Development of the pituitary adrenal axis in fetal sheep twins

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Schwartz, J effrey, and J ames C. Rose. Development of the pituitary adrenal axis in fetal sheep twins. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R1–R8, 1998.—Plasma cortisol increases in fetuses at term and is important for overall development. This study was designed to determine whether cortisol increases synchronously in twin fetal sheep and whether differences between twins contribute to the respective timing. Catheters were surgically implanted in fetal arteries in twins, the amniotic sac, and a maternal artery and vein. Blood was drawn daily until labor was imminent or the twins were delivered. Fetal pituitaries and adrenals were removed for in vitro measurements. Analyses included blood gases and cortisol (daily) and plasma cortisol, adrenocorticotropic hormone (ACTH), and estrogens (at completion). Twins were assigned retrospectively to group A or B, depending on which cortisol was first elevated (group A) above baseline. Group A fetuses consistently had higher cortisol until term. All group A fetuses also first had elevated ACTH. In four of four sets of twins of both sexes, the male was in group A. There were no differences between fetuses in plasma estrogens or pituitary ACTH response to stimulation, but adrenal cells from group A fetuses were more responsive. These data suggest that adrenal activity is increased in one twin consistently with the difference being attributable to the responsiveness of adrenal cells to ACTH rather than pituitary responsiveness to either corticotropin-releasing hormone or vasopressin. Difference between sexes may also be involved.

MATERIALS AND METHODS
Animals. All surgical and postsurgical procedures in these studies followed accepted veterinary medical practices and were approved by the Animal Care and Use Committee of this institution. Mixed-breed pregnant ewes with recorded dates of mating and bearing twins were used. In these sheep the length of gestation of singleton lambs typically runs 145 days. Surgery was performed on six ewes at 126 ± 1 days of gestation (Table 1). The sheep were anesthetized with ketamine and halothane and maintained under halothane anesthesia. With the use of strictly aseptic procedures, saline-filled polyvinyl catheters were implanted into the amniotic sac and into the descending aortas of both twins via both femoral arteries in each. After careful closure of the uterus, the catheters were led subcutaneously to the ewe’s flank, where they were exteriorized. Catheters were also placed in one femoral artery and vein of the ewe and exteriorized at the same site as the fetal catheters. All catheters were protected by being wrapped in a sterile gauze pad, covered with a latex glove, and held in place by protective elastic mesh. Propylactic intravenous antibiotics (1.5 mg/kg gentamicin and 20 mg/kg ampicillin) were administered at the time of surgery and once per day for 3 days afterward.

After recovery from anesthesia, the sheep were kept indoors in pens that allowed free movement. Food and water were available ad libitum. At no time did any of the sheep display any sign of illness or distress, and all procedures took place in the sheep pens.

Blood-sampling procedures. After at least 3 days of recovery (5 ± 1 days, mean ± SE), daily (between 0700 and 0900) sampling of fetal and maternal blood was commenced. Samples of 4 ml were drawn, and the volume was replaced by an equivalent amount of sterile isotonic saline. Partial pressures of O2 and CO2 in the blood and blood pH were measured immediately by automated blood gas analysis (Radiometer, Copenhagen, Denmark). Plasma cortisol concentration was...
also measured by radioimmunoassay (RIA) directly after sampling; remaining plasma was stored at −80°C until later analysis.

Time course of study. The study was initially designed to draw daily blood samples until as late in gestation as possible, but before the onset of labor, and then to recover the fetal pituitary and adrenals for in vitro studies. Based on previously published data (20, 28), the criterion that we established as being indicative of imminent labor was two successive measurements (i.e., 24 h) of plasma cortisol ≥50 ng/ml in either fetus, which experience has now taught us (see RESULTS) was not an appropriate indicator for twin sheep of this flock.

In vitro experiments. Fetuses, delivered by cesarean section, and neonates were killed by an overdose of intravenous pentobarbital sodium. The pituitary and adrenals were removed, dissociated, and cultured according to previously described methods (15, 34). Because there is evidence for an endogenous inhibitor of the adrenal responses to ACTH, the effects of which diminish with time in culture (14), and the function of pituitary cells is also altered by factors in the in vivo environment from which the tissue is obtained, we used cultured dissociated cells to examine inherent activity of the cells themselves. The left and right adrenals of each fetus were combined to yield a preparation of cells for each individual. The pituitary cells of each individual were cultured dissociated cells to examine inherent activity of the cells themselves. The left and right adrenals of each fetus were combined to yield a preparation of cells for each individual.

