Prolonged Low-Dose Dexamethasone, in Early Gestation, Has No Long-Term Deleterious Effect on Normal Ovine Fetuses

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Low-dose dexamethasone (D) treatment is used in pregnancies where the fetus is suspected to be at risk of congenital/virilizing adrenal hyperplasia. To study if this treatment had any immediate or long-term effects in normal fetuses, pregnant ewes were treated with D (20 μg/kg maternal body weight·d) or saline (S), from d 25–45 of gestation. Tissue was collected from fetuses killed at 45 d (S: 12.2 ± 0.7 mg; D: 6.5 ± 0.4 mg) and significantly decreased adrenal mRNA for P450scc. At 130 d, adrenal wt at 45 d (S: 12.2 ± 0.7 mg; D: 6.5 ± 0.4 mg) and mRNA levels measured using real-time PCR. D treatment reduced aldosterone to suppress fetal androgen production and mineralocorticoid receptor (MR) and GR mRNA levels were similar, and lambs were normotensive (S, 83 ± 3 mm Hg; D, 78 ± 3 mm Hg). Thus, there were no persistent, long-term effects of prolonged low-dose D treatment in normal ovine fetuses. (Endocrinology 143: 1159–1165, 2002)

Congenital adrenal hyperplasia is the term given to a group of autosomal recessive disorders in which one enzyme in the pathway of cortisol biosynthesis, most commonly P450–21-hydroxylase (P450c21), is deficient. In the absence of normal negative feedback on the hypothalamic-pituitary axis, excess ACTH secretion occurs, which causes the overgrowth of the fetal adrenal, and the secretion of excess amounts of cortisol precursors, which are androgenic steroids. Low-dose dexamethasone (D) treatment (20 μg/kg maternal body weight·d) is used to treat women suspected of carrying a fetus at risk for congenital/virilizing adrenal hyperplasia to suppress fetal androgen production before and during the period of sexual differentiation (1, 2). The treatment usually starts at 5 wk (~12.5% gestation) and ends, if amniocentesis proves the fetus to be male or not carrying the genetic defect, by 12 wk (~30% gestation). However, as only one in eight fetuses is likely to be an affected female, the other seven will receive this treatment unnecessarily. Concern has been raised that the treatment of normal fetuses may indeed have unwanted, deleterious consequences (2). These concerns have arisen because of three major lines of evidence: 1) the association of a higher incidence of adult disease in individuals who have been of inappropriately low birth weight for gestational age (3); 2) growth retardation (4, 5) and increased teenage blood pressure in children (5, 6) of mothers treated with antenatal corticosteroids for lung maturation before/after premature delivery and 3) animal studies in which large doses of D, given to the mother for various times during pregnancy, have led to offspring with cardiovascular and/or metabolic disease (7, 8).

There is now very convincing epidemiological evidence that links inappropriately low birth weight for gestational age, poor growth in the first postnatal year, and an increased risk for the development of hypertension, coronary heart disease, diabetes type 2, and dyslipidaemia in adult life (3). Low birth weight is thought to be a symptom of an unfavorable intrauterine environment, secondary to maternal undernutrition, placental insufficiency, or possibly excess stress in the mother (9). There is evidence that exposure of the fetus to excess natural or synthetic glucocorticoids can decrease birth weight and produce cardiovascular and metabolic disease in adult offspring (7–9). The mechanisms by which this occurs are not known, completely, but alterations in the expression of genes encoding the GR and mineralocorticoid receptors (MR), as well as corticotropin-releasing factor seem to be involved (7, 10).

Another system, potentially programmed by prenatal undernutrition and/or glucocorticoid exposure, is the renin-angiotensin system and its receptors (11–13). Angiotensin II receptors in the brain mediate both neuroendocrine and sympathetic responses to acute/chronic stress in adults (14) and the developmental attenuation of the baroreceptor vagal reflex in preweaned rats (15). The renal AT1 receptor expression has been shown to be increased, postnatally, in the offspring of rats exposed to excess glucocorticoid or a maternal low protein diet throughout pregnancy (16). In sheep undernourished from d 28–77 of gestation, there was also increased renal gene expression of the AT1 and GR, at term (17).

