Opioidergic control of gonadotrophin secretion in the prepubertal gilt during restricted feeding and realimentation

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Summary. Prepubertal gilts, having undergone a 7-day period of feed restriction to a maintenance ration, were allocated to one of 4 treatments; restricted feeding at 09:00 and 17:00 h for an 8th day both with (Group RN) and without (Group R) administration of the opioid antagonist naloxone hydrochloride ($1 \text{ mg} \cdot \text{kg}^{-1}$ at 09:30 h followed by $0.5 \text{ mg} \cdot \text{kg}^{-1}$ at hourly intervals for 7 h), or feed to appetite with (Group ALN) and without (Group AL) naloxone administration. Gilts were bled at 10-min intervals on Day 8 from morning to evening feed and plasma LH, FSH and prolactin concentrations were measured by radioimmunoassay. Compared with Group R gilts, Group AL gilts exhibited significantly ($P \leq 0.05$) higher mean and maximum LH concentrations and pulsatility, lower prolactin concentrations ($P < 0.05$) but no significant difference in FSH secretion. Naloxone significantly depressed the increase in LH after re-feeding (Group ALN) ($P < 0.05$). Once again there were no significant effects on FSH secretion. Naloxone also significantly depressed prolactin secretion in feed-restricted gilts ($P < 0.05$). These results confirm that re-feeding of feed-restricted prepubertal gilts stimulates an immediate increase in LH secretion and that this elevation is not mediated via a suppression of inhibitory endogenous opioidergic tone. Rather, naloxone treatment appeared to expose a latent inhibition of LH secretion. The control of LH secretion is distinct from that of FSH in this model.

Keywords: prepubertal gilt; realimentation; gonadotrophin; naloxone

Introduction

In a number of mammalian species a reduction of feed intake has been associated with a suppression of gonadotrophin secretion and reproductive capacity (sheep: Haresign, 1981; Foster *et al.*, 1989; rat: Kuderling *et al.*, 1984; Dyer *et al.*, 1985; macaque: Dubey *et al.*, 1986; pig: Armstrong & Britt, 1987; Booth, 1990). Several physiological mechanisms may contribute to these phenomena and present hypotheses centre about an impairment of the hypothalamic pulse-generator (see Steiner, 1987, for review), because the pituitary of the fasted animal has been shown to exhibit an undiminished response to gonadotrophin-releasing hormone (GnRH) challenges (Haresign, 1981; Dyer *et al.*, 1985; Armstrong & Britt, 1987). Booth (1990), using prepubertal gilts, has demonstrated that short-term, severe feed restriction will impair luteinizing hormone (LH) pulsatile secretion and reproductive tract development without concomitant alterations in body weight or composition. Re-feeding of previously feed-restricted gilts rapidly elevated LH pulsatile secretion and this response could be mimicked by use of a glucose injection regimen of energetic value similar to an ad-libitum feed.

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A report (Dyer *et al.*, 1985) that the opioid antagonist naloxone reversed fasting-induced depressions in LH secretion in ovariectomized female rats has implicated an elevation of endogenous opioid tone, inhibitory to LH secretion, as a potential mechanism by which feed-restriction may compromise reproductive capacity. The existence of opioid pathways inhibitory to LH secretion in the female pig is well established. Naloxone administration elevates LH secretion in the cyclic gilt (Barb *et al.*, 1985) and lactating sow (Barb *et al.*, 1986; Mattioli *et al.*, 1986) although responses appear to be dependent upon the steroid milieu of the animal and, in the prepubertal gilt, upon specific brain maturational processes (Barb *et al.*, 1988).

The objective of this study was to elucidate whether the suppression of LH secretion observed in the feed-restricted prepubertal gilt was due to an elevation of inhibitory endogenous opioidergic tone.

Materials and Methods

Groups of littermate gilts were obtained from the University of Nottingham piggery at approximately 90 days of age and 25 kg liveweight. Gilts were housed in pens of approximately 20 animals on straw bedding and received a commercial grower ration and water *ad libitum*. At 3 weeks before transfer to experimental procedures gilts were fed to appetite twice daily at 09:00 and 17:00 h over a 1-h period. Eight sets of 4 littermates, matched for growth rate, weight and age (78.3 ± 7.3 kg; 150.8 ± 3.2 days) were selected for final allocation to treatment.

