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Fetal Programming: Prenatal Testosterone Excess Leads to Fetal Growth Retardation and Postnatal Catch-Up Growth in Sheep

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Alterations in the maternal endocrine, nutritional, and metabolic environment disrupt the developmental trajectory of the fetus, leading to adult diseases. Female offspring of rats, subhuman primates, and sheep treated prenatally with testosterone (T) develop reproductive/metabolic defects during adult life similar to those that occur after intrauterine growth retardation. In the present study we determined whether prenatal T treatment produces growth-retarded offspring. Cottonseed oil or T propionate (100 mg, im) was administered twice weekly to pregnant sheep between 30–90 d gestation (term = 147 d; cottonseed oil, n = 16; prenatal T, n = 32). Newborn weight and body dimensions were measured the day after birth, and postnatal weight gain was monitored for 4 months in all females and in a subset of males. Consistent with its action, prenatal T treatment produced females and males with greater anogenital distances relative to controls. Prenatal T treatment reduced body weights and heights of newborns from both sexes and chest circumference of females. Prenatally T-treated females, but not males, exhibited catch-up growth during 2–4 months of postnatal life. Plasma IGF-binding protein-1 and IGF-binding protein-2, but not IGF-I, levels of prenatally T-treated females were elevated in the first month of life, a period when the prenatally T-treated females were not exhibiting catch-up growth. This is suggestive of reduced IGF availability and potential contribution to growth retardation. These findings support the concept that fetal growth retardation and postnatal catch-up growth, early markers of future adult diseases, can also be programmed by prenatal exposure to excess sex steroids. (*Endocrinology* 145: 790–798, 2004)

THE ENDOCRINE, nutritional, and metabolic milieu of the fetus programs its anatomy and physiology, and these changes persist into postnatal life (1–5). Some of this early programming can produce pathologies. For instance, prenatal programming by maternal diabetes or maternal malnutrition or maternal stress leads to metabolic and reproductive alterations in the offspring, the effects of which are manifested during adulthood (1–5). Intrauterine growth retardation, as typified by low birth weight is a marker of several adult disorders. Low birth weight has important links with infertility (6–9). For instance, low birth weight is a risk factor for cryptorchidism (10), male subfertility (11), pseudohermaphroditism (12), impaired ovarian development (13, 14), oligoovulation and anovulation (8), and polycystic ovarian syndrome (6). In addition, there is increasing risk of adult diseases, such as cardiovascular disease, type 2 diabetes, obesity, and hypertension, with decreasing size at birth (1, 3, 15). Birth weight links with disease risk markers, such as insulin resistance and obesity, are apparent particularly when low birth weight is followed by rapid postnatal weight gain (catch-up growth) and childhood obesity (15, 16). For example, the greatest risk for coronary heart disease is noted in small birth weight individuals who exhibit catch-up growth during childhood (17). Thus, changes in fetal metabolic and hormonal responses to intrauterine growth restraint and/or adaptations of the offspring in response to catch-up growth appear to be key to the early pathogenesis of adult disease.

Not only does prenatal growth retardation produce adverse lasting effects after birth, but prenatal exposure to steroids also has been shown to have serious consequences during adulthood. Studies in animals have shown that prenatal exposure to testosterone (T), similar to growth retardation, leads to infertility, behavior modifications, obesity, and insulin resistance during adulthood in the resultant offspring (18–22). In the classic forms of human congenital adrenal hyperplasia, fetal androgen excess causes external genital ambiguity in newborn females and progressive postnatal virilization in males and females, leading to reduced fertility, menstrual problems in women, and testicular and adrenal rests in men (23). In addition, decreased postnatal height and increased aggression behavior have been reported (24, 25). The severity and similarity of adult consequences in the prenatally growth-retarded and prenatally T-treated models suggest that common metabolic mediators may be involved. It could be argued that many of the adult consequences of prenatal T treatment may also be mediated via fetal growth retardation. If so, this would provide an early marker for assessing adult life consequences.

It is well documented that fetal growth retardation follows maternal malnutrition, diabetes, or stress, but information

Abbreviations: ADU, Arbitrary densitometric unit; IGFBP, IGF-binding protein; T, testosterone.

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about whether prenatal T treatment has the potential to cause intrauterine growth retardation is scanty. In view of the deleterious consequences of prenatal growth retardation and consequent catch-up growth of the offspring on adult well-being, programming of such features by prenatal exposure to steroids is of clinical relevance, especially because pregnant mothers can be inadvertently exposed to gonadal steroids during early gestation via continued use of contraceptive pills, environmental estrogenic pollutants, or anabolic steroids (26–30). Earlier studies addressing this issue suffered from small sample size or failed to consider litter size and/or sex distribution (22, 31–34). To gain a proper perspective on the effects of prenatal steroid exposure on fetal growth, it is essential to have a larger sample size and control for all variables, such as litter size and gender distribution of fetus when twins or triplets are involved. In this study we tested the hypothesis that prenatal exposure to T propionate (plasma half-life, 4.5 d) during early to midgestation (d 30–90; term = 147 d) programs fetal growth retardation and that the growth-retarded lambs will manifest postnatal catch-up growth.

