Pituitary and Leydig cell function in boars actively immunized against gonadotrophin-releasing hormone*

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Summary. Crossbred boars were (a) immunized against GnRH conjugated to human serum globulin (200 µg GnRH-hSG) in Freund's adjuvant at 12 weeks of age and boosted at weeks 18 and 20 (N = 10), (b) served as controls and received hSG only in adjuvant (N = 10), or castrated at weaning (N = 10). At 24 weeks of age (immediately before slaughter), the boars were challenged with saline or pig LH (1 µg/10 kg body weight). After slaughter, fresh testicular fragments were incubated with pig LH (0.05 and 0.2 ng/2 ml medium) to assess the effects of immunization on Leydig cell function. Pituitary contents of LH and FSH, and testicular LH receptor content were also measured. The results indicated that plasma LH and testosterone concentrations, pituitary LH content, testicular LH receptor content, testis and sex accessory organ weights were significantly reduced in GnRH-immunized boars compared to hSG-adjuvant controls. However, plasma and pituitary FSH content were not affected by high antibody titres generated against GnRH. The testicular testosterone response to exogenous LH *in vivo* and *in vitro* was significantly reduced ($P < 0.05$) in GnRH-immunized boars. These results indicate that active immunization against GnRH impairs pituitary and Leydig cell functions in boars.

Keywords: GnRH immunization; testosterone; LH; FSH; LH receptor; boars

Introduction

Gonadotrophin-releasing hormone (GnRH) has been shown to stimulate the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the adenohypophysis in both male and female mammals (Arimura & Schally, 1974). This observation has led to the widely accepted concept that GnRH is the primary neurohormone that regulates the release of both gonadotrophins. However, there have been a number of instances in which dichotomy in secretion of LH and FSH has been demonstrated, suggesting that there may be different mechanisms for the regulation of LH and FSH synthesis and secretion (Lorenzen *et al.*, 1981; Sherins *et al.*, 1982; Culler & Negro-Vilar, 1986, 1987).

In many studies conducted to elucidate the relationship existing between the hypothalamus and pituitary, and to study the physiology of the pituitary gland after deprivation of neurohormones, pituitary stalk transection and hypothalamic lesions have been imposed (Cronin *et al.*, 1982; Norman *et al.*, 1982; Clarke & Cummins, 1984). However, these methods can induce unintended and disruptive physiological consequences. Active immunization against GnRH in boars produced

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a selective immunological barrier between the hypothalamus and the anterior pituitary (Falvo *et al.*, 1986), and there are morphological changes in the boar testis after active immunization against GnRH (Awoniyi *et al.*, 1987). In the present study, we have examined the effects of active immunization against GnRH in boars on (i) the role of GnRH in the regulation of gonadotrophin secretion, (ii) the secondary effect of testicular steroidogenesis by stimulation of Leydig cells *in vivo* with pig LH and testicular testosterone production *in vitro* in response to exposure to pig LH, and (iii) the changes in the testicular LH receptor content.

Materials and Methods

Animals. Cross-bred boars (Duroc × Yorkshire × Hampshire) were randomly assigned to three groups (10 boars/group), and treated as follows: Group 1 boars were immunized with 200 µg GnRH-human serum globulin (GnRH-hSG) conjugate in complete Freund's adjuvant (CFA) at 12 weeks of age and were given booster injections using incomplete Freund's adjuvant in Weeks 18 and 20. Group 2 boars (adjuvant controls) received hSG only in adjuvant, and Group 3 boars were castrated at weaning (4–6 weeks of age). Purified GnRH was made immunogenic by conjugation to the carrier protein, hSG (Cohn fraction IV-1; Sigma Chemical Co., St Louis, MO, U.S.A.), by carbodiimide reaction. Briefly, 200 µg GnRH-hSG conjugate or hSG were dissolved in 1 ml sterile saline (900 mg NaCl in 100 ml distilled water; Abbott Laboratories, North Chicago, IL, U.S.A.) and 1 ml of Freund's adjuvant (Sigma Chemical Co.) and emulsified. The antigen was then injected subcutaneously (2 sites) at the basal side of the left and right axillary regions. Blood samples were taken from all boars in Weeks 8, 12, 16, 18, 20, 22 and 24 via anterior vena cava puncture without anaesthesia, and plasma was assayed for testosterone, LH and FSH by radioimmunoassays. At slaughter, the testes, sex accessory organs and pituitary glands were removed and weighed.

Antibody titre to GnRH. Antibody titres generated against GnRH in boars immunized with GnRH-hSG conjugate were determined from plasma samples collected in Weeks 16, 18, 20, 22 and 24 as previously described by Schanbacher (1982), using freshly radioiodinated GnRH.

