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Original article

Plasma cholecystokinin concentrations in 3-day-old lambs: effect of the duration of fasting preceding a sucking bout

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Summary – Cholecystokinin (CCK) is a regulatory peptide released into the circulation after the ingestion of food. Our knowledge concerning the factors affecting its pattern of secretion in the mammalian neonate is scanty. Therefore, in this work we studied plasma concentrations of CCK in 3-day-old lambs after a sucking bout that followed either a short (2 h) or a long fasting period (9 h). Plasma concentrations of CCK were measured by radioimmunoassay in blood samples collected from jugular veins 10 min before, immediately after a sucking bout, and then at 10-min intervals during the following hour. In the first experiment a period of fasting of 2 h before allowing the lambs to suck did not lower the levels of CCK. On the other hand, a significant decrease was recorded 10 min after feeding ended. Between 20 and 50 min after the end of sucking, the mean CCK concentration rose back to the pre-feeding level before decreasing again at 60 min. In the second experiment a period of fasting of 9 h, on the other hand, significantly lowered the levels of CCK. Plasma levels also tended to decrease immediately after sucking was ended. This was followed by a substantial rise and levels remained high during the rest of the sampling period. It was concluded that the decrease recorded immediately after a sucking bout could be due to an inhibitory effect of the oral phase while the following rise reflected the stimulatory effect of milk nutrients on intestinal CCK-producing cells.

lamb / new-born / cholecystokinin / sucking / fasting

Résumé – Effet de l'intervalle entre tétées sur les concentrations plasmatiques de cholécystokinine chez l'agneau nouveau-né. La cholécystokinine (CCK) est un peptide régulateur libéré dans la circulation après une prise alimentaire. La connaissance des facteurs influençant son profil de

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sécrétion chez le jeune mammifère reste assez limitée. C'est pourquoi nous avons mesuré les niveaux plasmatiques de CCK chez des agneaux âgés de 3 j après une tétée qui suivait une période de jeûne brève (2 h) ou longue (9 h). Les taux de CCK ont été détectés par dosage radio-immunologique à partir d'échantillons sanguins prélevés au niveau de la veine jugulaire 10 min avant, immédiatement après la tétée, puis toutes les 10 min au cours de l'heure qui suivait cette prise alimentaire. Une période de jeûne de 2 h n'a pas provoqué de diminution des taux de CCK. En revanche, une chute significative a été notée dans les 10 min qui ont suivi la fin de la tétée. Les taux de CCK sont ensuite revenus à leurs valeurs initiales avant de diminuer à nouveau 60 min plus tard. Un jeûne de 9 h, en revanche, a provoqué une chute significative des taux de CCK plasmatique. Une tendance à la baisse fut également observée immédiatement après la fin de la tétée. Elle a été immédiatement suivie par une augmentation importante des taux de CCK qui sont restés élevés par la suite. Nous avons conclu que la chute transitoire observée en fin de tétée pourrait être due à un effet inhibiteur de la phase orale alors que l'élévation qui suit reflète l'effet stimulateur des constituants du lait sur les cellules endocrines intestinales.

agneau / nouveau-né / cholécystokinine / tétée / jeûne

INTRODUCTION

The gastrointestinal hormone cholecystokinin (CCK) is produced in endocrine cells of the proximal intestine and is released into the circulation after the ingestion of food (Walsh, 1987). In the adult, its postprandial secretion varies according to the anatomical structure of the gastrointestinal tract. In monogastric mammals, plasma concentrations have been shown to rise in response to feeding (human: Liddle et al, 1984; Höcker et al, 1992; dog: Lindén and Uvnäs-Moberg, 1987; Reidelberger et al, 1989; cat: Backus et al, 1995; rat: Rodger et al, 1984; Lewis and Williams, 1990; pig: Cuber et al, 1990). On the other hand, in polygastric mammals, the concentrations do not change after a feed (cow: Furuse et al, 1991; goat: Furuse et al, 1992). This differential response might reflect the continuous flow of digesta from the rumen independently of the feeding pattern for the polygastric animals, whereas in monogastric animals the flow of digesta is directly linked to the meal size of the individual.

