UNIVERSITY OF CATANIA

INTERNATIONAL PhD PROGRAM IN NEUROSCIENCE

XXXI Cycle

GRAZIA MAUGERI

THE ROLE OF VIP FAMILY MEMBERS IN DIABETIC RETINOPATHY

PhD Thesis

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December 2018

SUMMARY

ACKNOWLEDGEMENTS

First of all, I would like to thank my mentor, Professor Velia D'Agata, for her inestimable support and for introducing me in the extraordinary research world.

I wish to express my sincere gratitude to Professor Filippo Drago and Professor Salvatore Salomone who gave me the opportunity to attend this prestigious PhD program allowing me to discover the fascinating world of neuroscience.

I would like to thank Doctor Agata Grazia for her precious help and friendship.

I am also grateful to all friends and colleagues of the Section of Human Anatomy and Histology with whom I shared time, energies, smiles and efforts.

Last, but certainly not least, I wish to to thank my family, for its love and nonpareil support.

LIST OF ABBREVIATIONS

AC	Adenylate Cyclase
AGE	Advanced Glycation End Product
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
BRB	Blood-Retinal Barrier
CTAD	Carboxy-Terminal Transactivation Domain
DME	Diabetic Macular Edema
DR	Diabetic Retinopathy
ERK 1/2	Extracellular Signal-Regulated Kinase 1/2
GCL	Ganglion Cell Layer
GPCR	G-Protein Coupled Receptor
HIFs	Hypoxia-Inducible Factors
HREs	Hypoxia Responsive Elements
IL-1α	Interleukin -1α
IL-1β	Interleukin-1β
INL	Inner Nuclear Layer
IPL	Inner Plexiform Layer
MAPK	Mammalian Mitogen Activated Protein Kinase
NFL	Nerve Fiber Layer
NPDR	Non-Proliferative Diabetic Retinopathy
NTAD	Amino-Terminal Transactivation Domain
ODD	Oxygen Dependent Degradation Domain
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
PACAP	Pituitary Adenylate Cyclase-Activatingpeptide
PDR	Proliferative Diabetic Retinopathy
PI3K/Akt	Phosphoinositide 3 Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PR	Photoreceptor
RGC	Retinal Ganglion Cells
ROS	Reactive Oxygen Species
RPE	Retinal Pigmented Epithelium
STZ	Streptozotocin
TNF- α	Tumor Necrosis Factor-A
VEGF	Vascular Endothelial Growth Factor
VIP	Vasoactive Intestinal Polypeptide
WHO	World Health Organization

ABSTRACT

Diabetic retinopathy (DR) is a microvascular complication of diabetes leading to blindness. Hyperglycemic/hypoxic microenvironment concurs to mechanical breakdown of blood-retinal barrier (BRB) as well as the aberrant angiogenesis activating many downstream target genes including inflammatory cytokines and vasoactive peptides, such as interleukin-1β (IL-1β) and vascular endothelial growth factor (VEGF). To date, intravitreal injection of agents directed against VEGF represents the elective DR therapy. However, they counteract efficaciently ocular neovascularization only in 50% of treated patients, suggesting that other pathogenic events may play an important role in non-responders. Therefore, there is an increased need for new molecule able to counteract microenvironmental alterations of DR.

It has been largely demonstrated that pituitary adenylate cyclase-activating peptide (PACAP) and vasoactive intestinal peptide (VIP) exert a protective effect against retinal injuries, including DR. However, until now, no study has investigated their protective role against the harmful combined effect of both hyperglycemia and hypoxia.

The main objectives of this PhD thesis have been evaluate whether PACAP protects retinal tissue of diabetic rats through modulation of hypoxia and inflammation, both converging on angiogenic process. Moreover, we have investigated the protective effects of PACAP and VIP on BRB integrity whose breakdown due to hypoxic and inflammatory event, leads to diabetic macular edema (DME).

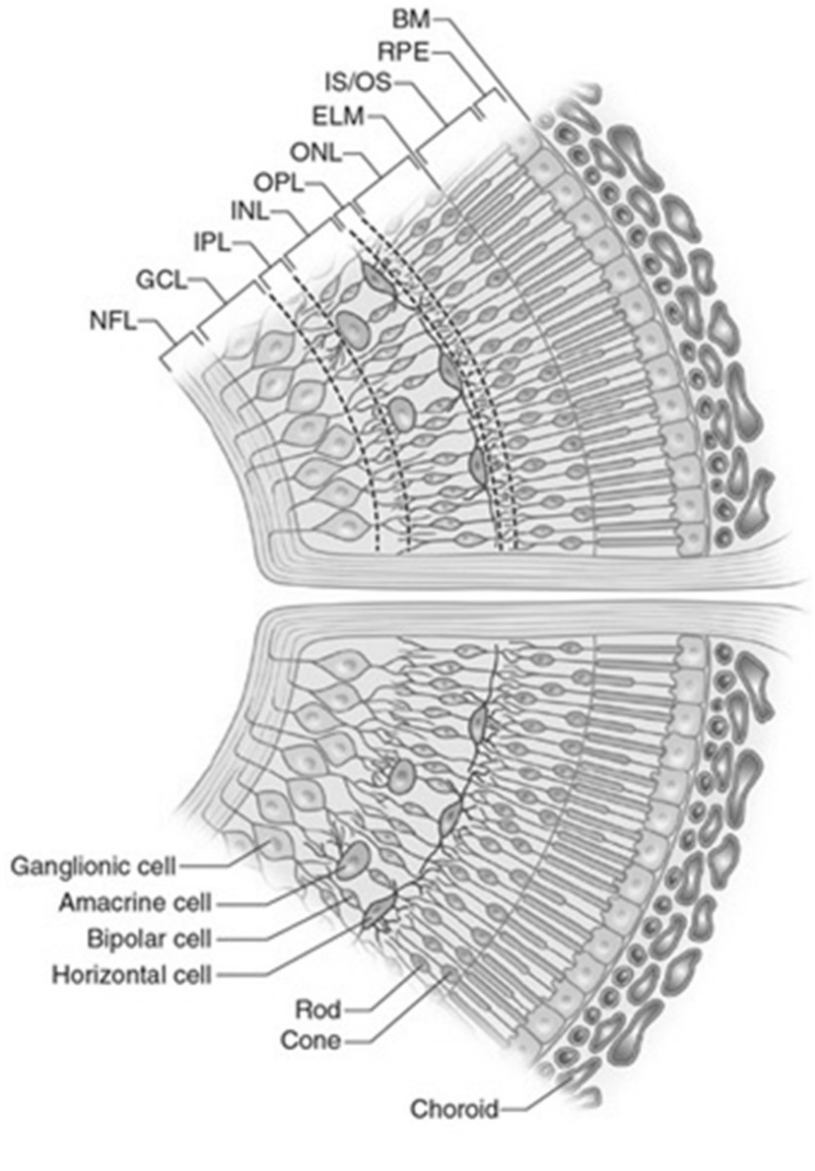
The results have demonstrated that a single intravitreal administration of PACAP in streptozotocin-injected rats modulated the expression of hypoxic, inflammatory and

angiogenic factors. Moreover, PACAP and VIP restored the integrity of BRB through the activation of phosphoinositide 3 kinase (PI3K)/Akt and mammalian mitogen activated protein kinase/Erk kinase (MAPK/ERK) survival signaling pathways.

Overall, these data indicate that these peptides, both *in vivo* and *in vitro*, perform a beneficial effect to attenuate some pathologic events occuring in DR.

CHAPTER 1

General Introduction & Aims



A FRAMEWORK FOR UNDERSTANDING DIABETIC RETINOPATHY

Diabetes is a progressive lifelong disease characterized by hyperglycemia occurring when the feedback loops between insulin action and insulin secretion do not function properly. It represents one of the principal challenges to current health care. Worldwide, there will be a progressive increase of diabetes from 382 million in 2013 to 592 million in 2035 (IDF diabetes, 2013).

Diabetes mellitus is classified into the following general categories: type 1 diabetes, which is caused by β-cell destruction, usually leading to absolute insulin deficiency; type 2 diabetes, characterized predominantly by insulin resistance with an inadequate compensatory increase in insulin secretion; gestational diabetes mellitus, defined as glucose intolerance with onset or first recognition during pregnancy; other specific types of diabetes caused by infections, drugs, endocrinopathies, pancreatic destruction, and genetic defects (Diabetes Care, 2018).

Complications of diabetes are divided into macrovascular and microvascular. The first include stroke, coronary and peripheral arterial disease. Instead the microvascular complications comprise diabetic nephropathy, neuropathy and retinopathy (Fowler, 2008).

Diabetic retinopathy (DR) represents the major cause of vision loss affecting almost 100 million people worldwide (Leasher et al., 2016). The microvascular lesions represent the most important criteria to asses and rank the retina in DR. Nevertheless, diabetic milieu induces changes also in non-vascular cell types exerting a prominent role in DR development in unison with the vasculature.

DR can be generally divided into two clinical stages: non-proliferative stage (NPDR) and proliferative diabetic retinopathy (PDR).

NPDR is characterized by microaneurysms, intraretinal hemorrhages, alteration in the venous caliber, lipid exudates from the damaged vasculature, capillary nonperfusion accompanied by neuronal infarcts represented as cotton-wool spots, and retinal neovascularization. Moreover, the hallmarks of early NPDR comprise vascular basement membrane thickening, the loss of pericytes and endothelial cells death. All of these features have been fully identified histologically in postmortem human eyes and in some preclinical models (Ljubimov et al., 1996; Yanoff, 1969; Mizutani et al., 1996).

The progressive capillary nonperfusion due to ischemia regions and impaired oxygenation leads to PDR. This stage is driven by hypoxia and overexpression of proangiogenic growth factors triggering new blood vessels formation and relentless abnormal epiretinal fibrovascular proliferation with tractional retinal detachment (Stitt et al., 2016).

At any time during the progression of DR, patients can also develop diabetic macular edema (DME), representing the major cause of visual impairment in diabetic patients. DME appears as a consequence of blood-retinal barrier (BRB) breakdown (Bhagat et al., 2009).

The BRB is constituted by an inner and an outer part. The inner part is formed by tight junctions between retinal vascular endothelial and retinal glial cells realizing a barrier impermeable to proteins (Cunha-Vaz and Travassos, 1984).

The outer BRB is formed by tight junctions between the retinal pigment epithelial (RPE) cells, separating the neural retina from a network of choriocapillaris supplying nutrients, water, ions, and promoting the elimination of metabolic wastes through blood flow. Furthermore, outer BRB regulates the recycle of fatty acids that represent the major components of photoreceptors (Campbell and Humphries, 2012; Gordon et al., 1992).

During BRB breakdown, proteins and other solutes move from capillaries into the extracellular space, causing an imbalance between hydrostatic and oncotic pressure. This event causes DME when fluid and protein deposits accumulate in the macular region, causing a thickening and swelling of the macula that can be either focal or diffuse (Cunha-Vaz and Travassos, 1984).

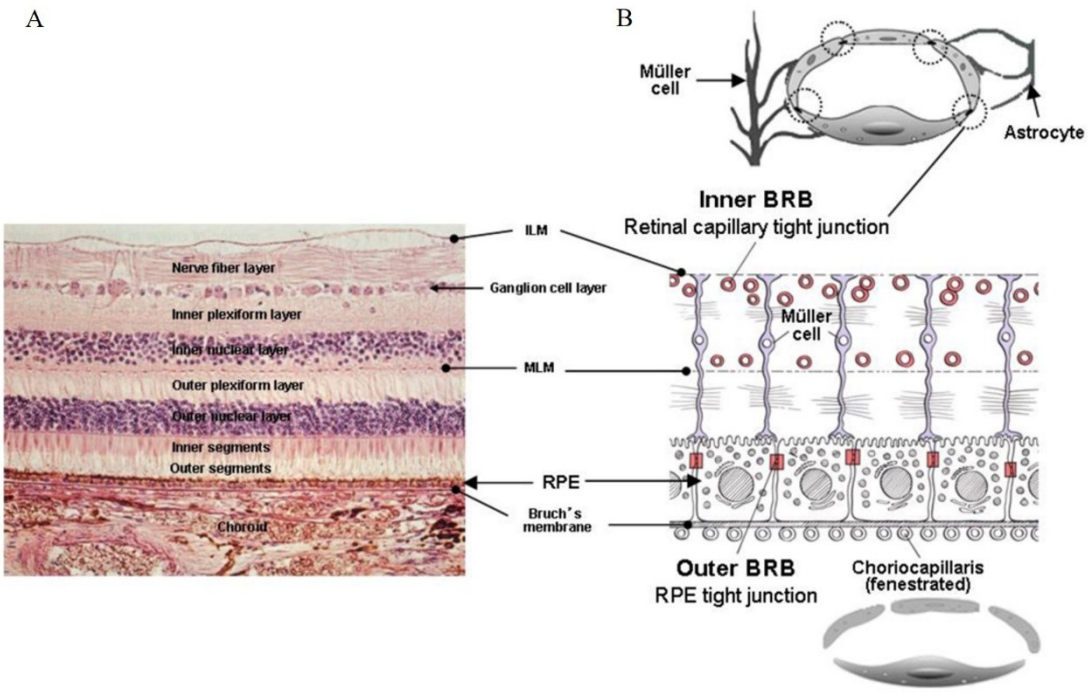


Figure 1. A) Cross-section of the human retina constituted by: ganglion cell layer, whose axons compose the optic nerve; the inner nuclear layer, formed by second-order neurons, such as bipolar, amacrine and horizontal cells; the photoreceptor (PR) layer, characterized by the cell bodies and nuclei of the rod and cone; the PR outer segments and the retinal pigment epithelium (RPE), a monolayer of cells containing tight junctions that separates the neural retina from the choroid. B) Schematic representation of the blood-retinal-barrier. The function of inner BRB is supported by Müller cells and astrocytes. Figure modified from Kuno and Fujii (2011).

FOCUS ON THE MAIN ACTORS INVOLVED IN DR DEVELOPMENT

In order to develop innovative strategies against DR and subsequent vision loss, most of the studies have focused on the clinically well-recognized features of microangiopathy and its underlying mechanisms. However, this condition appears only after an extended exposure to high glucose concentrations. Instead, the neural retina alterations may appear in early phase of diabetes since hyperglycemia increases metabolic activity of the neural retina by making it more susceptible to oxidative stress and other metabolic alterations. Moreover, high glucose levels induce tissue microenvironment alterations leading to retinal hypoxia and inflammation.

The reactive oxygen species (ROS) elicit the activation of several metabolic pathways involved in DR progression including: the polyol pathway, the advanced glycation end product (AGE) pathway, protein kinase C (PKC) pathway, the hexosamine biosynthesis pathway and mitochondrial dysfunctions (Giacco and Brownlee, 2010). The increased oxidative stress induces apoptotic cell death.

All neuronal cell types in the retina are susceptible to hyperglycaemia-induced apoptosis. The retinal neurons miss their capacity to adapt to cellular stress, as demonstrated by reduced axonal and dendritic branching, cell loss and consequently retinal layer thinning. Moreover, the early stage of DR is characterized by an imbalance between pro-apoptotic and survival signaling pathways in the neuroretina of diabetic patients.

Inflammation is a non-specific response to injury that triggers the recruitment and activation of different mediators. The role of inflammation in DR development has been discovered since diabetic patients, treated with ant-inflammatory drugs, showed a lower incidence of this pathology (Joussen et al., 2002). Moreover, several works have indicated an increased expression levels of pro-inflammatory cytokines,

chemokines and adhesion molecules in vitreous, serum and retina during diabetes. In particular, high levels of interleukin-1β (IL-1β), IL-6, and IL-8 have been found in the vitreous fluid of the diabetic patients and in the retina of rodents (Boss et al., 2017). Among these, IL-1β induced apoptotic cell death of retinal capillary cells via activation of NF-κB, which in turn strengthened the effect of hyperglycaemia by promoting retinal pericytes loss.

Hypoxia represents another key player acting in combination with hyperglycaemia to accelerate the onset and progression of DR. When capillary closure and nonperfusion become clinically apparent, the inner retinal tissue is hypoxic as shown in cats, primates or humans (Linsenmeier et al., 1998).

Hypoxic effects are mediated by hypoxia-inducible factors (HIFs), which are heterodimeric compounds consisting in α oxygen-dependent subunits (HIF-1α, HIF-2α and HIF-3α) and a constitutively expressed β subunit (Jiang et al., 1996; Makino et al., 2002; Maynard et al., 2007). Among these, HIF-1α is the key modulator of cellular response to low oxygen tension. On contrary, HIF-3α subunit acts as a negative regulator of the hypoxic response by reducing, in opposition, HIF-1α/2α overexpression (Maynard et al., 2007). These factors induce the activation of hypoxia responsive elements (HREs), such as many cytokines and growth factors including the vascular endothelial growth factor (VEGF) (Figure 2). This latter is the major angiogenic factor, playing a crucial role in normal and pathological angiogenesis. VEGF, synthesized by numerous retinal cells including RPE cells, pericytes, endothelial cells, glial cells, Müller cells, and ganglion cells, explicates its functions through the activation of VEGF 1 (VEGFR-1 or Flt-1) or 2 (VEGFR-2 or KDR/Flk-1) receptors. VEGF exerts a prominent role in the development of proliferative DR as well as in DME. Indeed, VEGF-induced breakdown of the BRB

is due to occludin phosphorylation and ubiquitination that contributes to TJ trafficking and subsequent vascular permeability (Saishin et al., 2003).

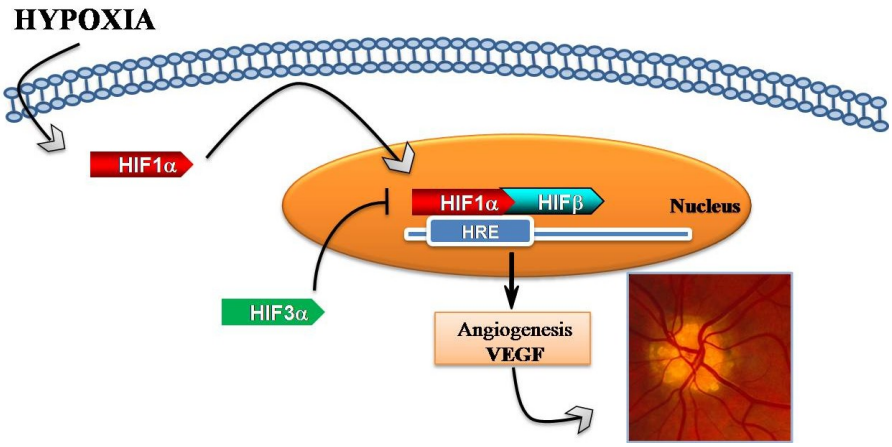


Figure 2. Schematic representation of hypoxia-signaling cascade involved in DR progression. During low oxygen tension levels, HIF-1α translocates to the nucleus, dimerizes with HIF-1β and through hypoxia-response element (HRE) it induces VEGF transcription, stimulating neovascularization.

PACAP AND VIP IN DIABETIC RETINOPATHY

Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are two structurally related neuropeptides that mediate different biological processes in the peripheral and central nervous system, acting either as neurotransmitters or neuromodulators.

PACAP has two isoforms, PACAP27 and PACAP38, containing 27 and 38 amino acids, respectively. PACAP and VIP perform their role through activation of three different G-protein coupled receptors known as PAC1, VPAC1 and VPAC2. Among these, PAC1 binds PACAP with high affinity, whereas VPAC1 or VPAC2 receptors recognize PACAP and VIP with similar affinities (Arimura and Shioda, 1995). PAC1 stimulation is associated to different signal transduction pathways, resulting among other in the activation of protein kinase A (PKA) or phospholipase C (PLC). VPAC1 and VPAC2 receptors are mainly coupled to the G-protein Gs and stimulate cellular adenylyl cyclase activity (Laburthe et al., 2002).

Different studies have described the presence of PACAP, VIP and their receptors in the retina. In particular, immunohistochemical studies have shown that PACAP is present in the nerve fiber layer (NFL), the ganglion cell layer (GCL), and the inner plexiform layer (IPL). In particular, PACAP and VIP immunoreactivity has been found to be positive in neuronal cell bodies of amacrine and horizontal cells localized in the inner nuclear layer (INL). Instead, no PACAP immunopositivity has been ever established in rods and cones (photoreceptors) of the outer nuclear layer (ONL) or in neighboring retinal pigmented epithelium it has not been found in photoreceptors in the outer nuclear layer (ONL) or RPE (D'Agata and Cavallaro 1998; Seki et al. 2000).

PAC1, VPAC1 and VPAC2 receptors have been detected in the developing mammalian retina, including the retinal progenitor cells (Njaine et al. 2010). A strong expression of PAC1 receptor has been shown in NFL, INL, GCL and also in photoreceptors layer (Seki et al. 1997, 2000b). In particular, PAC1 receptor has been described in the cell bodies and processes of ganglion, amacrine cells and Muller glial cells (Seki et al. 1997).

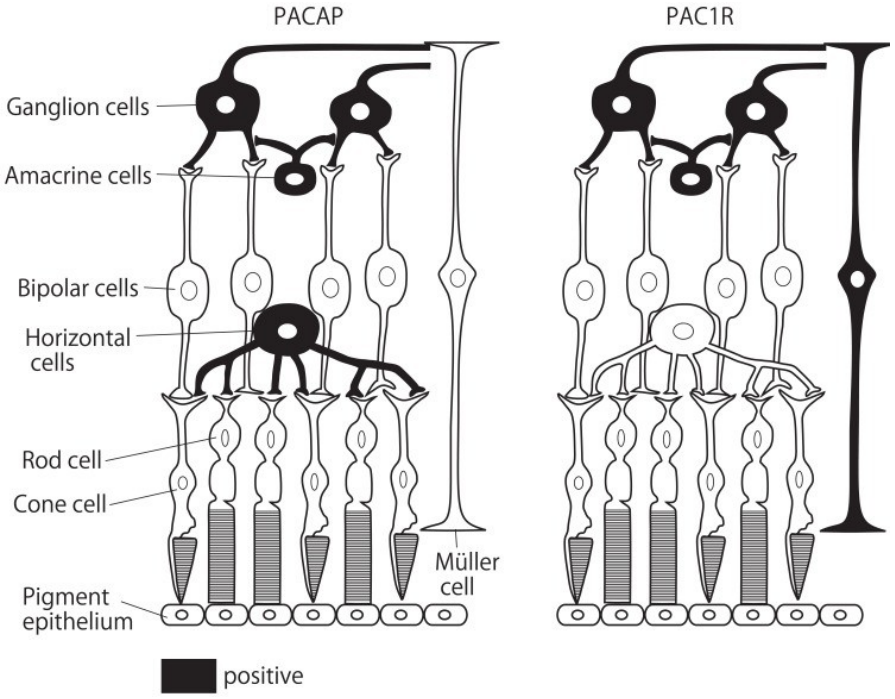


Figure 3. Schematic representation of PACAP and PAC1 receptor distributions in the rodent retina. Black color indicates PACAP or PAC1R-expressing cells. Figure from Nakamachi et al., (2012).

