
Postnatal developmental consequences of altered insulin sensitivity in female sheep treated prenatally with testosterone

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Recabarren, Sergio E., Vasantha Padmanabhan, Ethel Codner, Alejandro Lobos, Claudio Durán, Mónica Vidal, Douglas L. Foster, and Teresa Sir-Petermann. Postnatal developmental consequences of altered insulin sensitivity in female sheep treated prenatally with testosterone. *Am J Physiol Endocrinol Metab* 289: E801–E806, 2005; doi:10.1152/ajpendo.00107.2005.—Prenatally testosterone (T)-treated female sheep exhibit ovarian and endocrinological features that resemble those of women with polycystic ovarian syndrome (PCOS), which include luteinizing hormone excess, polyfollicular ovaries, functional hyperandrogenism, and anovulation. In this study, we determined the developmental impact of prenatal T treatment on insulin sensitivity indexes (ISI), a variable that is affected in a majority of PCOS women. Pregnant ewes were treated with 60 mg testosterone propionate intramuscularly in cottonseed oil two times a week or vehicle [control (C)] from days 30 to 90 of gestation. T-females weighed less than C-females or males ($P < 0.05$) at birth and at 5 wk of age. T-females had an increased anogenital ratio. An intravenous glucose tolerance test followed by an insulin tolerance test conducted after an overnight fast at 5, 20, and 30 wk of age ($n = 7$ –8/group) revealed that ISI were higher at 5 than 30 wk of age in C-females, reflective of a developing insulin resistance associated with puberty. T-females had higher basal insulin levels, higher fasting insulin-to-glucose ratio, and higher incremental area under the insulin curve to the glucose challenge. The ISI of T-females was similar to that of males. No differences in ISI were evident between groups at 20 and 30 wk of age. Mean basal plasma glucose concentrations and glucose disappearance and uptake did not differ between groups at any age. Our findings suggest that prenatal T treatment leads to offspring with reduced birth weight and impaired insulin sensitivity in early postnatal life.

prenatal programming; insulin sensitivity index; glucose metabolism; small for gestational age; polycystic ovary syndrome

POLYCYSTIC OVARY SYNDROME (PCOS) is the most common endocrine disorder affecting women of reproductive age (16, 26). PCOS is probably a multigenic disorder with unknown etiology, and it is characterized by hyperandrogenism and chronic anovulation in the absence of specific diseases of the ovaries, adrenals, and pituitary (16, 26). In addition, 60–65% of U.S. women with PCOS also exhibit peripheral insulin resistance, affecting predominantly muscle and adipose tissue, and a compensatory hyperinsulinemia independent of obesity (12, 16, 25).

Although there is no human model that can be used to experimentally produce PCOS, clinical observations in women

with congenital adrenal hyperplasia caused by classical 21-hydroxylase deficiency are suggestive of excess prenatal androgen action. Such women exhibit anovulation, ovarian hyperandrogenism, luteinizing hormone hypersecretion, polycystic-appearing ovaries, and insulin resistance despite normalization of adrenal androgen excess after birth (6, 21). Studies with animal models provide experimental evidence for prenatal testosterone (T) excess in the etiology of PCOS (1, 42). For example, experimentally produced prenatal T excess leads to the manifestation of metabolic and endocrine changes during adulthood in female monkeys that resemble those observed in human PCOS (1). Similarly, sheep treated with T during fetal life exhibit cycle anomalies, ovarian morphology, and gonadotropin secretion comparable to those of women with PCOS (9, 34–36, 42). These observations suggest that T excess during early life, whether derived from fetal or extrafetal (maternal or external environment) sources, may contribute to the PCOS phenotype during adulthood. Therefore, an experimental animal model in which the fetus is exposed to excess T can contribute to our understanding of the etiology of PCOS.