In the neonate, although interspecific comparisons are rather difficult to make since studies are not as numerous as in the adult, it has been shown that CCK is released into the plasma immediately after a

feed both in human babies, a monogastric species (Marchini and Lindén, 1992; Uvnäs-Moberg et al, 1993), and in preruminant calves, a polygastric species (Toullec et al, 1992; De Passilié et al, 1993). At first sight, the dichotomy observed in the adult is non-existent in the young and this would appear logical since in polygastric species the rumen is not functional as long as no solid food is ingested. Liquid food bypasses the reticulorumen, goes directly into the abomasum and is digested as in monogastric animals. On the other hand, one factor that could influence the profile of CCK in the new-born is the feeding frequency. In the work carried out on calves, the animals were fed twice a day (Toullec et al, 1992; De Passilié et al, 1993), which is far less than the feeding pattern of calves when milk is available *ad libitum* (Hammel et al, 1988). Studies of the dynamics of abomasal emptying in calves fed 4–6 kg of milk twice a day showed that although the digesta was not exempt of proteins and lipids 7 h after feeding, the outflow had returned to its pre-feeding value, which was much lower than within the hour of feeding (Toullec and Mathieu, 1973). Since the intensity of the abomasal flow declines over time, the endocrine cells of the duodenum will respond differently (Guilloteau et al, 1995)

and as a consequence, the postprandial release of CCK will be enhanced after the ingestion of a liquid meal

The young lamb is a good model to study the plasma concentration of CCK in relation to feeding frequency. In the first week of life the lamb sucks its mother as frequently as once an hour (Munro, 1956). Such a high frequency of feeding raises questions regarding the pattern of CCK concentration after food ingestion since in these conditions the lamb is likely to feed before gastric emptying is fully achieved. The aim of this work was to characterise plasma CCK responses in 3-day-old lambs after a sucking bout that followed either a short or a long duration of fasting. In the first experiment, lambs were fasted for 1 h, a duration chosen because it mimicked the interval between two sucking episodes. In the second experiment, lambs were fasted overnight (9 h) to ensure major emptying of the gastrointestinal tract.

MATERIALS AND METHODS

Animals

Ten Préalpes-du-Sud lambs were used in the first experiment (five males and five females) and eight lambs in the second experiment (four males and four females). They were all born after inducing parturition with 16 mg of dexamethazone on the 143rd day of gestation. None of these lambs experienced a difficult birth. Their mean (\pm sem) birth weights were 4.51 kg (\pm 0.13) and 4.48 kg (\pm 0.11), respectively, and were 3 days old at the time of the experiment.

Experimental procedure

Lambs were raised in individual pens with their mothers. In order to stimulate their feeding behaviour, lambs were fasted by being put in a small cage inside their mothers' pen. In the first experiment, lambs were fasted for 1 h. They were then released and allowed to suck ad libitum. Five lambs out of ten refused to feed and were

fasted for another hour so that the experiment had to be run with two groups of animals. The mean sucking times were 153 s (\pm 22) and 136 s (\pm 20) for the lambs fasted for 1 or 2 h, respectively. In the second experiment, lambs were fasted overnight (9 h). To standardise sucking times with the previous experiment, lambs were allowed to suck for a maximum of 3 min. Only two lambs out of eight were still sucking by the end of the 3-min period. The mean sucking time was 162 s (\pm 26). Lambs were put back into their small cages at the end of the sucking bout.

Samples collection and radioimmunoassay of CCK

Samples of 5 mL of blood were taken from jugular veins at the following times: before fasting started, 10 min before sucking, immediately after sucking ended, and then 10, 20, 30, 40, 50 and 60 min later. They were collected into glass tubes containing 10 iu heparin and 500 KIE of aprotinin (Trasylol, Bayer Diagnostics, Puteaux, France) per millilitre blood, kept on ice and centrifuged at 4 °C within 30 min of collection. Plasma was removed and stored at -20 °C until analysed for CCK by radioimmunoassay.

Before determination, CCK was separated from plasma proteins using SEP-PAK C-18 cartridges (Water Assoc Inc, Milford, PA, USA). The cartridges were equilibrated with 100 mL 100% acetonitrile (v/v), followed by 10 mL 0.1% acetic acid (v/v) before the application of 2 mL plasma samples. After washing with 5 mL 0.1% acetic acid, the samples were eluted with 6 mL of acetonitrile and 0.1% acetic acid (1:1, v/v). For determination of CCK, the purified samples were dissolved in half their original volume and thereby concentrated two times. This was later corrected for. Radioimmunoassay was performed as described by Himeno et al (1983) using anti-serum OAL-656 (Otsuka Pharmaceutical Co Ltd, Tokushima, Japan), which recognises the sulphated forms of CCK-8 and -33, -39 region specifically, and tracer 125 I-CCK (Dupont NEN Research Products, Boston, USA). Free and bound CCK were separated by precipitation of rabbit antibody using Decanting Suspension 3, a sheep anti-rabbit antibody (Kabi Pharmacia Diagnostics, Uppsala, Sweden). The cross reactivity with CCK-8 nonsulphated, tetragastrin, and gastrin is negligible. The limit of detection was 2.5

pmol/L. The intra-assay and inter-assay coefficients of variation were 8 and 11%, respectively.

