# Role of MAP kinases and PI-3-kinase/Akt pathway in the regulation of retinal degeneration

**Ph.D. thesis**

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**Pécs**

**2010**

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**LIST OF ABBREVIATIONS**

| | |
|---|---|
| AIF | apoptosis inducing factor |
| AMD | age-related macular degeneration |
| AMPA | aminomethyl-propionic-acid |
| ARPE-19 | human pigment epithelium cell line |
| BCCAO | bilateral common carotid occlusion |
| DRP | diabetic retinopathy |
| ERK | extracellular signal-regulated kinase |
| FCS | fetal calf serum |
| GCL | ganglion cell layer |
| GSK | glycogen synthase kinase |
| H2O2 | hydrogen-peroxid |
| HEPES | N-2-hydroxyethyl piperazine-N'-2-ethansulfonic acid |
| ILM | internal limiting membrane |
| INL | internal nuclear layer |
| IPL | internal plexiform layer |
| JC-1 | 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcar- bocyanine iodide |
| JNK | c-Jun-N-terminal kinase |
| DMEM | Dulbecco's modified Eagle's medium |
| LDL | low density lipoprotein |
| MAPK | mitogen activated protein kinase |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide |
| NAD | nicotinamid adenin dinukleotid |
| NFkB | nuclear factor kappa B |
| NMDA | N-metil-D-aspartat |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| OLM | outer limiting membrnae |
| OPL | outer plexiform layer |
| PACAP | pituitary adenylate cyclase activating polypeptide |
| PAR | poly(ADP-ribose) |
| PARP | poly(ADP-ribose) polymerase |
| PI-3K | phosphatidylinositol 3-kinase |

| | |
|---|---|
| ROS | reactive oxygen species |
| PI | propidium iodide |
| FITC | fluorescein isothiocyanate |
| RPE | retinal pigment epithelium |
| VEGF | vascular endothelial growth factor |
| VIP | vasoactive intestinal peptide |

# 1. INTRODUCTION

There are currently 45 million people blind and 135 million people with low vision. The global blindness prevalence was estimated to be 0.7 % in 1990. Of this global burden of blindness 90 % is born by developing countries and 80 % is avoidable (preventable or treatable) with applying existing knowledge and technology. Blindness is also more prevalent in the older age groups, largely as a result of non-communicable diseases. The number of blind increases every year by 2 million and is expected to double by the year 2020 (Cunninghem et al. 2001, Resnikoff et al. 2001, Thylefors et al. 2001).WHO data on blindness shows that except for developed countries, in spite of the progress made in surgical techniques in many countries during the last ten years, cataract (47.9%) remains the leading cause of visual impairment in all areas of the world. Other main causes of visual impairment in 2002 are glaucoma (12.3%), age-related macular degeneration (AMD) (8.7%), corneal opacities (5.1%), diabetic retinopathy (DRP) (4.8%), childhood blindness (3.9%), trachoma (3.6%), and onchocerciasis (0.8%). The causes of avoidable blindness such as primary cataract (50%), glaucoma (15%), corneal opacities (10%), trachoma (6.8%), childhood blindness (5.3%) and onchocerciasis (4%) are more common in the least-developed countries (such as countries of Sub-Saharan Africa) (Roodhoft et al 2002, www.who.int/blindness 2010), while in the developed countries (such as UK and USA) the most commonly recorded causes of blindness were degeneration of the macula and posterior pole which largely comprises AMD, glaucoma and DRP (Bunce et al. 2006, www.nlm.nih.gov/medlineplus/magazine/issues/summer08 2008).
It is remarkable that these latter-mentioned diseases (AMD, glaucoma, DRP) are all the diseases of the retina which is the most vulnerable tissue in the eye. Thus we can say that the main causes of visual impairment in the developed countries are the diseases affecting the retina.

Ischemia and oxidative stress alone or as a part of ischemia can be found in the pathogenesis of several disease affecting large populations in developed countries. Such diseases are particularly the cardiovascular, neurovascular diseases and neurodegenerative disorders. From the aspect of ophthalmology, various ocular and systemic diseases that lead to visual impairment or blindness (e.g., central retinal artery occlusion, ophthalmic ischemic syndrome, diabetic retinopathy, hypertension, glaucoma and AMD) are accompanied by retinal ischemia (Uckermann et al. 2005, Osborne et al 2004, Feigl 2009). Furthermore, the light absorption of the retina generates increased formation of oxidative/nitrosative agents, which may cause retinal injury as it can be observed in a vision-threathening retinal disease, the age-related macular degeneration (AMD) (Liang et al 2003, Winkler et al 1999, Cai et al 2000, Jarret et al 2008).

It is noticeable that ischemia and oxidative stress both can be found in the pathogenesis of diseases leading the “toplist” of blindness in the developed world (AMD, glaucoma, DRP). Hence, it is of utmost importance to understand the events involved in retinal injury caused by ischemia and/or oxidative stress, both from the pathological and the potential therapeutic point-of-view.

## 1.1. Retina

Embriologically the retina belongs to the central nervous system. Anatomically it is a very thin (180-560 micrometer), delicate, and transparent membrane, with a surface area of approximately 266 $mm^2$. The retina is localised between the choroid and vitreous body and consists of two distinct layers: the neurosensory retina and the retinal pigment epithelium (Fig.1.). The dual blood supply of the retina also reflects its embryological origins and its rightful inclusion as a specialised component of the central nervous system. The main function of the retina is transforming the light-stimuli from the outside world into a nerve impulse that reaches the brain via the optic nerve.

The retina is loosely attached to the choroid via the retinal pigment epithelium (RPE), that consists of a monolayer of hexagonal cells. The main functions of the RPE are: vitamin A metabolism, maintenance of the outer blood-retinal barrier, phagocytosis of the photoreceptor outer segments, absorption of light, heat exchange, formation of the basal lamina, production of the muccopolysaccharide matrix surrounding the outer segments, and active transport of materials in and out of photoreceptors (Grünwald 2009).

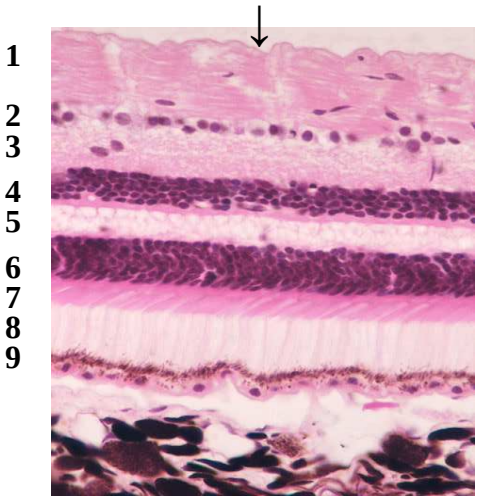


**Fig.1**. Light micrograph of human peripheral retina including portion of the choroid (HE, 40× objective, University of Pécs, Department of Anatomy). Inner limiting membrane (arrow): (1) nerve fiber layer, (2) ganglion cell layer, (3) inner plexiform layer, (4) inner nuclear layer, (5) outer plexiform layer, (6) nuclei of photoreceptors (outer nuclear layer), (7) rod and cone inner segments, (8) rod and cone outer segments, (9) pigment epithelium

## 1.2. Ischemia

The word ischemia was coined by Virchow, who combined the Greek iskho, meaning ‘‘I hold back’’, with háima, meaning ‘‘blood’’. Hence, ischemia refers to a pathological situation involving an inadequacy (not necessarily a complete lack of) blood flow to a tissue, with failure to meet cellular energy demands.

Retinal ischemia ensues when the retinal circulation is insufficient to meet the metabolic demands of the retina, the highest demands of any tissue (Cohen and Noell 1965, Anderson and Saltzman 1964; Ames 1992). It may be caused by general circulatory failure such as severe left ventricular failure and hypovolaemic shock, or more commonly by local circulatory failure. (Recchia et al. 2000, Brown 1991, Collaborators 1997, Jeffries et al. 1993, Robinson et al. 1988)

In practice, the clinicians observe the morphological alterations and determine the rate of function loss. Altough there are different signs of retinal ischemia depending on the strength and duration of the ischemic event, in general the main pathological features are the following: the loss of normal transparency, pallor or opacification, cotton wool spots, proliferative and degenerative changes (von Graefe 1859, Ashton and Harry 1963, Grünwald 2009). Parallel with the morphological changes there are functional alterations as well (e.g., worsening of visual acuity, ERG changes and defects of visual field) (Grozdanic et al. 2003, Chui et al. 2009). These functional and morphological changes are the consequence of retinal cell injury caused by ischemia.

The ischemic injury reflects the effect of a self-reinforcing destructive cascade called “ischemic cascade”, which is an extremely complex (not completely understood) succession or cascade of interrelated pathological changes and biochemical responses at the cellular and molecular level initiated by energy failure.

From the aspect of ischemia there are striking differences between the retina and the brain, although they have common embriological origin and shares many functional and structural characteristics. These striking differences are the relative resistance of the retina to an ischemic insult compared to the brain and the regionalised sensitivity of the retina to ischemia, with the outer layers less sensitive than the inner layers. This may reflect the peculiar metabolism and unique enviroment of the retina and the variability in the balance of excitatory and inhibitory neurotransmitter receptors on a given retinal cell (Osborne et al 2004).

Altough knowledge of ischemic cascade derives mainly from studies on brain tissues (Kristian and Siesjo 1998; Lee et al. 1999; Lipton 1999; Nishizawa 2001) the pathways leading to ischemic retinal damage have been reviewed in several excellent review papers (Osborne et al 1999, 2004, Bek et al. 2009, Roth 2004, Fulton et al. 2009). However, the most important potential

mediators of cell death during retinal ischemia may be the following (Fig.2.): hypoxia -as a component of ischemia- induces the expression of hypoxia inducible factor-1α (Iyer et al. 1998)and its target genes such as vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS) (Bernaudin et al. 2002). Increased production of VEGF results in disruption of the blood retinal barrier leading to retinal edema (Marmor et al. 1999, Kaur et al. 2007). Enhanced expression of NOS results in increased production of nitric oxide which may be toxic to the cells resulting in their death (Fukumura et al. 2001, Mishra et al. 2002, 2004, 2006; Zubrow et al. 2002a, 2002b, Neufeld et al. 2002, Brooks et al. 2001). Excess glutamate release in hypoxic-ischemic conditions causes excitotoxic damage to the retinal cells through activation of ionotropic and metabotropic glutamate receptors (Benveniste et al. 1984, Dreyer et al. 1998, Pin et al. 1995). Activation of glutamate receptors is thought to initiate damage in the retina by a cascade of biochemical effects such as neuronal NOS activation and increase in intracellular Ca2+ which has been described as a major contributing factor to retinal cell loss (Nicotera and Orrenius 1998; Sattler and Tymianski 2001). Excess production of proinflammatory cytokines (Hedtjärn et al. 2004, Joussen 2002, 2004) and free radicals (Hall and Braughler 1989, Chan 1994, 1996) also mediate cell damage.

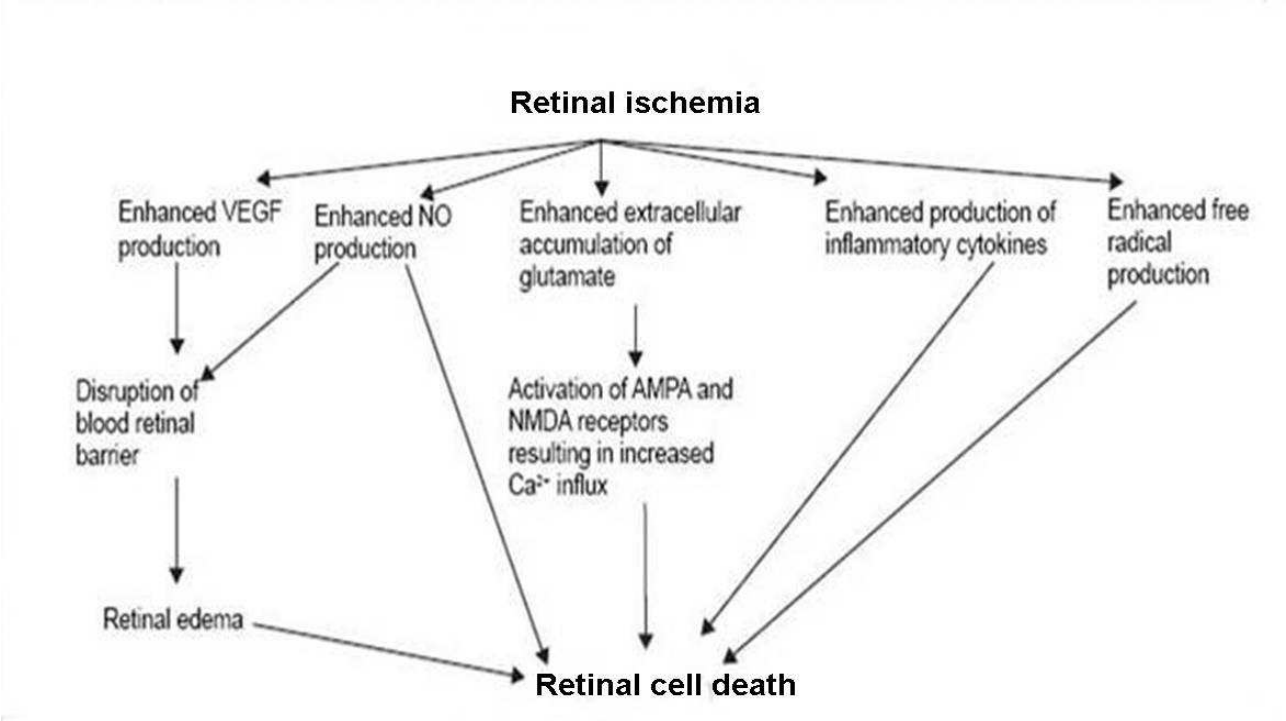


**Fig.2.** Potential mediators of retinal cell death in retinal hypoxia-ischemia

## 1.3. Oxidative stress

Oxidative stress represents an imbalance between the production of reactive oxigen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Oxidative stress contributes to tissue injury following irradiation, ATP depletion and hyperoxia.