Pituitary LH and FSH content. Boars in Groups 1 and 2 were exsanguinated and decapitated, and pituitaries were removed, weighed and stored frozen in 4 ml PBS at -20°C . Before assay, the pituitary was thawed to 4°C , homogenized and centrifuged for 30 min at 1200 g. The supernatant was decanted and assayed for LH and FSH.

Hormone assay. Plasma and pituitary concentrations of LH (Niswender *et al.*, 1970) and testosterone (Falvo & Nalbandov, 1974) were determined in duplicate samples. Sensitivities for the respective assays were 10 pg/tube for LH (LER-778-4, which has an LH biological activity of $0.50 \times \text{NIH-LH-SI U/mg}$) and 5 pg/tube for testosterone. The interassay coefficients of variation for LH and testosterone were $<10\%$.

Plasma and pituitary concentrations of FSH were quantified by a heterologous double antibody radioimmunoassay procedure. Purified pig FSH (USDA-FSH-PP1) was radioiodinated by the chloramine T method of Greenwood *et al.* (1963), and was used as the tracer. Pig FSH (pFSH-B-1) was utilized as a reference preparation, and antibodies developed against rat FSH (AFP-C0972881) were used. Although an homologous double-antibody radioimmunoassay using anti-pig FSH (USDA-10-1010) (Guthrie & Bolt, 1983) produced a linear standard curve, this antiserum was not sensitive enough to allow measurement of FSH in plasma from control or castrated boars at sample volumes of 500 µl, and so an anti-rat FSH was used. Various amounts of a plasma pool (from castrated boars) produced an inhibition curve which was parallel with the inhibition curve obtained with the pig FSH reference preparation. The slopes of the curve were -3.01 and -3.08 , respectively, which were not statistically different. This antiserum (anti-rat FSH) was used at a final dilution of 1:50 000. The bound radioactive antigen-antibody complex was precipitated by addition of goat anti-rabbit gamma globulin. The sensitivity of the assay was 5 pg/tube. The interassay coefficient of variation was 8.7%.

In-vivo testosterone release. At 24 weeks of age (immediately before slaughter), an in-vivo intravenous challenge to saline (0.5 ml) or USDA pig LH-B-1 (AFP-5400) ($1 \mu\text{g}/10 \text{ kg body weight}$) dissolved in 0.5 ml sterile saline was conducted.

In-vitro testosterone production. The ability of the testes of animals treated with hSG or GnRH-hSG to produce testosterone *in vitro* in response to gonadotrophin (pig LH) stimulation was tested using procedures previously described (Dufau *et al.*, 1971). Each testis was decapsulated, and fragments (about 200 mg, 3 fragments/boar) were obtained and placed in a beaker containing 2 ml PBS for a preincubation wash for 15 min. This preincubation wash was necessary to reduce the large quantities of preformed testosterone released into the medium which would tend to mask testosterone production when stimulated with LH. The fragments were then removed from the preincubation medium and placed in a 25 ml beaker containing 1.9 ml Krebs-Ringer bicarbonate buffer previously gassed with O_2 : CO_2 , (95:5 v/v) and containing glucose (1 mg/ml). Pig LH (USDA-pLH-B-1) was diluted with saline (0.05 ng, or 0.2 ng/0.1 ml) and added to the incubations in 0.1 ml volume. Saline (0.1 ml) was added to the incubations without pig LH. The incubations were carried out for 4 h at 32°C in a Dubnoff metabolic incubator. At the end of the incubation,

samples of the medium were removed for the measurement of testosterone by radioimmunoassay without purification, since these procedures are not required for valid determination of testosterone concentrations in incubation media (Van Damme *et al.*, 1973).

LH receptor assay. Testicular LH receptors were measured by radioreceptor assay using a procedure previously characterized for other species (Klemcke & Bartke, 1981; Klemcke *et al.*, 1981; Amador *et al.*, 1983). The content of protein in testicular membrane preparations used for measurement of LH receptors was determined by a modification of Lowry's procedure (Markwell *et al.*, 1978). Briefly each assay tube contained a membrane preparation concentration equivalent to that obtainable from 10 mg testes and 2.18 ng ^{125}I -labelled hCG (CR-121, NICHHD). This was found to be a saturating concentration of hCG for the receptors present in the membrane preparation obtained from 10 mg testis. The chloramine-T method was used to radioiodinate the hCG, and the resulting trace had an average specific activity of 30.9 $\mu\text{Ci}/\mu\text{g}$ and a maximum binding ability of 42.0%. The K_d for LH receptors (as determined by Scatchard plot) in pig testes was found to be 1.99×10^{-12} (Fig. 1).

