Effects of naloxone on circulating gonadotrophin concentrations in prepubertal heifers

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Summary. The pattern and opioidergic control of the secretion of gonadotrophins in prepubertal heifer calves were examined. Ten age-matched Hereford heifer calves were weighed and a blood sample was taken every 2 weeks from 2 to 25 weeks of age and then weekly until 60 weeks of age. At 60 weeks, a fertile bull was introduced and at 75 weeks of age pregnancy diagnosis was performed by transrectal ultrasonography. At 4, 12, 18, 24 and 32 weeks of age, the opioid antagonist naloxone was injected (i.v., $n = 5$; 1 mg kg^{-1} body weight) each hour for 12 h. Control heifers received sterile saline at the same ages. Blood samples were collected every 12 min for the 12 h treatment and serum samples were analysed for luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Samples taken once every 2 weeks from 2 to 60 weeks were analysed for LH, FSH and oestradiol, and weekly samples were taken for progesterone determination. There was no effect of naloxone on the age at puberty, which was 56.2 ± 0.7 weeks at a body weight of 388.5 ± 8.0 kg. The mean age at conception was 63.4 ± 0.5 weeks. On the basis of samples taken every other week, serum concentrations of LH were high at 10 weeks and between 40 and 60 weeks of age. From the periods of intensive blood collection, the early rise in mean serum concentrations of LH appeared later at 12 and 18 weeks of age and was caused by a rise in LH pulse amplitude. Serum FSH concentrations were increased between 20 and 22 weeks and oestradiol concentrations at 22, 56 and 58 weeks of age. At 4 weeks, naloxone increased mean LH concentrations, pulse amplitude and pulse frequency ($P < 0.01$) and thereafter only decreased LH pulse amplitude at 18 weeks ($P < 0.05$) and increased LH pulse frequency at 24 weeks ($P < 0.05$). The FSH secretion was pulsatile at all ages and naloxone only increased FSH pulse amplitude at 4 weeks.

From these data we conclude that (i) there is an early transient increase in gonadotrophin secretion in prepubertal heifers, (ii) significant opioidergic inhibition of gonadotrophin secretion occurs only in very young heifers and (iii) a decrease in endogenous opioid inhibition of LH secretion, particularly LH pulse amplitude, allows for the early rise in LH secretion.

Keywords: puberty; gonadotrophins; naloxone; heifer

Introduction

The mechanisms controlling the temporal pattern of circulating concentrations of gonadotrophins in prepubertal heifer calves are unknown. It appears that the pituitary and gonads in such calves can function at a very early age, since the pituitary is responsive to exogenous gonadotrophin-releasing hormone (GnRH) (Barnes *et al.*, 1980) and fertile ovulations can be induced in very

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young heifers using exogenous gonadotrophins (Onuman *et al.*, 1970; Seidel *et al.*, 1971). Early hypothalamic inactivity may prevent gonadotrophic drive to the ovaries, before the increase that precedes sexual maturation (Dyer *et al.*, 1990). Some authors suggest that there is an early rise in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) at about 20 weeks of age (Schams *et al.*, 1981); another report suggests that plasma LH concentrations decrease from birth to 15 weeks of age, and thereafter increase to 39 weeks and that FSH follows a similar pattern (Dodson *et al.*, 1988). In bull calves, we have observed an early transient rise in gonadotrophin secretion (Rawlings *et al.*, 1978).

There is convincing evidence that opioids play a role in regulating gonadotrophin secretion during the prepubertal period in rats (Wilkinson & Bhanot, 1982; Bhanot & Wilkinson, 1983). The results are not consistent between reports, but there is general agreement that there are sexual differences, and that opioids play a much greater role in female than in male rats (Schulz *et al.*, 1982; Bhanot & Wilkinson, 1983; Cicero *et al.*, 1986). Puberty has been induced in female rats by the administration of the opioid antagonist naloxone, and on the basis of this it was suggested that the removal of an inhibitory opioid 'brake' triggers the onset of puberty in female rats (Sirinathsinghji *et al.*, 1985). Studies on the role of opioids in sexual maturation in farm animals have been mainly confined to ewe lambs and have also produced inconsistencies (Haynes *et al.*, 1989; Rawlings & Churchill, 1990). In our data (Rawlings & Churchill, 1990), little opioidergic suppression of LH secretion was seen in ewe lambs until the last 10 weeks before first oestrus; in the study of Haynes *et al.* (1989), no opioidergic effect was seen until the ewes were sexually mature. Opioid receptors have been measured in fetal and newborn lambs and adult ewes; the concentrations increase through fetal life reaching concentrations in the newborn that are higher than those found in adults (Villiger *et al.*, 1982).

