Evidence for a local role of inhibin or inhibin α subunits in compensatory ovarian hypertrophy

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The aim of this study was to determine whether immunoneutralization of inhibin altered compensatory ovarian hypertrophy. Crossbred postpubertal gilts actively immunized with a synthetic bovine inhibin peptide fragment (bINH) conjugated to human alpha globulins (HAG, $n = 4$ gilts) or HAG alone (control; $n = 5$) were unilaterally ovariectomized at mid-cycle. After unilateral ovariectomy, the remaining ovary was removed between day 8 and day 12 of the subsequent oestrous cycle. The number of corpora lutea per ovary was determined at each ovariectomy. Blood samples were collected at frequent intervals beginning 1 h before and continuing until the first oestrus after unilateral ovariectomy, and serum concentrations of FSH, LH, progesterone and oestradiol were determined. Inhibin antibody titres were estimated from the percentage of ¹²⁵I-labelled bINH bound to serum diluted 1:4000. At unilateral ovariectomy, the number of corpora lutea per ovary was similar for bINH:HAG-immunized and control gilts (8.6 ± 0.7 versus 7.6 ± 0.6). During the next oestrous cycle after unilateral ovariectomy, the number of corpora lutea on each remaining ovary had doubled ($P < 0.05$) in controls compared with the number of corpora lutea per ovary in the previous cycle. In contrast, the number of corpora lutea remained unchanged in bINH:HAG-immunized gilts. Titre of anti-inhibin antibodies in bINH:HAG-immunized gilts was $9 \pm 1\%$ at unilateral ovariectomy compared with 0% for controls. Alterations in serum concentrations of hormones after unilateral ovariectomy did not differ between treatment groups. Compensatory ovarian hypertrophy was blocked after unilateral ovariectomy in immunized gilts independent of alterations in serum hormones, duration of oestrous cycle, or normal ovulation rate per ovary. Thus, it is concluded that inhibin or inhibin α subunits are positive local stimulators of compensatory ovarian hypertrophy in postpubertal gilts.

Introduction

After unilateral ovariectomy of litter-bearing species, the remaining ovary undergoes compensatory ovarian hypertrophy resulting in approximately the same number of ova being ovulated (Hermreck and Greenwald, 1964; Short *et al.*, 1969; Butcher, 1977) and a similar litter size (Hunter, 1787) compared with both ovaries before unilateral ovariectomy. Although mechanisms regulating compensatory ovarian hypertrophy are not precisely defined, a transient increase in FSH concentrations (Welshen *et al.*, 1978; Redmer *et al.*, 1984, 1985; Coleman *et al.*, 1984; Cox *et al.*, 1987; Ackland *et al.*, 1990) coincident with a transient decrease in inhibin bio- and immuno-activities in plasma (Redmer *et al.*, 1986; Ackland *et al.*, 1990) occurs within 2 days of unilateral ovariectomy compared with sham-operated

controls. These results imply that unilateral ovariectomy temporarily reduces the negative feedback effects of inhibin resulting in a transient increase in FSH which, in turn, leads, via an unknown mechanism, to a doubling of the ovulation rate in the remaining ovary. The present study used the unilateral ovariectomy model to investigate the role of inhibin in follicular growth. The objective of our study was to test whether immunoneutralization of inhibin altered compensatory ovarian hypertrophy after unilateral ovariectomy of postpubertal gilts.

Materials and Methods

Animals

A randomly selected subset of five gilts actively immunized against inhibin and five control gilts from a previous study (King *et al.*, 1993) were used in the present study as explained

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below. Gilts were housed in a curtain-sided building in individual stalls (0.8 m × 1.8 m) and fed 1.8–2.7 kg day⁻¹ of a 14% crude protein corn/soybean diet fortified with vitamins and minerals to meet National Research Council recommendations (NRC, 1988). All surgical procedures and animal care were approved by the Animal Care and Use Committee at North Carolina State University.