Table 1. Gestational ages of fetuses at important events in this study

<table>
<thead>
<tr>
<th>Set of Twins</th>
<th>Gestational age at surgery</th>
<th>Gestational age at first blood sample</th>
<th>Gestational age when group A fetus crossed cortisol threshold</th>
<th>ACTH Threshold</th>
<th>Gestational age at labor</th>
</tr>
</thead>
<tbody>
<tr>
<td>(M-M)</td>
<td>129</td>
<td>133</td>
<td>136</td>
<td>137</td>
<td>139</td>
</tr>
<tr>
<td>(M-F)</td>
<td>126</td>
<td>131</td>
<td>133</td>
<td>131</td>
<td>140</td>
</tr>
<tr>
<td>(M-F)</td>
<td>127</td>
<td>132</td>
<td>134</td>
<td>135</td>
<td>142</td>
</tr>
<tr>
<td>(M-F)</td>
<td>121</td>
<td>132</td>
<td>134</td>
<td>135</td>
<td>134</td>
</tr>
<tr>
<td>(M-F)</td>
<td>121</td>
<td>132</td>
<td>133</td>
<td>135</td>
<td>137</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>126 ± 1</td>
<td>131 ± 1</td>
<td>133 ± 1</td>
<td>135 ± 1</td>
<td>139 ± 2</td>
</tr>
</tbody>
</table>

Values are in days. M, male; F, female.
ear regression analysis of the curves. This was accomplished by fitting the curves to a sigmoid equation and determining the best fit by repeated iteration. The curves for adrenal responses in these cells were compared between cells obtained from group A fetuses and group B fetuses (see below) in terms of mean effective concentrations ($EC_{50}$) and maximum ACTH-secretory responses. Differences were considered significant if 95% confidence intervals did not overlap.

**RESULTS**

All 6 ewes and 12 fetuses survived the procedures of this study until the prescribed euthanasia. Maternal signs, such as appetite, indicated that the ewes remained healthy during the study.

Fetal well-being, as assessed by measurement of blood gases, was also maintained throughout the study. Partial pressures of $O_2$ and $CO_2$ in fetal blood averaged $18.9 \pm 5.1$ and $50.7 \pm 2.7$ mmHg (mean $\pm$ SD), respectively. Arterial pH averaged $7.32 \pm 0.05$. In individual animals there were occasional occurrences of low $O_2$ ($<15$ mmHg), high $CO_2$ (>53 mmHg), or low pH (<7.29), but in no case were there simultaneous measurements of abnormal $O_2$, $CO_2$, and pH indicating anything pathological.

Table 1 summarizes the gestational ages of the twins at various stages of the study. Because it was necessary to have a reference time point to compare data in all animals, day 0 was arbitrarily defined as the first day plasma cortisol was $\geq 50$ ng/ml in either fetus (Table 1). Elective delivery by cesarean section was performed, as originally planned, when plasma cortisol was $>50$ ng/ml for two successive daily measurements in only two sets of twins (sets 1 and 2). In twins (set 3), the fetuses were removed by cesarean section on the first day plasma cortisol was $\geq 50$ ng/ml because a later amniotic pressure reading indicated the ewe was in labor. In twins set 4, plasma cortisol went above 50 ng/ml in both twins, but for 1 day only before spontaneous birth, and there were no outward signs of labor on the evening before birth. Set 5 delivered spontaneously before plasma cortisol, based on the daily assay, exceeded 50 ng/ml in two successive daily measurements in only two sets of twins (sets 1 and 2). In twins (set 3), the fetuses were removed by cesarean section on the first day plasma cortisol was $\geq 50$ ng/ml because a later amniotic pressure reading indicated the ewe was in labor. In twins set 4, plasma cortisol went above 50 ng/ml in both twins, but for 1 day only before spontaneous birth, and there were no outward signs of labor on the evening before birth. Set 6 delivered spontaneously before plasma cortisol, based on the daily assay, exceeded 50 ng/ml in two successive daily measurements. In set 6, plasma cortisol was $>50$ ng/ml on the second day before birth but $<50$ ng/ml on the day before birth and $>50$ ng/ml again on the morning of birth.

