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Intrafetal Insulin-Like Growth Factor-I Infusion Stimulates Adrenal Growth But Not Steroidogenesis in the Sheep Fetus during Late Gestation

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We investigated the effects of an intrafetal infusion of IGF-I on adrenal growth and expression of the adrenal steroidogenic and catecholamine-synthetic enzyme mRNAs in the sheep fetus during late gestation. Fetal sheep were infused for 10 d with either IGF-I (26 μg/kg·h; n = 14) or saline (n = 10) between 120 and 130 d gestation, and adrenal glands were collected for morphological analysis and determination of the mRNA expression of steroidogenic and catecholamine-synthetic enzymes. Fetal body weight was not altered by IGF-I infusion; however, adrenal weight was significantly increased by 145% after IGF-I infusion. The density of cell nuclei within the fetal adrenal cortex (the zona glomerulosa and zona fasciculata), and within the adrenaline synthesizing zone of the adrenal medulla, was significantly less in the IGF-I-infused fetuses compared with the saline-infused group. Thus, based on cell-density measurements, there was a significant increase in cell size in the zona glomerulosa and zona fasciculata of the adrenal cortex and in the adrenaline-synthesizing zone of the adrenal medulla. There was no effect of IGF-I infusion on the adrenal mRNA expression of the steroidogenic or catecholamine-synthetic enzymes or on fetal plasma cortisol concentrations. In summary, infusion of IGF-I in late gestation resulted in a marked hypertrophy of the steroidogenic and adrenaline-containing cells of the fetal adrenal in the absence of changes in the mRNA levels of adrenal steroidogenic or catecholamine-synthetic enzymes or in fetal plasma cortisol concentrations. Thus, IGF-I infusion results in a dissociation of adrenal growth and function during late gestation. (*Endocrinology* 148: 5424–5432, 2007)

THROUGHOUT GESTATION, the timely development of the adrenal gland plays a critical role in fetal organ maturation and differentiation and parturition and in the successful transition to extrauterine life (1–3). During late gestation in the sheep, there is an increase in fetal adrenal growth and steroidogenesis resulting in a prepartum increase in fetal cortisol that is essential for the normal maturation of key organs including the fetal lungs, liver, kidneys, and brain and for the normal timing of parturition (4, 5). It is clear that the prepartum stimulation of adrenal growth and steroidogenesis is dependent on an intact fetal hypothalamus and pituitary (6–9), and it has been shown that intrafetal infusion of ACTH before the prepartum activation of the adrenal results in premature delivery of the fetus (10). It has been proposed that intraadrenal growth factors may be paracrine mediators or modulators of the trophic actions of ACTH on the fetal adrenal (3, 11, 12). In the mouse, overexpression of IGF-binding protein-2 results in increased adrenal weight by the third month after birth (13). In the primate fetus, we used metyrapone treatment to increase pituitary ACTH secretion, which resulted in a concomitant increase in adrenal IGF-II expression and adrenocortical growth (14). It has also been shown that administration of IGF-II to human fetal adrenal cells *in vitro* potentiated ACTH-stimulated expression of the steroidogenic enzymes cytochrome P450 cholesterol side-chain cleavage (CYP11A1), cytochrome P450 17-hydroxylase (CYP17), 3β-hydroxysteroid dehydrogenase (3βHSD), and steroid secretion (15). IGF-I and IGF-II have also been shown to be specific mitogens for human fetal adrenal cells *in vitro* (16). These growth factors have equipotent effects, suggesting that their mitogenic actions are mediated via a common receptor, most likely the type 1 IGF receptor (IGF1R) (16), which has been identified in bovine, human, and rhesus monkey adrenal cortical cells (3, 14, 17). It has also been shown that administration of IGF-I to ovine fetal adrenal cells *in vitro* enhances cAMP accumulation and the steroidogenic response to ACTH (12). IGF-II mRNA is also abundantly expressed in the steroidogenic cells of the fetal sheep adrenal from as early as 60 d gestation (18). Although these studies indicate that IGF-I and -II can stimulate fetal adrenal proliferation and steroidogenesis in adrenal cells *in vitro*, there have been no studies that have investigated the effects of these growth factors on the coordinate regulation of fetal adrenal growth and steroidogenesis *in vivo*.

Previous studies in the rat and cow have demonstrated that IGFs can also stimulate the growth of adrenomedullary cells (19–21). Other studies have shown that IGF-II mRNA colocalizes with 3βHSD-positive cells in the adrenal cortex and in groups of cells within the adrenal medulla (18) in the

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Abbreviations: b, Bovine; h, human; 3βHSD, 3β-hydroxysteroid dehydrogenase; IGF1R, type 1 IGF receptor; o, ovine; PNMT, phenylethanolamine *N*-methyltransferase; POMC, proopiomelanocortin; SSC, standard saline citrate; TH, tyrosine hydroxylase.

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fetal sheep, and thus, these data would suggest that intra-adrenal IGF would only mediate effects on adrenomedullary growth and function via a paracrine mechanism in the developing sheep adrenal gland. Thus, the aims of this study were to investigate the effects of administration of IGF-I to fetal sheep before the prepartum cortisol surge on adrenocortical and adrenomedullary growth, on the expression of adrenal steroidogenic and catecholamine synthetic enzyme mRNA levels, and on fetal plasma cortisol concentrations.

Materials and Methods

Animal protocols and surgery

All procedures were approved by the University of Adelaide Standing Committee on Ethics in Animal Experimentation. Twenty-four pregnant Border Leicester × Merino ewes with singleton fetuses were used in this study. The ewes were housed in individual pens in animal holding rooms, with a 12-h light, 12-h dark lighting regimen and fed once daily with 1 kg lucerne chaff and 1 kg Baramil joint stock rations between 0900 and 1300 h with water *ad libitum*. Surgery was carried out between 110 and 118 d of gestation under general anesthesia using aseptic techniques. Catheters were implanted into the fetal carotid artery, femoral artery, jugular vein, tarsal vein, umbilical vein, and maternal uteroovarian vein and filled with 50 IU/ml heparinized saline as described previously (23). There was a recovery period of at least 3 d after surgery before fetal blood samples were collected (for details of animal numbers in each experimental protocol outlined below, see Table 1).

