GH-receptor distribution in the ovine foetal adrenal gland: ontogenic and functional studies

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Abstract

In order to examine the role of GH in the regulation of foetal adrenal development and function, we have localized GH-receptor mRNA and protein in adrenal glands of ovine foetuses at specific stages of gestation. Adrenals from 60–75 day ($n=4$), 100–110 day ($n=4$) and 140–145 day ($n=3$) foetal sheep (term is 145–150 days) and non-pregnant adult animals ($n=3$) were dissected and fixed. GH-receptor mRNA localization was studied by *in situ* hybridization using a ³⁵S-labelled antisense cRNA probe, and protein by immunohistochemistry using a specific monoclonal antibody to the GH-receptor. At all ages studied, GH-receptor mRNA and immunoreactivity could be detected throughout the adrenocortical region. In adult adrenals, GH-receptor mRNA and immunoreactivity were also evident throughout the adrenocortical zone, with the strongest expression confined to a defined region of cells at the interface between the zona glomerulosa and zona fasciculata. Northern blot analysis of 100 day and 140 day foetal adrenals confirmed the presence of a 4.4 kb GH-receptor mRNA transcript, while immunoblotting of

foetal adrenals at approximately 110 days of gestation revealed a 55 kDa GH-receptor species. To study the effect of GH on the function and growth of the immature foetal adrenal gland *in vivo*, chronically catheterized ovine foetuses ($n=4$), between 100 and 110 days of gestation, were given a pulsatile infusion of recombinant bovine GH (125 µg/15 min, 24 pulses/24 h) for 72 h. Plasma cortisol and aldosterone levels were compared with age-matched controls receiving saline infusion alone ($n=4$). It was found that there was no difference in the basal plasma level of cortisol or aldosterone, and that infusion of GH did not alter steroid levels or gross adrenal size and morphology. These studies demonstrate strong expression of the GH-receptor in the developing ovine foetal adrenal cortex. However, in the immature foetus GH infusion is without effect on plasma steroid levels, suggesting that the steroidogenic action of GH in the ovine foetus may be gestationally dependent.

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Introduction

While adrenocorticotrophin (ACTH) is considered to be the principal regulator of adrenal growth and function, other pituitary hormones have been implicated in the control of adrenal steroid synthesis. In particular, growth hormone (GH) has been shown to potentiate the corticosterone response to ACTH in rats (Palmore *et al.* 1970). *In vivo*, acute administration of GH to hypopituitary children increases their dehydroepiandrosterone levels (Rudd *et al.* 1980), while prolonged treatment of GH-deficient children with GH potentiates the cortisol response to ACTH (Castro-Magana *et al.* 1983). GH has also been shown to increase aldosterone secretion in normal human subjects, although this response appears to be mediated by the activation of the renin–angiotensin system (Ho & Weissberger 1990). The involvement of GH in adrenal steroidogenesis may also extend to foetal

life. *In vitro*, addition of GH to culture medium stimulates corticosteroid production by foetal sheep and rabbit adrenocortical cells (Devaskar *et al.* 1981) and dehydroepiandrosterone and dehydroepiandrosterone sulphate formation by foetal baboon adrenocortical cells (Walker *et al.* 1988). However, information regarding the *in vivo* regulation of foetal adrenal function and growth by GH, particularly during early development, is limited.

The ovine foetus provides an appropriate animal model to study the steroidogenic action of GH, as the morphological and functional development of the adrenal gland has been well characterized in this species. In the present study, we have defined the pattern of adrenal GH-receptor gene and protein expression at specific stages of ovine gestation, using *in situ* hybridization and immunohistochemistry. We have also studied, *in vivo*, the effect of a sustained (72 h) infusion of recombinant bovine GH on the function (cortisol and aldosterone production) and

growth of the immature adrenal gland of foetal sheep, aged between 100 and 110 days of gestation (term is 145–150 days). At this age, circulating levels of GH in the ovine foetus are low (Gluckman *et al.* 1979) and cortisol production is at a nadir, due to a low level of pituitary drive and expression of key steroidogenic enzymes (Wintour *et al.* 1975, Glickman & Challis 1980, Hennessy *et al.* 1982, Tangelakis *et al.* 1989, 1990). Therefore, these studies would allow us to test the ability of exogenous GH to stimulate foetal adrenal activity and 'switch-on' steroid production at a time when the ovine foetal adrenal is normally in a quiescent state.

Methods

These studies were performed with the approval of the Animal Ethics Committee of the Howard Florey Institute of Experimental Physiology and Medicine, in accordance with the guidelines of the National Health and Medical Research Council of Australia.

