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Inhibin and activin in embryonic and fetal development in ruminants

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Inhibin, activin and follistatin are protein hormones with diverse physiological roles. The involvement of inhibin in the regulation of pituitary FSH production and secretion in adult males and non-pregnant females is well established. However, it is unlikely that inhibin plays a similar role in pregnancy in ruminants. Inhibin and activin molecules show a high degree of structural similarity to potent growth and differentiation factors of the transforming growth factor β (TGF-β) superfamily of peptides and their localization in a range of embryonic and fetal tissues indicates that they may thus play a role in development. Furthermore, the demonstration that follistatin is also present in a number of embryonic and fetal tissues and fluids has further implications for the actions of activin to which it binds. The role of inhibin, activin and follistatin in early development has yet to be established since gene knockout experiments have so far proved inconclusive. During mid- and late gestation, high concentrations of inhibin are found in the testes and plasma of male fetuses of sheep and cattle. Inhibin may play a role in regulating pituitary FSH release in late pregnancy, but the very high concentrations of this hormone in ovine fetal testes and in male fetal plasma compared with that observed in the fetal ovary and female fetal plasma has yet to be explained. The recent observation of high concentrations of inhibin, activin and follistatin in amniotic fluid surrounding the fetus is intriguing. Excretion via urine or lung liquid is partly responsible for the presence of these proteins in amniotic fluid. The fetal membranes and the placenta are also possible sources. It remains to be established whether these proteins constitute an inactive pool of secreted hormone or whether they have other actions in this fetal compartment.

Introduction

Inhibin was first isolated from bovine and porcine follicular fluid by Ling *et al.* (1985) and Robertson *et al.* (1985). Two isoforms of inhibin have been identified, inhibin A and inhibin B. These glycoproteins can suppress FSH production and secretion from rat anterior pituitary cells in culture. They are formed by dimerization of a common α subunit, derived from a pro α-c precursor molecule, and distinct β subunits termed β_A (inhibin A) and β_B (inhibin B). Both β_A and β_B subunits, as well as the α subunit, have been identified in most species studied, but it appears that only the α and β_A subunits are significantly expressed in adult ruminants since neither the β_B subunit sequence nor the cDNA clone has been identified in sheep or cattle (see Tilbrook *et al.*, 1992). The β subunits of inhibin also form disulfide linked dimers. These proteins, which have the capacity to stimulate FSH production and decrease inhibin biopotency (Robertson *et al.*, 1988), have been designated activin A ($\beta_A\beta_A$), activin B ($\beta_B\beta_B$) and activin AB ($\beta_A\beta_B$).

