The Protective Effects of Endogenous PACAP in Oxygen-induced Retinopathy

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Abstract

Purpose: Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide having trophic and protective functions in neural tissues including the retina. Previously we have shown that intravitreal PACAP administration can maintain retinal structure in the animal model of retinopathy of prematurity (ROP). The purpose of this study is to examine the development of ROP in PACAP-deficient and wild-type mice to reveal the function of endogenous PACAP.

Methods: Wild-type and PACAP-KO mouse pups at postnatal day (PD) 7 were maintained at 80% oxygen for 5 consecutive days then returned to room air on PD12 to develop oxygen-induced retinopathy (OIR). On PD15 animals underwent electroretinography (ERG) to assess visual function. On PD16 eyes were harvested for either immunohistochemistry to determine the percentage of central avascular retinal area and neovascular tuft formation or molecular analysis to assess angiogenesis proteins by array kit and antiapoptotic protein kinase B (Akt) change by western blot.

Results: The retina of PACAP deficient OIR mice showed greater central avascular area than that of the wild types. ERG revealed significantly decreased b-wave amplitude in PACAP KO compared to their controls. Several angiogenic proteins were upregulated as a results of OIR and 11 further proteins markedly increased in PACAP deficient mice.

Conclusion: This is the first study to examine the endogenous effect of PACAP in OIR model. Previously we have shown the beneficial effect of exogenous local PACAP treatment in the rat OIR model. Together with the present findings we suppose that PACAP could be a novel retinoprotective agent in ROP.

Introduction

Premature birth may come together with diseases that compromise future life quality, such as retinopathy of prematurity (ROP). Preterm infants with extreme retinal immaturity and incompletely vascularized retina are exposed to alternating oxygen concentration, which initiate new, abnormal vessel formation. These vulnerable vessels can grow into the vitreous cavity, causing bleeding and retinal detachment in worst cases. Despite advanced neonatal care and current therapeutic strategies, ROP still remains the main cause of preventable childhood visual impairment. Therefore, there is a need for developing new therapeutic agents with the help of the widely used oxygen-induced ischemic retinopathy (OIR) animal models.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a 38-amino acid pleiotropic peptide known to act as a neurotransmitter, neuromodulator and neurotrophic factor (Nakamachi et al. 2011; Johnson et al. 2020; Gargiulo et al. 2020; Ciranna and Costa 2019). PACAP consistently exerts protective effects in the nervous system and in peripheral organs (Martinez-Rojas et al. 2020; Nonaka et al. 2020; Toth et al. 2020). This can also be observed in the retina against hypoxic, mechanical and chemical injuries as reviewed by Nakamachi and colleagues and by our group also (Atlasz et al. 2016; Nakamachi et al, 2012). PACAP was also neuroprotective in diabetic retinopathy (D'Amico et al. 2021). Recently, we

showed the potentially positive effect of intravitreally given PACAP injection on the vascular changes in the rat OIR model (Kvarik et al, 2016).

