PH.D. THESIS

IN VITRO AND IN VIVO PROTECTIVE EFFECT OF PACAP IN THE KIDNEY

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1. INTRODUCTION

1.1. RENAL ISCHAEMIA-REPERFUSION

Ischaemia-reperfusion-induced kidney injury is a serious problem in the clinical practice, it can evoke an acut renal failure, can lead to the development of chronic kidney injury or promote the progression of existing kidney damage to end-stage renal failure. The pathophysiology of renal ischaemia-reperfusion is a complex process with apoptotic, inflammatory and oxidative stress-related components. Renal ischaemia-reperfusion leads to an inflammatory cascade. The main mediators of inflammation are chemokines, which regulate the activation of proinflammatory cytokines, adhesion molecules and leukocytes. Aggregation of several leukocyte-subtypes can be observed in peritubular capillaries, interstitium and tubules. The BMP (bone morphogenetic protein)-family plays a role in the regulation of inflammatory processes. BMPs are members of the TGF-β (transforming growth factor beta)-family, they are filogenetically conserved signalling molecules. They are important in the normal development of the kidney, but they also play a role in the maintenance of the renal structure and function. Although some studies have revealed that application of BMP-molecules in pharmacological doses is able to attenuate acute and chronic kidney injuries in animal models, the mechanism is not fully understood. Mitogen-activated protein kinases (MAPK) are important enzymes of signalling pathways, which are activated upon different stimuli, including ischaemia. It is known that inhibition of p38MAPK leads to attenuation of ischaemia-reperfusion-induced kidney injury. ERK (extracellular signal-regulated kinase) signalling pathway is also activated during renal ischaemia-reperfusion, leading to increased phosphorylation of ERK. ERK pathway plays a role in the cell proliferation and differentiation and contributes to damage and apoptotic cell death. Akt signalling pathway also plays a role in the protective response against hypoxia-ischaemia, as it has been proven in ischaemia-reperfusion-induced kidney injury as well. Activation of the complement system in ischaemia-reperfusion is a known phenomenon, resulting in several biologically active products (for example C3a, C4a, C5a), which have proinflammatory effects and upregulate the activation of adhesion molecules. The role of reactive oxygen species in the pathogenesis of

ischaemia-reperfusion-induced kidney injury has also been described. During ischaemia-reperfusion a high amount of reactive oxygen species is released from the damaged tissues, the mitochondrial oxidative phosphorylation changes, ATP (adenosine triphosphate) depletion develops, intracellular calcium level elevates and membrane phospholipid proteases activate. These changes lead to oxidative stress, which induces apoptosis through peroxidation of membrane lipids and oxidative damage of proteins and DNA. Downregulation of the antioxidant enzyme system (superoxide-dismutase, catalase, glutathion-peroxidase) also plays a role in the pathomechanism of ischaemia-reperfusion-induced injury. Inhibition of this pathway and decrease of the amount of free radicals is one possibility to reduce the alterations developing in ischaemia-reperfusion. It has been proven that apoptosis significantly contributes to renal dysfunction in ischaemia-reperfusion through destruction of tubular cells. Proximal tubular cells are very sensitive to acute ischaemia. Damage, death and detachment of these cells are, in addition to inflammation, primarily responsible for the pathophysiology of ischaemia-reperfusion-induced damage and the clinical aspects. The tubular epithelium is not only a passive sufferer of the injury, but it is an active participiant of the inflammatory response given to renal ischaemia-reperfusion. Injured tubular epithelium releases proinflammatory cytokines and chemokines, which promote the recruitment of immune cells.

1.2. GENTAMICIN NEPHROTOXICITY

Gentamicin belongs to the group of aminoglycoside antibiotics. It is widely used in the therapy of various infections caused by Gram negative bacteria. Gentamicin is one of the most clinically relevant drug toxicities in the kidney, it is responsible for 20% of acute kidney diseases. The toxicity is due to inflammatory and oxidative processes. Gentamicin accumulates in the kidney mainly in the proximal tubular epithelial cells. Through apoptosis/necrosis of epithelial cells it causes tubular damage. Furthermore, it is known that gentamicin is able to affect the activity of several membrane-bound enzymes and transport systems, among others of dipeptidyl-peptidase IV (DPPIV).

1.3. KIDNEY INJURY – GENDER DIFFERENCES

In clinical practice gender differences are observable in the progression of kidney injuries of different ethiologies. The outcome is usually more favourable in females. In animal experiments male animals are generally used, so only few data are available related to females. It has been shown that in ischaemia-reperfusion-induced acute renal failure differences are observable regarding to survival of animals, the vulnerability of the kidney is different in males and females, kidneys of males react more sensitively to ischaemia. In the background of the more favourable outcome of females different causes are supposed, but sex hormonal difference is one of the main candidate causes.

