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Effects of Labor on Pituitary Expression of Proopiomelanocortin, Prohormone Convertase (PC)-1, PC-2, and Glucocorticoid Receptor mRNA in Fetal Sheep

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We hypothesized that the concurrent prepartum rise in adrenocorticotropic hormone (ACTH) and cortisol in the plasma of fetal sheep might be attributable to altered expression of pituitary endoproteases, prohormone convertase (PC)-1, and PC-2, or to changes in pituitary expression of glucocorticoid receptor (GR) that would influence negative feedback potential. We obtained pituitary tissue from fetal sheep during late pregnancy (d 100–d 145, term) and at precise times during the process of labor and used *in situ* hybridization to localize and quantify mRNA levels. Proopiomelanocortin (POMC) mRNA was regionally distributed (pars intermedia > inferior pars distalis > superior pars distalis) and increased within the pars distalis during late pregnancy and with labor. At term, levels of PC-1 and PC-2 mRNA were higher in the pars intermedia than pars distalis; PC-1 but not PC-2 in the pars distalis increased with gestational age, although it did not change further at labor. GR mRNA levels in the pars distalis increased between d 135 and term, then decreased during labor. We suggest that the concomitant rise in plasma ACTH and cortisol of fetal sheep during late gestation may be attributable, in part, to increased expression of PC-1 leading to increased POMC processing. Furthermore, the negative feedback effects of cortisol on pituitary POMC synthesis and/or ACTH release during active parturition may be lessened by downregulation of anterior pituitary GR.

Key Words: Proopiomelanocortin; prohormone convertase 1; prohormone convertase 2; glucocorticoid receptor; adrenocorticotropic hormone; labor; fetal sheep.

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Introduction

Maturation and activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis provides the stimulus for the initiation of parturition in sheep. Plasma cortisol concentrations in the fetal lamb increase during late gestation in association with a concomitant rise in fetal plasma adrenocorticotropic hormone (ACTH) *(1)* and contribute to parturition onset. This occurs despite evidence for negative feedback effects of glucocorticoids on the synthesis of proopiomelanocortin (POMC), the ACTH precursor *(2,3)*, and pituitary ACTH output *(4,5)*. The mechanisms underlying the concurrent increases in fetal plasma ACTH and cortisol leading to the initiation of parturition are not clearly understood. Experiments involving fetal sheep suggest that the rise in plasma ACTH is associated with increased pituitary synthesis of the ACTH precursor, POMC, at term *(6,7)*. However, other studies have reported that POMC mRNA expression is suppressed at term *(8,9)*. This discrepancy in results among laboratories suggests that alterations in steady-state levels of POMC mRNA may not accurately predict ACTH output during late gestation. The prepartum increase in plasma ACTH may therefore reflect enhanced processing of POMC to ACTH as a result of changes in expression of the key POMC processing enzymes, prohormone convertases-1 and -2 (PC-1, PC-2).

PC-1 (also termed PC-3) and PC-2 are members of a family of endoproteases that cleave substrates at specific dibasic residues and that have tissue-specific expression in endocrine and neuronal tissues *(10)*. In the fetal sheep, pituitary PC-1 mRNA has been identified in the corticotrophs of the pars distalis, and both PC-1 and PC-2 mRNA have been localized to the melanotrophs of the pars intermedia *(11)*. In rodents, POMC is cleaved in a hierarchical order by PC-1 to form ACTH and β-lipotropin, followed by PC-2 cleavage of PC-1-generated ACTH to form α-MSH ($ACTH_{1–13}$) and corticotropin-like intermediate lobe peptide ($ACTH_{18–39}$) *(12)*. Therefore, altered processing of POMC by PC-1 and/or PC-2 could account, in part, for the late gestational rise in fetal plasma ACTH concentrations.

Alternatively, this rise in fetal plasma ACTH may reflect an attenuation of the negative feedback effects of cortisol on POMC synthesis, an effect mediated via changes in glucocorticoid receptor (GR) levels. GR mRNA *(13,14)* and binding *(15)* have been identified in the fetal sheep pituitary from 60 d of pregnancy to term. However, these studies have provided conflicting results regarding GR mRNA expression during late gestation.

