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Cholecystokinin in plasma and cerebrospinal fluid - a study in healthy young women

Kristina Lundberg[1]*, Susanne Hilke[1], Conny Nordin[2], Elvar Theodorsson[1], Ann Josefsson[3]

[1] Department of Clinical and Experimental Medicine, Division of Clinical Chemistry, Faculty of Health and Sciences, Linköping University, SE-581 85 Linköping, Sweden

[2] Department of Clinical and Experimental Medicine, Division of Neuroscience and Locomotion, Faculty of Health and Sciences, Linköping University, SE-581 85 Linköping, Sweden

[3] Department of Molecular and Clinical Medicine, Division of Obstetrics and Gynaecology, Faculty of Health and Sciences, Linköping University, SE-581 85 Linköping, Sweden

*Corresponding author
Susanne Hilke, PhD
Department of Clinical and Experimental Medicine (IKE),
Division of Clinical Chemistry
Lab 1, Floor 11
Linköping University Hospital
581 83 LINKÖPING, Sweden
Email; susanne.hilke@liu.se
Phone; +46-13-228111

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ABSTRACT:

Cholecystokinin (CCK) is widely distributed in the brain and is known to affect behavioral and physiological functions including anxiety and pain. The expression of CCK has been shown to be regulated by estrogen and to vary during the estrous cycle in rat brain. In the present study CCK was determined in plasma from 25 healthy women (age 25.0 ± 3.5) during the menstrual cycle, in the late luteal phase and in the follicular phase. In the follicular phase, a lumbar puncture was performed at the same time that a plasma sample was taken in 15 subjects. The participants had fasted and were nicotine-free for at least 8 h preceding the sampling.

We compared CCK-like immunoreactivity (CCK-LI) in plasma from 25 subjects in the late luteal phase (LLP) and the follicular phase (FP) and found that there was no difference during the menstrual cycle (n=25, R^2=89,60%, p= n.s.). In the follicular phase no significant difference was found between CCK-LI in plasma and in cerebrospinal fluid (CSF) collected at the same time (n=15, R^2=55,32%, p=n.s.).

1. Introduction

Cholecystokinin (CCK) is a peptide hormone and neurotransmitter with a wide range of biological effects [20]. This peptide was originally recognized as a gastrointestinal peptide released into the circulation from endocrine cells and neurons in the gastrointestinal tract Thus, CCK regulates gallbladder contraction, pancreatic enzyme secretion, intestinal motility, satiety signaling and inhibition of gastric acid secretion . CCK is also present in the central nervous system and is abundant in the cortex [22], where it induces excitation of central neurons [2] but also has inhibitory postsynaptic effects [13]. The CCK octapeptide (CCK-8) and tetrapeptide (CCK-4) are both implicated in behavioral and physiological functions such as satiety, anxiety and pain [1] and may also play a role in the pathogenesis of panic disorders

[10], schizophrenia [24] and abuse [4]. Whether or not CCK peptides are transferable between the central nervous system and the peripheral blood is a crucial question. In animal experiments, CCK-8 injected in the lateral ventricle diffuses rapidly into the blood while intravenous administration does not affect the cerebrospinal fluid (CSF) [16]. On the other hand, the fact that intravenous injection of CCK-4 induces panic-like attacks in humans [3] might be in accord with a transfer in the opposite direction , from the peripheral blood to the CNS.

Premenstrual symptoms are common and include psychological symptoms like anxiety and irritability [23]. Women with premenstrual dysphoric disorder have shown greater anxiety and panic response when injected with CCK-4 compared to controls [11]. The question arises if there is a connection between CCK and premenstrual symptoms in humans. In the rat brain CCK-levels vary during the normal estrous cycle. In contrast with what is observed during di-estrous and estrous phases, CCK-like immunoreactivity (CCK-LI) was significantly reduced in the hippocampus, striatum and hypothalamus during pro-estrous, the phase with the highest plasma estradiol levels [9]. Estrogen has also been found to influence CCK-1 and CCK-2 receptor expression [17, 18].

The concentration profile of CCK in plasma during the menstrual cycle in women is still unclear. In 1990, Frick et al found a variation with higher concentrations of CCK in plasma in the luteal phase compared to the follicular phase [5]. In contrast, Le Mellédo et al 1999 could not confirm this variation and found no significant differences between the basal CCK-like immunoreactivity (CCK-LI) in the luteal and follicular phases [11].

Based on the known effect of CCK as a mood peptide and because of a possible relation to the menstrual cycle, we investigated whether CCK-LI varies in plasma during the menstrual cycle in humans. The present study was also designed to investigate if CCK-LI in plasma correlates with CCK-LI in cerebrospinal fluid (CSF) in the follicular phase in healthy young women.

2. Material and methods

2.1 Subjects

Healthy female volunteers were recruited through advertisements at the Linköping University. Fifty-eight women replied. Pregnancy, ongoing severe somatic/physical disease, a history of mental illness, ongoing medication including hormonal contraceptives taken during the preceding three months or participation in other contemporary studies disqualified women from participation. The volunteers were given a medical check-up including physical examination and blood laboratory tests. Eleven were excluded because of a history of mental illness in themselves or in first-degree relatives. Three were excluded because of results from routine laboratory tests and one because of technical difficulties taking venous blood-tests. Forty-three women were included in the study.

