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CRH and AVP-Induced Changes in Synthesis and Release of ACTH from the Ovine Fetal Pituitary In Vitro: Negative Influences of Cortisol

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During late gestation in sheep, fetal plasma adrenocorticotrophin (ACTH) and cortisol levels increase, and these are associated with increased pro-opiomelanocortin (POMC) mRNA levels in the anterior pituitary. Corticotrophin-releasing hormone (CRH) and vasopressin (AVP) are the primary hypophysiotrophic factors regulating ACTH secretion from the fetal sheep pituitary corticotroph, but previous reports with term fetal tissue have failed to show effects on levels of POMC mRNA. The objectives of the present study were to establish the effects of CRH and AVP on both synthesis and secretion of ACTH before term, and to determine how cortisol affects these responses. Fetal pituitaries were removed at d 138 of gestation (term ~ d 147), the anterior pituitary was separated, and the cells dispersed and placed in monolayer tissue culture. After 4 d, cells were treated for 18 h with several different concentrations (10^{-6}–10^{-9} *M*) and combinations of CRH, AVP, and cortisol. Following incubation, the medium was removed for ACTH analysis, and the cells fixed for POMC mRNA measurement and immunoreactive (ir)-ACTH localization. Separately, CRH and AVP significantly ($p < 0.05$) stimulated ACTH secretion in a dose-dependent manner. Simul-taneous treatment of maximally stimulating levels of CRH and AVP augmented ($p < 0.05$) the output of ACTH. Cortisol did not affect basal (nonstimulated) ACTH output, but attenuated the neuropeptide-induced increases in ACTH secretion. This effect of cortisol was more pronounced in cells treated with CRH than in cells treated with AVP. POMC mRNA levels were increased by both CRH and AVP treatments in a dose-dependent manner, though there was no further increase in POMC mRNA when CRH and AVP were added together. Cortisol attenuated ($p < 0.05$) the neuropeptide-induced increases in POMC mRNA, though AVP-stimulated POMC mRNA levels were significantly higher than in cells treated with cortisol alone. Cortisol failed to alter non-stimulated POMC mRNA levels. We conclude that in late gestation:

1) **Fetal pituitary corticotrophs respond to CRH and AVP by increasing POMC mRNA levels and ACTH secretion**
2) **AVP is more potent than CRH at the level of ACTH secretion, but not POMC transcription**
3) **Cortisol attenuates the synthetic and secretory responses to CRH and AVP, but has little effect in the non-stimulated state**

Key Words: Fetus; pituitary; adrenocorticotrophin; pro-opiomelanocortin; vasopressin; corticotrophin-releasing hormone.

Introduction

Development of the fetal hypothalamic–pituitary–adrenal (HPA) axis is essential for fetal organ maturation and parturition in several species. It is also central to fetal adaptation to homeostatic challenge, such as hypoxic stress. In fetal sheep during late gestation, there is a simultaneous rise in circulating adrenocorticotrophin (ACTH) and cortisol concentrations *(1)*. These changes have been associated with increased expression of the ACTH precursor, pro-opiomelanocortin (POMC), in the anterior pituitary *(2–4)*, which, in turn, has been correlated with increases in corticotrophin-releasing hormone (CRH) mRNA *(5)* and immunoreactive (ir)-CRH in the hypothalamic paraventricular nucleus (PVN) *(6)*.

Vasopressin (AVP) and CRH have been shown to be the primary factors stimulating ACTH release from the anterior pituitary corticotroph of the fetal sheep in vivo *(7–10)* and in vitro *(11–14)*, whereas cortisol inhibited

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ACTH secretion *(12,15,16)*. In this connection, AVP was a more potent stimulator of ACTH secretion than CRH in adult *(17,18)* and fetal *(15)* sheep. Recent studies using dispersed cells from the fetal sheep anterior pituitary have demonstrated that CRH increases POMC mRNA levels at d120 *(19)*, but is without effect at d 142–144 of gestation *(14)* as in the adult sheep *(20)*. However, there is little information concerning the direct interactions of CRH, AVP, and cortisol on fetal pituitary corticotroph function at d 135–140, a time at which fetal pituitary growth is relatively static, and when there is a significant redistribution and upregulation of POMC mRNA levels within the anterior lobe *(4)*. Therefore, in the present study, we examined the hypothesis that during late gestation (d 135–140), when corticotroph POMC mRNA levels are increasing, CRH and AVP act to stimulate both pituitary ACTH output and POMC gene expression. Given that POMC mRNA levels and plasma ACTH levels increase at this time despite rising plasma cortisol concentrations, we further hypothesized a diminution in the negative feedback effects of cortisol on CRH- and AVP-induced ACTH synthesis and secretion.