Although changes in the fetal HPA axis are critical for parturition, there is little information available regarding changes in the activity of the fetal HPA axis immediately preceding the onset of labor and during the labor process. There has also been controversy concerning the pattern of pituitary POMC expression during the final weeks of gestation, and there is no information about fetal pituitary POMC expression during labor itself. Therefore, we have used *in situ* hybridization (ISH) to determine changes in the fetal pituitary expression of POMC, PC-1, PC-2, and GR mRNA at precise times during the last third of gestation and with the progression of spontaneous labor at term.

Results

Fetal Plasma Cortisol and ACTH

Cortisol concentrations in fetal plasma rose progressively from term not in labor (NIL) to active labor (L) (Table 1). The levels of plasma cortisol were significantly different among all three groups (all $p < 0.001$). Plasma ACTH rose progressively from the NIL to L group (Table 1). Plasma ACTH levels were significantly higher in the L than in the NIL and early labor (EL) groups ($p < 0.05$), but there was no significant difference between the NIL and EL groups (Table 1). When the data from all three stages of labor were pooled, there was a significant ($p < 0.001$) positive correlation between fetal plasma ACTH and plasma cortisol concentrations ($r = 0.62$).

POMC mRNA

POMC mRNA was present in both the pars distalis and the pars intermedia (Fig. 1). POMC mRNA expression in the inferior region of the pars distalis significantly increased from 135 d gestational age (GA) to term L ($p < 0.05$) (Fig. 2). However, there were no significant differences in levels of POMC mRNA in either the superior or inferior region of the pars distalis between the NIL, EL, and L groups. At all gestational ages studied, POMC mRNA expression was higher in the inferior region of the pars distalis than in the superior region (Fig. 2). POMC mRNA expression in the pars intermedia was always greater than in the pars distalis, but did not significantly differ among any of the groups (data not shown).

Ontogeny of PC-1 and PC-2 mRNA Expression

Prohormone Convertase-1

The PC-1 hybridization signal was detected in both the pars distalis and pars intermedia at all ages studied (Fig. 1).

Table 1
Terminal Fetal Plasma ACTH and Cortisol Concentrations During the Onset and Progression of Labor[a]

	ACTH (pg/mL^{-1})	Cortisol (ng/mL^{-1})
NIL	38.1 ± 10.5*	11.3 ± 3.7*
EL	59.7 ± 8.7*	37.7 ± 3.4^{+}
L	95.0 ± 8.7^{+}	75.2 ± 3.4$^{\pm}$

[a]Data are shown as the mean ± SEM. Values with different superscripts are significantly different ($p < 0.05$).

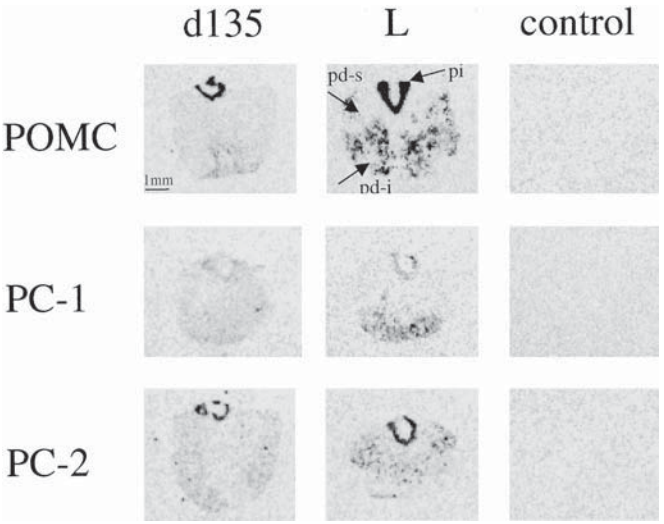

Fig. 1. Distribution of POMC, PC-1, and PC-2 mRNA in the ovine fetal pituitary taken from animals at 135 dGA and during L. Panels show representative computerized autoradiograms of mRNA expression as determined by ISH. A control 45-mer nonsensical sequence oligonucleotide probe was included to assess nonspecific binding.

PC-1 mRNA had a homogeneous pattern of distribution in the pars distalis prior to term (100–135 dGA), but at term (NIL, EL, L), PC-1 mRNA showed a regional distribution, with significantly higher levels in the inferior region (Fig. 1). There was a significant increase ($p < 0.05$) in PC-1 expression for the whole pars distalis during gestation, but there was no further increase in PC-1 mRNA levels at the time of labor compared with earlier in gestation (Fig. 3). Similarly, PC-1 mRNA levels in the pars intermedia did not change during the labor process (data not shown).

