Ocular Delivery of PACAP1-27 Protects the Retina From Ischemic Damage in Rodents

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Purpose. Pituitary adenylate cyclase activating polypeptide (PACAP) is neuroprotective in neuronal injuries. Bilateral common carotid artery occlusion (BCCAO) causes chronic hypoperfusion-induced degeneration in the rat retina, where we proved the retinoprotective effect of intravitreal PACAP. Although this route of administration is a common clinical practice in several diseases, easier routes are clinically important. Our aim was to investigate the potential retinoprotective effects of PACAP eye drops in BCCAO-induced ischemic retinopathy.

Methods. After performing BCCAO in rats, the right eyes were treated with PACAP1-27 eye drops (1 μg/drop, 2 × 1 drops/day for 5 days), containing different vehicles: saline, water for injections, thiomersal or benzalkonium solution for ophthalmic use (SOCB). Histology and immunohistochemistry were performed 2 weeks after surgery, while molecular analysis was performed 24 hours after BCCAO. Passage of PACAP1-27 through the ocular layers was tested with radioactive PACAP-SOCB in mice.

Results. Bilateral common carotid artery occlusion led to a severe degeneration of all retinal layers. Solution for ophthalmic use was the most effective vehicle for delivering PACAP (PACAP-SOCB), significantly ameliorating BCCAO-induced damage. The massive upregulation of GFAP was not observed in retinas treated with PACAP-SOCB eye drops. PACAP-SOCB treatment also increased activation of the protective Akt and ERK1/2 in hypoperfused retinas. The cytokine profile showing upregulation in different cytokines was attenuated by PACAP-SOCB. Radioactive PACAP reached the retina when delivered in SOCB-containing eye drops.

Conclusions. PACAP1-27, delivered in the SOCB vehicle as eye drops, was retinoprotective in ischemic retinopathy, providing the basis for future therapeutic administration.

Keywords: eye drops, ischemia, retina, neuropeptide

Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide with diverse biological actions. It elicits these actions by binding the G-protein-coupled receptors, PAC1 and VPAC1/2, which also bind vasoactive intestinal polypeptide (VIP).[1] Soon after the discovery of PACAP it became evident that the peptide has strong neuroprotective effects, in several in vitro and in vivo models.[2-6] The first in vivo proof of its neuroprotective action came from studies of global cerebral ischemia.[7] Subsequent studies confirmed the efficacy of PACAP in cerebral ischemic conditions and also in ischemic lesions of other organs, including kidney, intestine, and heart.[8-10]

In the retina, ischemic injury can be induced by ligating both carotid arteries transiently or permanently (bilateral carotid artery occlusion; BCCAO), leading to ischemia/reperfusion injury or chronic retinal hypoperfusion, respectively, methods that mimic several hallmarks of chronic retinal degeneration.[11] PACAP is protective in both types of ischemic retinal lesions.[5,12,13] Earlier we showed that PACAP1-38 ameliorates the reduction in thickness of different retinal layers as well as the loss of cells in the ganglion cell layer (GCL) in BCCAO-induced injury.[12,14] PACAP1-38 and 1-27 exert a similar degree of protection, while other fragments (PACAP6-38, PACAP6-27) and related peptides are either noneffective or deteriorate the injury.[15,16] We also found that PACAP counteracts the ischemia-induced cytokine changes and promotes antiapoptotic pathways.[17]

Altogether, these results provide strong evidence that PACAP has potential therapeutic value in ischemic retinopathy.

However, a major drawback of PACAP-induced retinal therapy is that in all these studies PACAP was given intravitreally. Although this route of administration is a common clinical practice in several diseases, like age-related macular degeneration (AMD) and diabetic retinopathy,[18] easier administration routes would be very important from the clinical point-of-view. Topical administration (eye drops) provides a suitable noninvasive method for treating ophthalmic diseases.[19] PACAP eye drops have been shown to have topical effects in the cornea,[20,21] but it is not known whether PACAP would penetrate the ocular barriers, reaching the retina. Therefore, the aim of the present study was to investigate the retinoprotective efficacy of PACAP given as eye drops in ischemic retinopathy.

MATERIALS AND METHODS

Surgery and PACAP-SOCB Treatment

In a preliminary experiment aimed to reveal the best vehicle for delivery of PACAP in eye drops, PACAP1-27 was dissolved in the following vehicles: saline, water for injections (aqua ad iniectabila), thiomersal solution for ophthalmic use (solvens viscosa pro oculoguttis cum thiomersalo), benzalkonium solution for ophthalmic use (solutio ophthalmica cum benzalkonio; SOCB). As SOCB was the most effective vehicle based on the histologic analysis, it was used in the subsequent experiments. Therefore, in the following sections, PACAP treatment indicated treatment with PACAP1-27 eye drops in SOCB in all cases.

Adult male Wistar rats ($n = 83$: $n = 28$ for histologic analysis, $n = 7$ for immunhistochemical analysis, $n = 19$ for immunoblot analysis, $n = 28$ for cytokine array) weighing 250 to 300 g were subjected to BCCAO. The animals were bred and kept in the Animal Facility of the Medical School University of Pecs (Pecs, Hungary). They were housed in individual cages, fed and watered ad libitum, under light/dark cycles of 12/12 hour. All 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. Under isoflurane anesthesia, common carotid arteries were exposed bilaterally through a midline incision and ligated with a 3-0 filament. Immediately following the BCCAO operation, the right eye was treated with PACAP1-27 eye drops (1 µg/drop). As in our past studies, PACAP1-27 was synthesized at the University of Szeged (Szeged, Hungary) as described in details elsewhere.[22] The left eye, serving as a control, was treated only with vehicle. A group of animals underwent anesthesia and all steps of the surgical procedure except ligation of the carotid arteries (midline incision of the neck and wound closure, sham group). Rats were treated for 5 consecutive days, twice a day with one drop.