Receptor studies

Tissues and tissue preparation Pregnant Merino ewes (of known mating dates) and non-pregnant adult sheep ($n=3$) obtained from the Howard Florey Institute of Experimental Physiology and Medicine Research Farm (Tooradin, Victoria, Australia) were killed with an overdose of pentobarbitone sodium (100 mg/kg body weight; Arnolds, Reading, UK). Three groups of foetal sheep were used for *in situ* hybridization and immunohistochemical studies: 60–75 days ($n=4$), 100–105 days ($n=4$) and 140–145 days ($n=3$), term in this species being 145–150 days. Tissues were removed, fixed in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 24 h at room temperature and then paraffin embedded. Sections of 5 μm were cut and mounted on aminoalkylsilane-coated slides and baked overnight at 37 °C. Adult and foetal tissue samples were also snap frozen in liquid nitrogen for Northern blot analysis and immunoblotting.

Labelling of RNA probes for *in situ* hybridization

Complementary (antisense) or non-complementary (sense) RNA probes were synthesized from an ovine GH-receptor cDNA clone, kindly provided by Dr Timothy Adams (CSIRO Division of Biomolecular Science, Parkville, Victoria, Australia), which encodes the full-length transmembrane ovine GH-receptor (Fiddes *et al.* 1992). RNA probes were synthesized in 20 μl reactions containing 100 μCi [^{35}S]cytidine 5'-triphosphate (1000–1500 Ci/mmol; Du Pont, Sydney, Australia), 40 mM Tris-HCl, pH 7.5, 6 mM MgCl_2 , 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 660 μM each of ATP, GTP, and UTP, 20 U RNasin, 100 ng linearized

DNA template and 20 U SP6 or T7 RNA polymerase. The reaction was incubated at 37 °C for 90 min, after which the DNA template was digested with 1 U DNase for 15 min at 37 °C. RNA probes were ethanol precipitated with yeast RNA as a carrier. Enzymes and the above reagents were obtained from Promega Corp. (Madison, WI, USA). Purified probes (antisense and sense) were adjusted to an average length of 150 bases by alkaline hydrolysis. The average specific activity of the RNA probes generated was 3×10^9 c.p.m./ μg RNA.

***In situ* hybridization** Paraffin sections were deparaffinized in xylene, hydrated in graded ethanol and ultrapure water (Liquipure; Continental Water Systems, Melbourne, Australia), equilibrated in buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 8.0) and treated with Pronase E (Sigma Chemical Co., St Louis, MO, USA) (125 $\mu\text{g}/\text{ml}$). After digestion, the sections were washed in 0.1 M sodium phosphate buffer, pH 7.2, ultrapure water, dehydrated in 70% ethanol and air dried. The ^{35}S -labelled RNA probes (5×10^5 c.p.m./25 μl hybridization buffer) were added to a hybridization buffer consisting of 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM Na_2HPO_4 , pH 6.8, 5 mM EDTA, pH 8.0, $1 \times$ Denhardt's solution, 50 mg/ml yeast RNA, 50% deionized formamide and 10% (w/v) dextran sulphate. The hybridization buffer containing labelled probe was preheated to 80 °C (5 min). Appropriate volumes were placed on the sections, which were then coverslipped and placed in humidified (50% formamide) chambers for 14–16 h incubation at 60 °C.

Slides were washed in $2 \times \text{SSC}$ (0.3 M NaCl, 0.33 M trisodium citrate dihydrate) containing 50% formamide preheated to 55 °C to remove coverslips, then washed in $2 \times \text{SSC}/50\%$ formamide at 55 °C for 1.5 h. Sections were then washed in several changes of RNase A buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 0.5 M NaCl), preheated to 37 °C and then treated with 150 $\mu\text{g}/\text{ml}$ RNase A in RNase A buffer for 1 h at 37 °C followed by a wash in $2 \times \text{SSC}$ at 55 °C for 45 min. After final dehydration through graded ethanol, slides were air-dried and autoradiographed with Kodak XAR film (Eastman Kodak, Rochester, NY, USA) for 1–3 days at room temperature.

Slides were then dipped in photographic emulsion (Amersham International, Amersham, Bucks, UK), stored with desiccant at 4 °C for approximately 14–21 days, developed in Kodak D19, fixed in Ilford Hypam (Ilford Pty Ltd, Mount Waverly, Australia), and stained with haematoxylin for evaluation.