2.2 Ethics

Approval for the study was given by the Ethics Committee of the Linköping University Hospital. All subjects gave their informed consent after having been fully informed about the study. The study was conducted according to the principles embodied in the Declaration of Helsinki.

2.3 Study design

Blood samples were drawn on two occasions at 8.00 A.M.. The subjects had fasted and were nicotine-free for at least 8 h (since midnight or earlier) prior to the sampling. The first sampling was done in the late luteal phase (LLP), 9-13 days after a demonstrated surge of luteinizing hormone (LH) in urine indicated by results from the Clearblue ovulation test (Unipath Ltd, Bedford, United Kingdom) The second sampling was done in the follicular phase (FP), 2-6 days after menstruation had started. In 15 subjects both a blood sample and a lumbar puncture sample were drawn at the same time on the second occasion. The lumbar puncture was carried out with local anesthesia with the subject in a left lateral position. First the intraspinal pressure was measured and thereafter 3 x 6 ml CSF was collected and tapping-time was noted. The neuraxis distance (from the external occipital protuberance to the site of puncture) in sitting (ND_S) and lying position (ND_L) were measured. To ensure that the subjects had ovulated, progesterone in plasma was collected 6-8 days after positive LH urine ovulation test as well as at the time of both samplings. Estradiol in plasma was collected at the time of both samplings.

2.4 Analyses

2.4.1 **Cholecystokinin-like immunoreactivity (CCK) in plasma**

Competitive radioimmunoassay of CCK was preformed using antiserum Ab 92128 which measures all molecular forms of CCK without binding any gastrins [19, 21].

500 µL of plasma was extracted with 1000 µL 96% ethanol, incubated, centrifuged, evaporated and resolved in 500 µL assay buffer (0.11 % bovine serum albumin in 0.05 M barbiturate buffer, pH 8.6). Samples of 200 µL were analyzed in duplicates and calibrators of 200 µL were analyzed in triplicates. After addition of 100 µL CCK antiserum , with a final dilution of 1/10 000 in assay buffer, samples were incubated at 6°C for 24 hours. A second incubation in the same manner was performed after addition of 100 µL ^{125}I-CCK (diluted to

5000 cpm ± 10 % in assay buffer). Free and bound tracer was separated using 100 µL anti-rabbit IgG (AA-Sac1 from IDS, Boldon Buisness Park, Boldon, England), incubation at room temperature for 30 min, addition of 1000 µL deionised water and centrifugation (2500 x g, 20 °C, 5 min). The supernatant was discarded and the precipitate measured in a gamma counter (Wizard 1470 Automatic gamma counter, Perkin Elmer, Wallac, Turku, Finland). The coefficient of variation (CV), inter assay was <13 % for controls.

2.4.2 Cholecystokinin-like immunoreactivity (CCK) in CSF

Samples of 200 µL were analyzed in duplicates and calibrators of 200 µL were analyzed in triplicates. After addition of 100 µL CCK antiserum (Ab 92128), final dilution 1/10 000 in assay buffer (0.11 % bovine serum albumin in 0.05 M barbiturate buffer, pH 8.6) samples were incubated at 6°C for 24 hours. A second incubation in the same manner was performed after addition of 100 µL ^{125}I -CCK (diluted to 5000 cpm ± 10 % in assay buffer). Radioactive ligands (CCK tracer) were synthesised using a modified chloramin-T method, purified by RP-HPLC using a µ-Bondapak C18 column; 3.9 x 300 mm, 125 Å, 10 *m, (Waters, Milford, Massachusetts, USA) and diluted 1/10 in acetonitrile and stored at -20°C until use. Free and bound tracer was separated using 100 µL anti-rabbit IgG (AA-Sac1 from IDS), incubation at room temperature for 30 min, addition of 1000 µL deionised water and centrifugation (2500 x g, 20°C, 5 min). The supernatant was discarded and the precipitate measured in a gamma counter (Wizard 1470 Automatic gamma counter). The CV, inter assay was <13 % for controls and the sensitivity for the assay was 0.3 pmol/L.

2.4.3 **Progesterone** was analyzed by chemiluminiscence immunoassay on the Immulite 2500 supplied by Siemens Medical Solutions Diagnostics AB.

2.4.4 **Estradiol** was analyzed by elechtrochemiluminescence immunoassay, ECLIA, on the Roche Modular E170 immunoassay analyser, all supplied by Roche Diagnostics Scandinavia AB, Sweden.

2.5 Statistics

We have used paired t-test, two way ANOVA and a general linear model (GLM) as statistical methods.

3. Results

Forty-three women were included in the study. Five of these women dropped out because of problems returning for the following visits, two because of medication and one due to infection on the second occasion. An additional ten dropped out because of problems with the menstrual cycle, irregular menses, menses started before the first occasion in late luteal phase or ovulation could not be demonstrated by LH urine ovulation test or plasma progesterone levels. The remaining subjects were 25 healthy women, age 25.0 ± 3.5 (Table 1).