Materials and Methods

Breeding and maintenance

Adult Suffolk ewes with proven fertility were purchased locally and moved to a nearby USDA-inspected and University of Michigan Department of Laboratory Animal Medicine-approved farm for breeding. All animal use procedures were approved by the University Committee for the Use and Care of Animals. Starting 2–3 wk before and continuing until the time of breeding, ewes were group-fed daily with 0.5 kg shelled corn and 1.0–1.5 kg alfalfa hay/ewe (see Table 1 for digestible energy and crude protein intake; for comparisons, Nutritional Research Council requirements are also provided) to increase energy balance in an attempt to increase ovulation rate. A total of 51 ewes were mated to rams with proven fertility, and the day of mating was recorded. After breeding, all ewes were housed under a natural photoperiod in the pasture and group-fed with a daily maintenance diet of 1.25 kg alfalfa/brome mix hay/ewe (see Table 1).

Prenatal treatment

Pregnant ewes were assigned to either a control (n = 16) or a prenatal T treatment (n = 32) group. Due to the reported high rate of fetal loss that follows prenatal T treatment (32), twice as many pregnant ewes were assigned to the treatment group compared with the control group. Treated ewes were administrated twice weekly im injections of 100 mg T propionate (Sigma-Aldrich Corp., St. Louis, MO) in 2.4 ml cottonseed oil (Sigma-Aldrich Corp.) from 30–90 d gestation (term = 147 d). Controls received an equal volume of vehicle.

Maternal nutrition and birth

Beginning 6 wk before lambing, when maximal fetus growth occurs, pregnant ewes were group-fed with 0.5 kg shelled corn, 2 kg alfalfa hay (see Table 1), and 250 mg aureomycin crumbles (chlortetracycline)/ewe daily. All lambs were born between March 15 and April 20, 2002. At birth, each lamb received oral vitamin E and selenium and injections for *Clostridium perfringens* types C and D and tetanus. Lactating ewes were fed 1 kg shelled corn and 2–2.5 kg alfalfa hay (see Table 1) while they were suckling the lambs.

Newborn and neonatal measures

Date of birth, number of offspring, sex of offspring, and fetal distribution during multiple births were recorded. The sex of the lambs was determined by examining the external genitalia. The prenatally T-treated females had a penis and an empty scrotum. Gender was later confirmed by the initiation of progestogenic cycles in biweekly samples obtained from 20 wk of age in those that lived and by examining the internal organs by autopsy in those that died. Lamb weights and growth parameters were measured 24 h after birth to allow sufficient time for maternal bonding. Growth measures included height, chest circumference, and head circumference. Height measures were determined with the lambs standing. Chest and head circumference measures were determined with a flexible plastic tape. Blood samples were obtained from all female lambs at 25 ± 1 d of age to determine circulating concentrations of insulin, IGF-I, IGF-binding protein (IGFBP), and cortisol, which are known metabolic mediators.

Postnatal nutrition and growth

Each mother and its lambs were individually housed for the first 3 d and then group-housed with other mothers and offspring in a barn

TABLE 1. Nutrient concentrations in diets of sheep [expressed on a 100% dry matter basis; for comparison National Research Council (NRC) requirements are provided]

Stage	Digestible energy, actual (Mcal/kg)	Digestible energy, NRC (Mcal/kg)	Crude protein, actual (%)	Crude protein, NRC (%)
Flushing (prebreeding)	2.8–2.68	2.6	16–16.6	9.1
Early gestation	2.31	2.4	11.7	9.3
Late gestation	2.6	2.9	11.3	17.1
Lactation	2.8–2.73	2.9	15.9–16.4	15
Lambs (rapid growth potential)	3.62	3.5/10 kg lamb	18	26.2 for 10-kg lamb[a]
		3.4/20 kg lamb		16.9 for 20-kg lamb[a]
		3.3/30 kg lamb		15.1 for 30-kg lamb
Lambs (reduced growth rate)	3.62	3.3/40 kg lamb	15	14.5 for 40-kg lamb

The ration comparisons presented provide ration concentrations for digestible energy (megacalories per kilogram of diet) and crude protein (% crude protein) at 100% dry matter. The differences noted between actual *vs.* NRC, namely −3.75%, −10.3%, and −3.4%, respectively, for the early gestation, late gestation, and lactation ration concentrations, are minimal and represent differences in the concentration of energy or crude protein in the feed provided, not deficiencies in the daily intake of energy (megacalories of digestible energy or pounds of total digestible energy) or protein (pounds or grams of crude protein ingested). Deficiencies in energy or protein intake would have been evident as 1) excessive loss of body condition in the ewe flock; 2) evidence of late pregnancy production disease, such as pregnancy toxemia; and 3) reduced birth weights associated with lambing, none of which was noted in either the control or treatment group of ewes. Additionally (see Fig. 4), the birth weights of the lambs in the control group averaged about 5.26 kg/lamb, which is slightly above the 4- to 5-kg birth weight typical of well-fed commercial ewes (Rook, J., College of Veterinary Medicine, Michigan State University, unpublished observation) and the 4.74 ± 0.14 kg birth weight reported from a large trial involving the Suffolk breed of sheep (35). As control and treatment groups were fed identical rations, any observed differences are independent of feed differences.

