The role of the pituitary gland and ACTH in the regulation of mRNAs encoding proteins essential for adrenal steroidogenesis in the late-gestation ovine fetus

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Abstract

To further understand the relative roles of the pituitary gland and ACTH in the regulation of mRNAs encoding proteins that are essential for adrenal development, we investigated the effects of, first, an ACTH infusion and labour in intact fetuses and, secondly, the effect of an ACTH infusion to fetuses with and without a pituitary gland, on the relative abundance of the mRNA encoding for the ACTH receptor (MC2R), steroidogenic factor 1 (SF-1), cholesterol side-chain cleavage enzyme ($P450_{scc}$), 3β-hydroxysteroid dehydrogenase (3βHSD) and 17α-hydroxylase ($P450_{C17}$) in the fetal adrenal gland. $ACTH_{1-24}$ infusion (14·7 pmol/kg per h) to intact fetuses was without effect on the abundance of mRNA encoding MC2R and SF-1, irrespective of whether the infusion was given for 18 (115–132 days of gestation) or 32 days (115 days to term (147 days of gestation)). Hypophysectomy (HX) did not alter the expression of MC2R mRNA; however, the abundance of SF-1 mRNA fell by approximately 50% following the removal of the pituitary gland. $ACTH_{1-24}$ infusion to HX fetuses failed to restore levels of SF-1 mRNA to that seen in intact animals. $P450_{scc}$ and 3βHSD mRNAs were increased by $ACTH_{1-24}$ infusion for 18 days in intact animals, although no effects of the

infusion were seen on $P450_{C17}$ mRNA levels. For all three of these mRNAs, there was a significant increase in their abundance between 132 days of gestation and term in intact fetuses. By term, $ACTH_{1-24}$ infusion was without any additional effect on their abundance. HX decreased the expression of $P450_{scc}$, 3βHSD and $P450_{C17}$ mRNAs, while $ACTH_{1-24}$ infusion to HX fetuses increased the expression of these mRNAs to levels seen in intact animals. There were significant correlations between the abundance of the mRNA for $P450_{scc}$, 3βHSD and $P450_{C17}$, but not MC2R and SF-1, and premortem plasma cortisol concentrations. These results emphasise the importance of the pituitary gland and ACTH in the regulation of the enzymes involved in adrenal steroidogenesis. Factors in addition to ACTH may also play some role, as the infusion was not always effective in increasing the abundance of the mRNAs. Surprisingly, the mRNA for MC2R and SF-1 did not appear to be regulated by ACTH in the late-gestation ovine fetus, though a pituitary-dependent factor may be involved in the regulation of SF-1 mRNA abundance.

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Introduction

A late-gestation increase in fetal glucocorticoid concentrations is a hallmark of fetal development in mammalian species, and plays a critical role in preparing the fetus for the transition to extrauterine life. In addition, for a number of species including the sheep, the increase in fetal cortisol production is also the critical trigger determining the timing of labour (Liggins & Thorburn 1994).

A number of factors are thought to contribute to the increase in cortisol concentrations in the late-gestation fetal sheep, though our understanding of their relative importance is incomplete. It is clear that an intact hypothalamo–pituitary–adrenal (HPA) axis is critical for the normal

cortisol surge and parturition, as disruption at any level of the axis results in decreased cortisol concentrations and a failure of parturition (Liggins *et al.* 1967, Drost & Holm 1968, Antolovich *et al.* 1991, Myers *et al.* 1993). Furthermore, the infusion of pituitary adrenocorticotrophin ($ACTH_{1-24}$) or adrenal (cortisol) hormones of the HPA axis induces premature labour in intact fetuses, and restores cortisol concentrations and parturition in fetuses that have had lesions of these respective organs (Liggins & Thorburn 1994, Poore *et al.* 1998). It is generally believed that increasing ACTH concentrations drive the increase in cortisol concentrations, though many studies have demonstrated only modest, linear increases in ACTH concentrations in the face of a large, exponential increase in

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cortisol (Norman *et al.* 1985, Deayton *et al.* 1994, Phillips *et al.* 1996, Canny *et al.* 1998, Poore *et al.* 1998). In addition, we have recently suggested that while the presence of ACTH is essential for the cortisol surge and parturition, an increase in the concentrations of the pituitary hormone is not a prerequisite for normal labour (Poore *et al.* 1998).

It is therefore clear that factors in addition to an increase in ACTH concentrations play a role in adrenal function in late gestation. Many studies have shown that the adrenal gland becomes more responsive to ACTH in late gestation, inasmuch as cortisol production in response to an acute increase in ACTH concentrations is greatly enhanced (Durand *et al.* 1981, 1982, Poore *et al.* 1998). The precise mechanisms controlling this increased responsiveness are not fully defined, but an increase in the number of ACTH receptors, or the efficiency of signal transduction, both in terms of second messenger production and the modulation of the steroidogenic process, have been suggested as possible means by which ACTH may become a more potent stimulus to cortisol production (Durand *et al.* 1981, 1982).

In the late-gestation ovine fetal adrenal gland, the mRNA concentration of a number of steroidogenic enzymes increases in a fashion that is somewhat parallel to the increase in fetal cortisol concentrations. These include the cholesterol side-chain cleavage enzyme (P450$_{scc}$), 3β-hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase (3βHSD), 17α-hydroxylase (P450$_{C17}$) and 21-hydroxylase (P450$_{C21}$) (Phillips *et al.* 1996). Changes in the expression of the enzyme responsible for catalysing the final step of cortisol biosynthesis (11β-hydroxylase) have not been demonstrated in the late-gestation ovine fetal adrenal gland, suggesting that this enzyme does not play an acute regulatory role in the increase in cortisol synthesis (Myers *et al.* 1992). In addition, the expression of P450$_{scc}$ and P450$_{C17}$, but not 3βHSD nor P450$_{C21}$, is decreased after lesions of the hypothalamic paraventricular nucleus (PVN), which destroy corticotrophin-releasing factor-containing neurones, resulting in decreased cortisol concentrations and a failure of parturition in the ovine fetus (Myers *et al.* 1993). Likewise, hypothalamo–pituitary disconnection (HPD), which disrupts the humoral and neural connections between the hypothalamus and pituitary gland, and hypophysectomy, which results in low cortisol concentrations and failure of parturition, both also decrease the expression of P450$_{scc}$ and P450$_{C17}$, as well as 3βHSD (Phillips *et al.* 1996, Ross *et al.* 1997).

