Dear Author

Here are the proofs of your article.

- You can submit your corrections **online,** via **e-mail** or by **fax**.
- For **online** submission please insert your corrections in the online correction form. Always indicate the line number to which the correction refers.
- You can also insert your corrections in the proof PDF and **email** the annotated PDF.
- For **fax** submission, please ensure that your corrections are clearly legible. Use a fine black pen and write the correction in the margin, not too close to the edge of the page.
- Remember to note the **journal title**, **article number**, and **your name** when sending your response via e-mail or fax.
- **Check** the metadata sheet to make sure that the header information, especially author names and the corresponding affiliations are correctly shown.
- **Check** the questions that may have arisen during copy editing and insert your answers/corrections.
- **Check** that the text is complete and that all figures, tables and their legends are included. Also check the accuracy of special characters, equations, and electronic supplementary material if applicable. If necessary refer to the *Edited manuscript*.
- The publication of inaccurate data such as dosages and units can have serious consequences. Please take particular care that all such details are correct.
- Please **do not** make changes that involve only matters of style. We have generally introduced forms that follow the journal's style.
- Substantial changes in content, e.g., new results, corrected values, title and authorship are not allowed without the approval of the responsible editor. In such a case, please contact the Editorial Office and return his/her consent together with the proof.
- If we do not receive your corrections **within 48 hours**, we will send you a reminder.
- Your article will be published **Online First** approximately one week after receipt of your corrected proofs. This is the **official first publication** citable with the DOI. **Further changes are, therefore, not possible.**
- The **printed version** will follow in a forthcoming issue.

Please note

After online publication, subscribers (personal/institutional) to this journal will have access to the complete article via the DOI using the URL:
http://dx.doi.org/10.1007/s12031-014-0387-6

If you would like to know when your article has been published online, take advantage of our free alert service. For registration and further information, go to: http://www.link.springer.com.

Due to the electronic nature of the procedure, the manuscript and the original figures will only be returned to you on special request. When you return your corrections, please inform us, if you would like to have these documents returned.

Metadata of the article that will be visualized in OnlineFirst

Please note: Images will appear in color online but will be printed in black and white.

1	Article Title	**Investigation of the Possible Functions of PACAP in Human Trophoblast Cells**	
2	Article Sub- Title		
3	Article Copyright - Year	**Springer Science+Business Media New York 2014 (This will be the copyright line in the final PDF)**	
4	Journal Name	Journal of Molecular Neuroscience	
5	Corresponding Author	Family Name	**Reglodi**
6		Particle	
7		Given Name	**D.**
8		Suffix	
9		Organization	University of Pecs
10		Division	Department of Anatomy MTA-PTE “Lendulet” PACAP Research Team
11		Address	Szigeti u 12, Pecs 7624, Hungary
12		e-mail	dora.reglodi@aok.pte.hu
13	Author	Family Name	**Horvath**
14		Particle	
15		Given Name	**G.**
16		Suffix	
17		Organization	University of Pecs
18		Division	Department of Anatomy MTA-PTE “Lendulet” PACAP Research Team
19		Address	Szigeti u 12, Pecs 7624, Hungary
20		e-mail	
21	Author	Family Name	**Brubel**
22		Particle	
23		Given Name	**R.**
24		Suffix	
25		Organization	University of Pecs
26		Division	Department of Anatomy MTA-PTE “Lendulet” PACAP Research Team
27		Address	Szigeti u 12, Pecs 7624, Hungary
28		e-mail	

29	Author	Family Name	**Halasz**
30		Particle	
31		Given Name	**M.**
32		Suffix	
33		Organization	University of Pecs
34		Division	Medical Microbiology and Immunology
35		Address	Pecs, Hungary
36		e-mail	
37	Author	Family Name	**Barakonyi**
38		Particle	
39		Given Name	**A.**
40		Suffix	
41		Organization	University of Pecs
42		Division	Medical Microbiology and Immunology
43		Address	Pecs, Hungary
44		e-mail	
45	Author	Family Name	**Tamas**
46		Particle	
47		Given Name	**A.**
48		Suffix	
49		Organization	University of Pecs
50		Division	Department of Anatomy MTA-PTE “Lendulet” PACAP Research Team
51		Address	Szigeti u 12, Pecs 7624, Hungary
52		e-mail	
53	Author	Family Name	**Fabian**
54		Particle	
55		Given Name	**E.**
56		Suffix	
57		Organization	University of Pecs
58		Division	Department of Anatomy MTA-PTE “Lendulet” PACAP Research Team
59		Address	Szigeti u 12, Pecs 7624, Hungary
60		e-mail	
61	Author	Family Name	**Opper**
62		Particle	
63		Given Name	**B.**
64		Suffix	

65		Organization	University of Pecs
66		Division	Department of Anatomy MTA-PTE “Lendulet” PACAP Research Team
67		Address	Szigeti u 12, Pecs 7624, Hungary
68		e-mail	
69	Author	Family Name	**Toth**
70		Particle	
71		Given Name	**G.**
72		Suffix	
73		Organization	University of Szeged
74		Division	Department of Medical Chemistry
75		Address	Szeged, Hungary
76		e-mail	
77	Author	Family Name	**Cohen**
78		Particle	
79		Given Name	**M.**
80		Suffix	
81		Organization	University of Geneva
82		Division	Department of Gynecology Obstetrics
83		Address	Geneva, Switzerland
84		e-mail	
85	Author	Family Name	**Szereday**
86		Particle	
87		Given Name	**L.**
88		Suffix	
89		Organization	University of Pecs
90		Division	Medical Microbiology and Immunology
91		Address	Pecs, Hungary
92		e-mail	

93	Schedule	Received	15 April 2014
94		Revised	
95		Accepted	16 May 2014

96 Abstract

Pituitary adenylate cyclase activating polypeptide (PACAP) is an endogenous neuropeptide having a widespread distribution both in the nervous system and peripheral organs including the female reproductive system. Both the peptide and its receptors have been shown in the placenta but its role in placental growth, especially its human aspects, remains unknown. The aim of the present study was to investigate the effects of PACAP on invasion, proliferation, cell

		survival, and angiogenesis of trophoblast cells. Furthermore, cytokine production was investigated in human decidual and peripheral blood mononuclear cells. For in vitro studies, human invasive proliferative extravillous cytotrophoblast (HIPEC) cells and HTR-8/SVneo human trophoblast cells were used. Both cell types were used for testing the effects of PACAP on invasion and cell survival in order to investigate whether the effects of PACAP in trophoblasts depend on the examined cell type. Invasion was studied by standardized invasion assay. PACAP increased proliferation in HIPEC cells, but not in HTR-8 cells. Cell viability was examined using MTT test, WST-1 assay, and annexin V/propidium iodide flow cytometry assay. Survival of HTR-8/SVneo cells was studied under oxidative stress conditions induced by hydrogen peroxide. PACAP as pretreatment, but not as co-treatment, significantly increased the number of surviving HTR-8 cells. Viability of HIPEC cells was investigated using methotrexate (MTX) toxicity, but PACAP1-38 could not counteract its toxic effect. Angiogenic molecules were determined both in the supernatant and the cell lysate by angiogenesis array. In the supernatant, we found that PACAP decreased the secretion of various angiogenic markers, such as angiopoietin, angiogenin, activin, endoglin, ADAMTS-1, and VEGF. For the cytokine assay, human decidual and peripheral blood lymphocytes were separated and treated with PACAP1-38. Th1 and Th2 cytokines were analyzed with CBA assay and the results showed that there were no significant differences in control and PACAP-treated cells. In summary, PACAP seems to play various roles in human trophoblast cells, depending on the cell type and microenvironmental influences.
97	Keywords separated by ' - '	Trophoblast - Invasion - Proliferation - Decidua - Lymphocyte - Angiogenesis
98	Foot note information	