Results

Effects of CRH and AVP on ACTH Concentrations

The mean basal output of (ir)-ACTH after 18 h of culture, in the absence of added hormones, was 2.88 ± 0.37 ng/mL (mean ± SEM). Both CRH and AVP caused dose-dependent increases in ACTH output (n = 6, Fig. 1A,B). Equimolar concentrations of AVP were more effective than CRH at inducing ACTH output, and this difference was significant ($p < 0.05$) at (10^{-7} *M*) (Fig. 1). When pituitary cells were treated with increasing doses of AVP (10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} *M*) in the presence of CRH (10^{-6} *M*), ACTH output was significantly greater than when treated with AVP (10^{-7} *M*, 10^{-8} *M*) alone (Fig. 1B). When augmentation occurred, the effects were additive rather than synergistic. A similar pattern was observed when cells were treated with CRH (10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} *M*) in the presence of AVP (10^{-6} *M*) (Fig. 1A). However, in this latter group, AVP significantly augmented the ACTH response to CRH in the highest concentration group only (CRH 10^{-6} *M*). In addition, the ACTH responses to simultaneous treatment with low doses of CRH (10^{-8} and 10^{-9} *M*) and AVP (10^{-6} *M*), were not different from that for AVP (10^{-6} *M*) alone.

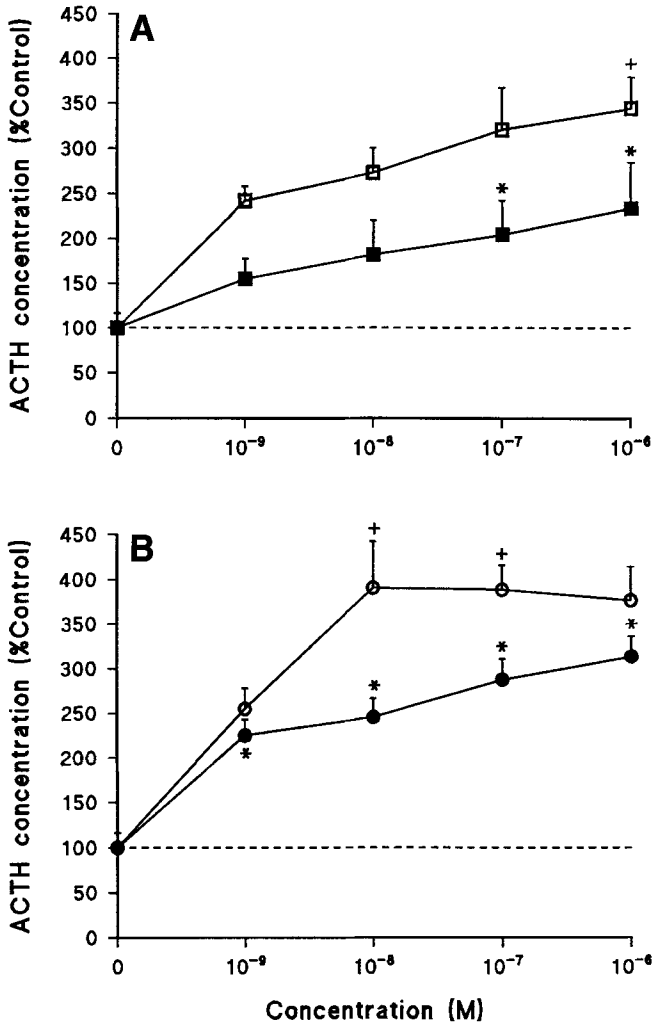

Fig. 1. (**A**) Concentrations (mean ± SEM) of ACTH in the culture media expressed as a percentage of control (n = 6) following 18 h of treatment with 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} *M* CRH (solid symbol) or simultaneous treatment with CRH (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} *M*) and AVP (10^{-6} *M*, open symbol). *Indicates significant differences ($p < 0.05$) from control; + indicates significant differences ($p < 0.05$) between CRH and CRH + AVP (10^{-6} *M*). Simultaneous treatment with CRH and AVP resulted in significantly elevated ACTH output compared to control, at all concentrations tested. (**B**) Concentrations (mean ± SEM) of ACTH in the culture media expressed as a percentage of control (n = 6) following 18 h treatment with 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} *M* AVP (solid symbol or simultaneous treatment with AVP (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} *M*) and CRH (10^{-6} *M*, open symbol). *Indicates significant differences ($p < 0.05$) from control; + indicates significant differences ($p < 0.05$) between AVP and AVP + CRH (10^{-6} *M*). Simultaneous treatment with AVP and CRH resulted in significantly elevated ACTH output compared to control, at all concentrations tested.

