INVESTIGATION OF THE ROLE OF PACAP AND PACAP RECEPTORS IN ANIMAL MODELS OF DIABETIC NEPHROPATHY AND NEUROGENIC INFLAMMATION

Ph.D. thesis

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Hypophysis adenylate cyclase activating polypeptide (PACAP)

The neuropeptide PACAP was originally isolated from bovine hypothalamus by professor Arimura and coworkers, based on its ability to activate adenylate cyclase in rat pituitary cells. PACAP is a member of the vasoactive intestinal polypeptide (VIP)/ secretin/ glucagon peptide family, occuring in two biologically active forms with either 27 or 38 amino acid residues. PACAP exerts its effects via G-protein coupled receptors. Its specific receptor is PAC1, while VPAC1 and VPAC2 receptors bind PACAP and VIP with the same affinity.

Besides its expression in the central nervous system, PACAP is present in the small sensory neurons of the spinal ganglia and in the autonomic pre- and postganglionic neurons. Moreover, PACAP is expressed in the endocrine glands, including the pancreas, in the gastrointestinal, cardiovascular and respiratory system, and in the whole urinary tract from the kidney to the urethra.

PACAP is a pleiotropic and multifunctional polypeptide, its neurotrophic and neuroprotective effects were also thoroughly investigated. Its endocrine effects, including its ability to regulate the pancreatic insulin production, are also reported. PACAP is a potent vasodilator and bronchodilator, and it regulates several physiological processes. The general cytoprotective effect of both the endogenous and exogenous PACAP, which was shown in several cells and tissues both *in vitro* and *in vivo*, is the result of its antiapoptotic and antiinflammatory effect.

The immunomodulatory effect of PACAP, which is exerted through the regulation of the anti- and proinflammatory factors, was reported in several models of non-neurogenic inflammation. This mechanism strongly contributes to the protective effect of PACAP in case of several diseases, like diabetes mellitus, septic shock, stroke, multiple sclerosis and colitis. Protective effects of PACAP in diabetic nephropathy, which is also partly considered to be of inflammatory origin, were investigated in a short term model of the disease, lasting for 2 weeks only.

Similarly, antiinflammatory effect of PACAP has also been reported in neurogenic inflammation. However, the exact receptorial mechanisms are unknown.

I. INVESTIGATION OF THE PROTECTIVE EFFECT OF PACAP-38 IN DIABETIC NEPHROPATHY

INTRODUCTION

1. Diabetic nephropathy

Diabetes is the major cause of the end stage renal disease (ESRD) being responsible for 25-55% of all ESRD. After 25 years of diabetes, diabetic nephropathy develops in 25-40% of the patients, meaning that 150 million people are estimated to suffer from diabetic nephropathy worldwide.

Molecular mechanisms of the development of diabetic nephropathy

The development and the progression of the disease are multifactorial; genetic and environmental factors are both involved. Therefore, only 30-40% of the diabetic patients develop nephropathy.

Besides metabolic factors, hemodynamic changes, like activation of the renin-angiotensin-aldosterone system (RAAS), also contribute to the development of the disease. These result in intraglomerular hypertension with consequences as follows:

(1) Oxidative stress
(2) Increased production of advanced glycation end products (AGE)
(3) Apoptosis
(4) Overproduction of prosclerotic factors
(5) Inflammation due to the excessive release of proinflammatory cytokines

Histological changes of diabetic nephropathy

The internationally approved pathological classification of diabetic nephropathy was described by Tervaert and coworkers. Class 1 includes cases with the thickening of the glomerular basement membrane (GBM), which can only be detected using electonmicroscopy. In Class 2, the mesangial matrix expands, while patients with nodular sclerosis in at least one glomerulus (Kimmelstiel-Wilson lesion) are diagnosed as Class 3. Class 4 designates advanced, end stage diabetic glomerulosclerosis.

Moreover, endothelial cell injury, as well as apoptosis, detachment of podocytes, extensive flattening and fusion of podocyte foot processes are also significant for the initiation and progression of the disease. Renal tubules are also affected, their basement membrane thickens, interstitial fibrosis and tubular atrophy may occur. Hyalinosis of the renal arterioles is also characteristic.

2. Role of PACAP in the carbohydrate metabolism

PACAP is released from the insulin-producing β-cells in the islets of Langerhans, however its presence was also reported in the secretory granules of the α-cells. Similarly to other organs, the dominant form of PACAP in the pancreas is the 38-

amino-acid form of the peptide. Regarding the endocrine effects of PACAP, pancreatic expression of PAC1 and VPAC2 receptors is of great importance. Both the 27 and the 38 amino acid residues belong to the most potent insulinotropic peptides. PACAP also stimulates the proliferation of the β-cells. Besides the beneficial effects mentioned above, PACAP also effectively enhances the insulin-sensitivity of adipocytes and protects the β-cells against gluco-and lipotoxicity. Despite this, according to a human study, intravenous infusion of the peptide does not influence the blood glucose level, which may be due to the PACAP-induced enhanced glucagon and adrenalin release, increased food intake and hepatic glucose production. However, the blood sugar levels of mice overexpressing PACAP in their pancreatic β-cells and rats treated with PACAP intraperitoneally are shown to be lower in case of streptozotocin-induced diabetes.