Infusion regimen and blood sampling protocol

Infusate preparation. Recombinant human IGF-I (animal/media grade, catalog no. IM 100; GroPep, Adelaide, Australia) was dissolved in saline solution (0.9% wt/vol) containing BSA (1 mg/ml, RIA grade; Sigma Chemical Co., St. Louis, MO) to make an IGF-I stock solution (1 mg/ml). The BSA preparation used in this study has been shown to be free of IGFs and IGF-binding proteins (24, 25). The BSA (1 mg/ml)/ saline (0.9% wt/vol) solution was prepared by dissolving 0.5 g BSA in 6 ml saline removed from a sterile 500-ml saline bag. This BSA/saline solution was then filter sterilized using a 0.22-μm filter (Millex-GS 0.22-μm filter; Millipore Australia, North Ryde, Australia), transferring the solution from one sterile syringe to another through a sterile three-way tap (Discofix; Braun Medical AG, Bella Vista, New South Wales, Australia) attached to the 0.22-μm filter before returning the filtered solution to the 500-ml saline bag. To make the IGF-I stock solution (1 mg/ml), 10 mg IGF-I was dissolved in 10 ml of the BSA (1 mg/ml)/saline (0.9% wt/vol) solution. The final IGF-I infusate (591 μg/ml) was made by diluting 5.91 ml IGF-I stock solution (1 mg/ml) to a total volume of 10 ml with the BSA (1 mg/ml)/saline (0.9% wt/vol) solution.

TABLE 1. Details of the numbers of animals included in each experimental group for the different analyses

	Saline-infused fetuses	IGF-I-infused fetuses
Surgery	10	14
Measurement of fetal plasma IGF-I concentrations	10	10
Measurement of fetal plasma cortisol concentrations	7	10
IGF-II and steroidogenic and catecholamine-synthetic enzyme mRNA expression	5	8
Morphometric analyses of the fetal adrenal	9	8
Measurement of fetal body and adrenal weights at 130 d	10	14

Not all animals had samples collected for histological and molecular analyses, and therefore, there are slight variations in the numbers for specific analyses.

Infusion regimen. IGF-I was infused at a rate of 81 μg/h, which was estimated to be an infusion rate equivalent to 30 μg/kg·h, where fetal weight was assumed to be 2.7 kg at 120 d of gestation, as based on previous studies. A total of 1944 μg/24 h of the IGF-I peptide was infused into each fetus in the IGF-I-infused group (n = 14 fetuses). Control fetuses received 3.3 ml/24 h of saline (n = 8 fetuses). The IGF-I or saline was infused into fetal sheep via the fetal tarsal vein for 10 d from 120–130 d of gestation, starting at 0900 h at 120 d of gestation (designated time zero) using an SP200 series syringe pump (World Precision Instruments, Berlin, Germany). Fetal arterial blood samples (2 ml) were collected into chilled collection tubes containing 125 IU lithium heparin every alternate day after surgery. Blood samples were centrifuged at 1800 × *g* for 10 min at 4 C before separation and storage of plasma at −20 C for subsequent assay. To monitor health and well-being of the fetus, arterial blood (0.5 ml) was collected on alternate days for measurement of fetal arterial p_aO_2, p_aCO_2, pH, O_2 saturation, and hemoglobin content using an ABL 550 acid-base analyzer and OSM2 hemoximeter (both from Radiometer Pacific, Blackburn, Victoria, Australia) and were in the range previously reported for healthy fetal sheep in late gestation (26).

Tissue collection

At 130 d of gestation, the ewe was killed with an overdose of sodium pentobarbitone and the fetus delivered via laparotomy, weighed, and then killed by decapitation. The adrenal glands from each fetus were quickly removed, weighed, and either snap-frozen in liquid N_2 and stored at −80 C until total RNA was extracted or cut in half and fixed by immersion in 0.1 M phosphate-buffered paraformaldehyde (4%) and embedded in paraffin.

RIAs

IGF-I RIA. Plasma samples were collected at 120 d of gestation before the IGF-I infusion, and at 130 d of gestation before the end of the infusion in IGF-I-infused (n = 10) and saline-infused (n = 10) fetuses for determination of plasma IGF-I concentrations. The specific IGF-I RIA was performed after removing IGF-binding proteins by acid chromatography of plasma as described previously (24). Briefly, the IGF-I RIA used rabbit antiserum PM87 raised against human IGF-I (27), which binds with equal affinity to recombinant human IGF-I and ovine IGF-I, which has been purified from sheep plasma (27). The cross-reactivity of the PM87 IGF-I antiserum for sheep IGF-II is less than 1% (27) and less than 0.01% for insulin. The intra- and interassay coefficients of variation, determined by repeated analysis of reference adult sheep plasma containing 330 ng/ml IGF-I were 3.2 and 12.4%, respectively (24).

Cortisol RIA. Cortisol concentrations were measured in fetal plasma samples from the IGF-I-infused group (n = 10 fetuses) and the saline-infused group (n = 7 fetuses). Plasma samples were collected at 128 or 130 d of gestation before the end of the infusion. Total cortisol concentrations in fetal sheep plasma were measured using a RIA, validated for fetal sheep plasma, as described previously (9). The intra- and interassay coefficients of variation were less than 10% and less than 20%, respectively.

