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PACAP-ADNP axis prevents outer retinal barrier breakdown and choroidal neovascularization by interfering with VEGF secreted from retinal pigmented epitelium cells

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ABSTRACT

During diabetic retinopathy (DR) progression, the retina undergoes various metabolic changes, including hypoxia-signalling cascade induction in the cells of retinal pigmented epithelium (RPE). The overexpression of hypoxic inducible factors causes transcription of many target genes including vascular endothelial growth factor (VEGF). The RPE cells form the outer blood retinal barrier (oBRB), a specialized structure that regulates ions and metabolites flux into the retina to maintain a suitable quality of its extracellular microenvironment. VEGF worsens retinal condition since its secretion from the basolateral compartment of RPE cells compromises the barrier's integrity and induces choroidal neovascularization. In this work, we hypothesized that PACAP prevents the damage to oBRB and controls choroidal neovascularization through the induction of ADNP. Firstly, we demonstrated that ADNP is expressed in Streptozotocin (STZ)-induced diabetic animals. To validate our hypothesis, we cultured endothelial cells (H5V) forming vessels-like structures, in a conditioned medium (CM) derived from ARPE-19 cells exposed to hyperglycaemic/hypoxic insult, containing a known VEGF concentration. The involvement of PACAP-ADNP axis on oBRB integrity was evaluated through the measurement of trans-epithelial-electrical resistance and permeability assay performed on ARPE cell monolayer cultured in CM and by analysing the expression of two tight junction forming proteins, ZO1 and occludin. By culturing H5V in CM, we demonstrated that PACAP-ADNP axis counteracted vessels-like structures formation promoted by VEGF. In conclusion, the results suggested a primary role of PACAP/ADNP axis in preventing oBRB damage and in controlling aberrant choroidal neovascularization induced by VEGF secreted from RPE cells exposed to hyperglycaemia/hypoxic insult in DR.

1. Introduction

Diabetic retinopathy (DR) represents the most common microvascular complication of diabetes leading to blindness in the late stages of disease [73]. During its development, the retinal tissue undergoes ~~to~~ various metabolic changes resulting in generation of reactive oxygen species (ROS), inflammation, hypoxia-signalling cascade induction and tissue structural damages, including thinning of the retinal layers and breakdown of blood retinal barrier (BRB) [36].

The BRB includes two different structures known as inner BRB (iBRB), constituted by endothelial cells of retinal capillaries, and outer BRB (oBRB) made by retinal pigmented epithelium (RPE) cells limiting

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the flux of molecules and water between capillaries of the choroid and retinal layers [15,14].

Long-term hyperglycaemia can damage the retinal microcirculation driving to activation of various pathogenic mechanisms including hypoxia in the (RPE) cells [9]. This event induces overexpression and translocation of hypoxic inducible factors (HIFs) 1α and 2α from the cytoplasmic to nuclear compartment of the cell where they bind to constitutively expressed 1β (HIF-1β) subunit. Here, these heterodimeric complexes activate transcription of many target genes including vascular endothelial growth factors (VEGF) [74]. This angiogenic factor, secreted by basolateral compartment of RPE cells, compromises barrier integrity acting directly to the junctional complexes between cells by inducing their cellular internalization [58]. By crossing the damaged barrier or spreading from the basal region of RPE cells, it also participates to choroidal neovascularization. The latter event further worsens oBRB breakdown and dysfunction of neurosensory retina [4,24]. In accord, many papers have previously demonstrated that this growth factor represents the main actor driving the uncontrolled intraocular neovascularization characterizing proliferative stage of retinopathy [7,26]. Considering this evidence, in the recent years an anti-VEGF therapy has been developed, however, it is not always able to counteract ocular neoangiogenesis by failing in half of treated patients, unfortunately. Then, the characterization of the molecular mechanisms involved in the maintenance of oBRB integrity as well as the identification of new therapeutic strategies counteracting uncontrolled retinal neovascularization represents the main targets of research to prevent DR development.

Several papers have demonstrated the retinal protective role exerted by pituitary adenylate cyclase activating peptide (PACAP) and vasoactive intestinal polypeptide (VIP) in different ocular disease including DR [16,19,2,22,29,46,48,60,61,64]. Many others evidences have widely demonstrated the retinoprotective effect of PACAP also in models of retinopathy of prematurity, hypertensive retinopathies, or in aging of the nervous system [34,38,40,56,57,59].

Further, PACAP has positive effects on ocular conditions such as dry eye disease [32,39,53,63], associated with diabetes, through modulation of aquaporins (AQPs) which are also regulates by oestradiol [28]. PACAP and VIP are widely expressed in central nervous system (CNS) as well as in peripheral organs [1,10,45,51,67,8]. Their biological functions are mediated by binding to three different types of G-coupled receptors, known as PAC1 (PAC1-R), VPAC-1, and VPAC-2 receptors (VPAC1-R and VPAC2-R) [70].

At least 10 PAC1R isoforms have been identified, to date [5,11]. By binding to PACAP, each variant activates a specific signalling pathway, such as PKC or PKA or MAPK/ERK signalling cascades, as well as L-type Ca^{2+} channels [71]. Moreover, previous papers have demonstrated that PACAP regulates some biological effects through the intracellular induction of a peptide, known as activity-dependent neuroprotective protein (ADNP) [43,52]. The smallest active element of this protein, known as NAP, is well known to exert various neuroprotective effect in different neurodegenerative disorders [3,30,31,33,37,44,65].

In our recent papers, we have demonstrated the protective effect of PACAP and NAP to counteract retinal damage induced by hyperglycaemia/hypoxia event as well as cytokines-mediated inflammatory process in DR [17,20,62]. We have demonstrated that either PACAP or NAP prevented oBRB damage, in a model in vitro of DR, represented by ARPE cells exposed to a hyperglycaemia/hypoxic insult. This effect was mediated by interfering with VEGF expression [21,50].

Considering the above-mentioned finding, we have hypothesized that PACAP prevents the damage to oBRB and controls choroidal neovascularization through the induction of ADNP. In the present work, we have confirmed this hypothesis by culturing human RPE cells (ARPE-19) or endothelial cell forming vessels-like structures, in a conditioned medium (CM) derived from ARPE-19 cells grown under hyperglycaemic/hypoxic condition, containing a known VEGF concentration. The results have demonstrated the involvement of PACAP-ADNP axis to maintain oBRB integrity and control aberrant choroidal neovascularization.

