The role of PACAP in the function of retinal pigment epithelium

Ph.D. thesis

Fábián Eszter

University of Pecs, Medical School
Department of Anatomy
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Doctoral school leader:	Júlia Szekeres MD., PhD., DSc.
Program leader:	Valér Csernus MD., PhD., DSc.
Supervisors:	Dóra Reglődi MD., PhD., DSc. Krisztina Kovács MD., PhD.

I. Introduction

1. The retina and retinal pigment epithelium

The retina is the innermost, photosensitive layer of the eye. The light is focused to the retina by the optic system of the eye, which induces several electric and chemical responses, through which the light, as electric impulse is projected to the primary visual cortex in the occipital lobe. We can distinguish ten layers within the retina. The retinal pigment epithelium (RPE) is a monolayer found between the neuroretina and the choroid, this is the outermost layer of the retina. The apical surface faces the outer segments of the photoreceptor cells, the basolateral surface is in strong contact with the Bruch's membrane, which separates the RPE cells from the fenestrated endothelium of the choriocapillary layer. The pigment epithelium contributes to the formation of the outer blood-retina barrier (BRB). The tight junctions between the RPE cells and the endothelial cells play a critical role in controlling the fluid flux through the BRB. They prevent the diffusion of toxic agent and plasma consituents into the retina.

The retinal pigment epithelium secretes different growth factors and proteins essential for the structural integrity of the retina and the choriocapillary. Thus, RPE produces molecules wich maintain photoreceptor survival and provide proper nutrition of the retina from the choroid. One of the most important factors secreted by the pigment epithelial cells is the vascular endothelial growth factor, VEGF. In the healthy eye it is produced in a low concentration, its function is to inhibit the apoptosis of the endothelial cells, thus playing a crucial role in maintaining the structural integrity of the choriocapillary. VEGF influences the cell permeability by stabilizing the fenestration of the endothelium. Overexpression of VEGF is one of the major inducers of the proliferative diabetic retinopathy (PDR). In western countries, among the working individuals diabetic retinopathy is the main cause of blindness. In type one diabetes the proliferative diabetic retinopathy, in type two diabetes the diabetic macular edema are the most common factors in visual impairment. The more common occurance of the type two diabetes makes it the leading cause of visual loss in diabetic patients. Moreover, it has been showed that patients suffering from type two diabetes along with PDR often develop macular edema as well.

The most important components of the pathogenesis of proliferative diabetic retinopathy is the neovascularization occurring after hypoxic conditions, and in case of macular edema the

influx of fluids due to the damage of the blood-retina barrier. Most studies dealing with diabetic retinopathy are concerned with the disfunction of the inner blood-retina barrier and the neuroretina, and very few are focusing on the effects of diabetes on the pigment epithelium. This thesis tries to emphasize the better understanding of the function and the signalling pathways of the pigment epithelial cells. The pathogenesis of macular edema is less understood, but it is known that VEGF and other inflammatory cytokines play a crucial role in the development of this contition. Thus inhibition of VEGF secretion could be an effective treatment for both diabetic retinopathy and macular edema.

2. PACAP

Pituitary adenylate cyclase activating polypeptide (PACAP) is a peptide with a wide range of functions, isolated first by Arimura from ovine hypothalamus in 1989. The first studies showed cAMP stimulating effects of PACAP in rat adenohypohysis. There are two biologically active forms of PACAP, the PACAP1-38 (constituted of 38 aminoacids) and the PACAP1-27 (27 aminoacids). The latter shows a 68% homology with the vasoactive intestinal polypeptide (VIP), so PACAP was classified as a member of the VIP/glucagon/GRF/secretin superfamily. Later PACAP was isolated from other animal species and they found that the aminoacid sequency is identical in all mammals, and there are only 1-4 aminoacid difference in lower vertebrates. This conservative structure of the peptide suggests that it plays major roles in basic physiological processes.

Presence of PACAP and receptors in the eye

PACAP-like immunoreactivity (PACAP-IR) was measured in the eye with radioimmunassay, and the highest concentrations were found in the sphincter pupillary muscle and the ciliary body outside the retina. PACAP positive nerve fibres were detected in the optic nerve layer, the ganglion cell layer and the internal plexiform layer of the retina. Besides, the perikarya of amacrine and horizontal cells in the internal nuclear layer and the ganglion cells were also stained with immunohistochemical methods.

In the retina and other tissues of the eye of rat, mouse and pig PACAP could cause a high cAMP increase. PACAP1-38 was found to be more effective than PACAP1-27, but both lead to a stronger cAMP activation than VIP. In certain groups of ganglion cells, wich project

to the suprachiasmatic nucleus, PACAP is stored together with glutamate and plays an important role is the regulation of circadian cycle.

PAC1 receptor and its mRNA was found in the highest concentration in the ganglion cell layer and the inner nuclear layer. In the pigment epithelium all three PACAP receptor mRNA were detectable.

