New possibilities of reducing ischemia/reperfusion injury during renal transplantation

Ph.D. Thesis

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Introduction

Parallel to increased life expectancy the prevalence of chronic diseases is also growing worldwide. The prevalence of end stage kidney disease superimposed on acute or chronic kidney injury is currently between 8-16% and is rapidly increasing worldwide; the number of patients increased tenfold in the last four decades. The same tendency is observable in Hungary, prevalence of the disease is increases by 6-7% every year. These patients require renal replacement therapy, the definitive treatment being kidney transplantation.

Improvement of long term graft survival after renal transplantation, as well as shortage of donor organs pose a major challenge. With the advancement of immunological and surgical techniques graft survival in the first year reaches 95%, but graft dysfunction is still a limiting factor of long term survival. Ischemia/reperfusion (I/R) injury of the kidney during the transplantation procedure is a major factor in both acute and chronic graft dysfunction.

Renal I/R injury also occurs in other clinical situations such as shock, cardiac failure or suprarenal aortic surgery. Mortality caused by I/R injury is high, and treatment options are limited.

Numerous investigations focus on this problem. A part of these study sex differences in ischemic diseases (e.g. myocardial infarct, stroke), in which the lower risk of females compared to males is well-known. However, the incidence of these diseases becomes similar after menopause. Our group recently confirmed sex differences in ischemic kidney injury and in the case of transplantation as well.

We previously showed that ischemia-induced kidney injury is less severe in female rats; and that dehydroepiandrosterone (DHEA), the most abundant pre-hormone of sex steroids improves the postischemic survival of males.

DHEA is not only a hormone, but is also known as the endogenic agonist ligand of sigma-1 receptor (S1R). Our experiments focused

on this largely unknown receptor. S1R is the receptor of selective serotonin reuptake inhibitor (SSRI) drugs among others, and is most abundant in the central nervous system. We examined its localization, function and role in the kidney. We proved the protective effect of S1R activation against renal I/R injury.

As estrogen is an endogenic agonist ligand of S1R, the possible role of the receptor in the development of sex differences emerged.

Our group previously studied various signaling mechanisms involved in the protective effects of both sex differences and S1R activation. We demonstrated increased activation and thus the protective effect of the heat shock response (heat shock protein-72 (HSP-72)) in female rats, as well as the renoprotective role of the protein kinase B (Akt) - endothelial nitric oxide synthase pathway. Heat shock proteins (HSPs) are members of the chaperone family of proteins and have protective role in cellular stress by stabilizing important molecules of cellular function and structure. Heat shock response is activated exclusively by heat shock factor-1 (*HSF-1*), which by the induction of stress translocates from the cytosol to the nucleus and activates the transcription of HSPs. *Akt* also plays a role in the activation of HSF-1. *HSP72* plays a role in preserving cell structure and polarity during renal I/R injury. Its most relevant function in the kidney is the stabilization of Na^+,K^+-ATPase (*NKA*) ion channel in the basal membrane. *HSP-27* is also activated during I/R insult and stabilizes microfilaments and membrane lipids.

Knowing the protective roles of female sex and S1R, this dissertation investigates the connection between S1R and sex-related differences in renal I/R injury. Furthermore, the protective effect of S1R through heat shock response activation was studied both *in vivo* in I/R injury and autotransplantation models, and *in vitro* using proximal tubular cell cultures.

Aims

1. Are there *sex differences* in the activation of S1R and the heat shock response during renal I/R injury?

2. Does the activation of *S1R* play a part in mitigated renal I/R injury?

3. Does *17β-estradiol* have a direct role in the activation of S1R and the heat shock response?

4. Is S1R agonist fluvoxamin (Flu) added to the *preservation solution* effective in reducing kidney damage during transplantation?

Methods

I/R and autotransplantation model and treatment protocol

Adult Wistar rats weighing 200±15g were used in all experiments. Ovariectomy was performed 7 days before the ischemic insult.
Renal ischemia was accomplished by cross-clamping the left renal hilus for 50 min with an atraumatic vascular clamp. The contralateral kidney was removed before the end of ischemia.
With this model we performed two series of experiments: first (I) we examined sex differences using female, male and ovariectomized (ovx) female animals; in the second series (II) we analyzed the effect of S1R agonist Flu pretreatment in males. Sham operated rats served as controls. (n=6-8/group)
In the transplantation model we removed the left kidney and placed it into ice cold preservation solution (Custodiol) for 2 hours. Warm ischemia time was 35 minutes in all cases. With this model (III) we examined the protective effectiveness of Flu added to the preservation solution. (n=8/group)

