Investigation of renin production in the collecting duct during the development of renal allograft dysfunction

Ph.D. thesis

Dr. Csohány Rózsa

Semmelweis University
Doctoral School of Clinical Medicine

Supervisor: Dr. Szabó J. Attila DSc

Official reviewers: Dr. Sulyok Endre DSc
Dr. Maurovich-Horváth Pál PhD

Head of the Final Examination Committee: Dr. Tordai Attila DSc
Members of the Final Examination Committee: Dr. Szíjártó Attila PhD
Dr. Szabó László DSc

Budapest
2016

INTRODUCTION

Due to its' increasing incidence, chronic kidney disease is worldwide a major problem of public health and economic. According to recent epidemiological study more than 10% of the developed countries' population are affected in one stage of chronic kidney disease. Renal replacement therapy is essential in the final stage of the disease. Renal transplantation ensures the patients better quality of life and longer survival compared to dialysis. In optimal cases the function of the transplanted kidney is physiologic for years, however the risk of the development of allograft dysfunction is rising after some years. In the background there are alloantigen-dependent and –independent processes described. In my PhD thesis I investigated the ischemia/reperfusion injury, which is always associated with transplantation, and the nephrotoxicity caused by the commonly used calcineurin inhibitors (CNI). According to the literature these processes leading to the development of graft dysfunction are alloantigen-independent factors. Ischemia/reperfusion injury and CNI nephrotoxicity exerts often together the transplanted kidney.

During renal ischemia/reperfusion the oxygen supply of the kidney tissue decreases, reactive oxygen species and calcium level increases in the cells, finally cell swelling and cell death occurs. It is well known that there are gender differences in the extent of injury: females are less susceptible for ischemia/reperfusion injury than males.

Tacrolimus (Tac) and Cyclosporin A (CyA) are two effective immunosuppressants which are essential to prevent allograft rejection, however due to their nephrotoxic side effect CNIs are one of the main factors of allograft loss in the long run. It is well known in the literature that renal blood flow decreases during CNI treatment.

It is important to note that increased activity of tissue renin-angiotensin system plays a pivotal role in both processes leading to allograft dysfunction. In the past decade it has been described that in certain pathophysiologic conditions, like hypertonia, diabetes mellitus or high salt

diet, the principal cells of the collecting ducts can regain their capability to produce renin which is characteristic for the embryonic period.

A recently identified receptor can draw the attention on the importance of renin synthesis in the collecting duct. Namely (pro)renin receptor [(P)RR], which is the renin's own, cell surface receptor has been described. The explanation of its name is that not only renin, but its precursor (pro)renin can also bound to the receptor (the term (pro)renin refers also to renin and prorenin). The (P)RR can be mainly found on the surface of the intercalated cells in the collecting duct. It is especially important that the renin producing principal cells and the renin sensing intercalated cells are located next to each other in the collecting duct, which enables a highly sensitive local renin-angiotensin system regulation. Due to studying (P)RR revealed the ligand function of renin beyond of the protease function. In addition, this double function is not only characteristic for renin but also for prorenin. After binding to its receptor, prorenin induces a conformational change that uncovers the active site, so that, on a cell surface, prorenin becomes enzymatically active. The binding of (pro)renin to its receptor induces an increase of the catalyctic efficiency of angiotensinogen conversion to angiotensin I. In addition (pro)renin binding to (P)RR results in phosphorylation of the intracellular subunit of the receptor and the mitogen activated protein kinase, and also the activation of the extracellular signal-regulated kinases 1 and 2. Finally, this leads to cell hypertrophy, hyperplasia and activation of profibrotic pathways. This is important because (pro)renin appears to play crucial role in the pathophysiology of nephropathy in diabetes mellitus or hypertension, at least in part via an angiotensin II independent mechanism.

We assume, that renin release beyond the juxtaglomerular apparatus can appear not only in the previously described stress condition, but also in other pathophysiological states and enhanced renin synthesis in the collecting duct could contribute to the development of renal allograft dysfunction.

