New pathophysiological pathways and therapeutical approaches in diabetic and ischemic renal injury

PhD Thesis

Nóra Fanni Bánki, M.D.

Semmelweis University
Clinical Medicine PhD School

Supervisor: Andrea Fekete, academic demonstrator, M.D., Ph.D.
Programleader: Tivadar Tulassay, professor, member of HAS

Reviewers: Tamás Szabó, associate prof., M.D., Ph.D.
Gábor Kökény, academic demonstrator, M.D., Ph.D.

Head of Exam. Comm.: Zoltán Prohászka, professor, M.D., Ph.D.
Exam. Committee: Norbert Fülöp, physician, M.D., Ph.D.
Pál Soós, academic demonstrator, M.D., Ph.D.

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Introduction

The growing prevalence of chronic kidney disease (CKD) is a serious economical and healthcare problem. This increasing tendency might be due to the epidemic occurrence of the main risk factor diabetes mellitus: according to WHO statistics, the number of diabetic patients will double within the year of 2030. The long term complication of diabetes, diabetic nephropathy (DNP) is the leading cause of end stage renal disease.

Two main hallmarks in the pathomechanism of DNP are glucotoxicitiy and the activated renin-angiotensin-aldosterone system (RAAS), which both induce fibrotic kidney destruction. One startpoint of fibrosis is the epithelial-mesenchymal transition (EMT), during which epithelial cells start to express alfa-smooth muscle actin (αSMA).

According to our previous studies angiotensin (Ang) II worsens the progression of DNP through the impairment of the sodium/potassium ATPase (NKA), which is crucial for renal function. Current data suggest that beside Ang II, aldosterone also might play a pivotal role in the progression of DNP, but the exact pathomechanism is still unknown.

Current international and national protocols suggest the usage of Ang II inhibitors in the treatment of DNP. However, several data indicate that these protocols do not slow the progression of renal damage sufficiently; most of the patients develop end stage renal disease.

Renal transplantation (NTx) is the obligate therapy for end stage renal disease. The long term outcome of NTx is influenced by several allogen independent factors, of which ischemia/reperfusion (I/R) injury is the most important. We have shown previously that female rats are more resistant against I/R caused by lower endothelin expression and more stable NKA function. Agonists of the Sigma-1 receptor (Sigma-1R) have been found to be protective against heart and brain ischemia recently.

The dissertation analyzes the pathomechanism of diabetic kidney damage emphasizing on the role of enhanced RAAS activity and reviews the main pathophysiological components of I/R, which plays a pivotal role in long term outcome of NTx. Our aim was to compare the efficacy of different RAAS inhibitors used in monotherapy and to analyze the role of a novel signaling pathway, the Sigma-1R – nitric-oxide synthase (NOS) cascade in the prevention of renal destruction.

Aims

Our aim was to analyze the processes influencing the progression of DNP and to study molecules which play a role in the pathomechanism of I/R and to evaluate novel therapeutic targets in both entities. Our questions were as follows:

1. DNP:
a. How do the different RAAS blockers used in monotherapy affect diabetes induced structural and functional kidney damage?
b. What is the impact of the RAAS blockers in diabetes on EMT and on the function of NKA?
c. Does hyperglycemia per se, independently from its osmotic effect influence these changes?

2. I/R:
a. Does the Sigma-1R agonist FLU prolong postischemic survival after renal I/R?
b. Does FLU ameliorate postischemic structural and functional kidney damage?
c. Has FLU any impact on the renal Sigma-1R – Akt - NOS system?
d. Are the effects of FLU exerted during renal I/R specifically Sigma-1R associated?

Methods

All experiments were performed on mature male Wistar rats.

1. DNP – after five weeks of streptozotocin (STZ, 65 mg/bwkg *ip.*) induced diabetes animals were treated for 2 weeks *per os* with: enalapril (40 mg/bwkg/die), losartan (20 mg/bwkg/die), spironolactone or eplerenone (50-50 mg/bwkg/die). Untreated diabetic and healthy rats served as controls (n=8/group).

Blood pressure and heart rate were measured before and after RAAS inhibitor treatment. Metabolic and renal parameters, renal histology and the expression of renal αSMA and NKA were evaluated after the treatment.

HK-2 proximal tubular cells were cultured in medium containing normal (5 mM), high concentrations of glucose (35 mM), or high concentration of mannitol (5 mM glucose + 30 mM mannitol), and treated for 72 hours with *enalapril* (1 μM), *losartan* (10 μM), *spironolactone* (200 nM) or *eplerenone* (10 μM). NKA protein expression was measured by Western-blot.

