RESEARCH ARTICLE

Inhibition of retinal ganglion cell apoptosis: regulation of mitochondrial function by PACAP

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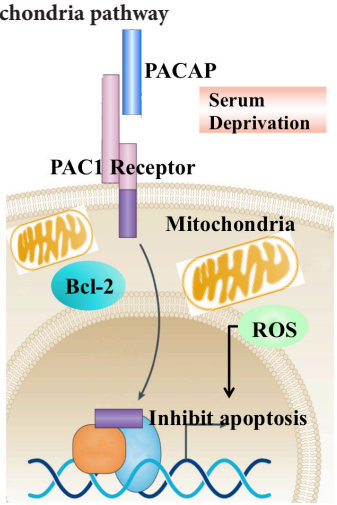
Graphical Abstract

PAC1 mediates the protection of pituitary adenylate cyclase-activating polypeptide (PACAP) against retinal ganglion cell apoptosis *via* mitochondria pathway



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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is an endogenous peptide with neuroprotective effects on retinal neurons, but the precise mechanism underlying these effects remains unknown. Considering the abundance of mitochondria in retinal ganglion cells (RGCs), we postulate that the protective effect of PACAP is associated with the regulation of mitochondrial function. RGC-5 cells were subjected to serum deprivation for 48 hours to induce apoptosis in the presence or absence of 100 nM PACAP. As revealed with the Cell Counting Kit-8 assay, PACAP at different concentrations significantly increased the viability of RGC-5 cells. PACAP also inhibited the excessive generation of reactive oxygen species in RGC-5 cells subjected to serum deprivation. We also showed by flow cytometry that PACAP inhibited serum deprivation-induced apoptosis in RGC-5 cells. The proportions of apoptotic cells and cells with mitochondria depolarization were significantly decreased with PACAP treatment. Western blot assays demonstrated that PACAP increased the levels of Bcl-2 and inhibited the compensatory increase of PAC1. Together, these data indicate protective effects of PACAP against serum deprivation-induced apoptosis in RGCs, and that the mechanism of this action is associated with maintaining mitochondrial function.

Key Words: *nerve regeneration; pituitary adenylate cyclase-activating polypeptide; pituitary adenylate cyclase-activating polypeptide receptor type 1; serum deprivation; apoptosis; retinal ganglion cell; retinal ganglion cell-5; glaucoma; mitochondria; neural regeneration*

Introduction

Glaucoma can cause permanent loss of vision and is characterized by initial changes to retinal ganglion cell (RGC) axons and secondary death of RGC cell bodies (Calkins et al., 2012). Glaucoma is typically associated with intraocular pressure (IOP). However, even with treatment to reduce IOP, the symptoms of a substantial fraction of glaucoma patients worsen with time. Furthermore, the IOP of some glaucoma patients never rises beyond the normal range (Pascale et al., 2012). Although the etiology of glaucoma is complex and enigmatic, RGC apoptosis is recognized as the ultimate reason for loss of vision (Tian et al., 2015). Neuroprotective treatments that directly target the injured RGCs are still in early stages of development. Therefore, there is an urgent need to develop therapeutic agents for glaucoma neuropathy and neurotrophic factors are promising candidates.

Pituitary adenylate cyclase-activating polypeptide (PACAP) exists in two forms (namely 27- or 38-amino acid neuropeptides) and belongs to the vasoactive intestinal polypeptide/glucagon/secretin family (Bourgault et al., 2009). PACAP and its receptors are expressed in various neural tissues and it exerts potent neuroprotective effects both

exogenously and endogenously (Endo et al., 2011). Most of PACAP's effects are mediated through the activation of its specific receptor, PAC1 (Zhou et al., 2002). PACAP is a pleiotropic molecule exerting effects on a wide array of physiological processes, including cell survival in neurodegenerative conditions, the stress response and cell division (Castorina et al., 2010; Giunta et al., 2012; D'Amico et al., 2013, 2015; Maugeri, et al., 2016). It also acts as a neurotransmitter and/or a neuromodulator in both the peripheral and central nervous systems (Jóźwiak-Bębenista et al., 2015; Yang et al., 2015; Shioda et al., 2016). The protective effects of PACAP in the retina have been investigated by numerous studies, including diabetic retinopathy induced by streptozotocin (Szabadfi et al., 2012), transient ischemia following high IOP (Seki et al., 2011) and ultraviolet-light-induced retinal damage (Atlasz et al., 2010). We have previously demonstrated that a PACAP derivative attenuates the apoptosis of RGC-5 cells induced by ultraviolet B irradiation and retinal excitotoxicity induced by N-methyl-D-aspartic acid (Cheng et al., 2014). However, the *in vitro* effects of PACAP and PAC1 on the apoptosis of RGCs and the underlying mechanism remain largely unknown. In the current study, the neuroprotective effects of PACAP against serum deprivation (SD)-induced apoptosis of RGC-5 cells were further explored.