Histologic Analysis of PACAP1-27

Rats ($n = 7$ sham, $n = 7$ sham + PACAP-SOCB and $n = 21$ BCCAO, $n = 21$ BCCAO + PACAP-SOCB retinas) were killed with an overdose of anesthetic 2 weeks after BCCAO and eyes were processed for histologic analysis. Histologic analysis was performed as described previously.[14] Briefly, eyes were removed and retinas were dissected in PBS, fixed in 4% paraformaldehyde dissolved in 0.1M phosphate buffer (PB), embedded in Durcupan ACM resin, and 2-µm thick sections were stained with toluidine blue (Sigma, Budapest, Hungary).

Four tissue blocks from at least four animals were prepared and central retinal areas within 1 mm from the optic nerve were used ($n = 5$ measurements from one tissue block). Photographs were taken with a digital CCD camera (Nikon) using the Spot program (Spot Imaging, Sterling Heights, MI, USA). The following parameters were measured: cross-section from the outer limiting membrane (OLM) to the inner limiting membrane (ILM), and width of all retinal layers (outer nuclear layer [ONL], outer plexiform layer [OPL], inner nuclear layer [INL], inner plexiform layer [IPL]). The number of cells in the ganglion cell layer (GCL) was also measured. Results are presented as mean ± SEM.

Radioactive Labeling of PACAP1-27

PACAP1-27 was labeled with ^{125}I using the lactoperoxidase method.[23] Briefly, 10 µg of PACAP1-27 (dissolved in 0.25M chloride-free PB) was mixed with 30 µL of 0.4M Na acetate (pH 5.6), 10 µL of lactoperoxidase (10 µg/mL dissolved in 0.1M Na acetate pH 5.6), and 1 mCi of ^{125}I. The reaction was started by adding 10 µL 30% H_2O_2 solution (prepared by adding 2 µL H_2O_2 in 30 mL H_2O). Ten minutes later, an additional 10 µL of the 30% H_2O_2 solution was added and the reaction allowed to progress 10 minutes more. At the end of this second 10-minutes incubation, the reaction solution was purified with HPLC (Shimadzu USA Manufacturing, Inc., Columbia, MD, USA) using a C18 column (P.J. Cobert Associates, Inc., St. Louis, MO, USA).

PACAP1-27 Eye Drop Preparation

The PACAP1-27 fraction collected from the HPLC was evaporated overnight in a fume hood using constant air flow to evaporate off the 0.1% trifluoroacetic acid in methanol solution that the fraction was collected in. The dried fraction was resuspended in 1% BSA Lactated Ringers' solution (Baxter Healthcare, Deerfield, IL, USA) and an acid precipitation was completed to assess the purity of the PACAP1-27 fraction. The acid precipitation was completed with 15% trichloroacetic acid. The percent of radioactivity precipitated was calculated using the following formula:

$$100 \times [(\text{CPM pellet})/(\text{CPM pellet} + \text{CPM supernatant})]$$

Only fractions that showed greater than 90% activity in the precipitate were used in the experiment. A 1×10^6 cpm solution was prepared in a 10-µL volume of benzalkonium-chloride. A 10-µL drop was placed on each eye delivering 1×10^6 cpm per eye.

Tissue Collection

Male CD-1 mice were anesthetized with urethane and administered PACAP1-27 as an eye drop (1×10^6 cpm/eye in benzalkonium-chloride). At various time-points, blood was collected from the carotid artery, and the eyes and whole brain removed. The time-points used in this study were 5, 30, 60, and 120 minutes. Mice ($n = 3–6$) were used at each time-point. The collected whole blood was allowed to clot at room temperature, centrifuged at 5400 g for 10 minutes at 4°C, and 50 µL of resulting serum was removed for use. The whole brain was collected and the eye dissected into the cornea, retina, and vitreous humor. The level of radioactivity in each of the regions and the serum was measured in a Wizard2 Automatic Gamma Counter (PerkinElmer, Waltham, MA, USA). The percentage of injected dose present in a milliliter of serum (%Inj/mL) and the percentage of the injected dose taken up per gram of tissue (%Inj/g) was calculated as previously described.[24,25]

Measurement of Glial Fibrillary Acidic Protein (GFAP) Activity in the Müller Glial Cells

For immunohistochemical analysis, 2 weeks after the induction of ischemia, animals ($n = 2$ sham, $n = 2$ sham + PACAP-SOCB, and $n = 5$ BCCAO; $n = 5$ BCCAO + PACAP-SOCB retinas) were killed with an overdose of anesthetic (120 mg/kg pentobarbital, Nembutal; Sanofi-Phylaxia, Budapest, Hungary), the eyes were immediately dissected in ice-cold PBS and fixed in 4% paraformaldehyde dissolved in 0.1M PB (pH 7.4) for 4 hours at room temperature, similarly to earlier descriptions.[14] Briefly, tissues were washed in 0.1M PB (6×10 minutes) and cryoprotected in 10% and 20% sucrose for 1 hour, followed by 30% sucrose in PBS overnight at 4°C. For cryostat sectioning, retinas were embedded in tissue freezing medium (Cryomatrix, Thermo Scientific, Waltham, MA, USA), and cut in a cryostat (Leica, Nussloch, Germany) at 10 to 12 μm. Central retinal areas within 2 mm from the optic nerve head were used for immunocytochemical analysis. Sections were mounted on chrome–alum–gelatin coated slides and stored at −20°C until use. Retinal sections were rinsed in PBS, permeabilized by incubation for 6×5 minutes in 0.1% Triton X-100 (Sigma) in PBS and incubated with 3% normal donkey serum and 0.1% Na-azide in PBS for 1 hour to minimize nonspecific labeling. Sections were incubated with the primary polyclonal antibody overnight at 4°C. We used the anti-GFAP antibody (rabbit anti-GFAP 1:1000; Sigma). The antibody was included in 1% donkey serum (Sigma). After 6 washes in PBS, sections were incubated for 2 hours at room temperature in the dark with the corresponding secondary fluorescent anti-rabbit antibody Alexa Fluor 488 (donkey anti-rabbit, 1:200; Life Technologies, Budapest, Hungary). Sections were then washed in PBS and propidium iodide (PI; 1:500; Sigma) was used to detect the nuclear components. After 3 washes in PBS, sections were coverslipped using Fluoroshield (Sigma). For control experiments, primary antibodies were omitted, resulting in no specific staining. Digital photographs were taken with a Nikon Eclipse Ci fluorescence microscope (Nikon, Melville, NY, USA). Photographs were further processed with the Adobe Photoshop CS6 program (Adobe Systems, Inc., San Jose, CA, USA). Images were adjusted for contrast only; they were aligned, arranged, and labeled by using the functions of the above program. An examiner blinded to the experimental treatment evaluated the images collected.