These intervention studies, PVN lesion and HPD, as well as the ontogenic studies have led to the hypothesis that diminished secretion of ACTH (and other pituitary factors) secretion leads to the decrease in adrenal steroidogenic enzyme expression (Myers *et al.* 1993, Phillips *et al.* 1996). Both surgical manipulations are, however, not completely specific, and the secretion of pituitary factors other than ACTH is also disrupted. While ACTH concentrations increase in the late-gestation fetus, so do the concentrations of a number of other factors (prostaglandin E$_2$, prolactin etc.) which may also play a role in the regulation of adrenal function (Deayton *et al.* 1994, Phillips *et al.* 1999). In the present study we have, therefore, directly examined the roles of ACTH, the pituitary gland and increasing gestational age in regulating the expression of adrenal steroidogenic enzymes. This was achieved by comparing the effects of an ACTH infusion at different gestational ages to intact fetuses, as well as examining the effect of the same infusion to fetuses with and without their pituitary glands. In addition to examining the expression of P450$_{scc}$, 3βHSD and P450$_{C17}$ we have also measured the relative abundance of the mRNA encoding the ACTH receptor (MC2R) and the transcription factor that plays a critical role in the regulation of the expression of a number of steroidogenic enzymes, steroidogenic factor 1 (SF-1) (Parker & Schimmer 1997).

Materials and Methods

All experiments were conducted in accordance with the requirements of the Victorian Prevention of Cruelty to Animals Act 1986 and the Code of Practice for the Care and Use of Animals for Scientific Purposes of the National Health and Medical Research Council, and received prior approval from the Monash University Standing Committee on Ethics in Animal Experimentation.

General methods

Pregnant Border Leicester–Merino cross-bred ewes of known gestational age were used in these experiments. Ewes were housed in individual metabolism cages with a 12 h light:12 h darkness cycle, fed once daily between 0900 and 1200 h and with water freely available. Surgery was performed between 113 days and 115 days of gestation using aseptic techniques. General anaesthesia was induced with thiopentone sodium in water (20 mg/kg; Pentothal, Bomac Laboratories Ltd, Asquith, NSW, Australia) and maintained by inhalation through an endotracheal tube with 0·5–2·0% halothane (Fluothane; ICI, Villawood, Australia) while vascular catheters were implanted into a fetal carotid artery and jugular vein.

The health of the fetuses was assessed by collecting a fetal arterial blood sample (1 ml) every second day for the measurement of PaO$_2$, O$_2$ saturation, PaCO$_2$ and haemoglobin concentration using an ABL5 blood gas analyser and an OSM2 haemoximeter (Radiometer, Copenhagen, Denmark).

At the end of each experiment the ewes were killed by an overdose of sodium pentobarbitone, and the fetal adrenal glands were rapidly dissected, weighed and immediately frozen in liquid nitrogen and stored at -80 °C.

Experiment 1: The effect of ACTH infusion and labour in intact fetuses on the relative abundance of the mRNA for MC2R, SF-1, P450$_{scc}$, 3βHSD and P450$_{C17}$

This experiment examined the hypothesis that a long-term continuous infusion of ACTH and labour regulate the expression of critical genes involved in fetal adrenal steroidogenesis. From the time of surgery, 20 fetuses received either an intravenous continuous infusion of saline (1 ml/h) or ACTH$_{1-24}$ (14·7 pmol/kg per h; Synacthen; Ciba Geigy Australia Ltd, Pendle Hill, NSW, Australia). Infusions were administered until either 132 days (intact (INT)/saline-treated (SAL) group, n=4; INT/ACTH group, n=5) or until the ewe was in established labour (INT/SAL, n=6; INT/ACTH, n=5) as determined by typical changes in the uterine electromyographic record (Harding *et al.* 1982). The dose of ACTH administered was increased every 5 days with estimated increases in fetal body weight (Cloete 1939). We have previously demonstrated that ACTH infusion at this dose to intact fetuses causes no increase in basal plasma cortisol concentrations, no enlargement of the adrenal glands and no advancement in the timing of labour. These fetuses, however, exhibited an enhanced cortisol response to the rapid intravenous administration of exogenous ACTH$_{1-24}$ (Poore *et al.* 1998). ACTH$_{1-24}$ was chosen as an experimental agent to mimic the actions of ACTH$_{1-39}$, as the two appear to have equivalent potency at the MC2R (Kapas *et al.* 1996).

ACTH challenge

At 120 days of gestation all fetuses received 2·5 µg/kg ACTH$_{1-24}$ by rapid intravenous injection. In addition, the animals receiving the ACTH$_{1-24}$ infusion for 32 days also had challenge experiments performed at 130–131 days or 140–142 days of gestation. Fetal arterial blood samples (2 ml) were collected for measurement of plasma cortisol concentrations at 30 min, 15 min and immediately prior to the ACTH injection (time 0) and at 10, 30, 60, 90 and 120 min after the ACTH challenge. This was performed to assess the effect of the continuous ACTH infusion on fetal adrenal responsiveness to the acute administration of ACTH. An additional fetal arterial blood sample (2 ml) was collected for the measurement of fetal cortisol concentrations the day before the scheduled post-mortem or once labour was evident. At 132 days, or when labour was detected, the ewes were killed and the fetal adrenal glands collected as described above.

