PRESENCE OF PACAP IN THE REPRODUCTIVE SYSTEM AND ITS EFFECTS ON TROPHOBLAST CELL SURVIVAL AND SIGNALING PATHWAYS

PhD. thesis

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Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP)

Pituitary adenylate cyclase activating polypeptide (PACAP) was originally isolated in 1989 from the hypothalamus, based on its cAMP-increasing effect in pituitary cells. It is a member of secretin/glucagon/vasoactive intestinal polypeptide family and shares 67% identity with VIP. PACAP occurs in two amino acid forms: PACAP38 and PACAP27, with the 38 amino acid form being predominant in human tissues. The peptide is degraded by dipeptidyl-peptidase IV (DPP IV) and it has a very short half-life time in body fluids. During the degradation more N-terminal shorter peptides arise, which are biologically not active any more. The N-terminal end is necessary for the biological activity, but not for the receptor binding. These peptides, for example PACAP3-38, in most cases are antagonists. The most effective antagonist fragment is PACAP6-38.

Occurrence of PACAP in the body

PACAP occurs not only in the central and peripheral nervous system, but also in other tissues, like the adenohypophysis, suprarenal gland, endocrine pancreas, parathyroid gland, and in the cardiovascular, gastrointestinal and the urinary tract and respiratory system.

PACAP-receptors

The PACAP receptors belong to the family of G-protein coupled receptors with seven transmembrane domains. There are two types of PACAP receptors: PAC1 receptor which binds PACAP with high affinity and VIP with a much lower affinity and VPAC1 and VPAC2 receptors which bind VIP and PACAP with similar affinities. PAC1 receptors are localized in the central nervous system (different parts of the hypothalamus, cortex, thalamus, mesencephalon, pons and cerebellum), adenohypophysis, suprarenal gland and in the testis.

Effect of PACAP on the gonadal functions

Several studies have shown the role of PACAP not only in the regulation of pituitary hormone secretion but also in other reproductive functions. PACAP is present in the uterus, where it supposedly plays a role in muscle contraction and blood supply. Little is known about the effects of PACAP in the placenta. Both forms of the peptide, PACAP38 and PACAP27, along with their receptors, are present in the human pregnant uterus and placenta. PACAP causes a relaxation on stem villous and intramyometrial arteries, suggesting a vasoregulatory role in the uteroplacental unit. PACAP is suggested to play a role in decidualization and the time-related localization of endometrial-uterine PACAP is implicated in facilitation of endometrial blood flow and increase the availability of metabolic substrates to the developing deciduoma or embryo. PACAP is an important trophic factor during the embryonic development and also in the mature nervous system. Based on the currently available data it seems that the placenta synthesizes PACAP in addition to the numerous growth factors and hormones important for the development and maintenance of pregnancy and for the fetal development.

Molecular mechanism of the protective effects of PACAP

PAC1 receptor is responsible for the cytoprotective effects of PACAP. PAC1 receptor is coupled to adenylate cyclase and phospholipase C. Through adenylate cyclase activation, it elevates cAMP, and activates protein kinase A (PKA), which can, among others, activate the mitogen-activated protein kinase (MAPK) and the extracellular signal-regulated kinases (ERK) pathways and down-regulate the C-Jun N-terminal kinases (JNK) and the p38 MAPK.

The aims were the following:

- to determine the concentration of PACAP38 and PACAP27 in human first trimester and full-term placentas (maternal central and peripheral; fetal central and peripheral) and in the umbilical cord.
- to determine, by means of mass spectrometry, whether PACAP is present in different samples from the human reproductive organs.
- to investigate the effects of PACAP on the survival and signaling pathways in JAR (human choriocarcinoma) cytotrophoblast cells exposed to cellular stressors such as H_2O_2, $CoCl_2$, lipopolysaccharide and ethanol induced *in vitro* hypoxia and after cytostatic treatment.
- to investigate the effects of PACAP1-38 and PACAP6-38 on the cell viability of HIPEC65 (human invasive proliferative extravillous cytotrophoblast) cells exposed to cytostatic treatment and on invasion-proliferation.

Materials and Methods

Occurrence of PACAP in the human placenta

Human placentas were collected from aborted (9 weeks, n=7) and full-term placentas (n=6). Samples were taken from the chorionic villi (fetal side) and from the decidua (maternal side). In case of full-term births, samples from the umbilical cord were also collected (n=6). Tissue samples were weighed and homogenized in ice-cold distilled water. The homogenate was centrifuged (12,000 rpm, 4 °C, 30 min) and the supernatant was further processed for radioimmunoassay (RIA) analysis of PACAP38 and PACAP27 content, as previously described.

Radioimmunoassay

Follicular fluid was collected as described above. The samples were weighed and centrifuged (12,000 rpm, 4ºC, 30 min), and supernatant was further processed for RIA

analysis of PACAP38-like immunoreactivity, as previously described. Briefly, the conditions were as follows: antiserum: PACAP38: "88111-3" (working dilution 1:10,000), tracer: mono-^{125}I-labelled ovine PACAP24-38 prepared in our laboratory (5,000 cpm/tube), standard: ovine PACAP38 were used as a RIA standard ranging from 0 to 1000 fmol/ml, buffer: the assay was prepared in 1 ml 0.05 mol/l (pH 7.4) phosphate buffer containing 0.1 mol/l sodium chloride, 0.25 % (w/v) BSA and 0.05 % (w/v) sodium azide. Incubation time: 48-72 h incubation at 4 °C. Separation solution: charcoal/dextran/milk powder (10:1:0.5 g in 100 ml distilled water).

