

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

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Abstract Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide with potent neurotrophic and neuroprotective effects. We have previously shown that PACAP protects against several types of retinal injuries in vivo, including retinal ischemia, glutamate-induced excitotoxicity, UV A-induced lesion, and diabetic retinopathy. We have also shown that PACAP activates antiapoptotic pathways and inhibits proapoptotic signaling in retinal lesions in vivo. PACAP receptors have been identified on the retinal pigment epithelial cells and PACAP has been shown to inhibit interleukin secretion from pigment epithelial cells. It is not known, however, whether PACAP is protective in these cells. Human retinal pigment epithelial cells (ARPE-19 cell line) were exposed to in vitro oxidative stress by hydrogen peroxide. Cell survival was decreased in cells exposed to oxidative stress, which could be significantly and dose-dependently attenuated by 10 pM–1 μ M PACAP treatment, as shown by MTT viability test. The protective effect of

PACAP could be blocked by the receptor antagonist PACAP6-38. In addition, flow cytometry and JC-1 assay revealed that oxidative stress-induced apoptosis in retinal pigment epithelial cells could be decreased by PACAP treatment. In summary, these results show, for the first time, that PACAP is antiapoptotic in the retinal pigment epithelial cells.

Keywords ARPE-19 · Apoptosis · Flow cytometry · Retinoprotection

Introduction

The biological actions of pituitary adenylate cyclase-activating polypeptide (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

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nerve transection, glutamate- and kainate-induced excitotoxic injury, and ischemic degeneration (Atlasz et al. 2007, 2009, 2010b; Babai et al. 2005; Racz et al. 2007a; Seki et al. 2006, 2008). We found that the protective effects of PACAP are not neuronal phenotype-specific but rather reflect the stimulation of a common protective pathway found in all examined neuronal cell types (Atlasz et al. 2008, 2010a).

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 1 β -stimulated expression of interleukin-6 and -8 and monocyte chemoattractant protein-1 in ARPE-19 human pigment epithelial cells (Zhang et al. 2005). In spite of the numerous studies showing the protective effects of PACAP in the retina, 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.

Materials and Methods

Cell Culture

ARPE-19 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 μ g/mL). The cells were grown at 37°C in a humidified 5% CO₂ atmosphere.

Cell Viability Test

ARPE-19 (1.5×10^4 /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 μ M 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 μ M PACAP1-38. Furthermore, the experiment was repeated in the presence of inhibitors of different signaling pathways. The inhibitors used were 10 μ M PD 98059 (ERK inhibitor), 2 μ M SB 203580 (p38 inhibitor), 5 μ M JNK Inhibitor II and 10 μ M 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 $p < 0.05$.

Annexin V and Propidium Iodide Staining of the Cells

Ratio of apoptosis was evaluated after double-staining with fluorescein isothiocyanate (FITC)-labeled annexin V (BD 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 10⁶ cells. One hundred microliters of buffer (10⁵ 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 FACS Calibur 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.

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 FACS Calibur 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/green fluorescence 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).

Results

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, ARPE-19 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. 1a). Furthermore, we showed that this effect was dose dependent: 1 pM 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 by 100 nM PACAP1-38 treatment (Fig. 1b). 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 co-application 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).

