Transient prenatal androgen exposure produces metabolic syndrome in adult female rats

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Polycystic ovary syndrome (PCOS) is among the most common disorders of premenopausal women, affecting ~7% of this population (35). Hyperandrogenemia is the cardinal reproductive feature of the syndrome (11, 28), whereas insulin resistance is the central metabolic phenotype (12). Abnormalities associated with insulin resistance are common in PCOS, such as obesity, dyslipidemia, and an increased risk for Type 2 diabetes mellitus and, possibly, cardiovascular disease (38). Hepatic steatosis, another finding associated with insulin resistance (22), is also increased in frequency in PCOS (20).

The mechanism for the association between these reproductive and metabolic abnormalities has been the subject of intense investigation since this phenomenon was first recognized in 1980 (8). There is extensive evidence that insulin functions as a cogonadotropin to stimulate ovarian androgen production (6). Furthermore, because lowering androgen levels results in, at best, modest improvements in insulin sensitivity (33), it has been widely accepted that insulin resistance causes hyperandrogenemia rather than vice versa (12). However, recent studies have challenged this view. First, androgen exposure during intrauterine life in nonhuman primates and in sheep produces many features of not only the reproductive but also of the metabolic phenotype of PCOS (1, 18). Human studies suggest that androgens play an important role in the abnormalities of feedback regulation of gonadotropin secretion (13) and that they contribute to certain metabolic defects characteristic of the syndrome (21). Consistent with an essential role of androgens in the development of PCOS, girls at high risk for hyperandrogenism at birth have early androgen excess (23, 31). Thus the developmental role of androgen excess in the etiology of PCOS has received renewed attention (1).

It is clear that androgens can act on the brain to induce major sex differences in neural structure and function (9). We have reported defeminization of the gonadotropin-releasing hormone (GnRH) neurosecretory system with accelerated basal GnRH pulse generator activity, similar to women with PCOS, in prenatally androgenized female rats (17). There has also been growing interest in their role in metabolic programming (43). Both transient neonatal and continuous pre- to postpubertal exposure resulted in insulin resistance and alterations in body composition in adult female rats (3, 29, 34). Accordingly, we sought to determine whether fetal exposure to androgen excess in female rats would also program a PCOS-like metabolic phenotype and to examine the mechanisms of such changes.
and water ad libitum. Body weights were monitored weekly from the day of weaning to tissue harvest at age 60 days.

**Study protocols.** At 35 days of age, female rats were either kept on RD (n = 37) or switched to the high-fat diet (HFD; n = 19) (5.24 kcal/g: kcal from fat 60%, carbohydrate 20%, and protein 20%, D12492 Test Diet; Purina Mills, Richmond, IN). There were 20 C RD, 17 PA RD, 11 C HFD, and 8 PA HFD female rats. Adult female rats (aged 60–63 days) on their respective diets were fasted overnight, anesthetized with CO2, weighed, and decapitated. To examine insulin signaling, one-half of the animals in all experimental groups were injected intraperitoneally with 5 U of regular human insulin (Novo Nordisk, Princeton, NJ) in 100 μl 0.9% sodium chloride (Abraxis, Schaumburg, IL), and half with 100 μl 0.9% sodium chloride 10 min before death. Trunk blood was collected and centrifuged, and serum was stored at −80°C. Glucose, insulin, cholesterol, and triglyceride (TG) levels were determined in samples from saline-treated animals only (10 C RD, 8 PA RD, 6 C HFD, 4 PA HFD). Liver, adipose tissue depots (parametrial and sc), and soleus muscles were excised, weighed, frozen, and stored at −80°C for further analyses. Tissues from both saline- and insulin-treated rats were used for analyses of body composition and hepatic TG content (15 C RD, 13 PA RD, 11 C HFD, 8 PA HFD), since administration of insulin would not acutely alter these end points (Fig. 1).

**Dynamic studies.** Dynamic studies of glucose homeostasis were performed in separate groups of animals at age 60 days after a 6-h fast with jugular catheters implanted 1 day before testing. Intraperitoneal (ip) glucose tolerance test (IPGTT) was performed in 5 C RD and 6 PA RD rats. A baseline blood sample was followed by intraperitoneal injection of 2 g/kg body wt dextrose and blood sampling at 2, 5, 10, 15, 30, 60, 90, and 120 min. Intraperitoneal insulin tolerance test (IPITT) was performed in a separate group of 4 C RD and 6 PA RD rats. A baseline blood sample was obtained followed by ip injection of insulin (5 U/rat) with blood sampling at 15, 30, 60, 90 and 120 min.

