THE ROLE OF INHIBITION OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM IN DIABETIC KIDNEY DISEASE: FOCUSING ON RENAL FIBROSIS

PhD Thesis

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INTRODUCTION

Diabetic Kidney Disease (DKD) is the leading cause of end-stage renal disease, whose incidence is constantly increasing worldwide. Its severity is indicated by the fact that cardiovascular morbidity is significantly higher in patients with DKD than in the healthy population.

Its severity is indicated by the fact that cardiovascular morbidity is significantly higher in patients with DKD than in the healthy population. During the progression of DKD, disruption of renal hemodynamics, endothelial dysfunction, structural (fibrotic) changes in the glomerulus, tubule and / or in the interstitium can be observed.

Currently, different therapies only slow the progression of DKD, but there is no effective treatment. In DM, it is known to increase RAAS activity both systemically and locally in the kidney, which plays a prominent role in the development of renal fibrosis. As a result of continuous RAAS activation, the renal arterioles contract, the external and internal resistance increases, resulting in an increased capillary pressure inside the glomeruli. As a result of the increased pressure, protein urea and oxidative stress is developed. The process leads to, among other things, endothelial dysfunction and proliferation of mesangial cells, resulting in the activation of proinflammatory signaling pathways and profibrotic processes. Inhibition of RAAS lowers blood pressure and proteinuria, prevents the development of renal fibrosis and slows the failure of renal function. RAAS inhibitors are divided into four classes according to their effect in the RAAS cascade: direct renin inhibitors, ACE inhibitors, ARBs and aldoseron antagonists. In clinical practice, an ACE inhibitor or ARB is primarily used when microalbuminuria appears. However, due to the phenomenon of so-called aldosterone escape, neither the ACE inhibitor nor the use of ARB may be not enough effective to reduce proteinuria. During aldosterone escape, aldosterone is also produced in an unclear manner, independently of AngII, which activates

proinflammatory and profibrotic signaling pathways, induces tubular interstitial fibrosis, and glomerulosclerosis, enhancing the progression of DKD. Aldosterone escape occurs in 20-40% of patients treated with an ACE inhibitor or ARB, and the use of aldosterone antagonists is recommended in such cases. The use of aldosterone antagonists as monotherapy is not currently included in the recommendations, but an increasing number of clinical trials demonstrate that their use as monotherapy improves the progression of DKD. Our group showed that in addition to routinely used ACE inhibitors, ARBs, and spironolactone, eplerenone, which has fewer side effects, is equally effective in ameliorating renal structural and functional damage during DKD.

In recent years, a number of new therapeutic options have been described that slow the progression of DKD, however, due to the complexity of the disease, none of the therapeutic options are perfect, so the topic of our work is to further investigate these therapies. In my PhD work, we examined how RAAS inhibitor treatments affect the progression of fibrosis in the pathomechanism of DKD. Using histological methods, we studied structural and fibrotic lesions, quantitative changes in renal fibrosis markers, localization of α-SMA protein, and an increase or decrease in fibrosis-induced growth factors expression. In addition, quantitative changes in PDGF protein were observed *in vitro* as a result of hyperglycemia and various RAAS inhibitor treatments.

OBJECTIVES

1. To investigate, how RAAS inhibitors affect functional parameters and structural and fibrotic tissue lesions in an experimental model of DKD?

2. To investigate, how do RAAS inhibitors affect the process of renal fibrosis, in particular the expression of profibrotic factors (TGF, PDGF, CTGF) in an experimental animal model of DKD?

3. To determine which of the RAAS inhibitors used is best suited to slow the progression of renal fibrosis

4. To demonstration of the central role of PDGF in the process of renal fibrosis *in vitro* in proximal tubule cell culture after high glucose treatment. How do RAAS inhibitors affect the PDGF production of proximal tubule cells?

METHODS

Study approval

All experiments were conducted with the approval of the Occupational Animal Welfare Committee of Semmelweis University (PEI / 001 / 1731-9-2015) and in compliance with the ethical permits for animal protection and animal experiments in Hungary (1998 / XXVIII.).

In vitro experiments

In our experiments, we used immortalized human kidney 2 (HK-2) proximal tubule cells. HK-2 proximal tubule cells were cultured in DMEM (Dulbeco's Modified Eagle Medium, containing 5.5 mM glucose) supplemented with 10% FBS, 1% penicillin / streptomycin antibiotic solution, and 1% L-glutamine. Cells were cultured at 95% humidity in a 5% CO2 thermostat at 37 ° C.

During treatment, HK-2 cells were maintained in normal (5.5 mM), high glucose (35 mM) or high mannitol (5.5 mM glucose + 29.5 mM mannitol) medium for 24 hours as an osmotic control, thus examining the direct effect of hyperglycemia on the proximal tubule. Cells maintained in high glucose medium were treated with RAAS inhibitors for 24 hours at the following doses:

1. ramipril: 10 μM
2. losartan: 10 μM
3. spironolactone: 200 nM
4. eplerenone: 10 μM

Control cells were maintained in DMSO vehicle (n = 6 wells / group).

