Inhibin and follistatin concentrations in fetal tissues and fluids during gestation in sheep: evidence for activin in amniotic fluid

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Abstract

The concentrations of inhibin and follistatin in amniotic fluid and in tissue extracts from the placenta, gonads and adrenals of fetal sheep were measured using radioimmunoassays. These tissue extracts were from whole fetuses from days 16 to 45 and from the individual organs from day 46 to 145 (term) and were assayed at multiple dilutions. The capacity of these extracts to alter FSH production of rat anterior pituitary cells in culture was also assessed at multiple dilutions.

Immunoreactive inhibin concentrations in amniotic fluid from both sexes increased during gestation and levels were significantly greater in males than females. Peak concentrations of immunoreactive inhibin of 11.2±1.9 ng/ml were found in males at 116–125 days of gestation. Follistatin concentrations did not change throughout gestation and no significant difference was noted between sexes. Mean follistatin levels throughout gestation were 3.0±0.9 ng/ml for males and 3.7±0.9 ng/ml for females.

Despite the potential for FSH inhibition by inhibin and follistatin, amniotic fluid from both sexes at all stages of gestation stimulated FSH secretion in the pituitary cell bioassays, suggesting the presence of activin which was confirmed by the measurement of immunoreactive activin (13.3±2.5 ng/ml) in a specific radioimmunoassay.

Maximum concentrations of immunoreactive and bioactive inhibin in placental extracts were observed in late gestation (2.2±0.6 and 3.8±1.6 ng/g respectively) and there was no significant difference between sexes. Follistatin concentrations in placental cotyledons ranged from 11.5 to 27.1 ng/g with no significant difference between sexes. In view of the higher follistatin concentrations compared with inhibin, it is likely that the capacity of placental extracts to suppress FSH production by pituitary cells in culture is due predominantly to follistatin.

Immunoreactive inhibin was observed in high concentrations in the fetal testis throughout gestation; with concentrations increasing to a maximum of 1993.0±519.7 ng/g at 126–135 days of gestation with a ratio of bioactive: immunoreactive inhibin of 1:20. Although bioactive and immunoreactive inhibin was also observed in fetal ovaries and adrenals from both male and female fetuses, concentrations were lower than those observed in fetal testes. Follistatin concentrations in the fetal testis were elevated between 70 and 95 days (97.6 ng/g) and then declined. Similar concentrations were found in the adrenal glands of both sexes (males 83.5–103.3 ng/g; females 55.3–95.8 ng/g). In both males and females, immunoreactive inhibin concentrations in fetal adrenals increased during gestation peaking at levels of 34.4±16.5 and 27.8±9.0 ng/g respectively. These data suggest that the capacity of adrenal extracts to suppress FSH production by pituitary cells is due to both inhibin and follistatin.

These studies demonstrated that significant concentrations of immunoreactive inhibin and follistatin are present in amniotic fluid, and the fetal gonads, adrenal glands and placenta in sheep. The role of these proteins during fetal development requires further study.

