Effects of a gonadotrophin-releasing hormone antagonist on gonadotrophin secretion and gonadal development in neonatal pigs*

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Summary. Male (N = 8) and female (N = 8) pigs were assigned to receive saline or a potent GnRH antagonist (\([\text{Ac-d}^2\text{Nal}^1,\text{d}^4\text{-Cl-Phe}^2,\text{d-Trp}^3,\text{d-Arg}^6, \text{d-Ala}^{10}]\)-GnRH*HOAc; 1 mg/kg body weight) at 14 days of age. The GnRH antagonist caused LH to decline \((P < 0.01)\) from 1.7 ng/ml at 0 h to <0.5 ng/ml during 4–32 h in males and females. Concentrations of FSH in gilts declined slowly from 75 ± 8 to 56 ± 5 ng/ml \((P < 0.05)\) at 32 h. In males FSH was low \((5.7 ± 0.5 \text{ ng/ml})\) at 0 h and did not change significantly.

To observe the effect of long-term treatment with GnRH antagonist, 10 male and 10 female pigs, 3 days of age, were treated with saline or 1 mg GnRH antagonist per kg body weight every 36 h for 21 days. Concentrations of LH were reduced \((P < 0.01)\) to 0.2–0.4 ng/ml throughout the experimental period in male and female piglets treated with GnRH antagonist. Plasma FSH increased in control females, but remained suppressed \((P < 0.001)\) in females treated with GnRH antagonist. Treatment with the GnRH antagonist suppressed FSH levels in males on Days 8 and 16 \((P < 0.05)\), but not on Day 24. Treatment of females with the GnRH antagonist did not influence \((P > 0.10)\) oestradiol-17\(\beta\) concentrations. Administration of GnRH antagonist to males suppressed testosterone and oestradiol-17\(\beta\) values \((P < 0.01)\) and reduced testicular weight \((P < 0.01)\). Concentration of LH/hCG receptors in testes of boars treated with GnRH antagonist was lower \((P < 0.10)\) than in controls, but concentration of FSH receptors was not affected. Basal and potassium-stimulated release of GnRH from the stalk median eminence and medial basal hypothalamus \textit{in vitro} did not differ between treatment groups. The amount of residual GnRH in hypothalamic tissue was not different in control gilts and in gilts receiving the GnRH antagonist, but it was lower \((P < 0.05)\) in boars treated with GnRH antagonist than in control boars.

Keywords: pig; GnRH antagonist; LH; FSH

Introduction

Secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland of neonatal pigs is controlled by hypothalamic gonadotrophin-releasing hormone (GnRH; Colenbrander et al., 1987; Elsaesser et al., 1988). Administration of GnRH induced release of LH and FSH from the pituitary gland as early as Day 70 of gestation and this responsiveness remained during the neonatal period (Colenbrander et al., 1982a). Circulating concentrations of LH and

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FSH are relatively high during the perinatal period in the female, but only LH is elevated in the male (Colenbrander et al., 1977, 1982b). Since the ontogeny of development of the hypothalamus and pituitary gland regulates the development of the gonads, interruption of the hormonal control of the pituitary gland by the hypothalamus should interfere with the normal development of the gonads. In rats, administration of a GnRH antagonist reduced gonadotrophin receptors in testes and delayed puberty in males (Kolho et al., 1988). The objectives of the present study were to examine the role of the hypothalamus in the regulation of gonadotrophin release in neonatal pigs and to investigate the role of gonadotrophins in gonadal development by giving a potent GnRH antagonist known to abolish gonadotrophin secretion in other species (Pineda et al., 1980; Kenigsberg et al., 1984; Grady et al., 1985; Kartun & Schwartz, 1987).

Materials and Methods

Experimental design. Part 1 of the study examined efficacy of the GnRH antagonist. Male (N = 8) and female (N = 8) pigs were assigned to receive saline (0-154 m-NaCl) or GnRH antagonist ([Ac-d^2]-Nal^1,d^4-Cl-Phe^2,d-Trp^3,d-Arg^6,d-Ala^10]-GnRH*HCl; Contraceptive Development Branch, National Institutes of Health, Bethesda, MD, USA) at a dose of 1 mg/kg body weight i.m. at 14 days of age. Blood samples for determination of LH and FSH were taken by jugular venepuncture on Days 7, 14, 21 and 28 of age. Blood samples were also collected at 0, 1, 4, 16 and 32 h after treatment.