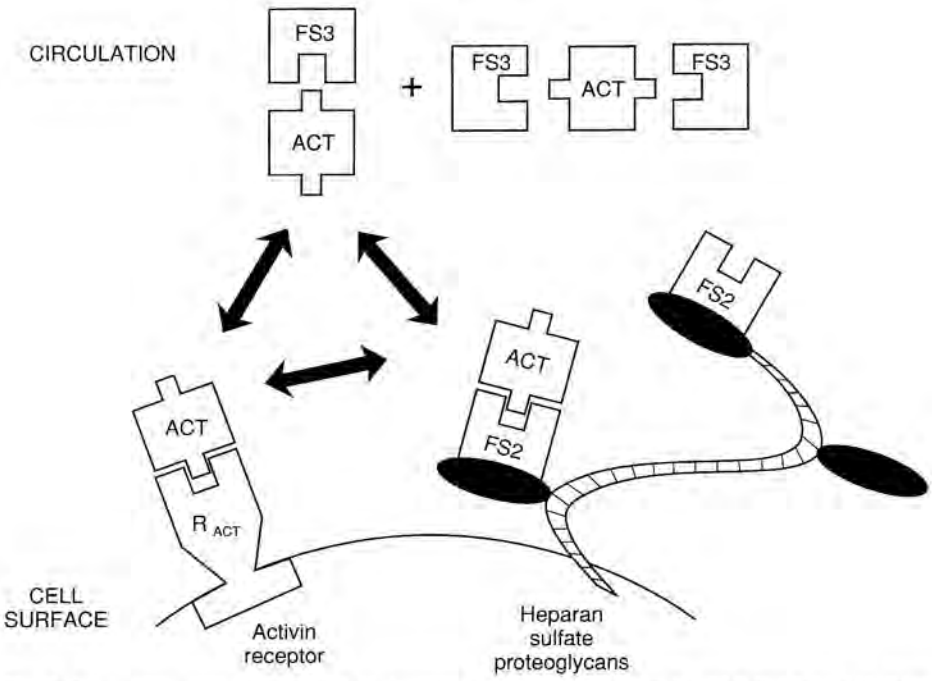

Fig. 1. Schematic representation of the interaction between activin (ACT) and follistatin (FS3). ACT can bind circulating FS3 in a 1:2 ratio. The major circulating forms of follistatin are FS303 and FS315. Activin also binds to cell surface receptors (R_{ACT}) either directly or via association with follistatin 288 (FS2) which is bound to cell surface heparan sulfate proteoglycans.

A protein, initially co-purified from follicular fluid but structurally distinct from the inhibins and activins, was subsequently identified as follicle stimulating hormone-suppressing protein (FSP) or follistatin by Robertson *et al.* (1987) and Ueno *et al.* (1987). This protein can suppress FSH production by rat anterior pituitary cells in culture with a potency of 10–30% that of the inhibins (Robertson *et al.*, 1990; De Paolo *et al.*, 1991). Although coded by a single gene, follistatin is now known to exist as several different isoforms arising from two precursor molecules generated by alternative splicing, together with different degrees of glycosylation (Michel *et al.*, 1993). At least three major isoforms of follistatin are thought to exist, all of which have been demonstrated by Nakamura *et al.* (1990) to bind activin with a K_d value of approximately 1 nmol l^{-1}. It has subsequently been revealed that activin has two binding sites for follistatin but that inhibin has only one binding site for follistatin. Since follistatin has only one binding site for activin and inhibin, it is likely that follistatin binds to these two proteins via their β subunits (Shimonaka *et al.*, 1991). The binding of activin to follistatin provides a potential role for this protein in neutralizing the bioactivity of circulating activin, as the K_d value for the binding of activin to its own receptor is reported to be approximately 0.1 nmol l^{-1} (Matthews and Vale, 1991; Attisano *et al.*, 1992). Furthermore, the presence of different isoforms of follistatin, all with the potential to bind activin, may be important in regulating the many actions of activin. Two full length core proteins, FS315 and FS303, containing 315 and 303 amino acids, respectively, constitute the circulating forms of follistatin, while a further carboxy-terminal truncated form containing 288 amino acids (FS 288) is not secreted but remains within the cell bound to cell surface heparan sulfate proteoglycans. Nakamura *et al.* (1991) proposed that, although circulating follistatin may neutralize the activity of activin, cell-bound follistatin could capture activin in the heparan sulfate proteoglycan matrix thus creating a reservoir of activin which potentially has the ability to control activin availability to its receptor (Fig. 1).

Initial studies on the role of inhibin, activin and follistatin have focused on their potential feedback regulatory role on the pituitary secretion of FSH in adults (de Kretser and Robertson, 1989). Subsequent studies have demonstrated that they possess diverse paracrine and autocrine biological roles and often

act as mutual antagonists. Thus, it has become recognized that these proteins have potent local regulatory functions in adult gonads (see Findlay, 1993) and possibly within the pituitary itself (Bilezikjian *et al.*, 1993). The identification of these proteins in embryonic and fetal tissues and their potential roles in the fetus is the subject of this review.