Immunoblot Analysis

For Western blot experiments, a separate group of retinas was removed 2 weeks ($n = 3$ sham, $n = 3$ sham + PACAP-SOCB, or $n = 9$ BCCAO, $n = 9$ BCCAO + PACAP-SOCB retinas) and 24 hours ($n = 4$ sham, $n = 4$ sham + PACAP-SOCB, and $n = 4$ BCCAO, $n = 4$ BCCAO + PACAP-SOCB retinas) after BCCAO. Samples were processed for Western blot analysis as described earlier.[17] Membranes were probed overnight at 4°C with anti-Akt-1 Ser473 (1:1000; R&D Systems, Budapest, Hungary), phospho-specific anti-ERK1/2 Thr202/Tyr204 (1:1000; Cell Signaling Technology, Danvers, MA, USA), total-Akt (tAkt) antibody was used as internal control for phospho-Akt (pAkt), actin antibody was used as control for phospho-ERK (pERK) 1/2. Membranes were washed six times for 5 minutes in TRIS-buffered saline (pH 7.5) containing 0.2% Tween prior to the addition of goat anti-rabbit or anti-mouse horseradish-peroxidase conjugated secondary antibody (1:3000; BioRad, Budapest, Hungary). The antibody–antigen complexes were visualized by means of enhanced chemiluminescence. After the scanning step, results were quantified using the ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The retina from each

rat was analyzed twice in two separate experiments. Pixel volumes of the spot were normalized to the internal controls. Data are represented by pixel density in arbitrary units.

Cytokine Array

For semiquantitative cytokine array, retinas ($n = 11$ sham, $n = 11$ sham + PACAP-SOCB, and $n = 17$ BCCAO, $n = 17$ BCCAO + PACAP-SOCB retinas) were removed after 24 hours of BCCAO operation 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%. 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 Cocktail for another 1 hour at room temperature, the membranes were incubated with 1.5 mL of tissue homogenates at 2°C to 8°C overnight on a rocking platform. After washing with buffer three times and addition of horseradish peroxidase-conjugated streptavidin to each membrane we exposed them to a chemiluminescent detection reagent (Amersham Biosciences, Budapest, Hungary) then to an X-ray film cassette.[17] The developed films were scanned, and the pixel volumes of the spot were determined by using ImageJ Protein Array Analyzer. Pixel volumes of the spot of interest were normalized to the control (sham retinas).[26] The array was repeated four times.

Statistical Analysis

Statistical comparisons were made using the 2-way ANOVA followed by Fischer's post hoc analysis.

Results

Retina Morphology and Morphometry

Bilateral common carotid artery occlusion resulted in severely reduced thickness of retinal layers 2 weeks after ligation, compared with sham animals (Figs. 1A, 1B). PACAP1-27 alone in sham animals did not result in alteration of any retinal layer (Figs. 1A, 1B). PACAP1-27 dissolved in solutio ophthalmica cum benzalkonio (PACAP-SOCB) led to significant protection in the retina in BCCAO. The BCCAO retinas treated with PACAP-SOCB had a more preserved structure compared with vehicle-treated retinas (Fig. 1A). Outer limiting membrane–ILM distance was reduced by 50.7% ($P < 0.0001$) in ischemic retinas compared with sham controls, but was reduced only by 36.1% ($P < 0.0001$) in the eyes treated with PACAP-SOCB. Similar protection could be observed in the OPL (BCCAO: 54.2%, BCCAO + PACAP1-27: 47.9%; $P < 0.005$), INL (BCCAO: 46.5%, BCCAO + PACAP1-27: 30.7%; $P < 0.0001$), and IPL (BCCAO: 61.4%, BCCAO + PACAP1-27: 38.3%; $P < 0.0001$), while no protection could be seen in the ONL (BCCAO: 35.3%, BCCAO + PACAP1-27: 36.3%) (Fig. 1B). The number of cells in the GCL was significantly decreased in ischemic retinas by 53.2% and was significantly increased by PACAP-SOCB (reduced only by 28.1%; Fig. 1C). Based on the findings, SOCB was the most effective vehicle for PACAP to exert neuroprotective effect in the retina; therefore, we used this solution for further experiments.
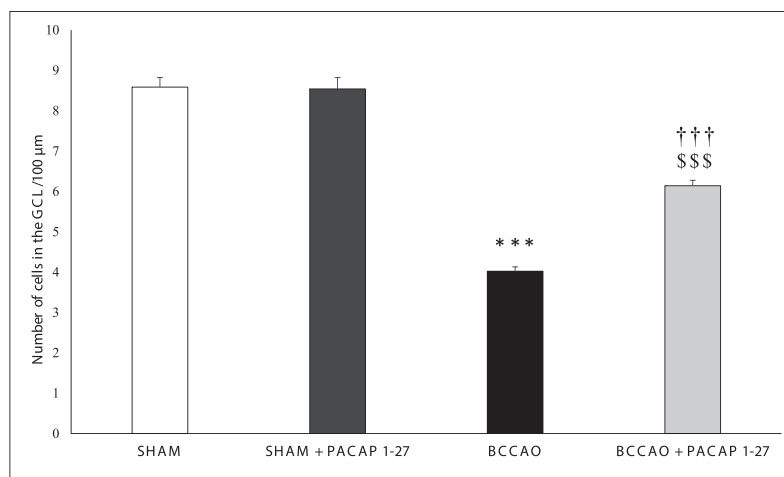
PACAP1-27 Uptake After Ocular Administration