2. I/R - Uninefrectomised male Wistar rats were treated 30 min. prior to a 50 min. renal I/R *i.p.* with vehicle (VEH), FLU (20mg/ttkg), or FLU + Sigma1-R antagonist NE100 (1mg/ttkg). Postischemic survival was followed for 1 week. Functional and structural kidney damage was analyzed after 24 hours of reperfusion in separate studies. The renal Sigma-1R – Akt - eNOS - nNOS pathway was evaluated by Western-blot and immunofluorescens techniques after 24 hours of reperfusion and without any ischemic insult 30 minutes after drug treatment. Changes in renal capillary diameters were measured *in vivo* using multiphoton-microscopy. To evaluate the role of NO further treatments were used: FLU + non-selective NOS blocker L-NAME (10mg/bwkg), FLU + selective eNOS blocker L-NIO (20mg/bwkg), FLU + selective nNOS blocker 7-NI (25mg/bwkg).

Results

DNP

Blood pressure of diabetic animals was the same as in controls and remained unchanged after RAAS inhibitor treatment.

Resting bradycardia of diabetic rats was restored by aldosterone antagonists only.

Bodyweight of diabetic rats was decreased compared to controls. Metabolic parameters (glucose, cholesterol, LDL-cholesterol, triglyceride) were higher in the diabetic group than in the control group. Losartan and enalapril lowered only glucose levels, while the aldosterone antagonists ameliorated every data.

Diabetic rats showed the signs of DNP: their higher kidney-to-bodyweight index suggested renal hypertrophy; they had elevated blood urea nitrogen, creatinine and potassium levels and decreased sodium levels. Enalapril lowered kidney-to-body weight ratio only, while losartan also decreased blood urea nitrogen and serum creatinine, moreover the aldosterone antagonists had a beneficial effect on almost every parameter.

Diabetic rats developed histological features typical for DNP: increased mesangial matrix expression and arteriolar hyalinization and occurrence of Armanni-Ebstein vacuolar tubular atrophy. Masson stained sections showed elevated tubulo-interstitial fibrosis of diabetic animals. Each RAAS inhibitor ameliorated structural kidney damage: the rate of mesangial matrix expression, arteriolar hyalinization and interstitial fibrosis was decreased.

As a first step of fibrosis, EMT, marked by elevated αSMA expression, might play a role in the pathomechanism of DNP and the efficacy of RAAS inhibitor. Diabetes induced increase of renal αSMA protein expression was diminished by each RAAS blocker.

Previously we have demonstrated that renal NKA expression is higher in diabetes, but the enzyme is mislocated and loses thereby its function. Currently we investigated the effect of different RAAS inhibitors on renal NKA. Renal protein expression of the enzyme

was higher in diabetes, which was lowered after each treatment except enalapril.

Controls showed a basolateral tubular localization of NKA in our immunohistological examinations. In the diabetic group the enzyme was also present at the apical membrane. This mislocation of NKA towards the apical membrane was prevented by each RAAS inhibitor. Cytoplasmic NKA staining was decreased by each treatment except enalapril and mostly by spironolactone.

To evaluate the effect of glucotoxicity per se on tubular cells separately from its osmotic feature and the efficacy of different RAAS blockers, *in vitro* studies were performed. HK-2 proximal tubular cells cultured with high glucose medium showed increased expression of NKA, which was decreased by each RAAS inhibitor except losartan. NKA protein level was higher in mannitol treated cells as well, but not as high as in glucose treated ones.

I/R

FLU treatment prolonged postischemic survival compared to VEH. Median survival was 36 hours (29-72) in the VEH, 67 hours (41-168) in the FLU and 49 hours (28-72) in the FLU+NE100 group. 70% of FLU treated animals were alive after 50 hours, while 90% deceased of the VEH treated ones at this time. The Sigma-1R antagonist NE100 decreased survival to the level of the VEH group.

Renal function was worse in each treatment group when compared to controls, thus serum creatinine and blood urea nitrogen levels were increased. FLU treated rats had lower creatinine and urea nitrogen levels than the VEH group. The FLU+NE100 groups showed as high levels as the VEH group.

Hematocrit and hemoglobin was decreased, white blood cell count increased after I/R. FLU treatment normalized hemoglobin and hematocrit levels and lowered white blood cell count. NE100 suspended the effect of FLU.

Compared to controls VEH treated animals showed severe tubular and glomerular kidney damage, consisting of glomerular hipercellularity, glomerular collapse and tubular epithelial vacuolization and necrosis. FLU treatment ameliorated both tubular and glomerular damage, while the FLU+NE100 group showed again severe lesions.

Evaluation of structural and functional kidney damage *in vivo* was performed using multiphoton microscopy, which revealed further structural changes. Controls presented fully intact brush borders, normal nuclear integrity and no necrotic cast formation. After I/R the tubular brush border vanished, nuclei were destroyed and the tubular lumen were filled with necrotic casts. FLU treated animals still showed some preserved brush border, nucleus integrity and less cast formation. NE100 neutralized the effect of FLU, structural damage was similar to the VEH group.

Since FLU prolonged postischemic survival, decreased systemic inflammation and functional kidney damage, and as a Sigma-1R agonist it could influence the NOS system, we postulated, that it could exerts its renoprotective effect through better circulation provided by vasodilatation. To prove this hypothesis we measured intrarenal capillary diameters using *in vivo* multiphoton microscopy. After 24 hours of I/R we experienced intrarenal vasoconstriction, while FLU treatment resulted in increased capillary diameters. NE100 caused again a decrease in diameters.