Immunization

Details of the immunization procedure are described by King *et al.* (1993). Briefly, each gilt received a primary immunization of α_c^{1-26} Gly-Tyr bovine inhibin peptide (bINH) conjugated to human alpha globulins (HAG; Sigma, St Louis, MO) or HAG mixed in Freund's complete adjuvant (Calbiochem-Novbiochem Corp., San Diego, CA) during the second post-pubertal oestrous cycle (week 0) followed by two boosters in Freund's incomplete adjuvant at weeks 8 and 12. Gilts immunized against bINH:HAG received unconjugated bINH in Freund's incomplete adjuvant at week 16 to stimulate antibody titres, whereas controls received adjuvant only.

Experimental protocol

On day 10 of the first oestrous cycle after week 19, which was approximately 4 weeks after the last booster, gilts were unilaterally ovariectomized. At unilateral ovariectomy, analgesia was induced with a 1:1 (v:v) mixture (i.v.) of xylazine (Rompum; Miles Laboratories, Shawnee, KS; 2.2 mg kg⁻¹ body mass) and ketamine hydrochloride (Ketaset; Bristol Laboratories, Syracuse, NY; 2.2 mg kg⁻¹ body mass). Halothane (Halocarbons Laboratories, Hackensack, NJ), oxygen and nitrous oxide were used to maintain a surgical plane of anaesthesia. Ovulation rate was estimated by counting the number of corpora lutea. Cystic follicles (> 10 mm) were observed in one bINH:HAG immunized gilt at unilateral ovariectomy; this gilt was removed from the study. After unilateral ovariectomy, gilts were observed for 30 min each day in the continuous presence of a boar until detection of the subsequent oestrus. To determine whether compensatory ovarian hypertrophy had occurred, the remaining ovary was removed by mid-ventral laparotomy between day 8 and day 12 of the first oestrous cycle after unilateral ovariectomy and the number of corpora lutea per ovary after unilateral ovariectomy was compared with the number per ovary before unilateral ovariectomy.

Blood sampling, antibody titres and radioimmunoassays

On day 9 of the first oestrous cycle after week 19, a catheter was installed into the anterior vena cava of each gilt (Britt *et al.*, 1991). To maintain patency, catheters were flushed with 0.12 mol sodium citrate l⁻¹ after each sample was collected. Blood was sampled 1 h before unilateral ovariectomy, every 2 h for 24 h after unilateral ovariectomy, and then every 6 h until oestrus. Blood samples were clotted overnight at 4°C, and serum was collected by decanting the supernatant after centrifugation at 1700 g for 30 min. Serum was stored at

– 20°C until assayed for LH, FSH, progesterone and oestradiol.

Antibody titres were determined at week 17 (7 days after the last booster) and at week 23 when unilateral ovariectomy was carried out by incubating serial dilutions of serum with 20 000 c.p.m. of the ¹²⁵I-labelled bINH at 5°C (Martin *et al.*, 1991a, b; King *et al.*, 1993). Titres were expressed as percentage of ¹²⁵I-labelled bINH bound at a 1:4000 dilution of serum.

Previously validated radioimmunoassays were used to quantify serum concentrations of oestradiol (Howard and Britt, 1990), progesterone (Stevenson *et al.*, 1981), LH (Stevenson *et al.*, 1981; Armstrong and Britt, 1987) and FSH (Guthrie and Bolt, 1983; Esbenschade and Britt, 1985). Inter- and intra-assay coefficients of variation ranged from 9% to 14% for each assay. For statistical analysis, FSH values below assay sensitivity were assigned a value equal to the limit of sensitivity of the assay.

Statistical analyses

All analyses were conducted using general linear models (SAS, 1985). Data for concentrations of LH, FSH, oestradiol and progesterone were subjected to split-plot ANOVA for repeated measures (Gill and Hafs, 1971). The model included treatment, gilt within treatment, time and time × treatment interaction. Main effect of treatment was tested using the gilt within treatment mean square as error term. Data were analysed as time (h) from unilateral ovariectomy and time (h) from day of oestrus because animals had a variable number of days between unilateral ovariectomy and oestrus. Data for duration of oestrous cycle were analysed by one-way ANOVA, whereas data for number of corpora lutea per ovary before and after unilateral ovariectomy were analysed by two-way ANOVA.