Studies in prenatal T-treated female sheep (T-females) have focused predominantly on the hypothalamic-pituitary-gonadal axis during prepubertal development (36, 43) and adult reproductive life (9, 10, 34, 35, 36, 42). However, investigations on the effects of prenatal T excess on insulin sensitivity index (ISI), a common perturbation in PCOS women, are lacking. Recent studies in rhesus monkeys (14) provide unequivocal evidence in support of impaired pancreatic cell function and insulin sensitivity in T-females. Because these studies were conducted with an older cohort of monkeys, the developmental onset and progression of this disruption are not known. The aim of the present study was to build upon the monkey studies (14) and determine the impact of prenatal T treatment on developmental changes in ISI.

MATERIALS AND METHODS

General management of mothers and lambs. Forty adult Suffolk female sheep were mated in March during the breeding season after a synchronized estrus produced by intravaginal progesterone pessaries (Eazy Breed; Pharmacia & Upjohn, Auckland, New Zealand) and prostaglandin (Genestren; Drug Pharma). Pregnant sheep were then allocated randomly to two treatments. One group of 20 females received 60 mg im testosterone propionate (Sigma, St. Louis, MO) dissolved in cottonseed oil two times weekly from 30 to 90 days of

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pregnancy. Dose and time of treatment were selected on the basis of previous studies of Kosut et al. (27). The other group of 20 pregnant sheep received the vehicle. Pregnant sheep were maintained under standard husbandry protocols at the sheep facility of the Faculty of Veterinary Medicine, University of Concepcion (Chillán, Chile). At birth, lambs were left undisturbed with their mothers for 4 h and then weighed. The distance between the anus and the root of the penis or the middle of the vulva (anogenital distance) was measured to record the amount of virilization. Newborns remained with their mothers until weaning at 10 wk of age. After being weaned, lambs were given free access to water and pasture and were supplemented two times daily with hay and commercial pellet food consisting of oat, corn, wheat, gluten feed, gluten meal, soybean meal, fish meal, sunflower meal, and mineral salts. The pelleted ration was 18% protein, 11% crude fiber, and 2% fat and it provided 2,450 kcal/kg (Glovigor; Compañía Molinera El Globo). Body weight was recorded weekly. All procedures were approved by the Faculty of Veterinary Medicine committee, University of Concepción, overseeing the Use and Care of Animals in Research.

Assessment of insulin secretion and insulin resistance. To compare developmental changes in circulating insulin levels and insulin resistance of T-females with control (C) females, three ages were studied (5 wk, when newborns were still nursing; 20 wk, which is 2 mo postweaning; and 30 wk of age, during the peripubertal period). The average onset of puberty was 28.4 ± 1.5 and 24.9 ± 1.8 wk of age in C and T-females, respectively. For comparison, a group of normal males born of the same flock were also included; they have not been treated before birth. It should be noted males begin puberty at 10 wk of age (43).

Glucose and insulin tolerance tests. To assess insulin sensitivity, an intravenous glucose tolerance test (IVGTT) was done on 8 C-females, 8 T-females, and 7 males at 5, 20, and 30 wk of age. Only singleton female or male offspring (no twins) were used. Before the experiment (1 or 2 days), lambs were placed in individual crates in the animal experimentation room to accommodate them to new surroundings. Indwelling catheters (18 gauge; Arrow International, Reading, PA) were placed under local anesthesia in both jugular veins (32). Food was withdrawn at 2000. To acclimatize the animals to handling and blood sampling, serial blood samples were withdrawn from the jugular vein catheter the day before the IVGTT. All IVGTT were initiated at 0800 the next day. During the IVGTT, 300 mg/kg glucose were infused over 2 min via one of the indwelling jugular catheter (33). Blood samples (1 ml) were collected from the contralateral jugular vein at -15, -10, 0, 3, 5, 7, 10, 13, 15, 17, and 20 min. Blood samples intended for insulin measurements were collected in heparinized tubes on ice. A second set of tubes containing heparin and sodium fluoride was used for plasma glucose determinations.

After the glucose challenge (20 min), 0.1 U/kg human insulin (Humulin; Elli Lilly) were given as a bolus, and blood samples were withdrawn at 23, 25, 27, 30, 33, 35, 37, 40, 50, 60, 80, 100, 120, 140, 160, and 180 min. Blood samples were collected and processed as during IVGTT and spun at 1,000 g at 4°C for 15 min, and plasma was harvested and kept frozen at -20°C.

