

Examination of PACAP38-like immunoreactivity in different milk and infant formula samples

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Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide with special importance in reproductive and developmental processes. PACAP is found in two bioactive forms: PACAP27 and PACAP38. Recently, we have described that PACAP38 is present in high levels in the milk of human and ruminant animals. Breastfeeding is of utmost importance in proper nutrition of the newborn, but artificial nursing with infant formulas is necessary when breastfeeding is not available. Composition of the breast milk varies during the whole period of nursing and it shows differences at the beginning (foremilk) and the end of an actual suckling (hindmilk). The aim of this study was to investigate PACAP38-like immunoreactivity (PACAP38-LI) in different milk and infant formula samples by radioimmunoassay and to prove the presence of PACAP38 in the infant formula by mass spectrometry. We found similar PACAP38-LI in human mature foremilk and hindmilk samples, in the fresh and pasteurized cow milk and also in formulas. However, we found significantly higher PACAP38-LI in the hypoantigenic formula undergoing extensive hydrolysis compared to the non-hypoantigenic ones. Our results suggest that PACAP38 is relatively stable in the milk and it can withstand the manufacturing processes.

Keywords: foremilk, hindmilk, infant formula, PACAP38, MALDI TOF/TOF, radioimmunoassay

Pituitary adenylate cyclase activating polypeptide (PACAP) is a multifunctional neuropeptide, belonging to the vasoactive intestinal polypeptide (VIP)/glucagon/growth hormone releasing hormone superfamily. Two bioactive forms are distinguished, such as PACAP27 and 38 (33). The 38-amino acid form was originally isolated from ovine hypothalamus, while PACAP27 is derived by an internal cleavage (9). The amino acid sequence of PACAP is highly conserved. PACAP can act on three different receptors, such as VPAC1-2 and PAC1 (33). The physiological role of PACAP is well known in the regulation of some processes, such as cerebral circulation (2), thermoregulation (11), circadian rhythm (13), feeding behaviour (21), inflammation (33). The roles of PACAP in reproductive processes and development are of particular importance (33).

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There are relatively few human studies describing the occurrence and functions of PACAP. Recently, we have provided evidence for the presence and changes of PACAP38 in human follicular fluid (4, 18) and plasma (3, 26). We have demonstrated an elevation of PACAP38-like immunoreactivity (PACAP38-LI) in the plasma during normal human pregnancy and a significant drop during delivery (26). We have also shown that similarly to the structurally closest VIP (34), PACAP38 is present in milk in much higher level than in the respective plasma samples (3). Also, we have found significantly higher levels of PACAP38 in human colostrum than in the mature milk and we have described its changes during the lactation period. The level of PACAP38-LI in the milk samples was stable during the first 6 months, but it showed a significant increase after the 10th month as a compensation of reduced milk production (8).

Breastfeeding is essential for the optimal development of the newborn. Several processes have been developed to modify cow milk in order to prepare cow milk-based infant formulas for artificial nursing. Dry and wet blending are used to make powdered cow milk-based formulas. Larger milk proteins are extensively hydrolysed in order to reduce allergic reactions, and hypoantigenic (HA) and non-hypoantigenic (non-HA) infant formulas can be distinguished (29).

The aim of the present study was to compare PACAP38-LI in foremilk and hindmilk, i.e. the first few streams of milk at the beginning of feeding and the following thicker milk, respectively. Furthermore, we aimed to compare PACAP38-LI in fresh cow milk, pasteurized cow milk and commercial infant formula samples by radioimmunoassay (RIA) and to investigate the presence of PACAP38 by mass spectrometry in cow milk-based formulas.

Materials and Methods

Sample collection

Samples of human foremilk and hindmilk (5 ml; $n = 4$) were derived from healthy, non-smoking, volunteer multipara mothers with healthy term infants, 3 weeks after delivery. All hindmilk samples were obtained from the mothers, who provided foremilk earlier. An approximate estimation of the daily total milk yield was carried out (900–1200 ml). Mothers were nursing 8–9 times/day for 20–30 minutes. The samples were collected into ice-cold polypropylene tubes without peptidase inhibitor and stored at $-20\text{ }^{\circ}\text{C}$. All human sample collections were carried out according to a protocol approved by the Institutional Ethic Committee (PTE KK 3117). In all cases, we obtained written consent of the volunteers. For the cow milk measurements, 5 ml unpasteurized, fresh bovine mature milk was collected during the 1–8 months of the postpartum period from Holstein Friesian and Hungarian Simmental mixed-bred dairy cows ($n = 4$; Nagybudmér, Baranya county). Commercially available non-hypoantigenic ($n = 6$) and hypoantigenic ($n = 2$) infant formula samples, as well as pasteurized cow milk ($n = 4$) were purchased.

