Maternal androgen excess reduces placental and fetal weights, increases placental steroidogenesis, and leads to long-term health effects in their female offspring

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1Institute of Neuroscience and Physiology, Department of Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; 2Department of Obstetrics and Gynecology, First Affiliated Hospital, Heilongjiang University of Chinese Medicine, Harbin, China; and 3Center for Pregnancy and Newborn Research, Department of Obstetrics and Gynecology, University of Texas Health Science Center, San Antonio, Texas

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Sun M, Maliqueo M, Benrick A, Johansson J, Shao R, Hou L, Jansson T, Wu X, Stener-Victorin E. Maternal androgen excess reduces placental and fetal weights, increases placental steroidogenesis, and leads to long-term health effects in their female offspring. Am J Physiol Endocrinol Metab 303: E1373–E1385, 2012. First published October 9, 2012; doi:10.1152/ajpendo.00421.2012.—Here, we tested the hypothesis that maternal androgen excess in late pregnancy reduces placental and fetal growth, increases placental steroidogenesis, and adversely affects glucose and lipid metabolism in adult female offspring. Pregnant Wistar rats were randomly assigned to treatment with testosterone (daily injections of 5 mg of free testosterone from gestational days 16 to 19) or vehicle alone. In experiment 1, fetal and placental weights, circulating maternal testosterone, estradiol, and corticosterone levels, and placental protein expression and distribution of estrogen receptor-α and -β, androgen receptor, and 17β-hydroxysteroid dehydrogenase 2 were determined. In experiment 2, birthweights, postnatal growth rates, circulating testosterone, estradiol, and corticosterone levels, insulin sensitivity, adipocyte size, lipid profiles, and the presence of nonalcoholic fatty liver were assessed in female adult offspring. Treatment with testosterone reduced placental and fetal weights and increased placental expression of all four proteins. The offspring of testosterone-treated dams were born with intrauterine growth restriction; however, at 6 wk of age there was no difference in body weight between the offspring of testosterone- and control-treated rats. At 10–11 wk of age, the offspring of the testosterone-treated dams had less fat mass and smaller adipocyte size than those born to control rats and had no difference in insulin sensitivity. Circulating triglyceride levels were higher in the offspring of testosterone-treated dams, and they developed nonalcoholic fatty liver as adults. We demonstrate for the first time that prenatal testosterone exposure alters placental steroidogenesis and leads to dysregulation of lipid metabolism in their adult female offspring.

testosterone; prenatal; maternal; placenta; polycystic ovary syndrome; insulin sensitivity; steroidogenesis; estrogen receptor; androgen receptor

THE MATERNAL ENVIRONMENT may influence epigenetic processes during placental and fetal development that have long-lasting effects and lead to diseases such as hypertension, obesity, type 2 diabetes, and endocrine and reproductive dysfunction in adult offspring (6, 24). Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age and is associated with hyperandrogenism, oligo/ovulatory

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androgen exposure has not, to our knowledge, been investigated.

Because PCOS is associated with excessive circulating androgen that further increases over the course of pregnancy (41), an increased risk of adverse pregnancy outcomes (33), and metabolic disturbances during adulthood, we tested the hypothesis that excess maternal androgen late in pregnancy reduces placent al and fetal growth, increases placental steroidogenesis, and adversely affects glucose and lipid metabolism in adult female offspring.

MATERIALS AND METHODS

Ethics Statement

Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals (www.sjv.se). The study was approved by the Animal Ethics Committee of the University of Gothenburg (Dnr: 70-2011).

Animals

Timed-pregnant Wistar rats purchased from Charles River Laboratories (Sulzfeld, Germany) arrived on day 7 of gestation and were housed one per cage under controlled conditions (21–22°C, 55–65% humidity, and a 12:12-h light/dark cycle). All rats were fed Harlan Teklad Global Diet (16% protein rodent diet no. 2016; Harlan Win kelmann, Harlan, Germany) and tap water ad libitum. Pregnant rats were treated from embryonic days 16 to 19 with daily injections of 5 mg of free testosterone (T-1500; Sigma) dissolved in 0.5 ml of a 1:1 mixture of sesame oil (S3547; Sigma) and benzyl benzoate (B6630; Sigma) or with 0.5 ml of sesame oil-benzyl benzoate vehicle as a control. The dose was selected to mimic the fetal testosterone surge that is observed in male rats (45, 48). Maternal weight gain and food intake were recorded from gestational days 16 to 21.

Experimental Design and Methods

Experiment 1. PLACENTAL SAMPLES. Seven control and eight testosterone-treated dams were anesthetized with thiobutabarbital sodium (130 mg/kg ip, Inactin; Sigma) and euthanized on gestational day 21. Maternal blood was collected by cardiac puncture and centrifuged, and the serum was stored at −80°C for further analysis. Laparotomy was performed, and the fetuses and placentas were collected and quickly dried on blotting paper to remove any remaining fetal membranes and weighed. Two placentas from each litter were fixed in Histofix containing 6% formaldehyde (Histolab, Gothenburg, Sweden) before final storage in 70% ethanol. The remaining placentas in each litter were pooled and cut in smaller pieces. Some placentas were pieces immediately frozen and stored at −80°C until later gene expression analysis, and the remaining tissue was quickly homogenized on ice in a Polytron in a buffer containing 10 mM Tris-HEPES, 250 mM sucrose, 1 mM EDTA, 1.6 mM antipain, 0.7 mM pepstatin, and 0.5 μg/ml aprotinin. The homogenate was frozen in liquid nitrogen and stored at −80°C until subsequent protein expression analysis.