Experiment 2: The effect of ACTH infusion to intact and hypophysectomised fetuses on the relative abundance of the mRNA for MC2R, SF-1, P450$_{scc}$, 3βHSD and P450$_{C17}$

This experiment examined the hypothesis that the expression of genes involved in fetal adrenal steroidogenesis is dependent on an intact pituitary gland and that a continuous ACTH infusion will restore any defect resulting from its removal. Surgery was performed on 18 pregnant ewes between 107 and 119 days of gestation (115 ± 1, mean ± s.e.m.) and, in addition to the insertion of vascular catheters, as described above, seven fetuses also had their fetal pituitary gland removed (hypophysectomy, HX) as previously described (Mesiano *et al.* 1987). HX was carried out under direct vision and completeness was confirmed by inspection of the empty sella turcica both at the time of surgery and at post-mortem. Fetuses received an infusion of saline (1 ml/h: INT/SAL, n=6; HX/SAL, n=4) or ACTH$_{1-24}$ (14·7 pmol/kg per h in 1 ml heparinised saline: INT/ACTH, n=5; HX/ACTH, n=3) from the time of surgery until the detection of labour or until 155 days of gestation (for the HX/SAL fetuses). Infusion of this dose of ACTH$_{1-24}$ results in normal cortisol profiles, adrenal morphology and enables labour to occur at the normal time in HX fetuses (Poore *et al.* 1998). The health of the fetuses was assessed by the measurement of fetal blood gases (see above); tests of adrenal responsiveness (see above) were conducted at 142 days of gestation and a fetal blood sample was collected on the day prior to post-mortem for the assessment of fetal cortisol concentrations. The experiment ended when labour was detected or at 155 days of gestation if labour did not occur. Note that intact fetuses receiving a saline or ACTH infusion are the same animals as the term animals described for experiment 1.

Cortisol radioimmunoassay

Cortisol was extracted from fetal plasma with dichloromethane and the concentration of cortisol was measured by radioimmunoassay as previously described (Bocking *et al.* 1986). Intra- and interassay coefficients were 5 and 18% respectively. The sensitivity of the assay was 1·2 ± 0·3 ng/ml.

Total RNA extraction from fetal adrenal glands

Frozen fetal adrenal glands were weighed and then homogenised in Tri-Reagent™ (Sigma, Castle Hill, NSW, Australia) and total RNA was isolated as described in the manufacturer's instructions. The concentration of nucleic acids was quantified by spectrophotometry (UV/VIS 918; GBC Scientific Equipment PTY Ltd, Dandenong, Victoria, Australia) at a wavelength of 260 nm. Ratios of 260/280 nm were used to determine the purity of the total RNA.

Probes

cDNA encoding bovine MC2R was generously provided by Dr K Mountjoy (University of Auckland, Auckland, New Zealand; Mountjoy *et al.* 1992). The human P450$_{scc}$

(Chung *et al.* 1986) and P450$_{C17}$ (Chung *et al.* 1987) cDNA probes were kindly provided by Professor W Miller (University of California at San Francisco, San Francisco, CA, USA). The human cDNA probe for 3βHSD (Lorence *et al.* 1990) was generously donated by Dr R Rodgers (Flinders University of South Australia, Bedford Park, Australia). Dr K Morohashi (Kyushu University, Fukuoka, Japan) kindly provided the cDNA probe for SF-1 (Ad4 BP; Honda *et al.* 1993). A cDNA probe for rat 18S rRNA was used. cDNA probes were random prime labelled with α-[^{32}P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA, USA; Oligolabelling kit; Pharmacia Biotech, Stockholm, Sweden) to the specific activity of 1×10^9 c.p.m./μg DNA.

The bovine MC2R probes shows 96% nucleotide identity to the ovine MC2R mRNA sequence within the coding region, the human P450$_{scc}$ probe shows 82% nucleotide identity to the ovine P450$_{scc}$ mRNA within the coding region, and the human P450$_{C17}$ probe has 80% identity to the ovine P450$_{C17}$ mRNA within the coding region. Nucleotide sequences for the ovine 3βHSD and SF-1 mRNAs are unknown. The identities of nucleotide sequences were determined using BLAST 2 sequences software (http://www.ncbi.nlm.nih.gov/gorf/wblast2.cgi).

Analysis of mRNA abundance

Northern blot analysis Adrenal gland total RNA (20 μg/sample) was denatured by incubation in formaldehyde (2·2 M) and 50% (v/v) formamide at 55 °C for 15 min and then separated by electrophoresis in a 1% agarose gel containing 2·2 M formaldehyde. Capillary blotting was used to transfer the RNA to Duralon nylon membrane (Stratagene, La Jolla, CA, USA).

Slot blot analysis Adrenal gland total RNA was applied to Duralon membrane using a slot blot apparatus (Hoeffer Scientific, San Francisco, CA, USA) (Phillips *et al.* 1996). In brief, total RNA from fetal adrenal glands (10 μg/slot) or wether adrenal glands (2·5–10 μg/slot) was denatured with 6·15 M formaldehyde and 10 × SSC at 55 °C for 15 min, and then applied to the nylon membrane. Membranes from Northern and slot blots were air dried and the RNA fixed to the membrane by baking at 80 °C for 1 h or by exposure to UV light (0·12 J/cm^2) for 30 s in a UV crosslinker (UVC 500; Hoeffer, San Francisco, CA, USA). The membranes were then incubated in hybridisation buffer (7% (w/v) SDS, 50% (v/v) deionised formamide, 5 × SSPE (50 mM sodium phosphate dibasic, 0·75 M sodium chloride, 5 mM EDTA and 100 μg/ml denatured salmon sperm DNA)) at 42 °C for 6 h, followed by an incubation with the relevant cDNA probe ($1–2 \times 10^6$ c.p.m./ml) in hybridisation buffer for 16 h at 42 °C. The membranes were then subjected to a series of 30-min washes (once in 1 × SSC, 0·1% SDS at room temperature,

then twice in 0·1 × SSC, 0·1% SDS at 42 °C). Finally, the membranes were air-dried and placed in an X-ray film cassette containing a storage phosphor screen (Kodak) for 16 h. The image was then visualised and signals quantified using ImageQuaNT (Molecular Dynamics, Sunnyvale, CA, USA). Membranes were stripped of cDNA probes in between hybridisations by washing in 0·01 × SSC, 0·5% SDS for 15 min at 90 °C. Even Northern and slot loading was verified by a final hybridisation with 18S rRNA cDNA probe.