Fig. 1. Scatchard plot of LH receptors in boar testicular membrane preparation using ^{125}I -labelled hCG.

Statistical analysis. Data were analysed by least-squares analysis of variance and corresponding F-test (Dixon & Massey, 1969). Post-hoc comparisons of means were done by Tukey (Kirk, 1968) and Scheffe tests (Scheffe, 1959).

Results

Antibody titre

The immunization schedule effectively induced antibody production capable of binding GnRH. Antibody titres in GnRH-immunized boars were detectable 4 weeks after primary immunization ($18.3 \pm 2.3\%$ at 1:1000 plasma dilution). The titre rose markedly in response to both the first and second boosters ($77.6 \pm 3.2\%$; $83 \pm 2.6\%$, at 1:1000 plasma dilution), and remained elevated during the remainder of the experiment ($87.2 \pm 3.4\%$).

Testes and accessory sex organs

Before slaughter, there was a correlation between the GnRH antibody titre, and the degree of testicular atrophy in GnRH-immunized boars. Testes of these boars were small, flaccid and retracted into the inguinal canal. The paired testes and accessory sex organ (Cowper's gland, prostate, and seminal vesicle) weights for the different groups are shown in Fig. 2. The weights of the testes in GnRH-immunized boars were significantly reduced. Similarly accessory sex organ

weights in GnRH-immunized boars and castrated controls were significantly different from each other ($P < 0.05$) and were significantly reduced when compared with those of adjuvant-injected controls.

Fig. 2. Paired testis and sex accessory gland weights in GnRH-immunized (Group 1), adjuvant control (Group 2), and castrated (Group 3) boars. Values are mean $\pm$ s.e.m. for 10 boars/group. ^a $P < 0.05$ compared with Group 2; ^b $P < 0.05$ compared with Group 3.

Testosterone, LH and FSH concentrations

The high antibody titre observed in response to the first booster at Week 18 (77.6%; 1:1000 plasma dilution) coincided with low plasma concentrations of both testosterone and LH in GnRH-immunized boars (Fig. 3), and showed a significant decrease compared to adjuvant-injected control boars. In GnRH-immunized boars, plasma concentrations of GnRH antibody, which were consistently $> 70\%$ binding after the first booster, impeded the synthesis of pituitary LH and caused a significant reduction in pituitary LH content as compared to adjuvant-treated controls (0.14 ± 0.01 vs 0.25 ± 0.02 ng/mg pituitary; $P < 0.05$).

In contrast to the low to non-detectable concentrations of testosterone and LH seen in response to high antibody titres to GnRH, FSH values in GnRH-immunized boars were unaffected and did not differ when compared with those of adjuvant-treated control boars throughout the experimental period. Concentrations of FSH in castrated boars were significantly elevated throughout the experiment and differed significantly from those in the GnRH-immunized and adjuvant control groups. There was no significant difference in pituitary FSH content in GnRH-immunized boars compared to the adjuvant-treated controls (3.1 ± 0.5 vs 5.1 ± 1.0 ng/mg pituitary).

Treatment with LH in vivo and in vitro

Intravenous injection of saline resulted in no significant alteration in plasma testosterone concentrations (Fig. 4). However, administration of LH significantly increased plasma testosterone concentrations at 60 min in both groups.

Fig LH at 0.05 or 0.2 ng/2 ml medium did not stimulate testosterone production by the testicular fragments of boars immunized against GnRH (0.04 ± 0.01 vs 0.32 ± 0.04 ng/mg; $P < 0.05$ at a dose of 0.05 ng LH/2 ml medium and 0.04 ± 0.01 vs 0.55 ± 0.05 ng/mg, $P < 0.05$ at a dose of 0.2 ng LH/2 ml medium). However, 0.2 ng LH significantly increased testosterone production in tissue from adjuvant-treated controls (saline = 0.27 ± 0.04 , LH = 0.55 ± 0.05 ng/mg; $P < 0.05$).

Fig. 3. Plasma concentrations of testosterone, LH and FSH in GnRH-immunized (Group 1), adjuvant control (Group 2), and castrated (Group 3) boars. Values are mean $\pm$ s.e.m. for 10 boars/group. Uppercase superscripts denote differences ($P < 0.05$) within each group over weeks; lower case superscripts indicate differences ($P < 0.05$) between groups. Pre-immunization levels of hormones are shown at 8 and 12 weeks of age.

LH receptor

Active immunization against GnRH did not significantly decrease the concentration of testicular LH receptors, but reduced the total content of the LH receptor compared to adjuvant-treated control boars (58.5 ± 18.0 vs 327.1 ± 44.1 pmol/pair of testes, $P < 0.001$).

