Prenatal androgen excess programs metabolic derangements in pubertal female rats

Xiaonan Yan, Xiaonan Dai, Jing Wang, Nannan Zhao, Yugui Cui and Jiayin Liu

State Key Laboratory of Reproductive Medicine, Clinical Center of Reproductive Medicine, First Affiliated Hospital, Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, China

Correspondence should be addressed to J Liu

Email
jyliu_nj@126.com

Abstract

Owing to the heterogeneity in the clinical symptoms of polycystic ovary syndrome (PCOS), the early pathophysiological mechanisms of PCOS remain unclear. Clinical, experimental, and genetic evidence supports an interaction between genetic susceptibility and the influence of maternal environment in the pathogenesis of PCOS. To determine whether prenatal androgen exposure induced PCOS-related metabolic derangements during pubertal development, we administrated 5 α -dihydrotestosterone (DHT) in pregnant rats and observed their female offspring from postnatal 4 to 8 weeks. The prenatally androgenized (PNA) rats exhibited more numerous total follicles, cystic follicles, and atretic follicles than the controls. Fasting glucose, insulin, leptin levels, and homeostatic model assessment for insulin resistance were elevated in the PNA rats at the age of 5–8 weeks. Following intraperitoneal glucose tolerance tests, glucose and insulin levels did not differ between two groups; however, the PNA rats showed significantly higher 30- and 60-min glucose levels than the controls after insulin stimulation during 5–8 weeks. In addition, prenatal DHT treatment significantly decreased insulin-stimulated phosphorylation of AKT in the skeletal muscles of 6-week-old PNA rats. The abundance of IR substrate 1 (IRS1) and IRS2 was decreased in the skeletal muscles and liver after stimulation with insulin in the PNA group, whereas phosphorylation of insulin-signaling proteins was unaltered in the adipose tissue. These findings validate the contribution of prenatal androgen excess to metabolic derangements in pubertal female rats, and the impaired insulin signaling through IRS and AKT may result in the peripheral insulin resistance during pubertal development.

Key Words

- Fetal origin of adult disease
- polycystic ovary syndrome
- prenatal androgen exposure
- insulin resistance
- insulin signaling

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Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous reproductive and metabolic disorder characterized by anovulation, hyperandrogenism, polycystic ovaries, LH hypersecretion, insulin resistance, and reduced fecundity. A heterogeneous clinical presentation prominently influenced by environmental factors indicates a variable etiology of PCOS. It has been proposed that the clinical manifestations of PCOS often emerge before or during

puberty (Rosenfield 2007, Sir-Petermann *et al.* 2009) and the first-degree female relatives of women with PCOS are at a high risk of developing PCOS (Kahsar-Miller *et al.* 2001, Sir-Petermann *et al.* 2007), suggesting an interaction between genetic susceptibility and the influence of the maternal environment (Abbott *et al.* 2002, Franks *et al.* 2006).

The emerging field of epigenetics has revealed that abnormal endocrine and metabolism in mothers perturb

the *in utero* environment and alter the developmental trajectory of multiple organ systems in the fetus, thus greatly enhancing the likelihood of diseases in the offspring. Clinical, experimental, and genetic evidence supports the hypothesis of a fetal origin of PCOS. Female fetuses exposed to high levels of androgens in the intrauterine environment, including women with virilizing congenital adrenal hyperplasia and congenital adrenal virilizing tumors, have an increased risk of PCOS in adolescence (Xita & Tsatsoulis 2006). In addition, prenatally androgenized (PNA) female nonhuman primates, sheep, rats, and mice manifest most of the reproductive and metabolic derangements observed in women with PCOS (Forsdike *et al.* 2007, Abbott *et al.* 2008a,b, 2009, Padmanabhan *et al.* 2010, Roland *et al.* 2010, Tyndall *et al.* 2012). PNA female nonhuman primates show reproductive and metabolic disturbances such as polycystic ovaries, hyperandrogenism, oligomenorrhea, oligo- or anovulation, and LH hypersecretion, which are consistent with the human PCOS phenotype (Abbott *et al.* 2008a,b). Furthermore, intrauterine androgen exposure in these monkeys leads to the development of insulin resistance associated with visceral adiposity, impaired glucose metabolism, and dyslipidemia (Abbott *et al.* 2009). These observations substantiate the hypothesis that an early perturbation due to *in utero* androgen excess resets the reproductive and metabolic trajectory of the growing fetus and programs target tissue differentiation and development, supporting a potential role of epigenetics and fetal programming in the pathogenesis of PCOS (Franks 2012).