In conclusion, these data furtherly elucidate the molecular mechanism exerted by PACAP-ADNP axis to prevent the pathogenetic event mediated by VEGF in neural retina during DR.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley (200 g each) were obtained from Charles River (Calco, Italy). Animal care and experimental procedures were carried out according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol (#279) has been approved by Institution Animal Care and Use Committee of the Catania University. The animals were fed with standard laboratory chow and were allowed free access to water in an air–conditioned room with a 12 h light/12 h dark cycle. Final sizes for all measurements were n = 6 animal/group.

2.2. Induction of diabetes

After 12 h of fasting, the animals received a single 60-mg/kg intravenous (i.v.) injection of streptozotocin (STZ) in 10 mM sodium citrate buffer, pH 4.5 (1 ml/ kg dose volume) as previously described [6]. Control (sham, non-diabetic) animals were fasted and received citrate buffer alone. After 24 h, animals with blood glucose levels > 250 mg/dl were considered diabetic, and randomly divided in groups of ten animals each. The diabetic state was confirmed by evaluating glycemia daily through a blood glucose meter (Accu-Check Active1, Roche Diagnostic, Milan, Italy). All the experiments were performed 21 days following the induction of diabetes. Rats were killed with CO_2 inhalation and the collected retinas were fixed in 4% paraformaldehyde for immunofluorescence analysis.

2.3. Intraocular administration of PACAP

One week after diabetes induction, 100 μM of PACAP-38 (Sigma-Aldrich, Milan, Italy) dissolved in PBS solution were injected intravitreally. Before intraocular administration of PACAP, animals were anesthetized by intravenous injection of 5 mg/kg agent (Zoletil, Virbac, Italy; 2.5 mg/kg tiletamine HCl, and 2.5 mg/kg zolazepam HCl) and 1 drop of local anaesthetic (Novesina; Novartis, Italy) administered into the eye. An equal volume of vehicle (PBS) was injected in the contralateral eye as control. Two weeks after the intraocular injection the animals were killed, and retinas collected.

2.4. Human RPE Cell Cultures

This study was performed on immortalized human retinal pigment epithelial cell line (ARPE-19), purchased from the American Type Culture Collection (Manassas, VA, USA). Briefly, the cells were cultured in T75 flasks using DMEM-F12 (1:1) medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Steinheim, Germany), 1 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Sigma Aldrich) and maintained at 37 °C in a humidified atmosphere of 95% air and 5%CO_2 and sub-cultured once a week. After 7 days of culturing in normal glucose (NG, 5.5 mM D-glucose) half of cells were maintained in 5.5 mM D-glucose (Control) whereas the others were switched to high glucose (HG, 25 mM D-glucose) for further 7 days. Cells cultured in HG were exposed to a hypoxia-mimetic agent 100 μM deferoxamine mesylate salt (DFX) (Sigma-Aldrich) with or without 10 nM NAP or 100 nM PACAP alone or in combination with PAC1 antagonist, 10 μM PACAP6–38 (Sigma-Aldrich), or with PLC inhibitor, 50μ M of U73122 [21,47] (New England Peptide, MA, USA) for 24 h. To rule out any osmotic effect, the controls were grown in low glucose medium addicted

with mannitol (5.5 mM D-glucose + 19.5 mM mannitol vs. 25 mM D-glucose) an osmotic control agent.

2.5. Conditioned medium (CM) collection

The ARPE-19 cells were cultured for 24 h at 37C in a medium containing 1% FBS, representing CM1 (control) or containing HG+DFX (CM2). Subsequently, the CMs were collected. VEGF-A concentration was measured in an aliquot (100 μl) of each CM by using the ELISA sandwich enzymatic method, as described below. Then, CMs were frozen at − 80C until use.

2.6. ELISA

VEGF-A release in conditioned media was measured by using the ELISA sandwich enzymatic method with a specific anti-VEGF-A antibody (human VEGF-A; cat. no. ELH-VEGF; RayBiotech, Inc.) coated on a 96-well plate, according to the manufacturer's guidelines. Briefly, confluent ARPE-19 cells cultured in media supplemented with 1% FBS were treated for 24 h with HG+DFX with or without with PACAP (100 nM) alone or in combination with PACAP6–38 (10 μM) or U73122 (50 μM), or with NAP (10 nM) alone. Standards or supernatants from samples were pipetted into the wells containing the immobilized anti-VEGF-A antibody. Then, wells were washed before adding biotinylated anti-human VEGF antibody. Following incubation, the unbound biotinylated antibody was washed off, and HRP-conjugated streptavidin was pipetted in each well. After an additional wash step, a 3,3',5,5'-tetramethylbenzidine substrate solution was added to each well, resulting in a blue coloration proportional to the amount of bound VEGF. The stop solution was added, and the colorimetric intensity of the blue substrate now turned yellow was measured at 450 nm. The mean absorbance was calculated for each set of duplicate standards, controls and samples, and the average zero standard optical density was subtracted.

2.7. Measurement of trans-epithelial-electrical resistance

ARPE-19 cells were seeded on transwell-clear permeable supports (Corning Costar, Cambridge, MA) at a density of 1.5×10^5cells/cm, and cultured in CM1 or CM2 alone or in combination with PACAP (100 nM) alone or in combination with PACAP6–38 (10 μM) or U73122 (50 μM), or with NAP (10 nM) alone for 24 h. The progress of epithelial barrier formation and polarization was monitored by measuring trans-epithelial-electrical resistance (TEER) using a Millicel-Electrical Resistance System (ERS2, Millipore, Epithelial Volt-Ohm Meter, Merck Millipore, Germany) as previously described [49]. TEER was recorded in ARPE-19 cells grown on transwell supports. Measurements started after 15 min equilibration period at room temperature. Values are expressed as Ω/cm^2. The combined resistance of the filter was subtracted to the values of filter-cultured ARPE-19 cells. TEER monolayer recordings were performed on three different wells for each experimental condition.

2.8. Permeability assay

ARPE-19 cells were seeded on transwell-clear permeable supports (Corning Costar, Cambridge, MA) at a density of 1.5×10^5cells/cm, and cultured in CM1 or CM2 alone or in combination with PACAP (100 nM) alone or in combination with PACAP6–38 (10 μM) or U73122 (50 μM), or with NAP (10 nM) alone for 24 h. Permeability assay was performed by measuring the apical-to-basolateral movements of FITC-dextran solution with average mol wt 70,000 (Sigma Aldrich) as previously described (28106278). PBS solution was added in the apical compartment of each transwell, then it was replaced with the FITC-dextran solution. Sixty minutes later, the solution from the lower chamber was collected and the absorbance was measured through a microplate reader (Biorad 680) using 480 nm for excitation and 535 nm for emission.