Morphometric analyses

Morphometric analysis was performed on sections (3–5 μm) of the left adrenal from IGF-I-infused (n = 8) and saline-infused (n = 9) animals. Adrenal sections were stained with hematoxylin and eosin to identify the nuclei and cytoplasm, respectively (28). The density of cells in the adrenal cortex (zona glomerulosa and zona fasciculata) and medulla (peripheral adrenaline-synthesizing cells and central noradrenaline-synthesizing cells) were measured in IGF-I- and saline-infused fetuses. The location and morphological characteristics of the adrenocortical and adrenomedullary cell types within the developing adrenal gland of the fetal sheep have been defined in previous studies (29–31). The total number of cell nuclei in a defined area of each zone of the cortex and medulla was counted in four random fields of view in each of three adrenal sections per fetus at a magnification of ×40 on an Olympus BHS microscope using a Panasonic KR222 camera connected to VideoPro imaging software (Leading Edge, Hove, South Australia, Australia). The

area measured (24,865 μm^2) was based on the degree of magnification required to identify cell nuclei (~150–450) and on the field of view of the video screen (512 × 719 pixels, where micrometers = pixels × 0.2604); the defined area was therefore [(512 × 0.2604) × (719 × 0.2604)] = 24,864.6 μm^2. Thus, for each animal, the mean of the total number of cell nuclei was expressed as total cell nuclei × 10^3 per square micrometer for each of the zona glomerulosa, zona fasciculata, adrenaline-synthesizing, and noradrenaline-synthesizing zones of the fetal adrenal.

Immunocytochemistry

Adrenal sections (3–5 μm) from IGF- and saline-infused control animals were prepared and mounted on pretreated slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) to localize 3βHSD (steroidogenic enzyme), phenylethanolamine *N*-methyltransferase (PNMT) (adrenaline-synthesizing enzyme), and Ki67 (proliferative marker) using immunocytochemical techniques as described previously. In brief, the 3βHSD, PNMT, and Ki67 proteins were each localized using a HistoPlus immunostaining kit (Zymed, South San Francisco, CA), which uses a broad-spectrum biotinylated secondary antibody that detects mouse, rabbit, guinea pig, and rat primary antibodies, and a streptavidin-horseradish peroxidase conjugate. The 3βHSD primary antibody was raised in rabbits against human 3βHSD (32, 33), was used at a dilution of 1:15,000, and has been previously shown to specifically localize 3βHSD in the fetal sheep adrenal cortex (33). The PNMT primary antibody was raised in rabbits against bovine PNMT, was used at a concentration of 1:15,000, and has been previously shown to localize PNMT in the peripheral-rim adrenaline-synthesizing cells of the fetal sheep adrenal medulla (31). The Ki67 antibody was a mouse antihuman monoclonal antibody (Dako-Cytomation, Glostrup, Denmark) and was used at a dilution of 1:200. The Ki67 clone-1 MIB-1 antibody (34) has been used extensively in a range of tissues to demonstrate the Ki67 antigen in formalin-fixed, paraffin-embedded specimens. Ki67 antigen clone-1 is a nuclear protein expressed by cells that are in all phases of the active cell cycle (G1, S, G2, and M phase) and is absent from resting (G0) cells. To detect the Ki67 in the cell nucleus of proliferating cells, a specific antigen retrieval step was included in the protocol, where the slides were incubated in sodium-citrate buffer (0.01 M, pH 6.0) for 10 min in an autoclave at 121 C. An immunopure, metal-enhanced diaminobenzidine substrate (Pierce, Rockford, IL) was then used as the chromogen to identify positive staining. To identify the cell nuclei within each of the sections immunostained for 3βHSD and PNMT, the slides were also lightly counterstained (Mayer's hematoxylin; Sigma Diagnostics, St. Louis, MO). Slides were then dehydrated, mounted, and coverslipped. The photomicrographic images were captured from an Olympus VANOX-AHT microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with a ×2 or ×10 objective using a Colorview I camera with AnalySIS 5 image analysis software (Soft Imaging Systems, Gulfview Heights, South Australia, Australia).

Northern blot analysis

Total RNA was extracted from one adrenal from IGF-I-infused (n = 8 fetuses) and saline-infused (n = 5 fetuses) fetal sheep by homogenization in 4 M guanidine hydrochloride solution and ultracentrifugation overnight at 36,000 rpm, through a cushion of 5.7 M CsCl in 100 mM EDTA (35, 36). Total RNA samples (20 μg adrenal RNA) were denatured by incubation in 2.2 M formaldehyde and 50% vol/vol formamide at 55 C for 10 min, separated by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde, and then transferred by gravity-feed blotting onto a Zetaprobe nitrocellulose membrane using 10× standard saline citrate (SSC). The Northern membrane was washed in 10× SSC, 0.1% SDS for 10 min at room temperature and exposed to UV light (12 sec at 120 J/cm^2) before overnight incubation at 42 C in 30 ml of either cDNA or antisense oligonucleotide hybridization buffer. The human (h)CYP11A and hCYP17 probes were generously provided by Professor W. Miller (Department of Pediatrics, University of California, San Francisco, San Francisco, CA), the h3βHSD cDNA probe by Professor R. J. Rodgers (Department of Obstetrics and Gynecology, University of Adelaide, South Australia, Australia), and the ovine (o)IGF-II cDNA probe by Dr. R. S. Gilmour (Institute of Animal Physiology and Genetics, Cambridge, UK). A full-length (1.73-kb) bovine (b) tyrosine hydroxylase (bTH) cDNA was used to detect TH mRNA (37) and an oligonucleotide antisense probe complementary to nucleotides 361–389 of the peptide coding region of bPNMT (29-mer; (38) was used to detect PNMT mRNA as described previously (39). The cDNAs were radiolabeled with α-[^{32}P]dCTP (3000 Ci/mmol) by the random priming oligomer method to a specific activity of 10^9 cpm/μg or greater. A 30-mer antisense oligonucleotide probe for rat 18S rRNA, complementary to nucleotides 151–180, was synthesized and end-labeled using T4PNK and γ-[^{32}P]ATP (4000 Ci/mmol) as substrate. The Northern membranes were then hybridized sequentially for 16 h at 42 C for cDNA probes (hCYP11A, hCYP17, h3βHSD, oIGF-II, and bTH) or 50 C for oligonucleotide probes (bPNMT and rat 18S) in 20–25 ml fresh hybridization buffer containing either 1–2 × 10^6 cpm/ml of the cDNA probe or 5 × 10^5 cpm/ml of the antisense oligonucleotide probe. The membrane was washed once (10 min) at room temperature in 1× SSC, 0.1% SDS and then twice (10 min each time) in 0.1× SSC, 0.1% SDS at 42 C and then briefly air-dried and sealed in a plastic bag. The membrane was exposed to phosphorimager plates in BAS 2040 cassettes for 24–48 h, and images were quantified on a Fuji-BAS 1000 phosphorimager scanner using Fuji MacBAS software (Fuji Photo Film Co., Tokyo, Japan). cDNA probes were stripped from membranes between hybridizations by washing in 0.01× SSC, 0.5% SDS for 10 min at 80 C. A ratio of the density of each specific band with the density of the corresponding 18S rRNA band was calculated before comparisons were made.