Peripubertal metabolic dysfunction is one of the first phenotypic traits observed in adolescent girls with PCOS (Coviello *et al.* 2006). Reportedly, metabolic derangements, including insulin resistance, hyperinsulinemia, and glucose intolerance, are present in adolescents with PCOS before the onset of puberty and persist during pubertal development (Sir-Petermann *et al.* 2009, Huang *et al.* 2010). Insulin resistance and hyperinsulinemia play a pivotal role in the pathogenesis of PCOS in adolescents. Insulin resistance is thought to be the metabolic abnormality most closely linked to an increased risk of obesity, type 2 diabetes mellitus (T2DM), metabolic syndrome, and cardiovascular disease (Teede *et al.* 2006). Owing to the significant overlap between these symptoms and the normal physiological changes associated with puberty, PCOS is possibly being under-evaluated in the adolescent population, raising serious concerns over the potential, long-term public health consequences (Broder-Fingert *et al.* 2009). Therefore, an improved understanding of

the pathogenesis of the metabolic derangements in adolescents with PCOS may be a key to minimizing the development of symptoms and preventing the onset of the long-term complications associated with this syndrome. However, the molecular mechanisms involved in insulin resistance in adolescents with PCOS are yet to be elucidated.

The pathogenesis of insulin resistance in PCOS involves the interaction of environmental factors with impaired peripheral insulin signaling (Diamanti-Kandarakis & Dunaif 2012). Women with PCOS do not typically have structural abnormalities of insulin receptors (IRs), fewer IRs, or altered insulin binding affinity. Therefore, a post-receptor mechanism in adolescents with PCOS is most likely responsible for insulin resistance (Bremer 2010). Defects in the IR substrate (IRS)–AKT (also called protein kinase B (PKB)) pathway, which plays a key role in the regulation of glycogen synthesis, gluconeogenesis, and glucose transport by insulin, have been found in T2DM and PCOS, suggesting that a defective IRS–AKT pathway underlies the clinical development of insulin resistance (Corbould 2008). Therefore, investigations have been focused on the IRS–AKT pathway in the skeletal muscles, liver, and adipose tissue in PCOS; however, the results seem to vary with different populations and animal models (Dunaif *et al.* 2001, Corbould *et al.* 2005, 2006, Hojlund *et al.* 2008, Rajkhowa *et al.* 2009). *In vivo* studies indicate an impaired insulin-mediated association of phosphatidylinositol-3 kinase (PI3K) with IRS1 in skeletal muscle (Dunaif *et al.* 2001), while studies of cultured fibroblasts and myotubes from PCOS patients suggest enhanced serine phosphorylation of IRs and IRS1 (Corbould *et al.* 2005, 2006). In a trial of patients with PCOS, insulin signaling for glucose transport through AKT and AKT substrate of 160 kDa was impaired in skeletal muscle (Hojlund *et al.* 2008). However, defects in IRS–AKT-mediated insulin signaling have not been found in the adipose cell lineage from women with PCOS (Corbould & Dunaif 2007). To date, limited investigations have focused on the potential link between insulin resistance and peripheral insulin signaling in adolescents with PCOS.

In this study, we investigated the role of prenatal androgen excess in the development of metabolic and reproductive derangements in female offspring rats at the peripubertal stage. The ovarian morphology, serum steroid concentrations, and metabolic parameters in the PNA rats were observed from postnatal 4 to 8 weeks. We also determined whether the PNA rats manifested impaired glucose tolerance (IGT) and insulin resistance

during the pubertal development stage and whether the resulting metabolic derangements were mediated by peripheral insulin signaling.

Materials and methods

Animals and study protocols

Sprague Dawley rats were purchased from the Lab Animal Center of Nanjing Medical University and housed at 25 °C under a 12 h light:12 h darkness cycle with a humidity of 65–70%. Water and food were available *ad libitum*. Animal care was conducted in accordance with the Animal Research Committee Guidelines of Nanjing Medical University, and experimental protocols were performed with the permission of the Institutional Ethics Committee of Nanjing Medical University.

Male and female rats were paired, and females were examined daily for copulatory plugs (day 1 of gestation). On days 16–19 of gestation, the rats were treated with daily s.c. injections of either 5 mg 5 α -dihydrotestosterone (DHT; 10255010, Dr Ehrenstorfer GmbH, Germany) dissolved in 500 μ l sesame oil (S3547, Sigma)/benzyl benzoate (B6630, Sigma) (PNA, $n=24$) or a vehicle as a control (C, $n=20$). Pups were culled from litters to equalize group sizes. All litters were weaned, and females were separated from males at 21 days of age.

