Prenatal exposure to excess testosterone modifies the developmental trajectory of the insulin-like growth factor system in female sheep

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Experimental elevation of maternal testosterone (T) from 30 to 90 days of gestation leads to intrauterine growth retardation (IUGR) and increased prepubertal growth rate in female lambs. This study tested the hypothesis that prenatal T treatment during mid-gestation alters the trajectory of the fetal insulin-like growth factor (IGF)–insulin-like growth factor binding protein (IGFBP) system to promote IUGR and subsequent postnatal catch-up growth in female lambs. Plasma IGF-I and IGFBPs were measured by radioimmunoassay and Western ligand blot, respectively, on 65, 90 and 140 days (d) of gestation, at birth, ∼5 months (prepubertal, the catch-up growth period), and ∼9.5 months (postpubertal). Northern blot analysis was used to measure hepatic mRNA content of IGF system components during fetal stages. At fetal 65 d, plasma protein and hepatic mRNA content of IGFBP-1, an inhibitor of IGF bioactivity, was elevated in prenatal T-treated fetuses although body weight did not differ. There was a transient increase in plasma IGF-I and IGFBP-3 concentrations at fetal 90 d in prenatal T-treated fetuses. Hepatic IGF-I mRNA and plasma IGFBP-3 content were reduced by 140 d when body weight was reduced in prenatal T-treated fetuses. Plasma IGFBP-2 content was significantly reduced in prenatal T-treated newborns, but by 4 months these females had significantly higher circulating IGF-I and IGFBP-3 concentrations and faster growth rates than control females. After puberty, plasma IGF-I remained elevated in prenatal T-treated females. These findings provide evidence that prenatal T excess programmes the developmental trajectory of the IGF/IGFBP system in female sheep to reduce IGF bioavailability during IUGR and increase IGF bioavailability during prepubertal catch-up growth.

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Epidemiological and experimental studies have provided evidence associating intrauterine growth retardation (IUGR) and subsequent postnatal catch-up growth with later onset of health disorders, including glucose intolerance, insulin resistance, type II diabetes, obesity, cardiovascular disease, and osteoporosis (Barker, 1994; Gluckman & Hanson, 2004; Ong & Dunger, 2004). Because the incidence of IUGR and postnatal catch-up growth are highly correlated, it is currently unclear whether the later onset of metabolic and cardiovascular disease is a result of IUGR, postnatal catch-up growth, or both (Cianfarani *et al.* 2001; Holt, 2002). Therefore, to design appropriate intervention strategies for preventing adult onset of diseases, it is essential to understand the nature of disruptions in key mediators of growth during both IUGR and catch-up growth, as well as the environmental factors that cause these disruptions.

The growth hormone (GH)–insulin-like-growth factor (IGF) axis is a complex system of ligands (IGF-I and IGF-II), receptors (type 1 and 2), and binding proteins (IGFBP-1 to -6) that primarily regulate fetal growth and mediate anabolic effects of GH after postnatal maturation of the GH–IGF axis (Baxter, 2000; Duan & Xu, 2005). Because IGFs bind to insulin receptors and insulin stimulates the GH–IGF axis, IGFs also play important roles in glucose metabolism (Holt *et al.* 2003). It has been proposed that the fetal environment can affect the expression and development of the GH–IGF axis in the fetus and permanently alter the growth trajectory throughout life (Holt, 2002). If the *in utero*

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environment programmes adult GH–IGF signalling, then alterations in the GH–IGF axis may provide a mechanistic link between IUGR and postnatal catch-up growth, and possibly between fetal growth and later-life metabolic disease (Cianfarani *et al.* 1999; Holt, 2002; Ong & Dunger, 2004).

Regulation of the IGF system in the fetus is extremely sensitive to environmental conditions. This is evident in adverse or stressful conditions such as maternal undernutrition (Gatford *et al.* 1997), fetal hypoxia (McLellan *et al.* 1992; Green *et al.* 2000), or infection (Tarantal *et al.* 2002) when an alteration in circulating IGF is believed to be involved in the inhibition of growth. IGF bioavailability is determined by the relative equilibrium between levels of circulating IGF and the various IGFBPs, some of which effectively increase while others decrease IGF signalling by regulating the transport, stability, tissue distribution and receptor binding of IGFs (Baxter, 2000; Duan & Xu, 2005). Specifically, IGFBP-1 reduces the amount of free IGF available to bind to receptors, and elevations in IGFBP-1 are associated with reductions in growth rates during adverse environmental conditions (Duan & Xu, 2005; Kajimura *et al.* 2005). IGFBP-2, the dominant binding protein during the fetal stage, has a positive association with growth; however, elevations in IGFBP-2, which reduce free IGF for receptor binding, have been associated with stress-induced growth retardation (Cianfarani *et al.* 1999). Plasma IGFBP-3, the dominant binding protein in circulation after birth, binds with acid-labile subunits to sequester IGF and increase IGF bioavailability primarily by extending IGF half-life in circulation (Baxter, 2000). It is therefore conceivable that the environmental effects on growth rate are mediated by the coordinated regulation of expression, secretion and stability of the IGFBP complex (Duan & Xu, 2005).

While it has been well established that sex steroids can modulate the GH–IGF system after birth (i.e. sexually dimorphic growth, Gatford *et al.* 1997; Golub *et al.* 2003; Rosenfeld, 2003), our recent studies found that prenatal exposure to excess testosterone (T) was associated with IUGR and postnatal catch-up growth at 2–4 months of age (prepubertal) in female lambs (Manikkam *et al.* 2004). Similarly, *in utero* exposure to diethylstilbestrol, an oestrogenic agent, also is associated with IUGR (Lang *et al.* 1996). Given that human fetuses may be exposed to excess steroid hormones via continued exposure to contraceptive steroids, use of anabolic steroids, or inadvertent exposure to steroid-mimicking environmental compounds (Smithells, 1981; Cotton, 1994; Bahrke *et al.* 1998), there is a need to understand how these hormones affect the development of the GH–IGF axis *in utero*.