Prohormone Convertase-2

The PC-2 hybridization signal in the pars intermedia was significantly higher than that in the pars distalis at all gestational ages ($p < 0.05$) except during L (Figs. 1 and 4). Levels of PC-2 mRNA in the pars distalis during L were significantly ($p < 0.05$) higher than at 100–110 dGA (Fig. 4). There were no significant changes in amounts of PC-2 mRNA in the pars intermedia with increasing gestational age, or with the onset and progression of labor (Fig. 4).

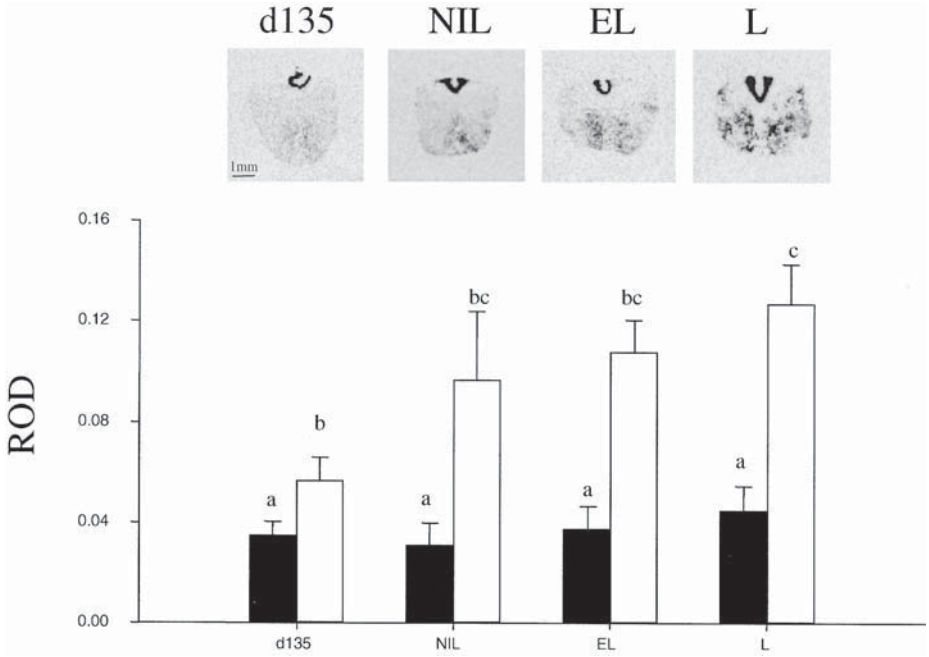

Fig. 2. Pituitary POMC mRNA levels in the ovine fetal pituitary gland from 135 dGA to L. **(Top)** Representative computerized autoradiograms of POMC expression as determined by ISH; **(bottom)** results from the computerized image analysis of the autoradiograms in the superior (j) and inferior (h) aspects of the pars distalis. Relative optical density (ROD) values are mean ± SEM. At all gestational ages, POMC mRNA expression is higher in the inferior than the superior aspect ($p < 0.05$). Values with different letters (a,b,c) are significantly different ($p < 0.05$).