In earlier studies in our laboratory, D (0.28 mg/kg·d) was administered to pregnant ewes, and thus their fetuses, for 2 d, early in pregnancy (26–28 d of gestation), and shown to

Abbreviations: CT, Cycle of threshold fluorescence; D, dexamethasone; P450c21, MR, mineralocorticoid receptor; P450–21-hydroxylase; S, saline.
have profound effects on the offspring. The female lambs, reproductively, developed high blood pressure (8, 12, 18), with an increased cardiac output altered baroreflexes and finally left-ventricular hypertrophy, and reduced functional capacity.

This 2-d D treatment causes significant changes in gene expression in the kidney, brain, and hearts of twin ovine fetuses, killed at 130 d of gestation (12, 19). In the kidney, there was increased mRNA for components of the renin-angiotensin system (angiotensinogen, angiotensin II receptors AT1, AT2) and for GR and MR. Angiotensinogen mRNA was decreased in the heart, whereas in the hypothalamus it was very significantly increased. AT1 mRNA was up-regulated in the brain stem (medulla oblongata).

Although the ovine fetal adrenal does not develop the specific fetal zone that characterizes the primate fetal adrenal, many studies on the development of the fetal hypothalamic-pituitary-adrenal axis have been carried out in the ovine fetus (20). It has been known for many years that the adrenal of the very early ovine fetus (40 d; term is approximately 150 d) can make very large amounts of cortisol, *in vitro*, when stimulated with exogenous ACTH (21, 22). All components of the hypothalamic-pituitary-adrenal axis are known to exist in the ovine fetus by at least 40 d of gestation (20), but it has not yet been established whether the pituitary is regulating the growth and function of the adrenal at this time.

In the current study, pregnant sheep were exposed to similar levels of D as used clinically (−20 µg/kgd), which was delivered over a similar period of pregnancy (−17–31% of gestation). The first aim was to examine whether the treatment had any immediate and/or permanent effects on the growth of the fetus and developing organs, particularly the fetal adrenal. Secondly, it was of great interest to determine if this treatment had any programming effects. Thus, gene expression levels of the angiotensin receptors, angiotensinogen, as well as the MR and GR, were examined in the kidney and brain (hippocampus, hypothalamus, and medulla oblongata) of fetuses in late gestation (130 d). In addition, some male lambs at 2 months of age were studied to see if this treatment resulted in alterations in blood pressure. To test these aims, one cohort of animals was killed immediately after the treatment at 45 d. A second cohort remained in utero until late in gestation (130–132 d) and was then killed for tissue collection, and a third cohort was allowed to lamb, and offspring were studied at 2 months of age.

**Materials and Methods**

**Animals**

All experiments were approved by the Animal Ethics Committee of the Howard Florey Institute in accordance with National Health and Medical Research Council of Australia guidelines. Thirty-three pregnant merino ewes weighing between 45 and 55 kg were used in this study. On d 22 or 23 of gestation, a SILASTIC cannula (Dow Corning, Midland, MI) (inner diameter 0.76 mm, outer diameter 1.65 mm) was inserted into a maternal jugular vein under local anesthesia. On d 25 of gestation, a maternal jugular vein and an endotracheal tube inserted. The anesthesia was further dissected and portions of the hippocampus, hypothalamus, and medulla oblongata taken frozen in liquid nitrogen.

A second cohort of ewes (S, n = 4; D, n = 4) were maintained until fetuses were at 130 d of gestation, at which time they were killed and fetuses (kidney, heart, lung, brain, adrenal) were weighed and collected. All ewes killed at this stage carried twin fetuses. Thus, there were tissues from 8 fetuses in each treatment group at 130 d. The brain was further dissected and portions of the hippocampus, hypothalamus, and medulla oblongata taken frozen in liquid nitrogen.