J Mol Neurosci
DOI 10.1007/s12031-014-0337-0

Investigation of the Possible Functions of PACAP in Human Trophoblast Cells

G. Horvath · D. Reglodi · R. Brubel · M. Halasz · A. Barakonyi · A. Tamas · E. Fabian · B. Opper · G. Toth · M. Cohen · L. Szereday

Received: 15 April 2014 / Accepted: 16 May 2014

Abstract Pituitary adenylate cyclase activating polypeptide (PACAP) is an endogenous neuropeptide having a widespread distribution both in the nervous system and peripheral organs including the female reproductive system. Both the peptide and its receptors have been shown in the placenta but its role in placental growth, especially its human aspects, remains unknown. The aim of the present study was to investigate the effects of PACAP on invasion, proliferation, cell survival, and angiogenesis of trophoblast cells. Furthermore, cytokine production was investigated in human decidual and peripheral blood mononuclear cells. For in vitro studies, human invasive proliferative extravillous cytotrophoblast (HIPEC) cells and HTR-8/SVneo human trophoblast cells were used. Both cell types were used for testing the effects of PACAP on invasion and cell survival in order to investigate whether the effects of PACAP in trophoblasts depend on the examined cell type. Invasion was studied by standardized invasion assay. PACAP increased proliferation in HIPEC cells, but not in HTR-8 cells. Cell viability was examined using MTT test, WST-1 assay, and annexin V/propidium iodide flow cytometry assay. Survival of HTR-8/SVneo cells was studied under oxidative stress conditions induced by hydrogen peroxide. PACAP as pretreatment, but not as co-treatment, significantly increased the number of surviving HTR-8 cells. Viability of HIPEC cells was investigated using methotrexate (MTX) toxicity, but PACAP1-38 could not counteract its toxic effect. Angiogenic molecules were determined both in the supernatant and the cell lysate by angiogenesis array. In the supernatant, we found that PACAP decreased the secretion of various angiogenic markers, such as angiopoietin, angiogenin, activin, endoglin, ADAMTS-1, and VEGF. For the cytokine assay, human decidual and peripheral blood lymphocytes were separated and treated with PACAP1-38. Th1 and Th2 cytokines were analyzed with CBA assay and the results showed that there were no significant differences in control and PACAP-treated cells. In summary, PACAP seems to play various roles in human trophoblast cells, depending on the cell type and microenvironmental influences.

Keywords Trophoblast · Invasion · Proliferation · Decidua · Lymphocyte · Angiogenesis

G. Horvath · D. Reglodi (✉) · R. Brubel · A. Tamas · E. Fabian · B. Opper
Department of Anatomy MTA-PTE "Lendulet" PACAP Research Team, University of Pecs, 7624 PecsSzigeti u 12, Hungary
e-mail: dora.reglodi@aok.pte.hu

M. Halasz · A. Barakonyi · L. Szereday
Medical Microbiology and Immunology, University of Pecs, Pecs, Hungary

G. Toth
Department of Medical Chemistry, University of Szeged, Szeged, Hungary

M. Cohen
Department of Gynecology Obstetrics, University of Geneva, Geneva, Switzerland

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) belongs to the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family. It was first isolated as a hypothalamic peptide based on its effect to influence adenylate cyclase activity in the pituitary gland (Miyata et al. 1989). PACAP is widely distributed throughout the entire body including female reproductive organs (Steenstrup et al. 1995; Ko et al. 1999; Reglodi et al. 2012b; Koves et al. 2014; Csanaky et al. 2014). It occurs in two forms, PACAP1-27 and PACAP1-38, with PACAP1-38 being the predominant form in mammals. PACAP exerts its effect through class II G-protein-coupled receptors. The specific PACAP receptor is called PAC1,

which binds VIP with much less affinity, while VPAC1 and VPAC2 receptors have similar high affinity for VIP and PACAP (Laburthe and Couvineau 2002; Laburthe et al. 2007; Lutz et al. 1999; Muller et al. 2007; Vaudry et al. 2009). Both PACAP1-38 and PACAP1-27, as well as their receptors have been found in the human pregnant uterus and placenta (Koh et al. 2005; Scaldaferri et al. 2000). PACAP has been shown to cause a concentration-dependent relaxation on stem villi and intramyometrial arteries, suggesting a vasoregulatory role in the uteroplacental unit (Steenstrup et al. 1996). PACAP has been suggested to play a role in decidualization, and the time-related localization of endometrial-uterine PACAP has been implicated in facilitation of endometrial blood flow (Spencer et al. 2001a, b). PACAP knockout mice have decreased fertility, described in numerous studies (Reglodi et al. 2012a). This is in part due to impaired implantation (Isaac and Sherwood 2008; Koh et al. 2003) in addition to other deficiencies described in mice lacking endogenous PACAP (Reglodi et al. 2012a).

Previously, we have investigated the levels of both PACAP isoforms in first trimester and full-term human placentas (Brubel et al. 2010). Both PACAP1-38 and PACAP1-27 could be detected in different parts of the full-term human placenta. Increasing PACAP1-38 content was measured in the placenta during pregnancy, both on the maternal and the fetal side. PACAP has been shown to influence cell survival of various cell types against harmful stimuli. In most experiments, PACAP enhances cell survival (Vaudry et al. 2009; Reglodi et al. 2011; Racz et al. 2010; Horvath et al. 2010; Fabian et al. 2012). However, investigating the effect of PACAP on survival of different cell types, cell survival-decreasing effect or no effect could also be detected depending on the examined cell type, PACAP concentrations, and other factors present (Li et al. 2006; Wojcieszak and Zawilska 2014; D'Amico et al. 2013; Horvath et al. 2011). Based on this background, it can be expected that PACAP has an effect on trophoblast cells of other origins and on placental growth. In our previous experiments, PACAP enhanced the survival-decreasing effect of H_2O_2-induced oxidative stress in JAR human choriocarcinoma cells (Boronkai et al. 2009). The first aim of our study was to examine whether the effect of PACAP in trophoblast cells depends on the type of the cells. Therefore, we tested the effect of PACAP on cell viability of HTR-8/Svneo, nontumorous primary trophoblast cells. Trophoblast cells, with their invasive capability, play a pivotal role during the implantation of blastocyst in the early phase of gestation. Therefore, the second aim of the present study was to investigate whether PACAP influences the invasiveness and angiogenesis of human trophoblast cells. Moreover, we examined the effect of PACAP on decidual lymphocytes and compared it with that of peripheral blood mononuclear cells. The common goal of our experiments was to examine whether there is a relationship between PACAP and different cell types related to human pregnancy.

Materials and Methods

Cell Lines

HTR-8/SVneo Cells

Human extravillous trophoblast-derived cell line HTR-8/SVneo was a generous gift of Charles Graham (Department of Anatomy and Cell biology, Queen's University, Kingston, ON, Canada L7L 3 N6). HTR-8/SVneo cells were cultured in RPMI (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (PAA, Csertex Kft. Hungary).

HIPEC 65

Human invasive, proliferative extravillous cytotrophoblast cell line (HIPEC) 65 was a generous gift from Pr. D Evain-Brion, Paris. These primary cells were transformed with simian virus 40 large T antigen for studying cell invasion (Pavan et al. 2003). HIPEC 65 cells were cultured in DMEM high glucose/Ham F-12 (1/1) supplemented with 10 % fetal bovine serum (FBS, Biochrom AG (Oxoid AG, Basel, Switzerland)) and antibiotic mixture (100 U/ml penicillin, 100 µg/ml streptomycin) (Invitrogen, Basel, Switzerland) at 37 °C in a humidified, 5 % CO_2 atmosphere.

Experiments with HTR-8/SVneo Cells

Cell Viability

HTR-8/SVneo cells were plated on 96-well microplate at a density of 3×10^4 per well. To investigate the effect of PACAP, PACAP1-38 was added either simultaneously or prior to oxidative stress evoking H_2O_2. Cells were assigned to one of the experimental groups: (1) control group of cells (no treatment); (2) and (3) cells exposed to 10 or 100 nM PACAP1-38 alone simultaneously or 2 h before starting the H_2O_2 treatment; (4) cells treated with 150 µM H_2O_2 for 24 h; (5) and (6) cells either cotreated with 10 or 100 nM PACAP1-38 and 150 µM H_2O_2 for 24 h or pretreated with 10 or 100 nM PACAP1-38 for 2 h then with 150 µM H_2O_2 for 24 h.