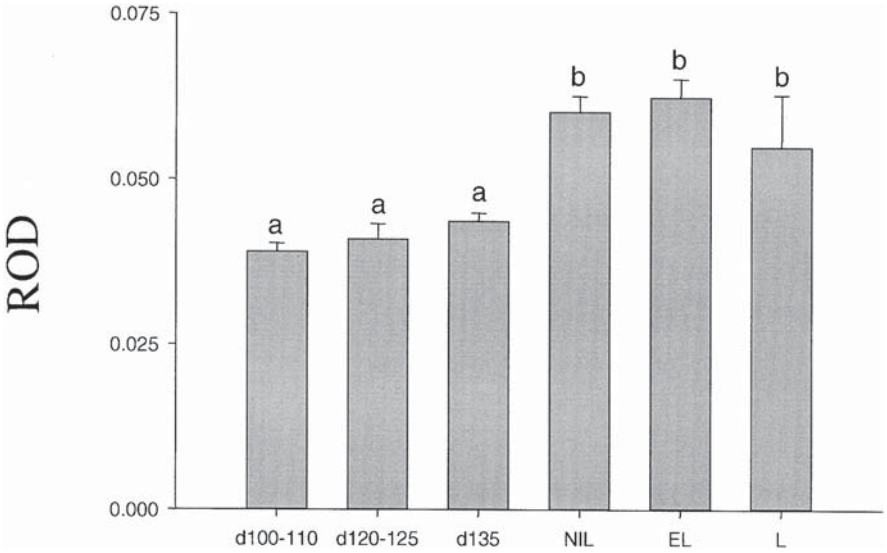

Fig. 3. Pituitary PC-1 mRNA expression in the ovine fetal pars distalis from 100 dGA to L as determined by ISH. Values are mean ± SEM. Values with different letters (a,b) are significantly different ($p < 0.05$).

GR mRNA

Pituitary GR mRNA expression was homogeneous in the whole pituitary at all ages studied (Fig. 5). Total pituitary GR mRNA expression increased between 135 dGA and term in NIL animals (Fig. 5). GR mRNA declined with the onset and progression of labor such that GR mRNA expression in the pars distalis of laboring animals was significantly decreased compared to the term NIL animals ($p < 0.05$), and was similar to the levels found earlier in gestation (135 dGA; $p > 0.05$).

Discussion

The present study clearly showed that there was an increase in POMC mRNA expression in the pars distalis of the fetal sheep at term, although pituitary POMC mRNA did not change further with the onset and progression of

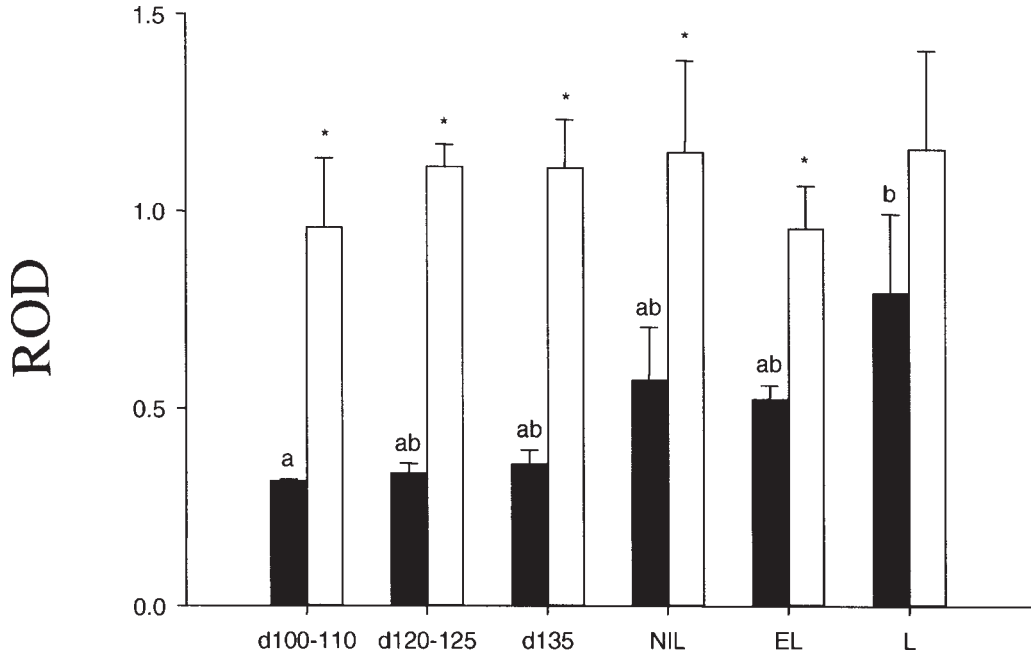

Fig. 4. Pituitary PC-2 mRNA expression in the ovine fetal pars distalis (j) and pars intermedia (h) from 100 dGA to L as determined by ISH. Values are mean ± SEM. Values with different letters (a,b) are significantly different ($p < 0.05$). PC-2 mRNA expression in the pars intermedia was significantly ($p < 0.05$) higher than in the pars distalis at all gestational ages (indicated by an asterisk except during L.

