Active immunization of heifers against inhibin: effects on plasma concentrations of gonadotrophins, steroids and ovarian follicular dynamics during prostaglandin-synchronized cycles

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We reported previously that active immunization of heifers using a synthetic peptide-based inhibin vaccine (bIA(1–29)Tyr38) can enhance ovarian follicular development and ovulation rate during spontaneous oestrous cycles. To extend this study, we investigated the effect of inhibin immunization more closely by monitoring plasma hormone profiles and ovarian activity in bIA(1–29)Tyr38-immunized and control (ovalbumin-immunized) heifers (n = 6 per group) over three consecutive oestrous cycles, which were synchronized and shortened by administering a PGF2α analogue at intervals of 14 days. Blood samples were collected at 2–8 h intervals for 40 days and the ovaries were examined daily using ultrasonography. Repeated-measures ANOVA showed that inhibin immunization significantly increased plasma FSH concentration (by 52% overall; P < 0.01) and ovulation rate (by 58%; P < 0.01). Both immunized and control heifers showed the same cyclic pattern of plasma FSH (treatment × time interaction; not significant), indicating that the increase in plasma FSH was sustained throughout the cycle. Immunization did not affect the concentration or pattern of secretion of LH, oestradiol or progesterone and had no influence on the timing of the LH surge or ovulation after PG injection. While inhibin immunization increased the number of ‘large’ (i.e. growing to ≥10 mm diameter) follicles that developed during both the preovulatory (by 90%, P < 0.02) and postovulatory (by 190%, P < 0.01) period, there was no difference between groups in the temporal pattern of growth or regression of large follicles or of corpora lutea. These observations confirm a physiological role for ovarian inhibin as a component of the ovarian feedback mechanism controlling FSH secretion in heifers, and support the hypothesis that active immunization of heifers against inhibin enhances ovarian follicular development and ovulation rate by promoting a sustained increase in pituitary FSH secretion.

Introduction

Active immunization of ewes against inhibin-enriched fractions of bovine follicular fluid (FF) (Henderson et al., 1984; Cummins et al., 1986; Al-Obaidi et al., 1987), synthetic peptide fragments of the α subunit of inhibin (Wrathall et al., 1990, 1992; Meyer et al., 1991; Schanbacher et al., 1991; Wheaton et al., 1992) or recombinant-DNA-derived α subunit (Forage et al., 1987; Findlay et al., 1989; Mizumachi et al., 1990) results in an increase in ovulation rate. In some (for example Wrathall et al., 1990, 1992; Mizumachi et al., 1990), but not all, of these studies, it was possible to relate the increase in ovulation rate to a small but significant rise in FSH concentration in plasma. Such an increase in plasma FSH would be expected as immunoneutralization of circulating inhibin should attenuate its negative feedback action on the anterior pituitary. Experiments involving passive immunization of sheep against inhibin have provided clearer evidence that endogenous inhibin contributes to the negative feedback regulation of FSH secretion in this species (Mann et al., 1989, 1990; Wrathall et al., 1990). The relative importance of inhibin in the regulation of FSH secretion in cows has been brought into question by the outcome of ovarian hormone replacement experiments in ovariectomized heifers which indicate that steroids alone can account for the inhibitory effect of the ovaries on FSH release (Price and Webb, 1988). However, as both crude (steroid-free bovine FF) and highly purified M, 32 000 bovine inhibin can suppress plasma FSH concentration in ovariectomized heifers (Ireland et al., 1983; Beard et al., 1989, 1990), it seems likely that inhibin does have a role in this regard.