Progesterone concentrations in plasma decreased significantly from luteal phase (LP, mean±SD, 31,8±12,6) and late luteal phase (LLP, 27,6±14,3) to follicular phase (FP 1,9±0,8) (paired t-test, $p<0,001$). Estradiol levels in plasma significantly decreased from LLP (664±446) to FP (157± 99) (paired t-test, $p<0,001$).

On comparing CCK-LI concentration in plasma in LLP (n=25, mean ±SD, 0,656±0,268 pmol/L) and FP (0,672±0,295 pmol/L) with two way ANOVA, no significant difference was found (R^2=89, 60%). Analyzing the inter-individual difference in CCK-LI in plasma in a general linear model with the factors tobacco use, month of testing and occasion as fixed

factors and age, height, weight, progesterone- and estradiol levels as covariates, weight showed a significant influence on the difference ($p<0,05$, $n=25$) but could not explain the whole inter-individual difference, R^2 was reduced to 18,55%, (Table 2).

The CCK-LI concentration in CSF in the follicular phase was 0,68±0,17 pmol/L (mean±SD, n=15). A comparison by means of two way ANOVA demonstrated that CCK-LI significantly increased from the first to the second and third 6ml CSF-fraction ($n=15$, $R^2=88,43$, $p<0,001$). By means of a general linear model analyzing tobacco use and month of testing as fixed factors and age, height, weight, progesterone- and estradiol levels, neuraxis distance in sitting (ND_S) and lying (ND_L) position and tapping-time as covariates, progesterone level and ND_S had a significant influence on CCK-LI in CSF ($n=15$, $p<0,05$ and $p<0,001$, respectively, $R^2=61,04\%$,), (Table 2).

In FP no significant difference was found comparing CCK-LI in plasma and CSF using two way ANOVA ($n=15$, $R^2=55,32\%$) (Table 2).

4. Discussion

To our knowledge this is the first study of possible relations of the concentrations between CCK-LI in plasma and CSF in healthy female humans. Thus, we found no significant difference when comparing CCK-LI in plasma and CSF in fifteen healthy young women in follicular phase (FP) (with low progesterone and estradiol levels in plasma). Since there are formerly established known connections between estradiol and CCK in the brain [7, 9, 14] we aimed to investigate if the CCK-LI in CSF varies during the menstrual cycle in healthy women. However, because an earlier study has shown a high frequency of post-lumbar-

puncture headaches in women in the luteal phase compared to follicular phase [15] we chose to do lumbar puncture only in the early follicular phase in this first study.

If the correlation of CCK-LI in CSF and plasma that we observed can be confirmed under other circumstances such as for instance during a menstrual cycle, this will indicate that CSF levels of CCK-LI concentration can be measured indirectly in the plasma of females. In earlier studies a diurnal variation of CCK has been seen both in CSF in healthy fasting men [8] and in plasma in both men and women, as well as a seasonal variation of CCK-LI in plasma at 12.00 noon but not at 08.00 a.m. [12]. In the present study the month of testing did not influence the CCK-LI concentration in plasma at 08.00 a.m., supporting earlier findings of no seasonal variations at 08.00 a.m.

No difference was found between CCK-LI concentrations in plasma at the different stages of the menstrual cycle. This supports the findings of Le Mellédo et al 1999, who described a stable baseline CCK-LI independent of menstrual phase [11]. Frick et al had earlier found a variation in plasma with higher concentrations of CCK in LP than in FP in women in 1990 [5], however, this study was small, made before more specific CCK analysis methods had been discovered, and may explain our divergent findings. In any case, neither we nor Le Mellédo [11] could repeat the findings of Frick.

Our results also indicate a possible effect of weight on plasma CCK-LI concentration in humans, with lower CCK-LI with increasing weight. This is in line with earlier findings of increased CCK-response to food intake in patients with anorexia nervosa and reduced weight compared to controls [6] as well as lower fasting CCK-levels and lower plasma CCK-

response to food intake in morbidly obese women (BMI=46,9±1,6) compared to lean controls (BMI=23,2±0,7) [25].

This study of healthy young women indicates a correlation between CCK-LI concentration in plasma and in CSF in the early follicular phase. One question is how much the CCK-LI in CSF can be considered to reflect the intricate activity of CCK in the brain - which could mean that CSF levels of CCK can be measured indirectly in plasma. Although CCK is the most abundant neuropeptide system in the human central nervous system the CSF concentrations are very low and many less abundant cerebral peptides have considerably higher CSF concentrations. Rehfeld hypothesised that a substantial degradation might occur on the way from the CCK-release to the analysis of CSF in the laboratory and that new methods are needed [21]. However, since the CCK-system is widespread in the brain and involved in many different psychological and physiological functions in human it would be a very interesting field for further investigation. As one future perspective it would be interesting to obtain more information about the concentration of CCK-LI in plasma and CSF in females to determine if, for example, delayed effects of estradiol and progesterone levels do occur.

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