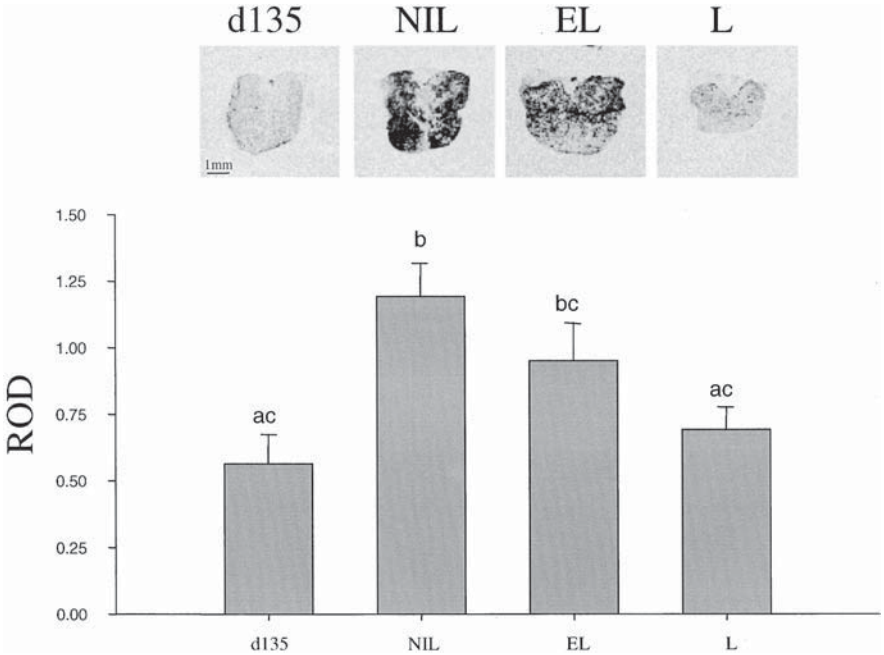

Fig. 5. Pituitary GR mRNA levels in the ovine fetal pituitary gland from 135 dGA to L. **(Top)** Representative computerized autoradiograms of GR expression as determined by ISH; **(bottom)** results from the computerized image analysis of the autoradiograms in the pars distalis. Values are mean ± SEM. Values with different letters (a,b,c) are significantly different ($p < 0.05$).

parturition. This gestational age-related increase in pars distalis POMC mRNA is consistent with previous observations by Yang et al. *(6)* and Matthews et al. *(7)*, but differs from those of McMillen et al. *(8)* and Merei et al. *(9)*, who used Northern blot analysis and reported a decline in POMC mRNA expression in the pars distalis in late gestation. Merei et al. *(9)* proposed that elevated circulating cortisol in late gestation acts to suppress POMC gene expression at

term. By contrast, the present study showed that POMC gene expression was not decreased either at term or during the labor process, despite continuously rising cortisol levels throughout the onset and progression of labor. However, pituitary POMC mRNA levels did not differ between the stages of labor, suggesting that the concurrent rise in fetal plasma ACTH during parturition may not be related simply to increased availability of its precursor.

We further hypothesized that elevated fetal plasma ACTH at term might reflect, in part, altered POMC processing as a result of changes in expression of PC-1 and PC-2 enzymes in the fetal pituitary. Saoud and Wood *(16)* reported an increase in pituitary content of $ACTH_{1–39}$ with increasing developmental age, and Carr et al. *(17)* reported an increase in the plasma concentrations of $ACTH_{1–39}$ approaching term such that the ratio of higher molecular weight POMC peptides to $ACTH_{1–39}$ was decreased. These changes in the output of POMC-derived peptides may be a reflection of changes in the activity of pituitary PCs, and would be consistent with the observed developmental change in the distribution of PC-1 mRNA in the pars distalis. We found that at term, PC-1 mRNA in the pars distalis was distributed in a heterogeneous pattern with higher levels in the inferior aspect of the pars distalis relative to the superior, similar to the distribution of POMC at term (140 dGA to parturition) *(7)*. Moreover, the gestational age–related increase in whole pituitary PC-1 mRNA in this study is consistent with an increase in PC-1 activity at term. However, these results differ from those of Bell et al. *(11)*, who reported a heterogeneous pattern of cells colocalizing POMC and PC-1 mRNA as early as 100 dGA. This discrepancy may be reflective of the difference between ISH techniques: Bell et al. *(11)* have reported PC-1 mRNA expression in POMC- and non-POMC-hybridizing cells, whereas we have reported steady-state PC-1 mRNA levels in the whole pars distalis.