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Introduction

The isolation of inhibin A and B from bovine and porcine follicular fluid (Ling et al. 1985, Robertson et al. 1985) and subsequently partial purification of inhibin A from ovine follicular fluid (Leversha et al. 1987) was followed by the development of radioimmunoassays to facilitate the determination of inhibin concentrations in biological fluids (McLachlan et al. 1986). Such assays have proved valuable in establishing the sites of production of inhibin and inhibin–related proteins in the adult of several species (see Hamada et al. 1989). Inhibin has also been identified in the placenta of women (McLachlan et al. 1986, Petraglia et al. 1987, 1990) and ovine and bovine fetal gonads (Albers et al. 1989, Torney et al. 1990) by bioassay or radioimmunoassay used either individually or in combination.
The specificity of the radioimmunoassays used in these studies has been questioned because of the recent identification of monomeric products of the α subunit which cross-react in the assays (Robertson et al. 1989, Sugino et al. 1989). When combined with measurements of bioactivity of the inhibin proteins, using a rat pituitary cell follicle-stimulating hormone (FSH) content assay, changes in bioactive to immunoreactive (B/I) ratio can, however, provide circumstantial evidence for the presence of molecules such as α subunit products of inhibin. Inhibin α subunit products do not affect FSH production in the bioassay but do decrease the B/I ratio by increasing the levels of immunoreactive inhibin. In contrast, activin, a close relative of inhibin composed of the two β subunits of inhibin A or B, stimulates FSH production in the bioassay, decreasing inhibin biopotencies and also lowering the B/I ratio (Robertson et al. 1988). Follistatin, which suppresses FSH with similar characteristics to inhibin but possesses no structural homology, will lead to a suppression of FSH production in rat pituitary cultures but does not cross-react in the inhibin radioimmunoassay, thus leading to a concomitant increase in the B/I ratio (Robertson et al. 1987, Ueno et al. 1987). Using combined bioassay and immunoassay for inhibin and protein purification techniques, the presence of α subunit products have been identified in bovine follicular fluid (Robertson et al. 1989, Sugino et al. 1989), media from cultures of rat Sertoli cells following FSH stimulation (Grotenhuis et al. 1990, Hancock et al. 1992) and in the bovine fetal testis in late gestation (Torney et al. 1990, 1992).

Several studies have demonstrated the presence of inhibin α, β_A, and β_R subunit mRNA in multiple sites in the primate, rat, and bovine fetus (Rabinovici et al. 1991, Roberts et al. 1991, Torney et al. 1992). However, the nature of the proteins secreted by these tissues has not been established nor has the presence of follistatin been assessed. The recent availability of follistatin (Klein et al. 1991) and activin radioimmunoassays (Robertson et al. 1992) makes such studies possible. In this study we have determined the concentrations of inhibin and follistatin in ovine fetal tissues and fluids during pregnancy using both immunoassay and bioassay and have shown the presence of activin in amniotic fluid.

Materials and Methods

Animals

The entire uterus was obtained from pregnant sheep of gestational age of 16–145 days which had been stunned and killed at a local abattoir. Each uterus was immediately placed on ice. Fetuses were sexed and gestational age was determined from a nomogram utilizing measurements of fetal weight, straight crown–rump length, vertebral column length, fore-limb and hind-limb length as described by Cloete (1939). An accuracy of estimation of ±5 days can be obtained using this method.

These investigations were approved by the Monash University Standing Committee on Ethics in Animal Experimentation and conform with the NHMRC/CSIRO/AAC Code of Practice for the Care and Use of Animals for Experimental Purposes.

Tissues and fluids

The myometrium of the uterus was carefully dissected to reveal the amniotic and allantoic fluid compartments. Amniotic fluid (10 ml) was collected from uteri containing fetuses judged to be 50 or more days of gestational age prior to removal of the fetus from the amniotic sac. The fluid samples were centrifuged at 3000 g for 10 min to remove particulate matter and the supernatants were aliquoted and snap-frozen in a mixture of ethanol/dry ice and stored at −20 °C until assay.

Placental cotyledons, gonads and adrenal glands were carefully dissected from the fetus. The tissues were frozen and stored at −20 °C until assayed. For fetuses prior to and including day 45 of gestation, sexing was not possible by inspection of the external genitalia (Mauléon 1961). In these animals the entire unsexed fetal membranes, consisting of amniotic, allantoic and chorionic membranes together with the overlying maternal endometrial cells were obtained for homogenization. Up to this stage of gestation, the entire fetus was also homogenized without dissection of gonads or adrenals. Tissue extracts were prepared as described below.

Before assay, the tissues were homogenized using a tissue homogenizer (Polytron; Lucerne, Switzerland) in Dulbecco's phosphate buffer (DPB), pH 7.0, in weight: volume ratios of between 1:1 and 1:5 depending on inhibin concentrations determined in a pilot study. The homogenates were centrifuged at 100 000 g for 60 min at 4 °C (Beckman Ultracentrifuge L8–70M). The supernatants were assayed at multiple dilutions by immunoassay and bioassay in the rat anterior pituitary bioassay. For the placental extracts of gestational age greater than 45 days, an extra step was introduced in which the cotyledons were chopped finely and washed thoroughly in DPB containing 0.1% (w/v) bovine serum albumin to remove blood contamination before homogenization (McLachlan et al. 1986).