The role of endogenous PACAP has been explored with the help of PACAP gene – deficient mice. These mice show several developmental and behavioural alterations, including altered neurobehavioural, bone and tooth development (Farkas et al. 2017; Fulop et al. 2019b; Jozsa et al. 2018), paradoxical age-dependent stress behavior (Biran et al. 2020), and altered light responses (Riedel et al. 2020). The strong neuro- and general cytoprotective role of PACAP are mainly due to its antiapoptotic, antioxidant and anti-inflammatory effects (Soles-Tarres et al. 2020; Toth et al. 2020). Due to the lack of these protective actions in PACAP deficient animals, increased vulnerability and pathological responses have been observed in different peripheral injuries, such as kidney ischemia, callus formation and cardiomyopathy (Jozsa et al. 2019; Mori et al. 2010; Reglodi et al. 2012) and also in injuries of the nervous system, such as spinal cord injury, ischemia and nerve degeneration (Armstrong et al. 2008; Maugeri et al. 2020; Ohtaki et al. 2006; Tsuchikawa et al. 2012). This endogenous protective effect of the peptide has also been confirmed in carotid artery occlusion-induced retinal injury (Szabadfi et al. 2012). It was found that PACAP gene deficient mice reacted with a higher degree of retinal cell loss and reduction of the retinal layers than their wild type mates. This could be counteracted by exogenous PACAP treatment (Szabadfi et al. 2012). Similarly, increased sensitivity has been observed in retinal inflammation induced by endotoxin (Vaczy et al. 2018). Levels of protective factors were decreased to a greater extent, while inflammatory cytokine increase was more intense along with a marker Muller glial cell activation in PACAP knockout mice (Vaczy et al. 2018). As the protective effects of PACAP are linked with aging processes, it is not surprising that PACAP deficient mice also show accelerated aging (Reglodi et al. 2018a). Accelerated systemic amyloidosis and increased levels of oxidative stress markers have been found in PACAP knockout mice (Ohtaki et al. 2010; Reglodi et al. 2018b). In the eye, age-related loss of lacrimal gland function as well as accelerated retinal aging have been described (Kovacs-Valasek et al. 2017; Nakamachi et al. 2016). These results clearly show that endogenously present PACAP reacts as a stress-response peptide that is necessary for protection against different retinal insults. The aim of the present study was to investigate whether PACAP exerts similar protective effects endogenously also in a model of retinopathy of prematurity.

Materials And Methods

Animals

Mice were obtained from breeding colonies maintained at the Animal Facility of Medical School, University of Pecs (Pecs, Hungary). Wild-type and homozygous PACAP-deficient mice were used. PACAP-deficient mice were generated and maintained on CD1 background as previously described (Hashimoto et al. 2001; Hashimoto et al. 2009); they were backcrossed for ten generations with the CD1 strain. Each nursing mum with their litters was housed in individual cages, fed and watered ad libitum under 12/12 hours light/dark cycles. Animal housing, care and application of experimental procedures were in accordance with the ethical guidelines approved by the University of Pecs (BA02/2000-31/2011) and

directives of the National Ethical Council for Animal Research, the European Communities Council (86/609/EEC), and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental design

To induce retinopathy, wild-type ($N_{\text{OIR-Wt}}=15$) and PACAP-deficient ($N_{\text{OIR-KO}}=14$) mice pups with their nursing mother were kept in an oxygen chamber (Biospherix Ltd., NY, USA) supplied by an oxygen sensor (ProOx 110, Biospherix Ltd., NY, USA) to monitor and maintained continuous 80 % of oxygen concentration from PD7 to PD12. Then they were returned to room air until PD16. Control litters of both genotypes ($N_{\text{Cont-Wt}}=15$, $N_{\text{Cont-KO}}=15$) were room-air reared during the whole experiment.

Electroretinography

Some of the animals from each group ($N_{\text{Cont-Wt}}=4$, $N_{\text{Cont-KO}}=3$, $N_{\text{OIR-Wt}}=3$, $N_{\text{OIR-KO}}=4$) underwent electroretinography (ERG) examinations to assess visual function on PD15 after an overnight dark adaptation. The measurements were performed as previously described by Danyadi and co-workers (Danyadi et al. 2014). Briefly, after systemic anesthesia, the pupils were dilated with 0.5 % cyclopentolate (Humapent-Teva, TEVA Ltd., Hungary) and topically anesthetized with Oxybuprocaine 0.4 % (Humacain-Teva, TEVA Ltd., Hungary) eye-drops. ERGs were recorded by surface electrodes from the center of the cornea with a negative electrode placed under the cheek and a ground electrode inserted under the skin of the neck. The responses to light flashes (5.0 cd/m², 0.25 Hz, 503 nm green LED light) were pre-amplified, amplified and recorded by an A/D converter (Ratsoft-Solar Electronic). Responses were averaged with the software of the A/D converter. The selected parameters were measured by OriginPro 2016 software (OriginLab Corporation, MA, USA) and statistically analyzed by ANOVA with Fisher's post hoc test after test for homogeneity of variance (STATISTICA, StatSoft Inc., OK, USA).