In vitro protecting effect of PACAP in pigment epithelial cells

The first results concerning the effects of PACAP came from Zhang et al. Presence of mRNA for PAC1 and VPAC1 receptors was confirmed in unstimulated human RPE cells (ARPE-19). VPAC2 mRNA was expressed in cells stimulated with IL-1β. PACAP treatment at 0,1-1 micromolar concentrations inhibited the IL-1 β-stimulated IL-6, IL-8, and MCP-1 mRNA and protein levels. ARPE19 cells exposed to PACAP1-38 could be rescued against oxidative stress induced by H_2O_2 in a dose-dependent manner. Highest efficacy was observed at 100 nM PACAP concentration, but it was effective in a range 10 pM- 1 μM, while not effective at 1 pM. Using flow cytometric and JC-1 assay, PACAP treatment was proven to reduce apoptotic cell death. The survival-promoting effect was inhibited by PI3K/Akt inhibitors, but not affected by MAPK inhibitors. A more recent study has reported that PACAP regulates semaphorin4A expression from RPE cells. The semaphorin protein family has members that influence brain and retinal development, by providing neural guidance. Semaphorin4A is expressed in the GCL, INL and RPE cell during the developmental period of contact-building between photoreceptors and RPE cells. Human ARPE cells were co-cultured with PC12 cells (prototype neuronal cells) based on a collagen vitrigel membrane. The authors found that in the presence of neural cells the protein and mRNA levels of semaphorin4A (but not other semaphorins) were increased, an effect mimicked by PACAP (but not VIP, NPY or enkephalin). Furthermore, this effect was also associated with ERK phosphorylation (but not that of JNK or p38 MAPK). All these effects of PACAP were blocked by the PACAP antagonist PACAP6-38. Furthermore, PACAP6-38 downregulated the cytokine IL-6 expression, indicating that endogenous PACAP participates in the neural cell-induced IL-6 production (other cytokines out of the tested 11 were not influenced by the neuronal co-culture). These results suggest that the PACAP secreted by neural cells regulates several factors secreted by RPE cells, important for retinal development and homeostasis.

Another study investigated the potential of PACAP and VIP against the disruption of tight junctions in the retina, a major factor in macular edema developing in diabetic retinopathy.

ARPE-19 cells were cultured either in normal or in high glucose in combination with the pro-inflammatory IL-1β. Effects on permeability were evaluated by measuring both apical-to-basolateral movements of fluorescein isothyocyanate (FITC) dextran and transepithelial electrical resistance. Results of these experiments demonstrated that normal glucose+IL-1β and, to a greater extent, high glucose+IL-1β significantly increased FITC-dextran diffusion, paralleled by decreased electrical resistance. PACAP or VIP reversed these effects. Furthermore, high glucose+IL-1β-induced reduction of claudin-1 and ZO-1 expression was reversed by PACAP and VIP. Occludin expression was not affected in any of the conditions tested. Altogether, these finding show that both peptides counteract high glucose+IL-1β-induced damage in ARPE19 cells, suggesting that they might be relevant to the maintenance of outer blood retinal barrier function in edema accompanying diabetic retinopathy. These results are in accordance with earlier observations in an in vitro blood-brain barrier model. The authors found that PACAP treatment improved the barrier properties of the brain endothelium. PACAP induced an increase in the transendothelial electrical resistance, which is the most important marker of the tightness of the tight junctions. Moreover, PACAP had a protective role against glucose deprivation- and oxidative stress-induced junctional damage in microvascular brain endothelial cells.

II. Aims

1. The robust degeneration following bilateral common carotid artery occlusion (BCCAO) and the significant morphological improvement after PACAP teratment was already reported by many authors. We investigated the morphological changes of pigment epithelial cells after serious severe ischemia, and whether it could be prevented or reversed by PACAP treatment.

2. Our aim was to better understand the effects of PACAP on pigment epithelial cells (ARPE-19 cell line), and to elucidate the molecular biological processes in case of oxidative stress, hypoxia and hyperosmosis.

3. VEGF and other angiogenic factors secreted by the pigment epithelium play an imoprtant role in the neovascularisation, which is crucial in the development of several eye diseases such as diabetic retinopathy and macular edema. We studied the angiogenic effects of PACAP on pigment epithelium in oxidative stress, hypoxia and hyperosmotic conditions.

III. Materials and methods

1. In vivo experiments

1.1.Animals and histological analysis

Male Wistar rats weighing 250–300 g were subjected to permanent bilateral common carotid artery occlusion (BCCAO). Experimental procedures were performed following institutional ethical guidelines (BA02/2000-24/2011). Under isoflurane anesthesia, bilateral common carotid arteries were exposed and ligated with a 3-0 filament. Immediately following the BCCAO operation, PACAP1-38 (100 nmol/3 μl saline) was intravitreally injected into the right eye with a Hamilton syringe. The left eye received the same volume of vehicle treatment. A group of animals underwent anesthesia and all steps of the surgical procedure, except ligation of the carotid arteries. These animals served as sham-operated saline- or PACAP-treated animals. Rats were sacrificed with an overdose of anesthetic; eyes were removed and immediately dissected in ice-cold phosphate buffered saline and fixed in 4% paraformaldehyde dissolved in 0.1 M of phosphate buffer (Sigma, Hungary). Tissues were embedded in Durcupan ACM resin (Fluka, Switzerland), cut at 2 μm and stained with toluidine blue (Sigma, Hungary). The sections were then mounted in Depex medium (Fluka, Switzerland) and examined in a Nikon Eclipse 80i microscope (Tokyo, Japan). Measurements were taken from the digital photographs with the NIH Image 1.55 program (Tokyo, Japan). Six tissue blocks from at least three animals were prepared, and central retinal areas within 1 and 2 mm from the optic disc were used for measurements (n=2–5 measurements from one tissue block). Sections where the ganglion cell layer (GCL) appeared thicker than a single cell row were excluded from evaluation. The following parameters were measured: (1) cross-section of the retina from the outer limiting membrane to the inner limiting membrane, (2) the width of individual retinal layers and (3) the number of pigment epithelial cells/100 μm of section length. In this thesis only the third parameter will be discussed.