Plasma cortisol concentrations. Cortisol concentrations increased steadily with advancing gestational age. For purposes of data analysis we defined baseline fetal plasma cortisol concentration as the mean of all fetal concentrations where fetal plasma cortisol concentration was less than the simultaneous maternal cortisol concentration ($=7.8$ ng/ml). We defined the threshold of increased fetal plasma concentration as being baseline $+2$ SD ($=13.4$ ng/ml). In each set of twins, the fetus in which plasma cortisol exceeded threshold first was designated as fetus A.

One important question addressed by these studies is whether activation of adrenal steroidogenesis occurs synchronously in twins and, if not, whether it occurs in parallel, but offset by a constant time quotient. Basically, we asked whether, once threshold was passed by one twin, that twin continued to produce higher plasma concentrations of cortisol than its sibling. When the simultaneous plasma concentrations of the twins are separated solely on the basis of which twin first crossed threshold, it becomes clear that adrenocortical activity of the twin that crossed first takes a lead over that of its sibling (Fig. 1). Considering the data from simultaneously drawn blood samples in all the pairs of twins, we found that once either twin crossed threshold, its plasma cortisol concentration was higher than the simultaneous concentration in the other twin 81% of the time (29 of 36 pairs of subsequent simultaneous measurements). In addition, analysis of variance of the average plasma cortisol concentrations in the two groups of twins, A and B, indicates significant effects of day (essentially gestational age) and group on plasma cortisol and a significant interaction between day and group (Fig. 1). In all sets of twins, plasma cortisol concentrations in group A fetuses reached 50 ng/ml before (5 of 6) or on the same day (1 of 6) as those in group B fetuses. Interestingly, it was also noted that plasma cortisol in group B twins often never attained the levels in their siblings. In two of three sets of twins that delivered live lambs, plasma cortisol levels in the B twin remained remarkably low. In one, plasma cortisol crossed 50 ng/ml (56.5) only within 24 h of birth, with the previously highest cortisol having been 37.8 ng/ml. In the other B twin, cortisol was only 45.3 ng/ml on the morning of birth (which occurred 3 h after the sample was drawn), with the previously highest cortisol having been 11.5 ng/ml and plasma cortisol having actually decreased (6.4 ng/ml) on the day before birth.

**Fig. 1.** Plasma cortisol concentrations as a function of gestational age, with the twins grouped according to which first crossed the threshold for increased cortisol (group A fetuses; ■). Solid horizontal line at 7.8 ng/ml represents baseline plasma cortisol concentration, defined as the average of all fetal plasma cortisol concentrations where fetal plasma concentration was less than simultaneous measurement of maternal cortisol. Hatched area represents $\pm$ 2 SD of those measurements. Threshold for increased cortisol was defined as the first measurement of cortisol greater than baseline $+2$ SD ($=13.4$ ng/ml). In each pair of twins, the twin whose cortisol was first over this mark was considered fetus A and the other, by default, fetus B (●). Curved lines represent third-order regression analysis of the data. Symbols represent means $\pm$ SE (maternal concentrations indicated by triangles). $^{*,*P < 0.05}$ for effect of time and difference between group A and B fetuses, respectively.
There was no correlation between plasma cortisol concentration and either blood $O_2$, $CO_2$, or pH (reckoned as such or as $H^+$ concentration).

Plasma ACTH-(1–39) concentrations. Daily changes and differences between twins in the concentration of plasma ACTH-(1–39) did not present as clear an outcome as did plasma cortisol. Over the course of the entire experiment (i.e., simply comparing the concentrations of ACTH in the plasma of each fetus at the first sample drawn with that in the last drawn before labor), there was a significant increase in plasma ACTH-(1–39) ($P < 0.01$). After defining the threshold, indicating stimulated plasma ACTH in a manner similar to that for plasma cortisol [mean $± 2 SD$ of all fetal ACTH-(1–39) measurements where fetal < maternal ACTH-(1–39) concentration], we found that all the fetuses that crossed the cortisol threshold before their sibling (group A) also crossed the ACTH threshold first. Group A fetuses did not cross the two thresholds on the same day, but five of six fetuses in group A crossed the cortisol threshold before they crossed the ACTH threshold (Table 1).