Female offspring were observed from 4 weeks (prepuberty) to 8 weeks (late puberty) (Fig. 1). During this period, body weight, ovarian weight, serum steroid hormones (testosterone and estradiol (E₂)), and fasting plasma biochemical profiles were determined weekly. Blood samples were collected by orbital puncture after

the rats were anesthetized with isoflurane, and sera were stored at –80 °C until use. In addition, the rats were fasted overnight, anesthetized, weighed, and decapitated at the age of 4–8 weeks. Their ovaries were isolated, weighed, fixed in 4% paraformaldehyde for 24 h, placed in 70% ethanol, dehydrated, and embedded in paraffin.

To investigate whether the PNA rats showed IGT and insulin resistance during different development stages, intraperitoneal glucose tolerance tests (IPGTTs) and intraperitoneal insulin tolerance tests (IPITTs) were performed weekly in the PNA rats ($n=45$) and control ($n=36$) from postnatal 5 to 8 weeks and 4 to 8 weeks respectively.

In addition, in order to explore insulin signaling, half of the animals in the experimental ($n=16$) and control groups ($n=16$) at the age of 6 weeks were i.p. injected with 2 U/kg regular human insulin (Humulin, Lilly Egypt) in 200 μ l 0.9% sodium chloride and half were injected with 200 μ l 0.9% sodium chloride, 10 min before death. The liver, soleus muscles, and adipose tissue depots (parametrial fat) were dissected, frozen in liquid nitrogen, and stored at –80 °C for further analyses.

Ovarian morphology

The ovaries were longitudinally and serially sectioned at 4 μ m intervals. Every tenth section was mounted on a glass slide, stained with hematoxylin and eosin, and scanned with a Zeiss Axioskop 2 microscope (Zeiss Fluorescent Microsystems, Göttingen, Germany). The slides were analyzed by two persons blinded to the origin of the sections. The quantitative analysis of ovarian follicles was performed as previously reported (Shi *et al.* 2009).

Steroid hormone analysis

The total serum testosterone and E₂ concentrations were determined using commercial double-antibody RIA kits (Beckman Coulter, Inc., Brea, CA, USA), according to the manufacturer's recommendations. The intra- and interassay coefficients of variation (CV) were 8.6 and 11.9% respectively for testosterone and 6.5 and 9.7% respectively for E₂.

Measurements of metabolic factors

Baseline blood samples were used to determine the fasting glucose, insulin, leptin, and plasma lipid levels. Blood glucose was measured using a glucometer (Accu-Chek Performa, Roche Diagnostics). The circulating total cholesterol, triglyceride, and free fatty acid (FFA) levels

Figure 1

Study schema with the number of animals used in each experiment. C_n, number of control; D_n, number of prenatally 5 α -dihydrotestosterone-treated rats; DHT, 5 α -dihydrotestosterone; IPGTTs, intraperitoneal glucose tolerance tests; IPITTs, intraperitoneal insulin tolerance tests. *IPGTTs were evaluated from postnatal 5 to 8 weeks.

were assessed by an enzymatic colorimetric method, according to the manufacturer's protocol (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Plasma insulin and leptin levels were measured using commercially available RIA kits (Linco Research, St Charles, MO, USA), according to the manufacturer's recommendations. The intra- and interassay CV were 5.8 and 10.8% respectively for insulin and 2.4 and 4.8% respectively for leptin. Homeostatic model assessment for insulin resistance (HOMA-IR) and β -cell function (HOMA- β) were obtained to evaluate insulin sensitivity and β -cell function respectively.

Intraperitoneal glucose tolerance test

From the ages of 5 to 8 weeks, IPGTTs were performed weekly after an overnight fasting (14 h). In brief, after a baseline (fasted) measurement, rats were i.p. injected with 2 g/kg body weight D-(+)-glucose (Sigma). Blood samples were obtained 15, 30, 60, and 120 min after the glucose load, by orbital puncture after the rats were anesthetized with isoflurane. Glucose levels were measured with the glucometer. In addition, blood samples were collected for insulin measurements.

Intraperitoneal insulin tolerance test

From postnatal 4 to 8 weeks, IPITTs were performed weekly after 6 h of fasting. After baseline blood samples were obtained, the rats were i.p. injected with 1 U/kg body weight of regular human insulin. Blood samples were collected at 15, 30, 60, and 120 min from a tail cut, and glucose levels were measured with the glucometer.

Western blot analysis

The liver, soleus muscle, and adipose tissue were homogenized in a lysis buffer at a proportion of 1 ml buffer/100 mg of tissues. Aliquots (30 μ g) of the protein extracts from each sample were loaded on 8% gels and separated using SDS-PAGE. The resulting proteins were then incubated with the primary antibodies to AKT (Cell Signaling, Beverly, MA, USA), phospho-AKT (Ser473; Cell Signaling), IRS1 (Merck Millipore), IRS2 (Cell Signaling), phospho-IRS1 (Tyr895; Cell Signaling), glycogen synthase kinase 3 β (GSK3 β) (Cell Signaling), phospho-GSK3 α/β (Ser21/9; Cell Signaling), or β -tubulin (Abcam, Inc., Cambridge, MA, USA) and appropriate secondary antibodies (goat anti-rabbit HRP conjugates; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The membrane was examined by an enhanced

chemiluminescence detection system and the signal intensity of each band was analyzed using AlphaEaseFC Imaging Software (Alpha Innotech, San Leandro, CA, USA). Phosphorylation was expressed as the ratio of phosphorylated to total protein.