Part 2 of the study was designed to observe the effect of long-term treatment of neonatal pigs with a GnRH antagonist on secretion of gonadotrophins, gonadal function and hypothalamic function. Male (N = 10) and female (N = 10) pigs were assigned to two groups at 3 days of age and given saline or 1 mg GnRH antagonist/kg body weight every 36 h for 21 days. Blood samples were taken by venepuncture at 8, 16 and 24 days of age and analysed for LH, FSH, oestradiol and testosterone. Animals were slaughtered at 25 days of age, 1 day after the last treatment with saline or GnRH antagonist. Testes and ovaries were excised, trimmed of excess tissue, weighed, frozen and stored in liquid nitrogen until measurement of LH and FSH receptors. Hypothalami were removed from the animals for study in vitro.

In-vitro incubation of hypothalami. The stalk median eminence and medial basal hypothalamus were dissected from each brain within 1–3 min after death and weighed and placed in glass vials (20 × 40 mm) containing 2 ml incubation medium (Krebs–Ringer bicarbonate buffer (Sigma Chemical Co., St Louis, MO, USA), pH 7.4, supplemented with 1 mg glucose/ml). Incubation vials were maintained in a water bath–shaker at 37°C with constant shaking (45 cycles/min) in an atmosphere of 95% O_2 and 5% CO_2. Tissues were incubated for 30 min, and after a change of medium, were incubated for another 60 min. Tissues were then exposed to 56 µM-potassium and release of GnRH into the medium was estimated during two 30-min periods. Immediately after incubation, the liquid contents were carefully transferred into plastic tubes and centrifuged at 3000 g for 20 min at 4°C. The supernatant was decanted and frozen at −20°C until assayed for GnRH. The hypothalamic tissue remaining in the incubation vials was weighed, homogenized in 2 ml 0.1 N HCl, centrifuged at 3000 g for 20 min at 4°C and the supernatant collected and stored at −20°C until determination of cellular content of GnRH.

Recoveries of GnRH in the incubation system were estimated from adding 400 µg synthetic GnRH (acetate salt, Sigma Chemical Co.) to vials containing 2 ml incubation buffer and a known amount of hypothalamic tissue. After 30 min of incubation under the above conditions, 373 ± 15 µg (mean ± s.e.; n = 5) or 93% of the GnRH was recovered.

Gonadal receptors. After thawing, testicular tissues were minced with scissors and homogenized in 5 volumes (w/v) of 25 mM-Tris–HCl (pH 7.4) at 4°C with a Polytron homogenizer (Brinkman Instruments, Westbury, NY, USA). The homogenates were filtered through cheesecloth and the filtrates saved for determination of receptors. The filtrates were incubated with 125I-labelled human chorionic gonadotrophin (hCG; CR-121; 13450 i.u./mg; Med-Tech, Inc., Elwood, KS, USA) in precoated (3% BSA) plastic tubes as previously described (Ziecik et al., 1988). Specific activity was 60 000 c.p.m./ng as measured by self-displacement analysis (Ketelslegers et al., 1975) in a radioligand receptor assay.

A one-point saturation analysis was conducted to estimate hCG binding to gonadal tissues: 50 µl of testicular homogenates were incubated in duplicate with 200 000 c.p.m. 125I-labelled hCG with (specific binding) or without (total binding) 20 i.u. hCG. Six-point Scatchard plots (Scatchard, 1949) for 125I-labelled hCG binding by 2 pools of testicular homogenates were conducted to confirm the validity of the one-point saturation analysis. Thus, 50 µl of the pool homogenates were incubated with increasing amounts of 125I-labelled hCG (20 000–800 000 c.p.m./tube) in the presence or absence of excess of unlabelled hCG.

Testicular FSH receptors were measured in the filtrates by previously reported and validated methods (Ziecik et al., 1988). The radiolabelled ligand was oFSH and non-specific binding was determined by adding excess pig FSH (0.5 mg; Burns-Biotec Lab., Inc., Omaha, NE, USA).

The specific activity (µCi/µg) and maximum binding (%) in the presence of receptor excess were 42 and 55 for the LH/hCG and 30 and 40 for the FSH receptor assays, respectively. Concentration of unoccupied binding sites and
affinity constants ($K_d$) were determined by Scatchard analysis with 6 sub-saturating quantities of unlabelled hCG or pig FSH (0.15–5.0 ng/tube) used for each receptor preparation.

