Isoforms and half-life of FSH from sheep with different reproductive states

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Abstract

The glycoprotein hormone FSH comes in many different isoforms. In humans and rats the charges of the FSH isoforms vary with reproductive state and these affect the half-life of FSH in plasma. In this study we examined the charge heterogeneity of FSH in pituitary extracts from sheep with different reproductive states. Also the half-life of clearance of pituitary FSH from the different reproductive states was determined in mice. Pituitaries were collected from: anoestrous, luteal phase, follicular phase, early-pregnant and late-pregnant ewes, ewe lambs, ram lambs, rams during the breeding and non-breeding seasons and wethers (5 per group). After extraction, FSH isoforms were fractionated by HPLC anion exchange chromatography. The volume at which half of the FSH had eluted was determined (HP50). It was found that FSH isoforms from ewes (HP50 = 96·7 ± 1·3 ml (s.e.m.)) eluted later (P<0·01) than those from rams (HP50 = 82·3 ± 1·3 ml) indicating that FSH isoforms in the ewes were more acidic than those from rams. There was a seasonal difference in ewes, with ewes in anoestrus (HP50 = 101·6 ± 2·6 ml) having more-acidic (P<0·01) FSH isoforms than the ewes during the oestrous cycle (HP50 = 95·3 ± 0·7 ml). There was an effect of age, with the FSH isoforms from cycling ewes (HP50 = 95·3 ± 0·7 ml) being more acidic (P<0·01) than those from ewe lambs (HP50 = 88·3 ± 1·9 ml). There was an effect of pregnancy, with late-pregnant ewes (HP50 = 107·3 ± 1·6 ml) having more-acidic FSH isoforms (P<0·05) than those from anoestrous ewes (HP50 = 101·6 ± 2·6 ml) and there was an effect of castration with the breeding season rams (HP50 = 80·7 ± 1·4 ml) having more-acidic (P<0·05) FSH isoforms than wethers (HP50 = 74·0 ± 0·5 ml). The half-life of pituitary FSH from animals in the different reproductive states was found to be negatively correlated with HP50 (r² = 0·56, P<0·01). The FSH isoforms from wethers were the least acidic and had the longest half-lives. Collectively, these findings show that in sheep, age, sex and reproductive state are all factors which influence the forms of FSH that are extracted from the pituitary gland. Moreover, these results demonstrate that FSH from sheep with the most-acidic FSH isoforms have the shortest half-life in plasma.

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Introduction

The pituitary hormone follicle-stimulating hormone (FSH) is composed of two polypeptide subunits and four carbohydrate side chains. FSH, like other glycoprotein hormones, exists as a large number of isoforms. The heterogeneity which generates these isoforms originates from the variation in the oligosaccharide side chains. These oligosaccharides often terminate in electrically charged sugars, such as sialic acid or sulphated N-acetyl galactosamine, which influence the properties of FSH (Chappel et al. 1983a, Cooke et al. 1996, Stanton et al. 1996). Because the sugars are charged, the isoforms can be separated using techniques such as electrophoresis (Wide 1985), isoelectric focusing (Chappel et al. 1982), ion exchange chromatography (Phillips et al. 1995) and chromatofocusing (Blum & Gupta 1985). These techniques have shown that the types of pituitary FSH isoforms that are produced are affected by reproductive status (Wide 1989, Beitins & Padmanabhan 1991, Ulloa-Aguirre et al. 1995). Studies in humans (Wide 1986) and rats (Blum & Gupta 1985) have shown that male FSH isoforms are more acidic and have longer half-lives than those from females of a reproductively active age. Also, in humans and rats when there is gonadal inadequacy such as before puberty, after menopause and after castration, FSH isoforms are more acidic and have longer half-lives than in other reproductive states (Cameron & Chappel 1985, Wide 1989). It is not known whether these changes in FSH isoforms occur in sheep. From the limited number of studies it appears that the relationship between the reproductive state of the sheep, the acidity of the isoforms and the half-life of the isoforms may be different from that in humans and rats (Blum & Gupta 1985, Wide 1986, Phillips et al. 1995,
Cooke et al. 1996). Currently, commercial preparations of ovine pituitary FSH (Ovagen; ICP Ltd, Auckland, New Zealand) are used for superovulating many different species including sheep, cattle and deer (McNatty et al. 1989, Henderson et al. 1990, Smith et al. 1993). However, little is known about the effect of age, sex or reproductive state on the properties of ovine FSH.