3. Renal effects of PACAP

Mass spectrometric and radioimmunoassay analysis revealed the presence of PACAP in the kidney, the dominating form being PACAP-38 in both the cortex and the medulla. Expression of PAC1 és VPAC1 receptors was proved in the tubular cells, while VPAC2 receptors are present in the renal vessels only. Vasodilatory effect of the peptide is also exerted in the kidney, however activation of the PAC1 receptors stimulates renin secretion *in vitro*. Renoprotective effect of the peptide has been proved in numerous *in vitro* and *in vivo* models, including cast nephropathy in multiple myeloma, cisplatin-, gentamicin- and ischemia/reperfusion-induced kidney injury.

AIMS OF THE STUDY

In the first part of my PhD research, we investigated the effect of exogenous PACAP treatment in a rat model of 8-week diabetic nephropathy. We then examined the molecular mechanisms underlying the nephroprotective effect of PACAP-38, including effects exerted on inflammatory, apoptotic, prosclerotic processes and oxidative stress.

MATERIALS AND METHODS

1. Animals

Experiments were carried out using male Wistar rats (n=33) weighing 250-300 grams. Animals were kept in 12/12 h light-dark cycles and provided standard rat chow and water ad libitum. Animal housing, care and application of experimental procedures were carried out in accordance with ethical regulations and approved university protocols (University of Pécs; BA02/2000- 15024/2011).

Animals were randomly divided in 4 experimental groups:

1) control (saline intravenously (i.v.) and intraperitoneally (i.p.), n=10)
2) PACAP-38-treated control (saline i.v., 20 µg PACAP-38 i.p. every second day, n=6)
3) diabetic (65 mg/kg streptozotocin i.v. and saline i.p., n=7)
4) PACAP-38-treated diabetic (65 mg/kg streptozotocin i.v. and 20 µg PACAP-38 i.p. every second day, n=10).

Animals were administered 65 mg/kg of bodyweight streptozotocin (STZ) solved in 200 µl saline intravenously to induce diabetes, while PACAP-38 was injected in 100 µl saline intraperitoneally.
Body weight and blood glucose level of the rats were measured before the experiment and weekly during the 8-week experiment. Blood used for blood sugar assessment was obtained from the tail vein of the animals. Blood sugar concentration was measured using an automatic glucometer.
Animals were sacrificed after 8 weeks of survival using overanesthesia of isoflurane, their kidneys were removed and the weight of the kidneys was measured.

2. Light microscopic and morphometric analysis of the kidneys
Following the fixation of the kidneys using 4% buffered formalin, 5 µm thin serial sections were cut using a rotator microtome. Sections were stained with hematoxylin-eosin, periodic acid-Schiff (PAS) or with diastase digested PAS reaction and digital photos were taken. Amount of tubular glycogen granules and extent of the intraglomerular PAS-positive area were assessed to evaluate the severity of tubulopathy and glomeropathy, respectively. Area of interest was marked using Adobe Photoshop CS6 software, while extent of the area was measured using Scion Image software. Arteriolar hyalinosis was determined on HE-stained slides according to a score system that extends from 0 to 4.

3. Electronmicroscopic analysis of the kidneys
5 pieces out of each 1 mm^3 sized part of the renal cortex were fixed in 5% glutaraldehyde for 24 hours at +4°C, then the samples were postfixed in 1% osmium tetroxide for 1 hour at +4°C. After dehydration, propylene oxide was used as intermedier for 2x15 minutes, followed by an incubation of 1 hour in propylene oxide – resin with a ratio of 1:1 for another hour. This was followed by a 1 hour incubation in resin (Durcupan A+B+C+D) under heating using infrared light. Finally, samples were embedded in the mixture of Durcupan resin into gelatin capsules.
Semithin slides were cut with ultramicrotome (LKB Type 4801) and stained with toluidin blue. Areas of interest were marked on these slides, and ultrathin sections were prepared from these areas (Leica Ultracut R), which were further enhanced by

uranyl acetate and lead citrate. During the electronmicroscopic analysis, thickness of the basement membrane and morphology of the podocytes were determined using JEOL 1200 EX-II electronmicroscope. Thickness of the basement membrane was assessed on images taken with 50.000x magnification by measuring the length of the perpendicular line connecting the membranes of the endothelial cell and podocyte foot processes in nanometers using Adobe Photoshop CS6 software.

4. Investigation of the nephroprotective mechanisms of PACAP-38

Specimens used for molecular biological assays were obtained from the renal cortex near the superior pole of the kidney, frozen in liquid nitrogen, and stored at -80°C until further processing.

4.1. Measurement of inflammatory cytokines and adhesion molecules using rat cytokine array kit and Luminex Multiplex Immunoassay

Expression of the renal cytokines was determined in a semiquantitative fashion from kidney homogenates using cytokine array kit (R&D Systems). Cytokines were examined as follows: cytokine-induced neutrophil chemoattractant(CINC)-1, -2α/β, -3, ciliary neurotrophic factor (CNTF), fractalkine, granulocyte-macrophage colony stimulating factor (GM-CSF), soluble intercellular adhesion molecule(sICAM)-1, interferon(IFN)-γ, interleukin(IL)-1α, -1β, -1ra, -2, -3, -4, -6, -10, -13, -17, IFNγ-induced protein(IP)-10, lipopolysaccharide-induced cystein-X-cystein chemokine (LIX), L-selectin, monokine induced by IFN-γ (MIG), macrophage inflammatory protein(MIP)-1α, -3α, regulated on activation normal T cell expressed and secreted (RANTES), thymus chemokine, tissue inhibitor of metalloproteinase(TIMP)-1, tumor necrosis factor(TNF)-α and vascular endothelial growth factor (VEGF). Briefly, kidney samples were homogenized in PBS with protease inhibitor, followed by the addition of 1% Triton X-100 and a 1 hour incubation with 15 µl of biotinylated antibodies. After blocking, 1.5 ml of the homogenate incubated with biotinylated antibodies was added to the nitrocellulose membrane, then following an overnight incubation, horseradish peroxidase-conjugated streptavidin was added to the membranes, and membranes were exposed to a chemiluminescent detection reagent. Pixel density of the immunopositivity was measured using ImageJ 1.40 software.