It is widely demonstrated that PACAP and VIP exert neuroprotective effects in different retinal injuries both *in vitro* and *in vivo* models.

Indeed, PACAP has shown to protects the retina against glutamate and kainate toxicity, and optic nerve transection (Racz et al., 2006 a and b). Moreover, PACAP has shown to preserve human retinal pigment epithelial cells against oxidative stress (Mester et al., 2011).

Recently, our research group has demonstrated an overexpression of PACAP, VIP, VPAC type receptors, but not of PAC1 receptor in diabetic rat retina after 1 week STZ injection. Instead, their levels were downregulated after 3 weeks of diabetes (Table 1). This result has suggested the involvement of these peptides in early response to hyperglycaemic insult (Giunta et al., 2012).

Table 1. Analysis of PACAP/VIP peptides and related receptors mRNA levels in rat retinas after 1 and 3 weeks streptozotocin treatment. Table from Giunta et al., (2012).

Gene	After 1 week		After 3 weeks	
	Nondiabetic Fold change ± S.E.M.	Diabetic Fold change ± S.E.M.	Nondiabetic Fold change ± S.E.M.	Diabetic Fold change ± S.E.M.
PACAP	1.06 ± 0.25	4.24 ± 0.33***	1.00 ± 0.05	0.76 ± 0.02**
VIP	1.01 ± 0.09	5.71 ± 0.24***	1.00 ± 0.06	0.74 ± 0.01**
PAC1	1.03 ± 0.18	0.6 ± 0.03	1.00 ± 0.02	0.45 ± 0.03***
VPAC1	1.03 ± 0.17	2.83 ± 0.28**	1.02 ± 0.03	0.79 ± 0.03**
VPAC2	1.02 ± 0.14	3.55 ± 0.43**	1.00 ± 0.03	0.76 ± 0.03**

In the same study, it has also been showed that PACAP intravitreal treatment downregulated the expression of proapoptotic genes indicating a key role of this peptide to counteract apoptotic process.

In accord, several studies have shown that PACAP administration markedly mitigated diabetic retinal injury by increasing the levels of the antiapoptotic p-Akt, pERK1, p-ERK2, PKC, and Bcl-2 signalling, while downregulating the expression of the proapoptotic p-p38MAPK pathway (Somogyvári-Vigh et al., 2004; Seaborn et al., 2011; Szabadfi et al., 2014).

Moreover, Atlasz et al., (2010) have demonstrated that PACAP prevented the dopaminergic amacrine cells degeneration induced in 1-month diabetes. In accord, PACAP and VIP treatment counteracted the early signs in a rat model of streptozotocin-induced DR including the decrease in the number of ganglion cells and the upregulation of GFAP as a sign of Müller glial cell activation (Szabadfi et al. 2012).

AIMS OF THE PhD WORK

The long-term purpose of the present PhD thesis is to provide a detailed framework on the protective role of PACAP and VIP to counteract the harmful effect of hyperglycemia/hypoxia/inflammation in an *in vivo* and *in vitro* model of DR.

The main aims addressed in the papers published during the doctoral work and included in the present thesis comprise:

AIM I

To investigate whether the protective effect of PACAP in the early stages of hyperglycaemia is mediated through the modulation of hypoxic process.

AIM II

To establish, whether retinoprotective role of PACAP is also linked to modulation of inflammatory process in DR.

AIM III

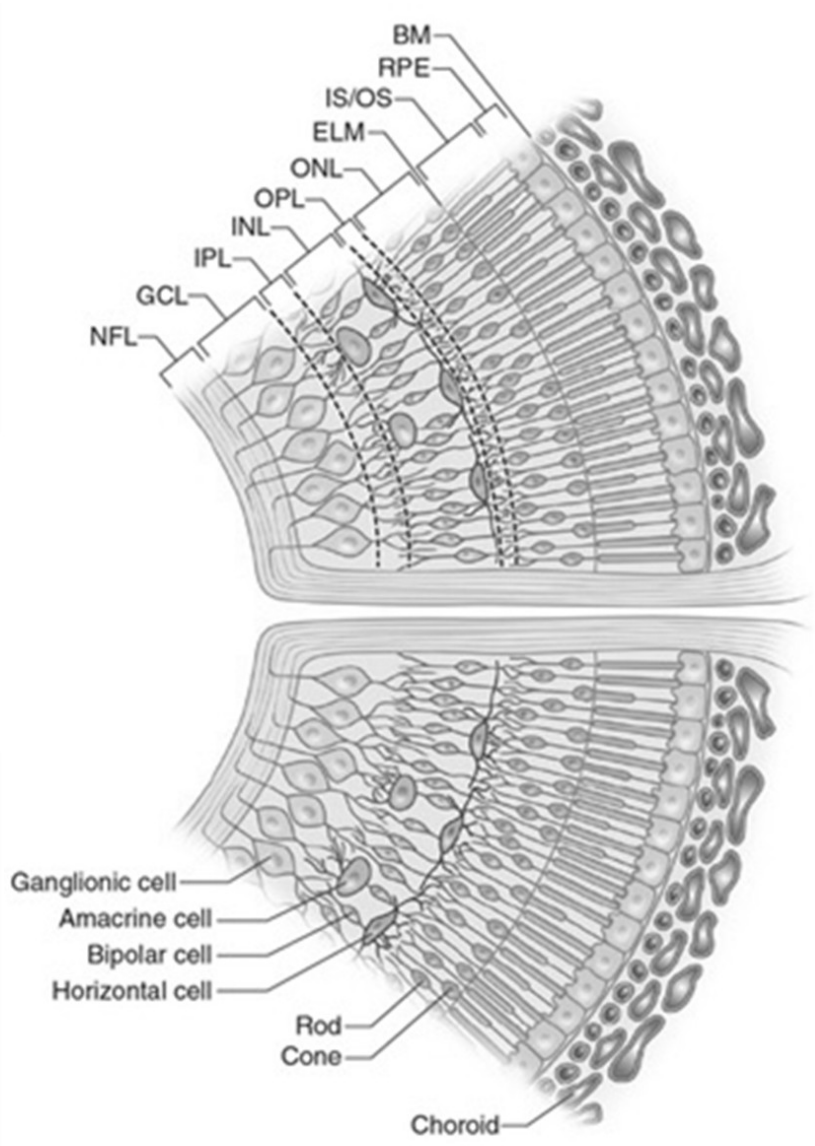
To assess the role of PACAP and VIP on permeability and inhibition of hyperglycemia/hypoxia-induced apoptosis of outer BRB.

AIM IV

To characterize the molecular mechanism played by PACAP and VIP against hyperglycemic/hypoxic insult, by analyzing their effect on HIFs, VEGF and activation of pro-apoptotic pathway p38 MAPK.

CHAPTER 2

PACAP Modulates Expression of Hypoxia-Inducible Factors in Streptozotocin Induced Diabetic Rat Retina



D'Amico et al., J Mol Neurosci (2015) doi: 10.1007/s12031-015-0621-7

PACAP MODULATES EXPRESSION OF HYPOXIA INDUCIBLE FACTORS IN STREPTOZOTOCIN-INDUCED DIABETIC RAT RETINA

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Abstract

Retinal hypoxia has been related to the pathogenesis of diabetic retinopathy. This event is mediated by the Hypoxia Inducible Factors (HIFs), including HIF-1α, HIF-2α and HIF-3α. Previously, we have demonstrated the protective role of pituitary adenylate cyclase-activating peptide (PACAP) in early phase of diabetic retinopathy. In the present work, we investigated whether PACAP effect in hyperglycemic retina is mediated through modulation of HIFs expression. Diabetes was induced with a single injection of streptozotocin (STZ) in rats. After one week, a group of diabetic animals was treated with a single intravitreal injection of 100 μM PACAP or saline

solution. Then, changes in HIFs expression levels were evaluated in the retina after 3 weeks of hyperglycemia. The expression of HIF-1α and HIF-2α was significantly ($p<0.001$ vs control) increased in diabetic rats as compared to controls. Instead, their expression levels were significantly ($p<0.001$ vs STZ) decreased after PACAP intraocular administration, as detected by western blot analysis. Conversely, the expression of HIF-3α was significantly ($p<0.001$ vs control) downregulated in retinas of STZ-injected rats and significantly ($p<0.001$ vs control) increased after PACAP treatment. These data were supported by the immunohistochemical analysis. HIFs were localized either in inner and outer retinal layers. Diabetes interferes with their distribution, which is changed following intravitreal injection of PACAP. The present results suggest that the protective effect of the peptide in diabetic retina might be also mediated through modulation of HIFs expression.

Keywords: Diabetic retinopathy; PACAP; Hypoxia Inducible Factors, STZ-injected rats.

Introduction

Diabetic retinopathy (DR) is a microvascular complication of diabetes leading to blindness (Saydah et al., 2004; Hoerger et al., 2008). Hypoxia is one of the major events involved in the progression of this pathology and hypoxia inducible factors (HIFs) are the principal regulators of hypoxic process (Wang et al., 1995; Semenza et al., 1999; Lin et al., 2011; Wright et al., 2011).

HIFs are heterodimeric transcription factors composed of α oxygen-related subunit (HIFα), including HIF-1α or HIF-2α, and an aryl hydrocarbon receptor nuclear translocator (ARNT) subunit, also known as HIF-1β, constitutively expressed independently to the presence of oxygen (Jiang et al., 1996; Maynard et al., 2004).

Indeed, HIFα subunits contain, besides two transactivation domains, the amino-terminal transactivation domain (NTAD) and the carboxy-terminal transactivation domain (CTAD), an oxygen dependent degradation domain (ODD) which makes these factors labile to the oxygen through ubiquitin proteasome pathway (Maynard et al., 2005; 2007). Under hypoxic condition, the HIFα subunits escape to the degradation by ubiquitin proteasome system, translocate into the nucleus and dimerize with β subunit. These complexes are able to bind hypoxia response elements (HREs) and induce the expression of many genes involved in different cellular mechanisms (Semenza et al., 1996; Kaelin et al., 2008; Elvidge et al., 2006; Scheurer et al., 2004). A third member of HIFα family, also known as HIF-3α, was identified by Gu et al., (1998). It has structural similarity with other HIF-α subunits but lacks of CTAD domain. It has been demonstrated that a human HIF-3α subunit is a negative modulator of both HIF-1α and HIF-2α expression, impairing their binding to HREs (Makino et al., 2002; Maynard et al., 2005; 2007). In particular, it has been showed that HIF-3α interacts with HIF-1α/ARNT complex leading to inhibition of its activity (Maynard et al., 2005). Furthermore, it competes with HIF-1α and HIF-2α for ARNT recruitment by suppressing their ability to modulate gene expression (Gu et al., 1998; Hara et al. 2001).

Many studies, both *in vitro* and *in vivo*, have demonstrated the involvement of such HIFs subunits in DR, such as HIF-1α and HIF-2α (Xiao et al., 2006; Ly et al., 2011; Li et al., 2012; Yan et al., 2012; Yan et al., 2014). In particular, some growth factors implicated in the pathogenesis of this disease are regulated by HIF-1α. Among these, the most studied is the vascular endothelial growth factor (VEGF), which is a regulator of intraocular vascularization and therefore it is involved in the proliferative retinopathy (Duh et al., 1999; Treins et al., 2005). However, to date anti-VEGF therapy has demonstrated its efficacy to counteract ocular

neovascularization only in 50% of treated patients, suggesting that other pathogenic factors may play an important role in non-responders (Lin et al., 2011; Rey et al., 2010; Brown et al., 2007). Therefore, the identification of other factors that regulate upstream HIF-1α expression could be more effective in preventing angiogenesis.

Considerable amounts of studies have focused on role of human retinal peptides in the pathophysiology of DR (D'Agata and Cavallaro, 1998; Giunta et al., 2012; Scuderi et al., 2013, Seki et al., 2008; Danyadi et al., 2014). Among these, pituitary adenylate cyclase-activating peptide (PACAP) has been identified as a possible candidate with therapeutic potential in treatment of this pathology (Gábriel, 2013). PACAP belongs to the vasoactive intestinal polypeptide (VIP)/secretin/glucagon peptide superfamily members. It plays different biological functions through interaction with two different receptor subtypes: PAC1 and VPAC, including VPAC1 and VPAC2 receptors (Arimura et al., 1995; Arimura, 1998; Zhou et al., 2002). It is widely demonstrated that PACAP exerts neuroprotective effect in different retinal injuries, including DR (Nakamachi et al., 2012; Szabadfi et al., 2012; Szabadfi et al., 2014). In particular, our previous study has demonstrated that expression of this peptide and its related receptors changes in rat retinas after 3 weeks of STZ injection, furthermore protective effect of PACAP38 intraocular administration has been also demonstrated at this time point (Giunta et al., 2012). On the other hand, many studies have described different biological changes during early phase of hyperglycemia. In particular, it was identified constriction of retinal arterioles and significant reduction in retinal blood flow after 3 weeks onset of diabetes (Lee et al., 2008). These alterations have been also associated to changes in HIFs expression (Wright et al., 2010; 2011). Therefore, in the present work, we investigated whether the retinoprotective effect of the PACAP is mediated through

the modulation of hypoxic process by analyzing the expression of HIFs after 3 weeks of hyperglycemia onset.

Materials and Methods

Animals

Male Sprague-Dawley rats, three months old, weighing approximately 200g each, obtained from Charles River (Calco, Italy) were used for the present study. All the animals were treated according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. 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 group sizes for all measurements were n = 6-9.

Induction of Diabetes

Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) as previously described (Bucolo et al., 2012). Briefly, after twelve hours of fasting, a group of animals received a single injection of STZ (60 mg/kg body weight) in 10mM sodium citrate buffer, pH 4.5 (1mL/kg dose/volume). Nondiabetic animals were fasted and received citrate buffer alone. Twenty-four hours post STZ injection blood glucose levels was measured and rats showing blood glucose levels greater than 250 mg/dL were considered as diabetic and selected for the study. We monitored throughout the study the diabetic state by evaluating glycemia daily using a blood glucose meter (Accu–CheckActive; Roche Diagnostic, Milan, Italy) and by the weight loss. All experiments were performed three weeks after the induction of diabetes. For subsequent experiments, rats were killed with a lethal intraperitoneal (i.p.) dose of sodium pentobarbital. Retinas were

immediately removed and homogenized in ice-cold buffer for Western blot analysis and others were fixed in 4% paraformaldehyde for immunofluorescence analysis.

2.3 Intravitreal administration of PACAP38

A single intraocular injection of 100 μM PACAP38 (Sigma-Aldrich) dissolved in PBS solution (final volume = 4 μL) was administered 1 week after intraperitoneal injection of STZ or sodium citrate buffer under general anesthesia with diethylether and topical anesthesia with a drop of 2% lidocaine applied to the eyes. PACAP38 was injected into the vitreous space of one eye chosen at random. An equal volume of vehicle (PBS) was injected in the other eye as control. Retinas were dissected 2 weeks after the intraocular injection.

Western Blot Analysis

Western blot analysis was performed to determine the expression levels of HIFs. Analysis was performed as previously described by D'Amico et al. (2014). 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) using a Teflon-glass homogenizer and then sonicated twice for 20 sec using an ultrasonic probe, followed by centrifugation at 10.000 g for 10 min at 4 °C. Protein concentrations were determined by the Quant-iT Protein Assay Kit (Invitrogen). Sample proteins (20 μg) were diluted in 2X Laemmli buffer (Invitrogen, Carlsbad, CA, USA), heated at 70°C for 10 min and then 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: mouse anti-HIF-1α (NB 100-105, 1:500), mouse anti-Arnt1 (A-3) (sc-17811, Santa Cruz Biotechnology; 1:200), rabbit anti-HIF-2α (NB 100-122, 1:500),

rabbit anti-HIF-3α (HIF3-H170, 1:200), rabbit anti-β-tubulin (sc-9104, 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 Odissey Infrared Imaging System (Odyssey). Densitometric analyses of signals 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-tubulin, which was used as loading control. No signal was detected when the primary antibody was omitted (data not shown).

Immunolocalization

Eyes were enucleated and fixed overnight with 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.6). Analyses was performed on whole retina, as previously described by Scuderi et al., (2015). Before immunofluorescence staining, paraffin-embedded retinas sections (6 μm) mounted on glass slides, were dewaxed in xylene and rehydrated through graded alcohols. Sections were put into Rodent Decloaker 1X retrieval solution (RD913, BIO-CARE MEDICAL) in a slide container and then incubated with the anti-HIF-1α, HIF-2α and HIF-3α primary antibodies. To establish signal specificity, in a preliminary experiment, each antibody was pre-incubated with an excess of antigen (blocking peptide). No signal was revealed in sections incubated with neutralized antibody. This confirmed that signal detected by each antibody was not an artifact (data not shown). Tissue sections were then incubated with Alexa fluor 488 goat anti-rabbit and Alexa fluor 594 goat anti-mouse secondary antibodies, respectively for 1h at room temperature and shielded from light. DAPI (diamidino-2-phenylindole) was used to stain nuclei (#940110 Vector Laboratories).

Images were taken with a confocal laser scanning microscope (CLSM) (Zeiss LSM700 as previously described (D'Amico et al., 2013). To compare similar regions of different groups, we analyzed, in each section, area close to the central retina (i.e. fovea centralis). Experiments were repeated at least three times to confirm results.

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 $p \leq 0.05$.

Results

The effect of PACAP on HIFs expression in diabetic retina.

The effect of PACAP on HIFs expression in hyperglycemic retina has been assessed by using western blot analysis. As shown in Figure 1 (A and B) hyperglycemia increases HIF-1α expression as compared to control animals (***$p<0.001$ vs control). Intraocular administration of PACAP significantly reduces HIF-1α levels in hyperglycemic retinas as compared to STZ injected rats (Figure 1 A and B, ###$p<0.001$ vs STZ), whereas it does not affect HIF-1α levels in control group. As predicted, ARNT expression is unchanged in all groups (Figure 1 A and C). The expression levels of HIF-2α are increased in diabetic rats as compared to control (Figure 2 A and B, ***$p<0.001$ vs control), whereas PACAP treatment reduces its levels in both control and diabetic animals, suggesting that expression of this hypoxic factor is affected by the peptide independently by hyperglycemia. This condition also affects HIF-3α protein expression, which is significantly lower in diabetic rat retinas when compared to the untreated group (Fig. 3a, b, ***$p<0.001$ vs control), whereas

intraocular injection of PACAP restores its level comparable to control animals (Fig. 3a, b, ###p<0.001 vs STZ). However, the PACAP effect is not limited to the pathological condition because its intraocular administration interferes with HIF-3α expression also in control retinas (Fig. 3a, b, ***p<0.001 vs control).