Radioactively labeled PACAP1-27 (I-PACAP) was administered ocularly in SOCB to male CD-1 mice for a period ranging from 5
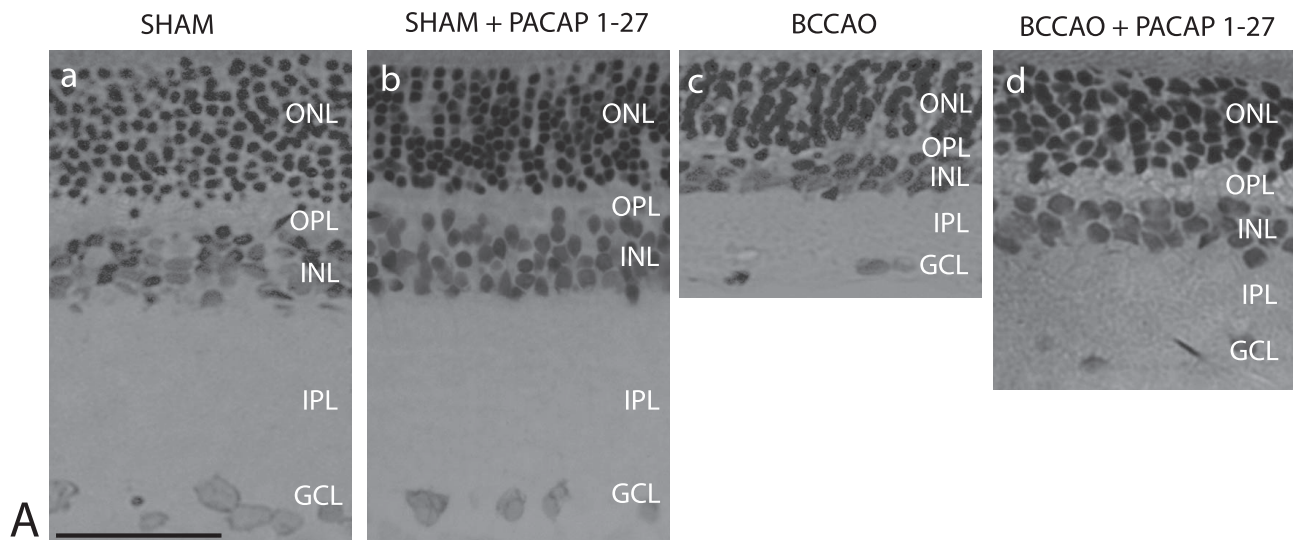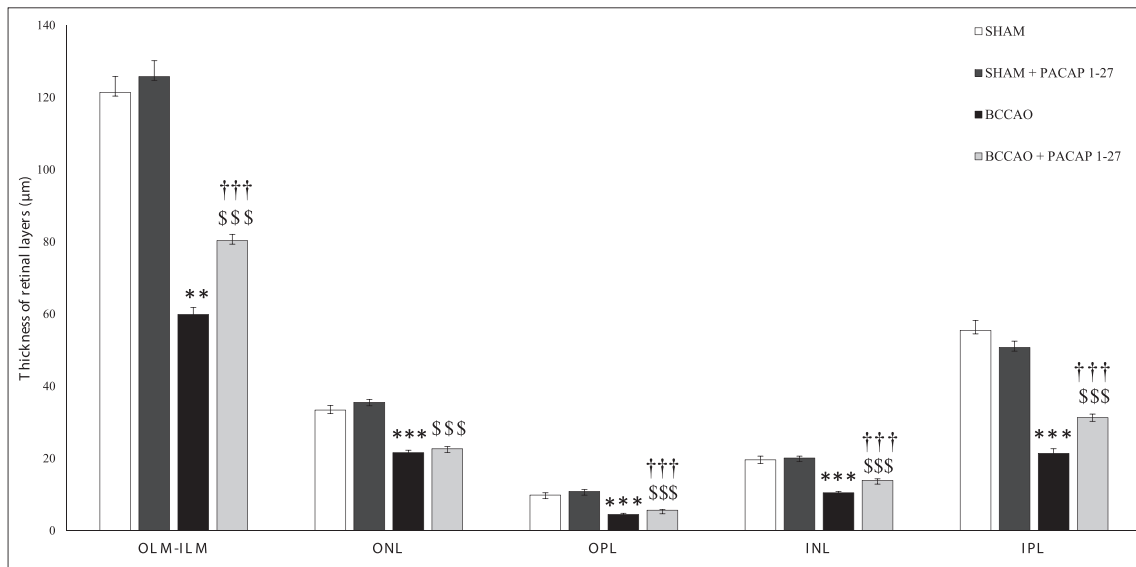
FIGURE 1. (**A**) Light microphotographs of retinal sections. Retinal tissue from BCCAO ($n = 21$) (**c**) showed severe degeneration compared with sham ($n = 7$) (**a**) and sham + PACAP-SOCB retinas ($n = 7$) (**b**). The retinal layers of BCCAO rats following treatment with eye drops containing PACAP-SOCB ($n = 21$) (**d**) showed only mild degeneration. (*Scale bar*: 50 µm). (**B, C**) Quantification of retinal layers in sham ($n = 7$ sham, $n = 7$ sham + PACAP-SOCB) and BCCAO ($n = 21$ BCCAO, $n = 21$ BCCAO + PACAP-SOCB) animals: the right eye was treated with PACAP-SOCB eye drops (PACAP1-27), the left eye served as controls receiving only