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Effects of Cortisol and Estradiol on Pituitary Expression of Proopiomelanocortin, Prohormone Convertase-1, Prohormone Convertase-2, and Glucocorticoid Receptor mRNA in Fetal Sheep

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We hypothesized that in the late-gestation sheep fetus there is an interaction between the prepartum rise in cortisol and the increase in placental estradiol production that allows expression of key components of the fetal hypothalamic-pituitary-adrenal (HPA) axis. Therefore, the goal of this study was to investigate the effects of cortisol on the fetal HPA axis in the presence and absence of increased placental estradiol production. We obtained fetal plasma samples and pituitary tissue from animals that had received an infusion of either cortisol, cortisol and 4-hydroxyandrostenedione (4OHA, an aromatase inhibitor), saline, or saline + 4OHA controls. Cortisol significantly decreased plasma adrenocorticotropic hormone concentrations, and in the presence of 4OHA reduced pituitary proopiomelanocortin (POMC) mRNA levels in the pars distalis. There was no effect of any treatment on the expression of the key POMC processing enzymes, prohormone convertase-1 or -2 in the fetal pituitary. Conversely, levels of glucocorticoid receptor (GR) mRNA in the pituitary were increased with cortisol treatment in the absence of increased estradiol. We suggest that in the late-gestation sheep fetus, cortisol and estradiol have opposite effects on pituitary POMC and GR mRNA expression, and interact to regulate these key components of the fetal HPA axis.

Key Words: Proopiomelanocortin; prohormone convertase-1; glucocorticoid receptor; adrenocorticotropic hormone; cortisol; estradiol.

Introduction

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 the onset of parturition. In sheep, as in other species, maturation and activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis provides the stimulus for the initiation of parturition *(2)*, and there have been a number of studies designed to investigate the development and regulation of the fetal HPA during late gestation. We reported recently that pituitary proopiomelanocortin (POMC) mRNA levels increased during late gestation in fetal sheep, but there was no further change with the onset or progression of labor *(3)*. By contrast, levels of glucocorticoid receptor (GR) mRNA in the fetal pituitary increased to term but then decreased with active labor *(3)*. These results suggested that the negative feedback effects of cortisol on pituitary POMC mRNA and ACTH output during active parturition might be attenuated. The mechanisms accounting for altered HPA activity in the late-gestation ovine fetus are currently unknown. However, there is an alteration in placental steroid hormone metabolism associated with parturition in sheep that causes a shift from progesterone to estrogen production *(4)*, and it is mediated via the induction of the placental cytochrome P450 17α-hydroxylase ($P450_{c17}$) enzyme. Increased expression of the $P450_{c17}$ enzyme is now thought to occur as a result of the increased production of prostaglandin E_2, effected through the induction of the prostaglandin H synthase type II enzyme in trophoblast cells by fetal cortisol *(5,6)*. The ovine placenta expresses P450 aromatase, and expression of $P450_{c17}$ in the same tissue leads to increased placental estrogen production *(7)*. It has been suggested that estradiol may promote increased fetal HPA activity and maturation *(3,8,9)*. The purpose of the present study was, therefore, to ascertain whether cortisol affects fetal HPA regulation in both the presence and absence of increased output of estradiol. Thus, we determined levels of mRNA encoding POMC, the POMC processing enzymes, prohormone convertase-1 (PC-1) and PC-2, and GR in fetuses treated with cortisol in the presence or absence of increased synthesis of estradiol.

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Results

Fetal Plasma Cortisol, ACTH, and Estradiol

After 80 h of intrafetal cortisol administration, fetal plasma cortisol concentrations were significantly elevated with

Table 1
Terminal Fetal Plasma ACTH, Cortisol, and Estradiol Concentrations[a]

	ACTH (pg/mL)	Cortisol (ng/mL)	Estradiol (pg/mL)[b]
Saline	57.1 ± 10.26[†]	7.2 ± 2.64	37 ± 14
Cortisol	18.1 ± 6.01[‡]	70.6 ± 20.34*	259 ± 33*
Saline + 4OHA	30.2 ± 9.21[†,‡]	6.1 ± 1.83	38 ± 11
Cortisol + 4OHA	21.7 ± 4.14[‡]	56.7 ± 11.06*	94 ± 11*

[a]Data are shown as the mean ± SEM. For plasma ACTH concentrations values with different superscripts are significantly different (two-way ANOVA followed by Tukey's pairwise comparison). For plasma cortisol and estradiol concentrations, values with an asterisk are significantly ($p < 0.05$) different from their respective control.