OBJECTIVES

The extrajuxtaglomerular renin synthesis is nowadays assumed to play an important role in the above mentioned renal injuries. Beyond the classical, systemic effect of renin-angiotensin system exerted via the receptor of angiotensin II, also tissue renin-angiotensin systems and the alternative function of the (P)RR in the collecting duct came in the focus of attention. We investigated the potential role of these effects in transplantation related processes, in renal ischemia/reperfusion injury and CNI nephropathy animal model. Our aim was to study the direct renin synthesis of the principal cells in these two pathophysiological states. Due to the aquaporin 2 receptor, which is characteristic for principal cells we had the opportunity to study separated the renin production of collecting duct. We aimed to answer the following questions:

Ischemia/reperfusion kidney injury:

1. Is there any renin production beyond the classical site of renin synthesis, in the collecting duct during renal ischemia/reperfusion injury?
2. Can gender influence renin production of the collecting duct during reperfusion?
3. Is there any difference in the renal blood supply during reperfusion compared the two genders?

CNI nephropathy:

1. Does CNI treatment increase renin release in the collecting ducts?
2. Do CNIs contribute to the development of nephropathy due to enhanced renin release in collecting duct?
3. What are the possible mechanisms through which locally produced renin participate in the development of CNI nephropathy?

METHODS

All rats and mice used in the study were kept in standard circumstances in a temperature-controlled room (22 ± 1°C) with a 12-hour day-night rhythm. Animals were fed by average diet and tap water *ad libitum*. All *in vivo* experiments were performed according to the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University (22.1/3491/003/2008).

Experiments on the renal ischemia/reperfusion model were performed on ten-week old male and female Wistar rats after anesthesia with an intraperitoneal dose of ketamine (100 mg/kg) and xylazine (10 mg/kg). Following 50 min left sided renal ischemia measurements were performed at the 1-30th minutes and at the 2nd (T2), 8th (T8), 16th (T16), 24th (T24) and 48th (T48) hour of the reperfusion.

In the model of CNI nephrotoxicity three-weeks old, male C57Bl6 mice were divided into five groups based on the following treatment schema. Mice were treated intraperitoneally for 3 weeks with:
1. saline – control group;
2. Cyclosporin A (2 mg/kg/day);
3. Tacrolimus (0.075 mg/kg/day) appropriate initiation doses used in kidney transplantation;
4. Cyclosporin A (2 mg/kg/day) + renin inhibitor Aliskiren (25mg/kg/day);
5. Tacrolimus (0.075 mg/kg/day) + renin inhibitor Aliskiren (25mg/kg/day).

The ratio of renin and VEGF positive cells in the aquaporin 2 positive principal cells were evaluated with flow cytometry. The localization of renin and the diameter of peritubular capillaries were detected by multi-photon microscopy. Structural damage and the amount of renal collagen was assessed by histological evaluation. Collagen I mRNA expression was determined real-time polymerase chain reactions. Whole-blood CNI concentrations were measured using high performance liquid chromatography coupled with tandem mass spectrometry. Plasma renin

activity was measured by angiotensin I radioimmunoassay kit. Serum creatinine was measured to describe the kidney function, in the ischemia/reperfusion model serum creatinine was determined by conventional photometric chemistry analyzer, while in the CNI nephropathy model high performance liquid chromatography coupled with tandem mass spectrometry was used. In CNI nephropathy animal model arterial blood pressure was determined via tail-cuff method. Statistical comparisons were evaluated using ANOVA (for normally distributed data) followed by Fisher-correction or Kruskal-Wallis test (for non-normally distributed data). A p value of <0.05 were considered to be significant. Data were presented as means ± SEM.

