Therapeutical options in prevention and treatment of diabetic and allograft nephropathy

Doctoral thesises

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Prevention of chronic disease in childhood

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INTRODUCTION

At present, every tenth person is suffering from kidney disease in Hungary and every year 500-600.000 patients with renal disease have to visit a nephrologist. By these days diabetic nephropathy is considered as the main underlying disease leading to chronic kidney disease, whose definitive therapeutic approach is the transplantation. Nowadays due to the immunosuppressive treatment, the incidence of acute rejection decreased and the chronic allograft nephropathy has become the leading cause of graft failure. In my Ph.D. dissertation, I investigate the relationship between high level of glucose and increased renin production in the collecting duct (CD) and hypothesize a crucial role of the G-coupled succinate receptor involved in this process. Afterwards from the therapeutic point of view I discuss three risk factors influencing the long-term outcome of the transplantation: the nephrotoxicity of the calcineurin inhibitors (CNIs), the post-transplant diabetes mellitus caused by immunosuppressive treatment as well as other risk factors and the protective effect of erythropoietin (EPO) in case of ischemia/reperfusion kidney injury.

AIMS

1. To validate the specificity of the FRET-based renin substrate and beyond the description of basolateral and luminal localization of the (pro)renin to demonstrate its release in CD principal cell.
2. To examine under diabetic conditions weather succinate through the GPR91 downstream signal pathway is one of the most important regulators of the local RAS in the CD revealing a new anti-fibrotic therapeutical target in diabetes mellitus.
3. To prove that especially in the connecting segment and CD CNIs take effect at least partially through the local RAS, supposing a potential promising terapeutical target in the prevention of chronic allograft nephropathy.
4. To investigate the protective effect of EPO following severe, unilateral renal I/R injury, to analyze different EPO effects in female

and male rats and finally to clarify the role of HSP72 and Na+/K+ATPase in the EPO signal pathway.

5. To evaluate the incindence of the IGT and PTDM among our transplant patients, the riskfactors characteristic for the pediatric renal transplanted population with special attention to the immunosuppression. Furthermore, to estimate the importance of OGTT in the transplanted children's look after.

METHODS

To address the abovementioned questions both *in vitro* and *in vivo* animal studies were performed, finally we applied some clinical studies:

- Cell lines and their treatment
- Animal models
- Gene expression studies
- Western blot
- Spectrofluorometry
- Fluorophore*s*
- Immunocitochemistry and immonohistochemistry
- Histological analysis
- Dissection of the collecting duct
- Micropunction
- *In vivo*, multi-photon fluorescent imaging of the kidney
- Spectrofluormetry for the urinary renin activity
- PGE_2 urinary secretion
- Renal function parameters
- Flow cytometry (FACS analysis)
- Human studies

RESULTS

Confirmation of the Specificity of FRET-Based Fluorogenic Renin Substrate by Spectrofluorometry and Multi-Photon Microscopy.

The pre-trypsinization activity measures endogenous active renin, and the post-trypsinization activity reflects the activity of both native renin and activated prorenin. Firstly, our results revealed that in control M1 cells (collecting duct derived cells) the prorenin content was 2.5-fold higher than the endogenous renin activity. While in the second set of experiment FRET-based renin substrate's specificity was confirmed for (pro)renin and not for other proteases, since pre-incubation of samples with 100 μmol/L of Aliskiren silenced the signal by sixty percent while 250 μmol/L of Aliskiren completely abolished the fluorescent sign. The specificity of FRET-based renin substrate was further conformed *in vivo*. Intact kidney was micropunctured and stained. While control db/db mouse showed robust granulation as well as luminal renin activity. 2-week Aliskiren treatment (50mg/kg i.p.) of the mice resulted in significant reduction in luminal renin activity.

Confirmation of FRET-Based Fluorogenic Renin Substrate Specificity Applying Confocal Microscopy.

