Proopiomelanocortin, prolactin and growth hormone messenger ribonucleic acid levels in the fetal sheep pituitary during late gestation

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We have measured the relative levels of proopiomelanocortin (POMC), prolactin (PRL) and growth hormone (GH) messenger ribonucleic acid (mRNA) in the anterior and neurointermediate lobes of the fetal pituitary during the last 2–3 weeks of gestation. The mean POMC mRNA/18S RNA ratio in the fetal anterior pituitary was significantly greater (p < 0.02) at 130–136 days (0.90 ± 0.08; N = 9) than at 141–143 days of gestation (0.67 ± 0.07; N = 6). In contrast, the mean PRL mRNA/18S RNA ratio increased significantly (p < 0.02) between 130 and 136 days (0.31 ± 0.05; N = 9) when compared with 141–143 days of gestation (0.58 ± 0.10; N = 6). There was no significant difference, however, between the mean GH mRNA/18S RNA ratio in fetal anterior pituitaries at 130–136 days (0.95 ± 0.04; N = 9) when compared with 141–143 days of gestation (1.08 ± 0.14; N = 6). The POMC mRNA/18S RNA ratio in the neurointermediate lobes was seven-, five- and tenfold higher than in anterior pituitaries at 130–134, 135–136 and 141–143 days of gestation, respectively. We hypothesize that elevated circulating cortisol levels after 140 days of gestation act in the slow time domain (i.e. over days) to suppress POMC gene expression and that the increase in fetal pituitary PRL mRNA levels may be a consequence of oestrogen stimulation in late gestation.

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Parturition in the sheep is dependent on the prepartum increase in the fetal plasma concentrations of ACTH and cortisol that occurs during the last 15–20 days of gestation (1). The relative roles of the fetal hypothalamus and adrenal in the regulation of ACTH synthesis and secretion in the fetal pituitary during late gestation are not fully understood. Several studies have demonstrated that short- or long-term intrafetal infusions of glucocorticoids can inhibit basal and stimulus-induced increases in fetal plasma concentrations of ACTH (2–5). There are conflicting data, however, on the effects of the prepartum increase in endogenous cortisol levels on the synthetic and secretory capacity of the corticotrophic cells of the fetal pituitary. McMillen and co-workers (6) reported that between 135 and 141 days of gestation there was a fall in the relative levels of mRNA for the ACTH precursor proopiomelanocortin (POMC) in fetal pituitaries. These findings would suggest that elevated cortisol levels act to suppress POMC gene expression in the fetal pituitary in late gestation. In contrast, Yang and colleagues (7) found that POMC mRNA levels were higher in fetal sheep pituitaries collected at 138–143 days than in pituitaries collected at 125–130 days of gestation. These authors suggested, therefore, that the presence of other mechanisms overcome the negative feedback influence of the elevated cortisol concentrations to result in an increase in fetal pituitary ACTH synthesis and secretion.

We have repeated the measurements of POMC mRNA in a large series of anterior pituitaries collected from fetal sheep at between 130 and 143 days of gestation in order to resolve the issue of the molecular basis of the prepartum changes in fetal ACTH concentrations. We have also measured POMC mRNA levels in the neurointermediate lobes of the fetal pituitaries collected in this gestational age range. Further, we have extended the study to determine whether there are changes in the mRNA levels for prolactin (PRL) and growth hormone (GH) in the fetal anterior pituitary in late gestation. Whilst there are gestational changes in the fetal plasma concentrations of PRL (8, 9) and GH (10), there have been no studies on the pattern of gene expression of these fetal pituitary hormones during late gestation in the sheep.

Whilst several groups have found that there is an increase in circulating PRL concentrations in late gestation (8, 9), it has been proposed that these changes are solely a consequence of the effect of lengthening of the
external photoperiod (11). We have determined whether there is a change in pituitary PRL mRNA levels in late gestation, which occurs in the absence of any alteration in the external photoperiod.

