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Effects of PACAP on Intracellular Signaling Pathways in Human Retinal Pigment Epithelial Cells Exposed to Oxidative Stress

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Abstract The integrity of retinal pigment epithelial cells is critical for photoreceptor survival and vision. Pituitary adenylate cyclase activating polypeptide (PACAP) exerts retinoprotective effects against several types of injuries in vivo, including optic nerve transection, retinal ischemia, excitotoxic injuries, UVA-induced lesion, and diabetic retinopathy. In a recent study, we have proven that PACAP is also protective in oxidative stress-induced injury in human pigment epithelial cells (ARPE-19 cells). The aim of the present study was to investigate the possible mechanisms of this protection. ARPE cells were exposed to a 24-h hydrogen peroxide treatment. Expressions of kinases and apoptotic markers were studied by complex array kits and Western blot. Oxidative stress induced the activation of several apoptotic markers, including Bad, Bax, HIF-1α, several heat shock proteins, TNF-related apoptosis-inducing ligand, and Fas-associated protein with death domain, while PACAP treatment decreased them. The changes in the expression of MAP kinases showed that PACAP activated the protective ERK1/2 and downstream CREB, and decreased the activation of the pro-apoptotic p38MAPK and c-Jun N-terminal kinase, an effect opposite to that observed with only oxidative stress. Furthermore, PACAP increased the activation of the protective Akt pathway. In addition, the effects of oxidative stress on several other signaling molecules were counteracted by PACAP treatment (Chk2, Yes, Lyn, paxillin, p53, PLC, STAT4, RSK). These play a role in cell death, cell cycle, inflammation, adhesion, differentiation and proliferation. In summary, PACAP, acting at several levels, influences the balance between pro- and anti-apoptotic factors in favor of anti-apoptosis, thereby providing protection in oxidative stress-induced injury of human retinal pigment epithelial cells.

Keywords ARPE-19 cells · PACAP · MAPK kinase · Apoptosis · Citokine array

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Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide first isolated from ovine hypothalamus but later shown in the entire nervous system (Vaudry et al. 2009). The neuroprotective effects of PACAP are intensively investigated. Retina, as an outgrowth from the prosencephalon brain vesicle, acts like the central nervous system in several aspects. Therefore, it is not surprising that PACAP is also neuroprotective in the retina (Atlasz et al. 2010b). The direct retinoprotective effects of PACAP have been investigated against

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various noxious agents and harmful stimuli. Among others, PACAP exerts retinoprotective effects in an optic nerve transection model (Seki et al. 2008), in neonatal monosodium glutamate-induced damage in vivo (Tamas et al. 2004; Babai et al. 2005, 2006; Atlasz et al. 2008, 2009), in ischemia (Atlasz et al. 2007, 2010a; Seki et al. 2011), in UV-induced damage (Atlasz et al. 2011), and in diabetic retinopathy (Szabadfi et al. 2012a). In addition, mice deficient in endogenous PACAP react to retinal injury with increased vulnerability (Endo et al. 2011; Szabadfi et al. 2012b).

Most studies on retinoprotective strategies focus on the retinal layers derived from the inner layer of the optic cup (Rojas 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 2008). Photoreceptor degeneration involves the closely associated retinal pigment epithelial cells in several ocular diseases, including age-related macular degeneration (Kook et al. 2008). In spite of the numerous studies showing the protective effects of PACAP in the retina, very limited data are currently available on the effects of PACAP on pigment epithelial cells. It has been published earlier that PAC1 and VPAC1 receptors are present in the pigment epithelial cells and that PACAP inhibits the interleukin 1β-stimulated expression of interleukin-6, -8, and monocyte chemotactic protein-1 (Zhang et al. 2005). This has drawn our attention to the possible involvement of retinal pigment epithelial cells in the protective effects of PACAP. Oxidative stress-induced apoptosis is a major contributor to cell death and it has been shown to play a role also in pigment epithelial cell degeneration (Kalariya et al. 2008; Kook et al. 2008; Antonelli et al. 2012; Kalonia et al. 2012). In our previous study, we have shown that PACAP protects human pigment epithelial cells (ARPE-19 cell line) against oxidative stress-induced apoptotic cell death (Mester et al. 2011).