SOCB. Comparison of retinal layers (**B**) and the number of cells/100 µm GCL length (**C**) in sham animals, rats with BCCAO and those receiving PACAP-SOCB (PACAP1-27) eye drops treatment after carotid occlusion. Morphometric analysis showed that treatment with eye drops improved the structure of all the retinal layers (except ONL). Statistical significance (***$P < 0.001$ versus sham retinas, $$$$P < 0.001$ versus sham + PACAP1-27 retinas, †††$P < 0.001$ versus BCCAO retinas) was calculated by 2-way ANOVA followed by Fischer's post hoc test.
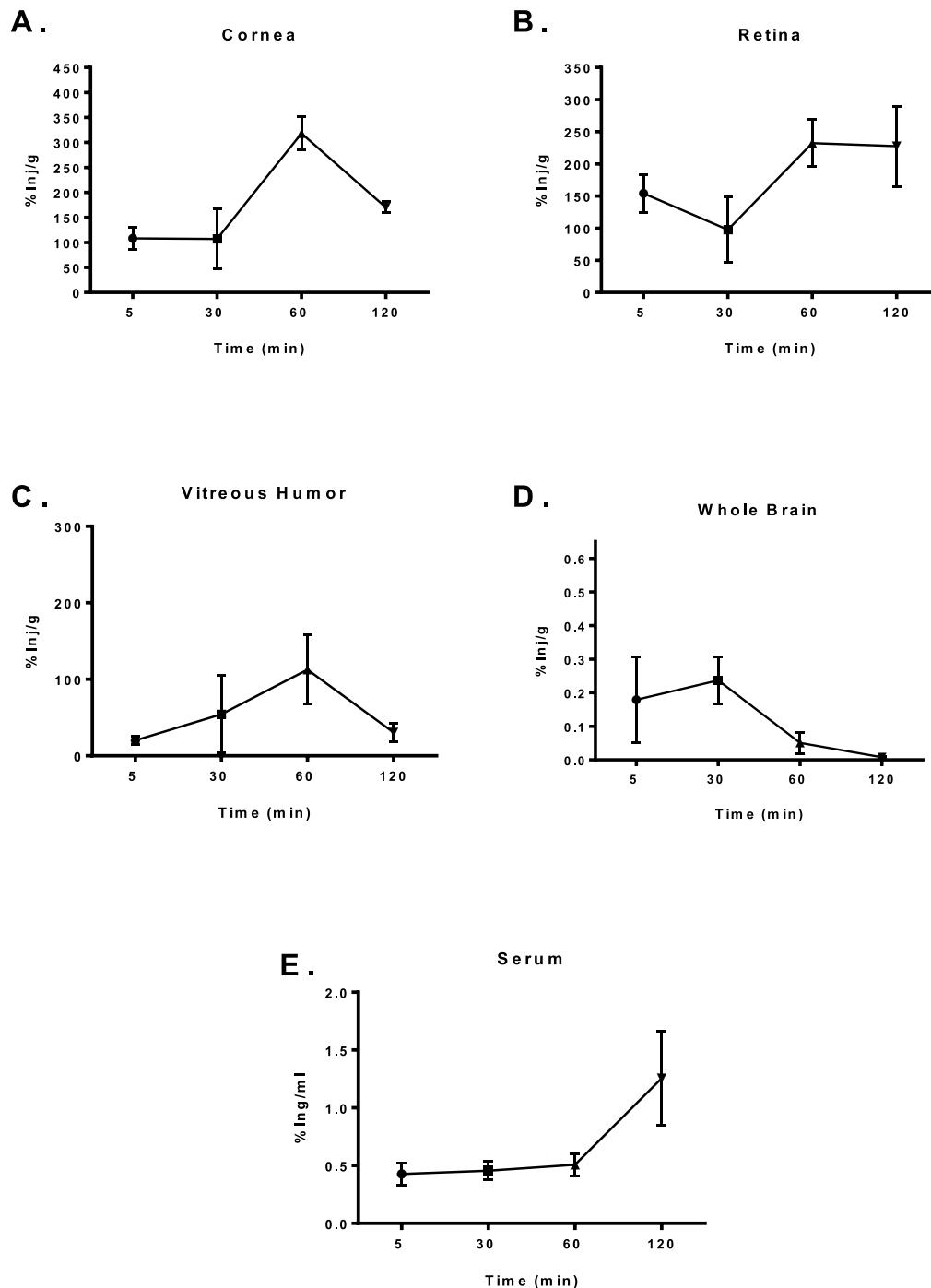
FIGURE 2. Distribution of radioactively labeled PACAP1-27 after ocular administration. Appearance of ^{125}I-labeled PACAP1-27 delivered to the cornea in SOCB (**A**), retina (**B**), vitreous humor (**C**), whole brain (**D**), and serum (**E**) at 5, 30, 60, and 120 minutes.