The present experiment investigated whether an early transient increase in circulating concentrations of gonadotrophins occurs in prepubertal heifers and studied the role of endogenous opioids in regulating the early onset and changes in gonadotrophin secretion.

Materials and Methods

Animals

Ten age-matched (± 5 days) Hereford heifer calves were maintained in a beef management system; calves were nursed at pasture until weaning at 21 weeks of age and then maintained in a paddock and fed a ration of 21% ground barley, 51% ground hay, 22% ground straw and 0.005% of a 1:1 calcium phosphorus mineral mix (w/w) *ad libitum*, with brome and lucerne hay and water also freely available. Each calf was weighed and a single blood sample drawn by jugular venepuncture every two weeks from 2 to 25 weeks of age; then samples were taken once a week until 60 weeks of age. At 60 weeks of age, a fertile bull was introduced and at 75 weeks of age pregnancy diagnosis was performed by transrectal ultrasonography and conceptus age was estimated.

Naloxone treatments

The calves received hourly intravenous bolus injections of naloxone in saline ($n = 5$; 1 mg kg⁻¹ body weight h⁻¹; Research Biomedicals Inc., Natick, MA, USA) or physiological saline solution ($n = 5$) for 12 h at 4, 12, 18, 24 and 32 weeks of age. On the day before treatment, the calves were fitted with indwelling jugular vinyl tubing catheters (SV-70; i.d. 1.0 mm, o.d. 1.5 mm; Dural Plastics and Engineering, Dural, NSW, Australia). Throughout the 12 h infusion period, 4 ml blood samples were drawn every 12 min. Cows were housed in stalls indoors overnight, with their calves loose and free to nurse, before the day of blood sampling. Weaned calves were cannulated in a similar way and housed unrestrained overnight.

Blood handling and radioimmunoassays

All blood samples were allowed to clot at room temperature for 12–18 h; the clots were removed and, after centrifuging at 1500 g for 20 min, the serum was poured off and stored at -20°C until analysis. Weekly blood samples were collected from the heifers in sodium heparinized vacutainers and spun within 20 min of collection; the plasma was aspirated and stored at -20°C until analysis.

LH, FSH and progesterone. Concentrations of LH, FSH and progesterone in serum were all determined in double-antibody radioimmunoassays using iodinated tracers (Rawlings *et al.*, 1984). LH concentrations are expressed in terms of NIDDK-bLH4. The range of the standard curve was from 0.06 to 8 ng ml⁻¹. The sensitivity of the assay was 0.1 ng ml⁻¹; this was the lowest concentration of unlabelled LH that could displace iodinated LH from the first antibody ($P < 0.05$ by *t* test). Intra- and interassay coefficients of variation (CVs) were 12.3 and 18.7%, respectively, (mean = 2.90 ng ml⁻¹ serum) and 11.2 and 18.6% (mean = 0.71 ng ml⁻¹ serum). For the FSH assay the first antibody used was NIDDK-anti-oFSH-1, and FSH concentrations are expressed in terms of USDA-bFSH-II. The range of the standard curve was 0.13–16 ng ml⁻¹. The sensitivity of the assay was 0.2 ng ml⁻¹ ($P < 0.05$). Intra- and interassay CVs were 9.1 and 14.0%, respectively, (mean = 3.10 ng ml⁻¹ serum) and 9.1 and 16.7% (mean = 1.69 ng ml⁻¹ serum). The range of the progesterone standard curve was 0.1–10 ng ml⁻¹; the sensitivity of the assay was 0.1 ng progesterone ml⁻¹ of plasma. Intra- and interassay CVs were 5.1 and 15.5% (mean = 5.19 ng ml⁻¹ plasma) and 4.5 and 15.8 (mean = 2.16 ng ml⁻¹ plasma).

Oestradiol. Oestradiol was measured by radioimmunoassay (Joseph *et al.*, 1992). The range of the standard curve was 1–100 pg ml⁻¹ and the assay sensitivity was 1 pg oestradiol ml⁻¹ serum. The intra- and interassay CVs were 9.4 and 11% (mean = 18.1 pg ml⁻¹ serum) and 15 and 22% (mean 6.9 pg ml⁻¹ serum).