The aim of this study was to determine whether there is an effect of age, sex or reproductive state on the charge and the half-life of pituitary FSH and to examine if the average charge of sheep FSH isoforms is correlated with the circulatory half-life of FSH.

Materials and Methods

Ethics
All experiments were performed in accordance with the 1987 animal protection (codes of ethical conduct) regulations of New Zealand and had been approved by the animal ethics committee of the Wallaceville Animal Research Centre, Upper Hutt.

Pituitaries
Pituitary glands were collected from: luteal phase ewes at day 10 of the oestrous cycle; follicular phase ewes 36 h after a prostaglandin F2 alpha (Estrumate; Pitman Moore, Upper Hutt, New Zealand) injection given at day 10 of the oestrous cycle; ewe lambs and ram lambs at 5 months of age; ewes at days 24 and 30 of pregnancy (early-pregnant); wethers at 2 years of age; and rams during the breeding season. Pituitaries from the above sheep were collected in May, which is about the middle of the breeding season in New Zealand. Pituitary glands were also collected from ewes between days 124 and 135 of pregnancy (late-pregnant) in August. Pituitaries from ewes in anoestrus and sexually inactive rams were collected in October, which is the middle of the non-breeding season in New Zealand.

Pituitary glands were collected from the sheep within 10 min of death, snap frozen in liquid nitrogen and stored at −70 °C until extraction. Individual pituitary glands were extracted in 6 ml 5 mM phosphate buffer (pH 7.5) containing 0·1% BSA (Imunno-Chemical Products Ltd, Auckland, New Zealand) and 1 mM phenylmethyl-sulphonyl fluoride (Sigma Chemical Co., St Louis, MO, USA). After centrifugation of the homogenate at 4 °C for 30 min at 30 000 g the precipitate was re-extracted as above. The supernatants were combined and then frozen and stored at −20 °C (Robertson et al. 1982).

FSH RIA
The ovine FSH RIA used the United States Department of Agriculture USDA-0FSH-19-SIAFP-I-2 preparation for the iodination reagent, USDA-0FSH-19-SIAFP-RP2 as the standard and the United States National Institute of Diabetes, Digestive and Kidney Diseases NIDDK-anti-0FSH-1 as the antiserum. The RIA was carried out as previously described (McNatty et al. 1987) with the exception that the iodinated FSH was purified by ion exchange chromatography (Moore et al. 1997). The mean intra- and interassay coefficients of variation were 8·8 and 6·6% respectively. The minimum detectable concentration of FSH was 0·2 ng/ml as defined by the concentration 2 × s.d. from the 0 standard. The ED50 value averaged 0·39 ng/ml and the ED50 1·29 ng/ml.

Half-life of FSH
The circulating half-life of the various FSH preparations was measured by injecting the sheep pituitary extracts i.v. into mice and measuring the rate of disappearance from the circulation as described by Phillips et al. (1995). Briefly, mice (three to five per time point) were injected with between 0·5 and 2 µg of immunoreactive pituitary FSH and were bled 5, 15, 30, 60 or 120 min later. Because ram and ram lamb pituitaries contained low amounts of FSH, two pituitary extracts were combined for each of these half-life determinations (n = 3). Individual pituitary extracts were used for all the other half-life determinations. Mouse FSH was not crossreactive in the ovine FSH RIA.