[b]These results were previously published in ref. *6*.

respect to the control groups (Table 1). The fetal plasma cortisol concentrations achieved with intrafetal infusion were comparable with cortisol concentrations observed in fetal sheep during active labor *(3)*. As previously reported *(6)*, treatment with cortisol for 80 h significantly increased fetal plasma estradiol ($p < 0.05$); this effect was attenuated in the presence of 4-hydroxyandrostenedione (4OHA) (Table 1). The rise in plasma cortisol concentration was unaltered by changes in fetal plasma estradiol; there was no significant difference in the plasma cortisol concentrations between the cortisol and cortisol + 4OHA groups. Based on two-way analysis of variance (ANOVA), there was a significant ($p < 0.05$) effect of cortisol to decrease fetal plasma ACTH. There was also a significant interaction effect ($p = 0.05$) between the two treatments (cortisol and 4OHA). When the plasma ACTH concentrations were compared (Tukey's pairwise comparison; $\alpha = 0.05$), cortisol and cortisol + 4OHA significantly decreased fetal plasma ACTH with respect to the saline treatment group.

Pituitary POMC mRNA

There was a significant overall effect of cortisol to decrease POMC mRNA expression in the inferior aspect of the pars distalis (two-way ANOVA; $p < 0.05$) (Fig. 1A). Cortisol and cortisol + 4OHA treatment decreased pituitary POMC mRNA levels in the inferior aspect of the pars distalis by 56 and 83%, respectively, relative to their respective control groups. However, when the levels of POMC mRNA expression in the cortisol and cortisol + 4OHA groups were compared (student's *t*-test; $\alpha = 0.05$) with their respective control groups (saline and saline + 4OHA), this effect of cortisol was significant ($p < 0.05$) in the absence (cortisol + 4OHA) but not in the presence (cortisol) of increased placental estradiol production ($p = 0.06$). Similarly, in the superior aspect of the pars distalis, cortisol and cortisol + 4OHA decreased POMC mRNA levels by 41 and 72%, respectively, and based on two-way ANOVA there was a significant main effect of cortisol treatment ($p < 0.05$). However, when the cortisol and cortisol + 4OHA groups were compared with their respective control groups (student's *t*-test; $\alpha = 0.05$) there was no significant effect of cortisol in the presence (cortisol vs saline; $p = 0.29$) or absence (cortisol + 4OHA vs saline + 4OHA; $p = 0.07$) of increased placental estradiol production. There was no change in POMC mRNA expression in the pars intermedia (Fig. 1B).

PC-1 and PC-2 mRNA

There was no effect of either cortisol or treatment with 4OHA on PC-1 mRNA expression in either the pars distalis or the pars intermedia (Table 2). Similarly, PC-2 mRNA levels were not altered in either the pars distalis or pars intermedia with any treatment (Table 3).

Pituitary GR mRNA

Pituitary GR mRNA expression in the pars distalis was increased significantly by cortisol infusion (two-way ANOVA; $p < 0.05$) (Fig. 2). However, when the GR mRNA expression in the cortisol and cortisol + 4OHA groups was compared (student's *t*-test; $\alpha = 0.05$) with their respective control groups (saline and saline + 4OHA), this effect of cortisol was only significant ($p < 0.05$) in the absence of increased placental estradiol (cortisol + 4OHA).

Discussion

Maturation and activation of the fetal HPA axis and a subsequent rise in circulating fetal plasma cortisol provide the stimulus for parturition in sheep. Elevated fetal glucocorticoids, acting via PGHS-II, are thought to increase expression and activity of the $P450_{c17}$ in the placenta *(5–7,10)*, resulting in a shift in the pattern of placental steroid hormone metabolism that allows pregnenolone to be used for estrogen production *(10)*. It has been proposed that estradiol may promote increased HPA activity in the fetal sheep, suggesting that HPA activity in response to endogenous or exogenous glucocorticoid administration might reflect the effects of both glucocorticoids and estradiol on fetal HPA development *(3,8,9)*. We developed a model wherein the effects of cortisol on fetal HPA function could be examined in both the presence and absence of an inhibitor of placental aromatase that resulted in an attenuation of the cortisol-induced rise in placental estradiol output. We found that there was an interaction between cortisol and 4OHA on levels of mRNA encoding POMC and GR in the fetal pituitary, although not on mRNA encoding PC-1 or PC-2, which one could attribute to differences in fetal estradiol concentrations.