Gilts were placed in individual crates within aural and visual contact of one another and received water *ad libitum*. All animals underwent 7 days (Days 1–7) of restricted feeding calculated to meet their maintenance requirements (approximately 30% of individual *ad-libitum* feed intake) split between two feeds at 09:00 and 17:00 h. On Day 6 animals were provided with indwelling Silastic jugular cannulae under general anaesthesia.

On Day 8 littermates were allocated according to weight to one of four treatments, ensuring genetic and weight balance across treatment groups. Group R gilts remained on restricted feeding (09:00 and 17:00 h) throughout Day 8; Group RN gilts also remained on restricted feeding on Day 8 but received a $1 \text{ mg} \cdot \text{kg}^{-1}$ injection of naloxone hydrochloride (Du Pont, Geneva, Switzerland; Sigma, St Louis, MO 63178, USA) at 09:30 h followed by $0.5 \text{ mg} \cdot \text{kg}^{-1}$ injections at 1-h intervals until 16:30 h. Group AL and ALN gilts were fed to appetite at 09:00 and 17:00 h on Day 8, and Group ALN gilts also received naloxone injections as described for Group RN gilts.

Blood samples (2.5 ml) were withdrawn at 10-min intervals from 09:00 until 17:00 h into heparinized tubes and centrifuged at 1500 g for 20 min. Plasma was stored at -20°C until analysis for LH, FSH and prolactin concentrations by radioimmunoassay. Following each sample cannulae were flushed with 2 ml sterile, heparinized ($10 \text{ IU} \cdot \text{ml}^{-1}$) saline.

Plasma LH concentrations were quantified by an homologous double-antibody RIA. The primary antiserum was raised in a goat immunized against a purified pig LH (pLH) preparation, SDG-2-65 ($0.96\text{--}1.18 \times \text{NIH oLH-S19}$, by bioassay), kindly provided by Dr S. D. Glenn. The first booster injection, 6 months after initial immunization using Freund's complete adjuvant, produced a series of antisera with good titres and specificity. The antiserum characterized in the present assay, GRF-G81-1 bound approximately 20% radiolabelled pLH in the absence of unlabelled antigen, at an initial dilution of 1:60 000. Other assay methodology was as described previously (Foxcroft *et al.*, 1987) with the following minor modifications. To enhance precipitation of antibody-bound hormone, the second antibody (donkey anti-goat gamma globulin (AGGG), raised locally) at 1:40 dilution was preincubated for 24 h with 10% (w/v) polyethylene glycol (PEG 6000: BDH Ltd, Poole, Dorset, UK); 200 μl PEG-AGGG were added and assay tubes were then incubated for a minimum of 8 h at 4°C before centrifugation and aspiration.

Reproducible standard curves were obtained using standard potencies from 0.01 to $0.5 \text{ ng} \cdot \text{tube}^{-1}$ of the pLH preparation and the overall limit of sensitivity, defined as 85% of total binding, was $0.13 \text{ ng} \cdot \text{ml}^{-1}$. A standard plasma pool, routinely assayed at 50, 100 and 200 μl , showed no significant lack of parallelism to the standard curve. The recovery of known amounts of pLH when added to pig plasma of known potency ranged from 92 to 102%, confirming accuracy. The specificity of the antibody was checked by carrying out cross-reaction studies with pig prolactin and pig FSH; at 50% binding these hormones showed 0.17 and 0.88% cross-reactivity, respectively. Samples from the present experiment were assayed at 200 μl in duplicate and intra- and inter-assay coefficients of variation (CV) for a series of assays ($n = 9$) including these samples were 11.5% and 15.4% respectively.

Plasma FSH concentrations were quantified by an homologous double-antibody RIA utilizing a rabbit antiserum to pig FSH (USDA-398-04P, USDA Animal Hormone Program, Reproduction Laboratory, Beltsville Agricultural Research Centre, Beltsville, MD 20705, USA) kindly supplied by Dr D. J. Bolt; purified pig FSH was used for reference standard (USDA pFSH-B1, $0.98 \times \text{NIH ovine FSH-S1}$) and for radioiodination (USDA pFSH-PP1) by the method of Greenwood *et al.* (1963). The radioiodinated hormone was purified by gel exclusion chromatography (Sephadex G100).

