Prenatal androgen exposure programs metabolic dysfunction in female mice

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Abstract

Polycystic ovary syndrome (PCOS) is a common fertility disorder with metabolic sequelae. Our laboratory previously characterized reproductive phenotypes in a prenatally androgenized (PNA) mouse model for PCOS. PNA mice exhibited elevated testosterone and LH levels, irregular estrous cycles, and neuroendocrine abnormalities suggesting increased central drive to the reproductive system. In this study, we examined metabolic characteristics of female PNA mice. PNA mice exhibited increased fasting glucose and impaired glucose tolerance (IGT) that were independent of age and were not associated with changes in body composition or peripheral insulin sensitivity. IGT was associated with defects in pancreatic islet function leading to an impaired response to high glucose, consistent with impaired insulin secretion. Exposure of isolated pancreatic islets to androgen *in vitro* demonstrated an impaired response to glucose stimulation similar to that in PNA mice, suggesting androgens may have activational in addition to organizational effects on pancreatic islet function. PNA mice also exhibited increased size of visceral adipocytes, suggesting androgen-programed differences in adipocyte differentiation and/or function. These studies demonstrate that in addition to causing reproductive axis abnormalities, *in utero* androgen exposure can induce long-term metabolic alterations in female mice.

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Introduction

Developmental programing by steroid hormones is important to establish sex differences in the reproductive tract and in other physiological systems. Androgen levels surge during gestation and postnatally in the male (Tapanainen *et al.* 1981, Quigley 2002), while in females androgens typically remain low during embryonic development, except in rare instances of pathologic exposure from intrinsic (fetal) or extrinsic (maternal or environmental) sources. Recent work on endocrine disruptors has demonstrated the existence of environmental substances with androgenic actions, such as 17-β-trenbolone and triclocarban, which are a potential cause of abnormal fetal androgenization (Gray *et al.* 2006, Hotchkiss *et al.* 2007, Chen *et al.* 2008). As hormonal perturbations during this critical time may have adverse effects that persist into adulthood, it is important to study the consequences of androgen exposure *in utero*.

Polycystic ovary syndrome (PCOS), the most common cause of infertility in women, is one disorder that may originate in prenatal androgen excess. PCOS is characterized by hyperandrogenemia, elevated central drive to the reproductive system, and irregular or absent menstrual cycles due to oligo- or anovulation (Dunaif 1997). PCOS also predisposes women to metabolic dysfunction characterized by impaired glucose homeostasis and abdominal adiposity (Sam & Dunaif 2003). Animal models have exhibited reproductive and metabolic abnormalities similar to PCOS following prenatal androgenization (Abbott *et al.* 2005, Dumesic *et al.* 2007, Demissie *et al.* 2008). The finding that prenatal androgen can lead to defects in both reproduction and metabolism suggests it may play a major role in the etiology of at least some cases of PCOS. Consistent with this idea, women with PCOS have elevated circulating androgens during late gestation (Sir-Petermann *et al.* 2002), potentially exposing their offspring, who are at increased risk for PCOS (Sir-Petermann *et al.* 2009). Alternatively, it has been proposed that the fetal ovary itself is the source of androgen due to abnormalities in genes controlling steroidogenesis (Legro *et al.* 1998).

To mimic the gestational androgen excess associated with PCOS and other inappropriate androgen exposures, our laboratory developed a mouse model treated with dihydrotestosterone (DHT) late in gestation. Previously, we described reproductive neuroendocrine abnormalities in this model (Sullivan & Moenter 2004), including elevated androgen and LH levels, irregular estrous cycles, and increased excitatory neurotransmission to GnRH neurons. In this study, we investigate metabolic phenotypes in prenatally androgenized (PNA) mice.