Following the treatments, viability of the HTR-8/SVneo cells was determined by colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Hungary), based on the reduction of MTT into a blue formazan dye by viable mitochondria. At the end of the treatments, cells were washed twice with phosphate-buffered

saline (PBS, Sigma), then incubated with PBS containing 0.5 mg/ml of MTT for 3 h at 37 °C in an atmosphere of 5 % CO_2. The solution was aspirated carefully and 200 μl of dimethylsulfoxide (Sigma, Hungary) was added to dissolve the blue-colored formazan particles and absorbance was measured by an ELISA reader (Dialab Kft., Hungary) at 570 nm representing the values in arbitrary unit (AU).

Invasion Assay of HTR-8/SVneo Cells

The invasiveness of HTR-8/SVneo cells was assessed by using Oris™ Cell Invasion and Detection Assay (Platypus Technologies, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 96-well plates were coated with basement membrane extract (BME, 3 mg/ml) and wells were populated with Cell Seeding Stoppers to restrict cell seeding to the outer annular regions; then seeded with 75,000 cells. Stoppers were removed after 24 h, resulting in an unseeded region in the center of each well (i.e., the detection zone). Cells were overlaid with 10 mg/ml BME containing 15 % FBS, then media with or without 1 μM PACAP1-38 was added to the wells. After 72 h, cells were labeled with calcein AM and the detection zone was analyzed by an Olympus Fluoview FV-1000 confocal microscope. Cell invasion was analyzed by measuring the area of the detection zones using ImageJ analysis software. Invasion was determined by area closure which was calculated as follows: invaded area of detection zone/full area of detection zone × 100.

Angiogenesis Array

HTR-8/SVneo cells were treated with 1 μM PACAP1-38 for 24 h. Supernatants of PACAP1-38 treated or untreated HTR-8/SVneo cells were analyzed by Human Angiogenesis Array (R&D Systems, Biomedica Hungaria, Budapest, Hungary). This array is based on binding between sample proteins and carefully selected captured antibodies spotted on nitrocellulose membranes. The supernatants were collected 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. 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 or 500 μl supernatant at 2–8 °C overnight on a rocking platform. After washing with buffer three times and adding of horseradish peroxidase-conjugated streptavidin to each membrane, we exposed them to a chemiluminescent detection reagent. Array data on developed X-ray film were quantitated by ImageJ software.

Experiments with HIPEC 65 Cells

Cell Viability Tests

HIPEC 65 cells were seeded into 96-well plates at a density of 10^4 cell/well and cultured in medium overnight before the experiment. HIPEC cells were randomly assigned to one of the four experimental groups: (1) control group of cells, (2) 100 nM PACAP1-38, (3) 10 μM MTX, and (4) pretreatment with 100 nM PACAP1-38 followed by 10 μM MTX.

After 48 h of treatment, viability of HIPEC cells was determined by colorimetric WST-1 assay. The medium was removed, and fresh DMEM/FCS containing 0.5 % of the water-soluble WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) solution was added. Cells were then incubated for 3 h at 37 °C in an atmosphere of 5 % CO_2. After 3 h of incubation, optical densities were determined by an ELISA reader (Anthos Labtech 2010; Vienna, Austria) at the wavelength of 550 nm representing the values in AU. All experiments were run in four parallels and repeated six times. Results are expressed as percentage of control values.

Cell viability was also investigated by annexin V–propidium iodide staining. By conjugating a fluorescent group to annexin V, apoptosis can be quantitatively detected using flow cytometry. The ratio of apoptosis was evaluated after double staining with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (Soft Flow, Hungary) using flow cytometry, as described previously (Gasz et al. 2006). First, the medium was discarded and wells were washed twice with isotonic sodium chloride solution. Cells were removed from plates using a mixture of 0.25 % trypsin (Sigma, Hungary), 0.2 % ethylene-diamin tetra-acetate (EDTA; Sigma, Hungary), 0.296 % sodium citrate, and 0.6 % sodium chloride in distillated water. This medium was applied for 15 min at 37 °C. Removed cells were washed twice in cold PBS and were resuspended in binding buffer containing 10 mM HEPES NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$. Cell count was determined in Burker's chamber for achieving a dilution in which 1 ml of solution contains 10^6 cells. One hundred microliters of cell suspension (10^5 cells) was transferred into 5 ml round-bottom polystyrene tubes. Cells were incubated for 15 min with FITC-conjugated annexin V molecules and propidium iodide (PI). After this period of incubation, 400 μl of annexin-binding buffer (BD Biosciences, USA) 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. Necrotic cells were identified as single PI-positive. 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 percentage of the total number of stained cells counted.

Invasion and Proliferation Assay

Cell invasion assay was performed in an invasion chamber based on the Boyden chamber principle. Each chamber contains an insert fitted with an 8 μM pore size polycarbonate membrane precoated with rat tail collagen I (5 $\mu g/cm^2$). The inserts were washed in DMEM and incubated for 30 min at room temperature. Each insert was filled with 5×10^5 cells in 400 μl of serum-free media. The following experimental groups were used: (1) cells with no treatment and (2) cells exposed to 100 nM PACAP1-38. Cells were incubated for 72 h at 37 °C in a CO_2 (5 %) incubator. After incubation, supernatant was discarded and viable cells invaded the collagen were stained with 400 μl of crystal violet for 20 min at room temperature. After washing, non-invading cells were removed from the insert. Each stained insert was transferred in a new chamber containing 200 μl of a solution of H_2O/ethanol/acetic acid (49:50:1) for 20 min at room temperature. One hundred microliters of the dye mixture was transferred to a 96-well microtiter plate for colorimetric measurement. Absorbance proportional to cell concentration was determined at 560 nm.

Proliferation assay was performed on cells found in the chambers simultaneously with invasion assay at the end of 72-h incubation. After removal of medium, 400 μl serum-free medium containing 20 % of CellTiter 96 Aqueous One solution reagent was added to the cells. This plate was incubated for 3 h at 37 °C in a humified, 5 % CO_2 atmosphere. After incubation, 100 μl of the medium of each well was transferred in a 96-well plate and absorbance proportional to cell concentration was determined at 560 nm using colorimetric test.

Experiments with Decidual and Peripheral Blood Mononuclear Cells of Healthy Pregnant Women

Patients and Human Samples

All subjects were patients of the Department of Obstetrics and Gynecology at the University of Pecs. Samples of decidual tissues and matched peripheral blood were obtained from nine healthy pregnant women underwent elective termination of apparently normal pregnancies at the 6–10 weeks of gestation. They did not have a history of spontaneous abortion, ectopic pregnancy, preterm delivery, or stillbirth.

Written informed consent was obtained from all participants. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Regional Ethical Committee at the Faculty of Medicine, University of Pecs.

Isolation of Decidual Mononuclear Cells

Isolation of decidual mononuclear cells (DMC) from decidual tissue was performed as previously described (Szereday et al. 2012). Briefly, decidual tissue was cut into pieces, exposed to collagenase IV digestion (equal volume of tissue and 0.5 % collagenase type IV, Sigma-Aldrich Kft., Hungary) at 37 °C for 60 min with gentle stirring on the magnetic stirrer. Cell suspension was passed through 100 μm nylon mesh (BD Biosciences, USA) for tissue debris elimination and centrifuged at $600\times g$ for 10 min. The pellet was resuspended and was passed through 70 μm nylon mesh (BD Biosciences, USA). The pellet was resuspended again in RPMI 1640 (Csertex Kft., Hungary) and overlaid on Ficoll-Paque gradient and centrifuged at $800\times g$ for 20 min. DMC were collected from the interface, washed, and passed through 40 μm mesh (BD Biosciences) and resuspended 10^6/ml of cell culture medium [RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (1×10^{-5} U/l), streptomycin sulfate (0.05 g/l), and 10 % fetal calf serum (all from GIBCO, Life Technologies, Hungary)].