For the assay, 300 μl plasma samples or standard (0.39, 0.78, 1.56, 3.25, 6.25, 12.5, 25, 50, 100, 200 ng FSH), 50 μl rabbit antiporcine FSH serum (at a dilution of 1:7500 in PBS containing 1:400 normal rabbit serum) and 50 μl ^{125}I -labelled pFSH (diluted in assay diluent to give 10 000–15 000 c.p.m.) were used as appropriate. All tubes were incubated at room temperature ($18\text{--}20^\circ\text{C}$) for 24 h followed by the addition of 50 μl of a 1:100 dilution of anti-rabbit

immunoglobulin antiserum (goat anti-rabbit Ig No. 539845: Calbiochem, San Diego, CA, USA) and incubated at 40°C for a further 24 h. To separate bound and free ^{125}I -labelled FSH, tubes were centrifuged at 2000 *g* for 30 min, the supernatant was aspirated and the radioactivity in the pellet counted. The first antibody bound 19% of ^{125}I -labelled FSH in the absence of unlabelled hormone. There was no significant lack of parallelism between the standard curve and three volumes (100, 200 and 300 μl) of a pooled standard plasma sample tested in each assay.

The recovery of known amounts of USDA pFSH-B1 added to pig plasma was 97.1%. As reported by the USDA Animal Hormone Program, the cross-reactivities of the antiserum to LH (USDA pLH-B1), GH (USDA pGH-B1) and prolactin (USDA pPRL-B1) were all <0.1%. The intra-assay CV was 8.3% and the overall limit of sensitivity of the assay was 3.3 ng pFSH·ml⁻¹.

Plasma concentrations of prolactin were measured by the methods described by Shaw & Foxcroft (1985). Intra- and interassay CVs were 13.4% and 11% respectively. The mean sensitivity of the assay defined as 85% of total bound was 1.8 ng·ml⁻¹.

Statistical analysis

LH profiles were analysed by the method of Shaw & Foxcroft (1985) using a sliding window technique to provide minimum, mean and maximum characteristics of LH secretion. The use of a frequent sampling regimen allowed the adoption of the following criteria to define an LH episode, enabling an estimation of LH pulse frequency by visual appraisal of individual profiles: any increase in LH concentration within 2 sampling intervals, followed by a decline, with at least 3 sampling intervals between the defined peak and succeeding baseline, occurring at a rate approximating the half-life of the hormone. (A full discussion of the validity and relative merits of visual appraisal for the estimation of LH profiles is available in Foxcroft *et al.*, 1988.) Analysis of treatment effects for hormone variables was undertaken by analysis of variance utilizing programs within the GENSTAT and SAS statistical packages. Due to the stratification by age and weight of the design, both these variables were fitted as covariates. Comparisons between treatment least-square means were made using Student's *t* test.

Results

Luteinizing hormone

Feed-restricted gilts (Groups R and RN) consumed all feed offered and gilts of Groups AL and ALN fed to appetite over a 30-min period before the first naloxone injection. Gilts receiving naloxone injections exhibited no observable behavioural responses to drug administration when compared with littermates.

Abnormally high LH and FSH secretion was observed in the gilts of Litter 215 (peak LH concentrations for each littermate being: AL 1.94; ALN 1.77; R 2.58; RN 1.68 ng·ml⁻¹) and data from this litter were excluded from all analyses. This did not alter the direction of treatment differences in any hormonal characteristics measured and hence the data presented are based on an analysis of 28 gilts from 7 litters.

LH profiles of two groups of littermates from the 4 treatment groups are presented in Fig. 1, representing the extremes of variability in LH pulsatility and the consistency of treatment effects within litters. Least-square treatment group means for minimum, mean and maximum LH secretion and number of LH pulses during the 8-h bleed period are presented in Figs 2 and 3(a). There were no differences ($P > 0.05$) in minimum LH secretion between any treatment groups, suggesting no effect of realimentation or naloxone administration on basal LH secretion. However, mean and maximum LH secretion in Group AL gilts was higher ($P \leq 0.05$) than in gilts of Group R, confirming previous findings that realimentation rapidly elevates LH secretion in the feed-restricted prepubertal gilt. Additionally, Group AL gilts exhibited higher ($P < 0.05$) mean and maximum LH secretion than did gilts of Group ALN, suggesting that naloxone inhibited the elevation of LH secretion that follows realimentation. Treatment effects on LH pulsatility during the 8-h bleed period, shown in Fig. 3(a), confirmed the above observations; re-feeding elevated LH pulsatility ($P < 0.05$) while naloxone injections exerted the opposite effect ($P < 0.05$).

