Developmental regulation of corticotrophin receptor gene expression in the adrenal gland of the ovine fetus and newborn lamb: effects of hypoxia during late pregnancy

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Abstract

Responsiveness of the fetal sheep adrenal gland to adrenocorticotrophin (ACTH) increases in late pregnancy, resulting in increased glucocorticoid production. Development of this responsiveness is an important determinant of fetal hypothalamic–pituitary–adrenal function and depends, in part, on the potential for ACTH binding to adrenal tissue. In the present study, we have examined the developmental pattern of ACTH receptor (ACTH-R) expression during the latter half of pregnancy and in neonatal and adult life. As hypoxaemia induces increases in cortisol and ACTH secretion, in addition to increasing fetal adrenal responsiveness, a further aim of this study was to investigate whether hypoxaemia was associated with altered expression of the *ACTH-R* gene.

Whole adrenal glands were removed from fetal sheep, lambs and adult sheep at different stages of development for measurement of ACTH-R mRNA. Moderate hypoxaemia was induced for 48 h beginning on days 124–128, or on days 132–134 of gestation, by decreasing the maternal fractional inspired oxygen. ACTH-R mRNA was detected by northern blotting using a cDNA cloned in our laboratory and by *in situ* bydridisation.

ACTH-R mRNA (3·6 kb major transcript) was detected in adrenal tissue at day 63 of gestation. Its relative abundance increased significantly ($P<0·05$) between days 126–128 and 140–141 of pregnancy, increased further with the onset of spontaneous labour, and remained increased in newborn lambs at 7 h–7 days after birth. ACTH-R mRNA levels then decreased in adrenal tissue from lambs and adult sheep ($P<0·05$). Hypoxaemia for 48 h significantly increased ACTH-R mRNA expression in adrenals of the older fetuses (days 134–136) compared with that in controls ($P<0·05$), but was without effect in younger fetuses.

We conclude that levels of ACTH-R mRNA in the fetal adrenal gland increase as term approaches, coincident with the endogenous prepartum surge in plasma ACTH and cortisol. Sustained hypoxaemia resulted in an upregulation of mRNA encoding for ACTH-R, but only in older fetuses and in association with a sustained increase in plasma cortisol. These results are consistent with cortisol, ACTH, or both, contributing to increased fetal adrenal responsiveness, by increasing expression of fetal adrenal receptors for ACTH.

Journal of Endocrinology (2001) **169,** 1–10

Introduction

In many species, control of cortisol output by the fetal adrenal gland is crucial, not only to the orderly maturation of fetal organ systems and the initiation of parturition, but also to the development of those homeostatic mechanisms that are a necessary part of the fetal adaptive response to stress (Challis & Brooks 1989). Increased output of adrenocorticotrophin (ACTH) from the fetal pituitary, and increased responsiveness of the fetal adrenal to ACTH are key to this sequence of events. However, the precise mechanisms by which responsiveness of the fetal adrenal gland to ACTH stimulation is increased during late pregnancy are not clear.

In the sheep, birth is preceded by a progressive increase in fetal plasma concentrations of ACTH and cortisol during the last 15–20 days of gestation (Liggins *et al.* 1973, Magyar *et al.* 1980, Challis & Brooks 1989). Fetal adrenal responsiveness to ACTH stimulation changes dramatically during gestation, being high at days 50–60, reduced at days 75–110 and then increased again as term (approximately days 145–150) approaches (Wintour *et al.* 1975, Glickman & Challis 1980). Many *in vivo* and *in vitro* studies have shown that ACTH increases adrenal cell

0022–0795/01/0169–001 *Printed in Great Britain*

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responsiveness (Mountjoy *et al.* 1994, Lebrethon *et al.* 1994*a*, Durand *et al.* 1985, Challis & Brooks 1989), but the extent to which altered expression of the ACTH receptor (*ACTH-R*) gene modulates this effect in fetal sheep is not known. Recent studies have shown that glucocorticoids exert a positive trophic effect on ovine adrenal cortical cells that involves enhancement of *ACTH-R* gene expression (Picard-Hagen *et al.* 1997), suggesting a role for glucocorticoids in the regulation of ACTH-induced increased adrenal responsiveness near term.

In the present study, our initial objective was to determine the developmental pattern of adrenal ACTH-R mRNA expression during the latter half of pregnancy and to relate these values to those in neonatal and adult sheep. Physiological stressors, such as hypoxaemia, are known to activate the fetal sheep pituitary–adrenal axis, resulting in increased plasma concentrations of ACTH and cortisol (Akagi & Challis 1990, Braems *et al.* 1996, 1998), but it is not known whether hypoxia results in changes in adrenal *ACTH-R* gene expression. Therefore, a further aim was to examine whether a period of sustained hypoxaemia led to altered *ACTH-R* gene expression in the fetal adrenal, and whether any response varied according to the gestational age of the fetuses.

Materials and Methods

Animals

Time-dated pregnant ewes and lambs of mixed breed were used. Experiments were performed according to procedures approved by the University Animal Care Committee, University of Toronto, in accordance with the guidelines of the Canadian Council for Animal Care.