We presumed the role of the NOS system in the vasodiltative effect of FLU, therefore we treated animals with NOS inhibitors as well. Each NOS blocker suspended the effect of FLU, but in different manners. The nNOS inhibitor 7-NI was the strongest; it resulted in a bigger vasoconstriction than the non-selective NOS blocker L-NAME or the eNOS blockr L-NIO, which showed nearly similar effects.

Since the role of the Sigma-1R–Akt–eNOS is considerable in the vasodilatative effect of FLU, protein amounts were analyzed. Renal Sigma-1R, Akt and eNOS protein expression increased after

I/R. FLU treatment lowered Akt and eNOS protein amounts to the level of controls, while the Sigma-1R antagonist NE100 elevated them again to the level of VEH. The expression of the Sigma-1R remained unchanged after the drug treatments.

The activation of the Sigma-1R could result in a translocation of the proteins, therefore immunhistochemistry was performed. Controls and FLU treated animals showed a nucleus associated pattern of all the three proteins, while in the VEH and FLU+NE100 groups a cytoplasmic staining was visible. The Sigma-1R, Akt and eNOS showed a colocalization. Within glomeruli eNOS was located at the nulcei, while Akt and Sigma-1R were hardly expressed in controls. After I/R Sigma-1R and Akt appeared within the glomeruli and eNOS was translocated towards the cytoplasm. FLU treatment decreased glomerular Akt and Sigma-1R expression and stabilized eNOS at the nuclei. Treatment with NE100 resulted in a staining similar to the VEH group.

Since the expression of the vasodilatative eNOS was decreased in the FLU group after 24 hours of I/R, but FLU resulted in vasodilatation, we hypothesized that another isoform of NOS, nNOS could be responsible for this phenomenon. Therefore we measured renal nNOS expression and found that its protein level was elevated after I/R, but much more in FLU than VEH or Sigma-1R antagoist NE100 treated rats.

Another explanation for the controversial between Western-blot and renal capillary diameter results could be a faster effect of FLU. We postulated that 30 minutes after FLU treatment, at the time point when ischemia started FLU had already resulted in vasodilatation, which was obtained even after 24 hours of I/R. Therefore we analyzed intrarenal capillary diameters for a period of 30 minutes after FLU and FLU+NE100 treatment without any ischemic insult. FLU elevated diameters after 10 minutes already and resulted in an average dilatation of 2 μm after 30 minutes. The Sigma-1R NE100 completely suspended this vasodilatation. When given NOS blockers to the system, we experienced that without

ischemia, 30 minutes after drug treatment, the selective eNOS blocker neutralized the vasodilatative effect of FLU the most, while the nNOS blocker had the mildest effect.

According to protein levels of the Sigma-1R – NOS system 30 minutes after drug treatment, without any ischemic insult we found that renal Sigma-1R – Akt – eNOS proteins had a higher expression after FLU treatment, while nNOS expression remained unchanged. The Sigma-1R antagonist NE100 suspended the effect of FLU.

Discussion

Diabetes induced renal failure is a rising problem worldwide, DNP is the main cause of chronic kidney disease. The number of patients developing end stage renal disease is also increasing, therefore more and more people need renal replacement therapy, NTx. Long term outcome of NTx is mostly influenced by the obligatory I/R injury. The better understanding of the pathomechanism of I/R could reveal novel therapeutic targets, which could revise life quality and long term survival of NTx patients.

Here we compared the efficacy of different RAAS inhibitors used in monotherapy in DNP. According to our result, aldosterone antagonists could be as or even more effective in slowing the progression of DNP as the routinely used ACE inhibitors or ARBs. Moreover, the pathophysiological role of EMT and NKA is assumable and they could serve as novel therapeutic targets of RAAS blockers. However, numerous clinical studies are needed to confirm our results and to introduce a monotherapy with aldosterone antagonists in the treatment of DNP.

Current protocols do not slow the progression of DNP adequately, a lot of patients still reach end stage renal disease. The ideal therapy for end stage renal disease is NTx, since it provides better life circumstances and longer survival compared to dialysis.

Despite a decrease in acute rejection, due to better immunosuppressive therapy, I/R, which is the main cause of chronic graft dysfunction, is still an unsolved problem.

In our study, treatment with the Sigma-1R agonist FLU prior ischemia prolonged postischemic survival, diminished structural and functional kidney damage, elevated intrarenal capillary diameter and influenced the Sigma-1R – NOS system.

According to our result, the chronically already used antidepressant FLU, without any notable side effects, is protective against renal I/R injury. We assume that FLU exerts this renoprotective effect through a Sigma-1R – NOS system mediated vasodilatation.

We hope that our results could provide novel therapeutic approaches for registered drugs enlarging thereby the number of medication probabilities in the treatment of chronic kidney disease.

Publication list

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