1.4. PACAP

1.4.1. General information

PACAP (pituitary adenylate cyclase-activating polypeptide) is a neuropeptide that was first isolated in 1989 by Professor Arimura and his team. PACAP is well-known for its neuroprotective and cytoprotective effects. It is a member of the secretin/glucagon/VIP (vasoactive intestinal peptide) peptide family, it is present in two biologically active forms in the body: the 38-amino-acid form (PACAP38) makes up 90% of PACAP in mammalians and the 27-amino-acid form (PACAP27). The sequence of the biologically active region of PACAP is filogenetically conserved, suggesting essential physiological functions of the peptide. The DPPIV enzyme causes the degradation of PACAP. It is responsible for the short half-life time of PACAP, being only a few minutes in the systemic circulation. Binding to phospholipid membranes causes higher stability of the peptide, inhibiting its degradation. Presence of PACAP and of its G-protein-coupled receptors (PAC1 (pituitary adenylate cyclase-activating polypeptide type I receptor), VPAC (vasoactive intestinal peptide receptor) 1 and 2) has been shown in the nervous system and in peripheral organs. VPAC1 and VPAC2 bind PACAP and VIP with the same affinity, but PAC1 shows 2-3 times higher affinity to PACAP. Activation of VPAC1 and 2 leads to elevation of the level of cAMP (cyclic adenosine monophosphate) or cGMP (cyclic guanosine monophosphate), activation of PAC1

receptor induces AC (adenylyl cyclase), PLC (phospholipase C) and PKC (protein kinase C) mediated signalling pathways. PACAP-specific PAC1 receptor has 8 splice variants, which cause activation of different second messengers. PACAP has widespread physiological effects, it influences the function of almost all organ systems. It plays an important role in the regulation of endocrine and exocrine glands, in the cardiovascular and gastrointestinal system. It is mainly known for its neuroprotective effects, but it has general cytoprotective effects as well. PACAP plays an important role in the development of different organs. The protective effects of PACAP are in connection with its function as a trophic factor in the nervous system and peripheral organs

1.4.2. Role of PACAP in the urogenital system

Presence of PAC1 receptor in the kidney has been demonstrated earlier by our team. The expression of this receptor was most pronounced in the cortical tubular epithelial cells. In a later study VPAC1 receptor has been found to be dominant in the human kidney, it was present in the proximal tubular epithelial cells and glomerular podocytes. Our team has shown the presence of PACAP38 in the rat kidney, changes of its level were also revealed during ischaemia-reperfusion. Both PACAP38- and PACAP27-immunoreactivity have been shown in rat ureteral-, bladder- and urethral cells. Presence of PACAP receptors (PAC1, VPAC1, VPAC2) has also been demonstrated in the lower urogenital tract. It has been proven that PACAP influences the micturition and sensory innervation. It is known that PACAP can induce renin secretion via PAC1 receptor and intravenous application of PACAP27 leads to vasodilatation and increase of blood flow in the kidney. In case of different kidney injuries (both *in vivo* and *in vitro*) protective effect of PACAP has been demonstrated. PACAP is protective in diabetic nephropathy, cyclosporin, cisplatin, contrast agent-induced nephrotoxicity and myeloma nephropathy. Furthermore, PACAP is able to ameliorate the decreased cell survival of rat kidney cells *in vitro* following oxidative stress.

1.4.3. PACAP-deficient mice

In PACAP-deficient mice (homozygous: PACAP -/- and heterozygous: PACAP +/-) the lack/decreased level of endogenous PACAP leads to increased sensitivity to

different *in vivo/in vitro* stimuli in the nervous system and also in peripheral organs. Some differences can be observed without outer stimuli compared to PACAP +/+ mice. Fertility of PACAP-deficient mice is lower and the mortality is higher. Temperature sensitivity, metabolic changes, increased insulin sensitivity, respiratory abnormalities, bladder dysfunction are characteristic to these animals. Early aging is also observable in the PACAP-deficient mice, onset of systemic senile amyloidosis is accelerated. They show depression-like behaviour, react abnormally to different stress situations, and hyperactivity is also a feature of these mice. Endogenous PACAP is important during the development, alterations found in teeth of PACAP--deficient mice show the role of PACAP in the tooth development. It has been demonstrated that PACAP-deficient mice react more sensitively to ischaemia-reperfusion-induced injury (cerebral, retinal, intestinal ischaemia). Endogenous PACAP can attenuate renal damage developing in oxidative stress and hypoxia. Furthermore, it is known that the level of reactive oxygen metabolites is significantly higher in PACAP-deficient mice and the antioxidant activity in the plasma is lower. This also confirms that in lack of endogenous PACAP the sensitivity against different harmful stimuli increases.

1.4.4. PACAP and gender differences

There are only few data available regarding gender differences and PACAP. Gender differences were found in an experimental model of Parkinson's disease, in castrated male rats PACAP did not considerably influence the 6-OHDA-evoked cell death compared to non-castrated rats. In the same experimental model PACAP did not influence the dopaminergic cell loss in female rats, but in ovariectomised animals it was able to reduce cell death.

2. AIMS OF THE STUDY

The aims of my PhD-research were to study the ischaemia-reperfusion-induced kidney injury and gentamicin-induced nephrotoxicity, both of them have clinical significance. We investigated the role of cytoprotective PACAP in these pathological conditions, with potential therapeutic significance in the future.

Specific aims were:

- examination of ischaemia-reperfusion-induced kidney injury *in vivo* in male and female rats and investigation of the effect of exogenous PACAP in both males and females, to reveal the possible protective effect and to explore gender differences
- investigation of the role of endogenous PACAP *in vivo* in renal ischaemia-reperfusion with the help of PACAP-deficient mice (PACAP -/- and PACAP +/-)
- examination of the effect of PACAP in gentamicin-induced nephrotoxicity *in vitro* on HK-2 cells

3. PROTECTIVE EFFECT OF EXOGENOUS PACAP ON ISCHAEMIA-REPERFUSION-INDUCED KIDNEY INJURY OF MALE AND FEMALE RATS

3.1. MATERIALS AND METHODS

3.1.1. Experimental animals

Adult male and female Wistar rats (n=112, 250-300 g) were divided into 12 groups:

- control (untreated) male rats (100 µl saline iv.) with 24-h, 48-h, or 14-day reperfusion
- control (untreated) female rats (100 µl saline iv.) with 24-h, 48-h, or 14-day reperfusion
- PACAP-treated male rats (100 µg PACAP/300 g dissolved in 100 µl saline iv.) with 24-h, 48-h, or 14-day reperfusion
- PACAP-treated female rats (100 µg PACAP/300 g dissolved in 100 µl saline iv.) with 24-h, 48-h, or 14-day reperfusion

To perform the operation in the same phase (oestrus phase) of oestrus cycle of female rats, vaginal smear was taken.