PLACENTA MORPHOLOGY. Paraffin-embedded histological placenta sections (6 μm) were mounted on glass slides and stained with hematoxylin and eosin. Each section was converted to a virtual slide with a Zeiss Mirax Desk scanning device (Zeiss, Oberkochen, Germany). In each section, the total placenta area, labyrinth zone area, and basal zone area were measured with MIRAX SCAN Control Software, and the ratios of labyrinth zone to total area and basal zone to total area were calculated.

IMMUNOHISTOCHEMISTRY. Immunohistochemical analysis was performed as described previously (17), with minor modifications. Paraffin sections were deparaffinized and rehydrated through a graded alcohol series followed by antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) for 18 min in a microwave oven at full power. Endogenous peroxidases and nonspecific binding were removed by preincubation with 3% H2O2 for 10 min, 0.25% Triton X-100 for 30 min at room temperature, and 2.5% normal horse serum for 1 h at 37°C. Sections were then incubated with commercially available primary antibodies [estrogen receptor-α (ERα) 1:400, estrogen receptor-β (ERβ) 1:200, androgen receptor (AR) 1:100, or 17β-hydroxysteroid dehydrogenase 2 (17β-HSD2) 1:200] from Santa Cruz Biotechnology (sc-542, sc-8974, sc-816, and sc-135042; Sigma) overnight at 4°C. After washing, the sections were incubated with the appropriate biotinylated secondary antibody for 1 h at 37°C. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used for the avidin-biotin peroxidase complex detection system according to the manufacturer’s instructions. Antibodies were visualized by reaction with the chromogen 3,3’-diaminobenzidine-tetrahydrochloride (Sigma) and hydrogen peroxidase for 1 min. Sections were viewed on an Olympus DP50 microscope using Image-Pro plus software or on an Axiovert 200 confocal microscope (Carl Zeiss, Jena, Germany) equipped with a laser-scanning confocal imaging LSM 510 META system (Carl Zeiss), and all sections were photographed. Rat testis tissue was used as a positive control for AR.

WESTERN BLOT ANALYSIS. Protein expression levels of ERα, ERβ, and 17β-HSD2 were determined in placenta homogenates using horseradish peroxidase-conjugated anti-rabbit IgGs (A0545; Sigma). Western blot analysis was performed as described (22). In brief, 20–50 μg of total proteins was separated by electrophoresis through an SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane. Membranes were rinsed in Tris-buffered saline with 1% Tween-20 (TBS-T), blocked in 3% BSA in TBS-T for 1 h at room temperature, and incubated with primary antibody overnight at 4°C. The blots were washed in TBS-T, incubated in secondary antibody for 1 h at room temperature, and washed again in TBS-T. Protein bands were developed with SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology) and photographed with an LAS-1000 camera system (Fujifilm, Tokyo, Japan). The intensities of the protein signals were quantified by densitometry with MultiGauge software version 3.0. β-Actin (A1978; Sigma) was used as a loading control and for normalization. Values are expressed in arbitrary densitometric units.

AR SEMIQUANTITATIVE PCR ANALYSIS. Total RNA from placent al tissues was extracted using the RNeasy Micro Kit (Qiagen) and RNase inhibitor (Applied Biosystems). Single-stranded cDNA was synthesized from each sample (0.5 μg) with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Oligo(dT)-primed cDNAs were synthesized from each sample (0.5 μg) with 2.5 units of HotStar Taq DNA polymerase (Qiagen) and 0.2 μM each of the sense and antisense primers used to amplify specific nucleotide sequences present in AR and β-actin transcripts. The primer pair used for amplification of a 29-bp fragment of AR cDNA corresponding to the ligand-binding domain of AR (NM_012502.1) was 5’-CCC ATTC GAC TAT TAC TTC CCA CC-3’ (sense) and 5’-TCCT TTC TTC TCT CTG TAG TTT GA-3’ (antisense). The primer pair for amplification of a 454-bp fragment of β-actin (NM_0131144.2) was 5’-CTG TGC CCA TCT ATG AGG GTT AC-3’ (sense) and 5’-AAT CCA CAC AGA GTA CTT GCG CT-3’ (antisense). Results were calculated as the ratio of the signal of the gene in each sample to its corresponding internal control (β-actin). To ensure reliability, PCR analysis for each gene was independently performed in duplicate for each tissue sample.

MATERNAL HORMONE LEVELS. Serum concentrations of cortico-sterone, 17β-estradiol, and testosterone were determined with double-antibody RIA kits (Siemens Healthcare Diagnostics, Los Angeles, CA). The intra- and interassay coefficients of variation were 6.4–10.6
and 5.9–11.9% for 17β-estradiol, 6.0–9.1 and 7.5–12.7% for testosterone, and 4.0–12.2 and 4.8–14.9%, respectively, for corticosterone. The sensitivities of the assays were 7.2 pg/ml, 0.03 ng/ml, and 5.7 ng/ml for 17β-estradiol, testosterone, and corticosterone, respectively.

**Experiment 2. Female offspring.** Spontaneous delivery was allowed for five control and five testosterone-treated dams. Four days after birth pups were counted and weighed, and the offspring were subsequently weighed twice/wk until 70 days of age. After 21 days, the female offspring were separated from the dam and their male littermates, and their weights, lengths, and anogenital distances were measured. Microchips (AVID, Norco, CA) with an identification number were inserted subcutaneously in their necks under light anesthesia (2% isoflurane in a 1:1 mixture of oxygen and air, Isoba Vet; Schering-Plough). Food intake was recorded from day 21 until 70 days of age.