Feedback Role of Inhibin in Pregnant Ewes

The classical feedback role of inhibin and steroids on pituitary production and secretion in adults is discussed in detail elsewhere (A. J. Tilbrook and I. J. Clarke) in this supplement. Although steroids act at the hypothalamus and the pituitary to inhibit GnRH and gonadotrophin synthesis and secretion respectively, inhibin, produced by granulosa cells in the female or Sertoli cells in the male, is thought to act solely at the anterior pituitary to inhibit FSH production and secretion preferentially (Tilbrook *et al.*, 1993) thus leading to an inverse relationship between FSH and inhibin.

During pregnancy in ewes, the relationship between FSH and inhibin is altered. Plasma gonadotrophin concentrations (LH and FSH) decrease to a nadir in late gestation (Chamley *et al.*, 1974a; Jenkin *et al.*, 1977). Furthermore, the release of LH and FSH in response to the administration of gonadotrophin releasing hormone (GnRH) is suppressed to minimal values in late gestation (Chamley *et al.*, 1974a,b; Jenkin and Heap, 1974; Jenkin *et al.*, 1977), while the release of prolactin in response to thyrotrophin releasing hormone is increased (Wright *et al.*, 1981). In contrast to the cycle, this suppression of gonadotrophin release is not associated with high plasma concentrations of inhibin, as Findlay *et al.* (1991) demonstrated that immunoreactive inhibin concentrations decline from values equivalent to those observed during the cycle from day 40 of gestation to barely detectable concentrations by late gestation. It would appear that the combined chronic negative feedback effect of oestradiol and progesterone, and not inhibin, is responsible for the redirection of the synthetic and secretory function of the anterior pituitary away from gonadotrophins in favour of prolactin (Jenkin 1975; Chamley *et al.*, 1976; Jenkin *et al.*, 1977; Wright *et al.*, 1978; see Fig. 2). A consequence of this redirection is that the surge of secretion of oestradiol associated with parturition (Challis, 1971) does not elicit the release of pituitary LH at this time.

Embryonic Growth and Development

Despite the low concentrations of circulating inhibin in maternal plasma during gestation in sheep and rats (Taya *et al.*, 1989; Findlay *et al.*, 1991; Yohkaichiya *et al.*, 1991), there is increasing interest in the possible roles of these proteins in embryonic and fetal growth and development. The cloning of inhibin and activin in the late 1980s (Mason *et al.*, 1985; Forage *et al.*, 1986) led to the observation that their subunits, particularly the β subunits, have a high degree of similarity to a range of growth and differentiation factors grouped together as the transforming growth factor (TGF) superfamily (Massagué, 1990). This similarity is particularly apparent in the carboxy-terminal amino acid sequence of the precursor polypepetide, as noted by the conservation and position of the cysteine residues. Members of the TGF-β superfamily include four structurally related subfamilies which all exert growth and differentiation regulatory activity: the TGF-β family, the inhibin family, the decapentaplegic gene complex/VGI family and the Müllerian inhibiting substance family.

One of the first demonstrations that indicated that inhibin and activin may be regulators of early embryonic development was the observation that activin A is a potent dorsal mesoderm inducing factor in animal cap explants from *Xenopus* and that activin has been isolated from *Xenopus* cells. Mesoderm induction is thought to be an initial step in early embryonic development. The early induction of mesodermal muscle cell types by activin, as well as notochord and neuro-ectodermal cells such as neuronal cells and melanocytes, provides strong evidence for a role of this protein in early embryonic development (for review see Huylebroeck *et al.*, 1993). A similar role for activin has been reported in chicken embryos where it can induce formation of axial structures (Mitrani *et al.*, 1990).