Isolation of Decidual Lymphocytes

Isolated decidual lymphocytes (DL) were obtained as non-adherent cell fraction of DMC cultured on 100 mm×20 mm tissue culture Petri dish in humidified 5 % CO_2 incubator at 37 °C, after the long adherence procedure (24 h).

Isolation of Peripheral Blood Mononuclear Cells

Ten milliliters of venous blood was taken. Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood on Ficoll-Paque gradient. Separated peripheral blood lymphocytes were obtained as non-adherent cell fraction of PBMC cultured on 100 mm×20 mm tissue culture Petri dish in humidified 5 % CO_2 incubator at 37 °C, after the long adherence procedure (24 h).

Cytometric Bead Array (CBA)

Concentration of cytokines from supernatants was determined using a human Th1/Th2 CBA kit (BD Biosciences, USA) which allowed for the simultaneous detection of IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ. Aliquoted samples were thawed and CBA analysis performed according to the manufacturer's protocol. Briefly, beads coated with capture antibodies were mixed. Fifty microliter of the capture bead mixture was added to 50 μl of sample. To these sample-bead

compounds, 50 μl of phycoerythrin conjugated detection antibody was added and this mixture was incubated for 3 h in dark at room temperature. The samples then were washed with 1 ml of wash buffer in 1,100 rpm for 5 min and the pellets were resuspended in 300 μl wash buffer. Cytokine standards were serially diluted to facilitate the construction of calibration curves necessary for determining protein concentrations of test samples. Flow cytometric analysis was performed on a BD FACSCalibur (BD Immunocytometry Systems, Erembodegen, Belgium) with Cell Quest software and data were analyzed with FCS Express V3 software.

Angiogenesis Array

Supernatants of PACAP1-38-treated decidual and peripheral blood mononuclear cells were also analyzed by angiogenesis array as described above.

Results

Experiments on HTR-8/SVneo Cells

Cell Viability Test

Viability of HTR-8/SVneo cells after H_2O_2 treatment was measured by MTT assay (Fig. 1). Exposure to 150 μM H_2O_2 for 24 h resulted in a significant decrease in cell viability. PACAP1-38 alone had no effect on cell viability. Pretreatment with 10 or 100 nM PACAP1-38 led to significant increase in the ratio of living cells, with no significant difference between the 10 and 100 nM concentrations of PACAP1-38 (Fig. 1a). In contrast to these findings, simultaneous PACAP1-38 treatment showed no effect against H_2O_2-induced oxidative stress (Fig. 1b).

Invasion Assay

To test the potential of PACAP on invasive behavior of HTR-8/SVneo human first trimester extravillous trophoblast cells, invasiveness of cells in the presence or absence of PACAP1-38 was analyzed by cell invasion assay (Fig. 2a). PACAP1-38 treatment did not significantly alter the invasiveness of HTR-8/SVneo trophoblast cells (Fig. 2b).

Angiogenesis Array

Cell-conditioned media of untreated or PACAP1-38 treated HTR-8/SVneo cells were subjected to angiogenesis array (Fig. 3a), suitable to measure protein levels of 51 angiogenesis-related molecules. Levels of several angiogenic factors were markedly decreased in the cell culture supernatants after 24 h PACAP1-38 treatment. Secreted levels of activin A, ADAMTS-1, angiogenin, angiopoietin-1, endocrine gland-derived vascular endothelial growth factor (EG-VEGF), and endoglin were reduced by 69, 79, 66, 91, 45, and 55 %, respectively (Fig. 3b).

Experiments on HIPEC65 Cells

Cell Viability Tests

Viability of HIPEC 65 cells after methotrexate treatment was measured by WST-1 assay (Fig. 4). Methotrexate treatment significantly decreased cell survival in HIPEC 65 cells, and this effect could not be altered by PACAP1-38. In order to distinguish apoptotic cells from necrotic and living cells, annexin V–propidium iodide staining was used. Similarly to results obtained from WST-1 assay, PACAP1-38 could not counteract the cell survival decreasing effect of MTX (Fig. 5).

Invasion and Proliferation Assay

Invasion assay performed on HIPEC 65 cells (Fig. 6) showed that addition of PACAP1-38 decreased the invasion of the HIPEC 65 cells and increased their proliferation.

Experiments on Decidual and Peripheral Blood Mononuclear Cells

Angiogenesis Array

Cell-conditioned media of untreated and PACAP1-38 treated (24 h) human peripheral blood and decidual mononuclear cells were also analyzed by angiogenesis protein array (Fig. 7). PACAP1-38 did not alter the secreted levels of tested angiogenic molecules by peripheral blood (Fig. 7a) or decidual (Fig. 7b) mononuclear cells.

We detected amphiregulin, endothelin-1, GM-CSF, IL-1β, IL-8, MIP-1α, matrix metalloproteinases (MMP)-8, MMP-9, TIMP1, CXCL4, and serpin E1 molecules in the supernatants of peripheral blood mononuclear cells (Fig. 7a). The decidual blood mononuclear cells also produced coagulation factor III, DPPIV, EG-VEGF, IGFBP-1, IGFBP-2, IGFBP-3, MCP1, and prolactin (Fig. 7b). Amphiregulin and CXCL4 were not present in the supernatants of decidual blood mononuclear cells (Fig. 7b).

Inflammatory Cytokine Production by Decidual and Peripheral Blood Mononuclear Cell in Healthy Pregnant Women

Th1 and Th2 cytokines were analyzed by CBA system. Inflammatory cytokines production by decidual and PBMC was not altered after PACAP1-38 or PACAP antagonist PACAP6-38 treatment compared to control samples (Table 1).