RESULTS

Ischemia/reperfusion kidney injury

Serum creatinine was measured to evaluate renal dysfunction in the different time points of reperfusion following ischemia. After ischemia/reperfusion kidney injury, level of serum creatinine significantly increased by the 8th hour of reperfusion and remained elevated also in later time points without any significant gender differences. These data suggested a marked deterioration in renal function and proved that our animal model was capable to investigate ischemia/reperfusion-induced acute renal failure.
The validation of our model, the progression of the ischemic injury and the existence of the gender difference were confirmed by **periodic acid-Schiff staining**. Already at T2 a significant damage has developed in both gender. In males already by T8 developed the severe ischemic injury, while in females this process took much prolonged and it reached its peak only at T24. A significantly worse histology of renal parenchyma in males could be seen at T8 which was present along the later examined time period.
Applying **flow cytometry** we investigated the percentage of the renin positive aquaporin 2 positive principal cells derived from the whole kidney. In control male group more principal cells contained renin than in females. The amount of renin positive cells decreased in collecting duct at T2 and T8 in both genders. From T16 there was an increase in the ratio of renin positive principal cells in both genders, however the increase was more pronounced in male and it reached the level of significance at T48.
Renin content in the juxtaglomerular apparatus (JGA) and collecting duct was visualized by **multi-photon microscopy**. The control group showed renin granules in traces in both JGA and collecting duct, this amount got even less in both localizations at T2 and T8. From T16, renin granulation increased in both JGA and collecting duct, which became even more pronounced at T24 and indeed robust at T48. At this time point the renin production was more pronounced in males in both localizations compared to females.
We observed that renin produced in the collecting duct after ischemia/reperfusion injury was secreted in two directions: to the systemic circulation and into the tubular lumen.

There was no significant difference in **peritubular capillary diameter** in control groups comparing the two genders. However, from the 2nd minutes of reperfusion a significant difference appeared between capillary diameters of males and females. There was a longer constriction in the capillary diameter of males. Then, at the 15th minutes of reperfusion, the difference turned around: more dilated capillaries could be seen in males than in females.

CNI nephropathy

To examine kidney function, we measured **serum creatinine** levels after 3 weeks of CNI treatment. Controls showed physiologic range of creatinine, while after CNI administration these values doubled, suggesting a significant deterioration of kidney function. Co-administration of CNIs with Aliskiren resulted in a remarkable amelioration of the decline of kidney function.

To exclude that the CNI treatment results in damage to the kidney via influencing the **blood pressure**, we measured the blood pressure and did not find any significant difference between the controls and treated groups.

To evaluate our treatment, we measured the **trough level of CyA** as well as **Tac**. Compared to the controls, we have seen a blood trough levels comparable to human target range.

We measured the **plasma renin activity** to determine whether CNI treatment results in only a local or as well as systemic increase in renin activity. In CyA and Tac-treated mice renin activity increased significantly compared to vehicle-treated animals. This increase was abolished when CNIs were given in combination with Aliskiren.

Applying **flow cytometry** we investigated the ratio of the renin positive aquaporin 2 positive principal cells derived from the whole kidney tissue. In vehicle-treated animals the percentage of renin-positive cells was approximately 3% of all the principal cells, which increased significantly in CyA- and Tac-treated mice. This effect was almost completely abolished by the direct renin inhibitor Aliskiren.

Multi-photon microscopy was performed to visualize the changes in renin content and its localization after 3 weeks CNI treatment. In the collecting duct the control group showed renin granules in trace, while the two CNI treated groups presented robust expression of renin granules densely populated close

to the apical and basal membrane of the principal cells. In case of combined treatment of CNIs with Aliskiren, the renin content remarkably decreased in this localization, almost to the level of controls.

Following 3 weeks of CNI treatment the **peritubular capillaries** showed significantly reduced peritubular capillary diameter compared to control animals. Aliskiren prevented the remarkable decrease of vessel diameter in both CNI-treated groups.