Data analysis

Prior to analysis, the abundance of an individual mRNA as determined by densitometry was calculated as a ratio of the amount of 18S rRNA in the same sample. The responsiveness of the fetal adrenal gland as tested by the ACTH challenge was measured by integrating the plasma cortisol response to ACTH (time points 10–120 min) above the mean preinjection cortisol concentrations (-30 to 0 min). All data were analysed by ANOVA (SPSS for Windows (version 8)) following the assessment of homogeneity of variances using Levene's test. Where appropriate, data were square root or log-transformed prior to analysis. For experiments 1 and 2 the results were compared using two-factor ANOVA (experiment 1 main effects: ACTH infusion and gestational age; experiment 2 main effects: HX and ACTH infusion). When statistically significant interactions between main factors occurred, differences between individual mean values were detected using Fisher's least significant difference test. The data from experiments 1 and 2 were also pooled and compared with the immediate premortem cortisol concentrations using regression analysis in the SPSS package. All results are presented as means ± S.E.M. and a probability level of 5% was considered significant.

Results

Fetal well being

All fetuses were considered healthy at the time of elective post-mortem. Immediately prior to euthanasia, the fetuses had haemoglobin values of $8·75 \pm 2·5$ g/dl, pH values of $7·40 \pm 0·02$ and PaO$_2$ values of $18·5 \pm 3·5$ mmHg.

mRNA transcript size

The bovine MC2R cDNA probe hybridised with a transcript at 4·0 kb, bovine SF-1 cDNA probe hybridised with two transcripts of 3·0 and 4·0 kb, human P450$_{scc}$ cDNA bound with a single transcript of size 1·9 kb, human 3βHSD cDNA hybridised with a single transcript of 1·6 kb, while human P450$_{C17}$ cDNA bound to a transcript of 1·7 kb. 18S rRNA cDNA probe hybridised to

a transcript at 2·0 kb. The sizes of these mRNA transcripts in the ovine fetal adrenal gland correspond in size to their human, rat or bovine counterparts (data not shown). There were no differences in the mean levels of 18S rRNA, measured in densitometric units between any of the treatment groups.

Experiment 1: The effect of ACTH infusion and labour in intact fetuses on the relative abundance of the mRNA for MC2R, SF-1, P450$_{scc}$, 3βHSD and P450$_{C17}$

The ACTH$_{1–24}$ infusion had no significant effect on the fetal adrenal to body weight ratio at either gestational age, though there was a significant ($P<0·05$) increase in this ratio between 132 days of gestation and term (data not shown). Consistent with our previous report (Poore *et al.* 1998), the ACTH$_{1–24}$ infusion increased ($P<0·05$) the cortisol response to a challenge with ACTH in the fetuses killed at 132 days of gestation (INT/SAL $187 ± 81$ vs INT/ACTH $1891 ± 459$ ng/min per ml).

Neither the ACTH infusion nor labour had a significant effect on the relative abundance of the mRNAs for SF-1 and MC2R (Fig. 1). ACTH$_{1–24}$ infusion significantly increased the abundance of the mRNA for P450$_{scc}$ and 3βHSD in the adrenal glands of 132 days of gestation fetuses, but was without effect in fetuses at the time of labour. Fetuses of ewes in labour, however, had a significantly greater abundance of mRNA for P450$_{scc}$ and 3βHSD than either group of fetuses at 132 days of gestation (Fig. 1). The ACTH infusion did not affect the abundance of the mRNA for P450$_{C17}$ at either 132 days or in labour, but the expression of the mRNA was greater in both groups of animals at labour (Fig. 1).

Experiment 2: The effect of ACTH infusion to intact and HX fetuses on the relative abundance of the mRNA for MC2R, SF-1, P450$_{scc}$, 3βHSD and P450$_{C17}$

The fetal adrenal to body weight ratio was significantly ($P<0·05$) decreased in the HX/SAL fetuses, while ACTH infusion restored the ratio to that seen in intact fetuses (Poore *et al.* 1998, data not shown). HX/SAL fetuses failed to generate a significant cortisol response to the ACTH challenge (data not shown).

The relative abundance of the mRNA for MC2R was unaffected by ACTH infusion or HX (Fig. 2). Following removal of the fetal pituitary gland there was a significant decrease in the abundance of the mRNA for SF-1, although ACTH infusion was without effect on the expression of this mRNA (Fig. 2). The abundance of mRNAs for both P450$_{scc}$ and 3βHSD decreased following HX. ACTH infusion restored the abundance of these mRNAs to values not different from that seen in intact fetuses; however, the infusion was without effect in intact animals (Fig. 2). The expression of the mRNA for P450$_{C17}$ was significantly decreased in the HX/SAL

group when compared with the INT/SAL animals. ACTH infusion to HX animals restored the amount of mRNA to values seen in the INT/SAL group. In contrast, the ACTH infusion to intact animals significantly decreased the amount of mRNA encoding P450$_{C17}$, although the values in this group were not different from the HX/ACTH animals (Fig. 2). This effect of ACTH on P450$_{C17}$ mRNA content in intact animals may be of limited physiological significance, as analysis of the same data in experiment 1 did not identify a significant effect.