To further explore this question, the aim of the present study was to gain some insight into the protective mechanism of PACAP in ARPE cells. Earlier, we have studied the molecular mechanism of the retinoprotective effects of PACAP, mediated predominantly by PAC1 receptors, involving protein kinase A (PKA) and C pathways. A major contribution to this effect has been shown to come from the PKA/mitogen activated protein kinase (MAPK) pathway and downstream, the inhibition of the apoptosis executor, caspase-3. In the retina, a part of this complex neuroprotective mechanism has been confirmed in an in vivo model, the MSG-induced degeneration. PACAP has been shown to upregulate the anti-apoptotic pathways, such as PKA, CREB, and extracellular signal-regulated kinase (ERK) phosphorylation, and the PKA/Bad/14-3-3 protein cascade resulting in increased expression of the protective Bcl-xL and Bcl-2 (Racz et al. 2006a,b, 2007). At the same time, PACAP treatment downregulates the pro-apoptotic signaling, such as c-Jun N-terminal kinase (JNK), apoptosis-inducing factor, caspase-3, and the release of mitochondrial cytochrome c into the cytosol (Atlasz et al. 2010b; Szabadfi et al. 2010). The mechanism of the protective effects of PACAP in pigment epithelial cells is not known. Given the known anti-apoptotic effects of PACAP, we first investigated whether PACAP acts on the apoptotic pathways in ARPE cells exposed to oxidative stress using a complex apoptosis array study. Furthermore, PACAP affects several kinases, cytokines, and other proteins, such as heat shock proteins, involved in apoptosis, cell death, and inflammation (Brenneman et al. 2002, 2003; Delgado et al. 2002; Somogyvari-Vigh and Reglodi 2004; Vaudry et al. 2009). In order to get a further insight into the mechanism of PACAP-induced protection against oxidative stress in ARPE cells, we also investigated the altered expression of kinases and cytokines using a complex kinase array and Western blot.

Materials and Methods

Cell Culture

Human ARPE-19 cells were obtained from the American type culture collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco's modified Eagle medium/F12 (DMEM/F12, Invitrogen) with 10 % fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C in 5 % CO_2.

Human Apoptosis Array and Human Phospho-Kinase Array

Apoptosis and phospho-kinase arrays were performed from cell homogenates using Human Apoptosis Array kit and Human Phospho-kinase Array kit (R&D Systems; Biomedica Hungaria, Budapest, Hungary). These arrays are based on binding between sample proteins and carefully selected captured antibodies spotted on nitrocellulose membranes. The ARPE-19 cells were treated with 0.25 mM H_2O_2 for 24 h and 100 and 10 nM PACAP38. Concentrations of H_2O_2 and PACAP38 were based on earlier descriptions (Mester et al. 2011). PACAP is strongly cytoprotective in the nanomolar

concentration range in most studies, and this is what we found in our earlier description using the same ARPE cell line (Mester et al. 2011; Somogyvari-Vigh and Reglodi 2004). The used H_2O_2 concentration was also found to be optimal for inducing cellular damage that is significant but still repairable (Mester et al. 2011). Cellular extracts were made as described by the manufacturer. The kit contains all buffers, detection antibodies, and membranes necessary for the measurements. The array was performed as described by the manufacturer, similarly to our previous study (Horvath et al. 2010). Briefly, after blocking the array membranes for 1 h and adding the reconstituted detection antibody cocktail for another 1 h at room temperature, the membranes were incubated with 1 ml of cellular extracts at 2–8°C overnight on a rocking platform. After washing with buffer three times and addition of horseradish peroxidase-conjugated streptavidin to each membrane, we exposed them to a chemiluminescent detection reagent.

