

Investigations on PACAP and its receptor in human milk, different milk-based samples, mammary gland, and cell cultures

PhD thesis

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INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptors

PACAP is a 38-amino acid C-terminally alpha-amidated peptide, which was isolated from ovine hypothalamus by Arimura and his coworkers in 1989. PACAP is named after its ability to stimulate adenylate cyclase activity and consequential cyclic adenosine mono-phosphate (cAMP) production in cultured pituitary cells. It is structurally most similar to vasoactive intestinal polypeptide (VIP). PACAP is member of the secretin/glucagon/growth hormone releasing hormone (GHRH) peptide family. The N-terminal domain of PACAP is evolutionary strongly preserved demonstrating the biological importance of the peptide. Shorter peptides arise during its degradation, which act as antagonists. Among these, PACAP6-38 is the most frequently used form in experimental studies.

The highest concentrations of PACAP occur in the hypothalamus, but it can be found in the cerebral cortex, mesencephalon, brainstem, amygdala, basal ganglia, hippocampus, pineal gland, cerebellum and pons as well. PACAP-immunoreactive (PACAP-IR) fibers are shown in the peripheral nervous system. PACAP can also be detected outside the central nervous system, such as in the ovary, uterus, placenta, adrenal gland, parathyroid gland, mammary gland, and the islets of Langerhans in the pancreas.

Three different receptors of PACAP are known, such as PAC1, VPAC1, and VPAC2. PACAP receptors are expressed almost in all of the human organs, but the distribution of their subtypes is variable in different organ systems.

Besides healthy tissues, PACAP and its receptors can also be detected in different tumors.

Physiological effects of PACAP

PACAP plays role in several physiological processes, such as feeding behavior, thermoregulation, circadian rhythm, and sleep regulation. Its endocrinological effects are also proven, thus it regulates parathyroid function, stimulates catecholamine synthesis in the adrenal gland, as well as production of insulin in the pancreas. From the point of view of our investigations, the effect of PACAP on reproduction, cell survival, proliferation, and differentiation deserves closer attention.

PACAP stimulates interleukin (IL)-6 production in hypophyseal follicular cells, and thereby, it increases the secretion of growth hormone (GH), prolactin (PRL), follicle-stimulating hormone (FSH), and luteinizing hormone (LH). PACAP increases steroid synthesis in gonadal stromal cells, promotes oogenesis in women, and spermiogenesis in men. Human experiments prove the role of PACAP/VIP in erection. PACAP has effect on tubal motility and increases the probability of fertilization. According to *in vitro* experiments, PACAP treatment results in relaxation of uteroplacental arteries, and it is supposed to influence blood supply this means.

The neuroprotective effect of PACAP was confirmed by several *in vitro* and *in vivo* experiments. It provides protection in cerebral ischemia, neurodegenerative diseases, and animal model of brain trauma.

The antiapoptotic property of PACAP was proven by experiments on cerebellar granule and pituitary adenoma cells shortly after its discovery. However, this effect can be observed not only on neural cells, but even on other cell types, such as T-lymphocytes, preovulatory follicles, lung alveolar cells, cardiomyocytes, hemangioendothelioma and retinal pigment cells. This effect is elicited by increasing the expression of antiapoptotic proteins (Bcl-2, ERK1/2, Akt) and decreasing the activity of proapoptotic factors (caspase-3, p38 MAPK, JNK, Bad, Bax, HIF-1 α) and several heat shock proteins.

All of the facts mentioned above are consistent with the cytoprotective properties of PACAP, but it can elicit reverse effects on tumor cells. Thus, it is proapoptotic on myeloma and myeloid leukemia cells, while antiapoptotic on insulinoma, prostatic and colonic carcinoma cells.

PACAP can induce dose dependent differentiation in *in vitro* experiments. In low, subnanomolar concentrations it causes proliferation, while in high doses, it leads to differentiation. Taking some examples, it should be mentioned that PACAP treatment results in astrocytic differentiation on cortical precursor and neural stem cells, and neural differentiation on cerebellar granular cells, cortical precursors, dorsal ganglia of chicken, neuroblastoma and mice embryonic stem cells, and P12 pheochromocytoma cells.

In case of non neural cell lines, it inhibits the differentiation of osteoblasts and blocks the transformation of single layered primordial follicle to cubic cell follicle, and on the contrary, it stimulates the maturation of T-cells.

PACAP inhibits cell differentiation via cAMP on osteoblasts. Receptor activator of nuclear factor- κ B (RANK) and its ligand (RANKL) play an important role both in bone metabolism and mammary gland development. The epithelium of the RANK and RANKL KO mice does not undergo lobulo-alveolar differentiation, and these mice fail to lactate. Therefore, an evolutionary relationship between these two organs is suggested.

PACAP in body fluids

Our research group proved the presence of PACAP38 in the follicle fluid of the ovary by mass spectrometry, and tried to detect PACAP38 in human aqueous humor, amniotic, cervico-vaginal, nasal, and seminal fluid, as well as in saliva samples, but the PACAP38 characteristic peak did not appear in these samples.

We adapted the specific and sensitive PACAP-radioimmunoassay (RIA) method, which was originally developed for tissue measurements, to investigations on blood plasma and other body fluids. The mean PACAP38-immunoreactivity (PACAP38-IR) of young healthy women and men volunteers was determined, and the changes of PACAP38-IR in connection with different physiological parameters were followed.

PACAP38-IR in peripheral blood of newborn babies was similar to young healthy adults. It was significantly lower in the umbilical vessels than in the peripheral blood. Lower PACAP38-IRs were measured in the umbilical vein than in the artery. Based on these results, the fetal synthesis of PACAP was presumed.