To further study the renal specificity of FRET-based renin substrate, As4.1 (JGA derived cells) and M1 cells were used. Both cell lines *de novo* produce renin. Forskolin, an adenylcyclase activator and IBMX, a phosphodiesterase inhibitor were conducted across multiple concentrations and time courses, with optimal effects at 10 μM or 500 μM, respectively, for 24 h. Immunocytochemistry confirmed the consequence of increased intracellular levels of cAMP, the activation of the renin synthetic machinery, the up-regulation of (pro)renin content in collecting duct derivated cells. The close proximity of the renin containing granules intracellularly and the released active renin on the surface of the cell validates the relevance of the application of the FRET-based fluorogenic renin substrate.

Direct Visualization of Renin Storage, Release and Activity in As4.1 and M1 Cells *in Vitro*.

Renin release was studied using living cell lines activated by forskolin and IBMX. FRET-based renin substrate, a fluorophore that selectively stains renin activity, was added to the media 10 minutes prior to the imaging to label renin activity. Reticular pattern of staining was observed using Lyso Tracker Red revealing the synthetic machinery of the endoplasmic reticulum and the Golgi apparatus. Gradually the vesicles become yellow since the maturation of (pro)renin is completed and the protein showed activity. While the granules close to the cell membrane were representative of cell-membrane fused vesicles ready to release active renin. One of the figures showed the two representative cell types of the collecting duct and their participation in the local RAS. On the two sides principal cells showed huge granulation which granules not just stored active renin but they were apparently released toward the apical membrane. Here the secreted renin contributes to the activation of the luminal RAS, furthermore, as part of that on the surface of the intercalated cells supposedly by the (pro)renin receptor [(P)RR] both prorenin and renin got bounded hence further activated.

Renin Activity in the Collecting Duct *in Vivo.*

Our state-of-the-art multi-photon fluorescence laser scanning microscopy has been utilized to visualize the *in vivo* renal tissue and doing so, provided exceptionally valuable scientific evidences of renal anatomy as well as (patho)physiology. In combination with our primary approach, we established the fine art of micropuncture technique in our research laboratory, which now allows the renin substrate to be injected directly into the collecting duct avoiding the systemic degradation and glomerular filtration barrier. We strongly believe that FRET-based renin substrate may provide more insight about the stored as well as the secreted (pro)renin in the collecting duct. Representative collecting duct of a diabetic mouse has renin producing capacity and shows abundant granulation in the principal cells.

Moreover, as the co-localization revealed, these granules not only store (pro)renin but are also filled with active renin. After the liberation of this active (pro)renin they accumulate in the tubular flow and may bind to the (P)RR appearing as a thin line on the surface of the intercalated cell. Nicely verifying the relevance of the application of the FRET-based fluorogenic renin substrate we were able to detect the renin activity from urine samples as well; the low levels of renin activity in wild type mice enhanced robustly in diabetic murine urine.

GPR91 mRNA and protein expression and localization.

In order to confirm the expression and localization of GPR91 mRNA and protein in cells of the CD, qPCR and immunocytochemistry were performed. The mouse CD-derived M1 cell line expressed high levels of GPR91 mRNA compared to other relevant renal cell types. Immunocytochemistry showed distinct cell membrane localization of GPR91 protein, but intense labeling was found also intracellularly, likely in the protein synthetic machinery in the endoplasmic reticulum and the Golgi apparatus. High level of GPR91 protein expression in cells of the distal nephron-CD and apical membrane localization were further confirmed in the mouse kidney tissue by immunohistochemistry, revealing the connecting tubule and the CD as the most important site of renal GPR91 expression.

GPR91 signaling in M1 cells.

It has been established that in cells of the macula densa (JGA) the succinate/GPR91 signaling cascade activates phosphorylation of MAP kinases ERK1/2 and p38, and consequently increases COX2 activity and PGE2 synthesis and renin secretion. Therefore, we tested if GPR91 signals via a similar mechanism in M1 cells. Immunoblots of M1 cells and densitometry analysis demonstrate that a 24 hour succinate treatment activated MAPK-COX2 signaling in a sustained, dose-dependent manner. Treatment of M1 cells with increasing concentrations of succinate (10μM, 100μM and 1mM) resulted in a