Insulin and glucose measurements. Plasma concentrations of insulin were determined by double-antibody RIA using commercial kits (DSL, Webster, TX). Increasing concentrations of ovine plasma were found to dilute in parallel with the human insulin standard. Recovery of added insulin averaged $96.4 \pm 3.4\%$ ($n = 5$). Results are expressed relative to the human insulin standard provided with the kit, which has been calibrated to the World Health Organization International Reference Reagent for Insulin (code 83/500). Minimal detectable dose, defined as 90% of buffer control, was 3 μ U/ml with intra- and interassay coefficients of variation of 6 and 10%, respectively. Plasma concentrations of glucose were determined by the glucose oxidase method using commercial kits (Weiner Laboratory). Intra- and inter-assay coefficients of variation were <5%.

ISI. The insulin response was determined by calculating the mean levels of insulin achieved during the first 20 min after glucose infusion and by calculating the incremental area under the curve (AUC) of insulin, calculated as the difference between the basal AUC and the AUC of insulin secreted in response to the glucose challenge during the first 20 min of the assay. AUC was calculated with the trapezoidal formula using a computer program based on an Excel spreadsheet.

Insulin sensitivity was assessed by measuring the fasting insulin-to-glucose ratio and composite insulin response (ISI-C), the glucose utilization constant, and the glucose disappearance after exogenous insulin. The insulin-to-glucose ratio was calculated from the mean values of insulin and glucose in plasma samples obtained at -15, -10, and 0 min of glucose infusion in each subject. ISI was calculated using the formula adapted from Matsuda and DeFronzo (29) where $ISI = 10,000/\text{square root of } [(fasting\ glucose \times fasting\ insulin) \times (\text{mean glucose} \times \text{mean insulin during the first 20 min of the IVGTT})]$ (29, 33).

The glucose utilization constant (%/min) was calculated using the slope of the log linear regression of plasma glucose concentrations between 10 and 20 min postglucose administration. The effect of exogenous insulin on plasma glucose disappearance was determined with the formula of Grulet et al. (20): $\Delta\text{glucose}/\text{glucose } 20$, where glucose 20 is the glucose concentration before exogenous insulin administration and $\Delta\text{glucose}$ is the variation between glucose 20 and the plasma glucose obtained at 40 min (20 min after the insulin administration), which is derived from the regression plot (20).

Statistical analysis. All variables were analyzed by ANOVA for repeated measures with treatment (C-female, T-female, male) as the main factor and age (5, 20, and 30 wk) as the repeated-measures factor using the GB-Stat version 6.5 statistical program. Pairwise post hoc comparisons were made by the Newman-Keuls test. Differences were considered statistically significant at a level of $P < 0.05$. Results are shown as means \pm SE.

RESULTS

The anogenital distances of the T-females were comparable to those of males, indicating that the T-females were heavily virilized. This served as a bioassay for excess exposure to prenatal T. The anogenital distance in males, C-females, and T-females were 15.9 ± 0.65 , 1.48 ± 0.13 , and 14.4 ± 0.26 cm, respectively. T-females weighed less than C-females at birth (C 4.8 ± 0.1 kg, T: 3.7 ± 0.2 kg; $P < 0.05$) and at 5 wk of age (C: 11.4 ± 0.8 kg; T: 8.8 ± 0.6 kg; $P < 0.05$); at 16 wk of age the weights were comparable, indicating catch-up growth in the T-females. The body weight of males paralleled that of C-females up to 16 wk of age when males began to grow faster (Fig. 1).

Basal insulin concentrations of C-females increased with age and were greater at 30 wk of age compared with the 5-wk-old C-females ($P < 0.05$; Fig. 2, left). Males manifested the opposite trend, with basal insulin secretion being higher in 5-wk-old male lambs compared with 30-wk-old males. Basal fasting insulin concentrations were higher in males and T-females at 5 wk of age compared with C-females of the same age (Fig. 2, left). There were no differences between males, C-females, and T-females at 20 or 30 wk of age (Fig. 2). In contrast, mean basal plasma glucose concentrations did not differ between ages within a given group or between groups (data not shown).