Effects of Cortisol on Basal and Stimulated ACTH Concentrations

Cortisol had no significant effect on ACTH output from nonstimulated fetal pituitary cells in culture (n = 6; Fig. 2A). Cortisol (10^{-7} *M*) significantly attenuated both CRH (10^{-6}, 10^{-7}, 10^{-8} *M*) and AVP (10^{-6} *M*) induced increases in ACTH output (n = 6; Fig 2B,C). The inhibition by cortisol was considerably more pronounced on CRH- than on AVP-stimulated ACTH output (Fig 2B,C). There was no effect of any of the treatments on the percentage of (ir)-ACTH-containing cells within the cultures (n = 6; control, 10.0 ± 1.9% of total; CRH (10^{-6} *M*), 11.9 ± 1.9% total; AVP (10^{-6} *M*), 11.37 ± 1.8% total, cortisol (10^{-6} *M*), 10.3 ± 0.92% control, mean ± SEM).

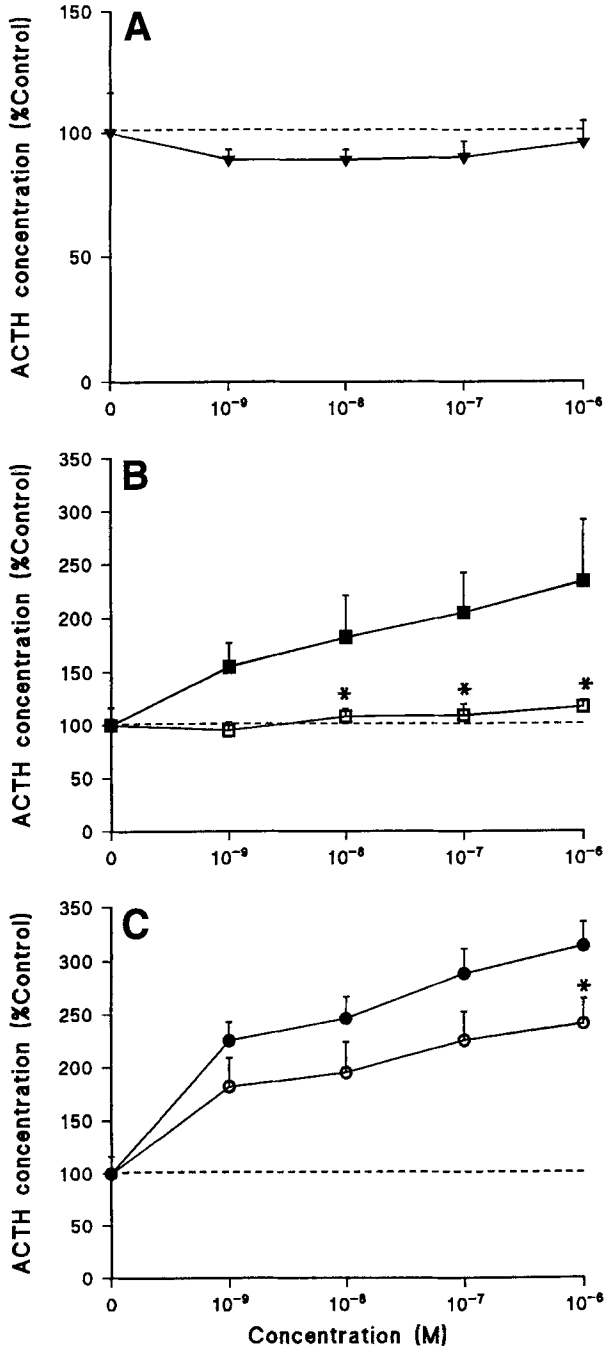

Fig. 2. **(A)** Concentrations (mean ± SEM) of ACTH in the culture media expressed as a percentage of control ($n = 6$) following 18 h of treatment with 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} *M* cortisol. There was no significant effect of cortisol alone on ACTH concentrations. **(B)** Concentrations (mean ± SEM) of ACTH in the culture media expressed as a percentage of control ($n = 6$) following 18 h treatment with 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} *M* CRH (solid symbol) or simultaneous treatment with CRH (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} *M*) and cortisol (10^{-7} *M*) (open symbol). Cortisol significantly (*$p < 0.05$) attenuated the CRH-induced increases in ACTH concentrations. **(C)** Concentrations (mean ± SEM) of ACTH in the culture media expressed as a percentage of control ($n = 6$) following 18 h of treatment with 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} *M* AVP (solid symbol) or simultaneous treatment with AVP (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} *M*) and cortisol (10^{-7} *M*) (open symbol). Cortisol attenuated the AVP-induced increases in ACTH concentrations. This was significant (*$p < 0.05$) at 10^{-6} *M* concentrations of AVP.