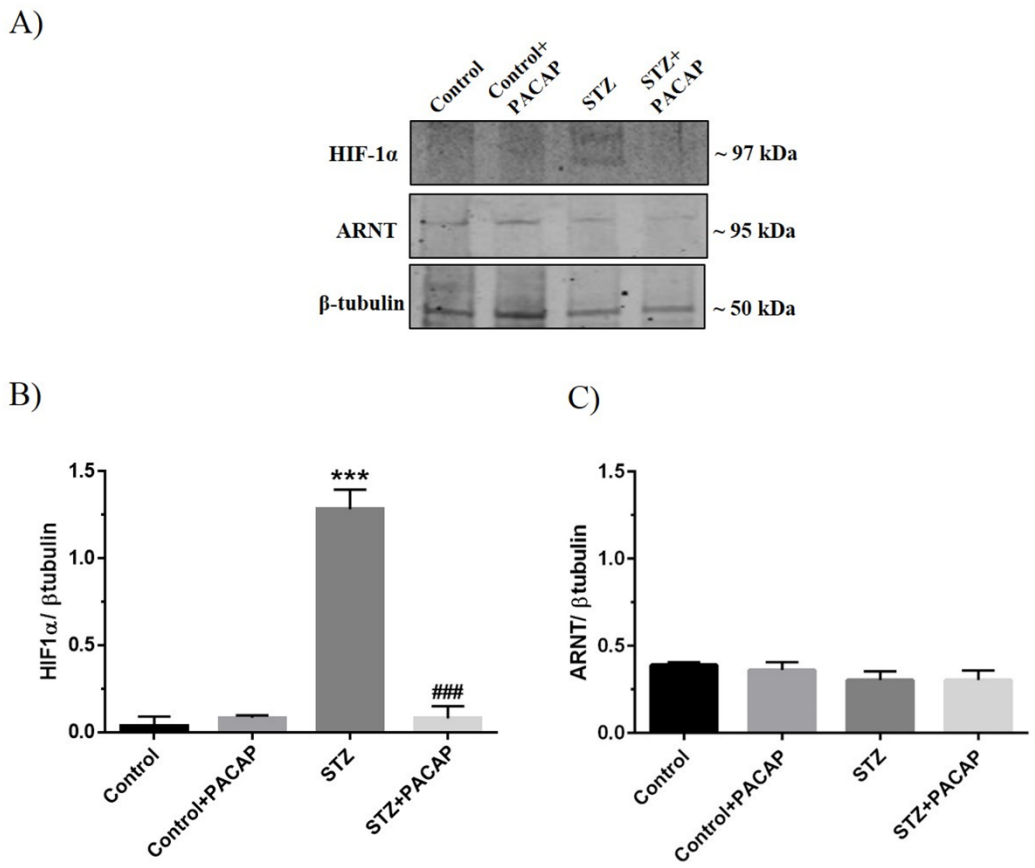

Fig. 1 Effect of PACAP38 intravitreal injection on HIF-1α and ARNT expression in the retina of STZ-injected rats. (A) Representative immunoblot of signals detected by HIF-1α and ARNT antibody, obtained using 20µg of tissue homogenates from retinas of both non diabetic (Control) and diabetic (STZ) rats intravitreally injected with PACAP38. (B-C) The bar graphs show the results of three independent experiments. Relative band densities were quantified by using ImageJ software. Protein levels are expressed as arbitrary units obtained after normalization to β-tubulin, which was used as loading control. Data are expressed as Mean ± SEM ***p<0.001 *vs* control group, $^{###}$p<0.001 *vs* STZ-injected animals, as determined by One-Way ANOVA followed by Tukey post-ho

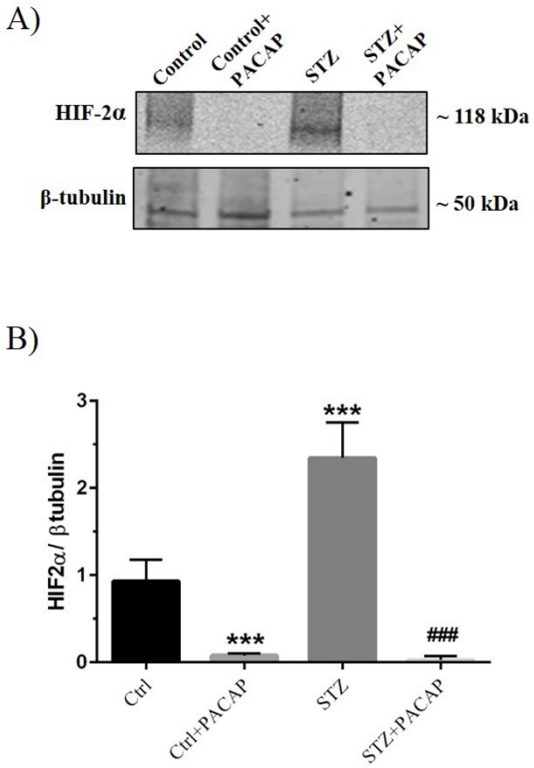

Fig. 2 Effect of PACAP38 intravitreal injection on HIF-2α expression in the retina of STZ-injected rats. (A) Representative immunoblot of signals detected by HIF-2α antibody, obtained using 20µg of tissue homogenates from retinas of both non diabetic (Control) and diabetic (STZ) rats intravitreally injected with PACAP38. (B-C) The bar graphs show the results of three independent experiments. Relative band densities were quantified by using ImageJ software. Protein levels are expressed as arbitrary units obtained after normalization to β-tubulin, which was used as loading control. Data are expressed as Mean ± SEM ***$p<0.001$ *vs* control group, $^{###}p<0.001$ *vs* STZ-injected animals, as determined by One-Way ANOVA followed by Tukey post-hoc test.

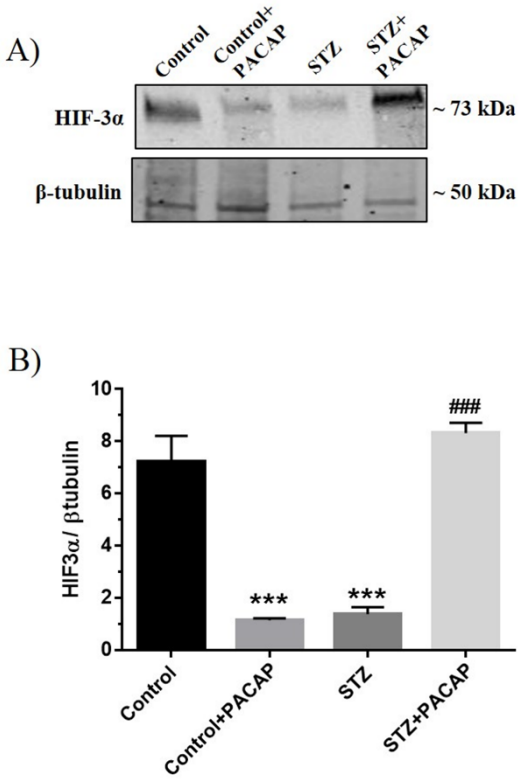

Fig. 3 Effect of PACAP38 intravitreal injection on HIF-3α expression in the retina of STZ-injected rats. (A) Representative immunoblot of signals detected by HIF-3α antibody, obtained using 20μg of tissue homogenates from retinas of both non diabetic (control) and diabetic (STZ) rats intravitreally injected with PACAP38. (B-C) The bar graphs show the results of three independent experiments. Relative band densities were quantified by using ImageJ software. Protein levels are expressed as arbitrary units obtained after normalization to β-tubulin, which was used as loading control. Data are expressed as Mean ± SEM ***$p<0.001$ vs control group, ###$p<0.001$ vs STZ-injected animals, as determined by One-Way ANOVA followed by Tukey post-hoc test.

Distribution of HIFs in diabetic retinas after PACAP treatment.

Based on the results obtained from protein expression analysis, we further investigated whether PACAP treatment influences retinal distribution of HIFs in hyperglycemic rats. To detect localization of HIF-1α, HIF-2α and HIF-3α in the retinal layers of diabetic and PACAP treated rats, we performed immunofluorescence analysis.

According with previous papers (Wright et al., 2011), HIF-1α is localized in the inner nuclear layer (INL), outer plexiform layer (OPL) and in photoreceptor layer (also known as rods and cones layer, RCL) both in control (Figure 4, A, A', A'') and diabetic group (Figure 4, B, B', B''). Diabetes induction increases HIF-1α fluorescence signal detected in INL and in RCL as compared to control rats (Figure 4, B, B', B''). However, PACAP treatment reduces its signal intensity (Figure 4, C, C', C'').

As showed in Figure 5, HIF-2α is weakly expressed in RCL and ganglion cell layer (GCL) of controls (Figure 5, A, A', A''), whereas in diabetic rats the intensity of its signals increases and is also visualized in INL and outer plexiform layer (OPL) (Figure 5 B, B', B''). PACAP administration reduces its expression (Figure 5, C, C', C'').

The HIF-3α is distributed in all retinal layers of control animals (Figure 6, A, A', A''), whereas hyperglycemia reduces its expression (Figure 6 B, B', B''). However, intravitreal PACAP treatment restores HIF-3α distribution to the control and increases its intensity as compared to STZ treated group (Figure 6, C, C', C'').

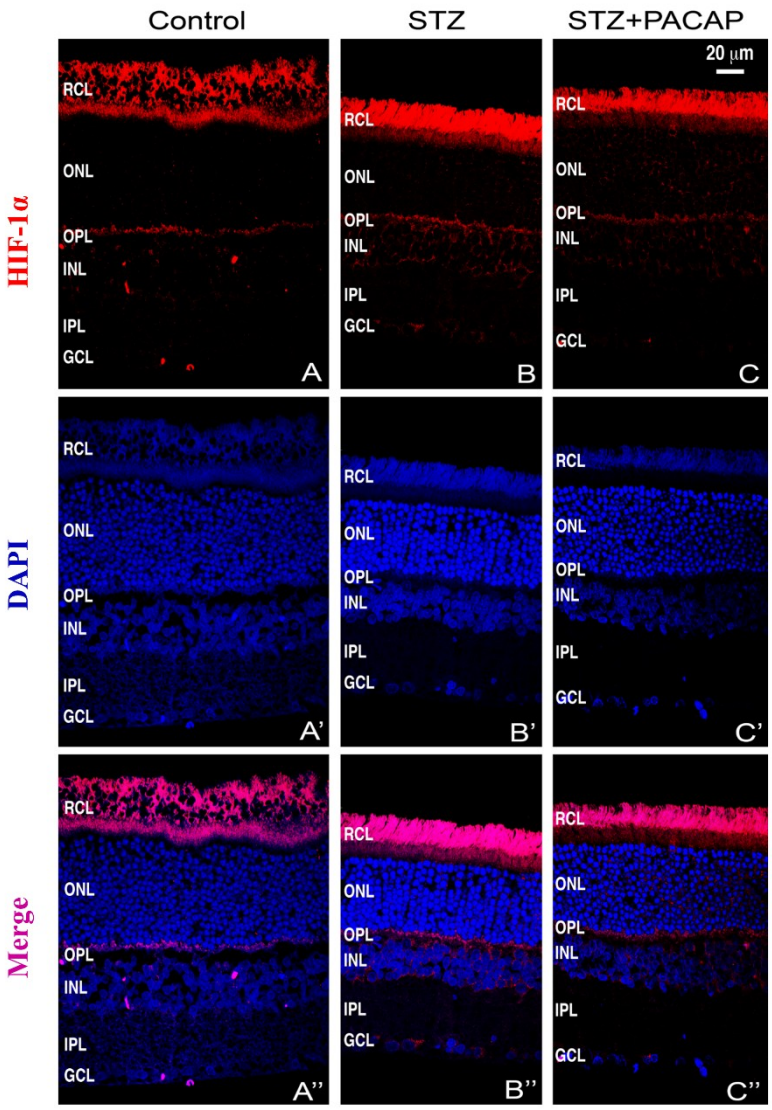

Fig. 4 Retinal distribution of HIF-1α in control and diabetic rat after PACAP treatment. Representative photomicrographs showing distribution of HIF-1α in the retinal layers of control (A, A', A"), diabetic (B, B', B") and PACAP intravitreally injected diabetic rats (C, C', C"). HIF-1α was detected using a mouse HIF-1α primary antibody and revealed with Alexa fluor 594 goat anti-mouse secondary antibodies (red fluorescence) (A, B, C). Cell nuclei were stained with DAPI (blue fluorescence) (A', B', C'). A", B", C" are merged images. Photomicrographs shown are representative results taken from different fields from randomly selected slides and scanned by CLSM. Retinal layers are indicated, on the left of the Figure, as follows: photoreceptors layer (also known as rods and cones layer, RCL); outer nuclear layer (ONL); outer plexiform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL); ganglion cell layer (CGL). Scale bar (20µm).

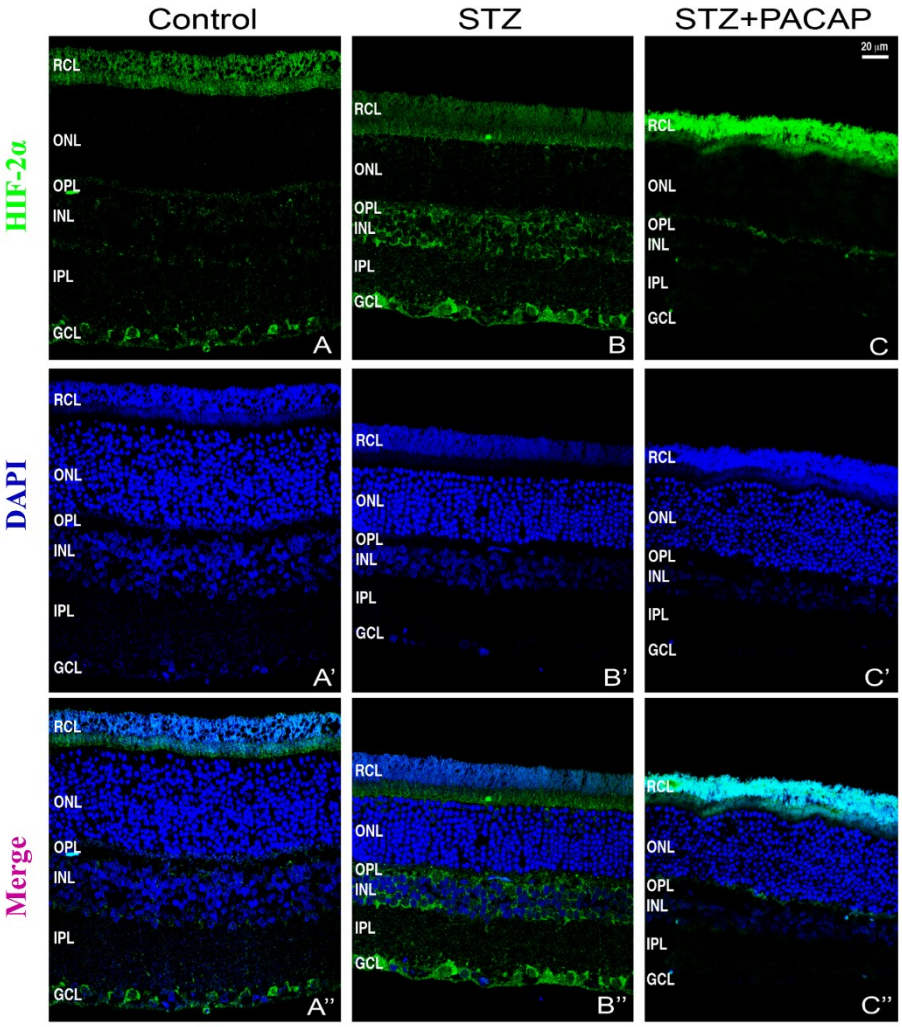

Fig. 5 Distribution of HIF-2α in the normal and diabetic rat retina after PACAP treatment.

Representative photomicrographs showing distribution of HIF-2α in the retinal layers of control (A, A', A"), diabetic (B, B', B") and PACAP intravitreally injected diabetic rats (C, C', C"). HIF-2α was detected using a rabbit HIF-2α primary antibody revealed with Alexa Fluor 488 mouse antigoat secondary antibodies (green fluorescence) (A, B, C). Cell nuclei were stained with DAPI (blue fluorescence) (A', B', C'). A", B", C" are merged images. Photomicrographs shown are representative results taken from different fields from randomly selected slides and scanned by CLSM. Retinal layers are indicated, on the left of the Figure, as follows: photoreceptors layer (also known as rods and cones layer, RCL); outer nuclear layer (ONL); outer plexiform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL); ganglion cell layer (CGL). Scale bar (20µm).

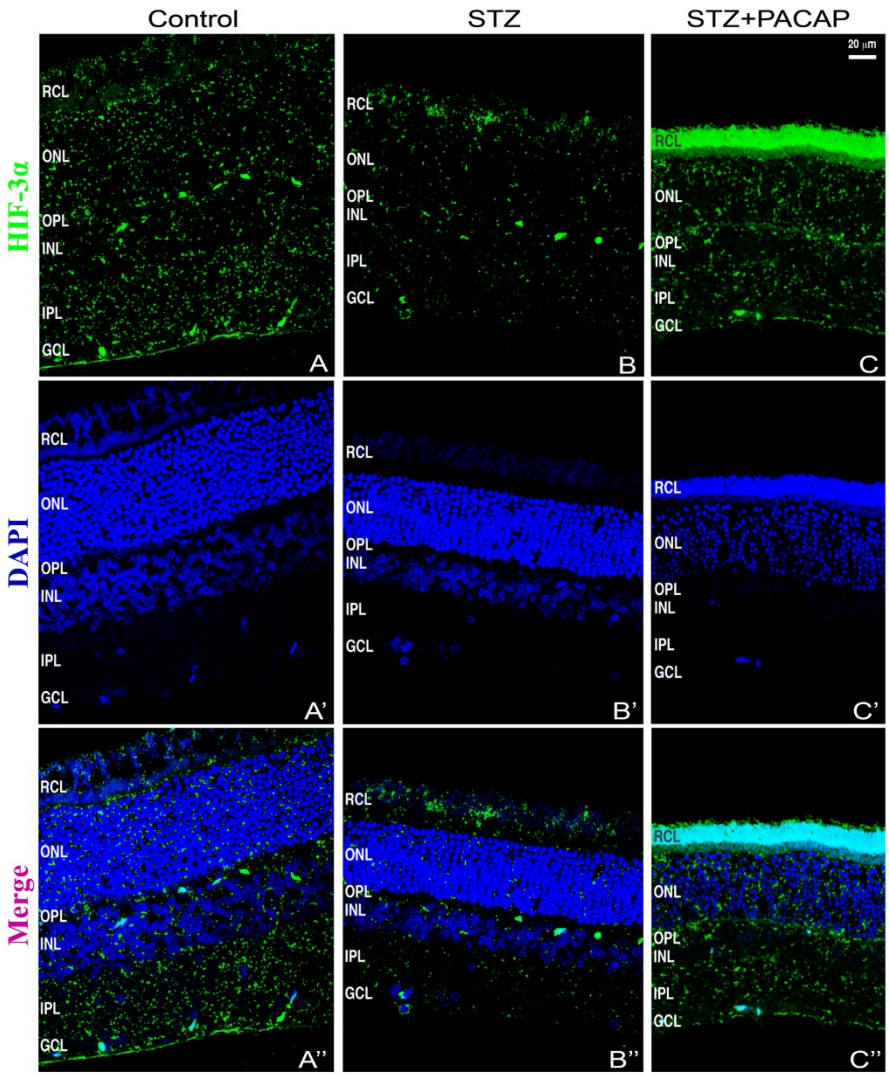

Fig. 6 Distribution of HIF-3α in the normal and diabetic rat retina after PACAP treatment.

Representative photomicrographs showing distribution of HIF-3α in the retinal layers of control (A, A', A''), diabetic (B, B', B'') and PACAP intravitreally injected diabetic rats (C, C', C''). HIF-3α was detected using a rabbit HIF-3α primary antibody revealed with Alexa Fluor 488 mouse antigoat secondary antibodies (green fluorescence) (A, B, C). Cell nuclei were stained with DAPI (blue fluorescence) (A', B', C'). A'', B'', C'' are merged images. Photomicrographs shown are representative results taken from different fields from randomly selected slides and scanned by CLSM. Retinal layers are indicated, on the left of the Figure, as follows: photoreceptors layer (also known as rods and cones layer, RCL); outer nuclear layer (ONL); outer plexiform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL); ganglion cell layer (CGL). Scale bar (20µm).

Discussion

The results of the present study have demonstrated that PACAP abrogates the over-expression of HIF-1α and HIF-2α and enhances HIF-3α expression in diabetic rat retina. These findings suggested that PACAP is able to modulate the hypoxic process opening new therapeutic perspectives to DR treatment.

A considerable number of studies have demonstrated that PACAP plays neuroprotective effect in different pathological conditions, including DR (Castorina et al., 2008; 2010; 2012, 2014; Atlasz et al., 2010). The pathogenesis of this disease is characterized by ischemic and metabolic changes that cause damage to endothelial cells and blood vessels with subsequent alteration in blood flow and microcirculatory damage (Aiello et al., 1998). This vascular complication leads to tissue hypoxia, with subsequent alteration of the expression of growth and transcription factors (Seki et al., 2004).

It has been widely demonstrated that PACAP treatment is able to counteract some pathological alterations that occur during retinal injuries. Indeed, it induces a slight improvement of retinal morphology during glutamate-induced damage, inhibits activation of pro-apoptotic pathway through downregulation of p53 expression and upregulation of Bcl2 level in diabetic rats and reduces loss of the GCL in rat retinas in early stage of hyperglycemia (Kiss et al., 2006; Atlasz et al., 2008; Giunta et al., 2012; Szabadfi et al 2012).

In line with these findings, for the first time, in this work we have shown that PACAP is able to downregulate retinal expression of HIF-1α and HIF-2α, already three weeks after administration of STZ. These factors, activated consequently to the reduction of oxygen tension, promote the transcription of growth factors leading to neovascularization typical of DR. Indeed, in agreement with previous studies, we observed an increased expression of these two factors in retinas of diabetic rats.

Instead, the intravitreal injection of PACAP is able to restore their levels to control values. Particularly, the PACAP effect on modulation of HIF-1α seems closely related to hyperglycemic condition, since its expression remains unchanged when the peptide is administered in control animals. Instead, its effect on the modulation of HIF-2α expression seems to be hyperglycemia-independent.

In the present work, we also provide data on the HIF-3α expression in retinas during the early stages of hyperglycemia. It is expressed in control retinas, as opposed to the low levels of HIF-1α and HIF-2α. Conversely, three weeks after STZ administration, the HIF-3α is significantly reduced, simultaneously with increase of the other two hypoxic factors analyzed. This confirms that it is a negative modulator of HIF-1α and HIF-2α, inasmuch its expression is inversely related to the levels of other two factors (Makino et al., 2002; Maynard 2007; Yang et al., 2015).

The mechanism responsible for this effect in control animals is unclear and requires further study. However, it is relevant the up-regulation of HIF-3α induced by the peptide in diabetic rats. We hypothesized that modulatory action of PACAP on hypoxic event hyperglycemia-linked might be mediated both through a direct mechanism acting on HIF-1α expression and, indirectly, through the regulation of HIF-3α, which in turn down-regulates HIF-1α levels (Zhang et al., 2014).

Our data were also confirmed by analyzing of HIFs distribution in the different retinal layers. We observed that HIF-1α and HIF-2α are distributed in INL in normal condition, whereas HIF-2α expression increases in GCL, INL and OPL three weeks after STZ treatment (Poulaki et al., 2004; Wright et al 2010; Wright et al 2011). In this study, we demonstrated, for the first time, that STZ injection decreases retinal distribution of HIF-3α as compared to control animals. Furthermore, we provide the first evidence on the ability of PACAP to interfere with retinal distribution of HIFs.

Indeed, following peptide injection, HIF-1α and HIF-2α expression decreases in some retinal layers, whereas, HIF-3α expression is restored to the control value confirming the evidence that it acts as negative modulator of HIF-1α and HIF-2α.

It has been previously shown the existence of direct correlation between HIF-1α and PACAP expression. Indeed HIF-1α binds to HRE domain of PACAP promoter, inducing increased expression of this peptide during cerebral ischemia (Lin et al., 2015). Through this mechanism PACAP protects neurons from degeneration in the penumbra area of stroke. Moreover, according with our data, Fabian and coworkers (2012) showed a relationship between PACAP treatment and the reduction of HIF-1α expression in an in vitro model of in human pigment epithelial cells exposed to oxidative stress.

In line with these evidences, we suggested that retinoprotective effect of PACAP could be mediated through inhibition of HIF-1α and HIF-2α, through HIF-3α activation, which competes with the other two hypoxic factors, inhibiting indirectly the transcription of genes involved in uncontrolled vascularization typical of DR. Therefore, these results suggest a possible action mechanism of PACAP as modulator of events triggered by low tissue oxygen tension.