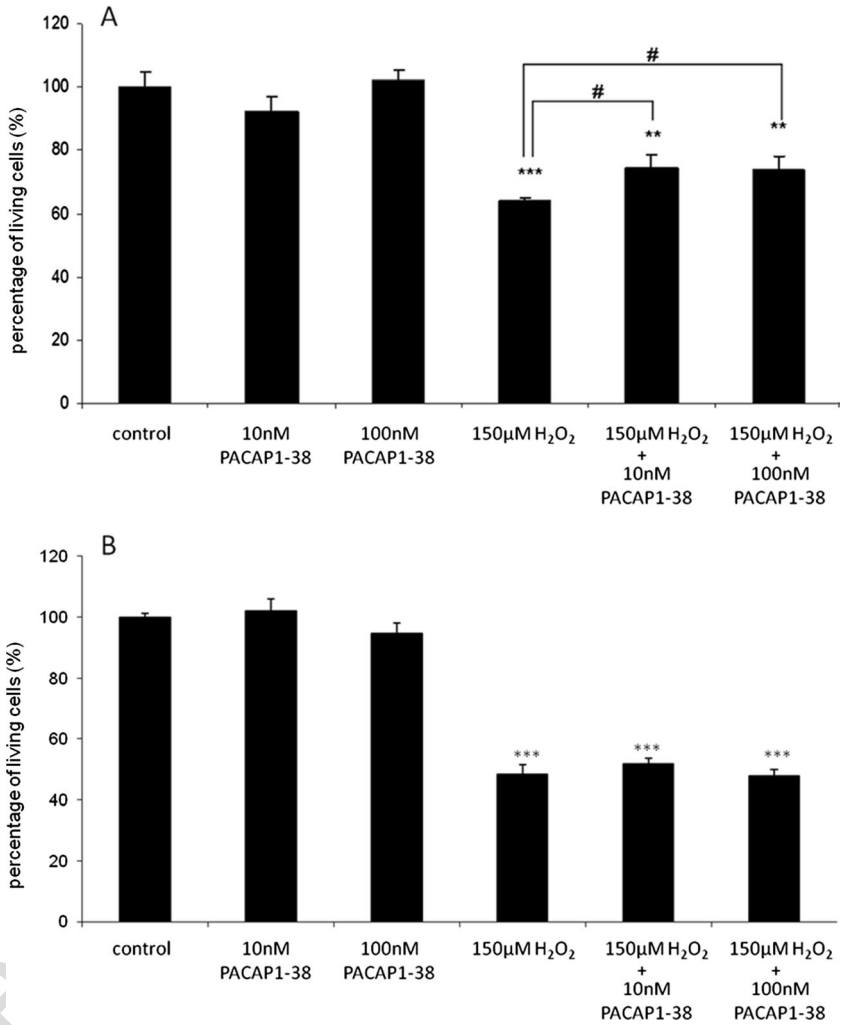


Fig. 1 Effect of PACAP1-38 (**a**) cotreatment and (**b**) pretreatment on viability of HTR-8/SVneo cells as measured by MTT assay exposed to H_2O_2 for 24 h. Data are expressed as percentage of living cells ± SEM. $^{**}p<0.01$, $^{***}p<0.001$, compared to control values; $^{\#}p<0.05$ compared to the H_2O_2-treated groups

Discussion

The common aim of our study was to use experiments examining the possible role of PACAP related to human pregnancy. In the first set of experiments, we investigated whether PACAP influences cell survival of primary trophoblast cell line exposed to H_2O_2-induced oxidative stress. Oxidative stress plays an important role in placental pathology. It was

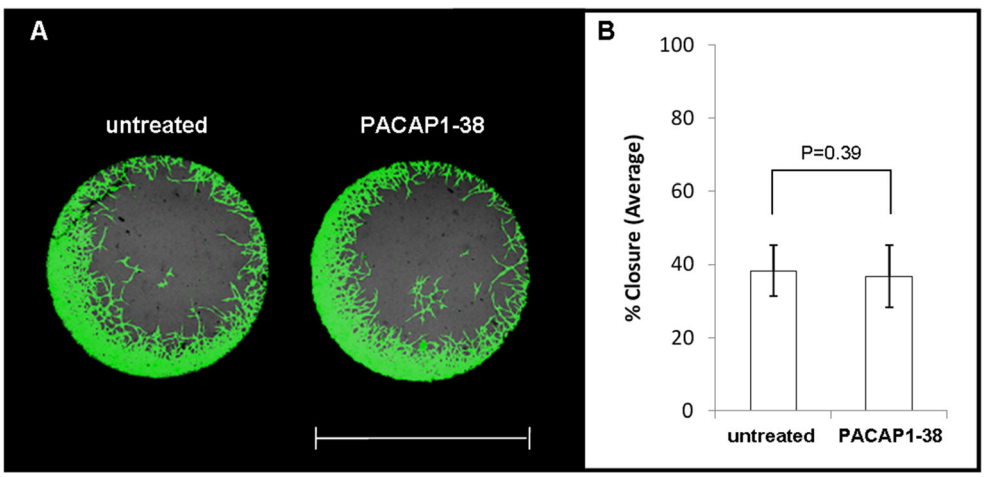


Fig. 2 PACAP1-38 does not alter invasiveness of HTR-8/SVneo trophoblast cells. **a** Invasion of untreated or 1 μM PACAP1-38 treated HTR-8/SVneo cells into the detection zones after 72 h is shown. Cells were labeled with calcein AM (confocal microscope, *bar* 2 mm). **b** Quantification of the invasion assay. Area closure (%) is calculated from measured areas of invasion at 72 h. Data are presented as mean ± SEM from three wells per condition

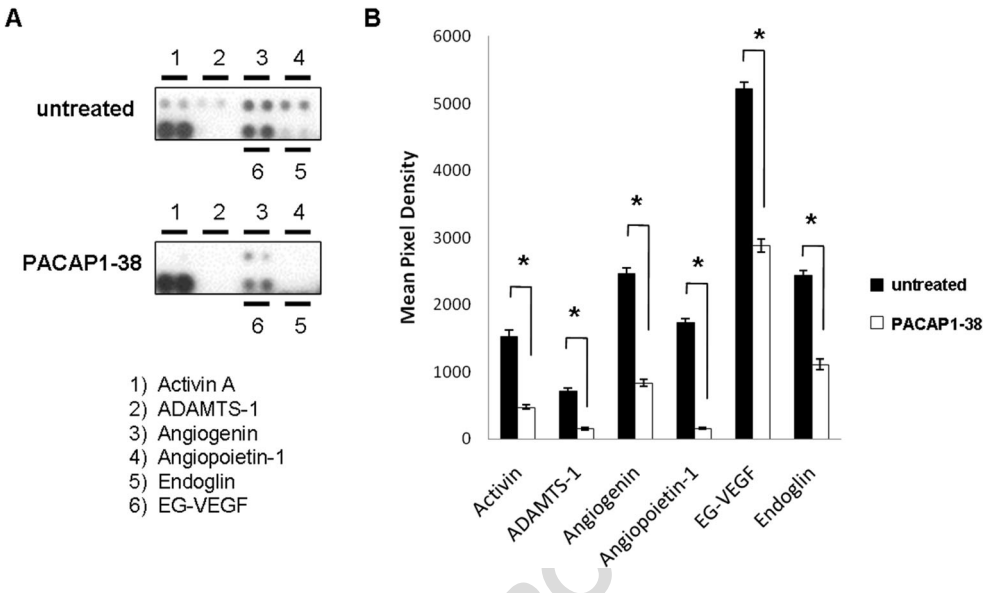


Fig. 3 PACAP1-38 decreases the secretion of several angiogenic factors in trophoblast cells. **a** Cell conditioned media of untreated or 1 μM PACAP1-38 (24 h) treated HTR-8/SVneo cells analyzed by protein array. **b** Densitometric evaluation of the protein array. Data are presented as mean ± SEM (N=2; *p<0.05)

suggested as a contributory factor in pathological events like miscarriage and preeclampsia (Burton and Jauniaux 2004; Poston et al. 2011). Previously PACAP has been shown to decrease cell viability of JAR cells originating from human choriocarcinoma (Boronkai et al. 2009). This was rather surprising given the common survival-promoting effect of PACAP in most cell types (Vaudry et al. 2009; Reglodi et al. 2011). Here, we tested whether this effect of PACAP on cell survival depends on the malignancy of cells. We found that PACAP pretreatment protected nontumorous HTR-8/SVneo trophoblast cells, hence we may conclude that PACAP shows different effects in malignant and nonmalignant trophoblast cells. This difference has also been described in other cells, for example in retinal cells, where PACAP is known to enhance survival of normal retinal cells under the influence of different stressors (Atlasz et al. 2010), while it reduces survival of retinoblastoma cells (Wojcieszak and Zawilska 2014). Based on our results, it seems that the effect of PACAP on trophoblast cells also depends on the normal versus tumorous nature of the cells, on environmental influences, timing of the treatment, and the nature of the stressor.