Changes in the fasting insulin-to-glucose ratio reflected changes in basal insulin and were higher in C-females at 30 wk than at 5 wk of age (Fig. 2, right). There were no developmental differences in the fasting insulin/glucose ratio in males

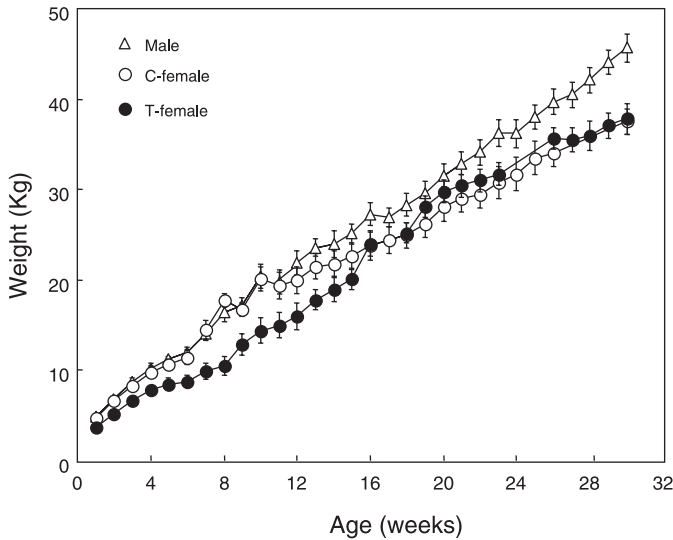


Fig. 1. Mean changes in body weight (\pm SE) of control \odot females (\circ), testosterone-treated (T) females (\bullet), and males (Δ) from birth to 30 wk of age.

or T-females. However, the fasting insulin-to-glucose ratio was higher ($P < 0.05$) in T-females at 5 wk of age compared with that in C-females but similar to that of males. There were no group differences in the fasting insulin-to-glucose ratio at either 20 and 30 wk of age.

AUC of basal insulin measurements over 20 min (imputed from 15-min measures) and incremental AUC of insulin response to glucose challenge during the first 20 min also increased with age in the C-females from 5 to 30 wk of age (Fig. 3). No age-related changes in basal and incremental AUC were observed in T-females and males. Basal and incremental AUC were of comparable magnitude at all three ages in T-females and males. Basal and incremental AUC of insulin was significantly higher in T-females compared with C-females at 5 wk of age but not at other ages. Basal and incremental AUC of insulin secretion were also higher in males

compared with C-females at 5 wk but similar between males and T-females.

Changes in ISI-C were inversely related to insulin secretion and were higher ($P < 0.05$) in C-females at 5 wk of age compared with 30 wk of age (Fig. 4). No age-related changes in ISI-C were evident in T-females and males. The ISI-C was lower in T-females compared with C-females at 5 wk but was similar to that of males. No treatment differences were evident at other ages.

Glucose utilization constant and glucose disappearance after insulin bolus did not differ between ages within a given group or between groups (data not shown).

DISCUSSION

Our findings revealed that prenatal T treatment from *days* 30 to 90 of pregnancy in sheep produces female offspring with smaller body weight and leads to alterations in ISI during early postnatal period. These effects wane during the later part of development, since no differences between C-females and T-females in body weight or ISI remained by 20 and 30 wk of age.

Effect of prenatal T treatment on birth weight. Reduced birth weights of T-females treated with 60 mg of T corroborate our earlier findings (28), where similar outcomes were achieved after prenatal treatment with a higher dose of T (100 mg two times weekly; see Ref. 28). The results of these two studies differ from an earlier study where a single administration of 100 mg T at 30, 40, or 50 days postcoitum had an opposite effect, resulting in the birth of heavier lambs (19). Such differences may stem from differences in timing and amount or duration of T exposure. Paradoxically, the C-males were not growth retarded. Absence of growth retardation in C-males as opposed to T-females may relate to exposure of male fetuses to endogenous T throughout gestation, with late-gestation T exposure facilitating tissue accretion. Recently, we found that males exposed to higher levels of T during midgestation have lower birth weight (28). The increase in proportional weight gain of males after 16 wk suggests that continued postnatal