The relationship between plasma cortisol concentration and the abundance of the mRNA encoding MC2R, SF-1, P450$_{scc}$, 3βHSD and P450$_{C17}$

For each animal in experiments 1 and 2, the individual cortisol concentrations obtained on the day before death or labour were correlated with the relative expression of each of the mRNAs measured (Fig. 3). Cortisol concentrations were not significantly correlated with the expression of mRNAs encoding MC2R (Pearson correlation coefficient, $r=0·025$) or SF-1 ($r=0·038$). There were highly significantly correlations between the plasma cortisol concentrations and the mRNAs encoding P450$_{scc}$ ($r=0·854$), 3βHSD ($r=0·811$) and P450$_{C17}$ ($r=0·58$) (Fig. 3). Multiple linear regression failed to significantly improve the relationship between the relative abundance of the mRNA for the steroidogenic enzymes and premortem cortisol concentrations. This was due, at least in part, to significant correlations between the individual enzymes (P450$_{scc}$ vs 3βHSD, $r=0·90$; P450$_{scc}$ vs P450$_{C17}$, $r=0·76$; 3βHSD vs P450$_{C17}$, $r=0·64$).

Discussion

The results reported in this paper suggest a complex regulation of the abundance of a number of mRNA transcripts encoding proteins that are important for the control of steroidogenesis in the adrenal gland of the late-gestation fetal sheep. Our experimental approaches allow us to begin to dissect the relative contributions of ACTH, the pituitary gland and advancing gestational age in the maturation of adrenal cortical function. The results we have obtained suggest that changes in the expression of genes encoding the steroidogenic enzymes are likely to be important in increasing the synthetic capacity of the adrenal gland. Their regulation, however, does not appear to be under the exclusive control of ACTH, further emphasising the complex regulation of adrenal maturation at this stage of fetal life.