Immunohistochemistry

Isolectin immunohistochemical staining was performed as previously described by Connor and co-workers (Connor et al. 2009). Briefly, after anesthesia on P16±1, eyes were removed and immediately placed into 4% PFA for fixation at room temperature. After 1 h, eyes were washed three times in PBS and retinas were isolated under dissecting microscope. To stain the retinal vasculature 500 µl fluoresceinated isolectin solution (Isolectin GS-IB4 from Griffonia simplicifolia, Alexa Fluor 568 conjugate; Thermo Fischer Scientific Inc., MA, USA) was added to the isolated retinas. After an overnight rocking in lectin solution at room temperature, retinas were rinsed three times in PBS. Finally, 4 incisions were made to flatten the retinas onto microscope slides and were covered with a coverslip with mounting media (Fluoromount, Sigma-Aldrich Co., MO, USA). Digital photographs were taken with a Nikon Eclipse Ci fluorescence microscope (Nikon, Melville, NY, USA).

Assessment of vessel morphology

A trained observer blinded for the groups evaluated the ratio of central avascular retinal territory, vessel density and neovascular tufts. Measurements were executed by AdobePhotoshop CS6 (Adobe Systems Inc., CA, USA) and ImageJ softwares (National Institutes of Health, Bethesda, MD, USA). Central avascular retina was outlined, measured and its percentage to the whole retina was given. Vessel density was calculated by marking out all the vessels in the intact retina and giving their portion to the whole vascularized territory. To define neovascular tufts formation, flat mounts were divided into 12 approximately equal areas - the so called "clock hours" - and were assigned a number 1-12 depending on the number of clock hours with neovascular tufts.

Results are represented in mean $\pm$ SEM. Statistical analysis was performed by independent T-test after Levene's F-test for equality of variance using STATISTICA software (StatSoft Inc., OK, USA).

Angiogenesis array analysis

After euthanasia on PD 17 $\pm$ 1 the eyeballs were removed, retinas were carefully separated and quickly frozen in dry ice. Angiogenesis proteins were investigated from pooled tissue homogenates by semi-quantitative Mouse Angiogenesis Array Kits (R&D Systems, Bio-Techne Ltd., MN, USA). In these arrays, the sample proteins bind to selected captured antibodies spotted on nitrocellulose membranes. The kits contain all buffers, detection antibodies and membranes necessary for the measurement. The arrays were performed as described by the manufacturer's protocol. In brief, after blocking the membranes for 1 hour and adding the detection antibody cocktail for another 1 hour at room temperature, the membranes were incubated with 1.5 ml tissue homogenates at 2-8 °C overnight on a rocking platform. After washing with buffer 3 times, membranes were incubated with horseradish peroxidase-conjugated Streptavidin at room temperature and exposed to chemiluminescence detection reagent to develop X-ray films. The arrays were repeated two times. For data analysis, films were scanned and mean pixel densities of interested proteins, selected by eye control, were measured by ImageJ software and were normalized to the reference spots. To compare the possible differences between angiogenetic profiles of the different groups, we determined the relative density change of the selected spots. Only those proteins are represented which showed at least 1.3-fold change.