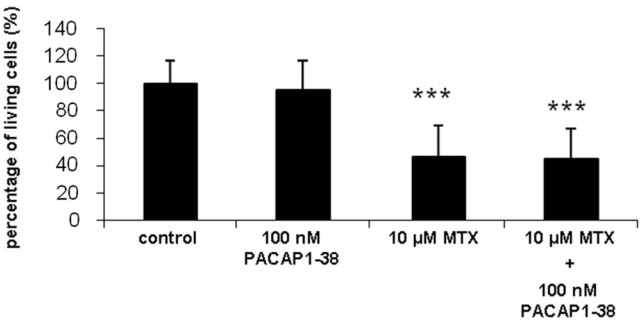
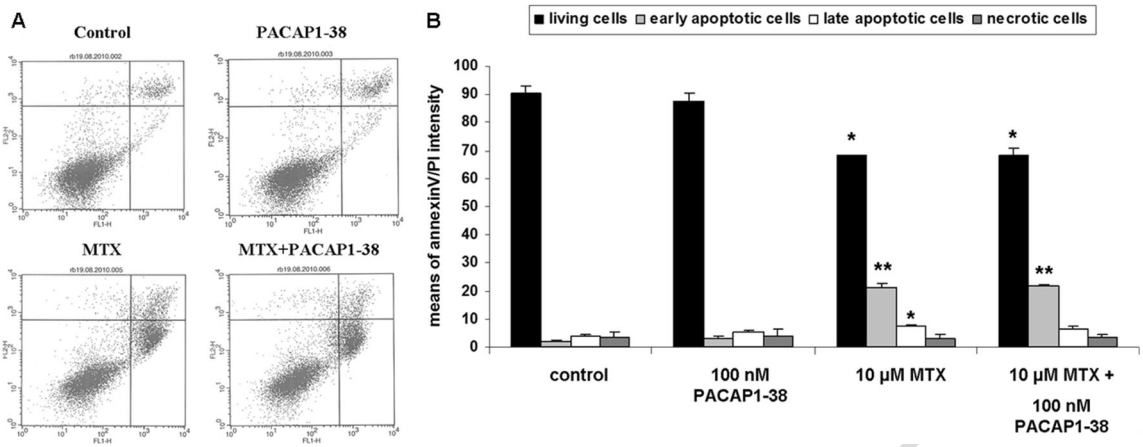


Fig. 4 Viability of HIPEC 65 cells as measured by WST-1 assay. Cells were exposed to 10 μM MTX and/or 100 nM PACAP1-38, as indicated in the figure. ***p<0.001 compared to control group

We also investigated if PACAP could enhance the invasive ability of HTR-8/SVneo and HIPEC65 trophoblast cells. We observed that PACAP had detectable effects on invasion and proliferation of HIPEC 65 cells, but it did not affect the invasiveness of HTR-8/SVneo cells suggesting that its effects depend on the cell type. Among others, MMPs, especially MMP-2, are involved in the invasive process responsible for implantation during pregnancy (Shaun-Ram et al. 2004). PACAP has been shown to enhance relaxin-induced secretion of MMP-2 in rats (Teng et al. 2000), raising the possibility of its contribution to the mechanisms of implantation. The possible explanation of its distinct effect in HTR-8/SVneo and HIPEC 65 cells could be the signaling mechanisms leading to cell death or survival and/or the presence/absence of different splice variants of the PACAP receptor.

We also investigated the effects of PACAP on angiogenetic factors. The human placenta is principally a vascular organ that functions to achieve a physiological union of the maternal and fetal blood supplies. A major physiological role of the placenta is to develop an extensive vascular network allowing for nutrient, waste, and gas exchange between the maternal and fetal circulations. To accomplish this, the placenta produces a variety of angiogenic factors. The regulation of angiogenesis in pregnancy is tightly controlled. Angiogenic growth factors such as angiogenin, angiopoietins, endoglin, EG-VEGF, VEGF, and placental growth factor play an important role in placental vasculogenesis and angiogenesis. Extravillous trophoblast-derived angiogenetic factors may also play a role in spiral artery remodeling as well as control of trophoblast invasion. The balance between proangiogenic and antiangiogenic factors modulates these processes.

Our result showed that HTR-8/SVneo cells secreted lower levels of activin, ADAMTS-1, angiogenin, angiopoietin-1, EG-VEGF, and endoglin after 24 h PACAP1-38 treatment.



Q5 **Fig. 5** Effect of MTX and PACAP1-38 on cell survival in HIPEC 65 trophoblast cells. Distinction between living, necrotic, early, and late apoptotic cells. Examples of *dot plots* (**a**) as determined by flow cytometry following annexin V and propidium iodide double staining. *Horizontal axis* represents annexin V intensity and *vertical axis* shows PI staining. The *lines* divide each plot into quadrants—*lower left quadrant* living cells (AnV−/PI−), *lower right quadrant* early apoptotic cells (AnV+/PI−), *upper left quadrant* necrotic cells (AnV−/PI+), *upper right quadrant* late apoptotic cells (AnV+/PI+). *Graphs* (**b**) demonstrate the mean percentage of living cells, ratio of cells in early and late apoptosis. *$p<0.05$, **$p<0.01$

Since placental oxygenation is an important factor in controlling angiogenic factor production, these data suggest that PACAP1-38 may play an important role in the fine regulation of angiogenesis by modulating the production of different angiogenic factors. However, regulation of these factors by PACAP1-38 in hypoxic conditions merit further investigation.

The immune microenvironment of the fetomaternal interface is crucial for the maintenance of pregnancy, and cytokines are considered to be key regulators. For many years, Th1/Th2 hypothesis has provided a useful framework for studies of the immunology of pregnancy. However, the findings that pregnancy itself is an inflammatory state have led to a revision of this hypothesis and now it is apparent that both arms of the immune response are intensified during healthy pregnancy, but with a stronger bias towards Th2 than Th1 responses. This Th2 cytokine polarization occurs both at systemic level and at the fetal–maternal interface and the cause behind this cytokine shift are not clearly defined.

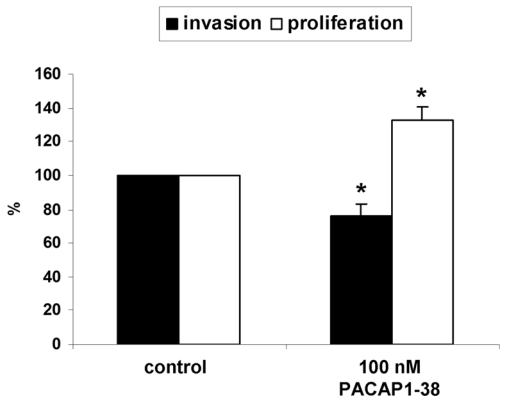


Fig. 6 Invasion and proliferation of HIPEC 65 cells as assessed by invasion assay based on Boyden chamber principle. Cells were treated with PACAP1-38. *$p<0.05$ compared to control group

Based on the well-known immunomodulatory actions of the VIP–PACAP peptide family (Ganea and Delgado 2002), we proposed that PACAP would be involved in cytokine production of decidual cells. Recently, VIP has been shown to be involved in recruitment of T cells during the decidualization process (Grasso et al. 2014). In this study, we analyzed the effect of PACAP1-38 and PACAP6-38 on the inflammatory and proinflammatory cytokine production of peripheral and decidual lymphocytes obtained from healthy pregnant women. Investigating Th1 and Th2 cytokine production by peripheral and decidual cells, we did not find any characteristic differences after PACAP1-38 or PACAP6-38 treatment compared to the control group. These results suggest that during healthy pregnancy neither in the periphery nor at the fetomaternal interface PACAP1-38 or PACAP6-38 regulates immune microenvironment by modulating cytokine production. Although PACAP has been described to modulate cytokine production in several experimental paradigms both in vitro and in vivo (Banki et al. 2013; Csanaky et al. 2014; Nakamachi et al. 2012), it seems that PACAP does not have such an effect on decidual lymphocytes, which further supports the finding that the actions of PACAP are dependent on cell types and several other factors such as cycle, hormonal status, and growth factors present in the microenvironment (Vaudry et al. 2009; Szabo et al. 2004; Somogyvari-Vigh and Reglodi 2004).