**Vaginal smears and blood sampling.** The estrous cycle stages of the female offspring were determined by microscopic analysis of the predominant cell type in vaginal smears. The smears were taken starting 1 wk prior to the collection of fasting tail blood samples at 10 wk of age and were continued until the euglycemic hyperinsulinemic clamp at weeks 11 and 12 (27). None of the testosterone-treated offspring had vaginal openings, so blood was collected, and the clamp was performed independent of cycle day. In control offspring, blood was collected and the clamp performed during estrous phase.

**Body composition by dual-energy X-ray absorptiometry.** A whole body dual-energy X-ray absorptiometry (DEXA) instrument (QDR-1000/W; Hologic, Waltham, MA) was used to assess the total body fat, lean body mass, and bone mineral content at 10 wk of age. Rats were lightly anesthetized with isoflurane during the procedure.

**Euglycemic hyperinsulinemic clamp and sample collection.** At 11–12 wk of age, rats were subjected to a euglycemic hyperinsulinemic clamp as described (27). In brief, rats were anesthetized with thiobutabarbital sodium (130 mg/kg ip, Inactin; Sigma). Body temperature was maintained at 37°C with a heating pad throughout the clamp. Insulin (100 U/ml Actrapid; Novo Nordisk) together with 0.2 ml of albumin and 10 ml of physiological saline was infused at 8 mU·min⁻¹·kg⁻¹ during the clamp after an initial bolus dose. The glucose infusion rate (GIR) was guided by blood glucose concentration measurements taken every 5 min with an Accu-Chek Compact Plus glucometer (Roche Diagnostics, Indianapolis, IN), and 20% glucose in saline solution was continuously administered to maintain plasma glucose at a constant euglycemic level (6.0 mM). At steady state (after 50–70 min), the mean GIR was normalized to body weight, and blood samples were taken to determine plasma insulin concentrations.

After the clamp, the rats were decapitated, and their ovaries, uteri, hindlimb muscles (tibialis anterior, extensor digitorum longus, and soleus), fat depots (inguinal, parametrial, retroperitoneal, and mesenteric), and livers were dissected and weighed. Collected tissues were divided into two halves, with one half being snap-frozen in liquid nitrogen and stored at −80°C until protein analysis and the other half being fixed in Histofix containing 6% formaldehyde (Histolab) before final storage in 70% ethanol.

**Ovarian morphology.** Paraffin-embedded histological sections (6 μm) of the ovaries were mounted on glass slides and stained with hematoxylin and eosin. Each section was converted to a virtual slide using a Zeiss Mirax Desk scanning device.

**Oil red O analysis of the liver.** Frozen 10-μm sections from liver samples in cryostat-embedding medium were cut with a Leica Cryostat microtome 3050S (Leica Microsystems Nussloch, Heidelberg, Germany) at −20°C, mounted onto glass slides, and air-dried. The tissue samples were rinsed with 60% isopropanol for 2 min and oil red O dissolved in 98% isopropanol for 15 min, 60% isopropanol for 30 s, and distilled water for 5 min. The tissue samples were counterstained with hematoxylin for 30 s and washed thoroughly with distilled water prior to mounting in glycerol gelatin. Unsaturated hydrophobic lipids were identified microscopically by the presence of a red stain and were subsequently classified into negative (−), possible early (+/−), and positive (+) fatty liver categories compared with predetermined reference sections (20).

**Computerized determination of adipocyte size.** The mean adipocyte size was determined by computerized image analysis according to Björnheden et al. (10). Adipocytes were obtained from −500 mg of inguinal or mesenteric adipose tissues. Small pieces of tissue were incubated with 0.2 U/ml type A collagenase (Roche) in 10 ml of minimum essential medium (Invitrogen) for 50 min at 37°C in a shaking water bath. Adipocytes were washed three times and suspended in fresh medium. The cell suspension was placed between a siliconized glass slide and a coverslip and transferred to the microscope (DM6000B, ×5 objective; Leica Microsystems). Twelve random visual fields were photographed with a charge-coupled device camera (DFC320; Leica Microsystems), and the adipocyte size was measured using the Leica QWin software package version 3. Uniform microspheres 98 μm in diameter (Dynal; Invitrogen) served as a reference.

**Hormone levels and biochemical analysis.** Serum concentrations of 17β-estradiol, testosterone, and corticosterone in the offspring were determined as described above for the dams. All lipid analyses were performed at an accredited laboratory at the Wallenberg Laboratory, Sahlgrenska University Hospital, Gothenburg, Sweden, on a Konelab 20 autoanalyzer (Thermo Fisher Scientific), with interassay coefficients of variation <3%. Plasma concentrations of total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides (TG) were determined enzymatically. HDL concentrations were determined after precipitation of apolipoprotein B-containing lipoproteins with magnesium sulfate and dextran sulfate (Thermo Fisher Scientific). Human insulin levels given during the clamp were measured in duplicate with an ELISA kit (Merckodia, Upplasa, Sweden), with intra- and interassay coefficients of variation of 3.4 and 3.0%, respectively, and a sensitivity of 1 mU/l.

**Statistical analysis.**

Data are reported as means ± SE. Body weight gains at each time point were analyzed by mixed between/within-subjects ANOVA followed by Student’s t-test. Remaining analyses were performed only with Student’s t-test. All statistical evaluations were performed with the SPSS software package (version 19.0; SPSS, Chicago, IL). P < 0.05 was considered significant.

**Results**

**Experiment 1.**

Maternal body weights were measured from gestation day (GD) 16 to GD 21. No significant differences were observed between testosterone-treated and control dams at any time point (Table 1).

**Maternal hormone levels.** Administration of testosterone to pregnant rats from GD 16 to GD 19 resulted in a 2.5-fold increase in 17β-estradiol, testosterone, and corticosterone concentrations.