Saphier et al. *(18)* have reported that in fetal sheep, plasma concentrations of the N-terminal POMC peptide, $N\text{-}POMC_{1–77}$, decline at term with a concomitant increase in γ^3MSH, suggesting increased formation of the latter. In vitro studies have shown that $N\text{-}POMC_{1–77}$ is cleaved by PC-2 to form γ^3MSH *(19)*, suggesting that the rise in γ^3MSH in fetal sheep plasma at term could reflect an increase in PC-2 activity, likely in the pars intermedia. However, we did not find gestational age–related changes in the steady-state levels of PC-2 mRNA in the pars intermedia. One possible explanation for the lack of an increase in PC-2 mRNA levels at the same time as an apparent increase in PC-2 activity might be the observation that a measure of PC-2 mRNA does not necessarily indicate its enzyme activity. Alternatively, this finding might be related to alterations in the levels of the neuroendocrine polypeptide 7B2. The polypeptide 7B2, identified in the rat and mouse pituitary gland *(20)*, has been implicated as a physiological inhibitor of PC-2 activity *(21)*. Therefore, PC-2 activity might be regulated during gestation via alterations in 7B2 levels, but this remains to be investigated.

Although the hybridization signal for PC-2 mRNA in the pars distalis was increased in the L group relative to earlier in gestation (100–110 dGA), Bell et al. *(11)* have reported that neither PC-2 mRNA nor protein co-localized with anterior lobe corticotrophs at any gestational age. Similarly, Day et al. *(22)* reported that rat anterior lobe corticotrophs express very little PC-2. Therefore, this change in steady-state PC-2 mRNA with L likely may not reflect changes in POMC processing in the pars distalis, which is associated with the progression of labor in the fetal sheep.

We further hypothesized that the late gestational rise in plasma ACTH could be associated with an attenuation of the negative feedback effects of cortisol on POMC synthesis, an effect mediated via alterations in the level of GR mRNA. This study clearly showed that although there is an increase in GR mRNA prior to parturition (term; NIL), consistent with earlier studies *(14)*, the abundance of GR mRNA decreased in the pituitary with the onset of labor (EL). Levels of GR mRNA were significantly lower during L. These data might suggest that during late pregnancy, prior to parturition, fetal pituitary GR mRNA is upregulated by circulating glucocorticoids, results that are consistent with earlier studies (unpublished observations; *[23]*). This relationship would appear to contrast with the classical feedback downregulatory mechanisms reported elsewhere *(23,24)*. However, during L, coincident with the terminal rise in fetal plasma cortisol, GR mRNA expression is decreased, suggesting that during L other hormones might act to downregulate GR mRNA in the presence of continued cortisol stimulation. One possible candidate is estradiol because it has been reported in rodents that pituitary GR mRNA is significantly decreased by treatment with estradiol *(25–27)*. There is a temporal relationship between the decline in fetal pituitary GR mRNA levels reported in the present study and the prepartum rise in maternal and fetal plasma estradiol concentrations associated with parturition *(28,29)*. This relationship needs to be investigated further.

In summary, the results from the present study suggest that the concomitant rise in plasma ACTH and cortisol during late gestation in the fetal sheep may be attributable, in part, to increased expression of the prohormone convertase enzyme PC-1, leading to increased POMC processing. Moreover, we suggest that the negative feedback effects of cortisol on pituitary POMC synthesis and/or ACTH release may be attenuated via a downregulation of pituitary GR, during L. However, further studies are required to evaluate the roles of estradiol and cortisol on the expression of POMC-processing enzymes and the regulation of pituitary GR.

Materials and Methods

Animals and Surgical Procedures

Pregnant mixed breed ewes of known gestational age were used in these studies. The experiments were performed according to protocols approved by the Animal Care Committee of the University of Toronto, in accordance with the guidelines of the Canadian Council for Animal Care. For ontogeny studies of PC-1 and PC-2 mRNA expression, fetuses were obtained at 100–110 dGA (n = 4), 120–127 dGA (n = 5), and 135 dGA (n = 5) from noninstrumented animals. To examine changes in pituitary hormone processing enzymes during the onset and progression of labor, tissues were collected from fetuses in three groups of pregnant sheep at three distinct times during the progression of labor. Vascular catheters were implanted into maternal and fetal sheep under general anesthesia between days 121 and 126 of gestation, and pregnancies were allowed to progress to term as previously described *(29)*. A catheter was inserted into the amniotic cavity to permit monitoring of intrauterine pressure and uterine electromyographic activity was monitored by stainless steel electrodes (Cooner, CA) sewn into the superficial layer of the myometrium. Fetal (2- to 3-mL) and maternal (5-mL) blood samples for hormone analysis were collected into heparinized tubes every other day until d 135 of gestation (term = 147 d), after which time samples were collected twice daily until the animals were sacrificed (*see* Tissue Collection). Blood samples were kept on ice until they were centrifuged at 1500*g* for 10 min at 4°C. Plasma samples were stored in aliquots at –20°C until analyzed.