Materials and methods

Animals

Eleven Border–Leicester × Merino ewes of known gestational age, carrying either twin or singleton fetuses, were used in this study. Ewes were killed by an overdose (20 ml) of sodium pentobarbitone, and fetuses (N = 15: seven males, eight females) were delivered by hysterotomy and killed by either decapitation or diffusion of sodium pentobarbitone from the mother. All fetal pituitaries were collected during a 2-week period in April (mid-Autumn, Southern Hemisphere). The whole fetal pituitary was removed immediately, and the anterior pituitary was dissected away from the neurointermediate lobe (NIL)—this process is relatively straightforward in the fetal pituitary because of clear separation between the lobes. Both lobes of the pituitary were then snap-frozen in liquid nitrogen and stored at −70°C until extraction of total cellular RNA.

Ribonucleic acid extraction

Ribonucleic acid was extracted from 15 individual fetal anterior pituitaries and from the NILs that were pooled into each of three age groups, i.e. 130–134 days (N = 5), 135–136 days (N = 4) and 141–143 days (N = 4). All pituitary tissue was weighed immediately prior to extraction of total cellular RNA, which was performed using guanidine thiocyanate and caesium chloride, as described previously (6). After precipitation with ethanol, the RNA pellet from each anterior pituitary and NIL was resuspended in Milli-Q water (i.e. deionized and sterile). Fractions (2 µl) of the RNA were read by a spectrophotometer (at 260 nm and 280 nm) to calculate the total amount of extracted RNA, and volumes were adjusted such that a final concentration of RNA of 5 µg/µl was used in subsequent experiments; until then, the RNA preparations were stored at −20°C. Prior to northern blot analysis, the RNA preparations were checked for degradation and correct concentration using non-denaturing gel electrophoresis. Fractions of each sample were subjected to electrophoresis on a 1% (w/v) agarose gel that was subsequently stained with ethidium bromide. The RNA preparations were used in subsequent experiments only if sharp ribosomal RNA bands were present and there was no evidence of degraded RNA at the level of the low-molecular-weight dye front.

Hybridization studies

Northern blot analysis was performed essentially as described by Thomas (12) and as reported previously (13). Aliquots of fetal anterior pituitary RNA (25 µg) and NIL RNA (8–27 µg) were denatured in 30% glyoxal for 1 h at 50°C before being electrophoresed in a 1.0% agarose gel in 10 mmol/l phosphate buffer. An RNA ladder (1.4–9.5 kb) was included in a separate well to act as a calibration marker. Following electrophoresis, the RNA was blotted onto a Biodyne nylon membrane (1.2 µm; Pall Biosupport Division, Glen Cove, New York) overnight at room temperature and then baked for 2 h at 80°C. The blot was prehybridized for 4 h at 42°C in prehybridization buffer: 50% formamide, 20 × SSC (1 × SSC = 0.15 mol/l NaCl + 0.015 mol/l sodium citrate), 200 mmol/l PO₄, 0.4% bovine serum albumin (BSA), 0.4% polyvinyl pyrrolidine, 0.4% ficoll, 80 µg transfer RNA/ml and 40 µg heat-denatured herring sperm DNA/ml. The blot was then hybridized with [1^-32P]-labelled human (h) POMC cDNA (10⁶ cpm/ml; generously donated by Dr P Whitfield, Australian National University, Canberra) for 24 h at 42°C. The hPOMC cDNA probe comprised an 800-base pair sequence from the exon III region of the POMC gene, and was labelled with [α-32P]CTP (Bresa Laboratories, South Australia) using an oligo-labelling technique. After hybridization, the blot was washed sequentially in 2 × SSC/0.1% sodium dodecyl sulphate (SDS) and in 0.2 × SSC/0.1% SDS before autoradiography using Fuji XAR film with an intensifying screen at −80°C for 1–4 days. Following autoradiography, the blot was stripped of the hPOMC cDNA probe to allow hybridization with the hPRL cDNA probe, which comprised a 686-base sequence containing the entire hPRL gene (donated by Drs N Cooke and J Shine, USA). The blot was then stripped and hybridized with an ovine (o) GH cDNA probe, which comprised an 800-base pair sequence, and was a gift from Dr D Catanzaro (Sydney, NSW, Australia). Both probes were oligo-labelled with [α-32P]CTP and hybridized with the blot as described for hPOMC cDNA.

To determine the relative amounts of RNA in each lane, an 18S ribosomal oligonucleotide (30-mer) probe was radiolabelled and hybridized to the blot, as described above.

