Effects of twinning, birth size, and postnatal growth on glucose tolerance and hypothalamic-pituitary-adrenal function in postpubertal sheep

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Bloomfield FH, Oliver MH, Harding JE. Effects of twinning, birth size, and postnatal growth on glucose tolerance and hypothalamic-pituitary-adrenal function in postpubertal sheep. Am J Physiol Endocrinol Metab 292: E231–E237, 2007. First published August 29, 2006; doi:10.1152/ajpendo.00210.2006.—Low birth weight is associated with postnatal physiological changes, including impaired glucose tolerance and increased cortisol secretion, that may predispose to disease in adulthood. Twins are born lighter than singletons, but there are conflicting data regarding the association between birth weight and postnatal physiology in twins. We studied glucose tolerance and ACTH and cortisol responses to a combined corticotropin-releasing hormone and arginine vasopressin (CRH + AVP) challenge in postpubertal female twin (n = 7 twin pairs) and singleton (n = 13) sheep from the same flock. There were no differences in glucose tolerance between twins and singletons and no association with birth weight. Twins had a greater ACTH (P < 0.05), but not cortisol, response to CRH + AVP than singletons. ACTH area under the curve was inversely related to birth weight in both singletons (R² = 0.31, P = 0.05; −8.311 (SD 3.736) pg·min·ml⁻¹·kg⁻¹) and twins (R² = 0.49); in twins, this was due to the within-twin pair rather than the between-twin pair coefficient in the regression analysis [P = 0.02, −26,856 (9,806) vs. P = 0.1, 8,619 (4,950) pg·min·ml⁻¹·kg⁻¹]. We conclude that the reduced fetal growth in twins has postnatal consequences for hypothalamic-pituitary-adrenal function and that this is determined by factors specific to the fetus (within-twin pair) rather than by shared maternal factors (between-twin pair). Studies investigating the associations between fetal growth and postnatal outcomes in twins benefit from an appropriate singleton control group and from analyses evaluating the contribution from both between- and within-pair coefficients in twins.

developmental origins of health and disease; twin pregnancy; hypothalamic-pituitary-adrenal axis

THE BIRTH WEIGHT DISTRIBUTION FOR TWINS is different from that for singletons (1, 61), with twins on average being born lighter and shorter (9). Twins, therefore, would be expected to have a greater risk of diseases that are associated with lower birth weight, such as coronary heart disease, hypertension and diabetes. However, epidemiological studies in twins have produced conflicting results (8, 30, 31, 39, 40, 53, 60, 63).

There may be several reasons why the literature is not consistent regarding the relationship between birth weight and later disease risk in twins (48). One reason may be selected samples in twin studies and the frequently small sample sizes. In some cases, the most affected subjects have been excluded, such as the exclusion of diabetic subjects in studies of glucose tolerance (50, 51).

Another reason may be that comparisons between twins of lighter and heavier birth weight may not reveal an association with later disease risk if both twins are in fact growth restricted and therefore show similar risk. There is some evidence that the incidence of diabetes, at least, is much higher in twins than in the general population (52, 56). However, few studies have compared twins with appropriate singleton controls. Those that have compared twin cohorts with either singleton control cohorts or singleton siblings did not find an association between being a twin and blood pressure (16) or between lower birth weight in twins and ischemic heart disease (57) or mortality (12, 13). However, a recent study in prepubertal subjects reported insulin sensitivity values in twins approximately one-half those in singleton controls, and this reduction was independent of birth weight, gestation length, and zygosity (34). We are not aware of any studies that have investigated postnatal hypothalamic-pituitary-adrenal (HPA) axis responsivity in twins compared with singletons.

Finally, several studies in singletons have reported the importance of interactions between prenatal and postnatal growth patterns in determining later disease risk (21–24, 28, 37, 55). We are not aware of any such studies in twins. However, there are data suggesting that postnatal growth patterns of twins are different from those in singletons (9, 11).

Recent studies have proposed that examining the relationships between birth weight and later disease risk within a cohort, rather than between cohorts, may provide more information on the role of the intrauterine environment (17, 18). Use of regression analyses that incorporate both a between-twin pair coefficient (accounting for the contribution of shared maternal factors, such as nutritional status, socioeconomic status, smoking, etc.) and a within-twin pair coefficient (accounting for individual fetal factors, such as individual nutritional, metabolic, or hormonal status) may facilitate greater insight into the relative contributions of maternal and fetal influences on birth weight and postnatal outcome (10).