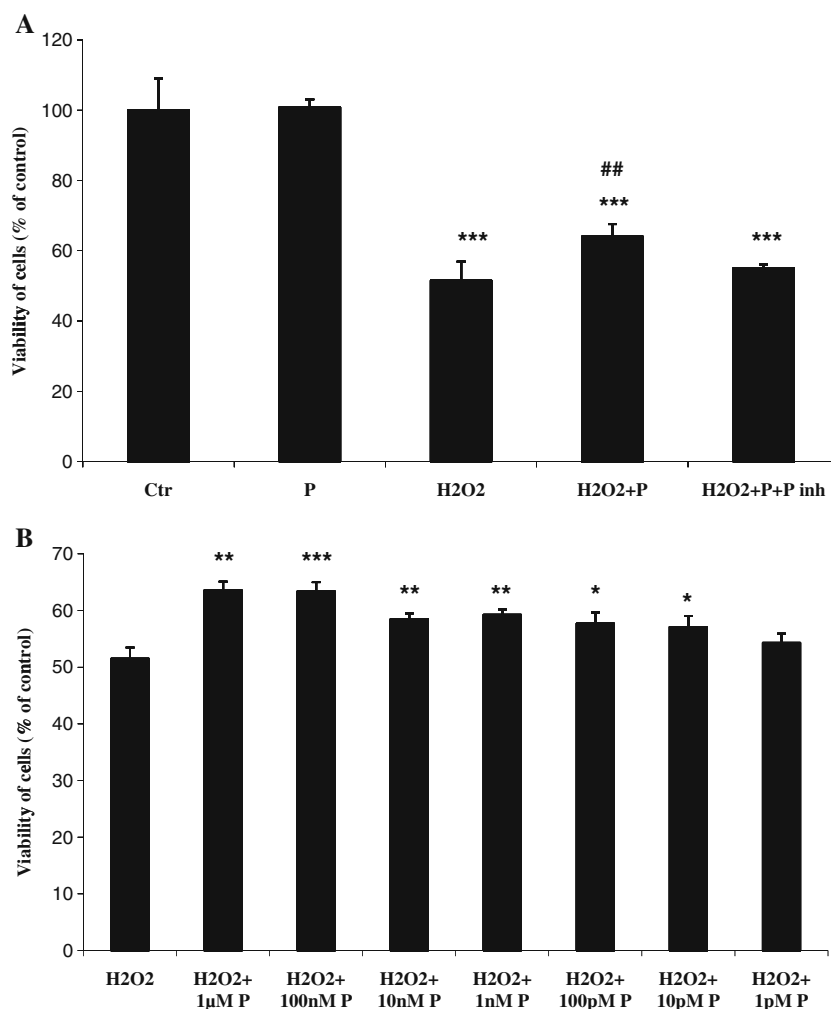
Annexin V and propidium iodide staining were used to detect apoptosis and necrosis in cultured cells. In the late phase of apoptosis, cells are stained with both dyes. Using this method, we found that the control group had more than 96% of intact, living cells and only less than 4% of cells in the necrotic and early, late phases of apoptosis (Figs. 2 and 3). 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. 2 and 3).

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. 4 and 5). 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. 4 and 5).

Discussion

The present 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

Figure 1 a Effect of PACAP on the viability on H_2O_2 -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 H_2O_2 alone, or with 10 nM PACAP1-38 (*P*), or PACAP1-38 and 1 μ M PACAP6-38 (PACAP inhibitor, *P inh*) for 3 h. **b** Concentration dependence of PACAP on H_2O_2 -treated ARPE-19 cells. ARPE-19 cells were treated with 0.25 mM H_2O_2 alone (H_2O_2) 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 \pm S.E.M. *** P <0.001 compared to control; ## P <0.01 compared to H_2O_2 and $H_2O_2 + P + P inh$ treatment



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 1 β -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