to 120 minutes. Transport of I-PACAP occurred rapidly as detection of radioactivity was present in the eye, the brain, and the serum 5 minutes post application. I-PACAP showed transport across the cornea (Fig. 2A), peaking 60 minutes after application. The vitreous humor showed a similar profile to the cornea (Fig. 2C). In the retina (Fig. 2B), I-PACAP uptake increased with time, plateauing at 60 minutes. I-PACAP was transported rapidly to the whole brain (Fig. 2D) before starting to decline after 30 minutes. By 120 minutes post application, the I-PACAP signal was barely detectable in the brain. Appearance of I-PACAP in the blood stream after ocular administration was delayed (Fig. 2E).

Analysis of Müller Glial Cells

Glial fibrillary acidic protein filaments are intermediate filaments expressed mainly by astrocytes and ependymal cells in the central nervous system. In the retina, they are normally present in the inner part of the Müller glial cells and their endfeet forming the ILM (Figs. 3A, 3B).[27] Glial fibrillary acidic protein was markedly upregulated following BCCAO with an altered distribution pattern: immunopositive signal was observed in the entire cell from the OLM to ILM (Fig. 3C). PACAP-SOCB eye drops appeared to ameliorate GFAP upregulation to some extent (Fig. 3D).
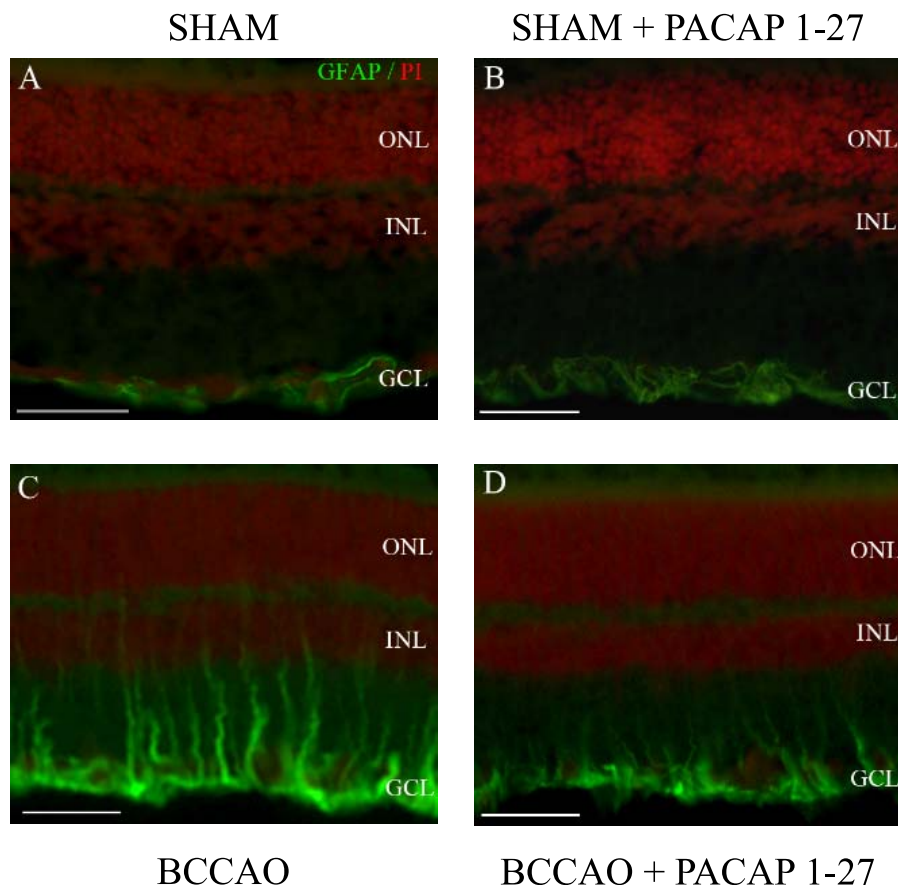
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SHAM

SHAM + PACAP 1-27

BCCAO

BCCAO + PACAP 1-27

FIGURE 3. Representative vertical retinal sections stained by GFAP antibody showing the effect of BCCAO ($n = 5$ BCCAO, $n = 5$ BCCAO + PACAP-SOCB) compared with sham ($n = 2$ sham, $n = 2$ sham + PACAP-SOCB) retinas. Propidium iodide was used to detect the nuclear components. In the sham (**A**) and sham + PACAP-SOCB (**B**) retina preparations GFAP-immunoreactivity was restricted to the inner part and internal endfeet of Müller cells. Retinal degeneration caused by BCCAO (**C**) showed strong upregulation of immunoreactivity. After PACAP-SOCB treatment (**D**) immunopositivity was reduced. *Scale bar*: 50 μm.