3.1.2. Operation

Animals were anaesthetized with intraperitoneal ketamine-xylazine. PACAP was injected into the jugular vein before total median laparotomy in half of the rats.

The left renal vessels were clamped for 60 min. Following 24 h, 48 h or 14 days, kidneys were removed and processed for further investigations.

3.1.3. Morphometric analysis

Following anaesthesia rats were perfused with PBS (phosphate buffered saline) and 4% paraformaldehyde under anaesthesia 24 h, 48 h or 14 days after surgery. Removed kidneys were embedded in paraffin, and 5-μm thick sections were stained with periodic acid–Schiff base (PAS) and haematoxylin or with haematoxylin–eosin. Evaluation was performed on digital photographs using Adobe Photoshop version 10.0 and Scion Image 1.47 programs. Minimum ten fields on each slide - where only tubules were seen - were examined. The ratio of tubular lumen area was measured, as its enlargement represents tubular epithelial damage. Tubular lumen area was expressed against the total tubular area.

3.1.4. Cytokine array – investigation of cytokine expression

Kidneys of male and female rats removed 24 h after ischaemia-reperfusion were processed for cytokine array (Proteome Profiler Rat Cytokine Array Kit, R&D Systems). After preparation and determination of the protein content of the samples the array was performed according to instructions of the manufacturers. The array is based on the binding between the proteins of the sample and antibodies of the nitrocellulose membrane. Chemiluminescent detection was used, pixel density of the immunopositive spots was measured using ImageJ 1.40 software.

3.1.5. Investigation of oxidative stress and antioxidant markers

24 h following ischaemia-reperfusion kidneys of male and female rats were removed to investigate oxidative stress and antioxidant markers. Malondialdehyde (MDA) was measured in kidney homogenates with addition of TBA (saturated thiobarbituric acid in 10% perchloric acid) – 20% trichloroacetic acid reagent. After incubation samples were centrifuged. MDA concentration was determined spectrophotometrically by measuring the absorbance at 532 nm. Data were expressed in μmol/g tissue weight. Glutathione (GSH) was quantified by adding 10% trichloroacetic acid to the samples and then the samples were centrifuged. 0.4 M tris(hydroxymethyl)-amino-methane (TRIS)-buffer was added to the supernatant and samples were measured at 412 nm after adding 5,5-dithiobis-2-

nitrobenzoic acid (DTNB) to the mixture. Values of renal glutathione were determined using a standard curve and expressed in µmol/g tissue weight. Kidney homogenates were centrifuged in order to determine the renal concentration of superoxide-dismutase, and the supernatant was used to measure the SOD concentration. SOD inhibited the transformation of adrenaline to adrenochrome which absorbed maximally at 480 nm. Quantification of SOD is based on the degree of the inhibition. The value of SOD was given in IU/g tissue weight.

3.1.6. Examination of signalling pathways

Examinations were performed using kidneys of male and female rats 24/48 h following ischaemia-reperfusion.

3.1.6.1. *RT-PCR (real time polymerase chain reaction)*

Total RNA was harvested, reverse transcriptase reaction was carried out, amplifications were performed in a thermal cycler. PCR products were analyzed by electrophoresis in 1.2% agarose gel containing ethidium bromide. Actin was used as internal control. Signals were developed with gel documentary system. Optical signal density was measured using ImageJ 1.40 g freeware and results were normalized to controls.

3.1.6.2. *Western blot*

Samples were collected in buffer containing protease inhibitors. Proteins were separated by 7.5% SDS-PAGE gel. Proteins were transferred electrophoretically to nitrocellulose membranes. After blocking membranes were exposed to primary antibodies (anti-PKA (protein kinase A), anti-P-PKA (phosphorylated protein kinase A), anti-BMPR1 (bone morphogenetic protein type I receptor), anti-BMP2 (bone morphogenetic protein 2), anti-BMP4 (bone morphogenetic protein 4), anti-Smad1 ("small" worm phenotype, "mothers against decapentaplegic"), anti-ColIV (collagen IV), anti-aktin, then incubated with the horseradish peroxidase conjugated secondary antibody, anti-rabbit or anti-mouse IgG. Signals were detected by enhanced chemiluminescence and developed with gel documentary system. Actin was used as internal control. The optical signal density was measured by ImageJ 1.40 g freeware and normalized to controls.

3.1.6.3. *Immunohistochemistry*

Perfused kidneys, removed 24 h or 48 h after surgery under anaesthesia, were washed and fixed in SaintMarie's fixative (99% ethanol, 1% anhydrous acetic acid) for 24 h. After embedding, 5-µm sections were made. Nonspecific binding sites were blocked, for ColIV, Smad1, BMPR1, and BMP4 immunohistochemistry, sections were incubated in anti-ColIV, anti-Smad1, anti-BMPR1 and anti-BMP4, primary antibodies were visualized with anti-rabbit Alexa 555 or anti-mouse Alexa 488 secondary antibodies. Slides were mounted in Vectashield Hard Set mounting medium containing DAPI (4',6-diamidino-2-phenylindole) to visualize cell nuclei. For investigation of subcellular localization of Smad1 and BMP4, fluorescent images were taken with an Olympus FV1000S confocal microscope.