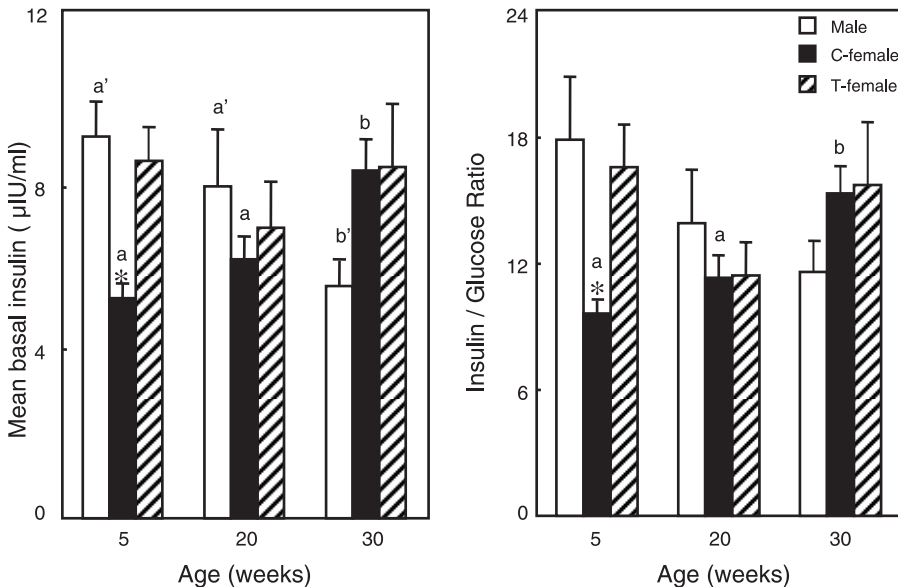


Fig. 2. Mean \pm SE basal plasma insulin (μ U/ml) and insulin-to-glucose ratio in males, C-females, and T-females at 5, 20, and 30 wk of age. *C-females are significantly different from T-females and males at 5 wk of age. Significant age differences between 5 and 30 wk in C-females are indicated by differing letters (females: a vs. b; males: a' vs. b').

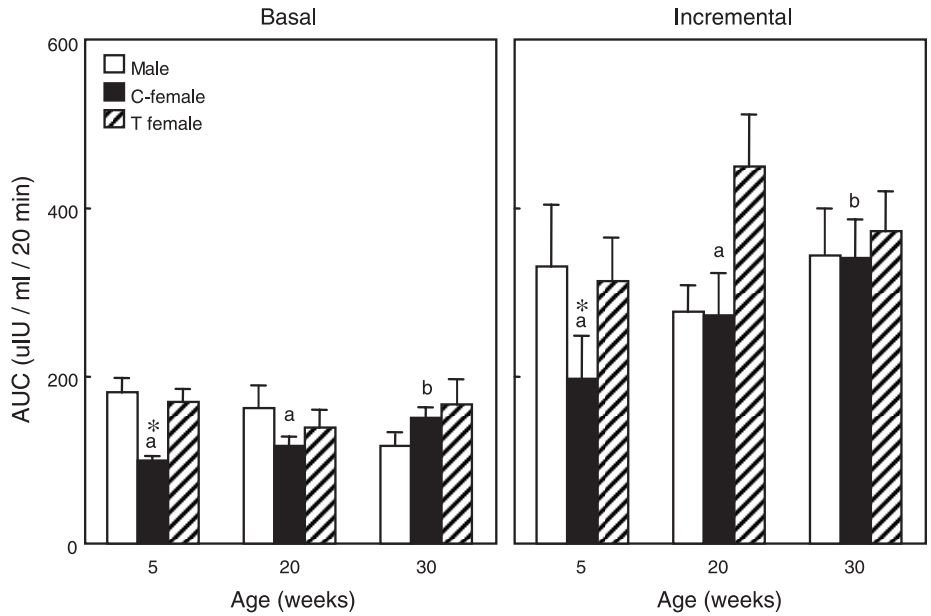


Fig. 3. Mean \pm SE area under the curve (AUC) of basal insulin secretion and incremental insulin release over 20 min (for basal AUC 20 min, AUC was imputed from 15-min assessment) after the glucose challenge in males, C-females, and T-females at 5, 20, and 30 wk of age. *C-females are significantly different from T-females and males at a given age. Significant age differences within a treatment group is indicated by differing letters (a vs. b).