Developmental study

Whole adrenal glands were collected from fetal sheep at day 63 ($n=2$), days 100–101 ($n=4$), days 120–128 ($n=4$), days 140–141 ($n=4$) and at term early spontaneous labour (days 143–146, $n=3$), from newborn lambs (5 h–7 days, $n=4$), prepubertal lambs (45–60 days, $n=4$) and late pregnant adult sheep ($n=4$) for measurement of ACTH-R mRNA by Northern analysis and *in situ* hybridisation. Early labour was determined by the uterine electromyogram (Lye *et al.* 1983) and was defined as uterine contractions lasting 0·5–1·0 min, occurring at a frequency of more than eight per hour. The animals were killed with an overdose of sodium pentobarbitone (Euthanyl, Abbott Laboratories, Montreal, Quebec, Canada). Adrenal glands were collected rapidly, trimmed, frozen (-80 °C) in liquid nitrogen or using dry ice and stored at -80 °C until required for processing for analysis of mRNA.

Effects of hypoxaemia

Vascular catheters were implanted into a fetal tibial vein or artery with the animal under general anaesthesia, in two groups of fetal sheep at either days 118–122 ($n=12$) or days 126–128 ($n=8$) of gestation, as described by Braems *et al.* (1996). Catheters were also inserted into the amniotic cavity and into the maternal femoral artery and vein. Experiments were commenced in the younger group of animals, beginning on days 124–128 (normoxia $n=6$, hypoxia $n=6$) of gestation, just before the major prepartum hypothalamo–pituitary–adrenal (HPA) activation, and in the older group of animals, during HPA activation, on days 132–134 (normoxia $n=4$, hypoxia $n=4$) of gestation. Fetal hypoxaemia for 48 h was produced as described by Braems *et al.* (1996). Control animals (normoxia) were maintained in room air. Blood samples were taken from a fetal artery (2·5 ml) and a maternal artery (5·0 ml) into chilled heparinised syringes at 8-h intervals before and during hypoxia, for measurement of blood gases, plasma immunoreactive (ir)-ACTH and cortisol (Norman *et al.* 1985, Jeffray *et al.* 1998). At the end of the experimental periods (days 126–130 and days 134–136), the animals were killed with an overdose of sodium pentobarbitone and the adrenal glands were removed immediately, frozen using liquid nitrogen, and stored at -80 °C for RNA extraction.

Generation of ACTH-R cDNA probe by RT-PCR

An ACTH-R cDNA probe was generated from adrenal total RNA from a 2-month-old lamb using Superscript II RNase$^-$ reverse transcriptase and Taq DNA polymerase (Canadian Life Technologies, Burlington, Ontario, Canada) according to manufacturer's instructions, with modifications. Primers (5′ tccgaattcccatgtacttttttcatctg and 5′ gaagaattccctgagctctgggctccg) corresponding to bp 297–316 and 977–960 of bovine ACTH-R cDNA (accession No. x74501) with added EcoRI sites (underlined) were used in the PCR reaction. Thirty-five cycles at 94 °C for 60s, 60 °C for 60s, and 72 °C for 90s produced sufficient product for subcloning into the EcoRI site of pBluescript KS$^+$ (Stratagene, La Jolla, CA, USA). The 643 bp EcoRI fragment was sequenced using a T7 Sequencing kit and [α^{35}S]dATP (Amersham Pharmacia Biotech, Baie D'Urfé, Quebec, Canada) and the result compared with the GenBank database.

A partial (643 bp) sequence of the 680 bp fragment of the ovine adrenal ACTH-R amplified using the primers indicated above was compared with the corresponding bases 317–959 (amino acid residues 65–279) of the bovine ACTH-R sequence (Fig. 1). Homology of the ovine ACTH-R fragment with the corresponding bovine ACTH-R sequence was 96% at the nucleotide level and 95% at the amino acid level.