In comparison with sheep, there have been relatively few reports of inhibin immunization studies in cattle. Price et al. (1987) reported that immunization of heifers against partially purified ovine FF results in an increase in circulating FSH concentrations and a transient rise in ovulation rate, although

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the response was inconsistent and highly variable among animals. Several groups have actively immunized heifers against immunogenic conjugates incorporating synthetic peptide fragments of the α subunit of inhibin (Sunderland et al., 1991; Glencross et al., 1992; Morris et al., 1993). In these studies there was a significant increase in the ovulation rate of immunized animals, although Glencross et al. (1992), who also measured plasma gonadotrophin concentrations, were unable to show a statistically significant increase in circulating FSH concentration. Active immunization of gilts against recombinant-DNA-derived inhibin α subunit (Brown et al., 1990) and of mares against a synthetic fragment of the inhibin α subunit (McCue et al., 1992) also results in an increase in ovulation rate. These observations suggest that inhibin may have a comparable role in the control of ovulation in at least four domesticated species. However, it is still unclear whether immunoneutralization of endogenous inhibin affects gonadotrophin secretion in these species, and the possibility remains that the ovulatory response to immunization occurs independently of a change in gonadotrophin secretion.

In this study, heifers that had previously been actively immunized against a synthetic peptide fragment of the inhibin α subunit (Glencross et al., 1992) were monitored closely over three consecutive oestrous cycles synchronized by PG administration. Blood samples were collected for hormone analysis at intervals of 2–8 h and ovarian ultrasonography was performed at intervals of 24 h throughout the 40 day experiment. The purpose of the study was to determine whether the presence of antibodies against inhibin affected the pattern of secretion of gonadotrophins, oestradiol or progesterone, to establish whether the timing of ovulation was affected and to ascertain whether the response of ovulation rate to inhibin immunization is maintained after repeated PG synchronization of cycles.

Materials and Methods

Animals

The 12 British Friesian heifers studied here were the same group described previously by Glencross et al. (1992). Six of the heifers had been immunized against a synthetic peptide fragment corresponding to the amino-terminal sequence of the α subunit of bovine inhibin (bIα(1–29)TyrAla) conjugated to ovalbumin, while six control animals were immunized against ovalbumin alone. The inhibin peptide was custom-synthesized by Peptide & Protein Research Consultants, Exeter. Booster immunizations were given at intervals of 1–2 months, one being given 2 weeks before the beginning of this experiment when the heifers were 19 months old (liveweight, 423 ± 10 kg). Oestrous cycles were synchronized by giving three i.m. injections (0.5 mg) of a PGF2α analogue, cloprostenol (Coopers Animal Health Ltd, Crewe) at intervals of 14 days. The day of the third injection was designated as day 0. Two further PG injections were given at 14 day intervals to shorten the ensuing oestrous cycles by inducing luteolysis on day 10–11 of the cycle. For the duration of the experiment, heifers were kept indoors, loosely tethered in individual standings. They were fed a maintenance ration of concentrates and straw and had free access to water at all times.

Blood samples and ultrasonography

Blood samples (20 ml) were taken every 8 h for 80 h before PG-induced luteolysis (time 0), every 2 h for the period 0–120 h after PG-induced luteolysis and then at intervals of 8 h for the remaining 9 days until the next PG injection. The same regimen of sampling every 2 h for 120 h after PG injection, followed by sampling every 8 h for the next 9 days was repeated during the next two cycles. Samples were taken by means of an indwelling jugular vein catheter. The patency of the catheter was maintained by flushing with a sterile solution of 5% (w/v) sodium citrate after sample withdrawal. An additional blood sample (50 ml) was withdrawn at 10:00 h each day for analysis of plasma steroids. Blood samples were collected into polystyrene tubes containing 50 µl 5000 IU heparin ml−1 and after centrifugation at 2000 g for 30 min the plasma was separated and stored at −20°C. Daily examination of the ovaries was carried out by transrectal ultrasonography (Pierson and Ginther, 1988) using a scanner fitted with a 7.5 MHz linear array transducer (Concept 2000; Dynamic Imaging, Livingstone). Images were recorded on videotape to facilitate sequential analysis.