[a] Still nursing.

under a natural photoperiod except for a 60-watt bulb in the lamb creep feed area during nights. Light intensity during the night at lamb head level ranged between 5 and 6 lux. When group-housed, lambs had access to commercial feed pellets (Shur-Gain, Elma, NY) containing 18% crude protein and alfalfa hay (see Table 1). All lambs were weaned at 8 wk of age. All female lambs were transferred to the Sheep Research Facility (Ann Arbor, MI; 42°, 18′N), where they were maintained outdoors under a natural photoperiod. Due to space constraints, only a subset of randomly selected control and prenatally T-treated male lambs (n = 6/group) were moved to the Sheep Research Facility for monitoring postnatal gain. All lambs were provided *ad libitum* access to commercial feed pellets (same as above). When they reached a weight of about 40 kg, all lambs were switched to a pellet feed with 15% crude protein to avoid fat deposition during the period of reduced growth rate (see Table 1). Trace mineralized salt with selenium and vitamins A, D, and E (Armada Grain Co., Armada, MI) were freely accessible throughout the study. The postnatal growth of all female lambs and the subset of males was monitored by determining body weights at biweekly intervals before feeding.

Effects of prenatal T treatment on ano-genital distance

The effect of prenatal T treatment of the lamb was assessed 8 wk after birth from the ratio of ano-urethral to ano-navel distances (36). With the animal standing, measurements were made from the middle of the anal opening to the navel or the urethral opening in the penis/vulva.

RIA

Plasma insulin concentrations were measured using the ImmuChem-coated tube insulin ^{125}I RIA kit (ICN Pharmaceuticals, Costa Mesa, CA). Duplicate volumes of 100 μl unextracted plasma samples were used in a single assay. The sensitivity of the assay was 3.86 μU/ml. The intraassay coefficient of variation was 4.8%. Circulating IGF-I levels were measured using a validated assay (37). Briefly serum samples were extracted with an acid-ethanol extraction solution (1:4 ratio), neutralized with 0.855 M Tris base. Neutralized serum extracts were assayed in duplicate volumes of 50 μl using recombinant human IGF-I (R&D Systems, Minneapolis, MN) as the assay standard. Standards were normalized by adding same volume of neutralized, extracted assay buffer. Increasing volumes of plasma from both control and prenatally T-treated animals were diluted in parallel in the IGF assay (Fig. 1). All samples from the study were assayed in a single assay. The sensitivity of the assay was 8 pg/tube (2.9 ng/ml). The intraassay coefficients of variation and recovery were 9.7% and 96%, respectively. Circulating cortisol concentrations were measured using a solid phase Coat-A-Count assay kit (Diagnostic Products, Los Angeles, CA) as previously described (38). Duplicate volumes of 50 μl unextracted plasma samples were used in a single assay. The sensitivity of the assay was 1.0 ng/ml. The intraassay coefficient of variation was 4.5%.

IGFBP Western ligand blot analysis

To measure the forms of IGFBP in plasma, which determine the net bioavailable IGF-I amount or action, Western ligand blot analysis was performed using a modification of a previously described method (39). A 1-μl plasma sample from each female lamb was mixed with 19 μl distilled water and 60 μl (3×) nonreducing SDS sample buffer (0.125 mol/liter Tris base, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, and 0.1% bromophenol blue). Samples were heated to 65 C and then loaded onto a 0.8% stacking/10% separating polyacrylamide gel for electrophoretic separation of proteins. Three microliters of rat plasma were also loaded in a separate lane as a positive control. Gels were run at 25 mA for 5–6 h. The proteins were then transferred (Trans-Blot SD, Bio-Rad Laboratories, Hercules, CA) to nitrocellulose membranes. After transfer, membranes were soaked in Ponceau S stain for 5 min and washed with water to determine whether protein transfer was successful and to determine loading differences among lanes. Membranes were blocked overnight in buffer with 1% BSA and were incubated in 5 ml buffer containing ^{125}I-labeled IGF-I at 200,000 cpm/ml buffer for 12 h. Autoradiography was carried out on Biomax film (Eastman Kodak Co., Rochester, NY) at −80 C for 5 d. Film images were digitized, and the resulting IGFBP bands were quantified using Scion Image software (Scion Corp., Frederick, MD). Band intensity, calculated in arbitrary densitometric units (ADU), was used as a measure of IGFBP quantity. Data on IGFBP densitometric units were expressed as the ratio of IGFBP ADU to the ADU value of 66,000 molecular weight protein (albumin), which was the prominent band in the Ponceau S-stained membrane (40).