Subsequent studies in rats have demonstrated that inhibin, activin and follistatin, as well as the activin-IIB receptor, are expressed in a range of embryonic and fetal tissues and localization is consistent with growth effects during embryogenesis. The α subunit of inhibin has been identified in somites as early as day 10–12 of pregnancy, while the β subunit is present in heart primordium, decidua capsularis and uterine blood vessel endothelium. The activin-IIB receptor is widely distributed

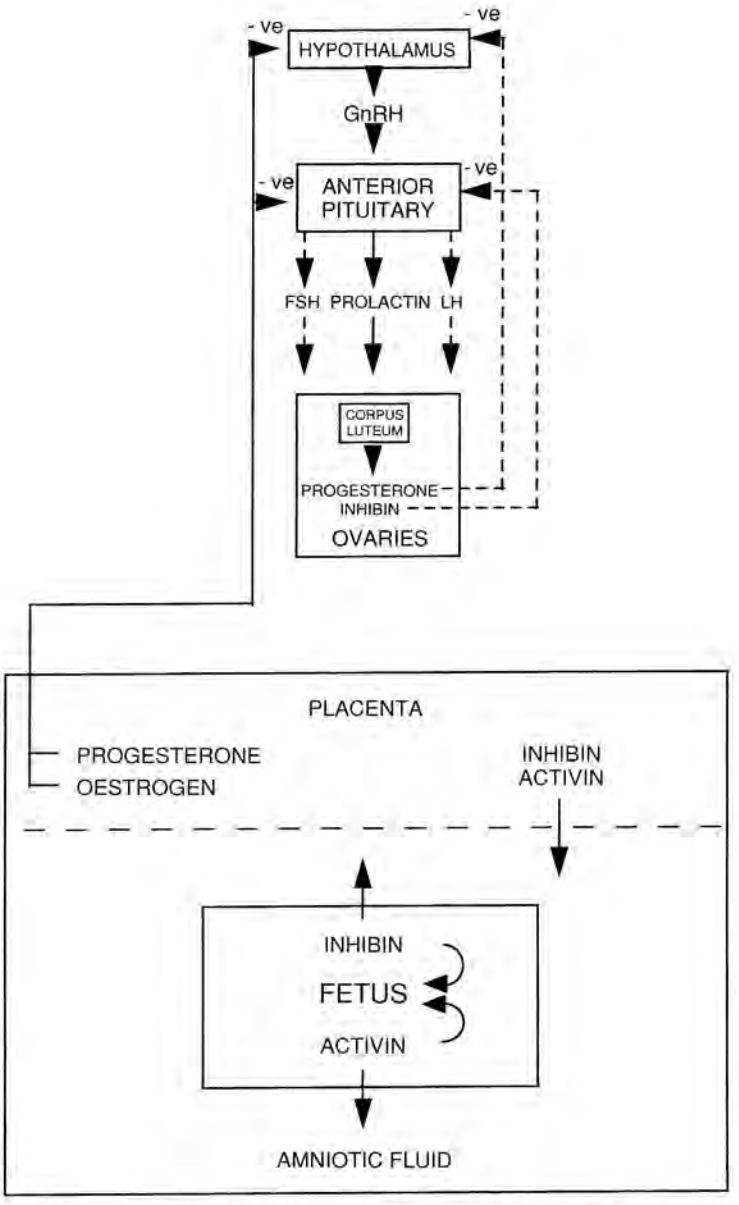

Fig. 2. Negative feedback mechanisms controlling maternal pituitary gonadotrophin and prolactin secretion in late pregnancy in ewes. It is proposed that progesterone and oestrogen, secreted primarily from the placenta of pregnant ewes, are responsible for inhibition of pituitary LH and FSH release and stimulation of prolactin release. In contrast to the oestrous cycle, relatively little inhibin is released from the ovary during pregnancy. Inhibin and activin produced by the placenta and fetus during pregnancy are not released into the maternal compartment but may have local effects on the fetus.

in the embryo and syncytiotrophoblast of the placenta at this time, whereas follistatin is localized in the embryonic neuronal system and the uterine myometrium (Roberts *et al.*, 1991; Roberts, 1993). Such localization provides indirect evidence for the involvement of these peptides in embryonic

growth and differentiation, although similar studies have not yet been undertaken in ruminants. Furthermore, the results of homologous recombination studies in mouse embryonic stem cells to create mouse strains carrying mutations in the genes encoding activin, inhibin and follistatin have been only partly successful in demonstrating their potential actions in embryos (Matzuk *et al.*, 1992, 1994; Vassalli *et al.*, 1994).