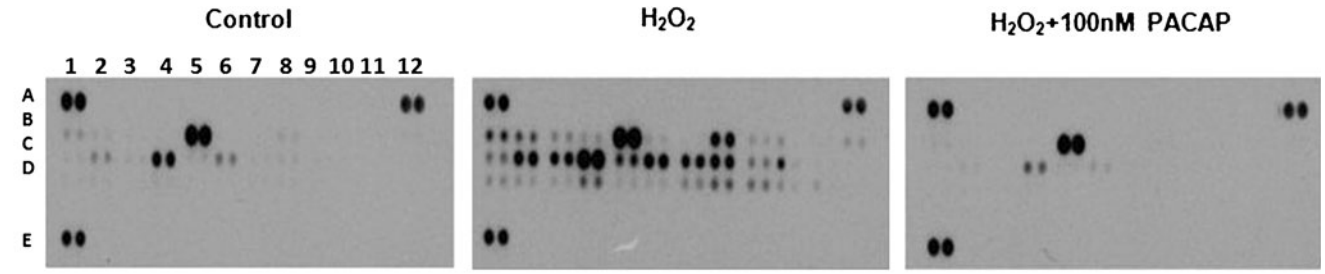
Western Blot Analysis

ARPE-19 cells (1×10^6/10 cm^2 dish) were cultured for 2 days. The cells were then incubated with 200 μM of hydrogen peroxide for 24 h with or without 100 or 10 nM PACAP38. After washing with ice-cold PBS, the cells were lysed in CelLytic M, mammalian cell lysis/extraction reagent (Sigma, Hungary) with a protease inhibitor cocktail (Sigma, Hungary). The lysates were centrifuged (15,000×*g* for 10 min at 4°C). The lysates were separated by SDS-polyacrylamide gel and transferred to a Protran Nitrocellulose Transfer Membrane (Whatman GmbH, Germany). The membranes were blocked in 5 % non-fat dried milk for 2 h at room temperature and were then incubated with primary antibodies. Membranes were probed overnight at 4°C with the following primary antibodies: phospho-specific anti-Akt-1 Ser473 (1:1,000 dilution; R&D Systems, Budapest, Hungary), phospho-specific anti-ERK1/2 Thr202/Tyr204, phospho-specific anti-SAPK/JNK Thr183/Tyr185, phospho-specific anti-p38 MAPK (1:1,000 dilution; Cell Signaling Technology, Beverly, USA) anti-total (t)Akt (1:1,000 dilution; Cell Signaling Technology, Beverly, USA) and anti-actin (1:5,000 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 (1:3,000; BioRad, Budapest, Hungary). The antibody–antigen complexes were visualized by means of enhanced chemiluminescence. The amount of protein expression was standardized against the amount of t-Akt and β-actin.

Results

Apoptosis Array

Under control conditions, only a few markers showed activation in ARPE cells. Oxidative stress increased the activation of Bad, Bax, cIAP1, TNF-related apoptosis-inducing



	1	2	3	4	5	6	7	8	9	10	11	12
A	PosC	-	-	-	-	-	-	-	-	-	-	PosC
B	Bad↓	Bax↓	Bcl-2	Bcl-x	Pro caspase3	Cleaved caspase3	Catalase	cIAP-1↓	cIAP2	Claspin	Clusterin	Cyto chrome-c
C	Trail R1 DR4	Trail R2↓ DR5	FADD↓	Fas TNFR↓ SF6	HIF1α↓	HO-1 HSP32↓	HO-2/ HMOX2↓	HSP 27↓	HSP 60	HSP 70	HTRA2/ Omi	Livin
D	PON2	P21	P27	Pp53 (S15)↓	Pp53 (S46)	Pp53 (S392)	PRad17 (S635)	SMAC Diablo↓	Survivin	TNF R1	XIAP	PBS
E	PosC	-	-	-	-	-	-	-	-	-	-	-

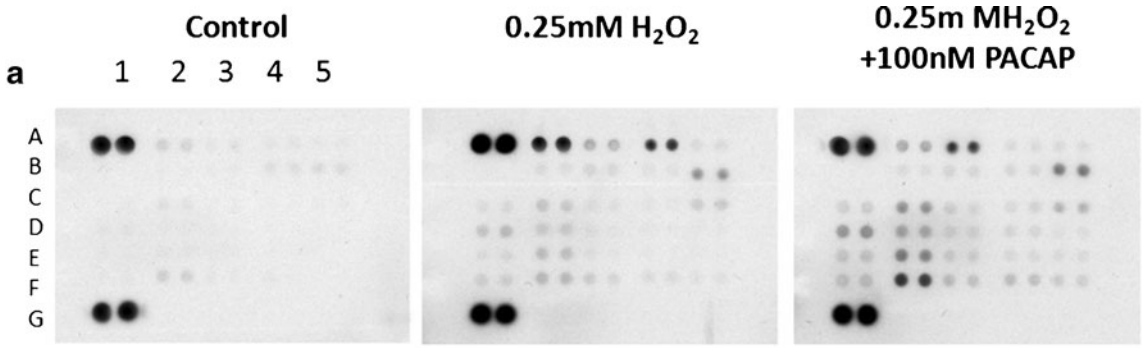
Fig. 1 Representative apoptosis array to detect the protective effects of 100 nM PACAP in ARPE cells exposed to oxidative stress by hydrogen peroxide. The *panels* show control (no treatment), H_2O_2-, and H_2O_2+100nM PACAP-treated arrays. The *table* indicates the examined factors; *arrows* indicate the changes observed after PACAP treatment

ligand (Trail) R2 DR5, Fas-associated protein with death domain (FADD), Fas TNFR SF6, HIF-1α, several heat shock proteins (HSP32, HMOX2, HSP-27), SMAC diablo, and pp53 (Fig. 1). Co-treatment with 100 nM PACAP38 could decrease the activation of all the above-mentioned markers (Fig. 1). Treatment with 10 nM attenuated the oxidative stress-induced changes in the expression of the same apoptotic markers (data not shown).