Effects of CRH, AVP, and Cortisol on POMC mRNA Levels

Effects of CRH and AVP (10^{-6} and 10^{-7} *M*) on levels of POMC mRNA were measured. Both peptides induced significant dose-dependent increases in cellular POMC mRNA compared to values in nontreated cells ($n = 6$; Fig. 3). CRH and AVP were equipotent with respect to upregulation of POMC mRNA levels. When CRH (10^{-6} *M*) and AVP (10^{-6} *M*) were administered simultaneously, there was no augmentation of the response on POMC mRNA (Fig. 3), in contrast to the effect on ACTH output (Fig. 1). Cortisol failed to affect nonstimulated levels of POMC mRNA, but significantly inhibited both CRH- and AVP-induced increases in POMC mRNA levels in the pituitary cells (Fig. 3). Cortisol had similar inhibitory effects on CRH- and AVP-induced increases in POMC mRNA levels, though attenuation appeared more complete in the CRH-treated cells. Further, when compared to the cortisol-only-treated cells, AVP (10^{-6} *M*) significantly stimulated POMC mRNA levels in the presence of cortisol, but CRH failed to induce a significant increase in POMC mRNA.

Discussion

This study has demonstrated for the first time that fetal pituitary corticotrophs, removed at d 138 of gestation, respond to CRH and AVP by upregulating POMC mRNA levels as well as ACTH output. This coincides with a period of high activity in the fetal anterior pituitary gland *(2–4)*. In addition, we have shown that CRH-induced ACTH output is more susceptible to direct pituitary glucocorticoid feedback than AVP-induced output, at this time. Cortisol also inhibited CRH and AVP-induced increases in POMC mRNA levels. These data clearly demonstrate that CRH and AVP drive both corticotroph synthesis and secretion in late gestation. Furthermore, glucocorticoid negative feedback on secretagog-induced corticotroph activity is maintained and may indicate that increased pituitary-adrenocortical activity in late gestation results from enhanced hypothalamic drive to the pituitary at this time.

At d 138 of gestation, we observed dose-dependent increases in ACTH output in response to both CRH and AVP treatment. AVP was more potent than CRH in this respect. Further, simultaneous administration of CRH and AVP resulted in a response that was significantly greater than when either neuropeptide was administered independently, and this was additive in nature. Augmentation of ACTH output by simultaneous administration of CRH and AVP has been demonstrated previously both in vivo and in vitro in near-term fetal sheep *(9–12)*. Interestingly, however, in the present study, the lower does of CRH (10^{-8} and 10^{-9} *M*) when administered simultaneously to AVP (10^{-6} *M*) did not result in an increase in ACTH output that was significantly different from AVP (10^{-6} *M*) alone. These data would suggest that low concentrations of CRH have little effect