It has an important role in the pathogenesis of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and Huntington's disease (Kumar et al. 2010, Bonda et al. 2010, Yuan et al. 2010). It is thought to be linked to certain cardiovascular disease, since oxidation of LDL in the vascular endothelium is a precursor to plaque formation. Futhermore it plays an important role in the ischemic cascade due to oxygen reperfusion injury following hypoxia. This cascade includes both strokes and heart attacks.(Sukla et al. 2010, Gamkrelidze et al. 2008)

In the eye oxidative stress can be found in the pathogenesis of diseases like keratoconus, keratopathia bullosa, Fuchs-dystrophia ( Buddi et al. 2002, Brown et al. 2004), cataracta in elderly (Varma and Hegde 2007), glaucoma simplex, age-related macular degeneration (AMD), retinopathia prematurorum, uveitis and in diabetic and other types of retinal vasculopathies and more (Szabó 2009, Augustin 2010).

Reactive oxygen species (ROS) are generated in the mitochondria, along the respiratory chain also under physiological conditions, responsible for a few percentage (2-5%) of ROS. However, in the lack of oxygen the rate of this ROS generation increases drastically. Sources of ROS can be the xanthine oxydase system, the leakage of electrons from the mitochondrial respiratory chain, the cyclooxygenase pathway of arachidonic acid metabolism, and the respiratory burst of phagocyte cells. Some of the less reactive of these species (such as superoxide) can be converted into more aggressive radical species (such as peroxynitrite) that can cause extensive cellular damage (Szabó 2009).

The excessive formation of ROS leads to lipid peroxidation, protein oxidation and DNA damage (Fig.3) (Packer et al. 1991, Pan and Hori 1994, Takeda et al. 1996) all of these effects in connection with the mitochondrial damage results in cell death: severe levels of oxidative stress can cause necrosis, while even moderate levels of oxidative stress can trigger apoptosis.

## 1.4. PARP

The PARP (poly(ADP-ribose) polymerase) -family currently comprises 18 members. PARP-1 is the best characterized and perhaps the most important member of the PARP-family

(Virag and Szabo 2002). The abundant nuclear enzyme PARP-1 is activated by single- and double-strand breaks of DNA. Upon binding to damaged DNA, PARP-1 forms homodimers and catalyses the cleavage of $NAD^+$ into nicotinamide and ADP-ribose to form long branches of ADP-ribose polymers on glutamic acid residues of a number of target proteins including histons and other nuclear proteins such as transcription factors and PARP-1 itself (Heller et al 1995, Alvarez-Gonzalez 1994) . This polymer has important signaling role in the guiding of the repairing enzymes of DNA to the site of DNA damage. For many decades, PARP was mainly viewed as an enzyme primarily involved in DNA repair and maintenance of genomic stability. However, over the last decade, an additional role of PARP has been identified in the sequale of oxidative and/or nitrosative stress. In this pathway (Virag and Szabo 2002), extensive oxidative and/or nitrosative stress triggers the extensive DNA breakage, overactivation of PARP and consequent depletion of the cellular stores of its substrate NAD+, impairing glycolysis, Krebs cycle, mitochondrial electron transport, eventually resulting in ATP depletion and consequent cell dysfunction and death by necrosis. Because the processes of apoptosis are ATP-dependent, it is understandable that over a certain extent of PARP activation, processes of apoptosis stop due to lack of ATP and, the cell death continues in necrosis instead of apoptosis. Hence the extent of PARP-1 activation is crucial to the outcome of the two types of cell death rate (Thies and Autor 1991, Radovits et al. 2007). However, several reports demonstrated the more complex role of PARP. PARP activation facilitates other components of the cell death machinery namely, destabilization of the mitochondrial membrane systems (Halmosi et al. 2001; Hong et al. 2004), nuclear translocation of apoptosis inducing factor (Yu et al. 2002), and activation of cell death promoting kinases such as c-Jun N-terminal kinase (JNK) (Xu et al. 2006). In addition, PARP activation suppresses the cytoprotective phosphatidyl-inositol-3 kinase (PI-3K)-Akt pathway (Veres et al. 2003). Furthermore, PARP-1 plays an important role in the transcriptional regulation of many inflammatory proteins including TNF-α, ICAM-1 and E-selectin (cell adhesion proteins), iNOS, MHC-II histocompatibility antigens. The transcription factor NFkB plays a key role in the regulation of these proteins. PARP activity is clearly required for the expression of this NFkB-dependent genes. Hence the PARP-1 is a cofactor of NFkB in this process (Peralta-Leal et al. 2009). In the transcriptional regulatory function of PARP-1 may be relevant in the loosening of chromatin, which achieved through the poly-ADP-ribosilation the histones. The electrostatic repulsion between the negatively charged polymers and the DNA relaxes the structure of chromatin, so that the genes become available for the transcription apparatus (Pacher and Szabo 2007).

The PARP-1 is involved in the development of diseases associated with oxidative stress with dual mechanism:

1. Excessive activation of the enzyme results in cell death caused by energy deficiency

2. The enzyme is involved in the regulation the NFkB-dependent transcription of inflammatory mediators

Therefore, in diseases where necrosis dominates (stroke, myocardial infarction, arteria centralis retinae occlusion) the former, while in the inflammatory type of diseases not accompanied by massive cell death (colitis, diabetes, uveitis, arthritis) rather the latter mechanism dominates.

The fact that pharmacological inhibition of the enzyme or the functional lack of PARP-1 in knock-out animals offer resistance and protection against oxidative injury highlights the importance of PARP-1 enzyme (Whalen et al. 1999, Mester et al. 2009). Numerous of data support the protective effect of PARP inhibitors against oxidative stress in different cell lines and in conditions like ischemia/reperfusion injury, neuronal ischemia, acute lung inflammation, acute septic shock, zymogen induced multi organ failure, diabetic pancreas injury. (Pan et Hori 1994, Eliasson et al. 1997, Liaudet et al. 2002, Soriano et al. 2006)

In the eye, blocking PARP activity has been shown to have protective effect in animal models of uveitis, ischemic retinal injury and against oxidative stress in retinal cells in vitro (Mabley et al. 2001, Goebel and Winkler 2006, Chiang et Lamm 2000, Jarrett and Boulton 2007).

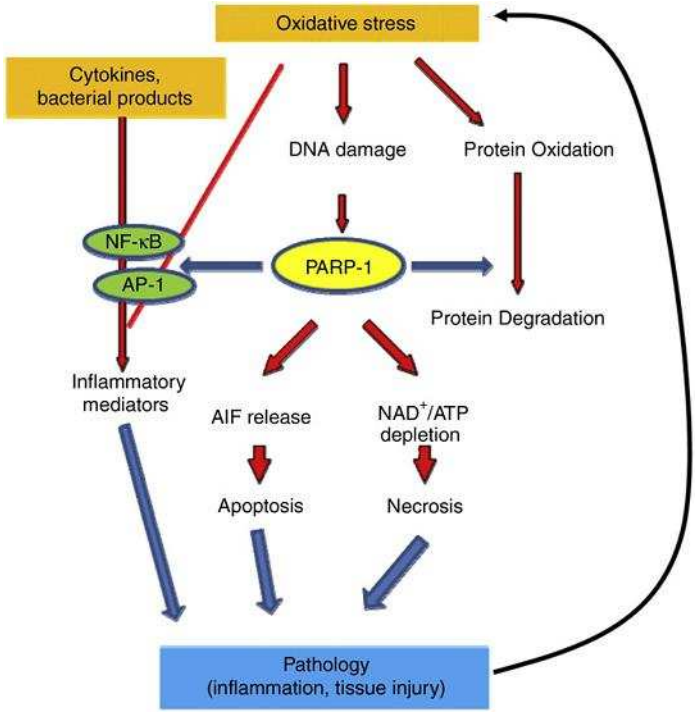


**Fig. 3**. The central role of PARP-1 in oxidative/nitrosative stress-related pathology.

## 1.5. PACAP

Pituitary adenylate cyclase activating polypeptide (PACAP) was first isolated from ovine hypothalami. However, PACAP is localized not only in the central but in the peripheral nervous system and also in non-neural tissues such as endocrine glands, cardiovascular and gastrointestinal tract (similar to other "brain-gut peptides") (Arimura 1998, Vaudry et al. 2009). PACAP belongs to the vasoactive intestinal peptide (VIP)/secretin/glucagon family of peptides and shares 68% identity with VIP. Despite the high similarity there are differences between VIP and PACAP: the adenylate cyclase stimulating activity of PACAP has been shown to be 1000-10000 times greater than that of VIP and the distribution of these peptides is quite different as well.

PACAP exists in two forms, with 27 and 38 amino acid residues. In mammalian tissues, the 38 amino acid form of PACAP is dominant, constituting approximately 90% of the peptide (Miyata et al. 1989, 1990). The primary structure of PACAP-38 is identical among all mammalian species examined, and it also shows marked similarity with lower vertebrates and nonvertebrates, with differences in only 1-4 amino acids (Arimura 1998). This suggests that the structure of PACAP has remained very conserved throughout phylogenesis and it may reflect its importance in fundamental functions in the nervous system.

The PACAP receptors belong to the family of G protein-coupled receptors with seven transmembrane domains. There are two types of PACAP receptors: PAC1 receptor which bind PACAP with high affinity and VIP with a much lower affinity and VPAC1 and VPAC2 receptors which bind VIP and PACAP with similar affinities (Arimura 1998, Vaudry et al. 2000). PAC1 receptor is coupled to adenylate cyclase and phospholipase C. Through adenylate cyclase activation, it elevates cAMP, and activates protein kinase A, which can activate the mitogen-activated protein kinase (MAPK) pathways (Vaudry et al., 1998).

The biological actions of PACAP are very diverse. Among others, the neuropeptide influences reproductive functions, circadian rhythm, thermoregulation, feeding, depression, memory, urinary reflexes, inflammatory reactions, and development (Falluel-Morel et al. 2008, Girard et al. 2008, Hagino 2008, Monaghan et al. 2008, Nagy and Csernus 2007, Racz et al. 2008b, Reichenstein et al. 2008, Vaudry et al. 2009, Yoshiyama and de Groat 2008).

PACAP has well-established neurotrophic and neuroprotective functions (Deguil et al. 2010, Dejda et al. 2008, Ohtaki et al. 2008, Scharf et al. 2008, Somogyvari-Vigh and Reglodi 2004, Vaudry et al. 2009). These effects have been proven also in the retina (Atlasz et al. 2010b). In vitro, PACAP is protective against glutamate toxicity in retinal neurons (Shoge et al. 1999). In retinal explants, PACAP has been shown to be protective against thapsigargin-induced photoreceptor cell

death and anisomycin-induced cell death in the neuroblastic layer (Silveira et al. 2002). PACAP-treated turtle eyecup preparations show electrical activity for a significantly longer time (Rabl et al. 2002). In vivo, PACAP has been shown to be protective against optic nerve transection, glutamate- and kainite-induced excitotoxic injury, and ischemic degeneration (Atlasz et al. 2007b, 2009, 2010b, Babai et al. 2005, Seki et al. 2006, 2008).

# 2. STUDY OBJECTIVES/AIMS AND HYPOTHESIS

Although involvement of PARP activation in various ischemia models has been thoroughly studied (Ferrer and Planas 2003; Meli et al. 2003; Ikeda et al. 2005), only circumstantial evidences are available for the role of PARP activation in chronic hypoperfusion-induced neurodegenerative processes (Cozzi et al. 2006). Therefore, the aim of the present study was the following :

- to demonstrate the activation of PARP as a major regulator of cell death in a chronic hypoperfusion-induced retinal degeneration model in rat (bilateral common carotid occlusion induced retinal degeneration)

- to evaluate the effect of PARP inhibition (by HO3089) in this model by assessing chronic hypoperfusion-induced morphological changes

- to determine the activation state of critical kinase cascades, such as MAP kinases and PI-3K-Akt in hypoperfusion-induced retinal degeneration

In spite of the numerous studies showing the protective effects of PACAP in the retina (Shoge et al 1999, Babai et al 2005, Rácz et al 2006), no data are currently available on the potential protective effect of PACAP against oxidative stress in pigment epithelial cells. Therefore, it seemed reasonable to study whether PACAP is able to increase cell survival in oxidative stress-induced apoptosis of human pigment epithelial cells. The aim of present study was the following:

- to elucidate the effect of PACAP on cultured human pigment epithelial cells (ARPE-19 cells) in oxidative stress

- to detect the effect of PACAP on apoptosis and necrosis on cultured ARPE-19 cells by Annexin V and propidium iodide staining

- to detect the effect of PACAP on mitochondrial depolarization occurring in apoptosis by using the JC-1 assay for flow cytometry

# 3. PROTECTION AGAINST CHRONIC HYPOPERFUSION-INDUCED RETINAL NEURODEGENERATION BY PARP INHIBITION

## 3.1. Background

Impaired cognitive function in the elderly and in degenerative neurological diseases, such as Alzheimer's disease and multiple sclerosis, has been associated with chronic cerebral hypoperfusion (de la Torre and Stefano 2000; Zlokovic 2005; Farkas et al. 2007; de Keyser et al. 2008). Moreover, the pattern of cerebral blood flow has emerged as a predictive marker for the progression into Alzheimer's disease (de la Torre and Stefano 2000; Zlokovic 2005), and was found to be involved in the development of a subtype of focal demyelinating lesions (type III lesions) in multiple sclerosis (de Keyser et al. 2008). Prolonged hypoperfusion secondary to carotid artery stenosis can also cause ocular ischemic syndrome (Dugan and Green 1991), which is a devastating disease seriously affecting quality of life in the elderly.