We have previously shown that the infusion of ACTH$_{1–24}$ (14·7 pmol/kg per h) to HX fetuses restores immunoreactive (ir)-ACTH concentrations to values seen in intact animals. Furthermore, when intact animals receive the ACTH$_{1–24}$ infusion there is no increase in
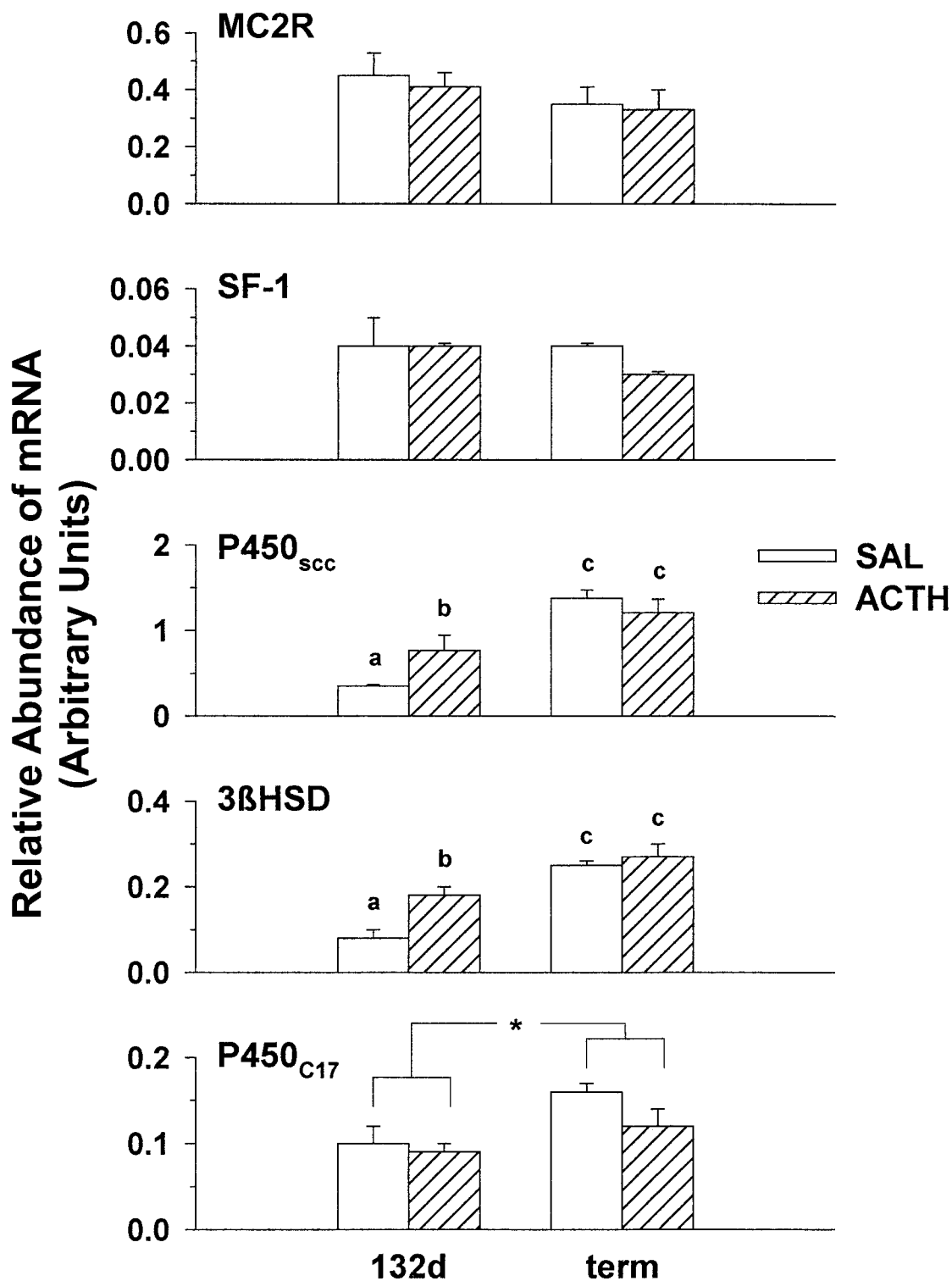
Figure 1 Effect of $ACTH_{1-24}$ infusion to intact fetal sheep on the relative abundance of the mRNA encoding MC2R, SF-1, $P450_{scc}$, 3βHSD and $P450_{C17}$ in adrenals from either saline- or $ACTH_{1-24}$-infused fetuses at either 132 days of gestation (132d) (INT/SAL, $n=4$; INT/ACTH, $n=5$) or at term (INT/SAL, $n=6$; INT/ACTH, $n=5$). Different letters indicate groups that are significantly different from each other; asterisk indicates significant difference between the 132-day and term groups.
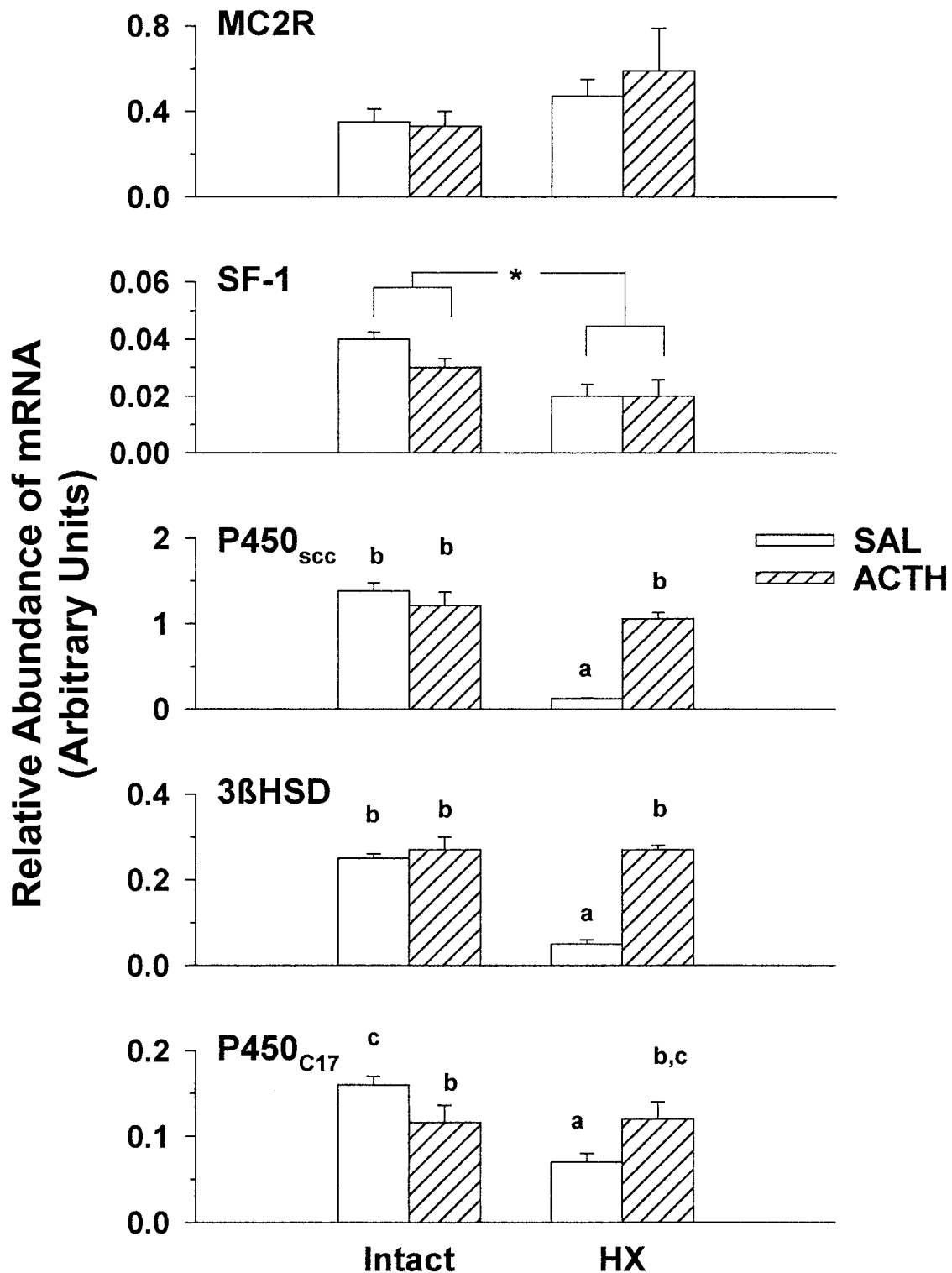
Figure 2 The expression of the mRNA encoding MC2R, SF-1, P450$_{scc}$, 3βHSD and P450$_{C17}$ in adrenals from intact or HX fetal sheep at term (or 155 days; HX/SAL group, $n=4$). Fetuses received either saline or ACTH$_{1-24}$ infusions (INT/SAL, $n=6$; INT/ACTH, $n=5$; HX/ACTH, $n=3$). Different letters indicate groups that are significantly different from each other; asterisk indicates significant difference between the intact and HX groups.
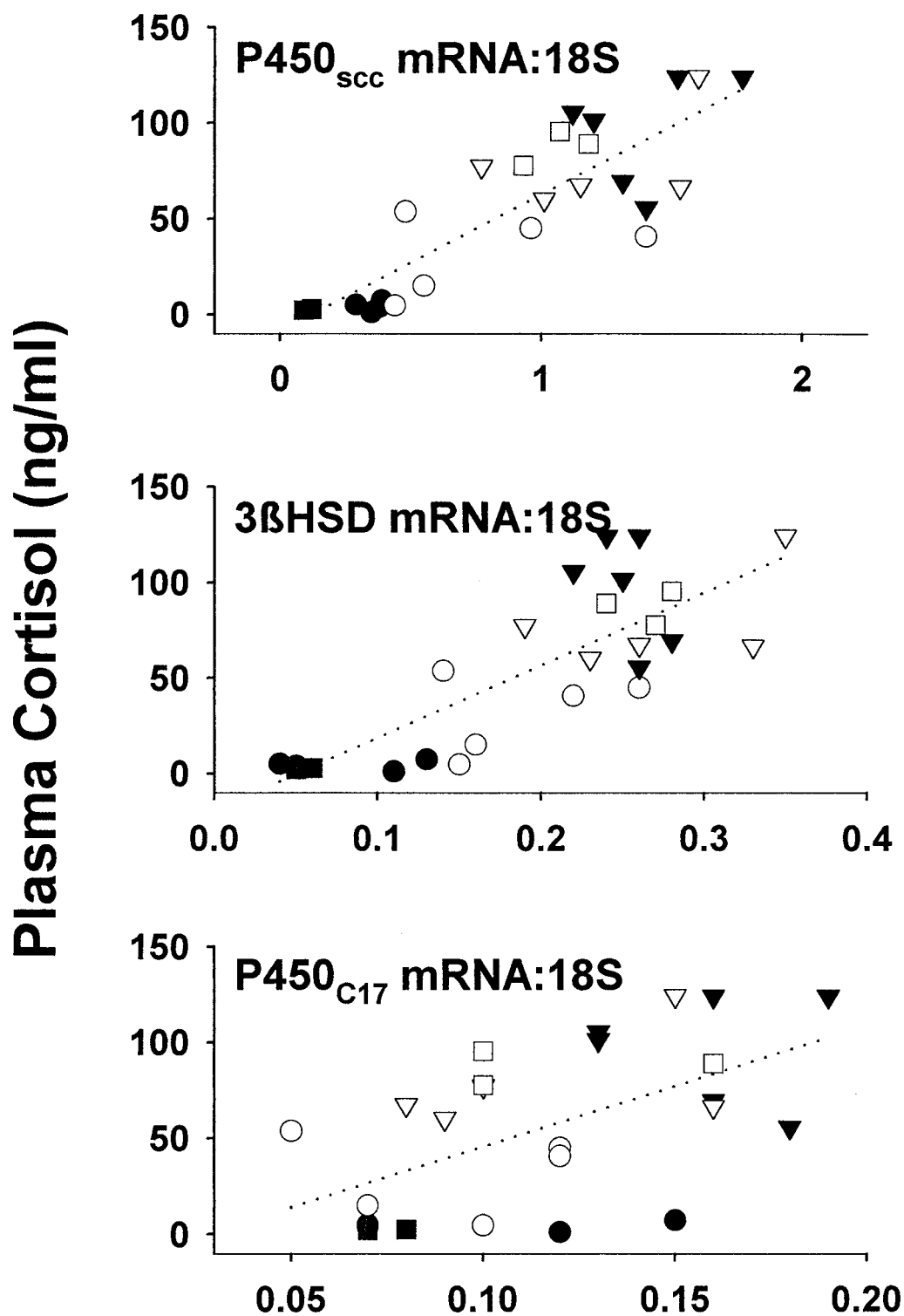
Figure 3 The relationship between premortem plasma cortisol concentration and the abundance of the mRNA encoding P450$_{scc}$, 3βHSD and P450$_{C17}$ in fetal adrenal glands. INT/SAL at 132 days of gestation (●); INT/ACTH at 132 days of gestation (○); INT/SAL at term (▼); INT/ACTH at term (▽); HX/SAL at 155 days of gestation (■); HX/ACTH at term (□). Cortisol concentrations were significantly correlated with P450$_{scc}$ ($r=0.85$), 3βHSD ($r=0.81$) and P450$_{C17}$ ($r=0.58$).