A third cohort of ewes was allowed to lamb and suckled their lambs until two months of age. From this cohort, 3 sets of twin lambs in both the S and D infused groups were killed and organs collected as described above. The remaining animals in this cohort (5 S-exposed and 6 D-exposed male singleton lambs) had a femoral arterial SILASTIC cannula (0.76 mm inner diameter, 1.65 mm outer diameter) with a SV70 vinyl end (1.00 mm inner diameter, 1.50 mm outer diameter) inserted under general anesthesia. General anesthesia was induced with 5% thiopentone sodium (Thiobarb, Jurox, Rutherford, Australia; 0.4 ml/kg) via the jugular vein and an endotracheal tube inserted. The anesthesia was maintained with 1.5% isoflurane/high oxygen mixture (4:1 oxygen to air) (isofl inhalation anaesthetic; Abbott, Kurnell, Australia).

**Lamb blood pressure and heart rate measurement protocol**

To measure blood pressure and heart rate, the cannula was connected via a pressure transducer (TD XII; Cobe Cardiovascular Laboratories, Arvada, CO) to a computer. The analog signal was digitally converted via a DT 301 Board Data Translation device (Marboro, MA) and the blood pressure and heart rate data collected at 100 Hz (HEM 3.1; Notocord, Kent Scientific Corp., Litchfield, CT). Heart rate was calculated by the software using the formula dP/dt Max, which calculates the maximum slope during systole of the pressure vs. time curve. The transducer was placed at heart level, and the lambs were suspended in slings, which supported their weight off the ground. Blood pressure and heart rate were recorded in the pen over a 5-h period, during which the lamb was removed from the sling after the first reading (−60 min) and left with the dam for 45 min. This was followed by other periods with the dam lasting 15 min three times an hour for 2 h and then for 25 min twice an hour for a further 2 h.

**Preparation of RNA**

Total RNA was extracted from fetal adrenal glands at 45 d using an extraction kit (RNAzol B, Bresatec, Adelaide, Australia). All other tissues were extracted using the phenol-chloroform method (23). Before use in real-time PCR, 1 µg of each sample was reverse transcribed in a 10 µl reaction containing 1× TaqMan RT buffer, 5.5 mM MgCl2, 300 µM each 2′-deoxynucleoside 5′-triphosphate, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor and 1.25 U/µl MultiScribe reverse transcriptase (PE Applied Biosystems, Melbourne, Australia). To ensure that there was no contaminating genomic DNA, control reactions that did not include reverse transcriptase were included in a separate RT reaction with all total RNA samples. The RT was performed in a GeneAmp PCR System 9600 (PE Applied Biosystems) at 25 C for 10 min, 48 C for 30 min and 95 C for 5 min. Upon completion, all samples were stored at −80 C until use.

**Real-time PCR**

A comparative C(T) (cycle of threshold fluorescence) method was used to determine relative expression levels in the adrenal gland of P450, P450c21, and P450 17a (along with an endogenous reference gene, 18S ribosomal RNA) at 45 and 130 d of gestation. In the kidney, hippocampus, hypothalamus, and medulla oblongata of the 130 d fetuses, mRNA expression levels for angiotensinogen, the AT1, and AT2 receptor as well as the MR and GR were assessed. This method has been described (Lethobarb, Arnolds, Reading, UK). In this cohort, all ewes receiving S were carrying twins (n = 6 fetuses) and of the ewes infused with D there were 3 sets of twins (total of n = 8 fetuses). Fetuses were removed from the amniotic compartment and weighed. The adrenal glands were dissected free from the fetus and also weighed. One adrenal gland was immediately frozen in liquid nitrogen and stored at −80 C until RNA extraction was performed. Other fetal organs (liver, meso- and metanephros, brain, heart, and lung) were dissected, weighed, and frozen. Fetal fluids (amniotic and allantoic) volumes were measured and a 2-ml sample taken for analysis of ionic composition.