There are few studies of the interactions among twinning, birth weight, postnatal growth, and postnatal physiology in experimental animals and none that have also studied singletons. This is despite the fact that animal studies can avoid many of the biases inherent in human population studies. The sheep is one of the few domestic species that is principally monotonous but with a reasonable incidence of twinning, which in almost all cases is dizygotic. We therefore examined the relationship between birth weight, postnatal growth, and glucose tolerance and HPA axis function after puberty in twin and singleton sheep.
singleton sheep from a single flock. We hypothesized that, in twins, the within-twin pair coefficient for the relationship between birth weight and postnatal outcome would be stronger than the between-twin pair coefficient, suggesting that factors specific to each individual twin that influence birth weight also influence postnatal physiology.

METHODS

Animals. This study was approved by the animal ethics committee of the University of Auckland. Multiparous Romney ewes in good nutritional condition were mated after synchronization of estrus with an intravaginal progesterone-containing CIDR (controlled internal drug release) (59). Ewes were maintained outdoors as a single flock on a good nutritional plane throughout pregnancy and the postnatal period. Ewes were weighed immediately prior to mating and at 60 and 120 days of pregnancy (term, 145 days). At delivery, lambs were weighed, measured, tagged, and identified as being a singleton or twin. Lambs were reared with their mothers until weaning at 3 mo when further measurements of size and weight were made. Thereafter, all female lambs were run as one flock until 10 mo of age. All female singleton (n = 13) and female twin pair (n = 7 twin pairs) offspring were then selected and acclimatized to a concentrate feed consisting of 65% lucerne, 30% barley and limestone, molasses, and trace elements (CamTech, Cambridge, New Zealand). Animals were weighed, brought indoors, and housed in individual large pens in a purposely built research barn. Estrus was synchronized as above. A polyvinyl catheter was inserted into a jugular vein by threading through a 12-gauge needle after the skin had been infiltrated with 2% xylocaine. The catheter was secured to the neck with tape and placed on the animal’s back under a meshed stocking. Animals were then fasted overnight, and the following morning a glucose tolerance test was performed. A 0.5 g/kg glucose bolus was given intravenously over 30 s, and blood samples were drawn at 0, 2, 5, 10, 15, 20, 30, 40, 50, 60, and 120 min. Animals were then fed. The following day, a combined corticotropin-releasing-hormone (CRH) and arginine vasopressin (AVP) challenge was performed. Equimolar amounts of CRH (0.1 μg/kg) and AVP (0.5 μg/kg) were injected rapidly into the jugular vein, and blood samples were drawn at 0, 15, 30, 45, 60, 120, and 240 min. The catheters were then removed and the animals returned to pasture. All blood samples were placed on ice until centrifugation, and plasma samples were stored at −80°C until analysis.

Hormone and metabolite analysis. Glucose concentrations were measured on a Hitachi 902 autoanalyzer (Hitachi Australia, North Ryde, NSW, Australia). Insulin concentrations were measured by an in-house radioimmunoassay (RIA), as described previously (43), except that ovine insulin (Sigma Chemical, St. Louis, MO) was used as the standard. The standard curve displaced in parallel with ovine plasma samples and cross-reactivity with IGF-I or IGF-II was <0.01%. The minimal detectable concentration was 40 pg/ml plasma, and the inter- and intra-assay coefficients of variation (CVs) were 14.1 and 5.3%, respectively. ACTH was measured with a commercial RIA kit (DiaSorin, Stillwater, MN), previously validated for use in sheep (35) and with inter- and intra-assay CVs of 7.5 and 9.2%, respectively. Cortisol was measured by mass spectrometry using a method developed in our laboratory and reported previously (33). The intra- and interassay CVs were 6.4 and 12.2%, respectively.

Statistics. Pregnancy data for mothers were compared by Student’s t-test. Twin offspring ewes were identified as being lighter or heavier twins in glucose or insulin response to a glucose tolerance test (Fig. 1, A and B). There were no significant differences between singleton and either heavier or lighter twins in glucose or insulin response to a glucose tolerance test (Fig. 1, A and B).

Baseline ACTH and cortisol concentrations were not statistically different among groups, although the lighter twins tended to have the lowest circulating ACTH and cortisol concentrations [ACTH: singleton 93.0 (66.7), heavier twin 63.5 (24.0), lighter twin 50.3 (7.3) pg/ml, P = 0.16; cortisol: singleton 9.7 (1.6), heavier twin 12.8 (7.7), lighter twin 5.3 (3.1) ng/ml, P = 0.06]. However, the baseline ACTH-to-cortisol ratio was not significantly different among groups [singleton 11.7 (7.5), heavier twin 9.5 (12.0), lighter twin 14.0 (11.6) pg/ng, P = 0.7]. The lighter twin had a significantly greater ACTH response to CRH + AVP than both heavier twin and singleton (Fig. 1C), but no significant difference in cortisol response (Fig. 1D). A plot of ACTH vs. cortisol concentrations during the CRH + AVP challenge (Fig. 2) demonstrates that maximal adrenal stimulation was achieved in all groups but that there were no differences in the relationship of ACTH to cortisol amongst the groups.