Our study was limited only to early stages of hyperglycemia, however in order to further clarify the role of PACAP in the hypoxic process of the DR, further studies should be carried out by using an in vivo models of longer lasting hyperglycemia.

Conclusion

In conclusion, our data have demonstrated that PACAP affects hypoxic process by modulating HIF-1α, HIF-2α and HIF-3α expression and distribution in diabetic rat retina. This evidence might open new therapeutic perspectives to DR treatment.

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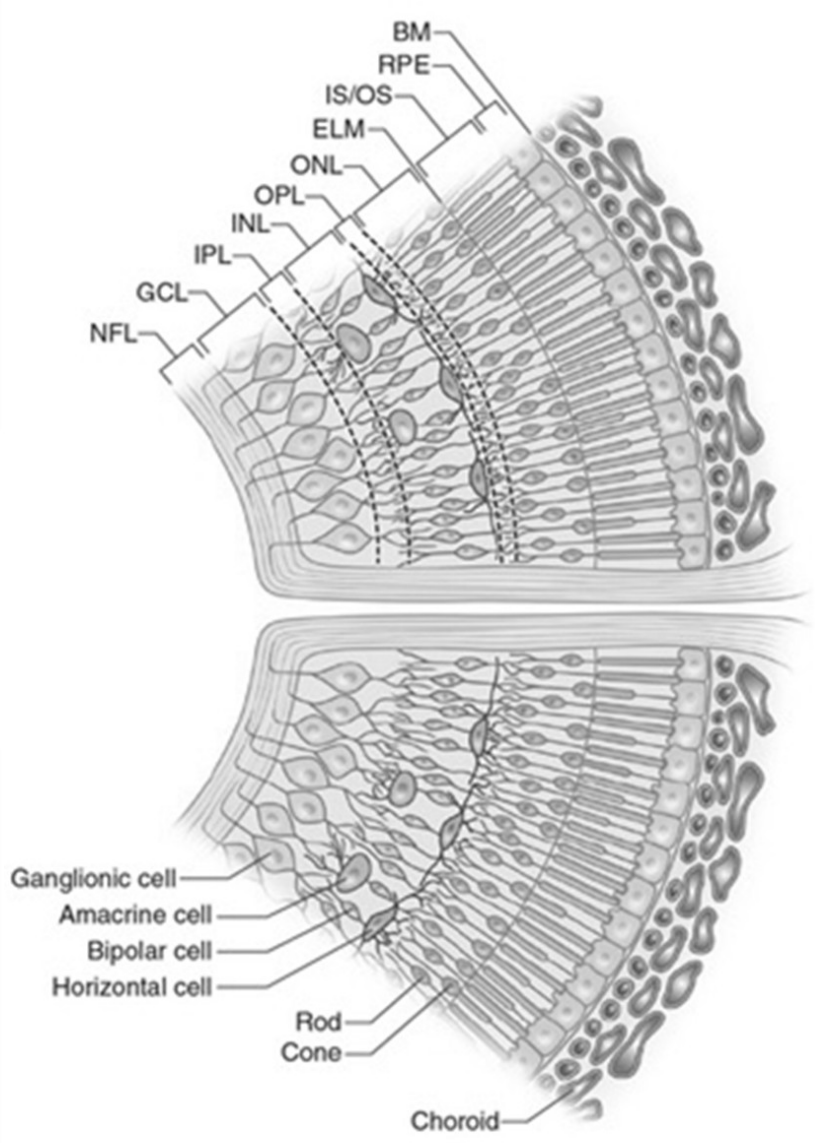
CHAPTER 3

Modulation of IL-1β and VEGF expression in rat diabetic retinopathy after PACAP administration



D'Amico AG. et al. Peptides (2017) doi: 10.1016/j.peptides.2017.09.014

MODULATION OF IL-1β AND VEGF EXPRESSION IN RAT DIABETIC RETINOPATHY AFTER PACAP ADMINISTRATION

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Abstract

Diabetic retinopathy (DR) is a microvascular complication of diabetes. Hyperglycemic/hypoxic microenvironment concurs to aberrant angiogenesis characterizing the pathology and activates many downstream target genes including inflammatory cytokines and vasoactive peptides, such as interleukin-1β (IL-1β) and vascular endothelial growth factor (VEGF).

It has been largely demonstrated that pituitary adenylate cyclase-activating peptide (PACAP) plays a protective effect in DR. In the present study, we investigated the role of PACAP to protect retinal tissue through IL-1β and VEGF expression.

Diabetes was induced in rats by streptozotocin (STZ) injection, and one week later a single intravitreal injection of 100 μM PACAP was administrated. Analyses of IL-1β and VEGF levels were performed three weeks after diabetes induction.

The results demonstrated that a single intraocular administration of PACAP significantly reduced the expression of IL-1β in diabetic animals. Moreover, it affects VEGF and its receptors (VEGFRs) levels and interferes with their retinal layers distribution as showed by confocal microscopy analysis. In particular, PACAP treatment downregulates VEGF and VEGFRs that are increasingly expressed in STZ-treated animals as compared to controls. These results indicate that PACAP plays an important role to attenuate the early phase of DR.

Keywords: Diabetic retinopathy, PACAP, VEGF, Interleukin-1β

Introduction

Diabetic retinopathy (DR) is a common complication of diabetes, leading to vision loss [1]. Hyperglycaemia triggers a sequential cascade of biological events [2-4], among others, the generation of reactive oxygen species (ROS) that causes a severe inflammatory process sustained by vasoactive peptides and inflammatory cytokines release [5, 6]. Retinal vascular permeability impairment is triggered by these factors, including interleukin-1β (IL-1β) [7]. This latter is involved in several ocular pathologies and it is considered a key mediator of retinal damage during early stage of DR [8].

We recently showed the expression profile of IL-1's family members in streptozotocin (STZ)-induced diabetic rats. We demonstrated that IL-1β plays a key role in the retinal inflammatory process [9]. We also demonstrated, by using an in

vitro model of diabetic macular edema (DME) that IL-1β is involved in outer blood retinal barrier dysfunction [10].

Hyperglycaemia triggers retinal hypoxic microenvironment, concurring to aberrant neoangiogenesis [11]. Low oxygen tension generates hypoxia inducible factors (HIFs), heterodimeric transcription factors formed by two different subunits: α oxygen-related subunits (HIF-1α, HIF-2α and HIF-3α) and constitutively expressed subunit, HIF-1β [12-14]. Hypoxia promotes HIF-1α and HIF-2α translocation into the nucleus with consequent dimerization with HIF-1β binding to hypoxia response elements (HREs). This latter induces the transcriptional activation of different genes, including vascular endothelial growth factors (VEGF) [15, 16]. Overexpression of HIF-1α during DR is responsible of VEGF upregulation sustaining uncontrolled neovascularization [17-19]. Based on the above considerations, intravitreal injection of agents directed against VEGF represents the elective DR therapy [20]. However this strategy is effective only in some patients, therefore the identification of other factors involved in DR pathogenesis could allow us to select helpful molecules for non-responders' patients [21]. In particular, compounds counteracting release of vasoactive peptides and inflammatory cytokines could be used to prevent neovascularization.

Several studies investigated the role of pituitary adenylate cyclase-activating peptide (PACAP), vasoactive intestinal polypeptide (VIP) and a small peptide derived from the activity-dependent neuroprotective protein (also known as NAP) in DR [21-26]. PACAP performs a wide array of actions binding two types of G protein coupled receptors: PAC1 and VPAC, including VPAC1 and VPAC2 receptors [27]. The expression of PACAP has been already described in the retina, where it displays a protective role during its degeneration [28, 29]. Recently, Amato et al., (2016) [30] tested its modulatory effect on VEGF release. They demonstrated that the peptide is

able to decrease expression of VEGF after different insults (e.g. high glucose and H2O2) in an in vitro model of DR.

We demonstrated that PACAP interferes with hypoxic event in diabetic retina by modulating HIFs expression [31]. However, we did not figure out whether this effect was releated to VEGF downregulation.

In the present study, we investigated the effects of PACAP to modulate IL-1β, VEGF and related receptors in the retina of diabetic rats.

Materials and Methods

Animals

In this study, we have used male Sprague-Dawley rats of three months old, weighing approximately 200g each, obtained from Charles River (Calco, Italy). All the animals were treated according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were fed with standard laboratory chow and were allowed free access to water in an air conditioned room with a 12h light/12h dark cycle. Final group sizes for all measurements were n = 6-9.

Induction of Diabetes

Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) as previously described [32]. Briefly, after twelve hours of fasting, a group of animals received a single injection of STZ (60 mg/kg body weight) in 10mM sodium citrate buffer, pH 4.5 (1mL/kg dose/volume). Nondiabetic animals were fasted and received citrate buffer alone. Twenty-four hours post STZ injection blood glucose levels were measured and rats showing blood glucose levels greater than 250 mg/dL were considered as diabetic and selected for the study. We monitored throughout the study the diabetic state by evaluating glycaemia daily using a blood glucose meter (Accu–CheckActive; Roche Diagnostic,

Milan, Italy) and by the weight loss. All experiments were performed three weeks after the induction of diabetes. For subsequent experiments, rats were killed with a lethal intraperitoneal (i.p.) dose of sodium pentobarbital. Retinal tissues were immediately collected and homogenized in ice-cold buffer for Western blot analysis and others were fixed in 4% paraformaldehyde for immunofluorescence analysis.

Intravitreal administration of PACAP38

A single intraocular injection of 100 μM PACAP38 (Sigma-Aldrich) dissolved in PBS solution (final volume = 4 μL) was administered one week after intraperitoneal injection of STZ or sodium citrate buffer. Before intravitreal injection, animals were anesthetized by intravenous injection of 5 mg/kg Zoletil (tiletamine HCl and zolazepam HCl, Virbac, Milano, Italy), and 1 drop in the eye of the local anesthetic 0.4% oxybuprocaine (Novesina, Novartis, Origgio, Italy). Same volume of vehicle (PBS) was injected in the other eye as control. Retinal tissues were collected two weeks after the intraocular injection.

Western Blot Analysis

Western blot analysis was performed to determine the relative expression levels of Interleukin 1β (IL-1β), VEGF and its related receptor, by using specific antibodies. Analysis was performed as previously described [33]. 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) using a Teflon-glass homogenizer and then sonicated twice for 20 sec using an ultrasonic probe, followed by centrifugation at 10.000 g for 10 min at 4°C. Protein concentrations were determined by the Quant-iT Protein Assay Kit (Invitrogen). Sample proteins (35 μg) were diluted in 2X Laemmli buffer (Invitrogen, Carlsbad, CA, USA), heated at 70°C for 10 min and then 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: goat anti-VEGF (sc-1836, Santa Cruz Biotechnology; 1:200), mouse anti-VEGF-R1 (sc-316, Santa Cruz Biotechnology; 1:200), mouse anti-VEGF-R2 (sc-6251, Santa Cruz Biotechnology; 1:200), goat anti-IL-1β (sc-1250, Santa Cruz Biotechnology; 1:200) and rabbit anti-β-tubulin (sc-9104, Santa Cruz Biotechnology; 1:500).

The secondary antibody donkey anti-goat IRDye 800CW (#926-32211; LI-COR Biosciences), 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 Odissey 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-tubulin, which was used as loading control.

No signal was detected when the primary antibody was omitted (data not shown).

Immunofluorescence analysis

Eyes were enucleated and fixed overnight with 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.6). Analyses was performed on whole retina, as previously described [34]. Before immunofluorescence staining, paraffin-embedded retinas sections (5 μm) mounted on glass slides, were dewaxed in xylene and rehydrated through graded alcohols. Sections were put into Rodent Decloaker 1X retrieval solution (RD913, BIO-CARE MEDICAL) in a slide container and then incubated with the anti-VEGF and ant VEGFR2 primary antibodies. To establish signal specificity, in a preliminary experiment, each antibody was pre-incubated with an excess of antigen (blocking peptide). No signal was revealed in sections incubated

with neutralized antibody. This confirmed that signal detected by each antibody was not an artifact (data not shown). Tissue sections were then incubated with Alexa fluor 594 goat anti-mouse and Alexa fluor 488 donkey anti-goat secondary antibodies, respectively, for 1h at room temperature and shielded from light. DAPI (diamidino-2-phenylindole) was used to stain nuclei (#940110 Vector Laboratories). Images were taken with a confocal laser-scanning microscope (CLSM), Zeiss LSM700. In order to compare similar regions among different groups, we analyzed the area close to the central retina for each sample (i.e. fovea centralis). Experiments were repeated at least three times to confirm results.

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

PACAP effect on IL-1β expression in diabetic rat retina.

To investigate whether PACAP treatment interferes with inflammatory process, we assessed IL-1β expression in the retina of STZ-injected rat before and after peptide treatment by using western blot analysis. As shown in Figure 1, diabetic animals have a significant upregulation of IL-1β in comparison with (***$p<0.001$ vs. Ctrl). Intravitreal injection of PACAP in hyperglycemic rats downregulates IL-1β to control levels (Figure 1; ###$p<0.001$ vs. STZ).

This effect is tightly related to pathological event because PACAP injection in normal glycemic animals does not affects IL-1β expression.

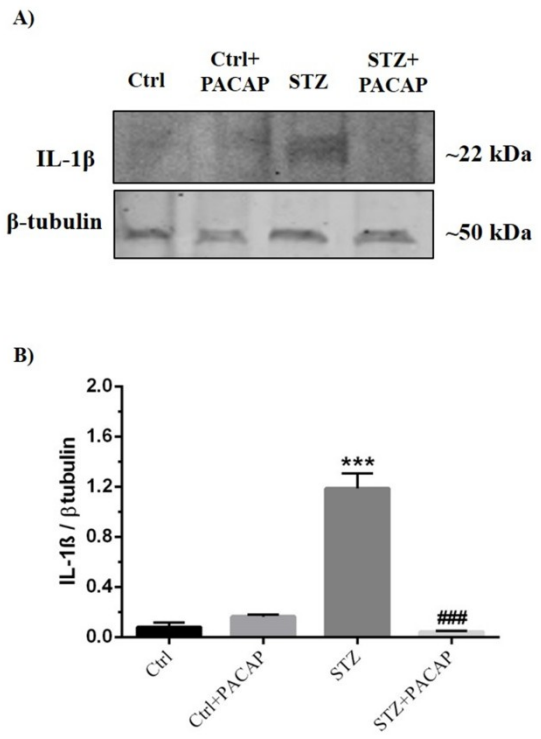

Figure 1. Effect of PACAP38 intravitreal injection on IL1β expression in diabetic rat retina. (A) Representative immunoblot of signals detected on 35 µg of tissue homogenate from non-diabetic (Control) and diabetic (STZ) rat retinas following PACAP38 intravitreal injection (STZ+PACAP) by using IL1β antibody. (B) The bar graph shows the results of three independent experiments. Relative band density was quantified by using ImageJ software and the protein levels were expressed as arbitrary units obtained after normalization to β-tubulin, which was used as loading control. Data are expressed as Mean ± SEM $^{***}p<0.001$ vs Control group (Ctrl), $^{###}p<0.001$ vs STZ-injected animals (STZ), as determined by One-Way ANOVA followed by Tukey post-hoc test.

Effect of PACAP intravitreal injection on retinal expression of VEGF and VEGFRs in STZ-treated rats.

The effect of PACAP treatment on VEGF expression in retina of hyperglycemic rat has been assessed by using western blot analysis. As shown in Figure 2, VEGF is significantly upregulated in STZ-treated animals as compared to controls (***p<0.001 vs. Ctrl). Intraocular injection of PACAP significantly reduces its expression in diabetic retinas (###p<0.001 vs. STZ), whereas it does not affect its level in normoglycemic animals.

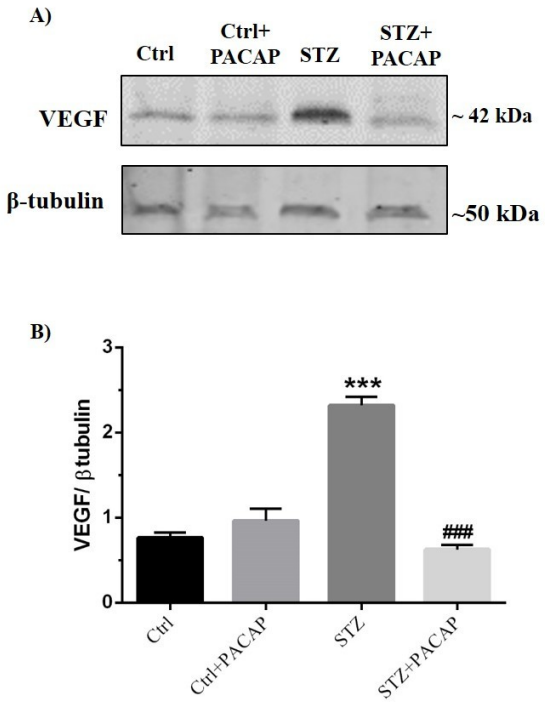

Figure 2. Effect of PACAP38 intravitreal administration on VEGF expression in diabetic rat retina. (A) Representative immunoblot of signals detected on 35 µg of tissue homogenate from non-diabetic (Control) and diabetic (STZ) rat retinas following PACAP38 intravitreal injection (STZ+PACAP) by using VEGF antibody. (B) The bar graph shows the results of three independent experiments. Relative band density was quantified by using ImageJ software and the protein levels were expressed as arbitrary units obtained after normalization to β-tubulin, which was used as loading control. Data are expressed as Mean ± SEM ***p<0.001 vs Control group (Ctrl), ###p<0.001 vs STZ-injected animals (STZ), as determined by One-Way ANOVA followed by Tukey post-hoc test.

The expression pattern of VEGFR2 significantly increased in diabetic retina as compared to control (Figure 2 ***p<0.001 vs. Ctrl). PACAP exogenous administration downregulates it by restoring control's level (***p<0.001 vs. Ctrl; ###p<0.001 vs. STZ). VEGFR1 is weakly expressed in all experimental conditions. However, it seems faintly increase after 3 weeks STZ-injection, but this is abrogated following PACAP administration (***p<0.001 vs. Ctrl; ###p<0.001 vs. STZ). In

control animals, VEGF receptors expression is unaffected by PACAP treatment suggesting that the effect of peptide is related to hyperglycemia.

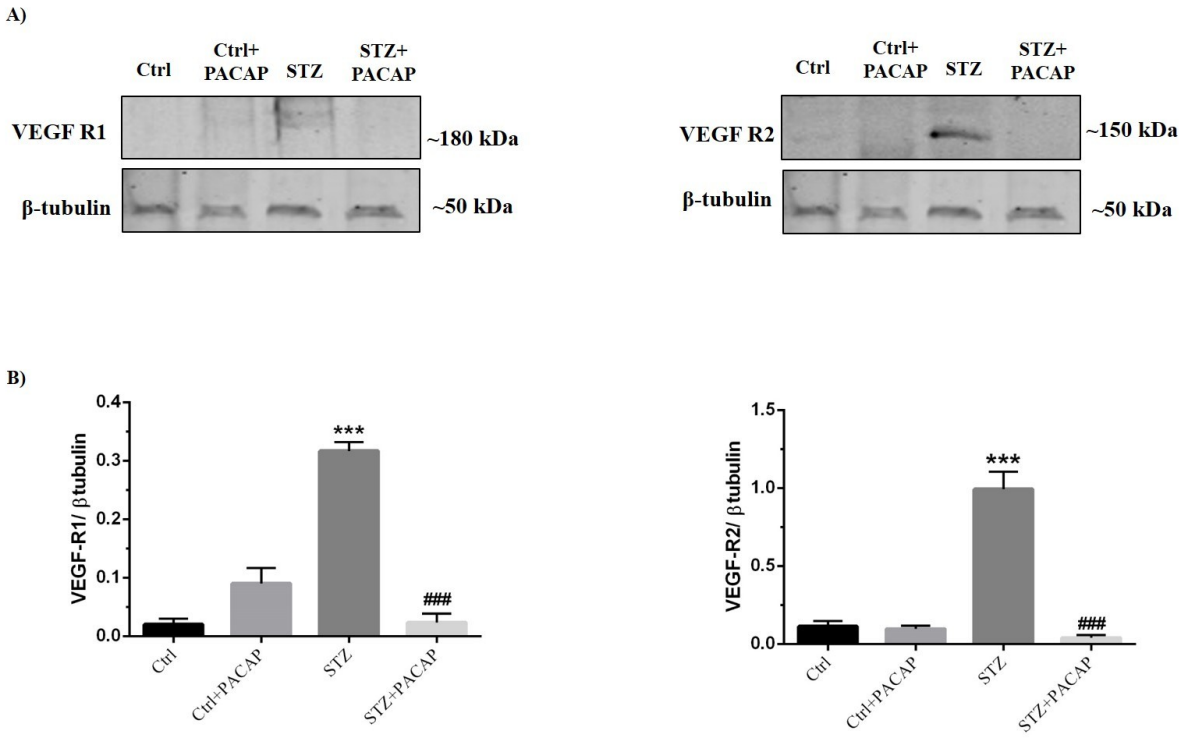

Figure 3. Effect of PACAP38 intravitreal administration on VEGFR1 and VEGFR2 expression in diabetic rat retina. (A) Representative immunoblot of signals detected on 35 µg of tissue homogenate from non-diabetic (Control) and diabetic (STZ) rat retinas following PACAP38 intravitreal injection (STZ+PACAP) by using VEGFR1 and VEGFR2 antibody. (B) The bar graph shows the results of three independent experiments. Relative band density was quantified by using ImageJ software and the protein levels were expressed as arbitrary units obtained after normalization to β-tubulin, which was used as loading control. Data are expressed as Mean ± SEM $^{***}p<0.001$ vs Control group (Ctrl), $^{###}p<0.001$ vs STZ-injected animals (STZ), as determined by One-Way ANOVA followed by Tukey post-hoc test.

Regional distribution of VEGF in diabetic retinas following PACAP treatment.

To confirm that PACAP treatment affects VEGF and VEGFR2 expression, we detected their distribution in retina of hyperglycemic rats by using immunofluorescence analysis. According to previous data [25], VEGF is weakly expressed in retinal layers (Figure 4 A', A'''), whereas its signal is higher after STZ treatment, especially in photoreceptors layer (RCL), outer plexiform layer (OPL) and ganglion cell layer (GCL) (Figure 4 B', B'''). Intravitreal injection of PACAP decreases the VEGF signal restoring it to control group (Figure 4 C', C''').