Follicle-stimulating hormone

There were no significant treatment effects upon FSH secretion as demonstrated in Fig. 3(b), suggesting that changes in LH secretion occurred independently of short-term changes in FSH secretion.

Fig. 1. Plasma LH profiles of 8 gilts of 2 litters exhibiting (a) low episodic secretion (Litter 211) and (b) high episodic secretion (Litter 217). Treatment abbreviations are as described in the text. Arrows indicate times of naloxone injection.

Fig. 2. Least squares means of minimum, mean and maximum LH secretion (s.e.d. above) for 4 treatments. Treatment abbreviations are as defined in the text. Bars with different superscripts are significantly different: * $P \leq 0.05$, ** $P < 0.01$.

Fig. 3. Least squares means of (a) LH pulsatility, (b) FSH secretion and (c) prolactin secretion (s.e.d. above) for 4 treatments. Treatment abbreviations are as defined in the text. Bars with different superscripts are significantly different: * $P < 0.05$.

Prolactin

Least-square treatment group means for prolactin are presented in Fig. 3(c). Prolactin secretion was elevated in feed-restricted gilts when compared with their ad libitum-fed counterparts ($P < 0.05$) and restrict-fed gilts receiving naloxone ($P < 0.02$). There was also a trend for ad libitum-fed gilts to secrete lower levels of prolactin than did ad libitum-fed animals receiving naloxone.

Discussion

The results of this study demonstrating a short-term effect of re-feeding in the 7-day feed-restricted prepubertal gilt model, evidenced by elevated LH secretion within 8 h of re-feeding, are confirmed by the observations of Booth (1990). The precise mechanism by which re-feeding operates to overcome LH suppression by restriction is unclear. In the female rat opioid peptides within the central nervous system have been implicated as modulators of LH secretion under stress conditions and during periods of restricted feeding (Corbet *et al.*, 1982; Dyer *et al.*, 1985; Dyer, 1988; Hollingsworth *et al.*, 1989). Briski *et al.* (1984) and Dyer *et al.* (1985) reported an elevation in LH secretion in the male and female feed-restricted rat following administration of naloxone. Other workers, however, have failed to demonstrate effects of naloxone on LH secretion in the underfed male rat (Kuderling *et al.*, 1984) and the nutritionally growth-limited female lamb (Ebling *et al.*, 1990).

The results of the present study suggest that the suppression of LH secretion seen in the feed-restricted gilt cannot be explained by an elevation of inhibitory opioid tone, because naloxone failed to elevate LH secretion in Group RN gilts. However, naloxone treatment (Group ALN) inhibited the LH response to re-feeding observed in littermates fed to appetite (Group AL) and this response to opioid antagonism during realimentation is both a novel and unexpected observation. The possibility that the dose of naloxone administered ($1 \text{ mg} \cdot \text{kg}^{-1}$ followed by $0.5 \text{ mg} \cdot \text{kg}^{-1}$ hourly) was too low to inhibit opioid tone seems unlikely, as earlier studies have adopted similar doses to elicit positive effects on LH secretion: additionally, a significant effect of naloxone was recorded albeit the reverse of that expected. Alternatively, the dose of naloxone chosen, coupled with the sub-chronic injection regimen, may have resulted in an agonistic effect of the drug as suggested by Armstrong *et al.* (1988) in the chronically infused lactating sow; this, however, also seems unlikely as a transient elevation in LH secretion would have been expected before suppression within the first hour following naloxone injection. This response is not apparent in the LH profiles presented in Fig. 1 and did not occur in any other gilts treated with naloxone (data not shown). Prolactin

responses to administration of the antagonist to feed-restricted gilts appeared to be in line with previous studies (Barb *et al.*, 1986; Mattioli *et al.*, 1986), i.e. a suppression of prolactin in this group, suggesting that this dosage of naloxone was capable of eliciting a predictable physiological response. The absence of a significant decrease in prolactin in Group ALN gilts may be due to the lower prolactin status of these gilts compared with their feed-restricted counterparts, Group R gilts exhibiting significantly higher prolactin secretion.