Levels of sICAM-1 and L-selectin were measured by Luminex Multiplex Immunoassay using Flurokine MAP Rat Base Kit. Analysis was performed with Luminex 100 device, median fluorescence intensity was determined using Luminex 100 IS software. Samples were homogenized with RPMI-1640 containing 1% protease inhibitor cocktail. Samples (20 mg/ml) and the standard were added to a 96-well plate containing 50 µl of antibody-coated fluorescent beads. Biotinylated secondary and streptavidin-PE antibodies were added to the plate. After the last washing step, 100 µl of the buffer was added to the wells. The plate was incubated and read on the Luminex 100 array reader. Data were analysed using MasterPlex software. Results are given in pg/g wet tissue.

4.2. Quantitative real-time polymerase chain reaction (qRT-PCR)

Expression of collagen IV was determined using *Col4a1A* primer, actin (*Actb*) was used as internal control. Primers used for the RT-PCR analysis of collagen IV are: Col4a1: 5' – TCG GCT ATT CCT TCG TGA TG – 3' and 5' – GGA TGG CGT GGG CTT CTT – 3' (GenBank ID: NM_009931.2, 52°C, amplimer size: 209 bp) and in case of the actin: Actb: 5' – GCC AAC CGT GAA AAG ATG A – 3' and 5' – CAA GAA GGA AGG CTG GAA AA – 3' (GenBank ID: NM_007393, amplimer size: 462 bp). Optical density of signals was measured using ImageJ 1.40g freeware and results were normalised to actin.

4.3. Western blot

Specimens from the cortical part of the kidney were homogenized in 100 µl ice cold homogenization buffer containing 50 mM Tris-HCl, and protease inhibitor coctail was added to the homogenates. SDS–polyacrylamide gel electrophoresis was followed by transfer of the proteins to Protran nitrocellulose membranes. After blocking, membranes were incubated with the following primary antibodies overnight at +4°C: anti-tAkt, phosphospecific anti-Akt-1 Ser473, phosphospecific anti-ERK1/2 Thr202/Tyr204, phosphospecific anti-p38MAPK (1:500), anti-TGF-β1, anti-collagen IV (1:400) and anti-actin (1:10000). Membranes were then incubated with goat anti-mouse IgG (1:1500) or anti-rabbit IgG (1:3000) for 30 minutes. Signals were detected by enhanced chemiluminescence according to the instructions of the manufacturer. Optical density of Western blot signals was measured using ImageJ 1.40g freeware.

4.4. Biochemical assay of malondialdehyde (MDA), glutathione (GSH) and superoxide-dismutase (SOD)

Malondialdehyde (MDA) was measured in kidney homogenates with addition of TBA (saturated thiobarbituric acid in 10% perchloric acid) – 20% trichloroacetic acid reagent. After incubation at 100 °C for 20 minutes, samples were placed in icecold water and centrifuged. MDA concentration was determined spectrophotometrically by measuring the absorbance at 532 nm. Data are expressed in µmol/g tissue weight.

Glutathione (GSH) was quantified by adding 10% trichloroacetic acid to the samples and then the samples were centrifuged. 4 ml of 0.4 M tris-(hydroxymethyl)-amino-methane (TRIS)-buffer (pH 8.7) was added to 2 ml of the supernatant and samples were measured at 412 nm after adding 100 µl of 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 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.

5. Statistical analysis

Statistical analysis was performed by Microsoft Office Excel and GraphPad software. Repeated measures analysis of variance (ANOVA) with Bonferroni correction was used to detect significant differences between groups. P value less than 0.05 was considered to be statistically significant.

RESULTS

1. Effect of PACAP-38 treatment on blood glucose level, body weight and kidney weight – body weight ratio of control and diabetic animals

Blood glucose level of the rats varied between 5.6-7.8 mmol/l before the experiment. A single injection of streptozotocin caused a significant rise in the blood sugar level in both the PACAP-38-treated and untreated diabetic group. 8-week PACAP-38 treatment did not result in any change of the blood glucose levels of either the control or diabetic animals.

Similarly, PACAP-38 treatment did not lead to any significant change of the body weight, however, diabetic animals exhibited a significant weight loss at the end of the 8th week. Decrease of body weight was milder in the PACAP-38-treated diabetic animals, but the difference did not result to be statistically significant.

Similarly, no significant changes were observed in the kidney weight – body weight ratio between the control and PACAP-38-treated control animals, while diabetic animals exhibited a significant increase in the ratio. PACAP-38 did not influence the increased kidney weight – body weight ratio in diabetes significantly.

2. Histological findings

2.1. Effect of PACAP-38 treatment on diabetes-induced glomarular alterations

In control animals, normal glomerular structure was observed, in which 8 weeks of PACAP treatment did not cause any changes. Kidneys from diabetic animals showed signs of severe diabetic glomerulopathy with mesangial matrix expansion and thickening of the glomerular basement membrane. Common consequence of these changes is the significantly increased intraglomerular PAS-positive area, which is a reliable indicator of the severity of glomerulopathy. This was found to be significantly increased in the diabetic rats, while PACAP-38 treatment could effectively counteract this lesion: PACAP-treated diabetic animals showed no significant difference compared to the intact control kidneys.