Adherent cells were harvested after 24 or 48 or 72 hours with 0.25% trypsin-EDTA and protein was isolated.

Diabetes rat model

Our experiments were performed on 8-week-old, mature, male, Wistar rats (Toxicoop Kft., Budapest, Hungary). The animals

were kept at a constant temperature (21 ° C), humidity (75%) and alternating illumination every 12 hours. Animals were allowed unrestricted consumption of tap water and general rodent feed during the study.

T1DM was artificially induced by a single intraperitoneal injection of high-dose streptozotocin (STZ, 65 mg / kg, Sigma Aldrich Kft, Budapest, Hungary) dissolved in citrate (0.1 M; pH 4.5). Control animals were also treated with citrate buffer without STZ. Blood glucose was measured with a Dcont Trend digital glucose meter (77 Elektronika Kft, Budapest, Hungary) from blood taken from a tail vein 72 hours after injection. Rats were considered diabetic if, after three randomized measurements, the peripheral blood glucose concentration exceeded 15 mmol/l. At lower blood glucose levels, animals were excluded from further experiments.

At 5 weeks after T1DM induction, animals were randomly assigned to groups (n = 7-8 rats / group) and treated *per os* with RAAS inhibitors dissolved isotonic saline for 2 weeks:

1. isotonic saline as a vehicle (D)
2. enalapril (D+ ENA; 40 mg/bwkg/day)
3. ramipril (D+ RAM; 10 μg/bwkg/day)
4. losartan (D + LOS; 20 mg/bwkg/day)
5. spironolactone (D + SPI; 50 mg/bwkg/day)
6. eplerenone (D + EPL; 50 mg/bwkg/day)

Based on our previous studies and literature data, the dose of RAAS inhibitors was chosen to effectively inhibit the expression and activity of some elements of RAAS, regardless of their antihypertensive properties.

Metabolic and renal parameters

Serum (glucose, creatinine and urea) and urine parameters (creatinine clearance) were determined at the end of the protocol.

Renal histology and immunocytochemistry and immunofluorescence

The extent of mesangial matrix expansion was assessed on Periodic Acid - Schiff stained sections. Tubulointerstitial fibrosis was examined in Masson's Trichome, and collagen accumulation was examined in Sirius-red stained kidneys. The amount of fibronectin was examined by fibronectin immunohistochemical staining. The localization of α-SMA was examined by confocal microscopy by immunofluorescence staining.

Quantitative RT-PCR

Tgfb1, Pdgfb, Ctgf, Mmp2, Timp1, Rn18S, TGFB1, CTGF, PDGFB, GAPDH and *RN18S* mRNA expressions were determined. Target gene expressions were normalized against *Rn18S* or *RN18S* mRNA or *GAPDH* housekeeping genes.

Flow cytometry

The level of PDGF-B were examinated in HK-2 cells.

Western blot

The amount of αSMA and the amount of PDGF protein in HK-2 cells were examined by Western blot. The resulting signal was densitometered and corrected for the amount of total protein obtained by Ponceau S staining and internal control.

Statistical analysis

Data are expressed as means±standard error of mean (SEM) or median±95% confidence intervals. Statistical analysis was performed using GraphPad Prism software. For normal distribution, one-way ANOVA and Bonferroni Post Test were used. For nonparametric data, Kruskal-Wallis ANOVA and Dunns correction were used. *P* values of <0.05 were considered significant.

RESULTS

RAAS inhibitors improved renal function in an animal model of T1DM

By the end of the two-week treatment period, blood pressure did not change in either group, confirming the selection of appropriate non-depressant doses, so their effects were evaluated independently of their antihypertensive properties. In the diabetic group, elevated serum glucose and fructosamine confirm the long-term existence of DM. In addition, there is a lack of weight gain in diabetic animals, as well as higher cholesterol and triglyceride levels, suggesting a disorder of lipid metabolism. Of the RAAS inhibitors, losartan, spironolactone, and eplerenone significantly improved cholesterol levels.

By the end of the study, diabetic animals showed functional impairments characteristic of DKD: increased renal / body weight ratio and serum creatinine, urea levels, increased glucose excretion and microalbuminuria, and decreased creatinine clearance. RAAS inhibitors reduced renal hypertrophy and glucose excretion, and improved urea nitrogen and creatinine clearance parameters, demonstrating their renoprotective effect.

RAAS inhibitors moderated DKD-specific structural and fibrotic lesions in the T1DM animal model

A healthy renal structure was observed in the kidneys of control animals, while in DM the capillary lumens narrowed and the amount of extracellular matrix inside the glomeruli was increased. RAAS inhibitors significantly reduced extracellular matrix accumulation.