An explanation for the observed depression of LH secretion by naloxone in this model is elusive. Opioids have been implicated in the control of LH secretion in the cyclic gilt (Barb *et al.*, 1985) and in the rat (Blank *et al.*, 1979; Bhanot & Wilkinson, 1983; Petraglia *et al.*, 1984; Sirinathsinghi *et al.*, 1985), sheep (Ebling & Lincoln, 1985; Brooks *et al.*, 1986; Currie & Rawlings, 1987), heifer (Short *et al.*, 1987) and women (Petraglia *et al.*, 1986). Although the effects of various doses of the opioid antagonist naloxone in the cyclic pig have generally been to elevate LH secretion, this response appears to be dependent upon both the steroid milieu of the animal (Barb *et al.*, 1986) and certain brain maturational events (Barb *et al.*, 1988). Barb *et al.* (1988) failed to show any LH response to naloxone in prepubertal gilts while chronologically mature, oestrogen/progesterone-treated ovariectomized gilts showed an elevation of LH secretion in response to the antagonist. In contrast, a depression of LH secretion following similar doses of naloxone ($1 \text{ mg} \cdot \text{kg}^{-1}$) in the 12-week-old gilt has been observed (H. Prunier, personal communication).

The noradrenergic/catecholaminergic systems have also been implicated in the control of gonadotrophin-releasing hormone (GnRH) in the rat (Dyer & Grossman, 1988; Hollingsworth *et al.*, 1989) and the pig (Parvizi & Ellendorff, 1978). It has been suggested that noradrenergic pathways both stimulatory and inhibitory to GnRH secretion are present in the rat and that the balance of these opposing inputs determines the level of GnRH and hence gonadotrophin release. The presence of circulating oestrogens may act centrally to bias this input to be stimulatory, while in the absence of this 'oestrogen switch', central noradrenaline release inhibits GnRH release (Dyer, 1988; Grossman & Dyer, 1989; Kalra *et al.*, 1989). Opioids have been shown to be inhibitory to central noradrenergic secretion in the rat (Grossman & Dyer, 1989).

Our results demonstrate an elevation in pulsatile LH secretion following ad-libitum feeding in the previously maintenance-fed gilt. Assuming that this rapid rise in LH secretion would have no immediate effect on ovarian oestrogen secretion, systemic oestrogen levels would remain low. If an 'oestrogenic switch' were to be present in the prepubertal gilt, under the above conditions the switch would be inoperable and noradrenergic secretion would act to inhibit GnRH and hence LH secretion. Naloxone may therefore block opioid inhibition of noradrenaline and hence further inhibit LH secretion by indirect actions on GnRH neurones. This hypothesis might explain LH responses to naloxone in Group ALN gilts and the trend for decreased LH secretion in Group RN gilts. Since LH secretion in feed-restricted gilts was already suppressed, significant reductions in response to naloxone would be difficult to demonstrate. A study of the LH response of oestrogen-primed re-fed prepubertal gilts to naloxone injection would be informative.

These results suggest that the LH response to re-feeding of the feed-restricted prepubertal gilt may be mediated by as yet unidentified cues, e.g. by metabolic hormone changes. Booth (1990) has suggested that insulin and/or insulin-like growth factor I (IGF-I) may be the modulators of enhanced LH secretion in such animals and Cox *et al.* (1987) have shown enhanced ovulation rate in gilts treated with long- and short-acting insulin. Insulin receptors have been identified in the vicinity of GnRH-producing neurones (Havrankova *et al.*, 1978) and Woods *et al.* (1979) have suggested that CSF levels of insulin represent integrated long-term reflections of humoral fluctuations and act as an effective central indicator of metabolic status. Therefore it seems likely that metabolic factors impinge upon the central regulation of LH secretion. Further study of central and ovarian responses is necessary.

In this study FSH secretion was not significantly affected by either treatment, underlining the differential secretion pattern and control of this hormone by LHRH. The study was conducted over a relatively short time period and it might be expected that short-term fluctuations in LHRH

release would not be reflected by FSH secretion. However, a previous study of the longer-term effects of re-feeding has also failed to establish significant effects on FSH secretion (Booth, 1990).

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