Immunohistochemistry Paraffin sections were deparaffinized in xylene, hydrated in graded ethanol and ultrapure water and subjected to immunohistochemical staining. Monoclonal antibody 263 (mAb 263; Agen Biomedical Ltd, Brisbane, Queensland, Australia)

was used at a concentration of 1:100 to detect the GH-receptor; this antibody recognizes an epitope on the external domain of the rat GH-receptor (Barnard *et al.* 1985), and has been shown to bind the GH-receptor of other species including the sheep (Breier *et al.* 1994). As no purified ovine GH-receptor protein was available, control slides were incubated with PBS or a mouse IgG of the same isotype as mAb 263 (Sigma). Briefly, sections were incubated in H_2O_2 in PBS (pH 7.4) to quench endogenous peroxidase activity and non-specific protein binding was eliminated by incubation with horse serum. Sections were then incubated with the diluted primary antibody (mAb 263 or mouse IgG) or PBS for 60 min at room temperature. After several washes in PBS, the tissues were incubated with an avidin-biotin complex (ABC) sequence (Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, CA, USA). The ABC sequence consisted of incubation with a biotinylated secondary antibody (horse anti-mouse) followed by incubation with an avidin-biotinylated peroxidase solution. The tissue was then exposed to the chromogen (0.033% 3,3'-diamino benzidine tetrachloride (DAB)) and 0.03% H_2O_2 as a source of peroxide substrate for 2–4 min. Following the DAB/peroxidase reaction sections were counterstained with haematoxylin, dehydrated, cleared and mounted. Slides were examined by bright-field microscopy to determine the extent and localization of the DAB staining (GH-receptor distribution).

To distinguish cortical and medullary regions of the adrenal gland, a rabbit polyclonal antiserum (Chemicon International, Temecula, CA, USA) developed against an internal domain of rat cytochrome P450 side-chain

cleavage was used on serial sections (1:200 dilution). The protocol was the same as that described above, although normal goat serum was used to eliminate non-specific protein binding and the biotinylated secondary antibody was goat anti-rabbit. Controls sections were incubated with PBS or normal rabbit serum.

Image analysis Bright- and dark-field photomicrographs were acquired using a Sony 3CCD colour video camera DXC-950P (Sony Australia Ltd, Melbourne, Australia) coupled to a Nikon Optiphot microscope (Laboratory Supplies, Melbourne, Australia). Images were analysed using the MCID (microcomputer imaging device) M4 image analyser (Imaging Research Inc., St Catherines, Canada).

Northern blot analysis Total RNA was isolated from maternal liver and 100 and 140 day foetal adrenals, using the guanidinium-thiocyanate method (Chomczynski & Sacchi 1987). Total RNA was then denatured in 1 M glyoxal, 54% dimethylsulphoxide, 10 mM sodium phosphate buffer (pH 7.0) at 50 °C for 1 h, fractionated on a 1% agarose gel with recirculating buffer (10 mM sodium phosphate, pH 7.0), transferred to Hybond-N membrane (Amersham) in $20 \times$ SSC (3 M NaCl, 0.3 M trisodium citrate dihydrate, pH 7.0) and UV cross-linked (2.5 J/cm^2). The cDNA for the ovine GH-receptor was labelled with [^{32}P]dCTP using a random priming kit (Boehringer Mannheim, Sydney, Australia) to an average specific activity of 1×10^9 d.p.m./ μg . Membranes were prehybridized at 42 °C (5 h) and hybridized with labelled

Figure 1 GH-receptor gene expression in the ovine foetal and adult adrenal as shown by *in situ* hybridization. Film autoradiographs of adrenal sections (60 day, 100 day, 140 day foetuses and adult tissue) hybridized to GH-receptor riboprobe: antisense on the left, sense on the right. Cx=cortex, M=medulla. Bar=1 cm.

Figure 2 Bright-field (A and E) and dark-field (B and F) photomicrographs showing GH-receptor gene expression in a 60 day (A and B) and 140 day (E and F) foetal adrenal. Corresponding bright-field photomicrographs of adjacent sections show GH-receptor immunoreactivity (brown stain) in the 60 day (C) and 140 day (G) adrenal, and side-chain cleavage immunoreactivity in the 60 day (D) and 140 day (H) adrenal. GH-receptor immunoreactivity (brown stain) in the adult sheep adrenal cortex (I). Immunoreactivity is found throughout the zona fasciculata and patches of cells in the zona glomerulosa. No staining is evident when non-specific mouse IgG is substituted for the primary antibody (J). c=capsule, ZG=zona glomerulosa, ZF=zona fasciculata, M=medulla. Magnification $\times 100$.

cDNA separately at 60 °C overnight in 0.05% (w/v) sodium pyrophosphate, 2.5 × SSC, 5 × Denhardt's solution, 25 mM sodium phosphate, 0.5% (w/v) SDS and 100 µg/ml boiled herring sperm DNA. Membranes were then washed twice in 2 × SSC, 0.1% SDS for 30 min at room temperature and once in 0.1 × SSC, 0.1% SDS for 30 min at 60 °C. Membranes were then exposed to X-ray film (Kodak, Rochester, NY, USA) for 3–12 days.