Mass spectrometry analyses

Human biological samples were collected according to a protocol approved by the institutional ethic committee (3117/2008, 3610/2009), during ophthalmological surgery or routine gynecological examinations, without extra intervention. Patients provided written approval of the sample collection in all cases. The samples were further processed for mass spectrometry analysis based on modifications of earlier descriptions. The peptidase inhibitor aprotinin was added to all samples (30 µl/ml), except for the cervico-vaginal fluid, nasal fluid and saliva on filter paper.

Follicular fluid was collected from female volunteers (age between 20-35, n=40) by follicular puncture after controlled ovarian hyperstimulation during the in vitro fertilization procedure.

Amniotic fluid specimens were collected at the 16th week of gestation from volunteering pregnant females undergoing amniocentesis as a prenatal diagnostic tool due to maternal age (age over 35 years, n=25).

Cervico-vaginal fluid samples were collected from female volunteers in the progesterone phase of the cycle (age between 25-35, n=10) by application of sterile filter paper strips (Schirmer paper) during colposcopic examination. Human nasal fluid (age between 20-40, n=10) and saliva (age between 20-40, n=10) were also collected by application of sterile filter paper strips (Schirmer paper) from healthy volunteers.

Human aqueous humor was collected from volunteers (age between 60-85 n=10) during cataract surgery. A 100 µl of the follicular fluid sample was centrifuged at 10,000 rpm for

5 min, followed by addition of 10 µl 72% trichloroacetic acid and 100 µl H_2O_2 to 90 µl supernatant. The samples were centrifuged at 13,000 rpm for 10 min after precipitation. The amniotic fluid (200 µl) was centrifuged at 10,000 rpm for 5 min. The supernatant (100 µl) was acidified by 100 µl 1% trifluoroacetic acid (TFA) and then centrifuged at 13,000 rpm for 10 min.

The solutions from the above described samples were desalted and cleaned using 0.1% TFA solution with $ZipTip_{18}$ pipette tips (Millipore Kft., Hungary). The purified proteins and peptides were eluted directly onto the MALDI target plate (MTP 384 massive target T, Bruker Daltonics, Bremen, Germany) by 3 µl of acetonitrile/0.1 % TFA (50/50, v/v) solution by mixing 1 µl of a saturated matrix solution, prepared freshly every day by dissolving a-cyano-4-hydroxycinnamic acid in acetonitrile/0.1% TFA (1/2, v/v).

The cervico-vaginal fluid, human nasal fluid and saliva were dissolved by using 100 µl of acetonitrile-0.1% TFA (5/95, v/v) mixture in an ultrasonic bath at 5 min. The samples, including the human aqueous humor were loaded onto the target plate (MTP 384 massive target T, Bruker Daltonics, Bremen, Germany) directly by mixing 1-1 µl of each solution with the same volume of a saturated matrix solution, prepared freshly every day by dissolving a-cyano-4-hydroxycinnamic acid in acetonitrile/0.1% TFA (1/2, v/v).

Identification of PACAP38 was performed with matrix-assisted laser desorption/ionization tandem time–of-flight (MALDI TOF/TOF) mass spectrometry. Briefly, the mass spectrometer used in this work was an Autoflex II TOF/TOF (Bruker Daltonics) operated in the linear detector for MALDI TOF or LIFT mode for high energy collision induced decay MALDI TOF/TOF with an automated mode using the FlexControl software. The ions were accelerated under delayed extraction conditions (200 ns) in positive ion mode with an acceleration voltage of 20.00 kV. The instrument uses a 337 nm pulsed nitrogen laser, model MNL-205MC (LTB Lasertechnik Berlin GmbH., Berlen, Germany). External calibration was performed in each case using Bruker Peptide Calibration Standard (#206195 Peptide Calibration Standard, Bruker Daltonics, Bremen, Germany). Protein masses were acquired with a range of m/z 1,000 to m/z 10,000. Each spectrum was proceded by accumulating data from 200 consecutive laser shots for standard PACAP38 solution and 1,000 for amniotic-, follicular-, cervico-vaginal-, nasal fluid, saliva and aqueous humor samples. The Bruker FlexControl 2.4

software was used to operate the instrument and the Bruker Flexanalysis 2.4 software for spectrum evaluation.

Cell culture

JAR human choriocarcinoma cells were from the American Type Culture Collection (Wesel, Germany). The cells were maintained as monolayer adherent culture in RPMI-1640 medium containing 1% antibiotic-antimycotic solution, 1% pyruvate solution (100 nM) and 10% fetal calf serum (FCS, Sigma, Hungary) in a humified 95% air and 5% CO_2 atmosphere at 37 ºC. HIPEC 65 cells are extravillous cytotrophoblast cells, they were cultured in DMEM medium.