2.9. Western blot analysis

Western blot analysis was performed according to the procedures previously described by [23]. Briefly, proteins were extracted with buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, 0.5 mM EGTA; 50 mM mercaptoethanol, 0.32 mM sucrose and a protease inhibitor cocktail (Roche Diagnostics, Monza, Italy) using a Teflon-glass homogenizer and then sonicated twice for 20 s through ultrasonic probe. Protein concentrations were determined by the Quant-iT Protein Assay Kit (Invitrogen). Sample proteins (32 μg) were diluted in 2X Laemmli buffer (Invitrogen, Carlsbad, CA, USA), heated at 70 °C for 10 min, separated on a Biorad Criterion XT 4–15% Bis-tris gel (BIO-RAD) by electrophoresis and then transferred to a nitrocellulose membrane (BIO-RAD). Blots were blocked using the Odyssey Blocking Buffer (LI-COR Biosciences) and probed with appropriate antibodies: rabbit anti-ADNP primary antibody (NBP1–89236; Novus Biologicals; 1:200), rabbit anti-ZO-1 (61–7300, Invitrogen; 2 m g/ml), rabbit anti-occludin (71–1500, Invitrogen; 2 m g/ml), mouse anti-β-actin (sc-47778, Santa Cruz Biotechnology; 1:500).

The secondary antibody goat anti-rabbit IRDye 800CW (#926–32211; LI-COR Biosciences) and goat anti-mouse IRDye 680CW, (#926–68020D; LI-COR Biosciences) were used at 1:20000. Blots were scanned with an Odyssey Infrared Imaging System (Odyssey). Densitometry analyses of blots were performed at non-saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Values were normalized to β-actin, which was used as loading control. No signal was detected when the primary antibody was omitted (data not shown).

2.10. Immunohistochemistry (IHC)

IHC analyses was performed on whole retina as previously described by [46]. Briefly, the sections were dewaxed in xylene, and by using graded ethanol were hydrated. In order to quench endogenous peroxidase activity, the sections were incubated for 30 min in 0.3% H2O2/methanol solution and rinsed with PBS. Then the sections were incubated with 1% bovine serum albumin (BSA) in PBS for 1 h, to reduce non-specific staining, and subsequently were incubated overnight at 4 °C with rabbit anti-ADNP primary antibody (NBP1–89236; Novus Biologicals; 1:100). After 24 h HRP conjugated was used as secondary antibodies and immune complexes were revealed with peroxidase labeled streptavin (LSAB+System-HRP, K0690, Dako, Glostrup, Denmark). The sections rinsed in PBS were incubated with diaminobenzidine (DAB) for 5 min. To counterstained the nuclei was used Hematoxylin. The stained sections were dehydrated through graded alcohols, cleared in xylene, and covered with neutral balsam. The sections were visualized with the light microscope (Carl Zeiss, Oberkochen, Germany) and the pictures were acquired with digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

2.11. Immunofluorescence analysis

To determine the cellular distribution of ADNP, immunofluorescence analysis was performed on ARPE-19 cells as previously described by [18]. Cells cultured on glass cover slips, were fixed in 4% paraformaldehyde in PBS (15 min at room temperature), permeabilized with 0.2% Triton X100, blocked with 0.1% BSA in PBS, and then probed with rabbit anti-ADNP primary antibody (NBP1–89236; Novus Biologicals; 1:50). Signals were revealed with Alexa Fluor 594 goat anti-rabbit for 1 h at room temperature and shielded from light. DNA was counterstained with DAPI (#940110, Vector Laboratories). After a series of PBS and double-distilled water washes, the fixed cells were cover-slipped with vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunolocalization was analyzed by confocal laser scanning

microscopy (CLSM; Zeiss LSM700). All acquisitions were performed with ZEN-2010 software.

2.12. Tubes formation assay

Murine micro-vascular endothelial cells (H5V), cultured overnight in a starved medium, were seeded into 24 well plate previously coated with 95 μl of Geltrex matrix. These cells were cultured in a medium containing 200 μl of CM1, CM2 alone or in combination with PACAP and NAP. Four randomly selected fields of view were captured with a digital camera (Canon) attached to a light inverted microscope (Axion Observer A1; Carl Zeiss AG). Tubes-like structure number per field was calculated as percentage of control (%).

2.13. Statistical Analysis

Data are reported as Mean ± S.E.M. One-way analysis of variance (ANOVA) was used to compare differences among groups, and statistical significance was assessed by the Tukey–Kramer post hoc test. The level of significance for all statistical tests was $p \leq 0.05$.

3. Results

3.1. PACAP-ADNP axis counteracted oBRB breakdown by interfering with the effect of VEGF secreted by ARPE-19 cells exposed to hyperglycemic/hypoxic insult

In a preliminary experiment, we have investigated the expression of ADNP in the RPE of diabetic animals. Considering that it is a very thin layer, it was not possible to evaluate the quantitative expression of the peptide. However, its expression was detected in diabetic animals following intravitreal injection of PACAP through IHC analysis (Fig. 1). To investigate the role of PACAP/ADNP axis on RPE during development of DR, we have used a common in vitro model represented by ARPE-19 cells, exposed to hyperglycemic/hypoxic insult (HG/DFX), mimicking the microenvironmental condition of this disease. In accord to in vivo result, ADNP was undetectable in basal condition as well as after HG exposure (Fig. 2). A weak ADNP expression was revealed in HG+DFX group respect to HG (Fig. 2A and B, $^{###}p < 0.001$ vs HG), whereas PACAP exogenous administration upregulated its levels respect HG+DFX group (Fig. 2A and B, $^{§§§}p < 0.001$ vs HG+DFX). The inhibition of PAC1R with PACAP6–38 counteracted ADNP overexpression as compared to PACAP-treated cells (Fig. 2A and B, $^{+++}p < 0.001$ vs HG+DFX+PACAP), suggesting that PACAP induced ADNP expression by binding with PAC1R. These results were corroborated by performing immunofluorescence analysis. As shown in Fig. 2C, ADNP was undetectable in NG and HG groups whereas its immunoreactivity was detected at perinuclear area in HG+DFX-treated cells. The treatment with PACAP increased ADNP immunosignal in the cytoplasm of ARPE-19 cells cultured in HG+DFX. On contrary, the administration of PAC1R antagonist has reduced ADNP immunoreactivity in cytoplasmatic compartment.