Statistical analysis

The episodic secretion of LH and FSH during the 12 h intensive bleeds were determined using PC-Pulsar (J. Gitzen & V. Ramirez, University of Illinois, USA). Tonic LH and FSH secretion is expressed in terms of basal and mean circulating concentrations, pulse amplitude and pulse frequency for the 12 h periods of intensive blood sampling. Episodes were identified using standard deviation criteria (Meriam & Wachter, 1982) and basal concentrations were determined by subtraction of episodes from the profile.

Comparison of the LH and FSH data from the intensive bleeds between control and naloxone-treated heifers at each age was performed by repeated measures ANOVA on the Statistical Analysis System (SAS, Version 6). Student–Newman–Keuls test for least-significant difference ($P < 0.05$) was used as the postANOVA test. The annual profiles for mean serum concentrations of FSH, LH and oestradiol were analysed using repeated measures ANOVA (True Epistat, Epistat Services, Richardson, Texas, USA) and Student–Newman–Keuls ($P < 0.05$ and $P < 0.01$) was used to determine age effects on the secretion of these hormones. The annual profiles for mean serum concentrations of LH, FSH and oestradiol were also blocked into 6-week periods and the analysis was repeated.

Results

The mean ($\pm$ SEM) birthweight was 40.3 ± 1.4 kg and the average growth rate between birth and 56 weeks was 0.97 ± 0.10 kg day⁻¹. The onset of puberty was taken to be the first age at which progesterone concentrations exceeded 0.4 ng ml⁻¹ of plasma and was 56.2 ± 0.7 weeks at a body weight of 388.5 ± 8.0 kg. Five of the ten animals showed concentrations of progesterone > 0.25 ng ml⁻¹ plasma for 1 to 2 weeks immediately before the onset of puberty. The mean age at conception was 63.4 ± 0.5 weeks.

Statistical analysis across all data points (Fig. 1) showed a significant effect of time ($P < 0.001$) in the FSH and oestradiol profiles, but not for the LH profile ($P = 0.09$). FSH concentrations at 20 and 22 weeks of age were different ($P < 0.05$) from 70% of all other points and the oestradiol concentration at 22 weeks was significantly greater ($P < 0.05$) than the nadirs at 2 and 38 weeks, but less than ($P < 0.05$) the concentrations at 56 and 58 weeks of age. When blocked into 6-week periods, a significant effect of time ($P < 0.001$) was noted in the annual profiles of LH, FSH and oestradiol. LH concentrations between 8 and 14 weeks of age were higher than those at 2 to 8 weeks of age ($P < 0.01$) and 14 to 36 weeks of age. LH concentrations from 38 to 60 weeks of age were higher ($P < 0.05$) than between 14 and 36 weeks. When the FSH data were blocked into 6-week periods, FSH concentrations between 18 and 24 weeks were higher than those at all other ages ($P < 0.01$).

Examination of the 12-h LH profiles for control heifers across ages showed that mean circulating concentrations of LH and LH pulse amplitude were higher ($P < 0.05$) at 12 and 18 weeks of age than at 4, 24 and 32 weeks of age (Fig. 2). There were no differences in FSH secretory patterns among ages ($P > 0.05$) (Fig. 3). At 4 weeks of age, the naloxone treatment resulted in an increase in mean LH concentrations ($P < 0.05$), LH and FSH pulse amplitude ($P < 0.01$) and LH pulse frequency ($P < 0.01$) (Fig. 4). Thereafter, naloxone administration decreased only LH pulse amplitude at 18 weeks ($P < 0.05$) and increased LH pulse frequency at 24 weeks of age ($P < 0.05$).

Fig. 1. Mean ($\pm$ SEM) concentrations of (a) luteinizing hormone (LH), (b) follicle-stimulating hormone (FSH), (c) oestradiol and (d) progesterone for Hereford heifers from 2 to 60 weeks of age ($n = 10$). P = puberty at 56.2 ± 0.7 weeks. Arrows indicate the ages at which intensive bleeds were performed.

Discussion

Available data suggest that the pituitary and ovaries of prepubertal heifers are functional before oestrous cycles are initiated, since pituitary responses to GnRH have been reported (Barnes *et al.*,

Fig. 2. Mean (a) and basal (b) luteinizing hormone (LH) concentrations and LH pulse amplitude (c) and frequency (d) (mean $\pm$ SEM) in heifers treated with naloxone (□, $n = 5$; 1 mg kg^{-1} body weight h^{-1}) or saline (■, $n = 5$) for 12 h at 4, 12, 18, 24 and 32 weeks of age; blood samples were taken every 12 min. Significant differences between treatment and control groups at an age are shown by * $P < 0.05$ and ** $P < 0.01$. Control groups with different superscripts are significantly different ($P < 0.05$).