Radioimmunoassay

PACAP38-LI in the milk whey and infant formula samples was determined with specific and sensitive RIA technique developed in our laboratory (14, 22). Standard dilution of infant formula samples was used for RIA analyses. 150 mg infant formula was dissolved in 1 ml hot distilled water. 10 μl 96% acetic acid was added to 1 ml sample and incubated in $40\text{ }^{\circ}\text{C}$ water bath for 5 min to precipitate the protein content. Centrifugation was performed at 4000 rpm for 10 min at $4\text{ }^{\circ}\text{C}$ to obtain a solid fat component on the top of the samples. The whey

localized between the precipitated protein and the fat fractions was then removed for RIA analysis. For the RIA measurement '88111-3' PACAP38 antiserum was raised against a conjugate of Cys23-PACAP24-38 and bovine thyroglobulin coupled by carbodiimide in rabbit (1). Tracer: mono-125I-labelled ovine PACAP24-38 C-terminal fragment was prepared in our laboratory. Standard: ovine PACAP38 was 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. Assay procedure: 100 µl antiserum (working dilution 1:10,000), 100 µl RIA tracer (5000 cpm/tube) and 100 µl PACAP38 standard or unknown samples were measured into polypropylene tubes with the assay buffer. After 48-72 h incubation at 4 °C, the antibody-bound peptide was separated from the free one by the 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). Following centrifugation (1800 g, 4 °C, 15 min) the tubes were gently decanted and the radioactivity of the precipitates was measured in a gamma counter (Gamma, type: NZ310). PACAP38-LI of the unknown samples were read from a calibration curve.

Mass spectrometry

To identify PACAP38 in the infant formulas matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF/TOF) mass spectrometry was used, as described previously (3, 8, 14). One ml standard dilution of infant formula samples was centrifuged (10 min, 13 000 rpm). The lipid fraction of the formula sample was precipitated by chilling (0-4 °C) and a 10-fold dilution was made of the liquid phase with 0.1% trifluoro-acetic acid (TFA). The solution was desalted and purified using 0.1% TFA and acetonitrile/0.1% TFA (2/98, v/v) solutions with ZipTip18 pipette tips (Millipore Kft). 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 post source decay MALDI TOF/TOF with an automated mode using the FlexControl software. Double-layer method was used with sinapinic acid (SA) as matrix, prepared freshly every day. A thin layer from SA-saturated solution in ethanol (1 µl) was made, and the diluted sample was loaded to the MALDI target plate (MTP 384 massive target T, Bruker Daltonics, Bremen, Germany). Elution was carried out by the matrix. 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., Berlin, Germany). External calibration was performed in each case using Bruker Peptide Calibration Standard (#206195 Peptide Calibration Standard, Bruker Daltonics). Protein masses were acquired with a range of m/z 1000 to m/z 10 000. Each spectrum was produced by accumulating data from 400 consecutive laser shots. The Bruker FlexControl 2.4 software was used for control of the instrument and the Bruker FlexAnalysis 2.4 software for spectra evaluation.

Statistical analysis

Data are expressed as percentage of the baseline \pm SEM. The baseline was calculated as the average of the values measured from mature human milk samples during the first 6 months of lactation (3, 8). For statistical analysis Student's *t*-test for unpaired comparison was used. In all cases $p < 0.05$ was considered to be statistically significant.

Results

Similarly to our previous measurements, PACAP38-LI could be detected with RIA analysis in all milk and infant formula samples. Comparing the PACAP38-LI in human foremilk and hindmilk samples, we did not show any significant change compared to human mature milk (Fig. 1).

