed to strengthen the relevance of novel urinary markers of ECM remodeling in early diagnosis and prognosis of renal fibrosis. Positive correlation was found between tubulointerstitial fibrosis (evaluated on Masson's trichrome-stained sections) and rPRO-C3 (R^2=0.4459, p=0.0003), uC3M (R^2=0.1922, p=0.0364) and TUM (R^2=0.2285, p=0.0182), respectively. Our experimental results support the use of these biomarkers in the diagnosis of renal fibrosis.

Hyperglycemia-induced *O*-GlcNAcylation and subsequent fibrosis is prevented by DAPA in HK-2 cells

Since SGLT2i act on proximal tubular cells, the direct effects of DAPA on *O*-GlcNAcylation were investigated in HK-2 proximal tubular cells cultured in hyperglycemic conditions. Both Western-blot and immunocytochemistry measurements revealed that protein *O*-GlcNAcylation is induced after 24 hours of high glucose (HG) treatment. DAPA prevented HG-induced protein *O*-GlcNAcylation. The changes were not detected in mannitol-treated cells confirming that the observed phenomenon is a consequence of hyperglycemia and not hyperosmolarity.

In parallel, levels of ncOGT and sOGT, the enzymes responsible for adding *O*-GlcNAc moiety were higher after 24 hours of HG treatment. DAPA prevented HG-induced ncOGT and sOGT upregulation in proximal tubular cells. OGA-L, which is responsible for removing *O*-GlcNAc residues remained unchanged in all of the groups.

We showed that diabetes-induced renal profibrotic growth factor increment was mitigated by DAPA; therefore, we investigated the effect of DAPA on *TGFB1*, *PDFGB* and *CTGF* in HK-2 proximal tubular cells cultured in hyperglycemic conditions. All growth factors were increased in HG conditions. DAPA significantly decreased the

level of *CTGF*, while the level of *TGFB1* and *PDGFB* remained unaltered. However, similarly to *in vivo* experiments a slight decrement was observed in *PDGF*.

DAPA moderates tubular response to hypoxia

To investigate the effect of DAPA independently of its glucose-lowering action HK-2 cells cultured in DMEM containing 25 mM glucose were placed into a hypoxic chamber (1% O_2 for 2h). Hypoxic injury was investigated using three different methods (qRT-PCR, Western blot, immunofluorescence analysis). In response to hypoxia, enhanced HIF-1α mRNA expression and protein level (by Western blot and immunocytochemistry as well) were observed. DAPA suspended HIF-1α elevation in both experiments indicating milder hypoxic injury. Moreover, DAPA treatment prevented HIF-1α translocation to the nucleus, thereby confirming abolished HIF-1α activation.

Hematopoietic growth factor EPO is produced by the kidneys and regulates the production of red blood cells, thereby it is one of the key determinants of physiological oxygen homeostasis. VEGF, an angiogenic factor is produced by glomerular and tubular epithelia and it may help to restore vascular supply to cells, thereby reducing hypoxia. The HIF system is the central transcriptional mediator of these processes, therefore downstream elements EPO and VEGF-A were investigated. Both EPO and VEGF-A mRNA expressions and protein levels were enhanced in response to hypoxic insult and DAPA prevented the induction of EPO.

Hypoxia triggers fibrotic response, thus TGF-β, PDGF and CTGF were investigated. *TGFB1*, *PDGFB* and *CTGF* mRNA expressions increased in hypoxic tubular cells. DAPA prevented the induction of *TGFB1* and *PDGFB*, however had no effect on *CTGF*.

CONCLUSIONS

1. Treatment with non-depressor doses of RAASi in monotherapy is renoprotective and antifibrotic in a rat model of T1DM.

2. SGLT2 inhibitor DAPA treatment is protective in T1DM and subsequent DKD. It substantially ameliorates functional and structural kidney damage.

3. Our *in vivo* and *in vitro* experiments revealed that DAPA has antifibrotic properties. It mitigates the level of novel urinary biomarkers of ECM remodeling, profibrotic growth factors and accumulation of collagen and fibronectin.

4. Hyperglycemia-induced protein *O*-GlcNAcylation in proximal tubules is minimized by DAPA.

5. DAPA directly moderates the tubular response to hypoxia independently of its antihyperglycemic property.

6. DAPA alone is as effective as the combination therapy with non-depressor LOS both concerning *in vivo* and *in vitro* experimental outcomes.

BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

Publications related to the Thesis

[*]Judit Hodrea, [*]**Dora B Balogh**, Adam Hosszu, Lilla Lenart, Balazs Besztercei, Sandor Koszegi, Nadja Sparding, Federica Genovese, Laszlo J Wagner, Attila J Szabo, Andrea Fekete; Reduced *O*-GlcNAcylation and tubular hypoxia contribute to the antifibrotic effect of SGLT2 inhibitor dapagliflozin in the diabetic kidney. *Am J Physiol Renal Physiol* 2020 **IF=3.323**

Sandor Koszegi, Agnes Molnar, Lilla Lenart, Judit Hodrea, **Dora Bianka Balogh**, Tamas Lakat, Edgar Szkibinszkij, Adam Hosszu, Nadja Sparding, Federica Genovese, Laszlo Wagner, Adam Vannay, Attila J Szabo, Andrea Fekete; RAAS inhibitors directly reduce diabetes-induced renal fibrosis via growth factor inhibition. *J Physiol* 00.0 pp 1-17 2018 **IF=4.950**

Gellai, R, Hodrea, J, Lenart, L, Hosszu, A, Koszegi, S, **Balogh, D**, Ver, A, Banki, NF, Fulop, N, Molnar, A, Wagner, LJ, Vannay, A, Szabo, AJ, Fekete, A.; The role of *O*-linked N-Acetylglucosamine modification in diabetic nephropathy. *Am J Physiol Renal Physiol 311: F1172–F1181*, 2016 **IF=3.611**

Hodrea Judit, **Balogh Dóra Bianka**, Lénárt Lilla, Kőszegi Sándor, Hosszú Ádám, Vannay Ádám, Wagner J László, Reusz György, Szabó J Attila, Fekete Andrea; A nátrium-glükóz-kotranszporterek szerepe a diabeteses nephropathiában. *Hypertonia és nephrologia* 19(4) pp.153-158., 6p. 2015

Other publications

Dora B Balogh, Agnes Molnar, Adam Hosszu, Judit Hodrea, Attila J Szabo, Lilla Lenart, Andrea Fekete; Antidepressant effect in diabetes-associated depression: a novel potential of RAAS inhibition. *Psychoneuroendocrinology* 2019 **IF=4.732**

Lilla Lenart, **Dora B. Balogh**, Nikolett Lenart, Adrienn Barczi, Adam Hosszu, Tamas Farkas, Judit Hodrea, Attila J. Szabo, Krisztian Szigeti, Adam Denes, Andrea Fekete; Novel therapeutic potential of angiotensin receptor 1 blockade in a rat model of diabetes-associated depression parallels altered BDNF signaling. *Diabetologia* 2019 **IF=7.518**

Adam Hosszu, Zsuzsanna Antal, Apor Veres-Szekely, Lilla Lenart, **Dora Bianka Balogh**, Edgar Szkibinszkij, Lilla Illesy, Judit Hodrea, Nora F. Banki, Laszlo Wagner, Adam Vannay, Attila J. Szabo, Andrea Fekete; The role of Sigma-1 receptor in sex-specific heat shock response in an experimental rat model of renal ischaemia/reperfusion injury. *Transplant International* 2018 **IF=3.526**

Adam Hosszu, Zsuzsanna Antal, Judit Hodrea, Sandor Koszegi, Lilla Lenart, **Dora B Balogh**, Nora F Banki, Laszlo Wagner, Adam Denes, Peter Hamar, Adam Vannay, Peter Degrell, Attila J Szabo, Andrea Fekete; σ1-Receptor Agonism Protects against Renal Ischemia-Reperfusion Injury. *J Am Soc Nephrol 27* 2017 **IF=8.655**

Lenart, Lilla; Hodrea, Judit; Hosszu, Adam; Koszegi, Sandor; Zelena, Dora; **Balogh, Dora**; Szkibinszkij, Edgar; Veres-Szekely, Apor; Wagner, Laszlo; Vannay, Adam; Szabo, Attila; Fekete, Andrea; The role of sigma-1 receptor and brain-derived neurotrophic factor in the development of diabetes and comorbid depression in streptozotocin-induced diabetic rats. *Psychopharmacology* 2016 **IF=3.308**

ACKNOWLEDGMENT

Firstly, I would like to express my deep sense of gratitude and profound respect to my supervisor Andrea Fekete for the continuous support of my PhD study and related research. Her tireless dedication, immense knowledge and motivation have given me power and spirit through the years.

I wish to sincerely thank Professor Attila Szabo for his support and help in completing my PhD work successfully in the Research Laboratory of the 1st Department of Pediatrics, Semmelweis University.

I express my gratitude and gratefulness to the postdocs of our group, Judit Hodrea, Adam Hosszu and Lilla Lenart for giving me encouragement and sharing insightful suggestions. Without their precious support it would not have been possible to conduct this research. Their endless guidance is hard to forget throughout my life.

I would like to thank all my colleagues at the lab for the stimulating discussions and the enthusiasm when we were working together before deadlines. Special thanks to Tamas Lakat for his invaluable help in molecular measurements and figure design, to Sandor Koszegi for preparing the histological evaluation and to Laszlo Wagner for his clinically relevant comments. I could not be more thankful to Maria Bernath for her constant help with *in vitro* experiments and laboratory work. I am privileged of having the fellow labmates as well: Nora Beres, Apor Veres-Szekely, Edgar Szkibinszkij, Agnes Molnar and Csenge Mezei, thanks to you for helping me to survive these years.

Last but not the least, all special thanks to my husband, Balazs Koczka and to my whole family for helping me throughout my PhD studies and writing this thesis. Without their constant support and love this work could not have been completed.