The changes in PACAP38-IR were also investigated in overstimulated conditions of hypothalamus-pituitary-gonadal axis, such as pregnancy, delivery, and lactation. A significant elevation of PACAP38-IR was observed in blood plasma in the second and third trimester compared to the first trimester and non pregnant women, and likewise higher PACAP-IRs were detected in placentas from full term pregnancies than in placentas of first trimester abortions. A 70 % decrease in PACAP38-IR was measured at the time of delivery, which returned to normal level after 1-3 days. A moderate, but significant elevation was seen in plasma PACAP38-IR in the first six months of lactation compared to healthy volunteers. Although definitive conclusions can not be drawn, these measurements prove that endogen PACAP-level reacts sensitively to hormonal changes.

PACAP38-IR was detected in human milk, and its level was 5-20-fold higher than in the respective plasma samples. Our studies on milk from domestic animals were in accordance with these results, the highest PACAP38-IRs were measured in sheep and goat milk.

Detection of PACAP in milk turned our attention to the feasibility of further investigations on milk and milk based products, and the question arose whether PACAP has any regulatory role in proliferative and differentiation processes of the breast.

Bioactive compounds in milk and milk-based products

Peptides in milk

The most important peptides in human milk are oxytocin, relaxin, galanin, neurotensin, VIP, somatostatin, GHRH, cholecystokinin, melatonin, substance P and thyrotropin releasing hormone (TRH). Among these, the expression of galanin, SOM, VIP, TRH, GHRH, CCK, neurotensin, oxytocin and relaxin was investigated by RT-PCR in normal, lactating, and postlactating rat mammary glands. Except SOM, the mRNA of these peptides could not be detected in the samples, and consequently, an active accumulation of neuropeptides from the maternal circulation to milk was supposed.

VIP, which is closely related to PACAP, was discovered in 1970, but its presence in human milk came to light only a decade and a half later. Concentration of VIP is lower in extracted plasma than in human milk, and it changes during lactation. Significantly higher levels of VIP are measured in human colostrum than in mature milk. The concentration of VIP in plasma is increased by vagal stimulation during suckling, while this increase cannot be detected after vagotomy. Milk ejection is oxytocin-independent in some species, and it can be hypothesized that VIP plays role in this process.

Changes in composition of milk

Composition of human milk changes dynamically during lactation and between the beginning and the end of the same suckling event. Human colostrum is produced during the first 3 days of lactation. It contains motilin, gastrin, leptin and β -casomorphin-5 and -7 in significantly higher concentrations than mature human milk. Foremilk, which is produced at the beginning of suckling, contains significantly higher levels of endothelin-1, ghrelin, and cholesterol than

hindmilk, which is produced at the end of suckling, while the triglyceride, leptin, and retinol levels are lower in foremilk than in hindmilk. Similar atrial natriuretic peptide and granulocyte colony-stimulating factor (G-CSF) concentrations were measured in foremilk and hindmilk.

Differences in composition of human, bovine milk, and milk based products

Bovine milk contains significantly lower level of parathyroid hormone-like protein (PLP) than human milk, but their epidermal growth factor (EGF) contents are similar. Pasteurized, commercially available milk contains significantly lower level of transforming growth factor (TGF)- β 1 than fresh cow milk. Composition of human milk differs from cow milk based infant formulas, i.e. gonadotropin releasing hormone (GnRH), insulin-like growth factor (IGF)-1, substance P and calcitonin gene-related peptide (CGRP) cannot be detected in the infant formulas. In contrast, other peptides, such as PLP, parathyroid hormone-related protein (PTHrP), EGF, insulin, and ghrelin, can be measured in infant formulas. Interestingly, even higher levels of ghrelin and leptin have been observed in the infant formulas than in human milk.

PACAP and its receptors and other neuropeptides in breast tissues

Neuropeptides, such as calcitonin, gene related peptide (CGRP), substance P, VIP, peptide histidine isoleucine (PHI), neuropeptid Y (NPY), galanin, and tyrosine hydroxylase (TH) positive fibers can be identified in subepidermal nerve endings, around smooth muscle cells of the nipple, in connective tissue adjacent to the lactiferous ducts, and around alveoli.

Nerve fibers in the breast not only contain the aforementioned neuropeptides, but even PACAP-like immunoreactivity can be detected in these structures. Measurement of tissue PACAP concentration shows significantly higher PACAP-IR in lactating mammary glands than in non lactating ones. However, this difference cannot be detected by simple immunohistochemistry.

The expression PACAP receptor was shown on mammary epithelial cells derived from normal and tumor samples, as well as in lactating mammary glands.

Regulation of lactogenic differentiation in mammary glands

Mammary cells undergo differentiation and proliferation and start secreting under the effect of estrogen, progesterone, and prolactin (PRL) in pregnancy. During lactogenic differentiation, binding of PRL to its own receptor induces homodimerization, which results in cross-phosphorylation of both the Janus kinase (JAK) and the PRL receptor itself. The signal transducer and activator of transcription (STAT)-5, which is present in the cytosol in form of monomers, undergo dimerization and phosphorylation, then the dimers are translocated into the nucleus and bind to GAS region (TTCCNGGAA) starting the transcription of milk proteins (β -casein, whey acidic protein/WAP, β -lactoglobulin) and STAT5. Engagement of PRL to its receptor induces other important kinases, such as mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K). The IL2 induced phosphorylation of suppressor of cytokine signaling (SOCS)-3 leads to selective, MAPK independent STAT5 inhibition, which calls attention to STAT5 independent pathways.

Interestingly, neuropeptides can also directly influence the lactogenesis. The neuropeptide, galanin, which is synthesized, stored, and secreted by the lactotrophs in the pituitary gland, not only influences the secretion of PRL, but has effect on the mammary epithelial cells by stimulating alveolar morphogenesis.