Immunoblotting analysis Ten micrograms of protein per lane, isolated from non-pregnant adult kidney, and 112 and 113 day foetal adrenal homogenates, were electrophoresed on a 7.5% polyacrylamide gel containing SDS and electrophoretically transferred to polyvinylidene fluoride filters. The filters were blocked with 5% non-fat dry milk powder in Tris-buffered saline for 2 h and incubated overnight at room temperature with the GH-receptor antibody (mAb 263) diluted 1:330 in 1% non-fat dry milk powder in 0.05% Tween-20 Tris-buffered saline. The filters were washed and incubated for 60 min at room temperature with affinity-purified goat anti-mouse IgG (Bio-Rad, Richmond, CA, USA) diluted 1:2000 with 1% non-fat dry milk powder solution. Afterwards, the filters were washed and incubated with enhanced chemiluminescence detection reagents (Amersham) for 1 min after which they were exposed to X-ray film (Kodak X-Omat AR) for 1 min.

In vivo studies

Animals The foetuses of eight Merino ewes (between 96 and 100 days of gestation) were cannulated under general anaesthesia as described previously (Lingwood *et al.* 1978). Silastic cannulae (0.76 mm internal diameter, 1.65 mm outside diameter) were inserted into one carotid artery, one jugular vein and transabdominally into the foetal bladder. The right jugular vein of the ewe was also cannulated at the time of surgery. Ewes were housed in individual metabolic cages following surgery, with food and water freely available. Vascular cannulae (foetal and maternal) were flushed daily with heparinized saline. Foetal arterial blood gases and urine osmolality were measured daily to assess foetal well-being.

Protocol Animals were allowed at least 4 days to recover following surgery. On the initial day of the experiment (Day 1, t=0), a sample of foetal blood was removed via the arterial cannula. From this sample, foetal blood gases were determined and plasma was assayed for cortisol and aldosterone. At all times, the volume of blood removed from the foetus was replaced with an equal volume of saline. A sustained (72 h) infusion of recombinant bovine GH (125 µg/15 min pulse, 24 pulses/24 h, n=4 foetuses) or isotonic saline (0.19 ml/h, n=4 foetuses) was then initiated via the foetal jugular cannula using a Braun

Perfusor Pump (Melsungen, Hassen, Germany), the infusion protocol designed to mimic the normal pulsatile pattern of GH secretion (Bassett & Gluckman 1986). Recombinant bovine GH was kindly provided by Dr William R Baumbach (American Cyanamid Company, Princeton, NJ, USA). On Day 2 (t=24 h), Day 3 (t=48 h) and Day 4 (t=72 h) blood was again taken from the foetus for the measurement of blood gases and plasma cortisol and aldosterone. At the end of the infusion (t=72 h) animals were killed with an overdose of pentobarbitone sodium and an immediate post-mortem examination conducted. Foetal adrenals were excised, weighed and processed to paraffin for histological examination.

Sample analysis Foetal arterial blood gases were measured on a Ciba Corning 278 Blood Gas System and a Corning 2500 Co-oximeter (Australian Diagnostics Corp., Melbourne, Australia). Urine osmolality was measured on an Advanced Instruments Osmometer (Advanced Instruments, Needham Heights, MA, USA).

Hormone assays Foetal blood samples were taken into pre-chilled tubes and centrifuged immediately, the plasma being stored at -20 °C until assayed.

Analysis of cortisol was carried out by RIA (Tangalakidis *et al.* 1989). This assay was shown to have an intra-assay coefficient of variation of 10.3% at 50 pg/tube. The inter-assay variation at 30 pg/tube was 13.1%, and 10.7% at 200 pg/tube. The sensitivity of this assay has been shown to compare favourably with the double-isotope dilution derivative assay, which has been validated for use with adult and foetal sheep plasma (Brown *et al.* 1978).

Aldosterone concentration was measured by use of a described RIA (Gogerly *et al.* 1993). This assay has intra- and inter-assay coefficients of variation of 7.8 and 11% respectively. Cross-reactivity with other steroids is less than 0.01%.