Permanent bilateral common carotid artery occlusion (BCCAO) in rats has emerged as the most successful animal model for studying the effects of chronic cerebral hypoperfusion on cognitive dysfunction and neurodegenerative processes (Farkas et al. 2007). It leads to a moderate reduction of blood flow, causing subtle morphological, biochemical, and behavioral changes (Osborne et al. 2004; Farkas et al. 2007; Kalesnykas et al. 2008). Among the numerous models of retinal ischemia, BCCAO induces functional and morphological damage in the rat retina such as electrophysiological alterations, loss of the pupillary reflex, detectable neurodegenerative changes both retinal or visual system level, as well as fundoscopic and fluorescein angiographic findings paralleling the retinopathy of carotid artery occlusive disease in humans (Spertus et al. 1984; Vidal-Sanz et al. 2000; Lavinsky et al. 2006). We have shown earlier that local treatment with pituitary adenylate cyclase-activating polypeptide or diazoxide counteracted mitochondrial dysfunction, and resulted in amelioration of BCCAO-induced retina degeneration in rats (Atlasz et al. 2007a, b).

In the retina, increased activation of PARP contributing to retinal ganglion cell death in response to optic nerve transection (Weise et al. 2001), is involved in photoreceptor degeneration in the retinal degeneration-1 transgenic mouse model (Paquet-Durand et al. 2007) and oxidative stress-induced apoptosis of ganglion cells (Li and Osborne 2008). PARP inhibition, on the other hand, has been demonstrated to decrease retinal damage in N-methyl-D-aspartate (NMDA)-induced cell death in

the retina (Goebel and Winkler 2006) and N-methyl-N-nitrosourea-induced photoreceptor cell apoptosis (Uehara et al. 2006).

## 3.2. Materials and methods

### 3.2.1. Animals

Animal housing, care, and application of experimental procedures were in accordance with institutional guidelines under approved protocols (No: BA02/2000-20/2006, University of Pecs). Animals were maintained under 12-h light/dark cycle with free access to food and water.

### 3.2.2. Bilateral Common Carotid Artery Occlusion and HO3089 Treatment

Adult male Wistar rats (n = 19) weighing 250–300 g were subjected to permanent bilateral carotid artery occlusion (BCCAO). Under isoflurane anesthesia, both common carotid arteries were ligated with a 3.0 filament through a midline cervical incision. Immediately following the BCCAO operation and 4 times in a 2-week-period (postoperative days 0, 3, 6, 9, and 12), HO3089 (175 mmol in 3 μl saline) was injected into the vitreous body of the right eye with a Hamilton syringe. Animals were anesthetized before each treatment and the same injection site was used in consecutive drug or vehicle administrations in order to minimize the complications. HO3089 (Alexy et al. 2004), a PARP inhibitor was a kind gift of Kalman Hideg (University of Pecs Medical School). The left eye received the same volume of vehicle treatment, serving as control bilateral carotid-occluded eyes. A group of animals underwent anesthesia and all steps of the surgical procedure, except ligation of the carotid arteries, with saline or HO3089 treatment. These animals served as sham-operated animals (n = 6). Animals with eye complications after consecutive treatments (cataract, endophthalmitis) were excluded from further evaluation (n = 4).

### 3.2.3. Histological Analysis

Two weeks after the carotid occlusion, rats were sacrificed under isoflurane anesthesia. The eyes were immediately dissected in ice-cold phosphate buffered saline and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (Sigma, Hungary). Tissues were embedded in Durcupan ACM resin (Fluka, Switzerland), cut at 2 μm, and stained with toluidine blue (Sigma, Hungary). Sections were mounted in Depex medium and examined in a Nikon Eclipse 80 i microscope. Photographs were taken with a digital CCD camera using the Spot program, from

central retinal areas of nearly same eccentricities. Files were then further processed with Adobe Photoshop 7.0 program. Measurements were taken from the digital photographs with the NIH Image 1.55 program. Samples for measurements derived from at least six tissue blocks prepared from at least four animals (n = 4–5 measurements from one tissue block). The following parameters were measured: (i) cross-section of the retina from the outer limiting membrane to the inner limiting membrane (OLM-ILM); (ii) the width of the outer and inner nuclear and outer and inner plexiform layers (ONL, INL, OPL, IPL, respectively); (iii) the number of cells in the ONL/500 lm2; (iv) the number of cells/100 μm section length in the ganglion cell layer (GCL). Results are presented as mean ± SEM. Statistical comparisons were made using the ANOVA test followed by Tukey-B's post hoc analysis.

### 3.2.4. Western Blot

Another group of rats underwent the same surgical procedure for bilateral carotid occlusion (n = 9) or sham operation (n = 5) and retinas were removed after 4 h in order to investigate the signaling pathways that are activated within the first few hours after an ischemic insult (Roth et al. 2003; Merienne et al. 2007; Roduit and Schorderet 2008). Samples were processed for Western blot analysis as described earlier (Racz et al. 2007). Membranes were probed overnight at 4°C with the following primary antibodies: phosphospecific anti-Akt-1 Ser473 (1:1000 dilution; R&D Systems, Budapest, Hungary), phospho-specific anti-GSK-3b Ser9, phospho-specific anti-ERK1/2 Thr202/Tyr204, phospho-specific anti-SAPK/JNK Thr183/Tyr185, phospho- specific anti-p38 MAPK (1:1000 dilution; Cell Signaling Technology, Beverly, USA), anti-poly(ADP-ribose) (1:1000 dilution; Alexis Biochemicals, Nottingham, UK), and anti-aktin (1:5000 dilution; Sigma-Aldrich Chemical Co., Budapest, Hungary). Membranes were washed six times for 5 min in Tris buffered saline (pH = 7.5) containing 0.2% Tween prior to addition of goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:3000; BioRad, Budapest, Hungary). The antibody–antigen complexes were visualized by means of enhanced chemiluminescence. After scanning, results were quantified by means of NIH ImageJ program. All experiments were performed at least four times. All data were expressed as mean ± SEM. Statistical comparisons were made using the ANOVA test followed by Bonferroni's post hoc analysis. Differences with P values below 0.05 were considered as significant.

## 3.3. Results

### 3.3.1. Histological Analysis

HO3089 treatment in sham-operated animals did not cause any morphological alteration. BCCAO resulted in severely reduced thickness of retinal layers compared to sham-operated saline-treated controls (Figs. 4,5). All retinal layers bore the marks of severe degeneration and were significantly thinner than sham-operated preparations (Fig. 4a, b). As a consequence, the distance between OLM and ILM was significantly decreased. Most marked reduction in thickness was found in the IPL, and a subtle, but significant change was observed in the OPL and ONL. Several empty cell body-shaped spaces were seen in the layer of photoreceptor perikarya (Fig. 4b). The number of cells in the ONL/500 μm2 was significantly reduced (Fig. 5b). In the IPL, evenly distributed dense dots were seen representing presumably degenerating bipolar cell terminals (Fig. 4b). Numerous cells in the GCL also displayed severe degeneration (Fig. 4b). This was well reflected in the reduced number of cells in the GCL (Fig. 5c).

Intraocular HO3089 treatment following BCCAO led to a nearly intact appearance of the retinal layers (Fig. 4c). This is well supported by the morphometric measurements (Fig. 5a, b, c). Although the overall thickness of the retina was not fully restored, the thickness of the major retinal layers was almost identical with that of the sham-operated animals and was markedly larger than that of the BCCAO retinas (Fig. 5a). Differences between BCCAO and HO3089-treated retinas were statistically significant in almost all retinal layers, except for the INL (Fig. 5a). The number of cells in the ONL in 10 fields of 500 μm2 was lower in the BCCAO group compared to the HO3089-treated group (Fig. 5b). Quantitative analysis demonstrated that HO3089 administration protected the cells also in the GCL (Fig. 5c).

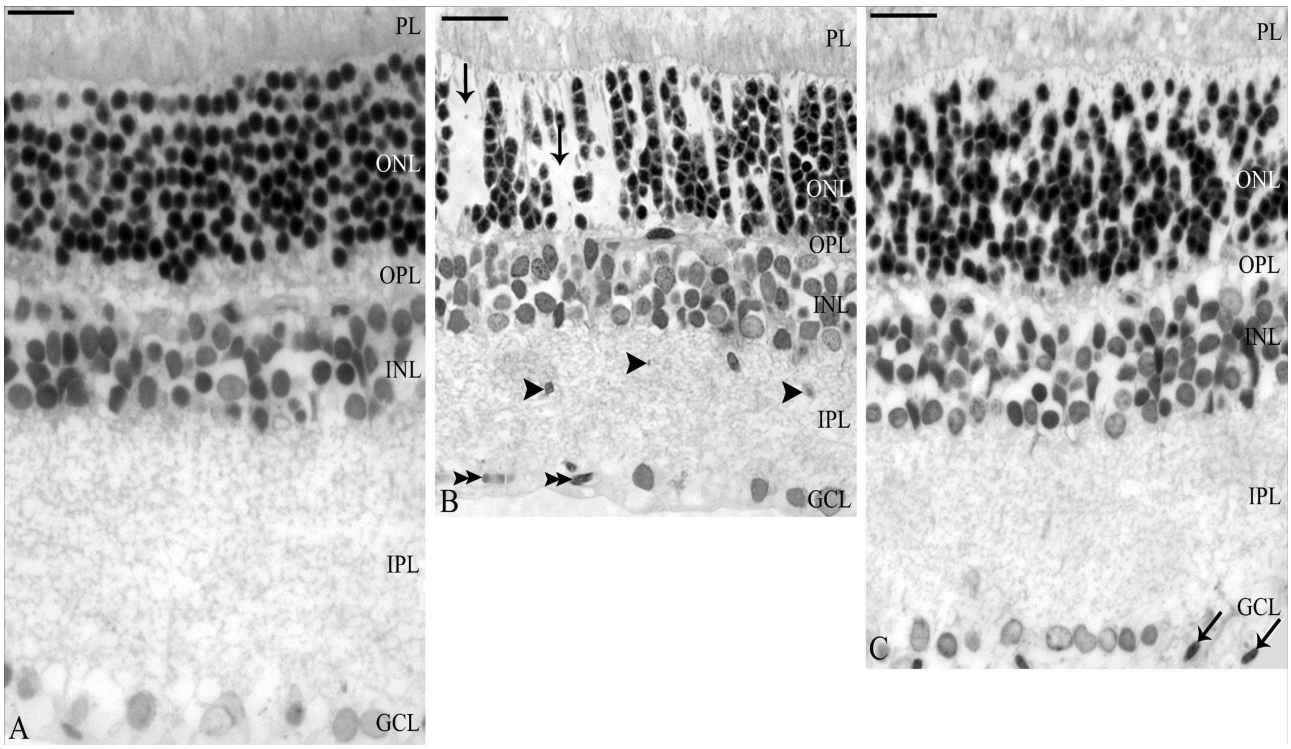


**Fig. 4.** Light microphotographs of retinal cross sections stained with toluidine blue. **A** Sham-operated retina treated with saline. **B** BCCAO induced retinal degeneration showing severe degeneration compared to sham retinas. Many cells in the ONL (arrows) and in the GCL (double arrowheads) suffered degeneration, shown by empty cell body shapes in these layers. Arrowheads indicate presumably the terminals of degenerated bipolar cells in the IPL. **C** Alleviation of BCCAO-induced retinal degeneration with HO3089. The retained retinal structure was similar to that of the sham retina. Arrows show some degenerated cells in the GCL. ONL Outer nuclear layer, OPL outer plexiform layer, INL inner nuclear layer, IPL inner plexiform layer, GCL ganglion cell layer. Scale bar: 20 μm