Besides hormones and other peptides, differentiation is modulated by growth-, transcriptional- and angiogenic-factors, and cytokines. During mammary cells differentiation, a similar Th1/Th2 cytokine switch is observed as it is seen in the differentiation of T-helper (Th) cells, i.e. the expression of IL-12, interferon (INF)- γ , and tumor necrosis factor (TNF)- α decreases, while that of IL-4, IL-13, and IL-5 increases. It is well known, that besides STAT5, STAT6 plays an important role in the differentiation of mammary cells, while STAT3 is involved in the regulation of involution. IL-4 and IL-13 exert their effects via STAT6. The role of cytokines in mammary gland differentiation is supported by the observation that the differentiation and alveolar morphogenesis are attenuated in STAT6 and IL-4/IL-13 double KO mice during pregnancy.

Aims and investigations

Based on the above mentioned data, we were curious whether, similarly to other milk proteins, PACAP shows any changes during lactation; it is possible to detect PACAP-IR in commercial milk, powdered milk, and infant formulas; PAC1 receptor shows any difference

in tissue and cell samples deriving from different species and under different hormonal influences; and lastly, PACAP that is present in both in mammary tissues and milk has any influence on the physiologic changes taking place in the breast, such as differentiation of mammary epithelial cells.

- PACAP38-IR was measured by PACAP-RIA in human colostrum, transitional and mature milk samples, and its changes were followed during a longer breastfeeding period. PACAP38-IR was also measured in foremilk and hindmilk.
- We tried to determine the PACAP38-IR in pasteurized and powdered milk, and infant formulas. Qualitative mass spectrometry was applied to prove that PACAP measured in infant formulas is really equivalent with PACAP38, and not the result of cross reaction with other similar molecules.
- PAC1 expression of lactating and non lactating sheep udders, as well as normal human mammary gland was compared to each other. We wondered whether PAC1 expression is a constitutional feature of mammary epithelial cells, and therefore, besides analyzing the PAC1 expression of mouse mammary cells used in this study, some other human tumor and non tumor cell lines were also microscopically studied.
- The differentiation induced by PRL in mouse mammary epithelial cells was proven by occurrence of β -casein production, and the potential effect of PACAP treatment on this process was followed by detection of changes in β -casein expression and phosphorylation of STAT5-dependent and independent (p38 MAPK and Akt) pathways on Western blots.
- The effect of PACAP on secretion of cyto- and chemokines, growth and angiogenic factors on differentiated and non differentiated mouse mammary epithelial cells was followed by mouse cytokine and angiogenesis array.

MATERIALS AND METHODS

Collection of samples

Human milk samples

Five ml mature milk samples (from the fourteenth day to the end of lactation; n = 167) were obtained from healthy, non-smoking mothers with term infants. One ml of human colostrum (milk produced in the first three days of lactation; n = 11) was collected. Additionally, 5 ml transitional milk (milk produced from the fourth day to the fourteenth day of lactation; n = 8) as well as foremilk and hindmilk samples (milk collected prior to the beginning of suckling and at the end of suckling; n = 4) were obtained. Total milk yield was estimated. The lactating mothers filled out a written consent and a questionnaire about their breastfeeding, complementary feeding habits, and lifestyles. The necessary ethical approval of laboratory experiments was also provided (PTE KK3117).

The samples were collected into ice-cold polypropylene tubes without peptidase inhibitor and stored at -20 °C.

Cow milk, powdered milk, and infant formulas

Five ml unpasteurized, fresh bovine mature milk was collected during 1-8 months of postpartum period from Holstein Friesian and Hungarian Simmental mixed-bred dairy cows (n = 4; Nagybudmér, Baranya). Commercially available non-hypoantigenic (non HA) and hypoantigenic (HA) infant formula samples, as well as pasteurized and powdered cow milk samples were purchased. Pasteurized milk: Tolle 2.8 %; Milli UHT 3.5 %; Pilos 2.8 %; Mizo UHT 1.5 %. Powdered milk samples: Marvel non-fat powdered milk; NATURBIT non-fat powdered milk 1.5 %; Bedeco instant non-fat powdered milk; MIZO fat powdered milk. Infant formula samples: Beba Pro 1; Beba Pro HA; Nestlé Alprem, Milupa; Hipp Plus; Hipp Follow; Milumil 2 6 hó; Aptamil HA; Milumil HA Start Optima.

Tissue and cell samples

Lactating and non-lactating udder biopsy samples were taken from adult Hungarian Merino ewes (n = 4) by minimal invasive needles between the seventh and thirtieth days. Human mammary gland samples derived from patients with breast cancer. Normal, tumor free parts

of the excisions were subjected to microscopy (n = 5; mean age: 36.3 yrs). Ethical approval: PTE KK4304.

Besides mouse mammary gland derived HC11 cells, human tumor (MCF-7 and MDA-MB-468) and normal (HMEC) mammary cell lines were investigated. The MCF-7, MDA-MB-468, and HMEC cell lines were used in some other experiments, which are not discussed in the present study, and therefore, the exact description of their culture condition will be omitted (requirements of ATCC and Lonza were followed in connection with culturing these cell lines).

Radioimmunoassay

The PACAP38 antiserum (88111-3) was raised in rabbits with synthetic peptides conjugated to bovine thyroglobulin with carbodiimide, and an antibody with high specificity and sensitivity to the C-terminus of the peptide was gained (Arimura, Tulane University, New Orleans). The mono-¹²⁵I-radiolabelled PACAP38 antigen was produced in the Department of Pharmacology and Pharmacotherapy, University of Pécs.

