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# Effect of PACAP on MAP kinases, Akt and cytokine expressions in rat retinal hypoperfusion

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## HIGHLIGHTS

- Effects of intravitreal PACAP was tested in hypoperfusion-induced retinal injury.
- PACAP attenuates inflammatory cytokine response upon ischemia in the retina.
- PACAP induces ERK1/2 and Akt activation in ischemic retina.
- PACAP inhibits JNK and p38 MAPK expression.

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## ABSTRACT

Pituitary adenylate cyclase activating polypeptide (PACAP) is known for its potent neuroprotective effects, including the retinoprotective actions in several types of retinal injuries. We have shown earlier that PACAP treatment causes activation of protective pathways and inhibition of pro-apoptotic signaling in excitotoxic retinal lesions. The aim of the present study was to gain insight into the in vivo protective mechanism of PACAP in retinal hypoperfusion injury induced by bilateral common carotid artery occlusion (BCCAO). Rats underwent BCCAO and received intravitreal PACAP (PACAP38) treatment. We investigated the activation level of the protective Akt pathway as well as the different mitogen activated protein kinases (MAPKs) by Western blot analysis and the expression of cytokines using a cytokine array kit. We found that PACAP treatment alone did not influence the phosphorylation of Akt or the MAPKs, but decreased the hypoperfusion-induced activation of both p38MAPK and JNK and increased the activation of the protective Akt and ERK1/2 in hypoperfused retinas. The cytokine profile was dramatically changed after BCCAO, with most cytokines and chemokines showing an increase, which was attenuated by PACAP (such as CINC, CNTF, fractalkine, sICAM, IL-1, LIX, Selectin, MIP-1, RANTES and TIMP-1). In addition, PACAP increased the expression of VEGF and thymus chemokine. The present results provide further insight into the neuroprotective mechanism induced by PACAP in ischemic retinal injuries, showing that PACAP ameliorates hypoperfusion injury involving Akt, MAPK pathways and anti-inflammatory actions.


## 1. Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide with miscellaneous actions in the nervous system and peripheral organs [38,41,49]. The effects of PACAP in the eye are also diverse. Actions on the pupillary reflex, tear secretion and retinohypothalamic circadian system, as well as trophic effects during retinal development have been described [7,17,20,24]. One of the well-studied effects of PACAP in the eye is itsretinoprotection

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action [4]. PACAP protects against various harmful stimuli in the retina, such as monosodium glutamate-, NMDA- or kainate-induced excitotoxic injury, UV light-induced retinal degeneration and diabetic retinopathy [3,5,6,16,28,40,43,44].

Retinal hypoperfusion or ischemia activates inflammatory reactions and induces apoptotic pathways that eventually lead to degeneration and visual impairment [33]. Ischemia is associated with several retinal vascular diseases including diabetic retinopathy, retinal artery occlusion and carotid artery stenosis [14]. Bilateral common carotid artery occlusion (BCCAO) causes mild cerebral hypoperfusion [18] and, depending on rat strain, it leads to retinal degeneration. We have previously studied different neuroprotective strategies using this rat model [42]. We have also shown that both PACAP and VIP are retinoprotective in chronic retinal hypoperfusion [2,45].

The neuroprotective effects of PACAP seem to be mediated by influencing signaling pathways at different levels. The main effects involved are the anti-apoptotic, anti-inflammatory and anti-oxidant effects. As a first step in investigating the PACAP-induced retinoprotective mechanism, we have studied signaling pathways in glutamate-induced excitotoxic degeneration. We have found that PACAP inhibits pro-apoptotic pathways, while it stimulates anti-apoptotic intracellular processes [35,36]. However, it is not known, which mechanisms are involved in the protective effects of PACAP in hypoperfusion-induced injury. Mitogen activated protein kinases (MAPKs) and Akt signaling play an important role in the neuroprotective effects of PACAP. Also, PACAP is known to influence levels of cytokines and chemokines after injuries, which are important mediators in ischemic processes [8,11,13]. Therefore, we investigated the activation level of MAPKs, Akt and cytokines in rat retina exposed to BCCAO.

## 2. Materials and methods

Male Wistar rats weighing 250–300 g were subjected to permanent BCCAO. Experimental procedures were performed following institutional ethical guidelines (BA02/2000-24/2011). Under isoflurane anesthesia, bilateral common carotid arteries were exposed and ligated with a 3-0 filament. Immediately following the BCCAO operation, PACAP38 (100 nmol/3 μl saline) was intravitreally injected into the right eye with a Hamilton syringe. The left eye received the same volume of vehicle treatment. A group of animals underwent anesthesia and all steps of the surgical procedure, except ligation of the carotid arteries. These animals served as sham-operated saline- or PACAP-treated animals.