Cell viability test

JAR CELLS

Viability of JAR cells was determined by colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Hungary). The assay is based on the reduction of MTT into a blue formazan dye by functional mitochondria of viable cells. The cells were seeded into 96-well plates at a density of 10^4 cell/well and cultured overnight before the experiment.

A., Effect of PACAP38 on JAR sells in case of several toxic agents

JAR cells were randomly assigned to one of the 12 experimental groups. 1.) a.) Control group of cells were incubated in RPMI (normal medium, Sigma, Hungary) without treatment. 1.) b.) 100 nM PACAP1-38, 2.) a.) 0.45 mM H_2O_2, b.) pretreatment with100 nM PACAP38 followed by 0.45 mM H_2O_2, 3.) a.) 0.9 mM H_2O_2, b.) 100 nM PACAP38pretreatment followed by 0.9 mM H_2O_2, 4.) a.) 75 µM $CoCl_2$, b.) pretreatment with 100 nM PACAP38 and followed by 75 µM $CoCl_2$, 5.) a.) 1 µg/ml lipopoliszacharid (LPS), b.) 100 nM PACAP38pretreatment, followed by 1 µg/ml lipopoliszacharid, 6.) a.) 200 mM etanol, b.) pretreatment with 100 nM PACAP38 followed by 200 mM etanol.

Control cells were not treated previously with PACAP38. Other groups of cells were pretreated with PACAP38 for 1 hour, and then exposed to different toxic agents for 9 hours.

B., Effect of PACAP38 on JAR cells exposed to chemotherapeutic agent

JAR cells were randomly assigned to one of the 24 experimental groups. Control group of cells were incubated in RPMI (normal medium, Sigma, Hungary) without treatment (group 1). Other groups were incubated in RPMI medium containing PACAP1-38 in the presence or absence of the PACAP receptor antagonist and/or methotrexate (MTX) (groups 5-12 and 17-24). Detailed treatment groups: (2): 100 nM PACAP1-38; (3) 1 μM PACAP6-38; (4) 100 nM PACAP1-38 and 1 μM PACAP6-38; (5) 10 μM MTX; (6) 100 nM PACAP1-38 pretreatment for 1 hour followed by 10 μM MTX; (7) pretreatment with 1 μM PACAP6-38 for 1 hour followed by 10 μM MTX; (8) 100 nM PACAP1-38 and 1 μM PACAP6-38 as pretreatment for 1 hour followed by 10 μM MTX; (9) 100 μM MTX; (10) 100 nM PACAP1-38 as pretreatment for 1 hour followed by 100 μM MTX; (11) 1 μM PACAP6-38 for 1 hour followed by 100 μM methotrexate; (12) 100 nM PACAP1-38 and 1 μM PACAP6-38 for 1 hour followed by 100 μM MTX; (13) control group; (14) 1 nM PACAP1-38; (15) 100 nM PACAP6-38; (16) 1 nM PACAP1-38 and 100 nM PACAP6-38; (17) 10 μM MTX; (18) 1 nM PACAP1-38 for 1 hour followed by 10 μM MTX; (19) 1 μM PACAP6-38 for 1 hour followed by 10 μM MTX; (20) 1 nM PACAP1-38 and 100 nM PACAP6-38 for 1 hour followed by 10 μM MTX; (21) 100 μM MTX; (22) pretreatment with 1 nM PACAP1-38 for 1 hour followed by 100 μM MTX; (23) 100 nM PACAP6-38 for 1 hour followed by 100 μM MTX; (24) 1 nM PACAP1-38 and 100 nM PACAP6-38 for 1 hour followed by 100 μM MTX. PACAP1-38 was synthetized as previously described. Cells were exposed to the mentioned concentrations of chemicals for 48 hrs. Evaluation of cell survival was performed immediately after termination of treatments. Experiments were repeated six times. The selected dose of MTX was based on our preliminary studies showing significant cell death with this concentration in JAR cells. The dose of PACAP was based on in vitro studies using PACAP as a protective agent against various toxic effects, where the range of 10-100 nM was proven to be the

most effective. After 48 hours of treatment, the medium was removed, and fresh RPMI/FCS containing 0.5% of the water-soluble yellow mitochondrial dye MTT was added.

Cells were then incubated for 3 hours at 37 ºC in an atmosphere of 5 % CO2. After 3 hours of incubation the medium was removed, and the water-insoluble blue formazan dye formed stochiometrically from MTT+ was solubilized by acidic isopropanol (Sigma, Hungary). Optical densities were determined by an ELISA reader (Anthos Labtech 2010; Vienna, Austria) at the wavelength of 550 nm representing the values in arbitrary unit (AU). All experiments were run at least four parallels and repeated six times. Results are expressed as percentage of control values.

HIPEC CELLS

Viability of HIPEC cells was determined by colorimetric WST-1 assay. The cells were seeded into 96-well plates at a density of 10^4 cell/well and cultured in DMEM medium overnight before the experiment.