Assays

Inhibin immunoassay

Amniotic fluid and fetal tissue extracts were assayed for inhibin using an heterologous double-antibody radioimmunoassay as previously published (Robertson et al. 1988). Briefly, this assay uses antiserum No. 1989 raised in our laboratory against 31 kDa bovine inhibin together with iodinated bovine
31 kDa inhibin as the tracer. The standard used in these studies was highly purified 31 kDa bovine inhibin preparation (BIS-3) isolated in our laboratory from bovine follicular fluid. The assay showed no cross-reactivity with activin A, follistatin, Mullerian-inhibiting substance and transforming growth factor-β but cross-reacted significantly (288%) with the α subunit precursor of inhibin pro-αc (Robertson et al. 1989). Consequently inhibin measurements using this assay are referred to as representing immunoactive inhibin. Since the pro-αc subunit of inhibin is not bioactive, the presence of this substance in samples is recognized by a decrease in the B/I ratio when immunoassay and bioassay are performed on the same sample (Hancock et al. 1992, Torney et al. 1992). The sensitivity of the assay ranged from 0.3 to 0.4 ng/ml and the intra-assay and interassay coefficients of variation were 4.6% to 7.5% respectively. Where possible, all samples of individual fluids or tissues over the gestational age range studied were measured in the same assay. Multiple dilutions of amniotic fluid and tissue extracts of placental cotyledons, fetal testes, fetal ovaries and adrenal glands from male and female fetuses were assayed to establish parallelism to the standard curve. All samples diluted in parallel to the inhibin standard (BIS-3).

**Follistatin immunoassay** Follistatin was assayed using a specific radioimmunoassay as previously described (Klein et al. 1991). This assay uses antiserum No. 202 which was raised in rabbits in our laboratory against bovine 35 kDa follistatin and iodinated bovine 35 kDa follistatin as tracer. Separation of bound from free was achieved by 6% polyethylene glycol (PEG)-facilitated second-antibody precipitation. The intra- and interassay coefficients of variation were 8 and 18% respectively. This assay detects 31, 35, 39 and 45 kDa forms of follistatin (Klein et al. 1991) and the results are expressed in terms of a bovine 35 kDa follistatin standard. Inhibin A and activin A showed no cross-reactivity in this assay.

**Activin Immunoassay** Activin was assayed using a radioimmunoassay previously described (Robertson et al. 1992), using a sheep antiserum (No. 64), kindly provided by Biotech Australia Pty Ltd, Rowville, NSW, Australia) raised against a βA subunit fusion protein and human recombinant activin A. Human recombinant activin A was used as tracer and standard. Separation of bound from free was achieved by a second-antibody precipitation in the presence of 6% PEG. All samples were measured in the same assay, and the intra-assay coefficient of variation was 9%. Inhibin A showed a 1–3% cross-reactivity but the cross-reactivity for inhibin B, activin AB and B could not be determined because of the lack of availability of pure substance. The βA subunit monomer cross-reacted 17% in this assay. Although follistatin shows no cross-reactivity in this assay, incubation of activin with follistatin diminishes the potency of activin as measured by this assay, such that 10 ng follistatin reduces the measurable levels of activin at 5.0 to 1.4 ng/ml. Further addition of follistatin to 30 ng caused no further change in the measured levels. Thus this assay is specific for activin and the β subunit monomer but, in the presence of follistatin, the potencies will be an underestimate. Because of the underestimate of activin potencies in the presence of follistatin, measurements of immunoactive activin in this study are only reported for amniotic fluid.

**Rat pituitary cell bioassay** Before bioassay, all supernatants were subjected to charcoal treatment to remove endogenous steroids. Samples (1 ml) were mixed with 10 mg activated charcoal (Merck, Darmstadt, Germany), vortexed and left at 4°C for 1 h. The solution was centrifuged in a Beckman Microfuge (Rotor 1–94) at full speed for 2 min and the supernatants were used for assay.