Statistical analyses

Statistical evaluations were performed using SPSS Software (version 16.0; SPSS, Inc., Chicago, IL, USA). The effects of DHT on body weight, ovarian weight, ovarian morphology, sex steroid levels, and fasting biochemical profiles at the same development stage were analyzed by two-tailed Student's *t*-tests. For IPGTTs and IPITTs, glucose and insulin values from the PNA rats and control were compared at each time point using repeated-measures ANOVA. The area under the curve (AUC) for the IPITTs at each age was analyzed by two-way ANOVA. All results are expressed as the mean $\pm$ S.E.M. $P < 0.05$ was considered significant.

Results

Baseline parameters

The ratio of female-to-male offspring, number of pups per litter, and birth weight did not significantly differ between the DHT-treated animals and the control (data not showed). Prenatal androgenization did not result in changes in body weight at weaning and at the age of 4 weeks. However, from 5 to 8 weeks, the body weight markedly increased in the PNA rats, compared with the controls ($P < 0.01$), and was notably higher in the PNA rats than in the controls at 8 weeks ($P < 0.001$) (Fig. 2A). No differences were observed in the ovarian weight and ovary/body weight ratio between the two groups during any development stage (Fig. 2B and C).

Ovarian morphology

According to their morphology, follicles were reported as normal, atretic, or cystic. Degenerated oocyte nuclei, wrinkling of the nuclear membrane, oocyte vacuoles, or degenerative changes in the granulosa layers were regarded as signs of follicular atresia. Cystic follicles were defined as follicles that were dilated, contained cavities filled with follicular fluid, and lined with one- to five-cell-thick layers of round-to-flattened granulosa cells (Shi *et al.* 2009). Ovaries from the PNA rats had an increased total number of follicles per section, compared with the number in the control animals ($P < 0.01$). The number of atretic

Figure 2

Effects of prenatal 5 α -dihydrotestosterone (DHT) treatment on body weight, ovary weight, ovarian morphology, and follicle number. (A) Growth curves of controls (open circles) and prenatally androgenized (PNA) female rats (closed circles) from birth to 8 weeks of age. (B and C) Ovary weight and ratio of ovary weight and body weight in control (open bars) and PNA rats (closed bars) from postnatal 4 to 8 weeks. (D) Number of total follicles, atretic follicles, and cystic follicles in control (open bars) and PNA rats (closed bars) at the age of 6 weeks. (E) An ovarian section from a 6-week-old control rat. Follicles at different stages are present in the cortex. (F) Higher magnification of (E). (G) An ovarian section from a 6-week-old PNA rat. Several cystic follicles lined by either a single-cell layer or a thin multicellular layer of granulosa cells are shown (*). (H) Higher magnification of (G), showing one cystic follicle with a large fluid-filled antrum and degenerated granulosa cells. Scale bar, 200 μ m. Values are expressed in mean $\pm$ S.E.M. * P < 0.05 and ** P < 0.01 vs control.

and cystic follicles was significantly greater in the PNA rats than in the control rats (P < 0.01; Fig. 2D). However, the number of normal follicles, including primary, secondary, and antral follicles, was not different between the two groups (data not showed). Cystic follicles appeared as large, fluid-filled cysts that were characterized by attenuated granulosa cell layers (Fig. 2G and H).

Steroid hormone levels

The plasma levels of total testosterone and E₂ did not significantly differ between the PNA and control rats

during any development stage (P > 0.05), whereas a trend toward elevated sex steroid hormone levels was observed in the PNA rats (Fig. 3).

Metabolic profile

The metabolic characteristics of the PNA and control rats at different development stages are shown in Table 1. At postnatal 4 weeks, the fasting glucose and insulin levels were not significantly different between the two groups (P > 0.05). However, from the ages of 5–8 weeks, the fasting glucose level was significantly higher in the experimental group (P < 0.01 or P < 0.05). Moreover, the PNA rats had remarkably increased fasting insulin concentrations at postnatal 6, 7, and 8 weeks (P < 0.01 or P < 0.05). In particular, the fasting insulin levels of 8-week-old PNA rats were threefold higher than the levels in the control rats (P < 0.01). HOMA-IR was elevated in the PNA group from postnatal 5 to 8 weeks

Figure 3

Total serum testosterone (A) and 17 β -estradiol (B) concentrations were detected weekly in control (open bars) and prenatally androgenized (PNA) rats (closed bars) from postnatal 4 to 8 weeks. Values are expressed in mean $\pm$ S.E.M.