Based on our results, PACAP has different effects on different placental cells. This differential effect may be the result of the nature of the cells (normal, tumorous or challenged) or other causes of the different functions can be the expression of different receptorial splice variants as well as the

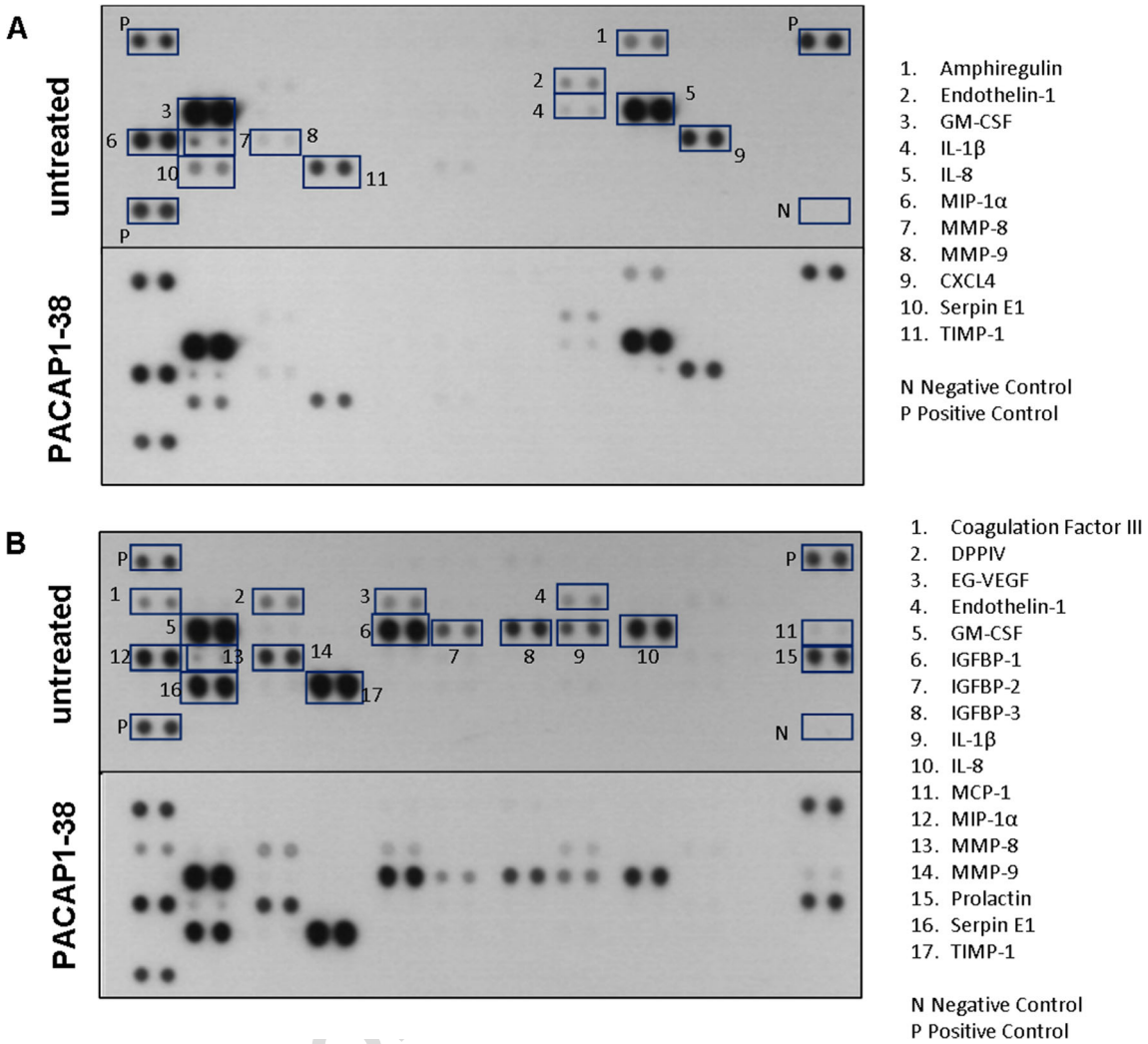


Fig. 7 Secreted angiogenic factors by peripheral blood and decidual mononuclear cells. **a** Angiogenesis array with cell-conditioned media of untreated or 1 μM PACAP1-38 treated (24 h) peripheral blood mononuclear cells. **b** Angiogenesis array with cell-conditioned media of untreated or 1 μM PACAP1-38 treated (24 h) decidual mononuclear cells

Table 1 Cytokine production by decidual and peripheral blood mononuclear cells in healthy pregnant women

pg/ml		Control	+PACAP1-38	+PACAP1-38+ PACAP6-38	+PACAP36-38
PBMC	IL-2	734.7	742.7	755.9	753.3
	IL-4	88.7	88.7	86.3	88.7
	IL-6	76.8	78.5	78.5	81.6
	IL-10	262.3	250	260.3	266.4
	TNF-α	1,016.1	1,017.9	1,068.4	1,064.8
	IFN-γ	653	644.3	729.4	665.1
Decidual lymphocytes	IL-2	562.8	563.6	575.3	577.8
	IL-4	25.9	25.9	29.3	29.3
	IL-6	99.5	96.5	95	99.5
	IL-10	184.5	160	160	164.1
	TNF-α	814.1	767.2	783.4	823.1
	IFN-γ	408.9	424.7	408.9	424.7

temporal and spatial distribution of the PACAP receptors in the placenta. The expression of the different PACAP receptors in the various placental cell types is not fully characterized yet. The expression of PAC1 and VPAC receptors has been shown in the placenta. The gene encoding VPAC receptors is weakly expressed in the human placenta (Sreedharan et al. 1995). In human placental tissues, Scaldaferri et al. (2000) found expression of the variants PAC1SV1 and SV2. Radioligand binding studies have confirmed that PACAP is able to bind to placental tissues (Scaldaferri et al. 2000). Koh et al. described that the expression of PAC1 receptors shows temporal and spatial variance—PAC1 receptor mRNA expression increases with the progression of pregnancy and varies between different cell types and cytotrophoblast and syncytiotrophoblast cells show weaker expression than decidual cells or chorionic vessels (Koh et al. 2005). Furthermore, a great interspecies difference also exists between rats and humans (Koh et al. 2003, 2005; Scaldaferri et al. 2000). A detailed mapping of the changes in receptor expression and their splice variants would be necessary to exactly verify the different effects of PACAP in the placenta throughout the entire pregnancy.

Our present results indicate that PACAP has some effects on mechanisms playing a role in invasiveness and implantation in certain cell types, while it has no effects on others. Our results could be a starting point to further investigation on the influence of PACAP on trophoblast cells.

Acknowledgments This study was supported by OTKA K104984, K104960, TAMOP (4.2.2.A-11/1/KONV-2012-0024), the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/2-11-1-2012-0001 "National Excellence Program", Magyary Zoltan Scholarship, Arimura Foundation, PTE-MTA "Lendület" Program, and the Bolyai Scholarship.

References

Atlasz T, Szabadfi K, Kiss P et al (2010) Pituitary adenylate cyclase activating polypeptide in the retina: focus on the retinoprotective effects. Ann NY Acad Sci 1200:128–139

Banki E, Degrell P, Kiss P et al (2013) Effect of PACAP treatment on kidney morphology and cytokine expression in rat diabetic nephropathy. Peptides 42:125–130

Brubel R, Boronkai A, Reglodi D et al (2010) Changes in the expression of pituitary adenylate cyclase-activating polypeptide in the human placenta during pregnancy and its effects on the survival of JAR choriocarcinoma cells. J Mol Neurosci 42:450–458

Burton GJ, Jauniaux E (2004) Placental oxidative stress: from miscarriage to preeclampsia. J Soc Gynecol Investig 11:342–352

Csanaky K, Doppler W, Tamas A, Kovacs K, Toth G, Reglodi D (2014) Influence of terminal differentiation and PACAP on the cytokine, chemokine, and growth factor secretion of mammary epithelial cells. J Mol Neurosci 52:28–36

D'Amico AG, Scuderi S, Saccone S, Castorina A, Drago F, D'Agata V (2013) Antiproliferative effects of PACAP and VIP in serum-starved glioma cells. J Mol Neurosci 51:503–513

Fabian E, Reglodi D, Mester L et al (2012) Effects of PACAP on intracellular signaling pathways in human retinal pigment epithelial cells exposed to oxidative stress. J Mol Neurosci 48:493–500

Ganea D, Delgado M (2002) Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) as modulators of both innate and adaptive immunity. Crit Rev Oral Biol Med 13:229–237