**Table 1. Body weights (g) in control and T-treated dams**

<table>
<thead>
<tr>
<th>Gestational Day</th>
<th>Control (n = 12)</th>
<th>T-treated (n = 13)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>294.9 ± 5.6</td>
<td>287.7 ± 5.5</td>
<td>0.372</td>
</tr>
<tr>
<td>17</td>
<td>311.4 ± 5.6</td>
<td>300.4 ± 5.8</td>
<td>0.184</td>
</tr>
<tr>
<td>18</td>
<td>325.1 ± 6.2</td>
<td>316.0 ± 6.2</td>
<td>0.309</td>
</tr>
<tr>
<td>19</td>
<td>339.6 ± 6.7</td>
<td>329.6 ± 6.3</td>
<td>0.292</td>
</tr>
<tr>
<td>20</td>
<td>356.1 ± 7.0</td>
<td>330.7 ± 9.2</td>
<td>0.117</td>
</tr>
<tr>
<td>21</td>
<td>373.0 ± 7.8</td>
<td>357.1 ± 6.9</td>
<td>0.138</td>
</tr>
</tbody>
</table>

Values are means ± SE. T, testosterone. P values were determined with Student’s t-test.
increase \((P = 0.032)\) in circulating testosterone levels on GD 21 (Table 2). There were no significant differences in circulating corticosterone or 17β-estradiol levels in testosterone-treated dams compared with controls (Table 2).

**Placental and fetal data.** Maternal administration of testosterone decreased fetal weight, fetal length, and placental weight compared with controls (Table 3). No significant differences in litter size or in the fetal-to-placental weight ratio were observed between testosterone-treated and control dams (Table 3).

**Placenta morphology.** The total placental area and the basal zone area were smaller in testosterone-treated dams compared with control dams at GD 21 (Table 3 and Fig. 1, A and D), but no significant differences were seen in the area of the labyrinth zone, the ratio of the labyrinth zone to total area, or the ratio of the basal zone to total area (Table 3). The labyrinth zones of the placentas from testosterone-treated dams had fewer red blood cells compared with controls (Fig. 1, B and E), and the basal zones of the placentas from testosterone-treated dams had larger and more dispersed trophoblast giant cells than controls (Fig. 1, C and F). No significant differences in the appearance of glycogen cells or spongiotrophoblasts in the basal zone were observed (Fig. 1, C and F).

**Changes in placental ER, AR, and 17β-HSD2 protein expression levels.** The placental distribution patterns of ERα, ERβ, AR, and 17β-HSD2 on GD 21 are shown in Fig. 2. The expression of all four proteins was higher in placentas from testosterone-treated dams than in controls (Fig. 2, A through E). The AR protein was very low and not detected in placental tissue from control dams (Fig. 2, A and F). Expression of the AR protein was higher in placentas from testosterone-treated dams (Fig. 2, G and H).

Western blot experiments were performed to confirm the immunohistochemical results (Fig. 3, A through D). The placentas of testosterone-treated dams showed 3.2-fold higher levels of expression of ERα, 2.5-fold higher ERβ levels, and 2.3-fold higher 17β-HSD2 levels compared with control placentas (Fig. 3, A through D). Expression of the AR protein was very low and could not be detected by Western blot. Semi-quantitative PCR of AR confirmed its expression in the placenta (Fig. 3, E and F). For ERα, measurements were based on the 52-kDa bands because the 66-kDa bands were too weak to be measured accurately (14).

**Experiment 2**

**Postnatal growth.** The offspring of dams treated with testosterone late in pregnancy weighed less than controls from postnatal days 4 to 35 (Fig. 4A). However, from postnatal day 42, female offspring from testosterone-treated and control rats did not differ in body weights (Fig. 4B), nor was there any difference in food intake during the study period (Fig. 4C).

On postnatal day 21, sex was determined, and females were separated from males. The mean anogenital distance was 26% longer in the female offspring of testosterone-treated dams compared with controls (11.5 ± 0.5 vs. 9.0 ± 1.5 mm, \(P < 0.001\)). There was no significant difference in body length between the groups (data not shown). Inspection of females revealed an absence of the vaginal opening in 100% of the offspring of the testosterone-treated dams, and some died during the study, most likely due to a large, distended, fluid-filled uterus and upper vagina (hydrometrocolpos).

**Female offspring hormone levels and biochemical analysis.** At 9 wk of age, circulating testosterone levels were higher in female offspring of the testosterone-treated dams compared with controls, whereas 17β-estradiol and corticosterone levels did not differ between the groups (Table 4). Fasting TG levels were higher in female offspring of the testosterone-treated dams compared with controls \((P < 0.01)\), whereas fasting levels of cholesterol, HDL, and LDL did not differ between the groups (Table 4).

**Body composition measured by DEXA.** At 10 wk of age there was no difference in body weight between the two groups (Table 5). Despite this, the female offspring of the testosterone-treated dams had less body fat, both in terms of total weight and as a percentage of body weight, and a tendency for reduced bone mineral density compared with controls as measured by DEXA.

**Insulin sensitivity and tissue weights.** There was no difference in mean GIR (Fig. 5A) or insulin sensitivity index (ratio of the mean GIR to the steady-state plasma insulin level; Fig. 5B) between the two groups. When steady state was reached, the glucose level was ~6 mmol/l in both groups, and the plasma insulin levels were 152.8 ± 12.3 mU/l in the control offspring and 151.6 ± 13.6 mU/l in the testosterone-treated offspring.