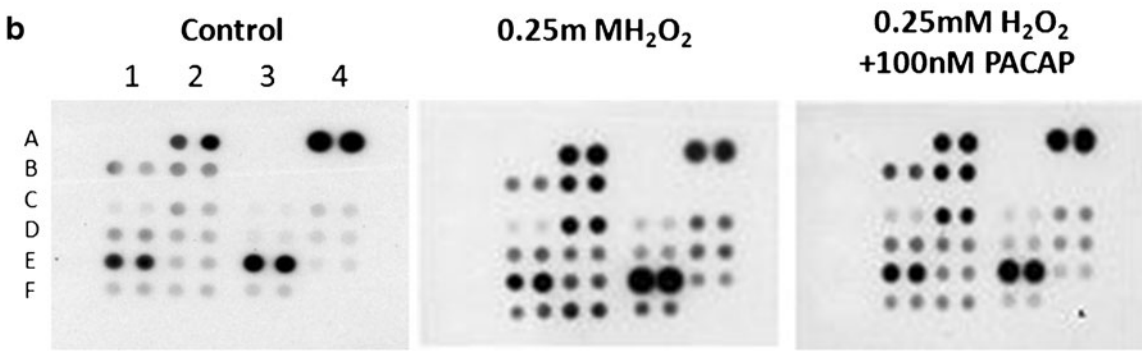
Phospho-Kinase Array

Array A

Among several phospho-kinases, p38alpha and JNK pan were strongly activated (phosphorylated) after hydrogen peroxide, while PACAP treatment could decrease these phosphorylations (Fig. 2a). The ERK1/2 as well as Akt



Panel A	1	2	3	4	5
A	Pos control	p38α ↓	ERK1/2 ↑	JNK pan ↓	GSK-3α/β
B	-	MEK 1/2	MSK 1/2	AMPKα2	Akt ↑
C	TOR	CREB ↑	HSP27	AMPKα2	ß-Catenin
D	Src ↑	Lyn ↑	Lck	STAT2	STAT5a
E	Fyn	Yes ↑	Fgr	STAT3	STAT5b
F	Hck	Chk-2	FAK	STAT6	STAT5a/b
G	Pos control	-	PBS(Neg control)	-	-



Panel B	1	2	3	4
A	-	p53 ↓	-	Pos control
B	Akt ↑	p53 ↓	-	-
C	p70S6kinase	p53 ↓	p27	Paxillin ↓
D	p70S6kinase	RSK1/2/3	p27	PLCγ-1 ↓
E	p70S6kinase	RSK1/2 ↓	c-Jun ↓	Pyk2
F	STAT1	STAT4 ↓	eNOS ↓	PBS(neg control)

Fig. 2 Representative phospho-kinase arrays (*panels A* and *B*) from control (no treatment), H_2O_2-, and H_2O_2+100nM PACAP-treated ARPE-19 cells. Markers, which changed expression levels after PACAP treatment, are highlighted in the table below

(S473) was found to be slightly activated after H_2O_2 treatment. PACAP coadministration led to a marked increase in ERK1/2 activation, while a slight increase in Akt activation. A slight activation of CREB, Src, Lyn, Yes pathways were found after PACAP treatments while it caused a strong activation of the Chk-2 pathway (Fig. 2a).

Array B

Akt (T303) activation occurred after the oxidative stress, but it was further increased in samples with 100 nM PACAP treatment (Fig. 2b). We could detect increased phosphorylation of p53 at three different sites (S392, S46, and S15) after H_2O_2 treatment. This activation was slightly decreased by the 100-nM PACAP treatment. Paxillin, p70S6 kinase, RSK1/2, PLCgamma-1, STAT4, and eNOS activation occurred after H_2O_2 treatment. All these changes were counteracted by PACAP treatment, except that of p70S6 kinase. Activation of c-Jun was observed under control conditions and this activation was further increased after the oxidative stress (Fig. 2b). PACAP treatment could decrease this activation. Treatment with 10 nM attenuated the oxidative stress-induced changes in the expression of the same cytokines (data not shown).