65	*Ser*	*Leu*	*Ala*	*Ile*	*Ser*	*Asp*	*Met*	*Leu*	*Gly*	***Ser***	*Leu*	*Tyr*	*Lys*	*Ile*	*Leu*	*Glu*	*Asp*	***Val***	*Leu*	*Ile*
317	cag	ctt	ggc	tat	ttc	cga	tat	gct	ggg	gag	cct	gta	caa	gat	ttt	gga	aaa	cgt	tct	gat
1	cag	ctt	ggc	tat	ttc	cga	tat	gct	ggg	**cag**	cct	gta	caa	gat	ttt	gga	aaa	**tgt**	tct	gat
85	*Met*	*Phe*	***Lys***	*Asp*	*Met*	*Gly*	*Tyr*	*Leu*	*Glu*	*Pro*	*Arg*	*Gly*	*Ser*	*Phe*	*Glu*	*Ser*	*Thr*	*Ala*	*Asp*	*Asp*
377	cat	gtt	caa	aaa	cat	ggg	tta	cct	cga	gcc	tcg	agg	cag	ctt	tga	aag	cac	agc	aga	tga
61	cat	gtt	**cag**	aaa	cat	ggg	tta	cct	cga	gcc	tcg	agg	cag	ttt	tga	aag	cac	agc	aga	tga
105	***Val***	*Val*	*Asp*	***Ser***	*Leu*	*Phe*	*Ile*	*Leu*	*Ser*	*Leu*	***Leu***	***Gly***	*Ser*	*Ile*	*Cys*	*Ser*	*Leu*	*Ser*	*Val*	*Ile*
437	tgt	ggt	gga	ctc	cct	gtt	cat	cct	ctc	cct	tct	cgg	ctc	cat	ctg	cag	cct	gtc	tgt	gat
121	**cgt**	ggt	gga	**ctt**	cct	gtt	cat	cct	ctc	cct	**cct**	**tgg**	ctc	cat	ctg	cag	cct	gtc	tgt	gat
125	*Ala*	*Ala*	*Asp*	*Arg*	*Tyr*	*Ile*	*Thr*	*Ile*	*Phe*	*His*	*Ala*	*Leu*	*Glu*	*Tyr*	*His*	*Arg*	*Ile*	*Met*	*Thr*	*Pro*
497	cgc	cgc	tga	ccg	cta	cat	cac	aat	ctt	cca	cgc	tct	gca	gta	cca	ccg	cat	cat	gac	ccc
181	cgc	cgc	tga	ccg	cta	cat	cac	aat	ctt	cca	cgc	tct	gca	gta	cca	ccg	cat	cgt	gac	ccc
145	*His*	*Arg*	***Ala***	*Leu*	*Val*	***Ile***	*Leu*	*Thr*	*Val*	*Leu*	*Try*	*Ala*	*Gly*	***Cys***	*Thr*	*Gly*	*Ser*	***Gly***	*Ile*	***Thr***
557	gca	ccg	tgc	cct	cgt	cat	cct	gac	ggt	cct	ctg	ggc	agg	ctg	cac	agg	cag	cgg	cat	tac
241	gca	ccg	**cgc**	cct	cgt	**cgt**	cct	gac	ggt	cct	ctg	ggc	agg	**cta**	cac	agg	cag	**tgg**	cat	**cac**
165	*Ile*	***Val***	*Thr*	*Phe*	*Ser*	*His*	*His*	*Val*	*Pro*	*Thr*	***Val***	*Ile*	*Ala*	*Phe*	*Thr*	*Ala*	***Leu***	*Phe*	*Pro*	***Leu***
617	cat	cgt	gac	ctt	ctc	cca	tca	cgt	ccc	cac	agt	gat	cgc	ctt	cac	agc	gct	gtt	ccc	gct
301	cat	**tgt**	gac	ctt	ctc	cca	tca	cgt	ccc	cac	**ggt**	gat	cgc	ctt	cac	agc	**gtt**	gtt	ccc	**cct**
185	*Met*	*Leu*	*Ala*	*Phe*	*Ile*	*Leu*	*Cys*	*Leu*	*Thr*	*Val*	*His*	*Met*	*Phe*	*Leu*	***Leu***	*Ala*	*Arg*	*Ser*	*His*	***Thr***
677	gat	gct	ggc	ctt	cat	cct	gtg	cct	cta	cgt	gca	cat	gtt	cct	gct	ggc	ccg	ctc	cca	cac
361	gat	gct	ggc	ctt	cat	cct	gtg	cct	cta	cgt	gca	cat	gtt	cct	**act**	ggc	ccg	ctc	cca	**cgc**
205	*Arg*	*Arg*	*Thr*	***Pro***	*Ser*	*Leu*	*Pro*	*Lys*	*Ala*	*Asp*	*Met*	*Arg*	*Gly*	*Ala*	***Val***	*Thr*	*Leu*	*Thr*	*Val*	*Leu*
737	cag	gag	gac	ccc	ctc	cct	tcc	caa	agc	caa	cat	gag	agg	ggc	cgt	cac	act	gac	tgt	cct
421	cag	gag	gac	**ctc**	ctc	cct	tcc	caa	agc	caa	cat	gag	agg	ggc	**cat**	cac	act	gac	tgt	cct
225	*Leu*	*Gly*	*Val*	*Phe*	*Ile*	*Phe*	*Cys*	***Try***	*Ala*	*Pro*	*Phe*	*Val*	*Leu*	*His*	*Val*	*Leu*	*Leu*	*Met*	*Thr*	*Phe*
797	gct	cgg	ggt	ctt	cat	ttt	ctg	ttg	ggc	acc	ctt	tgt	cct	tca	tgt	cct	ctt	gat	gac	att
481	gct	cgg	ggt	ctt	cat	ttt	ctg	**ctg**	ggc	acc	ctt	tgt	cct	tca	tgt	cct	ctt	gat	gac	att
245	*Cys*	*Pro*	***Ala***	*Asp*	*Pro*	*Tyr*	*Cys*	*Ala*	*Cys*	*Tyr*	*Met*	*Ser*	*Leu*	*Phe*	*Glu*	*Val*	*Asp*	*Gly*	*Val*	*Leu*
857	ctg	ccc	agc	tga	ccc	cta	ctg	tgc	ctg	cta	cat	gtc	cct	ctt	cca	ggt	gaa	tgg	tgt	gtt
541	ctg	ccc	**ggc**	tga	ccc	cta	ctg	tgc	ctg	cta	cat	gtc	cct	ctt	cca	ggt	gaa	tgg	tgt	gtt
265	*Ile*	*Met*	*Cys*	*Asp*	*Ala*	***Ile***	*Ile*	*Asp*	*Pro*	*Phe*	*Ile*	*Tyr*	*Ala*	*Phe*						
917	gat	cat	gtg	taa	tgc	cat	cat	cga	ccc	ctt	cat	ata	tgc	ctt						
601	gat	cat	gtg	taa	tgc	**cgt**	cat	cga	ccc	ctt	cat	ata	tgc	ctt	t					

Figure 1 Ovine adrenal ACTH-R partial cDNA sequence. A 643 bp fragment (numbering shown not in bold) corresponding to the bases 317–959 sequence (numbering shown in bold) of the bovine ACTH-R was cloned from 2-month-old lamb adrenal total RNA using RT-PCR. Nucleotide homology compared with bovine cDNA was 96%. Numbering shown in bold italics corresponds to amino acid residues 65–279 of the bovine sequence. Amino acid homology compared with bovine was 95%. All bases in bold refer to codons that are different when compared with the bovine sequence. All amino acid residues of the bovine sequence in bold indicate where there are differences compared with the ovine partial cDNA sequence.