Results

Compensatory ovarian hypertrophy

After unilateral ovariectomy, the mean (± SEM) number of corpora lutea per ovary increased ($P < 0.01$) over twofold in controls (7.6 ± 0.6 to 16.2 ± 1.2) but remained unchanged ($P > 0.10$) in gilts immunized against bINH:HAG (8.6 ± 0.7 versus 10 ± 2.1) compared with the number of corpora lutea per ovary at unilateral ovariectomy. Further statistical comparisons indicated that the number of corpora lutea per ovary for bINH:HAG-immunized gilts and controls was not different ($P > 0.10$) at unilateral ovariectomy, whereas the number of corpora lutea after unilateral ovariectomy was greater ($P < 0.05$) in controls compared with bINH-immunized gilts.

Duration of oestrous cycle

Mean (± SEM) duration of the oestrous cycle during which unilateral ovariectomy was performed did not differ between treatment groups (21.3 ± 1.6 for bINH:HAG versus 19.0 ± 0.9 days for control gilts).

Hormone profiles

Serum concentrations of FSH and LH increased ($P < 0.01$) within 24 h of unilateral ovariectomy (Fig. 1) followed by a

Fig. 1. Serum (a) FSH and (b) LH concentrations after unilateral ovariectomy of gilts immunized against human alpha globulins (Δ ; $n=5$) or bovine inhibin α^{1-26} Gly-Tyr conjugated to human α globulins ($\bullet$; $n=4$). Standard errors for LH ranged from 0.1 to 2.1 ng ml⁻¹, whereas standard errors for FSH ranged from 0 to 14.4 ng ml⁻¹.

sustained increase ($P < 0.01$) in oestradiol (Fig. 2) in control and bINH:HAG-immunized gilts. Serum concentrations of progesterone decreased after unilateral ovariectomy in both groups (Fig. 2). Serum LH, FSH, oestradiol and progesterone profiles did not differ between treatment groups after unilateral ovariectomy or when data were aligned based on day of oestrus after unilateral ovariectomy (data not shown).

Antibody titres

Antibody titres in bINH:HAG-treated gilts declined ($P < 0.05$) from $22 \pm 3\%$ at week 17 before unilateral ovariectomy to $9 \pm 1\%$ at week 23 when unilateral ovariectomy was carried out. In contrast, inhibin antibody titre was 0% in controls.

Discussion

The results presented in this study clearly show that active immunization of gilts against bINH:HAG blocks compensatory ovarian hypertrophy after unilateral ovariectomy without altering the patterns of gonadotrophin and ovarian steroid secretion or duration of the oestrous cycle compared with unilateral ovariectomy of controls immunized against HAG. In addition, the normal ovulation rate for a single ovary (in a gilt with both ovaries) was unaltered in bINH:HAG-immunized gilts, indicating that the negative effect of active immunization against bINH:HAG was specific to compensatory follicular growth and

Fig. 2. Serum (a) oestradiol and (b) progesterone concentrations after unilateral ovariectomy of gilts immunized against human alpha globulins (HAG; Δ ; $n=5$) or bovine inhibin α^{1-26} Gly-Tyr conjugated to HAG ($\bullet$; $n=4$). Standard errors ranged from 1.3 to 17.8 pg ml⁻¹ for oestradiol and from 0.4 to 8.9 ng ml⁻¹ for progesterone.

not the result of a general inhibitory effect of long-term immunoneutralization of inhibin or inhibin α subunits on follicular growth and function. These findings imply a specific, local, positive effect for inhibin or inhibin α subunits on compensatory follicular growth.