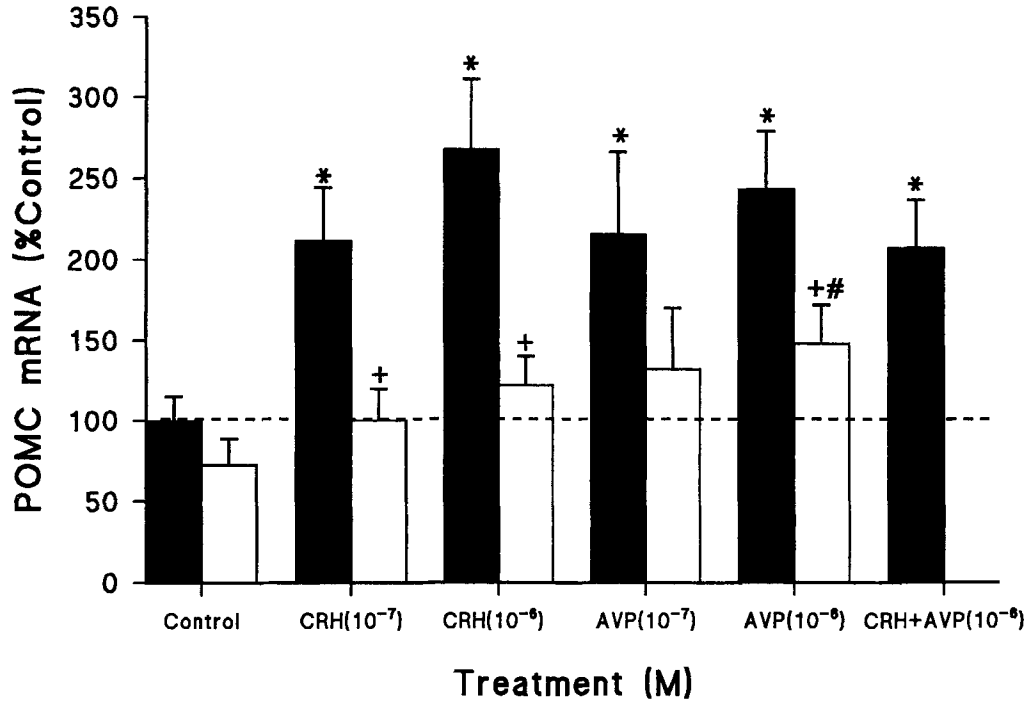

Fig. 3. Levels of POMC mRNA (mean ± SEM, $n = 6$) expressed as a percentage of control following various treatments with CRH and AVP either in the absence (solid bars) or presence of cortisol (10^{-7} *M*; open bars). Both CRH and AVP (10^{-7}, 10^{-6} *M*) and CRH + AVP(10^{-6} *M*) significantly (*$p < 0.05$) increased levels of POMC mRNA in the cultured cells compared to nontreated controls. Cortisol had no significant effect on nonstimulated levels of POMC mRNA but significantly (+ $p < 0.05$) attenuated CRH or AVP-induced increases in POMC mRNA levels compared to stimulated levels. However, AVP (10^{-6} *M*) significantly (#) increased POMC mRNA in the presence of cortisol when compared to cortisol alone.

in augmenting ACTH output in the presence of high AVP concentrations. In contrast, low doses of AVP effectively enhanced ACTH responses to high doses of CRH, in pituitary cells taken near term. The physiological significance of these observations remains unclear, because concentrations of AVP and CRH in the fetal hypophyseal portal circulation are not known at this time. However, further investigations of interactions between CRH and AVP at lower concentrations at this stage of gestation may help resolve this important issue.

CRH and AVP have been measured at high concentrations in sheep hypophyseal portal blood (basal; AVP, 0.05–5 n*M*; CRH, 0.02–0.25 n*M*), and levels of both hypophysiotrophic factors increase in response to stressful stimuli *(21)*. Although, there is no information available on concentrations of these neuropeptides in hypophyseal portal blood of fetal sheep, it is reasonable to suggest that CRH and AVP are secreted into the pituitary portal circulation and that levels increase in response to stress. The lower concentration (10^{-9} *M*) of secretagogs used in the present study is similar to those that would be expected following stressful stimuli in vivo. In this connection, AVP (10^{-9} *M*) significantly increased ACTH output, whereas CRH (10^{-9} *M*) just failed to elevate output significantly. This is consistent with a previous in vitro study using pituitary cells from late-gestation fetal sheep *(11)*, and further indicates that AVP is more potent than CRH at the pituitary corticotroph near term. Little is known of AVP receptor dynamics at this time. However, CRH binding in the pituitary increases to a maximum at around d 125–130, but declines thereafter *(22)*. Previous in vivo and in vitro studies have indicated that the magnitude of the responses to CRH and AVP change as a function of gestational age *(9–12)*. In vivo, corticotroph responsiveness to CRH and AVP has been shown to decrease near term *(10)*. This would be consistent with an increase in plasma cortisol (increased negative feedback) and a decrease in receptor populations *(22)*. However, in that study *(10)*, the same doses of AVP and CRH were administered at each gestational age. Thus, the effective dose would decline as gestation proceeds, and this may account, in part, for the decline in ACTH response. It has also been suggested following in vitro (12) and in vivo *(10)* investigation that CRH is more potent than AVP near term. However, more recent evidence has indicated that AVP is present at 5- to 10-fold the concentration of CRH in the hypophyseal portal blood of adult sheep *(21)*. If this is the case in the fetus, then AVP effects at the corticotroph likely exceed those of CRH in vivo.

There was no effect of cortisol on nonstimulated ACTH output, in vitro. A previous study using pituitary cells derived from younger (d 125) sheep fetuses also reported a lack of effect of cortisol on basal ACTH secretion *(13)*. This raises important issues concerning the different components of corticotroph function (i.e., activated

& nonactivated). In late gestation, exogenous cortisol infusion for 11 h inhibits basal ACTH secretion in fetal sheep in vivo *(5)*. This fact taken with the present in vitro observations, suggests that in the nonstimulated state, the majority of glucocorticoid feedback is mediated via the hypothalamus or higher brain centers. However, whether nonstimulated ACTH output, in vitro, and basal ACTH secretion, in vivo, are analogous situations is unclear, since tonic hypothalamic drive is likely maintained in the latter.