Studies in fetal sheep have suggested that estradiol treatment increased fetal HPA activity, as indicated by a rise in basal fetal plasma cortisol and ACTH concentrations *(8,9)*. This rise in fetal plasma cortisol with exogenous infusion was not different in the presence or absence of increased

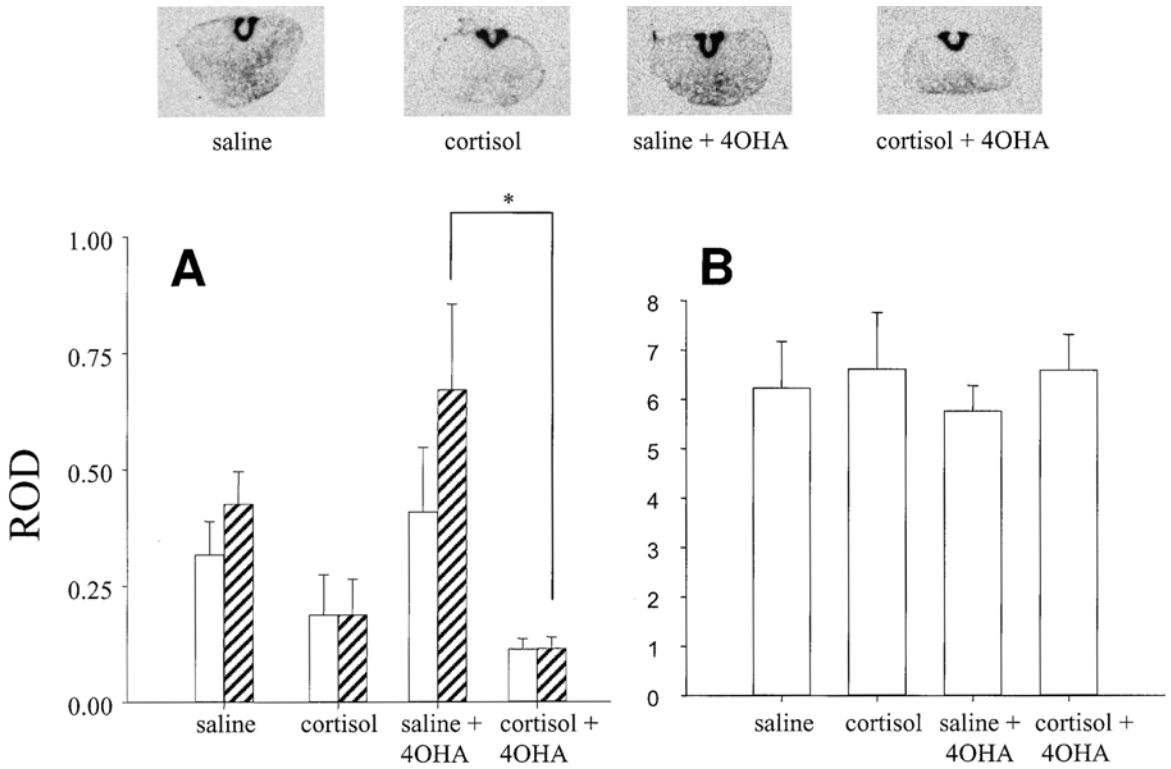

Fig. 1. Pituitary POMC mRNA expression in the ovine fetal pituitary gland. (**Top**) Representative computerized autoradiograms of POMC expression as determined by in situ hybridization (ISH); (**Bottom**) results from the computerized image analysis of the autoradiograms in the (**A**) superior (□) and inferior (▨) aspects of the pars distalis, and (**B**) pars intermedia. Values are the mean relative optical density (ROD) ± SEM. *$p < 0.05$.