1.28, 1.38, and 2.08-fold increase in pERK1/2, 1.31, 1.29, and 1.35-fold increase in pp38, and 1.45, 1.76, and 1.91-fold increase in COX2 protein levels compared to control, respectively. The same doses of succinate caused significant increases in M1 cell renin synthesis (1.3, 1.32, and 1.98-fold increase compared to control), whereas prorenin levels at the end of the 24 hour treatment were increased only after 1mM succinate administration (1, 0.98, and 1.36-fold increase compared to control). One of the newest RAS elements, the (P)RR showed also higher expression in response to the same increasing succinate concentrations (1.20, 1.56 and 1.86-fold increase compared to control). Next, the time-course of the succinate-induced elevations in M1 cell prorenin, renin, and (P)RR expression were studied using 1 mM succinate. At the end of the 1^{st} - 5^{th} hour after succinate treatment prorenin increased 1.07, 1.02, 1.46, 2.23, and 2.21-fold, renin increased 1.08, 1.24, 1.18, 1.76, 1.78-fold, and (P)RR levels increased 1.29, 1.26, 1.43, 2.64, and 2.72-fold, respectively. The stimulatory effects of 1mM succinate on M1 cell prorenin and renin expression were abolished in the presence of 10 μM PD98059, a MEK-1 inhibitor or 50 μM SC58236, a selective COX2 inhibitor (1.36, 0.76-, and 1-fold increase compared to control in succinate only, or with MEK-1, or COX2 inhibitor, respectively, for prorenin, and 1.32, 0.81,and 0.85-fold increase compared to control in succinate only, or with MEK-1, or COX2 inhibitor, respectively, for renin).

In vivo relevance of GPR91-mediated ERK1/2 activation in mouse CD.

Next, we assessed whether the GPR91-mediated signaling events observed in M1 cells can be extrapolated to conditions in the medullary region of the STZ-diabetic mouse kidney *in vivo*. Blood glucose levels were significantly and equally increased both in WT and $GPR91^{-/-}$ DM mice (>500mg/dl in all STZ-treated animals). Medullary CD segments, identified by intense aquaporin-2 staining, showed robust increase in phosphorylated ERK1/2 levels in diabetic WT mice compared to non-diabetic controls (56% of the total CD cells showed pERK1/2 labeling

in DM compared to zero in control). In contrast, pERK1/2 levels were below the limits of detection in kidneys of both diabetic and non-diabetic GPR91$^{-/-}$ mice.

Urinary PGE_2 excretion is GPR91-dependent in diabetes.

PGE_2 is a classic paracrine mediator of renin synthesis and its secretion in the JGA, and succinate-induced PGE_2 release in the renal cortex from macula densa cells was shown to be GPR91-dependent. Therefore, we tested if medullary PGE_2 levels, measured by urinary PGE_2 excretion, may reflect the GPR91-dependent regulation of CD function. Consistent with the data on MAPK-COX2 activation in M1 cells, urinary PGE_2 was elevated in diabetes, and this response was completely abolished in GPR91$^{-/-}$mice (1.6±0.8, 3.0±0.5, 1±0.4, and 1.3±0.4ng/24 hours in WT, DM,GPR91$^{-/-}$, and DM GPR91$^{-/-}$ mice, respectively).

Succinate / GPR91 signaling increases (pro)renin and (P)RR expression in the renal medulla in diabetes.

To further address the role of succinate/GPR91 in the activation of the local CD RAS, western blots were performed using renal medullary tissue to avoid the potential contaminants from the cortical JGA. The expression of both (pro)renin and (P)RR proteins showed a robust increase in response to STZ-diabetes (2.06, 4.12, and 3.26-fold increases compared to WT control in prorenin, renin, and (P)RR, respectively). In contrast, STZ-diabetes failed to increase the renal medullary expression of prorenin, renin, and (P)RR in GPR91$^{-/-}$ mice (0.62, 1.47, and 1.34-fold increases compared to WT control, respectively) suggesting the pivotal role of succinate/GPR91 signaling in the regulation of CD RAS.

Measurement of renin activity in the intact CD *in vivo* and in the urine.