A comparative CT (cycle of threshold fluorescence) method was used to determine relative expression levels in the adrenal gland of P450, P450c21, and P450 17a (along with an endogenous reference gene, 18S ribosomal RNA) at 45 and 130 d of gestation. In the kidney, hippocampus, hypothalamus, and medulla oblongata of the 130 d fetuses, mRNA expression levels for angiotensinogen, the AT1, and AT2 receptor as well as the MR and GR were assessed. This method has been described (Lethobarb, Arnolds, Reading, UK). In this cohort, all ewes receiving S were carrying twins (n = 6 fetuses) and of the ewes infused with D there were 3 sets of twins (total of n = 8 fetuses). Fetuses were removed from the amniotic compartment and weighed. The adrenal glands were dissected free from the fetus and also weighed. One adrenal gland was immediately frozen in liquid nitrogen and stored at −80 C until RNA extraction was performed. Other fetal organs (liver, meso- and metanephros, brain, heart, and lung) were dissected, weighed, and frozen. Fetal fluids (amniotic and allantoic) volumes were measured and a 2-ml sample taken for analysis of ionic composition.
was no difference in CT values when we compared any of these genes in Table 1. Additional preliminary experiments had shown that there was no difference in Ct values when we compared any of these genes in a nonmultiplex reaction to a multiplex reaction (containing 18S). Also, the amplification efficiency of these genes was equal to that of 18S over a range of template concentrations (50 ng to 0.5 pg). cDNA (50 ng) and no reverse transcriptase preparations were amplified at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Calculations for real-time analysis

One sample of adrenal from a 130 d fetus that had received a S infusion was assayed five times in each real-time PCR run and used to determine the intraassay coefficient of variation. The mean of these five samples was used as the calibrator. In addition, five aliquots of adult adrenal cDNA were run in each assay to determine the relative fetal/adult levels of expression of each gene. The ΔCt value (obtained by subtracting the Ct value for 18S from the Ct value of the gene of interest) was subtracted from the Ct value for 18S to obtain a final CT value. The equation of 2−ΔΔCt was used to obtain a final value for each sample relative to the calibrator. Coefficients of variation for one sample run five times in one assay were 7% (P450scc), 6% (P450c21) and 14% (18S). In brain samples, the coefficients of variation were 18%, 40%, 23%, and 15%, respectively, for angiotensinogen, AT 1, MR, and 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec and 60 C for 1 min.

Statistics

Comparisons of gene expression in the tissues of the two treatment groups at 130 d was made by an unpaired t test. To compare the effect of the treatment at two time points (45 and 130 d) on the expression of the adrenal enzymes, a two-way ANOVA was used. Composition and volumes of fluids as well as blood pressure data were also assessed by unpaired t test. Statistical significance was set at P < 0.05. Values are mean ± SEM.

Results

Effect of infusion on maternal plasma

Infusions of S and D did not cause any significant changes in the ionic composition of the maternal plasma over the 20 d (data not shown). Maternal plasma ACTH was significantly reduced by D infusion. Basal values in this group were 15 ± 3 pg/ml and on d 6 of infusion, all but two of the animals had a plasma ACTH less than 5 pg/ml (the sensitivity of the assay). The other two animals had values of 15 and 16 pg/ml. On d 14 and d 20, all animals receiving D had a plasma ACTH less than 5 pg/ml. In the S group, the basal value was 27 ± 9 pg/ml, and there was no significant change over the course of the S infusion.