Assays

Gonadotrophins. Plasma concentrations of FSH were measured using the heterologous radioimmunoassay described by Glencross et al. (1992). The detection limit of the assay, defined as the amount of standard (USDA-bFSH-BP-1; D.J. Bolt, USDA, Beltsville, MD) required to displace the binding of antibody to tracer by 20%, was 1.2 ng per tube. Inter- and intra-assay coefficients of variation based on repeated estimates of the potency of pooled bovine plasma ‘quality control’ samples were both less than 10%. Plasma LH concentrations were measured by radioimmunoassay (as described by Beard et al., 1989, 1990) using EHC-bLH-1 (Loeber et al., 1987) as the standard. The detection limit of the assay, as defined above, was 0.15 ng per tube and the inter- and intra-assay coefficients of variance were both less than 10%.

Oestradiol. Concentrations of oestradiol were measured in daily plasma samples using the immunoaffinity extraction and radioimmunoassay procedure described in detail by Glencross and Pope (1981). The detection limit of the assay, based on a sample volume of 5 ml, was equivalent to 1.7 pg ml−1 plasma. Inter- and intra-assay coefficients of variation were 15.0 and 10.5%, respectively.

Progesterone. Concentrations of progesterone in daily plasma samples were measured by the direct-addition enzyume-immunoassay of Sauer et al. (1986) as modified by Glencross et al. (1992) using ovine anti-progesterone serum (S1509/16) (Groves et al., 1990) and a progesterone standard range of 0–25 nmol l−1 in ovariecatomized cow plasma. Inter- and intra-assay coefficients of variation were 14.1 and 9.5%, respectively.

Anti-inhibin titres. Plasma anti-inhibin titres were assessed by the ability of diluted samples (1:2000; final dilution) to bind
125I-labelled M, 32 000 bovine inhibin (Knight et al., 1990) as reported by Glencross et al. (1992).

Statistical analysis and presentation of data. Statistical comparisons of mean daily hormone concentrations in immunized and control heifers over the entire 40 day period of blood sampling were performed by repeated measures analysis of variance (ANOVA), mean daily LH and FSH values having been calculated for each individual heifer before analysis. Owing to the inherent variability (both within and between heifers) in the interval between PG injection and the preovulatory LH surge, individual hormone profiles were realigned around the time of the preovulatory LH surge. Between-group comparisons of ovulation rate and plasma anti-inhibin titre were made using repeated measures ANOVA and Student’s unpaired t test. A probability value of \( P < 0.05 \) was regarded as statistically significant. To allow plasma hormone profiles for the whole experiment to be condensed and presented as a single cycle (see Fig. 2), results for each of the three consecutive cycles studied in each heifer were combined (i.e. superimposed temporally) after performing the statistical analyses.

Results

Retrospective ultrasound analysis of the hormone profiles and ovarian profiles revealed that four of the 36 individual cycles studied (three in the control group and one in the immunized group) were abnormal; a preovulatory LH surge did not occur within 5 days of PG injection, which resulted in ovulation failure and the absence of a functional corpus luteum at the time of the next PG injection. Data for these atypical cycles were excluded from the analysis.

Inhibin antibody titres

Antibodies capable of binding 125I-labelled bovine inhibin were detected in plasma from all blu(1–29)Tyr30-immunized heifers (overall mean: 22.9 ± 3.9% tracer binding at 1:2000 plasma dilution), whereas tracer binding in the carrier-immunized control heifers was similar to nonspecific binding values ( < 2%).

Plasma gonadotrophins

Overall mean plasma FSH concentrations for the 40 day experimental period were 52% higher (\( P < 0.01 \), t test) in the inhibin-immunized animals (17.13 ± 0.95 ng ml\(^{-1}\), \( n = 6 \)) than in the controls (11.30 ± 1.47 ng ml\(^{-1}\), \( n = 6 \)). Repeated measures ANOVA of mean daily FSH concentrations over 40 days (Fig. 1a) confirmed this significant effect of inhibin immunization on plasma FSH (effect of immunization, \( P < 0.02 \)). Plasma FSH concentrations in both treatment groups clearly showed cyclic changes during the 40-day period (effect of time, \( P < 0.001 \)). However, there was no detectable difference between immunized and control heifers in the temporal pattern of FSH secretion (treatment × time interaction, not significant). In both control and inhibin-immunized groups plasma FSH concentration decreased by approximately 40% after PG-induced luteolysis. This was followed by a preovu-