To evaluate whether PACAP-ADNP axis interfered with VEGF secretion from ARPE cells exposed to HG+DFX condition, we have measured the concentration of this angiogenic factor in the extracellular compartment of ARPE-19 cells grown for 24 h in a medium containing HG+DFX by using the ELISA sandwich enzymatic method. As shown in Table I, the amount of VEGF secreted in the growth medium increased in cells exposed to hyperglycemia/hypoxic insult as compared to NG group (Table I, $^{***}p < 0.001$ vs NG). Instead, the treatment with PACAP or NAP significantly reduced its levels in the culture medium of cells grown in HG+DFX (Table I, $^{###}p < 0.001$ vs HG+DFX). This effect was reverted by co-treating cells with PACAP+PACAP6–38 or with PACAP+U73122 (Table I, $^{$$$}p < 0.001$ vs HG+DFX+PACAP).

To evaluate the effect of VEGF secreted in extracellular compartment on oBRB integrity, we have seeded ARPE-19 cells on transwell-clear permeable supports, representing a model in vitro of oBRB, and grown them in conditioned medium (CM2) collected in the previous experiment, containing a known concentration of VEGF (536,95 pg/ml). The integrity of oBRB was evaluated by measuring TEER and the apical-to-basolateral movement of FITC-dextran solution between the upper and lower compartment of each transwell. As expected, TEER values significantly decreased in cells cultured in CM2 as compared to control (CM1) (Fig. 3A, $^{***}p < 0.001$ vs CM1), whereas PACAP or NAP administration on cells grown in CM2 significantly increased this value respect to CM2 group (Fig. 3A, $^{###}p < 0.001$ vs CM2). The co-treatment with PACAP+PACAP6–38 or PACAP+U73122 significantly reduced TEER values to levels of CM2 group (Fig. 3A, $^{$$$}p < 0.001$ vs CM2 +PACAP).

The permeability measurements to FITC-dextran solution are inversely related to TEER values. In accord, the permeability values to FITC-dextran solution were significantly higher in ARPE-19 cell monolayer cultured in CM2 respect to control cells grown in CM1 (Fig. 3B, $^{***}p < 0.001$ vs CM1). The treatment with PACAP or NAP significantly reduced permeability values as compared to CM2 group (Fig. 3B, $^{###}p < 0.001$ vs CM2), on contrary, co-treatment with PACAP+PACAP6–38 or PACAP+U73122 reported permeability values to levels of CM2 group (Fig. 3B, $^{$$$}p < 0.001$ vs CM2 +PACAP). These results suggested that PACAP prevented oBRB damage by binding to PAC1R, which in turn could induces ADNP expression by activating PKC signaling pathway.

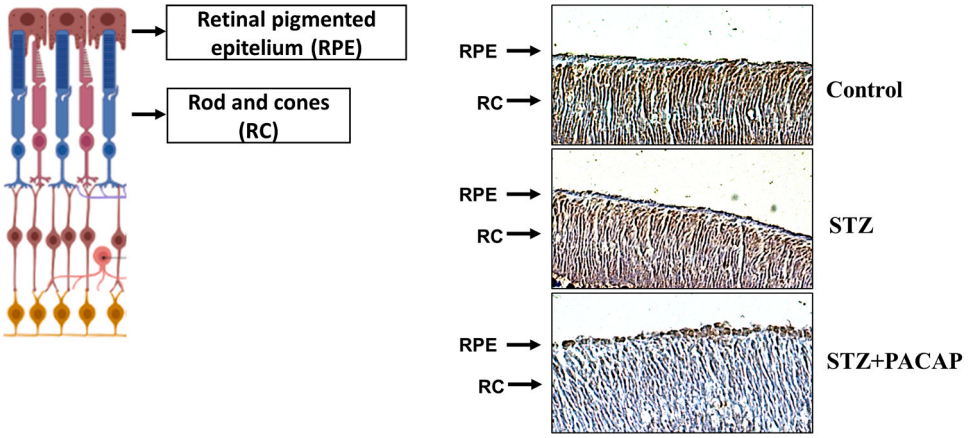

Fig. 1. Expression of ADNP in retinal pigment epithelium layer of diabetic rat retinas after intravitreal administration of PACAP. Representative retinal immunoreactivity of ADNP in control and diabetic rats (STZ) and after PACAP intravitreally administration (STZ+PACAP). In the left of the panel, we have indicated the retinal pigment epithelium (RPE). Magnification 40X, Scale bar 100 μm.