Instead, the VEGFR2 is faintly expressed in RCL, inner nuclear layer (INL) and in GCL of control animals (Figure 4 A, A'''), whereas its signal intensity increased in the STZ-injected rats, especially in outer nuclear layer (ONL) (Figure 4 B, B'''). PACAP administration induces a significant reduction of its expression in retinal layers of diabetic animals (Figure 4 C, C''').

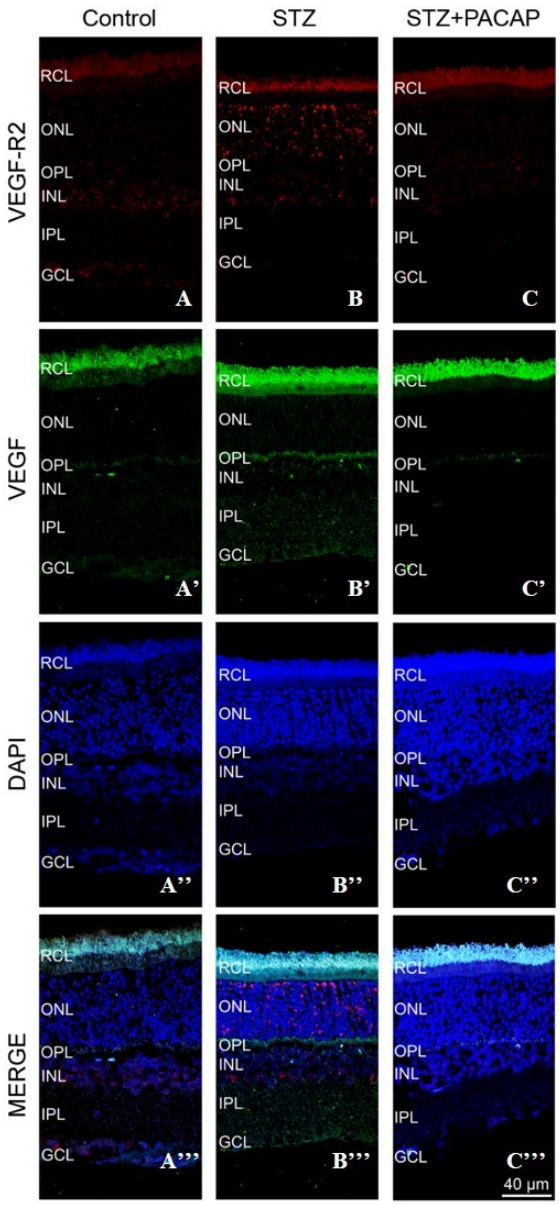

Figure 4. Retinal distribution of VEGF and VEGFR2 in diabetic rat retina following PACAP injection. The photomicrographs show the distribution of VEGF and VEGFR2 in the different retinal layers of control (A, A', A", A'''), diabetic (B, B', B", B''') and PACAP intravitreally injected diabetic rats (C, C', C", C'''). Their immunosignals were detected using a mouse VEGFR2 and a goat VEGF primary antibodies revealed with Alexa fluor 594 goat anti-mouse (red fluorescence) (A, B, C) and Alexa fluor 488 donkey anti-goat secondary antibodies (green fluorescence) (A', B', C'). Cell nuclei were stained with DAPI (blue fluorescence) (A'', B'', C''). The merged images are represented in A'", B'", C'". The photomicrographs are representative results taken from different fields in randomly selected slides and scanned by CLSM. The retinal layers are indicated in the figure as follows: photoreceptors layer (RCL); outer nuclear layer (ONL); outer plexiform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL); ganglion cell layer (CGL). Scale bar (40µm).

Discussion

Retinal microenvironment changes, occurring under hyperglycaemia, contribute to some biological events such as inflammation [35]. This process is tightly related to enhanced expression of tumor necrosis factor (TNF-α) and IL-1β both responsible of blood retinal barrier (BRB) impairment leading to retinal dysfunction [36]. Indeed, it has been demonstrated that IL-1β expression is increased in retina of STZ-induced diabetic rats [9-37].

In the present paper, we confirmed these results and provided the first evidence that intraocular injection of PACAP significantly reduces the retinal expression of IL-1β in hyperglycemic rats (Figure 1). Previous papers have shown the protective role of PACAP to retinal injury [38-42]. In particular, it has been demonstrated that PACAP ameliorates the organization of GCL and RCL compromised by hyperglycemia, protects ischemia-induced retinal damage and counteracts the proinflammatory pathways activated by retinal hypoxia [43-44].

Accordingly, Szabo et al., (2012) [45] have shown that PACAP modulates the expression of several cytokines, including IL-1β, in an in vivo model of retinal damage, whereas the PAC1 receptor agonist, maxadilan, reduces proinflammatory cytokines in ischemia-induced retinal impairments [46]. All these evidences suggest that the eye protective effect of PACAP is mediated through regulation of inflammatory pathway.

Hyperglycaemia also contributes to develop retinal hypoxic microenvironment that promotes HIFs activation by triggering a signalling cascade responsible of microvasculature dysfunction through abnormal VEGF release [47]. Molecules interfering either upstream or downstream to VEGF signalling represent the therapeutic approach to counteract the abnormal neovascularization in DR [48].

Under low oxygen tension, retinal and vascular cells, including pericytes and Müller cells, secrete VEGF [48, 49]. Moreover, it is well documented that STZ-induced diabetic condition leads to the significant VEGF increase [50]. In the current study, we confirmed this evidence showing the VEGF overexpression in retinal OPL and GCL but also in RCL (Figure 2 and 4), whereas PACAP treatment downregulates its level (Figure 2). Instead, PACAP treatment significantly reduces VEGF immunoreactivity in all cell layers, particularly abrogating its expression in GCL, as shown by immunofluorescence analysis (Figure 4).

VEGF binds to different receptors [51], among these, VGFR1 and VGFR2 are those mainly implicated in neovascularization process [52]. In particular, VEGFR2 is upregulated during DR [53, 54]. In agreement to previous papers, in the present study we detected higher expression of VEGFR2 as compared to VEGFR1 in hyperglycaemic animals (Figure 3). Moreover, for the first time, we demonstrated that PACAP modulates levels of both receptors. Therefore, PACAP interferes with the expression of VEGF as well as with the receptors VEGFR1 and VEGFR2 in diabetic retina (Figure 3). We have previously demonstrated that PACAP decreases VEGFRs level in an in vitro model of diabetic macular edema (DME) [42]. This evidence has been further supported by immunofluorescence analysis. VEGFR2 immunoreactivity is increased in retinal layers of diabetic animals, whereas PACAP treatment counteracts the receptor overexpression (Figures 3 and 4).

In the present work, we demonstrate, for the first time, that PACAP is able to counteract the IL-1β, VEGF and related receptor levels after retinal insult.

Our study has some limitation since rats do not develop retinal neovascularization in early stage of DR. A further study should be performed to investigate PACAP effect after long lasting diabetes where increased retinal vascular density has been demonstrated [55].

Conclusion

These results could provide considerable clues to develop a new strategy to counteract DR pathogenesis. These data suggest that PACAP treatment may be useful in the management of the early phase of DR, and that clinical translational studies are warranted.

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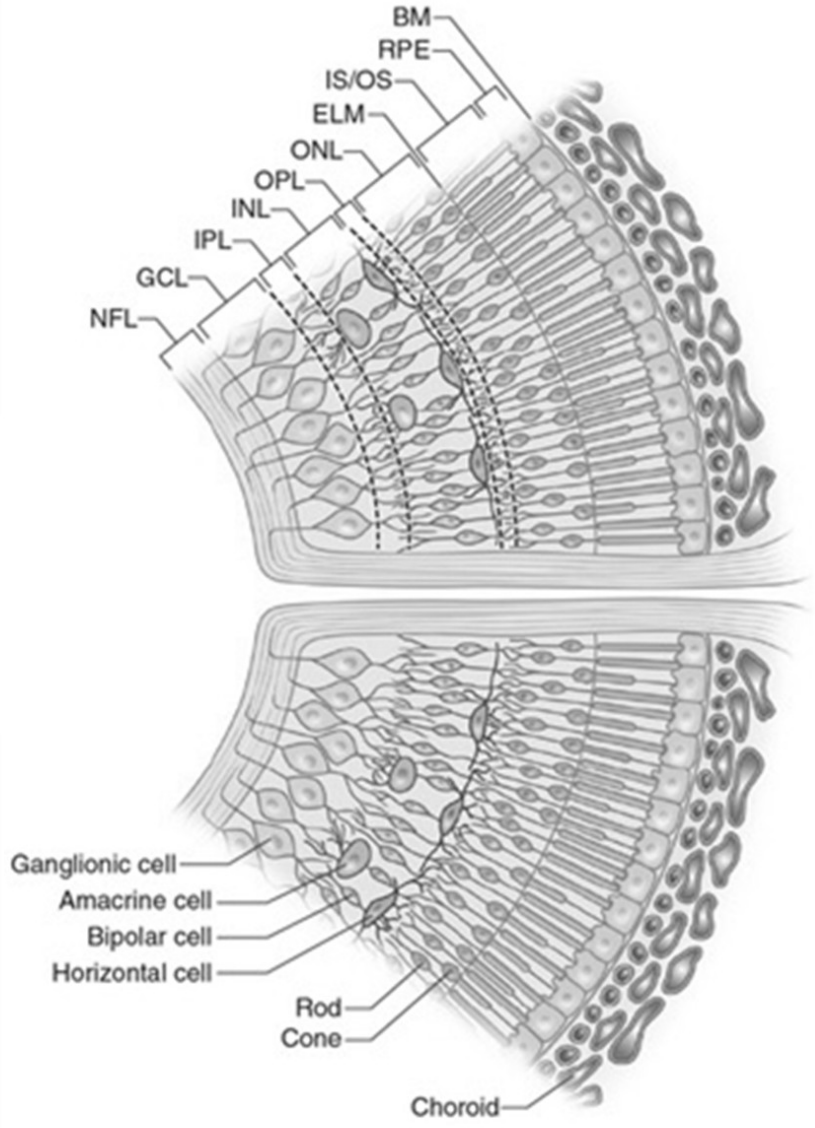
CHAPTER 4

VIP Family Members Prevent Outer Blood Retinal Barrier Damage in a Model of Diabetic Macular Edema



Maugeri G. et al. J Cell Physiol (2017); doi: 10.1002/jcp.25510.

VIP FAMILY MEMBERS PREVENT OUTER BLOOD RETINAL BARRIER DAMAGE IN A MODEL OF DIABETIC MACULAR EDEMA

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Abstract

Diabetic macular edema (DME), characterized by an increase of thickness in the eye macular area, is due to breakdown of the blood-retinal barrier (BRB). Hypoxia plays a key role in the progression of this pathology by activating the hypoxia-inducible factors. In the last years, various studies have put their attention on the role of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) in retinal dysfunction. However, until now, no study has investigated their protective role against the harmful combined effect of both hyperglycemia and hypoxia on outer BRB. Therefore, in the present study, we have analyzed the role of

these peptides on permeability, restoration of tight junctions expression and inhibition of hyperglycemia/hypoxia-induced apoptosis, in an experimental in vitro model of outer BRB. Our results have demonstrated that the peptides' treatment have restored the integrity of outer BRB induced by cell exposure to hyperglycemia/hypoxia. Their effect is mediated through the activation of phosphoinositide 3 kinase (PI3K)/Akt and mammalian mitogen activated protein kinase/Erk kinase (MAPK/ERK) signaling pathways.

In conclusion, our study further clarifies the mechanism through which PACAP and VIP perform the beneficial effect on retinal damage induced by hyperglycemic/hypoxic insult, responsible of DME progression.

Introduction

Diabetic retinopathy (DR) is one of the most important microvascular complications of diabetes mellitus and it is the leading cause of blindness in developed countries (Cheung et al., 2010). Advanced stages of DR are associated to aberrant growth of retinal blood vessels, which proliferate

in an attempt to supply oxygenated blood to the hypoxic retinal areas (Crawford et al., 2009). During progression of this pathology, patients may also develop diabetic macular edema (DME) characterized by thickness of macular area (Lightman et al., 2003). It is due to breakdown of the blood-retinal barrier (BRB), comprising both inner and outer compartments (Lightman et al., 2003). The first one is constituted by tight junctions (TJ) between vascular endothelial and retinal glial cells (Cunha-Vaz, 2004), instead the other is constituted by the retinal pigment epithelial (RPE) cell layer which represents a barrier regulating the movement of molecules from the choroid to the sub-retinal space. BRB breakdown triggers the extravasation of proteins and other solutes from capillaries to the extracellular space.

The pathological progression towards retinal neoangiogenesis begins with the breakdown of Bruch's membrane followed by local ischemia and hypoxia. In this condition the choroidal endothelial cells are activated and transmigrate across the retinal pigment epithelium (RPE) into neurosensory retina, where they proliferate into new vessels under stimulation of appropriate growth factors (Russell et al., 2000; Skeie and Mullins, 2008). This uncontrolled event puts hypoxia as a key player in the progression of DME. The primary response to hypoxia is mediated via the expression hypoxia-inducible factors (HIFs). These elements induce the transcription of a wide variety of genes, including vascular endothelial growth factor (VEGF), which is the master regulator in the formation of new abnormal blood vessels. Moreover, this latter is involved in maintenance of retinal damage because the aberrant vascularization induces mechanical disruption of the internal BRB during the proliferative stage of DR (Campochiaro et al. 2013; Das et al. 2003).

In the last years, various studies have focused their attention on the role of human retinal peptides in the pathophysiology of DME. Particularly, our group as well as other, have investigated the protective role of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) against different types of eye injuries, including DME (Atlasz et al. 2010; Castorina et al. 2010; D'Agata and Cavallaro 1998; D'Amico et al. 2015; Danyadi et al. 2014; Giunta et al. 2012; Scuderi et al. 2013; Seki et al. 2008).

PACAP and VIP belong to the secretine, glucagon, and peptide histidine-isoleucine (phi) superfamily. Their functions are mediated through binding to vasoactive intestinal peptide receptors (VIPRs), which include PAC1, VPAC1 and VPAC2 receptors (Harmar et al., 1998). VIPRs are characterized by a large N-terminal fragment comprising the binding site for PACAP/VIP and an intracellular C-terminal region coupled to heterodimeric G-proteins associated with various signal

transduction pathways (Laburthe et al., 2002). They are widely distributed in human organs and tissues, including the retina, an eye region highly sensitive to long-lasting glucose insult (Antonetti et al., 2006).

PACAP and VIP play cytoprotective action in neurons and other cells (Castorina et al. 2008; Giunta et al. 2010; Castorina et al. 2012; Maugeri et al., 2016). In particular, in the eye, they attenuate diabetic retinal injury both, in vitro, in cells exposed to oxidative stress and, in vivo, in a rat model of DR through the activation of phosphoinositide 3 kinase (PI3K)/Akt, mammalian mitogen activated protein kinase/Erk kinase (MAPK/ERK) signaling cascades and by decreasing the proapoptotic pathway (Castorina et al. 2008; Giunta et al. 2010; Castorina et al. 2012; Szabo et al. 2012; Fabian et al. 2012).

In our recent work, we have demonstrated that PACAP exerts a protective effect in diabetic rat retinas under hypoxic process by regulating HIFs expression (D'Amico et al. 2015). In light of this evidence, in this study we have analyzed if PACAP and VIP treatment protect the outer BRB against the harmful effects hyperglycemia/hypoxia, which represent key players in the pathogenesis of DME.

To this end, we have analyzed the action of both peptides on permeability, restoration of tight junctions expression and inhibition hypoxia-induced apoptosis, in an experimental in vitro model of outer BRB by using human RPE cultures (ARPE-19). Furthermore, we have evaluated whether their protective effect on barrier integrity is mediated through the activation of phosphoinositide 3 kinase (PI3K)/Akt and mammalian mitogen activated protein kinase/Erk kinase (MAPK/ERK) signaling cascades, since, as above described, these pathways are activated by PACAP to minimize apoptotic damage.

Our results have demonstrated that both peptides rescue the integrity of outer BRB during the hyperglycemic/hypoxic process. These results should be deepened to

understand whether PACAP and VIP may have beneficial effect in the clinical approach to DME.

Materials and methods

Human RPE Cell Cultures

This study was performed on human RPE cell culture (ARPE-19) purchased from the American Type Culture Collection (Rockville, MA, USA), as previously described by Scuderi et al. (2013). Briefly, the cells were cultured in T75 flasks using DMEM-F12 (1:1) medium supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, USA), 1 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Sigma Aldrich). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5%CO2 and sub-cultured once a week. Cells were cultured for 7 days on transwell-clear permeable supports (Corning Costar, Cambridge, MA) at a density of 1.5 x 105cells/cm2 in normal glucose condition (NG, 5.5 mM D-glucose). Subsequently, half of the transwells were maintained with 5.5 mM D-glucose (control) and the other half were switched to high glucose conditions (HG, 25 mM D-glucose) for a further 7 days. On the last week of the experiments, cells were exposed to hypoxia for 24 hours by treating them with 100 μM desferrioxamine mesylate salt (DFX) (Sigma-Aldrich), a hypoxia-mimetic agent, previously described (Maugeri et al., 2016), alone or in combination with 100 nM PACAP or 100 nM VIP (Sigma Aldrich, USA). To rule out a potential bias by an osmotic effect, the control cells were also 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.

Measurement of trans-epithelial-electrical resistance

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) as previously described (Scuderi et al., 2013). TEER recording were performed in ARPE-19 cells grown on transwell support. Measurements were commenced after a 15 min equilibration period at room temperature. Values are expressed as Ω/cm2. The combined resistance of the filter was subtracted to the values of filter-cultured ARPE19 cells in order to calculate the resistance of the cell layer. Measurements were performed every 3 days and at 24 hours after treatments on 3 different wells for each experimental condition.

Permeability Assay

Permeability experiments on filter-cultured ARPE19 cells were performed at 21 days by measuring the apical-to-basolateral movements of FITC-dextran with average mol wt 70,000 (Sigma Aldrich) as previously described (Scuderi et al., 2013). First, cells were allowed to equilibrate in phosphate-buffered saline (PBS), and then the solution in the apical compartment was replaced by the FITC-dextran solution. Sixty minutes later, the medium 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.

Western Blot Analysis

Western blot analysis was performed according to the procedures previously described by Maugeri et al., (2016). 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 sec using an ultrasonic probe, followed by centrifugation at 10,000 x g for 10 min at 4 °C. Protein concentrations were determined by the Quant-iT Protein Assay Kit (Invitrogen, Carlsbad, CA, USA). About 25 μg of protein homogenate were diluted in 2X

Laemmli buffer (Invitrogen), heated at 70°C for 10 min and then separated on a Biorad Criterion XT 4-15% Bis-tris gel (Invitrogen) by electrophoresis and then transferred to a nitrocellulose membrane (Invitrogen). Blots were blocked using the Odyssey Blocking Buffer (Li-Cor Biosciences, Nebraska, USA). The transfer was monitored by a prestained protein molecular weight marker (BioRad Laboratories, Segrate, MI, Italy). Immunoblot analysis was performed by using appropriate antibodies: rabbit anti-ZO-1 (cat n. 61-7300, Invitrogen; 2 μg/ml), rabbit anti-phospho Akt (Ser473 residue) (D9E, cat no. #4060, Cell Signaling, Denver, USA; 1:1000), rabbit anti-total Akt (C67E7, cat no. #4691, Cell Signaling; 1:1000), mouse anti-phospho Erk-1/2 (Thr202 and Tyr204 residues) (pT202/pY204.22A, cat no. sc-136521, Santa Cruz Biotechnology; 1:200), mouse anti-total Erk-1/2 (MK1, cat no. sc-135900, Santa Cruz Biotechnology; 1:200) and rabbit anti-β-tubulin (cat n.sc-9104, Santa Cruz Biotechnology; 1:500). The secondary antibody goat anti-rabbit IRDye 800CW (cat #926-32211; Li-Cor Biosciences) and goat antimouse IRDye 680CW (cat #926-68020D, Li-Cor Biosciences) were used at 1:20000 and 1:30000, respectively. Blots were scanned with an Odyssey Infrared Imaging System (Odyssey). Densitometric analyses of Western blot signals 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-tubulin, which served as loading control, as previously described by Maugeri et al., (2015).

Fluorescence microscopic analysis of cell death

Cells were treated with different glucose concentrations, 5.5 mM glucose (NG) or 25 mM glucose (HG) in the presence or absence of 100 μM DFX (HG+DFX), alone or in combination with PACAP (HG+DFX+PACAP) or VIP (HG+DFX+VIP) for 24 hours. The typical morphological features of apoptotic degeneration were analyzed

by the use of confocal microscopy with the nuclear dye Hoechst 33342 as previously described by Castorina et al., (2008). Cells were fixed with a solution of methanol/acetic acid (3:1 v/v) for 30 min, washed three times in PBS and incubated for 15 min at 37 °C with 0,4 µg/ml Hoechst 33342 dye. After being rinsed in water, cells were visualized for determination of nuclear chromatin morphology with the use of confocal laser scanning microscopy (CLSM; Zeiss LSM700). Each scanning was individually digitalized by a high sensitivity PMT using the following acquisition setup: Gain master: 776; digital offset: -202; digital gain: 1.0. All acquisitions were performed with ZEN-2010 software.

Apoptotic cells were recognized on the basis of nuclear condensation and/or fragmented chromatin. Each condition was reproduced in three dishes per experiment. Both apoptotic and normal cells were counted from three fields per dish in a fixed pattern.

Statistical analysis

Data are represented as mean ± standard error (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 set at $p \leq 0.05$.

Results

PACAP and VIP effect on ARPE-19 cell monolayer exposed to hyperglycemic and hypoxic insult

In order to evaluate outer BRB integrity, we have performed TEER measurement on ARPE-19 cell monolayer. This value significantly decreases after 24 h exposure to hyperglycemia and hypoxia as compared to cells grown in high (HG) or normal glucose (NG) (controls). Both peptides have significantly increased TEER values

(###p<0.001 vs HG+DFX). However, VIP effect was less efficacious than PACAP on the barrier integrity preservation (§§p<0.01 vs HG+DFX+PACAP) (Fig. 1).