Multiphoton fluorescence microscopy of the intact rat and mouse kidney *in vivo* has been established and used to quantitatively visualize various (patho)physiological parameters of kidney function including JGA and CD renin content and activity in the JGA. In the last set of experiments multiphoton imaging was performed to directly visualize the changes in renin activity in the tubular fluid of the CD in diabetes *in vivo* and to establish its GPR91-dependence. Local delivery of the fluorogenic renin substrate into the lumen of cortical tubules was achieved by using classic renal micropuncture techniques in order to study local renin activity and to avoid systemic cleavage and glomerular filtration of the renin substrate. Compared to control WT non-diabetic mice, STZ-diabetes caused a robust increase in renin activity in the CD tubular fluid. The labeling appeared to be specific for the CD since the lumen of other tubular segments surrounding the CD was negative. In addition, significant intracellular renin activity was found in cells of the CD in diabetic mice that appeared to be localized within acidic vesicles. In contrast, CD renin activity, both intracellular and in the tubular fluid remained at the low baseline levels in diabetic GPR91$^{-/-}$ mice (renin activity in the CD lumen increased 4.05, 1.08, and 1.20-fold in DM, KO, DM+KO mice compared to WT controls, respectively). Specificity of the fluorogenic renin substrate was tested by treating diabetic mice with the direct renin inhibitor Aliskiren. Majority of the signal in the CD lumen (54%) was lost in response to Aliskiren treatment (Fig.8F). Spectrofluorometry analysis of renin activity in urine samples collected from the same mice provided further support for the *in vivo* imaging results. In contrast to the low levels of renin activity found in WT and GPR91$^{-/-}$non-diabetic mice, STZ-diabetes resulted in robust elevations in urinary renin activity in WT but not in GPR91$^{-/-}$ mice (3.02,1.37, and 1.33-fold increase in DM, KO, and DM+KO mice, respectively, compared to WT).

The multi-photon imaging of renin secretion caused by immunosuppressants.

In our studies we have investigated the amount and the secretion of renin induced by the immunosuppressants in three different groups. In control JGA only very few granular cells were found being able to secrete renin, moreover in the collecting duct, as we expected, there was not any of the cells which showed renin granulation. On the contrary, in Tac treated group the JGA contained much more granular cells redifferentiated for production of renin. Apparently, even in the localization of collecting duct an increased granulation was observed. In CyA treated animals we revealed the same increased amount of renin in both localization.

The detection of immunosuppressants induced renin production measured by flow cytometry.

Using flow cytometry we sorted the abovementioned regions. AQP2 was applied for the separation of principal cells thus this cell population, responsible for water homeostasis, becomes possible to be visualized separately from every other kidney cells. After that in both cell populations we divided the renin producing cells from every others. While in controls only 2% of the principal cells produced renin, following three weeks of immunosuppressant administration it increased significantly, almost 4 times. In case of JGA the renin content followed similar dynamics.

Increased renin production induced vasoconstriction detected by multi-photon microscopy.

One of the direct renin effects, the vasoconstriction was monitorized by multi-photon microscopy. The approximately 7 μm diameter in control rodents decreased by 2 μm, thus the increased renin secretion resulted in a 50 % contraction in both investigated groups.

Description of the increased renin secretion induced vasoconstriction and hypoxia.

The Masson staining of control animals did not show any fibrosis, while in calcineurin treated groups there were a lot of collagen stripes running parallel with vessels in blue.

Immunosuppressives induced deterioration in kidney function followed by labor parameters.

As a last step in our experimental setting we investigated the functional deterioration of the kidney. both the CyA and Tac treated group showed a significant increase in the renal function parameters (creatinine levels) compared to the controls.

Renoprotective effect of erythropoietin in rats subjected to ischemia/reperfusion injury.

Survival.

The EPO administration resulted in a remarkable amelioration of male's post-ischemic survival. Although all untreated males died as a result of acute renal failure by the third day, those treated with EPO survived until the sixth day, almost twice as long as those untreated. EPO resulted in a slight improvement in females as well. To note, however, irrespective of the EPO administration, the 7-day survival was still better in females compared with their male counterparts.

Serum EPO level.