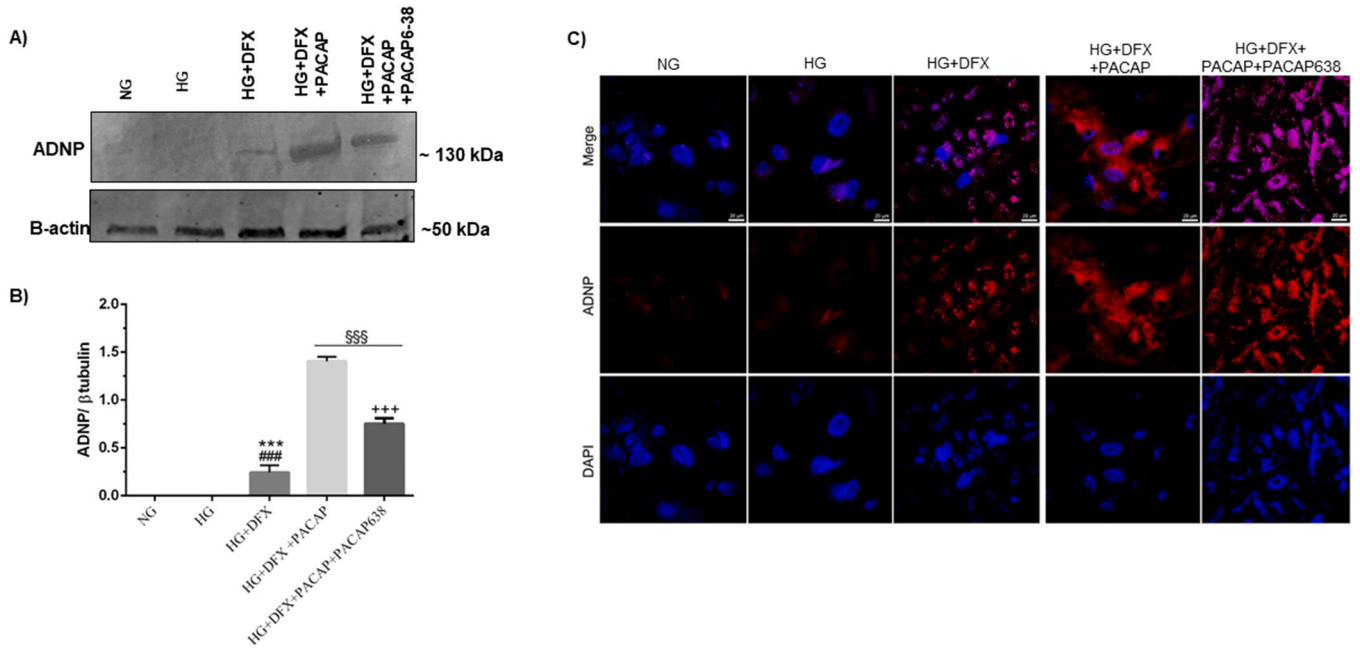

Fig. 2. Effect of PACAP on ADNP expression and distribution in ARPE-19 exposed to hyperglycemic/ hypoxic insult (A) Representative Immunoblot of signals detected by ADNP antibody obtained using 32 μg of cell homogenate from ARPE-19 cells cultured in 5.5 mM D-glucose + 19.5 mM mannitol (NG) or 25 mM D-glucose (HG) or HG plus desferrioxamine mesylate salt (HG+DFX) alone or in combination with PACAP (100 nM) or PACAP (100 nM) + PACAP6–38 (10 μM). (B) The bar graphs show the results of three independent experiments. The ImageJ software was used to quantify the relative band density obtained by normalize the protein levels to β-actin, which was used as loading control. Data are expressed as Mean ± SEM ($^{***}p < 0.001$ vs NG group, $^{###}p < 0.001$ vs HG, $^{§§§}p < 0.001$ vs HG+DFX, $^{+++}p < 0.001$ vs HG+DFX+PACAP as determined by One-Way ANOVA followed by Tukey post-hoc test). (C) Photomicrographs demonstrate the immunoreactivity of ADNP (red fluorescence) in ARPE-19 cells exposed to HG+DFX) alone or in combination with PACAP (100 nM) or PACAP6–38 (10 μM). Nuclei were stained with DAPI. Scale bar, 20 μm.

Table I
VEGF concentration in the extracellular compartment of ARPE-19 cells exposed to hyperglycemic/ hypoxic insult.

	NG (CM1)	HG+DFX (CM2)	HG+DFX +PACAP	HG+DFX +PACAP +PACAP6-38	HG+DFX +PACAP+ U73122	HG+DFX +NAP
VEGF (pg/ml)	341.13 ± 1.76	536.95 ± 2.00***	331.00 ± 2.07$^{###}$	498.07 ± 3.76$^{$$$}$	484.33 ± 4.68$^{$$$}$	334.52 ± 4.36$^{###}$

The VEGF levels were detected in supernatants and expressed in pg/ml. Data resulting from three independent experiments are represented as the mean ± SEM. ($^{***}p < 0.001$ vs NG, $^{###}p < 0.001$ vs HG+DFX, $$$p < 0.001$ vs HG+DFX+PACAP, as determined by One-Way ANOVA followed by Tukey's post-hoc test).

3.2. PACAP-ADNP axis prevented VEGF-induce damage on oBRB and neovascularization

As previously highlighted in the introduction, VEGF secreted from ARPE cells exposed to hyperglycemic/hypoxic insult damages oBRB by inducing intracellular internalization of tight junctions between ARPE cells.

To confirm this evidence, we analyzed the expression of two tight junction forming proteins, ZO-1 and occludin by western blot analysis. As represented in Fig. 4, the expression of these proteins was significantly decreased in ARPE-19 cells cultured in CM2 as compared to control (CM1) (Fig. 4A and B, $^{***}p < 0.001$ vs CM1). The exogenous administration of both PACAP and NAP induced upregulation of their expression respect to CM2-cultured cells (Fig. 4A and B, $^{###}p < 0.001$ vs CM2).

VEGF secreted from basolateral region of ARPE-19 cells exposed to hyperglycemic/hypoxic insult, by crossing damaged oBRB or spreading from basal membrane, reaches the choroid where it induces aberrant neovascularization [13,24,72]. Based on this evidence, in the present study we have also investigated whether the PACAP-ADNP axis exerts a modulatory role on choriocapillaris neovascularization process VEGF-mediated. By culturing H5V endothelial cells in CM2, we have demonstrated, for the first time, that VEGF secreted in the extracellular compartment of ARPE-19 cells exposed to HG+DFX insult increased number of vessels-like structures, representing a model in vitro of neoangiogenesis (Fig. 5A and B, $^{***}p < 0.001$ vs CM1). Their number was significantly reduced after PACAP or NAP exogenous administration as compared to CM2 group (Fig. 5A and B, $^{###}p < 0.001$ vs CM2). These results suggested that PACAP through ADNP activation could interfere with angiogenic process of choroidal capillaries VEGF-mediated.

4. Discussion

In the present investigation we have proved the evidences that PACAP-ADNP axis counteracts oBRB impairment induced by hyperglycemic/hypoxic condition.

Long lasting hyperglycemia induces damage to the retinal microcirculation through the activation of various pathogenetic mechanisms. In previous papers, we have demonstrated that PACAP and ADNP modulate hypoxic-angiogenic pathway activated by hyperglycemia responsible of retinal disfunction. In particular, the intraocular injection of both PACAP or NAP, a small peptide derived from ADNP, in diabetic animals modulated hypoxia-signaling by reducing expression of VEGF in the retina [17,20].