exposure to T from the active testes may be a contributing factor because T increases muscle mass (24).

Evidence exists in humans linking prenatal T exposure with growth retardation. Baby girls born to a Spanish American cohort of PCOS mothers were found to have lower weight compared with those born to mothers without PCOS (38). This raises the possibility that the chronic hyperandrogenemia present during pregnancy in PCOS (37) may have contributed to the reduced body weight. Higher levels of T have also been found in amniotic fluid of diabetic gestating mothers (4) and in

congenital adrenal virilizing disorders (6). A cordicentesis study of 114 pregnancies in humans also found that fetal serum T levels around midgestation (19–25 wk) were elevated in the male fetal range in ~4 of 10 female fetuses sampled (7).

Interestingly, the effect of prenatal T treatment on birth weight parallels the outcome achieved with maternal undernutrition. Food restriction during mid- to late gestation results in lower-birth-weight lambs (40). Small-for-gestational-age human infants in general appear to result from a restriction of the supply of nutrients and oxygen to the growing fetus arising from altered placental physiology (30). Whether prenatal T treatment impacts placental physiology and restricts nutrient transfer to fetuses remain to be determined. Consistent with this premise, preliminary studies at *days 60* and *90* of gestation found advancement of placental differentiation in sheep treated with T from *days 30* to *90* of gestation (3). Postnatally, the T-females in the present study exhibited catch-up growth similar to that found in our recent study (28) using a higher level of prenatal T exposure. Growth retardation and catch-up growth are viewed as risk factors for predisposition of adult diseases (5, 31).

Prenatal T treatment and postnatal insulin sensitivity. Our findings document that prenatal T alters ISI during early postnatal life. The extent to which this is an extension of perturbations in utero remains to be determined. The similarity of ISI between T-females and males at all ages coupled with differences in ISI between males and C-females suggest that T by androgenic or estrogenic action may mediate differences in the developmental trajectory of pancreatic differentiation or function. The increased insulin responses of the T-females may be the result of an increased percentage of islet tissue in the pancreas, a compensatory β -cell response to overcome compromised pancreatic differentiation, or alternatively the result of impaired glucose sensing by the pancreatic β -cell, as reported for women with PCOS (13). To what extent the reduced insulin sensitivity is related to reduced birth weight is also unclear. In humans, the percentage of islet tissue in the pan-

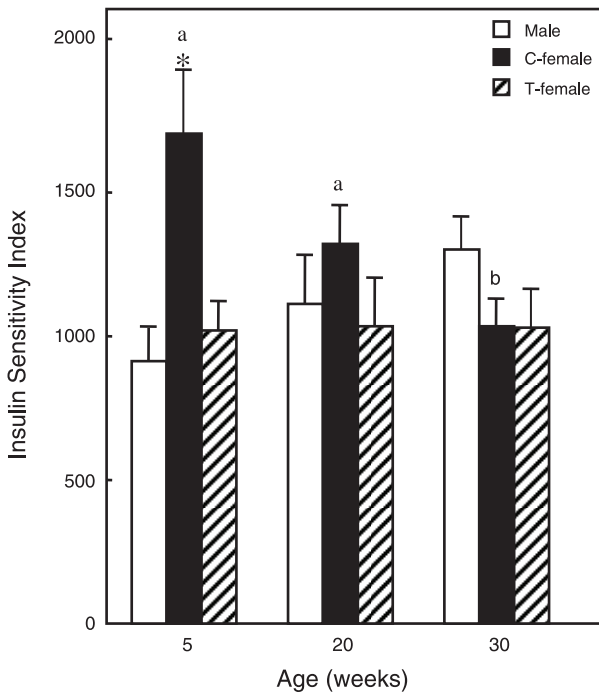


Fig. 4. Mean \pm SE insulin sensitivity index (ISI-C) in males, C-females, and T-females at 5, 20, and 30 wk of age. *C-females are significantly different from T-females and males at 5 wk of age. Significant age differences between 5 and 30 wk in control females are indicated by differing letters (a vs. b).