Statistical analysis

Data are presented as means \pm sem. Comparisons were made using the Kruskal–Wallis test for unrelated samples (1 h versus 2 h of fasting in experiment one). For related samples the Friedman test was chosen followed by the Wilcoxon test for pairwise comparison. The level of significance was set at $P = 0.05$.

RESULTS

In the first experiment, the hormonal profiles did not differ between the two groups of lambs nor between males and females; therefore data were pooled for statistical analysis. Consequently, data obtained at -120 min and -60 min were averaged and given a time value of -90 min (fig 1A). The Friedman test showed significant changes in CCK plasma levels over time ($P < 0.01$). The mean CCK concentration did not vary over the 1 or 2 h preceding the feeds: levels were $21.7 (\pm 3.2)$ and $21.1 (\pm 2.5)$ pmol/L before and at the end of fasting (fig 1A). On the other hand a significant decrease was recorded just after sucking finished and the level still remained low 10 min later ($P < 0.05$ in both cases). Thereafter the concentration rose (at 20, 30, 40 and 50 min it was not significantly different from the pre-feeding level) and decreased significantly again 60 min after sucking ($P < 0.05$).

In the second experiment, the hormonal profiles did not differ either between males and females and data were pooled for statistical analysis. Levels of CCK were significantly affected by the treatment (fig 1B; $P < 0.001$). The concentration was significantly lower after than before 9 h of fasting (20.1 ± 2.7 versus 5.5 ± 0.9 ; $P < 0.01$) and a further decrease, though not significant compared to the level 10 min before sucking started, was recorded immediately after

sucking ended (5.5 ± 0.9 versus 3.8 ± 2.2). This was followed by a substantial rise 10 min later and thereafter CCK plasma levels remained significantly higher than prior feeding or just after sucking had ended ($P < 0.05$ in all cases). Concentrations of CCK recorded at 30, 40, 50 and 60 min were not significantly different from the pre-fasting level.

DISCUSSION

This study clearly demonstrates that the postprandial concentration of CCK in the lamb varies according to the duration of the fasting period preceding the feed. Nine hours of fasting enhances the postprandial release of CCK into the plasma, whereas this response is characterised by very little change after 1–2 h of fasting. Three main points can be drawn from our experiment.

The first finding is that sucking is immediately followed by a transitory decrease in CCK after which the concentrations rise back to pre-fasting levels. This, however, was only significant in experiment one. Fasting is unlikely to be the direct cause of this decrease as levels did not vary during the 1–2 h preceding access to the mother's udder. Rather, this initial CCK response would reflect an activation of vagal efferents during the oral phase of sucking. Data obtained in other species have described a release of CCK due to a cephalic–vagal activation. In adult dogs, sham feeding causes a release of CCK (Schafmayer et al, 1988). In pre-ruminant calves, De Passillie et al (1993) found that non-nutritive sucking after consuming milk from a bucket further increases CCK concentrations in the portal vein. In new-born babies, Uvnäs-Moberg et al (1993) found two peaks of secretion (one immediately after completion of feeding, and another 30–60 min later) and conclude that sucking activation of the vagal efferents is responsible for the occurrence of the first. In contrast, the significant decrease