Statistics Values appearing in the text and figures are the means ± s.e. Plasma hormone data was analysed by two-way ANOVA, using the Prism GraphPad software package (GraphPad Software Inc., San Diego, CA, USA). For vertical pair-wise comparisons (between corresponding time-points in different treatment groups) the Bonferroni adjustment was made (Ludbrook 1994). *P*<0.05 was considered to indicate significance.

Results

Receptor studies

In situ hybridization There was abundant expression of GH-receptor mRNA throughout the cortical region of the developing ovine foetal adrenal gland (see Fig. 2), with a

Figure 3 (A) Northern blot analysis of 25 µg ovine foetal adrenal total RNA following hybridization with an ovine GH-receptor cDNA. GH-receptor mRNA is detected at 4.4 kb. Lane 1=100-day-old ovine foetal adrenal; Lane 2=140-day-old ovine foetal adrenal; Lane 3=ovine maternal liver. (B) Immunoblot analysis of ovine foetal adrenal tissue using an anti-GH-receptor antibody (mAb263). Lane 1=ovine adult kidney; Lane 2=112-day-old foetal adrenal; Lane 3=113-day-old foetal adrenal.

strong hybridization signal evident after 2–3 days exposure of sections to autoradiographic film (Fig. 1). The background, as identified by hybridization with sense probes under identical conditions, was minimal. The signal in the medullary region was not above background. In the adult adrenal, GH-receptor mRNA was also evident throughout the cortical region (albeit at a lower level than the foetal adrenal), with stronger expression confined to a region at the interface of the zona glomerulosa and zona fasciculata (Fig. 1).

Immunohistochemistry In foetal and adult tissues GH-receptor immunoreactivity was confined to the cortical region of the adrenal gland, as defined by the localization of cytochrome P₄₅₀ side-chain cleavage immunoreactivity (Figs 2 and 3). Immunoreactivity was abolished when non-specific mouse IgG was substituted for the primary antibody (in the case of mAb 263, Fig. 2J) or normal rabbit serum was used in place of the primary

antisera (in the case of P₄₅₀ side-chain cleavage, data not shown). While there was parallel expression of GH-receptor protein and mRNA throughout the foetal adrenal cortex, in some intracortical regions uniformity of expression was not apparent, particularly in the adrenals of 60 day animals (Fig. 2A–C). In the late foetal (140–145 day) and adult adrenal gland the immunoreactivity was apparent in scattered zona glomerulosa cells and in most, but not all, of the zona fasciculata cells. In the adult adrenal cortex the strongest expression of GH-receptor appeared in the cells between the glomerulosa and fasciculata.

Northern blot and immunoblotting analysis To further confirm the identity of locally synthesized GH-receptor in ovine foetal adrenal glands, total RNA (25 µg) extracted from 100 and 140 day foetal adrenals was subjected to Northern blot analysis. As shown in Fig. 3A, a 4.4 kb GH-receptor mRNA transcript was identified in 100 day (lane 1) and 140 day (lane 2) foetal adrenal total RNA. The 4.4 kb GH-receptor mRNA band was also detected in maternal liver (lane 3). Immunoblotting of 112 and 113 day foetal adrenal homogenates, using mAb 263 antibody, showed the presence of a GH-receptor species at approximately 55 kDa (Fig. 3B, Lanes 2 and 3). A 55 kDa band was also seen with non-pregnant adult kidney (lane 1).

In vivo studies

Animal status Foetal blood gases and urine osmolality were in the normal range for this age and did not change during the course of the experiment (data not shown).

Hormone levels Foetal plasma concentrations of cortisol and aldosterone are shown in Fig. 4. There was no difference in the basal level of cortisol or aldosterone between groups, nor did infusion of GH alter plasma levels.

Adrenal size and morphology At post-mortem, paired foetal adrenals weighed 0.16 ± 0.02 and 0.22 ± 0.04 g in the saline and GH groups respectively. Histological examination of adrenals from GH- and saline-infused animals revealed no significant change in gross anatomical structure.

