Luteinizing hormone response to an oestradiol challenge in 5 intersex pigs possessing ovotestes*

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Summary. After challenge with oestradiol benzoate, the mean maximum LH concentration in 5 XX intersex pigs possessing ovarian and testicular tissue, or only testicular tissue, was 2.10 (± 0.41) ng/ml compared with 8.9 ng/ml in mature domestic gilts. These results indicate that exposure of the pig brain to testosterone before Day 30 of gestation is important, or that early testicular secretions other than testosterone are involved in the determination of brain gender. The observation that some intersex pigs show normal oestrous cycles implies that the response to these prenatal factors is primarily quantitative rather than qualitative.

Keywords: LH; oestradiol; intersex; ovotestes; pigs

Introduction

The stimulatory (i.e. positive) oestrogen feedback mechanism that causes a surge of luteinizing hormone (LH) release, and hence ovulation in mature female mammals, has been shown to be absent in the male of several species, including the rat (Neill, 1972), hamster (Buhl *et al.*, 1978), sheep (Bolt, 1971; Karsch & Foster, 1975) and pig (Ford & Schanbacher, 1977). In rhesus monkeys, by contrast, administration of oestradiol benzoate to males and females can elicit an LH surge (Karsch *et al.*, 1973). Although sexually dimorphic responses to oestradiol are evident in miniature pigs at 14 days of age (Elsaesser *et al.*, 1978b), Foxcroft *et al.* (1984) suggested an ovarian-, possibly oestrogen-, dependent maturation of the feedback mechanism in domestic gilts. When oestrogen challenges were made at 160 days, gilts ovariectomized at 60 days of age showed a smaller LH peak and a greater interval between oestradiol administration and the gonadotrophin surge than did gilts ovariectomized at 130 days or ovariectomized at 60 days and treated with oestradiol to Day 130. Prenatal exposure of domestic pigs to testosterone (*via* the mother) significantly impairs the LH response to oestradiol benzoate in adult females (Elsaesser & Parvizi, 1979), as does prenatal exposure to androgens in mice (Barracrough, 1955) and sheep (Clarke & Scaramuzzi, 1978), implying that sexual differentiation of the oestradiol feedback mechanism occurs prenatally. In contrast, neonatal exposure of female rats to appropriate doses of androgen abolishes the positive feedback effect of oestradiol.

Intersex pigs occur at an estimated frequency of 0.1% to 1% in commercial pig herds (Bäckström & Henricson, 1971; Hunter *et al.*, 1982). Most of the animals are genetically XX

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(Breeuwsma, 1970; Miyake, 1973) but are identified due to partial masculinization of the genitalia caused by the steroid and protein hormone secretions of various amounts of testicular tissue. Although many intersex pigs are sterile, pregnancies have been recorded (Scofield *et al.*, 1969; Hunter *et al.*, 1985), providing evidence of a mature feedback response of gonadotrophins to oestradiol, despite the presumed exposure of the brain to prenatal testicular androgens.

A study of 5 intersex animals revealed different degrees of boar-like behaviour, but no detectable oestrous cycles, indicating masculinization of the brain. The present experiment was therefore designed to determine the brain gender of these pigs as defined by their LH responses to an oestradiol challenge.

Materials and Methods

Animals

The animals were all Landrace $\times$ Large White/Landrace pigs weighing between 90 and 150 kg at the time of the experiment. They had been identified as intersex animals at 2–3 months of age by examination of the vulva and clitoris. Karyotypes were XX as determined from blood leucocyte culture following the techniques of Lin *et al.* (1976) and Buckland *et al.* (1976). In the absence of detectable oestrous cycles, mid-ventral laparotomies were performed. Anaesthesia was induced with pentobarbitone sodium and maintained, after intubation, by a mixture of halothane, nitrous oxide and oxygen. The reproductive tract and gonads were exteriorized, when possible, and inspected for signs of previous cyclic activity. Tracts were recovered at slaughter for more detailed examination.