This is the first study to describe differential glucocorticoid feedback on corticotroph function following dose-dependent stimulation, in pituitary tissue removed from fetal sheep. Cortisol abolished CRH-induced ACTH output, but only partially attenuated AVP-stimulated output. This indicates a potential resistance of AVP-stimulated ACTH output to glucocorticoids in the late-gestation fetal sheep. If the ratio of AVP:CRH increases in the hypophyseal portal circulation near term, this may provide a mechanism by which ACTH secretion increases in the presence of elevated glucocorticoids. Investigation of these direct interactions at the pituitary is only possible using in vitro systems, where other secretagogs have been removed, and care must be maintained when extrapolating from the in vitro to the in vivo situation. Further, differences between in vitro studies may result from different treatment exposure times. In the present study, an 18 h treatment was used because we were interested in determining effects on POMC transcription as well as cellular ACTH output.

CRH and AVP affect the corticotroph via different second messenger systems. CRH exerts its effects by binding to an adenylate cyclase-linked membrane receptor, increasing intracellular generation of cAMP, and activating protein kinase A *(23)*. AVP binds to its receptors, which are linked with phosphatidylinositol turnover, stimulating phospholipase C and activating protein kinase C *(23)*. It is possible that glucocorticoids interact with these second messenger pathways selectively and, in this way, give rise to the differential effects of cortisol on stimulated ACTH output. In this connection, it has recently been demonstrated that glucocorticoid receptors are present in the pituitary by d 120 of gestation in fetal sheep *(24,25)* and are localized to areas of the anterior lobe known to contain corticotrophs *(25)*.

It has been proposed that several subpopulations of corticotrophs exist within the anterior lobe of the pituitary, and that these may differ in their affinity for CRH, AVP, and other ACTH secretagogs *(26)*. Recently, it has been demonstrated in pituitary tissue removed from adult sheep that ACTH secretion from a subpopulation of AVP-responsive corticotrophs is resistant to inhibition by glucocorticoids *(17)*, though the nature of this resistance is not clear. If such a subpopulation of corticotrophs exists in the fetal pituitary in late gestation, this may explain the glucocorticoid resistance of AVP-induced ACTH output observed in the present study. Another potential mechanism by which corticotroph-specific glucocorticoid resistance occurs may involve the enzyme 11β-hydroxysteroid dehydrogenase-1. This enzyme is responsible for the interconversion of cortisol to inactive cortisone, and is present at relatively high levels in the anterior pituitary during late gestation *(27)*. If this enzyme is selectively expressed in subpopulations of corticotrophs, it may provide an additional paracrine mechanism regulating glucocorticoid feedback.

Both CRH and AVP, in addition to increasing ACTH output, significantly increased cellular POMC mRNA levels. *In situ* hybridization has allowed us to identify changes in gene expression on a per-cell basis, which would not have been possible using Northern analysis. AVP and CRH were equipotent in stimulation of POMC mRNA levels, in contrast to their effects on ACTH output. Treatment with CRH for 18 h, as in the present study, has been shown to increase POMC mRNA levels in fetal sheep pituitary cells harvested at d 120 of gestation *(19)* and cultured adult rat pituitary cells *(28)*. However, CRH treatment (48 h) of pituitary cells removed at d 142-144 (term) and from adult sheep failed to affect POMC mRNA levels *(14,20)*. There are two possible interpretations of these differing results with sheep pituitary tissue. POMC mRNA levels were not measured until 48 h after treatment in the term gestation group *(14)*, and it is possible that stimulated POMC mRNA levels had declined by the time of analysis. Alternatively, there may be a switch in corticotroph responsiveness immediately prior to term, after d 138, when the pituitary corticotroph POMC gene is no longer responsive to CRH, perhaps an effect of receptor downregulation. A switch from "fetal" to "adult" type of corticotrophs has been described in late-gestation fetal sheep *(29)*. It is possible that only "fetal"-type ovine corticotrophs are capable of increasing POMC mRNA in response to CRH. Around d 138 of gestation, the fetal corticotroph is highly active *(2–4)*. We have previously reported a large increase in POMC mRNA levels in the anterior pituitary, prior to term, when levels are twofold higher than those in the adult sheep *(4)*.