The serum level of endogenous EPO was higher in males vs females not only in controls but post-ischemic ($P < .05$; .001). EPO administration increased control and T2 EPO levels in both genders (untreated vs treated $P < .05$; .01), whereas at T24, the effect of exogenous EPO

treatment disappeared. The dynamics of post-ischemic changes in serum EPO levels followed a remarkably different manner between males and females; in males, the already higher EPO decreased only at T24, whereas in females, the EPO level dropped to the third of the control value already at T2.

BUN and serum creatinine levels.

Fifty minutes of ischemia resulted in an acute renal failure as indicated by a progressive increase in renal function parameters in both sexes (control, T2 vs T24 $P < .001$). In males, the EPO treatment ameliorated the post post-ischemic kidney failure indicated by lower BUN and creatinine levels (at T24 vehicle vs EPO treated $P < .01$). EPO had no effect in females. Interestingly, at T24, the renal function parameters were even lower in EPO-treated males than in females with and without EPO treatment.

Renal histopathology.

Kidneys from control rats showed normal kidney structure in all groups. After 50 min of ischemia, a progression was observed in the extent of tubular epithelial cell damage (loss of brush border, tubular dissolving, cell death, and loss of the nucleus integrity) as well as in the amount of hyaline casts until T24 (control vs T2 vs T24 in all groups $P < .05$). EPO ameliorated each evaluated parameters in both sexes without any gender difference (EPO vs vehicle treated $P < .05$).

HSP72 protein levels.

The HSP72 level was higher in untreated females than in male counterparts at every time point. After the ischemic insult, HSP72 protein levels increased (control vs T2 $P < .05$) in a gender-dependent manner. In females, HSP72 reached its maximum at T2, whereas in males, the rate of increase was slower both with and without EPO treatment (female vs male $P < .05$; .01). EPO increased the HSP72

protein level in males at T24, whereas in females, the already higher HSP72 level was not elevated (male vs female $P < .01$).

Na+/K+ATPase-a1 subunit protein levels.

Similar to HSP72, the post-ischemic changes in Na+/K+ ATPase-a1 protein levels were different between the sexes. Na+/K+ATPase-a1 protein levels were higher in untreated females than in males at every time point ($P < .001$; .001; .05). EPO treatment was effective only in males by increasing the protein level at T24 (control and T2 vs T24 $P < .01$) to a level even higher than the EPO treated females ($P < .01$).

Immunolocalization of HSP72 and Na+/K+ATPase- a1 subunit.

Immunofluorescent staining was used to investigate the potential relationship between HSP72 and Na+/K+ATPase-a1. In the tubules of control rats, Na+/K+ATPase-a1 was localized on the basolateral membrane domain of tubular cells, with minimal staining in the cytosol or the apical domain. No gender differences were observed at this time point. In contrast, HSP72 staining was virtually undetectable in the tubular cells of control rats. After ischemic injury, Na+/K+ATPase-a1 became more prominent in the cytosol compared with the controls, but this internalization was less significant in untreated females vs males. After EPO treatment, however, the Na+/K+ATPase-a1 localization remained more pronounced on the basolateral membrane in males as well.

Post-transplant diabetes mellitus in pediatric transplants.

Between 1990 and 2006, 45 primary renal-only transplants were performed in our institution. The causes of transplantation were as follows: focal segmental glomerulosclerosis (n = 10), hypoplastic kidney (n = 9), polycystic kidney disease (n = 8), obstructive nephropathy (n = 5), interstitial nephritis (n = 3), Alport syndrome (n = 2), Prune belly syndrome (n = 2), juvenile nephronophthisis (n = 1),