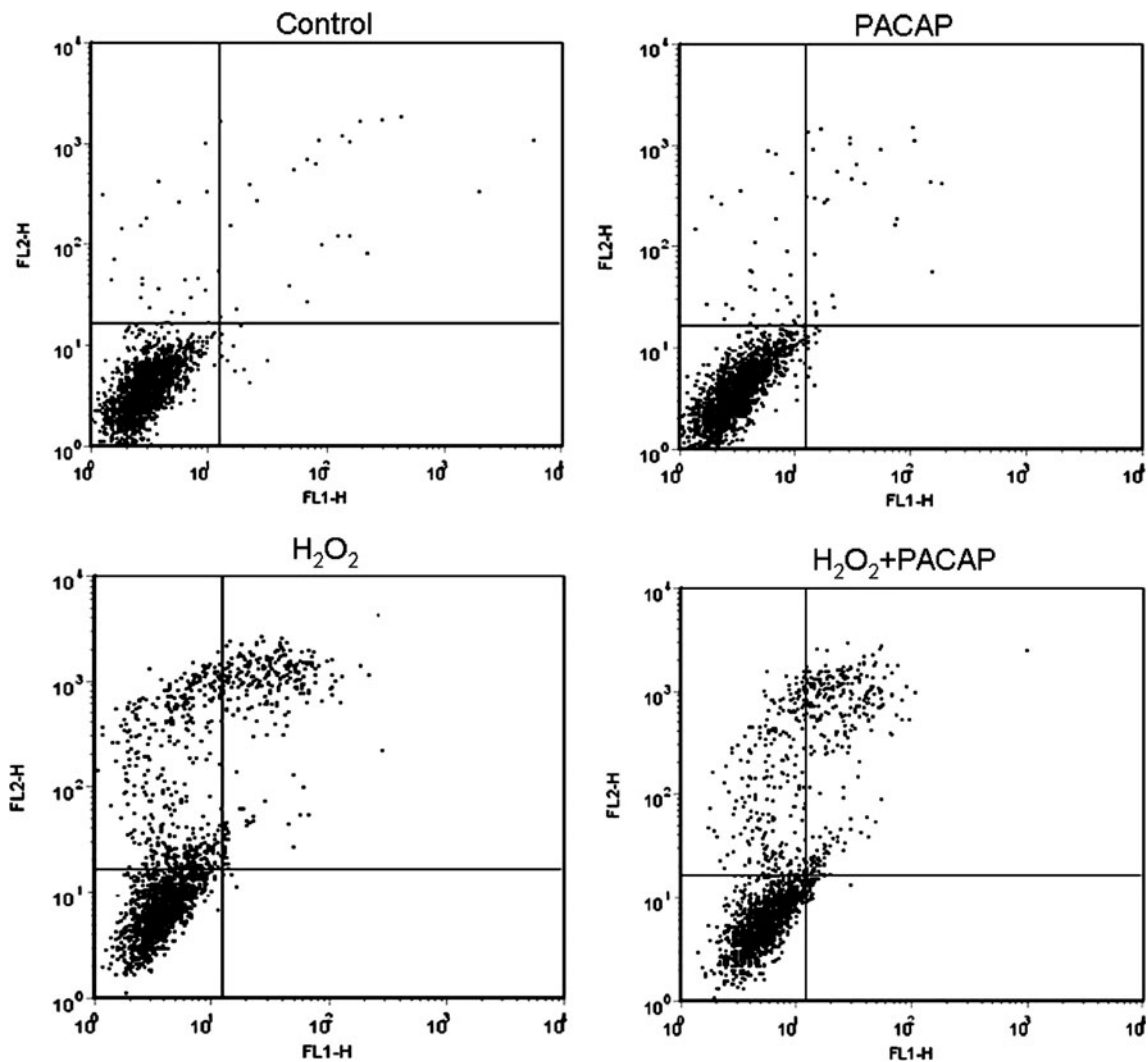


Figure 2 Distinction between living, necrotic, early, and late apoptotic cells in untreated control cells, PACAP-treated cells, cells exposed to 0.25 mM H_2O_2 for 3 h and H_2O_2 -treated cells co-incubated with 10 nM PACAP. The lines divide each plot into quadrants: *lower*