Animals received intramuscular injections (60 μ g/kg body weight) of oestradiol benzoate in ethyl oleate (Intervet Laboratories Ltd, Cambridge, UK) and were observed for signs of oestrus, vulval reddening and swelling being taken as an indication of an oestrogenic response.

Blood sampling

Indwelling jugular cannulae were fitted to permit frequent bleeding. Samples were collected into heparinized tubes, centrifuged at 1500 *g* for 10 min at 4°C, and plasma stored at –20°C until assay. For LH analysis, samples (4 ml) were taken every 24 h for 48 h before, and every 4 h for up to 112 h after, the oestradiol injection. Samples of 10 ml were collected every 12 h for oestradiol analysis, except for 6-h samples taken immediately after the challenge. Daily samples were also assayed for progesterone and testosterone.

Assays

LH radioimmunoassay. Plasma LH concentrations were determined by an homologous double-antibody RIA. The primary antiserum was raised in a goat immunized against a purified pig LH preparation. SDG-2-65 (0.96 – 1.18 $\times$ NIH-oLH-S19, by bioassay). The first booster injection, 6 months after initial immunization using Freund's complete adjuvant, produced a series of sera with good titres and specificity. The antiserum characterized in the present assays, GRF-G 81/1, bound approximately 20% of radiolabelled pig LH in the absence of unlabelled antigen, at an initial dilution of 1:60 000. Other assay methodology was as described previously (Foxcroft *et al.*, 1984) with the following minor modification. To enhance precipitation of antibody-bound hormone, a second antibody (donkey anti-goat gamma globulin, raised at Sutton Bonington) at 1:40 dilution was preincubated for 24 h with 10% (w/v) polyethylene glycol 6000 (PEG; BDH Ltd, Poole, UK); 200 μ l of the PEG anti-goat gamma-globulin were then added to assay tubes and a minimum period of 8 h incubation at 4°C then followed before centrifugation and aspiration. Reproducible standard curves were obtained with this assay with a range of standard potencies from 0.01 to 0.5 ng/tube and the overall sensitivity, defined as 90% of total binding, was 0.02 ng/tube. A standard plasma pool, routinely assayed at 50, 100 and 200 μ l showed parallelism to the standard curve. The recovery of pig LH 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. All samples were run at 100 μ l in duplicate in a single assay for which the intra-assay coefficient of variation was <10%.

Steroid assays. Radioimmunoassays for oestradiol, progesterone and testosterone were as described by Cook *et al.* (1977), except that bound and free steroids were separated using a second antibody (Scottish Antibody Production Unit, Law Hospital, Carlisle, Lanarkshire, UK). Oestradiol antiserum was obtained from Immunodiagnostics Ltd, Usworth Hall, Washington, Tyne and Wear, UK. Cross-reactions were 8.7% with oestrone for the oestradiol antiserum, 4.3% and 1.8% with deoxycorticosterone and corticosterone respectively for the progesterone antiserum and 33.8% and 6.4% with DHT and androstenedione respectively for the testosterone antiserum. Other cross-reactions were <1% (Cook *et al.*, 1977). The assay sensitivities were oestradiol 59 pmol/l, progesterone 2.2 nmol/l and testosterone 1.0 nmol/l.

Results

Table 1 summarizes the observations on the behaviour and morphology of the intersex pigs. Figure 1 shows the LH and oestradiol profiles before and after oestradiol benzoate injections. The maximum LH concentration after injection was $2.10 (\pm 0.41)$ ng/ml (mean $\pm$ s.e.) at $86 (\pm 6.8)$ h. In Pigs I2 and I4, higher concentrations were recorded before the oestradiol benzoate injection, than after. In all animals, plasma oestradiol rose after the oestradiol challenge. The mean concentration 84 h after the injection was $402 (\pm 115)$ pmol/l. Following the challenge, progesterone concentrations remained below 10 nmol/l in 4 animals, but in Pig I93, progesterone concentrations were between 40 and 75 nmol/l. Testosterone concentrations were, in all animals, < 3.0 nmol/l throughout sampling.