1980) and exogenous gonadotrophins can cause superovulatory responses in 4- and 8-week-old calves (Seidel *et al.*, 1971). Schams *et al.* (1981) describe prepubertal basal concentrations of gonadotrophins as exhibiting a biphasic profile, a rise and fall in LH and FSH occurring between birth and 20 weeks of age. However, Dodson *et al.* (1988) considered that serum LH and FSH concentrations decreased from birth to 15 and 19 weeks, respectively, and then increased to 39 weeks of age. Our data (Fig. 1) showed circulating LH concentrations to be high at 10 weeks of age, based on blood samples taken once every 2 weeks. An intensive bleed was not conducted at this time, but the intensive bleeds at 12 and 18 weeks showed an increase in mean LH concentrations as a result of an increase in LH pulse amplitude (Fig. 2). The reason that this is not evident in the annual profile is probably because of the inaccuracies involved in determining the concentrations of a pulsatile hormone based on single samples, particularly when very high-amplitude but low-frequency pulses are involved. In addition, the stress of obtaining single samples may have reduced LH secretion at this time by interfering with GnRH secretion (Rivier & Rivest, 1991). We have observed these kinds of discrepancies between LH patterns in blood samples collected weekly and during intensive sampling previously (Rawlings *et al.*, 1978). However, despite the variance in the data due to high-amplitude pulses, we consider that there is a short-lived early rise in mean circulating LH concentrations in heifer calves detected at 10 weeks in blood samples taken once every 2 weeks, but more accurately at 12 and 18 weeks by intensive sampling.

Fig. 3. Mean (a) and basal (b) follicle-stimulating hormone (FSH) concentrations and FSH pulse amplitude (c) and frequency (d) (mean $\pm$ SEM) in heifers treated with naloxone ($\square$, $n = 5$; 1 mg kg^{-1} body weight h^{-1}) or saline ($\blacksquare$, $n = 5$) for 12 h at 4, 12, 18, 24 and 32 weeks of age; blood samples were taken every 12 min. **Significant difference between treatment and control groups ($P < 0.01$).

Mean circulating concentrations of FSH also showed an early increase at about 22 weeks of age, based on single samples taken once in 2 weeks (Fig. 1). The intensive sampling periods did not occur during this rise, but sampling was done on the increase at 18 weeks and the decrease at 24 weeks of age. Mean FSH concentrations, determined during intensive bleedings, are slightly higher at these ages than at other ages (Fig. 3). The consistency in mean FSH data between samples collected once every 2 weeks and intensively collected blood samples compared with the LH data may be due to a higher pulse frequency in FSH than in LH profiles. The increase in FSH probably caused an increase in follicular activity resulting in the increase in oestradiol seen at 22 weeks of age (Fig. 1).

A short-lived early increase in LH and FSH secretion may aid ovarian development, as has been suggested for LH and normal testicular growth in bull calves (McCarthy *et al.*, 1979; Amann & Walker, 1983). Desjardins & Hafs (1969) conducted a study of follicle populations at slaughter in Holstein heifers and found that numbers of small ($< 5 \text{ mm}$) and large ($> 5 \text{ mm}$) ovarian follicles increased from birth to a maximum at 16 weeks of age, then decreased to 24 weeks of age and remained fairly constant until 1 year old. Prolonged exposure to high concentrations of gonadotrophins could have deleterious effects on the early developing ovary in the form of unwanted ovulations or cystic structures. Hence, a high sensitivity to the negative feedback effects of FSH-stimulated oestradiol may reduce the early rise in circulating concentrations of gonadotrophins at this age. It has been shown that, as puberty draws closer, there is a decline in oestradiol negative feedback on LH secretion and that this may be mediated by a decrease in oestradiol receptors at the

Fig. 4. Temporal pattern of serum concentrations of (a) luteinizing hormone (LH) and (b) follicle-stimulating hormone (FSH) in blood samples collected every 12 min for 12 h from two representative heifers at 4 weeks of age. Heifers were treated with saline (●) or naloxone (○; $1 \text{ mg kg}^{-1} \text{ body weight h}^{-1}$).

hypothalamus and pituitary (Day *et al.*, 1987). We consider that the early rise in LH and FSH, causing a transient rise in follicular activity, is an important event and suggest that this is a major component in the initiation and organization of cyclic ovarian activity, which persists throughout reproductive life.

