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Investigation of the Possible Functions of PACAP in Human Trophoblast Cells

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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

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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,

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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 co-treated 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 microliters 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 were then 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).

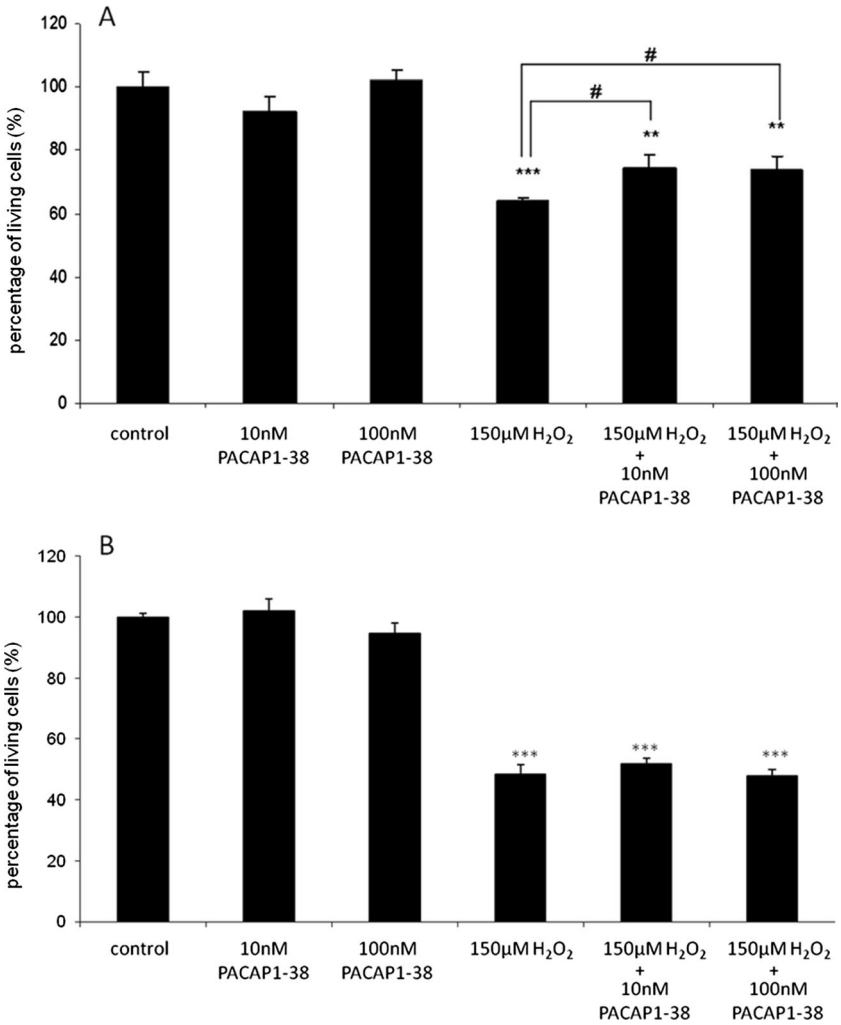

Fig. 1 Effect of PACAP1-38 (**a**) pretreatment and (**b**) co-treatment on viability of HTR-8/SVneo cells as measured by MTT assay exposed to H_2O_2 for 24 h. Data are expressed as percentage of living cells ± SEM. $^{**}p<0.01$, $^{***}p<0.001$, compared to control values; $^{\#}p<0.05$ compared to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