Western Blot Analysis

For Western blot experiments tissue homogenizates of four retinas per group were used. Frozen tissues were homogenized with ultra turrax and Potter homogenizer in 150 μ l lysis buffer (50 mM TRIS, 50 mM EDTA, 0.5% protease inhibitor cocktail (Sigma-Aldrich) and 0.5% phosphatase inhibitor cocktail (Sigma-Aldrich), pH = 7.4). The homogenate was sonicated and the protein concentration was determined with a DC™ Protein Assay kit (Bio-Rad) according to the manufacturer's description. The tissue lysate was diluted in Laemmli buffer, boiled for five minutes, centrifuged (13300 rpm, 10 min) and the clear supernatant was used for further investigations. Tissue extracts were separated with SDS-PAGE with protein loads of 20 μ g/lane and transferred onto nitrocellulose membrane. The membranes were blocked with 5% non-fat dried-milk proteins in Tris-buffered saline (TBS) and 0.1% Tween, incubated with anti-Akt (No:9272), anti-Akt1 Ser473 (No:9271) antibody (both from Cell Signaling Technology) at 4°C overnight

at a dilution of 1:1000. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG. Peroxidase labeling was visualized with the Pierce ECL Western Blotting Substrate (Thermo Scientific) detection system. Quantification of band intensities of the blots was performed by ImageJ software. Pixel volumes of the spot were normalized to the internal controls. Data are represented by pixel density in arbitrary units. Statistical analysis was performed by ANOVA test with Fisher's post hoc analysis after test for homogeneity of variance using STATISTICA software (StatSoft Inc., OK, USA).

Results

Vessel morphology

On PD16, the retinal vasculature of room-air raised pups of PACAP wild-type and KO groups reached to the ora serrata and no avascular area or abnormal vessel formation were observed (Figure 1). In OIR groups, avascular area was formed in the center of the retina and neovascular tuft formation was visible at the border of vascularized and non-vascularized area. The ratio of avascular territory to the whole retinal area was 10.77 ± 0.16 % in OIR-KO mice and 4.22 ± 0.37 % in OIR-Wt mice, which was a significant difference ($p < 0.000001$) (Figure 2). No difference was found in vessel density (27.31 ± 2.77 vs 29.77 ± 3.46 % respectively, $p = 0.59$).

Electroretinography

Visual function of mice was assessed by ERG examination after an overnight dark adaptation on PD15. Amplitudes of b-waves demonstrating the depolarization of Müller cells were significantly decreased in both KO groups compared to their controls (Figure 3A). The waves were not significantly different between control and OIR wild type groups. Average oscillatory potential amplitudes of OIR-KO mice were higher than those of the OIR-Wt (Figure 3B).

Angiogenesis array

Altogether 53 angiogenesis-related proteins were tested using a semi-quantitative angiogenesis array (Figure 4A,B). As a result of oxygen-induced retinopathy 9 angiogenic factors (i.e. CYR61, ADAMTS-1, IP-10, Osteopontin, Proliferin) were upregulated in wild-type mice (Figure 5b). In the retina of PACAP-deficient mice with retinopathy we observed an increase in altogether 11 more factors, including proteins related to extracellular matrix reorganization (i.e. MMP-3, MMP-8), inflammation (i.e. IL-10, IP-10), growth factors (i.e. FGF-7, IGFBP-1, PIGF-2) and the decrease of 4 proteins, such as CXCL-16, Endoglin, Endothelin-1 and Serpin E1, also known as Plasminogen activator inhibitor-1 (PAI-1) (Figure 5C). Under control circumstances only a few proteins (Endothelin-1, IGFBP-1, IP-10) showed alteration in KO animals (Figure 5A).

Western blot analysis

Phosphorylation level of antiapoptotic Akt did not change in control KO mice. A strong Akt activation was detected in wild-type retinopathic animals (OIR-Wt), whereas results of retinopathic KO mice (OIR-KO)

showed a significant decrease in Akt phosphorylation (Figure 6A,B).

