Effects of ovariectomy and *FecB^B* genotype on the median charge and circulating half-life of pituitary FSH isoforms of ewes

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This study investigated the effects of short-term (20 days) ovariectomy, the effects of FSH assay (radioimmunoassay, receptor assay or *in vitro* bioassay) and of *FecB^B* genotype on the characteristics of pituitary FSH from Booroola ewes. Pituitary extracts were obtained from ovariectomized homozygous carriers (BB) and non-carriers (++, $n = 8$ per genotype) and ovary-intact controls ($n = 4$ per genotype). The extracts ($n = 4$ per genotype per treatment) were subjected to agarose suspension electrophoresis and the eluates were assayed by the three FSH methods. There were significant effects of ovariectomy ($P < 0.01$) and assay system ($P < 0.05$) but not of genotype on the median charge of FSH isoforms. The mean $\pm$ SEM migration rates for FSH in intact and ovariectomized ewes were 0.469 ± 0.006 and 0.439 ± 0.006 albumin mobility units, respectively ($P < 0.01$), indicating a shift to more basic isoforms after short-term ovariectomy. When the pituitary extracts were subjected to anion-exchange HPLC, there was a significant ($P < 0.01$) shift to more basic isoforms in the ovariectomized ewes as shown using agarose electrophoresis, and no gene effects were noted. When the pituitary extracts ($n = 4$ –8 per group) were injected into mature female mice, there were no significant effects of ovariectomy or genotype on the circulating half-lives of the pituitary FSH isoforms. These results indicate that after short-term ovariectomy, the increase in plasma FSH concentrations is accompanied by a shift in the median charge of pituitary FSH isoforms without any observable change in their metabolic clearance rates. Moreover, the *FecB^B* gene has little effect on the median charge or half-life of pituitary FSH.

Introduction

The plasma concentrations of immunoreactive and bioactive FSH increase within a few hours of ovariectomy (McNatty *et al.*, 1989; Phillips *et al.*, 1993a). Over the next 20–40 days the plasma concentrations of immunoreactive and bioactive FSH increase to values 4 to 15 times higher than those before ovariectomy. It is not clear whether the rapid increases in concentration in the short term are due to the presence in the pituitary or plasma of FSH isoforms with longer circulating half-lives, as has been noted in ewes ovariectomized for more than six months (Fry *et al.*, 1987). However, there is a marked decrease in the immunoreactive:bioactive ratios of plasma FSH during the first few weeks after ovariectomy, although no such differences were detected in the pituitary gland (Phillips *et al.*, 1993a). Collectively, the evidence suggests that, in the first weeks after ovariectomy, there may be qualitative changes in the FSH isoforms in plasma, but whether the forms in the pituitary are altered is unknown.

The study of Wide and Hobson (1986) showed that the median charge of human pituitary FSH was affected by the

method of measurement. The median charge was found to be more basic using an *in vitro* bioassay than when the measurement was made by radioimmunoassay. Thus, when examining FSH isoforms, the assay system used may be an important factor.

Booroola ewes possess a major gene, *FecB^B*, that influences their ovulation rate (i.e., the number of ovulations per oestrous cycle; for review see McNatty *et al.*, 1991). Homozygous (BB), heterozygous (B+) and non-carriers of the *FecB^B* gene have ovulation rates of ≥ 5 , 3–4 and 1–2, respectively. Although the *FecB^B* gene is known to be expressed in early fetal life and to affect the pituitary–gonadal axis in neonatal and adult life, its mechanism of action remains unknown (McNatty *et al.*, 1995). One possibility is that the differences in ovulation rate between the Booroola genotypes are in some way affected by the circulating concentrations of bioactive FSH; BB ewes have higher plasma concentrations of bioactive FSH and immunoreactive FSH both before and after short-term ovariectomy, compared with ++ ewes (Phillips *et al.*, 1993a). In a study of ovary-intact heterozygous (B+) and ++ ewes, Robertson *et al.* (1984) report no significant changes in the electrofocusing profiles of pituitary FSH isoforms. Although the plasma and pituitary immunoreactive FSH concentrations are consistently higher in the B+ than in the ++ ewes, the differences are small and, frequently, not statistically significant (McNatty *et al.*,

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1987). Thus, it is possible that there are differences in FSH isoforms but they are more likely to be evident when comparing plasma or pituitary extracts from BB and ++ ewes.