Statistical analysis

Data are presented as the mean ± SEM. Total adrenal weight (the sum of the weights of the left and right adrenals), the ratio of adrenal to fetal body weight and the ratios of adrenal steroidogenic enzyme mRNA to 18S rRNA, IGF-II mRNA to 18S rRNA, and the adrenal catecholamine synthetic enzyme mRNA to 18S rRNA between the IGF-I- and saline-infused fetuses were compared using Student's *t* test. The mean plasma concentrations of IGF-I and cortisol during the infusion period were also compared between the IGF-I- and saline-infused fetuses using the Student's *t* test. A value of $P < 0.05$ was considered to be significant.

Results

Fetal plasma IGF-I and cortisol concentrations

Infusion of IGF-I resulted in a significant increase in the fetal plasma concentrations of IGF-I (IGF-I, 380 ± 69 ng/ml; saline, 120 ± 12 ng/ml). There was no difference in the fetal plasma cortisol concentrations during the infusion period between the IGF- and saline-infused fetuses (IGF-I, 7.0 ± 1.3 nmol/liter; saline, 10.0 ± 3.2 nmol/liter).

Total fetal adrenal weight and fetal body weight

Fetal body weights were not significantly different after intrafetal infusion of IGF-I (3.29 ± 0.14 kg) or saline (3.28 ± 0.15 kg). IGF-I infusion did, however, result in a significant increase ($P < 0.05$) in total adrenal weight (IGF-I, 414 ± 30 mg; saline, 286 ± 17 mg) (Fig. 1) and in the ratio of total adrenal to fetal body weight (IGF-I, 131 ± 14 mg/kg; saline, 88 ± 4 mg/kg) (Fig. 1).

Adrenal morphometry and immunocytochemistry

The density of cell nuclei within the zona glomerulosa and zona fasciculata, were significantly less in the IGF-I-infused fetuses (9.6 ± 0.5 × 10^3 and 7.6 ± 0.7 × 10^3 nuclei/μm^2, respectively) compared with the saline-infused group (12.2 ± 0.8 × 10^3 and 10.3 ± 0.7 × 10^3 nuclei/μm^2, respectively) (Fig. 2). The density of cell nuclei within the adrenaline-synthesizing zone was also significantly less in the IGF-I-infused fetuses (7.5 ± 0.4 × 10^3 nuclei/μm^2) compared with the saline-infused group (8.7 ± 0.4 × 10^3 nuclei/μm^2) (Fig. 2). There was no effect of IGF-I infusion, however, on

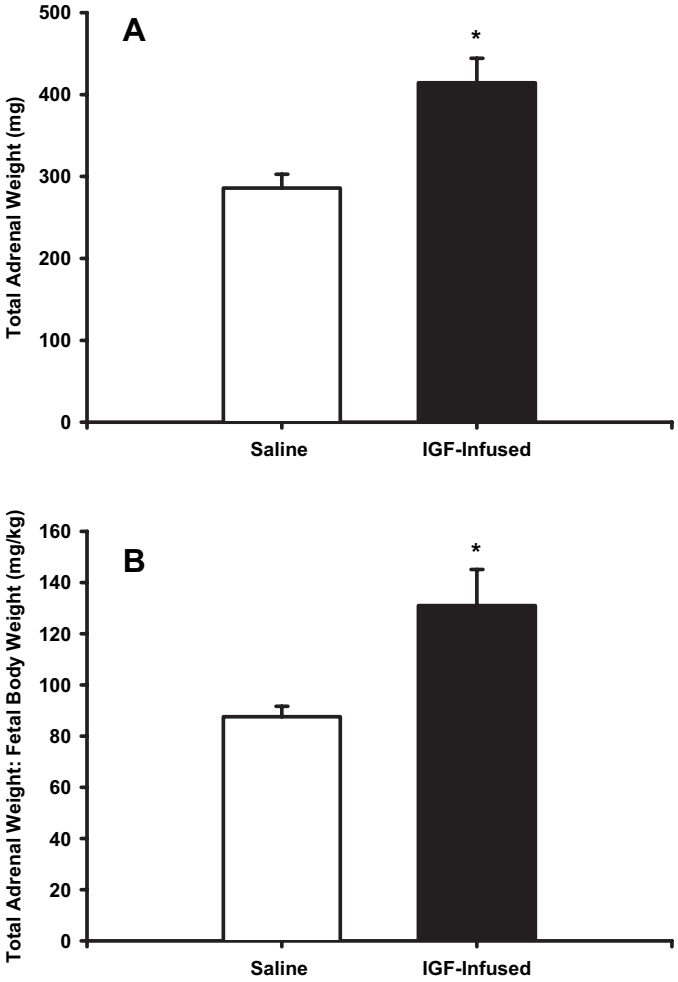

FIG. 1. The effect of IGF-I infusion on total fetal adrenal weight (A) and the ratio of adrenal to fetal body weight (B). The IGF-I-infused group is represented by the *black bars* and the saline-infused group by the *white bars*. *, Significant difference at $P < 0.05$.

the density of cells within the noradrenaline-synthesizing zone (IGF-I, 9.1 ± 0.6 × 10^3 nuclei/μm^2; saline, 9.4 ± 0.8 × 10^3 nuclei/μm^2) (Fig. 2).