Effects on body and organ weights

Table 2 shows the body and organ weights of fetuses at 45 d, 130 d, and the lambs killed at 2 months postpartum after exposure to S or D from d 25–45 of gestation. At 45 d, D treatment had no effect on the weight of the fetus, or any organ, with the exception of the adrenal. The weight of the adrenals was significantly reduced (P < 0.001) by maternal D treatment. At 130 d, the body weight and that of most organs were reduced, but only body weight (P < 0.001), heart (P < 0.001), and lung (P < 0.05) reached statistical signifi-

<table>
<thead>
<tr>
<th>TABLE 1. Primer and probe concentrations and sequences used in real-time PCR</th>
<th>Position</th>
<th>Conc. (nM)</th>
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<tbody>
<tr>
<td>Probe sequence</td>
<td>17α</td>
<td>AGCTGTTCCTAAACAAGACATATGCTGCC</td>
</tr>
<tr>
<td></td>
<td>e21</td>
<td>ACCCGGAGATTCCGGCCGC</td>
</tr>
<tr>
<td></td>
<td>SCC</td>
<td>ATGGGGCAGAGCCCTCCGTCTTCTT</td>
</tr>
<tr>
<td></td>
<td>AT1</td>
<td>ACCGGTCCGTCTCTGCCGA</td>
</tr>
<tr>
<td></td>
<td>A/ogen</td>
<td>CCACGGACCCAAATCTCGCTGC</td>
</tr>
<tr>
<td></td>
<td>MR</td>
<td>TCCCTCATTTCTCCAAAAGCCAGCCTTG</td>
</tr>
<tr>
<td></td>
<td>GR</td>
<td>AAAGAAGATTATATCGAATCTCGACCCCCTG</td>
</tr>
<tr>
<td>Forward primer sequence</td>
<td>17α</td>
<td>GGGCCAGAGAAGATCCA</td>
</tr>
<tr>
<td></td>
<td>e21</td>
<td>GCGGTTGCCTCTCTACTTC</td>
</tr>
<tr>
<td></td>
<td>SCC</td>
<td>CTGTTTCAATGGCCATCTAT</td>
</tr>
<tr>
<td></td>
<td>AT1</td>
<td>GGGCTGTCGCTGATGTGAGGAA</td>
</tr>
<tr>
<td></td>
<td>A/ogen</td>
<td>CTCTCCACAGCTCCATACTAGTGG</td>
</tr>
<tr>
<td></td>
<td>MR</td>
<td>TCCAAAGATGTGCCCACAAA</td>
</tr>
<tr>
<td></td>
<td>GR</td>
<td>ACTGCCCCAATAGTAAAACACA</td>
</tr>
<tr>
<td>Reverse primer sequence</td>
<td>17α</td>
<td>CCCGAAGATGTCCGCCTT</td>
</tr>
<tr>
<td></td>
<td>e21</td>
<td>TCTGGATCCAACTCCTCTGGA</td>
</tr>
<tr>
<td></td>
<td>SCC</td>
<td>GCCACCTCGGTTGGGTCAA</td>
</tr>
<tr>
<td></td>
<td>AT1</td>
<td>CCGGAAGCATTTACATAGGTA</td>
</tr>
<tr>
<td></td>
<td>A/ogen</td>
<td>CTCTTCTAGTGTTCGACTGAT</td>
</tr>
<tr>
<td></td>
<td>MR</td>
<td>TTTAATGTTAGTCTCGACTCTTCTA</td>
</tr>
<tr>
<td></td>
<td>GR</td>
<td>TACCTGTCTTTACCGCATGTGAAAAT</td>
</tr>
</tbody>
</table>

All primers and probes are from 5’ to 3’ and all concentrations are in nM (the final reaction).
TABLE 2. Body and organ weights of fetuses killed at 45 and 130 d of gestation and at 2 months of age (postpartum) after maternal treatment with saline or dexamethasone between 25 and 45 d of gestation