When plasma ACTH data are considered, as were the cortisol data, on a daily basis across the time frame of the experiment, there is neither an effect of day nor any significant difference between fetuses in groups A and B (Fig. 2). Even if days 6–2 are considered, wherein it appears that plasma concentrations of ACTH-(1–39) are consistently increasing in group A fetuses, there are no statistically significant effects. Although there was a correlation between plasma concentrations of cortisol and ACTH-(1–39), the relationship between the variables was weak ($r^2 = 0.35$).

Consideration of sex differences and altered time alignment. In contrast to the first two sets of twins, the last four provided an unexpected opportunity for further analysis of the data. In each of these fetal pairs there was a male and a female, and in all cases the pregnancy went through to delivery or confirmed onset of labor. The former factor permitted analyses of sex differences and the latter outcome permitted analysis in terms of a different physiological parameter for day 0, namely the day of labor or the day before birth (with the plasma sample on day 0 having been drawn on the day of, but before the onset of labor). In all four of these pregnancies there was a significant sex difference, with the male twin having a significantly higher plasma cortisol over the last days before day 0 (Fig. 3). In addition, using the criteria defined above for the crossing of plasma cortisol or ACTH thresholds, in all these pregnancies the group A fetuses were the males. Thus, despite use of a different criterion for day 0, there was still a significant increase in plasma cortisol with time in these four sets of twins. Analyses on these bases did not substantially alter plasma ACTH results (Table 2).

Plasma estrogens. With the last three sets of twins, sufficient plasma remained after the analyses of cortisol and ACTH for extraction and measurement of plasma total estrogens ($17\beta$-estradiol and estrone). The result was very consistent and indicated a significant increase in estrogens with time, although there was no indication of any increase until the day before birth (Table 2). There was no significant difference in plasma concentrations of estrogens between the male (group A) and female (group B) fetuses.

Physiometric measurements. There were no differences in whole body, adrenal, or kidney weights or in crown–rump lengths between fetuses designated A or B (Table 3). Any apparent potential difference in kidney weights between fetuses disappears when the kidney weights are normalized to individual body weights ($P = 0.242$, data not shown).

In vitro secretory activity of pituitary and adrenal cells. There was no difference in the ACTH-secretory responses in vitro to maximum stimulation with the hypothalamic peptides CRH and AVP by anterior pituitary cells from groups A and B fetuses (Fig. 4). Similarly, responses to lesser concentrations of CRH or AVP, albeit incomplete, offer no suggestion that the responses differed between cells from groups A and B fetuses.
In marked contrast, cultured adrenal cells from group A fetuses had a greater maximum response to ACTH than did cells from group B fetuses (Fig. 4). Analysis of variance of the concentration-response relationship of ACTH and secreted cortisol between groups A and B fetuses indicated significant effects of concentration of ACTH and of group (A or B). Further analyses of the concentration-response data indicated a significant difference in maximum response (13.35 ± 3.49 vs. 4.04 ± 0.88) but not in EC50 between the two groups. Interestingly, the responses of pituitary and adrenal cells from tissues obtained from the fetuses in labor (twins set 3) were close to the mean of the responses from all the experiments, and the inclusion or exclusion of these data did not alter the statistical outcome of the experiments.

**DISCUSSION**

The immediate object of the present study was to determine whether concentrations of plasma cortisol increase at the same time and to the same extent in twin sheep fetuses as they approach term. Given that each set of twins is of similar genetic composition and subject to nearly identical maternal and environmental factors in utero, these findings imply that in sheep the ultimate trigger and mechanisms for the onset of the increase in fetal plasma glucocorticoids resides in the fetus and not the mother or placenta. Kitts et al. (20) performed a study with mixed-breed twin pregnancies achieved by embryo transfer. They observed that activation of C-21 adrenal steroidogenesis occurred first and remained ahead in the sibling of the breed with the shorter period of
gestation. In the earlier study, the timing of steroidogenic activation was thus attributed to the genetic composition of the fetuses; it is noteworthy that a similar phenomenon was observed in the present study despite the greater genetic similarity of the twins.