2.2. Effect of PACAP-38 treatment on diabetes-induced tubular changes

PAS-positive granules were present in the tubular epithelial cells of the kidney samples of the diabetic animals. The diastase digested PAS reaction performed for the analysis of these deposits proved that these tubular granules contained glycogen.

Accumulation of glycogen deposits in the tubules, also called Armanni-Ebstein phenomenon, is an important characteristic of diabetic nephropathy. These PAS-positive deposits were completely absent from the kidneys of control and PACAP-38-treated control animals. Severe Armanni-Ebstein phenomenon was detected in the untreated diabetic kidneys, excessive amount of glycogen granules was present in these samples. PACAP-38 significantly decreased the degree of glycogen granule formation in the renal tubules of the diabetic animals.

2.3. Effect of PACAP-38 treatment on diabetes-induced arteriolar hyalinosis

Vascular hyalinosis, a characteristic, but not pathognomic alteration in diabetic nephropathy, was sparingly present in the PACAP-38-treated and untreated control animals. The degree of arteriolar hyalinosis was significantly higher in the untreated diabetic group compared to control animals, while PACAP-38 treatment in diabetic animals was able to completely prevent the development of arteriolar hyalinosis.

3. Effect of PACAP-38 on electronmicroscopic changes of diabetic nephropathy

Electronmicroscopy revealed segmental thickening of the glomerular basement membrane (GBM) in several parts of the untreated diabetic glomeruli. The thickness of these parts of the GBM was significantly greater than the GBM in control, PACAP-38-treated control and PACAP-38-treated diabetic kidneys. However, we could not detect any changes between the non-thickened part of the GBM in diabetic animals and the thickness of the GBM in control animals. PACAP-38-treated diabetic animals did not show this segmental thickening; there was no difference between values of PACAP-treated or untreated control or PACAP-treated diabetic animals.

Severe podocyte injury was observed in the diabetic glomeruli with fusion, marked broadening and extensive flattening of the podocyte foot processes. Podocytes in the PACAP-38-treated diabetic kidneys did not show any morphological alterations compared to the control groups, electronmicroscopic images showed intact podocyte foot processes.

4. Mechanism of the nephroprotective effect of PACAP-38

4.1. Effect of PACAP-38 treatment on the expression of cytokines, chemokines and adhesion molecules

PACAP-38 treatment had no effect on the level of numerous cytokines in control animals, however, it elevated the expression of a few cytokines: TIMP-1, MIG, MIP-3α, RANTES, L-selectin, while the peptide decreased the levels of LIX and CNTF. Diabetes markedly increased the expression of CINC-1, TIMP-1, LIX, MIG, MIP-3α, RANTES, CNTF and levels of L-selectin and sICAM adhesion molecules. Treatment with 20 μg PACAP every second day markedly decreased the

expression of the above-mentioned cytokines and chemokines, with some of them reaching the levels of control animals.

4.2. Effect of PACAP-38 on the expression of collagen IV

Diabetes significantly elevated the renal expression of type IV collagen, a collagen playing an outstanding role in the structure of the basement membrane. 8-week PACAP-38 treatment effectively counteracted the upregulation of collagen IV.

4.3. Effect of PACAP-38 treatment on the level of prosclerotic and apoptotic factors

Diabetes excessively upregulated the renal expression of collagen IV and TGF-β1, which are key fibrotic markers in the pathogenesis of diabetic nephropathy. PACAP-38 significantly attenuated the increased production of these factors.

PACAP alone increased the level of the phosphorylated form of Akt in control animals. Diabetic nephropathy is accompanied by excessive apoptosis of the renal cells, shown by the upregulation of the phosphorylated form of the proapototic pp38MAPK. However, the antiapoptotic Akt and ERK1/2 were also activated in the untreated diabetic kidneys. In the PACAP-38-treated diabetic animals, activation of the antiapoptotic factors, like pAkt and pERK1/2 was significantly increased compared to their untreated diabetic mates. Moreover, PACAP significantly inhibited the activation of p38MAPK and decreased the diabetes-induced elevation in the cleaved caspase-3 levels. Diabetes resulted in the upregulation of the p60 NFκB, a protein known to have an important role in both cytokine production and cell survival. PACAP treatment in diabetic animals resulted in a significantly attenuated activation of p60NFκB.

4.4. Effect of PACAP-38 treatment on the levels of malondialdehyde (MDA), glutathione (GSH) and superoxide-dismutase (SOD)

Biochemical assay of the oxidative stress markers revealed a significant elevation in the renal GSH concentration of the PACAP-treated diabetic group compared to their untreated diabetic mates. PACAP treatment caused a significant increase in the SOD level of the control animals, however, diabetes did not result in significant changes in the renal expression of SOD and MDA.

DISCUSSION, CONCLUSIONS

In the first part of our study, we revealed evidence that *in vivo* PACAP-38 treatment exerts protective effect in 8-week diabetic nephropathy. Moreover, we applied 8-week long PACAP treatment for the first time, proving the efficiency of the long-term PACAP treatment.

Our histological analysis showed that PACAP-38 treatment significantly attenuated the characteristic glomerular, tubular and vascular alterations of diabetic nephropathy without influencing the blood glucose level of the rats.

In the second part of our study, we revealed the underlying molecular mechanisms of the nephroprotective effect by proving the antiinflammatory, antiapoptotic, antifibrotic and antioxidative effects of PACAP.