Selected tissue extracts and amniotic fluid samples were assessed for their capacity to suppress FSH cell content in the rat anterior pituitary cell assay described by Scott et al. (1980) as modified by Au et al. (1983). This assay detects inhibin or follistatin by suppression of FSH and activin by a stimulation of FSH. Samples were assayed in triplicate at multiple dilutions and the FSH inhibitory potency estimates were made using parallel line bioassay statistics (Finney 1978) in comparison with a bovine follicular fluid standard (bFF 2/4) for inhibin. It is recognized that the FSH suppression referred to in the Figures as 'inhibin bioactivity' may represent the action of follistatin or a combination of follistatin and inhibin. Multiple dilutions of all samples from all tissues examined showed parallelism to the inhibin standard in the bioassay. One ng/ml of the inhibin radioimmunoassay standard BIS-3 is equivalent to 1-9 UI bFF 2/4. The between-assay and within-assay coefficients of variation were 18.6% and 14% respectively. When FSH stimulation was detected in the bioassay, human activin A standard was used as the standard and the samples were assessed for parallelism to this standard.

**Analysis of data**

Results were pooled in gestational age groups of 10 days from 16 to 145 days (term) for the purpose of analysis. All data were log transformed to establish homogeneity of variance as established by Cochran’s or Bartlett’s tests. The transformed data were subjected to analysis of variance followed by the Student–Newman–Keuls procedure for multiple range testing (Snedecor & Cochran 1980). Comparison between inhibin concentrations in tissues and fluids derived from male and female fetuses at 136–145 days of gestation were made by Student’s t-test. All results are presented as means ± S.E.M.
Results

Inhibin, follistatin and activin concentrations in amniotic fluid

Immunoreactive inhibin concentrations in amniotic fluid from male fetuses were 0.7 ± 0.1 ng/ml at the gestational age range of 46–55 days and increased 12-fold (P<0.001) to a maximum of 11.2 ± 1.9 ng/ml at 116–125 days of gestation. There was no further increase in amniotic fluid immunoreactive inhibin concentrations from this stage of gestation to term (Fig. 1). The levels of immunoreactive inhibin in amniotic fluid from female fetuses at 46–55 days of gestation (0.4 ± 0.2 ng/ml) increased fivefold (P<0.001) during gestation to reach maximum concentrations of 3.9 ± 1.5 ng/ml at 126 ± 135 days of gestation. Concentrations at term in amniotic fluid from female fetuses were significantly (P<0.001) lower than those observed in males at the same gestational ages (Fig. 1).

When amniotic fluid samples from male or female fetuses were subjected to bioassay for inhibin, FSH levels in the rat pituitary cell bioassay were significantly stimulated in all samples from both sexes and at all gestational ages studied. In view of the unexpected stimulation of FSH, no activin standard had been used in this assay and in order to provide an index of the degree of stimulation produced by these samples, the percentage FSH rise caused by the highest dose-interval used in the bioassay (100 µl neat amniotic fluid) was calculated for all samples. No significant difference in the degree of FSH stimulation between samples for different gestational ages or for either sex was found (Table 1). In subsequent assays, potency estimates against a human recombinant activin standard were carried out to determine that the rise in FSH stimulated by amniotic fluid was parallel to this standard. Pooled samples of amniotic fluid from 100 to 130 days of gestation from both male and female fetuses ran parallel to the standard curve and the potency was 3.6 ± 0.6 ng/ml (n=15). No significant differences were found between sexes. To confirm further that this FSH stimulatory activity was due to activin, pools of amniotic fluid from 100 to 130 days of gestation were measured in the activin

<table>
<thead>
<tr>
<th>Age of fetus (days)</th>
<th>Response in male fetuses</th>
<th>Response in female fetuses</th>
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<tr>
<td>60</td>
<td>168 ± 32 (4)</td>
<td>146 ± 3 (6)</td>
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<tr>
<td>90</td>
<td>162 ± 21 (5)</td>
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<td>100</td>
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<td>130–140</td>
<td>145 ± 17 (7)</td>
<td>152 ± 11 (5)</td>
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TABLE 1. Response of rat anterior pituitary cell FSH content to amniotic fluid. The effect of ovine amniotic fluid from male and female fetuses of differing gestational age on FSH content of cultured rat anterior pituitary cells are shown. Results are expressed as % increase above basal concentrations (= 100%) of FSH in unstimulated cells caused by 100 µl amniotic fluid. Values are means ± S.E.M. The numbers in parentheses are the number of samples from individual animals assayed at each gestational age.