The protein content of the milk samples was precipitated by acetic acid, and the precipitated proteins were removed by repeated centrifugations. Standards in duplicates (100 µl) and 200 µl from the unknown samples, 100 µl antiserum, and 100 µl (5000 cpm/tube) ¹²⁵I-radiolabelled antibody were added in polypropylene RIA-tubes, and this mixture was diluted in 1 ml assay buffer [0.05 mol/l (pH 7.4) phosphate buffer containing 0.1 mol/l NaCl, 0.25 % (w/v) BSA and 0.05 % (w/v) NaN₃]. Then the samples were mixed and incubated for 48-72 h at 4 °C. The antibody-bound peptide was separated from the free peptide by addition of 100 µl separating solution (10 g charcoal, 1 g dextran, and 0.5 g commercial fat-free milk powder in 100 ml distilled water). After centrifugation (1800 x g, 4 °C, 15 min), the tubes were gently decanted, and the radioactivity of the precipitate was measured in a gamma counter (Gamma type: NZ310). The PACAP38-IR of the unknown samples was read from the calibration curve.

Mass spectrometry

The same sample preparation method was applied for mass spectrometry as in the RIA measurements. Ten-fold dilutions were made from the whey fraction with 0.1 % trifluoro-

acetic acid (TFA). Then the solution was desalted and purified using ZIP-TIP18 pipette tips. One μ l of sinapinic acid (SA)-saturated solution in ethanol was layered on the MALDI target plate. Then the PACAP38 bound to the surface of the pipette tips was eluted by matrix solution [mixture of SA in 50 % acetonitril/0.1% TFA (1/2, v/v)], and it was also loaded to target plate. Calibration was performed in each case using Bruker Peptide Calibration Standard.

Bruker Autoflex II type matrix-assisted laser desorption/ionization time of flight (MALDI TOF/TOF) mass spectrometer was used. This instrument utilizes a 337 nm pulsed nitrogen laser, model MNL. Ions were accelerated under delayed extraction conditions (200 ns) in positive ion mode with an acceleration voltage of 20 kV. Protein masses were acquired with a range of m/z 1000 to m/z 10 000. Each spectrum was produced by data accumulated from 400 consecutive laser shots. Bruker FlexControl 2.4 software was used for control of the instrument, and Bruker FlexAnalysis 2.4 software for spectra evaluation.

Histological and cytological investigations

Mammary gland tissues were fixed in 4 % paraformaldehyde (PFA). After cryostat sectioning, slides were incubated with anti-PAC1 antibody (1:100) raised in rabbits (courtesy of Seiji Shioda, Showa University, Tokyo) then with the corresponding Alexa Fluor “568” labeled anti-rabbit secondary antibody (1:1000). Negative controls were used in each case. Digital photographs were taken with a Nikon Eclipse 80i microscope on 568 nm with help of SPOT Basic 4.04 software.

For fluorescent immunocytochemical examinations, Thermanox coverslips were cut to size and shape and placed into 96-well plates then they were pretreated with ethanol, poly-lysine, and UV light. The cells were incubated on the pretreated plates for 24 hours at 37 °C then fixed in 4 % PFA at room temperature for 30 min, then at 4 °C in freezer. After removing the PFA and washing the cells with PBS, the unspecific binding sites were blocked with BSA. The same anti-PAC1 antibody (1:100) was used as in the histological investigations. Overnight incubation on 4 °C with the primary antibody was followed by treatment with Cy3-conjugated secondary anti-rabbit IgG-antibody. After several washes, the samples were mounted onto coverslips, and the PAC1 expression was visualized by Olympus FluoView 1000 confocal laser scanning microscope.

Culturing of HC11 cells

HC11 mouse mammary epithelial cells were maintained in growth medium (GM), which contained RPMI-1640, 10 % heat-activated fetal calf serum (FCS), 5 µg/ml insulin (I), and 10 ng/ml EGF, was supplemented with 50 µg/ml gentamicin and glutamine. Cells were grown in 5 % CO₂ at 37 °C and passaged every 3–4 days.

In the differentiation studies, cells were plated in six-well plates and grown to confluence for 2–3 days. After the cells reached confluent state, they were washed twice with PBS to remove EGF, and an additional 2-day-incubation was carried out in pre-hormone medium (PHM) containing RPMI-1640, 2 % FCS, 5 µg/ml insulin, and 50 µg/ml gentamicin and glutamine. Then PHM was changed to DIP medium by adding 1 µM dexamethasone (D) and 5 µg/ml prolactin (P) to the PHM.

The DIP medium was changed daily in the next four days according to the respective experiments. Cells were incubated in DIP, DIP + PACAP38, and DI + PACAP38. PACAP38 was applied in dose of 100 nM. Cells were harvested for β-casein Western blot on the fifth day. β-casein expression of DIP + PACAP38 and DI + PACAP38 treated cells were compared to DIP-treated-only cells.

Lysates were taken 20 min after treatment with different combinations of hormone and PACAP mixes (DIP, DIP + PACAP38, DI + PACAP38, I, and I + PACAP38) for Western blot analysis of differentiation pathways. Results of PACAP treatments were compared to that of untreated cells.

Cell culturing for the arrays were carried out as it was described with the β-casein Western blot experiments. Expression of secreted factors of non differentiated, untreated cells (GM alone) was compared to that of PACAP38-treated non differentiated and untreated differentiated cells (GM + PACAP38 and DIP alone, respectively). Secretion of untreated differentiated cells was also compared to cells treated with PACAP38 (DIP + PACAP38).