Inflammatory processes, as previously mentioned in the introduction, are key pathogenetic factors of diabetic nephropathy leading to tubulointerstitial fibrosis, tubular atrophy and vascular damage. Similarly to our findings, immunomodulatory effect of PACAP on cytokine expression has also been proved in several earlier studies.

Moreover, PACAP-38 treatment decreased the diabetes-induced increase in the renal NFκB levels, which has key role in the development of diabetic nephropathy. Numerous studies reported that PACAP, similarly to the structurally related VIP, inhibits the translocation of NFκB to the cell nucleus by counteracting the phosphorylation of IκB both *in vivo* és *in vitro*. Activation of NFκB results in tubular damage and excessive expression of proinflammatory cytokines, chemokines and adhesion molecules. Furthermore, NFκB is also supposed to be involved in the signal transduction of the apoptotic events induced by reactive oxygen species. PACAP exerted an inhibitory effect on these processes by decreasing the activation of NFκB.

In the present study, similarly to numerous earlier studies, we found that PACAP stimulated the phosphorylation of the antiapoptotic Akt and ERK1/2, while decreased that of the proapoptotic p38MAPK and inhibited the activation of caspase-3. Caspase-3 is known to be involved in the signal transduction of the hyperglycaemia-induced apoptosis of podocytes.

TGF-β1 leads to excessive production and decreased breakdown of fibronectin, collagen IV and laminin in the mesangial matrix, glomerular and tubular basement membrane and in the interstitium, resulting in severe morphological and functional changes of the kidney. The PACAP-induced decreased expression of TGF-β1 and collagen IV contributes to the observed histological improvement in the PACAP-treated animals.

Significance of the oxidative stress on the development of diabetic complications is well known. Decreased antioxidative protection due to the decreased level of the active form of gluthatione also contributes to the oxidative damage of the renal cells besides the overproduction of mitochondrial free radicals. In our study, PACAP

counteracted the diabetes-induced decrease in the GSH level, proving the antioxidative effect of PACAP.

Based on these results, PACAP seems to be a promising candidate in the therapy of diabetic nephropathy. However, several aspects have to be considered before its clinical application, including the possible side-effects and poor bioavailability. In the present study, chronic intraperitoneal PACAP-38 treatment did not cause any remarkable side-effects in rats. A previous human study also proved that systemic infusion of the peptide does not evoke significant side effects. The other drawback of systemic PACAP administration is the poor bioavailability, since the half-life of the peptide in the circulation is only 2-10 minutes due to its rapid degradation by dipeptidyl peptidase IV (DPP IV). However, nowadays several studies aim at finding an easy and reliable way of PACAP treatment. In the present study, intraperitoneal injection of the peptide every second day resulted in a significant protective effect despite its short half-life. This prolonged duration of the effect can be presumably due to the long-term activation of the signal transduction pathways, however, its half-life is short in the circulation. Inhibitors of the DPP IV enzyme are widely used in the clinical therapy of type 2 diabetes, exerting their effects by increasing the level of glucagon-like peptide-1 (GLP-1) and according to recent studies, by upregulation of PACAP also.

In summary, with the present study we proved the protective effect of PACAP-38 treatment against the diabetes-induced glumerulopathy, tubulopathy and vasculopathy in a rat model of 8-week diabetic nephropathy. Moreover, we demonstrated that antiinflammatory, antifibrotic, antioxidative and antiapoptotic mechanisms are involved in the protective effect of PACAP. Due to the fact, that PACAP stimulates beta cell proliferation and insulin secretion, and has been shown to be protective against diabetic retinopathy, PACAP inhibits the development and progression of the diabetic complications at multiple levels, thus providing a promising opportunity for the complex treatment of diabetes mellitus.

II. EXAMINATION OF THE ROLE OF PAC1 AND VPAC1/2 RECEPTORS IN THE INHIBITORY EFFECT OF PACAP ON NEUROGENIC INFLAMMATION

INTRODUCTION

1. Transient Receptor Potential Ankyrin 1 (TRPA1) channels and their role in neurogenic inflammation

Transient Receptor Potential Ankyrin 1 (TRPA1) is a member of TRP ion channel receptor family, which is involved in pain sensation and inflammatory processes. TRPA1 receptors are expressed in the central and peripheral nerve endings of nociceptive primary sensory neurons in trigeminal and nodose ganglia, where they uptake and amplify nociceptive stimuli. Most important role of the receptors is to provide sensitivity against external irritants, therefore they are located in sensory nerve endings of the skin, respiratory and gastrointestinal tract. Numerous natural and synthetic stimulants of the receptor are known, including mustard oil, chemically named as allyl-isothiocyanate. Mustard oil induces acute neurogenic inflammation by specifically activating TRPA1 receptors on sensory nerve endings, which is accompanied by erythema, edema, pain and mechanical and thermal hyperalgesia.

TRPA1 and TRPV1 channel expressing sensory nerve terminals possess a triple function; afferent function, meaning the orthodromic, central conduction of the pain, resulting in nociception and pain sensation. Local efferent function refers to the release of proinflammatory neuropeptides, like calcitonin gene-related peptide (CGRP) and tachykinins: substance P (SP) and neurokinins (NK) from the sensory nerve terminals. The released CGRP causes arteriolar vasodilation, while tachykinins induce the accumulation of leukocytes and plasma extravasation, accompanied by local inflammatory hypersensitivity, a process called neurogenic inflammation. Finally, the systemic efferent function means the release of systemic antiinflammatory peptides, like somatostatin and PACAP.