Figure 1. Concentrations of immunoreactive inhibin concentration in amniotic fluid from (a) male and (b) female ovine fetuses during gestation. Values are the mean ± s.e.m. of inhibin concentrations. Samples (n=3–12) were analysed for fetuses at the gestational age shown. Different letters indicate significant differences (P<0.05) between gestational age groups.
radioimmunoassay and showed levels of $13.3 \pm 2.5$ ng/ml ($n=3$) of immunoactive activin.

There was no significant difference in follistatin levels in amniotic fluid throughout gestation or between sexes (Fig. 2), and the overall mean levels were $3.0 \pm 0.9$ ng/ml for males and $3.7 \pm 0.9$ ng/ml for the females.

**Inhibin and follistatin concentrations in placental cotyledons**

Homogenates of the entire fetal membrane consisting of amniotic, allantoic and chorionic membranes and developing fetal cotyledons from fetuses prior to gonadal differentiation between days 26 and 45 of gestation contained detectable levels of immunoactive inhibin of $4.3 \pm 0.6$ and $4.6 \pm 1.0$ ng/g at days 26–35 and 36–45 respectively.

Placental cotyledons from male fetuses contained immunoactive inhibin concentrations of $2.2 \pm 0.6$ ng/g at days 46–55 of gestation and there was no significant change in immunoactive inhibin concentrations at any gestational age range studied. Immunoactive inhibin concentrations were not significantly different between sexes (Fig. 3). A similar pattern of immunoactive inhibin concentrations was observed in female fetuses.

When placental extracts from male fetuses were added to the pituitary cell bioassay they caused a suppression of the FSH cell content which was equivalent to $1.00 \pm 0.1$ ng inhibin/g at days 46–55 and increased significantly ($P<0.05$) to $3.8 \pm 1.6$ ng/g at days 116–125 of gestation. A similar pattern in the capacity of placental extracts from female fetuses to suppress FSH was observed (Fig. 3). There was no significant difference between sexes in the capacity of extracts to suppress FSH production in the pituitary cell bioassay.

To determine whether the FSH suppression was due entirely to inhibin or in part to the presence of follistatin, the placental cytosols were assayed in the follistatin radioimmunoassay. In female fetuses, placental follistatin concentrations ranged from $14.8 \pm 1.7$ ng/g at 135 days to $27.1$ ng/g at 75 days with no significant differences at each of the time-points. In male fetuses, placental cotyledon follistatin levels ranged from $11.5 \pm 1.0$ ng/g at 65 days to $25.2 \pm 2.4$ ng/g at 105 days with no group showing a significant change.

**Inhibin and follistatin concentrations in whole fetuses at days 20–50 of gestation**

Immunoreactive inhibin concentrations increased significantly ($P<0.01$) in whole fetuses between days 16–25 and 46–55 of gestation from $1.9 \pm 0.3$ ng/g at days 16–25 to $5.0 \pm 0.8$ ng/g at days 46–55 of gestation. A significant ($P<0.05$) increase was also measured in the capacity of fetal extracts to suppress FSH between days 26–35 and 46–55 of gestation with levels, expressed as an inhibin potency, rising from $2.6 \pm 0.4$ ng/g to $12.4 \pm 3.8$ ng/g respectively (Fig. 4).

In contrast to the rise found in inhibin concentrations, follistatin concentrations in the whole fetus decreased from $65.4 \pm 4.2$ ng/g at 26–45 days to $14.1 \pm 0.7$ ng/g at 46–55 days (Fig. 5).