High performance liquid chromatography (HPLC) anion exchange chromatography
The HPLC anion exchange chromatography was performed as described by Phillips et al. (1995). Briefly, 12% of each pituitary extract was filtered through a 0·45 µm Sartorius Minsart filter (Gottingen, Germany) and loaded onto an MA7Q anion exchange column (100 × 19 mm; Biorad, Richmond, CA, USA) connected to an HPLC (Hewlett Packard 1050, Waldbronn, Germany). The column was eluted at 4 ml/min with 20 mM Tris–HCl (pH 8·6) for 2 min. This was followed by a linear gradient from 0 to 400 mM KCl in 20 mM Tris–HCl (pH 8·6) over 50 min. Finally, the concentration of KCl was increased to 2 M for 4 min. Three-millilitre fractions were collected in test tubes containing 100 µl 10% (w/v) BSA in 20 mM Tris–HCl. The fractions were stored at −20 °C until they were assayed. Prior to RIA the buffer in each fraction was exchanged for 50 mM Tris–HCl (pH 7·4) containing 0·3% BSA and 10 mM MgCl2 by running 2 ml from each fraction through a PD10 column (Pharmacia, Uppsala, Sweden). The average recovery of FSH from the anion exchange column was 70·1 ± 2·1% (s.e.m.).

Comparison of chromatofocusing and HPLC anion exchange chromatography
A sheep pituitary extract was run on an HPLC anion exchange column and then the fractions which eluted at
analysed using linear regression. Statistical analyses

After assaying the fractions from the HPLC anion exchange column for FSH, the average charge of pituitary FSH was determined by calculating the volume at which 50% of the FSH had eluted (HP50). The results were analysed by ANOVA and the following contrasts were made: ewes during the luteal phase with ewes during the follicular phase; ewes during anoestrus with ewes during the oestrous cycle; ewes in the breeding season with ewes in early pregnancy; ewes in anoestrus with ewes in late pregnancy; ewes in the breeding season with ewe lambs; ewes in the breeding season with rams in the breeding season; ewes in anoestrus with rams from the non-breeding season; ewe lambs with ram lambs; rams in the breeding season with ram lambs; rams in the breeding season with rams from the non-breeding season; and wethers with rams from the breeding season.

The half-lives of ovine FSH were determined by fitting the decay rate to a single exponential component curve using the Sigma Plot (Jandel, San Rafael, CA, USA) graphical package.

Comparison of pituitary weights, FSH half-lives, FSH pituitary concentrations and FSH contents was done by ANOVA with Tukey’s post hoc test.

The relationship between the HP50 and half-life was analysed using linear regression.

Results

Pituitary FSH concentration and content

Sheep pituitary weights, FSH concentrations and FSH contents are shown in Table 1. Ewe lambs, ewes during early pregnancy and wethers had pituitary FSH concentrations which were higher than in the other groups (P<0.01). The three ram groups had pituitary FSH contents which were lower than (P<0.05) those in the wethers and in all groups of ewes except for late-pregnancy. The non-pregnant ewes had heavier (P<0.05) pituitaries than the wethers and the ewe and ram lambs.

Comparison of chromatofocusing and HPLC anion exchange chromatography

The FSH from an HPLC anion exchange column that eluted at 75, 90, 105 and 120 ml eluted at average pHs of 5.30, 4.93, 4.54 and 4.50 respectively when run on a chromatofocusing column.

HPLC ion exchange reproducibility

A quality control pituitary extract, which was run approximately every tenth HPLC run, had an HP50 of 73±7·0±7 ml (s.e.m., n=6). Rerunning fraction 24 from the HPLC run resulted in a defined peak of FSH activity centred on fraction 24 (Fig. 1).

HPLC ion exchange

Mean FSH profiles for the ewes during the follicular and luteal phases and the rams during the breeding season following HPLC ion exchange are shown in Fig. 2. No difference between the mean profiles of the follicular phase and the luteal phase ewes was observed. However, both groups of ewes contained FSH isoforms which eluted later than the isoforms from rams in the breeding season.