Discussion

Improved perinatal care leads to an increased survival rates of very low birth weight preterm infants. These premature babies are at the risk to develop retinopathy of prematurity, a disease leading to vision impairment or eye problems with various degree. The estimated worldwide incidence of ROP among babies weighing less than 1500 grams is approximately 30% (Cavallaro et al. 2014). The current therapeutic options might halt the progress of the disease in mild cases but the real cure for this condition still awaits to be discovered. Animal models can mimic the development and characteristics of ROP so they help to study potential retinoprotective agents, such as PACAP. Its strong neuroprotective and neurotrophic effects have been well described in several retinal pathologies. Exogenously applied PACAP attenuates retinal excitotoxic injury caused by monosodium glutamate (Atlasz et al. 2009; Tamas et al. 2004), ischemic retinal lesion (Atlasz et al. 2007; 2010) and UV-light induced retinal degeneration (Atlasz et al. 2011). Previously, we have shown that three times intravitreal PACAP administration during the first two weeks of life ameliorates the retinopathy seen in the ROP model of neonatal rats (Kvarik et al. 2016).

In the present study, we used an established mouse model of retinopathy of prematurity to evaluate the effect of lacking endogenous PACAP with the help of PACAP-deficient mice. Here, we first showed that retinal flat mounts of KO mice demonstrated a significant increase of central avascular area on PD 16 suggesting that PACAP-deficiency leads to a more severe form of retinopathy. This assumption is further confirmed by functional electroretinography examination where the b-wave amplitudes – reflecting the activity of bipolar and third order retinal cells – are decreased in the retinopathic PACAP-deficient mice.

Several studies have described that under physiological conditions there is no remarkable difference in the gross morphology of PACAP KO mice compared to wild types (Kovács-Valasek et al. 2017; Szabadfi et al. 2012; Vaudry et al. 2005). However, slight biochemical, synaptic and ultrastructural alterations were observed in the nervous system regarding axonal arborization, myelination process, inner ear structure and cerebellar migration (Allais et al. 2007; Tamas et al. 2012; Vincze et al. 2011; Yamada et al. 2010). Data obtained from PACAP deficient mice provide evidence that lacking the neuropeptide leads to an increased vulnerability to stressors. PACAP KO mice exhibited more severe pathology of experimental autoimmune encephalomyelitis, delayed axonal regeneration in peripheral nerve injury and an increased infarct size in cerebral ischemia (Armstrong et al. 2008; Chen et al. 2006; Nakamachi et al. 2010; Tan et al. 2009). Recent studies have shown that PACAP knockout animals display early onset and a more generalized form of systemic amyloid deposition throughout the body in various organs (Reglodi et al. 2018b) and PACAP deficiency makes the articular cartilage structure more prone to degenerate (Szegeczki et al. 2019). PACAP deficient mice also display accelerated hearing impairment compared to wild-type mice (Fulop et al. 2019a). The protective effect of endogenous PACAP has also been described in the retina, in retinal toxic, ischemic, metabolic and inflammatory lesions (Kawaguchi et al. 2010; Szabadfi et al. 2012; Vaczy et al. 2018). Endo and colleagues have demonstrated that even the partial lack of endogenous PACAP leads to aggravation of the toxic neuronal damage. They have shown that

after 7 days of intravitreal NMDA injection the number of retinal ganglionic cells decreased significantly in PACAP heterozygous mice relative to their wild-type counterparts (Endo et al. 2011). In the mouse model of transient retinal ischemia Szabadfi et al has reported that all retinal layers suffered more severe damage after a 10 minutes ischemia followed by 2 weeks reperfusion period in PACAP deficient mice than in wild-type mice (Szabadfi et al. 2012). Recently, Vaczy and co-workers have revealed that intraperitoneal injection of lipopolysaccharide (LPS) led to a markedly more severe eye inflammation in PACAP KO mice with a decrease in antiapoptotic protein kinase B (pAkt) level and a more expressed elevation of sICAM-1, JE, TIMP-1 cytokines than in wild-type mice (Vaczy et al. 2018). The changes in the protective protein profile are in accordance with the generally observed PACAP-induced proteomic and transcriptomic changes in neuronal injuries (Rivnyak et al. 2018). Moreover, the use of PACAP and PACAP derivative eye-drops in ischemic retinopathy model was found to be retinoprotective as it passes the blood-retinal barrier (Atlasz et al. 2019; Werling et al. 2016; Werling et al. 2017). These results are in accordance with our observations that under normoxic conditions there is no gross morphological aberration in retinal vessel development but after a 5-day hyperoxic insult PACAP deficient mice had a significantly greater avascular central retinal territory than their wild-type littermates.