The aim of this study was to establish whether there were differences in pituitary FSH isoforms that could be detected between BB and ++ ewes. The pituitary FSH isoforms were examined in BB and ++ animals, both before and after short-term (i.e. 20 days) ovariectomy, using both agarose suspension electrophoresis and anion-exchange HPLC. Their median charges were determined by measuring the above eluates using three different assay systems: radioimmunoassay, radioreceptor assay and *in vitro* bioassay. The half-life of clearance of FSH in these pituitary extracts was examined in mature mice.

Materials and Methods

Animals and samples

The experimental procedures reported in this study were carried out in accordance with the 1987 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand after approval was granted by the Animal Ethics Committee of the Wallaceville Animal Research Centre.

Detailed descriptions of the animals, and plasma and pituitary bioactive and immunoreactive FSH concentrations are given in Phillips *et al.* (1993a). The pituitary extracts from these animals were used because there were significant differences in plasma concentrations of both bioactive and immunoreactive FSH between the BB and ++ groups. Breed (Booroola Merino/Romney) and age (about 7 years of age) matched homozygous carriers (BB) and non-carriers (++) of the *FecB^B* gene ($n = 8$ per genotype) were killed by cervical exsanguination 20 days after ovariectomy. The mean ($\pm$ SEM) day of the oestrous cycle at the time of ovariectomy was 6.3 ± 1.1 for the BB group and 9.6 ± 1.5 for the ++ group. Pituitary glands were collected from the ewes within 5 min of death, snap-frozen in liquid nitrogen, and stored at -70°C until processing. Another group of cyclic BB and ++ ewes ($n = 4$ per genotype) was killed between day 10 and day 13 of the oestrous cycle and pituitary glands were collected and stored as above. After thawing and weighing, individual pituitary glands were extracted as described by Robertson *et al.* (1982) and stored at -20°C until required. For electrophoresis and HPLC analysis, the extracts were dialysed at 4°C against distilled water (4×5 l) using a Visking 24/32 membrane (London). The retentates were freeze-dried. The mean ($\pm$ SEM) recovery of ^{125}I -labelled FSH after dialysis and freeze-drying was $94.8 \pm 3.3\%$. Pituitary extracts ($n = 8$ per genotype from the ovariectomized group and $n = 4$ per genotype from the ovary-intact group) were also injected into the bloodstream of mice to determine the circulating half-lives of the FSH isoforms.

Agarose zone electrophoresis

Zone electrophoresis was performed in 0.17% (w/v) agarose suspension (Isogel, FMC, Rockland) as described by Wide (1985), using a $2 \text{ cm} \times 70 \text{ cm}$ glass column. Before loading, approximately 30% of each freeze-dried pituitary extract was dissolved in 1.5 ml hypophysectomized ewe serum, 0.5 ml of

0.075 mol barbitone buffer l^{-1} containing 30 000 c.p.m. ^{125}I -labelled BSA (Sigma, St Louis, MO) and 1 ml 0.17% (w/v) agarose suspension. The column was run for 25 h at 75 mA. Fractions of approximately 2.3 ml were collected and the amount of ^{125}I -labelled BSA was determined; the start zone was identified by measuring the absorbance of the fractions at 340 nm. The fractions were centrifuged at 3000 g for 20 min to remove the agarose, and 50 μl of 10% (w/v) BSA was added to each supernatant before storage at -20°C . Fractions were assayed by immunoassay, receptor assay and bioassay. The mean ($\pm$ SEM) recovery of FSH from the electrophoretic technique was $90 \pm 3\%$ for immunoreactive FSH, $81 \pm 5\%$ when measured by receptor assay, and $83 \pm 7\%$ for the *in vitro* assay. A sample that was run three times had mean $\pm$ SEM of 0.495 ± 0.004 albumin mobility units (AMUs).

HPLC analysis

Approximately 2.5% of each freeze-dried pituitary extract was dissolved in 20 mmol Tris-HCl l^{-1} (pH 8.6) and was loaded with ^{125}I -labelled ovine (o) FSH (30 000 c.p.m.) onto an MA7Q anion exchange column ($10 \text{ cm} \times 1.9 \text{ cm}$; Biorad, Richmond, CA) connected to an HPLC (Hewlett Packard 1050). The column was eluted at 4 ml min^{-1} with 20 mmol Tris-HCl l^{-1} for 2 min. This was followed by a linear gradient from 0 to 400 mmol KCl l^{-1} in 20 mmol Tris-HCl l^{-1} over 50 min. Finally, the concentration of KCl was increased to 2 mol l^{-1} for 4 min. Fractions of 3 ml were collected from the column, to which 100 μl of 10% (w/v) BSA in 20 mmol Tris-HCl l^{-1} was added before storage at -20°C . At the assay dilutions used, the KCl gradient did not have a significant effect on the assay. Each fraction was assayed using the radioimmunoassay. The average recovery of immunoreactive FSH from the anion-exchange column was $83 \pm 7\%$.