Northern blot hybridisation

Total cellular RNA was prepared using the lithium chloride–urea method as described previously (Auffray & Rougeon 1980). Absorption at 260 and 280 nm was measured; the ratios of the two absorbancies at were greater than 1·5.

For Northern blot analysis, total RNA (20 μg) was denatured and subjected to electrophoresis as described by Fraser *et al*. (1994). Blots were hybridised overnight with the ACTH-R cDNA probe labelled with [α^{32}P]deoxy-CTP by the random priming technique to specific activity of $1–2 \times 10^9$ c.p.m./μg using an Oligo-labelling kit (Amersham Pharmacia Biotech Inc. Canada, Baie D'Urfé,

Quebec, Canada). The blots were washed and subjected to autoradiography as described previously (Fraser *et al.* 1994). Confirmation of the relative amounts of total RNA loaded onto each lane was determined by stripping the blots and probing with a [α^{32}P]-labelled cDNA for mouse 18S rRNA. The relative optical densities (RODs) of the autoradiographic signals were within the linear range of the film, and were quantified after subtraction of background using a computerised image analysis system (Imaging Research Inc., St Catherines, Ontario, Canada). Values obtained represent an average optical density over the area measured, which was then expressed as a ratio to that of the 18S rRNA band. Integrity of RNA samples was determined before Northern analysis by electrophoresis of 10 μg RNA in a 1% (w/v) agarose–formaldehyde gel. Gels were stained with ethidium bromide to visualise the 28S and 18S rRNA. Degraded samples were discarded.

In situ *hybridisation*

In situ hybridisation was performed using a previously described method (Matthews & Challis 1995) with some modification. Coronal sections (10 μm) of fetal adrenal tissue were cut using a cryostat (CM 3000 cryostat, Leica Microsystems (Canada) Inc., Willowdale, Ontario, Canada). Tissue sections were hybridised with [α^{35}S]UTP-labelled antisense or sense ACTH-R RNA (cRNA) probe (SA 1250Ci/mmol, NEN Life Science Products, Boston, MA, USA) generated from the ACTH-R cDNA described above subcloned into pBluescript KS^+ vectors. All adrenal sections analysed for ACTH-R mRNA localisation were processed simultaneously. Synthesis of radiolabelled probes was performed as described previously (Matthews & Challis 1995), to a specific activity of 10^9 c.p.m./μg, and used at a concentration of 10×10^6 c.p.m./ml.

Adrenal sections were exposed to autoradiographic film for 8 h. Signals were within the linear range of the film, as verified by simultaneous exposure of the film to carbon-14 standards (American Radiochemical Co., St Louis, MO, USA). Sections were then coated with a silver liquid emulsion (Ilford K5, Ilford Ltd, Mobberley, Cheshire, UK) and exposed for 2 days at room temperature. The silver emulsion was then developed using standard procedures, and the sections counterstained with methylene blue (1%) and mounted with Permount (Fisher Scientific, Toronto, Ontario, Canada). There was absence of hybridisation signal in fetal adrenal sections exposed to radiolabelled ACTH-R sense cRNA probe.

Data analysis

Results are expressed as the mean ± S.E.M. Group comparisons were performed by one- and two-way ANOVA. If significance was obtained ($P<0{\cdot}05$), further analysis was conducted using Duncan's multiple range test or Student's *t*-test for specific contrasts.

Results

Developmental changes in ACTH-R mRNA in ovine adrenal gland

A major ACTH-R transcript of 3·6 kb and two minor mRNA transcripts of 4·2 and 1·3 kb were identified in the ovine fetal adrenal gland by Northern blot analysis. ACTH-R mRNA was detected in fetal adrenal tissue at day 63 of gestation (Fig. 2A). Abundance of ACTH-R mRNA (3·6 kb transcript) increased between days 126–128 and days 140–141 ($P<0{\cdot}05$; Fig. 2B). Levels of ACTH-R mRNA increased further in fetal adrenal tissue collected during spontaneous labour (days 143–146, $P<0{\cdot}05$; Fig 2B), but decreased by 45–60 days of postnatal age ($P<0{\cdot}05$), to values that were similar to those in adult animals.

Localisation of ACTH-R mRNA

ACTH-R mRNA localised to cells of both the zona glomerulosa and fasiculata–reticularis of the adrenal cortex and to groups of steroidogenic cells present in the adrenal medulla (Fig. 3). Developmental changes in the intensity of the *in situ* hybridisation signal (Fig. 3) were similar to that of the Northern hybridisation signal (Fig. 2). Specificity of ACTH-R hybridisation was determined by the absence of silver grains in adrenal glands from fetal sheep at term spontaneous labour after incubation with sense probe.