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**Fig. 1.** Comparison of (a) mean (±SEM) daily plasma FSH concentrations and (b) mean (±SEM) ovulation rates in six control (□) and six blu(1–29)Tyr30-immunized (■) heifers during a 40-day period encompassing three consecutive PG-shortened oestrous cycles. The times of the PG injections are indicated by arrows. In (b) cycle 1 refers to the initial synchronized cycle, which was terminated on day 0 of the study. Statistical analyses were performed by repeated measures ANOVA. Plasma FSH data: effect of treatment, \( P < 0.02 \); effect of time, \( P < 0.001 \); treatment × time interaction, \( P = 0.5 \) (not significant). Ovulation rate data: effect of treatment, \( P < 0.01 \); effect of time, \( P = 0.8 \); treatment × time interaction, \( P = 0.5 \).

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Plasma steroids

Repeated measures ANOVA showed that there was no significant effect of inhibin immunization on plasma concentrations of oestradiol, or in the temporal pattern of oestradiol secretion during the three consecutive cycles studied (effect of treatment, not significant; treatment × time interaction, not significant). A significant (\( P < 0.001 \)) variation over time in
plasma oestradiol concentration was observed in both groups, with values rising immediately after PG-induced luteolysis to reach a peak within 3 days, shortly before the preovulatory LH surge. The concentration then fell and remained relatively low until after the next PG injection, with the exception of a smaller peak (P < 0.05 in control group; not significant in immunized group) 3–5 days after the preovulatory LH surge (Fig. 2b).

Despite a tendency for plasma progesterone to be higher (about 20%) in immunized heifers, repeated measures ANOVA showed that there was no difference in the secretion profile or the concentration of progesterone between the two groups. In both groups, plasma progesterone concentration fell by over 80% within 1 day of PG-induced luteolysis. Progesterone concentration remained low until day 6–7 after PG injection, when it started to rise again, confirming that ovulation and corpus luteum formation had occurred (Fig. 2c).

**Ovarian observations**

Ovulation rate was recorded over four consecutive cycles (including the initial synchronized cycle, which was terminated on day 0 of the study). The combined ovulation rate data for the four cycles showed that heifers immunized against inhibin had a 58% higher ovulation rate (P < 0.01) than the control animals (Figs 1b, 3). Likewise, significantly more large (≥10 mm diameter) follicles were observed on the ovaries of inhibin-immunized heifers compared with control heifers during both the preovulatory (about 90% more, P < 0.05) and postovulatory (about 190% more, P < 0.01) waves of follicular development.

There was no discernible difference in the temporal pattern of growth and regression of either corpora lutea or large follicles between inhibin-immunized and control heifers (Fig. 4). In both groups, a proportion of ovariogenous follicles (i.e. those that were identified as having ovulated) were identifiable as large follicles already present at the time of PG-induced luteolysis (immunized, 57%; control, 58%). Other ovariogenous follicles emerged and grew after PG-induced luteolysis (immunized, 43%; controls, 42%). Those large follicles present at the time of PG injection that did not go on to ovulate regressed at a similar rate in both groups. Likewise, the time of emergence and rate of growth of large follicles associated with the preovulatory and postovulatory wave of development

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**Fig. 3.** Mean (± SEM) number of large follicles (diameter ≥10 mm) recorded during the preovulatory and postovulatory phase of the cycle, and mean number of corpora lutea in six control (○) and six inhibin-immunized (●) heifers studied over three prostaglandin-shortened cycles. *P < 0.05, **P < 0.01 compared with controls (unpaired t test).
**Inhibin immunization raises FSH in heifers**

![Image of follicle growth and regression](https://via.placeholder.com/150)