In the adrenal gland of saline- and IGF-infused fetuses, Ki67 immunoreactivity was localized to a few cells within the zona glomerulosa and in the zona fasciculata, with fewer Ki67-positive cells nearer to border of the zona fasciculata with the adrenal medulla (Fig. 3, B and D). We observed few cells in the adrenal medulla that were positively stained for Ki67 (Fig. 3F).

In the adrenal gland of saline- and IGF-infused fetuses, 3βHSD immunoreactivity was localized to the cells of the zona glomerulosa and zona fasciculata and also present in some cells close to the central vein (Figs. 4 and 5). The photomicrographs of 3βHSD immunostaining highlight the effect of the IGF treatment on increasing the growth of both the adrenal cortex and adrenal medulla (Fig. 4, A and B). In the adrenal gland of saline- and IGF-infused fetuses, PNMT immunoreactivity was localized predominantly to the peripheral rim of adrenomedullary cells (Fig. 5, A and C). At the higher magnification, the photomicrographs of 3βHSD and

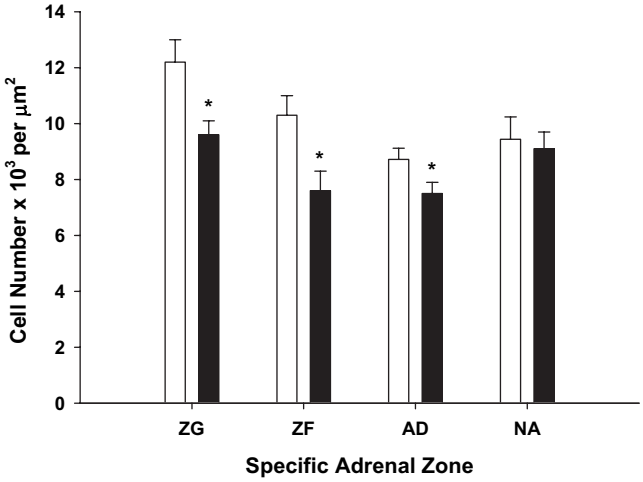

FIG. 2. The effects of IGF-I infusion on adrenal cell density were determined in each specific adrenal zone by morphometric techniques. The density of cell nuclei within the zona glomerulosa (ZG), zona fasciculata (ZF), adrenaline-synthesizing zone (AD), and noradrenaline-synthesizing zone (NA) is expressed as cells times 1000 per square micrometer of adrenal tissue. The IGF-I-infused group is represented by the *black bars* and the saline-infused group by the *white bars*. *, Significant difference between treatment groups at $P < 0.05$.

PNMT immunostaining have been used to differentiate between the specific zones of the adrenal gland and further demonstrate the impact of IGF treatment on the growth of the zona glomerulosa and zona fasciculata (Fig. 5).

Adrenal steroidogenic and catecholamine-synthetic enzyme mRNA expression

There was no difference in the ratio of CYP11A1 mRNA (1.9-kb transcript), CYP17 mRNA (2.2-kb transcript), or 3βHSD mRNA (1.6-kb transcript) to 18S rRNA in fetal adrenals of IGF-I-infused fetuses (CYP11A1, 0.158 ± 0.028; CYP17, 0.281 ± 0.044; 3βHSD, 0.234 ± 0.020) compared with saline-infused fetuses (CYP11A1, 0.160 ± 0.034; CYP17, 0.277 ± 0.039; 3βHSD, 0.211 ± 0.029). There was also no difference in the ratio of TH mRNA (1.8-kb transcript) or PNMT mRNA (1.1-kb transcript) to 18S rRNA in fetal adrenals of IGF-I-infused fetuses (TH, 0.028 ± 0.006; PNMT, 0.067 ± 0.008) compared with saline-infused fetuses (TH, 0.029 ± 0.011; PNMT, 0.085 ± 0.017).

Adrenal IGF-II mRNA expression

The oIGF-II cDNA identified six transcripts in the range of 1.8–6.0 kb by Northern analysis of total RNA from fetal adrenals. The ratio of total IGF-II mRNA to 18S rRNA was not significantly different in the fetal adrenals from IGF-I-infused fetuses (3.35 ± 0.13) compared with saline-infused fetuses (3.42 ± 0.52).

Discussion

In the present study, we have demonstrated that intrafetal infusion of IGF-I between 120 and 130 d in fetal sheep, resulting in an approximately 3-fold increase in free IGF-I in the fetal circulation, stimulated an increase in absolute and relative fetal adrenal weight, without concomitant changes in