Table 1. Maternal weights before and during pregnancy and weight gain over pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Singleton-Bearing Ewes</th>
<th>Twin-Bearing Ewes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 13)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Weight at mating</td>
<td>54.5 (4.0)</td>
<td>57.6 (2.6)*</td>
</tr>
<tr>
<td>Weight at day 60</td>
<td>58.0 (4.5)</td>
<td>61.6 (3.3)*</td>
</tr>
<tr>
<td>Weight at day 120</td>
<td>64.4 (4.6)</td>
<td>69.5 (3.5)</td>
</tr>
<tr>
<td>Pregnancy weight gain</td>
<td>9.9 (3.8)</td>
<td>11.9 (2.0)</td>
</tr>
</tbody>
</table>

Data are means (SD) in kg. *P < 0.05 vs. singleton-bearing ewes.
Regression analyses showed no significant associations between birth weight and glucose and insulin AUC in response to a glucose load for either singletons or twins (Table 3). Including weight gain from birth to weaning and from weaning to 10 mo increased the regression coefficients for insulin AUC in both singletons ($R^2$ 0.08 to 0.55) and twins ($R^2$ 0.10 to 0.43), and for glucose AUC in twins ($R^2$ 0.09 to 0.29), suggesting that postnatal weight gain is more important than birth weight in determining glucose and insulin responses to a glucose load, although none of the coefficients was statistically significant.

The regression coefficients for the association between birth weight and ACTH and cortisol AUC in response to CRH + AVP were of borderline significance in singletons ($R^2$ 0.33 and 0.26, both $P = 0.05$; Table 3). In twins, the regression coefficient for the association between birth weight and ACTH AUC was stronger than in singletons ($R^2$ 0.49); the within-twin pair, but not the between-twin pair, coefficient was statistically significant ($P < 0.02$, $B$, $P = 0.11$; Table 3). There was no significant association between birth weight and cortisol AUC in twins ($R^2$ 0.06; $P = 0.8$, $B$: $P = 0.5$; Table 3). Including measures of postnatal growth did not substantially alter the correlation coefficient for ACTH AUC for either singletons or twins, and the within-twin pair coefficient remained statistically significant in twins. For cortisol, the correlation coefficients increased with inclusion of postnatal growth variables, but there were no statistically significant associations (Table 3).

**DISCUSSION**

These data demonstrate that, in postpubertal sheep, twins have an accentuated ACTH response to a CRH + AVP challenge, and this is more pronounced in the within-twin pair than the between-twin pair. However, there was no significant association between birth weight and cortisol AUC in twins.

**Table 2. Growth measurements at birth, weaning, and 10 mo of age**

<table>
<thead>
<tr>
<th></th>
<th>Singletons ($n = 13$)</th>
<th>Heavier Twin ($n = 7$)</th>
<th>Lighter Twin ($n = 7$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Birth</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td>4.86 (0.69)</td>
<td>4.24 (0.67)</td>
<td>3.76 (0.53)†</td>
</tr>
<tr>
<td>Crown-rump length, cm</td>
<td>52.9 (2.7)</td>
<td>52.1 (2.3)</td>
<td>51.3 (2.4)</td>
</tr>
<tr>
<td>Hindlimb length, cm</td>
<td>36.7 (1.8)</td>
<td>35.9 (1.9)</td>
<td>34.9 (1.7)</td>
</tr>
<tr>
<td>Forelimb length, cm</td>
<td>30.6 (1.8)</td>
<td>29.0 (1.8)</td>
<td>28.4 (1.5)*</td>
</tr>
<tr>
<td>Chest girth, cm</td>
<td>41.6 (2.4)</td>
<td>39.2 (1.7)</td>
<td>37.6 (2.4)†</td>
</tr>
<tr>
<td><strong>Weaning</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td>30.4 (2.0)</td>
<td>23.2 (3.3)‡</td>
<td>22.9 (2.4)‡</td>
</tr>
<tr>
<td>Weight gain, birth to weaning, kg</td>
<td>25.5 (1.6)</td>
<td>19.0 (2.9)‡</td>
<td>19.2 (2.2)‡</td>
</tr>
<tr>
<td>Crown-rump length, cm</td>
<td>93.8 (7.2)</td>
<td>85.9 (4.9)*</td>
<td>88.3 (3.7)</td>
</tr>
<tr>
<td>Hindlimb length, cm</td>
<td>52.7 (3.3)</td>
<td>49.3 (3.8)</td>
<td>48.9 (2.6)*</td>
</tr>
<tr>
<td>Forelimb length, cm</td>
<td>42.2 (2.2)</td>
<td>40.7 (2.3)</td>
<td>40.0 (3.4)</td>
</tr>
<tr>
<td>Chest girth, cm</td>
<td>78.7 (4.7)</td>
<td>71.1 (6.2)‡</td>
<td>70.4 (2.6)‡</td>
</tr>
<tr>
<td><strong>10 mo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td>52.1 (4.0)</td>
<td>46.1 (3.6)‡</td>
<td>43.6 (3.1)‡</td>
</tr>
<tr>
<td>Weight gain, weaning to 10 mo, kg</td>
<td>21.7 (3.8)</td>
<td>22.9 (5.3)</td>
<td>20.6 (2.4)</td>
</tr>
</tbody>
</table>