Looking at the change in vessel diameter closely, we could recognize a very disproportional vascular growth in the CNI-treated groups mostly in the proximity of collecting ducts. There were areas where remarkable reduced capillary diameter was present, in other cases highly dilated vessel assemblies and turbulent blood flow were observed by multi-photon microscopy. To gain a closer insight into the underlying pathomechanism, **vascular endothelial growth factor (VEGF) content** in the aquaporin 2 positive principal cells was investigated by flow cytometry. Following 3 weeks of CNI treatment there was a significant elevation in VEGF production in principal cells, which was abolished by co-treatment with direct renin inhibitor Aliskiren.

Interstitial fibrosis is one of the main histological hallmarks of CNI nephropathy. We evaluated the amount of collagen I – one marker of the histological changes – by measuring the **mRNA expression of collagen I** in whole kidney. There was a significant increase in collagen I mRNA expression in both CNI treated groups compared to controls. In contrast, Aliskiren treatment prevented the increased collagen I synthesis in the kidney.

We investigated the structural changes concerning the whole kidney with **Masson's trichrome staining** which is appropriate for collagen dyeing. To evaluate the pro-fibrotic effect of CNI treatment and the beneficial effect of Aliskiren on the progression of CNI nephropathy we evaluated the extent and the exact location of collagen. In controls there was a weak collagen staining around the arterioles, but we could hardly detect any interstitial staining. Three week CNI treatment resulted in significantly elevated amount of collagens mostly in the close proximity of collecting ducts. The increased rate of collagen deposition was abolished when CNIs were administrated in combination with Aliskiren.

CONCLUSIONS

The aim of my PhD thesis was to gain a better understanding of the underlying processes of allograft dysfunction developed as a consequence of kidney transplantation (Figure 1.). We investigated the renin production of principal cells in the collecting duct during ischemia/reperfusion injury, which is always associated with transplantation, and in the nephropathy caused by the commonly used CNIs. Based on our results we can draw the following conclusions.

1. We described the renin response in the acute model of renal ischemia/reperfusion injury not only in the JGA, but also in the collecting duct.

2. We demonstrated that in males the renin response is more pronounced compared to females. Males possess not only more renin content in physiological states, but renin synthesis is more pronounced also during renal ischemia/reperfusion injury.

3. Females show more balanced peritubular capillary diameters in the kidney cortex compared to males, which enables a more stable blood supply in the kidney cortex.

4. We demonstrated that beyond the previously known pathophysiological states, CNI treatment also induce renin production in the collecting duct.

5. The enhanced renin activity in the collecting duct can contribute to the development of CNI nephropathy. This is confirmed by the beneficial effect of Aliskiren in our CNI nephropathy animal model: we assume that Aliskiren is able to prevent at least some course leading to chronic allograft nephropathy.

6. Parallel with the increased renin production in the collecting duct, the diameter of peritubular capillaries results in decreased renal blood flow. Due to local hypoxia, principal cells in the collecting ducts respond with

pathophysiological VEGF production resulting in disproportional vessel growth, further worsening the local hypoxia, and striped fibrosis.