Neurogenic inflammation plays a significant role in the pathogenesis of several diseases, like rosacea, allergic contact dermatitis, atopic dermatitis, migraine, allergic rhinitis, sarcoidosis, rheumatoid arthritis, psoriasis, asthma and chronic obstructive pulmonary disease (COPD). However, neurogenic inflammation cannot be alleviated by the conventional nonsteroid antiinflammatory drugs, therefore, presently there is no available therapy to eliminate the primary pathogenetic factor of these diseases.

2. Role of PACAP in neurogenic inflammation

PACAP is expressed in the small and medium-sized neurons of the dorsal root ganglion, in the trigeminal ganglion, in capsaicin-sensitive sensory neurons and in the superficial layers of the dorsal horn of the spinal cord. Protective effect of PACAP against diseases associated with neurogenic inflammation was proved in

numerous conditions, like asthma, rheumatoid arthritis, allergic contact dermatitis and Crohn's disease. Enhanced release of PACAP-38 in response to capsaicin- and electric field stimulation has been reported from the myenteric plexus of the stomach, eye and sensory nerve terminals of the trachea *in vitro* and from rat spinal cord *in vivo*. Systemic TRPV1 agonist resiniferatoxin induces the release of PACAP from capsaicin-sensitive nerve terminals, resulting in a detectable increase in the PACAP-like immunoreactivity of the blood plasma. This effect is absent after local stimulation of the nerve endings. Antiinflammatory action of the peptide has been observed also *in vivo*, intraperitoneal PACAP-38 significantly reduces the neurogenic edema and plasma extravasation induced by 1% mustard oil, capsaicin or resiniferatoxin. This effect may be due to the local antiinflammatory effect of the released PACAP-38 and its concentration-dependent inhibitory effect on the CGRP and SP release.

3. Role of maxadilan and VIP in inflammation

Maxadilan, a peptide consisting of 61 amino acids is the specific agonist of the PAC1 receptor. Besides its potent vasodilatory effect, numerous studies have reported its significant antiinflammatory action. However, its effect on neurogenic inflammation remained unknown.

The 28-amino acid peptide, VIP is co-expressed with the structurally similar PACAP in the perikarya of the parasympathetic, dorsal root, otic, sphenopalatine and nodose ganglia. Immunomodulatory actions of VIP are mainly mediated via VPAC1 receptors through activation of adenylate cyclase. VIP knockout mice are more susceptible to several inflammatory diseases, including septic shock, bronchial asthma and pulmonary hypertension. Involvement of VIP in neurogenic inflammation has been demonstrated by several experiments. The neuropeptide inhibits the glutamate- and oxidative stress-induced pulmonary edema, capsaicin-triggered bronchoconstriction and respiratory inflammation. Similarly to PACAP and maxadilan, intradermal injection of VIP also stimulates plasma extravasation in non-inflamed tissues, proving that in lack of mustard oil stimulation, activation of both the PAC1 and VPAC1/2 receptors is associated with increased vascular permeability.

AIMS OF THE STUDY

In the second part of my Ph.D. research, I investigated the involvement of PAC1, VPAC1 and 2 receptors in the inhibitory action of PACAP in neurogenic inflammation. In the present study, effect of the selective PAC1 receptor agonist maxadilan, and the VPAC1/2 agonist VIP was examined on mustard oil-induced neurogenic edema, plasma protein extravasation and vasodilation. The late, non-neurogenic phase of the inflammation, characterized by the infiltration of neutrophil granulocytes, was assessed by measuring the tissue myeloperoxidase enzyme activity.

MATERIALS AND METHODS

1. Animals

Experiments were performed using 3-month-old female CD1 mice. All experimental procedures were carried out in accordance with ethical regulations and approved protocols (University of Pécs; BA02/2000- 15024/2011). Experimental groups were as follows:

1) I.p. maxadilan-treated (100 μg/kg; n=48)
2) I.p. VIP- treated (100 μg/kg; n=52)
3) I.p. saline-treated (10 ml/kg; n=52).

Intraperitoneal drug administrations were performed at least 15 minutes prior to the experiments. Core body temperature of the animals was maintained at 38ºC with a heating pad.

2. Measurement of mustard oil- and formalin-induced neurogenic edema formation in the mouse ear

Ear thickness was measured with engineer's micrometer under ketamine and xylazine (100 mg/kg and 5 mg/kg, i.p., respectively) anesthesia. After the control measurements, either 10-10 μl 1 or 5% mustard oil dissolved in paraffin oil or 5% formalin dissolved in distilled water was applied topically on both surfaces of the ear at the beginning of the experiment and 1 hour later. Ear thickness was assessed before the treatment as control and 30 minutes after the application of mustard oil or formalin, and later every hour until the end of the 6-hour experiment. Data are shown as means ± SEM of percentage increase of ear thickness compared to the initial controls.

3. Measurement of Evans blue-bound albumin extravasation in the mouse ear

Mice were anesthetised with urethane (1.2 g/kg) and Evans blue (25 mg/kg) was injected intravenously at least 10 minutes prior to experiment. 3 images were taken as control using Nikon intravital videomicroscope (1x objective and 2x optical zoom) before the topical application of 20 μl 5% mustard oil or paraffin – in case of control animals – on the dorsal surface of the ears. Pictures were taken in every 30 sec for 30 minutes after paraffin or mustard oil treatment. Intensity was determined using Image-Pro Plus 7.0.0.591 software. Data were expressed in % ± SEM of the control values.