**Blood samples and radioimmunoassays.** Blood samples were collected with heparinized syringes, transferred to test tubes, placed on ice and centrifuged within 1 h. Plasma was separated by decanting the supernatant and stored at -20°C until assayed.

Plasma FSH was quantified by radioimmunoassay procedures (Guthrie & Bolt, 1983) using anti-pFSH (USDA-10-1010) and $^{125}$I-labelled pFSH (USDA-FSH-Ppl) for the radiolabelled ligand and pFSH (USDA-FSH-B1) for standards as modified by Esbenshade & Britt (1985). Average sensitivity of the assay was 4 ng/ml. All samples were analysed in one assay with an intra-assay coefficient of variation of 7%.

Plasma concentrations of LH were determined by radioimmunoassay (Stevenson et al., 1981; Armstrong & Britt, 1987) with pig LH (LER-786-3) used as the radioiodinated antigen and standard. Assay sensitivity at 95% binding was 0.2 ng/ml. Samples were quantified in one assay with an intra-assay coefficient of variation of 5%.

A previously reported and validated radioimmunoassay was used to quantify plasma concentrations of oestradiol-17ß (Cox & Britt, 1982). Total testosterone in plasma samples was estimated by solid-phase radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA, USA). The sensitivity of one assay was 0.2 ng/ml and the intra-assay coefficient of variation was 10%.

GnRH was determined in media by using antiserum R-42 (Nett et al., 1973) at a dilution of 1:60 000. Synthetic GnRH was radioiodinated using the Iodogen method (Fraker & Speck, 1978) and iodinated hormone was separated from free iodine with ion-exchange chromatography. For the assay, 0.2 ml samples of antiserum in 0.1% BSA-PBS were added to 0.2 ml unknown or standard samples diluted in PBS followed by 0.1 ml $^{125}$I-labelled GnRH in 0.1% BSA-PBS (~25 000 c.p.m.). After incubation for 24 h at 4°C, free labelled GnRH was separated from bound by adding 1.5 ml ice-cold 95% (v/v) ethanol, centrifuging and decanting the supernatant. Bound $^{125}$I-labelled GnRH was estimated by counting the pellet. The range of standards used was 0.01 to 10 pg/tube with an average assay sensitivity of 4 pg/ml at 95% binding. Intra- and interassay coefficients of variation were 7 and 12%, respectively. The GnRH antagonist used in the present experiment did not cross-react in the assay at concentrations up to 1000 ng/tube.

**Statistical analysis.** Analysis of variance for repeated measures using general linear models (SAS, 1985) was used to determine differences in concentration of hormones in the blood after treatment with saline or GnRH antagonist. Other differences between groups were compared by one way analysis of variance.

**Results**

**Response to single injection of GnRH antagonist**

Plasma concentrations of LH fell rapidly in male and female piglets after administration of GnRH antagonist (Fig. 1). Concentrations were suppressed ($P < 0.01$) from pre-treatment values of 1.8 ± 0.2 and 1.6 ± 0.2 ng/ml in male and female piglets, respectively, to less than 0.5 ng/ml in both sexes by 4 h after treatment. In control gilts, LH declined 1 and 4 h after venepuncture and saline injection ($P < 0.05$), but returned to normal levels at 16 h. LH concentrations returned to normal values by 1 week after treatment in males and by 2 weeks after treatment in females.

Plasma concentration of FSH (ng/ml) in gilts declined slowly from 75.0 ± 7.5 to 58.3 ± 7.5 ($P < 0.05$) by 32 h after GnRH antagonist administration (Fig. 2) and were different from controls ($P < 0.05$) at 16 and 32 h after a single injection. In males the level of FSH (ng/ml) was low (5.7 ± 0.5) at 0 h and remained unchanged during the experimental period.

**Hormone responses to multiple injections of GnRH antagonist**

In neonatal females and males treated every 36 h with GnRH antagonist plasma LH concentrations were significantly reduced by the 5th day of treatment (8 days of age) and remained suppressed throughout the experiment (Table 1). Average concentration of LH in piglets treated with saline ranged from 0.6 to 1.3 ng/ml during the treatment period, but LH was suppressed to close to the sensitivity of the LH assays in animals given GnRH antagonist.