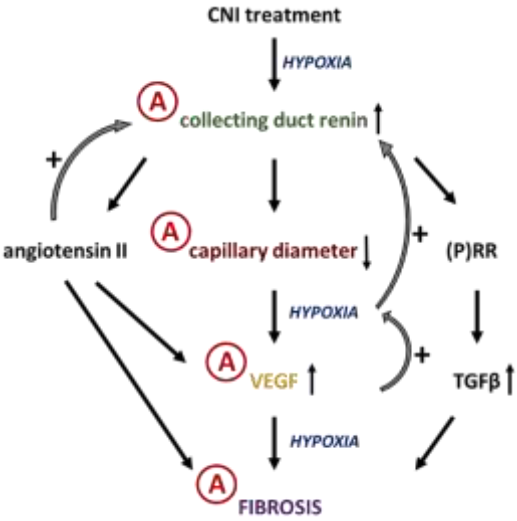

Figure 1. Potential pathophysiological pathways leading to CNI nephropathy. Treatment with CNIs are known to decrease renal blood flow resulting in local hypoxia in the kidney, which leads to enhanced renin production beyond in the JGA, also in the collecting duct. Renin increases the formation of angiotensin II, which leads directly and also through pernicious VEGF synthesis to fibrosis. The high amount of renin resulting in local hypoxia is partly also responsible for pernicious VEGF synthesis. It is important to note, that renin can also play a role in the development of CNI nephropathy via its own receptor. In our study Aliskiren was able to prevent the deteriorative renin and VEGF production, the reduction of peritubular capillary diameter and the development of fibrosis. A: Aliskiren; CNI: calcineurin inhibitor; JGA: juxtaglomerular apparatus; (P)RR: (pro)renin receptor; TGF β: transformation growth factor β; VEGF: vascular endothelial growth factor.

THE BIBLIOGRAPHY OF THE CANDIDATES'S PUBLICATIONS

Publications related to the PhD thesis:

Csohány R, Prókai Á, Sziksz E, Balicza-Himer L, Pap D, Kosik A, Sugár D, Vannay Á, Kis-Petik K, Fekete A, Szabó AJ. Sex differences in renin response and changes of capillary diameters after renal ischemia/reperfusion injury. *Pediatric Transplantation.* 2016 Apr. doi: 10.1111/petr.12712. *(IF: 1,441)*

Prókai Á and **Csohány R**, Sziksz E, Pap D, Balicza-Himer L, Boros Sz, Vannay Á, Kis-Petik K, Fekete A, Peti-Peterdi J, Szabó AJ. Calcineurin-inhibition results in up-regulation of local renin and subsequent VEGF production in renal collecting ducts. *Transplantation.* 2016 Feb;100 (2):325-33 *(IF:3,828)*

Csohány R, Prókai Á, Kosik A, Szabó JA. A gyűjtőcsatorna meghatározó szerepe a vese lokális renin-angiotenzin rendszerében. *Orvosi Hetilap.* 2013 154 (17):643-9.

C**sohány R**, Prókai Á, Kosik A, Szabó JA. Multi-foton mikroszkópia a vesekutatásban. *Gyermekgyógyászat* 2013; 64. Vol. 2.

Other – not to the PhD thesis related – publications:

Szebényi K, Füredi A, Kolacsek O, **Csohány R**, Prókai Á, Kis-Petik K, Szabó AJ, Bősze Zs, Bender B, Tóvári J, Enyedi Á, Orbán TI, Apáti Á and Sarkadi B. Visualization of calcium dynamics in rat kidney proximal tubules. *JASN.* 2015 Nov;26(11):2731-40. *(IF: 9,343)*

Kerti A, **Csohány R**, Wagner L, Jávorszky E, Maka E, Tory K. *NPHS2* homozygous p.R229Q variant: potential modifier instead of causal effect in FSGS. *Pediatr Nephrol*. 2013 28(10):2061-4. *(IF: 2,856)*

Kerti A, **Csohány R**, Szabó A, Árkossy O, Sallay P, Moriniére V, Vega-Warner V, Nyírő G, Lakatos O, Szabó T, Lipska BS, Schaefer F, Antignac C, Reusz G, Tulassay T, Tory K. *NPHS2* p.V290M mutation in late-onset steroid-resistant nephrotic syndrome. *Pediatr Nephrol*. 2013 28(5):751-7. *(IF: 2,856)*

Kerti A, Jávorszky E, **Csohány R**, Varga N, Szabó A, Árkossy O, Sallay P, Balogh L, Szabó T, Reusz Gy, Tulassay T, Tory K. A szteroid-rezisztens nephrosis szindró, a genetikai vizsgálata Magyarországon. *Gyermekgyógyászat* 2013; 64. évfolyam 1. szám