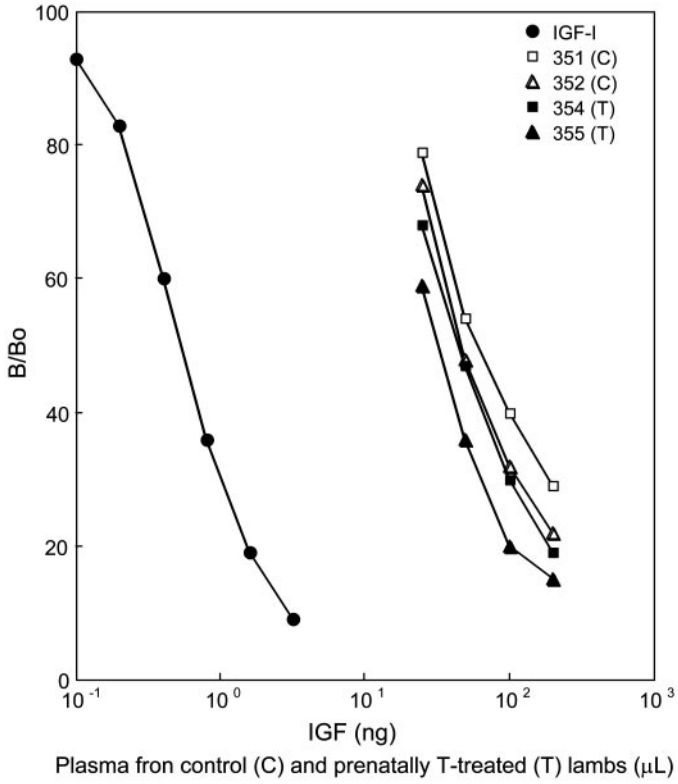

FIG. 1. Studies of parallelism in the IGF-I assay. Addition of increasing volumes (25–100 μl) of plasma from control and prenatally T-treated lambs displaced [^{125}I]IGF in a parallel manner to that of the recombinant human IGF-I standard.

Statistical analysis

The primary outcome measures were ano-genital distance (the ratio of ano-urethral to ano-navel distance); body weight; height; chest and head circumferences; insulin, IGF-I, and cortisol levels; and the proportion of IGFBP ADU to the ADU value of albumin. Two types of analyses were conducted, one that included all litter sizes and the other with twins only. When studying only twins (which avoids confounding with litter size), each outcome measure was compared between groups using only the mean value from the two siblings from twin gestations by a general linear model that included treatment and number of males in the twin pair as a covariate. This is similar to a repeated measures ANOVA where the two siblings are repeated measures, with gender and treatment as grouping variables (covariates). We repeated the analysis using a random effects model with all offspring; treatment, gender, and number of offspring per ewe (as a categorical variable) were used as covariates. Both analyses yielded similar results.

To determine the effect of prenatal T treatment on postnatal growth, weight was measured biweekly from 32 female lambs and 11 male lambs (one died), the 2- and 4-month weights were imputed by linear interpolation between the two closest measurements in time to 2 and 4 months. The rates of growth of the prenatally T-treated lambs were compared with those of control lambs by a two-sample *t* test.

Results

Gestational length and pregnancy outcome

Prenatal T treatment from d 30–90 of gestation decreased gestational length from 148.33 ± 0.43 to 146.93 ± 0.30 d ($P <$

0.01). The number of offspring had no effect on gestational length.

Litter size and gender distribution

Eleven female and 18 male lambs were born from the 15 control ewes. Twenty-four female and 27 male lambs were born from the 28 prenatally T-treated females. One control and four prenatally T-treated ewes did not produce offspring (either aborted or absorbed *in utero*). The ratio of males to females born was 1.64 for control and 1.12 for prenatally T-treated groups; the difference between these was not statistically significant. For controls, 13.3% of births were single live births, 80% were twins, and 6.67% were triplets. In the prenatally T-treated group, 32.1% of births were single live births, 53.6% were twins, and 14.29% were triplets. Although there were more singleton births and fewer twin births in the prenatally T-treated ewes, the differences were not statistically significant. The total number of offspring born and the gender distribution are summarized in Table 2.

Degree of masculinization

The effect of prenatal T treatment was determined by computing ano-genital distances, an index of the degree of masculinization. As expected, prenatal androgenization resulted in phenotypic changes in the external genitalia of female lambs, which included the presence of a penis and an empty scrotum. Consistent with the androgenic action of T, prenatally T-treated females displayed a greater ano-genital distance ($P < 0.01$; Fig. 1) compared with the control females. The prenatally T-treated males ($P < 0.01$) also displayed greater ano-genital distances compared with the control males; these distances were similar to those of prenatally T-treated females (Fig. 2).

Newborn lamb weight and body measures

As expected, when all lambs from both treatment groups were considered, the greater the number of offspring, the lower the average weight and growth measures of the lamb ($P < 0.0001$; Fig. 3). Comparison of weights adjusted for number of offspring per ewe, which used all animals from all litter sizes, revealed that prenatally T-treated lambs weighed less than controls ($P < 0.0001$; Fig. 4). There were no differences in any of the measured variables between males and females (Fig. 4). Overall, the chest circumference and height measures were significantly smaller in prenatally T-treated animals than controls ($P < 0.01$). When different sexes were analyzed separately, prenatally T-treated females were found to have reduced body weight ($P < 0.01$), height ($P < 0.005$), and chest circumferences ($P < 0.005$) compared with control females. Similarly, prenatally T-treated males weighed less ($P < 0.01$) and were shorter ($P < 0.005$) than control males. Chest circumferences of prenatally T-treated males were similar to controls ($P = 0.078$). Head circumferences were similar in control and prenatally T-treated females as well as in males. Restricting the analyses to twins while accounting for sex distribution also revealed that the prenatally T-treated females had reduced birth weight ($P < 0.01$), height ($P < 0.01$), and chest circumferences ($P < 0.01$). Prenatally T-treated males, despite the small sample size, also had reduced weight ($P < 0.01$) and height ($P < 0.0001$).