3.1.7. Statistical analysis

Statistical analysis was performed with use of SPSS 15.0 software. Results of morphometric analysis, cytokine expression, antioxidant and oxidative stress markers were evaluated with analysis of variance (ANOVA) with Bonferroni//Tamhane's correction, the normal distribution was investigated with Kolmogorov-Smirnov- and Shapiro-Wilk-test, the homogenity with Levene's probe. Results of Western blot and RT-PCR were analyzed with two-sample t-test. $p<0.05$ was considered statistically significant.

3.2. RESULTS

3.2.1. Morphometry

In case of renal ischaemia-reperfusion damage of the tubular epithelium is observable in the proximal tubules, the tubular epithelial cells loose on their height, therefore partly virtual dilatation of the tubular lumen can be seen. This alteration was markedly greater in the control than in the PACAP-treated groups. In control males, the tubular damage already reached its maximum at 24 h, and there was no remarkable deterioration at 48-h or 14-day ischaemia-reperfusion. Histological results of female control rats were significantly better following 24-h reperfusion, tubular damage became more severe with time, gradually reaching the level observed in males at 24-h ischaemia-reperfusion. Significantly less severe histological alterations were found in the PACAP-treated groups in both genders. PACAP treatment significantly decreased the severity of histological damage in male rats in case of all intervals. With time significant progression was observable compared to the 24-h group. Results of PACAP-treated females were significantly better compared to males and control females at 48 h and 14 days. In contrast with the PACAP-treated male and control female rats the duration of the reperfusion had no influence on the severity of the damage in PACAP-treated females.

3.2.2. Cytokine expression

In the expression of fractalkine, L-selectin, RANTES (regulated on activation, normal T cell expressed and secreted) and sICAM-1 (soluble intercellular adhesion molecule) no significant difference was found between the genders following ischaemia-reperfusion. After PACAP treatment expression of fractalkine, L-selectin and RANTES showed a significant decrease in both genders, but this decrease was more pronounced in females. Decrease of sICAM-1 expression was similar in males and females, it did not reach the significant level. Marked gender differences were found in the expression of TIMP-1 (tissue inhibitor of metalloproteinases) and MIP-3α (macrophage inflammatory protein), it was significantly higher in both control and PACAP-treated males. Following PACAP treatment decrease of the expression was observable in both genders, in case of TIMP-1 it was significant in both males and females, in case of MIP-3α only in females. Presence of CNTF (ciliary neurotrophic factor) could be observed almost only in the kidneys of control

females, the expression was significantly higher compared to the males and PACAP-treated females.

3.2.3. Oxidative stress and antioxidant markers

Higher SOD activity was measured after PACAP treatment in both sexes, but a significant difference was only found in females. No significant alterations were seen in GSH and MDA levels between any groups.

3.2.4. Signalling pathways (RT-PCR, Western blot and immunohistochemistry)

Binding of PACAP to its receptors activates PKA, the mRNA and protein expression of which increased after 24-h ischaemia-reperfusion in the PACAP-treated females and males. Phosphorylation (activation) of PKA also showed a significant elevation in both genders after PACAP treatment following 24-h ischaemia-reperfusion. However, after 48-h ischaemia-reperfusion, the expression profiles of PKA showed a significant decrease in the case of PACAP treatment. BMP2 mRNA was significantly elevated in males, while no alteration was detected in females following PACAP treatment at 24 h; BMP2 protein expression did not change in either sex. On the contrary, BMP4 mRNA and protein were significantly elevated in both genders. The immunopositivity of this transcription factor was stronger in the PACAP-treated groups accumulating mainly in the cortical tubules. PACAP altered the mRNA expression of BMPs in the 48-h ischaemia-reperfusion group: BMP2 and BMP4 increased in males, while no significant changes were detected in females. Protein expression of BMP2 and BMP4 was elevated only in females, but no alterations were observed in the PACAP-treated males after 48-h ischaemia-reperfusion. BMPR1 was detected in both genders, and PACAP increased its mRNA and protein expression in male and female rats after 24 h. In the cortical tubules, BMPR1 showed stronger signals following PACAP administration. Interestingly, BMPR1 mRNA expression was reduced after PACAP treatment at 48 h in both sexes, although its protein level remained constant. One of the downstream targets of BMPR1 is Smad1, the mRNA expression of which was significantly elevated in the PACAP-treated groups following 24-h ischaemia-reperfusion. The protein level of Smad1 in PACAP-treated males and females was increased 24 h after ischaemia-reperfusion. Moreover, immunopositivity of tubules dramatically increased in the

PACAP-treated groups. In contrast, the mRNA expression of Smad1 was reduced after 48-h ischaemia-reperfusion in PACAP-treated animals, but surprisingly, the protein expression still showed an elevated level. One of the target genes of this signalling pathway is collagen type IV, the mRNA expression of which was also augmented by PACAP treatment after 24-h ischaemia-reperfusion in male and female rats equally. Moreover, a strong elevation in their protein expression was detected in both genders. The tubules and the renal corpuscles were strongly surrounded by a collagen type IV-immunopositive line in PACAP-treated groups. On the contrary, PACAP treatment resulted in reduced collagen type IV mRNA after 48-h ischaemia-reperfusion, but its protein expression was still higher than in the control group in both females and males.