In the present paper, we investigated the role of these peptides on RPE consisting in a cellular monolayer located between the photoreceptors layer of the retina and the choroid [41]. Firstly, we demonstrated that intravitreal injection of PACAP induced ADNP expression in RPE of hyperglycemic animals (Fig. 1), corroborating previously published data that the latter is an intracellular mediator of some PACAP

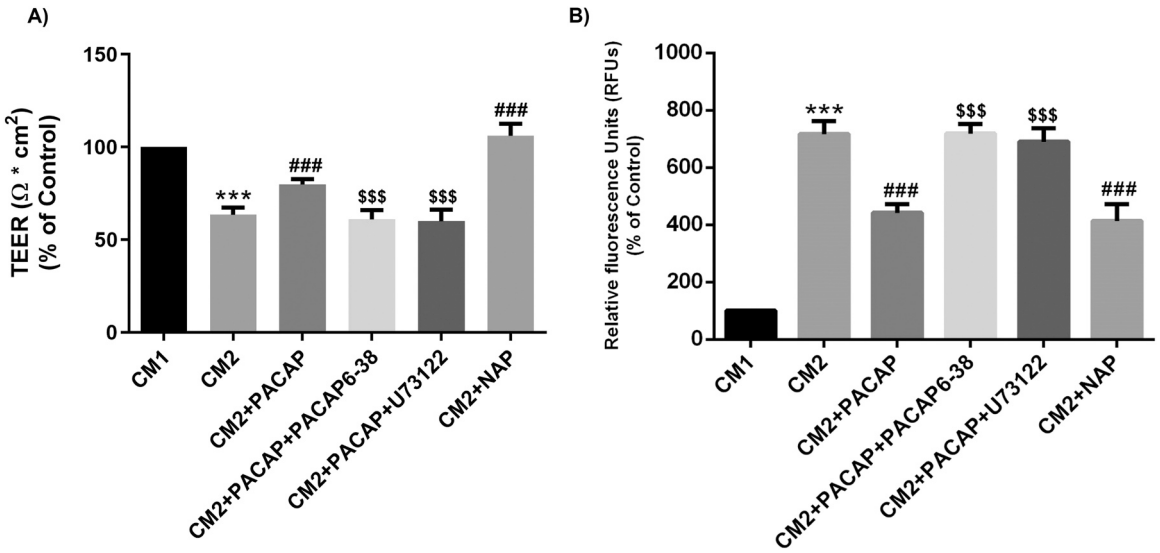

Fig. 3. Effect of PACAP and NAP on TEER and permeability of ARPE-19 cell monolayer cultured in conditioned medium (CM) deriving from cells exposed to hyperglycemic/ hypoxic insult (A) TERR values were measured in ARPE-19 cell monolayer cultured on transwell-clear semipermeable supports with medium containing 1% FBS, representing CM1 (control) or with CM2 (HG+DFX) alone or in combination with PACAP, PACAP+PACAP6–38 or PACAP+U73122, or with NAP. (B) Permeability values were determined by measuring the apical to the basolateral movements of FITC-dextran solution of these cells. ($^{***}p < 0.001$ vs CM1, $^{###}p < 0.001$ vs CM2, $^{§§§}p < 0.001$ vs CM2 +PACAP, as determined by One-Way ANOVA followed by Tukey's post-hoc test).

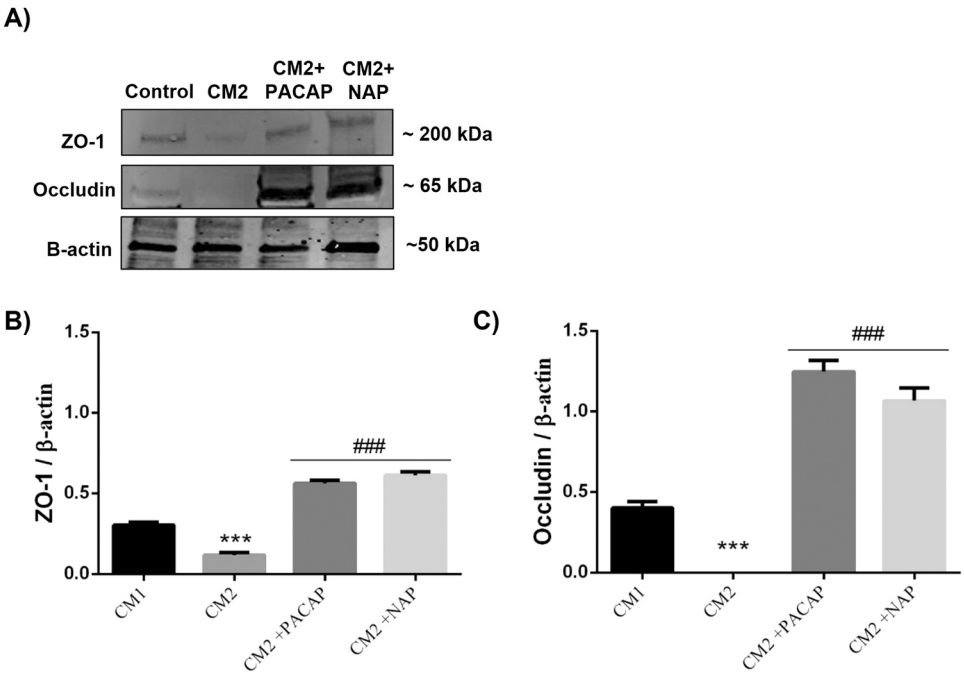

Fig. 4. ZO-1 and occludin expression in ARPE-19 cells cultured in conditioned medium (CM) deriving from cells exposed to hyperglycemic/ hypoxic insult (A) Representative Immunoblot of signals detected by ZO-1 and occludin antibodies obtained using 32 μg of cell homogenate from ARPE-19 cells cultured in CM1 (control) or with CM2 (HG+DFX) alone or in combination with PACAP or NAP. (B) The bar graphs show the results of three independent experiments. The ImageJ software was used to quantify the relative band density obtained by normalize the protein levels to β-actin, which was used as loading control. Data are expressed as Mean ± SEM ($^{***}p < 0.001$ vs CM1, $^{###}p < 0.01$ vs CM2, as determined by One-Way ANOVA followed by Tukey post-hoc test).

neuroprotective actions (Nakamachi et al., 2008). This result was also confirmed in an in vitro model of RPE, represented by ARPE-19 cells, exposed to hyperglycemic/hypoxic insult (HG/DFX) that is involved in retinal disfunction of DR. In particular, we have demonstrated that PACAP by binding to PAC1R stimulates ADNP expression in RPE cells exposed to the hyperglycemic/hypoxic insult.