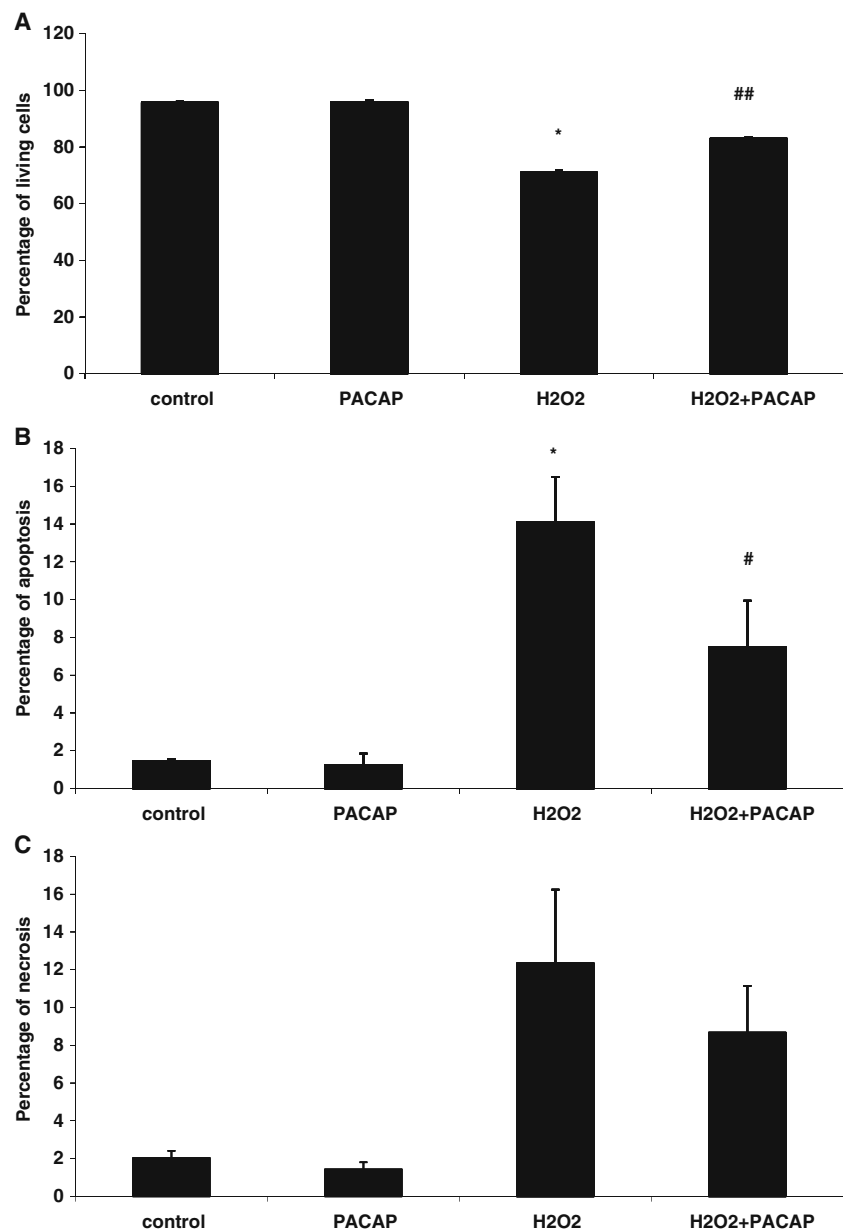
left quadrant, living cells; *lower right quadrant*, early apoptotic cells; *upper left quadrant*, necrotic cells; *upper right quadrant*, late apoptotic cells

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 cardio-

Figure 3 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 \pm S.E.M. * P <0.05 compared to control group; # P <0.05, ## P <0.01 compared to H₂O₂ group



myocytes (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.

In summary, our present results show that PACAP has antiapoptotic effects against oxidative stress-induced cell