Western blot

Cell lysates were prepared by washing cells three times with ice-cold PBS followed by lysis for 30 min at 4 °C in lysis buffer [(50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EGTA, 2 mM EDTA, 25 mM β-glycerophosphate, 1.5 mM MgCl₂, 10 % glycerol, 1 % Triton X-100, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM

dithiothreitol, 1.19 mM Na₃VO₄, and 2.5 mM NaF)]. The lysates were centrifuged (15,300 rpm) at 4 °C for 10 min to remove insoluble parts. Protein concentrations were determined by Bradford.

Proteins were separated by SDS-PAGE and blotted on Odyssey membranes. The membranes were blocked with 5 % milk for 1 hour at room temperature, incubated overnight at 4 °C with the primary antibodies: antiphospho-Akt (1:500); anti Akt-1 (1:500); antiphospho-p38 MAP Kinase (1:1000); anti-p38 MAP Kinase (1:1000); antiphospho-STAT-5 (1:900); anti-STAT5 (1:900); anti- α -tubulin (1:1000); and anti- β -casein (1000). On the following day after washing 3 x 10 min with TBS-T, the membranes were incubated for 30 min at room temperature with the appropriate secondary antibodies.

Reactions were detected with ECL Plus Western blotting detection system (GE Healthcare, Little Chalfont, UK). X-rays were scanned in transmission mode and densities were analyzed by ImageJ software. Experiments were repeated three times under same conditions.

Mouse cytokine and angiogenesis array

This is a semiquantitative method, which is based on binding of captured antibodies spotted in duplicate on nitrocellulose membranes and proteins of the investigated samples. The kits contain all the buffers, detection antibodies and membranes necessary for the measurements. The arrays were performed as described by the manufacturer.

Briefly, after blocking the array membranes for 1 hour, 500 μ l medium was added and incubated overnight at 2–8 °C on rocking platform with detection antibody cocktail. After washing with buffer three times and adding HRP conjugated streptavidin, the membranes were exposed to chemiluminescent detection reagent.

The densitometry was performed as previously described. Results of three independent experiments were taken into account in the statistical analysis.

Statistical analysis

In case of human milk samples Friedman, while in case of fresh and pasteurized milk, powdered milk, and infant formula samples unpaired Student-t test were used. Results of the

array kits were analyzed by one- and two way ANOVA test and Bonferroni post hoc analysis. Variations in the measurements were expressed as standard error of the mean (SEM).

RESULTS AND DISCUSSION

Changes of PACAP38-IR in the 1-17 months period of lactation

The PACAP38-IR concentration remained fairly constant (112 ± 13 fmol/ml) in the first six months of exclusive breastfeeding (1-6 months). These samples were also analyzed on monthly basis, and the lowest values ($100 \pm 8,8$ fmol/ml) were measured from samples collected in the sixth month, while the highest ones ($119 \pm 14,2$ fmol/ml) in the samples from the second month.

The PACAP38-IR levels increased significantly after the tenth month. Compared to the mean value of the first six months, the PACAP38-IR showed a 2.3-fold increase ($\Delta\% = +128$; $p < 0.001$) in the 11-13 months, while almost a 3-fold increase ($\Delta\% = +177$; $p < 0.001$) was observed in the 14-17 months ($\Delta\% = \frac{(X - X_{ref})}{X_{ref}} \times 100$, X = the mean PACAP38-IR in the investigated samples, X_{ref} = the mean of PACAP38-IR in the mature milk samples).

In other experiments, similarly to our findings, the PTHrP concentration increased in rat and bovine milk during lactation, and it decreased after separation of the pups from their mothers. The concentration of other hormones and growth factors, such as PRL and EGF decreased during prolonged lactation, while the CGRP remained unchanged.

As breastfeeding prolongs so does the milk yield decrease, but the total protein content remains relatively stable. The increased PACAP38-IR after the tenth month in our experiments suggests accumulation due to increased PACAP production.

PACAP38-IR in human colostrum

The PACAP38-IR showed a 1.5-fold increase in human colostrums compared to milk samples collected in the first six months ($\Delta\% = +56$), which proved to be a statistically significant difference ($p < 0.05$).

Higher levels of immunoglobulins, growth factors, cytokines, oligosaccharides, antimicrobial, and immune-regulating factors occur in colostrum than in mature milk. According to the colostrum measurements, one may suppose that some proteins are not only required for the development of neonatal gut, they are also indispensable for other organs of the newborns.

PACAP38-IR in foremilk and hindmilk

The PACAP38-IR did not show any significant difference in foremilk and hindmilk compared to the six mature human milk samples, which were used as controls ($\Delta\% = -2.5$ and $\Delta\% = +5.1$, respectively).

Changes in the composition of foremilk and hindmilk are signals to terminate suckling. Ghrelin, which increases appetite, and leptin, which decreases food intake, are the most important regulatory peptides of feeding. The concentration of the fast-acting, orexigen ghrelin is significantly lower in hindmilk than that of the anorexigen leptin, and this may terminate suckling.

As it was mentioned earlier, PACAP influences the regulation of food intake. The anorexigen effect of PACAP is exerted via inhibition of α -melanocyte-stimulating hormone (MSH) and corticotropin-releasing hormone (CRH) pathways. The food intake of PACAP KO mice is decreased, and their early postnatal death is related to their impaired carbohydrate metabolism. We could not detect any changes in the PACAP38-IR in foremilk and hindmilk, and therefore, PACAP seems not playing any role in the regulation of food intake of newborns.

PACAP38-IR in bovine milk samples

There was no significant difference in the PACAP38-IR of fresh and pasteurized cow milk samples compared to mature human milk ($\Delta\% = +11.8$ and $\Delta\% = -9.9$, respectively).