**Hormone assays and hepatic TG content.** Whole blood glucose levels were measured using a Prestige Smart System Glucose Monitor (Home Diagnostics, Ft. Lauderdale, FL). Insulin levels were measured by a rat insulin ELISA kit (Crystal Chem, Downers Grove, IL). Circulating TG and cholesterol levels and hepatic TG content were assessed by spectrophotometric assay (Infinity Triglyceride Reagent Kit; Thermo Electron, Pittsburgh, PA). Hepatic TG content was assessed by spectrophotometric assay (Infinity Triglyceride Reagent Kit; Thermo Electron, Pittsburgh, PA).

**MATERIALS AND METHODS**

**Animal treatments and diet.** All animal procedures were approved by the Animal Care and Use Committee at Northwestern University (Evanston, IL). Rats were housed at 23–24°C on a 10:14-h light-dark cycle.

Time-pregnant female Sprague-Dawley rats were obtained from Charles River (Portage, WI) at day 14 of gestation and treated from embryonic day (E) 16 to E19 with daily subcutaneous (sc) injections of 5 mg of free testosterone (T-1500; Sigma, St. Louis, MO) dissolved in 500 μl sesame oil (S3547; Sigma)/benzyl benzoate (B6630; Sigma) (prenatally androgenized: PA n = 8) or of 500 μl sesame oil vehicle as a control (C, n = 12). This hormonal paradigm mimics the fetal testosterone surge that is observed in male rats (17). In preliminary experiments, we found no significant difference in either the female-to-male offspring ratio, number of pups per litter, or birth weights between PA and C animals (unpublished observations). Pups were weaned, and females were separated from males at 21 days of age and culled from control litters to equalize group sizes. All litters were between PA and C animals (unpublished observations). Pups were fed regular chow diet (RD) (3.30 kcal/g: kcal from fat 15%, carbohydrate 20%, and protein 20%).

**Fig. 1.** Study schema with the number of animals used in each experiment.
samples run together on the same gels. Bands were visualized and expressed as percent of protein content as described previously (41). Frozen livers were stained with Oil red-O for lipids (19).

**Western blotting.** Liver and soleus muscle lysates (100 μg protein) were resolved using SDS-PAGE on 7.5% gels and incubated with specific antibodies to insulin receptor substrate (IRS)-1 (Upstate Biotechnology, Lake Placid, NY), IRS-2 (gift of Dr. M. White; Joslin Diabetes Center, Boston, MA), protein kinase B (Akt), phospho-Akt (Ser473), extracellular signal-regulated kinase (ERK)1/2, phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling, Beverly, MA), and appropriate secondary antibodies (goat anti-rabbit and -mouse horseradish peroxidase conjugates; Cell Signaling), with PA and C and appropriate secondary antibodies (goat anti-rabbit and -mouse horseradish peroxidase conjugates; Cell Signaling), with PA and C samples run together on the same gels. Bands were visualized and analyzed as described previously (10). Phosphorylation was expressed as the ratio of phosphorylated to total protein.

**Statistical analysis.** Repeated-measures two-way ANOVA with time and treatment as factors was applied to body weight, IPITT, and IPGTT with a Bonferroni’s post hoc test to determine which groups differed significantly. For all other analyses, comparisons of interest, 1) C RD vs PA RD, 2) C RD vs C HFD, 3) C HFD vs PA HFD, and 4) PA RD vs PA HFD, were made separately using the appropriate parametric (Student’s t-test) or nonparametric (Mann-Whitney) tests according to the sample size (n ≤ 6, nonparametric test used) and/or the normality of the data. A Bonferroni correction for multiple testing was used to adjust the threshold for statistical significance to P < 0.0125 for these four comparisons. The data were transformed when necessary to achieve homogeneity of variance. Data are reported as means ± SE. All analyses were performed using GraphPad Software (GraphPad Software, San Diego, CA).

**RESULTS**

**Baseline features.** PA treatment did not result in changes in body weight at weaning (Fig. 2). Beginning at 42 days, body weight began to increase in PA compared with C rats (Fig. 2). PA rats had significantly increased body weight compared with C rats at 60 days (P < 0.01, Fig. 2). The parametral and subcutaneous fat depots were increased by 30% at 60 days in PA compared with C rats (P < 0.0125, Fig. 2). HFD produced similar changes in C rats in body and fat pad weights to those observed in PA rats with RD and did not further change these parameters in PA rats (Fig. 2). PA rats exhibited ~12-fold higher fasting insulin levels compared with C rats at 60 days (P < 0.005, Fig. 3). However, fasting glucose levels at 60 days did not differ in PA compared with C rats (Fig. 3). HFD produced a significant ~2.5-fold increase in fasting insulin levels in C rats (C RD vs C HFD, P < 0.0125). In PA rats, HFD did not further increase circulating insulin levels (Fig. 3).