Table 2
Pituitary PC-1 mRNA Expression Analyzed by ISH[a]

	Pars distalis (inferior aspect)	Pars distalis (superior aspect)	Pars intermedia
Saline	6.7 ± 0.28	6.4 ± 0.20	8.7 ± 0.98
Cortisol	7.0 ± 0.28	6.4 ± 0.37	11.5 ± 2.42
Saline + 4OHA	7.0 ± 0.19	5.9 ± 0.16	9.3 ± 0.99
Cortisol + 4OHA	6.7 ± 0.35	6.0 ± 0.10	10.1 ± 0.84

[a]Data are shown as the mean ROD ± SEM ($\times 10^{-2}$).

Table 3
Pituitary PC-2 mRNA Expression Analyzed by ISH[a]

	Pars distalis	Pars intermedia
Saline	5.6 ± 0.46	25 ± 6.2
Cortisol	5.2 ± 0.34	24 ± 4.4
Saline + 4OHA	5.0 ± 0.03	28 ± 5.1
Cortisol + 4OHA	5.8 ± 0.25	30 ± 4.8

[a]Data are shown as the mean ROD ± SEM ($\times 10^{-2}$).

fetal plasma estradiol, suggesting that the rise in estrogen does not appreciably alter the metabolism of exogenous cortisol, even though it does increase levels of hepatic CBG and 11β-hydroxysteroid dehydrogenase type 1 *(11)*.

The rise in fetal plasma cortisol inhibited fetal plasma ACTH, consistent with previous observations *(12–14)*, and decreased expression of POMC mRNA in the inferior aspect of the pars distalis. However, the decline in POMC mRNA during cortisol infusion was only statistically significant in the absence of increased plasma estradiol (cortisol + 4OHA). These data suggest that estradiol and cortisol might have opposite effects on POMC mRNA expression in the fetal pars distalis such that the cortisol inhibition of POMC mRNA levels is attenuated by estrogen, leading to a sustained or an increased availability of the ACTH precursor.

The role of estradiol in the regulation of fetal plasma ACTH has not been resolved. Previously, Wang et al. *(11)* reported no significant change in the mean concentration of ir-ACTH in the plasma of sheep fetuses during estradiol infusion, whereas a stimulatory effect of estradiol on basal plasma ACTH has been reported *(8,9)*. In the present study, there did not appear to be any influence of increased placental estradiol production on the cortisol inhibition of plasma ACTH concentrations. However, from the present study it is difficult to interpret the role of estradiol on the regulation of fetal plasma ACTH, because there was a significant interaction effect between the two treatments (cortisol and 4OHA). Although the data from this study do not provide strong evidence of a stimulatory effect of estradiol on fetal plasma ACTH concentrations, the presence of in-