The main difference between human and cow milk is that cow milk contains more proteins and the ratio of casein to other whey proteins are different (82:18 and 40:60), and it can result in protein overload to the newborn kidney and a higher, poorly digested protein uptake in case of cow milk fed babies. The essential fatty acid concentration of cow milk is

lower than that of human milk. Several industrial procedures have been developed in order to make the composition of cow milk similar to human milk and provide the best quality infant formulas to the newborns. Even under these conditions, the amount of important factors is less in the infant formulas than in human milk.

It is an interesting observation that consumption of pasteurized milk reduces the incidence of atopic children. Besides other neuropeptides (CGRP, neuropeptide Y/NPY, VIP), PACAP can also alleviate the symptoms of atopic dermatitis. Therefore, not only the changes in microbial composition of the pasteurized milk and difference in the quality of fatty acids and cytokines, but the preserved PACAP in pasteurized milk may support the protection against atopic phenotype.

PACAP38-IR in powdered milk and infant formulas

Presence of PACAP was proved by mass spectrometry in infant formulas. The PACAP38-IR was unchanged in the powdered milk samples and in the HA infant formulas ($\Delta\% = +6$ and $+8$, respectively), while in the non-HA infant formulas, it decreased nearly by half compared to the fresh cow milk samples, which were used as controls ($\Delta\% = -42$; $p < 0.05$).

In industrial production of powdered milk and infant formulas, the pretreated cow milk is completely or incompletely dehydrated and a powdered product is gained after heat-treatment. The nutrients and vitamin-content of cow milk are only partially preserved. A considerable part of the lost minerals, vitamins, and fibers is supplemented. HA infant formulas are free from large proteins because they are extensively hydrolyzed, and therefore, the number of allergic reactions is minimized. Only peptides above 6000 Da are cleaved during processing of infant formulas, and PACAP with its 4535 Da molecular weight might withstand the manufacturing processes, and that is why PACAP can be detected in infant formulas.

The difference between PACAP38-IR in HA and non-HA infant formulas might be attributed to the binding of PACAP to its preserved carrier molecules in HA formulas. The carrier-ligated PACAP is supposed not being accessible to the antibody, and therefore, it cannot be detected by RIA-measurement. On the contrary, after extensive hydrolysis, the PACAP38-IR is relatively high in HA type infant formulas.

Interestingly, the rapidly degradable PACAP is relatively stable in human milk and in milk-based products. Human milk is rich in serinproteases (α 1-antitrypsin and antichymotrypsin), and the concentration of inhibitors is the highest at the beginning of lactation, while it decreases as the time passes. Consequently, the concentration of several physiologically important, secreted factors is high at the first few months of lactation. The protective effect of the protease inhibitors can explain the detection of short life time PACAP in human milk and milk-based products.

PACAP38 is selectively bound to ceruloplasmin in blood plasma, but its carrier protein in milk is not known. Ceruloplasmin and lactoferrin form a complex in milk, and PACAP38 can eluate the lactoferrin from the ceruloplasmin. This observation suggests that ceruloplasmin can indeed be the carrier molecule of PACAP38 in milk.

Origin and suspected physiological effects of PACAP in human milk

According to our unpublished PCR data, neither the 288 bp, nor the 247 bp of PACAP mRNA can be detected in the mammary glands of lactating natal multimammate mice. Similarly to VIP, PACAP is not synthesized in the mammary gland, but it originates from blood plasma.

We do not have supportive experimental data, but it can be hypothesized that PACAP in milk (1) may be required for proper development of the nervous system in newborns; (2) it may be an important immunomodulator at the early stages of postnatal development; and (3) may influence the neonatal PRL production.

PAC1 receptor on mammary epithelial cells

The presence of PAC1 was directly demonstrated on each of our mouse and human mammary cell line by microscopic investigations. However, the studied cell lines showed a variable staining intensity. There was no difference in the PAC1 receptor expression between the differentiated and the non differentiated HC11 cells. In the tissue samples, the terminal lobular units of the non lactating mammary glands showed a weak PAC1 expression, while the connective tissue remained negative. In the lactating glands, the PAC1 receptor expression was increased compared to the non lactating glands.

The effect of VIP-like peptides on HMEC and HC11 cells had already been investigated before VPAC1-2 and PAC1 were isolated. After the discovery of PACAP receptors, the mRNA of VPAC1 and PAC1 was demonstrated on MCF-7 cells by PCR method. These results were supported by Western blot analysis as PAC1 receptor could be detected at 60 kDa.

PACAP receptors were demonstrated by PCR, Western blot, and immunohistochemistry in human mammary biopsy samples earlier. VPAC1 and VPAC2 were expressed on ductal and glandular epithelial cells in normal mammary gland. A similar distribution, but weaker intensity was observed in case of PAC1.

PACAP receptors on normal and neoplastic mammary cells are functionally active, and therefore, they are suspected to play role in regulation of physiological and pathological processes of the mammary gland. Binding of PACAP to its receptors stimulates the proliferation of breast cancer cells. The inhibitory effect of the PAC1 receptor antagonist, PACAP6-38 is also well proven. VIP-hybrids, such as VPAC antagonist, can potentiate the effect of chemotherapeutic drugs. Based on autoradiographic investigations, metastatic axillary lymph nodes show a 2-fold higher VPAC1 receptor density than primary tumor cells. These observations provide opportunities to develop PACAP/VIP radioligands. The increasing knowledge about the PACAP receptors can be advantageously utilized in the diagnosis and therapy of breast cancers.

Investigations on DIP-induced differentiation of HC11 cell cultures

In our experiments, the PACAP co-incubation without PRL did not induce β -casein expression, and it did not modify the DIP evoked β -casein expression either. The possible effect of PACAP was detectable neither on STAT5, Akt, nor p38 MAPK signaling pathways.