The differences in plasma cortisol between the twins also suggest that in multiple pregnancies there is little communication between the fetuses in this regard. Even before labor, differences of >20 ng/ml between twin fetuses were not uncommon, and in one set of twins there were 2 days when the difference was >50 ng/ml. These data, with naturally occurring changes in plasma cortisol, resemble those of a study by Brooks et al. (8) in which ACTH was infused into one fetus in twin pregnancies. In that study, plasma cortisol increased only in the infused fetus. Similarly, Kitts et al. (20) measured significant differences in cortisol between fetal twins in their embryo-transfer experiments. The widely divergent values for plasma cortisol obtained at simultaneous samplings from maternal and both fetal arterial circulations is consistent with the observations of Belton et al. (6) in their direct studies of resistance to diffusion by the ovine placenta. They concluded that negligible amounts of cortisol cross from the maternal to the fetal circulation and that the small amount that crosses in the opposite direction contributes very little to the maternal cortisol concentration.

If the trigger for the stimulation of adrenal steroidogenic activity is resident in the fetus, then analysis of the differences between twins may yield clues to the nature of the trigger. In the present study this was noted in two ways, in terms of the anatomic level at which the timing mechanism may reside and in terms of the mechanisms that might operate to implement or act as a result of the trigger.

The responses of cells cultured from the individual twins (fetuses and neonates) postmortem shed light on the level at which key mechanisms for the timing of the increase in cortisol may occur. The difference between the cortisol responses to ACTH of the adrenal cells of the two groups suggests that inherent differences in the development of fetal adrenals, rendering them more responsive to ACTH, contribute more to the increase in plasma cortisol than do changes in the responsiveness of pituitary cells to hypothalamic factors. Studies of cortisol responses to exogenous ACTH in vivo indicate that adrenals become more responsive with age (33), and it is not unreasonable to infer that with twins it is possible for the onset of the increase in responsiveness to occur in the adrenals of one fetus earlier than the other. The finding that group A adrenal cells had a greater cortisol response to ACTH than did those of group B also supports the concept that the elevated plasma cortisol measurements in group A were more likely due to increased secretion by the adrenals rather than decreased clearance of cortisol, although these results by themselves are not conclusive.

Interestingly, in all four sets of twins with male and female fetuses, the males crossed the cortisol and ACTH thresholds first and had significantly higher plasma cortisol concentrations (group A) than the female siblings (all group B). This apparent sex difference suggested a potential mechanism for the timing of the increase in cortisol involving sex steroids. The absence of a difference between twins in concentrations of total estrogens argues against a role for total estrogens. The present findings are similar to more extensive results reported by Kitts et al. (19, 20), in which there was a difference in plasma cortisol concentrations between the Rambouillet and Finnish Landrace siblings but no difference in unconjugated estrone, estradiol, androstenedione, or estrone sulfate and in which plasma concentrations of the estrogens also increased after those of cortisol.

The precipitous increase in plasma cortisol measured in the twins in the present study is consistent with previous measurements made in singleton fetuses approaching term (5). In twins, the differences over time and between groups were significant whether the timing of the crossing of plasma cortisol threshold or sex was the determining factor for grouping the siblings. Similarly, the differences were significant whether the day of the first plasma value of ≥50 ng cortisol/ml or the day before labor was used as day 0. These observations support the idea that the increases in cortisol are indeed different between the twins.

The measurements of plasma ACTH are somewhat more difficult to interpret. Although there was an overall correlation between simultaneous plasma ACTH-(1—39) and cortisol values, in contrast to the case with cortisol, there was no significant increase in plasma daily ACTH over the course of the study nor any significant difference between twins. The absence of any effect of time (i.e., gestational age) on plasma ACTH in the presence of an effect of time on cortisol may reflect increasing responsiveness with time of the adrenals to stimulation by ACTH. Thus ACTH could be increasing on a daily basis, with changes too small to be detected, at the same time that adrenal responses to ACTH are also increasing, the net effect being increases in cortisol of greater magnitude than those of ACTH and thereby significant. Another factor to be considered is that the pulsatility of ACTH secretion (2, 18) may have increased the variability of measurements and obscured daily changes. A third factor to be considered is the effect of steadily increasing cortisol on ACTH secretion. Negative feedback by glucocorticoids, even if attenuated at this stage of gestation, might provide enough inhibition of ACTH secretion to render daily changes in ACTH indistinguishable. With regard to this last consideration, it may be worth contrasting Figs. 1 and 2 over days −1 to +1, during which time plasma cortisol is clearly still increasing while ACTH concentrations are not.