Table 1. Summary of observations on 5 intersex pigs

Pig	Behaviour	External morphology	Morphology of tract	Morphology of gonads
I2	Not assessed	Upturned vulva, hypertrophied clitoris	Fluid-filled uterus of normal morphology; Wolffian duct development expressed as epididymis	Testis on right and left
I4	Aggressive with boar; no interest in oestrous gilt despite inducing 'standing response'	Upturned vulva; hypertrophied clitoris; prominent tusks; mid-ventral penile sheath	Fluid-filled tract; epididymis associated with right gonad	Testis on right; ovotestis (90% testicular) on left
I5	Not aggressive with boar; no detectable response to oestrous gilt	Upturned vulva; hypertrophied clitoris; fat-filled scrotal sacs	Immature dimensions but normal morphology	Not located on right; underdeveloped (could not be identified as ovarian or testicular) on left
I7	Aggressive with boar; excessive salivation; mounted oestrous gilt	Upturned vulva; hypertrophied clitoris; prominent tusks	Normal uterus; ampullary portion of oviducts rudimentary; pampiniform plexus associated with each gonad	Ovotestis (70% testicular; > 4 follicles 8–10 mm; no corpora lutea) on right; ovotestis (90% ovarian; > 12 cystic follicles; no corpora lutea) on left
I93	Not assessed	Upturned vulva; hypertrophied clitoris	Normal uterus; Wolffian duct development on right; normal oviduct on left	Testis on right; ovary (16 cystic follicles) on left

Discussion

By 160 days of age, domestic gilts are able to respond to an oestradiol benzoate challenge with a surge release of LH 37–55 h later (Elsaesser & Foxcroft, 1978; Foxcroft *et al.*, 1984), the average surge concentration being 8.9 ng/ml. None of the intersex animals in the present study showed an LH surge comparable to this despite raised oestradiol concentrations. The mean pretreatment LH concentration in the present study was 1.62 ng/ml and the mean post-treatment concentration was 2.10 ng/ml. In all animals, after the oestradiol benzoate challenge, peripheral oestradiol remained within the range of peak preovulatory concentrations reported for cyclic gilts (Henricks *et al.*, 1972; Van de Wiel *et al.*, 1981).

Pig I5 showed an LH rise above pretreatment levels, reaching a maximum 96 h after the oestradiol dose. Such a response indicates the operation of an immature feedback mechanism similar to

Fig. 1. Plasma concentrations of LH and oestradiol in 5 intersex pigs before and after an injection of oestradiol benzoate given at time 0 (arrow). (For comparison, oestrogen injection gave a peak concentration of 8.9 ng LH/ml at 37 h in normal gilts (Foxcroft *et al.*, 1984), whereas in normal males a maximum of 1.7 ng/ml was reached at 90 h (Ford & Schanbacher, 1977).)

that seen in 60-day-old domestic gilts (Foxcroft *et al.*, 1984), a mechanism which the authors suggest matures due to exposure to ovarian follicular secretions, possibly oestrogens. The low preinjection concentrations of oestradiol in Pig I5 support this view, but the masculinized external genitalia indicate prenatal exposure to testosterone, which may also be responsible for the diminished LH release.

In 4 animals, progesterone concentrations were within the range corresponding to follicular-phase concentrations in cyclic gilts (Stabenfeldt *et al.*, 1969; Edqvist & Lamm, 1971). In Pig I93, however, a high progesterone concentration (presumably secreted by luteal tissue discovered during histological examination) may have prevented an LH surge after the oestradiol injection; oestradiol given during the luteal phase of the oestrous cycle will not elicit an LH surge in rats (Tapper *et al.*, 1974), sheep (Karsch *et al.*, 1978) or women (Leyendecker *et al.*, 1972). In all 5

intersexes, the pattern of the LH release was closer to that seen in castrated adult male pigs (Ford & Schanbacher, 1977) than to normal females (Elsaesser & Foxcroft, 1978; Foxcroft *et al.*, 1984) despite the XX karyotypes.