Nevertheless, the results of this unilateral ovariectomy study were surprising because a previous study (King *et al.*, 1993), using the same gilts with both gonads, showed that active immunization against bINH:HAG increased the ovulation rate by 38% which was associated with transiently higher concentrations of FSH in the oestrous cycle before unilateral ovariectomy. In addition, injections of follicular fluid, which is rich in inhibins, block compensatory ovarian hypertrophy in gilts (Redmer *et al.*, 1985). Thus, it was expected that active immunization against inhibin would have a positive effect on compensatory ovarian hypertrophy. Although the mechanism explaining how active immunization against bINH:HAG inhibits compensatory ovarian hypertrophy is not understood, results of several recent studies indicate a positive role for inhibin or inhibin α subunits in follicular growth and function. Specifically, ovarian mRNAs encoding inhibin increase in newly recruited follicles after unilateral ovariectomy of rats (D'Agostino *et al.*, 1989) and during development of follicles in cattle (Ireland and Ireland, 1994). The immuno- and bio-activity of inhibin increases after unilateral ovariectomy of gilts and rats (Redmer *et al.*, 1986; Ackland *et al.*, 1990) and during follicular growth in cattle (Martin *et al.*, 1991b; Ireland *et al.*, 1994). Inhibin injected into an ovary enhances growth of medium-size follicles in rats (Woodruff *et al.*, 1990) and stimulates androgen

production by thecal cells of rats *in vitro* (Hillier *et al.*, 1991). Inhibin α subunits bind the FSH receptor in rat granulosa cells (Schneyer *et al.*, 1991) and may act as agonists to FSH, promoting follicular growth. On the basis of the results of the present study and others, we hypothesize that active immunization against bINH:HAG blocks the local stimulatory effects of inhibins or inhibin α subunits on the growth of a subpopulation of follicles that would normally undergo compensatory ovarian hypertrophy after unilateral ovariectomy of gilts.

While the reason FSH concentrations were not increased in gilts actively immunized against bINH:HAG in the present study, despite high titres of neutralizing inhibin antibodies (King *et al.*, 1993), is unknown, other studies report similar results after active (Wrathall *et al.*, 1990; Knight *et al.*, 1991) or passive immunization (Mann *et al.*, 1989) of sheep. In addition, our previous study (King *et al.*, 1993) indicates a positive effect of actively immunizing gilts against bINH:HAG on FSH secretion and ovulation rate. Thus, the results of the present study appear paradoxical. Although the reason for these inconsistent results following active immunization against fragments of inhibin α subunits is unknown, there are numerous forms of inhibins and inhibin α subunits (Miyamoto *et al.*, 1985, 1986; Sugino *et al.*, 1992; Ireland *et al.*, 1994) with different biological actions and potencies (Ghosh *et al.*, 1994; Padmanabhan *et al.*, 1994) in follicular fluid that are secreted into the peripheral circulation (Knight *et al.*, 1989) in cattle. Thus, it is difficult to predict which forms of inhibin or inhibin α subunits in the peripheral circulation or follicular fluid are neutralized after active immunization against bINH:HAG, or whether the forms of inhibin or inhibin α subunits recognized by bINH:HAG antiserum change with decreasing titre and/or time. Either, or both, of these possibilities could explain the different effects of active immunization against bINH:HAG on FSH secretion and ovulation rate in our previous (King *et al.*, 1993) compared with our present study. Alternatively, the different effects of active immunization against bINH:HAG on FSH secretion and ovulation rate may be unique to the two different animal models (intact versus unilateral ovariectomy) used in our studies.

Because compensatory ovarian hypertrophy was blocked after unilateral ovariectomy of gilts actively immunized against bINH:HAG, independent of alterations in serum concentrations of gonadotrophins or ovarian steroids, the duration of the oestrous cycle, or a reduction in the normal ovulation rate per ovary, we conclude that inhibins or inhibin α subunits act locally to stimulate positively compensatory ovarian hypertrophy in postpubertal gilts.

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