This study addressed the developmental changes in the IGF system induced by prenatal T excess and tested the hypothesis that IUGR and postnatal catch-up growth in prenatal T-treated females are a function of changes in IGF bioavailability manifested as changes in circulating IGF levels or the IGFBPs, regulators of IGF bioavailability. We recently found that T injections given twice weekly from 30 to 90 days (d) of gestation (term = 147 d) caused IUGR and significant changes in IGF-I and IGFBP profiles measured on postnatal day 25 (Manikkam *et al.* 2004). In this study, the same dosage of T was used to determine the effects of elevated maternal T on the IGF axis in female offspring throughout fetal and postnatal development. The prediction was that IGF/IGFBP changes would be consistent with the inhibition of IGF signalling during fetal development (e.g. lower IGF-I and/or higher IGFBP-1, 2) and the converse during prepubertal catch-up growth (e.g. higher IGF-I and IGFBP-3 and/or lower IGFBP-1).

Methods

Breeding and maintenance

Two- to three-year-old Suffolk ewes were purchased from local breeders and moved to a nearby United States Department of Agriculture-inspected and University of Michigan Department of Laboratory Animal Medicine-approved farm for breeding. Ewes were randomly assigned to treatment. Starting 2–3 weeks 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 per ewe to increase energy balance. Day of mating was determined by visual confirmation of a paint mark left by an intact ram on the hindquarter of bred ewes. After breeding, all ewes were maintained on pasture under natural photoperiod and supplemented with 1.25 kg alfalfa–brome mix hay per ewe. After birth, mother and lambs were individually housed for the first 3 d and then group-housed in a barn under natural photoperiod except for a 60-watt bulb in the lamb feed area at night. When group housed, lambs had *ad libitum* access to commercial feed pellets (Shur-Gain, Elma, NY, USA) containing 18% crude protein and alfalfa hay. All lambs were weaned at 8 week and transferred to the Sheep Research Facility (Ann Arbor, MI, USA) where they were maintained outdoors and fed commercial feed pellets *ad libitum*.

Prenatal treatment

Pregnant ewes were injected with 100 mg T propionate (Sigma-Aldrich Corp., St Louis, MO, USA; T) in cottonseed oil (2 ml volume) twice weekly from either 30–90 d or 60–90 d of gestation. The first set of control breeders received an equal volume of vehicle. The second set of breeder sheep did not receive vehicle due to lack of postnatal differences in the growth trajectory between purchased controls and controls receiving vehicle from first breeding. Changes in circulating IGF-I and IGFBP

Table 1. Sample sizes for each developmental stage and molecular analysis in this study

	IGFBP Western blot and IGF RIA (protein)			Northern blot (mRNA)	
	Control	T 30–90	T 60–90	Control	T 30–90
Prenatal Samples	7 (6)	9 (7)	—	6 (6)	7 (7)
Fetal day 65	7 (6)	9 (7)	—	6 (6)	6 (6)
Fetal day 90	8 (6)	8 (6)	—	6 (6)	7 (7)
Fetal day 140	7 (6)	8 (7)	—		
Postnatal Samples	6 (4)	5 (4)	15 (9)	—	—
Birth	6 (4)	5 (4)	15 (9)	—	—
Prepubertal	18 (17)	5 (4)	12 (9)	—	—
Postpubertal	12 (12)	—	11(9)	—	—

Numbers in parenthesis indicate the number of dams (mothers) the offspring came from (dam is the experimental unit for all analyses). For mRNA measures, only one of two randomly selected fetuses was used. Due to fewer number of control females born at the facility, an additional set of female lambs born around the same time were purchased within two weeks of birth from the same breeder, who provided the experimental dams. They were reared in parallel with the treatment groups.

levels were determined at all developmental stages: 65, 90 and 140 d of fetal life, birth, as well as the prepubertal and postpubertal periods. Changes in hepatic IGF-I/II and IGFBP mRNA levels were determined only during fetal life.

Blood/tissue samples for this study were generated over a 2-year period and involved two sets of breeding ewes (see Table 1 for sample sizes). The first group of ewes were bred in October 2002 and blood samples were collected from fetuses (umbilical artery) on day 140 of gestation, 24 h after birth, and during prepubertal (20.0 ± 0.4 weeks of age) and postpubertal (38.5 ± 0.2 weeks of age) periods (jugular). Postnatal samples (birth, pre- and postpubertal) were also collected from a second group of sheep bred in parallel and exposed prenatally to T from days 60–90 of gestation (no fetal samples). Due to fewer number of control dams delivering female offspring ($n = 4$), an additional set of female lambs born around the same time was purchased from the same breeder who provided the experimental dams, and was brought to the Sheep Research Facility at 4 weeks of age to be reared in parallel with the treatment groups. Growth trajectories of these animals and controls reared at the Sheep Research Facility were similar. Blood samples from purchased controls were not available at birth. Weekly weights were also taken from animals to monitor changes in growth rate over time starting 24 h after birth until 35 weeks of age.

The second group of ewes was bred in October 2003 and fetal umbilical arterial samples were collected under anaesthesia on days 65 and 90 of gestation (during T treatment). Anaesthesia was initiated by administering 20–30 ml of pentobarbital I.V. (Nembutol Na solution 50 mg ml^{-1}; Abbott Laboratories, Chicago, IL, USA) and animals intubated to maintain a plane of anaesthesia with 1–2% halothane (Halocarbon Laboratories, Riveredge, New Jersey). Following collection of blood samples, pregnant ewes (65, 90 and 140 d of gestation) were killed with a barbiturate overdose (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI, USA) and fetuses removed. After recording body weight and body measures, liver tissue was harvested from each individual and frozen at -80°C until Northern blot analysis (see Table 1 for sample sizes). The University Committee on Use and Care of Animals (UCUCA) approved all procedures used in this study.

IGF-I RIA

Plasma IGF-I concentrations at each developmental stage were measured using a validated radioimmunoassay (RIA) as previously described (Manikkam *et al.* 2004) based on the methods of Berrie *et al.* (1995). The sensitivity of the IGF-I assay was < 3 ng ml^{-1}, and intra- and interassay coefficients of variation were $< 10\%$ ($n = 3$ assays). IGF-I was measured because it is the dominant IGF in circulation during late gestation and postnatal periods. Lack of availability of reagents precluded measurement of IGF-II, the dominant IGF in fetal circulation (Dupont & Holzenberger, 2003). However, earlier studies have found that directionality of changes in liver IGF-I expression and secretion during fetal life track changes in IGF-II (Ali & Cohen, 2003).