Regarding the absence of differences in plasma ACTH concentrations between twins, the mechanisms described in the previous paragraph also may be responsible. In particular, the differences in sensitivity between the adrenal cells to ACTH of groups A and B may be sufficient to explain why there can be a difference on plasma cortisol concentrations without a simultaneous significant difference in plasma ACTH.
One unexpected finding was the extent to which plasma cortisol remained low in some of the individual twins in group B. This was true even among twins that were allowed to continue to birth, in all cases of which the neonates were healthy, breathing, and nursing. The precise role of plasma cortisol for maturation of various organ systems in terms of concentrations and duration of exposure is still a matter of study. Prolonged survival studies of the live-born lambs to test whether all organ systems were fully developed were not performed in the present study. Research suggests that for maturation of the lungs, at least, glucocorticoids, as a single component, may be more critical at earlier deliveries than at term (3). That would appear to be the case in the present studies.

Another interesting finding was the absence of correlation between fetal blood gas parameters and plasma concentrations of cortisol. Among the known stimuli in adult and fetal animals of HPA axis activity are hypoxia, hypercapnia, and acidemia (e.g., Refs. 24, 29, 30, 36). None of these conditions by themselves were associated with increased plasma cortisol in the present study. The elevations of H+ concentration and CO2 levels were modest. As such, the results are consistent with those of Chen and Wood (13), who reported that fetal sheep (123–127 days gestational age), whose CO2 tensions were elevated to 55.2 and pH levels were decreased to 7.257 as a result of increasing the fraction of CO2 in the maternal inspired air, maintained normal plasma ACTH and cortisol concentrations. Hypoxemia is a more common cause of increased pituitary-adrenal activity in fetuses, and several experimental models have been employed to study the effects of decreased oxygen (1, 7, 12, 17). Over a period of up to 24 h, there is a clear and sustained increase in cortisol in response to hypoxia (1, 7, 12, 17). In contrast, in a model of chronic fetal hypoxia, Harvey and co-workers (16) found no effect on plasma ACTH or cortisol concentrations. This would suggest the existence of dissociable response mechanisms to hypoxia in fetuses, which may operate in separate time domains. The results of the present study would be entirely consistent with this description. Interestingly, the one fetus with the lowest average measurements of oxygen also had the lowest average measurements of cortisol, with both measurements being consistent over an entire period of 5 days of measurements. The responses in this animal would appear to fit a model of chronic hypoxia as in the experiments of Harvey et al. (16).

Perspectives

The role of adrenal glucocorticoids in signaling maturation of other organs is rather clear, but the ultimate trigger for activation of adrenal steroidogenic activity and interactions between the ACTH-glucocorticoid system and other factors is not as well characterized. A growing body of evidence, including the present results, points to increased adrenal responsiveness to ACTH as a major component of the triggering mechanism, and further efforts will, no doubt, focus on the changes within the adrenal that yield this outcome. Some clues to the interactions with other factors were also suggested by the present results. The limited data on male-female sets of twins are consistent with some factor(s) in sexual development that hasten or retard activation of adrenal cortisol responses in males or females, respectively. On the other side of the cause-and-effect relationship, there may be something in sexual development, perhaps an interaction with other hormones, that permits equivalent effects of glucocorticoids at lower concentrations in female fetuses. The lungs of the neonatal female lambs in the present study were apparently adequate for postnatal life, despite lesser exposure to cortisol in utero. It is known that in experimental animals and humans, fetal female lungs are apparently more mature than age-matched male lungs, and treatment with exogenous glucocorticoid at a given dose is more effective on lungs in female fetuses (21, 25, 26). Future work is indicated to determine the specific sex-related factor(s) and the mechanisms by which they generate apparently enhanced adrenal maturation in male fetuses and enhanced effects of glucocorticoids in female fetuses.

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