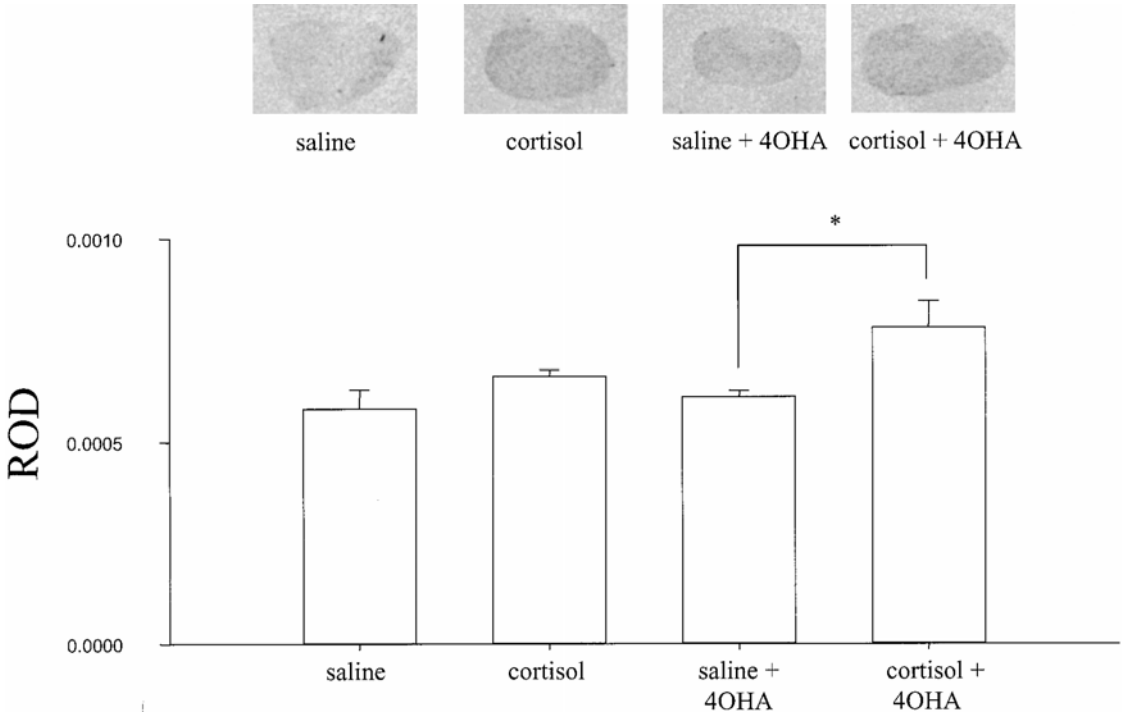

Fig. 2. Pituitary GR mRNA expression in the ovine fetal pituitary gland. (**Top**) Representative computerized autoradiograms of GR expression as determined by ISH; (**Bottom**) results from the computerized image analysis of the autoradiograms in the pituitary. Values are mean ROD ± SEM. $*p < 0.05$.

creased fetal plasma estradiol appears to attenuate the inhibitory effect of cortisol on the synthesis of the ACTH precursor, POMC.

Therefore, we hypothesized that altered processing of POMC by PC-1 and/or PC-2 could account, in part, for the discrepancy between estradiol effects on POMC synthesis and plasma ACTH concentrations. 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 *(15)*. 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 ($ACTH_{18–39}$) *(15)*. In the fetal sheep pituitary, PC-1 and PC-2 have been identified in both the pars distalis and the pars intermedia *(3,16)*, and there is a gestational age–related increase in fetal pituitary PC-1 mRNA at term *(3)* that is temporally associated with the rise in both fetal plasma cor-tisol and estradiol concentrations *(1,17)*. However, cortisol infusion did not alter PC-1 or PC-2 mRNA expression in either the presence or absence of increased plasma estradiol. Thus, the apparent differential effects of estradiol on HPA activity are unlikely to be owing to alterations in POMC processing. However, the absence of changes in PC-1 and PC-2 mRNA does not preclude cortisol or estradiol effects on either PC protein expression or enzyme activity.

We further hypothesized that the presence of increased fetal plasma estradiol might alter the negative feedback effects of cortisol on POMC synthesis, via changes in the expression of pituitary GR. In rodents, pituitary GR mRNA is significantly decreased by treatment with estradiol *(18–20)*. In sheep, we reported previously that there is a temporal relationship between decreased fetal pituitary GR mRNA and increased fetal plasma estradiol at the time of advanced labor *(3)*. The present study established a main effect of cortisol to increase pituitary GR mRNA significantly, consistent with a prepartum rise in pituitary GR mRNA in fetal sheep as endogenous cortisol rises *(3)*, and with earlier studies in rats *(21)*. Prolonged exposure to cortisol appears necessary since shorter times of administration did not affect GR mRNA expression in the fetal pituitary *(22)*. The increase in GR mRNA after cortisol treatment was only significant in the presence of 4OHA. These data suggest that estradiol may downregulate pituitary GR mRNA expression in the fetal sheep, a result consistent with the later decline in GR mRNA in the sheep fetal pituitary during active labor when endogenous estrogen is highest *(3)*, and with previous studies in the rat *(18,19)*. Moreover, ongoing studies in our laboratory suggest that estradiol infusion to the late-gestation ovine fetus significantly decreases pituitary GR mRNA (unpublished observations).

We propose that the cortisol-induced decrease in fetal plasma ACTH concentrations is attributable, in part, to negative feedback effects of cortisol at the pituitary, mediated

via the increase in pituitary GR mRNA expression. However, as plasma estrogen rises, further increases in pituitary GR mRNA expression are restrained, with subsequent diminution of glucocorticoid-mediated negative feedback on POMC expression. Therefore, cortisol and estradiol appear to interact to regulate pituitary POMC and GR expression in the ovine fetus at term; however, we cannot exclude the possibility that cortisol and estradiol interact at the hypothalamus to regulate the expression of corticotropin-releasing hormone or GR mRNA *(20,23,24)*.