JAR cells were randomly assigned to one of the 24 experimental groups. 1.) Control group of cells were incubated in DMEM medium. 2.) 100 nM PACAP1-38 3.) 1 µM PACAP6-38, 4.) 10 µM MTX, 5.) pretreatment with 100 nM PACAP1-38 followed by 10 µM MTX, 6.) pretreated with 1 µM PACAP6-38 followed by 10 µM MTX.

Control cells were not treated previously with PACAP1-38 or PACAP6-38, but in taht case, where the cells were exposed to cytostaticum for 48 hours, they were pretreated with PACAP1-38 or PACAP6-38 for 1 hour. After 48 hours of treatment, the medium was removed, and fresh DMEM/FCS containing 0.5% of the water-soluble WST-1 sollution was added. Cells were then incubated for 3 hours at 37 ºC in an atmosphere of 5 % CO2. After 3 hours 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 arbitrary unit (AU). All experiments were run at least four parallels and repeated six times. Results are expressed as percentage of control values.

Invasion and proliferation assay

Cells invasion assay was permormed in an invasion chamber based on the Boyden chamber principle. Each insert is fitted with an 8-µm pore size polycarbonate membrane. The membrane filter of inserts were coated with rat tail collagen I (5 µg/cm^2). After the inserts were washed in DMEM medium and incubated for 30 minutes at room temperature. For each well, we added 5 x 10^5 HIPEC cells in 100 µL of serum-free media to the upper compartment of the transwell chambers. We also added 5 x 10^5 HIPEC cells respactively, in 400 µL of serum-free media to the lower chamber. We incubated the cells for 72 hours at 37 ºC in an atmosphere of 5 % CO2 incubator. After incubation, we discard the supernatant and stain the viable cells that invaded collagen with 400 µL of crystal violet cell stain during 20 minutes at room temperature. Then we rinsed the cells several times in water and while the insert was still moist, we removed non-invading cells from the insert using a cotton-tipped swab. After we transfered the stained insert to a clean well containing 200 µL of a solution of H_2O:ethanol:acetic acid (49:50:1) for 20 minutes at room temperature. Then we transfered 100 µL of the dye mixture to a 96-well microtiter plate for colorimetric measurement at 560 nm. Express data as the percentage of invading cells relative to the untreated (control: cultured alone) cells.

Western-blot

The JAR cells were seeded into 6-well plates at the starting density of 5 x 10^5 cells/ml.

A.)Effect of PACAP1-38 and PACAP6-38 on JAR cells

JAR cells were randomly assigned to one of the 4 experimental groups. 1.) control group, 2.) 1 hour treatment with 100 nM PACAP38, 3.) 1 hour treament with 1 µM PACAP6-38, 4.) 1 treamtnet with 100 nM PACAP38 and 1 µM PACAP6-38.

B.) Effect of PACAP1-38 on JAR cells exposed to *H_2O_2*

JAR cells were randomly assigned to one of the 4 experimental groups. 1.) control group, 2.) cells were exposed to 0.6 mM H_2O_2 for 9 hours, 3.) cells were exposed to 0.6 mM

H_2O_2 for 9 hours following to 1 hour pretreatment with 100 nM PACAP1, 4.) 1 hour treament with 100 nM PACAP38.

The control groups remained without PACAP1-38 exposition. After exposition the cells were harvested in ice-cold lysis buffer containing the phosphatase inhibitor Na_3VO_4 (0.5 mM) and protease inhibitor cocktail in PBS. The cells were disrupted by sonication and centrifuged at 10,000 x g for 15 min. Protein contents of the supernatant fractions were measured using the BioRad assay and the protein concentration of the samples were equalized. Even loads (10 µg each) of cell extracts were separated on 8, 12 or 15 % sodium dodecyl sulphate polyacrylamide gels, transferred to nitrocellulose membrane, and were probed with rabbit polyclonal or mouse monoclonal IgGs (Cell Signaling Technology Inc, Beverly, Massachusetts, USA) against the following antigens: protein kinase B (Akt), phospho-protein kinase B, (P-Akt, Ser473), phospho-p44/42 mitogen activated protein kinase/extracellular signal regulated kinase-1/2, (P-ERK1/2, Thr202/Tyr204), phospho-stress activated protein kinase/c-Jun N-terminal kinase (P-JNK, Thr183/Thr185), phospho-p38-mitogen activated protein kinase (P-p38-MAPK, Thr180/Tyr182). Horseradish peroxidase labelled anti-rabbit and anti-mouse IgG were used as secondary antibodies. Protein bands were visualized by the ECL chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Results were demonstrated by representative blots of at least three experiments.

Flow Cytometry

The JAR cells were seeded into 6-well plates at the starting density of 10^5 cells/2ml/well. JAR cells were assigned to 6 experimental groups: 1.) control group without treatment, 2.) 100 nM PACAP1-38, 3.) 10 µM MTX, 4.) 100 µM MTX, 5.) 100 nM PACAP1-38 and 10 µM MTX, 6.) 100 nM PACAP1-38 and 100 µM MTX.

The HIPEC cells were also seeded into 6-well plates at the starting density of 10^5 cells/2ml/well. HIPEC cells were assigned to 6 experimental groups: 1.) control group

without treatment, 2.) 100 nM PACAP1-38, 3.) 1 μM PACAP6-38, 4.) 10 μM MTX, 5.) 100 nM PACAP1-38 and 10 μM MTX, 6.) 1 μM PACAP6-38 and 10 μM MTX.