Materials and Methods

RGC-5 cell culture

The RGC-5 cell line (Li et al., 2011) was provided by Dr. Neeraj Agarwal from the Department of Cell Biology and Genetics, UNT Health Science Center, Fort Worth, TX, USA. The RGC-5 cell line is a transformed RGC line that has been widely used, expresses RGC markers, and exhibits ganglion cell-like behavior in culture (Wood et al., 2010). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) in a humidified incubator at 37°C for 24 hours. The culture medium was then exchanged for DMEM containing normal 10% FBS or FBS-free DMEM with or without PACAP1–38 at different concentrations (1 nM, 10 nM, 100 nM, 1 μM, 10 μM PACAP). Cells were further incubated for 48 hours only because a previous study (Fuma et al., 2016) demonstrated an approximately 50% loss of cell viability after serum withdrawal for 48 hours. After determining the optimal concentration of PACAP, cells were divided into three groups: control, serum deprivation (SD) and SD + PACAP groups. In the SD + PACAP group, cells were exposed to SD and 100 nM PACAP for 48 hours, while SD group was exposed to SD without PACAP.

Cell viability assay

Forty-eight hours after treatment with SD or SD + PACAP, cell viability was assessed with Cell Counting Kit-8 (Dojindo, Japan). Briefly, cells were stained with 10 μL Cell Counting Kit-8 solution for 3 hours. Optical density (OD) of each well was measured with a microplate reader (Tecan Safire2, Männedorf, Switzerland) at 450 nm. Wells with only culture medium were used as the blank control. Cell viability was equal to $(OD_{SD\ group\ or\ SD+PACAP\ group} - OD_{blank\ control})/(OD_{control\ group} - OD_{blank\ control}) \times 100\%$.

Cell cycle analysis

RGC-5 cells were deprived of serum with or without 100 nM PACAP for 12 or 24 hours. The phase distribution of DNA content in the cells was then detected with propidium iodide (PI) staining and flow cytometry. Following 100 nM PACAP treatment for 12 or 24 hours, RGC-5 cells were collected, fixed in 70% ethanol and stored overnight at −20°C. The next morning, cells were washed and stained with PI staining solution (50 μg/mL PI and 10 μg/mL RNase) for 30 minutes in the dark. The cell cycle was then analyzed by flow cytometry using Cell-Quest software (FACSAria™, BD, San Jose, CA, USA). The percentages of cells in S, G0/G1 and G2/M phases were analyzed by pairwise comparisons.

Annexin V/PI staining and JC-1 assays

RGC-5 cells (5×10^5) were collected following treatment for 48 hours and suspended in 200 μL binding buffer. Then cells were stained with 10 μL Annexin V-FITC and 10 μL PI for 15 minutes. The apoptosis of cells was subsequently detected by flow cytometry (FACSAriaTM, BD).

After SD treatment with or without PACAP for 48 hours, cells were incubated with 200 μL JC-1 solution for 15 minutes. Then cells were washed with phosphate buffered saline (PBS), pelleted by centrifugation, resuspended in 500 μL PBS and analyzed with a flow cytometer (FACSAriaTM, BD). The percentage of apoptotic cells with mitochondrial depolarization was analyzed.

Hoechst 33342 staining

Briefly, RGC-5 cells were seeded on 6-well plates at a density of 5×10^4 cells/mL. After treatment, cells were washed with PBS and fixed with 4% paraformaldehyde for 20 minutes. After removing paraformaldehyde, cells were stained with