Discussion

In the present study we have shown the GH-receptor (mRNA and protein) to be localized to the cortical region of the developing ovine foetal adrenal gland throughout gestation. At 60 days gestation the cortex contains one cortical cell type with both vesicular and lamellar cristae in the mitochondria. By 100 days there are two distinct zones – the glomerulosa with a mature phenotype and the fasciculata, with still an immature phenotype (Robinson *et al.* 1979). Future medullary cells are present by 60 days,

Figure 4 Plasma levels of cortisol and aldosterone in ovine foetuses during a 72 h infusion of recombinant bovine GH (■, $n=4$) or isotonic saline (□, $n=4$). Values are means $\pm$ S.E.

and form a distinct inner medulla by 80 days. Medullary cells did not express the GH-receptor mRNA or protein. Northern blot analysis of 100 and 140 day foetal adrenals confirmed the presence of a 4.4 kb GH-receptor mRNA transcript, consistent with that previously identified in the adult and foetal sheep liver (Klempt *et al.* 1993). Immunoblotting using foetal adrenal tissue at approximately 110 day of gestation identified a GH-receptor species at the predicted size of 55 kDa (Freemark *et al.* 1987).

Previous studies have shown that human GH is capable of directly stimulating cortisol production by freshly isolated late-gestation ovine foetal adrenocortical cells (Devaskar *et al.* 1981). Our finding that the GH-receptor is also expressed in the immature adrenal cortex, from at least 60 days of gestation, suggests that GH may play a role in foetal adrenal steroidogenesis from early in development. Post-natally, insulin-like growth factor-I (IGF-I) is the classical mediator of GH action. IGF-I and IGF-II are also expressed throughout the human (Han *et al.* 1988) and ovine foetal adrenal (Han *et al.* 1992), and may be local mediators of GH action, having been shown to stimulate proliferation, differentiation and steroid production by cultured adrenocortical cells (Penhoat *et al.* 1994). Although no information is available regarding the GH-dependent expression of the IGFs in the foetal adrenal gland, other foetal tissues (from a number of species) have been shown to release IGF-I and IGF-II in response to GH (Swenne *et al.* 1987, Werther *et al.* 1990, Goodyer *et al.* 1994), suggesting that the GH-receptor is functional in other non-adrenal tissues during foetal life. In the

present study, Northern blot analysis and immunoblotting of ovine foetal adrenals demonstrate a GH-receptor mRNA transcript and protein band of equivalent size to that expressed by the adult sheep liver (Freemark *et al.* 1987, Klempt *et al.* 1993). Furthermore, chronic administration of recombinant bovine GH to foetal sheep at approximately 110 days of gestation has been shown to stimulate foetal growth and placental and spleen weights (Bauer 1996), suggesting that recombinant bovine GH is at least 'biologically active' in the ovine foetal system, at an age equivalent to that used in our studies.

While GH has been shown to stimulate adrenal cortisol production in mature foetal sheep between 137 and 143 days of gestation (Devaskar *et al.* 1981), GH infusion was without an effect on steroid production in the present study. The activity of the ovine foetal adrenal shows a triphasic pattern, corresponding to the circulating levels of ACTH and the level of expression of key steroidogenic enzymes (Wintour *et al.* 1975, Glickman & Challis 1980, Hennessy *et al.* 1982, Tangalakakis *et al.* 1989, 1990). Between approximately 90 and 120 days of gestation, plasma levels of ACTH are low and the adrenal is quiescent and does not respond to stimuli in the short-term. This is in contrast to earlier (<90 days) and later (>120 days) in gestation when the adrenal is active. Given the assumption that a functional GH-receptor is expressed by the 100 day ovine foetal adrenal, the lack of an effect of GH in the present study may relate to the quiescent state of the foetal adrenal at this age, the action of GH requiring a critical level of adrenocortical activity or priming by ACTH. Indeed, several studies in adult animals or human subjects have shown a synergistic effect of GH on ACTH-stimulated steroid biosynthesis (Palmore *et al.* 1970, Castro-Magana *et al.* 1983). Therefore in the foetus, GH may also play a more permissive role (facilitating the response to ACTH), the steroidogenic effect of GH seen in the late gestation ovine foetus relating to the higher level of ACTH stimulus and adrenocortical activity at this time.

A unique pattern of GH-receptor localization was seen in the adrenal glands of adult animals. Although the GH-receptor was present throughout the adrenocortical region, as seen in the foetal adrenal, a relatively higher level of gene and protein expression was confined to a distinct region at the interface of the zona glomerulosa and zona fasciculata. Studies in the adult rat have identified a novel cell layer devoid of both mineralocorticoid- and glucocorticoid-synthesizing enzymes between the zona glomerulosa and zona fasciculata, which is believed to be the progenitor cell zone of the rat adrenal cortex (Mitani *et al.* 1994). A thin layer of cells (which may be analogous to the progenitor zone of the rat adrenal) has also been identified between the zona glomerulosa and zona fasciculata of the adult sheep adrenal cortex (McDougall *et al.* 1980), the so called 'intermediate cells' of this zone possessing unique cellular morphology intermediate

between typical zona glomerulosa and zona fasciculata cells. The localization of the GH-receptor to this region suggests that GH may play a role in the differentiation and/or growth of the cells of this zone.