After application of the clamp, the animals were euthanized, and various tissues were dissected and weighed (Table 6). The weights of the tibial muscle and of the inguinal and retroperitoneal fat depots were reduced in the offspring of the testosterone-treated dams, whereas the ovarian, uterine, and liver tissue weights did not differ between the two groups (Table 6).

**Morphology of ovary and liver.** The offspring of the testosterone-treated dams displayed normal ovarian morphologies and had antral follicles at different stages and fresh corpus luteum.
luteum, just as in the control ovaries (Fig. 6A). However, there were signs of early nonalcoholic fatty liver in 37.5% of the offspring of the testosterone-treated dams compared with 10.0% in controls ($P < 0.05$), and there were signs of fatty liver in 50% of the offspring compared with 30% in controls ($P < 0.01$) (Fig. 6B).

**Adipocyte size.** Mean adipocyte size and size distribution were determined in inguinal and mesenteric adipose tissues. In female offspring of the testosterone-treated dams, the size distribution curve of inguinal adipocytes was shifted to the left (Fig. 7, A–C), with smaller mean inguinal adipocytes than in controls ($61.9 \pm 2.4$ vs. $70.9 \pm 7.6 \mu m$, $P < 0.05$). In the mesenteric fat depot, the mean adipocyte size and size distribution did not differ compared with controls (data not shown).

**DISCUSSION**

This study is, to our knowledge, the first to demonstrate enhanced placental ER, AR, and $17\beta$-HSD2 protein expression levels in rats after maternal exposure to high levels of testosterone during late pregnancy. The increase in placental $17\beta$-HSD2 protein expression suggests altered placental steroidogenesis, and the increased expression of ER and AR indicates altered estrogen and androgen activity that occurred together with decreased placental weight, altered placental morphology, and decreased fetal weight. The female offspring of the testosterone-treated dams were born with intrauterine growth restriction (IUGR), but by puberty no difference in body weight was observed between the two groups. Furthermore, female offspring of testosterone-treated dams displayed normal insulin sensitivity but had less adipose tissue, smaller adipocytes, and high levels of circulating TG with clear signs of nonalcoholic fatty liver. These results indicate that prenatal androgen exposure in rats induces early signs of metabolic dysfunction in female offspring as in human PCOS.

**Effect of Prenatal Testosterone Exposure on the Placenta and Fetal Growth**

Administration of testosterone to rats in late pregnancy did not affect maternal weight gain, food intake, metabolic status, or circulating estradiol or corticosterone concentrations, and
these results are consistent with previous reports in rodents (36). This suggests that the effect of testosterone on placental and fetal growth in rats is a direct effect of the testosterone and not a secondary consequence of maternal malnutrition or alterations in metabolic and steroid hormones. However, these results may not be extrapolated directly to humans since similarly treated monkeys show altered maternal weight gain, metabolic status, and circulating conjugated estrogens (2).

Testosterone is a lipophilic hormone and can diffuse from the amniotic fluid into the fetal circulation and across the placenta (16, 28, 44). However, previous studies have reported that a significant increase in maternal testosterone level in rats is not associated with a significant increase in testosterone in the fetus (35, 50), whereas in both sheep and monkeys it has been shown that elevated maternal testosterone levels also elevate fetal testosterone levels in female offspring (2, 47). Also, a recent preliminary report indicates altered enzymatic regulation in PCOS placentas, as demonstrated by higher 17β-HSD1 placental expression and lower P450 aromatase activities compared with controls (11). Thus, maternal testosterone may exert an indirect or direct effect on the fetus and alter critical placental functions that support fetal growth. One hypothesis is that a high maternal testosterone level alters placental metabolism of testosterone, although the pathway by which this may occur is still unknown. It has been reported recently that high maternal testosterone concentrations do not directly cross the placenta to suppress fetal growth but instead decrease amino acid nutrient delivery to the fetus by downregulation of the activity and expression of specific placental amino acid transporters (35). Although it is unclear whether the maternal environment in women with PCOS directly influences the development of PCOS in their offspring, it has been demonstrated that female offspring of mothers with PCOS are exposed to testosterone levels comparable with male levels in utero (7).

Altered birth weight with both small-for-gestational-age (40) and large-for-gestational-age infants (33) has been reported in women with PCOS. Whether the placental weight or the placental metabolism of testosterone is altered in women with PCOS has not been investigated to our knowledge, but our finding that high maternal testosterone during late pregnancy in rats affects placenta weight, size, morphology, and steroidogenesis as well as androgen and estrogen activity may be relevant to human PCOS. Excess maternal androgen levels in rats decreased placental size, increased circulating androgens, and tended to increase circulating 17β-estradiol and the expression of AR and the ERs and altered placental morphology, and this may adversely affect the ability of the placenta to deliver nutrients to the fetus. Importantly, blockade of AR action has not been performed, and thus an alternative explanation may exist. Maternal stress (i.e., food restriction) and maternal glucocorticoid excess also inhibit placental and fetal development in rats (9). In the present study, it is clear that prenatal exposure to high levels of
testosterone inhibits placenta growth, especially in the basal zone. Maternal food restriction is also known to cause a reduction in placental weight, with associated hypotrophy in the basal and labyrinth zones of the placenta (9). In the maternal stress model, maternal circulating corticosterone concentrations are increased (9), but no such increase after maternal testosterone exposure was seen in our experiments. This indicates that the effects on placental and fetal development that we have observed are not due simply to a stress response.