Circulating half-life of FSH

The circulating half-life of FSH isoforms from individual pituitary extracts was measured as the rate of disappearance from the circulation of mice. Mature, virgin CF1 females, between 20 and 27 g, were injected in the tail vein with between 0.5 and 1.8 μg of immunoreactive pituitary FSH extract in 0.1 ml physiological saline; these amounts of FSH were determined from a knowledge of the pituitary content in individual sheep and the necessity to treat a minimum number of mice. For each extract, mice were anaesthetized (0.2 ml Nembutal i.p., 12 mg ml^{-1} , Techvet Laboratories, Auckland) before being bled by cardiac puncture out at 5, 15, 30, 60, 120 and 180 (± 1) min after injection of the pituitary extract (3–5 mice per timepoint). The blood was collected into heparinized tubes, kept at room temperature until centrifugation at 4000 g for 10 min, and the plasma obtained was stored at -20°C . Each plasma sample plus a 1:5 and 1:25 dilution was assayed by radioimmunoassay, using hypophysectomized ewe plasma as the diluent. There was no crossreaction or interference of mouse plasma in the ovine FSH assay.

Assays

Immunoreactive FSH concentrations were measured using radioimmunoassay kits supplied by the National Hormone and

Pituitary Program (NHPP). For the assays of FSH in the pituitary extracts and in eluates following gel electrophoresis, the oFSH used for iodination was NIAMDD-oFSH-I-1, the reference preparation was NIAMMD-oFSH-RP-1 (biopotency $75 \times \text{NIH-FSH-S1}$) and the oFSH antiserum was NIAMDD-anti-oFSH-1. Owing to the release of a new radioimmunoassay kit by NHPP, eluates from the HPLC column and samples from the half-life studies were assayed using USDA-oFSH-19-SIAFP-I-2 for iodination, USDA-oFSH-19-SIAFP-RP-2 (biopotency $94 \times \text{NIH-oFSH-S1}$) as the reference preparation and NIAMDD-anti-oFSH-1 as the antiserum. In both assays, the sensitivity was 0.1 ng ml^{-1} and the intra- and interassay coefficients of variance (CVs) were $< 8\%$.

FSH concentrations measured by the receptor assay were quantified using membrane fractions from bovine testes as described by Phillips *et al.* (1993b). The assay sensitivity was 2 ng ml^{-1} and the intra- and interassay CVs were both $< 8\%$. Concentrations were expressed relative to units of NIAMDD-oFSH-RP-1.

Bioactive FSH concentrations were quantified using the production of oestradiol *in vitro* from Sertoli cells isolated from immature male rats (Padmanabhan *et al.*, 1987). This assay has been validated for ovine biological fluids (Phillips *et al.*, 1992). The radioimmunoassay used to measure the production of oestradiol (McNatty *et al.*, 1984) has an assay sensitivity of 5 pg ml^{-1} , and the intra- and interassay CVs were both $< 10\%$. The reference standard for FSH was NIAMDD-oFSH-RP-1; the assay sensitivity of the *in vitro* bioassay was 1 ng ml^{-1} ; and the intra- and interassay CVs were 8 and 14%, respectively.

Statistical analyses

The median charge of FSH isoforms after electrophoresis was expressed relative to the median mobility of ^{125}I -labelled BSA (in AMUs). FSH migration after agarose electrophoresis was analysed separately for effects of genotype, ovariectomy, assay system and interactions by split-plot analysis of variance. Animal (nested within level of genotype and ovariectomy) was used as the error term for genotype and ovariectomy effects and their interaction, and the residual error was used to test the significance of assay system effects and all other interactions. The SAS General Linear Models procedure and regression sum of squares (SAS Type III) were used for these calculations. After anion-exchange HPLC, the average charge of pituitary FSH was expressed as the volume collected from the column at which 50% of the FSH had been eluted. Differences were determined by two-way ANOVA followed by Tukey's test. Comparison of the 50% value obtained from the HPLC system was compared with the AMU value of the same extract using the agarose electrophoretic system, by linear regression analysis. Concentrations of oFSH with time after injection of extracts into mice were analysed by fitting the decay rate to a single exponential component curve using the Sigma Plot graphical package on a personal computer. Overall, the single exponential curve gave a better fit in all instances than a two exponential equation.