Table 1 Metabolic parameters in control and prenatally DHT-treated rats at peripubertal stages. Values are expressed as mean $\pm$ S.E.M.

	Postnatal 4 weeks		Postnatal 5 weeks		Postnatal 6 weeks		Postnatal 7 weeks		Postnatal 8 weeks	
	C (n=10)	DHT (n=12)	C (n=10)	DHT (n=12)	C (n=10)	DHT (n=12)	C (n=10)	DHT (n=12)	C (n=10)	DHT (n=12)
Glucose (mg/dl)	78.48 $\pm$ 4.66	80.25 $\pm$ 6.16	81.00 $\pm$ 6.52	89.85 $\pm$ 6.71 [†]	94.26 $\pm$ 3.62	102.32 $\pm$ 8.31 [†]	93.27 $\pm$ 3.93	99.97 $\pm$ 7.19 [*]	93.44 $\pm$ 6.05	100.70 $\pm$ 7.18 [*]
Insulin (mU/l)	0.75 $\pm$ 0.28	0.49 $\pm$ 0.33	0.75 $\pm$ 0.37	0.96 $\pm$ 1.16	1.03 $\pm$ 0.29	2.45 $\pm$ 1.87 [*]	1.77 $\pm$ 1.30	3.86 $\pm$ 2.48 [*]	2.76 $\pm$ 2.28	8.94 $\pm$ 5.32 [†]
Leptin (ng/ml)	1.08 $\pm$ 0.09	1.10 $\pm$ 0.15	1.04 $\pm$ 0.13	1.16 $\pm$ 0.19	1.13 $\pm$ 0.17	1.33 $\pm$ 0.17 [*]	1.26 $\pm$ 0.23	1.80 $\pm$ 0.31 [†]	1.56 $\pm$ 0.21	2.40 $\pm$ 0.46 [†]
Cholesterol (mg/dl)	94.05 $\pm$ 11.28	97.67 $\pm$ 15.47	98.68 $\pm$ 12.27	103.27 $\pm$ 12.55	93.81 $\pm$ 20.57	100.02 $\pm$ 13.69	97.46 $\pm$ 18.4	98.56 $\pm$ 19.18	107.57 $\pm$ 16.82	103.57 $\pm$ 21.40
Triglyceride (mg/dl)	170.09 $\pm$ 53.09	216.07 $\pm$ 59.55	169.86 $\pm$ 27.41	144.55 $\pm$ 33.11	106.4 $\pm$ 31.16	94.68 $\pm$ 24.32	66.49 $\pm$ 13.17	63.67 $\pm$ 13.89	61.20 $\pm$ 17.48	71.17 $\pm$ 26.54
Free fatty acids (mEq/l)	1.54 $\pm$ 0.34	1.73 $\pm$ 0.4	1.54 $\pm$ 0.25	1.36 $\pm$ 0.30	1.31 $\pm$ 0.30	1.38 $\pm$ 0.22	0.99 $\pm$ 0.34	1.13 $\pm$ 0.24	0.98 $\pm$ 0.15	1.17 $\pm$ 0.25 [*]
HOMA-IR	0.14 $\pm$ 0.05	0.10 $\pm$ 0.06	0.15 $\pm$ 0.09	0.34 $\pm$ 0.24 [*]	0.24 $\pm$ 0.07	0.67 $\pm$ 0.47 [†]	0.41 $\pm$ 0.30	0.99 $\pm$ 0.64 [*]	0.65 $\pm$ 0.56	2.06 $\pm$ 1.16 [†]
HOMA- β (%)	19.61 $\pm$ 11.66	12.62 $\pm$ 9.65	16.06 $\pm$ 7.75	24.39 $\pm$ 22.77	12.07 $\pm$ 3.81	25.35 $\pm$ 20.13	21.76 $\pm$ 16.93	40.01 $\pm$ 29.42	31.25 $\pm$ 23.31	88.85 $\pm$ 59.82 [*]

C, control; DHT, 5 α -dihydrotestosterone. [†]t-Tests were performed between age-matched animals; ^{*}P<0.05 and [†]P<0.01.

($P<0.01$ or $P<0.05$), whereas HOMA- β was significantly higher in the PNA rats at the age of 8 weeks ($P<0.05$).