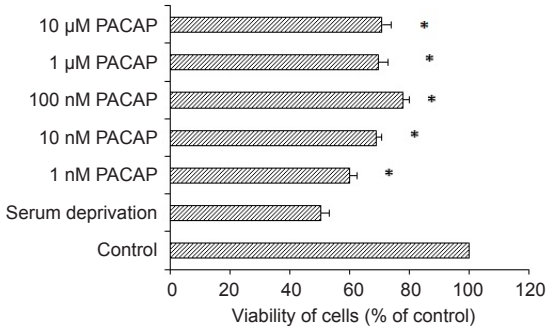

Figure 1 The effect of PACAP on the viability of RGC-5 cells subjected to serum deprivation for 48 hours.
Serum deprivation for 48 hours reduced the viability of RGC-5 cells to approximately 50% compared to control. This negative effect of serum deprivation was attenuated by treatment with 1 nM to 10 μM PACAP for 48 hours. $*P < 0.05$, *vs.* serum deprivation group (mean ± SD, one-way analysis of variance followed by the Student-Newman-Keuls test). Each experiment was conducted six times. PACAP: Pituitary adenylate cyclase-activating polypeptide; RGC: retinal ganglion cell.

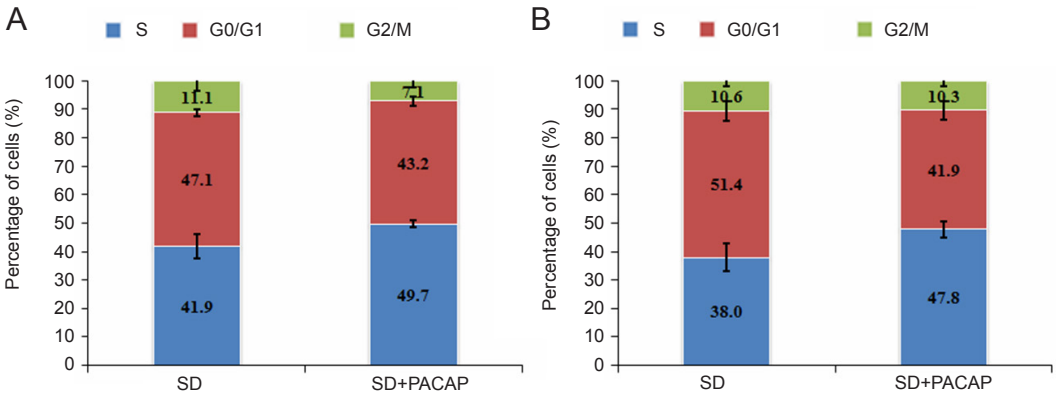

Figure 2 Effects of 100 nM PACAP on the cell cycle of RGC-5 cells at various time points using flow cytometric analysis.
(A, B) 100 nM PACAP induced a marked increase in the percentage of cells in S phase compared to cells subjected to SD only at both 12 hours (A) and 24 hours (B). Each experiment was conducted six times. PACAP: Pituitary adenylate cyclase-activating polypeptide; RGC: retinal ganglion cell; SD: serum deprivation.

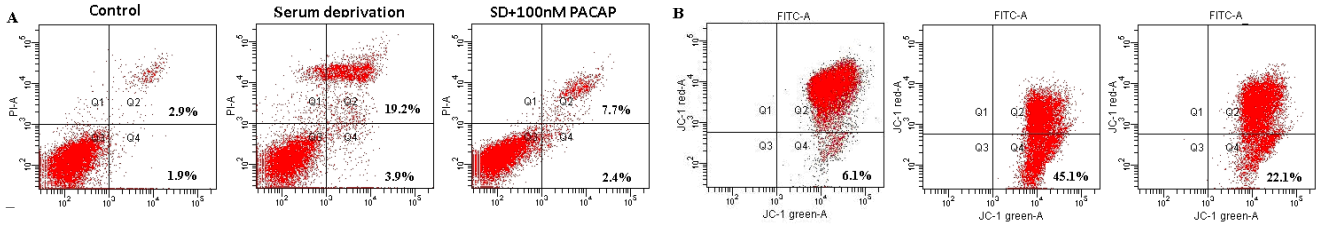

Figure 3 Representative dot plots determined by flow cytometry following Annexin V/PI staining.
(A) Lower-left quadrant (Q3), living cells; upper-left quadrant (Q1), necrotic cells; lower-right quadrant (Q4), apoptotic cells. (B) Dot plots of JC-1 staining: lower-right quadrant (Q4), apoptotic cells with mitochondrial depolarization; upper-right quadrant (Q2), living cells. PI: Propidium iodide; PACAP: pituitary adenylate cyclase-activating polypeptide; SD: serum deprivation.