IGFBP Western ligand blot analysis

To measure the relative concentrations of circulating IGFBPs at each developmental stage, Western ligand

blot analysis was performed as previously described (Manikkam *et al.* 2004) and based on the method of Hossenlopp *et al.* (1986). Briefly, 2 μl plasma from each individual mixed in 3× non-reducing SDS buffer was loaded onto a 0.8% stacking/10% separating polyacrylamide gel for electrophoretic separation of proteins. Proteins were transferred to nitrocellulose membranes, blocked overnight in buffer with 1% bovine serum albumin, and then incubated in ^{125}I-labelled IGF-I at 200 000 c.p.m. (ml buffer)$^{-1}$ for 12 h. Autoradiography was carried out on Biomax film (Kodak, Rochester, NY, USA) at −80°C for 24–48 h. Film images were digitized and IGFBP bands were quantified using ScionImage software. Band intensity, calculated in arbitrary densiometric units (ADU), was used as a measure of IGFBP quantity.

Northern blot analysis

Total RNA was isolated from frozen liver samples (150–200 mg) harvested from 65, 90 and 140 d fetuses with TRIzol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Fifteen micrograms of liver RNA from each individual was loaded on a 1% formaldehyde gel for electrophoresis and Northern blot analysis. Primer sequences of Hastie *et al.* (2004) were used to generate cDNA probes for ovine IGF-I, IGF-II, IGFBP-1, IGFBP-2, IGFBP-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, housekeeping gene). The focus was on IGFBP-1 to -3 because of differences found in these binding proteins in circulation and because their expression patterns have been shown to change in other IUGR models (McLellan *et al.* 1992; Tapanainen *et al.* 1994). Nylon blots were hybridized with Hybrisol I (50% formamide; Intergen, Purchase, NY, USA) with ^{32}P-labelled probes overnight at 42°C and washed initially at low stringency (2× SSC, 0.1% SDS, 0.1% sodium pyrophosphate; 2 × 5 min) at room temperature, then at intermediate stringency (6.25 ml 20× SSC + 25 ml 10% SDS + 20 ml 2.5% sodium pyrophosphate; 2 × 30 min) at 65°C. The blots were exposed to Biomax film (Eastman Kodak) for 6–24 h at −80°C. Prior to hybridization with a different probe, blots were stripped a maximum of 3 times with 1× TE, 1% SDS at 80–90°C. Densitometry was conducted as described above; ADU values for each IGF system gene were divided by GAPDH densitometric values to normalize for loading and transfer differences among samples.

Statistical analyses

For all statistical analyses, dam (mother) was used as the experimental unit. To assess the effect of elevated prenatal T on fetal growth, body mass of prenatal T-treated and control fetuses at 65, 90 and 140 d of gestation was compared using analysis of variance (ANOVA). To assess changes in postnatal growth trajectory, body weights of each lamb were adjusted for age and the growth trajectories among the control, 30–90 d and 60–90 d prenatal T-treated groups were compared using linear regression analysis to determine the relationships between prenatal T treatment and postnatal growth trajectory. A significant time–treatment interaction was taken to reflect a significant effect of treatment on growth rate. In addition, weight gains between 4 and 6 months (prepubertal) and 6 and 8 months (postpubertal) were calculated. The growth rates of prepubertal and postpubertal growth of the prenatal T-treated lambs (since there were no differences in growth rates between the 60–90 and 30–90 T-treatment groups, the two groups were combined for this analysis) were compared to those of control lambs by a two-sample Student's *t* test.

Significant differences in IGF and IGFBP protein and mRNA expression (densitometric values of bands on blots) between control and prenatal T-treated groups at 65, 90 and 140 d of fetal development were analysed by ANOVA. Body size was used as a covariate in the ANOVA for the 140 d analyses since this factor significantly varied between prenatal T-treated and control fetuses. For IGFBP protein analyses, because Ponceau-S staining of nitrocellulose membranes after transfer showed that sample loading was equivalent over all samples, no adjustments were made for loading differences. For mRNA analyses, arcsine–square root transformed ratios were used and statistical differences in gene expression between groups were assessed using *t* tests. To increase statistical power to detect treatment effects in the birth and postpubertal analyses of plasma IGF and IGFBPs, data from 30 to 90 d and 60–90 d prenatal T-treated groups were combined after establishing that there were no significant differences between them. Because prepubertal studies included two groups of controls and prenatal T-treated (30–90 d and 60–90 d prenatal treatment) animals, a two-way ANOVA was performed with treatment and group as main factors.

Results

Effect of prenatal T treatment on fetal growth

Body mass did not vary between control and prenatal T-treated fetuses at 65 and 90 d of gestation, but prenatal T-treated fetuses were significantly smaller than controls at 140 d gestation (*t* test, $P = 0.0002$; Fig. 1). Body measures of 140 d fetuses have been published previously (Steckler *et al.* 2005*a*) and are included for completeness of changes in growth trajectory. Prenatal T-treated 140 d fetuses also had significantly smaller head circumferences and a lower body weight/head circumference ratio, consistent with IUGR (Steckler *et al.* 2005*a*). There were no

differences in body weight at birth (C: 4.31 ± 0.53 g, $T_{30–90d}$: 4.51 ± 0.60 g, $T_{60–90d}$: 4.86 ± 0.25 g, ANOVA $P = 0.603$). However, regression analysis revealed that prenatal T-treated lambs tended to be heavier than control lambs throughout postnatal growth ($P = 0.079$), and there was a significant treatment by time interaction ($P < 0.001$). Individual analyses of the change in body weight during specific postnatal periods showed that prenatal T females grew faster than controls during the prepubertal period (t test, $P < 0.05$), but not during the postpubertal period (Fig. 2).