TABLE 2. Ewes classified according to number of offspring and gender distribution

Type	Control (n = 15)	Prenatal T (n = 28)
Single male	2	4
Single female	0	5
Twin, female-female	2	1
Twin, male-male	4	5
Twin, male-female	6	9
Triplets, male-male-female	1	0
Triplets, female-female-male	0	4
Total no. of female offspring	11	24
Total no. of male offspring	18	27

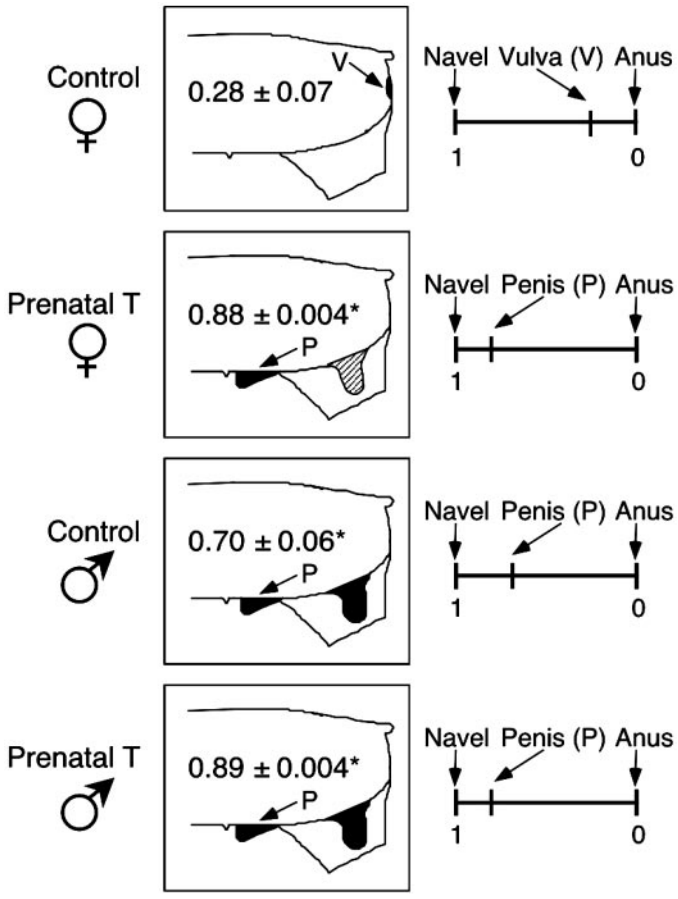

FIG. 2. Effects of prenatal T treatment on the external genitalia of postnatal lambs. *Darkened areas* represent the penis and scrotum. The *hatched area* indicates an empty scrotum. Values indicate the mean (±SE) position of urethral opening (penis or vulva) relative to the distance between the anus (0) and the navel (1) in control and prenatally T-treated males (controls, 18; T-treated, 27) and females (controls, 11; T-treated, 24). *Asterisks* denote significant differences from normal females ($P < 0.01$).

Hormones

Circulating cortisol levels were not significantly different between control (9.4 ± 1.9 ng/ml) and prenatally T-treated (7.3 ± 0.8 ng/ml) female lambs. Circulating insulin levels were similar between newborn control (14.2 ± 2.9 μU/ml) and prenatally T-treated (11.4 ± 1.6 μU/ml) females. Cir-

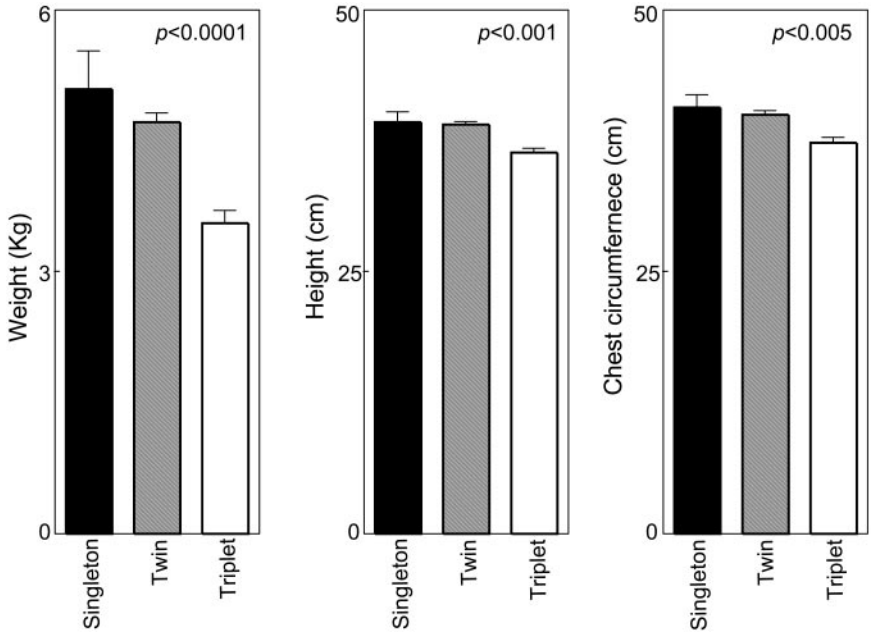

FIG. 3. Newborn body weight, height, and chest circumference as a function of litter size of lambs born (□, singletons; ▨, twins; ■, triplets). The analysis included both control and prenatally T-treated females and is independent of treatment. Measures include data from all males (n = 45) and females (n = 35) born.