In the kidneys of diabetic animals, the collagen-rich connective tissue deposition present in the interstitium was significantly increased compared to healthy kidney tissue in control animals. RAAS inhibitor treatments reduced the amount of connective tissue deposits.

Elevated renal collagen levels in untreated diabetic animals were significantly reduced by RAAS inhibitors.

RAAS inhibitors, with the exception of enalapril, significantly reduced fibronectin accumulation therefore they proved to be anti-fibrotic.

No differences were observed between RAAS inhibitors during mesangial matrix expansion and collagen studies (Masson's Trichome and Sirius-red method) however, aldosterone antagonists had a more significant inhibitory effect on fibronectin synthesis.

RAAS inhibitors reduced the amount of α-SMA in the T1DM animal model

In the kidneys of control animals, the intensity of α-SMA is weak, in contrast, in the kidneys of untreated diabetic animals, the intensity of α-SMA increases in the interstitial space.

The intensity was reduced with each RAAS inhibitor treatment, and the intensity of the intensity was not quantified due to the complex settings of the confocal microscope. However, immunofluorescence results were confirmed by western blot analysis: we demonstrated that the levels of α-SMA in the kidneys of diabetic animals were significantly elevated compared to healthy control animals, and all RAAS inhibitor treatments moderated this increase.

RAAS inhibitors moderated the amount of fibrosis-induced growth factors in the T1DM animal model

Growth factors, such as TGFβ, PDGF, and CTGF, play a key role in the pathomechanism of DKD-induced renal fibrosis.

The expression of TGFβ, PDGF and CTGF in control, untreated diabetic animals and diabetic animals treated with RAAS inhibitors was examined using a real-time reverse transcription polymerase chain reaction. Our studies confirmed that mRNA expression of all three growth factors is significantly increased in the kidneys of diabetic animals. RAAS treatment did not affect TGFβ

mRNA expression, PDGF and CTGF expression were significantly reduced by aldosterone antagonists.

RAAS inhibitors reduced the amount of extracellular matrix components in the T1DM animal model

The degradation and synthesis of ECM proteins in the kidney are regulated by MMPs and their inhibitors, TIMPs. In our studies, we examined the mRNA expression of gelatinase-type MMP2 and its inhibitor, TIMP1.

Both MMP2 and TIMP1 mRNA expression were increased in the kidney of diabetic animals. RAAS inhibitors reduced MMP2 mRNA expression, with the exception of ramipril. The inhibitory effect of aldosterone antagonists on MMP2 expression is stronger than that of the angiotensin II receptor blocker losartan. TIMP1 mRNA expression was significantly reduced by losartan and eplerenone.

High glucose increased the amount of PDGF protein in proximal tubule cells

Among growth factors, PDGF has a prominent role in DM-induced renal fibrosis. Mesenchymal cells with the PDGF receptor (fibroblasts, vascular smooth muscle cells) are activated by PDGF and initiate cell proliferative signaling pathways which contribute to the progression of fibrotic processes.

The amount of PDGF protein in human proximal tubule (HK-2) cells was examined *in vitro* after treatments with normal (5.5 mM) and high (35 mM) glucose concentrations. The amount of PDGF protein was significantly increased after 24 hours with high (35 mM) glucose treatment, so 24 hours of treatment was used for further studies. Flow cytometry showed that cells treated with high (35 mM) glucose concentration had significantly higher amounts of PDGF protein compared to the normal control group (5.5 mM glucose). There was no difference in the amount of PDGF protein between the

35 mM isoosmotic mannitol-treated and the control group, confirming the direct glucose effect.

RAAS inhibitors reduced the amount of PDGF mRNA in proximal tubule cells

After 24 h of high glucose (35 mM) treatment, PDGF mRNA expression was increased in HK-2 cells. Ramipril and spironolactone significantly reduced this increase, and losartan and eplerenone tended to reduce it.

CONCLUSIONS

1. We demonstrated that in the experimental model of DKD, RAAS inhibitors improve renal functional parameters in monotherapy; and RAAS inhibitors reduce structural and fibrotic tissue lesions

2. In our series of experiments, we were the first to show that the monotherapy of aldosterone antagonists, including eplerenone, is the most suitable RAAS inhibitor to slow the progression of renal fibrosis by inhibiting fibronectin synthesis and reducing PDGF, CTGF expression.

3. We have shown that aldosterone antagonists (spironolactone and eplerenone) are more effective in reducing the expression of the profibrotic factor PDGF and CTGF than ACE inhibitors or ARBs in the experimental animal model of DKD. However, TGF expression did not change in the experimental model we used with RAAS inhibitory treatments.

4. In our *in vitro* studies, we demonstrated that RAAS inhibitors reduce the increased PDGF production in proximal tubule cells by hyperglycemia, which may contribute to their antifibrotic effect.

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