1.2.Cytokine array

For semiquantitative cytokine array, retinas (n=18 from 9 animals/ group) in the ischemic and sham-operated groups were removed after 24 h of ischemia and homogenized in PBS with protease inhibitors. Samples were pooled in three replicates (n = 3 per replicate).

Triton X-100 was added to a final concentration of 1%. The samples were stored at −80 ◦C prior to use. Cytokine array from tissue homogenates was performed using Rat Cytokine Array Panel A Array kit from R&D Systems (Biomedica, Budapest, Hungary). After blocking the array membranes for 1 hour and adding the reconstituted Detection Antibody Coctail for another 1 h at room temperature, the membranes were incubated with 1.5 ml of tissue homogenates at 2–8 ◦C overnight on a rocking platform. After washing with buffer 3 times and addition of horseradish peroxidase-conjugated Streptavidin to each membrane we exposed them to a chemiluminescent detection reagent (Amersham Biosciences, Hungary) then side up to an X-ray film cassette. We analysed the films using Image J software.

2. In vitro experiments

Human adult retinal pigment epithelium (ARPE-19) cells were obtained from the American type culture collection (ATCC, Manassas, VA). ARPE-19 is a spontaneously arising retinal pigment epithelia (RPE) cell line derived from the normal eyes of a 19-year-old male, who died from head trauma in a motor vehicle accident. The cells were cultured in Dulbecco's modified Eagle medium/F12 (DMEM/F12, Invitrogen) with 10 % fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified incubator at 37°C in 5 % CO2.

2.1. MTT test

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a colorimetric assay for assessing cell metabolic activity. The cells are capable of reducing the yellow MTT dye which penetrates the mitochondria to insoluble formazan, which has a purple color. The measured absorbances correlates with the number of viable cells. We put 15 000 cells in 100 µl medium pro well in a 96 well plate. Hypoxia was induced with 200 µM $CoCl_2$, hyperosmotic stress was triggered by 200 mM sucrose treatment and oxidative stress with 250 µM H_2O_2. We analysed the following groups:

1. Control
2. 100 nM PACAP
3. 200 µM $CoCl_2$
4. 200 µM $CoCl_2$ + 100 nM PACAP

5. 40 mM glucose
6. 40 mM glucose + 100 nM PACAP
7. 200 mM sucrose
8. 200 mM sucrose + 100 nM PACAP
9. 250 μM H_2O_2
10. 250 μM H_2O_2 + 100 nM PACAP

We put the samples of each group into 12 wells and all of them received a 24 hours long treatment. The next day we added 10 μl 5 mg/ml MTT solution to each well, so the final concentration was 0,45 mg/ml. We incubated the samples for 4 hours in thermostate, then dissolved the reducted formazan dye with 100 μl DMSO (dymethyl-sulfoxide). After 30 minutes shaking we measured the absorbance on 630 nm with ELISA reader. Data was analysed with a two-way ANOVA and Bonferroni post-test.

2.2. Apoptosis array

Apoptosis array was performed from cell homogenates using human apoptosis array kit (R&D Systems; Biomedica Hungaria, Budapest, Hungary). The ARPE-19 cells were treated with 0.25 mM H_2O_2 for 24 h and 100 nM PACAP1-38. Concentrations of H_2O_2 and PACAP1-38 were based on earlier descriptions. PACAP is strongly cytoprotective in the nanomolar concentration range in most studies, and this is what we found in our earlier description using the same ARPE cell line. The used H_2O_2 concentration was also found to be optimal for inducing cellular damage that is significant but still repairable. Cellular extracts were made as described by the manufacturer. We performed the array as described earlier by the cytokine array.

2.3. Phospho-kinase array

For the phospho-kinase array we used the human phospho-kinase array kit (R&D Systems; Biomedica Hungaria, Budapest, Hungary). Similarly to the apoptosis array, we treated the cells with 0,25 mM hydrogen peroxide and 100 nM PACAP1-38 for 24 hours. We prepeared cell homogenates and performed the protocoll as described above.

2.4. Angiogenesis array

We investigated the quantitative changes of angiogenic factors secreted to the supernatant after different injuries. For modelling oxidative stress and hypoxia, we administered 0,25 mM hydrogen peroxide and 200 mM $CoCl_2$ respectively. To observe the effect of hyperosmotic stress, we added 200 mM sucrose or 100 mM NaCl. High saline concentrations change the osmotic conditions, which can cause damage of the blood-retina barrier, leading to macular degenaration and edema formation. We performed the human angiogenesis array kit (R&D Systems; Biomedica Hungaria, Hungary) from the supernatant following the above mentioned protocol.