![Figure 2](image-url)
Inhibin and follistatin concentrations in fetal testes

There was a significant ($P<0.001$) and approximate 12-fold increase in concentrations of immunoactive inhibin in testes obtained from fetuses from days 46–55 to 136–145 of gestation (Fig. 4). Concentrations increased from 167.1 ± 38.8 ng/g at 46–55 days of gestation to maximum levels of 1993.0 ± 519.7 ng/g at 126–135 days of gestation. A significant correlation ($P<0.001$, $r=1.0$) was found to...
exist between the increases in inhibin concentrations in the amniotic fluid and the rise found in the testis concentrations.

The capacity of testis extracts to suppress FSH in the bioassay (Fig. 4) also increased significantly with gestational age (P<0.05) and, as with immunoactive values, reached maximum concentrations, expressed in terms of an inhibin standard, at 126–135 days of gestation (98.5 ± 10.1 ng/g). The increase in ‘bioactive inhibin’ concentrations during gestation was not, however, as pronounced as that observed for immunoactive inhibin content (Fig. 4).

As the amount of testicular cytosol available to measure follistatin levels in this study was limited, we made two pools, each consisting of equal aliquots from four animals at each time-point. Follistatin concentrations increased from 14.1 ng/g at 55 days to peak at 97.6 ng/g at 95 days decreasing thereafter to 25 ng/g at 115 days (Fig. 5). To confirm this initial observation, a separate series of testicular cytosols were obtained from individual fetuses. This series demonstrated that testicular follistatin concentrations were elevated at 75 days of gestation and declined thereafter (Fig. 5).

### Inhibin concentrations in fetal ovaries

Because of the very small size of the fetal ovary during gestation, measurements of inhibin immunoactivity were not possible at early stages of ovarian development. Pools of three ovaries from 46 days of gestation to term were, however, assayed for inhibin over the gestational age ranges studied. Immunoactive inhibin was only detectable in these pools from days 126–135 and 136–145 of gestation, being 6.5 ng/g and 7.4 ng/g respectively. FSH-suppressing capacity expressed as bioactive inhibin concentrations in these pools was 5.9 and 5.3 ng/g respectively.

### Inhibin and follistatin concentrations in fetal adrenals

In male fetuses, adrenal immunoactive inhibin concentrations increased significantly (P<0.01) during gestation to reach maximum concentrations of 34.4 ± 16.5 ng/g at days 126–135 of gestation (Fig. 6). The capacity to suppress FSH expressed as bioactive inhibin levels was determined for the 76–85, 106–155 and 136–145 days of gestation age groups and levels were 14.7 ± 4.4, 18.9 ± 5.9 and 15.7 ± 3.5 ng/g respectively. These values did not differ statistically between the different age groups.

In female fetuses, adrenal immunoactive inhibin concentrations also increased significantly (P<0.05) from a nadir at days 76–85 to reach maximum concentrations between 136 and 145 days of gestation (27.8 ± 9.0 ng/g). These were not significantly higher than those observed early in gestation (Fig. 6). FSH suppression expressed as ‘bioactive inhibin’ levels at 76–85, 86–95 and 126–135 days of gestation was 7.0 ± 2.3, 5.0 ± 2.8 and 9.2 ± 2.1 ng/g respectively and was not significantly different between groups (Fig. 6). No statistically significant differences were found between immunoactive or bioactive inhibin concentrations in the adrenals of different sexes.

To determine if the FSH suppression seen in the bioassays was due to inhibin alone or in part to the presence of follistatin, the levels of follistatin were measured in pooled samples of adrenal cytosol extracts. The results obtained from samples from male fetuses at gestational ages 76–85, 106–135 and 136–145 days were 103.3 ± 5.8, 83.5 ± 0.9 (P<0.05 compared with 103.3 ± 5.8) and 96.1 ± 2.2 ng/g respectively, the results in the 106–135 day group being significantly (P<0.05) lower than in the 76–85 day group. In the female samples, the results from age groups at 76–85, 86–95 and 126–135 days were 95.9 ± 2.0, 80.8 ± 3.8 and 55.3 ± 3.5 ng/g (each significantly (P<0.05) different).