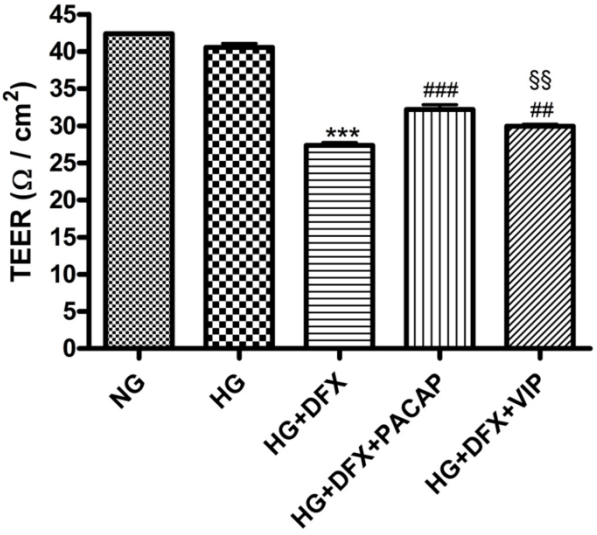

Figure 1. Effect of PACAP and VIP on TEER of ARPE19 cell monolayer exposed to hyperglycemia and hypoxia. Effect of PACAP and VIP on TEER values in ARPE19 cells grown on transwell-clear permeable supports in the presence of 5.5 mM d-glucose + 19.5 mM mannitol (NG) or 25 mM d-glucose (HG) or HG plus desferrioxamine mesylate salt (HG+DFX) or HG + DFX + PACAP (100 nM) or HG + DFX + VIP (100 nM) at 24 hours after treatments. Results are expressed as the mean ± SD (n = 3). ***p < 0.001 vs HG; ##p<0.01 and ###p<0.001 vs HG+DFX; §§p<0.01 vs HG+DFX+PACAP as determined by one-way ANOVA followed by the Tukey post hoc test.

To further confirm these data, we have also performed a permeability assay by measuring apical-to-basolateral movements of FITC-dextran on ARPE-19 cells monolayer. These values are inversely correlated to TEER measurements. Indeed, as shown in Fig. 2, through FITC-dextran diffusion, cell monolayer is significantly increased after exposure to high glucose and desferoxamine as compared to controls (***p < 0.001 vs HG). The treatment with PACAP or VIP significantly reduce permeability as compared to HG+DFX cultured cells (###p < 0.001 vs HG + DFX). Moreover, in accord to TEER data, VIP showed a less efficacy in preserve the barrier permeability as compared to PACAP treatment (§§p<0.01 vs HG+ DFX+PACAP) (Fig. 2).

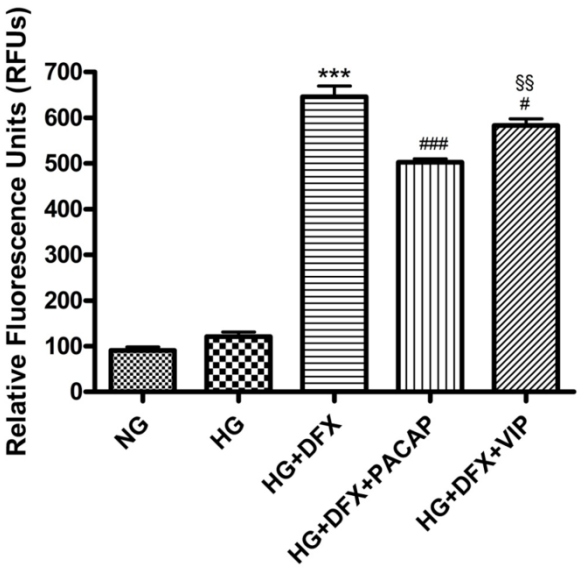

Figure 2. Effect of PACAP and VIP on ARPE19 cell monolayer permeability exposed to hyperglycemia and hypoxia. The effect of PACAP and VIP on outer BRB integrity following exposure to high glucose plus desferrioxamine mesylate salt (DFX) was evaluated by measuring apical-to-basolateral movements of FITC-dextran in ARPE19 cells grown on transwell-clear permeable supports. They were cultured in the presence of 5.5 mM d-glucose + 19.5 mM mannitol (NG) or 25 mM d-glucose (HG) or HG plus desferrioxamine mesylate salt (HG+DFX) or HG + DFX + PACAP (100 nM) or HG + DFX + VIP (100 nM). Results are presented as mean of relative fluorescence units (RFUs) ± SD obtained from measurements on 3 different wells for each treatment in three separate experiments (n = 3). ***$p < 0.001$ vs HG; #$p<0.05$ and ###$p < 0.001$ vs HG + DFX; §§$p<0.01$ vs HG+ DFX+PACAP as determined by one-way ANOVA followed by the Tukey post hoc test.

Alterations of the barrier are associated to changes in the intercellular spaces due to dysfunction of Tight Junction-related proteins. Therefore, to assess the protective effect of PACAP and VIP on the outer BRB integrity, we have analyzed the expression of the zonula occludens (ZO-1) tight junction protein. As shown in Fig. 3, ZO-1 expression is significantly reduced in cells exposed to HG plus hypoxia as compared to controls (***$p<0.001$ vs HG). PACAP or VIP treatment has

significantly increased the levels of this protein in cells grown in presence of high glucose and desferoxamine (###p<0.001 vs HG+DFX).

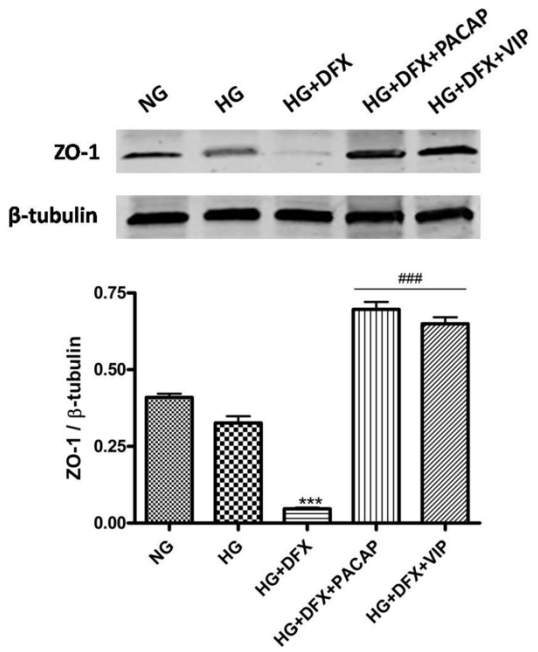

Figure 3. Effect of PACAP and VIP on ZO-1 tight junction protein expression in ARPE-19 cells exposed to hyperglycemia and hypoxia. Representative immunoblot of ZO-1 expression in ARPE19 cells cultured in 5.5 mM d-glucose + 19.5 mM mannitol (NG) or 25 mM d-glucose (HG) or HG plus desferrioxamine mesylate salt (HG+DFX) or HG + DFX + PACAP (100 nM) or HG + DFX + VIP (100 nM). The bar graph shows quantitative analysis of signals obtained by immunoblots resulting from three independent experiments. Relative band densities were quantified by using ImageJ software. Protein levels are expressed as arbitrary units obtained after normalization to β-tubulin which was used as loading control. Data represent means ± SEM. $$$p<0.001 vs NG; ***p<0.001 vs HG; ###p<0.001 vs HG+DFX, as determined by one-way ANOVA followed by the Tukey post hoc test.

The protective role performed by these peptides is related to their antiapoptotic effect which preserves monolayer alteration during hypoxic process. The presence of morphological signs of nuclear damage and/or chromatin fragmentation has been analyzed by using the Hoechst 33342 staining technique. As reported in Fig. 4A-B, about 18% of cells grown in high glucose, die by apoptosis. In the figure we have reported, as a control, only data regarding HG group since the NG group has given

similar result. The apoptotic death is significantly increased to about 80%, when cells are grown in high glucose and DFX. This value is significantly reduced at around 20% following PACAP or VIP treatment, confirming the protective and anti-apoptotic effect played by these peptides.

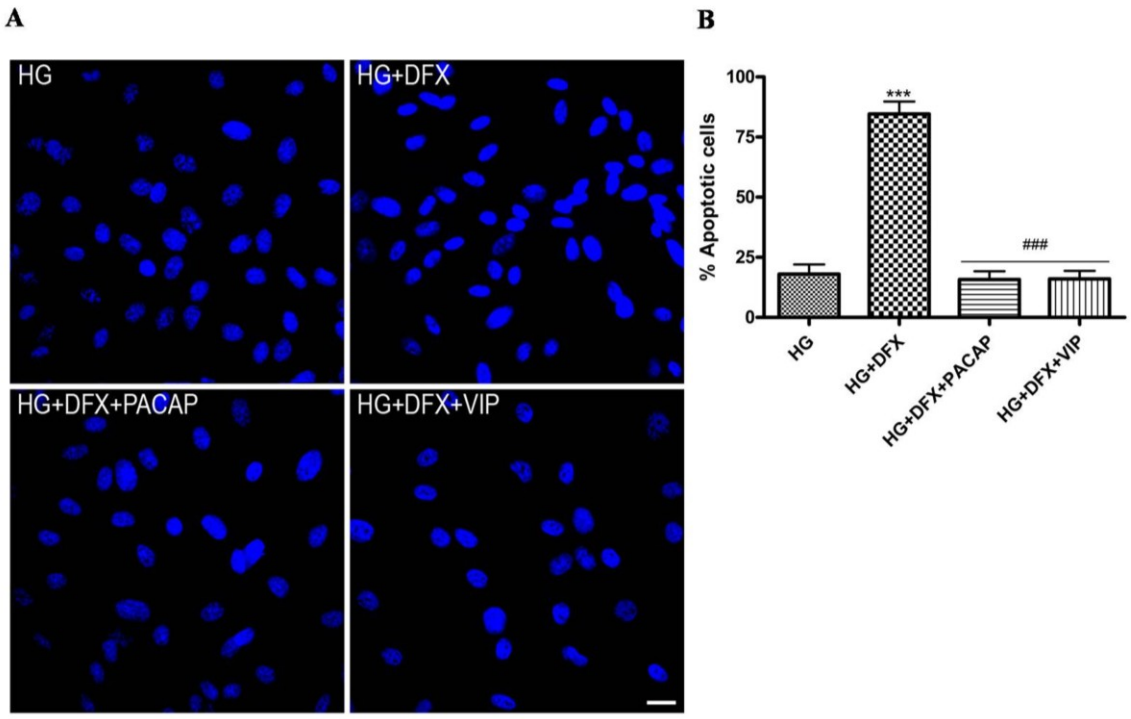

Figure 4. Effect of PACAP and VIP on hyperglycemia/hypoxia-induced apoptosis in ARPE-19 cells. Cells were stained with the fluorescent nuclear dye Hoechst 33342 and viewed at ×100 magnification; Scale bar = 20 μm. The bar graph represents mean ± SD of apoptotic cells percentages calculated counting cells from seven fields per dish, in a fixed pattern. $^{***}p<0.001$ vs HG; $^{###}p<0.001$ vs HG+DFX, as determined by one-way ANOVA followed by the Tukey post hoc test.

Effect of PACAP and VIP on activation of PI3K/Akt and MAPK/ERK survival signaling pathways

In order to investigate the molecular mechanisms involved in PACAP and VIP protection against outer BRB breakdown, we have analyzed the activation of PI3K/Akt and the MAPK/ERK survival signaling cascades. As shown in Fig. 5, both peptides have significantly increased the level of phosphorylated Ser473 AKT and ERK1/2 as compared to cells grown under hyperglycemia and hypoxia ($^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs HG+DFX).

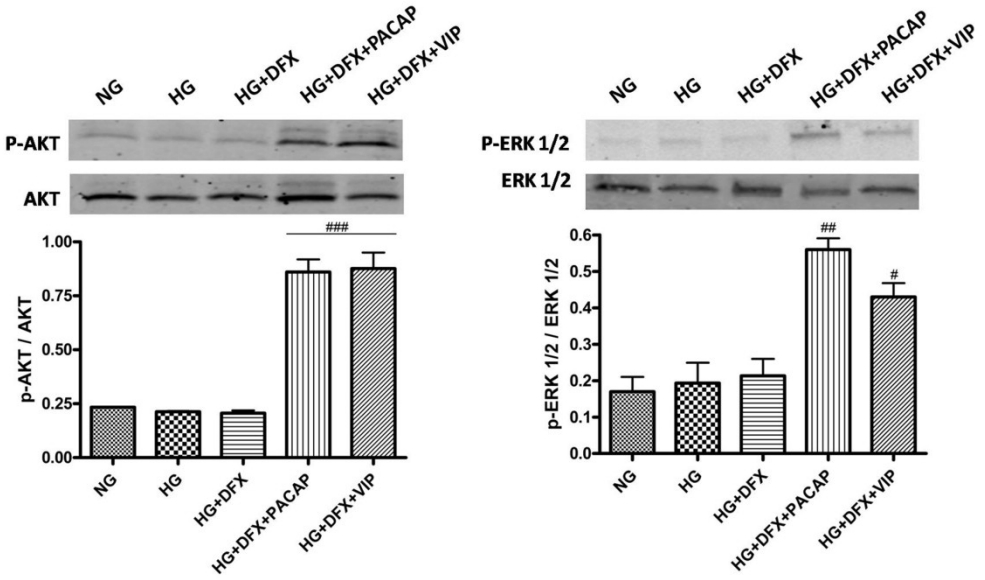

Figure 5. Effect of PACAP and VIP on ARPE19 cell monolayer exposed to hyperglycemia and hypoxia is mediated through activation of PI3K/Akt and the MAPK/ERK survival pathways. Representative immunoblots of Ser473-p Akt or p-Erk1/2 expression in ARPE-19 cells cultured in the presence of 5.5 mM d-glucose + 19.5 mM mannitol (NG) or 25 mM d-glucose (HG) or HG plus desferrioxamine mesylate salt (HG+DFX) or HG + DFX + PACAP (100 nM) or HG + DFX + VIP (100 nM). The bar graphs show quantitative analysis of signals obtained by immunoblots resulting from three independent experiments. Relative band densities were quantified by using ImageJ software. Each signal of phosphorylated protein was normalized to total protein expression. Data are expressed as mean ± S.E.M. $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs HG+DFX, as determined by one-way ANOVA followed by the Tukey post hoc test.

To confirm that alterations of cell monolayer were due to imbalance of PI3K/Akt and MAPK/ERK signaling pathway, we have analyzed ZO-1 expression in cells treated with PACAP or VIP in the presence of wortmannin or PD98059, a PI3K and MEK1 inhibitors, respectively. As shown previously, ZO-1 expression is significantly reduced in cells exposed to hyperglycemia and hypoxia as compared to control (***$p<0.001$ vs HG), whereas treatment with PACAP or VIP significantly increased its expression as compared to cells grown in presence of high glucose and desferoxamine (###$p<0.001$ vs HG+DFX). Instead, PACAP and VIP treatment in presence of PI3K or MEK1 inhibitors, drastically reduced ZO-1 expression ($$$$p<0.001$ vs HG+DFX+PACAP; §§§$p<0.01$ vs HG+DFX+VIP), confirming that both peptides act through activation of PI3K/Akt and MAPK/ERK survival signalling pathways (Fig. 6).

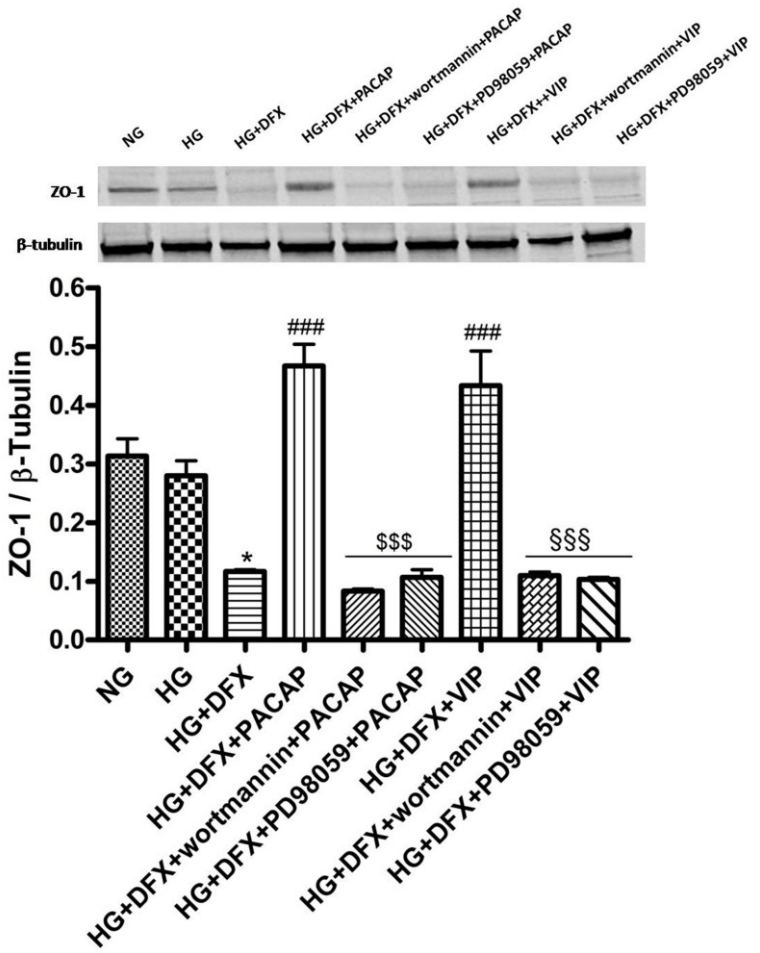

Figure 6. Expression of ZO-1 protein following inhibition of PI3K/Akt or MAPK/Erk kinase signaling pathways. Representative immunoblot of ZO-1 expression in ARPE-19 cells cultured in the presence of 5.5 mM d-glucose + 19.5 mM mannitol (NG) or 25 mM d-glucose (HG) or HG plus desferrioxamine mesylate salt (HG+DFX) or HG + DFX + PACAP or VIP (100 nM), or HG + DFX + PACAP or VIP with Wortmannin (10 μM) or PD98059 (50 μM). The bar graph shows quantitative analysis of signals obtained by immunoblots resulting from three independent experiments. Relative density of each band was quantified using ImageJ software. Each signal was normalized on correspondent β-tubulin signal. Data are expressed as mean ± S.E.M. $^{*}p<0.001$ vs HG; $^{\#\#\#}p<0.001$ vs HG+DFX; $^{\$\$\$}p<0.001$ vs HG+DFX+PACAP; $^{\S\S\S}p<0.001$ vs HG+DFX+VIP as determined by one-way ANOVA followed by the Tukey post hoc test.

Discussion

PACAP and VIP exert protective effect against several types of retinal injuries, including DME, which occurs after the breakdown of the BRB due to leakage of dilated hyperpermeable capillaries and microaneurysms (Ciulla et al., 2003). The involvement of hyperglycemia/hypoxia in the development and maintenance of DME has been widely investigated. It is now clear, that dramatic reduction of oxygen levels affects not only the neuroretina, but also the RPE. In fact, even if this latter is near to the choriocapillaries, in response to hyperglycemic insult, below Bruch's membrane partial pressure of O2 (PO2) falls dramatically by exposing the apical surface of RPE and the photoreceptors to low oxygen tension (Yu and Cringle, 2001). Therefore, hyperglycemia/hypoxia associated to fall in perfusion pressure of the retinal capillaries might play a key role on DR progression. The present study has been performed in a model, in vitro, of outer BRB that more closely mimics the situation in vivo occurring in DME. Human RPE cells have been cultured in high glucose on transwell-clear permeable supports, and exposed to hypoxia following DFX administration, a hypoxia-mimetic agent. Our results have shown that hyperglycemia alone weakly affects the cellular monolayer permeability, instead, concomitant exposure to hypoxia significantly compromised its integrity. This evidence, in accord with the clinical hypothesis above described, shows that low oxygen tension is one of mains factors that promotes DME progression (Wang et al., 2012).

Previous papers have shown that both PACAP and VIP have a protective role on various retinal dysfunctions (Scuderi eta al., 2013; Zhang et al., 2005; Fabian et al., 2012). However, until now, data regarding their effect on maintenance of outer BRB under hyperglycemic/hypoxic insult have not been reported.

In the present study, it has been demonstrated that both peptides prevent damage induced by hyperglycemia and hypoxia, by increasing, in our model, TEER values, as well as decreasing diffusion of FITC-dextran solution from the apical to the basolateral compartment of the transwell. Interestingly, PACAP seems to be more efficient as compared to VIP treatment (Figs. 1 and 2).

Essential in the structural maintenance of cell monolayer are the tight junctions. As described in previous paper, they are involved in the strict control of fluids and solutes crossing BRB, as well as in preventing the entrance of toxic molecules and plasma components into the retina (Strauss et al., 2005). Among tight junction-associated proteins, ZO-1 belongs to a group named membrane-associated guanylate kinase homologs that are positioned on the cytoplasmic surface of junctional contacts. A previous study has shown that the increase of vascular permeability is concomitant to reduction of ZO-1 content in the vitreous of patients with DR (Deissler et al., 2013). Furthermore, its expression in the cell membrane is correlated with improved BRB function (Muthusamy et al., 2014). In the present study, we have found that in cells exposed to hyperglycemia and hypoxia, ZO-1 expression is drastically decreased as compared to control, on contrary, treatment with PACAP or VIP is able to significantly increase its levels (Fig. 3). Therefore, these peptides improve barrier integrity, by increasing the contact between adjacent retinal epithelial cells, and by preventing hyperglycemia/hypoxia-induced apoptotic cell death as shown by Hoechst analysis (Fig. 4). This evidence is in accord to previous studies showing that PACAP prevents different types of retinal injuries through activation of several protective pathways. Indeed, Fabian et al., (2012) have demonstrated that PACAP prevents the process of programmed cell death in human RPE cells following oxidative stress-induced injury. Furthermore, Szabadfi et al., (2014), have shown that intraocular PACAP injection strongly attenuates diabetic

retinal injury by increasing the levels of the anti-apoptotic p-Akt, p-ERK1 and p-ERK2, which are necessary to maintain the structural and functional integrity of the retina during the diabetic challenge.