Fig. 1. Mean $\pm$ SEM immunoreactive FSH activity recovered after electrophoresis of pituitary extracts from ovary-intact ($\bullet$; $n = 8$) and ovariectomized ($\circ$; $n = 8$) ewes. The data from the ovary-intact BB ($n = 4$) and ++ ($n = 4$) and ovariectomized BB ($n = 4$) and ++ ($n = 4$) were each pooled.

Results

After agarose suspension electrophoresis of pituitary extracts, no significant effects of genotype and no interactions were noted for the median charge of the pituitary FSH isoforms. However, there were significant effects of treatment ($F_{1,12} = 14.2$, $P = 0.003$) and assay system ($F_{2,24} = 4.34$, $P = 0.025$). The mean $\pm$ SEM migration rates for FSH in intact and short-term ovariectomized ewes were 0.469 ± 0.006 and 0.439 ± 0.006 AMU, respectively ($P < 0.003$, Fig. 1), thus indicating a shift to more basic isoforms after ovariectomy. Moreover, the mean $\pm$ SEM migration rates, irrespective of treatment and genotype as measured by radioimmunoassay, receptor assay or bioassay were 0.458 ± 0.002 , 0.451 ± 0.002 and 0.453 ± 0.002 AMU, respectively. The mean AMU value measured by radioimmunoassay was significantly ($P < 0.05$, Tukey's test) higher than when measured by radioreceptor assay, whereas the values given by the bioassay were not different from those of the other two assay systems.

When the same pituitary extracts were subjected to anion-exchange HPLC, there was a significant ($P < 0.001$) shift to more basic isoforms in the ovariectomized ewes (Fig. 2) but no genotypic differences between BB and ++ groups (Fig. 2). The mean ($\pm$ SEM) volume at which 50% of immunoreactive FSH eluted was 99.3 ± 3.6 ml and 98.0 ± 1.2 ml for the ovary-intact ++ and BB ewes, respectively, and 85.7 ± 1.8 ml and 85.6 ± 3.3 ml for the ovariectomized ++ and BB ewes. The 50% value for each pituitary extract from the HPLC system was strongly correlated with the AMU value obtained for that extract with the electrophoretic technique ($r = 0.80$, $P < 0.001$, linear regression analysis).

When pituitary extracts ($n = 8$ per genotype from ovariectomized ewes and $n = 4$ per genotype from ovary intact ewes per group) were injected into mature female mice, there were no significant effects of either genotype or ovariectomy on the circulating half-lives of the pituitary FSH isoforms (Table 1). No significant relationship was noted between the estimated half-life of oFSH in mice and the amount of immunoreactive pituitary FSH extract that was injected.

Fig. 2. Mean $\pm$ SEM immunoreactive FSH activity recovered after anion-exchange HPLC of pituitary extracts from ovary-intact ($\bullet$; $n = 8$) and ovariectomized ($\circ$; $n = 8$) ewes. The data from the ovary-intact BB ($n = 4$) and ++ ($n = 4$) and ovariectomized BB ($n = 4$) were each pooled.

Table 1. Mean $\pm$ SEM half-life (min) of pituitary extracts of ovine FSH in the circulation of mature female mice

Treatment	Genotype of sheep	
	++	BB
Ovary-intact	8.9 ± 0.7 (4)	9.3 ± 1.0 (4)
Ovariectomized	10.8 ± 0.9 (8)	11.2 ± 1.3 (8)

FSH values were determined by an ovine FSH radioimmunoassay.

Values in parentheses represent number of sheep.

The differences between the genotypes or treatments were not significantly different from one another (two-way ANOVA).

Discussion

This study reports a significant alteration in the median migration rate of pituitary FSH isoforms after short-term ovariectomy. This finding extends the results of a change in the bioactivity of plasma FSH isoforms after short-term ovariectomy (Phillips *et al.*, 1993a). In the study reported here, the alteration in FSH isoforms was demonstrable using analytical techniques that separate on net charge. In another study on sheep pituitaries from intact and ovariectomized ewes, no significant changes in FSH isoforms were noted using an electrofocusing technique that separated isoforms according to their isoelectric point (Robertson *et al.*, 1991). The charge of plasma forms of oFSH has not yet been determined, as current assays are not of sufficient sensitivity to detect FSH activity in eluates after plasma or serum is subjected to electrophoretic or HPLC techniques. Moreover, attempts to increase circulating FSH concentrations using a GnRH challenge are unlikely to overcome the problem, as GnRH is known to shift FSH isoforms to more basic forms (Wide and Albertsson-Wikland, 1993).