4. Determination of the cutaneous microcirculation in the ear by laser Doppler method

Cutaneous blood flow was recorded by laser Doppler perfusion imaging (Periscan PIM-II, Perimed, Sweden) under ketamine and xylazine anesthesia. Three images were taken as control at the beginning of the experiment and then 20 μl 5% mustard oil was applied topically to the dorsal surface of the right ear, while the left ear was

treated with the solvent, paraffin oil. Scan of the ears was performed in every 2 minutes. Two regions of interest (ROIs) were chosen representing the total area of both ears. Blood flow of the ears was calculated by comparing mean microcirculation values of the ROIs to those measured on the three baseline images. In order to exclude systemic perfusion changes, blood flow values of the vehicle-treated ears were subtracted from those of the ones treated with 5% mustard oil.

5. Determination of the myeloperoxidase (MPO) activity of the ears

6 hours after the first topical application of 5% AITC, ears were removed, and stored at -80°C until further processing. Ears were homogenized in potassium-phosphate buffer. After centrifugation, the pellet was resuspended in potassium-phosphate buffer containing 0.5% hexadecyl-trimethylammonium (HTAB). MPO activity was determined from the supernatant after another centrifugation with the addition of the substrate of MPO, H_2O_2-3,3′,5,5′-tetramethylbenzidine (TMB/H_2O_2). The optical density (OD) was measured twice with 5 minutes difference at 620 nm using a Labsystems microplate reader and plotted against the human standard samples. Change of optical density per unit time (ΔOD/time) was used to determine the activity of MPO enzyme in the samples. Data were expressed in U/g wet tissue.

6. Statistical analysis

Statistical analysis was performed by GraphPad software. One-way or Two-way repeated measures analysis of variance (ANOVA) with Bonferroni correction was used to detect significant differences between groups in all experiments. P value less than 0.05 was considered to be statistically significant.

RESULTS

1. Effect of maxadilan and VIP on mustard oil- and formalin-induced neurogenic edema

Maximal increase of ear thickness was ~20%, ~45% and ~80% after topical application of 1 and 5% mustard oil and 5% formalin, respectively.

Topical application of 1% mustard oil led to a maximum of 21.2% increase in ear thickness after 4 hours in the control, saline-treated animals. The mustard oil-induced edema was absent in the maxadilan-treated group: maxadilan significantly counteracted the neurogenic edema formation both 3 and 4 hours after the topical application of 1% MO with a maximum of 4.8% increase in ear thickness. VIP treatment did not lead to significant inhibiton of the neurogenic edema.

5% MO caused markedly greater edema compared to the 1% solution with a maximum of 45.9% increase in the saline-treated animals. Maxadilan significantly decreased this neurogenic edema formation in the first 5 hours of the experiment with a maximal ear swelling of 25.6%. VIP also inhibited the development of neurogenic edema, reaching statistical significance after 2 and 4 hours. However,

similarly to the results obtained with 1% MO, the inhibitory effect of maxadilan was also significantly greater than that of VIP throughout the whole experiment.
No difference was observed in the 5% formalin-induced neurogenic edema between the vehicle- and the VIP-treated animals, maximal increase of ear thickness was 79.4% and 77%, respectively. Maxadilan significantly counteracted the formalin-induced neurogenic edema during the 6 hours of experiment.
Based on these results, 5% MO was found to be the most suitable compound to examine the effect of maxadilan and VIP on neurogenic inflammation. Therefore, further experiments were performed using 5% MO.

2. Effect of VIP and maxadilan on plasma extravasation induced by 5% mustard oil in the mouse ear

In the control, solvent-treated animals, 5% mustard oil increased the albumin-leakage of the postcapillary venules significantly with 48.3% compared to the paraffin oil-treated ears. Systemic VIP treatment did not prevent the plasma protein extravasation during the 30 minutes of experiment, increase in plasma leakage was 43.7% in VIP-treated animals. MO-evoked albumin leakage was significantly decreased by maxadilan with 15.8% of maximal swelling.

3. Effect of VIP and maxadilan on 5% mustard oil-induced vasodilation in the mouse ear

Maxadilan induced a basal vasodilation in the mouse ear, while VIP did not change the baseline perfusion. Increase of neurogenic cutaneous vasodilation reached its maximum 6-10 minutes after the topical application of 5% mustard oil with a peak value of 87.2% in the vehicle-treated groups. Systemic administration of both VIP and maxadilan significantly counteracted the MO-induced vasodilation with a maximum of 39.4% and 19.2% inhibition of the cutaneous blood flow, respectively. The inhibitory action of the PAC1 receptor agonist was greater, showing statistical significance at 4-8. minutes of the experiment.

4. Effect of VIP and maxadilan on the myeloperoxidase activity

6 hours after the stimulation, MPO activity was significantly increased in the mustard oil-treated ears compared to the respective paraffin oil-treated controls. However, neither VIP, nor maxadilan exerted any effect on this parameter suggesting that activation of VPAC1/2 or PAC1 receptors does not influence the accumulation of granulocytes in either inflamed or non-inflamed tissues.