<table>
<thead>
<tr>
<th></th>
<th>45 d</th>
<th>130 d</th>
<th>2 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline (6)</td>
<td>Dex (7)</td>
<td>Saline (8)</td>
</tr>
<tr>
<td>Body weight</td>
<td>8.97 ± 0.13 g</td>
<td>8.27 ± 0.55 g</td>
<td>3.2 ± 0.1 kg</td>
</tr>
<tr>
<td>Meconephros (mg)</td>
<td>38 ± 3</td>
<td>31 ± 1</td>
<td>17.4 ± 0.8</td>
</tr>
<tr>
<td>(g/kg BW)</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>5.4 ± 0.2</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>0.10 ± 0.01</td>
<td>0.098 ± 0.008</td>
<td>28.0 ± 1.8</td>
</tr>
<tr>
<td>(g/kg BW)</td>
<td>0.37 ± 0.02</td>
<td>0.34 ± 0.03</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>0.65 ± 0.03</td>
<td>0.59 ± 0.03</td>
<td>78 ± 6</td>
</tr>
<tr>
<td>(g/kg BW)</td>
<td>25 ± 2</td>
<td>26 ± 1</td>
<td></td>
</tr>
<tr>
<td>Adrenal (mg)</td>
<td>12.2 ± 0.7</td>
<td>6.3 ± 0.4a</td>
<td>315 ± 25</td>
</tr>
<tr>
<td>(mg/g BW)</td>
<td>1.36 ± 0.07</td>
<td>0.74 ± 0.07a</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>Brain</td>
<td>50.6 ± 1.5</td>
<td>46.7 ± 1.1</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>(g/kg BW)</td>
<td>15.8 ± 0.5</td>
<td>18.7 ± 0.4a</td>
<td></td>
</tr>
</tbody>
</table>

Number in each group is shown in parentheses.

α P < 0.001.
b P < 0.05.
c P < 0.01.

cancence. The kidney (metanephros) at 130 d was protected from the overall decrease occurring in the D-treated group, and as a ratio to body weight, was significantly greater than in the S-treated group (P < 0.05). Similarly, the brain weight in the D-infused group was similar to the S group, making it significantly larger in relation to body weight (P < 0.01). The adrenal was not significantly different in the D and S groups, either in absolute values or as a proportion of body weight.

At birth, singleton lambs from the D exposed group weighed 3.9 ± 0.3 kg (n = 6) compared with the 4.2 ± 0.3 kg (n = 5) in the S group. The tendency for lower body weight in the D exposed group was still apparent in 2 months of age with twins in this group weighing 10.9 ± 1.0 kg, whereas in the S group, twins weighed 11.6 ± 1.0 kg. When the twin lambs were killed at this stage of development all organs except the kidney were of similar weight between the groups. The kidney was smaller in the D exposed group (P < 0.05) and tended to be a lower proportion of the total body weight (P = 0.06).

**Fetal fluids**

There was no difference in the volume or composition of amniotic and allantoic fluid between the treatment protocols at either age. The volume of amniotic fluid at 45 d was 62 ± 13 ml (S) and 58 ± 10 ml (D) and was 314 ± 40 ml (S) and 266 ± 44 ml (D) at 130 d. Allantoic fluid volume was 69 ± 8 ml (S) and 67 ± 7 ml (D) at 45 d and 514 ± 79 ml (S) and 562 ± 57 ml (D) at 130 d. There was no significant difference in osmolality or the concentration of any individual solute (data not shown).

**Male singleton basal blood pressure and heart rate at 2 months**

On the day of measurement of blood pressure the lambs were 57 ± 2 d (S group, n = 5) and 59 ± 1 d (D group, n = 6). No significant changes in blood pressure or heart rate occurred during the recording period in individual lambs. The mean arterial pressure in the S exposed lambs was 83 ± 3 mm Hg and heart rate 114 ± 7 beats/min. In the D-exposed lambs, the pressure was not significantly different (78 ± 3 mm Hg), nor was the heart rate (113 ± 9 beats/min).