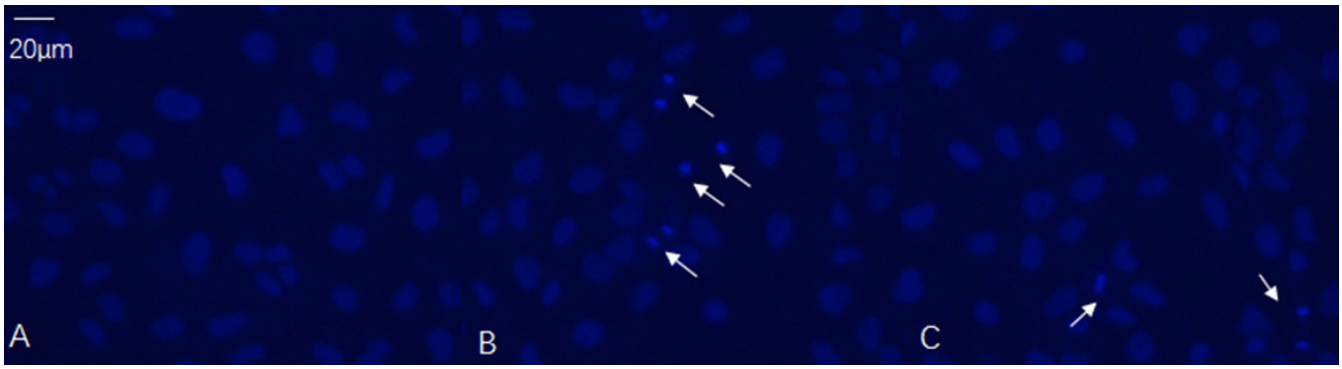

Figure 4 Representative micrographs of RGC-5 cells stained with Hoechst 33342.
(A–C) Normal control cells show normal nuclear morphology (A). Cells subjected to serum deprivation show nuclear shrinkage and condensation (B). 100 nM PACAP reduced this negative effect (C). White arrows point to apoptotic cells showing high intensity blue fluorescence and nuclear condensation. Scale bar: 20 μm. PACAP: Pituitary adenylate cyclase-activating polypeptide; RGC: retinal ganglion cell.

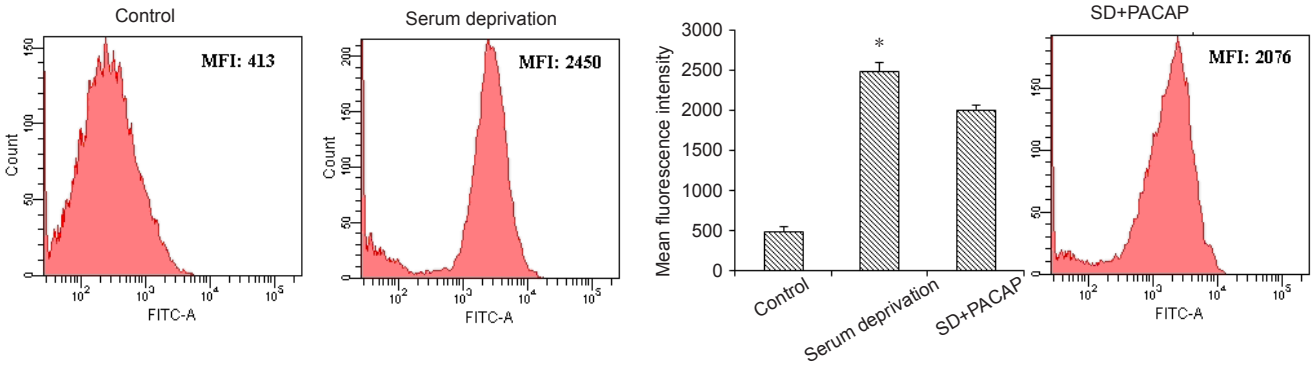

Figure 5 Effect of PACAP on ROS levels in RGC-5 cells subjected to SD for 48 hours.
RGC-5 cells exposed to SD exhibited intense fluorescence. 100 nM PACAP clearly blunted the accumulation of ROS in serum-deprived cells. $*P < 0.05$, *vs.* SD + PACAP group (mean ± SD, one-way analysis of variance followed by Student-Newman-Keuls test). Each experiment was conducted six times. PACAP: Pituitary adenylate cyclase-activating polypeptide; RGC: retinal ganglion cell; SD: serum deprivation; ROS: reactive oxygen species.