2.5. Flow cytometric measurements

We carried out the measurment of VEGF concentrations with an Accuri C6 type flow cytometry using CBA human soluable protein kit (R&D Systems; Biomedica Hungaria, Hungary). The kit contains VEGF antibody conjugated microbeads, which can bind to the corresponding binding sites. The VEGF concentrations become measurable after binding it to a phycoerythrin conjugated secondary antibody. We mixed 50 µl supernatant with 50 µl VEGF conjugated microbeads, and incubated it for an hour in dark on room temperature. We added 50 µl phycoerythrin conjugated antibody and further incubated it for 3 hours. We centrifuged the cells in 1 ml wash buffer on 1100 rpm for 5 minutes. After carefully discarding the supernatant, we added 300 µl buffer to the pellet. We quantified our measured results with the calibration curve derived from the standard row. The statistical analysis was performed with a two-way ANOVA and Bonferroni post-test.

2.6. Cell stress array

We investigated not only the effects of oxidative stress, but also of hypoxia. Oxidative stress was induced by 0,25 mM hydrogen peroxide, while hypoxic conditions were modelled with 24 hours long 200 µM $CoCl_2$ treatment. The ischemic injury of the retina plays a critical role in the pathomechanism of several ocular diseases, such as diabetic retinopathy, age related macular degenaration and retinopathy of prematurity. With $CoCl_2$ administration we were able to mimic these conditions and examine the possible protective effects of PACAP. The human

cell stress array was performed as the cytokine array described above (R&D Systems; Biomedica Hungaria, Hungary).

IV. Results

1. In vivo experiments

1.1 Histological analysis

We counted the pigment epthelial cells in 100 μm of the retina of animals after bilateral common carotid artery occlusion. On the control retina we found 5,8 cell bodies in average. In the right eye of the control animals PACAP was injected. PACAP alone did not cause any changes in the number of pigment epithelial cells, we found 5,4 cell bodies in 100 μm. In the left eye of BCCAO operated animals (saline treated) the thickness of the whole retina, the thickness of each individual layer, and the number of ganglion cells strongly decreased. The pigment epithelial cells did not suffer any numerical changes, we counted 5,4 cell bodies in average. While PACAP treatment could significantly improve the other parameters of the retina, as suggested, the number of pigment cells did not change (5,6 in average).

1.2. Cytokine array

The cytokine array made from the retinas of the BCCAO operated animals showed, that ischemic conditions increase the expression of several cytokines, as the family members of the chemoattractant CINC and MIP, namely: CINC-1, CINC-2 α/β and MIP-1α, MIP-3α. The level of numerous interleukins was elevated: IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-6, IL-10, IL-13 és IL-17. In the BCCAO operated animals the concentration of ciliary neurotrophic factor (CNTF), fractalkine, sICAM-1 (intercellular adhesion melecule), LIX (lipopolysaccharide inducable CXC chemokine), L-selectin, RANTES (regulated on activation, normal T cell expressed and secreted), tymus chemokine and TIMP-1 (tissue inhibitor of metalloproteinases) were significantly higher than the control. We found that in the cytokine array PACAP could decrease the level of all above mentioned cytokines, except for VEGF and thymus chemokine, the expression of these was further increased by PACAP.

2. In vitro studies

2.1.Results of the MTT test

We investigated the changes in cell survival upon different harmful stimuli, such as hypoxia, oxidative stress and hyperosmotic condition.

The effect of hyperosmosis on survival

We treated the cells with 200 mM sucrose overnight to establish a hyperosmotic model. Almost 36 % of the cells died due to the sucrose treatment, while PACAP could protect them from the damage caused by hyperosmotic conditions, and 95 % survived.

Effect of oxidative stress

The ARPE-19 cells were treated with 250 μM hydrogen peroxide for 24 hours. This strong oxidative stress resulted in a significant cells loss (31 %). Co-treatment with PACAP could protect most of the ARPE cells (86 %).

Effect of hypoxia

Cobalt-chloride caused the strongest damage of the cells. After 24 hours long 200 μM cobalt-chloride administration, approximately 50 % of the pigment epithelial cells died. In this model PACAP was also able to increase the cell survival up to 90 %.

2.2. Results of the apoptosis array

We treated the cell line with 250 μM hydrogen peroxide and 100 nM PACAP for 24 hours. In control conditions there were just a few markers expressed in the ARPE-19 cells. Oxidative stress induced the levels of Bad, Bax, cytochrome-c, Trail R1 DR4, Trail R2 DR5 (TNF related apoptosis inducing ligand), FADD (Fas associated death domain), Fas TNFR SF, HIF-1α (hypoxia inducible factor), some heat schock proteins (HSP32, HO-2, HSP-27, HSP-60, HSP-70), p21, p27, pp53, TNF R1 and SMAC diablo. PACAP co-administration could decrease the activation of all above mentioned pro-apoptotic markers.

2.3. Results of the phospho-kinase array

Hydrogen peroxide increased the concentration of several phospho-kinases, such as p38 and JNK pan, while PACAP lowered the expression of these factors. Moreover, PACAP further increased the levels of ERK ½ and Akt, the activation of which was already elevated due to the oxidative stress. Furthermore, the levels of CREB, Src, Lyn, Yes and Chk-2 were also significantly higher after PACAP administration. The phosphorylation of three different sites (S392, S46 and S15) of p53 increased after H_2O_2 treatment, 100 nM of PACAP could slightly decrease this. Oxidative stress caused by hydrogen peroxide resulted in the activation of paxillin, p70S6 kinase, RSK1/2 (ribosomal S-6 kinase), PLCγ-1, STAT4 and eNOS. These changes, except the activation of p70S6 were counteracted by PACAP. The c-Jun expression was already markedly higher in control conditions, hydrogen peroxide further increased the concentration, while PACAP attenuated these effects.