Phosphorylation of Akt and ERK1/2 After PACAP Treatment

No changes were detected between groups in retinas removed 2 weeks after BCCAO, however, marked alterations were seen in retinas 24 hours after the induction of ischemia. Ischemia itself caused a decrease in the expression of pAkt, which was not only reversed by PACAP-SOCB, but a robust increase could be observed (Figs. 4A, 4B). PACAP-SOCB treatment alone led to a slight decrease of pERK1/2 compared with the sham group. Ischemia induced a strong phosphorylation of ERK1/2 compared with sham retinas. This was further increased by PACAP-SOCB (data not shown).
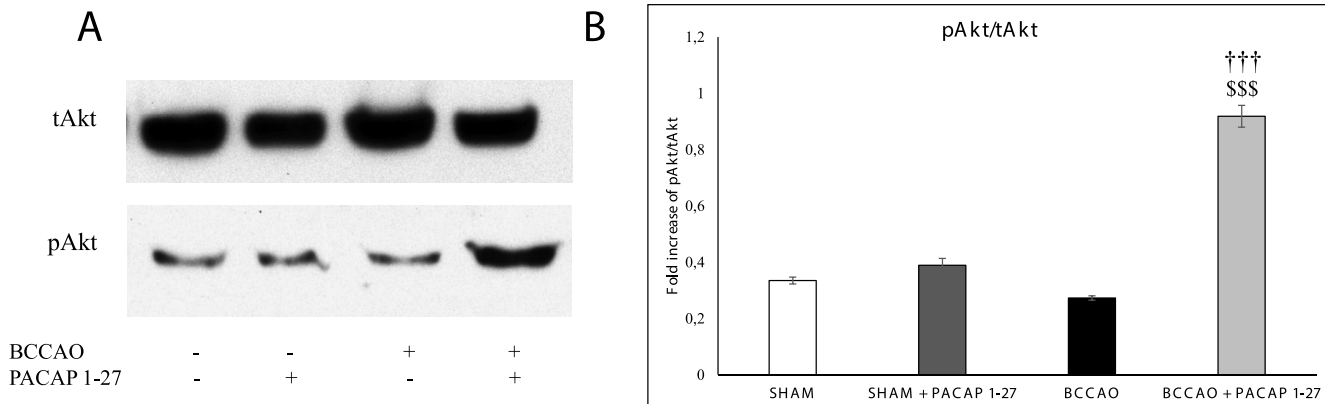
FIGURE 4. Representative Western blots showing activation (phosphorylation) of Akt (**A**) in sham ($n = 4$), sham + PACAP-SOCB ($n = 4$), BCCAO ($n = 4$), and BCCAO + PACAP-SOCB ($n = 4$) retinas. tAkt was used as internal control for pAkt. Statistical analysis of Western blot results for pAkt (**B**). Data are given as mean ± SEM. \$\$\$$P < 0.001$ vs. sham + PACAP-SOCB animals, †††$P < 0.001$ versus BCCAO animals. Statistical significance was calculated by 2-way ANOVA followed by Fischer's post hoc test.

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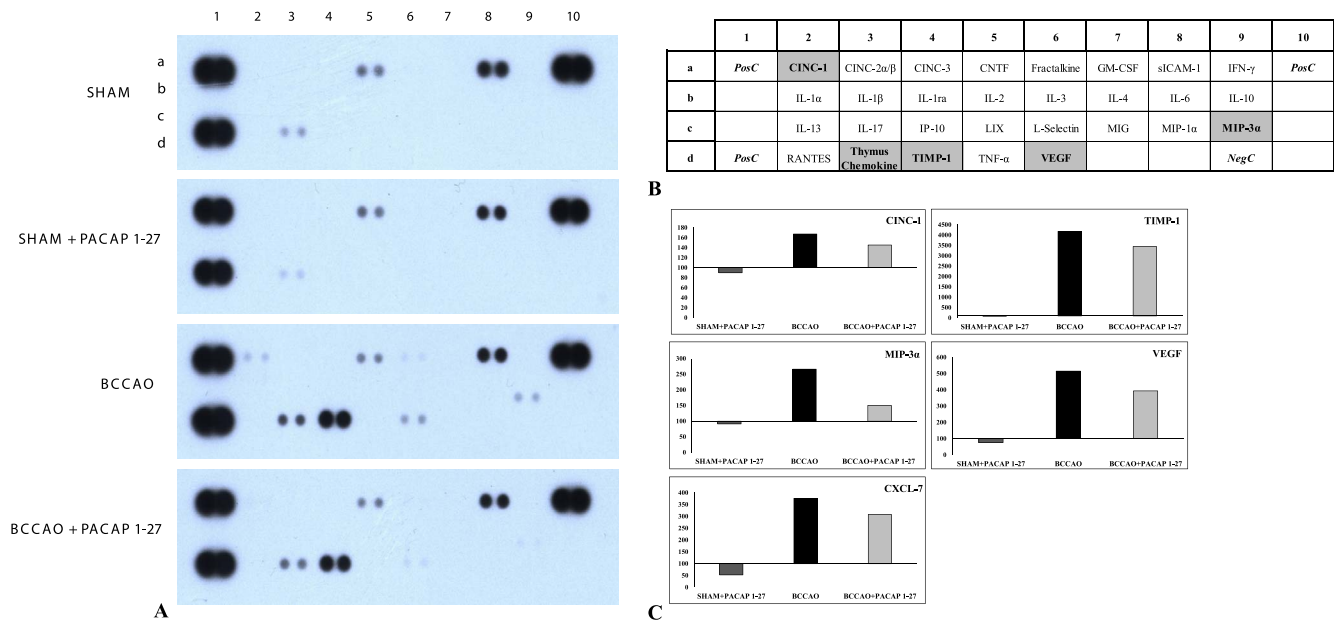|	1	2	3	4	5	6	7	8	9	10
a	*PosC*	CINC-1	CINC-2α/β	CINC-3	CNTF	Fractalkine	GM-CSF	sICAM-1	IFN-γ	*PosC*
b		IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-6	IL-10	
c		IL-13	IL-17	IP-10	LIX	L-Selectin	MIG	MIP-1α	MIP-3α	
d	*PosC*	RANTES	Thymus Chemokine	TIMP-1	TNF-α	VEGF			*NegC*	

B

C

FIGURE 5. Representative blot from the cytokine array measurements (A), where *dots* show cytokine expression detected by the array. The *panels* show arrays from sham (*n* = 11), and sham + PACAP-SOCB (*n* = 11), BCCAO (*n* = 17), and BCCAO + PACAP-SOCB (*n* = 17) retinas. The table (B) indicates the examined factors in each *box*, highlighting the changes observed after PACAP-SOCB treatment. Image analysis of cytokines (C) showing more than 20% change after treatments compared with the sham group, analyzed by the semiquantitative cytokine array. The different arrays were normalized to the sham controls, shown at baseline (100%). The *bar charts* show the relative changes in protein expressions based on four independent measurements given in percentage (%).