Developmental changes in IGF-I

Developmental changes in circulating IGF-I concentrations are shown in Fig. 3. Circulating IGF-I concentrations were approximately 2- to 3-fold higher during postnatal stages than prenatal stages. Prenatal T treatment from 30 to 90 d of gestation had no effect on plasma IGF-I concentration at 65 d gestation ($P = 0.16$), but significantly increased plasma IGF-I at 90 d of gestation ($P < 0.003$). Circulating concentrations of IGF-I were not significantly different between prenatal T-treated and control fetuses at 140 d ($P = 0.45$). At birth, plasma IGF-I concentrations did not differ between treatment groups, but prenatal T-treated females had higher IGF-I levels than control females during the prepubertal ($P = 0.0073$) and postpubertal ($P = 0.0078$) stages (Fig. 3). Hepatic IGF-I mRNA content did not differ between prenatal T-treated and control groups at fetal 65 or 90 d, but was significantly lower in prenatal T-treated fetuses at 140 d of gestation ($P = 0.02$; Fig. 3*B*, Table 2). There were no treatment differences in hepatic IGF-II mRNA expression at any fetal developmental stage (Table 2).

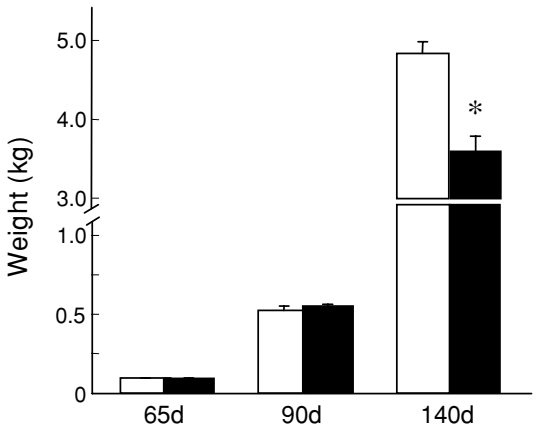

Figure 1. Effects of prenatal T treatment on fetal growth
Mean (± S.E.M.) body weight of female fetuses after 65, 90 and 140 d gestation of control (white bars) and T-treated (black bars) dams ($n = 6$–7/treatment). T-treated fetuses weighed significantly less than controls at 140 d (*$P < 0.05$; ANOVA).

Developmental changes in plasma IGFBPs

Western ligand blot analyses of fetal and postnatal samples revealed a 36–42 kDa (doublet, IGFBP-3), 32 kDa (IGFBP-2), 27–28 kDa (phosphorylated and non-phosphorylated IGFBP-1, IGFBP-5), and 24 kDa (IGFBP-4) band at all developmental stages (Fig. 4). ANOVA showed that relative expression (percent total protein) significantly differed through development for IGFBP-1 ($P < 0.0001$), IGFBP-2 ($P < 0.0001$) and IGFBP-3 ($P < 0.003$). The IGFBP-1 and/or IGFBP-5 concentration increased at 140 d of gestation relative to earlier fetal time points, decreased during prepuberty when growth is rapid, then increased during post-puberty when growth slowed (see Fig. 2). IGFBP-2, the dominant IGFBP during fetal development, was at its highest concentration during 140 d of gestation. IGFBP-3 became the dominant IGFBP during postnatal growth and was at its highest concentration during postpuberty. IGFBP-4 concentration remained relatively constant throughout fetal development and tended to decrease during postnatal development. Because of the low sample sizes ($n = 3$/stage/treatment), treatment effects were not determined from these blots; effects of prenatal T treatment on IGFBP expression at each development stage were determined separately and discussed below.

Effect of prenatal T treatment on IGFBPs

Prenatal T treatment significantly affected plasma concentrations and liver mRNA expression of IGFBPs in female fetuses; however, the specific IGFBP affected changed with developmental stage (Figs 5 and 6, Table 2).

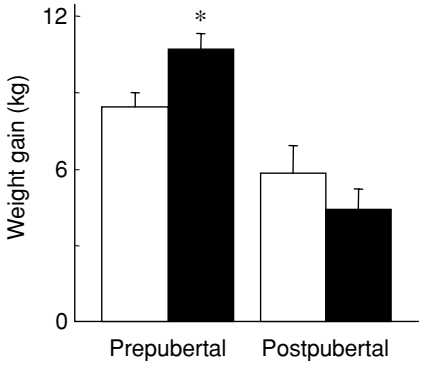

Figure 2. Effect of prenatal T treatment on prepubertal and postpubertal growth
The change in weight of control (white bars) and prenatal T-treated (black bars) lambs was calculated during the prepubertal period (between 18 and 26 weeks of age) and the postpubertal period (between 26 and 34 weeks of age). Because there was not a significant difference between the two prenatal T groups (30–60 d and 60–90 d treatments), these groups were combined in this analysis (refer to Table 1 for sample sizes). Bars indicate mean ± S.E.M.; *$P < 0.05$, t test).

Table 2. Densitometric results of IGF/IGFBP mRNA expression in fetal liver from Northern blot analysis

Day of gestation	Treatment	*n*	IGF-I	IGF-II	IGFBP-1	IGFBP-2	IGFBP-3
65	Control	6	1.20 ± 0.07	0.93 ± 0.07	0.67 ± 0.14*	0.94 ± 0.07	1.08 ± 0.06
	T-treated	6	0.98 ± 0.09	1.00 ± 0.08	1.20 ± 0.16	1.02 ± 0.09	0.97 ± 0.05
90	Control	5	1.05 ± 0.14	1.15 ± 0.15	1.15 ± 0.19	1.11 ± 0.06*	0.99 ± 0.03
	T-treated	6	0.98 ± 0.06	0.90 ± 0.09	0.89 ± 0.14	0.91 ± 0.07	1.01 ± 0.04
140	Control	5	1.29 ± 0.18*	1.07 ± 0.08	1.07 ± 0.08	1.01 ± 0.12	1.18 ± 0.16
	T-treated	7	0.84 ± 0.06	1.03 ± 0.09	0.97 ± 0.13	1.01 ± 0.7	0.92 ± 0.09

Numbers represent the mean ± S.E.M. of the ratio of IGF/IGFBP mRNA and GAPDH mRNA densitometric values. n = sample size; asterisks indicate significant differences in treatment means ($P < 0.05$).

At 65 d of gestation, prenatal T-treated fetuses had greater circulating IGFBP-1, 5 ($P = 0.380$) (Fig. 5, top panel) and liver IGFBP-1 mRNA concentrations ($P = 0.032$) (Table 2) than control fetuses (Fig. 5, top panel). Prenatal T treatment did not alter circulating protein levels (Fig. 5, top panel) or hepatic mRNA content (Table 2) of IGFBP-2 or -3 at this time. The increase in plasma IGFBP-1 in prenatal T fetuses was associated with an increase in a larger, secondary band on the Western ligand blot (Fig. 5, top panel), which likely indicates the phosphorylated IGFBP-1 isoform.