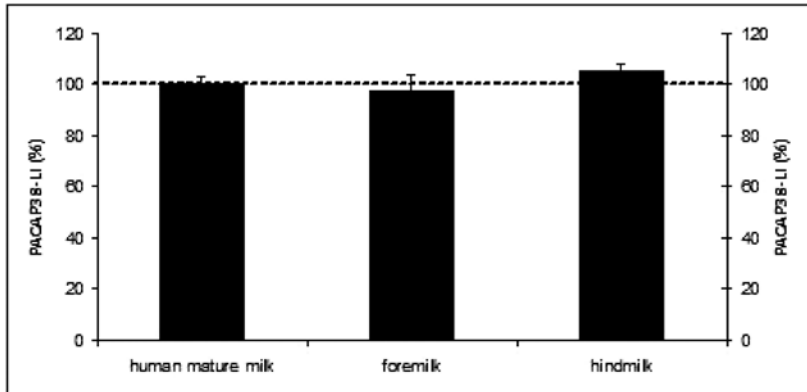


Fig. 1. PACAP38-like immunoreactivity determined by radioimmunoassay from human mature milk, hindmilk and foremilk. Values are expressed as percentage of the baseline \pm SEM

Next we compared PACAP38 content in different milk products. Fresh cow milk, pasteurized cow milk and cow milk-based infant formula samples (pooled from hypoantigenic and non-hypoantigenic samples) showed similar PACAP38-LI levels to human milk samples. Although in pasteurized cow milk and cow milk-based formulas PACAP38-LI had a decreasing tendency compared to fresh samples, no statistical significance between the different samples could be demonstrated (Fig. 2).

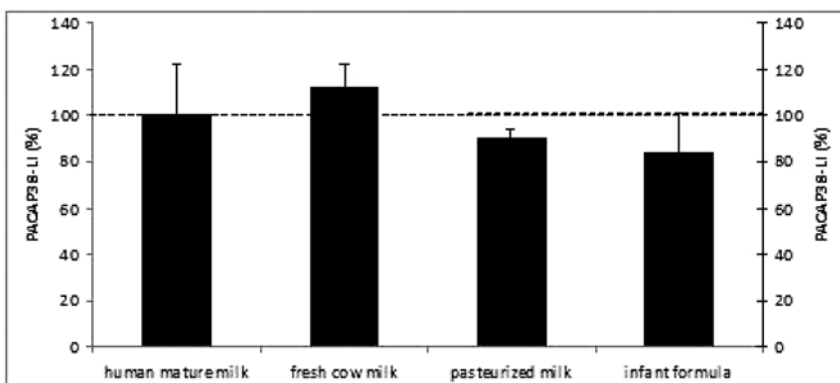


Fig. 2. PACAP38-like immunoreactivity determined by radioimmunoassay from human mature milk, fresh cow milk, pasteurized milk and infant formula samples. Values are expressed as percentage of the baseline \pm SEM

Mass spectrometry was utilized to prove that the PACAP38-LI measured by RIA corresponds to the PACAP peptide and not to a similar molecule cross-reacting with the antibody. Similarly to our earlier descriptions on fresh milk, the presence of PACAP38 could be detected in the infant formula samples. The average peak of protonated quasimolecular ion $[M+H^+]$ of PACAP38 was indicated by the MALDI TOF/TOF spectrum of a hypoantigenic infant formula sample in positive ion mode and linear detection. The result of mass spectrometry indicates that the measured PACAP38-LI presents PACAP38 molecule in the infant formula (Fig. 3).