There was an increase in both fasting TG and cholesterol levels in PA compared with C rats (P < 0.0125, Fig. 4). HFD increased TG levels in C rats and did not further influence them in PA rats (Fig. 4). Cholesterol levels in C and PA rats on HFD did not differ from those in C RD rats. Hepatic TG content was increased in PA rats (Fig. 4). Cholesterol levels in C and PA rats on HFD did not differ from those in C RD rats. Hepatic TG content was increased in PA rats (Fig. 4).
androgens during late gestation programs adult female rats assessed by IPITT in PA and C rats (Fig. 5). There were no differences in insulin sensitivity as well as glucose or insulin responses between PA and C groups in both PA and C rats than those seen with PA alone (Fig. 4). HFD produced greater increases in hepatic TG content. Increased lipid content was also visualized by oil red-O stain in liver or skeletal muscle with either diet (Fig. 6). There were also no differences in insulin-stimulated activation of Akt compared with C rats on either diet (data not shown). There were also no significant changes in IRs protein abundance in PA compared with C rats or of ERK1/2 (not shown) in PA compared with C rats. There were no significant changes in adult female offspring. Furthermore, PA also resulted in hepatic steatosis, a novel finding that has not been reported previously with either pre- or postnatal androgen treatment in any species (1, 18, 43).

The contribution of body weight and composition to the metabolic phenotype resulting from exposure to testosterone at critical developmental periods has not been investigated in prior studies (1, 3, 18, 29). Our findings suggest that increased adiposity mediates most of the observed metabolic changes since they were reproduced in control animals by HFD, which resulted in similar increases in weight and visceral adiposity. Thus one of the major mechanisms for PA-mediated metabolic changes may be through increased food intake and/or decreased energy expenditure, resulting in greater adiposity (32, 40).

The only prominent metabolic change specific to PA was the increase in circulating insulin levels, despite similar increases in body weight in control rats during HFD. These findings suggest that prenatal testosterone has an impact on glucose homeostasis that is independent of increased adiposity. Because glucose levels did not fall despite increased circulating insulin levels, these findings suggest the presence of insulin resistance (4), despite our failure to detect significant decreases in insulin sensitivity or signaling. Indeed, it is quite plausible that modest decreases in insulin action escaped detection because the IPITT is less sensitive than the glucose clamp technique for assessing this parameter (5). The observation that PA had a more substantial effect on circulating insulin levels than HFD may be due to androgen-mediated decreases in insulin clearance, such as those that have been reported with postnatal testosterone treatment (27). However, we detected no decreases in IR-β abundance in livers of PA females, regardless of the diet, suggesting that there were no alterations in receptor-mediated insulin clearance due to PA treatment. It is
also possible that PA caused increases in pancreatic β-cell mass similar to those observed in other models for metabolic programming (36). These possibilities will be directly assessed in future studies.

Studies of postnatal androgen exposure suggest that androgen acting directly through activation of the androgen receptor (3, 29) or by aromatization to estrogen (3) can result in metabolic abnormalities in rodents. The timing of androgen
exposure may also be critical. Testosterone treatment earlier during gestation results in defects in insulin secretion, whereas treatment later in gestation results in decreased insulin sensitivity in adult female rhesus monkeys (2). In female rats, both transient neonatal (3) and continuous pre- to postpubertal (29) dihydrotestosterone treatments result in insulin resistance, whereas only the pre- to postpubertal treatment results in increased total body and fat weight. Moreover, other prenatal insults, such as maternal caloric restriction, nutrient excess, or stress, result in metabolic abnormalities in adult offspring, such as increased circulating lipid levels, hepatic steatosis, and insulin resistance (15, 16). These observations suggest that prenatal androgen treatment could cause other changes in the maternal-fetal environment that contribute to the phenotype.

In conclusion, hyperandrogenemia is the cardinal reproductive feature of PCOS (11, 28), and its development may be one of the earliest harbingers of the disorder (24). We have recently mapped a susceptibility variant for hyperandrogenemia in PCOS to an intron of the fibrillin-3 gene (42), consistent with the hypothesis that hyperandrogenemia is a fundamental defect in this disorder. Increased endogenous androgen levels are also associated with metabolic syndrome in epidemiological studies in both pre- and postmenopausal women (26, 39). Our study suggests that androgen excess at critical prenatal developmental periods could play a role in the pathogenesis of PCOS, obesity, and metabolic syndrome.

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