Treatment of female piglets with GnRH antagonist resulted in a significant reduction in FSH concentrations. Mean concentration of FSH in females treated with saline increased from 62 ng/ml at Day 8 to 134 ng/ml at Day 24, but gradually declined in treated females from 13.8 ng/ml at Day 8 to 9.8 ng/ml at Day 24. In control males FSH was lower ($P < 0.01$) than in control females;
however, treatment with GnRH antagonist suppressed FSH concentration at Days 8 and 16, but not at Day 24.

Treatment with GnRH antagonist for 21 days did not influence concentrations of oestradiol in gilts, but significantly suppressed concentrations of oestradiol in males to levels comparable to those observed in females. Concentration of testosterone in control boars gradually decreased ($P < 0.05$) from Days 8 to 24 of age. In contrast, treatment of males with GnRH antagonist suppressed testosterone to 0.2–0.4 ng/ml.

**Gonadal responses to multiple injections of GnRH antagonist**

Injections of GnRH antagonist dramatically reduced testicular weight ($3.34 \pm 0.29$ control vs $0.74 \pm 0.18$ g treated; $P < 0.001$) while the weight (g) of ovaries was not changed ($0.053 \pm 0.004$ vs $0.053 \pm 0.003$, respectively). Concentrations of LH/hCG receptors in the testes of boars treated with GnRH antagonist were lower than in boars treated with saline when measured at the end of the study ($43.1 \pm 4.5$ fmol/mg protein, treated vs $55.8 \pm 4.5$ fmol/mg protein, control, $P < 0.09$). The concentration of FSH receptors in testicular tissue of controls was $20.4 \pm 1.9$ fmol/mg protein and this was not different ($P < 0.10$) from $20.8 \pm 1.9$ fmol/mg protein for boars treated with the GnRH antagonist. The affinity of the receptors for hCG in males treated with GnRH antagonist was $3.5 \pm 0.2 \times 10^{10}$ M$^{-1}$, not different from $2.7 \pm 0.2 \times 10^{10}$ M$^{-1}$ for males treated with saline. Likewise, treatment with GnRH antagonist did not affect the $K_a$ of FSH receptors ($2.0 \pm 0.2 \times 10^{10}$ M$^{-1}$ treated vs $1.9 \pm 0.2 \times 10^{10}$ M$^{-1}$ control).
Fig. 2. Effect of a single i.m. injection of GnRH antagonist on concentration of FSH in neonatal gilts (a) and boars (b). Values are means ± s.e. of 4 observations.

Response of hypothalami in vitro

Basal and potassium-stimulated release of GnRH did not differ between treatment groups in females or males (Fig. 3). The amount (ng/mg) of residual GnRH in tissue after incubation was not different in gilts (1.88 ± 0.33, control vs 1.62 ± 0.14, treated), but was lower (P < 0.05) in boars treated with GnRH antagonist (1.13 ± 0.05) than in controls (1.79 ± 0.54). Concentration of GnRH in the median eminence was approximately 150 times higher than in the medial basal hypothalamus and did not differ between control and treatment groups (data not shown).

Discussion

The present study shows that a single injection of a GnRH antagonist results in an immediate suppression of blood LH concentrations to near the sensitivity of the LH assays when given to young male and female pigs. The disappearance of LH from the blood was similar to that observed after passive immunization of pigs against GnRH (Esbenshade et al., 1986), indicating that the GnRH antagonist was effective in blocking LH secretion from the pituitary gland. Similar results have been shown for the rat (Grady et al., 1985; Kartun & Schwartz, 1987), primate (Pineda et al., 1980) and sheep (Matwijew & Faiman, 1987) fetus. Potent GnRH antagonist analogues bind tenaciously to the GnRH receptor on the gonadotroph with slow dissociation rates resulting in altered ED$_{50}$ values and depressed gonadotrophin secretion rates (Heber et al., 1982; Hsueh et al., 1986).
Table 1. Serum concentrations (mean ± s.e.m. for 10 piglets/group) of LH, FSH, oestradiol-17β and testosterone in female and male piglets given saline or a GnRH antagonist

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Sex</th>
<th>Treatment</th>
<th>Age (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>Female</td>
<td>Saline</td>
<td>1.3 ± 0.3*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GnRH antagonist</td>
<td>0.2 ± 0.0*</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Saline</td>
<td>1.3 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GnRH antagonist</td>
<td>0.4 ± 0.1**</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>Female</td>
<td>Saline</td>
<td>62 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GnRH antagonist</td>
<td>14 ± 3**</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Saline</td>
<td>12 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GnRH antagonist</td>
<td>4 ± 1*</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>Female</td>
<td>Saline</td>
<td>NM</td>
</tr>
<tr>
<td>(pg/ml)</td>
<td></td>
<td>GnRH antagonist</td>
<td>NM</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Saline</td>
<td>NM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GnRH antagonist</td>
<td>NM</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Male</td>
<td>Saline</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td></td>
<td>GnRH antagonist</td>
<td>0.2 ± 0.1**</td>
</tr>
</tbody>
</table>

NM = not measured.