Tissue Collection

Animals were euthanized with an overdose of Euthanyl (MTC Pharmaceuticals, Cambridge, Ontario, Canada), and the fetal pituitaries were rapidly dissected out, slow frozen on dry ice, and stored at –80°C for later ISH. The timing of tissue collection was determined by continuous monitoring of intrauterine pressure and electromyographic activity as previously reported by Gyomorey et al. *(29)*. Briefly, animals were grouped as term, not in labor (NIL) (n = 6; 140–145 dGA); early labor (EL) (n = 6, 143–149 dGA); and active labor (L) (n = 6; 145–149 dGA). Progression into labor was determined by the pattern of uterine electrical and mechanical activity during the last 6 h before animal sacrifice as previously described *(29)*, and was based on criteria published by Lye et al. *(30)*. For analysis of GR and POMC mRNA expression during the onset and progression of labor, pituitaries were analyzed from animals at 135 dGA, NIL, EL, and L. Pituitaries from animals at earlier gestational ages were not included, because the ontogeny of POMC *(7)* and GR *(14)* mRNA expression has previously been well characterized.

Plasma Hormone Analysis

Fetal plasma immunoreactive cortisol concentrations were determined as described by Norman et al. *(1)*. Fetal plasma ACTH levels were assessed using a commercial radioimmunoassay (Diasorin, Stillwater, MN) shown to be specific for $ACTH_{1–39}$ *(31)*.

In Situ *Hybridization*

Frozen fetal pituitary glands were cut in coronal sections (12 μm) on a cryostat (Jung CM 300; Leica, Nussloch, Germany), freeze-thaw mounted onto slides coated with poly-L-lysine (Sigma, St. Louis, MO) and air-dried. Slides were then postfixed in 4% paraformaldehyde (pH 7.4, 4°C, 5 min), rinsed twice in phosphate-buffered saline (pH 7.4, 1 min), dehydrated in an ascending ethanol series, and stored in 95% ethanol at 4°C until analysis by ISH.

The ISH technique used has been described in detail previously *(7,32)*. Briefly, 45-mer oligonucleotide probes complementary to bases 231–275 of porcine PC-1 *(33)*, bases 153–197 of porcine PC-2 *(34)*, bases 269–313 of ovine GR characterized by Matthews et al. *(14)*, and bases 504–549 of ovine POMC characterized by Broad et al. *(35)* were labeled using terminal deoxynucleotidyl transferase (Pharmacia Biotech, Baie d'Urfe, PQ) and [α-^{35}S]dATP (NEN Dupont Canada, Mississauga, Ontario). The sections were hybridized overnight in a moist chamber (42°C) with the radiolabeled probes. After hybridization, the sections were washed and exposed to autoradiographic film (Biomax; Kodak, Rochester NY). The autoradiographic films were developed using standard methods. Linearity was established by simultaneous exposure of the film to ^{14}C standards, and a control 45-mer nonsensical sequence oligonucleotide probe was included to assess nonspecific binding. The autoradiograms were then analyzed using computerized image analysis software (Imaging Research, St. Catherines, Ontario). When appropriate, the superior (region around the pars intermedia) and inferior (region at the base of the pars distalis) aspects of the pars distalis, as previously described *(5)*, were analyzed separately. The relative optical density (OD) of pituitary POMC, PC-1, PC-2, and GR mRNA levels was assessed using a minimum of 12 sections for each animal.

Statistical Analysis

Plasma hormone data were subjected to one-way analysis of variance (ANOVA). When significance was indicated ($\alpha = 0.05$), data were analyzed using post-hoc pairwise comparisons (Tukey's). Similarly, pituitary mRNA levels reported as relative OD were subjected to an ANOVA

followed by Tukey's HSD analysis when appropriate. Results are expressed as the mean ± SEM.

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