2.4. Results of the angiogenesis array

The effect of hyperosmosis

We used two different models to simulate hyperosmotic conditions. First, we used 100 mM NaCl. After the treatment the concentratios of various pro-angiogenic factors increased, such as angiogenin, artemin, EG-VEGF (endocrine gland derived VEGF) pentraxin 3, platelet factor 4 and VEGF. Artemin, pentraxin and platelet factor 4 play an important role in inflammatory processes, while the other three are classical angiogenic proteins. The 100 nM PACAP administration could decrease the expression of all six above mentioned factors, thus proved to be protective in this model.

In our next experiment we treated the ARPE-19 cells with 200 mM sucrose for 24 hours to mimic hyperosmosis. As we could see it in the MTT test, hyperosmosis induced by sucrose damaged the cells and increased the number of apoptotic cells, while PACAP treatment could prevent this process. We expected similar results with the angiogenesis array. After analysing the membranes of the array, we saw that sucrose increased the concentration of several angiogenic factors in the supernatant. The levels of DPP IV, TIMP-1, MCP-1, VEGF, EG-VEGF, IL-8, IGFBP-1 and 3, MMP-9, PIGF, serpin B5 and uPA were elevated after sucrose treatment. PACAP was protective, it could decrease all of the above mentioned factors.

The effect of oxidative stress

The 24 hours long hydrogen peroxide treatment strongly elevated the concentrations of different pro-angiogenic markers. Regarding neovasculogenesis the most important ones are VEGF, FGF, angiopoietin-1 and 2, platelet derived growth factor and thrombospondin-1. The increased levels of these and other factors in the supernatant after oxidative stress were lowered upon PACAP treatment.

The effect of hypoxia

We modelled the hypoxic conditions with 24 hours long 200 μM cobalt-chloride treatment. The concentration of several angiogenic factors, such as the members of the IGFBP family, PDGF, thrombospondin-1, μPA and VEGF was elevated due to the lack of oxygen. PACAP could significantly decrease the concentrations of all above mentioned cytokines. The endothelin-1 is a peptide produced mainly by endothelial cells. The high levels of these peptides play a critical role in the development of hypertension and type 2 diabetes. Cobalt-chloride administration upregulated the expression of endothelin, while PACAP significantly decrease it. All of these prove the protective effect of PACAP in cobalt-chloride induced hypoxia on ARPE-19 cells.

2.5. The results of flow cytometric experiments

We measured the VEGF concentrations secreted by the ARPE cells with flow cytometry after sucrose administration, thus investigated the effects of hyperosmotic conditions. Our results showed that 24 hours long 200 mM sucrose treatment significantly increased the levels of VEGF in the supernatant. Coadministration with 100 nM PACAP attenuated the VEGF concentrations to the control levels.

2.6. The results of the cell stress array

The effect of oxidative stress

We used the cell stress array on two different models. First we concentrated on the oxidative stress induced changes. Hydrogen peroxide activated several heat shock proteins,

cytochrome-c, phosphorylated p53 and p38. All these factors play a crucial role in the induction of apoptotic processes. PACAP lowered the expression of all above mentioned proteins, thus proved to be anti-apoptotic in this model as well.

The effect of hypoxia

Cobalt-chloride treatment did not cause much change in the concentrations of the factors measured by cell stress array. The carbonic-anhydrase plays an essential role in regulating the acid-base balance. Hypoxic conditions decreased the levels of this enzyme, which could be counteracted by PACAP. The lack of oxygen significantly increased the expression of hif1-α and SIRT1, PACAP could not alter these changes. The concentrations of HSP70 and thyoredoxin had slightly elevated concentrations in hypoxia, PACAP further stimulated the expression of both factors.

V. Discussion

Discussion of the in vivo experiments

The present study showed that PACAP counteracted changes in the phosphorylation of MAPKs and levels of cytokines after ischemic injury in the rat retina. PACAP has been shown to influence cell survival and inflammatory pathways in several ischemic injuries, including ischemia of the brain and different peripheral organs. The overall picture of cytokine expression shows that ischemia dramatically induced several cytokines. Less activation was found in most cytokines after PACAP treatment, except for VEGF and thymus chemokine, the concentrations of which were elevated after PACAP treatment. The alterations induced by PACAP strongly suggest an anti-inflammatory role of the peptide in ischemic retinal injury, keeping in mind that this was checked at one time-point, namely 24 hours after the induction of ischemia.

The family of CINC (CINC1-3) is a pro-inflammatory chemokine family, involved in several inflammatory processes, usually during the acute inflammatory response phase. This is the first report showing that PACAP attenuates elevated CINC levels after ischemia induction in the retina.

The present results provide further insight into the neuroprotective mechanism induced by PACAP in ischemic injuries, showing that PACAP ameliorates ischemic retinal injury involving Akt, MAPK pathways and anti-inflammatory actions.