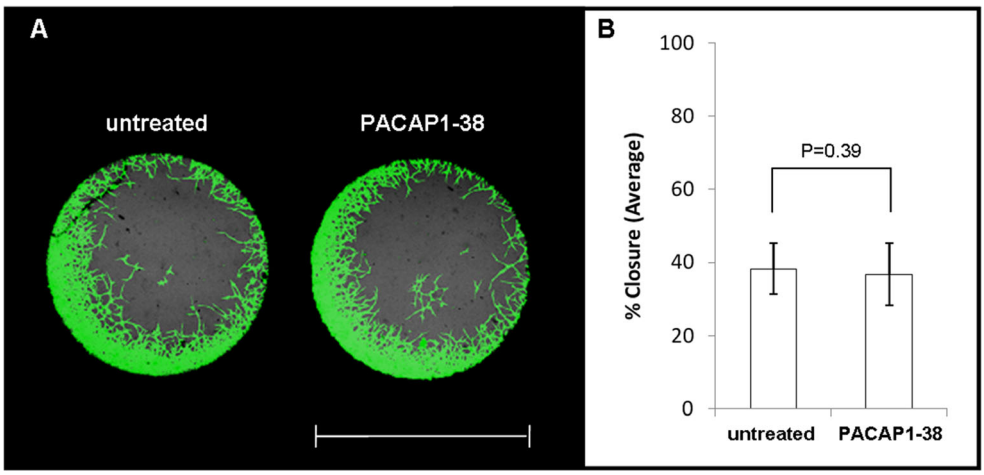

Fig. 2 PACAP1-38 does not alter invasiveness of HTR-8/SVneo trophoblast cells. **a** Invasion of untreated or 1 μM PACAP1-38 treated HTR-8/SVneo cells into the detection zones after 72 h is shown. Cells were labeled with calcein AM (confocal microscope, *bar* 2 mm). **b** Quantification of the invasion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

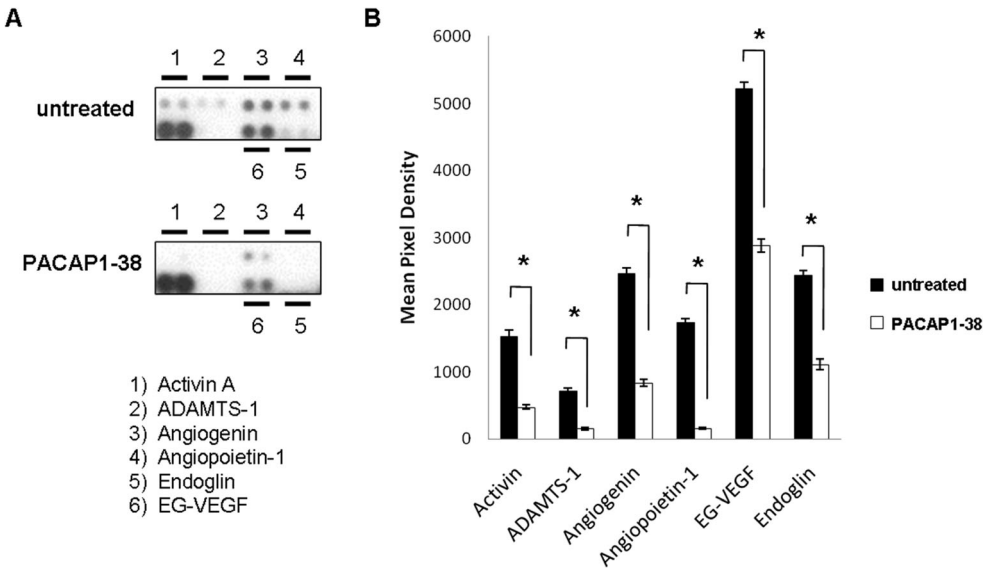

Fig. 3 PACAP1-38 decreases the secretion of several angiogenic factors in trophoblast cells. **a** Cell conditioned media of untreated or 1 μM PACAP1-38 (24 h) treated HTR-8/SVneo cells analyzed by protein array. **b** Densitometric evaluation of the protein array. Data are presented as mean ± SEM (N=2;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