Cyto-, chemokine, and growth factor secretion of HC11 cells

Insulin-like growth factors and their binding proteins

Besides other proteins, the secretion of insulin-like growth factor-binding proteins (IGFBPs) of non differentiated and differentiated HC11 cells was investigated by angiogenesis array. It is noteworthy that significantly decreased IGFBP3 were measured after DIP treatment

($p < 0.05$), which is compatible with the physiological changes of IGFbps during lactation. Reduced carrier proteins can increase the level of free IGFs, which can potentiate the effect of insulin and stimulate the PRL-induced alveolar differentiation.

Contradictory results were reported on the secretion of IGFBP3 by non differentiated cells. Other investigators did not observe IGFBP3 secretion in cell cultures of non differentiated HC11 cells. However, similarly to the investigation on Comma-1D cells (progenitors of HC11 cell line), IGFBP3 was present in the media of our non-differentiated HC11 cells.

Interleukines

The IL series showed very weak densities in our experiments, and therefore they were not analyzed further.

According to investigations on KIM2 cells, a cell line, which is similar to HC11 cells, the expression of IL-13R α and IL-4R α increased during differentiation, and the STAT6 was phosphorylated after IL-4 and IL-13 treatment suggesting the role of ILs in the process of differentiation. Similarly, an IL-4 mediated STAT6 activation and a β -casein promoter induction were observed in case of HC11 cells. A higher IL-4 and a lower G-CSF and IL-6 secretion was observed with cytokine array measurement in the media of differentiated KIM2 cells, but the other Th2 cytokines (IL-2, IL-3, IL-5, IL-9, IL-10 and IL-13) did not show any difference. Simultaneously with the induction of differentiation, a Th1/Th2 cytokine switch was observed: the IL-12 and TNF α were downregulated, while the IL-4, IL-5 and IL-13 were upregulated.

We could not detect similar changes in the release of IL-4, IL-6, and G-CSF from differentiated HC11 cells, and this may reflect cell line specific differences and/or differences in the differentiation protocols used in the studies.

Chemokines

The secretion of the chemokines, interferon gamma-induced protein (IP)-10 and regulated upon activation normal T cell expressed and presumably secreted (RANTES) significantly decreased during the lactogenic differentiation ($p < 0.05$ and $p < 0.005$, respectively).

IP-10 and RANTES are responsible for the recruitment of T-lymphocytes and some other leucocytes. They are present in the mammary gland and milk, and they are supposed to

maintain the balance of lymphocyte homing to the mammary gland at different stages of differentiation. Colonization of the mammary gland is dominated by T-cells during pregnancy, while IgA-containing B-cells are abundant during lactation. Therefore, the decreased release of T-cell attractants, such as IP-10 and RANTES in our experiment, may reflect the shift of T- to B-cells in lactating glands.

Growth factors

The secretion of some EGF receptor (EGFR) ligands, such as amphiregulin (AREG) and EGF decreased significantly in the media of differentiated HC11 cells ($p < 0,001$).

EGFR ligands are structurally and functionally related proteins resulting in cell proliferation and differentiation.

AREG is a glycosylated heparin binding protein, and the amino acid sequence of its C-terminus is identical in 38 % with the C-terminus of EGF. AREG was isolated as an EGFR ligand on MCF-7 cells. AREG is specifically required for ductal morphogenesis, while EGF and transforming growth factor (TGF)- α are dispensable in this process. AREG exists as a membrane-associated precursor, which is released into the stroma, where it binds to EGFR, and plays an important role in stromal fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and IGF1 production, and consequently stimulates the proliferation of the surrounding mammary epithelial cells.

It is known that EGF blocks differentiation and/or results in dedifferentiation. Differentiation of the mammary gland is influenced by the balance between stimulatory (PRL, insulin, glucocorticoids) and inhibitory effects (TGF β , progesterone). The increased EGF in blood plasma and the enhanced expression of its receptor during pregnancy, as well as their decrease prior to lactation call attention to the role of EGF. The differentiated cells become flat, the amount of intracytoplasmic fat droplets decreases, the expression of WAP shows a dramatic and the β -casein a moderate decline, and similarly, the level of mammary-derived growth inhibitor (MDGI) decreases in response to EGF. Furthermore, EGF stimulates TGF α suggesting that the effect of EGF is mediated by TGF α .

The decreased AREG and EGF release from differentiated cells in our experiments may reflect the switch from proliferative to lactogenic phase, and it is consistent with the above mentioned data. Transcription of AREG is regulated by PRL, but to our knowledge, no data are available about the effect of PRL on its secretion. In our experiments, PRL was a

standard component of the hormone mix, and therefore, it cannot be excluded that it might have a direct effect on AREG secretion.

Effect of PACAP on secretion of AREG and EGF of non differentiated mammary cells

Regulation of EGF, AREG, and TGF α secretion

PACAP induced a significant decrease in AREG and EGF secretion of non differentiated HC11 cells ($p < 0.001$ and $p < 0.05$, respectively), which can be attributed to decreased ligand shedding and/or decreased expression of these EGFR ligands.

The metalloproteinase, „a disintegrin and metalloproteinase domain (ADAM)-17” is responsible for shedding of AREG, EGF, and TGF α , and the consequential EGFR activation. ADAM17 can be inhibited by TGF β . TGF β inhibits ADAM17 via upregulating metalloproteinase inhibitors, such as tissue inhibitor of metalloproteinase (TIMP)-3. TGF β is downregulated in PACAP KO mice, and experimental data suggest that PACAP can directly stimulate TGF β . Based on these facts, it can be hypothesized that PACAP induces TGF β and consequential TIMP3 upregulation, which inhibits ADAM17 leading to decreased AREG and EGF shedding. Our observation on PACAP-induced reduction of secreted EGF and AREG is a potential starting point of new experiments to reveal the exact mechanism of EGFR-ligand shedding from mammary cells.