Materials and Methods

Animal Preparation

Singleton fetuses of pregnant mixed breed ewes of known gestational age were used. The studies 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. Venous and arterial catheters were implanted into mothers and fetuses under general anesthetic, as previously described *(25)*, between 120 and 123 d of gestation. In addition, stainless steel electrodes (Cooner, CA) were sewn into the superficial layer of the myometrium to monitor uterine electromyographic activity.

After 5 d of postoperative recovery (125–128 d of gestation), animals were given an infusion of either saline (3 mL/h; $n = 10$) or cortisol (1.35 mg/h in the same volume of infusate; $n = 10$) (Steraloids, Wilton, NH). Following 24 h of infusion with either saline or cortisol, each group of animals was further subdivided to receive either Lentaron™, an inhibitor of the P450 aromatase enzyme (4OHA) (1.44 mg/h × 3 mL/h; $n = 5$) (CIBA-Geigy, Basel, Switzerland), or vehicle, thereby creating four treatment groups: saline, saline + 4OHA, cortisol, and cortisol + 4OHA. Administration of 4OHA has been shown previously to inhibit the cortisol-induced rise in fetal and maternal plasma estradiol concentrations *(6)*. Fetal (3 mL) and maternal (5 mL) arterial blood samples were collected at 12-h intervals beginning 24 h prior to the start of the infusion protocol and continued throughout the infusion period. A final pair of blood samples was taken immediately prior to sacrifice. Blood samples were collected in heparinized syringes and kept on ice until they were centrifuged at 1500*g* for 10 min at 4°C. Plasma samples were stored at −20°C until analyzed. Following 80 h of infusion, the animals were euthanized with an overdose of Euthanyl™ (MTC Pharmaceuticals, Cambridge, Ontario, Canada). The fetal pituitary was rapidly dissected out, slow frozen on dry ice, and stored at −80°C for later ISH.

Plasma Hormone Analysis

Fetal plasma immunoreactive cortisol concentrations were determined as described by Norman et al. *(1)*. Fetal plasma ACTH concentrations were assessed using a commercial radioimmunoassay (Diasorin, Stillwater, MN) shown to be specific for $ACTH_{1\text{-}39}$ *(26)*. Fetal plasma estradiol concentrations were determined using a commercially available radioimmunoassay (ImmuChem™ Double Antibody 17βEstradiol ^{125}I RIA kit; ICN, Costa Mesa, CA), as previously described *(6)*. Samples were measured in a single assay. Intraassay coefficients of variation were 3–7%.

In Situ Hybridization

Frozen fetal pituitary glands were cut into coronal sections (12 μm) on a cryostat (Jung CM 300; Leica, Nussloch, Germany), freeze-thaw mounted onto Fisher Superfrost glass slides (Fisher, Nepean, Ontario, Canada), 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 previously in detail *(27,28)*. Briefly, 45-mer oligonucleotide probes complementary to bases 504–549 of ovine POMC, characterized by Broad et al. *(29)*; bases 231–275 of porcine PC1 *(30)*; bases 153–197 of porcine PC2 *(31)*, and bases 269–313 of ovine GR, characterized by Matthews et al. *(22)*, were labeled using terminal deoxynucleotidyl transferase (Pharmacia Biotech, Baie d'Urfe, Canada) and [α-^{35}S] dATP (NEN Dupont Canada, Mississauga, Canada). 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 slides were exposed to autoradiographic film for 6 h to determine the hybridization signal for POMC mRNA levels in the pars intermedia and then reexposed for 4 d to measure the levels of POMC mRNA in the pars distalis. The two exposure times were necessary for the hybridization signal to be within the linear range of the film for the two regions of the pituitary. 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 hybridization. The autoradiograms were then analyzed using computerized image analysis software (Imaging Research, St. Catherines, Ontario, Canada). The ROD of pituitary POMC, PC-1, PC-2, and GR mRNA levels were assessed using a minimum of 12 sections for each animal.

Statistical Analyses

Plasma hormone data were analyzed using two-way ANOVA. When significance was indicated, data were compared in a pairwise manner using appropriate post-hoc analysis (student's *t*-test or Tukey's pairwise comparison; $\alpha = 0.05$). Similarly, pituitary mRNA levels, reported as ROD, were subjected to an ANOVA followed by *t*-test when appropriate. Results are expressed as the mean ± SEM.

Acknowledgments

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