Fig. 4. Patterns of growth or regression of (a) corpora lutea and (b) 'large' follicles (growing to a diameter of ≥ 10 mm) observed in six control (○) and six inhibin-immunized (●) heifers. Observations are based on amalgamated data from three consecutive prostaglandin (PG)-shortened cycles. The time of PG injection is indicated. In an attempt to convey more clearly the cyclic nature of events, observations for days + 4 to + 8 have been re-plotted in the shaded area on the left as days − 10 to − 6. Three separate cohorts of growing large follicles (A, B, C) and one cohort of regressing large follicles (D) were distinguished during the cyclic 14 day period in both groups. Ovulatory follicles (open box) emerged either during the previous luteal phase (Wave C: 57% of ovulatory follicles in immunized group, 58% in control group) or after luteolysis (Wave A: 43% of ovulatory follicles in immunized group, 42% in control group). Note that there was no apparent difference between immunized and control heifers in the pattern of turnover of large follicles or corpora lutea.

Discussion

This study is the first to show that active immunization of heifers against inhibin leads to an increased number of large follicles and an increased ovulation rate, both of which are associated with a significantly raised concentration of circulating FSH. Such an association has already been demonstrated in sheep (Mizumachi et al., 1990; Wrathall et al., 1990, 1992; McLeod et al., 1992; Wheaton et al., 1992). Other recent experiments in cattle (Sunderland et al., 1991; Glencross et al., 1992; Morris et al., 1993) and other large domesticated species, such as mares (McCue et al., 1992) and gilts (Brown et al., 1990) confirm that active immunization against inhibin or inhibin peptide fragments results in an increase in ovulation rate, although FSH concentration was not measured in most of these studies. Of those studies in cattle in which plasma FSH was measured (Glencross et al., 1992; Morris and Grealy, 1993) neither was able to detect a significant FSH response to inhibin immunization, although both groups reported a tendency for plasma FSH concentration to be higher in immunized animals. It should be mentioned that some previous studies in heifers contradict the present finding, in that active immunization against inhibin peptides failed to increase ovulation rate (Scanlon et al., 1990; Rhind et al., 1991). However, in these studies heifers were immunized against different peptide conjugates, and production of antibodies capable of binding native (whole molecule) inhibin was not confirmed.

In this study, observations over four consecutive luteal phases showed that the presence of inhibin antibodies was associated with a significant 58% increase in mean ovulation rate. Thus, it is evident that the ovulatory response of heifers to inhibin immunization persists despite the repeated use of PG to synchronize oestrus by promoting premature luteolysis on day 10–11 of the cycle. Mean plasma FSH concentration was substantially (52%, *P* < 0.01) higher in immunized heifers compared with controls. Repeated measures ANOVA of longitudinal data derived from six heifers per group sampled over three consecutive PG-shortened oestrous cycles revealed that inhibin immunization significantly (*P* < 0.02) increased plasma FSH concentration in a uniform manner throughout the entire 40 day period. As expected, plasma FSH concentration varied according to the stage of the cycle (*P* < 0.001). However, the same cyclic pattern of change in plasma FSH was observed in both immunized and control groups (treatment × time interaction, not significant). On the basis of this finding, it could be argued that the ovarian output of inhibin in heifers is relatively constant throughout the oestrous cycle and that peripheral concentrations of inhibin are mainly involved in setting the overall tonic level of FSH secretion, whereas cyclic changes in ovarian output of progesterone and oestradiol are responsible for driving cyclic changes in FSH secretion. Certainly, the fall in plasma FSH concentration that occurs after luteolysis is most likely attributable to the observed increase in plasma oestradiol concentration, rather than inhibin, since it occurred in both immunized and control groups. In the absence of a satisfactory assay system for measuring the peripheral concentration of dimeric inhibin in cattle (see Knight, 1991; Knight et al., 1991a), the pattern of inhibin secretion during the bovine oestrous cycle remains obscure. Only when this information becomes available will a thorough evaluation of the role of inhibin at different stages of the cycle be possible.