### Discussion

The most striking observation in this study was the capacity of amniotic fluid from fetuses of both sexes at all stages of gestation studied to increase FSH production in the pituitary cell culture system. This FSH stimulation, in the presence of relatively high concentrations of immunoactive inhibin levels and follistatin, suggests that the...
amniotic fluid may contain substantial concentrations of FSH-releasing substances which interfere in the assay. The presence of detectable activin concentrations by radi-immunoassay and bioassay confirms that amniotic fluid contains activin, although the levels measured may be an underestimate due to the potential interference of follistatin in the radi-immunoassay and both follistatin and inhibin in the bioassay. These results do, however, confirm that activin is present in amniotic fluid and can express its bioactivity as shown by its FSH-stimulatory activity in the presence of inhibin and follistatin. Since activin has been implicated in mesodermal induction (Thomsen et al. 1990) and in other developmental processes (Rabinovici et al. 1991, Roberts et al. 1991), there is a need to determine the nature of the molecular species in amniotic fluid. Recently completed purification studies have shown that the predominant protein associated with the capacity to stimulate FSH is activin A (de Kretser et al. 1993). Detailed analyses of the changes in immunoreactive activin concentrations throughout gestation in amniotic fluid and other tissues were not possible due to the interference of follistatin in the immunoassays because of its capacity to bind activin (Nakamura et al. 1990). Accurate potencies require methods to dissociate activin and follistatin or to enable their separation prior to the assay of activin.

The source of the immunoreactive inhibin observed in amniotic fluid was not directly addressed in this study. The increase in immunoreactive fluid concentration of inhibin and fetal testis content of immunoreactive inhibin in late gestation would, however, suggest that the fetal gonads may make a significant contribution to amniotic fluid immunoreactive inhibin concentrations. It is possible that inhibin is either secreted from the fetus into the amniotic fluid compartment via fetal urine or via lung liquid secretions. This study has, however, also confirmed that placental cotyledons from both male and female fetuses contain detectable concentrations of both immunoreactive and bioactive inhibin. It is thus possible that the placenta, via the amnio-chorionic circulation, may contribute to the observed inhibin content in amniotic fluid. Further studies are presently being undertaken to determine the source of this protein in amniotic fluid.

As with studies on human placental extracts (McLachlan et al. 1986, Petraglia et al. 1987) the sex of the fetus had no significant effect on placental immunoactive inhibin content. Although inhibin has been localized to both the cytotrophoblast and the syncytiotrophoblast in man (Petraglia et al. 1987, 1990, 1992), further studies using immunocytochemistry or in situ hybridization are required to determine the cellular site of inhibin observed in ovine placenta. Furthermore, no significant increase in immunoreactive inhibin content on a per gram wet weight basis was found in the placenta with increasing gestational age but there was a significant increase in the capacity for placental extracts to suppress FSH. Since the follistatin content of the cotyledons did not change during gestation, this increase in FSH-inhibitory activity is not due to follistatin, although, in view of the higher concentrations of follistatin to inhibin in placental extracts, a major part of the FSH inhibition is likely to represent the bioactivity of follistatin. Since activin can antagonize the FSH inhibition caused by inhibin and follistatin, the data suggest that the concentrations of activin in the placenta in sheep decline...
with gestation. No information as yet is available regarding activin concentrations in the placenta of sheep; however, our unpublished data from man indicate that the term placenta contains activin. Further studies are required to resolve this issue.

The observation that immunoactive and bioactive inhibin is present in sheep fetal testis, ovary and adrenal is not unexpected in view of findings described in other species (McLachlan et al. 1986, Torney et al. 1990, 1992, Rabinoivici et al. 1991). The observed increase in immunoactive inhibin concentrations in the sheep fetal testis with gestational age is almost identical to the pattern described for cattle during fetal testicular development (Torney et al. 1990). The similarity also extends to the observation that the inhibin B/I ratio decreases significantly during the later stages of gestation. Part of the decline in bioactivity may be due to changes in follistatin concentrations in the testis which peak between 75 and 100 days and decline thereafter. Alternatively, our recently published study of inhibin content in the late gestation bovine testis demonstrated that the decline in the B/I ratio of inhibin was due to the increased secretion of α subunit products which are immunoactive but do not suppress FSH production in the bioassay (Torney et al. 1992). It is possible that a similar explanation pertains to the ovine testis.