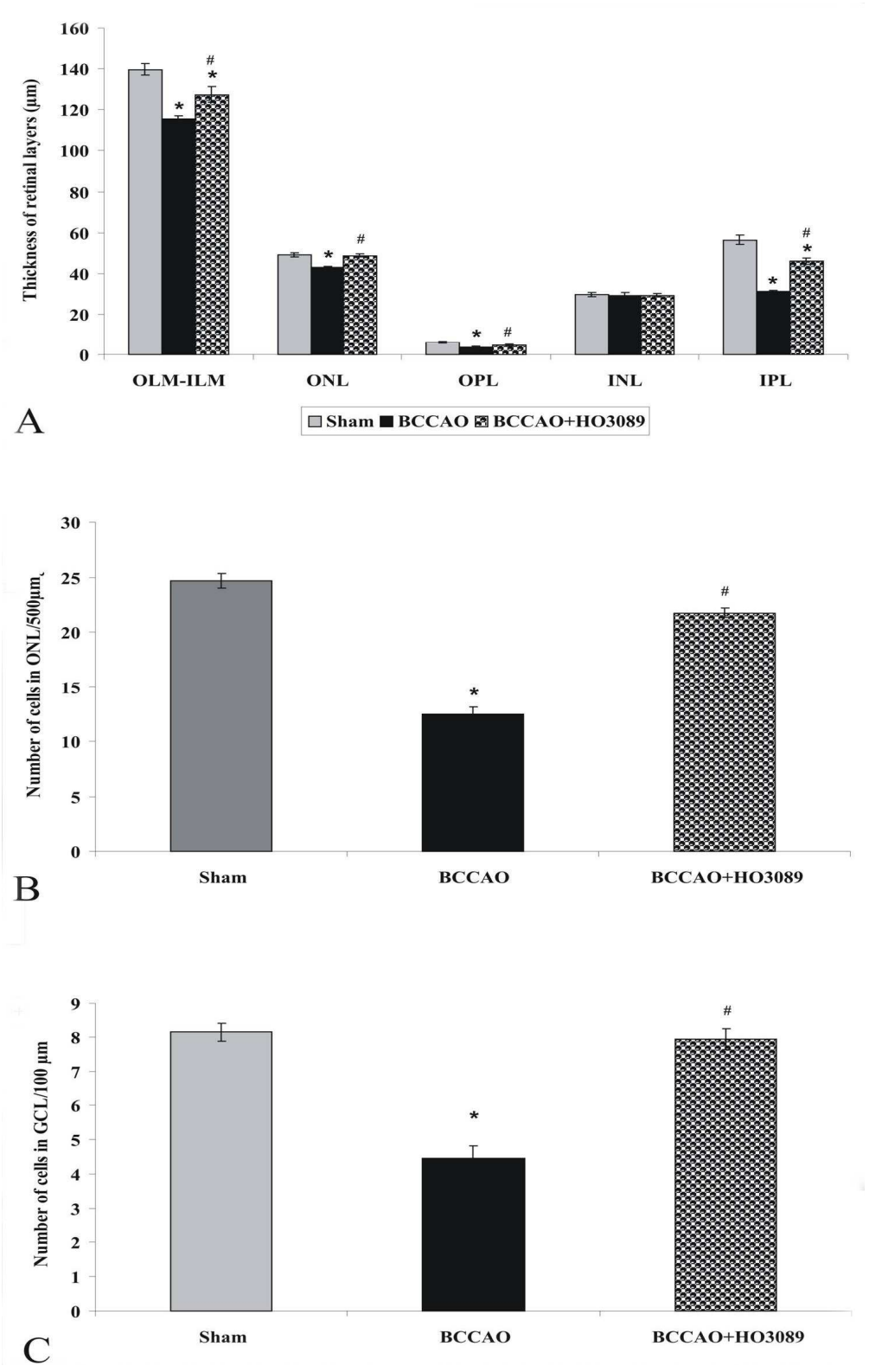


**Fig.5.** Comparison of **A**: retinal layers, **B**: the number of cells/500 µm2in the ONL, and **C**: the number of cells/100 µm GCL length in shamoperated control rats (saline-treated) and those receiving HO3089 treatment after the BCCAO. OLM-ILM: Cross-section of the retina from the outer limiting membrane to the inner limiting membrane, ONL outer nuclear layer, OPL outer plexiform layer, INL inner nuclear layer, IPL inner plexiform layer. Data are expressed as mean ± SEM. *P<0.05 compared to sham-operated retinas; #P<0.05 compared to BCCAO-induced ischemic retinas

### 3.3.2. Effect of PARP Inhibitor Treatment on Ischemia-Induced Poly-ADP-Ribosylation

Activation of PARP in the retina was revealed by assessing poly-ADP-ribosylation of target proteins. Treatment of the eye with the PARP inhibitor (HO3089) attenuated the BCCAO-induced self-poly-ADP-ribosylation of PARP (Fig. 6) as well as that of other nuclear proteins (not shown) as it was detected by Western blotting utilizing an anti-poly (ADP-ribose) antibody.

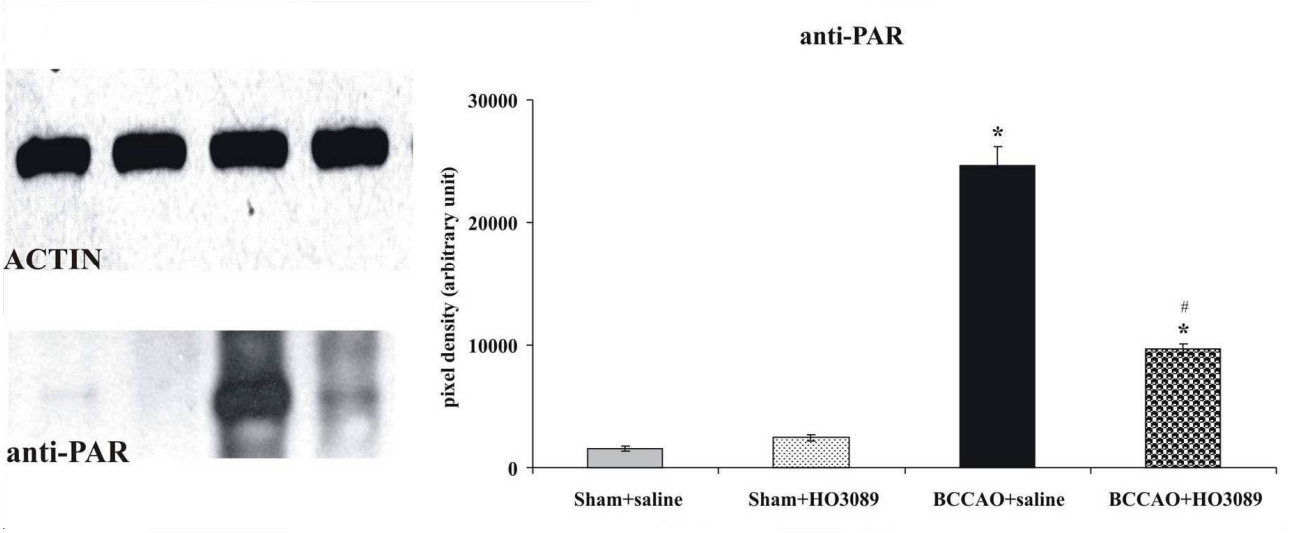


**Fig. 6.** Effect of BCCAO and HO3089 treatment on PARP activation in the retina. PARP activation was assessed by self-ADP-ribosylation of the PARP enzyme detected by anti-PAR immunoblotting. Representative blots of three experiments as well as quantitative evaluation of the pixel densities are shown. Values are given as mean ± SEM. Aktin was used as a loading control. *P<0.05 versus sham + saline and sham + HO3089-treated retinas; #P<0.05 versus BCCAO + saline-treated retinas

### 3.3.3. Effect of PARP Inhibitor Treatment on Ischemia-Induced Signaling Pathways

Phosphorylation of Akt-1 was significantly elevated following HO3089 treatment in sham-operated retinas, while that of its downstream target GSK-3b was slightly increased (Fig. 7). Ischemia itself did not change the phosphorylation of these proteins. However, HO3089 treatment increased the activation (phosphorylation) of Akt-1 and GSK-3b in ischemic retinas (Fig. 7).

ERK1/2 phosphorylation was close to the detection limit in sham-operated saline-treated retinas, while HO3089 treatment resulted in increased activation of this MAPK kinase. Ischemia induced a strong phosphorylation of ERK1/2 compared to sham-operation and was further increased by HO3089 treatment (Fig. 8). In the sham-operated eyes, we observed very low phosphorylation of SAPK/JNK. Ischemia induced the activation of this kinase as it was revealed by its increased phosphorylation (Fig. 8). However, administration of HO3089 caused a dramatic decrease in phosphorylation of SAPK/JNK. A phosphorylation pattern similar to that of JNK was detected in

case of p38 MAPK. Minimal phosphorylation of this kinase was observed in sham-operated animals, and we found no difference between saline and PARP inhibitor-treated retinas. Ischemia induced a strong phosphorylation of p38 MAPK that was significantly attenuated by HO3089 treatment (Fig. 8).

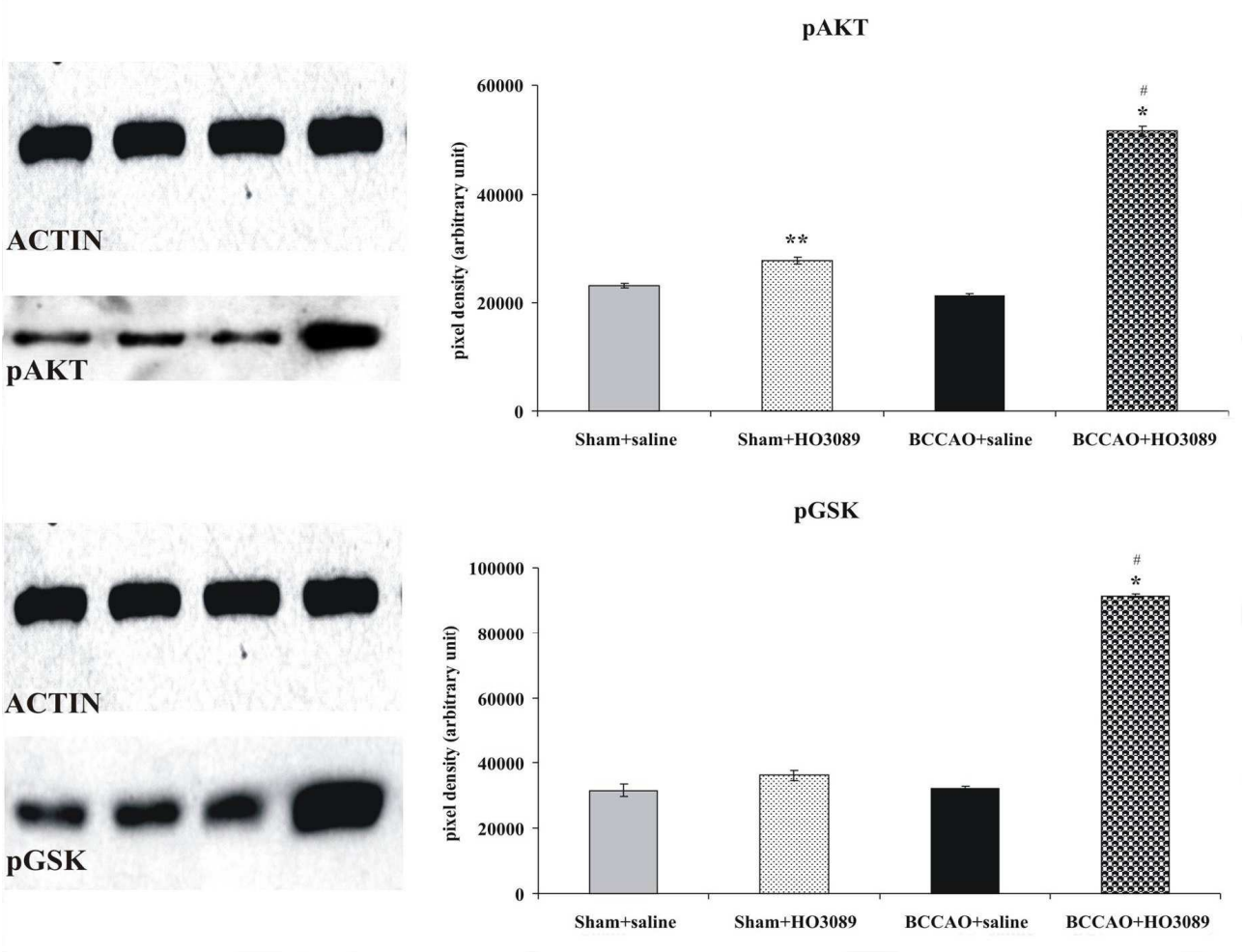


**Fig. 7.** Effect of BCCAO and HO3089 treatment on Akt activation in the retina. Akt activation was demonstrated by its phosphorylation and phosphorylation of its down-stream target, GSK detected by immunoblotting utilizing phosphorylation-specific primary antibodies. Representative blots of three experiments as well as quantitative evaluation of the pixel densities are shown. Values are given asmean ± SEM. Aktin was used as a loading control. *P<0.05 versus sham + saline and sham + HO3089-treated retinas; **P<0.01 versus sham + saline-treated retinas; #P<0.05 versus BCCAO +saline-treated retinas