No difference was observed in the serum leptin levels between the two groups at postnatal 4 and 5 weeks. At 6, 7, and 8 weeks, however, the serum leptin concentrations were significantly higher in the PNA rats than in the control rats ($P<0.05$). The serum total cholesterol and triglyceride levels were unaltered in both the experimental and the control groups during pubertal development. At 8 weeks of age, the PNA rats had higher serum FFAs concentrations than those in the control rats. These results suggest that young adult rats with PCOS began to exhibit dyslipidemia, as characterized by increased plasma concentrations of FFAs.

IPGTT and IPITT

To determine whether the PNA rats showed IGT and insulin resistance during puberty, IPGTTs and IPITTs were performed weekly from postnatal 5 to 8 weeks and 4 to 8 weeks respectively. After glucose load, serum glucose and insulin levels did not significantly differ between the two groups at the age of 5–8 weeks (Fig. 4). Although no difference was detected in the glucose levels at 15, 30, and 120 min after insulin administration between the two groups, the 60-min glucose levels were significantly higher in the PNA group than in the control group at postnatal 5 and 6 weeks ($P<0.01$ or $P<0.05$; Fig. 5B and C). Moreover, prenatal DHT treatment induced significantly elevated glucose levels at 30 and 60 min after IPITT ($P<0.05$; Fig. 5D and E) and significantly increased 0- to 120-min AUC values ($P<0.05$; Fig. 5F) in 7- and 8-week-old PNA rats.

Insulin signaling pathway

To determine whether the insulin resistance induced by prenatal DHT treatment correlated with defects in the insulin-signaling pathway during pubertal development, we investigated the phosphorylation of different components of the IRS-AKT pathway in the liver, soleus muscles, and parametrial fat of 6-week-old PNA rats injected with insulin. The abundance of IRS1 and IRS2 was significantly decreased in the skeletal muscles and liver of the PNA rats after stimulation with insulin ($P<0.01$; Fig. 6C, D, E and F). Prenatal DHT treatment remarkably decreased insulin-stimulated phosphorylation of AKT in the skeletal muscles ($P<0.01$; Fig. 6G), whereas phosphorylation of AKT was unaltered in the liver of PNA rats (Fig. 6H). The phosphorylation of IRS1 and GSK in the skeletal muscles and liver after insulin administration did not differ between the two groups (Fig. 6A and B). In the

Figure 4

Intraperitoneal glucose tolerance tests (IPGTTs) were performed weekly from postnatal 5 to 8 weeks. (A, B, C and D) Plasma glucose levels during IPGTTs in control (open circles) and prenatally androgenized (PNA) rats (closed circles) at the age of 5–8 weeks. (E, F, G and H) Plasma insulin levels during IPGTTs in control (open circles) and PNA rats (closed circles) at the age of 5–8 weeks. Values are expressed in mean $\pm$ s.e.m. * $P < 0.05$ vs control.

adipose tissue of PNA rats, no decreases were detected in the phosphorylation of insulin signaling proteins (data not shown).

Discussion

In this study, we evaluated ovarian morphology, serum steroid concentrations, and metabolic parameters during the peripubertal period in the PNA female rats. PNA rats exhibited an increased number of atretic follicles and cystic follicles compared with control animals. The cystic follicles were lined with attenuated granulosa cell layers and the granulosa cells were flat and epithelioid. These changes are consistent with the typical ovarian

morphological changes observed in human PCOS patients and DHT-treated rodent models of PCOS (Manneras *et al.* 2007, Leonie *et al.* 2012). However, ovarian weight and ovary/body weight ratio did not differ between the two groups during any development stage. This observation is different from the clinical phenotype of PCOS and may be attributable to the heterogeneity of PCOS or to inter-species differences.

Our study showed that the plasma total testosterone and E_2 concentrations did not significantly differ between the controls and the PNA rats during any development stage. To date, whether prenatal DHT treatment *in utero* results in increased levels of testosterone in female offspring remains controversial. The testosterone levels of androgen-treated models of PCOS appear to vary between different studies. Some previous studies have reported that plasma testosterone levels did not differ between rodent models of PCOS and controls (Foeking *et al.* 2005, Manneras *et al.* 2007), whereas other studies

Figure 5

Prenatal 5α -dihydrotestosterone (DHT) treatment induced insulin resistance during puberty in prenatally androgenized (PNA) rats. Intraperitoneal insulin tolerance tests (IPITTs) were performed weekly from postnatal 4 to 8 weeks. (A, B, C, D and E) Plasma glucose levels during IPITTs in control (open circles) and PNA rats (closed circles) at the age of 4–8 weeks. (F) Corresponding 0- to 120-min area under the curve (AUC) for control (open bars) and PNA (closed bars) rats from postnatal 4 to 8 weeks. Values are expressed in mean $\pm$ s.e.m. * $P < 0.05$ and ** $P < 0.01$ vs control.