*P < 0.05 compared with saline values.

**P < 0.01 compared with saline values.

1983; Jennes et al., 1984; Vickery, 1986). Although not tested in this study, it would appear that the GnRH antagonist acts in the pig in a way similar to that in other species as shown by the decline in concentration of LH in the peripheral circulation after administration.

Newborn male and female pigs treated for a period of 21 days with GnRH antagonist had suppressed LH secretion for the duration of treatment. This finding is consistent with previous observations in the rat (Huhtaniemi et al., 1984a) and in the monkey (Kenigsberg et al., 1984) when a GnRH antagonist was given for a prolonged period of time. In the present study, FSH concentrations in gilts decreased more slowly over the treatment period and were suppressed to 5% of the values in control animals by the end of the experiment. The differential suppression of LH and FSH after disruption of the hypothalamic–pituitary axis has been shown previously in rats given GnRH antagonists (Grady et al., 1985; Kartun & Schwartz, 1987) and in numerous species, including the pig, after passive immunization against GnRH (Lincoln & Fraser, 1979; Fraser et al., 1982; Esbenshade et al., 1986).

Concentrations of FSH in males were unaffected by GnRH antagonist. The lower concentrations of FSH observed in the neonatal male compared to neonatal females probably result from negative feedback from compounds of testicular origin. One possible compound contributing to the low FSH values in neonatal male pigs is inhibin, which may be produced in high concentrations by the neonatal testes (Colenbrander et al., 1987).

Treatment with a GnRH antagonist suppressed circulating testosterone and oestradiol-17β concentrations and reduced testicular weight in male piglets, but did not alter gonadal weight or ovarian steroids in female piglets. This difference probably reflects the difference in ontogeny of ovarian and testicular development in the pig. Testicular development and hormonal synthesis and secretion by the testes occurs throughout the neonatal period, whereas the ovaries are quiescent until about the 8th week after birth (Oxender et al., 1979; Hennen et al., 1982; Colenbrander et al., 1987). Although concentrations (fmol/mg protein) of LH and FSH receptors were not significantly changed in testes during treatment with GnRH antagonist, it can be assumed that the total content of gonadotrophin receptors (fmol/testis) decreased proportionately to the loss of testicular mass.
Fig. 3. Effect of in-vivo administration of GnRH antagonist on basal and potassium-stimulated release of GnRH in vitro from the stalk median eminence. Values are means ± s.e. of 5 observations.

(GnRH antagonist in neonatal pigs) GnRH antagonists and large doses of GnRH agonists have been reported to reduce testicular LH/hCG receptors in male rats (Hsueh & Erickson, 1979; Huhtaniemi et al., 1984b), but this reduction may depend upon a direct gonadal effect (Clayton et al., 1980; Hunter et al., 1982; Sundaram et al., 1984). In contrast to the rat, we have not been able to detect specific GnRH binding sites in crude cell membrane fractions prepared from neonatal boar testes (A. J. Ziecik, K. L. Esbenshade & J. H. Britt, unpublished data).

When median eminence fragments taken from rats were incubated in vitro in the presence of a potent GnRH antagonist, basal secretion of GnRH was enhanced in a dose-dependent manner (Valenca et al., 1987). This suggests that GnRH exerts an inhibitory effect on its own secretion via the ultrashort loop feedback as proposed by Hyppa et al. (1971). In contrast to the rat, the results of the present study demonstrated that long-term treatment of neonatal pigs with a GnRH antagonist did not affect release of GnRH from the median eminence when incubated in vitro. This is the first evidence that the proposed negative ultrashort loop feedback mechanism observed in rats is not functional in the neonatal pig.

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Lincoln, G.A. & Fraser, H.M. (1979) Blockade of episodic secretion of luteinizing hormone in the ram by the administration of antibodies to luteinizing hormone releasing hormone. Biol. Reprod. 21, 1239–1245.


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