Fetal Development

In a series of studies on the ontogeny of inhibin, activin and follistatin in ovine and bovine fetal tissues and fluids, we have demonstrated that both immunoactive and bioactive inhibin is present in the fetal testes throughout gestation with concentrations increasing to a maximum at approximately 130 days of gestation in sheep and between 210 days and term in cows (Torney *et al.*, 1990, 1992; Wongprasartsuk *et al.*, 1994). The increased concentrations of immunoactive versus bioactive inhibin concentrations observed in these tissues indicates that α subunit products of inhibin, which are immunoactive but do not suppress FSH production in the bioassay (Torney *et al.*, 1992), predominate in the fetal testes of these species. An alternative explanation is that follistatin, concentrations of which reach a maximum in the ovine testes at between day 75 and day 95 of gestation (Wongprasartsuk *et al.*, 1994), but which is present in testicular extracts at all stages of gestation, may inhibit biological but not immunological activity. Both bovine and ovine fetal ovaries also contain significant concentrations of inhibin during gestation but concentrations of immunoactive inhibin are lower than those observed in the testes at all stages of gestation studied.

The physiological significance of high concentrations of inhibin in the fetal gonads is not clear at present. In sheep, the pattern of inhibin concentrations in fetal testes is inversely proportional to that of testosterone (M. Goodman, D. M. de Kretser, J. McFarlane and G. Jenkin, unpublished). Whether this is a direct effect of inhibin within the testes, or whether a change in FSH-induced testosterone production is involved is not clear. The ovine fetal testes will certainly produce inhibin in response to FSH which, in turn, inhibits testosterone synthesis (Albers *et al.*, 1989a). Furthermore, inhibin-rich pig follicular fluid can suppress FSH secretion by the pituitary in late gestation (Albers *et al.*, 1989b). We demonstrated that inhibin concentrations are high in fetal plasma in late gestation, and that concentrations in the plasma of male fetuses are significantly higher than those in female fetuses (Wongprasartsuk *et al.*, 1991). The pattern of inhibin secretion into the circulation bears an inverse relationship to circulating FSH, in which concentrations in female fetuses are significantly higher than those observed in male fetuses in mid-gestation, with concentrations decreasing in both sexes before term (Sklar *et al.*, 1981). The inverse relationship between circulating inhibin and FSH concentrations has been re-examined by Phillips *et al.* (1992), who demonstrated differences in bioactive versus immunoactive FSH. Although female fetuses have significantly higher concentrations of both bioactive and immunoactive FSH than do male fetuses in the circulation in late gestation, in contrast to immunoactive FSH, bioactive FSH does not decline towards term in either of the sexes. Phillips *et al.* (1992) also demonstrated that the plasma concentrations of inhibin were negatively correlated with those of immunoactive, but not bioactive, FSH in female fetal plasma but were not correlated with either form of FSH in male fetal plasma. This data, taken together with our observations of gonadal and circulating inhibin concentrations in the fetus, indicate feedback mechanisms between the gonads and the pituitary or hypothalamus may be active in the fetus in late gestation in a similar way to that in adults with high concentrations of inhibin in late gestation opposing FSH release by the pituitary. However, the role of inhibin versus that of circulating steroid concentrations may be different because in adults FSH secretion in female fetuses is relatively unopposed despite high concentrations of circulating steroid hormones (Nathanielsz *et al.*, 1982), whereas in male fetuses, unlike in adults, the major negative feedback effect is likely to be due to inhibin and not testosterone, since testis and circulating concentrations of testosterone in the late gestation male fetus are low (Yu *et al.*, 1983) and do not significantly decrease on castration, whereas FSH concentrations are high after castration in male but not in female fetuses (Mesiano *et al.*, 1991, and see Fig. 3).