In summary, the ovine foetus has high circulating GH levels (Gluckman *et al.* 1979) and we have shown in the present study that the GH-receptor is strongly expressed in the developing ovine foetal adrenal cortex, suggesting a possible involvement of GH in the growth and function of this organ. Interestingly, the profile of GH in the ovine foetal circulation during pregnancy correlates well with the triphasic pattern of adrenocortical activity in the ovine foetus previously described – GH levels are high between 60 and 90 days (when the adrenal is active and undergoing rapid growth), low between 90 and 120 days (when the adrenal is quiescent), with another GH surge prior to term (Gluckman *et al.* 1979). However, our *in vivo* studies, using immature animals between 100 and 110 days of gestation, demonstrate a lack of an effect of GH infusion on adrenocortical activity or growth at this age. This is in contrast to the situation in the late gestation animal, where GH stimulates cortisol production *in vivo* and *in vitro* (Devaskar *et al.* 1981), suggesting that the steroidogenic action of GH in the ovine foetus may be gestationally dependent, possibly requiring a critical level of ACTH exposure.

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References

- Barnard R, Bundesen PG, Rylatt DB & Waters MJ 1985 Evidence from the use of monoclonal antibody probes for structural heterogeneity of the growth hormone receptor. *Biochemical Journal* **231** 459–468.
- Bassett NS & Gluckman PD 1986 Pulsatile growth hormone secretion in the ovine fetus and neonatal lamb. *Journal of Endocrinology* **109** 307–312.
- Bauer M 1996 Treatment of fetal sheep with growth hormone results in increased placental and fetal weights. Paper presented at The Tenth Annual Workshop on Fetal and Neonatal Physiology, The University of Adelaide, March 22–23, 1996.
- Breier BH, Funk B, Surus A, Ambler GR, Wells CA, Waters MJ & Gluckman PD 1994 Characterization of ovine growth hormone (oGH) and ovine placental lactogen (oPL) binding to fetal and adult hepatic tissue in sheep: evidence that oGH and oPL interact with a common receptor. *Endocrinology* **135** 919–928.
- Brown EH, Coghlan JP, Hardy KJ & Wintour EM 1978 Aldosterone, corticosterone, cortisol, 11-deoxycortisol and 11-deoxycorticosterone concentration in the blood of chronically cannulated ovine fetuses: effect of ACTH. *Acta Endocrinologica* **88** 364–374.
- Castro-Magana M, Maddaiah VT, Collipp PJ & Angulo M 1983 Synergistic effects of growth hormone therapy on plasma levels of 11-deoxycortisol and cortisol in growth hormone-deficient children. *Journal of Clinical Endocrinology and Metabolism* **56** 662–667.
- Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159.
- Devaskar UP, Devaskar SU, Voina S, Velayo N & Sperling MA 1981 Growth hormone stimulates adrenal steroidogenesis in the fetus. *Nature* **290** 404–405.
- Fiddes RJ, Brandon MR & Adams TE 1992 Functional expression of an ovine growth hormone receptor in transfected Chinese hamster ovary cells. *Molecular and Cellular Endocrinology* **86** 37–47.
- Freemark M, Comer M, Korner G & Handwerger 1987 A unique placental lactogen receptor: implications for fetal growth. *Endocrinology* **120** 1865–1872.
- Glickman JA & Challis JRG 1980 The changing response pattern of sheep fetal adrenal cells throughout the course of gestation. *Endocrinology* **106** 1371–1376.
- Gluckman PD, Mueller PL, Kaplan SL, Rudolph AM & Grumbach MM 1979 Hormone ontogeny in the ovine fetus. I. Circulating growth hormone in mid- and late gestation. *Endocrinology* **104** 162–168.
- Gogerly RL, Coghlan JP, Morgenroth P & McDougall JG 1993 Two-compartment model of acute stimulation of aldosterone secretion *in vivo* by angiotensin II. *American Journal of Physiology* **264** E456–E464.
- Goodyer CG, Torban E, Zogopoulos G, Ferretti E, Khalil A, Guyda HJ & Goodyer PR 1994 Role of growth hormone during human fetal kidney development. In *Program of the 76th Annual Meeting of the Endocrine Society, Anaheim, CA, 1994*, p 433, abstract 930.
- Han VKM, Lund PK, Lee DC & D'Ercole AJ 1988 Expression of somatomedin/insulin-like growth factor messenger RNAs in the human fetus: identification, characterization and tissue distribution. *Journal of Clinical Endocrinology and Metabolism* **66** 422–429.
- Han VKM, Lu F, Bassett N, Yang KP, Delhanty PJD & Challis JRG 1992 Insulin-like growth factor-II (IGF-II) messenger ribonucleic acid is expressed in steroidogenic cells of the developing ovine adrenal gland: evidence of an autocrine/paracrine role of IGF-II. *Endocrinology* **131** 3100–3109.
- Hennessy DP, Coghlan JP, Hardy KJ & Wintour EM 1982 Development of the pituitary–adrenal axis in chronically cannulated ovine fetuses. *Journal of Developmental Physiology* **4** 339–352.
- Ho KY & Weissberger AJ 1990 The antidiuretic action of bio-synthetic human growth hormone in man involves activation of the renin–angiotensin system. *Metabolism* **39** 133–137.
- Klempf M, Bingham B, Breier BH, Baumbach WR & Gluckman PD 1993 Tissue distribution and ontogeny of growth hormone receptor messenger ribonucleic acid and ligand binding to hepatic tissue in the midgestation sheep fetus. *Endocrinology* **132** 1071–1077.
- Lingwood B, Hardy KJ, Horacek I, McPhee ML, Scoggins BA & Wintour EM 1978 The effects of antidiuretic hormone on urine flow and composition in the chronically cannulated ovine foetus. *Quarterly Journal of Experimental Physiology* **63** 305–320.
- Ludbrook J 1994 Repeated measures and multiple comparisons in cardiovascular research. *Cardiovascular Research* **28** 303–311.
- McDougall JG, Butkus A, Coghlan JP, Denton DA, Müller J, Oddie CJ, Robinson PM & Scoggins BA 1980 Biosynthetic and morphological evidence for inhibition of aldosterone production following administration of ACTH to sheep. *Acta Endocrinologica* **94** 559–570.
- Mitani F, Suzuki H, Hata J-I, Ogishima T, Shimada H & Ishimura Y 1994 A novel cell layer without corticosteroid-synthesizing enzymes in rat adrenal cortex: histochemical detection and possible physiological role. *Endocrinology* **135** 431–438.
- Palmore WP, Anderson R & Mulrow PJ 1970 Role of the pituitary in controlling aldosterone production in sodium-depleted rats. *Endocrinology* **86** 728–734.