At 90 d of gestation, plasma IGFBP-3 protein content was significantly higher in prenatal T-treated fetuses ($P = 0.0162$), but IGFBP-1 or -2 did not vary between groups (Fig. 5, middle panel). Hepatic IGFBP-2 mRNA was reduced ($P = 0.045$) in prenatal T-treated 90 d fetuses, but IGFBP-1 and IGFBP-3 expression were not affected (Table 2). By contrast, at 140 d of gestation plasma IGFBP-3 was significantly lower in prenatal T-treated fetuses ($P = 0.0016$; Fig. 5, bottom panel). Hepatic IGFBP-3 mRNA expression tended to be lower in prenatal T-treated fetuses, but did not reach statistical significance (Table 2). No differences in liver IGFBP-1 or -2 mRNA content were detected at this developmental stage (Table 2).

Circulating IGFBP-2 levels were significantly lower at birth in prenatal T-treated females compared to controls ($P = 0.049$; Fig. 6, top panels), despite the small sample size. Circulating concentrations of other IGFBPs also tended to be lower in prenatal T-treated females but did not achieve statistical significance. During the prepubertal stage when prenatal T-treated females exhibited a faster growth rate, plasma IGFBP-3 concentrations in prenatal T-treated females were significantly higher compared to

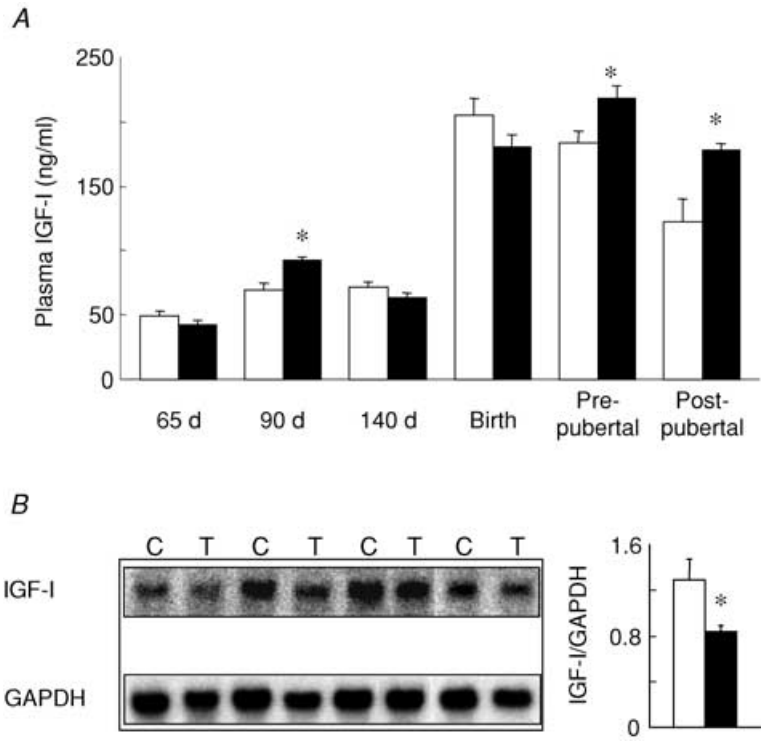

Figure 3. Plasma IGF-I concentration varies with developmental stage and prenatal T treatment
A, circulating IGF-I concentrations in control (white bars) and prenatal T-treated (black bars) female lambs throughout development (see Table 1 for sample sizes). *B*, Liver IGF-I mRNA (7.5 kb transcript, the dominant band) in control and prenatal T-treated female lambs at fetal day 140 (n = 6–7/treatment); there were no significant differences in IGF-I on fetal day 65 or 90. Asterisk indicates significant differences in concentrations as determined by ANOVA ($P < 0.05$).

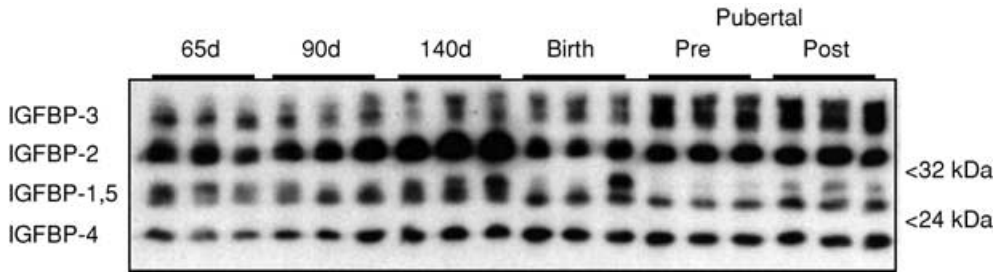

Figure 4. Developmental changes in plasma IGFBP content
The plasma samples in this analysis were a randomly selected subsample from each developmental stage. (Prenatal T-treated females shown on this blot; control females showed similar trends in IGFBP concentration and are not shown.)

controls ($P = 0.01$; Fig. 6, middle panel). No significant differences were detected in the other plasma IGFBPs during prepubertal period, when catch-up growth occurs or any of the IGFBPs during the postpubertal period (Fig. 6, lower panel).