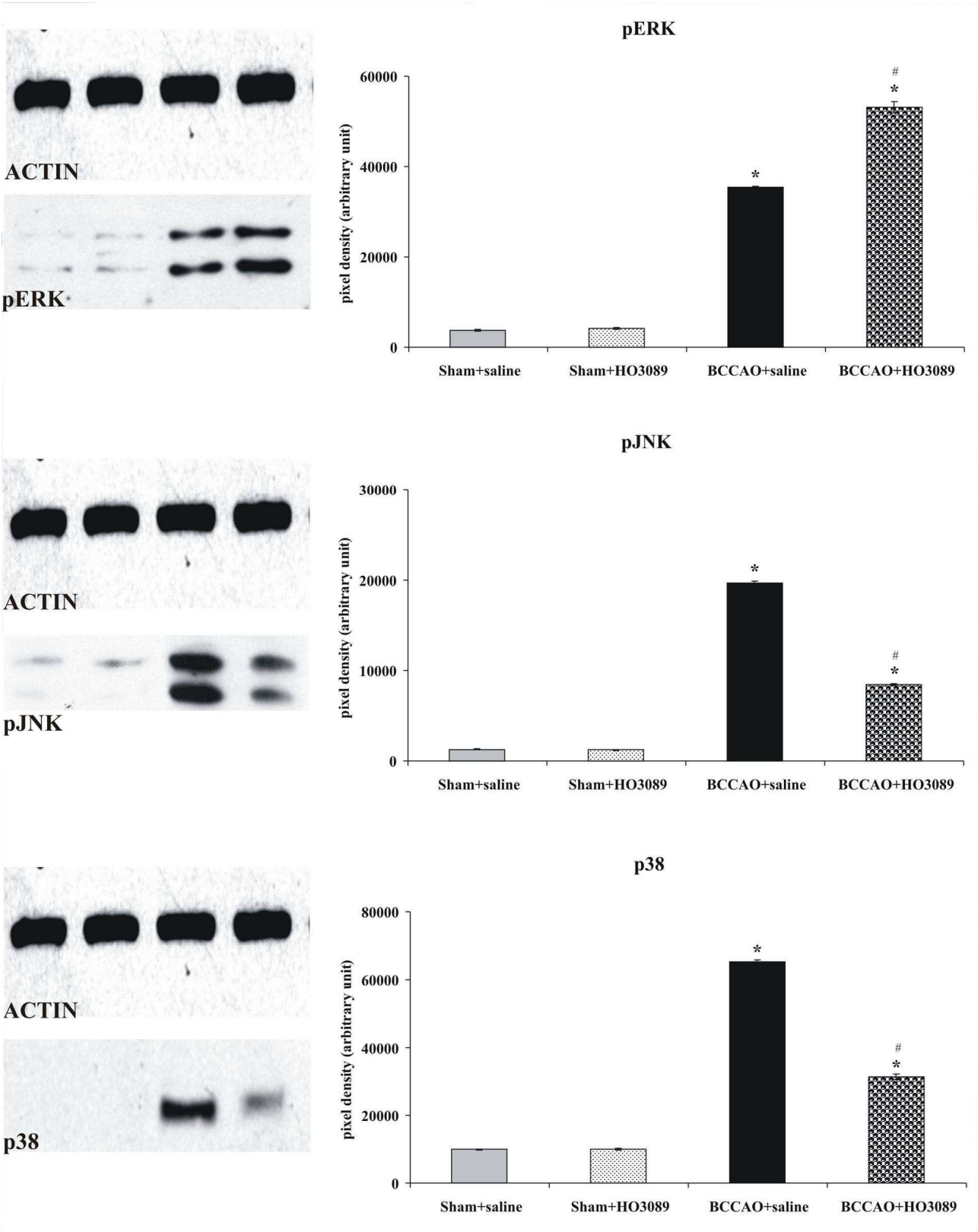


**Fig. 8.** Effect of BCCAO and HO3089 treatment on MAP kinase activation in the retina. Activation of ERK, JNK, and p38 MAPK was demonstrated by their phosphorylation detected by immunoblotting utilizing phosphorylationspecific primary antibodies. Representative blots of three experiments as well as quantitative evaluation of the pixel densities are shown. Values are given as mean ± SEM. Aktin was used as a loading control. *P<0.05 versus sham + saline and sham + HO3089-treated retinas; #P<0.05 versus BCCAO + saline-treated retinas

# 4. PACAP IS PROTECTIVE AGAINST OXIDATIVE STRESS IN HUMAN RETINAL PIGMENT EPITHELIAL CELLS

## 4.1. Backgrounds

Most studies on retinoprotective strategies focus on the retinal layers derived from the inner layer of the optic cup (Mester et al. 2009; Rojas et al. 2009; Szabadfi et al. 2009), since these layers contain the neurons arranged in three vertical layers. However, the outermost layer of the retina, the pigment epithelial cell layer, is also a very important part of the retina. The integrity of the pigment epithelial cells is critical for the photoreceptor survival and vision (Bazan 2006, 2008). Photoreceptor degeneration involves the closely associated retinal pigment epithelial cells in several ocular diseases, including age-related macular degeneration (Bazan 2006; Kook et al. 2008).

Oxidative stress is one of the most common apoptosis-inducing factors in several organs from the intestinal and cardiovascular systems to neuronal cells, including the extremely vulnerable sensory organs (Ferencz et al. 2002; Racz et al. 2007b, 2010; Vaudry et al. 2002). Not surprisingly, oxidative stress-induced apoptosis has been shown to play a role also in pigment epithelial cell death (Kalariya et al. 2008; Kook et al. 2008). Human pigment epithelial cells possess PAC1 and VPAC receptors, as shown by PCR studies (Zhang et al. 2005). Vasoactive intestinal peptide, a peptide related to PACAP, has been shown to have effects on pigment epithelial cells: it induces cAMP formation (Koh and Chader 1984), influences proliferation (Kishi et al. 1996; Troger et al. 2003) and induces differentiation of retinal pigment cells from mesenchymal cells (Vossmerbaeumer et al. 2009). A previous study has shown that PACAP inhibited the interleukin 1β-stimulated expression of interleukin-6 and -8 and monocyte chemotactic protein-1 in ARPE-19 human pigment epithelial cells (Zhang et al. 2005).

## 4.2. Materials and Methods

### 4.2.1. Cell Culture

ARPE19 is an immortalized cell line of human retinal pigment epithelium (RPE) that is used widely to draw inferences about the behavior of adult human RPE (ahRPE)(Cai and Del Priore 2006). Cells were obtained from American Type Culture Collection (Manassas, VA, USA). These cells were cultured in DMEM/Ham’s F12 supplemented with 10% FCS, penicillin (100 U/mL), and

streptomycin sulfate (100 μg/mL). The cells were grown at 37°C in a humidified 5% CO2 atmosphere.

### 4.2.2. Cell Viability Test

ARPE-19 (1.5×104/well) cells were seeded in 96-well microculture plate and cultured overnight before treatment with increasing concentration of H2O2 (0.2 to 0.3 mM) and 10 nM PACAP1-38. Untreated control cells were handled in a similar fashion without H2O2. In order to test whether the action of PACAP is specific, separate groups of 0.25 mM H2O2-treated cells were incubated with 1 μM PACAP6-38 alone or together with 10 nM PACAP1-38. After the first set of experiments proving the protective effects of PACAP, we tested the dose dependency of PACAP treatment on the viability of ARPE-19 cells. Cells were exposed to 0.25 mM H2O2 in the presence of 1 pM to 1 μM PACAP1-38. Furthermore, the experiment was repeated in the presence of inhibitors of different signaling pathways. The inhibitors used were 10 μM PD 98059 (ERK inhibitor), 2 μM SB 203580 (p38 inhibitor), 5 μM JNK Inhibitor II and 10 μM Ly 294002 (Akt inhibitor).
Viability of cells was determined by the addition of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Hungary) at a 1:10 volume ratio for 4 h at 37°C, according to the manufacturer's instruction (Sigma, Hungary). The assay is based on the reduction of MTT into a blue formazan dye by the functional mitochondria of viable cells. Samples from duplicate wells were transferred to a 96-well plate and absorbance was measured by an ELISA reader (Anthos Labtech 2010, Austria) at 550 nm, representing the values in arbitrary unit. Results are expressed as percentage of control values. Statistical analysis was performed by ANOVA test and results were considered significant at $p < 0.05$.

### 4.2.3. Annexin V and Propidium Iodide Staining of the Cells

Ratio of apoptosis was evaluated after double-staining with fluorescein isothiocyanate (FITC)-labeled annexin V (BD150 Biosciences, Hungary) and propidium iodide (PI) (BD Biosciences, Hungary) using flow cytometry (FacsCalibur, BD Biosciences, USA). First, the medium was discarded and the wells were washed twice with isotonic NaCl solution. Cells were removed from the plates using a mixture of 0.25% trypsin (Sigma, Hungary), 0.2% ethylene-diamin tetra-acetate (Serva, Hungary), 0.296% sodium citrate, and 0.6% sodium chloride in distilled water for 15 min at 37°C. Removed cells were washed twice in cold phosphate-buffered saline and were resuspended in binding buffer containing 10 mM Hepes NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl2. Cell count was determined in Burker's chamber for achieving a dilution in which 1 ml of solution

contains cells. One hundred microliters of buffer ($10^5$ cells) was transferred into 5-ml round-bottom polystyrene tubes. Cells were incubated for 15 min with fluorescein isothiocyanate-conjugated annexin V molecules and PI. After this period of incubation, 400 μl of annexin-binding buffer (BD Biosciences, Hungary) was added to the tubes as described by the manufacturers. The samples were immediately measured by BD FacsCalibur flow cytometer (BD Biosciences, USA).

Results were analyzed by Cellquest software (BD Biosciences, USA). Quadrant dot plot was introduced to identify living and necrotic cells and cells in early or late phase of apoptosis. Living cells were identified as annexin V-FITC and PI-negative. Apoptotic cells were branded as annexin V-FITC-positive only and cells in late apoptosis were recognized as double-positive for annexin V-FITC and PI. Cells in each category were expressed as the percentage of the total number of stained cells counted.

### 4.2.4. JC-1 Assay for Flow Cytometry

JC-1 assay kit for flow cytometry was used to detect apoptosis in cultured cells (Invitrogen, Molecular Probes, Hungary). Cells were incubated with 10 μl of 200 μM JC-1 (2 μM final concentration) for 30 min. Cells were washed once by adding 2 ml of warm PBS to each tube of cells. The cells were pelleted by centrifugation. Cells were resuspended by gently flicking the tubes and 500 μl PBS was added to each tube. The samples were immediately measured by BD FacsCalibur flow cytometer (BD Biosciences, USA). The red/green fluorescence ratio was introduced to identify living and apoptotic cells. JC-1 exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (≈529 nm) to red (≈590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/greenfluorescence intensity ratio. Cells in each category were expressed as the percentage of the total number of stained cells counted. Results were analyzed by Cellquest software (BD Biosciences, USA).

## 4.3. Results

### 4.3.1 Cell viability tests

Initial experiments were performed to assess the rate of cell viability loss on exposure of the cells to oxidative stress. Cells were treated with various doses of H2O2 for 3 h, and cell viability was determined with MTT assay. Similarly to findings by others, ARPE19 cells were resistant to low concentrations of H2O2 but rapidly lost viability with an increase in H2O2 concentration (Qin et al. 2006). Exposure of the cells to 0.25 mM H2O2 for 3 h reduced viable cells to approximately 50%

of control. Therefore, we chose this concentration for our further experiments. PACAP1-38 or PACAP6-38 administration alone caused no changes in cell viability. Treatment with 10 nM PACAP for 3 h significantly increased the percentage of viable cells after exposure to oxidative injury, which could be blocked by PACAP6-38 co-application (Fig. 9). Furthermore, we showed that this effect was dose dependent: 1 pM 217 PACAP1-38 did not lead to a significant increase in cell survival; 10 and 100 pM could significantly decrease the effect of oxidative stress. Best result was achieved by100 nM PACAP1-38 treatment (Fig. 10). We repeated the experiments with 10 nM PACAP in the presence of various inhibitors of the MAPK and PI3K/Akt pathways. It was found that the coapplication of different MAPK inhibitors did not influence the protective effect of PACAP, while the presence of PI3K/Akt inhibitor significantly reduced this protective effect (data not shown).

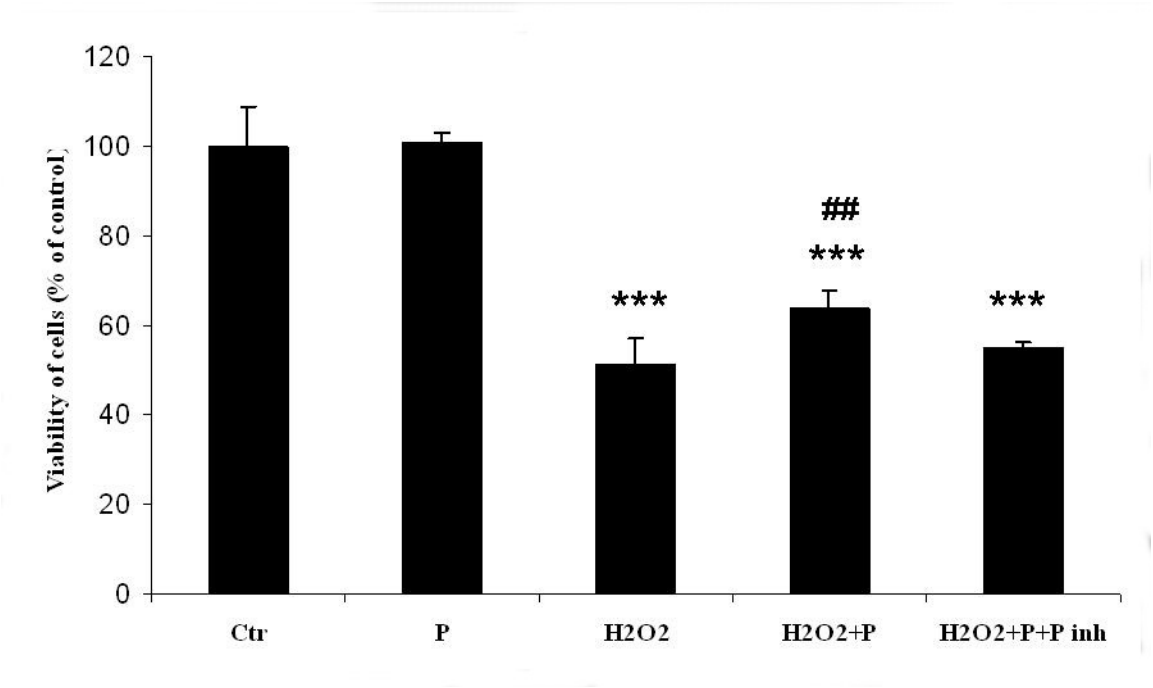


**Fig. 9.** Effect of PACAP on the viability on H2O2-treated ARPE-19 cells. ARPE-19 cells were left untreated (control, ctr), were treated with 10 nM PACAP1-38 alone (P), with 0.25 mM H2O2 alone, or with 10 nM PACAP1-38 (P), or PACAP1-38 and 1 μM PACAP6-38 (PACAP inhibitor, P inh) for 3 h. Cell viabilities were detected by MTT assay and expressed as a percentage of untreated control cells. Data are expressed as mean percentage ± S.E.M. ***P<0.001 compared to control; ##P<0.01 compared to H2O2 and H2O2 + P + P inh treatment