Table 1 Mean ± s.e.m. pituitary weights, FSH concentrations and FSH contents from sheep of different reproductive states (n=5 per group)

<table>
<thead>
<tr>
<th>Reproductive State</th>
<th>Pituitary weight (g)</th>
<th>Pituitary FSH concentration (µg/g)</th>
<th>Pituitary FSH content (µg/pit)</th>
<th>Half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular phase ewes</td>
<td>0.89 ± 0.09de</td>
<td>26.1 ± 5.0a</td>
<td>21.6 ± 3.0a</td>
<td>8.8 ± 0.9a</td>
</tr>
<tr>
<td>Luteal phase ewes</td>
<td>0.91 ± 0.09de</td>
<td>21.1 ± 3.1a</td>
<td>19.2 ± 2.9a</td>
<td>9.6 ± 0.4a</td>
</tr>
<tr>
<td>Early-pregnant ewes</td>
<td>0.62 ± 0.04abcd</td>
<td>66.6 ± 7.7b</td>
<td>41.9 ± 7.1a</td>
<td>10.2 ± 1.1a</td>
</tr>
<tr>
<td>Late-anoestrus ewes</td>
<td>0.66 ± 0.03fde</td>
<td>24.0 ± 4.9a</td>
<td>16.4 ± 4.1ab</td>
<td>8.3 ± 0.8a</td>
</tr>
<tr>
<td>Breeding season rams</td>
<td>1.02 ± 0.15</td>
<td>29.3 ± 4.3a</td>
<td>27.9 ± 2.3a</td>
<td>7.2 ± 1.5a</td>
</tr>
<tr>
<td>Ewe lambs</td>
<td>0.25 ± 0.02</td>
<td>96.8 ± 9.8b</td>
<td>23.1 ± 0.9a</td>
<td>11.0 ± 1.0a</td>
</tr>
<tr>
<td>Ram lambs</td>
<td>0.39 ± 0.11abc</td>
<td>17.4 ± 5.4a</td>
<td>5.3 ± 1.1b</td>
<td>9.0 ± 1.3a</td>
</tr>
<tr>
<td>Non-breeding season rams</td>
<td>0.68 ± 0.06fde</td>
<td>9.8 ± 1.6a</td>
<td>6.5 ± 1.1b</td>
<td>11.5 ± 1.7a</td>
</tr>
<tr>
<td>Wethers</td>
<td>0.73 ± 0.09fde</td>
<td>7.6 ± 1.8a</td>
<td>5.7 ± 1.7b</td>
<td>12.3 ± 2.6ab</td>
</tr>
<tr>
<td></td>
<td>0.28 ± 0.05ab</td>
<td>80.1 ± 3.0b</td>
<td>22.4 ± 2.5a</td>
<td>18.2 ± 2.0b</td>
</tr>
</tbody>
</table>

abcdeWithin a column means with different superscripts are significantly different (P<0.05).
significantly higher than for ewes during anoestrus (101.6 ± 2.6 ml) (P<0.05). There was a significant effect of sex with the ram lambs (81.6 ± 2.7 ml), rams during the breeding (80.7 ± 1.4 ml) and non-breeding seasons (84.5 ± 2.5 ml) having significantly lower mean HP50 values than ewe lambs (88.3 ± 1.9 ml) (P<0.05), ewes during the breeding (95.3 ± 0.7 ml) (P<0.01) (Fig. 2) and non-breeding seasons (101.6 ± 2.6 ml) (P<0.01). The mean HP50 of the rams during the breeding season (80.7 ± 1.4 ml) was higher than those of the wethers (74.0 ± 0.5 ml) (P<0.05), indicating an effect of castration.

**Half-life and correlation of HP50 with half-life**

FSH from wethers had a longer (P<0.05) mean half-life in mice than FSH from all the other groups except for extracts from rams during the non-breeding season (Table 1).

The half-life of FSH was negatively correlated with HP50 (r²=0.56, P<0.01) (Fig. 4). The wethers had the longest half-lives and the smallest HP50 values.

**Discussion**

This study has shown that in sheep that there are significant differences in pituitary FSH isoforms between...
Figure 4 The relationship between the half-life in mice and the HP50 of pituitary extracts of FSH. $r^2=0.56$, $P<0.01$.
higher oestrogenic states of late pregnancy compared with anoestrous ewes and in ewes compared with rams. However, when the ewes were exposed to high concentrations of oestrogen during the follicular phase compared with the luteal phase there was no effect on the FSH isoform profile. Also when anoestrous ewes were compared with cycling ewes it was the anoestrous ewes, with the lowest oestrogen levels, which had the most-acidic FSH isoforms. Therefore it seems that the FSH isoform distribution pattern in sheep is not controlled solely by oestrogen.