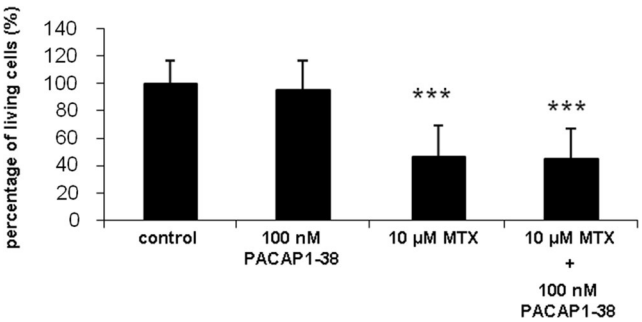

Fig. 4 Viability of HIPEC 65 cells as measured by WST-1 assay. Cells were exposed to 10 μM MTX and/or 100 nM PACAP1-38, as indicated in the figure. ***p<0.001 compared to control group

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 (Staun-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.

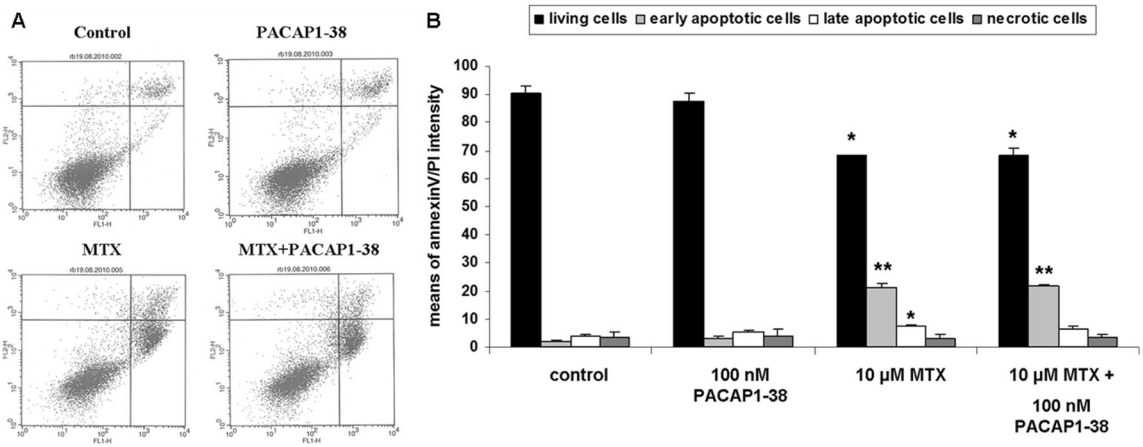

Fig. 5 Effect of MTX and PACAP1-38 on cell survival in HIPEC 65 trophoblast cells. Distinction between living, necrotic, early, and late apoptotic cells. Examples of *dot plots* (**a**) as determined by flow cytometry following annexin V and propidium iodide double staining. *Horizontal axis* represents 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.

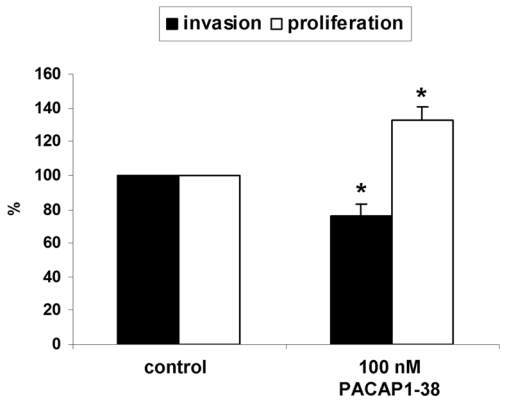

Fig. 6 Invasion and proliferation of HIPEC 65 cells as assessed by invasion assay based on Boyden chamber principle. Cells were treated with PACAP1-38. *$p<0.05$ compared to control group