DISCUSSION, CONCLUSIONS

In the present study, we provided the first evidence that PAC1 receptor activation by the selective agonist maxadilan inhibits acute neurogenic inflammation. This effect is due to the inhibition of both arterial vasodilation and plasma protein leakage from the veins and capillaries as demonstrated by the significantly attenuated tissue perfusion with laser Doppler imaging, as well as diminished plasma protein extravasation with micrometry and intravital microscopy. The edema-inhibiting action of the VPAC1/2 receptor agonist VIP was milder, reaching the level of statistical significance only after topical application of 5% MO. Edema formation is predominantly due to plasma protein extravasation, which was not influenced by VIP treatment. The later, basically non-neurogenic, cellular phase of the mustard oil-induced inflammation occurs 6 hours after the topical application of MO in the mouse ear and capsaicin-sensitive sensory nerve endings are not involved in its development. PACAP-38 has been reported to decrease only the neurogenic phase of the inflammation, however, the peptide did not influenced the infiltration of leukocytes indicated by the unchanged myelopreoxidase activity. In the present study, we could not detect any changes in the extent of cellular infiltration either in case of PAC1R or VPAC1/2R activation.

PACAP inhibits acute neurogenic inflammation in a concentration-dependent manner, the neuropeptide was found to exert significant inhibitory action on MO-induced neurogenic inflammation in a dose of 100 μg/kg. Moreover, PACAP exhibits a local immunomodulatory function by inhibiting capsaicin- and electric field stimulation-induced CGRP, SP and somatostatin release from the sensory nerve terminals of the trachea *in vitro* and thus counteracts neurogenic edema and plasma albumin extravasation. However, our present results are the first to reveal the receptorial mechanism responsible for the inhibitory effect of PACAP-38 in neurogenic inflammation.

Evidence has accumulated over the last decade suggesting that VPAC1 receptor is primarily responsible for the anti-inflammatory actions of VIP and PACAP in experimental arthritis and Crohn's disease, while PAC1R was found to mediate the protective effects against septic endotoxemia. Involvement of VPAC1/2 receptors was also reported in pressure-induced vasodilation, a process associated with the activation of capsaicin-sensitive nerve fibers and CGRP-release. VIP induces neurogenic inflammation and degranulation of mast cells more potently than PACAP, which also suggests the importance of VPAC1/2 receptors. Even in a human study, VPAC2 receptor was shown to exert inhibitory action against bronchial asthma. However, based on our results, involvement of VPAC1 and 2 receptors in the inhibitory action of PACAP on neurogenic inflammation is limited only to arterial vasodilation.

A great amount of publications reported the vasoregulatory effect and immunomodulatory properties of maxadilan in non-neurogenic inflammation, which were also shown to be essential for the transmission of Leishmania.

However, its involvement in neurogenic inflammation has not been investigated yet. Our results demonstrate that maxadilan – despite its strong vasodilator effect on paraffin-treated ears – diminishes neuropeptide-mediated increased blood flow under inflammatory conditions.

The presently known signaling mechanisms linked to PAC1 receptor stimulation all lead to cAMP and calcium increase, which cannot give an appropriate molecular explanation for the observed potent inhibitory actions on the sensory nerve endings. Since we have previously shown that PACAP6-38, which is widely used as a PAC1/VPAC2 antagonist in several systems, acts as a potent agonist on the sensory nerve terminals, the presence of a not yet identified PAC1-related novel receptor or a splice variant can be suggested on the capsaicin-sensitive afferents.

In summary, we found that maxadilan significantly attenuated neurogenic edema, reduced vascular permeability and increase of microcirculation, while the inhibitory effect of VIP was limited to neurogenic vasodilation only. These data indicate that inhibitory effect of PACAP on the mustard oil-induced neurogenic inflammation is mediated via PAC1 receptors. Our present results raise the possibility of the use of PAC1 receptor selective agonists in the therapy of neurogenic inflammation associated diseases.

SUMMARY OF THE NEW FINDINGS

In the first part of my Ph.D. research, I investigated the effect of PACAP in diabetic nephropathy with histological and molecular biological methods. In the second part, I examined the receptorial mechanism responsible for the antiinflammatory effect of PACAP in neurogenic inflammation.

1. Intraperitoneal PACAP-38 treatment significantly diminished glomerulopathy, tubulopathy and vasculopathy induced by the 8-week diabetes. Electronmicroscopy revealed the protective effect of PACAP against diabetes-induced basement membrane thickening and podocyte injury. We proved the antifibrotic and antiapoptotic effects underlying the nephroprotective effect of the peptide by PCR and Western blot methods. Antiapoptotic action of the PACAP treatment was shown by decreased phosphorylation of the proapoptotic and increased activation of the antiapoptotic factors. Antiinflammatory and antioxidative effects of the peptide, indicated by the reduced expression of proinflammatory cytokines and elevated glutathione levels, also contribute to the renoprotection. Our present study explored an other beneficial aspect of PACAP treatment, which suggests that PACAP could be a promising candidate in the complex treatment of diabetes.

2. Our results demonstrate the inhibitory action of the PAC1 receptor agonist maxadilan on mustard oil-induced neurogenic edema, plasma extravasation and vasodilation. However, the VPAC1/2 receptor agonist VIP mainly decreased the neurogenic vasodilation, while it did not influence the permeability of the postcapillary venules. Based on these findings, PACAP exerts its antiinflammatory action against neurogenic inflammation via PAC1 receptors. Our results may contribute to the specific treatment of neurogenic diseases.

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PUBLICATIONS

Publications related to the thesis:

Bánki E, Degrell P, Kiss P, Kovács K, Kemény Á, Csanaky K, Düh A, Nagy D, Tóth G, Tamás A, Reglődi D. (2013) Effect of PACAP treatment on kidney morphology and cytokine expression in rat diabetic nephropathy. *Peptides.* 42:125-130. (IF:2.614)

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