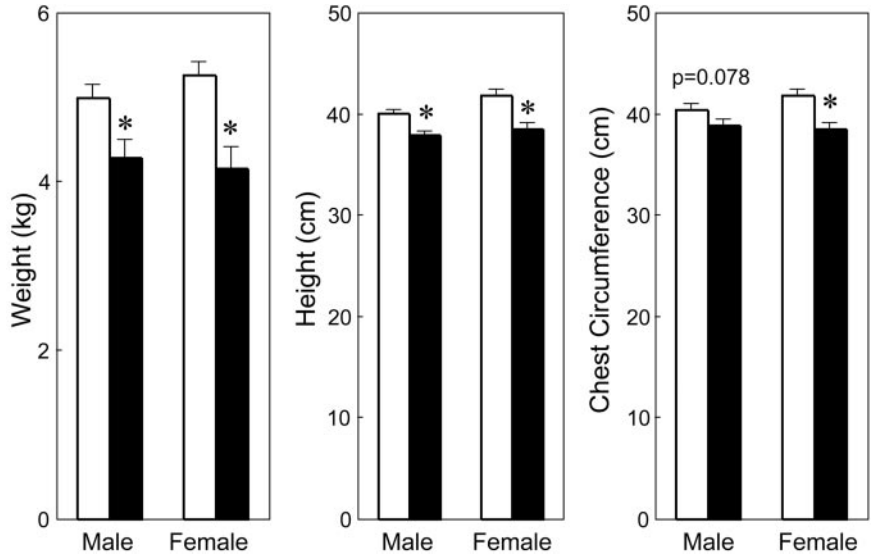

FIG. 4. Newborn body weight, height, and chest circumference adjusted for litter size (mean ± SE) of female (F) or male (M) lambs born to ewes treated with cottonseed oil (control; □; 18 males and 11 females) or sheep prenatally treated during d 30–90 of pregnancy with T propionate (prenatal T; ■; 27 males and 24 females). *Asterisks* denote significant differences from controls ($P < 0.01$) within each gender.

culating IGF-I concentrations were also not statistically different between control and prenatally T-treated females (Fig. 5), nor were they affected by the number of offspring per ewe. Ligand blotting analysis revealed a 29% increase in IGFBP-2 ($P < 0.001$), a 33% increase in IGFBP-I in prenatally T-treated females (Fig. 5), and no differences in IGFBP-3 levels (not shown). As expected, albumin, the most prominent band in the Ponceau S-stained membrane, which was used as the reference protein, did not change between control and prenatally T-treated females.

Postnatal growth

The rate of growth of female lambs from prenatally T-treated ewes was greater than that from control female lambs ($P < 0.05$ from birth to 4 months; Fig. 6). This catch-up growth, defined as growth faster than the mean of the control, occurred mainly between 2 and 4 months of age ($P < 0.01$). The subset of prenatally T-treated male lambs that were monitored had a reduced rate of growth during the first 2 months compared with those of control males ($P < 0.05$), but this difference was not evident during the next 2 months.

Discussion

The results of the present study of the sheep provide an example of the deleterious postnatal consequences of adverse changes in uterine environment. Exposure of fetuses from d 30–90 of gestation to T, by either androgenic action or conversion to estradiol, produced fetal growth retardation, manifested as reduced birth weight, height, and chest circumferences, as well as a postnatal increase in IGFBP-1 and IGFBP-2 levels in the females (not measured in males). Furthermore, the prenatally T-treated females, but not the males, exhibited catch-up growth between 2–4 months of their birth. Previous studies addressing the effects of prenatal

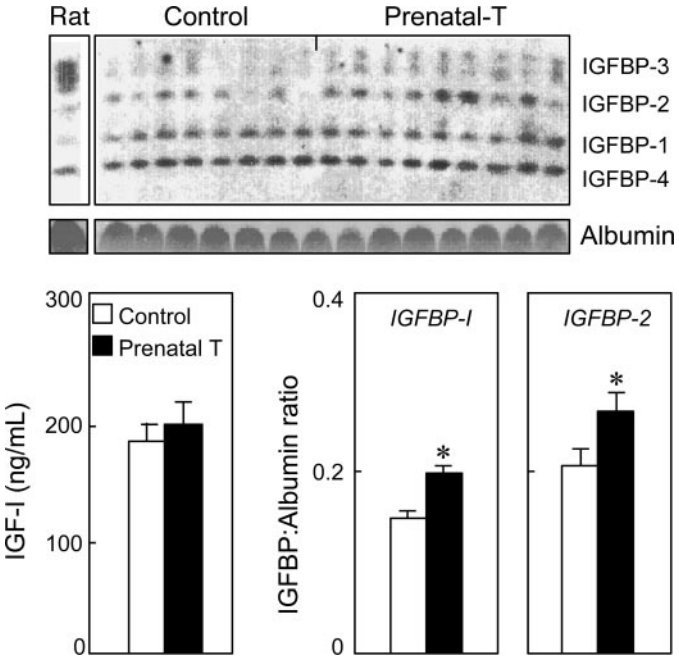

FIG. 5. Changes in circulating IGF-I concentrations and IGFBPs in female lambs born to ewes treated with cottonseed oil (control; □; n = 11) or sheep prenatally treated during d 30–90 of pregnancy with T propionate (prenatal T; ■; n = 22). Plasma concentrations of IGF-I were measured by RIA (*bottom left panel*). Mean levels of IGFBP-1 and IGFBP-2 measured by ligand blotting are expressed as the ratio of IGFBP-1 ADU and IGFBP-2 ADU to the ADU value of albumin, the prominent band in the Ponceau S-stained membrane that was not affected by prenatal T treatment (*bottom right panel*). A repeat ligand blot confirmed these findings. The *top panel* shows a representative ligand blot. Note that measures include data from all females. Blood samples were not obtained from the subset of males.