Figure 6

Prenatal 5 α -dihydrotestosterone (DHT) treatment impaired insulin signaling in the skeletal muscles and liver of 6-week-old prenatally androgenized (PNA) rats. Control and PNA rats were fasted for 14 h and then injected with 2 U/kg insulin 10 min before killing. Insulin-stimulated phosphorylation of insulin-signaling proteins in the skeletal muscles (A) and liver (B) of control and prenatally DHT-treated rats was measured by immunoblot at the age of 6 weeks. (C, D, E and F) Quantification of IRS1 (C) and IRS2 (E) protein levels in the skeletal muscles of control (open bars) and PNA rats (closed bars); quantification of IRS1 (D) and IRS2 (F) protein levels in the liver of control (open bars) and PNA rats (closed bars). The protein levels were normalized to tubulin. (G and H) Quantification of phosphorylated AKT levels in the skeletal muscles (G) and liver (H) of control (open bars) and PNA rats (closed bars). Phosphorylation was expressed as the ratio of phosphorylated to total protein. Values are expressed in mean $\pm$ S.E.M. ** $P < 0.01$ vs control.

showed elevated levels of testosterone in PNA animals (Sullivan & Moenter 2004, Abbott *et al.* 2009, 2012). Androgen excess is considered to be the key feature of PCOS; however, only 80–85% of women with PCOS have clinical hyperandrogenism (Azziz *et al.* 2009). Some metabolic abnormalities have been shown in children and adolescents with PCOS before the onset of

hyperandrogenism (Sir-Petermann *et al.* 2007, 2009). In our study, evidence of hyperandrogenism was not apparent in PNA rats during early puberty, possibly because androgen levels were low during pubertal development and difficult to be evaluated in adolescent (Rosenfield 2007). In addition, most commercial RIA and immunoassay kits lack the sensitivity, precision, and accuracy to measure the low total testosterone concentrations in children and adolescents (Matsumoto & Bremner 2004, Rosner *et al.* 2007). Therefore, the use of RIAs for testosterone measurements in PNA rats during the peripubertal period may be a potential limitation of our study.

In this study, compared with the control rats, the PNA rats exhibited elevated fasting glucose, fasting insulin, and leptin levels from postnatal 5 to 8 weeks and increased FFAs concentrations at 8 weeks. In addition, HOMA-IR, a predictor of insulin sensitivity, was significantly higher in the PNA group than in the control group during puberty. Our data indicate that prenatal androgen excess programs metabolic abnormalities such as insulin resistance, hyperinsulinemia, and hyperleptinemia during pubertal development in the PNA rats, followed by dyslipidemia at the end of pubertal development. Our observations confirm the concept that insulin resistance and hyperinsulinemia are early features during the ontogeny of PCOS (Sir-Petermann *et al.* 2009). Animal models have shown that prenatal testosterone exposure may induce insulin resistance in early postnatal life (Padmanabhan *et al.* 2010), which is in accordance with the present results.

In our study, we observed that the PNA rats gained notable amounts of body weight between 4 and 8 weeks postnatally, especially at 8 weeks of age. This finding verifies the hypothesis that exposure to excess androgens *in utero* mediates the postnatal development of body weight. The serum levels of total cholesterol, triglyceride, and FFAs were unaltered in both the experimental and the control groups during pubertal development. However, at the end of pubertal development (8 weeks postnatally), the PNA rats had higher serum concentrations of FFAs than the control rats. These results suggest that young adult rats with PCOS began to exhibit dyslipidemia, as characterized by increased plasma concentrations of FFAs. In a recent study, the rats treated prenatally with testosterone had significantly lower body weights than untreated controls at postnatal 21 days; however, at 60 days of age, body weights were not different between two groups. In addition, the levels of lipids were elevated in the 60-day-old PNA rats compared with controls (Heber *et al.* 2012).

The disagreement between these studies indicates that the effects of DHT and testosterone in the intrauterine environment on body weight and the lipid profile of female offspring are indeed different. It has been proposed that hyperleptinemia, an indicator of leptin resistance, may contribute to the pathogenesis of obesity and is strongly connected with insulin resistance (Sahu 2004). In this study, we also observed that the plasma leptin levels of the PNA rats were higher than those of the controls, suggesting that a possible dysfunction of adipose tissue is involved in the establishment of insulin resistance.