For Western blot experiments, retinas from 33 animals were removed after 5, 30 and 60 min in order to investigate the signaling pathways activated within the first few minutes after an ischemic insult ($n = 6$ animal/group in the ischemic groups and 5 animals/group in the sham-operated groups). Samples were processed for Western blot analysis as described earlier [35,36]. Membranes were probed overnight at 4 °C with the primary antibodies: phospho-specific anti-Akt-1 $Ser^{473}$ (1:1000; R&D Systems, Hungary), phospho-specific anti-ERK1/2 $Thr^{202}/Tyr^{204}$, phospho-specific anti-SAPK/JNK $Thr^{183}/Tyr^{185}$, phospho-specific anti-p38 MAPK (1:1000; Cell Signaling Technology, USA), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000; Millipore, 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, Hungary). The antibody–antigen complexes were visualized by means of enhanced chemiluminescence. After scanning, results were quantified by means of Scion Image Beta 4.2 program. All experiments were performed at least four times. Data are expressed as the mean ± SEM. Statistical analysis was performed by analysis of variance and unpaired Student's $t$-test. Differences with $p$ values below 0.05 were considered to be significant.

For semiquantitative cytokine array, retinas (n=18 from 9 animals/group) in the ischemic and sham-operated groups) were removed after 24 h of ischemia and homogenized in PBS with protease inhibitors. Samples were pooled in three replicates ($n = 3$ per replicate). Triton X-100 was added to a final concentration of 1%. The samples were stored at −80 °C prior to use. Cytokine array from tissue homogenates was performed using Rat Cytokine Array Panel A Array kit from R&D Systems (Biomedica, Budapest, Hungary). After blocking the array membranes for 1 h and adding the reconstituted Detection Antibody Coctail for another 1 h at room temperature, the membranes were incubated with 1.5 ml of tissue homogenates at 2–8 °C overnight on a rocking platform. After washing with buffer 3 times and addition of horseradish peroxidase-conjugated Streptavidin to each

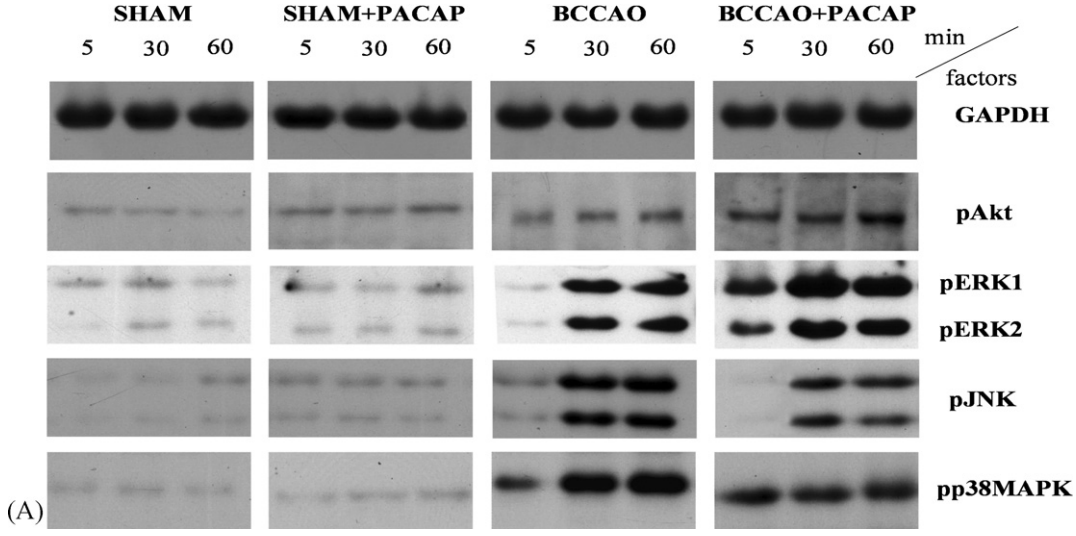



**Fig. 1.** (A) Representative Western blots showing activation (phosphorylation) of Akt and MAP kinases in sham-operated, sham+PACAP-treated, ischemic (induced by BCCAO) and BCCAO+PACAP-treated retinas. Samples were taken 5, 30 and 60 min after treatments. GAPDH was used as internal control. (B) Statistical analysis of Western blot results. Data are given as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ vs control, $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ vs ischemic retinas.