creas at birth is directly correlated to birth weight (15, 41), leading to reduced basal insulin levels (15, 23).

Developmentally, the decline in insulin sensitivity in C-females from 5 to 30 wk of age is consistent with findings of Gatford et al. (17) in sheep. Maturational decreases in insulin sensitivity in C-females also parallel what has been found in rats (18) and humans (2). Lack of sex differences at 38 days of life (~5 wk of age) in the study by Gatford et al. (17) as opposed to our study may relate to the high variability in basal insulin levels of males in their study and consequent masking of differences or, alternatively, a function of altered developmental trajectories in the two breeds (Suffolk in our study and Merino in the study by Gatford et al.).

The disappearance of differences in ISI between C-females and T-females at 20 and 30 wk of age appears to be a reflection of a reduction in insulin sensitivity of C-females as they approached puberty, since the ISI of T-females did not change with age. The age-related increase in basal insulin concentrations in C-females, which confirms recent work of Gatford et al. (17), and the corresponding decline in ISI-C are supportive of developing insulin resistance in C-females as they approach puberty. Interestingly, ISI of 30-wk C-females were comparable to that of 5-wk-old males. The directionality of changes in ISI in males and C-females may relate to the marked differences in the timing of puberty. Male lambs reach puberty at ~10 wk of age and females at ~30 wk (43). The similarity of the ISI in males and T-females may relate to the reported early onset of neuroendocrine puberty in T-females (43). The extent to which changes in peripheral ISI contribute to the neuroendocrine changes that subserve puberty or, alternatively, the extent to which puberty-associated changes in neuroendocrine and gonadal function impact on ISI remain to be understood.

Although the observed hyperinsulinemia in T-females is suggestive of developing insulin resistance, this was not evident from the glucose disappearance rate after insulin bolus or fasting glucose levels. The absence of changes in fasting glucose levels in our study differs from other studies (11, 17), which found an age-related decline in basal glucose levels in both sexes. It is possible that an early elevation in basal glucose may have been missed in our study as a consequence of advancement in developmental trajectory of mechanisms governing glucose homeostasis in this breed of sheep. Sheep, like other ruminants, are perceived as more insulin resistant than nonruminants, and they obtain most of their glucose supply by gluconeogenesis in the liver, using as substrate short-chain fatty acids (propionate, butyrate and acetate) synthesized by microorganisms in the rumen (8). As such, the glucose disappearance rate obtained by the change in plasma glucose concentrations in response to an insulin challenge in this study may not be a good measure of insulin-mediated changes in sheep and may require hyperinsulinemic-euglycemic clamp studies.

Our findings raise several questions to be addressed. What are the late-life consequences of such early perturbation in ISI? Are these perturbations a continuum of disruptions that occurred in utero? What is the critical period for pancreatic programming, and is this facilitated by androgenic or estrogenic action of T? On the basis of epidemiologic data and other experimental models (22, 39), growth retardation and catch-up growth observed in our T-females suggest that these animals may be at risk of developing insulin resistance during their

adult life, especially as they get older. This is corroborated by the documentation of impaired pancreatic β -cell function and insulin resistance in older Rhesus monkeys treated prenatally with T (14). As such, the monkey and sheep studies complement each other, with sheep studies addressing developmental changes and monkey studies documenting adult disease onset. Life span studies comparing C-females and T-females are needed to establish the chronology of development of diseases involving the insulin/glucose homeostasis. In summary, our findings document that prenatal T excess leads to fetal growth retardation and impaired insulin sensitivity early in life in the growing female sheep.

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