Effect of hypoxaemia on ACTH-R mRNA expression

Mean changes in fetal plasma ACTH and cortisol in response to hypoxaemia at the two times in gestation were reported by Braems *et al.* (1996). Briefly, in animals at days 124–128, plasma ir-ACTH increased from basal values of approximately 15 pg/ml to maximum concentrations of >200 pg/ml at 16 h, then declined towards baseline; at days 132–134, basal ir-ACTH values of approximately 20 pg/ml increased to >350 pg/ml at approximately 16 h hypoxaemia, then decrease d to mean concentrations that remained slightly greater than, although not statistically different from, controls for the remainder of the experiment. The effects of hypoxaemia on changes in ACTH-R mRNA expression are illustrated in Fig. 4. Gestational age-related changes in response to hypoxaemia were observed. In the younger group of fetuses, levels of ACTH-R mRNA in the adrenal gland were not significantly altered after 48 h of hypoxaemia, when compared with normoxaemic controls. However, in the older group

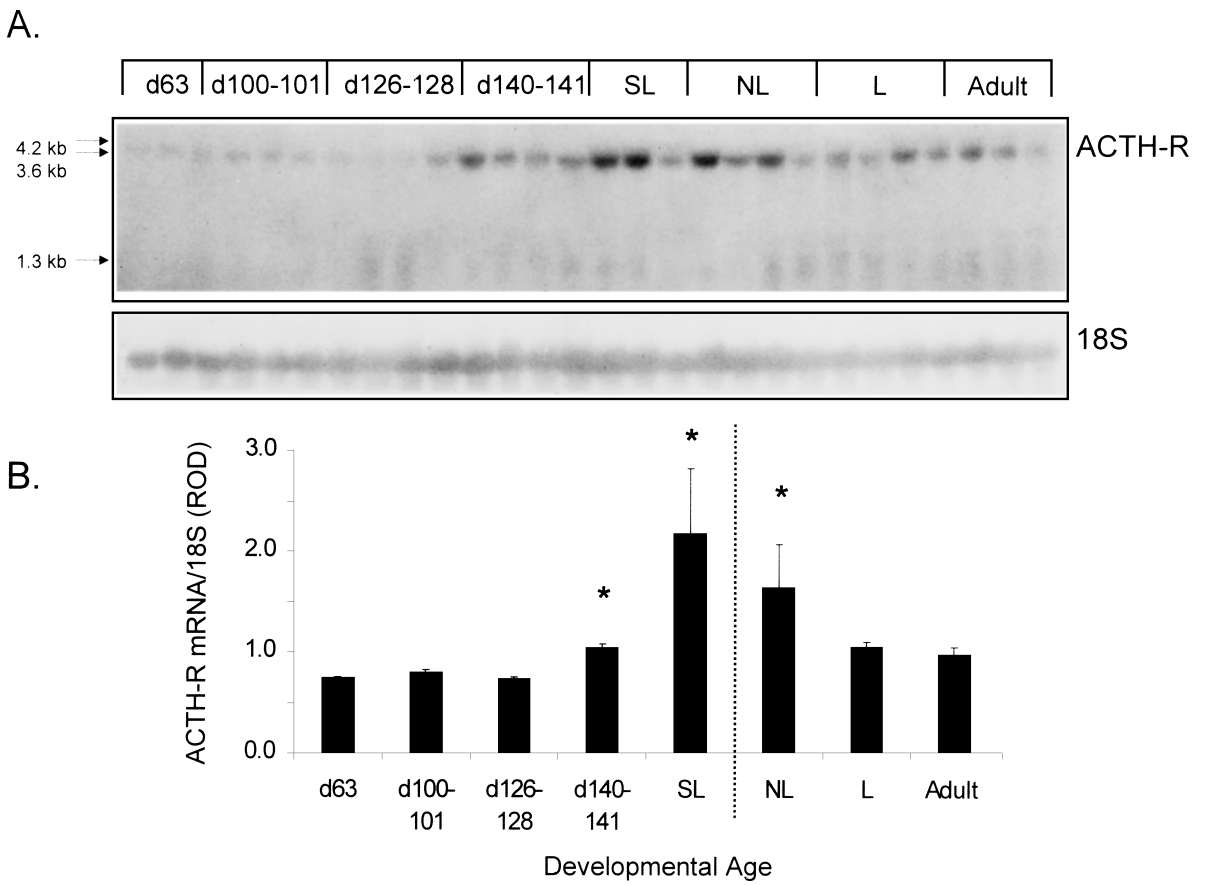

Figure 2 (A) Northern blot hybridisation of total RNA from adrenal glands of fetal sheep, lambs and late gestation pregnant ewes (Adult). The blot was probed with a ^{32}P-labelled ovine cDNA. The autoradiograph was exposed for 4 days at −80 °C. Three ACTH-R transcripts of 4·2, 3·6 and 1·3 kb were identified at each gestational age and postnatally. (B) Ratio of ACTH-R mRNA (3·6 kb transcript)/18S rRNA (mean ± S.E.M. ROD) at different developmental ages. d, day(s); SL, spontaneous labour; NL, newborn lambs; L, prepubertal lambs. *Significantly (P<0·05) greater than other groups.

of fetuses, levels of ACTH-R mRNA were significantly greater than in controls (P<0·05; hypoxaemia 2·84 ± 0·30 ROD, n=4; normoxaemia 1·98 ± 0·18 ROD, n=4). Basal and hypoxaemia-induced levels of ACTH-R mRNA in the adrenal tissue of the older fetuses were significantly greater (P<0·05) than those in the younger animals.

Discussion

We have demonstrated that ACTH-R mRNA is detectable in the ovine fetal adrenal gland at about day 60 of gestation, then increases after days 126–128 to attain greatest levels at the onset of spontaneous labour. Levels of ACTH-R mRNA in the fetal adrenal increased significantly in response to hypoxaemia at days 134–136, but not in younger fetuses before HPA activation. Both the response to hypoxaemia in older fetuses and the prepartum increase in ACTH-R occurred in association with the known increase in plasma concentrations of ACTH and cortisol (Bassett & Thorburn 1969, Magyar *et al.* 1980, Norman *et al.* 1985, Braems *et al.* 1996), and seem likely to contribute to an enhanced fetal adrenal response.