Although we have demonstrated that inhibin is present in fetal tissues other than the gonads, such as the adrenals and placenta (Wongprasartsuk *et al.*, 1994), the major source of circulating inhibin in male

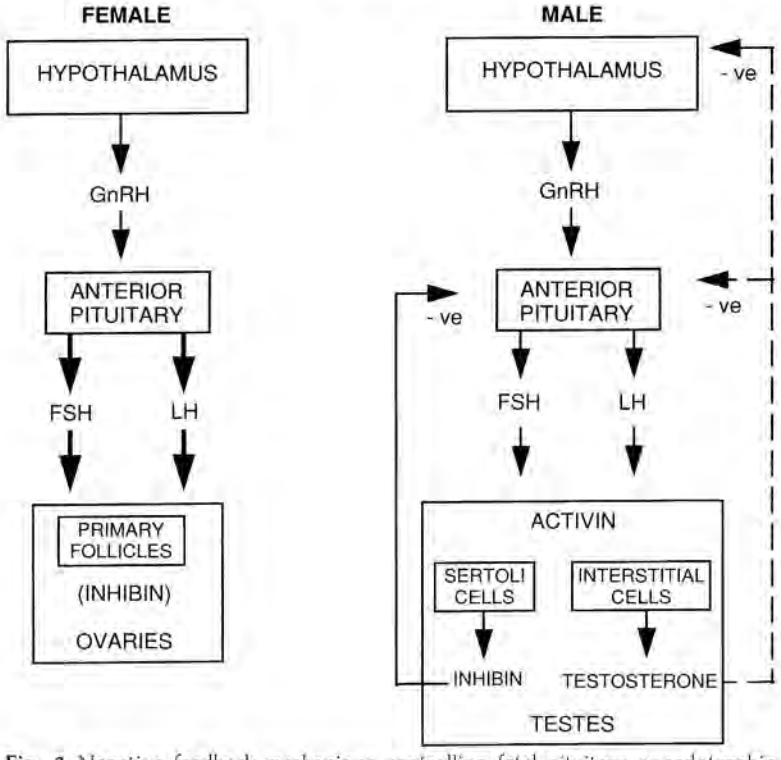

Fig. 3. Negative feedback mechanisms controlling fetal pituitary gonadotrophin secretion during late pregnancy in sheep. Secretion of pituitary gonadotrophins in the female fetus is relatively unopposed in late gestation, while secretion of gonadotrophins in the male fetus may be under the influence of inhibin produced by the fetal testes. Testosterone does not appear to play a major role in the control of hypothalamic or pituitary gonadotrophin secretion in the late gestation male fetus.

fetuses was demonstrated to be the gonads. Castration of male fetuses in late gestation leads to a precipitous decrease in circulating inhibin with concentrations reaching basal values within 12 h after castration. Analysis of decay curves of inhibin concentrations after castration indicates that inhibin has a half-life of approximately 3.5 h in fetal plasma (Jenkin *et al.*, 1993). As determination of arterio–venous differences across the placenta indicates that inhibin is not secreted into the maternal plasma, it is not known whether inhibin is metabolized within the fetus or is secreted either via the placenta or the fluids surrounding the fetus (allantoic and amniotic). A study of the concentrations of immunoactive inhibin in ovine amniotic fluid throughout gestation shows a similar pattern to that observed in the fetal gonads, with concentrations in amniotic fluid from male fetuses greatly exceeding those from female fetuses in late gestation and with maximum concentrations being observed at approximately 120 days of gestation (Fig. 4, Wongprasartsuk *et al.*, 1994). Concentrations of inhibin in amniotic fluid decreased only slightly after castration of male fetuses and remained high throughout the remainder of gestation. Subsequent experiments indicate that either a source other than the fetal gonads is partly responsible for the high concentrations of inhibin, and/or that inhibin is not rapidly metabolized in late gestation in this compartment. The high concentrations of follistatin, which have the potential to bind inhibin, observed in amniotic fluid of both male and female fetuses in late gestation (Wongprasartsuk *et al.*, 1994) may account for this potential lack of metabolism.