**Gene expression using real-time PCR**

**Fetal adrenal at 45 d.** Figure 1 shows the relative expression levels of A) P450ecC, B) P450c17α, and C) P450c17β in the adrenals of S- and D-treated fetuses at 45 and 130 d of gestation. The D treatment did not cause any significant alteration in gene expression except for the 45d adrenal P450ecC, which was halved (P < 0.05). The levels of expression of the P450c17α and P450c17β, in the S-treated animals, were the same at both gestational ages. However, there was a significant increase in the level of expression of P450c17α in both the S and D-treated fetuses at 130 d, when compared with 45 d. Results using the adult adrenal cDNA as a calibrator are shown in Table 3. In all cases, the level of expression in fetal adrenals was lower than that in adult adrenal, but P450c17α was more highly expressed (at ~30%) than P450c17β or P450c21 (~10% at 45 d).

**Fetal kidney and brain at 130 d.** Figure 2 shows gene expression levels of the AT1 receptor, angiotensinogen, MR and GR in the fetal kidney, hippocampus, hypothalamus, and medulla oblongata. Similar levels of expression of all these genes was seen in the kidney, hypothalamus and medulla oblongata. In the hippocampus, there was reduced expression of the MR (P < 0.05) and GR (P < 0.05) in the D-treated group.

**Lambs at 2 months.** Due to the differences observed in the expression levels of the MR and GR in the hippocampus at 130 d of gestation, expression levels of these genes were examined in the hippocampus of the 2 month lambs. Expression level of the MR tended to be lower in the D-exposed group (0.8 ± 0.1 compared with 1.2 ± 0.2 in the S group), but this did not reach statistical significance. Expression of the GR was similar (1.1 ± 0.2 in the S group and 1.1 ± 0.1 in the D group).
Discussion

This is the first animal study to replicate the D exposure commonly applied to some normal human fetuses who had been suspected of being at risk for congenital adrenal hyperplasia. The reassuring finding is that, in spite of some effects shown to be due to the treatment in intrauterine life, the long-term outcome was benign, at least as far as 2 months of age. While there have been follow-up studies of human babies who did carry the genetic defect (25), there have been relatively few studies of individuals who did not have the defect, but were exposed to the early glucocorticoid treatment (26). The current studies confirm that such a dose of D is having a dramatic effect on the growth of the adrenal, early in gestation, and suggests that such growth is dependent on ACTH production by the fetal pituitary. It has been known for many years that the early ovine fetal adrenal is capable of producing cortisol when incubated in vitro with ACTH (21, 22), and that there are relatively high levels of adrenal enzyme mRNA present (27).

In the current study, it was confirmed that the expression levels of three steroidogenic enzymes were high early in gestation, although still lower than that of the adult adrenal. With a low dose of D, only a reduction in P450scc mRNA was apparent with ACTH suppression, whereas with a shorter exposure to a higher dose of D, later in gestation both P450scc and P45017α were reduced (28).

ACTH-immunoreactive cells occur in the ovine fetal pituitary by at least 38 d of gestation (29). At the onset of

Table 3. Expression of adrenal enzyme mRNAs in the fetus as a fraction of the adult

<table>
<thead>
<tr>
<th></th>
<th>45 d</th>
<th>130 d</th>
</tr>
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<tbody>
<tr>
<td>P450scc</td>
<td>0.36 ± 0.03</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>P45017α</td>
<td>0.11 ± 0.05</td>
<td>0.16 ± 0.09</td>
</tr>
<tr>
<td>P45021</td>
<td>0.11 ± 0.04</td>
<td>0.24 ± 0.06</td>
</tr>
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</table>

Fig. 1. Ratio of expression of mRNA from ovine fetal adrenals with respect to a calibrator (one sample of 130-d fetal adrenal) for (A) P450scc, (B) P45021, and (C) P45017α. Fetuses were killed at 45 d or 130 d after exposure to S or D from d 25–45 of gestation. *, P < 0.05, n = 6 at each age and for each treatment.