The common finding of a decline in the B/I ratio of inhibin during gestation in two species raises the possibility that this phenomenon may have an important biological action. There is evidence from adult female sheep that immunization against the NH2-terminal region of the α subunit of the 58 kDa inhibin molecule decreases fertility (Findlay et al. 1989). Furthermore, Schneyer et al. (1991) have provided evidence that α subunit products can interfere with the binding of FSH to its receptor. Thus during late fetal testicular development it is possible that increased α subunit secretion protects the fetal testis from the increased secretion of FSH from the fetal pituitary which was observed in late ovine gestation by Sklar et al. (1981). Furthermore, since human recombinant inhibin alone is able to lower FSH to the normal range in castrated adult rams (Tilbrook et al. 1993), fetal inhibin may have a role in the control of fetal FSH secretion.

Our studies provide only limited information concerning inhibin levels in the fetal ovary, due predominantly to the small size of the ovary during development. However, the pooled samples obtained at 126–135 and 136–145 days of gestation confirm the presence of bioactive and immunoactive inhibin in these tissues. At these stages of gestation, both the bioactive and immunoactive inhibin levels were, however, substantially lower than those found in the fetal testis, the ovarian: testicular ratio at 130 days being 1:16:7 for bioactivity and 1:306:6 for immunoactivity. Similar results were obtained in the bovine fetal gonads (Torney et al. 1990).

It is of interest that the fetal adrenal glands of both sexes contain immunoactive and bioactive inhibin. It has been recognized that the ovine fetal adrenal contained mRNA for the α subunit of inhibin (Crawford et al. 1987) but evidence for the secreted protein has only recently been obtained from studies on adrenocortical cells in culture (Voutilainen et al. 1991). It was proposed, from the investigations of Voutilainen et al. (1991), that adrenocorticotrophic hormone (ACTH) stimulation of the fetal adrenal led to inhibin secretion as detected by immunoassay and that, in dedifferentiated adrenal cells, activin was likely to be the major secreted molecule since β subunit mRNA increased. The data from this present study represent the first systematic study of adrenal inhibin content during development. In the male and female fetuses, the immunoactive inhibin levels increased significantly after 120 days of gestation, but to levels that were considerably less than those found in the fetal testis. Since the capacity of adrenal extracts to suppress FSH were not significantly different between the sexes and did not change with gestational age, the increase in immunoactive inhibin concentrations in the late gestation adrenal may indicate that the fetal adrenal contains inhibin α subunit products. Recently, Spencer et al. (1992) demonstrated that human adrenal cortical cells contained mRNA for α, βA and βB subunits and ACTH stimulated α and βA subunit message. They also demonstrated that human recombinant activin A inhibited mitotic division and enhanced ACTH stimulated cortical secretion by adrenal fetal zone cells but not by adult cells, whereas inhibin A had no such effect. The identification of α and β subunit mRNA in the fetal adrenal cortex (Spencer et al. 1992) supports the view that at least a proportion of the FSH-inhibitory capacity of adrenal extracts is due to inhibin. However, our preliminary data indicate that high levels of follistatin are present in the adrenal of both sexes (55–103 ng/g). The levels found in the adrenal strongly suggest that a significant portion of the FSH-inhibitory activity in the bioassay of adrenal tissue extracts must reside with follistatin even if its biopotency relative to inhibin in the system is assessed as between 10 and 30% (Robertson et al. 1987, Ueno et al. 1987).

As a consequence of the observations reported in this study, it is clear that further detailed studies of each tissue are required to assess the relative roles of each of the FSH-suppressing proteins in bioassay measurements of FSH inhibition. This view is particularly important since our studies have shown the co-presence of inhibin and follistatin in all tissues studied, albeit with varying patterns. Furthermore, in addition, activin is also present in amniotic fluid making accurate determinations of bioassay potencies for each substance impossible to achieve. Clearly future studies must involve techniques to separate these substances or the development of specific immunoassays. Even with the latter, a multipronged approach may be necessary since the monomeric α and β subunit proteins...
may have specific physiological actions and there is thus a need to explore the physiology of these substances in each tissue during development.

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