Discussion

This study tested the hypothesis that prenatal exposure to excess T during mid-gestation programmes the developmental trajectory of the fetal IGF-IGFBP system to

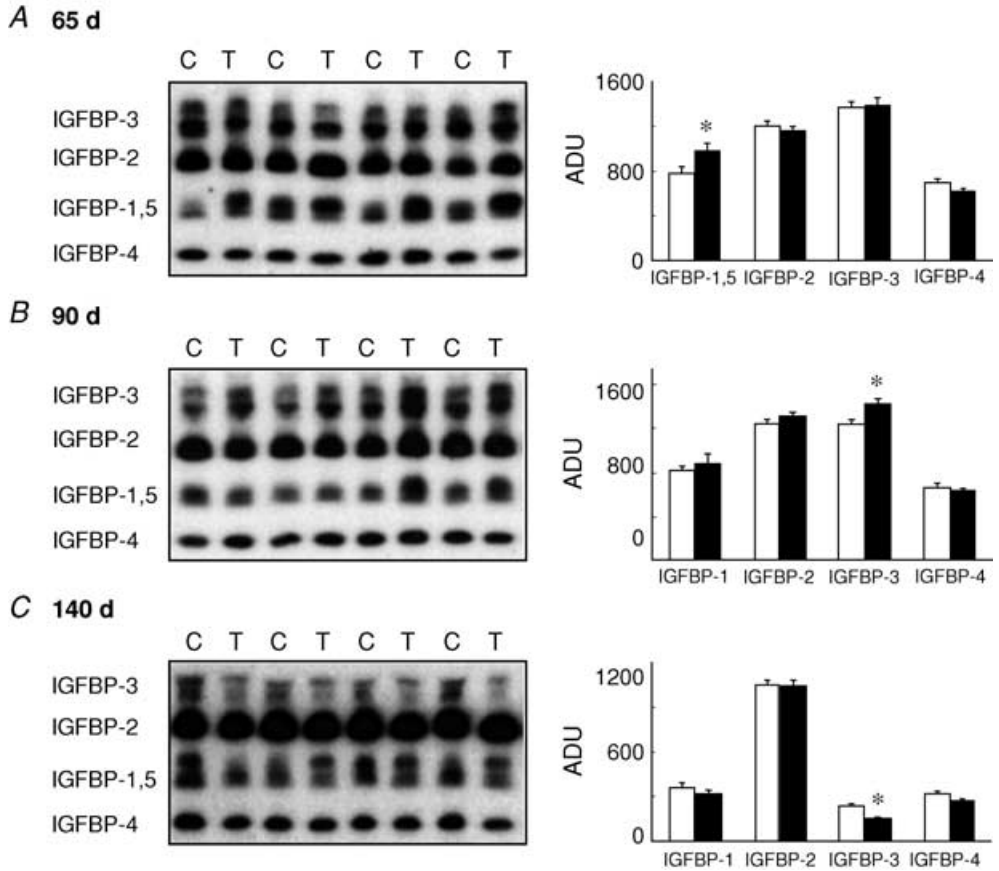

Figure 5. Effects of prenatal T treatment on plasma IGFBPs in 65 d (*A*), 90 d (*B*) and 140 d (*C*) gestation female fetuses
Plasma IGFBP content was determined by Western ligand blot analysis (representative blots for each stage shown, $n = 6$–7/treatment) and densitometric analysis of band intensities (ADU = arbitrary densitometric units). Refer to Table 1 for sample sizes of each developmental stage. Bars indicate mean ± S.E.M. (control – white bars, T-treated – black bars). Asterisks indicate significant differences in IGFBP protein as determined by ANOVA ($P < 0.05$).

promote IUGR and subsequent postnatal catch-up growth in female lambs. Previously, we found that plasma IGFBP-1 was elevated and IGFBP-2 was reduced at postnatal 25 d in prenatal T-treated female lambs that were growth-retarded at birth (Manikkam *et al.* 2004). Detailed analysis of the IGF system throughout fetal and postnatal development in this follow-up study allowed us to unequivocally determine that *in utero* exposure to excess T perturbs the fetal IGF system to reduce IGF bioavailability in a manner consistent with IUGR, and induces postnatal changes in the IGF system to increase IGF bioavailability to account for the increased growth rate during the prepubertal period.

Impact of prenatal T excess on fetal IGF system and IUGR

Based on our findings that prenatal T excess leads to IUGR (Manikkam *et al.* 2004; Steckler *et al.* 2005*a*), we predicted that IGF bioavailability would be reduced. Findings from this study are consistent with this prediction. An increase in liver mRNA expression and plasma concentration of IGFBP-1, a binding protein that reduces IGF bioavailability, were evident as early as fetal 65 d of prenatal T-treated females, well before IUGR was observed on fetal 140 d. Results from Western ligand analysis also suggests that prenatal T treatment may have caused increased phosphorylation of IGFBP-1, which dramatically increases binding affinity to IGF-I in humans (Baxter, 2000). Considering that IGF signalling is involved with early developmental processes, including bone differentiation, muscle formation and angiogenesis (Dupont & Holzenberger, 2003), a reduction in IGF bioavailability on fetal 65 d will reduce growth potential that is likely to manifest later as IUGR.

Increased hepatic IGFBP-1 expression seen on fetal 65 d may be the result of direct steroid action (T treatment spans days 30–90 of gestation) on hepatic tissues. *In vitro* studies with cultured hepatocytes and *in vivo* studies