The present study demonstrated an effect of the assay system on the median charge of the pituitary FSH isoforms, with the radioreceptor assay and the *in vitro* bioassay tending to give more basic values than those given by the radioimmunoassay. Although significant, the effect of assay system was small and possibly a consequence of the small numbers of animals used. Nevertheless, these findings are in accordance with those of Wide and Hobson (1986). Irrespective of the effects of assay type, all three assays revealed that the median charge of the pituitary isoforms was more basic after short-term ovariectomy than in ovary-intact ewes.

The FSH isoforms in intact ewes were studied during days 10–13 of the oestrous cycle, whereas the isoforms studied after ovariectomy were made on ++ and BB ewes ovariectomized at a mean of 6.5 and 9.2 days of the cycle, respectively. Four peaks of bioactive FSH are observed throughout the oestrous cycle, with two during the luteal phase, one after the LH surge and the other either during the follicular phase or associated with the LH surge (Phillips *et al.*, 1994). It is therefore possible that time of ovariectomy may have influenced the result obtained in the present study. However, it needs to be stressed that the changes observed in plasma may not be observed in the pituitary but, to our knowledge, there are no data available to address this. In the case of LH no detectable changes in pituitary isoforms were noted between the follicular and luteal phases (Zalesky *et al.*, 1992).

Studies examining the circulating half-life of FSH in ewes ovariectomized for more than one month (Fry *et al.*, 1987; Robertson *et al.*, 1991) suggested that there would be a significant change in half-life following ovariectomy; however, none was noted in the study reported here. It is possible that the mouse model used was not sensitive enough to detect any changes in half-life. However, Wide (1986), using this technique, reported that differences in the median charge of pituitary FSH isoforms similar to those found in the present study caused detectable changes in half-life. Another possibility

is that with more animals per group a significant difference may have become apparent. However, the most likely explanation is the difference in the time after ovariectomy. In the study reported here, the ewes had been ovariectomized for 20 days, whereas in the study of Robertson *et al.* (1991), in which the ewes were ovariectomized for more than one month, there was a threefold increase in the half-life of FSH. Moreover, in the study of Fry *et al.* (1987), in which ewes were ovariectomized for at least six months, a tenfold increase in the half-life of FSH was observed. It is not known how soon after ovariectomy the clearance rate of FSH isoforms changes, although circulating concentrations of FSH increase significantly within a few hours of ovariectomy (McNatty *et al.*, 1989). It seems likely that during the first weeks after ovariectomy there is an increase in FSH secretion rate, a decrease in the bioactivity of circulating FSH, and that there are changes in the median charge of the pituitary isoforms before clearance rates are affected.

In the study reported here, no significant effects of the *FecB^B* gene in terms of either pituitary FSH isoforms or metabolic clearance rate were detected. Moreover, there was no evidence to suggest that the clearance rates were influenced by the amount of oFSH injected into the mice. These results in homozygous carriers of the gene confirm previous findings in animals with only one copy of the gene (Robertson *et al.*, 1984; Fry *et al.*, 1987). Consequently, it is unlikely that the *FecB^B* gene influences the molecular structure or glycosylation of FSH within the gonadotrophs. It has been determined that there are no gene-specific differences in the characteristics of GnRH secretion (McNatty *et al.*, 1993), the GnRH content in different areas of the brain (Gale *et al.*, 1988) or in GnRH receptor-binding characteristics (Fleming *et al.*, 1990). Moreover, the genotypic differences in plasma FSH persist in hypothalamic-pituitary disconnected ewes, in which both genotypes are given the same pattern of exogenous GnRH (McNatty *et al.*, 1993). The most likely explanation for the documented gene-specific increases in both plasma bioactive and immunoreactive FSH (McNatty *et al.*, 1991; Phillips *et al.*, 1993a) is an increased secretion of all forms of FSH from the pituitary gland.

In summary, short-term ovariectomy resulted in a significant shift to more basic isoforms of pituitary FSH, but no differences were detected in the half-lives of these forms. Furthermore, no *FecB^B*-specific differences were noted before or after short-term ovariectomy in Booroola ewes in the characteristics of pituitary FSH isoforms.

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