3.3. DISCUSSION

Morphometric analysis revealed that the histological alterations were more severe in control males than in females, with the exception of the 14-day reperfusion group. This corresponds to descriptions showing that kidney diseases are more severe, the damage progrediates more rapidly and leads earlier to end-stage renal failure in males. Significantly less severe histological alterations were found in the PACAP-treated rats in both sexes. PACAP has already been proven protective in different kidney injuries, but all these studies were performed on male animals. We proved for the first time, that PACAP is protective in renal ischaemia-reperfusion also in females. In this study, we found less severe tubular damage in PACAP-treated female rats. Similar gender-dependent protective effect of PACAP has been described in Parkinson's disease.

We tested the effect of PACAP on the cytokine profile, PACAP treatment decreased the expression of several cytokines (fractalkine, L-selectin, RANTES, sICAM-1, thymus chemokine, CNTF, TIMP-1 and MIP-3α), showing overlap with findings in diabetic nephropathy. In retinal hypoperfusion, PACAP also attenuated increased cytokine expression. Decrease of the expression of fractalkine, L-selectin, RANTES, TIMP-1, MIP-3α and CNTF was more pronounced in females in case of PACAP treatment. Remarkable gender difference was also observed in TIMP-1 and MIP-3α,

with higher levels in males. In hepatic ischaemia-reperfusion of mice similar observations were made in case of most cytokines.

Oxidative stress also plays an important role in the pathomechanism of renal ischaemia-reperfusion. We did not find significant difference between the groups in MDA (side product of lipid peroxidation) and antioxidant GSH level, but higher activity of the antioxidant SOD was measured in the PACAP-treated groups, similar to earlier findings in intestinal ischaemia. Difference between SOD activity of PACAP-treated and control group was significant only in females, confirming the gender-dependent protective effect of PACAP.

Members of BMP pathway can be participiants of inflammatory processes. Our team has earlier demonstrated that PACAP increases the expression of BMPs in osteoblasts and chondrifying cells, where BMP signalling can induce extracellular matrix production. BMP, binding to its receptor (BMPR1), can induce nuclear translocation of Smad1, which may induce production or modification of basal membrane component collagen type IV. Collagen type IV is necessary to the normal activation of BMP signalling in kidney tubules. Here, we demonstrated that PACAP had gender- and time-dependent effects on BMP expression. BMP2 was unaltered in males, but PACAP increased its protein expression after 48 h in females. BMP4 protein expression was elevated in both sexes after 24 h of PACAP administration, but an increase was detected after 48 h only in females. There are some BMPs the expression and function of which can be gender-dependent. The present results suggest that BMP2 does not have an important function in PACAP-induced mechanism in renal ischaemia-reperfusion. BMP4 can have a direct correlation with the collagen type IV synthesis, resulting in the thickening of the basement membrane of the tubules. In our experiments, we detected gender-dependent elevation of BMP4-BMPR1-Smad1, resulting in increased collagen type IV. This result interestingly suggests a signalling cascade very precisely regulated by PACAP, which can be disturbed during kidney injury. Addition of PACAP may have a dual effect as it can prevent the inflammatory effects directly and shift the balance of BMP pathway resulting a thickening of basement membrane.

Conclusively, the present study showed that PACAP is able to decrease the damage in renal ischaemia-reperfusion in both males and females, with markedly less

pronounced injury in females. Whether this difference between sexes is due to hormonal alterations, as it has already been described in earlier studies, or further factors play a role in this gender-dependent effect of PACAP requires further investigation preceding the potential therapeutic use of PACAP in kidney injuries.

4. EFFECT OF ENDOGENOUS PACAP IN ISCHAEMIA-REPERFUSION INDUCED KIDNEY INJURY

Earlier our team has studied the ischaemia-reperfusion-induced kidney injury of PACAP +/+ and PACAP -/- mice, concentrating to the histological alterations. During my PhD research I investigated the SOD activity and the cytokine expression in addition to the earlier study. Furthermore all investigations were performed also in PACAP +/- mice, supplemented with examination of an other antioxidant marker and the signalling pathways. In all cases results of PACAP -/- (homozygous) and PACAP +/- (heterozygous) groups were compared with the results of PACAP +/+ (wild) group.

4.1. MATERIALS AND METHODS

4.1.1. Experimental animals

CD1 adult male mice were used, experimental groups were the followings: control (sham operated) PACAP +/+ (n=9) and PACAP +/- (n=9), furthermore PACAP +/+ (n=9 and 6) and PACAP +/- (n=9 and 6) following 60-min and 45-min ischaemia-reperfusion. During investigation of cytokine expression and SOD activity groups were studied in addition to the earlier histological evaluation: control PACAP +/+ (n=5) and PACAP -/- (n=5), furthermore PACAP +/+ (n=5) and PACAP -/- (n=5) following 60-min ischaemia-reperfusion.

4.1.2. Operation

Animals were anaesthetized with combination of ketamine-xylazine. Total median laparotomy was performed, renal vessels were freed and warm ischemic damage was induced by cross-clamping of renal pedicles for 45 or 60 minutes. Clamping was performed only on the left side, while the other side was left intact.

4.1.3. Morphometric analysis

Animals (PACAP +/+ and PACAP +/- mice) were terminated 14 days following ischaemia-reperfusion. Mice were perfused with 4% paraformaldehyde containing 15% picric acid under ketamine-xylazine anaesthesia, kidneys were removed and further processed for routine haematoxylin-eosin and periodic acid Schiff (PAS)-haematoxylin staining on serial, 10-μm thick sections. Histological evaluation was performed in a blinded fashion, the following parameters were graded on a scale 0–2 (0-absent, 1-mild-moderate, 2-severe): tubular dilatation, damage of the glycocalyx in the tubules, thyreoidisation (cylinders in the tubules), lymphocyte infiltration, macrophage infiltration and dilatation of the Bowman's capsule.