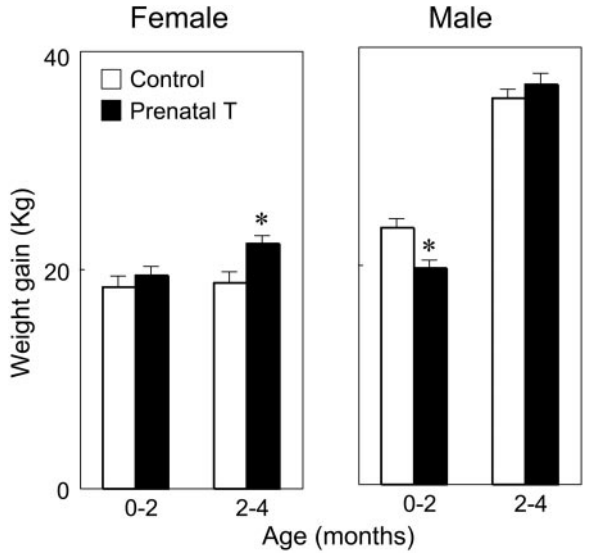

FIG. 6. Postnatal growth rate from 0–2 and 2–4 months of female and male lambs born to ewes treated with cottonseed oil (control; □; 11 females and five males) or sheep prenatally treated during d 30–90 of pregnancy with T propionate (prenatal T; ■; 22 females and six males). *Asterisks* denote significant differences from controls ($P < 0.01$).

T treatment on fetal growth have yielded mixed outcomes. Without taking into consideration litter size and gender, earlier studies found that fetal exposure to T reduces birth weights in rats (22), but not in mice (31). Using a limited number of animals and not considering gender, studies in sheep have found increased fetal mortality and reduced birth weight in sheep exposed to the short-acting T enanthate from d 75 of gestation to term (32). A follow-up study using T propionate without adjusting for gender composition of twins reported a reduction in birth weight of females in two studies and of males in only one of two studies (33). In contrast, acute exposure to T propionate on d 30, 40, or 60 of gestation (34) was found to increase birth weight. Most of these studies did not undertake a detailed assessment of growth parameters. This study, accounting for gender and multiple births as well as undertaking a detailed assessment of growth, clearly documents that prenatal T treatment from d 30–90 of gestation results in growth-retarded offspring.

What are the possible means by which prenatal T treatment programs growth retardation? Previous studies in the human have found an association between high cortisol concentrations during fetal life and low birth weight (41, 42). In rodents and other model species, antenatal exposure to glucocorticoids was found to reduce offspring birth weight (42, 43). In our study, although circulating levels of cortisol did not differ between control and prenatally T-treated offspring during neonatal life, the possibility that prenatal T treatment activates the maternal or fetal stress axis during fetal differentiation cannot be eliminated. Alternatively, the growth retardation observed in the stress models may be facilitated via increased sex steroid levels. In subhuman primates, increases in plasma androgen and estrogen metabolites were noted after the induction of hypoxic conditions through arterial ligation, suggesting that fetal and maternal stress have the potential to cause significant increases in sex steroid levels (44). In these studies it is also unclear whether the effects of prenatal T treatment, if any, are due to its androgenic action or are the result of its aromatization to estradiol. Several studies have shown that fetal exposure to estrogenic compounds results in fetal growth retardation (45–47). Low birth weight is one of the adverse outcomes of pregnancy in women exposed to diethylstilbestrol *in utero* (48).

Another possibility is that the growth retardation observed in both the prenatally T-treated and prenatal stress models may be facilitated through a common mediator and involve changes in metabolic hormones. There is evidence linking maternal metabolic alteration with fetal growth retardation (49). Animal models have also provided support for a role of the metabolic environment during prenatal life in mediating fetal growth retardation (49, 50). Insulin sensitivity and metabolic actions of insulin have been altered in these situations. Although we did not detect alterations in plasma insulin levels in prenatally T-treated lambs neonatally, whether the same holds true in fetal life remains to be explored.

Another metabolic alteration that would mediate growth retardation is a change in the IGF system. Abnormalities in the GH-IGF axis have been noted in other models of fetal growth retardation (51). Although we did not detect differences in plasma concentrations of total IGF-I, we found significant elevations of two IGFBPs in prenatally T-treated

lambs that have been associated with fetal growth retardation: IGFBP-1 and IGFBP-2. The plasma concentration of IGFBP-2, the dominant growth-regulating IGFBP during fetal development, has been found to be inversely associated with intrauterine growth (52). Elevations in IGFBP-1 also have been correlated with low birth weight (53). In this study we were unable to determine whether the band that we identified as IGFBP-1 also contains the similarly sized IGFBP-5. Western blot analysis using specific antibodies will be required to assess the relative levels of the two IGFBPs (IGFBP-1 and IGFBP-5). Levels of IGFBP-3 were relatively low in the 25-d-old females. An earlier study (54) reported a stronger IGFBP-3 signal in female lambs of comparable age. The faint signal in the present study does not appear to be a function of detection deficiency for the following reasons. First, the expected strong IGFBP-3 signal was found in the rat sample (positive control) in each blot (Fig. 5). Second the, results reported by Gatford *et al.* (54) are based on a single pooled sample as opposed to the multiple measures carried out in our study. Third, the breed of sheep used in this study (Suffolk) was different from the mixed breed of sheep [(Border Leicester × Australian Merino) × Romney] that was used in the study by Gatford *et al.* (54). Detailed developmental studies are required to gain a more thorough understanding of the progression of changes in IGFBP-3.