EGF, AREG, and other neuropeptides in the activation of EGFR

The PACAP-induced reduction in EGFR ligand secretion draws attention to the issue of EGFR transactivation, which plays a special role in induction and maintenance of cell proliferation. Activation of EGFR is primarily regulated by secretion of EGF, AREG, and TGF α , and this process was investigated by other researchers on HC11 cells. HC11 cells express EGFR and HGF receptor/MET, but neither short, nor long term incubation causes EGF induced MET and HGF mediated EGFR activation. Therefore, this model of EGRF transactivation can be ruled out.

The process of EGRF activation can be initiated even by neuropeptides. Human epidermal growth factor receptor (HER)-2 and EGFR transactivation were observed on prostatic, colon, estrogen dependent, and independent breast cancer cell lines after VIP treatment. VIP could stimulate the phosphorylation of both HER2 and EGFR, which could be

inhibited by VIP receptor antagonist. According to investigations on small cell carcinoma cell lines derived from the lung, the G protein-coupled receptor (GPCR) agonist, bombesin, and neuromedin B could transactivate EGFR via TGF α secretion.

EGFR transactivation was detected after PACAP treatment on cultures of small cell carcinoma of the lung, which could be blocked by VIP, PACAP6-38, and anti TGF α administration. Therefore, this PAC1 and TGF α mediated process was described as it follows. PAC1/PACAP interaction via cAMP/PKA pathway stimulates Src/ADAM17 and increases TGF α secretion, which finally results in EGFR transactivation. Administration of anti-AREG did not reverse the transactivation suggesting that shedding of AREG was not elevated.

Role of AREG in proliferation of breast cancer cells

AREG is an important growth factor in regulating proliferation of breast cancer cells via EGFR transactivation. Namely, EGFR dependent breast cancers utilize ADAM mediated EGFR ligand shedding, and therefore, AREG can be a promising target of breast cancer therapy. A higher level of AREG was detected in invasive breast cancers compared to *in situ* ductal carcinomas and tissues from normal breasts, which seems to prove the role of AREG in the development and progression of breast cancer. *In vivo* suppression of AREG secretion results in decreased tumor formation. Interestingly, AREG plays role in the development of bone metastasis, because it stimulates parathyroid hormone-like hormone (PTHrH) production via EGFR, and promotes bone resorption. Considering EGFR transactivation, our results may indicate that PACAP has an inverse effect on mammary epithelial cells compared to VIP. The observed PACAP mediated reduction of secreted AREG and EGF in the media of our cultured HC11 cells seems to be valuable even from the viewpoint of experimental oncology.

SUMMARY

Summary of the results

- A significantly higher PACAP38-IR was measured in human colostrum than in human mature milk. PACAP38 accumulated in human milk after the tenth month of lactation. No differences were seen in the PACAP38-IR in transitional milk, foremilk, and hindmilk.

- A similar level of PACAP38-IR was measured in pasteurized and fresh cow milk, and in powdered milk samples. According to our mass spectrometry analysis, PACAP38 was present in the commercially available milk-based infant formulas. The PACAP38-IR was significantly higher in the extensively hydrolyzed, hypoallergenic infant formulas than in the less hydrolyzed ones.
- PAC1 was expressed in all the investigated histological and cytological samples. The expression of PAC1 was higher in the samples of lactating than in the non lactating mammary glands.
- PACAP did not influence the mammary cell differentiation, which is reflected by the expression of β -casein and proteins of the differentiation-related pathways (STAT5, Akt and p38 MAPK).
- The secretion of some chemokines and growth factors, such as RANTES, IP-10, AREG, EGF, and IGFBP3 was significantly lower in the media of the differentiated compared to the non-differentiated HC11 cells.
- The PACAP-treated non-differentiated HC11 cells secreted significantly lower level of AREG and EGF than the non-treated ones.

Assessing the value of our results

Our results prove that PACAP is present in biologically effective concentration in human milk and in different milk-based products. It can be supposed that PACAP has effect not only on the development of newborn, but on the mammary gland itself.

The concentration of PACAP changes characteristically in human milk. It is high at the early (colostrums) and late stages of lactation, while it is stable in the intermediate period (mature milk). These changes seem to follow the well known proliferative and differentiation processes of the mammary gland.

Our microscopic investigations on PAC1 show that PAC1 receptor is constitutionally expressed on mammary epithelial cells, but its actual level depends on the origin (species, normal, or tumor cells) and differentiation state of the investigated cells.

Although we could not detect any PACAP effect on lactogenic differentiation of the mammary epithelial cells, we gained valuable data about some secreted cyto- and chemokines, and growth factors.

The changes in secretion of different cyto- and chemokines, and growth factors related to differentiation of mammary cells are not only noteworthy as phenomena following proliferation and differentiation, but they may even reveal some important cell regulatory mechanisms. From this point of view, the hypothetic relation of PRL and AREG might be particularly interesting.

We could not find any data in the literature about PACAP-related EGF and AREG secretion, and therefore, we regard these observations as new results. It is not clear, whether these are only cell line, or mammary-specific changes, or eventually represent a phenomenon, which may have wider significance in cell biology.

Based on the presented results of this study, further interesting experiments can be designed about the regulatory functions of PACAP (identification of its carrier protein in milk, investigations on its influence on the PRL secretion of newborns and on neonatal intestinal flora, as well as defining its potential role in EGFR activation).

LIST OF PUBLICATIONS

Publications serving as base for this study

Csanaky K, Doppler W, Tamás A, Kovács K, Tóth G, Reglődi 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. **IF: 2,891**

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