Annexin V and propidium iodide staining were used to detect apoptosis and necrosis in JAR cells. In the early stages of apoptosis, cells typically have an intact cell membrane. Thus, they are not stained with propidium iodide, whereas externalization of phophatidylserine can be detected by annexin V. In the late phase of apoptosis, cells are stained with both dyes. Ratio of apoptosis was evaluated after double staining with fluorescein isothiocyanate (FITC)-labeled annexin V (BD Biosciences, Hungary) and propidium iodide (BD Biosciences, Hungary) using flow cytometry. First, the medium was discarded and the wells were washed twice with isotonic sodium chloride solution. JAR cells were removed from the plates using a mixture of 0.25 % trypsin (Sigma, Hungary), 0.2 % ethylene-diamin tetra-acetate (EDTA; Serva, Hungary), 0.296 % sodium citrate, 0.6 % sodium chloride in distilled water. This medium was applied for 15 min at 37 ^{o}C. Removed JAR cells were washed twice in cold PBS and were resuspended in binding buffer containing 10 mM Hepes NaOH, pH 7.4, 140 mM NaCl, 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 buffer (10^5 cells) was transferred into 5 ml round-bottom polystyrene tubes. Cells were incubated for 15 min with fluorescein isothiocyanate (FITC) conjugated annexin V molecules and propidium iodide (PI). After this period of incubation, 400 μl of annexin-binding buffer (BD Biosciences, Hungary) 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, according to previous descriptions. 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.

Statistical analysis

Data were presented as mean ± S.E.M. from three independent experiments, analyzed with one-way ANOVA followed by Neumann-Keul's *post hoc* analysis, and were considered significant at $P<0.05$.

Results

Occurrence of PACAP in the human placenta

The presence of both PACAP38 and PACAP27 could be detected in the placentas using RIA. PACAP38 had significantly higher concentrations in every part of the full-term placenta, with a slightly higher concentration in the chorionic villi. The level of both peptides was very low in the umbilical cord, but concentration of PACAP38 was significantly higher than that of PACAP27 also in the umbilical cord. Both forms of PACAP could be measured in both the maternal and fetal parts of the placenta already at 9 weeks (in the aborted placentas). The levels of PACAP27 showed a significant increase on the maternal side toward the terminus, but not on the fetal side. Levels of PACAP38 significantly increased in both parts.

Mass spectrometry analyses

The sample preparation procedure optimized for each biological fluid was suitable for measuring and identifying low molecular weight peptides by mass spectrometry. Based on our previous and current results, sensitive and reproducible identification of PACAP38 can be carried out using linear MALDI TOF MS. The characteristic peak of PACAP38 is 4534.6 Da, as verified in the PACAP standard solutions. Mass spectrometry analysis revealed that PACAP38 was present in all of the stimulated ovarian follicular fluid samples. The collision induced decay MALDI TOF/TOF fragmentation of PACAP38 standard yielded mainly y fragment ions of the PACAP38 parent ion (4535 kDa). On the contrary, mass spectrometry results could not prove the presence of the

unmodified PACAP38 in any of the amniotic fluid, cervico-vaginal fluid, nasal fluid, human saliva or aqueous humor samples.

Effect of PACAP on choriocarcinoma cells

A., Effect of PACAP38 on JAR sells in case of several toxic agents

PACAP treatment alone did not influence the survival rate of JAR cells. Surprisingly, no protective effect was seen when cells were exposed to different concentrations of H_2O_2 after incubation with PACAP. On the contrary, cells exposed both to oxidative stress and PACAP showed a decreased survival rate, which was more robust in cells exposed to higher H_2O_2 concentration. A similar effect was observed in cells undergoing chemically induced hypoxia. PACAP treatment significantly reduced survival rate of cells exposed to $CoCl_2$. PACAP treatment did not have significant influence on the survival of cells exposed to LPS or ethanol.

B., Effect of PACAP38 on JAR cells exposed to cytostatic agent

To establish whether PACAP influences viability of cells exposed to MTX, mitochondrial viability in JAR cells was measured by MTT assay. Administration of MTX alone resulted in manifest decrease in cell viability compared to control values, with no difference between the two applied doses. Treatment with PACAP1-38 and PACAP6-38 alone did not alter cell viability. Co-incubation with PACAP1-38 and/or PACAP6-38 did not significantly affect cell viability compared to that of the control MTX-treated cells. It was observed with both PACAP1-38 and PACAP6-38 doses.

Effects of PACAP on the cell survival of HIPEC cells

PACAP1-38 or PACAP6-38 treatment alone did not influence the survival rate of HIPEC cells. Co-incubation with PACAP1-38 and/or PACAP6-38 did not affect cell viability compared to that of the control MTX-treated cells.

Effect of PACAP on the invasion and proliferation of HIPEC cellsThe invasion of the HIPEC cells was decreased by PACAP1-38, but it increased the proliferation. PACAP6-38 did not influence the invasion, but it significantly increased the proliferation.