The pattern of change in ACTH-R mRNA levels observed in this study is closely associated with the final phase of adrenocortical growth (Boshier & Holloway 1989), and occurs simultaneously with the known increase in sensitivity of the fetal sheep adrenal gland to ACTH stimulation late in gestation (Rose *et al.* 1982, Glickman & Challis 1980). Recently, Wang *et al.* (1999), using ribonuclease protection assay analysis, also reported an increase in ACTH-R mRNA between day 100 and day 140 of gestation, but did not include time periods in between, or the time of parturition and the neonatal period. Our results also show a close correlation with those obtained for ACTH binding capacity (Durand 1979), suggesting

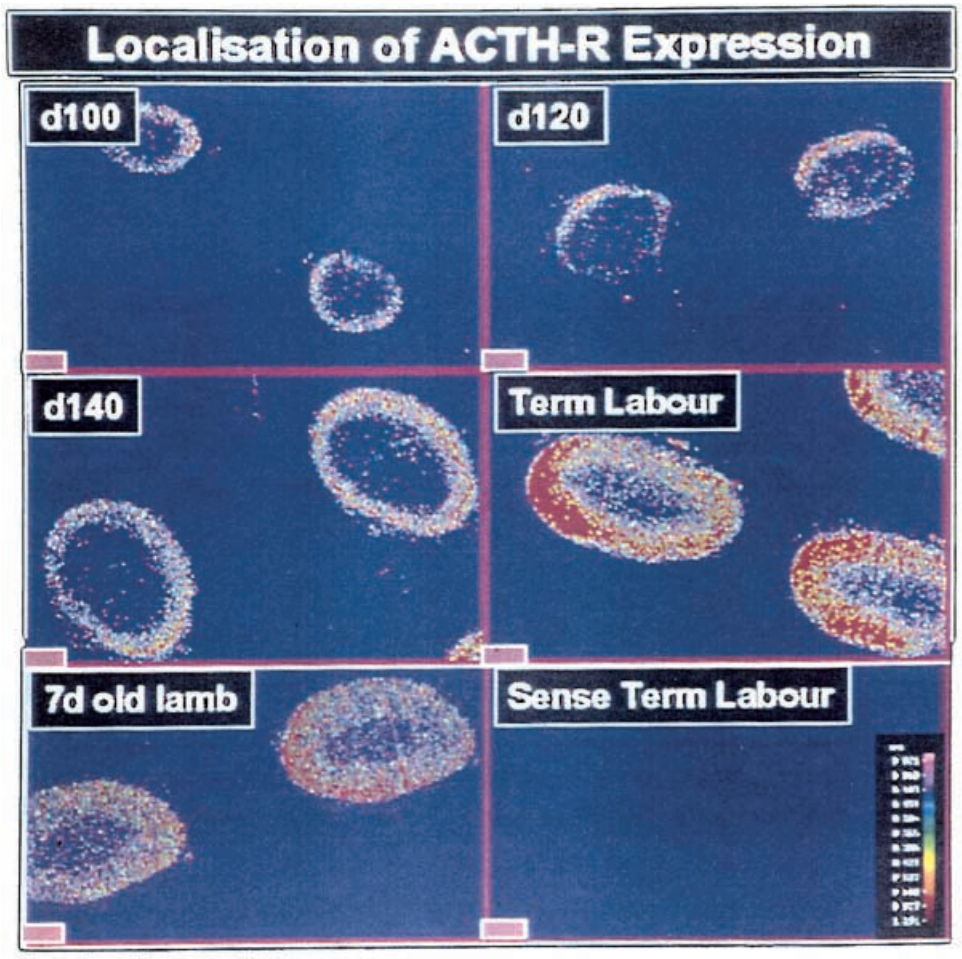

Figure 3 Localisation of ACTH-R mRNA in the adrenal after *in situ* hybridisation of sections of adrenal glands from fetal sheep at day 100, 120 and 140 of pregnancy, at term spontaneous labour and from newborn lamb, hybridised with ^{35}S-labelled antisense cRNA probe for ACTH-R. The specificity of hybridisation with antisense cRNA probe for ACTH-R was demonstrated by the lack of hybridisation with sense cRNA probe for ACTH-R.

parallel changes in mRNA and protein. As the values measured in the present study represent steady-state levels of ACTH-R mRNA, it is possible that alterations in the turnover of ACTH-R mRNA may contribute to the enhanced ACTH-R mRNA expression in the perinatal period. However, we did not observe any change in transcript sizes that might reflect altered processing or polyadenylation. Our finding of increased ACTH-R expression by *in situ* hybridisation is consistent with greater ACTH-R mRNA per adrenocortical cell, but also indicates recruitment of additional cells expressing ACTH-R in later pregnancy.