In contrast to the human placenta, the rat placenta normally secretes only small amounts of progesterone and testosterone (46) and cannot synthesize estrogens since they do not express aromatase, and therefore, it is dependent on ovarian estrogen since it cannot synthesize any estrogen itself (3, 43). High estrogen concentrations are known to inhibit placental growth, whereas estrogen deficiency induces placental hypertrophy (8, 12). Estrogen exerts its biological effects by binding to the ER, and the ER exists as two different isoforms, ERα and ERβ. In our present study, high maternal testosterone levels increased both ERα and ERβ expression in the placental basal zone during late gestation. Furthermore, placental expression of 17β-HSD2 was increased in response to maternal administration of testosterone. In the rat placenta, 17β-HSD2 is expressed mainly in the decidual and basal zones (the functional zone), and this enzyme converts the active 17β-hydroxy forms of estradiol and testosterone to their less active 17-keto forms (29, 49). Mustonen et al. (29) found that the distribution of 17β-HSD2 expression in the placenta changes as gestation ad-

Fig. 3. ER, AR, and 17β-HSD2 protein expression in placentas of control (n = 7) and testosterone-treated (n = 8) dams. A: representative blots of ERα, ERβ, AR, and 17β-HSD2 protein expression. B–D: densitometric analysis of ERα (B), ERβ (C), and 17β-HSD2 protein expression (D). E: semiquantitative PCR analysis. Values are means ± SE. *P < 0.05 vs. control; **P < 0.01 vs. control.
advances, and this enzyme is expressed mainly in both the giant
cells at the interface of the chorioallantoic placenta and decid-
ual zone as well as in the functional zone during gestation, and
this is consistent with our observations. It is well known that
the amniotic fluid surrounding the fetus contains large amounts
of various steroids among other compounds (38). Hence, 17β-
HSD2 in the fetus, together with its oxidative activity, suggests
that this enzyme protects the embryo from excessive action of
the 17-hydroxysteroids present in the amniotic fluid (29). This
may explain why previous studies found less testosterone in
fetal serum than in testosterone-treated maternal serum (35,
50). Based on these collective results, we speculate that the
increased expression of placental ER may contribute to the
inhibition of placental growth in the current study. Further-
more, we propose that the increased expression of 17β-
HSD2 in the placenta in response to maternal administration of

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

Fig. 4. Body weight development in the offspring of control and testosterone-
gain from days 4 to 70 after birth. C: food intake corrected by weight gain from
days 21 to 70 after birth. Values are means ± SE. *P < 0.05 vs. control; **P <
0.01 vs. control; ***P < 0.001 vs. control.

Table 4. Serum T, 17β-estradiol, and lipid profiles in
T-exposed and C offspring

<table>
<thead>
<tr>
<th></th>
<th>C Offspring (n = 11)</th>
<th>T Offspring (n = 9)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T, ng/ml</td>
<td>0.01 ± 0.001</td>
<td>0.03 ± 0.015</td>
<td>0.012</td>
</tr>
<tr>
<td>17β-Estradiol, pg/ml</td>
<td>103.6 ± 11.3</td>
<td>146.5 ± 29.9</td>
<td>0.209</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>846.4 ± 68.1</td>
<td>663.2 ± 86.5</td>
<td>0.109</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>2.30 ± 0.08</td>
<td>2.28 ± 0.09</td>
<td>0.859</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>0.64 ± 0.04</td>
<td>0.89 ± 0.08</td>
<td>0.008</td>
</tr>
<tr>
<td>HDL, mmol/l</td>
<td>1.68 ± 0.06</td>
<td>1.70 ± 0.05</td>
<td>0.815</td>
</tr>
<tr>
<td>LDL, mmol/l</td>
<td>0.22 ± 0.02</td>
<td>0.26 ± 0.03</td>
<td>0.258</td>
</tr>
</tbody>
</table>

Values are means ± SE. C, control. P values were determined with Student’s t-test.

![Graph A](image1)

Fig. 5. Glucose infusion rate (GIR) and insulin sensitivity index (ISI) in female
offspring from control (n = 9) and testosterone (T)-treated (n = 8) dams at
11–12 wk of age. There was no significant difference in GIR or ISI between
the 2 groups. Values are means ± SE.

Table 5. Body composition as determined by DEXA
in T-exposed and C offspring

<table>
<thead>
<tr>
<th></th>
<th>C Offspring (n = 11)</th>
<th>T Offspring (n = 9)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>264.9 ± 8.9</td>
<td>260.1 ± 10.3</td>
<td>0.733</td>
</tr>
<tr>
<td>Bone mineral content,</td>
<td>6.8 ± 0.1</td>
<td>6.4 ± 0.2</td>
<td>0.120</td>
</tr>
<tr>
<td>%BW</td>
<td>2.59 ± 0.03</td>
<td>2.49 ± 0.05</td>
<td>0.130</td>
</tr>
<tr>
<td>Bone mineral density,</td>
<td>0.136 ± 0.002</td>
<td>0.132 ± 0.001</td>
<td>0.052</td>
</tr>
<tr>
<td>g/cm²</td>
<td>40.1 ± 3.2</td>
<td>27.4 ± 2.4</td>
<td>0.007</td>
</tr>
<tr>
<td>Fat mass, g</td>
<td>15.3 ± 1.1</td>
<td>10.9 ± 1.0</td>
<td>0.008</td>
</tr>
<tr>
<td>Fat mass, %BW</td>
<td>215.8 ± 8.1</td>
<td>218.4 ± 9.0</td>
<td>0.833</td>
</tr>
<tr>
<td>Lean body mass, g</td>
<td>81.5 ± 1.4</td>
<td>84.0 ± 1.0</td>
<td>0.180</td>
</tr>
<tr>
<td>Lean body mass, %BW</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. DEXA, dual-energy X-ray absorptiometry; %BW, %body weight. P values were determined with Student’s t-test.
hypothesis that the overexpression of placental AR, together with increased expression of ER and 17β-HSD2 protein, that is observed in response to high maternal testosterone levels is involved in the development of adult diseases in the offspring.