ir-ACTH concentrations when compared with intact fetuses receiving saline (Poore *et al.* 1998). In addition, the concentrations of ir-ACTH were not significantly different in the intact fetuses receiving $ACTH_{1-24}$ or saline for 18 days to 132 days (data not shown). The similarity of ACTH and cortisol profiles between intact fetuses with saline or $ACTH_{1-24}$ and HX fetuses infused with $ACTH_{1-24}$ indicates that the biological response to exogenous ACTH was not adversely influenced by the constant mode of infusion of $ACTH_{1-24}$ used in these studies. There is some evidence that endogenous secretion of ACTH may have been slightly reduced in intact fetuses receiving the $ACTH_{1-24}$ infusion but the occurrence of a prepartum ir-ACTH and cortisol increase in these fetuses indicates that the $ACTH_{1-24}$ infusion was not associated with a severe depression of HPA activity (Poore *et al.* 1998). It is formally possible that if the endogenous secretion of $ACTH_{1-39}$ is decreased in intact fetuses receiving the $ACTH_{1-24}$ infusion, then the secretion of other pro-opiomelanocortin (POMC)-derived products from the pituitary will also be decreased. These compounds may affect the development of the fetal adrenal gland and the expression of adrenal steroidogenic enzymes (Estivariz *et al.* 1982), but definitive studies concerning this issue have not been undertaken. Notwithstanding, the constant infusion of the dose of $ACTH_{1-24}$ employed in the current study appears to be without profound effect on the basal activity of the ovine fetal HPA axis.

These results clearly demonstrate that the pituitary gland and ACTH positively regulate the expression in the adrenal gland of three of the enzymes involved in cortisol biosynthesis. The effects of the $ACTH_{1-24}$ infusion, and labour, on the abundance of the mRNA for $P450_{scc}$ and 3βHSD were similar. When $ACTH_{1-24}$ was infused into intact animals for 18 days until 132 days of gestation (approximately 2 weeks prior to normal delivery) there was a significant increase in levels of both mRNAs. This increase was not maximal since, by the time of labour, further significant increases in mRNA abundance had occurred. At term, the $ACTH_{1-24}$ infusion was without any additional effect on the expression of mRNA for $P450_{scc}$ or 3βHSD. It is not clear whether this lack of additional effect reflects the fact that steady-state mRNA levels had reached a maximum by the time of labour in the intact animals, if ACTH secretion was at a maximum in the saline-infused animals at this time, or if factors in addition to ACTH influence the expression of these enzymes by term. Hypophysectomy, on the other hand, dramatically reduced the expression of both enzymes, an effect which was completely restored by the $ACTH_{1-24}$ infusion. This observation confirms the critical role of pituitary-derived ACTH in regulating the expression of $P450_{scc}$ and 3βHSD.