Effect of PACAP on the cell signalling pathways of JAR cells

A.) Effect of PACAP1-38 and PACAP6-38 on Jar cells

ERK1/2

The active, phosphorylated form of ERK1/2 could be detected in control cells. The phosphorylation of ERK1/2 showed a slight increase after the administration of PACAP1-38. Interestingly, a similar, but more expressed increase in intensity was observed after the administration of a 10-fold higher concentration of PACAP6-38, and a similar increase was observed when both PACAP1-38 and PACAP6-38 were present.

p38 MAPK

Activation of p38 MAPK showed the opposite pattern as that of ERK1/2. Phosphorylation of p38 MAPK decreased in the presence of PACAP1-38, which was even more expressed when cells were incubated with PACAP6-38. In case of co-application of PACAP1-38 and PACAP6-38, the phosphorylation of p38 MAPK showed a marked further decrease.

JNK/SAPK

Phosphorylation of JNK/SAPK was only slightly detected in control cells with no treatments. Phosphorylation of JNK/SAPK showed an increase in the presence of PACAP1-38 alone, a stronger increase was observed in the presence of PACAP6-38 alone and an even more expressed increase was seen when both PACAP1-38 and PACAP6-38 were present. Our results show that PACAP6-38 did not behave as an

antagonist, but it had similar effects to PACAP1-38 on the MAP kinases in choriocarcinoma cells.

B.) Effect of PACAP1-38 on JAR cells exposed to H_2O_2

Akt

Initially, the total amount of Akt was examined, and no differences were found between any of the groups. The active form of Akt (phospho-Akt) was present in control cells, the amount of which was considerably lower if cells were exposed to PACAP1-38. The same effect was observed in case of oxidative stress alone. When cells were exposed to oxidative stress and PACAP, the activation of this well known protective pathway was extremely suppressed.

ERK1/2

Phosphorylation of ERK1/2 showed a slight increase after the administration of PACAP1-38, but the decreased phosphorylation of ERK1/2 after H_2O_2 was further decreased after simultaneous treatment with PACAP.

p38 MAPK

Activation of p38-MAPK showed the same pattern as that of Akt. Phosphorylation of p38 decreased in the presence of PACAP alone. In case of co-application of H_2O_2 and PACAP1-38, the phosphorylation of p38 showed a marked further decrease.

Investigation of apoptosis in JAR cells

Using this method, we found that the control group had more than 93% of intact, living cells and only less than 7% of cells in the apoptosis. PACAP1-38 administration alone caused no changes in the percentage of living, necrotic and apoptotic cells compared to control values. An increase of apoptotic and necrotic cells was observed in the MTX-treated group with a lower number of living cells. PACAP and MTX co-incubation

caused no significant changes in the percentage of living cells, necrotic and apoptotic cells compared to the cells treated with MTX alone.

Investigation of apoptosis in JAR cells

PACAP1-38 or PACAP6-38 administration alone caused no changes in the percentage of living, necrotic and apoptotic cells compared to control values. We found an increase of apoptotic and necrotic cells in the MTX-treated group. PACAP1-38 or PACAP6-38 and MTX co-incubation caused no significant changes in the percentage of living cells, necrotic and apoptotic cells compared to the cells treated with MTX alone.

Discussion

Occurrence of PACAP in the human placenta

In the present study we found high levels of PACAP38 and lower levels of PACAP27 in different parts of the full-term human placenta. The difference between the two forms of the peptide found in our study is in accordance with previous observations in the placenta. The concentration range of PACAP measured in the placenta is close to the range found in the brain, where the levels of PACAP are the highest. Our results also show that PACAP content increases during pregnancy in the placenta, both in the maternal and the fetal side. This is in accordance with earlier studies showing that PACAP mRNA and PAC1 receptor mRNA increases as gestation advances. In the rat, PACAP and PAC1 receptor mRNAs are expressed in decidual cells, chorionic vessels and stromal cells of the villi, with decreased expression mRNAs in the decidua and strong upregulation in the chorionic villi with advanced gestational age. A similar temporal and spatial distribution has been described in the human placenta. Our results also show that placental PACAP immunoreactivity is much stronger in later gestational age, although we found it in all parts of the placenta.

The high concentrations of PACAP imply that the peptide plays an important role in the placenta, which is further supported by the decreased fertility in mice lacking PACAP or PAC1 receptors, most probably due to impaired implantation in PACAP knockout mice.

Also, the expression of PACAP shows alteration during pregnancy in the cervix and spinal cord and a temporal relationship has been demonstrated between endometrial PACAP expression and proliferation of the deciduoma during decidualization and gestation.

Mass spectrometry analyses

In this study, we found the presence of PACAP38 in ovarian follicular fluid, obtained from forty stimulated female patients. Here, all of the samples contained PACAP38. The follicular fluid serves as a culture medium for the developing oocyte, and it is important for the morphological and functional integrity for the germ cell development. PACAP has been shown to have several functions in follicular development. PACAP is expressed stage-specifically in granulosa cells of large mature follicles before ovulation, but weaker expression has also been shown in the wall of immature antral and pre-antral follicles. PACAP receptors have also been demonstrated in developing follicles. Both PACAP and PAC1 receptor have been demonstrated in the corpus luteum. The PACAP found in the follicular fluid may derive from granulose and/or theca cells. The peptide is thought to play a role in primordial germ cell proliferation, cyclic recruitement of immature follicles, follicular apoptosis, meiotic maturation of the oocytes and ovarian hormone and enzyme production. The finding that PACAP occurs in all of the ovarian follicular fluid samples indicates an important biological role for PACAP in this culture medium for the developing oocytes, the exact determination of which awaits further investigation.