Gasz B, Racz B, Roth E et al (2006) Pituitary adenylate cyclase activating polypeptide protects cardiomyocytes against oxidative stress-induced apoptosis. Peptides 27:87–94

Grasso E, Paparini D, Auero M, Mor G, Perez Leiros C, Ramhorst R (2014) VIP contribution to the decidualization program: regulatory T cell recruitment. J Endocrinol 221(1):121–31

Horvath G, Reglodi D, Opper B et al (2010) Effects of PACAP on the oxidative stress-induced cell death in chicken pinealocytes is influenced by the phase of the circadian clock. Neurosci Lett 484:148–152

Isaac ER, Sherwood NM (2008) Pituitary adenylate cyclase activating polypeptide (PACAP) is important for embryo implantation in mice. Mol Cell Endocrinol 280:13–19

Ko C, In YH, Park-Sarge OK (1999) Role of progesterone receptor activation in pituitary adenylate cyclase-activating polypeptide gene expression in rat ovary. Endocrinology 140:5185–5194

Koh PO, Kwak SD, Kim HJ et al (2003) Expression patterns of pituitary adenylate cyclase activating polypeptide and its type I receptor mRNAs in the rat placenta. Mol Reprod Dev 64:27–31

Koh PO, Won CK, Noh HS, Cho CJ, Choi WS (2005) Expression of pituitary adenylate cyclase activating polypeptide and its type I receptor mRNAs in human placenta. J Vet Sci 6:1–5

Köves K, Kántor O, Lakatos A et al. (2014) Advent and recent advances in research on the role of pituitary adenylate cyclase-activating polypeptide (PACAP) in the regulation of gonadotropic hormone secretion of female rats. J Mol Neurosci (in press) Q8

Laburthe M, Couvineau A (2002) Molecular pharmacology and structure of VPAC receptors for VIP and PACAP. Regul Pept 108:165–173

Laburthe M, Couvineau A, Tan V (2007) Class II G protein-coupled receptors for VIP and PACAP: Structure, models of activation and pharmacology. Peptides 28:1631–1639

Li M, Cortez S, Nakamachi T, Batuman V, Arimura A (2006) Pituitary adenylate cyclase activating polypeptide is a potent inhibitor of the growth of light chain-secreting human multiple myeloma cells. Cancer Res 66:8796–8803

Lutz EM, MacKenzie CJ, Johnson M et al (1999) Domains determining agonist selectivity in chimaeric VIP2 (VPAC2)/PACAP (PAC1) receptors. Br J Pharmacol 128:934–940

Miyata A, Arimura A, Dahl RR et al (1989) Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun 164:567–574

Muller JM, Debaigt C, Goursaud S et al (2007) Unconventional binding sites and receptors for VIP and related peptides PACAP and PHI/PHM: an update. Peptides 28:1655–1666

Nakamachi T, Tsuchida M, Kagami N et al (2012) IL-6 and PACAP receptor expression and localization after global brain ischemia in mice. J Mol Neurosci 48:518–525

Pavan L, Tarrade A, Hermouet A et al (2003) Human invasive trophoblasts transformed with simian virus 40 provide a new tool to study the role of PPARγ in cell invasion process. Carcinogenesis 24:1325–1336

Poston L, Igosheva N, Mistry HD et al (2011) Role of oxidative stress and antioxidant supplementation in pregnancy disorders. Am J Clin Nutr 94:1980S–1985S

Racz B, Reglodi D, Horvath G et al (2010) Protective effect of PACAP against doxorubicin-induced cell death in cardiomyocyte culture. J Mol Neurosci 42:419–427

Reglodi D, Kiss P, Lubics A, Tamas A (2011) Review on the protective effects of PACAP in models of neurodegenerative diseases in vitro and in vivo. Curr Pharm Des 17:962–972

Reglodi D, Kiss P, Szabadfi K et al (2012a) PACAP is an endogenous protective factor-insights from PACAP-deficient mice. J Mol Neurosci 48:482–492

Reglodi D, Tamas A, Koppan M, Szogyi D, Welke L (2012b) Role of PACAP in female fertility and reproduction at gonadal level—recent advances. Front Endocrinol (Lausanne) 3:155

Scaldaferri ML, Modesti A, Palumbo C et al (2000) Pituitary adenylate cyclase activating polypeptide (PACAP) and PACAP-receptor type 1 expression in rat and human placenta. Endocrinology 141:1158–1167

Somogyvari-Vigh A, Reglodi D (2004) Pituitary adenylate cyclase activating polypeptide: a potential neuroprotective peptide. Curr Pharm Des 10:2861–2889

Spencer F, Chi L, Zhu MX (2001a) Temporal relationships among uterine pituitary adenylate cyclase activating polypeptide, decidual prolactin-related protein and progesterone receptor mRNAs expressions during decidualization and gestation in rats. Comp Biochem Physiol C 129:25–34

Spencer F, Chi L, Zhu MX (2001b) A mechanistic assessment of 1,3-butadiene diepoxide-induced inhibition of uterine deciduoma proliferation in pseudopregnant rats. Reprod Toxicol 15:253–260

Sreedharan SP, Huang JX, Cheung MC, Goetzl EJ (1995) Structure, expression, and chromosomal localization of the type I human vasoactive intestinal peptide receptor gene. Proc Natl Acad Sci U S A 92:2939–2943

Staun-Ram E, Goldman S, Gabarin D, Shalev E (2004) Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. Reprod Biol Endocrinol 2:59

Steenstrup BR, Alm P, Hannibal J et al (1995) Pituitary adenylate cyclase-activating polypeptide: occurrence and relaxant effect in female genital tract. Am J Physiol 32:E108–E117

Steenstrup BR, Jorgensen JC, Alm P et al (1996) Pituitary adenylate cyclase activating polypeptide (PACAP): occurrence and vasodilatory effect in the human uteroplacental unit. Regul Pept 61:197–204

Szabo E, Nemeskeri A, Arimura A, Koves K (2004) Effect of PACAP on LH release studied by cell immunoblot assay depends on the gender, on the time of day and in female rats on the day of the estrous cycle. Regul Pept 123:139–145

Szereday L, Miko E, Meggyes M et al (2012) Commitment of decidual haematopoietic progenitor cells in first trimester pregnancy. Am J Reprod Immunol 67:9–16

Teng CH, Ke FC, Lee MT, Lin SW, Chen L, Hwang JJ (2000) Pituitary adenylate cyclase-activating polypeptide acts synergistically with relaxin in modulating ovarian cell function in rats. J Endocrinol 167:61–69

Vaudry D, Falluel-Morel A, Bourgault S et al (2009) (2009) Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. Pharmacol Rev 61:283–357

Wojcieszak J, Zawilska JB (2014) PACAP38 and PACAP6-38 exert cytotoxic activity against human retinoblastoma Y79 cells. J Mol Neurosci 2014 (in press)

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

Q1. Please check if the affiliation 1 is presented correctly.
Q2. The citation “Isaac et al., 2008” (original) has been changed to “Isaac and Sherwood 2008”. Please check if appropriate.
Q3. “Horvath et al., 2011” is cited in text but not given in the reference list. Please provide details in the list or delete the citation from the text.
Q4. “Boronkai et al., 2009” is cited in text but not given in the reference list. Please provide details in the list or delete the citation from the text.
Q5. Figure 5 contains poor quality of resolution(small text below standard size)Please provide replacement/s. Otherwise, please advise if it's okay to proceed with the figures as is.
Q6. The citation “Burton et al., 2004” (original) has been changed to “Burton and Jauniaux 2004”. Please check if appropriate.
Q7. “Shaun-Ram et al. 2004” is cited in text but not given in the reference list. Please provide details in the list or delete the citation from the text.
Q8. Please provide complete bibliographic details of this reference.
Q9. Staun-Ram et al. (2004) was not cited anywhere in the text. Please provide a citation. Alternatively, delete the item from the list.
Q10. Please provide complete bibliographic details of this reference.