The autoradiographs were analysed using an LKB Ultrascan XL densitometer (Bromma, Sweden), which produced graphic representations of the band densities and automatically integrated the areas under these curves. Where more than one autoradiograph was available from each blot, the density readings for each pituitary were meaned prior to statistical analysis. The density readings of all pituitaries were compared at the same dilution of the pituitary total RNA, and results were standardized by expressing the density readings from each tissue as a percentage of the density reading of the anterior pituitary collected from a 143-day fetus. The relative amount of POMC mRNA was then
expressed as a ratio of the relative amount of 18S RNA for each sample.

Statistics
All results are expressed as means ± SEM or as pituitary hormone mRNA/18S RNA ratios, and the weights of the anterior pituitaries, the RNA yields from the pituitaries and the anterior pituitary hormone ratios for the two age groups, i.e. 130–136 and 141–143 days of gestation, were compared statistically using the Mann–Whitney test. Differences between age groups were taken as significant if the probability level was less than 5% (p < 0.05).

Results
Anterior pituitary weights and total RNA content
There was an increase in the mean body weight of fetal sheep at 130–136 days of gestation (3.64 ± 0.38 kg) and 140–143 days of gestation (4.61 ± 0.38 kg). There was no difference, however, between the mean weight of anterior pituitaries collected at 130–136 days of gestation (99.1 ± 7.7 mg; N = 9) and 141–143 days of gestation (102.5 ± 11.7 mg; N = 6). There was also no significant difference in the yield of total RNA from the anterior pituitaries in the two age groups (130–136 days of gestation, 1.2 ± 0.1 μg RNA/mg tissue; 141–143 days of gestation, 1.3 ± 0.2 μg RNA/mg tissue).

Fetal pituitary POMC, PRL and GH mRNA
Autoradiographs of the northern blots of RNA from the fetal pituitaries showed that a single RNA transcript hybridized with each of the hPOMC cDNA, hPRL cDNA, oGH cDNA and 18S probes (Fig. 1); whilst the fetal sheep NILs contained POMC mRNA, there was no PRL or GH mRNA signal in the NIL lanes. The size of the POMC mRNA transcript in the fetal anterior pituitaries was 1.25–1.4 kb, whereas the size of the PRL and GH mRNA transcripts was 1–1.2 kb. The mean POMC mRNA/18S RNA ratio was significantly greater (p < 0.02) at 130–136 days (0.90 ± 0.08; N = 9) than at 141–143 days (0.67 ± 0.07; N = 6) (Fig. 2A). In contrast, the mean PRL mRNA/18S RNA ratio increased significantly (p < 0.02) between 130–136 days (0.31 ± 0.05; N = 9) and 141–143 days (0.58 ± 0.10; N = 6) (Fig. 2B). There was no significant difference, however, between the mean GH mRNA/18S RNA ratio at 130–136 days of gestation (0.95 ± 0.04; N = 9) when compared with that at 141–143 days of gestation (1.08 ± 0.14; N = 6) (Fig. 2C). There was no difference between male and female fetuses in the mean ratios of POMC mRNA, PRL mRNA and GH mRNA/18S RNA in the anterior pituitaries.

Hybridization with the hPOMC cDNA probe showed that the POMC mRNA/18S RNA ratio was always greater in the NIL tissue than in anterior pituitaries in the same age range. The POMC mRNA/18S RNA ratio in the NILs at 130–134, 135–136 and 141–143 days were 6.9, 3.8 and 6.8, respectively. These values were seven-, tenfold and higher than the POMC mRNA/18S RNA ratio in the anterior pituitaries at these gestational age ranges.

Discussion
We have demonstrated that in the fetal sheep there is a decrease in POMC mRNA and an increase in PRL mRNA.
levels in the anterior pituitary between 136 and 141 days of gestation.