When amniotic fluid samples from either male or female fetuses were subjected to bioassay, FSH concentrations in the rat pituitary cell bioassay were significantly stimulated at all gestational ages studied. It was subsequently demonstrated that this stimulation of FSH was due to the presence of high

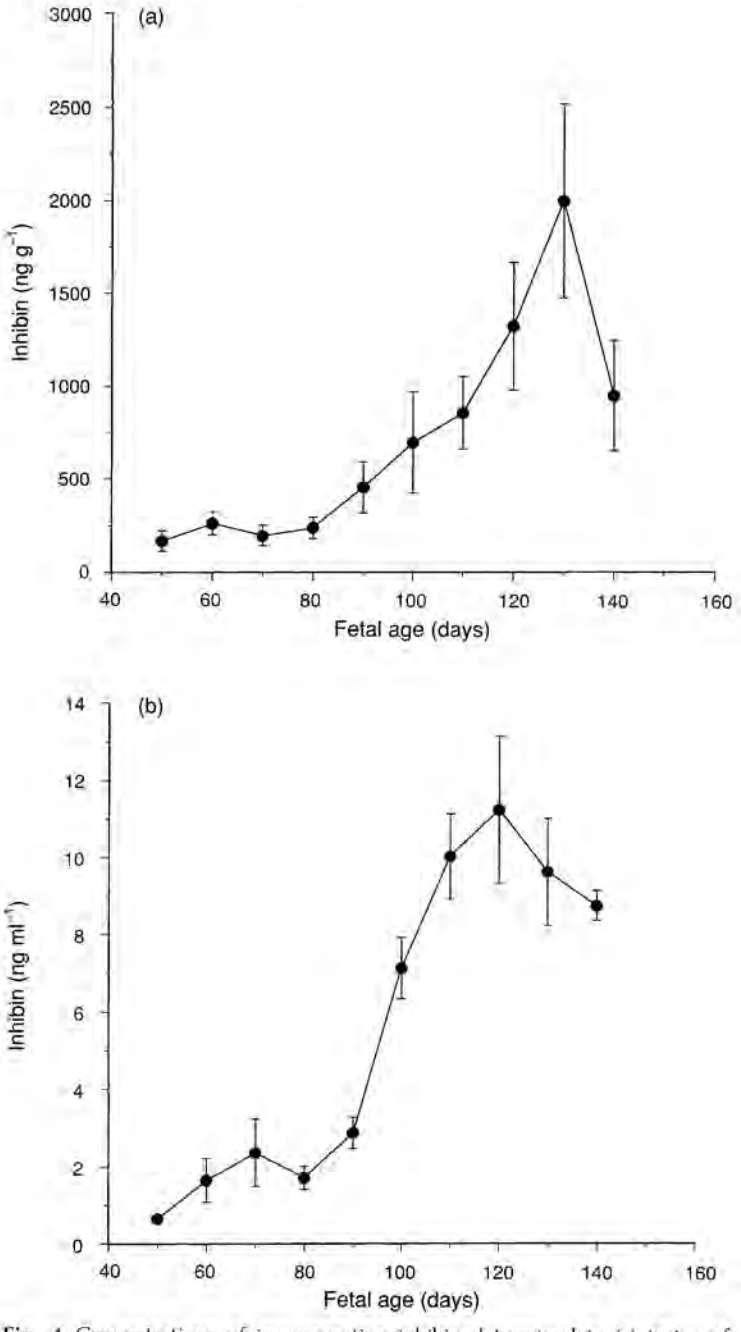

Fig. 4. Concentrations of immunoactive inhibin determined in (a) testes of fetus and (b) amniotic fluid of male fetuses during gestation in ewes. Values are means ± SEM of between three and 12 samples at each gestational age. Redrawn from Wongprasartsuk *et al.* (1994) by permission of the Journal of Endocrinology Ltd.

concentrations of bioactive activin in the amniotic fluid samples (de Kretser *et al.*, 1994) which far exceeded those of follistatin during most of gestation. Activin from sheep amniotic fluid has

subsequently been isolated and purified and the amino-terminal amino acid sequence shown to be identical to the known sequence of sheep activin A (de Kretser *et al.*, 1994).