Prenatal exposure of the brain to testosterone is thought to be the cause of sexual differentiation of the feedback response to oestradiol in rats, treatment with testosterone propionate during pregnancy abolishing the LH response in the female offspring (Neill, 1977). In rhesus monkeys, however, prenatal testosterone did not affect adult LH secretion; both sexes responded to an oestradiol injection with a surge release of LH (Steiner *et al.*, 1976). In the sheep, the effect of prenatal testosterone treatment on the female may be quantitative rather than qualitative, since the LH response to oestradiol in the adult is reduced but not abolished (Clarke & Scaramuzzi, 1978). This may also be the case in pigs (Elsaesser & Parvizi, 1979).

Fetuses treated with testosterone injection into the dam between Days 30 and 70 of gestation are more likely to show a masculinized LH response profile as adults than fetuses similarly treated during the second half of gestation (Elsaesser & Parvizi, 1979). Maximum testosterone content of the fetal pig testis is reached between Days 35 and 38 (>4 ng/pair) (Raeside & Sigman, 1975) and in umbilical arterial serum on Day 35 (>4 ng/ml), concentrations in the female fetus being undetectable at this stage (Ford *et al.*, 1980). These changes in testosterone concentration follow the morphological development of Leydig cells within the testes (Moon & Hardy, 1973; Pelliniemi, 1975). It is therefore assumed that differentiation of the brain in the pig is due to exposure to testosterone around Day 35 of pregnancy (Ford *et al.*, 1980). However, exposure of the 30-day-old fetus to testosterone, either *via* the pregnant sow (Elsaesser & Parvizi, 1979) or directly (Elsaesser *et al.*, 1978a), does not completely abolish the LH response to oestradiol, or ovulation, in the adult. The presence of testicular tissue in the intersex pigs of this study apparently masculinizes the brain, despite testosterone concentrations being below those reported for mature intact (miniature) or castrated (domestic) pigs (Ellendorff *et al.*, 1975; Ford & Schanbacher, 1977). This indicates (1) that the brain exposure to testosterone before Day 30 of gestation is important, or (2) that testosterone is not the sole determinant of brain gender. Since development of a normal male reproductive tract involves regression of the Müllerian hormone (Jost, 1947), this hormone could also be implicated in brain defeminization.

Foxcroft *et al.* (1984) have suggested that the lack of an LH surge mechanism in the male pig is due, not to the presence of neonatal androgen secretion, but to the absence of an ovary during prepubertal life. Although the presence of a sexually dimorphic LH response to oestradiol in newborn pigs (Elsaesser *et al.*, 1978b) would seem to refute this, prenatal ovarian influences may be important in counteracting the defeminizing/masculinizing effects of testicular secretions. Such influences may result in the development of a mature feedback response and ovulation in some intersex pigs (Hunter *et al.*, 1982, 1985). In the intersex pigs used in the present study, testicular secretions were presumably sufficient to cause substantial masculinization of the feedback mechanism.

The mechanism involved in masculinization is not clear. It is known that oestrogen is as effective as testosterone in masculinizing neonatal rat brains, aromatization of testosterone to oestradiol in the brain playing a crucial role (MacLusky & Naftolin, 1981). An LHRH challenge in male pigs (Pomerantz *et al.*, 1974), and in the intersexes of the present study (results not shown), invokes an LH response, implying that pituitary insensitivity is not a problem. The lack of hypothalamic/pituitary steroid receptors has been suggested (Elsaesser & Parvizi, 1979) with reference to the rat, in which the stimulatory oestrogen feedback mechanism does not operate until 22 days of age (Caligaris *et al.*, 1972) when maturation of hypothalamic receptors occurs (Plapinger & McEwen, 1973). Oestrogen receptors may be deficient in the intersex pigs in that no oestrous behaviour was observed after the oestradiol benzoate injection, but inhibition of LH secretion was evident in all animals, indicating at least some hypothalamic/pituitary sensitivity to the steroid, albeit as a negative feedback effect. Oestrogen insensitivity is one explanation for infertility in intersex pigs, although the failure of attempts to induce, with PMSG, ovulation from an ovotestis

in an anoestrous intersex (Hunter *et al.*, 1985) suggests that these gonads may be unresponsive to gonadotrophins.

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