Western Blot Analysis

We used t-Akt and actin as internal controls. We could not detect differences between the protein levels of the different samples. PACAP alone caused increase in the phosphorylation of the protective ERK and Akt. Oxidative stress also led to increased phosphorylation of these molecules, but they were further activated when PACAP was coadministered with hydrogen peroxide (Fig. 3). Interestingly, PACAP treatment alone could increase the activation of JNK, while that of p38 did not change. H_2O_2 treatment caused a strong activation of the pro-apoptotic p38MAPK and JNK, marked by the increased phosphorylation levels. Co-treatment with PACAP could decrease this oxidative stress-induced increased phosphorylation of the pro-apoptotic kinases (Fig. 3).

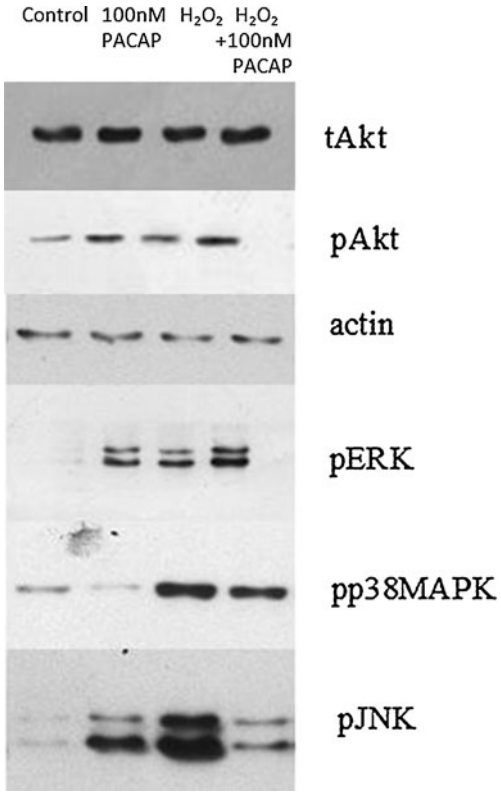


Fig. 3 Representative blots of the Western blot analysis Akt and MAP kinases in untreated, control ARPE cells (*first column*), in cells exposed to PACAP treatment alone (*second column*), in cells exposed to oxidative stress (*third column*), and in cells exposed to oxidative stress and PACAP co-treatment (*fourth column*). Total Akt (*tAkt*) and actin were used as internal controls

Discussion

In the present study, we showed that PACAP counteracted the oxidative stress-induced changes in apoptotic and kinase signaling molecules of human pigment epithelial cells, providing details for the molecular mechanism of the protective effects of PACAP in ARPE cells.

We have previously described that PACAP protects pigment epithelial cells of the retina against oxidative stress-induced apoptosis (Mester et al. 2011), but the molecular background has not been investigated. In the present study, we found that exposure of human ARPE cells to hydrogen peroxide induced a marked increase in several apoptotic markers, as shown by the complex apoptosis array. Coadministration with 100 nM PACAP38 led to an apoptotic profile similar to control conditions. A marked increase was observed in the mitochondrial pro-apoptotic proteins, Bax and Bad in cells under oxidative stress, while PACAP abolished this increase entirely. Also, PACAP counteracted the oxidative stress-induced upregulation of Trail, FADD, Fas, SMAC diablo, and several heat shock proteins. Apoptosis involves a series of cascades and is mediated by a diverse range of extracellular and intracellular signals. These include processes like TNF and Fas activation, which is followed by further activation of pro-apoptotic signals. The balance between pro- and anti-apoptotic processes is very important not only during development, but also