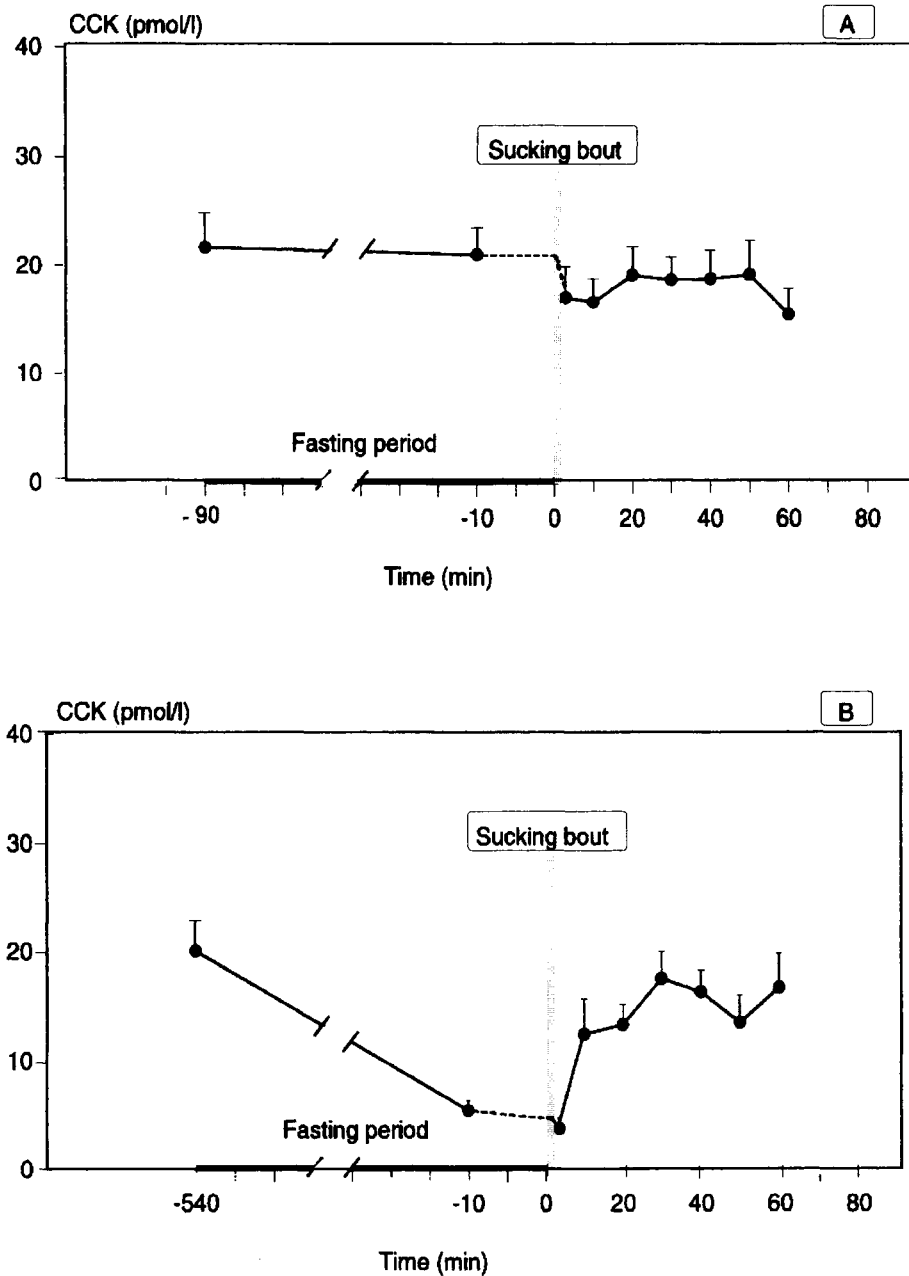


Fig 1. Mean (\pm sem) concentrations of CCK in the 3-day-old lamb before and after sucking. (A) In the upper graph the lambs had been fasted for 1–2 h before having access to their mothers' udder. (B) In the lower graph the lambs had been fasted for 9 h. Sucking started at $t = 0$ min and ended at $t = 3$ min at which time the first post-feeding blood sample was taken.

observed in 3-day-old lambs could well reflect a cephalic–vagal inhibition on the CCK secreting cells. This would not be abnormal as a similar temporary decrease in plasma CCK has been described in adult goats after a meal supplemented with dietary proteins (Furuse et al, 1992). Furthermore, it must be stressed that in the work by Furuse et al (1992) this effect was obtained only with oral and not with intraduodenal infusion of amino acid solutions. Also, work carried out on adult rats showed that administration of atropine causes a rise of basal CCK levels indicating the presence of a cholinergic inhibitory tone on CCK release (Uvnäs-Moberg et al, 1992). The vagal nerve would therefore play a modulatory role, causing either a stimulation or an inhibition of CCK release, in a similar way to that which has already been described for gastrin (Walsh, 1987). In our situation the decrease in plasma CCK was only clearly recorded in lambs fasted for 1–2 h when pre-feeding CCK levels were high. After 9 h of fasting, plasma levels might have been too low to lead to the same effect, although a small non-significant decrease was also recorded.