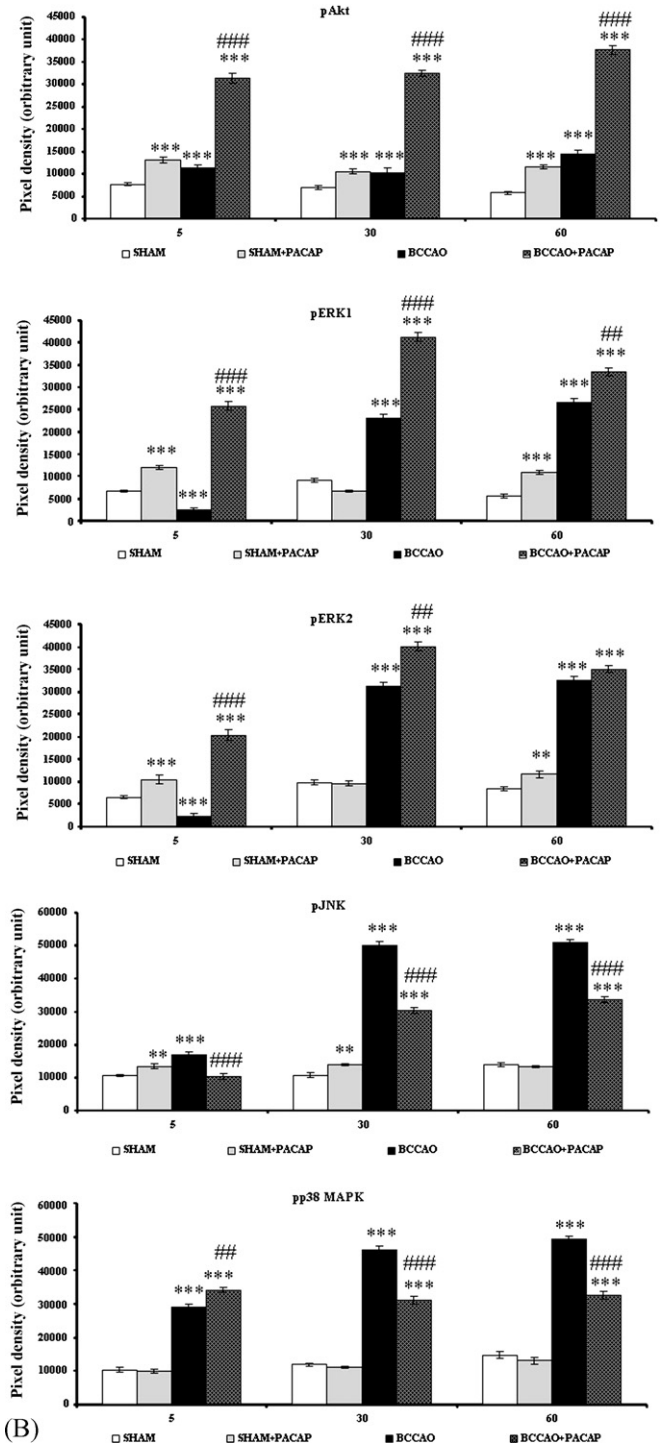


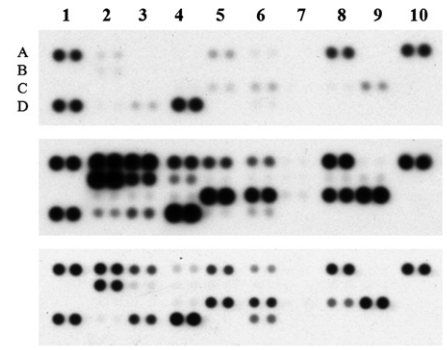

**Fig. 1.** (A) (*Continued*).

membrane we exposed them to a chemiluminescent detection reagent (Amersham Biosciences, Hungary) then side up to an X-ray film cassette [46].