In humans, reproductive states which have a high gonadotrophin-releasing hormone (GnRH) pulse frequency are associated with increases in the amount of the least-acidic FSH isoforms in serum (Wide & Albertsson-Wikland 1990, Phillips & Wide 1994, Wide et al. 1996). Consistent with this pattern, pituitary FSH isoforms from ovariectomized ewes are less acidic than those from ewes during the oestrous cycle (Phillips et al. 1995). Also FSH isoforms from wethers are less acidic than those from rams, and ewe lambs are less acidic than those from adult ewes. However, in other comparisons where the secretion of GnRH is different, such as between luteal phase and follicular phase ewes, between ram lambs and adult rams or between rams during the non-breeding season and breeding season, no differences in the FSH isoform distributions were observed. When anoestrous ewes were compared with cycling ewes it was the anoestrous ewes with the lowest GnRH pulse frequency which had the most-acidic FSH isoforms. Overall, there does not appear to be a consistent effect of GnRH on the ovine FSH isoform distribution. In nutritionally restricted ovariectomized lambs, Hassing et al. (1993) found no evidence of an effect of GnRH on the pituitary, secreted or serum FSH isoform distributions. However, in intact ewe lambs pulsatile GnRH increased the amount of the least-acidic serum FSH isoforms (Padmanabhan et al. 1992). This suggests that in the absence of gonadal hormones GnRH is unable to alter the FSH isoform pattern.

In this study we found that in sheep the most-acidic isoforms have the shortest half-lives. Consistent with this result is that FSH from ovariectomized sheep is less acidic (Phillips et al. 1995) and has a longer half-life than FSH from intact ewes (Robertson et al. 1991). In sheep there are more of the less-acidic FSH isoforms in serum than in the pituitary (Padmanabhan et al. 1992) despite the pituitary secreting a similar profile of FSH isoforms to that found in pituitary extracts (Hassing et al. 1993). A possible explanation for this result is that the least-acidic isoforms are more abundant in serum than in the pituitary because the least-acidic isoforms have longer half-lives in serum than the acidic isoforms. The observation that sheep with the least-acidic FSH isoforms have the longest half-lives is the opposite to what happens in humans (Wide 1986) and rats (Blum & Gupta 1985) where the most-acidic FSH isoforms have the longest half-lives. The half-life of human FSH isoforms is correlated with sialic acid content (Morell et al. 1971). Sheep FSH is not as rich in sialic acid as human FSH but it contains more sulphated residues (Green & Baenziger 1988), which also make FSH isoforms more acidic. These acidic sulphated FSH residues may cause a decrease in the half-life of ovine FSH (Fiete et al. 1991, Baenziger et al. 1992). However, it is worth noting that the least-basic forms of ovine LH, which is also highly sulphated, have the longest half-lives (Nakamura et al. 1993) and desulphation of bovine LH causes a decrease in its half-life (Baenziger et al. 1992). Nevertheless, a desulphated glycoprotein may have different properties to the native and less-acidic FSH isoforms found in the pituitary, and moreover ovine FSH may not behave in the same way as bovine LH.

Lower pituitary levels of FSH in rams compared with ewes has also been observed by Robertson et al. (1984) and is consistent with low plasma levels of FSH in rams (Bremner et al. 1980). In humans, males also have lower pituitary levels of FSH than females (Wide 1989). We think that this lower level of FSH in males than females may be due to negative feedback effects of testicular inhibin and androgens because we found that wethers have higher concentrations of pituitary FSH than rams.

In summary, the average charge of sheep FSH isoforms that are present in different reproductive states are often the opposite to what are present in humans. In humans and in sheep, gonadectomy causes the production of FSH isoforms with long half-lives. But whereas in humans the long half-life FSH isoforms are the most acidic, in sheep the long half-life FSH isoforms are the least acidic ones.

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