It is interesting that the IGFBP ligand blot profile we measured in 25-d-old lambs is similar to that in fetal sheep (55) rather than that in adult sheep, in which IGFBP-3 is dominant (56). Furthermore, the IGFBPs typically associated with growth retardation in the fetus are expressed in a similar pattern in our 25-d-old lambs. It is possible that the IGF/IGFBP environment observed in this study during the suckling phase, when the prenatally T-treated lambs are growth retarded, is an extension of what is observed during fetal growth. Furthermore, if prenatal T treatment mediates growth retardation via increases in IGFBP-1 and IGFBP-2 levels and consequent reduction in IGF bioavailability, IGFBP increases are likely to dissipate when the females are exhibiting catch-up growth. Unfortunately, we did not collect blood samples during fetal life or the catch-up growth phase of the offspring to test this premise.

A common sequel to intrauterine growth retardation is catch-up growth during the postnatal period, and it is a risk factor for childhood obesity, insulin resistance, diabetes, and coronary heart disease (16, 17). The prenatally T-treated females in our study, in addition to being growth-retarded at birth and 0–2 months of age, exhibited catch-up growth between 2–4 months of age. Animal studies have shown that when fetal growth impairment is followed by catch-up growth postnatally, the life span is significantly shortened (57).

What are the likely consequences of the growth retardation that stems from prenatal T treatment? Epidemiological studies have found that fetal growth retardation and postnatal catch-up growth pose threats to the well-being of the offspring, often leading to adverse postnatal health consequences (1, 3–17). Fetal growth retardation in humans has been found to be linked to a disruption of the reproductive axis, namely a reduction in primordial follicles (58), an early age of menarche and menstrual disorders (59), and development of hyperandrogenism and adolescent polycystic ovary syndrome (6, 7, 9). Interestingly, prenatal T treatment from 30–90 d gestation, which results in growth retardation at birth and postnatal catch-up growth from 2–4 months in females (this study), also results in reproductive anomalies during adulthood, such as progressive deterioration of reproductive cycles leading to anovulation (19, 20), reduced sensitivity to the negative feedback actions of progesterone and estradiol (60, 61), LH surge defects (62), hypergonadotropism (61, 62), functional hyperandrogenemia manifested as polyfollicular ovaries (63), and absence of behavioral estrus and manifestation of male mounting behavior (20, 64, 65). To what extent fetal growth retardation contributes to such adult reproductive consequences remains to be determined.

The growth retardation seen after prenatal T treatment may also contribute to other metabolic dysfunctions. Prenatally T-treated female sheep (same model as that used in this study) also manifest metabolic anomalies during the prepubertal period and adulthood, as evidenced by an increase in fasting insulin levels and a heightened insulin response to a glucose challenge (33, 66).

Our current findings that excess prenatal steroids program low birth weight in conjunction with the epidemiological and experimental studies linking low birth weight to adult diseases highlight the health concerns that exposure of pregnant mothers to exogenous steroids pose to human health. Human fetuses are exposed to exogenous steroids for a variety of reasons: failed contraception and continued exposure to contraceptive steroids, use of anabolic steroids, or inadvertent exposure to environmental compounds with estrogenic or androgenic activity (26–30). Similarly, female body builders and athletes may intentionally or unintentionally use anabolic steroids during early pregnancy (30). In addition to exposure to steroids unknowingly/unintentionally during pregnancy, there is a natural variation in androgens and estrogens produced by the mother *in utero*. A cordiocentesis study of 114 pregnancies in humans in which we participated found that fetal serum T levels around midgestation (19–25 wk) were elevated into the male fetal range in approximately four of 10 female fetuses sampled (67). Similarly, pregnant mothers with polycystic ovary syndrome have higher circulating levels of androgen (68). Considering the potential for androgens to be aromatized to estrogens, these findings also bear upon our understanding of the consequences of prenatal exposure to phytoestrogens (69) and xenoestrogens (28, 29). The devastating consequences of *in utero* exposure to diethylstilbestrol (70) bears testimonial to such concerns being a reality. Considering that much of the programming that occurs at a critical period of early development may go unrecognized until adulthood, early markers of such risks would be helpful in planning intervention. Low birth weight and catch-up growth may serve as such markers. Our finding opens up several areas of investigations to pursue. Does exposure to excess prenatal steroids program different developmental trajectories in the fetus? Are there other early recognizable measures that can project consequences later in life? Do androgens and estrogens have differential effects on programming growth retardation and catch-up growth? In summary, findings from this study clearly document that

prenatal T treatment during fetal growth and development leads to growth-retarded male and female offspring and subsequent catch-up growth during the neonatal life of female offspring. The metabolic and hormonal responses associated with growth retardation and subsequent catch-up growth may contribute to the development of the adult reproductive, metabolic, and behavioral deficits that have been observed in this model.

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