## 3. Results

### *3.1. Effect of PACAP treatment on ischemia induced signaling pathways*

In sham-operated groups, no marked differences in the phosphorylation level of the examined factors were found upon PACAP treatment, only phosphorylation of Akt was slightly elevated by PACAP in sham-operated retinas. Ischemia itself also slightly increased the phosphorylation state of Akt, but PACAP treatment led to a marked significant further increase at all time-points following ischemia (Fig. 1A and B). ERK1/2 phosphorylation was close to the detection limit in sham operated saline-treated retinas with little effect after PACAP treatment. Ischemia induced a strong phosphorylation of ERK1/2 compared to sham-operation, and this was further increased by PACAP. We found the activation slightly more intensive after 30 than after 60 min, suggesting a fast response after an ischemic insult. In PACAP-treated retinas, the significant




| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| A | PosC | CINC-1 ↓ | CINC-2αβ ↓ | CINC-3 ↓ | CNTF ↓ | Fractalkine ↓ | GM-CSF | sICAM-1 ↓ | IFN-γ | PosC |
| B | | IL-1α ↓ | IL-1β ↓ | IL-1ra ↓ | IL-2 | IL-3 | IL-4 | IL-6 | IL-10 | |
| C | | IL-13 | IL-17 | IP-10 | LIX ↓ | L-Selectin ↓ | MIG | MIP-1α ↓ | MIP-3α ↓ | |
| D | PosC | RANTES ↓ | Thymus Chemokine ↑ | TIMP-1 ↓ | TNF-α | VEGF ↑ | | | NegC | |

**Fig. 2.** Representative cytokine array to detect the protective effects of 100 nM PACAP in retina exposed to ischemic injury by bilateral common carotid artery occlusion (BCCAO). The panels show arrays from sham-operated control (upper panel), BCCAO ischemic (middle panel) and BCCAO + PACAP-treated (lower panel) retinas. The table indicates the examined factors in each box, highlighting with arrows the changes observed after PACAP treatment.

activation of ERK1/2 appeared already 5 min after the induction of ischemia, further supporting this rapid reaction.

In the sham-operated eyes, we could not detect marked phosphorylation of SAPK/JNK. Ischemia induced the activation of this kinase as it was revealed by its slightly increased phosphorylation after 5 min and strong elevation after 30 and 60 min. However, administration of PACAP caused significant decrease in the phosphorylation state of SAPK/JNK at all time-points after ischemia. A phosphorylation pattern quite similar to that of JNK was detected in case of p38 MAPK. No phosphorylation of this kinase was observed in sham-operated animals and we found no difference between saline and PACAP-treated retinas. Ischemia induced a strong phosphorylation of p38 MAPK that was attenuated by PACAP treatment 30 and 60 min after BCCAO (Fig. 1A and B).

### 3.2. Cytokine array

The expression of several cytokines was increased after ischemia, including chemoattractant proteins, the chemokines of the CINC (cytokine-induced neutrophil chemoattractant) and MIP (macrophage inflammatory protein) families: CINC-1, CINC-2α/β, CINC-3 and MIP-1α, MIP-3α. Several interleukins were also activated: IL-1α, IL-1β and IL-1ra, while other interleukins remained unchanged, such as IL-2, IL-3, IL-4, IL-6 and IL-10. The activation of the ciliary neurotrophic factor (CNTF), fractalkine, sICAM-1 (intercellular adhesion molecule), LIX (lipopolysaccharide induced CXC chemokine), L-selectin, RANTES (regulated on activation, normal t cell expressed and secreted), thymus chemokine and TIMP-1 (tissue inhibitor of metalloproteinase) was increased in the retinas that underwent BCCAO compared with the control groups (Fig. 2). PACAP treatment attenuated activation of all the above-mentioned cytokines, as measured by a cytokine array system. Exceptions were VEGF and thymus chemokine, the activation of which was further increased upon PACAP treatment. The expression of other cytokines analyzed by the array did not show any marked changes (Fig. 2).

## 4. Discussion

The present study showed that PACAP counteracted changes in the phosphorylation of MAPKs and levels of cytokines after ischemic injury in the rat retina. PACAP has been shown to influence cell survival and inflammatory pathways in several ischemic injuries, including ischemia of the brain and different peripheral organs [19,34,38,46]. The MAPK family seems to play an important role in the PACAP-induced cellular protection in several models [31,47]. In cerebellar granule cells, the balance between ERK and JNK MAPKs is critical for survival, and PACAP induces ERK phosphorylation while it inhibits JNK phosphorylation, thereby attenuating ceramide-induced cell death [48]. Our present results are also in accordance with previous observations in glutamate-induced retinal injury, where PACAP had opposing effects on ERK and JNK phosphorylation [35,36]. p38 MAPK, the third main member of the MAPK family, is typically considered to be a pro-apoptotic MAPK, the phosphorylation of which is induced by many cellular injuries. PACAP has been shown to inhibit the phosphorylation of p38 MAPK in several studies [35,36]. Akt signaling is generally associated with increased cellular protection, including retinal ischemia [14]. Akt phosphorylation was also induced by PACAP in the present study, similarly to our earlier observations in excitotoxic retinal injury and other descriptions on PACAP-mediated neuronal protection [31,37].