Effect of PACAP-SOCB Eye Drops on Ischemia-Induced Changes in Cytokine Expression

Cytokine expression was tested using a cytokine array (Figs. 5A, 5B). Expression of several cytokines increased after ischemia, including chemoattractant proteins, chemokines of the cytokine-induced neutrophil chemoattractant (CINC), and macrophage inflammatory protein (MIP) families: CINC-1 and MIP-3α. The activation of the thymus chemokine, tissue inhibitor of metalloproteinase (TIMP-1), and VEGF was also increased in the retinas that underwent BCCAO. PACAP-SOCB treatment attenuated activation of all the above mentioned cytokines (Fig. 5C). Expression of other cytokines analyzed by the array did not show any marked changes (Figs. 5A, 5B). Although several other cytokines showed slight alterations, only those are displayed in graphs and discussed, where we detected more than 20% change in the normalized data compared with sham group.

DISCUSSION

In the present study, we showed that PACAP administration delivered in the vehicle SOCB in form of eye drops is protective in ischemic retinal lesion. Thus, these data not only confirm the retinoprotective effects of PACAP in ischemic retinopathy, but prove that PACAP, in an appropriate vehicle, can cross the ocular barriers and reach the retina in a concentration sufficient to stimulate the protective pathways leading to reduced retinal damage.

One major disadvantage of in vivo PACAP treatments is the poor bioavailability of the natural peptide due to its fast cleavage by the dipeptidyl-peptidase (DPPIV) enzyme.[1] Its half-life in the serum is a few minutes only. Although PACAP crosses the blood–brain barrier,[28] the passage through the blood–retina barrier has not been investigated. These properties can explain why little or no retinal protective effect was observed

in a monosodium glutamate-induced excitotoxic retinal injury model in newborn rats when PACAP was administered systemically[29] compared with local, intravitreal treatment. Despite its rapid metabolism, PACAP has several advantages that may be responsible for its widespread effects. For example, the peptide shows high conformational stability and its binding to lipid membranes is very stable, preventing enzymatic degradation.[30,31] Furthermore, binding to its receptor induces long-term effects despite of the short half-life.[32]

PACAP has systemic effects when given intravenously or intraperitoneally, such as a decrease in blood pressure, facial flushing, migraine-like attacks in migraineurs, and hormonal changes.[33-36] Given these side effects and the fact that as far as ocular diseases are concerned, the first choice of treatment is topical application of drops or ointments, we aimed at investigating whether topical PACAP administration would be beneficial in a model of chronic retinal hypoperfusion. The morphologic results of the present study showed that PACAP, dissolved in SOCB, significantly ameliorated the BCCAO-induced retinal damage, indicating that applying the appropriate vehicle, PACAP can reach the retina from the ocular surface. Indeed, using radioactive labeling, we provided evidence that PACAP passes through the cornea, vitreous body to the retina. In light of the above mentioned side effects, an important finding of our study is that very little of the PACAP administered in SOCB as eye drops reached the systemic circulation or the brain. Specifically, the concentration of PACAP in the blood was 0.5% and in brain was 0.1% of the retina.

In addition to the usual intravenous or intraperitoneal routes, several alternative routes of PACAP administration have been applied in various experimental paradigms. Intranasal administration is suggested for treatment of neurodegenerative diseases.[23,37] Topical application to skin was used to achieve dermal vasodilation,[38] while buccal delivery for treatment of type 2 diabetes.[39] Topical ocular application of PACAP has already been used to test local, corneal effects of the peptide. It

has been found that PACAP, given in eye drops, enhances corneal wound recovery,[20,40,41] stimulates lacrimal secretion,[40,41] and accelerates nerve regeneration.[21] However, the effects of topical PACAP on the retina have never been investigated. Some other growth factors have already been used as potential topical treatments, such as nerve growth factor (NGF). For NGF, the best proven effects of corneal administration are for corneal disorders, but NGF eye drops reaches the retina and protective effects have been suggested.[42,43] The pharmacokinetics of topical NGF treatment seems different from that of PACAP: while NGF reaches its peak in the retina 6 hours after treatment, we found that PACAP reaches its maximum level in the retina 1 hour after administration. The reason for this difference may relate to the molecular size, as PACAP is a short neuropeptide in contrast to the high molecular weight NGF protein.

Our present study also showed that PACAP exerts its retinoprotective effects by similarly complex protective mechanisms as previously shown in intravitreal treatments. It has antiapoptotic/antioxidant effects and promotes an anti-inflammatory status.[44–47] We have previously shown that PACAP, binding to its receptors present in the retina, stimulates antiapoptotic, whereas inhibits proapoptotic pathways,[17,44,45,48] including the pathways studied here: ERK1/2 and Akt. PACAP not only has strong antiapoptotic properties, but also has anti-inflammatory and antioxidant actions. In the retina, we have previously described that PACAP shifts the injury-induced inflammatory cytokine/chemokine profile toward an anti-inflammatory profile[17] in a very similar manner to our present findings, where the BCCAO-induced increase of the cytokines CINC-1, MIP-3α, thymus chemokine, TIMP-1, and VEGF was attenuated by PACAP treatment. Glial cell activation is a pathologic sign typically present in lesions, including retinal ischemia. Here, we showed that Müller glial cell activation was markedly increased after induction of ischemia, whereas ocular PACAP-SOCB attenuated it, similarly to our earlier descriptions in ischemic and diabetic retinal lesions.[12,49–51] Wada and co-workers[47] have demonstrated that PACAP influences another glial cell in the retina: they showed that the neuropeptide attenuates N-methyl-D-aspartate (NMDA)-induced retinal damage in association with modulation of the microglia/macrophage status into an acquired deactivation subtype.[47]

In summary, we found that PACAP dissolved in SOCB and delivered as eye drops exerted retinoprotective effects and deserves further investigation in order to exploit its therapeutic potential in retinal degenerations in an easy-to-use form of application.

Acknowledgments

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