In a normal pregnancy, the red blood cell mass increases during the latter half of gestation and is associated with increased erythropoietin production. Adequate blood flow to the placenta is essential for the successful outcome of the pregnancy (31). Here, we observed fewer red blood cells in the labyrinth zone in the testosterone-treated dams compared with controls, which may indicate decrease of placental blood flow but also a diminution of red blood cells. Thus, high maternal testosterone may play an adverse role in the control of placental blood flow.

**Effect of Maternal Testosterone on Birth Weight, Postnatal Growth, and Offspring Metabolism**

It has been proposed that PCOS has its origin in fetal life based on reports that prenatal exposure to testosterone in sheep, monkeys, and rodents causes endocrine and metabolic dysfunction in the offspring when they reach adulthood (1, 4, 13, 15, 20, 23, 30, 37). The etiology of PCOS remains uncertain, but there is increasing evidence for a genetic basis. PCOS manifests during adolescence along with maturation of the hypothalamic-pituitary-ovarian axis, but the genesis of the syndrome may occur anywhere from very early fetal development of the ovary to the onset of puberty (19). However, at present, it is unclear whether the maternal environment directly influences the development of PCOS in the offspring.

In this study, we found tissue-specific abnormalities in the female offspring of testosterone-treated dams. A novel finding is that these offspring had no vaginal openings, and it was not possible to observe estrous cycle changes in these rats. This birth defect, together with an increased anogenital distance, appears to be a direct effect of prenatal androgen exposure. On the other hand, inspection of ovarian morphology demonstrated clearly that the female offspring of the testosterone-treated dams have a normal estrous cycle independent of higher circulating testosterone levels. The uterus was large and fluid filled, which also indicates that normal estrus cycles were occurring. Furthermore, the female offspring of testosterone-treated dams had less fat mass and smaller adipocyte size together with normal insulin sensitivity. The decrease in fat may reflect disturbances in water balance regulation as indicated by the DEXA results, since a 5% variation in fat-free mass hydration can change the DEXA-determined body fat percentage by nearly 3% (32). Other possible factors are the large fluid-filled uteri, which also may reflect this difference. Regarding smaller adipocytes, this may not always be considered to be healthy. It has been demonstrated recently that adipocytes taken on days 1 and 21 after birth from rats that were born small for their gestational age exhibited enhanced adipogenesis and lipogenesis (52), and this may increase the risk of development of obesity and insulin resistance in adulthood.

The offspring of the testosterone-treated dams in the present study were born intrauterine growth restricted, but their body weights increased until they were similar to the offspring of the control group from 6 wk of age onward. However, the mechanisms underlying fetal growth restriction in response to maternal testosterone treatment remain to be established. Antenatal exposure to glucocorticoids has been found to reduce offspring birth weight, and this is followed by catchup growth (39). In our study, although circulating corticosterone levels did not differ in maternal serum, we cannot completely exclude that maternal exposure to testosterone activates the fetal hypothalamus-pituitary-adrenal axis, resulting in increased fetal corticosterone levels. Testosterone may stimulate the placental transfer of corticosterone by modulating the expression of both 11β-hydroxysteroid dehydrogenases, as has been shown in

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**Table 6. Dissected tissue weights in T-exposed and C offspring**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>C Offspring (n = 10)</th>
<th>T Offspring (n = 8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>285.7 ± 10.1</td>
<td>287.9 ± 10.0</td>
<td>0.877</td>
</tr>
<tr>
<td>Ovary</td>
<td>g</td>
<td>0.135 ± 0.008</td>
<td>0.155 ± 0.013</td>
</tr>
<tr>
<td>Liver</td>
<td>g</td>
<td>0.047 ± 0.002</td>
<td>0.055 ± 0.006</td>
</tr>
<tr>
<td>Muscles, g/kg BW</td>
<td></td>
<td>0.533 ± 0.019</td>
<td>0.908 ± 0.399</td>
</tr>
<tr>
<td>Liver</td>
<td>g/kg BW</td>
<td>0.188 ± 0.007</td>
<td>0.311 ± 0.128</td>
</tr>
<tr>
<td>Uterus</td>
<td></td>
<td>11.33 ± 0.49</td>
<td>11.69 ± 0.70</td>
</tr>
<tr>
<td>Liver</td>
<td>g/kg BW</td>
<td>3.96 ± 0.09</td>
<td>4.05 ± 0.16</td>
</tr>
<tr>
<td>Muscles, g</td>
<td>Tibialis</td>
<td>1.095 ± 0.052</td>
<td>1.022 ± 0.032</td>
</tr>
<tr>
<td>EDL</td>
<td>0.255 ± 0.012</td>
<td>0.246 ± 0.009</td>
<td>0.558</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.266 ± 0.010</td>
<td>0.260 ± 0.010</td>
<td>0.719</td>
</tr>
<tr>
<td>Muscles, g/kg BW</td>
<td>Tibialis</td>
<td>0.383 ± 0.010</td>
<td>0.356 ± 0.007</td>
</tr>
<tr>
<td>EDL</td>
<td>0.089 ± 0.003</td>
<td>0.085 ± 0.002</td>
<td>0.325</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.093 ± 0.002</td>
<td>0.091 ± 0.002</td>
<td>0.453</td>
</tr>
<tr>
<td>Fat depots, g</td>
<td>Inguinal</td>
<td>1.34 ± 0.08</td>
<td>1.03 ± 0.10</td>
</tr>
<tr>
<td>Parametrial</td>
<td>3.79 ± 0.39</td>
<td>3.00 ± 0.36</td>
<td>0.174</td>
</tr>
<tr>
<td>Retropertioneal</td>
<td>2.93 ± 0.34</td>
<td>1.85 ± 0.20</td>
<td>0.022</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>2.42 ± 0.13</td>
<td>2.18 ± 0.15</td>
<td>0.246</td>
</tr>
<tr>
<td>Fat depots, g/kg BW</td>
<td>Inguinal</td>
<td>0.48 ± 0.03</td>
<td>0.36 ± 0.04</td>
</tr>
<tr>
<td>Parametrial</td>
<td>1.33 ± 0.13</td>
<td>1.08 ± 0.14</td>
<td>0.210</td>
</tr>
<tr>
<td>Retropertioneal</td>
<td>1.02 ± 0.11</td>
<td>0.66 ± 0.08</td>
<td>0.019</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>0.85 ± 0.04</td>
<td>0.76 ± 0.05</td>
<td>0.189</td>
</tr>
</tbody>
</table>