I. Experiment: sex differences
Sample collection 2 (T2) and 24 (T24) hours after reperfusion
1. **female**
2. **male**
3. **ovx**

II. Experiment: activation of S1R
Pretreatment 30 min before ischemia, sample collection 24 (T24) hours after reperfusion
1. 20 mg/kg **Flu** intraperitoneally (*ip.*)
2. 20 mg/kg **Flu** + 1 mg/kg S1R antagonist N,N-dipropil-2-[4-methoxy-3-(2-fenilethoxy)-fenil]-thylamin-monohidroklorid (**NE-100**) *ip.*
3. saline (vehicle **- veh)**

4. sham-operated rats (**control**)

III. Experiment: transplantation
sample collection 24 (T24) hours after reperfusion
1. **Custodiol**
2. **Custodiol** + 0,003 mg/ml **Flu**
3. sham-operated rats (**control**)

For sample collection rats were re-anaesthetized, blood samples were collected from the abdominal aorta, kidneys were harvested and snap-frozen in liquid nitrogen for further investigation.

In vitro cell culture and treatment protocol

We examined the direct effect of *17β-estradiol* in human proximal tubular epithelial cell line (HK-2). Cells were treated for 24 hours.

Treatment groups:
1. 10 nM **17β-estradiol**
2. 10 nM **17β-estradiol** + 3 μM **NE-100**
3. saline (vehicle - **K**)

Experiments

Renal functional parameters
Serum creatinine, blood urea nitrogen (BUN) and aspartate transaminase (AST) levels were photometrically determined.

Histology
Kidney sections were stained with hematoxilin eosin (HE) and Periodic acid-Schiff (PAS) reagents and evaluated semi-quantitatively. In the first series glomerular collapse, tubular necrosis, hyalinization, interstitial lesions and leucocyte infiltration were evaluated. In the second series only tubular lesions were

scored, while in the third series we measured tubular lumen dilatation which correlates with the severity of injury.

Western blot analysis
We analyzed S1R, Akt, HSF-1, HSP-72, HSP-27 and NKA levels both in the *in vivo* and *in vitro* experiments. Bands of interest were factored for Ponceau red staining to correct for any variations in total protein loading.

Fluorescent immunohistochemistry
HK-2 cells were stained with immunohistochemistry to localize HSF-1 and S1R.

Apoptosis TUNEL test
In the transplantation series we examined terminal deoxynucleotidyl transferase dUTPnick-end labeling (TUNEL) on kidney sections to evaluate the proportion of apoptotic cells.

Quantitative real-time PCR
Bcl2 and *Bax* mRNA expressions were determined using quantitative real-time polymerase chain reaction (PCR) and normalized against *Rn18s* as a housekeeping gene.

Statistical analysis
Statistical analyses were performed using GraphPad Prism Software. Parametrical data was expressed as means ± SEMs, while non-parametrical data as median ± range. Multiple comparisons and possible interactions were evaluated by one-way ANOVA followed by Bonferroni's *post hoc* test. Histological changes were analyzed using the Kruskal-Wallis test followed by multiple pairwise comparisons according to Fisher's test. The criterion for significance was $p<0.05$ in all experiments.

Results

Postischemic renal function is better, structural kidney damage is milder in female rats

Renal functional parameters were massively increased at T24 in all groups, but both serum creatinine and BUN levels of females were significantly lower compared to both male and ovx animals.
I/R injury caused less severe glomerular collapse, leukocyte infiltration and interstitial lesion in females compared to males.

Sex differences in S1R, pAkt, HSF-1, HSPs and NKA protein levels

Renal S1R and pAkt (the phosphorylated, active form of Akt) levels remained unaltered after I/R injury in males and ovx, but there was a marked increase in females at T2. Proteins possibly activated by S1R showed similar dynamics. Baseline HSF-1, HSP-72, HSP-27 and NKA protein levels were already higher in females and elevated further after the insult. NKA level reached its maximum at T2, while the others at T24. Protein levels of the ovx group followed the levels of males in most cases confirming the role of female sex hormones.

S1R agonist Flu abates renal functional and structural damage

In animals pretreated with Flu both functional laboratory parameters and structural tubular lesions were mitigated compared to the untreated group.