- Penhoat A, Rainey WE, Viard I & Saez JM 1994 Regulation of adrenal cell-differentiated functions by growth factors. *Hormone Research* **42** 39–43.
- Robinson PM, Rowe EJ & Wintour EM 1979 The histogenesis of the adrenal cortex in the foetal sheep. *Acta Endocrinologica* **91** 134–149.
- Rudd BT, Rayner PHW, Bassett RM & Williams JW 1980 Serum dehydroepiandrosterone (DHA) and sulphate (DHAS) after acute growth hormone therapy. *Acta Paediatrica Scandinavica* **69** 287–292.
- Svenne I, Hill DJ, Strain AJ & Milner RDG 1987 Growth hormone regulation of somatomedin C/insulin-like growth factor I production and DNA replication of fetal rat islets in tissue culture. *Diabetes* **36** 288–294.
- Tangalakis K, Coghlan JP, Connell J, Crawford R, Darling P, Hammond V, Penschow J & Wintour EM 1989 Tissue distribution and levels of gene expression of three steroid hydroxylases in ovine fetal adrenal glands. *Acta Endocrinologica* **120** 225–232.
- Tangalakis K, Coghlan JP, Crawford R, Hammond VE & Wintour EM 1990 Steroid hydroxylase gene expression in the ovine fetal adrenal gland following ACTH infusion. *Acta Endocrinologica* **123** 371–377.
- Walker ML, Pepe GJ & Albrecht ED 1988 Regulation of baboon fetal adrenal androgen formation by pituitary peptides at mid- and late gestation. *Endocrinology* **122** 546–551.
- Werther GA, Haynes K & Johnson GR 1990 Insulin-like growth factors promote DNA synthesis and support cell viability in fetal hemopoietic tissue by paracrine mechanisms. *Growth Factors* **3** 171–179.
- Wintour EM, Brown EH, Denton DA, Hardy KJ, McDougall JG, Oddie CJ & Whipp GT 1975 The ontogeny and regulation of corticosteroid secretion by the ovine foetal adrenal. *Acta Endocrinologica* **79** 301–316.

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