Discussion

This study demonstrates that young boars are capable of generating high levels of GnRH antibodies after active immunization against GnRH. Binding of 125 I-labelled GnRH with sera of

Fig. 4. Plasma testosterone concentrations in GnRH-immunized (Group 1) and adjuvant control (Group 2) boars after an in-vivo saline and pig LH challenge. Values are mean $\pm$ s.e.m. for 10 boars/group. Uppercase superscripts denote differences ($P < 0.05$) within each group over time; lower case superscripts indicate differences ($P < 0.05$) between groups.

immunized boars (at a dilution of 1:1000) suggests that GnRH antibodies effectively neutralized endogenous GnRH.

When GnRH antibody titres reached high levels, plasma LH concentrations became undetectable, as a result of insufficient free GnRH binding to pituitary gonadotrophes. Low to non-detectable concentrations of plasma testosterone resulted, with a reduction in both the size and weight of testes and sex accessory organs. Similar findings for the effect of GnRH immunization have been reported for males of other species such as rats (Sharpe & Fraser, 1978, 1979; Shiota *et al.*, 1981), rabbits (Arimura *et al.*, 1973), monkeys (Chappel *et al.* 1980), and ram lambs (Schanbacher, 1982). Accessory sex organ weights were reduced in castrated compared to GnRH-immunized animals, most probably due to the increased period of androgen deprivation.

In this study, FSH concentrations were not affected in spite of the high antibody titres to GnRH, which leads us to suggest that there may be a component for FSH secretion independent of GnRH in boars. In addition, this divergent effect of GnRH immunization on LH and FSH secretion supports the hypothesis of a separate releasing hormone for FSH. Nevertheless, the possibility of reduced FSH concentrations in these boars after chronic GnRH immunization cannot be ruled out.

The differential regulation of LH and FSH in other species has been demonstrated (Thompson *et al.*, 1979; Sherins *et al.*, 1982; Culler & Negro-Vilar, 1986, 1987). Since inhibin secreted by the testis has a role in FSH secretion, it is possible that inhibin was not affected in GnRH-immunized boars, contributing to the unaltered plasma FSH values in this study. However, providing proof of the factor(s) that control the synthesis and secretion of FSH (other than GnRH) in boars requires further investigation.

Changes in testicular responsiveness to LH are usually monitored by quantifying testosterone production after gonadotrophin stimulation *in vivo* (Lincoln *et al.*, 1982) and *in vitro* (Wolfe *et al.*, 1981). However, in-vitro testosterone production by incubating pig testicular tissue with LH has yet to be reported. In this study, testosterone production was demonstrated only *in vivo* but not *in vitro* after treatment of immunized boars with pig LH. The severe damage of Leydig cells in boars after active immunization against GnRH (Awoniyi *et al.*, 1987) may explain the inability of the testes to produce testosterone *in vitro*. On the other hand, the ability of the testes to produce testosterone *in vivo* suggests that there may be another factor(s) acting synergistically with pig LH

when injected to augment testosterone release. Other factors implicated to produce testosterone include intratesticular factors (Sharpe, 1984), and a thymus factor (Pedernera *et al.*, 1986). FSH promotes the development and increased sensitivity of LH receptors (MacKinnon *et al.*, 1978), therefore the presence of normal FSH concentrations in the immunized boars also may have increased the sensitivity of LH receptors to LH. Since the testosterone concentrations after administration of pig LH in GnRH-immunized boars were still below the baseline values observed in adjuvant-treated controls, it may be concluded that the testes of these boars have less steroidogenic capacity.

Morphometric analysis showed a drastic reduction in the volume percentage of Leydig cells in the testes of GnRH-immunized boars (Awoniyi *et al.*, 1987). This observation may explain the decrease in the total content of the LH receptor in spite of a normal concentration of the receptor, since similar changes have been observed in Syrian and Siberian hamsters treated with anti-GnRH serum (Bartke *et al.*, 1987).

Uniformity and a high percentage of response to immunization must be demonstrated before using this method as a practical alternative to surgical castration. However, this type of uniform response has not been seen in previous studies in rats (Fraser *et al.*, 1974), monkeys (Chappel *et al.*, 1980), rabbits (Arimura *et al.*, 1973) and dogs (Schanbacher *et al.*, 1983) immunized against GnRH. Combined with the high success rate of immunocastration in boars observed in our previous study (Falvo *et al.*, 1986), our present findings provide an added incentive to use this approach both experimentally and practically as an alternative to surgical castration.

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