For this reason, in our study, we have also evaluated if protective effect of these peptides on outer BRB during hyperglycemic/hypoxic insult, is mediated through the activation of PI3K/Akt and MAPK/ERK signalling pathways. In accord to previous data, our results suggest that both PACAP or VIP reduce RPE barrier breakdown induced by hyperglycemia/hypoxia by increasing Akt and ERK1/2 phosphorylation (Fig. 5).

In order to further confirm the involvement of these pathways in the maintenance of membrane integrity, we have treated cells exposed to hyperglycemia and hypoxia, with PACAP and VIP in the presence of a specific PI3K/Akt (wortmannin) or MAPK/ERK (PD98059) signalling pathway inhibitors. As shown in Fig. 6, ZO-1 expression levels are significantly reduced, confirming that PACAP and VIP maintain RPE cellular integrity under conditions mimicking the DME through the activation of Akt and ERK1/2 protective pathways.

In conclusion, our study elucidates the mechanism of action by which PACAP and VIP contribute to protect the outer BRB from damage induced by combined action of hyperglycemia and hypoxia, which represent some of the key factors in the progression of DME.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

These experiments were supported by the international PhD program in Neuroscience, University of Catania, Medical School.

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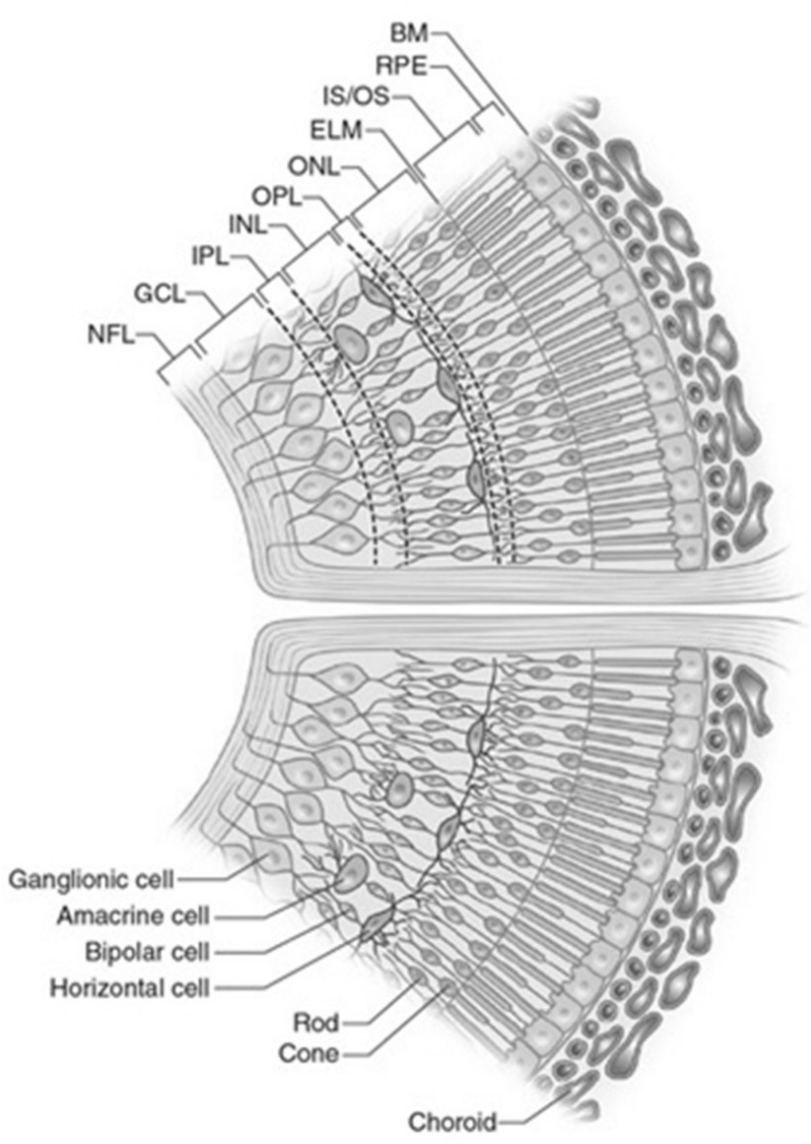
CHAPTER 5

PACAP and VIP Inhibit HIF-1α-Mediated VEGF Expression in a Model of Diabetic Macular Edema



Maugeri G. et al. J Cell Physiol (2017); doi: 10.1002/jcp.25616.

PACAP AND VIP INHIBIT HIF-1α-MEDIATED VEGF EXPRESSION IN A MODEL OF DIABETIC MACULAR EDEMA

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) exert a protective role against retinal injuries, including diabetic macular edema (DME). The macular damage is induced by hyperglycemia, which damages vessels supplying blood to the retina and induces hypoxia. The microenvironmental changes stimulate the expression of hypoxia-inducible factors (HIFs), which promote the choroidal endothelial cell transmigration across the retinal pigmented epitelium (RPE) into neurosensory retina, where they proliferate into new vessels under stimulation of the vascular endothelial growth factor (VEGF). In the present study, we have investigated whether PACAP and VIP prevent retinal damage by modulating the expression of HIFs, VEGF and its receptors. In accord to our hypothesis, we have shown that both peptides are able to significantly reduce HIF-1α

and increase HIF-3α expression in ARPE19 cells exposed to hyperglycemic/hypoxic insult. This effect is also related to a reduction of VEGF and its receptors expression. Moreover, both peptides also reduce the activation of p38 mitogen-activated protein kinase (MAPK), a pro-apoptotic signaling pathway, which is activated by VEGFR-1 and 2 receptors.

In conclusion, our study has further elucidated the protective role performed by PACAP and VIP, against the harmful combined effect of hyperglycemia/hypoxia characterizing the DME microenvironment.

INTRODUCTION

Diabetic retinopathy (DR) is the most common complication of type 1 and type 2 diabetes. It is the main cause of blindness in the working-age population worldwide (Sivaprasad et al., 2012). DR progression is associated to diabetic macular edema (DME), which is characterized by retinal thickness in the macula, the central area of the retina. It occurs when hyperglycemia damage the small capillaries which supply blood to the retina and it is due to breakdown of the blood-retinal barrier (BRB) (Lightman et al., 2003). This latter comprises two compartments: the inner one is represented by tight junctions between vascular endothelial and retinal glial cells (Cunha-Vaz et al., 2004), and the outer includes the retinal pigment epithelial (RPE) cell layer, which regulates the movement of molecules from the choroid to the sub-retinal area. The breakdown of the BRB promotes the disease progression. In particular, the damage of Bruch's membrane is followed by local ischemia and hypoxia, which converges towards retinal neoangiogenesis. In fact, in this condition, the activation of hypoxia-inducible factors (HIFs) promotes the choroidal endothelial cells transmigration across the RPE into neurosensory retina, where they proliferate into new vessels under stimulation of the vascular endothelial growth factor (VEGF)

(Russell et al., 2000; Skeie and Mullins, 2009). This final event contributes to maintenance of retinal disruption, because the aberrant vessels induce mechanical damage to the internal BRB during the proliferative stage of DR (Campochiaro et al. 2013; Das et al. 2003; Simo et al., 2008).

The primary response to hypoxia, is mediated via the induction of transcriptional regulator HIFs (Lin et al., 2011; Wright et al., 2010). They are constituted by heterodimeric complexes consisting of an oxygen labile α-subunit, and a stable nuclear β-subunit (HIF-1β/ARNT) (Semenza et al., 1996; Semenza et al., 1999; Maynard et al. 2007). Humans express three HIF-α genes: HIF-1α, HIF-2α and HIF-3α. Among these, HIF-1α is the key modulator of cellular response to low oxygen tension. During hypoxic process, HIF-1α dimerizes with HIF-1β, translocates to the nucleus, and it induces the transcription of a wide variety of genes, including VEGF.

The production and secretion of VEGF is promoted in various cells, including RPE (Wang et al., 2011; Geisen et al., 2006; Forooghian et al., 2007; Xiao et al., 2006; Ikeda et al., 2006). The peptide actions are mediated through its binding to VEGF 1 (VEGFR-1 or Flt-1) or 2 (VEGFR-2 or KDR/Flk-1) receptors, which trigger the activation of p38 mitogen-activated protein kinase (MAPK) pathway (Shibuya et al., 1990; Holmes et al., 2007; Gee et al., 2010). This signaling cascade is one of the three major MAPK pathways implicated in vascular hyperpermeability in diabetic retinas, but also in VEGF-induced permeability and damage of RPE cells (Poulaki et al., 2002; Miranda et al., 2012).

In light of these evidences, the identification of molecules able to regulate the mechanisms described above, might open new therapeutic perspectives to treatment of DME. In the last years, different studies have focused on the role of two peptides, pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal

peptide (VIP), in retinal dysfunction during DR (D'Agata et al., 1998; Atlasz et al., 2010; Giunta et al., 2012; D'Amico et al., 2015; Danyadi et al., 2014).

PACAP and VIP belong to the secretine, glucagon, and histidine-isoleucine (PHI) superfamily peptides. They have sequence homology of about 80%, and explicate their functions through the activation of vasoactive intestinal peptide receptors (VIPRs) including PAC1, VPAC1, and VPAC2 receptors (Arimura and Shioda, 1995). Particularly, PAC1 receptor binds PACAP with high affinity rather than VIP, instead VPAC1 and VPAC2 receptors (VPAC1R, VPAC2R) bind both peptides with similar affinities (Harmar et al., 2012). PACAP and VIP are largely distributed in different organs and tissues, where they play cytoprotection (D'Agata and Cavallaro, 1998; Castorina et al. 2008; Giunta et al. 2010; Castorina et al. 2012; Maugeri et al., 2016; Castorina et al., 2010). In particular, several studies have suggested a beneficial role of PACAP against retinal injuries, including DME (Giunta et al. 2012; Szabadfi et al., 2014; Fabian et al., 2012; Szabo et al., 2012; D'Amico et al., 2015).

In our recent study, we have shown that both peptides prevent outer BRB damage in a model of DME, through the activation of phosphoinositide 3 kinase (PI3K)/Akt and mammalian mitogen activated protein kinase/Erk kinase (MAPK/ERK) signaling pathways (Maugeri et al., 2017).

To deeply characterize their action mechanism, in the present work, we have investigated if PACAP and VIP are able to protect retina against hyperglycemic/hypoxic insult characterizing DME, by modulating HIFs and indirectly VEGF expression. Furthermore, in order to get insight into the mechanism exerted by these peptides against hyperglycemic/hypoxic insult, we have also analyzed the activation of pro-apoptotic pathway p38 MAPK. Indeed, VEGF through the interaction with its VEGF receptors, induces the activation of this signaling pathway in ARPE-19 cells following exposure to hyperglycemia and hypoxia

(Shibuya et al., 1990; Holmes et al., 2007; Gee et al., 2010). Furthermore, previous studies, have shown that PACAP performs its protective effect in the retina, by inhibiting the phosphorylation of p38 protein (Szabo et al., 2012; Fabian et al., 2012).

Our results have suggested that both peptides exert retinoprotective effect in ARPE19 cells exposed to hyperglycemia/hypoxia by modulating HIFs, VEGF and its receptors expression and activation of p38 MAPK pathway.

In conclusion, this study allow to further clarify the mechanisms mediating the beneficial effects of PACAP and VIP on retinal damage during DME progression.

MATERIALS AND METHODS

Human RPE Cell Cultures

This study was performed on human RPE cell culture (ARPE-19) purchased from the American Type Culture Collection (Rockville, MA, USA), as previously described by Scuderi et al. (2013). Briefly, the cells were cultured in T75 flasks using DMEM-F12 (1:1) medium supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, USA), 1 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Sigma Aldrich). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5%CO2 and sub-cultured once a week. Cells were cultured for 7 days on transwell-clear permeable supports (Corning Costar, Cambridge, MA) at a density of 1.5 x 105cells/cm2 in normal glucose condition (NG, 5.5 mM D-glucose). Subsequently, half of the transwells were maintained with 5.5 mM D-glucose (control) and the other half were switched to high glucose conditions (HG, 25 mM D-glucose) for a further 7 days. On the last week of the experiments, cells were exposed to hypoxia for 24 hours by treating them with 100 μM desferrioxamine mesylate salt (DFX) (Sigma-Aldrich), a hypoxia-mimetic agent, previously described

(Maugeri et al., 2016), alone or in combination with 100 nM PACAP or 100 nM VIP (Sigma Aldrich, USA). To rule out a potential bias by an osmotic effect, the control cells were also 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.

Western Blot Analysis

Western blot analysis was performed according to the procedures previously described by Maugeri et al., (2016). 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 sec using an ultrasonic probe, followed by centrifugation at 10,000 x g for 10 min at 4 °C. Protein concentrations were determined by the Quant-iT Protein Assay Kit (Invitrogen, Carlsbad, CA, USA). About 25 μg of protein homogenate were diluted in 2X Laemmli buffer (Invitrogen), heated at 70°C for 10 min and then separated on a Biorad Criterion XT 4-15% Bis-tris gel (Invitrogen) by electrophoresis and then transferred to a nitrocellulose membrane (Invitrogen). Blots were blocked using the Odyssey Blocking Buffer (Li-Cor Biosciences, Nebraska, USA). The transfer was monitored by a prestained protein molecular weight marker (BioRad Laboratories, Segrate, MI, Italy). Immunoblot analysis was performed by using appropriate antibodies: mouse anti- HIF-1alpha (cat.n. NB100-105, Novus Biologicals, 1:500), rabbit anti-HIF-3alpha (H-170) (cat n.sc-28707, Santa Cruz Biotechnology; 1:200), goat anti-VEGF (cat n.sc-1836, Santa Cruz Biotechnology; 1:200), rabbit anti-VEGF-R1 (cat n.sc-316, Santa Cruz Biotechnology; 1:200), mouse anti- VEGF-R2 (cat n.sc-6251, Santa Cruz Biotechnology; 1:200), rabbit anti-p-p38 (cat n.sc-17852-R, Santa Cruz Biotechnology; 1:200), rabbit anti-p38α/β (cat n.sc-7149, Santa Cruz Biotechnology; 1:200) and rabbit anti-β-tubulin (cat n.sc-9104, Santa Cruz

Biotechnology; 1:500). The secondary antibody goat anti-rabbit IRDye 800CW (cat #926-32211; Li-Cor Biosciences) and goat anti-mouse IRDye 680CW (cat #926-68020D, Li-Cor Biosciences) were used at 1:20000 and 1:30000, respectively. Blots were scanned with an Odissey Infrared Imaging System (Odyssey). Densitometric analyses of Western blot signals 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-tubulin, which served as loading control, as previously described by Maugeri et al., (2015).

Immunolocalization

To determine the cellular distribution of HIF-1α proteins, immunofluorescence analysis was performed on ARPE-19 cells as previously described by Maugeri et al., (2016). Cells cultured on glass cover slips, were fixed in 4% paraformaldehyde in PBS (15' at room temperature), permeabilized with 0.2% Triton X100, blocked with 0.1% BSA in PBS, and then probed with anti-HIF-1alpha (1:50) antibody. Signals were revealed with Alexa Fluor 594 goat anti-mouse for 1.5 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, Inc., Burlingame, CA, USA). Immunolocalization was analyzed by confocal laser scanning microscopy (CLSM; Zeiss LSM700). Red and blue signals were detected with laser 594nm/10mW and 405nm/5mW respectively, and using the objective "PLAN-APOCHROMAT" 63X/1,40 OIL DIC M27. Each scanning was individually digitalized by an high sensitivity PMT using the following acquisition setup: Gain master: 776; digital offset: -202; digital gain: 1.0. All acquisitions were performed with ZEN-2010 software.

Statistical analysis

Data are represented as mean ± standard error (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 set at $p \leq 0.05$.

RESULTS

PACAP and VIP treatment interfere with HIF-1α and HIF-3α expression in ARPE-19 cells exposed to hyperglycemia/ hypoxia

In this study, we have firstly evaluated the effect of PACAP or VIP on HIF-1α and HIF-3α expression. As shown in Fig.1 and Fig.2, when cells are grown in normal (NG) or high glucose (HG), HIF-1α is weakly expressed, whereas HIF-3α levels increase. Instead, in cells exposed to hyperglycemia/hypoxia, the expression pattern of these proteins is completely reversed (***$p<0.001$ vs HG). Finally, PACAP or VIP treatment significantly decreases HIF-1α and increases HIF-3α expression as compared to cells grown in presence of high glucose and desferoxamine (###$p<0.001$ vs HG+DFX).

These results have been also confirmed through the evaluation of cellular distribution of HIF-1α by immunofluorescence analysis. As shown in Fig. 1B, HIF-1α is highly expressed either in the cytoplasm or nucleus of ARPE-19 cells grown in high glucose and DFX. The treatment with PACAP or VIP significantly has reduced its levels in both intracellular compartments.

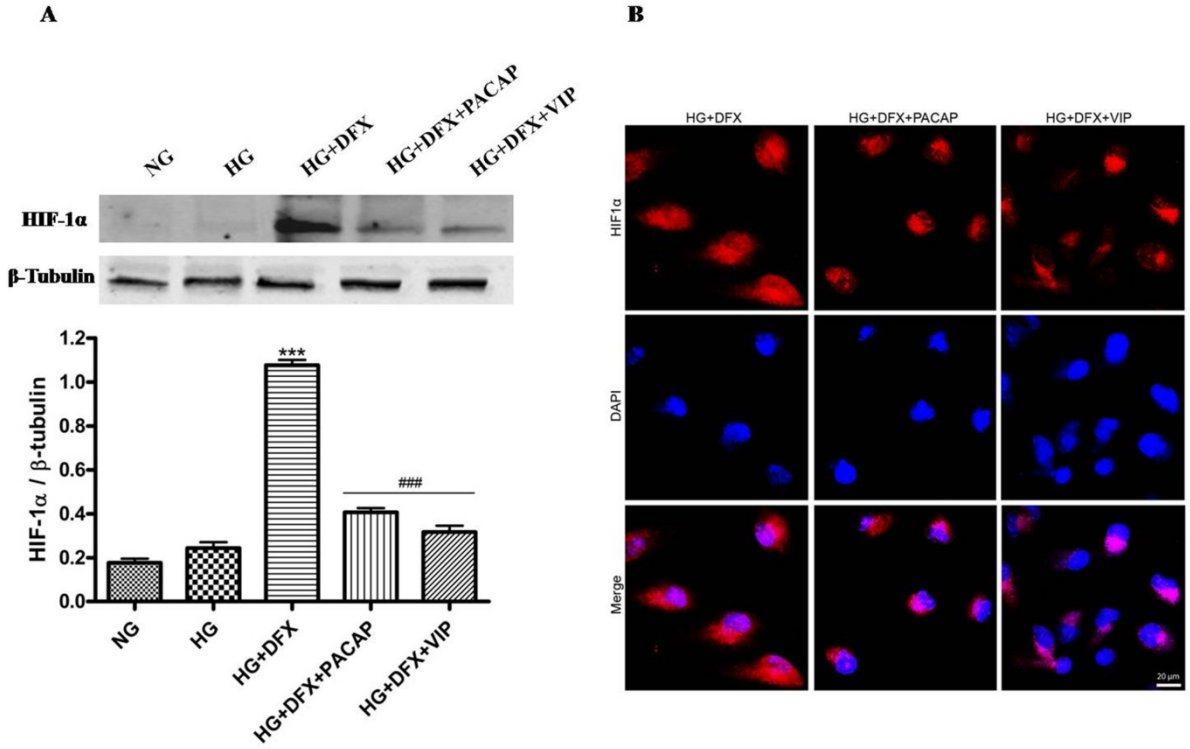

Figure 1. Effect of PACAP and VIP on HIF-1α expression and distribution in ARPE-19 cells exposed to hyperglycemia/ hypoxia. A) Representative immunoblots of HIF-1α expression in ARPE19 cells cultured in 5.5 mM d-glucose + 19.5 mM mannitol (NG) or 25 mM d-glucose (HG) or HG plus desferrioxamine mesylate salt (HG+DFX) or HG + DFX + PACAP (100 nM) or HG + DFX + VIP (100 nM). The bar graph shows quantitative analysis of signals obtained by immunoblots resulting from three independent experiments. Relative band densities were quantified by using ImageJ software. Protein levels are expressed as arbitrary units obtained following normalization to β-tubulin, which was used as loading control. Data represent means ± SEM. ($^{***}p<0.001$ vs HG or NG; $^{###}p<0.001$ vs HG+DFX, as determined by one-way ANOVA followed by the Tukey post hoc test). B) Representative photomicrographs showing HIF-1α expression (red) in ARPE19 cells cultured in HG+DFX or HG + DFX + PACAP or HG + DFX + VIP. Nuclei were stained with DAPI. Photomicrographs are representative results of fields taken randomly from each slide and scanned by confocal laser scanning microscopy (CLSM; Zeiss LSM700).