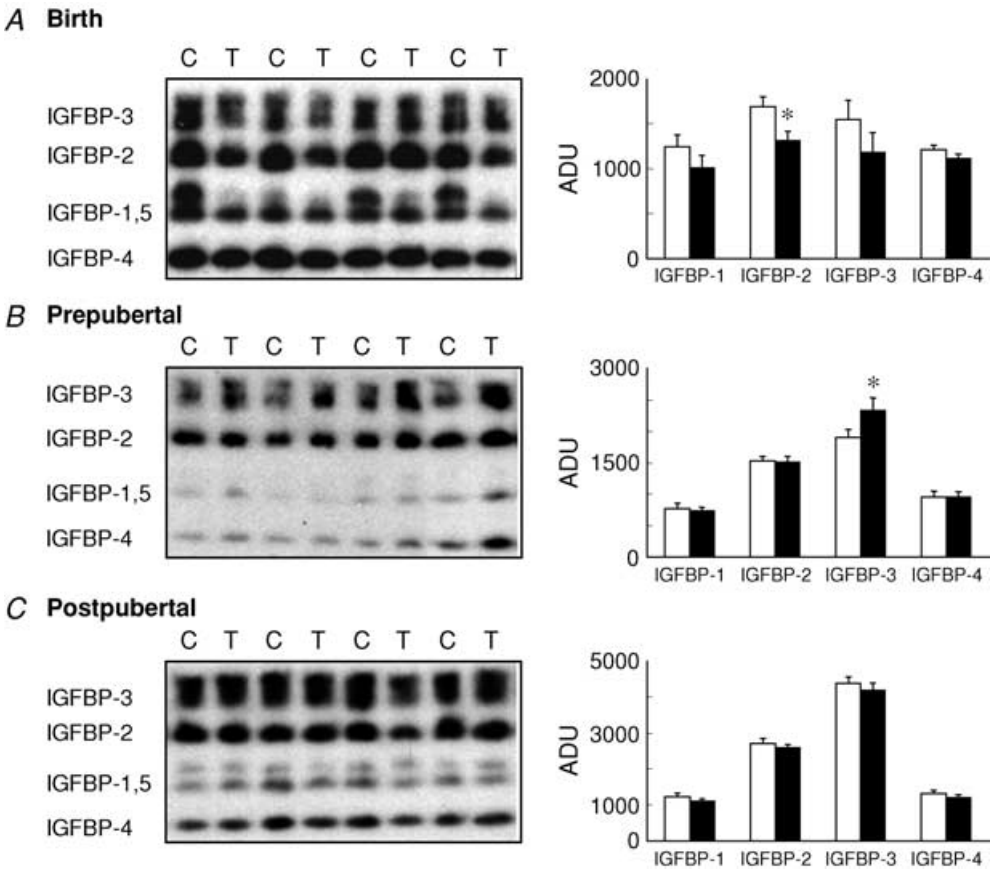

Figure 6. Effects of prenatal T treatment on plasma IGFBPs at birth (*A*), and prepubertal (*B*) and postpubertal (*C*) periods
Representative Western ligand blots and densitometric analyses are shown. Bars indicate mean ± S.E.M. (control – white bars, T-treated – black bars), ADU = arbitrary densitometric units. Asterisks indicate significant differences in IGFBP protein by ANOVA ($P < 0.05$).

in adult humans and other mammals have found that oestradiol treatment increases IGFBP-1 expression (Goya *et al.* 2002; McCarty, 2003; Veldhuis *et al.* 2005). This raises the possibility that the altered IGFBP-I programming may stem from oestrogenic but not androgenic actions of T. Alternatively, increased IGFBP-1 expression may be an indirect response to T-induced reductions in nutrient transfer from the placenta, as IGFBP-1 expression has been shown to increase in response to decreased insulin (Holt, 2002) and amino acid deprivation (Takenaka *et al.* 2000). In prenatal T-treated female sheep on day 65 of gestation, placentome differentiation was advanced and placentome efficiency increased (Astapova *et al.* 2005), suggesting that fetal tissue is responding to a reduced supply of nutrients.

In contrast, increased IGFBP-3 and IGF-I concentrations seen on fetal 90 d, while not consistent with IUGR, may be reflective of a compensatory fetal response to overcome the reduced IGF bioavailability seen in 65 d fetuses. However, the decrease in hepatic IGFBP-2 mRNA expression in the 90 d prenatal T-treated fetuses suggests reduced IGF signalling at the tissue level. Because paracrine actions of IGFBP-2 have been associated with cell differentiation and antiapoptotic effects in embryonic or fetal tissues (van Kleffens *et al.* 1999; Allan *et al.* 2001; Wood *et al.* 2005), a decrease in IGFBP-2 expression in liver (and potentially other tissues) may partially explain why growth increases in prenatal T-treated fetuses were not seen in the face of increased IGF-I and IGFBP-3 at this stage.

At 140 d gestation, when significant IUGR was observed in prenatal T-treated fetuses, the significant reduction in hepatic IGF-I mRNA expression and circulating IGFBP-3 concentration are consistent with reduced IGF bioavailability. These fetuses also had significantly reduced circulating glucose concentrations (Steckler *et al.* 2005*b*) and decreased placentome efficiency (Astapova *et al.* 2005), suggesting that changes in the IGF system and growth may be secondary to reduced nutrient transfer across the placenta. Indeed, the expression and secretion of IGFs and IGFBP-3 have been shown to be nutritionally dependent (Greenwood & Bell, 2003). Paradoxically, plasma IGFBP-1 (this study) and cortisol (Steckler *et al.* 2005*b*), two potential indicators of nutrient stress (Langford *et al.* 1994; Cianfarani *et al.* 2001; Ali & Cohen, 2003), were not altered in 140 d prenatal T fetuses, but it is conceivable that IGFBP-1 expression is altered only in more severe states of nutrient restriction and hypo-insulinemia (Ozkan *et al.* 1999). In addition, given that circulating GH concentrations were not monitored, it is possible that excess T treatment delayed the maturation of the neuroendocrine GH–IGF axis, which typically develops after 100 d of gestation in sheep (Rhoades *et al.* 2000).

Impact of prenatal T excess on postnatal IGF system and prepubertal growth

While exposure to excess prenatal T caused a significant reduction in plasma IGFBP-2 and a general tendency for decline in other IGFBPs and IGF-I at birth, body weights of prenatal T females were not reduced at this time. This result contrasts with the significant reduction in body weight found in prenatal T-treated fetuses right before birth (140 d gestation), and significant IUGR measured at birth in a previous study in which larger sample sizes were used (Manikkam *et al.* 2004). Failure to detect a reduction in birth weight in this study may be due to the combination of small sample size and the confounding effects of multiple births and sex distribution on fetal body size: the control and $T_{30–90}$ samples each contained 1 singleton, 2 twin, and 1 triplet birth, and the $T_{60–90}$ sample had three of each litter size. Yet, the reduced plasma IGFBP-2 concentration at birth in T-treated lambs is in general agreement with our previous study (Manikkam *et al.* 2004), as well as others (Albertsson-Wikland *et al.* 1998; Ozkan *et al.* 1999) that reported reduced plasma IGFBP-2 in IUGR lambs shortly after birth.

During the prepubertal stage, however, T females grew faster than control females, as previously shown (Manikkam *et al.* 2004), and this acceleration in growth was associated with greater IGF bioavailability. It is well accepted that during postnatal growth, IGF-I and IGFBP-3 are positively correlated with GH secretion and are good indicators of liver responsiveness to GH (Ali & Cohen, 2003) and circulating insulin (Holt, 2002). Therefore, the prepubertal increase in plasma IGF-I and IGFBP-3 concentrations of prenatal T females likely represents a hyper-activation of the entire GH–IGF axis. While manipulations of sex steroid concentrations during the perinatal period have been shown to alter GH–IGF function after weaning (Gatford *et al.* 1997), our findings definitively show that the sex steroid milieu experienced as early as mid-gestation also programmes postnatal growth axis function.