Considering the direct link between the two peptides, we have then investigated the role of PACAP-ADNP axis on hyperglycemic/hypoxic cascade of diabetes in RPE cells. Previously, we have demonstrated that PACAP or NAP treatment protect cells by modulating the expression of the downstream key factor of this pathogenetic pathway, that is VEGF [17,20]. The identification of substances able to stimulate endogenous peptides leading to reduction of VEGF secretion could lead to advance in DR therapeutic treatment. To date, many molecules have been identified as potent modulators of vascular permeability as well as endothelial proliferation including Pegatinib, bevacizumab, ranibizumab, and aflibercept [75]. However, their use is limited by the side effects linked to their treatment, such as retinal detachment, hemorrhage, inflammation, and increased intraocular pressure [27,68].

In the present investigation, we have shown that PACAP and NAP reduced VEGF secretion in the culture medium of cells exposed to hyperglycemic/hypoxic condition. Because PACAP 6–38 addition reduced VEGF secretion, it could be hypothesized that PACAP effect is mediated by PAC1R. However, since PACAP 6–38 is not a selective PAC1R

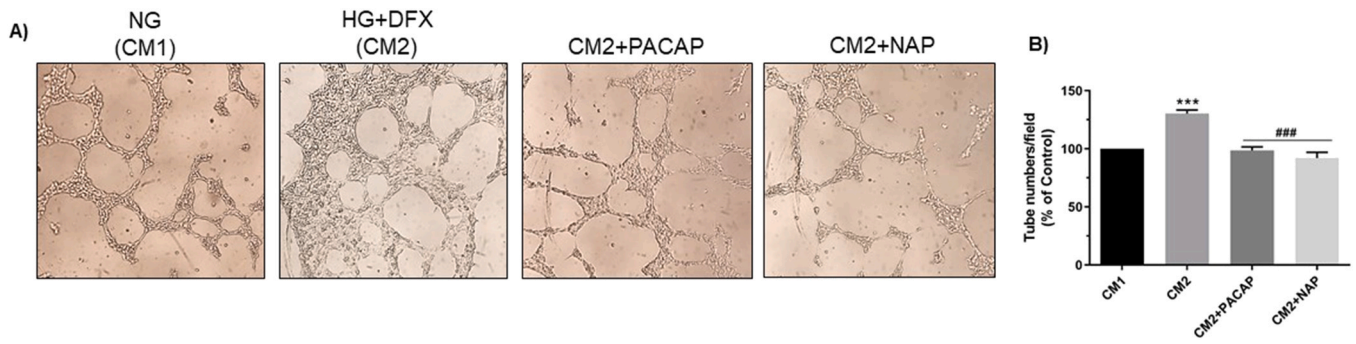

Fig. 5. Effect of PACAP and NAP on new vessels formation (A) Microphotographs show tubes formed by H5V cells cultured in CM1, CM2 alone or in combination with PACAP or NAP. (B) In the bar graph, the number of tubes/fields is expressed as the mean ± SEM. ($^{***}p < 0.001$ vs CM1, $^{###}p < 0.01$ vs CM2, as determined by One-Way ANOVA followed by Tukey post-hoc test).

antagonist, we cannot rule out this effect is also mediated trough the involvement of others VPAC receptors [42,66].

Under hyperglycemia/hypoxia insult of diabetes, RPE cells secrete VEGF from their basolateral region into extracellular compartment. Here, this growth factor acts directly on the junctional complexes between cells compromising oBRB integrity and functionality [58]. In the present work, we simulated in vitro this event by treating ARPE19 cells with the CM2, collected from cells exposed to hyperglycemic/hypoxic insult and containing therefore a known concentration of VEGF. Exposure to this angiogenic factor altered the integrity of the barrier formed by the ARPE cell monolayer, instead peptides treatment counteracted its harmful effect on barrier (Fig. 3). In particular, the protective effect of PACAP was mediated by binding to PAC1R as the addition of the antagonist PACAP 6–38 counteracted its effect on oBRB integrity.

Today, it has been identified various isoforms of PAC1R, each of them activating specific a signaling cascade [12,25,35,55]. In particular, it has been previously demonstrated that PACAP binding to specific PAC1-R variants, such as its short isoform, stimulates PKC pathway activation leading to ADNP expression in neurons (PMID: 17942168; PMID: 16564114). In this work, we demonstrated that the PAC1R isoforms activating this pathway mediated the protective effect of PACAP on barrier because the addition of the PKC inhibitor, U73122, abrogated its effect. To date, few studies have described the expression profile of PAC1R splice variants in the retina, and the presence of PAC1R-short and PAC1R-HOP isoform was detected in this tissue by Cavallaro et al., [11]. Moreover, it was shown that the treatment of retina with different concentrations of PACAP induced, in a dose-dependent (1–100 nM) manner, the stimulation of PKC and PKA signaling cascade (D'Agata et al., 1998). Recently, the distribution of PACAP and PAC1 receptor was recently demonstrated in the human eye. More specifically their immunoreactivity was detected in the corneal epithelium and endothelium, in the stroma and muscles of the iris and ciliary body. Remarkable, their immunosignals were detected in several layers of retina with very strong expression in the pigmented epithelial cells [54].

Although, we did not investigate the PAC1R splice variants expression pattern in ARPE19 cells, the present data suggested that the protective effect of PACAP on oBRB integrity is mediated through PAC1R activating PKC signaling pathway which in turn induced ADNP expression.

To confirm that the protective effect of PACAP/ADNP axis on oBRB integrity occurred by counteracting the harmful effect of VEGF, we analyzed the expression of TJ proteins, such as ZO1 and occludin in ARPE cells cultured in CM2. Under this experimental condition the TJ proteins were weakly expressed, whereas either PACAP or NAP exogenous addition significantly increased their levels, suggesting that VEGF secreted from RPE cells damaged oBRB by destroying the junctional structures between cells. These results let us to speculate that PACAP through the stimulation of its intracellular mediator, ADNP, maintained barrier integrity (Sragovich et al., 2019). In accord to this hypothesis, it has been largely demonstrated that NAP, the octapeptide sequence of ADNP, provides neuroprotection by inducing microtubules (MTs) stabilization. The latter is mediated through peptide interaction with end-binding protein (EB3) which facilitates EB3-tubulin binding in the microtubules as well as it induces Tau recruitment to microtubules (Gozes et al., 2019; Magen et al., 2013; Glotfelty et al., 2014). MTs contribute to formation and stabilization of TJs between cells. In particular, MTs form a track system along which MT-associated membrane vesicles transport TJ proteins to cell-cell contact sites by ensuring the barrier formation. In accord to this hypothesis, it has been demonstrated that occludin moves along MT tracks [69].