The sustained presence of ACTH-R mRNA in fetal adrenal tissue between day 65 and day 120 of ovine pregnancy contrasts with the decline in responsiveness of the gland to ACTH stimulation *in vitro*, between day 60 and day 100 (Wintour *et al.* 1975, Glickman & Challis 1980), which would therefore appear to be independent of altered ACTH-R expression. Thus altered adrenal responsiveness probably reflects either low levels of $ACTH_{1–39}$ in the circulation of the fetal sheep between days 80–115, as suggested by Wintour *et al.* (1995), or a greater proportion of large-molecular-weight peptides, derived from pro-opiomelanocortin (POMC), that are inhibitory to the adrenal effects of ACTH (Saphier *et al.* 1993, Jones & Roebuck 1980, Schwartz *et al.* 1995). There is diminished expression of P450 C17 in fetal adrenal tissue at days 80–100 of gestation, but the present studies show that the limitation to upregulation of P450 C17 in late pregnancy is apparently not due to the absence of ACTH-R, and reflects more the inadequate or inappropriate stimulus to the fetal adrenal cortical cells.

The prepartum increase in plasma ACTH and cortisol in fetal sheep occurs over the final 25 days of pregnancy

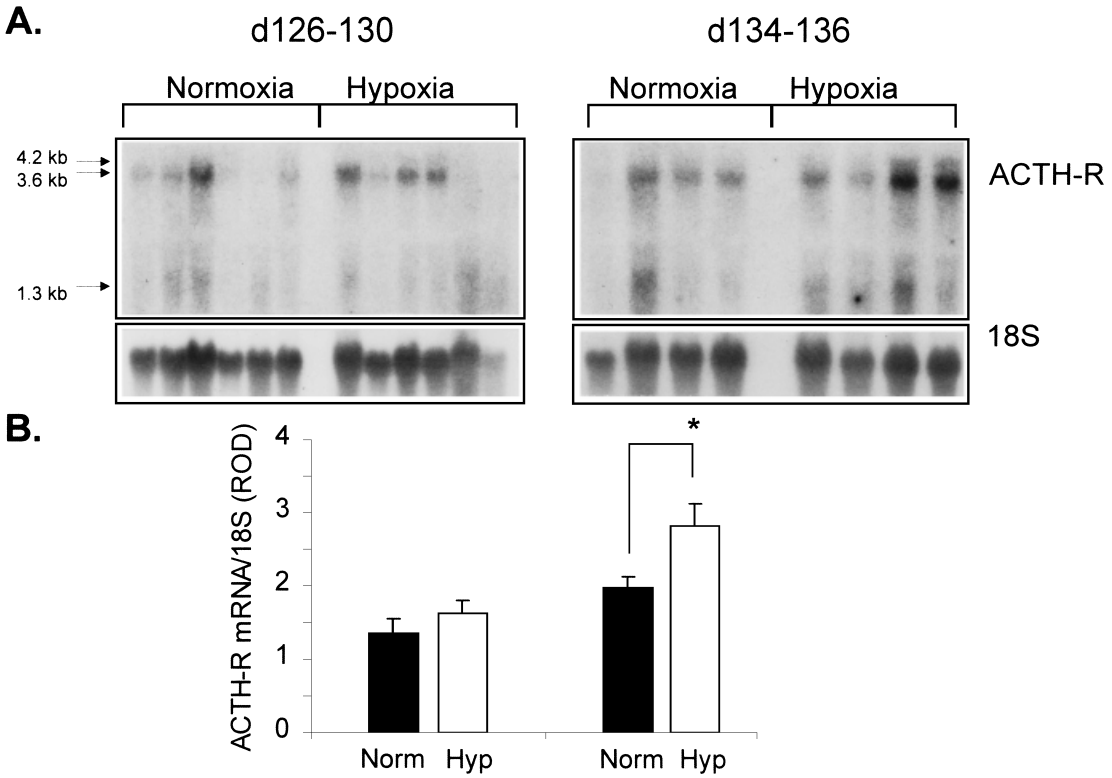

Figure 4(A) Northern blot hybrisation of total RNA from adrenal glands of fetal sheep between days (d) 126–130 and days 134–136 previously exposed to normoxaemia (solid bars in (B)) or hypoxaemia (open bars in (B)). In the younger gestational group (days 126–130), fetuses were exposed to normoxaemia ($n=6$) or hypoxia ($n=5$), beginning on days 124–128. In the older gestational age group (days 134–136), fetuses were exposed to normoxaemia ($n=4$) or hypoxia ($n=4$), beginning on days 132–134. The blots were probed with a ^{32}P-labelled ovine cDNA, then stripped and rehybridised with a labelled cDNA for mouse 18S rRNA. (B) Ratio of ACTH-R mRNA 3·6 kb transcript/18S rRNA (mean ± S.E.M. ROD). *Significant difference ($P<0·05$) between normoxaemia and hypoxaemia.

(Norman *et al.* 1985), consistent with a role for either ACTH or cortisol in the regulation of adrenal ACTH-R expression. *In vivo* and *in vitro* studies show that a characteristic of the *ACTH-R* gene is upregulation by its own ligand through a cAMP-dependent pathway (Pepe & Albrecht 1990, Lebrethon *et al.* 1994*a*,*b*, Mountjoy *et al.* 1994, Mesiano *et al.* 1996, Picard-Hagen *et al.* 1997). This effect is associated with increased production of cortisol. Studies of human, bovine and ovine adrenocortical cells in culture suggest that ACTH upregulates ACTH-R mRNA expression at both transcriptional and post-transcriptional level (Lebrethon *et al.* 1994*b*, Penhoat *et al.* 1994, Picard-Hagen *et al.* 1997). There is also evidence for glucocorticoid regulation of ACTH-R protein and mRNA. Glucocorticoids enhance both ACTH-induced cAMP production and ACTH or [^{8}Br]cAMP-induced steroidogenesis in ovine fetal and adult adrenocortical cells and this involves increased ACTH receptor activity (Picard-Hagen *et al.* 1997, Darbeida & Durand 1987, Darbeida *et al.* 1987). Picard-Hagen *et al.* (1997) showed that dexamethasone increased ACTH-R mRNA expression in cultured ovine adrenocortical cells, and that treatment with RU38486, an antiglucocorticoid, blocked this effect.