To account for the failure of several previous inhibin immunization studies in ewes and heifers to detect increased plasma FSH concentration in animals with raised ovulation rates, it seems likely that a relatively small increase in circulating FSH concentration may be sufficient to increase ovulation rate by increasing the number of developing follicles exposed to their individual 'threshold' concentration of FSH, which prevents atresia and allows full maturation and hence ovulation (Ireland, 1987). Given the considerable variation in plasma FSH concentration, both within and between animals, it would thus seem that failure to detect a significant FSH response to inhibin immunization is largely a problem of inadequate sampling.
In contrast to our observations on plasma FSH concentrations, there was no difference in mean plasma LH concentrations between the control and immunized heifers. Likewise, the pattern of secretion of LH (repeated measures ANOVA: treatment x time interaction, not significant) and the timing of the preovulatory LH surge after PG administration were not affected. Similarly, no change in plasma LH was observed in ewes actively immunized against inhibin (Henderson et al., 1984; Wheaton et al., 1992; Wrathall et al., 1992). However, studies in anoestrous ewes have shown that bovine FF can cause a transient increase in the circulating LH concentration (McLeod and McNelley, 1987; Knight and Castillo, 1988), and that active immunization against a synthetic peptide fragment of inhibin can lead to a decrease in the circulating LH concentration during seasonal anoestrus (Knight et al., 1991b). Lower concentrations of LH after active immunization against inhibin have also been noted in cyclic ewes (Meyer et al., 1991), and Wrathall et al. (1990) showed that the LH response to a GnRH challenge was reduced in inhibin-immunized ewes. There is also evidence from studies in vitro involving ovine pituitary cells that both bovine FF and purified inhibin can stimulate GnRH-induced release of LH (Muttukrishna and Knight, 1990). This finding suggests that, in sheep at least, inhibin may have a role to play in modulating LH secretion. The possibility of such a role in cattle should not be excluded on the basis of the present findings.

Consistent with previous observations in heifers (Dobson, 1978; Glencross and Pope, 1981; Quirke and Fortune, 1986; Turzillo and Fortune, 1990), the plasma concentration of oestriol rose immediately after PG-induced luteolysis and reached a peak within 2–3 days; as anticipated, progesterone concentration fell abruptly after PG administration. There was no significant effect of inhibin immunization on either the plasma concentrations of oestriol or progesterone (repeated measures ANOVA: effect of immunization, not significant) or on their secretory patterns (treatment x time interaction, not significant), although the progesterone concentration tended to be about 20% higher in immunized than in control heifers (perhaps reflecting a greater mass of luteal tissue in the former group). Similarly, retrospective analysis of sequential ultrasound images revealed no discernible difference between immunized and control groups with respect to the patterns of growth or regression of corpora lutea or ‘large’ follicles (i.e. those that grew to ≥10 mm diameter). However, previous studies in which heifers were treated with bovine FF at about the time of PG-induced luteolysis, revealed a delay in the timing of the increase in oestriol concentration, follicular development and ovulation, which was probably due to a reduced concentration of FSH that led to a delay in the maturation of oestrogenic follicles (Quirke and Fortune, 1986; Turzillo and Fortune, 1990; Wood et al., 1993). To our knowledge, there have been no previous studies reporting oestriol concentration in plasma of heifers actively immunized against inhibin. It would perhaps have been expected that the amount of oestriol would be higher in immunized heifers since their ovaries clearly had more large (presumably oestrogenic) follicles. However, no such difference in plasma oestriol concentration was detected in the present study.

In conclusion, the results of this study clearly show that active immunization of heifers against a synthetic fragment of inhibin leads to a sustained rise in plasma FSH concentration that is associated with an increase in the number of large follicles developing during both the preovulatory and postovulatory waves of follicular development, and with an increase in ovulation rate. However, immunization had no discernible effect on plasma concentration or patterns of secretion of LH, oestradiol or progesterone, and did not affect the timing of oestrus or the patterns of growth or regression of large follicles and corpora lutea.

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