In amniotic-, cervico-vaginal-, nasal fluid, saliva and in the aqueous humor we could not detect unmodified PACAP38 by mass spectrometry. This could possibly be due either to the complete lack of PACAP in the samples, or levels below detection limit. The rapid digestion can be excluded as explanation, since we added peptidase inhibitor to the samples. It is also conceivable that in some samples PACAP, or its fagments, would occur in a modified form, the determination of which would require further experiments. Another possibility is that the different biological fluids exhibit different quali- and quantitative composition. This could also be reflected in suppression effects, which is often observed in MALDI spectra of complex protein-peptide mixtures. The lack of PACAP found in the present study does not necessarily exclude the possibility that

PACAP could be found under pathological circumstances. However, based on the present findings, we can conclude that under physiological circumstances PACAP, in its original form, is not present in the amniotic-, cervico-, vaginal-, nasal fluid, saliva or in the aqueous humor.

Effect of PACAP on the cell survival and signalling pathways of JAR cells

Effect of PACAP1-38 on JAR cells exposed to different harmful stimuli

The present study described various effects of PACAP1-38 on survival and signal transduction in JAR choriocarcinoma cells. It was found that 1 hour pretreatment with PACAP1-38 did not significantly influence the survival of trophoblast cells. However, the survival rate of cells exposed to oxidative stress and in vitro hypoxia showed a significant further decrease in PACAP-treated cells, implying that PACAP1-38 sensitizes the cells to these stressors. This was not observed in case of LPS or ethanol and MTX treatment.

Effect of PACAP1-38 or PACAP6-38 on JAR cells

Our present data show that PACAP1-38 treatment alone did not influence the phosphorilation of ERK1/2 and p38MAPK. In case of ERK1/2, PACAP6-38 treatment significantly increased the activation of this pathway and the same effect was observed following combination treatment. Opposite effect was found in case of p38MAPK, where PACAP1-38 and PACAP6-38 combination treatment lead to a significant decrease in phosphorylation. In JNK/SAPK pathway, PACAP1-38 treatment significantly increased avtivation which was also found following PACAP6-38 treatment. More signifacant phosphorylation was found after combination treatment.

Effects of PACAP on cell survival on JAR cells following MTX treatment

We found that neither PACAP1-38 nor PACAP6-38 altered the survival of the cell line. MTX alone decreased survival by approximately 50%. The same results could be obtained in case of combination treatment of MTX and PACAP1-38 or PACAP6-38. It suggests that the peptide or its antagonist does not have either protective or toxic effect.

Effects of PACAP of cell signaling pathways of JAR cells exposed to toxic agents

Our results showed that PACAP38 did not alter phosphorylation of ERK1/2 and p38MAPK, but decreased Akt activation. Combination of H_2O_2 and PACAP38 resulted in a decreased phosphorylation of these pathways. In case of Akt PACAP38 alone could also decrease activation, so the decreased activation found in combination treatment is an additive affect. PACAP38 alone could not decrease the activation of ERK1/2 and p38MAPK, suggesting that the decrease found in combination treatment is due to a potenciating effect.

Effects of PACAP on HIPEC cells

Under the same circumstanses PACAP1-38 and PACAP6-38 had the same effect on immortalized extravillous cytotrophoblast cells as in tumorous choriocarcinoma cells. Survival of HIPEC cells was not influenced by PACAP1-38 or PACAP6-38 treatment alone, while MTX significantly decreased survival. These toxic effects of MTX could not be prevented or potenciated by PACAP1-38 or PACAP6-38. Invasion and proferation of the HIPEC cells was previously observed with the administration of PPARγ. In contrast to PACAP1-38 and PACAP6-38 PPARγ did not increase proliferation but PPARγ and PACAP1-38 decreased invasion, which implies that these compounds modulate cytotrophoblast invasion in the placenta. It still remains an interesting question why PACAP6-38 does not act as antagonist on cytotrophoblast cells. We plan to perform PCR experiments to explore a potencial new type of receptor in placenta.

Different, non-protecting effects of PACAP on various cell lines

PACAP is generally described as a cytoprotective agent. This effect was first shown in neuronal cells exposed to different toxic agents. Among others, PACAP exerts neuroprotection in vitro against toxicity induced by glutamate, ethanol, ceramide, 6-hydroxydopamine, HIV envelope protein, rotenone, hypoxia and nitric oxide. The protective effects of PACAP in cerebellar granule cells have also been shown against oxidative stress. Similar degree of protection against oxidative stress has been found in other cell types, such as endothelial cells and cardiomyocytes. In spite of the vast

majority of data showing the neuroprotective effects of PACAP, this effect may be related to cell types, possibly expressing different splice variants of the receptors. No protective effect has been described in cultured rat myenteric neurons.