The present study is also the first demonstration that fetal corticotroph POMC mRNA levels can be increased by AVP, though AVP had a tendency to increase POMC mRNA levels in cells derived from d 120 fetuses *(19)* and increased POMC mRNA in adult sheep pituitary cells *(30)*. There was no augmentation of the POMC mRNA response following simultaneous administration of CRH and AVP. In AtT20 cells, CRH induction of POMC gene transcription is mediated by c-*fos*-dependent and c-*fos*-independent pathways *(31)*, and presumably this is the same in fetal sheep. However, the molecular mechanisms by which AVP stimulates POMC transcription remains to be determined. Taken together, these studies indicate that the fetal pituitary responds to CRH and AVP by increasing both

secretion and synthesis of ACTH, though there may be a change in synthetic responsiveness to CRH immediately prior to term.

Cortisol alone failed to affect nonstimulated POMC mRNA in pituitary cells from fetuses at d 138 of gestation. However, simultaneous administration of cortisol significantly attenuated both the CRH- and AVP-induced increases in POMC mRNA levels. The glucocorticoid inhibition of agonist-induced POMC synthesis appeared to be of lesser magnitude in the AVP stimulated cells. In this connection, AVP (10^{-6} *M*) significantly increased POMC mRNA levels when administered simultaneously with cortisol (comparison between AVP + cortisol and cortisol alone). In contrast, there was no significant effect of CRH on POMC mRNA levels in the presence of cortisol. This may indicate that as for ACTH output, AVP-induced POMC mRNA expression is less susceptible to glucocorticoid feedback than CRH-stimulated synthesis. Thus, an increase in hypophyseal portal AVP near term may facilitate the increase in POMC mRNA levels, known to occur *(4)* in the presence of elevated cortisol. These results are consistent with those observed in late-gestation fetal sheep in vivo *(32)*, where basal POMC mRNA levels were unaffected by cortisol infusion, but stress-induced increases in POMC mRNA levels were abolished (32). Further, these data may suggest that nonstimulated POMC mRNA levels are glucocorticoid-resistant, but stimulated POMC mRNA levels are glucocorticoid-sensitive.

In summary, we have identified and characterized a number of mechanisms involved in the regulation of fetal sheep corticotroph function in vitro. These mechanisms may be, in part, responsible for the paradoxical increase in ACTH synthesis and secretion, in the presence of elevated plasma cortisol, that occur in the late-gestation fetal sheep. Cortisol selectively inhibits CRH-induced ACTH output from pituitary cells removed at d 138 of gestation, whereas AVP-induced output is relatively glucocorticoid-resistant. Both CRH and AVP increase POMC mRNA levels in fetal corticotrophs at this stage of gestation, and as for ACTH secretion, cortisol is less effective at inhibiting AVP-stimulated POMC synthesis. This finding is consistent with the extremely high activity of the sheep pituitary corticotrophs in late gestation.

Materials and Methods

Animals and Tissues

Fetuses of mixed-breed sheep with single insemination dates were used. At d 138 of gestation, sheep were killed by an iv overdose of euthanyl (MTC Pharmaceuticals, Cambridge, Ontario, Canada). The fetuses were delivered, their pituitaries removed and placed in Dulbecco's phosphate-buffered saline (DPBS; pH 7.4) supplemented with calcium chloride (0.9 mmol/L), magnesium chloride (0.5 mmol/L), glucose (7.5 mmol/L), 0.1% bovine serum albumin (BSA), and gentamycin (50 mg/L). Media and reagents were obtained from Canadian Life (Gibco-BRL, Mississauga, Ontario, Canada). These studies were performed according to protocols approved by the Animal Care Committees of St. Joseph's Health Centre, the University of Western Ontario and the University of Toronto, in accordance with the Canadian Council for Animal Care.

The anterior lobe was dissected from the neurointermediate lobe, as described previously *(14)*. Tissue culture was based on previously described techniques *(33)*. Briefly, the anterior lobe was chopped into blocks and washed in DPBS. The tissue blocks were then incubated in DPBS containing trypsin (0.5%) at 37°C for 30 min. Tissues were then incubated in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with stripped fetal calf serum (10%), glutamine (2 mmol/L), transferrin (5 mg/L), and Pen-Stre-Fungizone solution (PAFS; 5 mg/L) at 37°C for 30 min. The tissue fragments were then dispersed mechanically in supplemented DPBS (calcium- and magnesium-free). Cells were then filtered and centrifuged (10 min, 460*g*, 22°C). The pellet was resuspended in DMEM supplemented as above, and the cells were counted and their viability tested with trypan blue exclusion. Viability was generally >90%. Cells were plated onto poly-L-lysine-coated eight-well tissue-culture slides (NUNC, Gibco-BRL, Mississauga, Ontario, Canada) at a concentration of 100,000 cells/well (0.5 mL). The cells were maintained in a humidified incubator at 37°C containing carbon dioxide (5%) and oxygen (95%).