The second finding is the demonstration of a release of plasma CCK in response to a sucking bout. In experiment two, the rise 10 min after feeding was particularly notable since CCK plasma levels were depleted by 9 h of fasting. In experiment one, plasma levels only rose slightly between the onset of sucking and 20 min later because a temporary decrease was observed at the onset of sucking. Previous work carried out on pre-ruminant calves fed twice a day has demonstrated that plasma CCK, which rises sharply after milk ingestion, falls back to its pre-feeding levels between 6 and 7 h later. Therefore, it is not surprising that no major variation was observed in experiment one as the lambs' secreting cells were still on a stimulatory phase after 1–2 h of fasting. In both experiments, however, postprandial levels of CCK rose back to pre-fasting con-

centrations and never above. This postprandial increase most likely reflects the stimulatory effect of milk on the endocrine cells as digesta passes through the duodenum within minutes after feeding has ended (Newhook and Titchen, 1974). Very little is known about the factors in milk that are responsible for the releasing effect in the young. As in the adult, digestion products of lipids and proteins are likely to be the effective stimulant as it has been shown in the calf that caseinomacropptide, which is a fragment of kappa-casein released during the gastric digestion of milk, stimulates CCK secretion (Guilloteau et al, 1994). The absence of a substantial increase in CCK induced by feeding when lambs were fasted for 1–2 h could be ascribed to the high basal concentrations of CCK that are present during the neonatal period. Plasma CCK concentrations increase tremendously in the first day of life owing to the lambs' sucking activity (Nowak et al, 1997). Such postprandial response is not seen on the third day unless lambs are previously fasted for 9 h. This suggests that the high plasma concentrations recorded before feeding in three-day-old lambs reflect a high degree of stimulation of the CCK producing cells during a period of life when the intake of nutrients is important. In this regards, the response obtained in lambs is very different to that described in human babies. Plasma CCK levels increase after feeding in 3-day-old infants who are breast-fed on demand (Marchini and Lindén, 1992; Uvnäs-Moberg et al, 1993). Under such conditions, the feed-to-feed intervals are very short, ranging from 1–3 h, which is very similar to those observed in lambs of the same age (Munro, 1956). The reason why the postprandial response is so dissimilar lays in the fact that woman's milk does not contain as much casein as ewe's milk and clots much less in the stomach (Luquet, 1985). Gastric emptying occurs more rapidly and human babies are more likely to have an empty stomach 3 h after the previous feed than would lambs

be. The decrease observed 60 min after feeding in experiment one is unclear. It could be that most nutrients ingested during the sucking bout (at $t = 0$ min) and acting on CCK release might have passed through the duodenum by the end of our sampling period (at $t = 60$ min). However, this does not appear logical since CCK levels were not previously affected by 1 or 2 h of fasting. A temporary variation in plasma CCK release due to irregular outflow of digesta from the abomasum could be another hypothesis.

The third finding is that a period of fasting of 2 h does not lower plasma levels of CCK. This could be explained by the important milk intake of such young lambs. In the first week of age a lamb sucks its mother hourly (Munro, 1956) and ingests an average of 1200 g milk per day (Villette and Thériez, 1983). Milk clots in the abomasum, which slows down the release of protein and fat into the duodenum (Frantzen et al, 1973). As a consequence, the abomasum is refilled with milk before being fully emptied and the CCK endocrine cells are permanently stimulated by the regular flow of nutrients. Therefore 1–2 h of fasting were insufficient to empty the stomach of our lambs and lower their levels of CCK. On the other hand, the low concentrations of CCK observed in experiment two reflect well the fact that the gastrointestinal tract was much less stimulated by nutrients after 9 h of fasting. In experiment one, the high pre-feeding levels of plasma CCK did not prevent the lambs from sucking. Although it has been suggested that CCK might be a factor regulating food intake in the new-born infant (Marchini and Lindén, 1992) as it does in the adult (Crawley and Corwin, 1994), there is evidence in animal models that hunger mechanisms are not fully functional in the suckled young or do not involve CCK (Blass et al, 1979; Lorenz, 1994). In any case, CCK is unlikely to be a satiation factor in the young lamb since in experiment two most

lambs stopped feeding before any increase was recorded in the plasma.

In conclusion, the present work demonstrates that postprandial plasma concentrations of CCK in 3-day-old lambs vary greatly whether the sucking bout is followed either by a short or a long fasting period. This response so dissimilar to the one recorded in human babies of the same age (Marchini and Lindén, 1992; Uvnäs-Moberg et al, 1993) shows that important interspecific variations exists even in the neonatal period.

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