In the present study we observed a single POMC mRNA transcript in the anterior and NILs of the fetal pituitary and its size was consistent with previous studies in the fetal sheep (7, 13) and in the adult cow (14). The demonstration of a decrease in the relative level of POMC mRNA in the fetal anterior pituitary after 141 days of gestation confirms our previous finding of a significant fall in the POMC mRNA/poly (A⁺) RNA ratio in the anterior pituitary of fetal sheep between 135 and 141 days of gestation (6). Our findings are, however, in direct contrast to those of Yang et al. (7), who reported that there was a three- to fourfold increase in the mean POMC mRNA levels in the fetal anterior pituitary between 125–130 days and the last week of gestation. One possible reason for the differences between the two studies may be the considerable variability in the intensity of the POMC mRNA signal that Yang observed in several of the key age groups (notably at 125–130 days and term). It should also be noted that the relative increase in pituitary POMC mRNA levels measured in late gestation by Yang and co-workers exceeds that measured in the fetal sheep anterior pituitary after fetal bilateral adrenalectomy (13, 15).

Yang and co-workers (7) noted that the timing in the increase in the relative levels of the POMC mRNA in the fetal anterior pituitary coincided with the prepartum increase in the fetal plasma concentrations of ACTH. These authors concluded therefore that the negative feedback actions of the high prepartum concentrations of cortisol on POMC gene expression are obscured by other mechanisms that increase POMC mRNA accumulation near term. In contrast, given our current and previous findings, we would conclude that elevated circulating cortisol acts in the slow time domain (i.e. over days) in late gestation to suppress POMC gene expression. It is clear that further studies on the turnover of POMC mRNA in the corticotrophs of the fetal sheep pituitary are required to elucidate the molecular basis of the prepartum increase in fetal ACTH and cortisol concentrations.

It may be of relevance that the POMC mRNA signal in the NILs of the fetal pituitaries was at least five- to tenfold higher than in the anterior lobes throughout late gestation. There was no decrease in the POMC mRNA levels in fetal NILs after 141 days of gestation, and this is consistent with the previously demonstrated absence of a negative feedback effect of cortisol on POMC synthesis in the adult sheep NIL (16). Whether the fetal NIL is a source of the prepartum increase in circulating ACTH concentrations remains to be established.

In the present study we have measured a significant increase in the relative abundance of PRL mRNA in the fetal sheep anterior pituitary during the last week of gestation. Several groups have found that there is an increase in the fetal plasma concentrations of PRL during the last 7–10 days of gestation (8, 9). Leisti and co-workers (17) also reported that PRL synthesis in explants of fetal anterior pituitaries incubated with [³⁵S]methionine increased steadily during the latter two-thirds of gestation.

It has been suggested that an increase in circulating oestrogens stimulates both maternal and fetal pituitary PRL secretion in the sheep in late gestation (8). Bassett and his colleagues (11), however, have proposed that increases in circulating PRL concentrations in the pregnant ewe and her fetus are coincident with and dependent on the lengthening of the external photo-period.

In the present study, fetal pituitaries were collected from pregnant ewes who were all exposed to an 11–12-h
photoperiod during the same preceding 2-week period. It would seem unlikely, therefore, that the increase in the PRL mRNA observed in the fetal pituitaries in this study is related to a photoperiod effect.

It may be, therefore, that the increase in pituitary PRL concentrations in late gestation is associated with an increase in circulating oestrogens. A number of studies in the rat have demonstrated that oestrogens act to increase PRL gene expression and PRL synthesis in the pituitary lactotrophs (18, 19). In the late pregnant sheep there is an increase in fetal and maternal plasma oestrogens (20). This increase, however, occurs predominantly in the last 48–72 h before birth and we have found that PRL mRNA levels in the fetal pituitary were consistently elevated from as early as 141 days, i.e. within a week of delivery (term = 147 ± 3 days of gestation). One possibility is that the fetal pituitary lactotrophs are more sensitive to the stimulatory actions of oestrogens in the week before delivery.

Plasma GH concentrations are five- to tenfold higher in the fetal sheep than in the pregnant ewe and have been reported to fall in the 24–48 h preceding delivery (8, 11). We have found that there appears to be no change in GH gene expression in fetal pituitary somatotrophs up until 3–4 days before delivery.

In conclusion, our study has demonstrated that there are changes in POMC and PRL mRNA levels in the fetal sheep pituitary in the week before delivery. These changes may form part of the sequence of neuroendocrine events upon which the process of successful delivery and transition to extraterine life depend.

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References

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