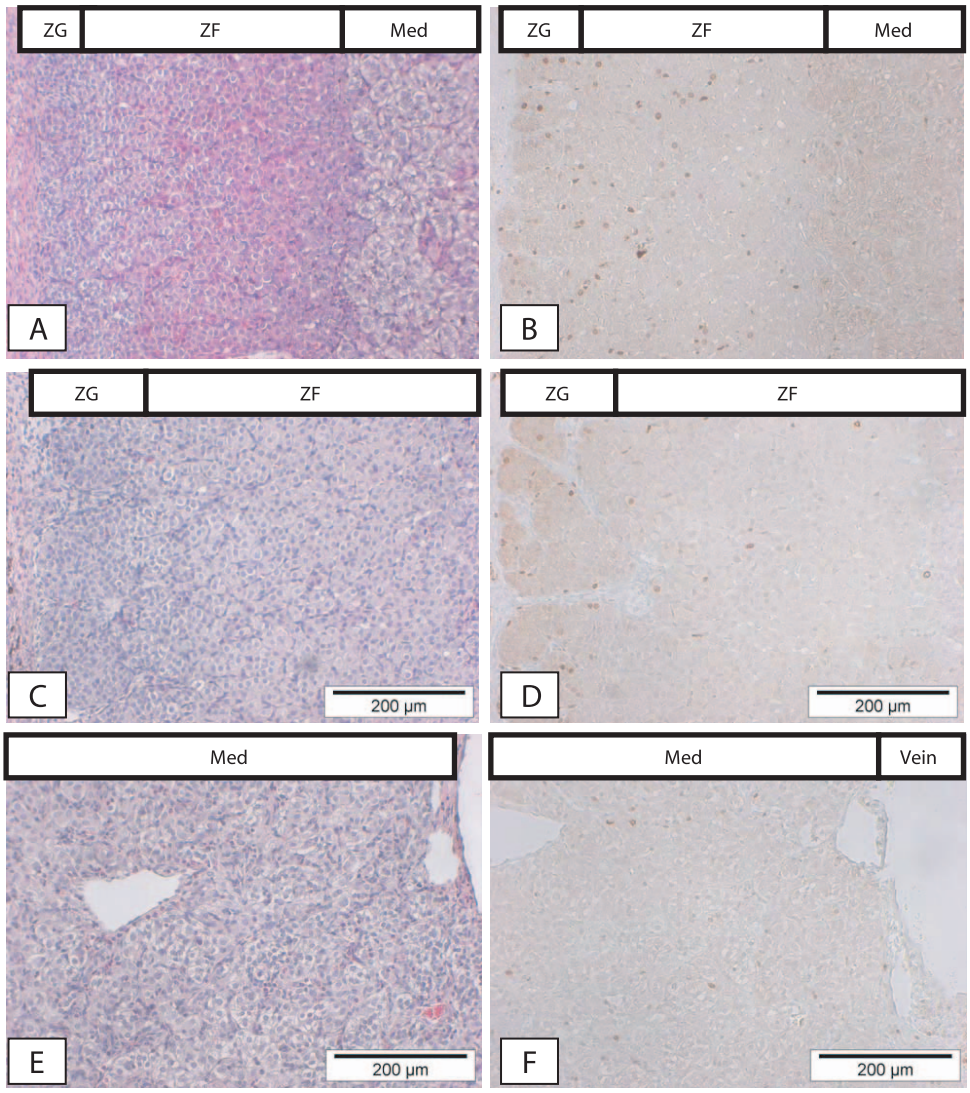

FIG. 3. Photomicrographs of adrenal glands from saline-infused (A and B) and IGF-infused (C–F) fetal sheep at 130 d gestation stained with hematoxylin and eosin (A, C, and E) to identify the various zones of the adrenal gland and the serial section immunostained for Ki67 (B, D, and F) to identify any cells undergoing proliferation within the adrenal cortex or adrenal medulla. The *brown* staining indicates cell nuclei positive for Ki67 immunoreactivty. All photomicrographs are taken at the same magnification: *scale bar*, 200 μm. Med, Adrenal medulla; ZF, zona fasciculata; ZG, zona glomerulosa.

the adrenal mRNA levels of the steroidogenic or catecholamine-synthetic enzymes. In the current study, the increased level of plasma IGF-I after exogenous infusion was similar to that which can be achieved physiologically through endogenous production. As previous studies have shown, fetal plasma IGF-I concentrations increase from approximately 120 ng/ml to 180 ng/ml just before delivery and then to 200 ng/ml by 10–15 d after birth (40). In the current study, fetal plasma IGF-I concentrations were slightly above the level achieved in response to chronic glucose supplementation of the pregnant ewe and fetus (22). We have also shown that hypertrophy of the cells in the zona glomerulosa, zona fasciculata, and the adrenaline-synthesizing zone of the adrenal glands contributed to the increase in adrenal weight in the IGF-infused group. To investigate the impact of IGF treatment, we used immunocytochemistry to localize Ki67 as a marker of proliferating cells and found no evidence that the increase in adrenal growth after IGF treatment was due to an increase in proliferation of either adrenal cortical or medullary cells. Previously, it has been reported that chronic overexpression of GH in the adult mouse *in vivo*, resulting in a 2-fold increase in serum IGF-I levels, also led to increased growth of the adrenal cortex (41). In this mouse model, the adrenal enlargement involved both hypertrophy and hyperplasia of the zona fasciculata cells of the adrenal cortex (41). IGF-I also induces proliferation of fetal sheep and rat adrenocortical (12, 42) and fetal rat medullary chromaffin cells (43) *in vitro*. In the primate fetus, we previously showed that metyrapone treatment for 3–7 d to increase pituitary ACTH secretion in late gestation resulted in a concomitant increase in adrenal IGF-II expression and adrenal growth that was solely a consequence of hypertrophy of the adrenocortical cells (14).

IGF-I and IGF-II have equipotent mitogenic actions on human fetal adrenal cells, suggesting that these actions are most likely mediated via the IGF1R (16). The IGF1R has been shown to be expressed in bovine, human, and rhesus monkey adrenal cortical cells (3, 14, 17). Metyrapone infusion in the fetal primate, which increased adrenocortical growth, also resulted in an increase in the expression of the IGF1R in the fetal adrenal cortex (14). Although we found that intrafetal infusion of IGF-I stimulated adrenal growth, there was no change in the expression of steroidogenic enzymes and circulating cortisol levels after the 10-d infusion period. It has