Fig. 2. Ratio of expression of mRNA to calibrator in kidney, medulla oblongata, hypothalamus, and hippocampus for AT1, angiotensinogen (A'ogen), MR, and GR for 130-d fetuses treated with either S (open bars, n = 8) or low-dose D (solid bars, n = 8) between d 25 and 45. Calibrator in each case is mean of S group. Values are mean ± SEM. *, P < 0.05.
treatment, at d 25 there is no discrete identifiable pituitary, and it is likely that the effects of treatment result from the latter stages of D exposure. Growth of the human fetal adrenal in the first half of gestation is determined by pituitary ACTH (30).

There were no immediate effects of the D treatment on fetal body growth. However, there was a distinct and statistically significant reduction in body and some organ weights in late gestation. By 2 months after birth, there had been some catch-up growth in the D-exposed animals and all organ weights, except for the kidney, were of similar size. As seen in the male singleton lambs, blood pressure and heart rate were not significantly different. In children at risk of congenital adrenal hyperplasia who were treated prenatally with similar levels of D, there was no effect on birth weight (1).

The growth retardation that did occur between the end of the treatment and late gestation was, in many ways, typical of growth retardation that occurs in sheep that have placental insufficiency or whose mothers are nutrient deprived (31). The brain and kidney were spared, whereas other organs—liver, heart, lung—were not. The mechanisms underlying the growth retardation in the late gestation twins from the D treatment group are not well understood. It would be useful, perhaps, to investigate the IGF hormones, binding proteins and receptors in such twin fetuses, as there is good evidence for this system influencing the somatic growth of ovine fetuses (32, 33). The ovine placenta expresses mRNAs for IGFs, receptor and binding proteins at high levels before 50 d of gestation (34). Fetuses undernourished early in gestation show significant alterations in components of the IGF system, late in gestation (32). Thus, the growth trajectory in late gestation could have been programmed in the D-treated twins in this study by a long-lasting effect of early D treatment on the IGF system.

In the studies in which a higher dose of D was given for a shorter period, at 26–28 d of gestation, there were significant increases in the renal gene expression of GR, MR (19), and components of the RAS (12) in the late gestation fetus. These did not occur in the current protocol, and these data are consistent with the lack of increase in MAP seen in the lambs at 2 months, although it is not yet established whether hypertension will develop at a greater age. In this study, kidney weight, although not compromised in utero, was significantly reduced by the D in lambs at 2 months of age. Follow-up studies are required to determine if this has any long-term effect on renal, cardiovascular, or hormonal function. When ovine fetuses are treated with higher doses of D, for 2 d early in gestation, the offspring become hypertensive, with increased cardiac outputs, reset baroreflexes, and eventually develop left ventricular hypertrophy and impaired cardiac function (8, 18).

Acknowledgments

We thank Jehan Jeyaseelan and Irene Koukoulas for designing some of the probes and primers used in real-time PCR.

Received November 1, 2001. Accepted December 17, 2001.

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This work was supported by a Block Grant from National Health and Medical Research Council of Australia (963001) and a grant-in-aid from Broken Hill Propriety. The PE Applied Biosystems PRISM sequencing detector system was purchased with donations from the Philip Bushell Foundation, the Harold and Cora Brennen Benevolent Trust, the Viertel Foundation, and the Ramaciotti Foundation.

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Conclusion

When ovine fetuses are exposed to a low dose of D over the period 17–31% of gestation, there are immediate effects in reducing adrenal growth and the expression of the rate limiting enzyme P450scc. Twin fetuses from such treatment, investigated late in gestation, were symmetrically growth retarded, had altered hippocampal gene expression, but had normal adrenal steroidogenic gene expression. However, by 2 months after birth the offspring from the low-dose early D treatment were essentially normal in all measured parameters. Long-term monitoring of adult animals exposed to this treatment is required to assess if any subtle abnormalities develop with age. With the advent of noninvasive methods to determine fetal sex (35), fewer fetuses should be treated unnecessarily in the future. However, the results of these animal studies should prove reassuring to those children/adults so treated in the past.
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