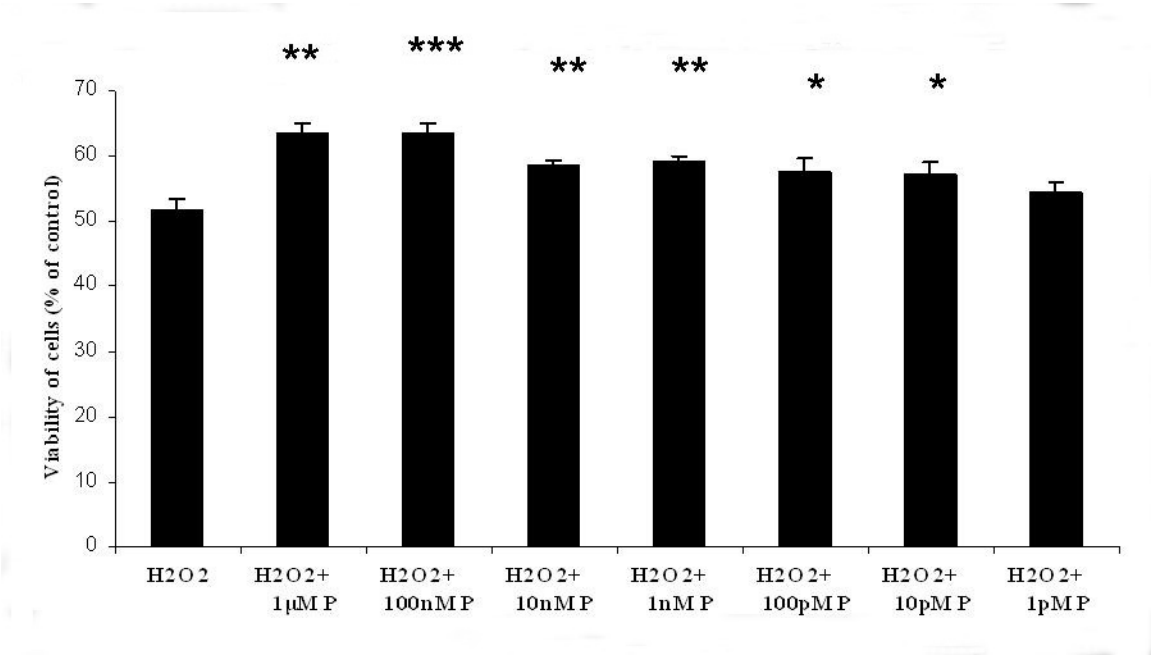


**Fig. 10.** Concentration dependence of PACAP on H2O2-treated ARPE-19 cells. ARPE-19 cells were treated with 0.25 mM H2O2 alone (H2O2) or with the mentioned concentrations of PACAP1-38 (P) for 3 h. Cell viabilities were detected by MTT assay and expressed as a percentage of untreated control cells. Data are expressed as mean percentage ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 compared to H2O2

### 4.3.2. Annexin V and propidium iodide staining of the cells

Annexin V and propidium iodide staining were used to detect apoptosis and necrosis in cultured cells. In the late phase of apoptosis, cells are stained with both dyes. Using this method, we found that the control group had more than 96% of intact, living cells and only less than 4% of cells in the necrotic and early, late phases of apoptosis (Figs. 11 and 12). An increase of apoptotic and necrotic cells was observed in the H2O2-treated group with a lower number of living cells. PACAP administration alone caused no changes in the percentage of living, necrotic, and apoptotic cells compared to control values. Necrosis was slightly decreased upon PACAP treatment, but differences were not statistically significant. However, PACAP administration led to a significant increase in the percentage of living cells and a reproducible decrease in the rate of apoptosis (Figs. 11 and 12).

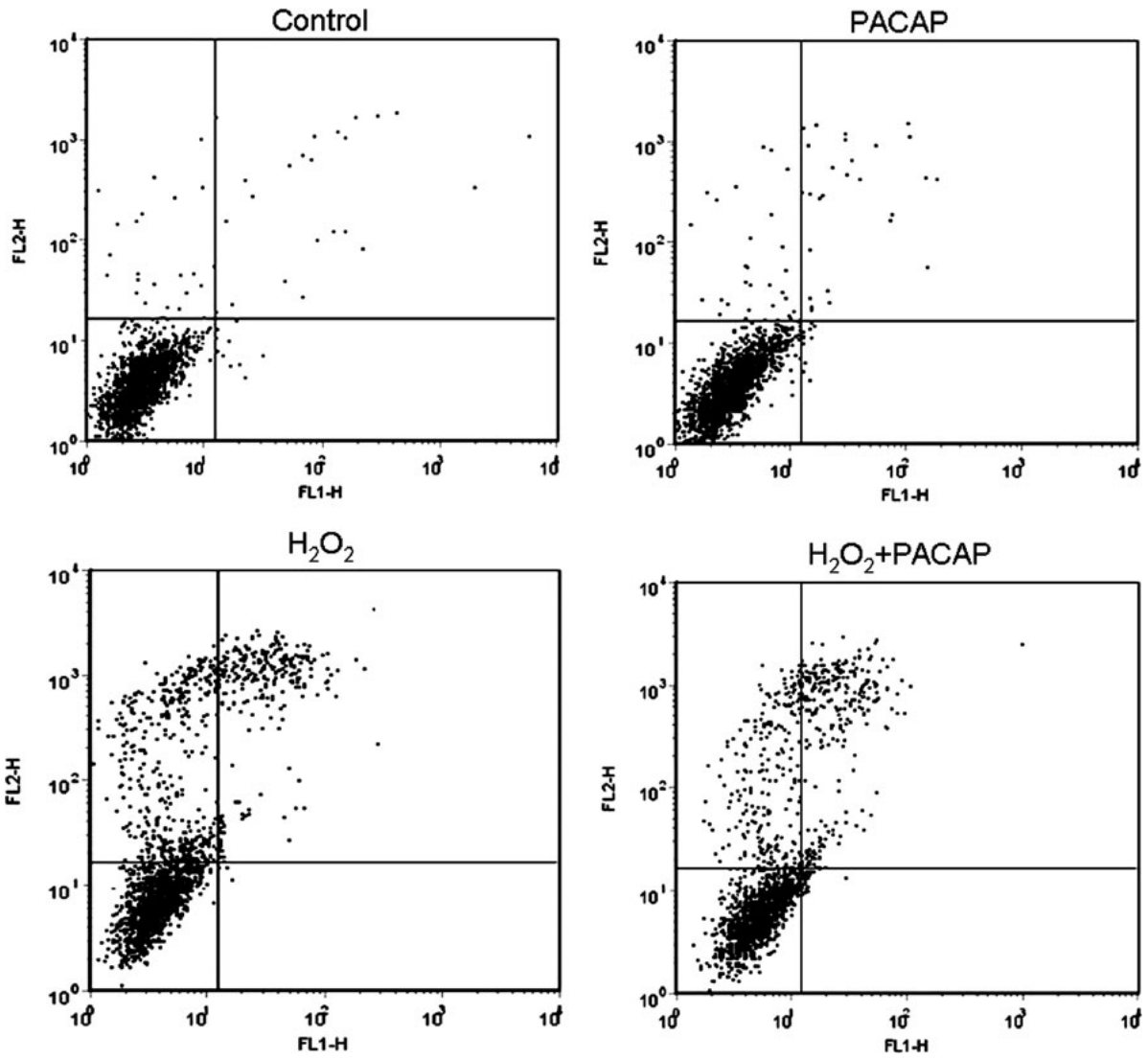


**Fig. 11.** Distinction between living, necrotic, early, and late apoptotic cells in untreated control cells, PACAP-treated cells, cells exposed to 0.25 mM H2O2 for 3 h and H2O2-treated cells co-incubated with 10 nM PACAP. The lines divide each plot into quadrants: lower left quadrant, living cells; lower right quadrant, early apoptotic cells; upper left quadrant, necrotic cells; upper right quadrant, late apoptotic cells

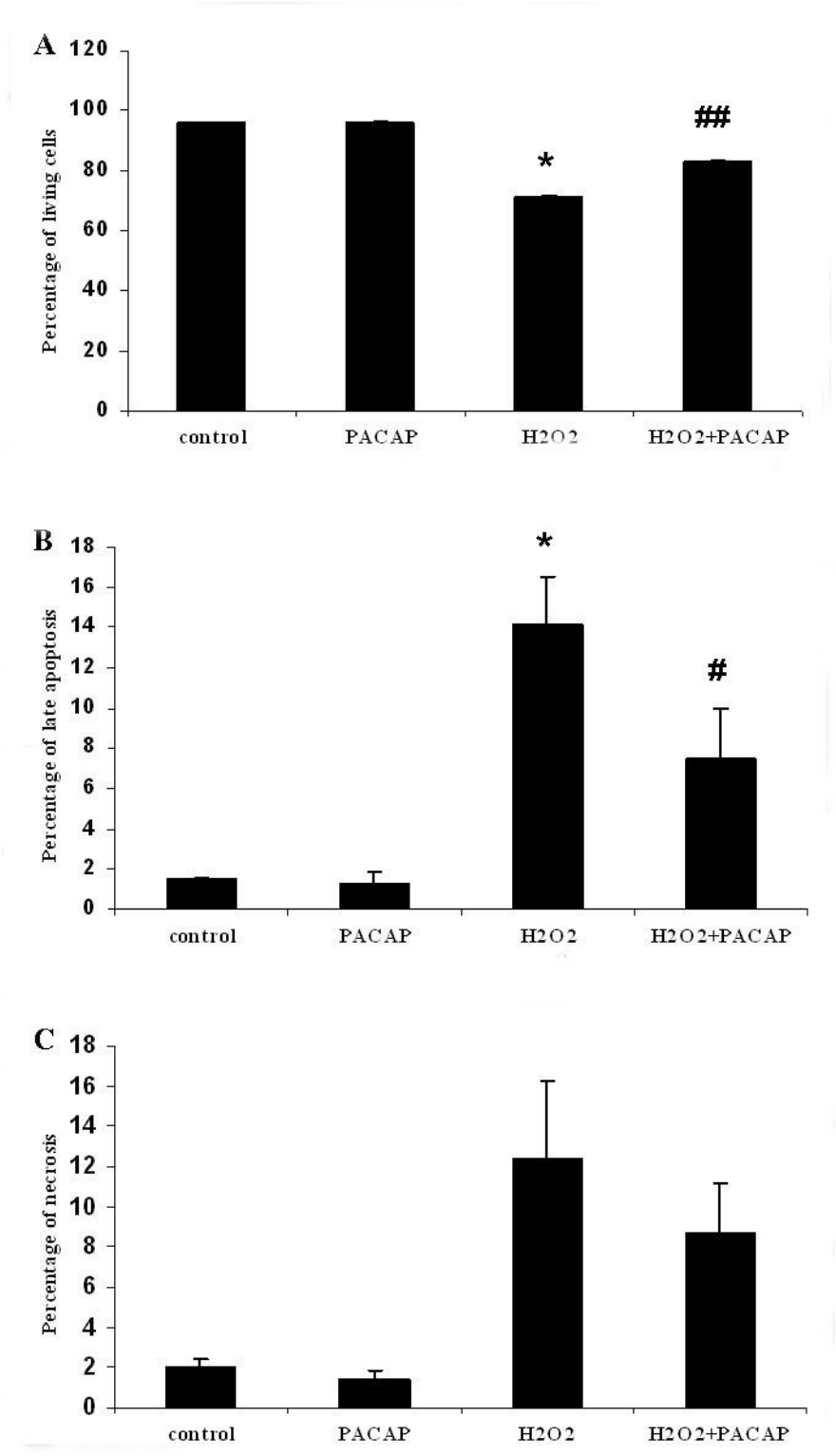


**Fig. 12.** Graphs demonstrate the mean percentage of living cells (**A**), ratio of cells in apoptosis (**B**), ratio of necrotic cells (**C**). ARPE-19 cells were treated with 0.25 mM H2O2 and 10 nM PACAP for 3 h and cell death was assessed by annexin V and propidium iodide staining. Data are expressed as mean percentage ± S.E.M. *P<0.05 compared to control group; #P< 0.05, ##P<0.01 compared to H2O2 group

### 4.3.3. JC-1 Assay for Flow Cytometry

JC-1 is for the detection of mitochondrial depolarization occurring in apoptosis. Using the JC-1 assay for flow cytometry, we found that the control group and the group treated with PACAP alone had more than 98% of living cells (Figs. 13 and 14). An increase of apoptotic cell number was observed in the H2O2-treated group with a lower number of living cells. PACAP administration led to a significant increase in the percentage of living cells and a decrease in the percentage of apoptotic cells exposed to H2O2 (Figs. 13 and 14).