throughout life. Disturbed balance can be detected in most diseases characterized by increased cell death, including retinal pathologies. PACAP has been shown to influence apoptotic processes at several levels. Early studies have established the role of PACAP in the regulation of MAP kinases, resulting in an anti-apoptotic profile of most examined cells (Vaudry et al. 2002; Somogyvari-Vigh and Reglodi 2004; Dejda et al. 2008). In the present study, we also found that PACAP counteracted the hydrogen peroxide-induced changes in the balance of the members of the MAPK family: elevated levels of the protective ERK1/2, while decreased expression of the pro-apoptotic JNK and p38 MAPK were observed after PACAP treatment, as confirmed by both kinase array and Western blot measurements. Thus, these results are in accordance with earlier observations in other neuronal and non-neuronal cells (Somogyvari-Vigh and Reglodi 2004; Racz et al. 2007; 2008; 2010). The involvement of MAP kinases is crucial in mediating apoptosis also in pigment epithelial cells of the retina (Glotin et al. 2006). The activation of the anti-apoptotic signaling can lead to elevated CREB phosphorylation, which has already been described in various cell types after PACAP treatment, and our present results further support these observations in ARPE cells (Bhave and Hoffman 2004; Racz et al. 2006b). Furthermore, we found that PACAP counteracts the elevated expression of Trail, FADD, and Fas, initiators of the apoptotic cascade. Balanced effect of PACAP and FasL has already been shown on cerebellar granule cell death during development (Allais et al. 2010). Also, PACAP-induced downregulation of Fas has been demonstrated in peripheral cells, such as colonic tumor cells and lymphocytes (Delgado and Ganea 2001; Le et al. 2002). Our observations in ARPE cells support the general aspect of this effect induced by PACAP.

The involvement of the mitochondrial Bcl-family in the PACAP-induced cytoprotection has been described in several cell types exposed to different harmful stimuli. For example, we have described that PACAP induced the anti-apoptotic members of this family, while it decreased the pro-apoptotic signaling, including Bad, in the retina (Racz et al. 2007). Similar results have been described in cerebellar granule cells and other neuronal cell types (Aubert et al. 2008; Seaborn et al. 2011). The inhibiting action of PACAP on the pro-apoptotic Bax and Bad has been described not only in neuronal cells but also non-neuronal cell types, such as in insulinoma cells upon streptozotocin-induced apoptosis (Onoue et al. 2008) and in serum-deprivation-induced apoptosis of Schwann cells (Castorina et al. 2008).

During the activation of the apoptotic cascade, the mitochondrial membrane becomes more permeable and several pro-apoptotic factors are released to the cytosol, including cytochrome c and SMAC. Our present results show that PACAP could decrease the induced levels of SMAC, an effect not previously reported. p53 also plays a critical role in apoptosis. Any disruption to the regulation of the p53 will result in impaired apoptosis (Di Giovanni and Rathore 2012). p53-dependent Bax synthesis induces apoptosis by binding to Bcl-2 and antagonizing its non-apoptotic ability, a mechanism observed in different forms of oxidative stress (Cheng et al. 2001; Yang et al. 2003). We found that PACAP could slightly decrease the increased expression of p53 after oxidative stress. Other factors were also found to be decreased after PACAP treatment. These include PLC, eNOS, STAT, RSK, and cIAP-1. Altered levels of STAT in the brain have already been reported in PACAP gene-deficient mice (Ohtaki et al. 2006), while others have found no influence of PACAP on STAT expression in microglial cells (Lee and Suk 2004). At the moment, too little is known about the relationship between PACAP and these markers to draw conclusion from our observations, but the retinoprotective effects of PACAP along with the present observations indicate that PACAP can definitely counteract increased expressions of markers induced by oxidative stress.

Some other markers were found to be elevated after PACAP treatment. In addition to the already-described ERK1/2, Akt was also found to be elevated in our study. The Akt-induced processes are generally associated with increased tolerance to apoptosis, and PACAP-associated Akt phosphorylation has been reported in other cell types (Racz et al. 2008). Further factors, that have not yet been reported to be affected by PACAP treatment, were found to be increased after PACAP treatment. Src family kinases regulate an array of cellular processes, including growth factor signaling, cytoskeletal dynamics, and cell proliferation. Lyn, a tyrosin kinase belonging to the src family, is usually associated with anti-apoptotic processes and preservation of mitochondrial integrity (Gringeri et al. 2009; Tibaldi et al. 2011; Takadera et al. 2012). Another widely expressed member of the src family, Yes, was also found to be elevated after PACAP treatment. In addition, paxillin, a signal transduction adaptor protein playing a role in focal adhesion (Turner et al. 1990), and Chk2, a checkpoint protein important in cell cycle, were upregulated following PACAP administration to ARPE cells exposed to oxidative stress. The exact role of these markers in ARPE cell apoptosis is not known yet, and the relation of PACAP and these markers needs further investigation.

In summary, our present results further support the effects of PACAP in oxidative stress-induced apoptotic processes and show that PACAP acts at several levels in the apoptotic-kinase pathway in human pigment epithelial cells.

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