Data are means (SD). *$P < 0.05$, ‡$P < 0.01$, †$P < 0.001$ vs. singleton ewes.
challenge compared with singletons. We believe this is the first study comparing postnatal HPA function between twins and singletons in either humans or animals. Consistent with human studies (14), including one of adult male twins (62), we found an inverse association between birth weight and HPA response. This was similar in singletons and twins, but the effect in twins was due to the within-pair rather than the between-pair coefficient of the analysis, that is, to factors specific to individual fetuses rather than to those such as maternal and environmental influences common to both twins of the pair.

Given that twins overall have reduced growth compared with singletons (1, 61), both twins may be at increased risk of adverse outcome compared with singletons. This is consistent with a report of insulin resistance in twin children compared with singletons (34) and with a greater reported incidence of diabetes in twin populations (52) compared with general populations (56). Therefore, we suggest that the use of a co-twin alone as a control is not appropriate when one is investigating whether reduced growth in twins is related to long-term outcome and that an appropriate singleton control group should also be included whenever this is feasible.

These data also suggest that, at least in twins, changes in postnatal HPA axis function in relation to birth weight are not likely to be due to the maternal factors that have been proposed as possible intermediaries, such as maternal nutritional status (47) or glucocorticoid levels (58). In our study, twin-bearing ewes were heavier at conception [a well-recognized association (36)] and gained more live weight during pregnancy, consistent with the good nutritional plane on which all ewes were maintained. However, the regression analyses incorporating both between- and within-twin pair coefficients control for any differences there may be between singleton- and twin-bearing ewes. If the association between postnatal HPA function and birth weight had been due to maternal influences, then in the twin analysis the between-twin, rather than the within-twin, pair coefficient should have shown greater statistical significance. However, we found that the converse was true, suggesting that factors associated with the fetal growth of individual twins of the pair, rather than maternal factors common to both twins, were responsible for the observed associations (10).

Although the regulation of fetal growth is poorly understood, there are now several strands of evidence suggesting that fetal growth trajectory may be set early in gestation. Human ultrasound and animal post mortem studies suggest that fetal growth trajectory in twins may be set as early as the end of the first trimester (3, 29, 38). In multiple pregnancies that have undergone either spontaneous or interventional fetal reduction, birth weight and gestational age are significantly associated with the original number of fetuses rather than the number that survive in utero (2, 49). We have previously reported that fetal growth trajectory in late gestation singleton fetal sheep depends upon nutritional status at the time of conception (45) and that the fetal growth response to a late-gestation nutritional insult depends on prior growth rate (27). Periconceptional nutrition may also affect fetal endocrine development (44), maturation of the fetal HPA axis (6), timing of birth (5), and fetal muscle development (41, 54). We therefore speculate that the factors that alter postnatal physiology in relation to size at birth may also be determined very early in gestation (4) rather than reflecting maternal constraint of fetal growth for reasons of space and nutrient supply in late gestation, as has generally been assumed (26).