Culture Protocol

The cells from the anterior pituitary were preincubated for 96 h. The medium was changed after 48 h and immediately prior to experimental treatment (96 h). At the latter change, the medium was replaced with DMEM/F12 containing glutamine (2 mmol/L), transferrin (5 mg/L), PAFS (5 mg/L), and 0.2% BSA. Various concentrations (0, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} *M*) of ovine CRH, AVP, and cortisol were added in duplicate. After 18 h of treatment, the medium was removed and stored at –20°C. The cells were fixed *in situ* on the culture slides (4% paraformaldehyde, 4 min), washed (phosphate-buffered saline), dehydrated, and stored under ethanol (95%) until *in situ* hybridization or immunohistochemical analysis.

Radioimmunoassay and Immunohistochemistry

Concentrations of ACTH in the cell-culture media were measured by a double-antibody RIA (Incstar, Stillwater, MN), as described previously *(5,14)*. The primary antibody showed <0.01% crossreactivity with β-lipotropin, α-melanocyte-stimulating hormone (MSH) *(14)*, pro-ACTH, and POMC (kindly provided by J. Schwartz, Wake Forest University, NC).

Immunohistochemical detection of (ir)-ACTH was carried out using a specific antibody for ACTH in con-

junction with avidin–biotin–peroxidase reagents from Vectastain ABC kits (Vector Laboratories, Burlingame, CA), as previously described *(34)*. Briefly, this primary antibody does not recognize β-endorphin, ACTH (1–17) and ACTH (1–13; α-melanocyte-stimulating hormone) but does recognize ACTH (1–24), ACTH (18–39; CLIP), and ACTH (1–39) *(34)*. Results are expressed as the mean ± SEM of percentage of pituitary cells positive for (ir)-ACTH.

In Situ *Hybridization*

The method for *in situ* hybridization has been described previously *(4,5)*. Briefly, the antisense POMC oligonucleotide probe (45-mer) was labeled using terminal deoxynucleotidyl transferase (Gibco BRL, Burlington, Ontario, Canada) and [^{35}S]-deoxyadenosine 5'-(α-thio)triphosphate (1300 Ci/mmol, NEN, Du Pont Canada Inc., Mississauga, Ontario, Canada) to a specific activity of 1.0×10^9 cpm/μg. After the slides had been allowed to air-dry, labeled probe in hybridization buffer (200 μl) was applied at a concentration of 1.0×10^3 cpm/μl. The hybridization buffer used for these experiments contained 4X saline sodium citrate (SSC) (1X SSC contains 150 m*M* sodium chloride and 15 m*M* sodium citrate), 50% deionized formamide, 50 m*M* sodium phosphate (pH 7.0), 1 m*M* sodium pyrophosphate (pH 7.0), 0.02% BSA, 200 μg/mL hydrolyzed salmon sperm DNA, 0.02% Ficoll, 0.02% polyvinylpyrolidine, 10% dextran sulfate, and 40 m*M* dithiothreitol (DTT). Slides were incubated overnight in a moist chamber at 42°C. After washing in 1X SSC (30 min at room temperature), 1X SSC (30 min. at 55°C), the slides were rinsed once with 1X SSC and with 0.1X SSC, then dehydrated in ethanol, dried, exposed to X-ray film (Biomax, Kodak), and dipped in Ilford K5 liquid emulsion. The X-ray films and emulsion-coated slides were developed using standard procedures. The sections were counterstained with Haematoxylin to permit identification of nuclei.

Northern blot analysis of total and poly (A^+) RNA extracted from ovine pituitary tissue was performed to verify the specificity of the probe, and a control 45-mer sense oligonucleotide demonstrated no hybridization when incubated with sections known to contain POMC mRNA *(35)*.

Data Analysis

All results are expressed as mean ± SEM for six different experiments carried out in duplicate using tissues from different animals. The concentrations of (ir)-ACTH, number of ACTH-positive cells and POMC mRNA levels in each individual treatment group are expressed as a percentage of the untreated (control) cultures. POMC mRNA levels were determined by measuring the relative optical density (ROD) of autoradiographic film that had been exposed to the culture wells, using computerized image analysis *(4,5)*. The exact number of cells (total cells) underlying each region of film analyzed was counted by computerized cell counting. The ROD value was corrected for the number of underlying cells. Therefore, the POMC mRNA values obtained represent the amount of POMC mRNA/cell. Since the corticotroph population (indexed by ACTH-immunopositive cells) did not change following these treatments, this measure gives an accurate indication of cellular POMC mRNA levels. Differences were assessed by analysis of variance followed by Duncan's multiple-range test, with $p < 0.05$ taken as the level of significance.

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