Although the major source of inhibin, activin and follistatin is likely to be the fetus, the fetal membranes and placenta cannot be excluded as a potential source of these proteins in amniotic fluid. It is important that further studies on the source of, and possible role of, these multipotent proteins in the fetus and fetal fluid compartment surrounding the fetus are undertaken.

Conclusions

Initial studies on the role of inhibin and activin in adult ruminants have focused on their feedback regulatory function in nonpregnant animals, as a regulator of follicular recruitment and in the control of ovulation rate. Follistatin has been promoted as a factor responsible for neutralizing activin in the circulation and the ability of specific molecular forms of follistatin to bind to heparan sulfate proteoglycans on the cell surface has led to the suggestion that it may present activin to its cell surface receptor.

The maternal plasma of ruminants contains basal concentrations of inhibin throughout gestation. Furthermore, unlike during the oestrous cycle, circulating inhibin concentrations in maternal plasma are not inversely related to circulating gonadotrophin concentrations, or to pituitary responsiveness to GnRH, which are all minimal in late gestation. It is unlikely, therefore, that inhibin has a negative feedback role on gonadotrophins in the mother during gestation.

The cloning of inhibin and activin has led to the observation that the β subunit exhibits a high degree of similarity with a number of potent growth promoting and differentiation factors. Studies in rats have demonstrated that inhibin, activin and follistatin are expressed in embryonic and fetal tissues, as well as in the placenta, and that activin affects the growth and differentiation of a number of cell types in a variety of species, including mesoderm induction, regulation of anterior–posterior polarity and induction of the body axis. Follistatin antagonizes many of these biological actions. In ruminants, immunoactive and bioactive inhibin and follistatin are present in the fetal testes, ovaries, adrenals and placenta throughout gestation. Fetal testicular inhibin concentrations increase markedly from mid-gestation, resulting in high concentrations of circulating inhibin in male fetal plasma in late gestation which rapidly decrease to basal values after castration. Although the stimulus for these high concentrations of inhibin in the male fetus is not known, they are associated with a decrease in testicular testosterone and a decrease in circulating FSH concentrations suggesting that, as in adults, inhibin may be directly or indirectly involved in the regulation of testicular androgen and pituitary FSH secretion during late gestation.

Inhibin concentrations in ovine amniotic fluid, in fetuses of both sexes, increase during gestation, and concentrations in male fetuses are greater than those in female fetuses. The fetal gonads account for most of the inhibin observed in amniotic fluid and it is secreted into this compartment from both urine and tracheal fluid. However, an extra-gonadal source, probably the fetal membranes, also contributes to inhibin concentrations in amniotic fluid in late gestation. Follistatin is detectable in amniotic fluid but its concentration does not appear to alter during gestation, nor is there a significant difference between the sexes. Recent studies have demonstrated that concentrations of activin are much greater than those of follistatin in amniotic fluid for most of gestation. Activin has been purified from amniotic fluid and sequenced and shown to be identical to the known sequence of ovine activin A.

The role of these potentially potent regulatory proteins in this fetal fluid compartment is not known. However, it is possible that they constitute an inactive reservoir of hormone secreted from the fetus or that they are partly produced and secreted from the fetal tissues surrounding the amniotic fluid. The regulatory role, if any, of these potentially potent growth and differentiation factors in the fetus and amniotic fluid has yet to be established.

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