4.1.4. Western blot – examination of signalling pathways

Kidneys of PACAP +/+ and PACAP +/- mice were removed following 60-min ischaemia and 24-h reperfusion, the controls were sham operated PACAP +/+ and PACAP +/- mice. After preparation of the samples the protein content was measured to use the same concentration. Following separation in 7,5% SDS-PAGE gel the samples were transferred to nitrocellulose membrane. After blocking the membranes the following primary antibodies were applied: anti-tAkt, anti-pAkt, anti-tERK1/2, anti-pERK1/2, anti-p38MAPK, anti-pp38MAPK and anti-aktin, as secondary antibody horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG were used. Antigen-antibody complexes were visualized with enhanced chemiluminescence. Actin was used as internal control. The optical signal density was measured by ImageJ 1.40 g freeware and normalized to actin.

4.1.5. Cytokine array - investigation of cytokine expression

Kidneys of PACAP +/+, PACAP +/- and PACAP -/- mice were removed after 60-min ischaemia and 24-hour reperfusion, the controls were sham operated PACAP +/+, PACAP +/- and PACAP -/- mice. After preparation and determination of the protein content of the samples the array was performed according to instructions of the manufacturers. The array (Proteome Profiler Mouse Cytokine Array Kit, R&D Systems) is based on the binding between the proteins of the sample and antibodies of the nitrocellulose membrane. Chemiluminescent detection was used, pixel density of the immunopositive spots was measured using ImageJ 1.40 software.

4.1.6. Investigation of antioxidant markers

Kidneys of PACAP +/+, PACAP +/- and PACAP -/- mice were removed after 60-min ischaemia and 24-hour reperfusion, the controls were sham operated PACAP +/+, PACAP +/- and PACAP -/- mice. SOD was measured in homogenates using SOD assay kit, the array was performed according to instructions of the manufacturers. One reagent of the kit underwent alkaline autooxidation, which was accelerated by SOD. Autooxidation of this reagent yielded a chromophore, which absorbed maximally at 525 nm. Level of glutathione was measured according to instructions of Sedlak and Lindsay described in the chapter 3.1.5.

4.1.7. Statistical analysis

Statistical analysis was performed with use of SPSS 15.0 software. Results were evaluated with analysis of variance (ANOVA) with Bonferroni//Tamhane's correction, the normal distribution was investigated with Kolmogorov-Smirnov- and Shapiro-Wilk-test, the homogenity with Levene's probe. $p<0.05$ was considered statistically significant.

4.2. RESULTS

4.2.1. Morphometric analysis of PACAP +/+ and PACAP +/- mice

Kidneys of control PACAP +/+ and PACAP +/- mice showed normal histological appearance. Following ischaemia-reperfusion differences were observable between the PACAP +/+ and PACAP +/- mice. Lymphocyte and macrophage infiltration were significantly more severe in the PACAP +/- animals (after both 60- and 45-min ischaemia). Thyreoidisation (cylinders in the tubules) was more pronounced in the PACAP +/- mice following 45- and 60-min ischaemia, this alteration was only in mild form present in PACAP +/+ animals, the difference between the groups was significant. Damage of the glycocalyx was more severe in the PACAP +/- mice in case of both ischaemic interval. We did not find significant difference between the PACAP +/+ and PACAP +/- mice in the tubular dilatation and dilatation of the Bowman's capsule.

4.2.2. Western blot – signalling pathways in PACAP +/+ and PACAP +/- mice

In expression of tAkt no significant difference was observable between the groups. Expression of pAkt showed significant increase in case of ischaemia-reperfusion in both PACAP +/+ and PACAP +/- mice, the level of pAkt was significantly higher in PACAP +/+ mice compared to PACAP +/- animals following ischaemia-reperfusion. In p38MAPK expression no significant difference was found between the groups, but expression of the phosphorylated form of p38MAPK showed significant elevation during ischaemia-reperfusion in PACAP +/- mice. Following ischaemia-reperfusion the expression of phosphorylated p38MAPK was significantly higher in the PACAP +/- than in the PACAP +/+ group. Expression of tERK1,2 was similar in both control groups, significant decrease was observable following ischaemia-reperfusion in the PACAP +/- group. tERK1,2 expression measured after ischaemia-reperfusion was significantly lower in the PACAP +/- group compared to the PACAP +/+ group. pERK1,2 was almost not detectable in the control groups, no marked difference was found between the groups. After ischaemia-reperfusion significant increase of the expression was shown in both PACAP +/+ and PACAP +/- mice, significantly higher level was measured in the PACAP +/- group compared to the PACAP +/+ group.

4.2.3. Cytokine expression

4.2.3.1. *Results of PACAP +/+ and PACAP +/- mice*

No significant difference was found in the cytokine expression pattern of control PACAP +/+ and PACAP +/- mice. Following ischaemia-reperfusion level of several cytokines showed an increase, resulting in differences between the PACAP +/+ and PACAP +/- group. Expression of IL-1ra (interleukin-1 receptor antagonist), KC (keratinocyte chemoattractant), M-CSF (macrophage colony-stimulating factor), RANTES and TIMP-1 was significantly higher in the PACAP +/- mice compared to PACAP +/+ animals following ischaemia-reperfusion. In the PACAP +/+ mice significant increase of the expression due to ischaemia-reperfusion was found in case of BLC (B lymphocyte chemoattractant), IL-1ra, IL-27 (interleukin-27) and TIMP-1. In the PACAP +/- animals increase of the expression reached the significant

level in case of IL-1ra, KC, M-CSF, MCP-1 (monocyte chemoattractant protein), MIP-2 and TIMP-1.