The mechanism of protective effect of PACAP have been studied extensively mainly in the nervous system and in the retina. It has been revealed that PACAP influences anti-apoptotic pathways, like ERK, CREB, Bcl-2, Bcl-xl, Akt; while inhibits pro-apoptotic proteins, such as caspases and Bad in glutamate-induced retinal injury in neonatal rats (Racz et al. 2006, 2007). In a rat retinal hypoperfusion model the MAPKs and Akt signaling pathways were studied after PACAP injection. It was shown that PACAP treatment led to a marked significant increase in the level of phosphorylated Akt (Szabo et al, 2012). The same observations have been reported in connection with excitotoxic retinal injury and other ischemic/reperfusion lesions (May et al. 2010; Racz et al. 2008). The antiapoptotic action of endogenous PACAP through Akt signaling has been further strengthened by Vaczy et al in their retinal inflammation model. They have reported a decrease in pAkt levels in LPS-injected PACAP KO mice compared to wild-type mice (Vaczy et al. 2018). Our western blot results correlate with these findings. We observed a robust increase in the phosphorylation state of Akt in wild-type retinopathic mice, while it markedly decreased in PACAP-deficient retinopathic mice. This indicates a disturbance in protective mechanisms against apoptosis leading to an increased vulnerability in case of PACAP deficiency.

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest

Availability of data and material

The data presented in this study are available on request from the corresponding author

Code availability (not applicable)

Ethics approval

Animal housing, care and application of experimental procedures were in accordance with the ethical guidelines approved by the University of Pecs (BA02/2000-31/2011)

Consent to participate (not applicable)

Consent for publication (not applicable)

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Figures

Figure 1

Representative pictures of mouse retinas for retinal vasculature. The presence of OIR is indicated with yellow area. PACAP deficiency affects the extent of retinopathy, control animals have completely vascularized retina on PD16.

Figure 2

Percentage of retinal avascular area of mice with retinopathy is represented as mean $\pm$ SEM. PACAP-deficient mice developed bigger central avascular retinal area. *** $p < 0.000001$

Figure 3

Measurement of electroretinography amplitudes in PACAP wild-type (Wt) and knock-out (KO) room-air reared controls (Cont) and mice with oxygen-induced retinopathy (OIR). Amplitudes of b-wave (a) and averaged oscillatory potential (b) were measured on PD15 after overnight dark adaptation. Values are expressed as mean ± SEM, *p<0.05.

Figure 4

(A) Representative panels show cytokine arrays from homogenates of control (I), control-PACAP-deficient (II), wild type OIR (III), and PACAP-deficient OIR retinas (IV). (B) The table indicates the examined cytokines in each box.

Figure 5

Semiquantitative analysis of angiogenesis related proteins. The pro- and antiangiogenic factors whose expressions in the retina of control PACAP-deficient (A) or wild-type OIR (B) or PACAP-deficient OIR mice (C) showed more than 30 % relative change compared to either control wild-type or OIR wild-type mice are represented in bar charts. The results are based on two independent measurements.

A **B**

Figure 6

Activation of AKT was determined in mouse retinas on PD16. Total proteins (non-phosphorylated) were used as loading controls. Representative blots (A) and bar chart (B) of the quantified blots are presented. Bars represent mean $\pm$ SEM of pixel densities; * indicates significant difference between PACAP knock out (OIR-KO) and wild-type (OIR-Wt) retinopathic mice ($p=0.027$).