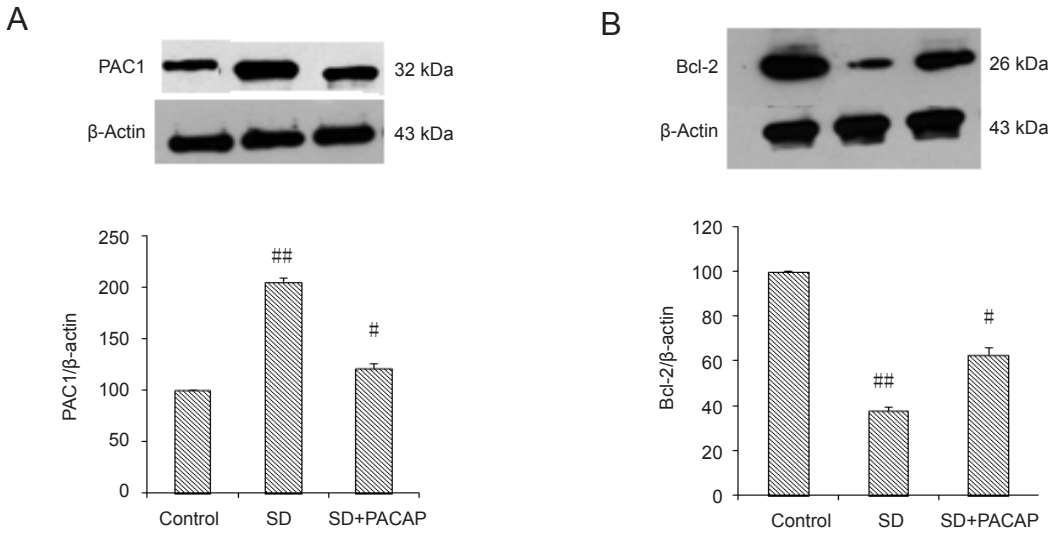

Figure 6 Densitometric analysis of PAC1 and Bcl-2 levels in RGC-5 cells exposed to SD with or without 100 nM PACAP.
SD caused a significant decrease in the level (optical value ratio) of Bcl-2 (B) and a concomitant increase of PAC1 (A), which was counteracted by PACAP treatment. #$P < 0.05$, ##$P < 0.05$, *vs*. SD group (mean ± SD, one-way analysis of variance followed by the Student-Newman-Keuls test). Each experiment was conducted three times. PACAP: Pituitary adenylate cyclase-activating polypeptide; RGC: retinal ganglion cell; SD: serum deprivation.

10 μM Hoechst 33342 solution (Sigma, Shanghai, China) for 20 minutes and observed under a fluorescence microscope (BZ X700, Keyence, Osaka, Japan).

ROS quantification

Following treatment, RGC-5 cells were stained with 10 μM DCFH-DA for 15 minutes in the dark at 37°C. Cells were then washed with PBS and analyzed within 30 minutes by flow cytometry (FACSAriaTM, BD, equipped with an air-cooled argon laser at 488 nm). The intensity of green fluorescence, which indicates the level of intracellular ROS accumulation, was detected and compared between groups.

Western blot assay

To control cell number, RGC-5 cells were seeded at a density of 5×10^4 cells/mL in 6-well plates and then subjected to various treatments. Forty-eight hours after treatment with SD in the presence or absence of 100 nM PACAP, cells were collected, lysed with RIPA buffer sup-plied with protease and phosphatase inhibitor cocktail and sonicated on ice. The sonicated cell samples were then centrifuged for 20 minutes at 15,000 × *g* at 4°C. After centrifugation, the supernatant was collected. Proteins (10 μg) were then separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were incubated at 4°C with primary antibodies as follows: mouse monoclonal anti-Bcl-2 antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-PAC1 antibody (1:1000; Santa Cruz Biotech nology), and mouse monoclonal anti-β-actin antibody (1:3000; Cell Signaling, Danvers, MA, USA). After over-night incubation with primary antibodies, secondary an-tibodies conjugated with horseradish peroxidase (1:3000; Santa Cruz Biotechnology) were applied for 1 hour at room temperature. The signals were detected with enhanced chemiluminescence (BeyoECL Plus). Protein levels were evaluated by measuring the optical value ratios of Bcl-2 and PAC1 to β-actin, and then normalized to the control group. The immunoblot band of each protein was quantified and normalized to that of β-actin.