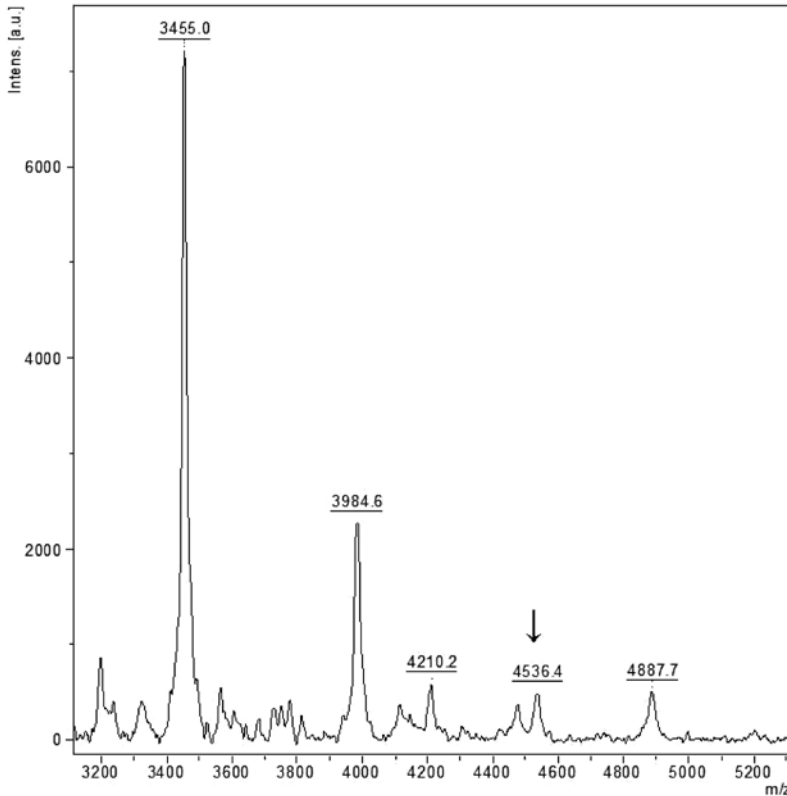


Fig. 3. MALDI TOF/TOF spectrum of a hypoantigenic infant formula sample. The average peak of the protonated quasimolecular ion $[M+H^+]$ of PACAP38 at 4536 Da (arrow)

In the next step we aimed to compare the PACAP38-LI in the two basic infant formulas, the hypoantigenic and non-hypoantigenic infant formula samples by RIA. During the preparation process, hypoantigenic infant formula is extensively hydrolysed in order to minimize the risk of larger proteins to induce allergic reactions in infants. Our measurements revealed that the hypoantigenic formula samples contained significantly higher level of PACAP38-LI than the non-hypoantigenic samples (Fig. 4).

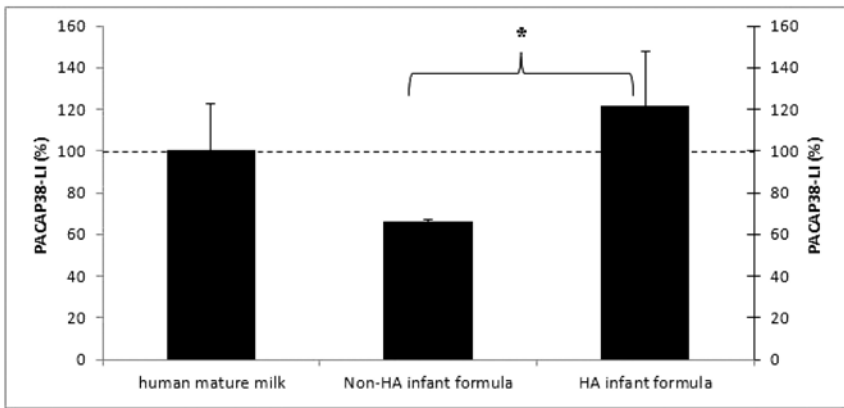


Fig. 4. PACAP38-like immunoreactivity determined by radioimmunoassay from human mature milk, hypoantigenic (HA) and non-hypoantigenic (non-HA) infant formula samples. Values are expressed as percentage of the baseline \pm SEM. * $p < 0.05$ PACAP38-LI in non-HA infant formula vs. HA infant formula

Discussion

In the present study we investigated PACAP38-LI in different milk and infant formula samples. We have found that it does not show alteration between the beginning and the end of the lactation and that there is no difference in PACAP38 content between fresh human, fresh cow milk and infant formula samples. However, extensively hydrolysed, hypoantigenic infant formulas contain higher levels of PACAP38.