The mechanisms through which *in utero* elevations in T affect later-life hyperactivity of the IGF axis are not clear at this time. One possibility is that excess T exposure *in utero* causes permanent changes in pancreatic function. In support of this premise, Recabarren *et al.* (2005) found that prenatal T-treated females lambs were hyperinsulinaemic at 5 weeks of age, a comparable window to the prepubertal group in this study. Alternatively, prenatal T may cause postnatal catch-up growth via the masculinization of social behaviour. Excess prenatal T or early postnatal T exposure has been associated with androgen-mediated masculinization of female behaviour in humans (Berenbaum & Resnick, 1997; Ramirez, 2003; Cohen-Bendahan *et al.* 2005). Likewise in this study, prenatal T-treated and control females were housed

together with a common food source after weaning, and prenatal T-treated females exhibited social dominance (Lee *et al.* 2003). Therefore, increased growth and hyperactivity of the GH–IGF axis of prenatal T-treated females may have resulted from greater food intake or decreased energy expenditure needed to access food relative to the subordinate control females. Although food intake was not monitored, other studies have found that dominant social position is associated with faster growth rates (Blanchard *et al.* 1993; Pohorecky *et al.* 2004).

Prenatal T programming of late-onset of disease

The link between IUGR, postnatal catch-up growth, and adult onset of metabolic disease is thought to be the result of fetal development in a poor *in utero* nutritional environment followed by exposure to a postnatal environment of abundant food supply (i.e. the thrifty phenotype hypothesis; Barker, 1994). Mechanistically, this hypothesis predicts that exposure to chronically low levels of insulin and IGFs during the fetal stage will lead to insulin resistance when insulin/IGF concentrations are elevated shortly after birth (the catch-up growth hypothesis; Cianfarani *et al.* 1999, 2001). Indeed, female sheep exposed to excess T *in utero* also exhibit hyperinsulinemia and increased insulin response to glucose (Recabarren *et al.* 2005; Rosser *et al.* 2003). In addition, because IGFs can bind to the insulin receptor (Holt *et al.* 2003), the increase in IGF-I throughout pre- and post-puberty in prenatal T-treated sheep may contribute to insulin resistance and hyperglycaemia later in life. Taken together, these studies support the hypothesis that, like prenatal nutrient restriction, prenatal T excess predisposes females to late-onset insulin resistance and altered glucose metabolism (e.g. type II diabetes) due in part to the persistent hyperactivation of the IGF axis throughout postnatal life.

Effects of prenatal T exposure on growth trajectory: a model

Our findings for the first time document the impact of prenatal T excess in altering the developmental trajectory of the IGF system in a manner consistent with the timing of IUGR and postnatal catch-up growth. Figure 7 summarizes the potential mechanisms by which excess prenatal T exposure may programme the IGF axis to produce an altered growth trajectory in female lambs. Prenatal T excess, either through androgenic or oestrogenic pathways, reduces IGF bioavailability by increasing IGFBP-1 expression during T treatment (65 d) before growth differences are expressed and/or decreasing hepatic IGF-I expression and plasma IGFBP-3 concentration during late gestation when growth is reduced. The effects of T in reducing IGF bioavailability and causing IUGR may be mediated by direct actions at the level of the fetus or involve reduced placental nutrient transfer. Postnatal catch-up growth in prenatal T-treated females is associated with increased circulating IGF-I and IGFBP-3, resulting from either direct effects of T on the development of brain and peripheral organs or indirect effects of T on social behaviour that may increase food intake or decrease energy expenditure. Future work is needed to test these hypothetical pathways that link

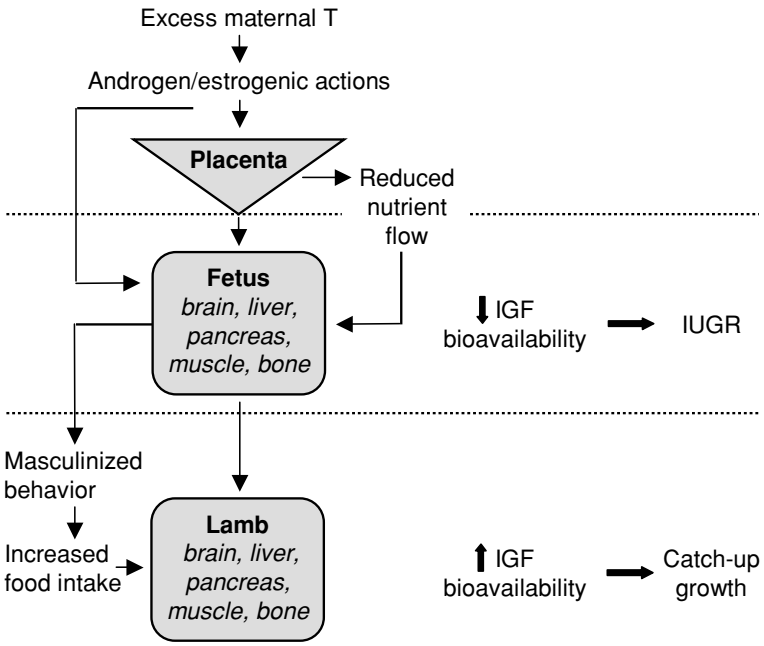

Figure 7. Schematic diagram of hypothetical mechanisms through which excess maternal T is associated with IUGR and postnatal catch-up growth
Prenatal T excess, by androgenic or estrogenic (aromatization of T to estradiol) effects, may act directly at the fetal level or indirectly via the placenta (reduced placental nutrient transfer to the fetus) to decrease fetal IGF bioavailability leading to IUGR. Postnatal catch-up growth and increased IGF bioavailability in prenatal T-treated female may be the result of T programmeed changes in the developmental trajectory of the fetal brain and/or peripheral organs. This may involve increased food intake and/or decreased energy expenditure stemming from increased dominance behaviour (Lee *et al* 2003) displayed by prenatal T treated females.

prenatal T exposure to life-long growth patterns, none of which are mutually exclusive, and identify whether these effects are the result of androgenic or oestrogenic signalling.

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