Table 3. Regression coefficients and parameter effect size (ΔAUC per kg increase in birth wt) for singletons and twins

<table>
<thead>
<tr>
<th>Factor</th>
<th>ACTH AUC (pg·min·mL⁻¹) per kg increase in birth wt</th>
<th>Cortisol AUC (ng·min·mL⁻¹) per kg increase in birth wt</th>
<th>Insulin AUC (pmol·min·mL⁻¹) per kg increase in birth wt</th>
<th>Glucose AUC (mmol·min·mL⁻¹) per kg increase in birth wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect size</td>
<td>ΔACTH</td>
<td>ΔCortisol</td>
<td>ΔInsulin</td>
<td>ΔGlucose</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>b_n</td>
<td>b_w</td>
<td>n</td>
</tr>
<tr>
<td>P value</td>
<td>0.05</td>
<td>0.11</td>
<td>0.02</td>
<td>0.05</td>
</tr>
</tbody>
</table>

ΔAUC, change in area under the curve; b_n, between-pair coefficient of twin analysis (mean birth wt of the twin pair); Adj R², coefficient of correlation including weight gain from birth weight to weaning and from weaning to time of study as covariates. Parameter estimates and P values are given only for birth weight.
If the factors that alter postnatal HPA function in twin sheep are determined early in gestation, then differences in HPA function between singletons and twins may already be present in fetal life. Twin fetal sheep have been reported to have similar baseline and hypoxia-induced ACTH but reduced cortisol concentrations compared with singletons, consistent with adrenal resistance (25). The prepartum rise in ACTH and cortisol have also been reported to be delayed in twin fetal sheep compared with singletons (19). In both of these studies, only one twin of a pair was studied; in a study in which both twins of a pair were studied simultaneously, we (7) recently found both adrenal resistance and reduced cortisol negative feedback sensitivity in twin fetal sheep compared with singletons. In the study of postnatal twin sheep that we report here, both explanations for our findings are possible, but an effect on negative feedback or a greater pituitary corticotropin response to higher input seems more likely. The fact that the basal ACTH-to-cortisol ratio was not different between twins and singletons, and the very similar relationship among groups between cortisol and ACTH concentrations in response to CRH + AVP injection (Fig. 2), suggests that significant adrenal resistance is unlikely. We have not been able to investigate potential mechanisms at the molecular level, as these animals are still alive.

Although the lack of associations between birth weight and the glucose and insulin responses to a glucose tolerance test in this study may seem surprising, this is consistent with other studies in animals and with human data. We (42) have previously reported similar results in postpubertal singleton sheep that had been subjected to 10 or 20 days of severe late-gestational undernutrition in utero. In that study, glucose tolerance at 5 mo of age (prepubertal) was related to birth weight rather than to the duration of nutritional deprivation, but the association was no longer present at 3 yr of age. In children, the effects of birth weight on insulin sensitivity are largely mediated by weight gain in childhood and current body mass index (BMI), and an independent effect of birth weight on insulin sensitivity was evident only in children with the highest BMI at the time of study (32, 46). Similar associations between glucose tolerance, birth weight, and postnatal growth have been shown in large studies in adults from Finland (22, 24), with the worst glucose tolerance being seen in those born small who then had slow weight gain in the first few months of life followed by rapid weight gain later in childhood. Our findings that insulin AUC was related positively to weight gain to weaning but negatively to weight gain from weaning to 10 mo of age are consistent with these human data. The small size of the effect of lamb growth on insulin response may reflect the relatively young age at which these animals were studied. A previous study in young twin sheep showed small effects of lamb size on insulin sensitivity at 6 mo of age, with lighter twins having slower growth and greater insulin sensitivity, but no effect by 12 mo of age (15). This complex interaction between pre- and postnatal growth and glucose tolerance clearly has important clinical implications, particularly in the light of the current concern about trends in obesity and its origins early in life (20). If growth rate in the first few months of life has an impact on later glucose tolerance, this may have particular implications for twins, when competition between two infants for a single milk supply may impact on growth rate. Furthermore, the inclusion of a singleton control group is essential when one is studying the postnatal consequences of intrauterine development in twins, not only because of differences in intrauterine growth rate but also because of the potential impact of differences in postnatal growth trajectory between singletons and twins.

In conclusion, we have demonstrated that, in postpubertal ewes, HPA axis responsiveness is similarly related to birth weight in both singletons and twins. However, in twins this relationship is determined by factors associated with fetal growth trajectory that are specific to each fetus rather than by common maternal factors. We speculate that such factors may operate early in gestation and are not those presumed to operate via maternal constraint of fetal growth in late gestation. In contrast, glucose tolerance in postpubertal ewes is related more strongly to postnatal growth than to birth weight. We suggest that animal studies investigating the interactions between fetal development and postnatal physiology need to be cognizant of the potential confounder of different in utero growth trajectories in multiples that may influence postnatal physiology. Obversely, detailed physiological studies comparing in utero growth in twins vs. singletons may provide insights into the determination of the fetal growth trajectory.

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REFERENCES

BIRTH WEIGHT AND POSTPUBERTAL HPA FUNCTION IN TWINS