Statistical analysis

Data were analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA) and are presented as the mean ± SD. One-way analysis of variance and the Student-Newman-Keuls test were used to determine statistical comparison. $P < 0.05$ was considered statistically significant.

Results

PACAP increased the viability of RGC-5 cells

The cell viability of RGC-5 cells deprived of FBS was significantly increased by 1 nM to 10 μM PACAP38 (**Figure 1**). The optimum protection was achieved with 100 nM PACAP. DNA content analysis showed that 100 nM PACAP treatment resulted in remarkably more cells at S phase compared to cells subjected to SD only. As shown in **Figure 2**, PACAP increased the percentage of cells in S phase and reduced the percentage of cells in G_0/G_1 phase compared to RGC-5 cells subjected to SD only. The proportion of cells in S-phase was significantly higher in the SD + PACAP group than in the SD group at both 12 hours (49.65 ± 1.17%, *vs*. 41.85 ± 4.13%) and 24 hours (47.80 ± 2.78%, *vs*. 38.00 ± 4.94%).

PACAP inhibited SD-induced apoptosis in RGC-5 cells

PACAP treatment significantly decreased the rate of apoptosis, which was 10.50 ± 1.23% in the SD + PACAP group and 25.14 ± 1.84% in the SD group. With JC-1 staining, we discovered that PACAP treatment remarkably decreased the proportion of cells with mitochondrial depolarization to

22.57 ± 2.24%, while the percentage of depolarized cells in the SD group was 64.17 ± 1.70% (**Figure 3**). Hoechst 33342 staining showed that the nuclei of serum-deprived cells were shrunken and condensed, while PACAP treatment attenuated nuclei change (**Figure 4**).

ROS, PAC1 and Bcl-2 levels

The mean intensity of ROS fluorescence was elevated over 5-fold in RGC-5 cells subjected to SD but was considerably decreased with 100 nM PACAP treatment (**Figure 5**).

To further investigate the protective mechanism of PACAP, we analyzed the expression levels of PAC1 and Bcl-2. Forty-eight hours after SD, a significant increase in PAC1 and a simultaneous decrease in Bcl-2 levels were seen. Treatment with PACAP dramatically inhibited such changes (**Figure 6**).

Discussion

PACAP is an important growth factor capable of preventing apoptosis in neuronal cells (Maino et al., 2015). Protection against RGC apoptosis by PACAP has been investigated using ultraviolet B irradiation (Ding et al., 2012), N-methyl-D-aspartic acid excitotoxicity (Cheng et al., 2014), ischemic retinal degeneration (Szabadfi, 2012) and experimental diabetic retinopathy (Szabadfi et al., 2014, 2016). On the basis of our previous *in vivo* and *in vitro* studies (Cheng et al., 2014), the question emerged as to whether PACAP can attenuate RGC apoptosis induced by other unexplored insults. Protective effects of PACAP on serum deprived neuronal cells have been reported for cerebellar granule cells (Maino et al., 2015), schwannoma cells (Castorina et al., 2008) and rat cortical neurons (Frechilla et al., 2001). SD is a well-established model to investigate apoptosis in RGCs (Sun et al., 2012; Majid et al., 2013; Miki et al., 2013). However, to our knowledge, no previous study has assessed the protection of PACAP on RGC apoptosis induced by SD. The present study demonstrated that PACAP attenuates SD-induced apoptosis in RGC-5 cells.

PACAP is neuroprotective in the retina and 100 nM was the optimal concentration (Seki et al., 2008). In our study, the anti-apoptotic effect of PACAP was not dose-dependent and peaked at a concentration of 100 nM. The SD + PACAP group exhibited a higher percentage of cells in S-phase compared with the SD group, indicating that PACAP promoted cell viability partly by accelerating the cell cycle. Neuronal damage in glaucoma has been associated with increased free radical production and to a low level of endogenous antioxidant defense (Izzotti et al., 2006). In the present research, SD led to ROS over-generation. The over generation of ROS is indicative of compromised antioxidant capacity and makes cells vulnerable to injury (Kang et al., 2010). Numerous stressors capable of causing excessive ROS production are involved in glaucoma (Pinazo-Durán et al., 2012). Mitochondria are the major site for superoxide production, and are vulnerable to direct attack by ROS (Orrenius, 2007). Mitochondrial dysfunction leads to increased production of ROS, which in turn aggravates oxidative stress (Jezek et al., 2005). PACAP prevents the decrease of mitochondrial activity in astroglial cells (Masmoudi-Kouki et al., 2011) and inhibits the excessive generation of ROS in ultraviolet B irradiated RGC-5 cells (Cheng et al., 2014). However, whether PACAP reduces ROS production in serum-deprived RGC-5 cells has not been elucidated. Our study revealed that the rise in ROS levels in response to SD was markedly quenched by 100 nM PACAP treatment.