Effect of Flu on apoptosis

In the Flu-treated group anti-apoptotic *bcl-2* gene expression had an increasing tendency, while apoptotic *bax* gene expression remained

unchanged. In animals treated with Flu and S1R antagonist NE-100 the expression of *bcl-2* was significantly lower.

Flu increases S1R and pAkt protein levels

Flu treatment increased the level of its receptor, S1R, which was inhibited by the antagonist NE-100. Similarly, the protein level of pAkt was elevated in the Flu-treated group, and this effect was inhibited by the addition of NE-100.

Flu is renoprotective in kidney transplantation

Flu added to the preservation solution mitigated renal functional decline.
Moreover, tubular lumen dilatation, and indicator of tubular damage was less prominent in the Flu-treated group.

Flu is anti-apoptotic in kidney transplantation

In the transplantation model anti-apoptotic *bcl-2* gene expression was increased in the kidney samples of Flu-treated animals.
In kidney sections of these animals the TUNEL apoptosis test showed increased number of apoptotic cells is all groups, but in the Flu treated group it was milder.

The role of 17β-estradiol in the S1R mediated heat shock response

In the *in vitro* experiments estrogen treatment had no effect on the cells' S1R protein level, although the receptor's localization was altered. S1R showed perinuclear localization in control cells, but was detected everywhere intracellularly and also in the nucleus after estrogen treatment, which is consistent with activation of the receptor.

In parallel, estrogen promoted the increase of HSF-1 protein level and also its translocation to the nuclei of proximal tubular cells, meaning the activation of the factor.
The addition of S1R antagonist NE-100 inhibited the estrogen mediated translocation of both S1R and HSF-1. Only HSP-72 had an estrogen mediated protein level elevation, HSP-27 levels remained unchanged.

Conclusions

1. We showed the role of female sex in moderating functional and structural lesions of the kidney and the negative effect of ovariectomy on renal function in case of I/R injury.

2. We were the first to show the higher baseline protein level of S1R and HSP-27 in the kidneys of female rats, and the further increase of these proteins after I/R injury.

3. *In vitro* results confirmed the role of 17β-estradiol in activating S1R and HSF-1 by their translocation in tubular epithelial cells. This activation was suspended by the addition of a S1R antagonist. We proved that 17β-estradiol stimulates the production of S1R, HSF-1 and HSP-72 proteins.

4. We showed the anti-apoptotic effect of S1R activation both in I/R and autotransplantation models.

5. We proved the protective effect of S1R agonist Flu added to the preservation solution in a kidney transplantation model.

Bibliography of the candidate's publications

Publications related to the theme of the Ph.D. thesis

1. Hosszu A, **Antal Z**, Veres-Szekely A, Lenart L, Balogh DB, Szkibinszkij E, Illesy L, Hodrea J, Banki NF, Wagner L, Vannay A, Szabo AJ, Fekete A. The role of Sigma-1 receptor in sex-specific heat shock response in an experimental ra tmodel of renal ischemia/reperfusion injury. TRANSPLANT INTERNATIONAL 31:(11) pp. 1268-1278. (2018)
   IF: 3,196

2. Hosszu A, **Antal Z**, Lenart L, Hodrea J, Koszegi S, Balogh DB, Banki NF, Wagner L, Denes A, Hamar P, Degrell P, Vannay A, Szabo AJ, Fekete A. Sigma 1-Receptor Agonism Protects against Renal Ischemia-Reperfusion Injury. JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY 28:(1) pp. 152-165. (2017)
   IF: 8,655

Other publications

1. **Antal Z**, Kovacs T, Voros P, Verebely T. Gastric Transposition after Duodenoduodenostomy in Infants with Combined Esophageal and Duodenal Atresia - Report of Three Cases. EUROPEAN JOURNAL OF PEDIATRIC SURGERY 22:(6) pp. 470-472. (2012)
   IF: 0,839

2. **Antal Zsuzsanna**, Pintér András, Verebély Tibor, Molnár Dénes, Tulassay Tivadar. Újszülöttkori urológiai beavatkozások: Két gyermeksebészeti centrum tízéves beteganyagának feldolgozása és a műtéti indikációk áttekintése. GYERMEKGYÓGYÁSZAT 60:(4) pp. 149-153. (2009)

3. Verebély Tibor, Kálmán Attila, Kiss Imre, Vörös Péter, **Antal Zsuzsanna**. Új eljárások a gyermeksebészetben. GYERMEKGYÓGYÁSZAT 60:(1) pp. 91-97. (2009)

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