4.2.3.2. *Results of PACAP +/+ and PACAP -/- mice*

In point of the cytokine expression no significant difference was found between the control PACAP +/+ and PACAP -/- mice. Following ischaemia-reperfusion level of several cytokines was higher both in PACAP +/+ and PACAP -/- group compared to the controls. Significant increase in the expression of BLC, C5a (complement 5a), G-CSF (granulocyte-colony stimulating factor), IL-16, IL-1ra, KC, MCP-1, M-CSF, MIP-2 and TIMP-1 was shown in the PACAP +/+ and PACAP -/- mice. After ischaemia-reperfusion level of BLC, C5a, G-CSF, IL-1ra, KC, MCP-1, MIP-2, TIMP-1 and TREM (triggering receptor expressed on myeloid cells) was significantly higher in the PACAP -/- mice compared to the PACAP +/+ animals.

4.2.4. Antioxidant markers

4.2.4.1. *Results of PACAP +/+ and PACAP +/- mice*

SOD activity of the kidneys of control PACAP +/+ and PACAP +/- mice was similar. Following 60-min ischaemia and 24-h reperfusion significantly lower SOD activity was found in the PACAP +/- mice compared to the PACAP +/+ animals, in comparison with the control significant decrease was shown in the PACAP +/- mice. In GSH expression no significant difference was observable between the groups.

4.2.4.2. *Results of PACAP +/+ and PACAP -/- mice*

SOD activity did not show significant difference in the kidneys of control PACAP +/+ and PACAP -/- mice. Following ischaemia-reperfusion significant decrease of SOD acitivity was observable in the kidneys of PACAP -/- mice, the level measured following ischaemia-reperfusion was significantly lower than in the PACAP +/+ animals.

4.3. DISCUSSION

Increased sensitivity of PACAP -/- mice in renal ischaemia-reperfusion has been proven in earlier investigation of our team. In the kidneys of PACAP +/+ and PACAP -/- mice normal histological appearance could be observed. In our present study similar observations were made, we did not find marked difference between the control PACAP +/+ and PACAP +/- mice. However, following ischaemia-reperfusion differences between the groups were shown. Similar findings were reported in retinal ischaemia. We have earlier confirmed, that the histological damage is more severe in the PACAP -/- mice compared to the PACAP +/+ animals. This also was observable in the PACAP +/- mice, resulting in significant difference compared to PACAP +/+ mice in case most of the parameters, such as thyreoidisation, lymphocyte and macrophage infiltration and damage of the glycocalyx.

Furthermore, expression of several proinflammatory cytokines/chemokines was found more pronounced following ischaemia-reperfusion in both of the PACAP +/- and PACAP -/- mice. Level of KC, MCP-1 and TIMP-1 was significantly higher compared to the PACAP +/+ mice. Significantly higher expression of BLC, C5a, G-CSF, MIP-2 and TREM-1 was shown in the PACAP -/- mice, level of RANTES and M-CSF was more pronounced in the PACAP +/- mice. This confirms the results described in previous studies, according to which PACAP treatment is able to decrease the level of several proinflammatory cytokines/chemokines in case of different models of kidney injuries.

Significant decrease of SOD activity was shown during ischaemia-reperfusion in the PACAP +/- and PACAP -/- mice, its level measured following ischaemia-reperfusion was significantly lower in the PACAP-deficient mice compared to the PACAP +/+ animals. This can be an indicator of the decreased antioxidant capacity in the PACAP-deficient mice. Similar alterations were described in case of intestinal ischaemia.

It is known that inhibition of p38MAPK activity leads to amelioration of the ischaemia-reperfusion-induced kidney injury. In our study pp38MAPK expression measured in the PACAP +/- mice was significantly higher than in the PACAP +/+ mice. Increasing number of evidences confirm, that ERK activation contributes to

the damage and the apoptotic cell death. In our study higher pERK expression was found in the PACAP +/- animals. Expression of pAkt was found significantly higher in the PACAP +/+ mice compared to the PACAP +/- animals. Several studies have revealed, that the ischaemia-reperfusion-induced kidney injury can be attenuated through activation of the Akt signalling pathway.

All these are in accordance with several studies, which have confirmed that PACAP-deficient mice react more sensitively to different harmful stimuli, leading to more severe alterations. It was shown in intestinal, retinal and also in cerebral ischaemia. In our present study it was proved that PACAP present in the body is an important member of the endogenous protective system, its partial or total lack have severe consequences in the protective capacity against different harmful stimuli. Antiapoptotic, antiinflammatory and antioxidant effect of the peptide can be in the background of the protective function.

5. EFFECTS OF PACAP ON HUMAN PROXIMAL TUBULE CELLS AGAINST GENTAMICIN TOXICITY

5.1. MATERIALS AND METHODS

5.1.1. Cell culture

HK-2 human renal proximal tubule epithelial cell line (ATCC) was cultured in DMEM high glucose/F-12 supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Cells were passaged by trypsinization, followed by dilution in DMEM high glucose/F-12 medium containing 10% fetal bovine serum. Experiments started 24 h after incubation in humified 95% air and 5% CO_2 mixture at 37 °C in the medium.

5.1.2. MTT assay

Effect of PACAP on cell proliferation and survival was investigated using cells plated into 96-well microplates at 10,000/well.

Experimental groups:

- control (without treatment)
- 10 nM PACAP treatment
- 100 nM PACAP treatment
- 1 μM PACAP treatment

For investigating the effect of PACAP against gentamicin toxicity, cells were randomly assigned to one of the following experimental groups:

- control (without treatment)
- cells exposed to 100 nM PACAP for 24 h
- cells treated with 2 μg/ml gentamicin for 24 h
- cells treated with 100 nM PACAP and 2 μg/ml gentamicin simultaneously for 24 h

Viability was assessed by colorimetric MTT assay (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) detecting the absorption of blue formazan dye particles produced by viable mitochondria.