Discussion of the in vitro experiments

In the present study, we showed that PACAP counteracted the oxidative stress induced changes in apoptotic and kinase signaling molecules of human pigment epithelial cells, providing details for the molecular mechanism of the protective effects of PACAP in ARPE cells. We have previously described that PACAP protects pigment epithelial cells of the retina against oxidative stress induced apoptosis, but the molecular background has not been investigated. In the present study, we found that exposure of human ARPE cells to hydrogen peroxide induced a marked increase in several apoptotic factors, as shown by the complex apoptosis array. Co-administration with 100 nM PACAP1-38 led to an apoptotic profile similar to control conditions. A marked increase was observed in the mitochondrial pro-apoptotic proteins, Bax and Bad in cells under oxidative stress, while PACAP abolished this increase entirely. Also, PACAP counteracted the oxidative stress-induced upregulation of Trail, FADD,

Fas, SMAC diablo, and several heat shock proteins. Apoptosis involves a series of cascades and is mediated by a diverse range of extracellular and intracellular signals. These include processes like TNF and Fas activation, which is followed by further activation of pro-apoptotic signals. The balance between pro- and anti-apoptotic processes is very important not only during development, but also throughout life. Disturbed balance can be detected in most diseases characterized by increased cell death, including retinal pathologies.

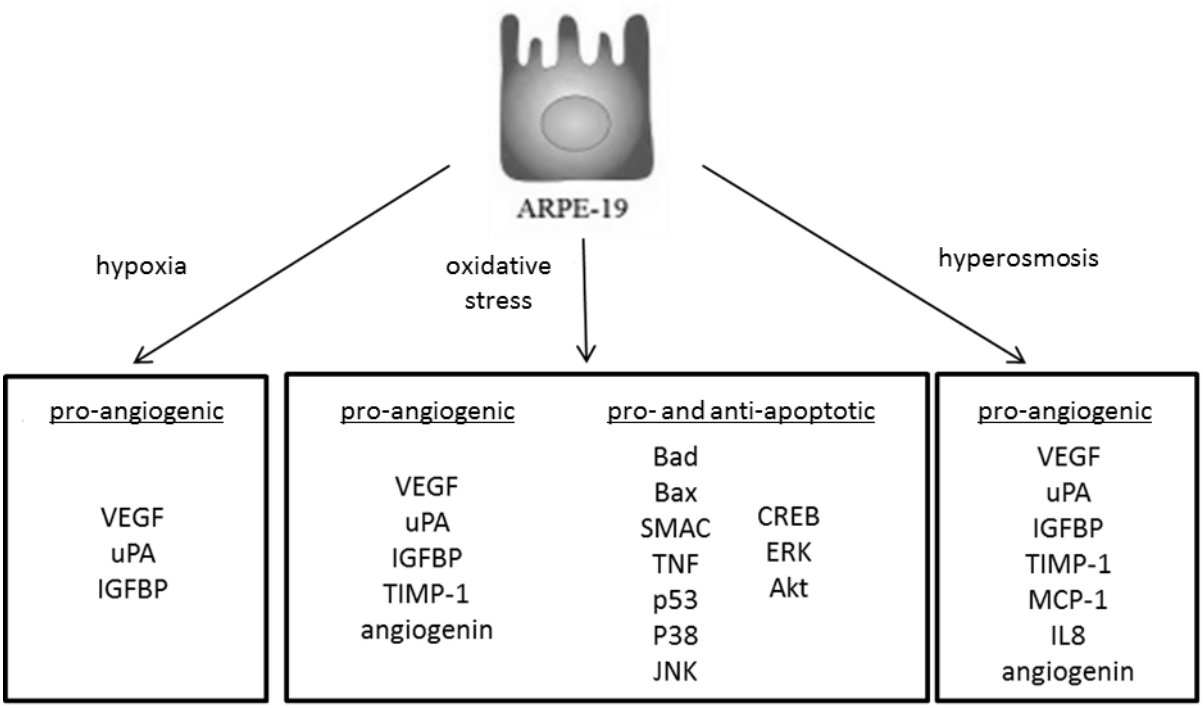
PACAP has been shown to influence apoptotic processes at several levels. Early studies have established the role of PACAP in the regulation of MAP kinases, resulting in an anti-apoptotic profile of most examined cells. In the present study, we also found that PACAP counteracted the hydrogen peroxide induced changes in the balance of the members of the MAPK family: elevated levels of the protective ERK1/2, while decreased expression of the pro-apoptotic JNK and p38 MAPK were observed after PACAP treatment, as confirmed by both kinase array and Western blot measurements. Thus, these results are in accordance with earlier observations in other neuronal and non-neuronal cells. The involvement of MAP kinases is crucial in mediating apoptosis also in pigment epithelial cells of the retina. The activation of the anti-apoptotic signaling can lead to elevated CREB phosphorylation, which has already been described in various cell types after PACAP treatment, and our present results further support these observations in ARPE cells. Furthermore, we found that PACAP counteracts the elevated expression of Trail, FADD, and Fas, initiators of the apoptotic cascade. Balanced effect of PACAP and FasL has already been shown on cerebellar granule cell death during development. Also, PACAP-induced downregulation of Fas has been demonstrated in peripheral cells, such as colonic tumor cells and lymphocytes. Our observations in ARPE cells support the general aspect of this effect induced by PACAP.