The anti-inflammatory effects of PACAP offer another mechanism against ischemic injury in the retina. The involvement of PACAP in inflammatory reactions has long been known, but the exact effects of PACAP in inflammation depend on cell type, tissue, age, pathological conditions and several other factors. However, PACAP is usually considered to be an anti-inflammatory peptide [12]. In the present study, we also found that addition of PACAP counteracted changes in several cytokines and chemokines in ischemic retinal injury. The overall picture of cytokine expression shows that ischemia dramatically induced several cytokines and less activation was found in most cytokines after PACAP treatment, except for VEGF and thymus chemokine, which were elevated after PACAP treatment. The strong activation of several cytokines found in our study is in agreement with other reports showing induced cytokine expression after retinal ischemia [23,50]. The alterations induced by PACAP strongly suggest an anti-inflammatory role of the peptide in ischemic retinal injury, keeping in mind that this was checked at one time-point, namely 24 h after the induction of ischemia.

The family of CINC (CINC1-3) is a pro-inflammatory chemokine family, involved in several inflammatory processes, usually during the acute inflammatory response phase [21]. Compromise in blood flow has been shown to induce expression of the cytokine CINC also in the retina [52] and substances decreasing CINC levels are usually associated with decreased tissue damage [1,27]. This is the first report showing that PACAP attenuates elevated CINC levels after ischemia induction in the retina. Fractalkine, another chemoattractant, was also reduced after PACAP treatment. The effect of PACAP on interleukins was variable. Several interleukins were not affected, while the increased expression of others was attenuated by PACAP. Taken together, PACAP decreased levels of IL-1 subclasses, the reduction of which has been shown to be beneficial in retinal ischemic injuries [15,25].

RANTES also participates in retinal inflammatory reactions. The chemokine RANTES has the potential to influence the migration of memory T cells and monocytes across the blood-retinal barrier during inflammatory eye disease [10]. Here we showed that PACAP was able to downregulate the ischemia-induced upregulation of RANTES in the retina. Earlier studies found that PACAP induced secretion of RANTES, for example in astrocytes [8] and in cortical neurons [39]. In these studies, release of RANTES has been

shown to be associated with increased neuroprotection. The role of RANTES in retinal ischemia, however, seems to be the increase in inflammatory reactions, against which PACAP may protect according to our findings. This is, therefore, in accordance with findings of others showing that PACAP and VIP inhibit chemokine release in microglial cells [11].

Several other factors were found to be altered upon PACAP administration. Little is known about the effect of PACAP on these factors. Adhesion molecules have been demonstrated to be involved in several ophthalmic pathologies [26]. Similarly, the involvement of matrix metalloproteinases in retinal ischemic injury has been reported by many authors [30]. MIP-1 alpha has also been found to be elevated after retinal ischemia [51]. In the present study, PACAP prevented the increased activation of ICAM, selectin, MIP-1 alpha and TIMP-1. VEGF, on the other hand, was increased after PACAP treatment. The inducing effect of PACAP on the levels of VEGF has been reported earlier in lung cancer cells [32] and in pituitary folliculostellate cells [22]. VEGF is a well-studied angiogenetic factor playing a role in several retinopathies, including retinopathy of prematurity and diabetic retinopathy, but it also has a neuroprotective function [53]. In retinal ischemia, VEGF expression is altered depending on the time, reperfusion and arterial or neuronal localization of VEGF [9,29]. Whether this PACAP-induced increase in VEGF has a neuroprotective function, a role in reestablishing circulation after ischemic retina injury, or it plays a role in inflammatory processes, is not known at the moment. Altogether, the exact quantification of the changes in cytokine expression and activation is needed to be performed in future experiments focusing on the cytokines and chemokines showing the most marked changes.

Taken together, the dramatic changes in the cytokine profile is counteracted by PACAP treatment. The present results provide further insight into the neuroprotective mechanism induced by PACAP in ischemic injuries, showing that PACAP ameliorates ischemic retinal injury involving Akt, MAPK pathways and anti-inflammatory actions.

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