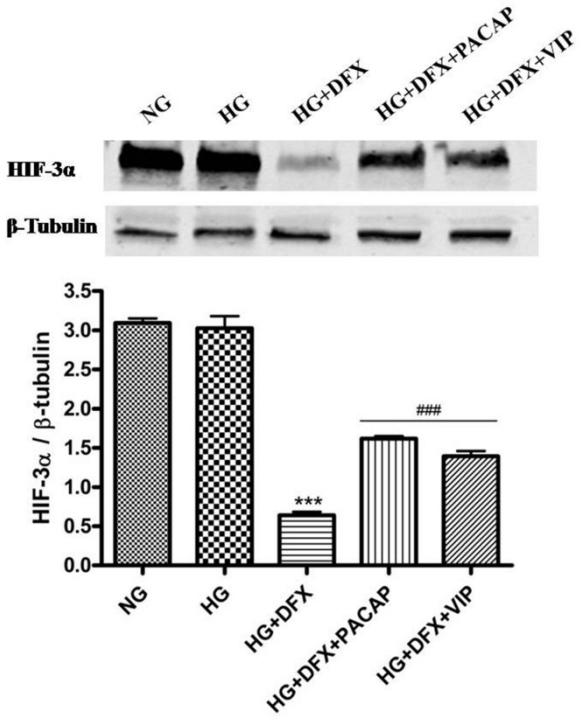

Figure 2. Effect of PACAP and VIP on HIF-3α expression in ARPE-19 cells exposed to hyperglycemia/ hypoxia. A) Representative immunoblots of HIF-3α expression in ARPE19 cells cultured in 5.5 mM d-glucose + 19.5 mM mannitol (NG) or 25 mM d-glucose (HG) or HG plus desferrioxamine mesylate salt (HG+DFX) or HG + DFX + PACAP (100 nM) or HG + DFX + VIP (100 nM). The bar graph shows quantitative analysis of signals obtained by immunoblots resulting from three independent experiments. Relative band densities were quantified by using ImageJ software. Protein levels are expressed as arbitrary units obtained following normalization to β-tubulin, which was used as loading control. Data represent means ± SEM. (***p<0.001 vs HG or NG; $^{###}$p<0.001 vs HG+DFX, as determined by one-way ANOVA followed by the Tukey post hoc test).

PACAP and VIP modulate expression of VEGF and its receptors in ARPE-19 cells exposed to hyperglycemic/hypoxic insult

To assess whether the effect of PACAP and VIP on HIFs changes is related to modulation of pro-angiogenic factor levels VEGF, the expression of this peptide and its receptors has been evaluated in ARPE-19 cells exposed to hyperglycemic/hypoxic insult. As expected, VEGF expression is weakly expressed in NG control cells, instead its levels are significantly increased in cells grown in HG ($^{\$\$}$p<0.01 vs NG) (Figure 3). Its levels have furtherly increased following hyperglycemia/hypoxia exposure (***p<0.001 vs HG), however peptides treatment has significantly reduced

its expression by restoring HG control values ($^{###}p < 0.001$ vs HG + DFX). Similarly, the expression of VEGF receptors is significantly increased in cells grown in presence of high glucose and DFX ($^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ vs NG; $^{*}p<0.05$ and $^{***}p<0.001$ vs HG) whereas PACAP or VIP treatment of cells exposed to hyperglycemia and hypoxia is able to significantly reduce their levels ($^{###}p<0.001$ vs HG+DFX) (Figure 3).

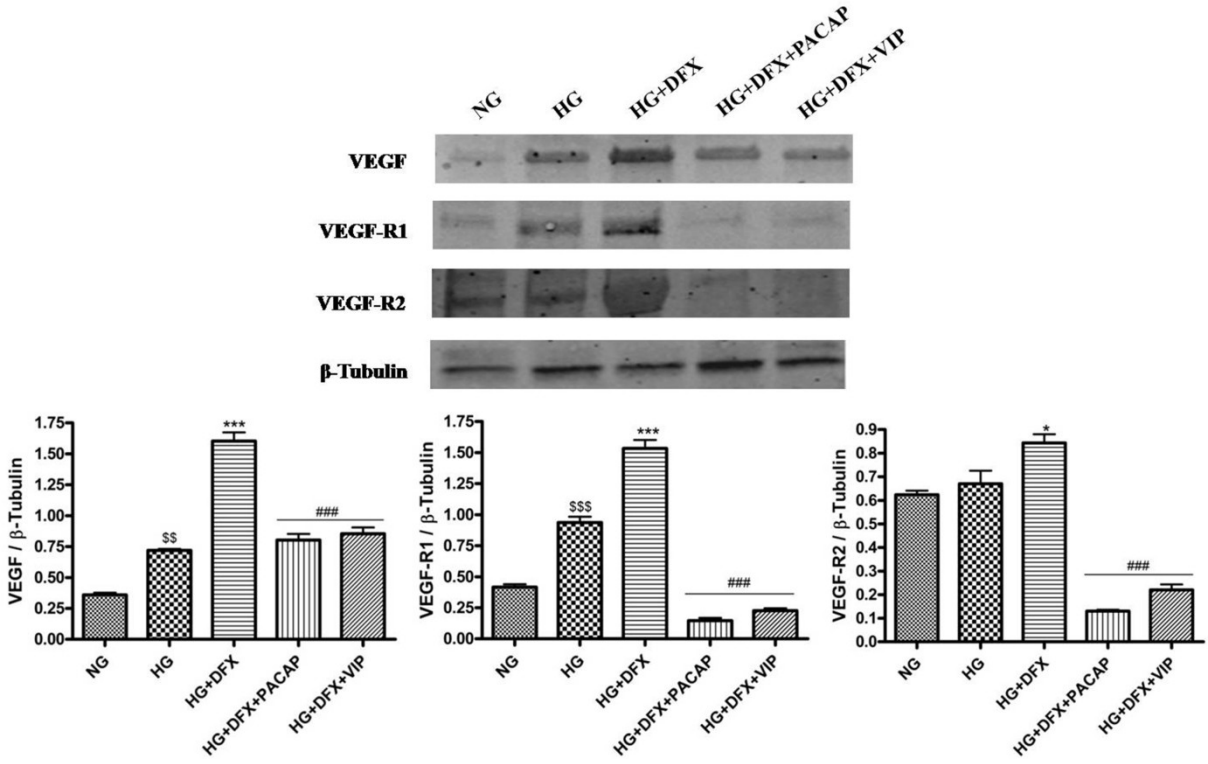

Figure 3. PACAP and VIP treatment interferes with VEGF and its receptors expression in ARPE-19 cells exposed to hyperglycemia/ hypoxia. Representative immunoblots of VEGF, VEGF-R1 and VEGF-R2 expression in ARPE19 cells cultured in 5.5 mM d-glucose + 19.5 mM mannitol (NG) or 25 mM d-glucose (HG) or HG plus desferrioxamine mesylate salt (HG+DFX) or HG + DFX + PACAP (100 nM) or HG + DFX + VIP (100 nM). The bar graph shows quantitative analysis of signals obtained by immunoblots resulting from three independent experiments. Relative band densities were quantified by using ImageJ software. Protein levels are expressed as arbitrary units obtained following normalization to β-tubulin, which was used as loading control. Data represent means ± SEM. ($$p<0.01 and $$$p<0.001 vs NG; *p<0.05 and ***p<0.001 vs HG; ###p<0.001 vs HG+DFX, as determined by one-way ANOVA followed by the Tukey post hoc test).

Effect of PACAP and VIP on hyperglycemia/hypoxia-induced p38 MAPK activation in ARPE-19 cells

To confirm the indirect modulatory role performed by PACAP and VIP on VEGF levels, we have analyzed their activity on regulation of p38 MAPK signaling pathway, which is activated by VEGF-R receptors. To this end, we have investigated the effect of peptides treatment on the phosphorylation of p38 in ARPE-19 cells after exposure to hyperglycemic/hypoxic insult. As shown in Figure 4, this protein is significantly activated in cells exposed to hyperglycemia, or hyperglycemia/hypoxia-treated cells as compared to controls ($$$p<0.001 vs NG; ***p<0.001 vs HG). Following treatment with PACAP or VIP, its phosphorylation is significantly decreased as compared to cells grown under hyperglycemia/hypoxia ($^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 vs HG+DFX).

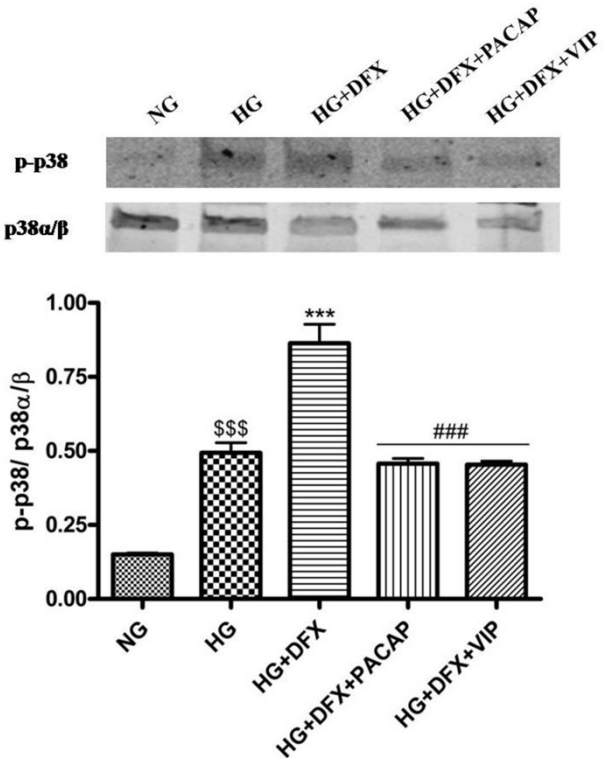

Figure 4. PACAP and VIP treatment prevents hyperglycemia/ hypoxia-induced p38 MAPK phosphorylation in ARPE19 cells. Representative immunoblots of p38 MAPK phosphorylation expression in ARPE19 cells cultured in the presence of 5.5 mM d-glucose + 19.5 mM mannitol (NG)

or 25 mM d-glucose (HG) or HG+ DFX (100 μM) or HG + DFX + PACAP (100 nM) or HG + DFX + VIP (100 nM). The bar graph show quantitative analysis of signals obtained by immunoblots resulting from three independent experiments. Relative band densities were quantified by using ImageJ software. Protein levels are expressed as arbitrary units obtained following normalization to total p38 which was used as loading control. Data represent means ± SEM ($^{\$\$\$}p<0.001$ vs NG; $^{***}p<0.001$ vs HG; $^{\#\#\#}p<0.001$ vs HG+DFX, as determined by one-way ANOVA followed by the Tukey post hoc test).

DISCUSSION

Different papers have suggested that PACAP and VIP through the binding of VIPRs perform a protective role against several types of retinal injuries, including DME, which is the leading cause of blindness in the diabetic population (Yonekura et al., 2005). In our recent paper, we have demonstrated that these peptides through the interaction with their own receptors prevent the damage to the outer BRB by activating two protective signaling pathways: the PI3K/Akt and the MAPK/ERK (Maugeri et al., 2016). In the present study, we have furtherly deepened the characterization of the mechanism exerted by these peptides on RPE, when this latter is exposed to the hyperglycemia/hypoxia insult, a condition mimicking tightly the microenvironment which triggers the DME pathology.

The hyperglycemia damages the blood vessels and leads, long term, to vascular dysfunction and occlusion. As consequence, the hypoxic microenvironment induces the activation of HIFs. These latter upregulate in the affected tissues, the production of some growth factors, mainly the VEGF, which is produced in the eye, not only by RPE, but also ganglion, Müller, perycites, endothelial, glial, neuronal and smooth muscle retinal cells. The VEGF action on small blood vessels induces leakage of fluid into the retina and obliterates capillaries, causing local regions of extra hypoxia and further increasing VEGF production in a vicious cycle (Wang et al., 2010; Aiello et al., 2000). In light of these evidences, to restore the normal retinal environment by

preventing tissue damage, strategies against hyperglycemia/hypoxia inducing retinal cell damage are needed.

Our results have shown that hypoxia induces an upregulation of HIF-1α, the primary player in cellular response to low oxygen tension. This evidence is in accord to previous papers showing that under hypoxia, this factor dimerizes with β-subunit, translocates to the nucleus and transactivate their target genes (Semenza et al., 1996; Hu et al., 2003). Conversely, here, we demonstrate that PACAP or VIP treatment is able to reduce not only its protein expression (Fig. 1A), but also its translocation to the nuclear compartment as demonstrated by immunofluorescence analysis (Fig. 1B). The expression profile of HIF-1α is usually inversed as compared to HIF-3α levels. In our study, the expression of the third member of HIFs family is significantly reduced in cells exposed to hyperglycemia/hypoxia as compared to cells grown in NG or HG (Fig.2). This evidence is in agreement with previous data showing that under normal oxygen tension, HIF-3α competes with HIF-1α to form the heterodimer complex with HIF-1β and consequently, it inhibits HIF-1α activity, whereas under hypoxia, the increased levels of HIF-1α overwhelms the modulatory effect of HIF-3α (Makino et al., 2002; Maynard et al., 2007; Yang et al., 2015). The treatment with PACAP or VIP is able to significantly revert the HIFs profile, when RPE cells are exposed to high glucose and desferoxamine. Hypoxia, through the activation of HIF-1α stimulates VEGF expression by increasing its gene transcription and stabilizing its mRNA (Treins et al., 2001). In accord to previous papers, in ARPE-19 cells exposed to hyperglycemia/hypoxia, is increased expression of VEGF and its receptors. Furthermore, in this study, we have demonstrated that PACAP and VIP, through the modulation of HIFs, interfere with VEGF and its receptors expression when ARPE-19 cells are exposed to hyperglicemic/hypoxic insult. The previous data regarding PACAP effect on VEGF expression in retina are controversial in relation

to the experimental condition considered. Indeed, Szabo et al. (2012), have reported that this peptide increases VEGF expression in rats underwent to retinal hypoperfusion injury, instead, more recently, Amato et al., (2016), have shown that in the early phase of DR, PACAP decreases VEGF production in the retina limiting the risk, in the long term, of pathologic angiogenesis. In accord to this latter paper, we have shown that in RPE cells exposed to hyperglycemia/hypoxia, PACAP or VIP treatment is able to drastically reduce VEGF, VEGF-R1 and VEGF-R2 expression (Fig.3). Although further studies are needed, we hypothesize that these peptides could act through a direct downregulation of HIF-1α expression, and indirectly, through this mechanism, they modulate VEGF and its receptors expression. VEGF, through the interaction with VEGFR-1 and 2, induces the activation of p38 MAPK signaling pathway. This signaling cascades is involved in triggering ARPE-19 cell damage induced by hyperglycemia/hypoxia (Shibuya et al., 1990; Holmes et al., 2007; Gee et al., 2010; Miranda et al., 2012). Moreover, previous studies have shown that PACAP decreases the activation of p38 MAPK following to retinal injuries (Szabo et al., 2012; Szabadfi et al., 2014). Our results have shown that hyperglycemia alone or in combination with hypoxia increases phosphorylation of p38 protein. Instead, the treatment with PACAP or VIP reduces its activation and, therefore, inhibits this signalling pro-apoptotic pathway (Fig.4).

In conclusion, here, we further provided new insight to better understand the protective role of these peptides on the DME progression.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

These experiments were supported by the international PhD program in Neuroscience, University of Catania, Medical School.

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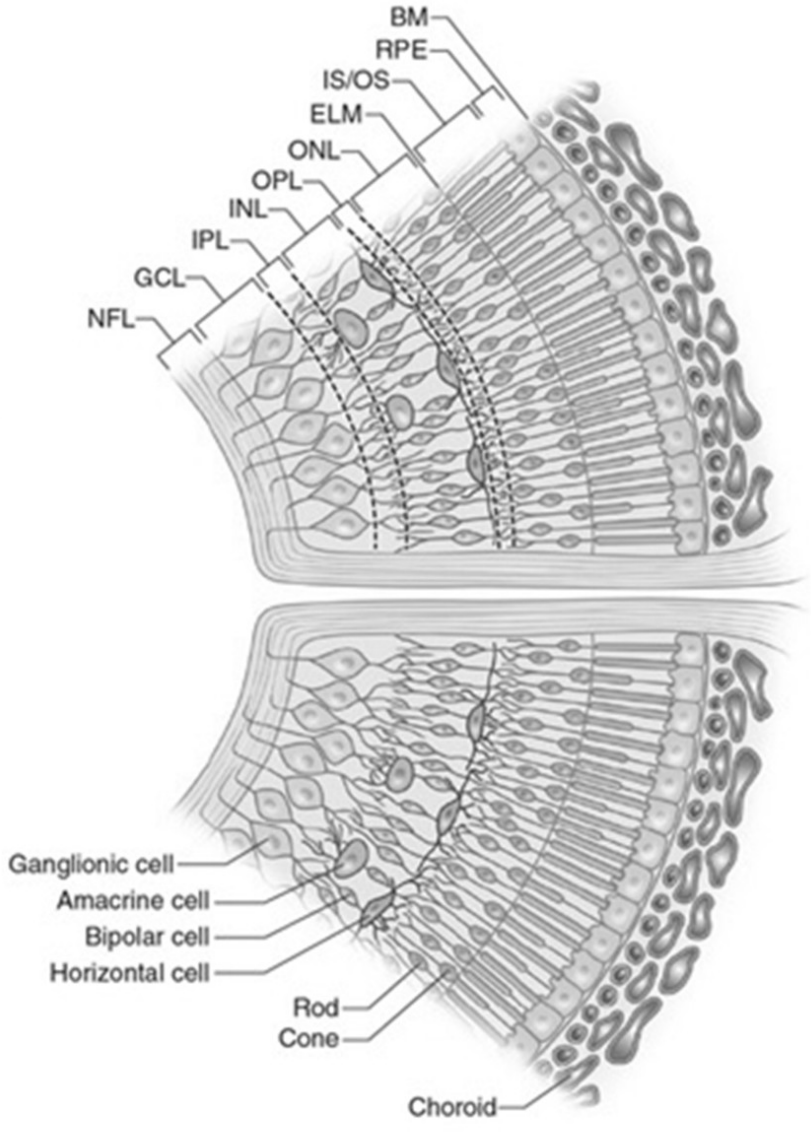
CHAPTER 6

Concluding remarks



GENERAL DISCUSSION AND CONCLUSION

DR is one of the most significant and disabling chronic complications of diabetes mellitus. The hallmarks of this disease are hypoxic-ischemic insult and metabolic alteration causing endothelial cells loss and blood vessels damage leads to increased vascular permeability and breakdown of the blood–retina barrier (Aiello et al. 1998). However, a growing body of evidence suggest that DR also results in damage to nonvascular cells of the retina, which can precedes vascular cell changes. Apoptotic death of retinal ganglion cells (RGC) and amacrine cells occurs in diabetic animal models and it has been also identified in postmortem diabetic eyes (Barber et al., 1998; Carrasco et al., 2007). Moreover, studies of structural changes have proved a reduction in thickness of the inner retinal layers in type 1 diabetics with minimal diabetic retinopathy (van Dijk et al., 2009 and 2010). The neurosensory retina alterations could contribute to the progression of vascular DR pathology. Indeed, retinal neurons, including photoreceptors, may be an important source of oxidative stress driving the proinflammatory environment in DR (Du et al., 2013).

In the current scenario, the focus of the pharmacotherapy against the development and progression of DR is still mainly concentrated on anti-VEGF agents. However, this approach is effective only in some patients. Therefore, the identification of molecules able to counteract other factors involved in DR pathogenesis could represent a valid alternative for non-responders'patients (Brown et al., 2006; Rey and Semenza, 2010).

Works from others and our laboratory have indicated that PACAP and VIP are able to counteract some pathological alterations that occur during retinal injuries (Giunta et al., 2012; Scuderi et al., 2013; Atlasz et al. 2010). Indeed, these peptides induce a

slight improvement of retinal morphology during glutamate-induced damage, inhibit activation of pro-apoptotic pathway through downregulation of p53 expression and upregulation of Bcl2 level in diabetic rats, and reduce GCL loss in rat retinas in the early stage of hyperglycemia (Kiss et al. 2006; Atlasz et al. 2008; Giunta et al. 2012; Szabadfi et al., 2012). Moreover, PACAP treatment attenuates photoreceptor degeneration observed in 1-month diabetic retinas (Szabadfi et al., 2016), and it ameliorates the vascular changes in the animal model of retinopathy of prematurity (Kvarik et al., 2016).

However, data regarding the role of PACAP and VIP against the harmful combined effect of hyperglycaemic/hypoxic insult have not been reported, yet.

In Chapters 2 of this PhD thesis, by using an *in vivo* model of early stages of hyperglycaemia, we have demonstrated that PACAP exerted a modulatory role on hyperglycemia-linked hypoxic event, acting directly on HIF-1α expression and, indirectly, through the regulation of HIF-3α, which in turn downregulates HIF-1α levels.

We have also showed in Chapter 3 that a single intraocular administration of peptide attenuated the early phase of DR, by regulating downstream target genes of hyperglycemic/hypoxic microenvironment such as IL-1β, VEGF and its related receptors. Noteworthy, for the first time it has been shown that PACAP treatment counteracted VEGFR2 overexpression found in retinal layer of diabetic rats.

In Chapter 4 and 5, we have further characterized the protective role of PACAP and VIP in an experimental *in vitro* model of outer BRB during the hyperglycaemic/hypoxic insult that closely mimics the *in vivo* condition occurring in DME. The obtained data have suggested that both peptides improved barrier integrity, by increasing the contact between adjacent retinal epithelial cells,

preventing hyperglycemia/hypoxia-induced apoptotic cell and by modulating HIFs, VEGF and its receptors expression. All of these PACAP/VIP-mediated effects are due to activation of PI3K/Akt and MAPK/ERK survival signaling pathways, and inhibition of pro-apoptotic pathway p38 MAPK.

Although the incidence of DR continues to increase, the past decade has seen the emergence of novel treatment options, which have greatly improved DR prognosis. Nevertheless, a pressing need remains for efficacious new treatments for all stages of DR.

A complete characterization of the pathological event triggered by hyperglycemia and leading to hypoxia/inflammation may open new therapeutic perspective.

Here, we have provided evidences regarding the protective effect of PACAP and VIP in the early phase of DR, suggesting that they may be attractive candidates to counteract these pathological events.

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Professional Honors & Recognitions

Best Oral Presentation Award - 69° Congresso Nazionale S.I.A.I. - Società Italiana di Anatomia e Istologia, 2015, Ferrara, Italy

Conference proceedings

Oral communication

1. **Maugeri G**, D'Amico AG, Rasà DM, Federico C, Saccone S, Cavallaro S, D'Agata V. (2018) Molecular mechanism of PACAP-induced EGFR transactivation in an in vitro model of ALS. 72° CONGRESSO NAZIONALE SIAI Società Italiana di Anatomia Umana e Istologia. Parma 20-22 Settembre 2018.

2. **Maugeri G**, Reitano R, Cavallaro S, D'Agata V. (2016) PACAP and VIP counteract glioblastoma cells invasiveness. 70° CONGRESSO NAZIONALE SIAI Società Italiana di Anatomia Umana e Istologia. Roma 15-17 Settembre 2016.

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Poster

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