idiopathic rapidly progressive glomerulonephritis (n = 1), membranous glomerulonephritis, nephrocalcinosis (n = 1), cystinosis (n = 1), acrorenal syndrome (n = 1) and acute tubular necrosis (n = 1). Table 1 summarizes demographic data of our kidney-transplanted patients. Thirty-two of 45 children (71%, 20 boys/12 girls) had normal glucose tolerance. IGT was found in seven cases (16%, 3 boys/4 girls). In IGT patients during OGTT serum glucose level was 5.3 ± 0.95 mmol/L (at 0 min), 10.9 ± 1.3 mmol/L (at 60 min) and 9.6 ± 0.6 mmol/L (at 120 min). PTDM developed in six patients (13%, 2 boys/ 4 girls). Mean duration of onset after PTDM after transplantation was 25.8 months (range 0.5–119); however, the duration time was shorter than one yr in 70% of the patients. Glucose metabolic disorder (PTDM + IGT) was reversible in six patients. The primary cause of transplantation was autosome recessive polycystic kidney disease in three of six PTDM patients requiring continuous anti-diabetic therapy. Patients with PTDM + IGT are younger (14.1 ± 4 yr) than those with normal glucose tolerance (18.4 ± 6 yr; $p = 0.02$). There was no difference in the type and duration of dialysis, or in the ratio of deceased/living grafts. Family history was positive in more patients with PTDM + IGT than with normal glucose tolerance ($p = 0.03$). Systolic blood pressure and serum triglyceride level were elevated in patients with PTDM + IGT compared with levels of children with normal glucose tolerance ($p = 0.02$ and 0.039, respectively). No significant difference was found in the other investigated parameters (Table 1). Patients demographics in the different immunosuppressant groups have been summarized. Tac-treated patients were younger ($p = 0.0001$), with higher incidence of diabetes in family history ($p = 0.002$). Tac treated children had higher GFR ($p = 0.04$) and HOMA-R ($p = 0.05$) and lower serum cholesterol level ($p = 0.05$) compared with CyA-treated group, while there was no difference in the other parameters investigated. To assess the association between immunosuppressive treatment and glucose metabolic disorder (PTDM + IGT), we analyzed the onset of PTDM + IGT in the different immunosuppressive regimes. Fig. 1 shows that 12 of 34 Tac treated patients (35%) developed PTDM + IGT compared with one of 11 CyA-treated children (9%; $p < 0.05$). Moreover, Tac

trough level was higher in patients with PTDM + IGT than in children with normal glucose tolerance ($p = 0.04$). After multiple stepwise regression analysis Tac trough level showed significant association with insulin level ($T = 0$ min, $p = 0.015$; $T = 120$ min, $p = 0.041$, respectively), which refers to decreased insulin secretion in these patients. To evaluate the importance of steroid therapy in the development of glucose metabolic disorder, the daily dosage and the number of steroid pulses were compared in transplanted children. Fig. 3 demonstrates that the number of steroid pulses was higher in patients with PTDM +IGT than with normal glucose tolerance ($p = 0.036$); however, the difference in the daily dosage of steroids was not significant. By multiple stepwise regression analysis independently of other factors, there was a significant association between steroid daily dosage and elevated fasting blood glucose level ($p = 0.0002$), and between the number of steroid pulses and HOMA-R ($p = 0.038$) level.

THESISES

1. Using multi-photon microscopy and the recently developed FRET-based renin substrate validated by our lab we have identified and demonstrated the basolateral localization of the CD (pro)renin.
2. Our study established succinate and GPR91 signaling as novel and important regulators of CD (pro)renin and (P)RR, the first key elements of the local RAS. *In vivo* relevance and functional significance of this new signaling mechanism were demonstrated by finding GPR91-dependent up-regulation of pERK1/2, (pro)renin and (P)RR expression in the renal medullary CD, urinary PGE_2 excretion, and increased renin activity in the tubular fluid of the CD in diabetes that was directly and quantitatively visualized for the first time using multiphoton microscopy of the intact mouse kidney *in vivo*. Succinate and GPR91 signaling are novel pathophysiological mechanisms in DM that regulate the activity of the local CD RAS and may serve as new therapeutic targets in diabetic nephropathy.

3. Beyond the JGA we have revealed the production and secretion of renin in deeper layers of the kidney, in the CD possessing a much prominent cell population. Based on our studies both the CyA and the Tac are able to enhance the renin secretion in the CD resulting in chronic renin effects and deterioration of kidney function.
4. Our studies have showed a protective effect of EPO against the severe, unilateral renal I/R injury, especially in male rats. This beneficial effect partly might be the result of EPO's HSP72 mediated impact on Na+/K+ATPase- α1.
5. Our results indicate that the incidence of glucose metabolism disorder is relatively high in renal transplanted children and the development of PTDM+IGT seems to be clearly associated with Tac and corticosteroid therapy.

PUBLICATIONS

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