Values are means ± SE. EDL, extensor digitorum longus. P values were determined with Student’s t-test.
adipose tissue in children (53). However, the circulating corticosterone level had a tendency to be lower in the female offspring of the testosterone-treated offspring. In addition to higher circulating testosterone, an indication of enhanced placental steroidogenesis and reduced placental and fetal weight in testosterone-treated dams indicate that it is the high maternal testosterone exposure rather than activation of the fetal stress axis that causes the low birth weight and the catchup growth.

Interestingly, the offspring of the testosterone-treated dams had lower body fat, tended to have lower bone mass, had smaller adipocytes, and had higher circulating TG levels than the offspring of the control rats. The lower fat mass and adipocyte size may or may not indicate a more favorable metabolic status, as discussed above. To elucidate the reason for increased circulating TG but no other lipids, we examined the liver and found that the female offspring of the testosterone-treated dams showed signs of early nonalcoholic liver. A recent report showed an increased incidence of nonalcoholic fatty liver independent of obesity in the offspring of sheep that had been treated with testosterone (20). This indicates that adult liver metabolism and signaling could be altered by early exposure to testosterone and implicates an epigenetic regulation of metabolic disturbances.

The results in the present study are somewhat contradictory to what has been demonstrated previously in the female offspring of testosterone-treated rats (13, 18, 37). In these previous studies, the offspring of pregnant rats treated with the same dose of testosterone as used in our current study exhibited metabolic disturbances at 9 wk of age, including increased body weight and fat mass, increased serum insulin, cholesterol, and TG levels, increased hepatic TG content, and hypertension. They also showed signs of endocrine/reproductive disturbances, including irregular estrus cycles, polycystic ovaries, and neuroendocrine changes. In contrast, the offspring of the testosterone-treated dams in

![Fig. 6.](image-url)

**Fig. 6.** A: morphology of ovarian changes in female offspring of control (n = 10) and T-treated (n = 8) dams. The morphologies did not differ significantly between the 2 groups, and both groups had follicles (F) at different stages and fresh corpus luteum (CL). Magnification: 500 μm. B: the morphology of the livers (oil red O and hematoxylin and eosin (H & E) staining) in the offspring of control (n = 10) and T-treated dams (n = 8). C: the offspring of the T-treated dams displayed signs of early nonalcoholic fatty liver compared with controls. *P < 0.05, and signs of fatty liver; **P < 0.01. Magnification: 50 μm. Representative staining for lipids in the liver sections illustrating negative (−), early nonalcoholic fatty liver (−/+), or fatty liver (+) and associated analysis show an increased fatty liver in the offspring of T-treated dams compared with controls.
the present study had normal insulin sensitivity, decreased fat mass, smaller adipocytes, and normal estrus cycles. The only differences between the previous and current studies are the rat strains used in the current study. Also, all measurements, including clamp studies in the testosterone-treated offspring, were performed independent of estrus cycle day since we assumed that they were acyclic due to the lack of vaginal opening. These differences may lead to a different phenotype. The dose of prenatal testosterone exposure may also determine the extent of metabolic and endocrine alterations since it has been demonstrated recently that different doses of free testosterone cause different endocrine and metabolic phenotypes, with the dose of free testosterone (5 mg) used in the present study causing a more severe phenotype (4).

In conclusion, we demonstrate for the first time that prenatal testosterone exposure in rats increases the placental expression of ER, 17β-HSD2, and AR proteins, which is indicative of enhanced placental steroidogenesis. The offspring of the testosterone-treated dams were born with IUGR but showed catchup growth at puberty. These rats also developed early signs of metabolic dysfunction with increased TG levels and nonalcoholic liver at adult age despite less adiposity, smaller adipocytes, and normal insulin sensitivity. In addition to these metabolic effects, the female offspring of the testosterone-treated dams suffered from severe birth defects and were born with a lack of vaginal openings and an increased anogenital distance.

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DISCLOSURES

The authors confirm that there are no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

M.S., M.M., J.J., R.S., and E.S.-V. performed the experiments; M.S. and E.S.-V. analyzed the data; M.S., M.M., A.B., J.J., R.S., T.J., and E.S.-V. interpreted the results of experiments; M.S. and E.S.-V. prepared the figures; M.S. and E.S.-V. drafted the manuscript; M.S., M.M., A.B., J.J., R.S., L.H., T.J., X.W., and E.S.-V. edited and revised the manuscript; M.S., M.M., A.B., J.J., R.S., L.H., T.J., X.W., and E.S.-V. approved the final version of the manuscript; A.B., J.J., L.H., T.J., X.W., and E.S.-V. did the conception and design of the research.

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