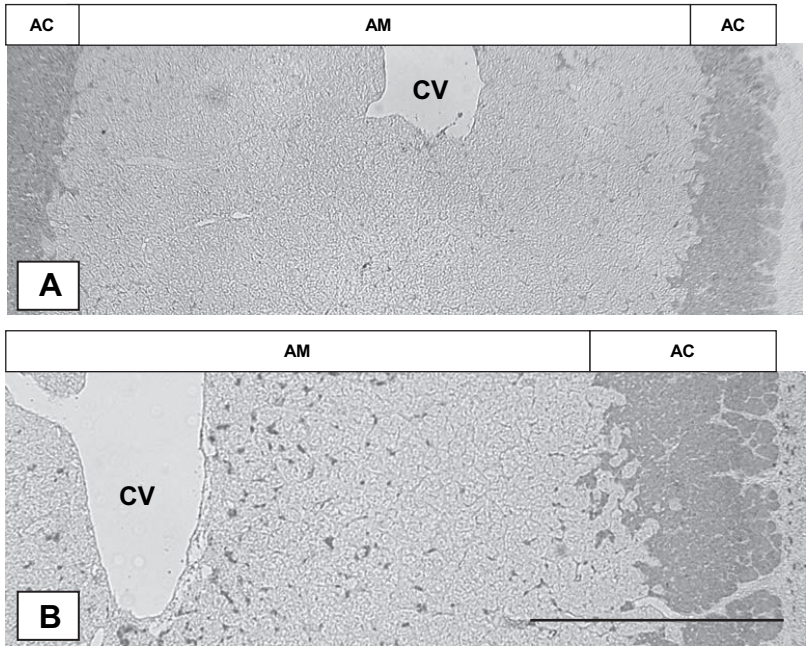

FIG. 4. Photomicrographs of adrenal glands from saline-infused (A) and IGF-infused (B) fetal sheep at 130 d gestation immunostained for 3βHSD to identify the steroidogenic-synthesizing cells within the adrenal gland. The *dark* staining indicates cell cytoplasm positive for 3βHSD immunoreactivity and is present throughout the adrenal cortex (AC) and absent from cells in the adrenal medulla (AM). Some cells near the central vein (CV) are adrenocortical cells and stain positively for 3βHSD. These photomicrographs were taken using a ×2 objective and demonstrate the effect of IGF on increasing the growth of the adrenal cortex and adrenal medulla (B) when compared with the adrenal gland from the saline-infused control (A). *Scale bar*, 1000 μm.

been shown that IGF-I enhances the steroidogenic response of adult and fetal adrenocortical cells to ACTH *in vitro* (12, 44), through mechanisms that include an increase in the number of adrenal ACTH receptors (12, 45–47), an increase in the expression of stimulatory G protein subunits (46), and increased activity of specific steroidogenic enzymes (12, 44, 45). It is interesting that whereas IGFs consistently enhance the steroidogenic responsiveness of adrenocortical cells to ACTH *in vitro*, infusion of IGF-I into the fetal circulation *in vivo* did not alter the expression of the mRNAs for the adrenal steroid-synthesizing enzymes or the circulating level of cortisol. It is possible that a systemic infusion of IGF-I does not result in a sufficiently high intracellular concentration of IGF-I to modulate ACTH-stimulated adrenal steroidogenesis. Alternatively, it is possible that the potentiating effects of IGF-I on adrenal steroidogenesis require prepartum levels of ACTH, which are greater than those present *in vivo* between 120 and 130 d gestation in the sheep fetus. Interestingly, at this concentration of ACTH, IGF-I infusion was effective at stimulating adrenal growth. Thus, although the potentiating action of IGF-I on steroidogenesis *in vitro* may require higher levels of IGF-I and/or ACTH, the actions of IGF-I on adrenal growth *in vivo* either are independent of ACTH or the concentrations of ACTH present in the sheep fetus from 120–130 d gestation are sufficient to mediate the affects of IGF-I on adrenal growth.

The specific intracellular pathways by which IGFs stimulate growth and functional differentiation within the adrenal gland have not been clearly defined. In other cell types, activation of the IGF1R activates a variety of intracellular signaling pathways, enabling the IGFs to play a complex array of roles in stimulating cell growth, promoting cell survival, and stimulating functional differentiation. In addition, cross-talk between the signaling pathways of IGF and other growth factor receptors has been recently shown to play a critical role in various cellular responses to ligands. For example, in recent studies in other cell types including anterior pituitary and skeletal muscle cells, IGF-I has been shown to activate two separate intracellular signaling pathways; the MAPK pathway, which triggers proliferation, and the phosphoinositol-3-kinase pathway, which regulates cell survival and differentiation (48, 49). Therefore, in the current study, it may be the predominance of activation of one of these specific intracellular pathways that has led to dissociation of adrenal growth and steroidogenesis after IGF-I infusion *in vivo*.

It is also possible that exogenous IGF-I acts to modulate the responsiveness of the fetal adrenal to intra- or extraadrenal trophic hormones other than ACTH. For example, IGF-II is abundantly expressed within the steroid-secreting cells of the fetal ovine adrenal cortex (22). Although IGF-I has limited ability to bind to IGF2R, the IGF2R has been postulated to be a tumor suppressor gene owing to its ability to bind and degrade IGF-II. Therefore, in these studies, we determined the expression of IGF-II mRNA after IGF-I infusion. However, the exogenous IGF infusion did not result in an upregulation of intraadrenal IGF-II expression in the fetal sheep adrenal, suggesting a limited involvement of the IGF2R in modulating adrenal growth in this model.

There are a number of pituitary-derived candidates that have been shown to stimulate adrenal growth. For example, circulating concentrations of the proopiomelanocortin (POMC)-derived peptide, *N*-POMC (1–77), are 20- to 50-fold higher than ACTH (1–39) in the sheep fetus during late gestation (50), and biologically active peptides cleaved from *N*-POMC (1–77) are mitogenic for adrenal cells both *in vitro* (51, 52) and *in vivo* (53). In previous studies, we have demonstrated that a 48-h infusion of *N*-POMC (1–77) at 136–138 d gestation significantly stimulated the growth of the fetal sheep adrenal (54). Importantly, in these studies, circulating cortisol concentrations were the same in both *N*-POMC (1–77)- and vehicle-infused fetuses (54), thereby demonstrating a dissociation between the actions of *N*-POMC (1–77) on adrenal growth and steroidogenesis. Therefore, it is possible