The observations for $P450_{C17}$ were somewhat more complex. When given to intact animals, the $ACTH_{1-24}$ infusion appeared to be generally without effect on the

abundance of the mRNA, irrespective of whether the infusion had been given for 18 or 32 days. In intact animals, however, there was a significant increase in the abundance of the mRNA between 132 days and term. Conversely, hypophysectomy caused an approximately 50% reduction in the expression of $P450_{C17}$, and $ACTH_{1-24}$ infusion restored the abundance to levels not different from those of intact fetuses at term. These observations suggest that while ACTH plays a critical role in regulating the expression of $P450_{C17}$, other factors may be necessary to explain the increase in expression that is seen with advancing gestational age.

Taken together, these findings confirm and extend previous observations regarding the ontogeny and regulation of steroidogenic enzymes in the late-gestation sheep fetus. As the present study included animals that were confirmed to be in established labour, we have been able to demonstrate that the expression of 3βHSD increases with advancing gestational age, an observation at odds with a previous report (Phillips *et al.* 1996). This difference may be accounted for by the facts that our earlier study did not include animals in labour and that the capacity of the fetal adrenal to secrete cortisol increases dramatically in the last 48 h of fetal life. Our finding of increased expression of the mRNA encoding 3βHSD in the present study is not unexpected, given the dramatic increase in cortisol production that is seen over the last 2 weeks of gestation, and our previous observation that HPD decreased the expression of the enzyme. Furthermore, it is consistent with observations made for $P450_{scc}$ and $P450_{C17}$. The effects of hypophysectomy were remarkably similar to those we have previously reported for the HPD procedure (Phillips *et al.* 1996, Ross *et al.* 1997). By infusing $ACTH_{1-24}$ to HX fetuses, at a rate sufficient to restore basal cortisol concentrations and maintain the correct timing of labour, we have been able to directly confirm the pivotal role played by ACTH in regulating the expression of $P450_{scc}$, 3βHSD and $P450_{C17}$ mRNA. This is an important finding, as previous studies that have employed the use of the HPD procedure or bilateral hypothalamic PVN lesions may have induced a range of effects on pituitary hormone secretion, which are not necessarily restricted to the HPA axis.

Neither surgical manipulations nor ACTH infusion nor advancing gestational age had an effect on the abundance of the mRNA for MC2R. This was a somewhat surprising finding given previous reports that have suggested that the expression of MC2R mRNA in ovine adrenocortical cells is regulated by ACTH and glucocorticoids (Picard-Hagen *et al.* 1997), and that ACTH binding and the efficiency of ACTH signal transduction increases with advancing gestational age in the ovine fetal adrenal (Durand *et al.* 1981, 1982). Our Northern blot analysis revealed a single transcript of approximately 4 kb, whereas other reports, using the same probe as that used in this study, have suggested multiple transcripts of 1·3, 1·8, 3·6 and 4·2 kb in

adult ovine adrenocortical cells (Picard-Hagen *et al.* 1997). If multiple transcripts also exist in the ovine fetal adrenal, and their abundance was below the detection limit for our initial Northern blot analysis, it is possible that any changes in their expression induced by our experimental procedures may have been obscured by the contribution of other transcripts when analysed by slot blot techniques. In addition, we extracted total RNA from both the cortex and medulla of the fetal adrenal gland, and again this approach may have obscured significant changes occurring only in the relevant cells of the cortex.

The expression of SF-1 was also not affected by $ACTH_{1-24}$ infusion or advancing gestational age. Fetal hypophysectomy reduced SF-1 mRNA levels by approximately 50% and $ACTH_{1-24}$ infusion was without any effect in HX fetuses. This suggests that SF-1 levels are controlled, at least in part, by a pituitary-derived factor, distinct from ACTH. There are a number of candidate pituitary molecules that have been implicated in adrenal development, including a number of fragments of the POMC molecule and prolactin (Estivariz *et al.* 1982, Phillips *et al.* 1999). Whether any one of these has a role in the regulation of SF-1 levels is a matter of speculation. Furthermore, the physiological effects of such a modest reduction in SF-1 mRNA levels, in terms of actual protein levels, is unclear.

As the production of cortisol is an important function of the ovine fetal adrenal gland, we correlated the abundance of each mRNA in individual adrenal glands with the plasma cortisol concentrations in that animal on the day before tissue collection. Not surprisingly, no significant relationship between the mRNA for SF-1 or MC2R and cortisol concentrations was observed; however, highly significant correlations between the three steroidogenic enzymes and cortisol were seen. In the case of $P450_{scc}$ and $3\beta HSD$ the *r* values were in excess of 0.81, suggesting that the expression of these enzymes plays a critical role in determining cortisol concentrations. The correlation between $P450_{C17}$ and cortisol was not as strong, although it was still highly statistically significant. Multiple regression analysis did not reveal any significant increase in the predictive value for the mRNA levels on cortisol concentrations, suggesting that the expression of the mRNA for the steroidogenic enzymes is somewhat interrelated. This is not surprising, given the parallel findings we observed in response to our experimental manoeuvres.

In conclusion, the results of the present study confirm the critical role of the pituitary gland and ACTH in regulating the function of the ovine fetal adrenal gland. The expression of mRNA encoding proteins critical in the steroidogenic pathway was clearly affected by our experimental manipulations, while the expression of MC2R and SF-1 was largely unaffected. ACTH did not appear, however, to be the only factor involved in regulating the steroidogenic enzymes as, in a number of cases, $ACTH_{1-24}$ infusion was unable to increase enzyme expression in intact animals, or fully restore the effects of hypophysectomy. Despite the fact that ACTH did not fully restore the effects of hypophysectomy on $P450_{C17}$ expression, these animals had normal cortisol concentrations and precipitated labour at the appropriate time. These observations are consistent with our previous suggestions that ACTH plays an essential, but largely permissive, role in the regulation of cortisol secretion in the late-gestation ovine fetus, with the potential for the involvement of other factors. The identity of these factors awaits elucidation.

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