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

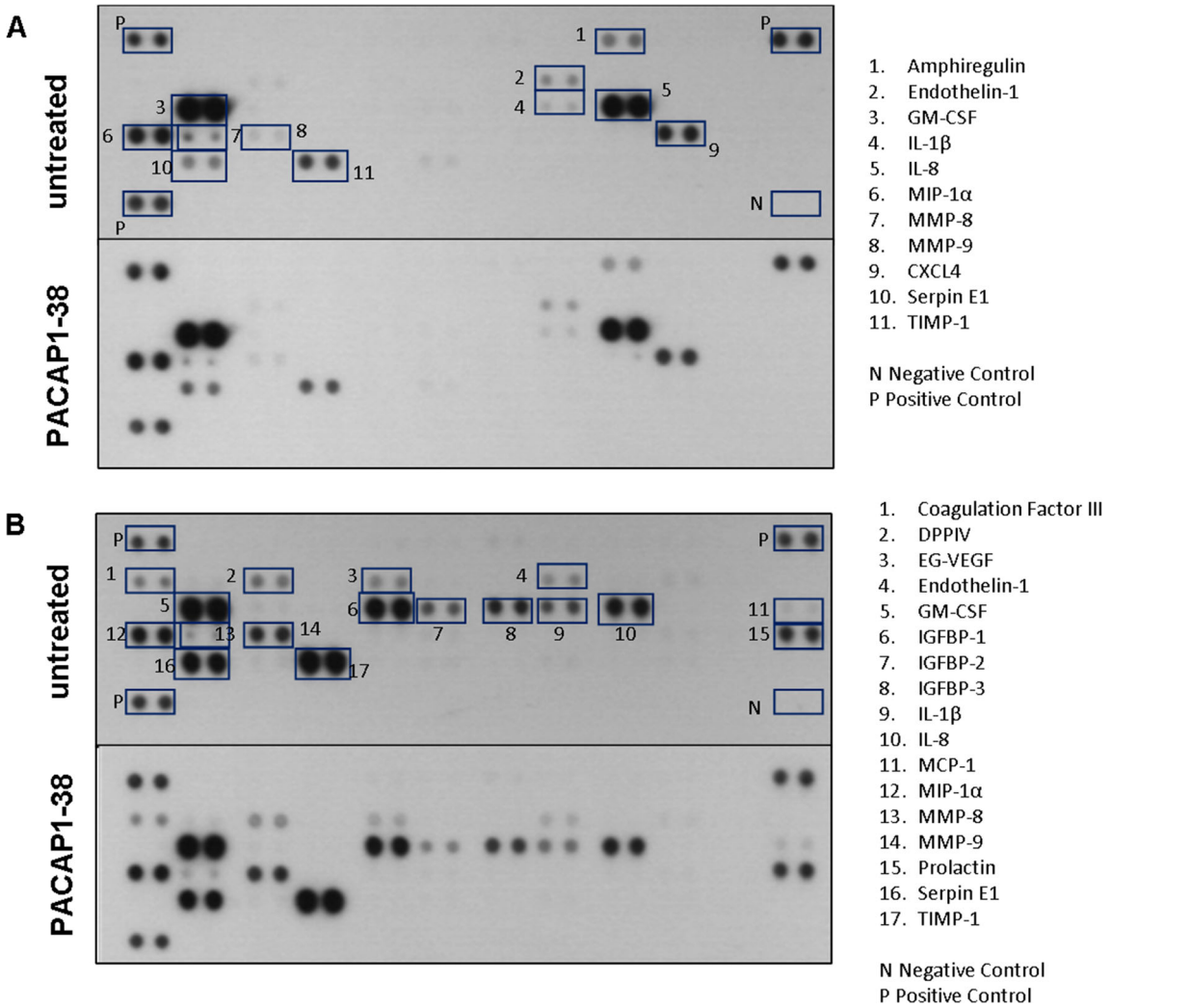

Fig. 7 Secreted angiogenic factors by peripheral blood and decidual mononuclear cells. **a** Angiogenesis array with cell-conditioned media of untreated or 1 μM PACAP1-38 treated (24 h) peripheral blood mononuclear cells. **b** Angiogenesis array with cell-conditioned media of untreated or 1 μM PACAP1-38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

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