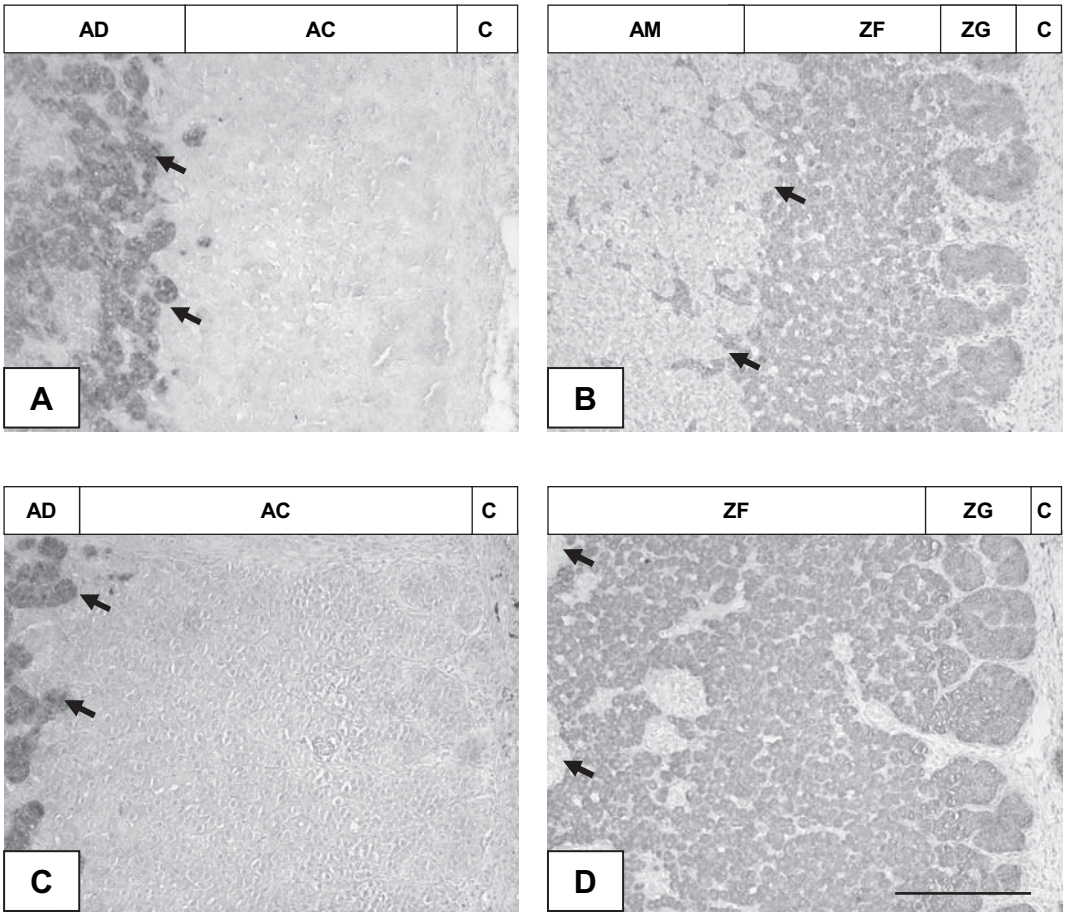

FIG. 5. Photomicrographs of adrenal glands from saline-infused (A and B) and IGF-infused (C and D) fetal sheep at 130 d gestation immunostained for PNMT (A and C) and 3βHSD (B and D) to identify the adrenaline-synthesizing (AD) and steroidogenic-synthetic cells, respectively. The *dark staining* indicates positive immunostaining within the cell cytoplasm for PNMT (A and C) or 3βHSD (B and D). The *arrows* identify the border between the adrenal medulla (AM) and adrenal cortex (AC) and highlight the impact of IGF treatment on increasing the growth of the zona glomerulosa (ZG) and zona fasciculata (ZF). All photomicrographs are taken at the same magnification: *scale bar*, 200 μm.

that *N*-POMC (1–77) and IGF-I act through similar mechanisms within the fetal adrenal to stimulate cellular hypertrophy, or alternatively, IGF-I may act indirectly to stimulate *N*-POMC (1–77) secretion from fetal corticotrophs within the anterior pituitary. Previous studies have shown, however, that IGF administration to fetal sheep pituitary cells *in vitro* has no effect on POMC mRNA expression, corticotroph cell number, or ACTH secretion, suggesting that it is unlikely that the IGFs act to stimulate corticotroph function *in vivo* (55).

In the present study, IGF-I infusion also stimulated the growth of the adrenaline-containing adrenomedullary cells. The effects of IGF-I on these adrenomedullary cells may be either direct or indirect through the paracrine actions of the neighboring adrenocortical cells. IGF-I stimulates proliferation of the adrenomedullary cells of the fetal rat *in vitro*, suggesting that these cells express IGF1R (42). In a previous study in the rhesus monkey, however, it was demonstrated that whereas IGF1R mRNA was present in all zones of the fetal adrenal cortex, it was not present within the adrenal medulla (14). This suggests that in this species, the stimulatory effect of IGFs on the adrenaline-synthesizing cells is indirect. Although intrafetal IGF infusion stimulated the growth of the adrenaline-synthesizing cells, there was no effect on adrenal expression of two of the key enzymes in adrenaline synthesis, TH and PNMT, suggesting that IGF-I infusion results in a dissociation of adrenal medullary growth and function. Previous studies using rat and bovine adrenal cells *in vitro*, however, have found that IGFs stimulate both the proliferation of adrenal medullary cells and their capacity to synthesize and secrete catecholamines (19–21). The differences in these findings may be due to the different concentrations of IGFs achieved *in vitro* compared with *in vivo* and the threshold concentrations required to stimulate catecholamine biosynthesis.

In summary, we have demonstrated that chronic exposure to an increase in circulating IGF-I in late gestation promotes adrenal growth but does not alter the expression of mRNA for the steroidogenic enzymes, catecholamine-synthetic enzymes, or circulating cortisol levels in the fetal sheep. The dissociation of the effects of IGFs on adrenal growth and steroidogenesis in this study indicates that an increase in circulating IGF alone is insufficient to stimulate adrenal steroidogenesis. This is evidence that when ACTH concentrations are relatively low, IGF-I stimulates adrenocortical growth through intracellular pathways that do not result in

an associated up-regulation of adrenal steroidogenic enzymes. It may be that interactions between the growth-promoting and steroidogenic actions of IGFs require higher ACTH concentrations than those present at 120–130 d gestation, such as can be achieved in experiments *in vitro*, or as a consequence of fetal pituitary activation during either exposure to intrauterine stress or in the prepartum period (5), intraadrenal IGFs may then act to enhance the sensitivity of the adrenal to the steroidogenic actions of ACTH.

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