To further investigate whether the pubertal PNA rats showed altered glucose tolerance and insulin resistance, we conducted weekly IPGTTs and IPITTs from postnatal 4 to 8 weeks. After glucose load, the glucose and insulin levels did not differ between the two groups. Following IPITTs, however, the PNA rats exhibited significantly higher 30- and 60-min glucose levels than the control rats at 5–8 weeks. Our results indicated that the PNA rats displayed impaired insulin sensitivity during pubertal development and a trend toward more severe insulin resistance during and after puberty. In addition, these results showed that the decreased insulin sensitivity induced a compensatory rise in insulin secretion in pubertal PNA rats and led to hyperinsulinemia. This observation is in agreement with data reported in adolescents with PCOS, who exhibited twofold higher fasting insulin levels and a 70% higher first-phase and a 44% higher second-phase insulin secretion during hyperglycemic clamp (Lewy *et al.* 2001). In another study on adolescents with PCOS, the daughters of women with PCOS showed higher insulin levels from the beginning of puberty than those found in control girls; moreover, a high prevalence of glucose intolerance during late puberty was observed in the girls whose mothers had PCOS (Sir-Petermann *et al.* 2009). However, in our study, IGT was not found in the PNA rats during pubertal development. Variable prevalence of IGT has been reported in studies of adolescents with PCOS. In a study of 22 obese adolescents with PCOS, only one participant (4.5%) had IGT (Bridger *et al.* 2006), whereas other studies of obese adolescents with PCOS have reported IGT rates as high as 33–52% (Arslanian *et al.* 2001, Palmert *et al.* 2002). By contrast, nonobese adolescents with PCOS have not been reported to have an increased risk of IGT (Silfen *et al.* 2003), suggesting the underlying relationship between IGT and obesity.

In our study, HOMA- β , a useful index of β -cell function, was not different between the two groups from postnatal 4 to 7 weeks, suggesting that the PNA rats did

not manifest defects in β -cell function and insulin secretion at pubertal development stage. This result is in disagreement with the hypothesis that *in utero* androgen excess directly impairs the pancreatic islets and programs insulin resistance in offspring with PCOS. Therefore, our findings have raised the possibility that prenatal androgen excess may impact peripheral insulin sensitivity in PCOS through some other mechanisms. To determine whether the insulin resistance induced by prenatal DHT administration correlated with defects in IRS–AKT-mediated insulin signaling during pubertal development, we investigated different components of this pathway in the liver, soleus muscles, and parametrial fat of 6-week-old PNA rats injected with insulin. A pronounced reduction in the insulin-stimulated phosphorylation of AKT was detected in the skeletal muscles of the PNA rats. In addition, the abundance of IRS1 and IRS2 was markedly decreased in both skeletal muscles and liver of the PNA rats after stimulation with insulin compared with control. Our findings indicate that impaired insulin signaling through IRS and AKT explain, in part, the molecular mechanism of insulin resistance in the liver and skeletal muscles of PNA rats. These results are consistent with the data reported in PCOS patients who displayed impaired insulin-mediated phosphorylation of AKT in skeletal muscles (Hojlund *et al.* 2008). Moreover, in accordance with studies on adipose cell lineage derived from women with PCOS (Corbould & Dunaif 2007), the phosphorylation of insulin-signaling proteins was not decreased in the adipose tissue of the PNA rats, indicating a difference in the pathogenesis of insulin resistance in the peripheral target tissues of the PNA rats. However, our observations differ from those reported by some studies of animal models of PCOS. A study of female sheep prenatally treated with testosterone found increased mRNA expressions of the *IRS1* and *GSK3* genes in skeletal muscles and the *IRS2*, *PI3K*, and *AKT* genes in adipose tissue (Nada *et al.* 2010). In a rodent study, no difference was found in the expression levels of the IR, IRS1, IRS2, and AKT proteins in the skeletal muscles or liver, after prenatal testosterone exposure (Demissie *et al.* 2008). These conflicting observations may result from differences between the action of DHT and testosterone, interspecies differences, and the expression of phosphorylated protein distinct from mRNA in insulin signaling pathway. In summary, our findings provide correlative evidence that prenatal DHT excess may contribute to tissue-specific alterations in the expression of key factors of insulin signaling in PNA rats during pubertal development. Further studies are warranted to determine how an early perturbation from *in utero* androgen excess programs the

peripheral target tissue of the fetus during critical prenatal developmental periods and leads to defects in insulin signaling, resulting in insulin resistance in the skeletal muscles and liver during puberty.

In this study, we investigated the role of prenatal DHT exposure in the development of metabolic abnormalities during the peripubertal period in female rats. Our results demonstrated that prenatal androgen excess programmed both the metabolic and the reproductive derangements of PCOS in pubertal female rats, including polycystic ovaries, increased body weight, hyperinsulinemia, and insulin resistance, similar to the development of PCOS in adolescents. These findings substantiate a direct relationship of *in utero* androgen excess with the metabolic features of PCOS in adolescents. In addition, impaired insulin signaling through IRS and AKT resulted in insulin resistance in the liver and skeletal muscles of PNA rats, suggesting that prenatal DHT excess may contribute to defective insulin signaling in the peripheral tissues of female offspring during pubertal development.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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