The composition of the human milk depends on the time that passes between the successive sucklings. Mothers produce two types of milk, such as foremilk (the thinner milk with lower fat content) and hindmilk that follows (creamier milk with higher fat content). At the beginning of suckling, the babies first receive the foremilk, which quenches their thirst. The hindmilk mixes with the high-lactose foremilk, and the baby receives the perfect food for growth and development (20, 28). According to our measurements, PACAP38-LI level does not show any changes in the foremilk compared to the hindmilk during an actual suckling. Similar investigations have been performed concerning the concentrations of different lipids and peptides. Similarly to PACAP, the concentrations of atrial natriuretic peptide (17) and granulocyte colony-stimulating factor (G-CSF) are similar in the foremilk and hindmilk (7). However, other factors show different levels in the foremilk and hindmilk. For example foremilk contains significantly higher concentrations of endothelin-1 (17), ghrelin and cholesterol (16) compared to hindmilk, while triglyceride, leptin (16) and retinol (27) levels are higher in the hindmilk than in the foremilk.

The differences concerning the peptide content of human milk, cow milk and cow milk-based infant formulas are also well studied. In the present study we found that the fresh cow milk contained similar PACAP38-LI to the human milk. This is in accordance with epidermal growth factor (EGF), which has been described to have the same level in the cow milk and human mature milk (35). Higher immunoreactivity of parathyroid hormone-like protein (PLP) has been measured in the human milk compared to the bovine one (5).

To preserve the nutritional value of the cow milk and to kill pathogenic microorganisms heat treatments are used: sterilization and pasteurization. Two types of pasteurization are

known: continuous-flow HTST (high temperature, short time) and UHT (ultra-high temperature) (6). The pasteurized milk samples used in our investigations were of UHT-treated ones. Transforming growth factor beta-1 (TGF-beta1) has been found to be significantly lower in the commercial pasteurized milk than in the cow milk (24). In contrast, we did not see any difference in the PACAP levels of unpasteurized and pasteurized milks.

Artificial nursing with cow milk-based infant formulas is necessary when breastfeeding is not available. There are some peptides, which can be detected in the milk, but not in the infant formulas: gonadotropin-releasing hormone (GnRH) (12), substance P, calcitonin gene-related peptide (CGRP) (10) and insulin-like growth factor (IGF-1) (15). In contrast, other peptides can be detected in infant formulas, such as PLP (5), parathyroid hormone-related protein (PTHrP) (23), EGF, insulin (25) and ghrelin (a growth hormone-releasing peptide) (30). Our present results show that PACAP, similarly to these latter peptides, can be detected in cow milk-based infant formulas. Interestingly, even higher levels of ghrelin (30) and leptin (19) have been observed in the infant formulas and commercial pasteurized milk than in the human milk.

Peptides above 6000 Da are cleaved during the processing of the hypoantigenic infant formulas (35). PACAP38 with its relatively low molecular weight (4535 Da) might withstand hydrolysis and it could be detected in both the non-hypoantigenic and hypoantigenic infant formulas similarly to the human milk samples. It might be supposed that a part of the PACAP molecule is not detected by our RIA method, i.e. the antigen is not accessible to the binding sites of the antibody due to its binding to a carrier protein in the milk. In the plasma, PACAP38 is selectively bound to ceruloplasmin (32), but its carrier protein in the milk is not known. However, it has been shown that ceruloplasmin and lactoferrin form a complex in the milk, and PACAP38 can eluate lactoferrin from ceruloplasmin (31). This observation suggests that ceruloplasmin can indeed be the carrier of PACAP38 in the milk. As the hypoantigenic formula is made by extensive hydrolysis, PACAP38 might be released from its carrier during the preparation process. This could explain the higher PACAP38-LI in the hydrolyzed hypoantigenic infant formulas compared to the non-hypoantigenic ones. Our present data also show that PACAP38 is relatively stable in the milk and it can withstand the manufacturing processes.

The function of PACAP along with other peptides (growth factors, gastrointestinal- and neuropeptides) in the milk and the significance of their changes during lactation have not yet been precisely elucidated. Many of them (i) may be required for the development of the newborn (ghrelin, leptin, EGF, IGF-1, VIP); (ii) can be immunomodulators during postnatal period (G-CSF, TGF-beta1); (iii) are important in the growth of the mammary gland itself (PTHrP); and (iv) may play a role in lactation at local or at hypothalamo/pituitary level (GnRH) (7, 12, 15, 19, 23, 24, 34). As PACAP is an important trophic factor during development, has well-known immunoregulatory functions, is a pituitary hormone and has strong cytoprotective effects, it is plausible that PACAP is involved in all these above-mentioned functions. Further investigations are necessary to elucidate the exact function of PACAP in these regulatory processes.

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