5.1.3. Kidney biomarker array

For investigating the effect of PACAP on expression of various kidney-related proteins, Proteome Profiler Human Kidney Biomarker Array (R&D Systems) was

used. Cells were plated in six-well plates and were treated for 24 h according to the following experimental groups:

- control (without treatment)
- cells exposed to 100 nM PACAP for 24 h
- cells treated with 2 μg/ml gentamicin for 24 h
- cells treated with 100 nM PACAP and 2 μg/ml gentamicin simultaneously for 24 h

After incubation, supernatants were collected and the procedure was performed as described by the manufacturer, the kit contains all necessary contents. The array is based on the binding between the proteins of the sample and antibodies of the nitrocellulose membrane. Chemiluminescent detection was used, pixel density of the immunopositive spots was measured using ImageJ 1.40 software.

5.1.4. Statistical analysis

Each experiment was repeated minimum three times. Statistical analysis was performed by two-way analysis of variance. $p<0.05$ was considered as significant.

5.2. RESULTS

5.2.1. Cell survival – MTT assay

None of the applied concentrations (10, 100 nM, 1 μM) of PACAP alone resulted in significant changes in cell proliferation. Treatment of HK-2 cells with 2 μg/ml gentamicin led to a significant decrease in cell viability. PACAP treatment was able to alleviate the toxic effect of gentamicin, whereas PACAP alone did not influence the cell survival rate of proximal tubule cells.

5.2.2. Kidney biomarker array

PACAP alone significantly increased the expression of DPPIV and VEGF (vascular endothelial growth factor). Expression of DPPIV and VEGF was significantly decreased by gentamicin treatment, but PACAP was able to counteract this decrease.

5.3. DISCUSSION

Aminoglycoside toxicity is one of the most clinically relevant drug toxicities in the kidney, the main target of the aminoglycoside toxicity is the tubular system

especially the proximal tubule. Our results showed that PACAP protects HK-2 cells against gentamicin-induced toxicity. PACAP was able to counteract the decrease of cell survival caused by gentamicin.

We demonstrated that PACAP could alleviate the gentamicin-induced changes of the serine dipeptidase DPPIV. We detected DPPIV decreasing effect of gentamicin in vitro, which was counteracted by PACAP treatment. Our observations about the DPPIV suppressing effect of gentamicin are in accordance with data available in the literature. DPPIV is found in two forms: a soluble form playing a major role in the degradation of circulating PACAP in vivo and in a membrane-linked form. The latter has already been shown in many epithelial cell types, but the major source is the brush border of proximal tubules of renal cortex, suggested to be part of renal peptide transport system.

Our present data showed that PACAP significantly increased the expression of VEGF and could alleviate the VEGF decreasing action of gentamicin. Among others, VEGF affects endothelial cell proliferation and differentiation, acts on vascular permeability and blood vessel diameter. It has been shown to be present in the kidney where it has a peculiar distribution suggesting its regulatory role in glomerular permeability. Impaired function of VEGF system has been described in a wide variety of kidney diseases indicating its clinical relevance. In our present study, we found that in vitro gentamicin toxicity decreases the VEGF expression of human proximal tubule cells, which could be counteracted by PACAP co-treatment. This effect of PACAP is in accordance with previous studies where PACAP is labeled as a nonclassic regulator of angiogenesis.

In a previous study presence of PAC1R and VPAC1R in HK-2 cells was detected, from which PAC1R can be responsible for protective effect of PACAP against gentamicin toxicity.

In summary, present results showed that exogenous PACAP acts against gentamicin-induced injury in human proximal tubule cells: it enhances cell survival and counteracts the toxicity-induced decrease in the expression of DPPIV and VEGF. Based on our observations, PACAP could serve as a possible therapeutic option to reduce incidence of gentamicin-induced nephropathy.

6. SUMMARY, NEW FINDINGS

- ✓ We proved the protective effect of exogenous PACAP in renal ischaemia-reperfusion is male and female rats. PACAP decreased the histological damage, expression of inflammatory cytokines and increased the activity of antioxidant SOD. In the background of the effect of PACAP the importance of BMP4-BMPR1-Smad1-collagenIV signalling pathway was highlighted. Gender differences in renal ischaemia-reperfusion and regarding the PACAP treatment were also shown, in females less severe injuries were found.
- ✓ We confirmed the protective function of endogenous PACAP in ischaemia-reperfusion-induced kidney injury with the help of PACAP-deficient mice. Endogenous PACAP present in the human body was able to diminish histological damage, reduce expression of inflammatory cytokines and influence favourably the antioxidant system during renal ischaemia-reperfusion. In the background of the alterations of PACAP-deficient mice higher activity of pp38MAPK and pERK1,2, furthermore lower activity of pAkt was found.
- ✓ We showed the protective effect of PACAP in the kidney in case of the application of the widely used, nephrotoxic antibiotic, gentamicin. PACAP improved the survival of proximal tubule cells and influenced positively the expression of renal proteins in case of gentamicin toxicity.

In summary, our results confirmed the protective effects of both endogenous and exogenous PACAP in the kidney, its beneficial function was proved in gentamicin- and ischaemia-reperfusion-induced injury, both of which have clinical importance. In the future PACAP may have a possible significance in the treatment of these pathologies.

7. PUBLICATIONS

Publications related to the thesis

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Publications not related to the thesis

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