The involvement of the mitochondrial Bcl-family in the PACAP-induced cytoprotection has been described in several cell types exposed to different harmful stimuli. For example, we have described that PACAP induced the anti-apoptotic members of this family, while it decreased the pro-apoptotic signaling, including Bad, in the retina. Similar results have been described in cerebellar granule cells and other neuronal cell types. The inhibiting action of PACAP on the proapoptotic Bax and Bad has been described not only in neuronal cells but also non-neuronal cell types, such as in insulinoma cells upon streptozotocin-induced apoptosis and in serum-deprivation induced apoptosis of Schwann cells. During the activation of the apoptotic cascade, the mitochondrial membrane becomes more permeable and several pro-apoptotic factors are released to the cytosol, including cytochrome-c and SMAC. Our present results show that PACAP could decrease the induced levels of SMAC, an effect not previously reported.

The tumor protein p53 also plays a critical role in apoptosis. Any disruption to the regulation of the p53 will result in impaired apoptosis. The p53-dependent Bax synthesis induces apoptosis by binding to Bcl-2 and antagonizing its non-apoptotic ability, a mechanism observed in different forms of oxidative stress. We found that PACAP could slightly decrease the increased expression of p53 after oxidative stress. Other factors were also found to be decreased after PACAP treatment. These include PLC, eNOS, STAT, RSK, and cIAP-1. Altered levels of STAT in the brain have already been reported in PACAP gene deficient mice, while others have found no influence of PACAP on STAT expression in microglial cells. At the moment, too little is known about the relationship between PACAP and these markers to draw conclusion from our observations, but the retinoprotective effects of PACAP along with the present observations indicate that PACAP can definitely counteract increased expressions of markers induced by oxidative stress.

The results of the phospho-kinase array are also in accordance with our previous finding, that PACAP increases ERK, while decreases JNK levels. Some other markers were found to be elevated after PACAP treatment. In addition to the already-described $ERK_{1/2}$, Akt was also found to be elevated in our study. The Akt-induced processes are generally associated with increased tolerance to apoptosis, and PACAP-associated Akt phosphorylation has been reported in other cell types. Further factors, that have not yet been reported to be affected by PACAP treatment, were found to be increased after PACAP treatment. Src family kinases regulate an array of cellular processes, including growth factor signaling, cytoskeletal dynamics, and cell proliferation. Lyn, a tyrosin kinase belonging to the src family, is usually associated with anti-apoptotic processes and preservation of mitochondrial integrity. Another widely expressed member of the src family, Yes, was also found to be elevated after PACAP treatment. In addition, paxillin, a signal transduction adaptor protein playing a role in focal adhesion, and Chk2, a checkpoint protein important in cell cycle, were upregulated following PACAP administration to ARPE cells exposed to oxidative stress. The exact role of these markers in ARPE cell apoptosis is not known yet, and the relation of PACAP and these markers needs further investigation.

In summary, our present results further support the effects of PACAP in oxidative stress-induced apoptotic processes and show that PACAP acts at several levels in the apoptotic kinase pathway in human pigment epithelial cells.



Proangiogenic, proapoptotic and antiapoptotic factors upregulated by hypoxia, oxidative stress and hyperosmosis The concentration of the mentioned factors were lowered to contol levels with 100 nM PACAP traetment.

The role of PACAP in angiogenic processes is not clear yet. The action of PACAP on vascular endothelial growth factor levels is one of the most studied effects. The expression of VEGF expression is elevated at the beginning of angiogenesis, while in normal conditions it is lowered. In lung cancer cells PACAP1-27 and VIP induced VEGF activation, while in an other study VEGF mRNA expression was upregulated by PACAP in prostate cancer cells. In contrast, in our experiments we found opposite effects. Hyperosmosis caused a strong elevation in the VEGF levels both with NaCl and with sucrose treatment. PACAP attenuated these effects. We could further verify the results of sucrose administration with flow cytometric measurments. In sodium-azid induced retinal ischemia a recent study investigated the effects of PACAP on metabolic changes in an ex-vivo mouse model. PACAP caused different changes, it could decrease ischemia induced cell death, glutamate secretion and VEGF expression, thus it was in accordance with our findings.

The elevated glucose levels in the blood in diabetic retinopathy causes not only hyperglycemic but also hyperosmotic conditions. According to the results of the angiogenesis array, PACAP reduced the expression of the pro-angiogenic factors activated by hyperosmosis.

NaCl treatment raised the activity of several angiogenic factors. Angiogenin is described as a factor, which can stimulate neovascularization. Moreover, angiogenin plays a critical role in the regulation of survival, cell proliferation and cell migration. In a previous study of our

PACAP research team we found that PACAP attenuated the elevated levels of angiogenin in human trophoblast cells. After PACAP administration the concentration of several other factors was reduced to the control levels: angiopoietin, EG-VEGF, IL-8 and TIMP-1. In the present experiments we got similar results. Expression of angiogenin decreased after co-treatment with PACAP and NaCl or PACAP and hydrogen peroxide. Levels of EG-VEGF were attenuated by PACAP in all three experimental models (NaCl, sucrose and H_2O_2), while PACAP could lower IL-8 concentration only in sucrose induced hyperosmosis.

TIMP-1, as a matrix metalloprotease inhibitor also posesses pro-angiogenic characteristics. We found that PACAP counteracted the inducing effects of sucrose and hydrogen peroxide on TIMP-1 levels. In addition, NaCl promoted artemin, pentraxin-3 and platelet factor-4 activation, while PACAP could reduce it to control levels. We have to further clarify the proper functions of these molecules in the process of angiogenesis.