SD induces apoptotic cell death of transformed rat RGCs *via* mitochondrial signaling pathways (Charles et al., 2005). The number and morphology of mitochondria vary widely among cell types and are regulated by intrinsic and extrinsic mechanisms (Davis et al., 2014). RGCs probably have more mitochondria than any other neuronal cell type and efficient intraocular axon mitochondrial function is essential to maintain overall function of RGCs (Osborne et al., 2014). Mitochondrial stress in individual RGCs has been proposed as a major trigger of glaucoma and pharmacological agents that maintain mitochondrial functions might, therefore, provide a novel way of delaying RGC death (Osborne et al., 2013). As indicated by JC-1 assays, PACAP compensated the loss of mitochondrial membrane potential caused by SD in RGC-5 cells. PAC1 is abundant in the retina (Dénes et al., 2014) and we have previously reported the expression of PAC1 in RGC-5 cells, which was increased in ultraviolet B-induced apoptotic cells (Ding et al., 2012). In our present study, there is a direct involvement of PAC1 in the anti-apoptotic function because PAC1 expression is significantly increased in RGC5 cells exposed to SD. Similarly, expression of the gene encoding PAC1 increases markedly during inflammation and disease to alleviate inflammation, oxidative stress and apoptosis (Xu et al., 2016). We suggest that the protective effect of PACAP is mediated *via* PAC1 and an exogenous PACAP supply counteracts the compensational increase of PAC1 signaling. The activation of PAC1 by PACAP modulates cell death in the retina through the intracellular cAMP/cAMP-dependent protein kinase pathway (Silveira et al., 2002) and the protective effect of PACAP involves complex kinase signaling pathways related to cAMP/ERK/CREB activation (Racz et al., 2006). PACAP, through activation of its receptor, PAC1, and the protein kinase A, protein kinase C, and MAP-kinases signaling pathways, prevents accumulation of ROS, which allows the preservation of mitochondrial membrane integrity (Han et al., 2014; Douiri et al., 2016). Further studies are needed to gain deeper insights into the mechanism underlying the protective effect of PACAP against SD-induced apoptosis in RGC-5 cells.

Bcl-2 levels in RGC-5 cells subjected to SD were significantly lower than those in RGC-5 cells. The expression of anti-apoptotic Bcl-2 increases greatly in apoptotic RGC-5 cells in response to other insults like H_2O_2. Members of the Bcl-2 family participate in the initiation of the mitochondrial signal pathway, thus regulating cell apoptosis (Wang et al., 2013). Therefore, PACAP may inhibit the apoptosis of RGC-5 cells *via* the mitochondrial pathway by reducing ROS levels and, at the same time, by increasing Bcl-2 levels. Although there is controversy over the validity of RGC-5

cells, they are widely accepted as retinal neuronal precursor cells (Van Bergen et al., 2009). Furthermore, in our previous study, the RGC-5 cells that we use have been proven to express specific markers for RGCs. Our results suggest that PACAP may protect RGCs from apoptotic death by inhibiting the mitochondrial apoptosis pathway. However, as intracellular signaling pathways are very complex, the involvement of other pathways cannot be excluded. Furthermore, the protective mechanism of PACAP mediated by PAC1 is complicated and varies among cell types. The protective effect of PACAP and its underlying mechanism should be further investigated in animal models.

In conclusion, PACAP attenuates SD-induced apoptosis in RGCs and might play an important role in the neuroprotection of RGCs. Perturbations in the PACAP/PAC1 pathway are involved in abnormal stress responses underlying post-traumatic stress disorder (Ressler et al., 2011). We cannot help but wonder whether the PACAP/PAC1 pathway is associated with glaucomatous neuropathy, which will be the direction of our future research.

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Author contributions: *HHC and YD designed the study. HHC and RPP performed experiments. JD and HY analyzed data. HHC wrote the paper. All authors approved the final version of the paper.*

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