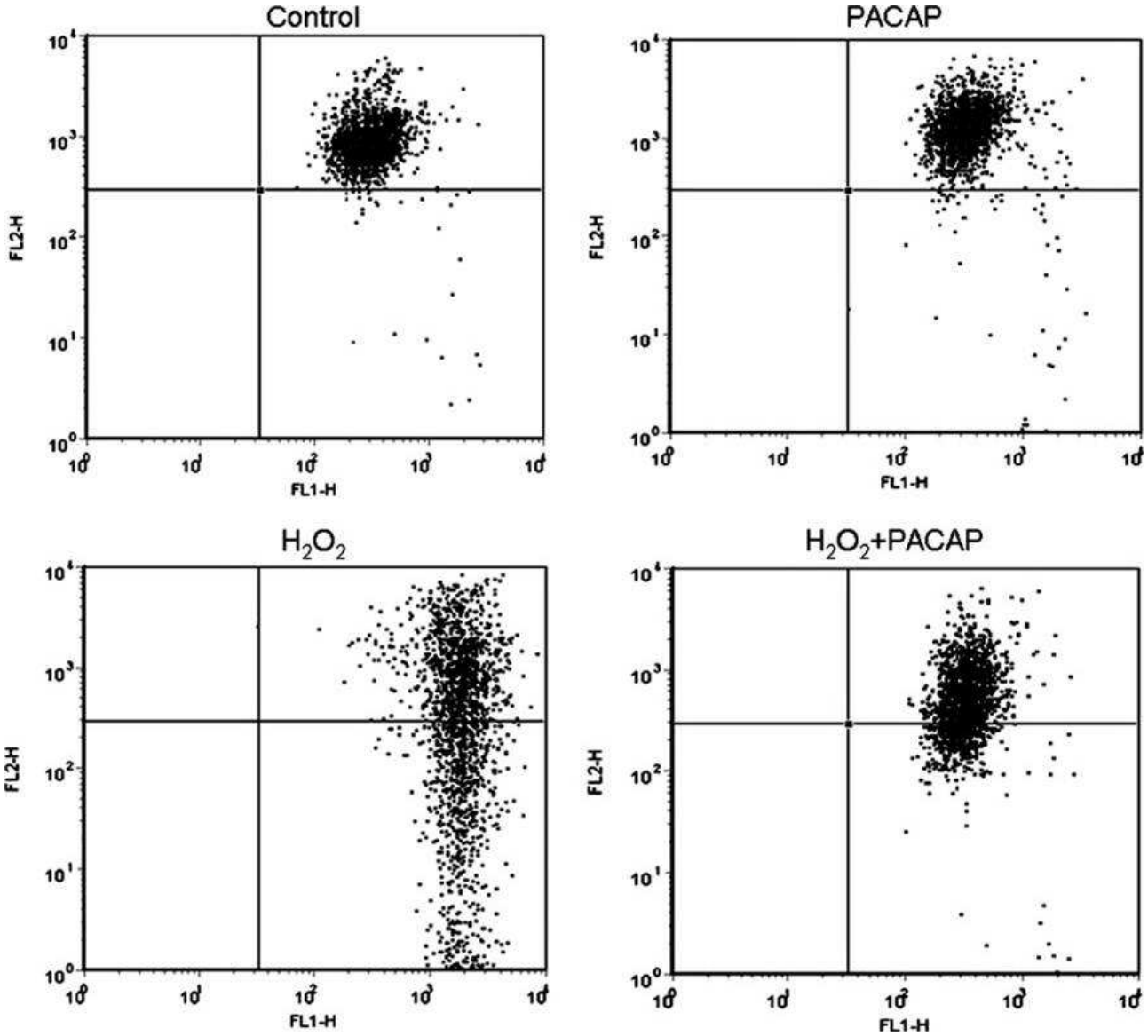


**Fig. 13.** Examples of dot plots as determined by flow cytometry following JC-1 staining. Horizontal axis represents JC 1 green intensity and vertical axis shows JC-1 red staining. The lines divide each plot into two quadrants: lower right quadrant, apoptotic cells; upper right quadrant, living cells

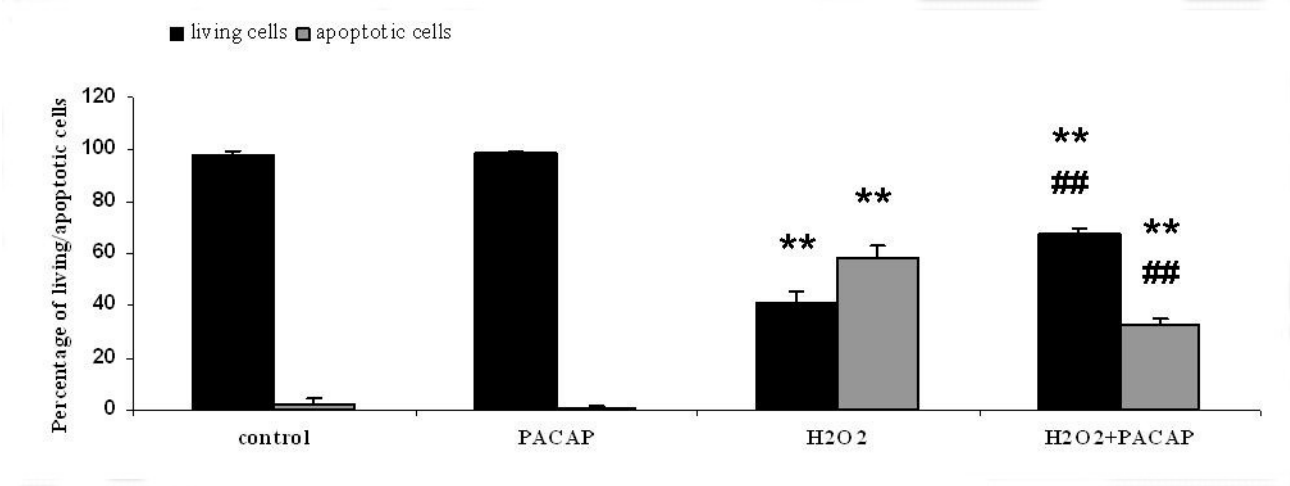


**Fig. 14.** Graphs demonstrate the mean percentage of living and apoptotic cells assessed by JC-1 assay. ARPE-19 cells were treated with 0.25 mM H2O2 and 10 nM PACAP for 3 h. Data are expressed as mean ± S.E.M. **P<0.01 compared to corresponding control values; ##P<0.01 compared to corresponding H2O2 values

# 5. DISCUSSION

Our first study shows that PARP inhibition is able to exert protective actions against ischemia induced retinal degeneration. Oxidative stress-induced DNA damage triggers PARP activation that was found to be a major regulator of cell death in various pathological conditions (Halmosi et al. 2001; Pacher and Szabo 2008) including diseases affecting the central nervous system (Kauppinen and Swanson 2007). In the eye, increased activation of PARP has been shown to contribute to several types of retinal degeneration, such as retinitis pigmentosa, diabetic retinopathy, and NMDA-, N-methyl-N-nitrosourea- or ischemia-reperfusion-induced cell death (Lam 1997; Shojaee et al. 1999; Chiang and Lam 2000; Patil and Sharma 2004; Goebel and Winkler 2006; Uehara et al. 2006). We used a murine BCCAO model in order to determine the role of PARP activation in chronic hypoperfusion-induced retinal degeneration. BCCAO was suggested as an adequate model for chronic cerebral hypoperfusion-related neurodegenerative diseases (Farkas et al. 2007) including ocular ischemic syndrome (Lavinsky et al. 2006; Yamamoto et al. 2006; Kalesnykas et al. 2008). Additionally, the possibility of direct unilateral administration of the substances to the vitreous body eliminated most of the pharmacokinetic complications. Ischemic insults are known to cause variable infarct/degeneration depending on several factors. Great variability of ischemic outcome, in the brain and in the retina as well, has been reported using different strains of rats, mainly depending on the anastomosis network (Oliff et al. 1997; Davidson et al. 2000). We did not observe the lack of retinal degeneration following carotid occlusion in our rat strain, and using the two sides of the same animal, as described in the present study, diminishes individual differences by allowing comparison to the untreated contralateral eye.

We observed an about 15-fold increase in self-ADP-ribosylation upon BCCAO indicating that PARP activation may have mediated the chronic cerebral hypoperfusion-induced injuries. We anticipated this finding since various aspects of oxidative stress but DNA-breaks and PARP activation during chronic hypoperfusion were published previously (Aliev et al. 2003; Kasparova et al. 2005; Kim et al. 2008; He et al. 2009). Unilateral administration of the carboxaminobenzimidazol PARP inhibitor HO3089 attenuated the BCCAO-induced PARP activation, demonstrating that it could exert its pharmacological effect in the retina under our experimental conditions. Besides suppressing PARP activation, HO3089 treatment alleviated the morphological changes associated with retinal degeneration caused by BCCAO. This finding suggests that PARP activation was a causative factor behind the chronic hypoperfusion-induced retinal degeneration, that is a major regulator of the cell death process exactly as it was found in various other models (Lam 1997; Shojaee et al. 1999; Chiang and Lam 2000; Halmosi et al. 2001;

Patil and Sharma 2004; Goebel and Winkler 2006; Uehara et al. 2006; Kauppinen and Swanson 2007; Pacher and Szabo 2008). Specificity and possible side effects of a pharmacological agent are always an issue; however, PARP inhibitory property of HO3089 was characterized previously (Kovacs et al. 2006). We found that it effectively inhibited PARP enzyme at nanomolar concentrations; therefore, the neuroprotective effect of HO3089 in this model was most likely the result of its inhibitory effect on PARP rather than a side effect.

Besides inducing NAD+ and ATP depletion leading to necrotic cell death, PARP activation was shown to influence other components of cell death machinery. Namely, PARP activation leads to destabilization of mitochondrial membrane systems (Halmosi et al. 2001; Hong et al. 2004; Tapodi et al. 2005), facilitates nuclear translocation of apoptosis inducing factor (Yu et al. 2002; Xiao et al. 2004; Li and Osborne 2008), activates cell death promoting kinases such as JNK1 (Xu et al. 2006), and modulates transcription factors and gene expression (Aguilar-Quesada et al. 2007; Cohausz and Althaus 2009; Krishnakumar et al. 2008). Inhibition of PARP can revert these processes, and in addition, it can activate one of the most important cytoprotective pathway, the PI-3-kinase-Akt system (Vereset al. 2003; Tapodi et al. 2005). We found enhanced activation of this pathway upon HO3089 treatment, indicated by increased phosphorylation of both Akt and its downstream target GSK-3beta. This is in agreement with our previous data that treatment with PARP inhibitor led to increased Akt and GSK-3beta activation in oxidative stress- and ischemia-induced cellular damage (Tapodi et al. 2005; Kovacs et al. 2006). Akt-mediated pathways are major cytoprotective signaling pathways in the retina (Lai et al. 2002). Inhibition of Akt activation increased the degree of retinal injury (Fontaine et al. 2002; Nakazawa et al. 2003; Park et al. 2008), whereas increasing the phosphorylation of Akt and its downstream target, GSK-3beta by neuroprotective strategies has been demonstrated to protect the retina against various types of injuries, including retinal ischemia (Lai et al. 2002; Weishaupt et al. 2004; Russo et al. 2008). The signal transduction pathways involving MAP kinases play key roles in cell survival and adaptation in various tissues including the retina (Zhang et al. 2002; Roth et al. 2003). According to our data, activation of ERK1/2 was dramatically enhanced by BCCAO. ERK1/2 is involved in cell differentiation and proliferation, and it is generally considered to be a survival-promoting agent. Postischemic elevation of ERK is usually associated with the stimulation of endogenous protective mechanisms.Elevated activation of ERK generally induces cytoprotection, although contradictory data also exist showing proapoptotic effects of the kinase pathway or no contribution to cell death (Roth et al. 2003; Munemasa et al. 2005). In the retina, inhibiting ERK activation has been reported to increase the degree of ganglion cell death following ischemia/reperfusion injury or optic nerve transection (Akiyama et al. 2002; Kilic et al. 2005). Other reports also support a protective role of ERK1/2 in the retina under different stress conditions (Chavarr´ıa et al. 2007; Luo et al. 2007). We found that similar to Akt,

ERK1/2 phosphorylation was increased upon HO3089 treatment over that of caused by BCCAO alone; therefore, it is very likely that the PARP inhibitor-induced overactivation of these pathways were important components of the neuroprotective effect observed in this model.

Retinal ischemia induced by elevation of intraocular pressure leads to activation of JNK and p38 MAP kinases within the first 6 h (Roth et al. 2003; Merienne et al. 2007; Roduit and Schorderet 2008). Long-term activation of MAP kinases has also been reported in excitotoxic retinal damage, with activation beginning 1–6 h after the NMDA treatment or the ischemic insult (Zhang et al. 2002; Munemasa et al. 2005). Our observations are in accordance with these data since we found activation of JNK and p38 MAP kinases 2–4 h after BCCAO indicated by increased phosphorylation of these kinases. JNK and p38 MAP kinases generally mediate the effect of cellular stress in the retina (Roth et al. 2003). Upon stressor effects, activation of these kinases is observed, while their inhibition is generally protective against various types of injuries. Blockade of p38 has been shown to provide significant protection against ischemic retinal damage (Roth et al. 2003). Similarly, blocking the activation of JNK and p38 MAPK decreased retinal ganglion cell death in NMDA retinotoxicity and in elevated intraocular pressure-induced ischemia (Ettaiche et al. 1999; Munemasa et al. 2005). PARP inhibition has also been shown to protect cells against alkylating agent-induced stress via JNK inactivation, and thus, decreasing photoreceptor apoptosis (Uehara et al. 2006). According to our data, the PARP inhibitor suppressed the BCCAO-induced activation of JNK and p38 MAPK; therefore, these mechanisms could also contribute to the alleviation of chronic hypoperfusion-induced damages of the retina.