The results presented here show that in 4-week-old heifer calves there is strong opioid inhibition of mean circulating LH concentrations, LH pulse amplitude and LH pulse frequency as demonstrated by its reversal with naloxone ($P < 0.01$). The higher LH pulse amplitude seen in control animals at 12 and 18 weeks than in controls at other ages ($P < 0.05$) is likely to be the reason for the increase seen in mean LH concentrations ($P < 0.05$). Since no effects of naloxone were seen during this time (except for the anomalous decrease in LH pulse amplitude at 18 weeks), and as naloxone had strong effects at 4 weeks of age in increasing LH secretion, it would appear that the removal of opioidergic inhibition between 4 and 12 weeks of age allowed for the increase in LH secretion seen in the control animals. Apart from an increase in LH pulse frequency at 24 weeks, no evidence of opioidergic suppression of LH secretion was noted after 18 weeks of age. It therefore appears that, in the immediate postnatal period, there was opioidergic suppression of LH secretion, but that this effect diminished after 4 weeks of age and was minimal up to at least 32 weeks of age. It has been suggested that in rats diminished opioidergic suppression of LH secretion may be involved in the initiation of ovarian cyclicity (Sirinathsinghji *et al.*, 1985); this was not apparent in studies of ewe lambs (Haynes *et al.*, 1989; Rawlings & Churchill, 1990). Haynes *et al.* (1989) reported that

prepubertal heifers treated monthly with an opioid antagonist, WIN-3, showed enhanced LH secretion every month from 6 to 12 months of age.

Circulating patterns of FSH in heifer calves were pulsatile at all the ages studied. However, opioidergic inhibition of FSH secretion was demonstrated only at 4 weeks of age in the form of enhanced pulse amplitude ($P < 0.01$). Hence, opioidergic control of FSH secretion occurs to a small extent at 4 weeks of age and is not demonstrable thereafter until at least 32 weeks of age. This is in agreement with studies in sheep, where there does not appear to be opioid involvement in the control of FSH secretion in adult ewes (Currie & Rawlings, 1989) or ewe lambs (Rawlings & Churchill, 1990).

Opioids control the pattern of gonadotrophin secretion to different extents at different ages in heifer calves. The mechanism of opioidergic regulation is unknown, but two possibilities must be considered. First, opioidergic systems may act in concert with, or relay information from, higher centres in the brain which control reproduction; second, opioids may partially or wholly mediate feedback of agents of systemic origin (e.g. steroids from the gonads) on the GnRH-producing areas of the hypothalamus or both of these actions may occur (Kalra, 1985). Deaver & Peters (1988) conducted a study on the changes in prepubertal hypothalamic metabolism of amines in bull calves and related these to changes in LH secretion. They concluded that the hypothalamic mechanisms regulating the secretion of LH are not fully functional until 12 weeks of age. Is the neonatal hypothalamus therefore dysfunctional or is its activity depressed? The results of this study tend to suggest that opioidergic mechanisms suppress an otherwise functional postnatal hypothalamus in heifer calves. Later on in the prepubertal period, opioidergic control of gonadotrophin secretion alters, possibly reflecting changes in the role of feedback agents of gonadal origin or maturational modifications in the organization of hypothalamic structures. A possible explanation for this is that opioids are involved in gonadotrophin suppression during the early postnatal period, but that, soon after, strong negative feedback by oestradiol, working through routes not involving opioids, overrides any opioidergic control mechanisms. Then, just before puberty, the role for opioids reappears to 'fine tune' LH secretion during subsequent oestrous cycles (Haynes *et al.*, 1989). A study with ovariectomized oestradiol-implanted ewe lambs suggested that this was not the case, as opioidergic suppression of LH secretion was reversed with naloxone, despite high oestradiol concentrations (Ebling *et al.*, 1989), which possibly reflects a species difference. It would appear that opioids are involved in the early organization of LH, and perhaps FSH, secretion in heifer calves, and that they resume some regulatory significance in the later stages of sexual maturation.

We have demonstrated that, in heifer calves 10–22 weeks old, there is an increase in circulating gonadotrophin concentrations, which stimulates follicular activity. Opioidergic control of gonadotrophin secretion occurs in 4-week-old calves, but thereafter is much reduced or absent. A decrease in endogenous opioid inhibition appears to allow for the early transient rise in LH secretion.

The authors thank S. J. Cook for technical assistance, W. M. Kerr and his staff for care and management of the animals and NIDDK and USDA for the provision of purified hormones. This research was funded by the National Sciences and Engineering Research Council of Canada.

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Received 19 December 1991