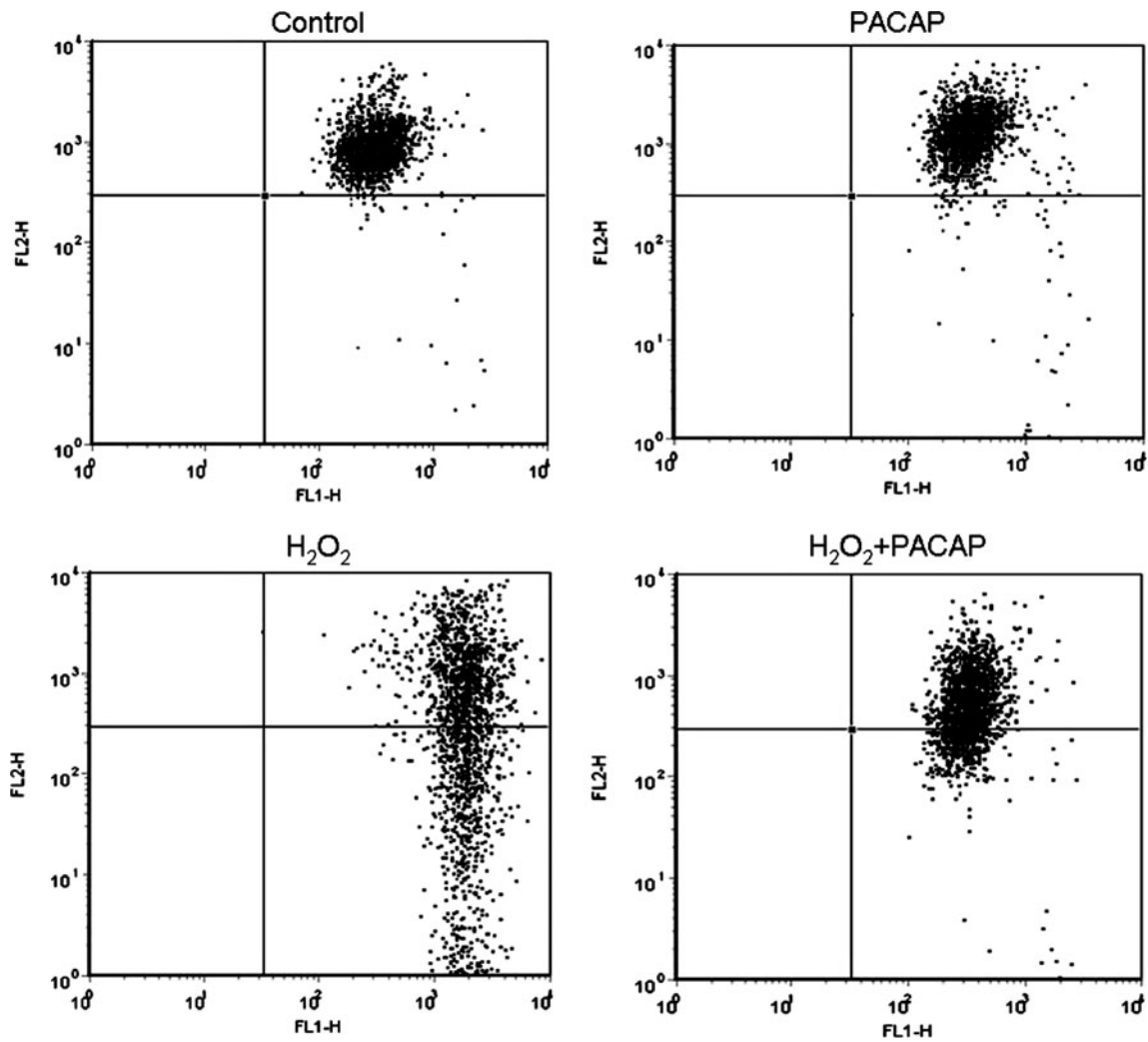
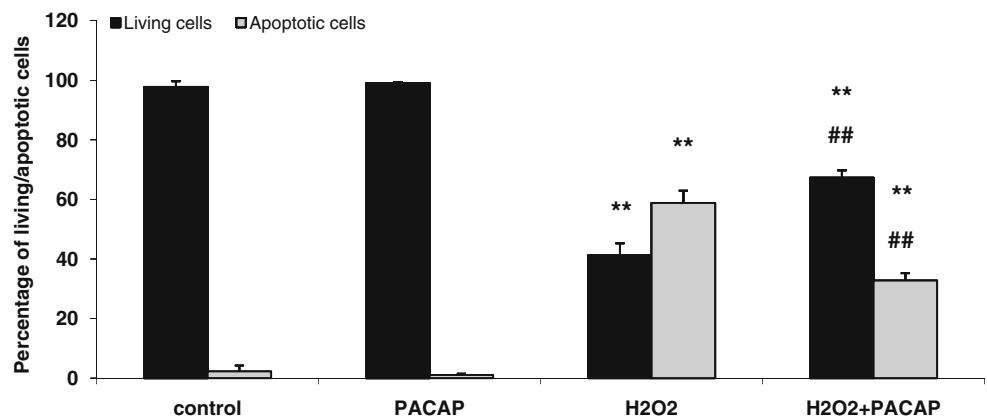


Figure 4 Examples of dot plots as determined by flow cytometry following JC-1 staining. *Horizontal axis* represents JC-1 green intensity and *vertical axis* shows JC-1 red staining. The lines divide

each plot into two quadrants: *lower right quadrant*, apoptotic cells; *upper right quadrant*, living cells

Figure 5 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



death in retinal human pigment epithelial cells, providing an additional piece of evidence for the retinoprotective effects of PACAP.

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