Sucrose and hydrogen peroxide induced the expression of many more factors than NaCl, such as insulin-like growth factor binding proteins (IGFBP). IGFBP-1 was found to stimulate angiogeneis in glioblastoma cells. Our results are in accordance with this study. Both sucrose and hydrogen peroxide treatment increased the activation of IGFBP-s, while PACAP proved to be protective against them. We received similar results with an other pro-angiogenic factor, the urokinase plasminogen activator (uPA). It is already proven that the binding of uPA to VEGF receptor activates several signalling pathways, which lead to angiogenesis. In our case PACAP decreased the elevated urokinase expressions, thus proved to be anti-angiogenic in this model.

The relationship between PACAP and several molecules measured by the angiogenesis array is still unclear, but taken together it can be concluded that PACAP reduced the secretion of many pro-angiogenic factors in retinal pigment epithelial cells. Thus, PACAP could be a potent drug against retinopathies with angioenic processes in the background.

We investigated the effect of PACAP on stress markers with the cell stress array. D'Amico and coworkers analysed the hypoxia inducible factors (hif) in the third week of streptozotocin induced diabetes. They found that the expression of hif-1 alpha and hif-2 alpha significantly increased in diabetic rats, but not after 1 intravitreal PACAP treatment. Conversely, the expression of hif-3 alpha was significantly downregulated in retinas of diabetic rats, and increased after PACAP treatment. These results suggest that the retinoprotective effects of PACAP are partially mediated by interfering with the expression of HIFs that play an important role in pathological vasculogenesis occurring in diabetic retinopathy. According to our experiments oxidative stress did not, but hypoxia did induce the expression of hif molecules, but PACAP could not decrease their levels. Oxidative stress elevated the concentrations of heat

schock proteins 60 and 70, cytochrome-c, p38 and p53. Hypoxia did not cause any notable changes.

Carbonic anhydrase IX is essential in the acid-base homeostasis. Several studies dealt with the role of carbonic anhydrase IX in cancer. They found that elevated levels of this enzyme worsen the prognosis of several carcinomas, such as prostate cancer. In our experiments the concentration of carbonic anhydrase was decreased by cobalt-chloride, and elevated by PACAP. Further examinations are necessary to elucidate this effect of PACAP.

SOD2 is a member of superoxide-dismutase family, which removes mitochondrial reactive oxygen species (ROS) and, as a result, protects against cell death. Hypoxic conditions did not cause any changes in the expression of this protein, but PACAP could significantly elevate its levels, thus proved to be protective.

Taken together, these results, in accordance with our previous studies, show that PACAP is cytoprotective against different harmful agents in retinal pigment epithelial cells. The peptide could decrease the levels of several pro-apoptotic factors (Bad, Bax, cytochrome-c, Fas, JNK, p38 and p53) and induce the expression of many anti-apoptotic molecules (ERK, Bcl-2, CRB, Akt, Src, Lyn and Yes) both in oxidative stress and hypoxia induced injuries.

PACAP could reduce the concentration of VEGF in several models, which is one of the most important factors in the angiogenic processes. This alone is a very important finding, because PACAP exerts opposite effects on other cell types. Thus, we are the first to prove that PACAP inhibits VEGF activation in retinal pigment epithelial cells. Moreover PACAP was able to attenuate the levels of some other pro-angiogenic proteins (uPA, IGFBP, TIMP-1, EG-VEGF, angiogenin and angiopoietin). As a conclusion, PACAP is among the emerging molecules to fight diabetic complications and macular degeneration, similarly to VEGF antagonists, antioxidants, anti-inflammaotry agents and other neuropeptides.

Publications related to the thesis

Fábián E, Reglődi D, Mester L, Szabó A, Szabadfi K, Tamás A, Tóth G, Kovács K. Effects of PACAP on intracellular signaling pathways in human retinal pigment epithelial cells exposed to oxidative stress. Journal of Molecular Neuroscience 2012, 48:(3) 493-500. (IF: 2,891)

Szabó A, Dányádi B, Bognár E, Szabadfi K, **Fábián E**, Kiss P, Mester L, Manavalan S, Atlasz T, Gábriel R, Tóth G, Tamás A, Reglődi D, Kovács K. Effect of PACAP on MAP kinases, Akt and cytokine expressions in rat retinal hypoperfusion. Neuroscience Letters 2012, 523:(2) 93-98. (IF:2,026)

Atlasz T, Váczy A, Werling D, Kiss P, Tamás A, Kovács K, **Fábián E**, Kvárik T, Mammel B, Dányádi B, Lőkös E, Reglődi D. In Pituitary Adenylate Cyclase Activating Polypeptide – PACAP book. New York: Springer Nature 2016 – in press.

Impact factors related to the thesis: **4,917**

Further publications:

Varga B, Szabadfi K, Kiss P, **Fábián E,** Tamas A, Griecs M, Gabriel R, Reglődi D, Kemeny-Beke A, Pamer Z, Biro Z, Tosaki A, Atlasz T, Juhasz B. PACAP improves functional outcome in excitotoxic retinal lesion: an electroretinographic study. Journal of Molecular Neuroscience 2011, 43:(1) 44-50. (IF:2,504)

Szabadfi K, Dányádi B, Kiss P, Tamás A, **Fábián E**, Gábriel R, Reglődi D. Protective effects of vasoactive intestinal peptide (Vip) in ischemic retinal degeneration. Journal of Molecular Neuroscience 2012, 48:(3) 501-507. (IF:2,891)

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Cumulative impact factors: **12,655**