Similarly, different effects can be observed depending on the mechanism of toxicity, as it has been described in cerebellar granule cells against beta-amyloid (25-35)-induced apoptosis, where PACAP failed to protect against cell death. Based on our present data, PACAP does not enhance survival of trophoblast cells. PACAP alone did not influence survival rate, however, it enhanced the effects of oxidative stress and in vitro hypoxia. The reason for this sensitizing effect is not known. A recent study has shown that the PACAP-induced cAMP formation was significantly reduced in neuronal cells and astrocytes exposed to in vitro hypoxia.

The effects of PACAP on the survival of non-neuronal tumor cells are also divergent. Most studies show that PACAP enhances survival not only of normal cell types but it stimulates proliferation and promotes survival of cancerous cells. This has been shown in colonic tumor cells, lung cancer cells and a prostate cancer cell line. In contrast, PACAP suppresses the growth of myeloma cell line, a myeloid leukemic cell line and PACAP KO mice develop colorectal tumors more frequently. Our present results show that PACAP suppresses the survival of choriocarcinoma cells exposed to H_2O_2 toxicity and chemically induced hypoxia. Whether these effects are specific to trophoblast cells, or show tumor-dependent effects (choriocarcinoma), requires further investigation.

PACAP mediated signal transudction pathways

PACAP has been shown to influence multiple signal transduction pathways. The involvement of various members of the MAPK family in the PACAP-mediated actions on cell survival has been shown earlier. PACAP stimulates the production of the generally anti-apoptotic ERK1/2, and inhibits the production of the generally pro-apoptotic JNK1/2 and p38 MAPK. PACAP has also been shown to counteract the ischemia-, oxidative stress- or glutamate-induced changes in these signaling molecules. The present results show that PACAP acts partly differently on trophoblast cells. Although the phorphorylation of ERK1/2 was slightly increased after PACAP treatment alone, the decreased phosphorylation of ERK1/2 after H_2O_2 was further decreased after

simultaneous treatment with the peptide. The observed changes in p38 MAPK activity were in accordance with previous findings showing that the oxidative stress-induced elevations can be reduced by PACAP. JNK was also activated by the PACAP alone, but the H_2O_2 toxicity-induced elevation was reduced. In the present study a decrease in cell survival could be observed following oxidative stress or hypoxia if PACAP1-38 pretreatment was applied. Based on Western-blot results, the effects of PACAP were divergent. Attenuation of protective mechanisms (ERK-1/2 activation, Akt and GSK-3β phosphorylation) was evident, but PACAP also attenuated some of the pro-apoptotic pathways. Concerning SAPK/JNK and p38 MAPK, members of the MAPK family which can act either as proapoptotic or anti-apoptotic mediators in different stages, the same inactivation occurred as in case of protective factors. The overall effect seems to be a sensitizing effect in almost all examined pathways when oxidative stress was applied, which may explain the enhancing effect of PACAP on cell death. PACAP signaling has been shown to be cell-specific depending on the expressed receptor splice variant, PACAP concentration and other factors. Based on these observations, PACAP is suggested to promote adaptation to stress and coordinate diverse environmental signals influencing cell survival under different circumstances both during developing and in mature tissues. Our present results should lead to further experiments on trophoblast cells to elucidate the mechanism and the possible physiological and pathophysiological significance of PACAP-mediated signaling in trophoblast cells and choriocarcinoma.

Summary of new findigs

- We detected both PACAP38 and PACAP27 in the human full-term placenta and in 9 weeks abortions. Levels of PACAP38 significantly increased toward the terminus.
- We detected PACAP38 in the human follicular fluid.
- PACAP1-38 pretreatment significantly decreased the cell viability on JAR cells exposed to oxidative stress or hypoxia.
- Combination of PACAP1-38 and PACAP6-38 increased the phosphorylation of ERK1/2 és JNK/SAPK signal transduction pathways, but it decreased the activation of p38MAPK.

- Neither PACAP1-38, nor PACAP6-38, nor combined application had antiapoptotic effect on JAR cells against MTX treatment.
- PACAP1-38 and H_2O_2 together decreased the activation of ERK1/2 and p38MAPK in JAR cells.
- Neither PACAP1-38, nor PACAP6-38, nor combined application had antiapoptotic effect on HIPEC65 cells against MTX treatment.
- PACAP1-38 decreased the invasion but increased the proliferation of HIPEC65 cells. PACAP6-38 significantly increased the proliferation of HIPEC cells.

Publications related to the thesis:

Brubel R, Reglődi D, Jámbor É, Koppán M, Várnagy Á, Bíró Zs, Kiss P, Gaál V, Matkovits A, Farkas J, Lubics A, Bódis J, Bay Cs, Veszprémi B, Tamás A, Németh J, Márk L. Investigation of pituitary adenylate cyclase activating polypeptide in human gynecological and other biological fluids by using MALDI TOF mass spectrometry. J Mass Spectr 2011;46: 189-94 (IF: 3.411)

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Impact factor of all publications (abstracts not included): **23,194**