The recent demonstration of a glucocorticoid response element in the promoter region of the human and mouse *ACTH-R* genes supports an effect of glucocorticoid (Cammas *et al.* 1997, Naville *et al.* 1999). Naville *et al.* (1999) have also shown that there are three SF-1 binding sites within the *ACTH-R* promoter region, and that binding of SF-1 protein to all three sites is required for full constitutive activity of this gene. The present data lend support to the view that upregulation of ACTH-R mRNA expression by ACTH, cortisol, or both, may be an important maturational event near term, to ensure optimal adrenal responsiveness in preparation for labour and delivery. However, this conclusion should be tempered by the observation that exogenous glucocorticoid decreases *ACTH-R* expression in the primate fetal adrenal *in vivo* (Leavitt *et al.* 1997, Aberdeen *et al.* 1998). Whether these

are true species differences or differences between *in vivo* and *in vitro* experiments is unclear at present.

Other hormones and growth factors have been reported to be involved in the regulation of ACTH-R mRNA expression and receptor activity in the adrenal cortex. These include angiotensin II (Ang II), insulin-like growth factor II (IGF-II) and transforming growth factor β1 (TGFβ1). Ang II inhibits adrenal steroidogenesis in ovine fetal adrenocortical cells (Bird *et al.* 1992), but enhances responsiveness to ACTH and increases ACTH-R mRNA expression in human fetal (Lebrethon *et al.* 1994*a*, Mountjoy *et al.* 1994) or adult bovine adrenocortical cells (Penhoat *et al.* 1994). IGF-II is an important local regulator of fetal adrenal development (Mesiano & Jaffe 1997) that increases ACTH responsiveness (Naaman *et al.* 1989) and increases expression of the ACTH-R (Penhoat *et al.* 1989). In contrast, TGFβ1 inhibits basal and ACTH-stimulated cortisol output and reduces ACTH-R number in ovine adult adrenal cortical cells (Rainey *et al.* 1988, 1989). Clearly, the effects of IGF-II and TGFβ1 on ACTH-R and adrenal function could be exerted through circulatory peptide, or through intra-adrenal peptide, under the influence of circulating ACTH, cortisol, or both.

We recognise that altered expression of ACTH-R is only one step in the potential regulation of fetal adrenal function. We and others (Challis & Brooks 1989) have discussed the multifactorial regulation of fetal adrenal responsiveness, and particularly the critical part that up-regulation of P450 C17 and enhanced coupling of ACTH-R through $G_{\alpha s}$ to adenylate cyclase play in the late gestational changes in adrenal sensitivity. Expression of several of the genes required for cortisol synthesis is increased by ACTH infusion to the chronically catheterised ovine fetus (Tangalakis *et al.* 1990) even early in gestation, when expression of ACTH-R mRNA is at the lowest levels determined in the present study.

It is well established that hypoxaemia results in acute upregulation of fetal HPA function, with increased expression of hypothalamic corticotrophin-releasing hormone mRNA and pituitary POMC mRNA (Matthews & Challis 1995) and ACTH-dependent increases in fetal adrenocortical blood flow (Carter *et al.* 1993). In the present experiments, we found that 48 h of hypoxia, in the absence of a change in fetal pH or partial pressure of carbon dioxide (Braems *et al.* 1996), led to a significant increase in levels of ACTH-R mRNA in adrenals from fetuses at days 134–136, but not at days 126–130. Plasma ACTH increased in response to hypoxaemia in both groups of fetuses, but a sustained increase in cortisol was maintained only in the older animals (Braems *et al.* 1996). It is possible that the increase in cortisol contributes to regulation of ACTH-R mRNA expression. Alternatively, increased ACTH-R could enhance adrenal responsiveness and cortisol output, as mRNA levels encoding key steroidogenic enzymes were also increased in these fetuses (Braems *et al.* 1998). Thus, with hypoxaemia, a feed-forward loop may be established, with increased cortisol synthesis driving enhanced adrenal responsiveness (Lye & Challis 1984), potentially resulting in preterm birth.

In summary, we have demonstrated that, although there is no lack of ACTH-R expression in the adrenal of the mid-gestation ovine fetus that would explain diminished ACTH responsiveness, the late-gestation increase in ACTH-R probably contributes to enhanced adrenal cortisol output at term. The finding that ACTH-R mRNA levels increase in response to hypoxaemia is consistent with these observations. We suggest that ACTH/cortisol may contribute to the stimulus to ACTH-R expression, which in turn results in increased fetal adrenal steroidogenesis, establishing a feed-forward loop that is broken only at the time of birth.

Acknowledgements

This work was supported by the Medical Research Council of Canada (Canadian Institutes for Health Research, CIHR; Group Grant in Fetal and Neonatal Health and Development). We thank Dr Geoffrey Hammond (Director, CIHR Group Molecular Core) for assistance in preparing the ACTH-receptor cDNA, and Mr Doug Johnson for his help with supplying animals.

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Received in final form 13 November 2000
Accepted 28 November 2000