VEGF secreted from basolateral compartment of ARPE cells exposed to hyperglycemic/hypoxic insult reaches the choroid~~s~~ layer either by crossing the damaged oBRB as well as directly spreading from the basal region of these cells. Here, it induces neovascularization. This evidence was here confirmed by treating endothelial cells with CM2. The angiogenic efficacy of VEGF, collected by ARPE cells exposed to hyperglycemic/hypoxic insult, was demonstrated by increased number of tubes-like structure mimicking the neoangiogenic event accomplishing in the choroid. Both PACAP and NAP treatment counteracted angiogenesis VEGF driven by significantly reducing microvessels formation.

The results reported so far indicate that PACAP or NAP interfere on VEGF-induced neoangiogenesis by reducing its secretion from ARPE cells exposed to hyperglycemic/hypoxic insult as well as by directly interfering with the effect of this angiogenic factor on endothelial cells. In accord to this evidence, we have previously demonstrated that both PACAP and NAP opposed to tumor malignancy as well as DR development by counteracting the angiogenic pathway stimulated by hypoxia ([17]; Maugeri et al., 2018). In particular, the effects of these peptides focused on modulation of HIFs and VEGF expression triggered in RPE by the hypoxic insult [21,50]. The effect of these two peptides is also relevant on endothelial cells where they slow down their proliferation stimulated by hyperglycemia (Castorina et al., 2010; [23]) and protected them from the damage induced by cytokine TNF-α (Bian et al., 2017) or oxidative stress (Rácz et al., 2007). In particular, results described here for the first time demonstrated that both peptides reduced abnormal angiogenesis promoted by HIF-VEGF axis on endothelial cells. Although further studies should be performed to determine the specific contribution of PACAP-ADNP axis on maintaining the physiological function of the retina, overall, our data suggests that PACAP by inducing endogenous peptide ADNP modulates VEGF secretion. This growth factor induces retinal damage by destroying oBRB and by inducing choroidal neovascularization. The suggested molecular mechanism is detailed in Fig. 6.

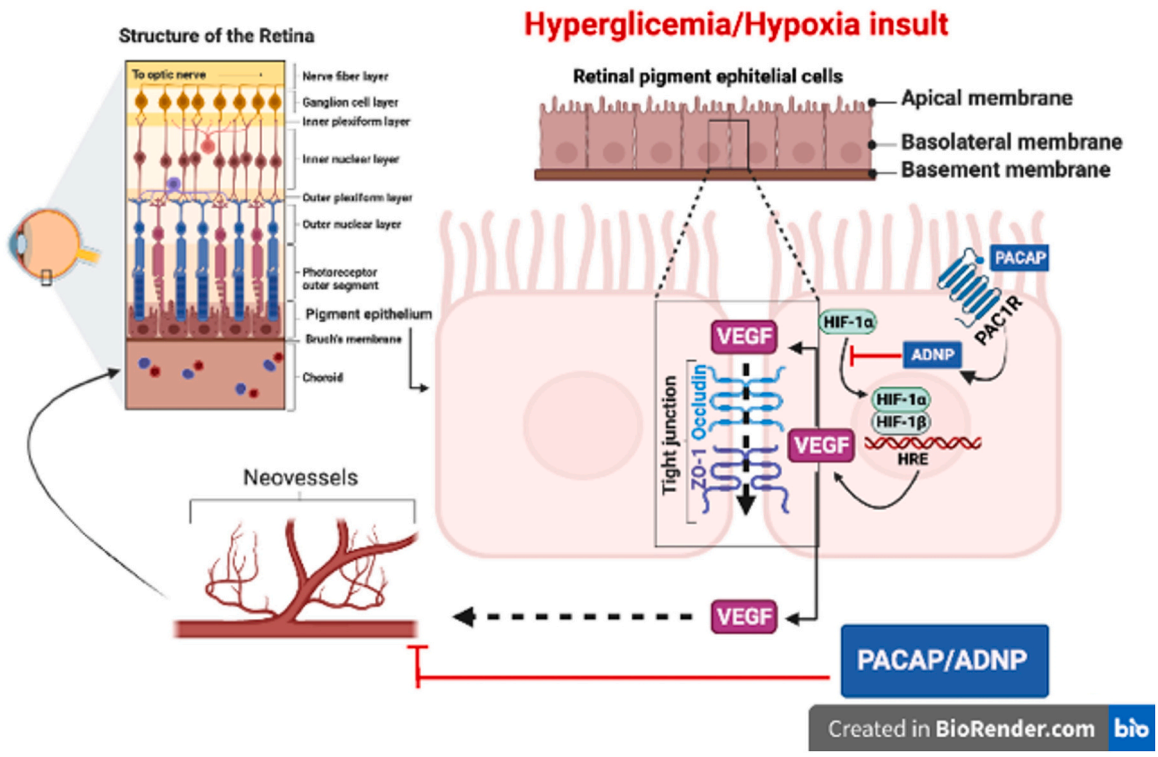

Fig. 6. Graphical representations showing the effect of PACAP-ADNP axis on VEGF secreted from RPE exposed to hyperglycemic/hypoxia insult of DR. Hyperglycemia induces hypoxia in RPE cells. This event triggers the HIF/VEGF signaling cascade. VEGF secreted in the extracellular compartment is responsible of oBRB impairment since it damages TJ by inducing their intracellular internalization. PACAP prevents the damage to oBRB by binding to PAC1R and activating ADNP expression. The latter stabilizes microtubules by preventing TJ proteins internalization. Furthermore, VEGF secreted from the basal compartment of RPE cells reaches choroidal layer where it induces neovascolarization. PACAP-ADNP axis acts on endothelial cells by counteracting formation of new vessels.

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Ethical approval

The protocol (#279) has been approved by Institution Animal Care and Use Committee of the Catania University.

Informed consent

Not Applicable.

CRediT authorship contribution statement

All authors reviewed the manuscript. Conceptualization, V.D. and A. G.D.; Funding acquisition, S.G., A.G.D., G.M. and C.B.; Investigation, A. G.D, S.S., C.F., G.M., B.M, P.C.; Resources, C.B.; Visualization, A.G.D.; Writing – original draft, V.D. and A.G.D.; Writing – review & editing, V. D., P.C. and C.L. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data Availability

No data was used for the research described in the article.

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