Our second study shows that PACAP is able to exert protective actions against oxidative stress in human retinal pigment epithelial cells. In addition to the numerous studies providing evidence for the protective effects of PACAP in the neuronal retina, this is the first study that demonstrates such effects in the pigment epithelial cells. PACAP receptors have been identified earlier in several neuronal layers of the retina. The selective PACAP receptors are responsible for approximately 80% of PACAP binding in the retina (Nilsson et al. 1994). Radioligand binding studies have revealed the existence of PACAP receptors also in the human fetal retina and in retinoblastoma cells (Olianas et al. 1996, 1997). Detailed localization studies have revealed a strong expression of PAC1 receptor mRNA in the ganglion cell layer, inner nuclear layer, and nerve fiber layer, while a weaker expression in the inner and outer plexiform and the outer nuclear layers and the outer segments of the photoreceptors was observed (Seki et al. 1997, 2000). In culture, PAC1 receptor expression has been shown in the Muller glial cells, where PACAP exerts several functions (Kubrusly et al. 2005; Nakatani et al. 2006). In retinal pigment epithelial cells, PAC1 and VPAC1

receptors have been identified by RT-PCR studies (Zhang et al. 2005). The same study has shown that PACAP inhibited the interleukin 1β-stimulated expression of interleukin-6 and -8 and monocyte chemotactic protein-1 (Zhang et al. 2005). The authors used the same human pigment epithelial cell line (ARPE-19) that we used also in our present study.

Pigment epithelial cells perform a wide variety of functions that are critical during the embryonic development of the retina as well as throughout adult life to maintain normal vision (Grunwald 2009). Originally, the pigment layer of the retina was thought to function mainly as an absorptive layer of stray light to enhance visual acuity. However, these cells play several other important roles, such as formation of the blood–retinal barrier, elimination of waste products, selective transport to photoreceptors, processing of vitamin A in the visual cycle and phagocytosis of the photoreceptor outer segments disks facilitating the renewal of the photoreceptors. By building a barrier between blood and photoreceptors and by possessing specialized apical, basal, and lateral surfaces, pigment epithelial cells also provide a protective layer against toxic and oxidative damage that would be harmful for the photoreceptors. However, the retinal pigment epithelium itself is constantly exposed to external injuries, including oxidative stress. This may lead to degeneration, dysfunction, or loss of pigment epithelial cells. The balance between RPE cell death and proliferation may be responsible for several diseases of the underlying retina (Roduit and Schorderet 2008). High oxygen tension, exposure to light and the biochemical events of vision generate significant oxidative stress in the retina and the retinal pigment epithelium (Maeda et al. 2005). Retinal pigment epithelial cells have been implicated in several retinal diseases, including age-related macular degeneration, which is the leading cause of blindness among adults in developed countries, and in proliferative vitreoretinopathy, which is a major complication resulting from retinal detachment (Grunwald 2009).

Our results indicate that PACAP has antiapoptotic effects in oxidative stress-induced cell death in retinal pigment epithelial cells. This is in accordance with other studies showing that PACAP protects cells of different origin against oxidative stress. Not only neuronal cells but also non-neuronal cells have been demonstrated to react with decreased apoptotic rate when exposed to PACAP. Such effects have been described in cerebellar granule cells (Vaudry et al. 2002), cochlear cell culture (Racz et al. 2010), endothelial cells (Racz et al. 2007b) and cardiomyocytes (Gasz et al. 2006; Racz et al. 2008a). The exact mechanism of the observed protective effect is not known at the moment, but our results indicate the involvement of the Akt signaling pathway, since the survival-promoting effect of PACAP was significantly reduced in the presence of the PI3K/ Akt inhibitor. This is in agreement with previous observations showing the involvement of the Akt signaling pathway in the protective effects of PACAP (Racz et al. 2007a, 2008a).

Based on our present results, it can be hypothesized that such an effect is also present endogenously. Numerous studies have shown that PACAP-deficient mice react to damaging insults with increased susceptibility. For example, cerebellar granule cells isolated from PACAP knockout mice react to oxidative stress with a higher apoptotic rate (Vaudry et al. 2005). In addition, PACAP-deficient mice have increased neuronal damage in brain ischemia (Ohtaki et al. 2008) and axonal regeneration is delayed in a model of axotomy (Armstrong et al. 2008). Recently, we have shown that even kidney tubular cells isolated from PACAP-deficient mice are more sensitive to oxidative stress (Horvath et al. 2010). Retinal pigment epithelial cells possess PACAP receptors, and an earlier study has revealed that PACAP inhibits some inflammatory cytokine expression in these cells (Zhang et al. 2005). It is possible that PACAP is a survival-promoting factor also in retinal pigment epithelial cells.

# 6. CONCLUSION

**The activation status of PARP in BCCAO model**

- We provided evidence for establishing PARP activation as a major regulator of the cell death process in chronic hypoperfusion-induced neurodegeneration. Activation of PARP in the retina was revealed by assessing poly-ADP-ribosylation of target proteins. Treatment of the eye with the PARP inhibitor -HO3089- attenuated the BCCAO-induced self-poly-ADP-ribosylation of PARP.

**The effect of PARP inhibitor –HO3089- on retinal morphology in BCCAO model**

- We provide histological evidence for the retinoprotective effect of PARP inhibition. Intravitreal PARP inhibition -HO3089- treatment following BCCAO led to a nearly intact appearance of the retinal layers.This is well supported by the morphometric measurements.

**The involvement of different cell signaling pathways in the mechanism of PARP-inhibition-induced neuroprotection in this model.**

- We determined that activation of PI-3K-Akt and ERK1/2 was cytoprotective, and inhibition of JNK and p38 MAPK cytotoxic signaling pathways were very likely involved in the mechanism of PARP-inhibition-induced neuroprotection in this model.

In summary, based on these results PARP inhibition may represent a molecular target in the clinical management of ocular ischemic syndrome, and in a broader sense, chronic hypoperfusion-induced neurodegenerative diseases.

**Effect of PACAP on cell viability of human pigment epithelial cells**

- We showed that PACAP treatment diminished the effect of cell death caused by $H_2O_2$ treatment in retinal human pigment epithelial cell line. Furthermore, the effect of PACAP1-38 could be blocked by PACAP6-38 (inhibitor of PACAP1-38) co-application

**Concentration-dependency of PACAP**

- We found that the protective effect of PACAP was concentration dependent. From the concentration range of 1pM to 1µM, the best result was achived by 100 nM PACAP1-38 treatment.

**Effect of PACAP in cell death**

- PACAP administration led to a significant increase in the percentage of living cells and a reproducible decrease in the rate of apoptosis in cells treated with $H_2O_2$.

**Effect of PACAP on mitochondrial depolarization**

- An increase of apoptotic cell number was observed in the $H_2O_2$-treated group with a lower number of living cells. PACAP administration led to a significant increase in the percentage of living cells and a decrease in the percentage of apoptotic cells exposed to $H_2O_2$.

In summary, our present results show that PACAP has antiapoptotic effects against oxidative stress-induced cell death in retinal human pigment epithelial cells, providing an additional piece of evidence for the retinoprotective effects of PACAP. Thus PACAP may take part in future clinically effective treatments of retinal diseases caused by oxidative stress.

# 7. PRACTICAL MEANING / FUTURE PROSPECTS

Clinical observations often provide clues that assist in directing research regarding the pathogenesis of disease. Clinicians have known for many years that there is a strong correlation between retinal ischemia and retinal neovascularization. This led to the hypothesis that ischemic retina releases a substance(s) that stimulates retinal neovascularization. Investigators began searching for retina-derived factors that could stimulate proliferation and migration of endothelial cells in vitro and angiogenesis in vivo. Vascular endothelial growth factor (VEGF) fit the profile. Several in vitro and in vivo expermients are performed and confirmed the major role of VEGF in the process of angiogenesis. As the biochemical role of VEGF was elucidated, the antibody of VEGF was introduced as a therapeutic agent in the clinical practice.

Undoubtedly, the inhibition of the growth factor VEGF was only the first step. There is already a growing arsenal of new substances of different classes in preclinical and clinical studies. Although VEGF is not always the primary point of attack, the main goals are the inhibition or modulation of the members of VEGF-induced cascade.

There are extremely complex molecular mechanisms in intracellular signaling that we still do not know. Thus the identification and the elucidation of the role of these pathways are particularly important since it leads to identification of new drug targets and this can be the first step in the development of new therapeutic agents. On the other hand, the “deeper” exploration of the mode of action of new and existing drugs give the clinician the chance to treat the patient more effectively to save his/her vision.

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# 9. PUBLICATION OF THE AUTHOR

## This work is based on the following articles:

**L Mester**, A Szabo, T Atlasz, K Szabadfi, D Reglodi, P Kiss, B Racz, A Tamas, F Gallyas Jr, B Sumegi, E Hocsak, R Gabriel, K Kovacs

*Protection Against Chronic Hypoperfusion-Induced Retinal Neurodegeneration by PARP Inhibition via Activation of PI-3 kinase Akt Pathway and Supression of JNK and p38 MAP kinases*

Neurotox Res (2009) 16:68-76

**IF: 2,439 (2009)**

**L Mester**, K Kovacs, B Racz, I Solti, T Atlasz, K Szabadfi, A Tamas, D Reglodi

*Pituitary Adenylate Cyclase-Activating Polypeptide is Protective Against Oxidative Stress in Human Retinal Pigment Epithelial Cells*

J Mol Neurosci (2010) (In Press, PMID: 20645022)

**IF 2,720 (2009)**

K Szabadfi , **L Mester** , D Reglodi , P Kiss , N Babai , B Racz , K Kovacs , A Szabo , A Tamas, R Gabriel, T Atlasz

*Novel Neuroprotective Strategies in Ischemic Retinal Lesions*

Int. J. Mol. Sci. (2010), Feb 3;11(2):544-61

**IF: 1,387 (2009)**

## Further publications:

V Mester, F Kuhn, **L Mester**

*Epiretinal membranes: current management concepts*

Expert Review of Ophthalmology (2007), Feb Vol. 2, No.1, Pages 131-141

E Hocsak, B Racz, A Szabo, **L Mester**, E Rapolti, Sz Javor, Sz Bellyei, F Gallyas Jr., B Sumegi, A Szigeti

*TIP47 protects mitochondrial membrane integrity and inhibits oxidative-stress-induced cell death*

FEBS Letters (2010) Jul 2;584(13):2953-60.

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Viktoria, F Kuhn, **L Mester**
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Kovács Bálint professzor a Pécsi Szemklinika Igazgatója 1988-2008, Emlékkönyv
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## Abstracts

A Szigeti ., E Hocsák., **L Mester,** A Szabó , K Kovács , Sz Bellyei, B Sümegi
*A SOUL-fehérje mitokondriális permeabilitás-tranzícion keresztül nekrotikus sejthalált indukál*
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K Szabadfi, T Atlasz, D Reglődi, P Kiss, **L Mester**, R Gábriel, B Sümegi, M Obsahl, N Braaten, Alíz Szabó, F Jr. Gallyas, K Kovács
*Effects of PARP-inhibitor (HO3089) and SOD-mimetic (H2545) in bilateral common carotid artery occlusion induced retinal degeneration*
Magyar Idegtudományi Társaság Konferenciája (MITT) 2009. január 22-24.

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*Protection Against Chronic Hypoperfusion-Induced Retinal Neurodegeneration by PARP Inhibition via Activation of PI-3 kinase Akt Pathway and Supression of JNK and p38 MAP kinases*
Membrán-Transzport Konferencia, Sümeg, 2009. május 19-22.

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*Pituitary adenylate cyclase activating polypeptide is protective against oxidative stress in human retinal pigment epithelial cells*
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Solti I, Molnár E, **Mester L**, Rácz B, Kovács K, Agócs A, ifj. Gallyas F, Sümegi B
*A β-kriptoxantin védő hatásának vizsgálata oxidatív stressz-indukálta apoptózisban*
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Membrán-Transzport Konferencia, Sümeg, 2010. május 18-21.

## Presentations

**Mester L**, Hocsák E., Szabó A., Kovács K., Bellyei Sz., Szigeti A., Gallyas F., Sümegi B.
*Egy új-16.2 kD-kis hősokk fehérje azonosítása és a jelátviteli folyamatokra gyakorolt szerepének a vizsgálata*
Biokémiai és Orvosi Kémiai Intézet, Általános Orvostudományi Kar, Pécsi Tudomány Egyetem
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# 10. ACKNOWLEDGEMENTS

I would like to thank to my program leader Prof. Dr Balázs Sümegi for having given the possibility to work at the Department of Biochemistry and giving me support. I am grateful to Dr Dóra Reglődi and Prof. Dr Ferenc Gallyas Jr. for their tutorial work.

I would like to thank the following people who worked with me during the past 3 years and helped my work not only with giving excellent professional advice, but with maintaining friendly atmosphere:

Dr Krisztina Kovács, Alíz Szabó, Enikő Hocsák, Dr Péter Kiss, Krisztina Szabadfi, Tamás Atlasz from University of Pécs and Prof. Dr Bálint Kovács, Dr Balázs Kovács and all from the Department of Ophthalmolgy of the Kaposi Mór County Hospital.