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Materials and Methods

Generation of PNA mice

Adult (2–4 months) female GnRH-green fluorescent protein (descended from CBB6/F1 founder, currently ∼75% C57Bl/6J by speed congenics) transgenic mice were used to generate PNA mice. Mice were housed under a 14 h light:10 h darkness cycle with chow (2916, Harlan, Indianapolis, IN, USA) and water made available *ad libitum*. Females were paired with males and checked for copulatory plugs. The date of plug was considered day 1 of gestation. Pregnant mice were injected daily s.c. with 50 μl sesame oil containing 250 μg of DHT on days 16–18 of gestation. DHT was used to eliminate the possibility of aromatization to estradiol (E_2), thereby permitting the study of primarily androgen receptor-mediated effects. Of note, DHT can be metabolized to 5α-androstane-3β,17β-diol, which can bind estrogen receptor (ER)β (Handa *et al.* 2008); however, the levels of this metabolite attained in the fetal compartment are unknown. Female offspring were subjected to glucose tolerance tests (GTTs) beginning at 1 month of age; all other studies were performed at 3–6 months. Control mice (CON) were offspring of either oil-injected dams or untreated mice; no differences were observed between these groups, and they were combined for analysis. Three rounds of PNA mice were generated for use in this study: 9 CON and 7 PNA mice for repeated glucose tolerance testing; 22 CON and 21 PNA mice for dual energy X-ray absorptiometry (DEXA), adipocyte studies, islet studies, and hormone measurements; and 10 CON and 10 PNA mice for insulin tolerance testing. For *in vitro* examination of steroid effects on pancreatic islets, C57BL/6J female mice 8–12-weeks old were purchased from Jackson Laboratories (Bar Harbor, ME, USA). These mice were ovariectomized (OVX) 3 days before islet isolation under isoflurane anesthesia (Burns Veterinary Supply, Westbury, NY, USA). Long-acting postoperative local analgesia was provided by 0·25% bupivacaine (Abbott Laboratories). All procedures were approved by the University of Virginia Animal Care and Use Committee and conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Glucose tolerance tests

Mice were singly housed on Sani-Chip bedding (Harlan) and fasted overnight for 16 h (1600–0800 h) prior to the test. The tail was anesthetized with the skin refrigerant ethyl chloride (Gebauer, Cleveland, OH, USA) and the tip removed with a sterile scalpel blade. Tail blood (∼1 μl/sample) was collected for glucose measurement with a OneTouch Ultra glucometer (Lifescan, Milpitas, CA, USA). Following a fasting glucose measurement, mice were injected i.p. with a bolus of 1 g/kg glucose in 0·9% NaCl. Blood glucose was assessed at 10, 20, 30, 45, 60, 75, 90, and 120 min post injection. GTTs were performed monthly from 1 to 6 months.

Insulin tolerance tests

Studies were performed 10 h after lights-on in singly housed fed mice. Although mice had free access to food prior to testing, their active (feeding) period normally ends at the time of lights-on. Following an initial glucose measurement, mice were injected i.p. with a bolus of 0·75 U/kg insulin in sterile 0·9% NaCl. Blood glucose was determined at 10, 20, 30, 45, 60, and 75 min post injection as described above.

Fasting insulin measurements

Fasting insulin was measured following an overnight fast (1600–0800 h). Five- to six-month-old mice were restrained, and ∼50 μl blood was collected from the tail vein using a heparinized capillary tube. Plasma insulin was determined by radioimmunoassay (Millipore, Billerica, MA, USA, cat# SRI-13K). All samples were determined in a single assay with a sensitivity of 0·1 ng/ml and intra-assay coefficient of variation (CV) of <10%.

Dual energy X-ray absorptiometry

Mice were anesthetized by i.p. injection of 100–150 μl of a ketamine/xylazine mix in saline (ketamine at 20 mg/ml and xylazine at 2 mg/ml). Anesthetized mice were introduced into the DEXA machine (GE Lunar Piximus II, GE Healthcare, Waukesha, WI, USA) and were subjected to total body imaging. Lean body mass and fat mass were determined using the Lunar Piximus II software and percent fat mass was calculated. Abdominal fat percentage was calculated by selecting a region of interest in the scanned image. Mice were scanned at age 3, 4, and 5 months.

Measurement of adipocyte size

Mice were euthanized with CO_2. The left parametrial fat pad and the adjacent uterus were fixed in 10% formalin for 48 h and embedded in paraffin. Sections (5 μm) were cut and stained with hematoxylin–eosin. Pictures of stained sections were taken at ×10 magnification with a Zeiss Axioplan Universal Microscope (Thornwood, NY, USA); the uterus was used for photographic orientation and the adipocytes sized were in the same frame as the uterus to minimize bias due to regional differences. Cell areas (in μm^2) of 50 adipocytes were determined for each mouse using Scion Image Corporation software (Frederick, MD, USA). Only 40 cells were used for one animal in which tissue damage precluded analysis of 50 cells.

Glucose uptake assays in adipocytes

The right parametrial adipose tissues were dissected, and adipocytes were isolated and subjected to glucose uptake assays as previously described in detail (Liu *et al.* 1999). In brief, isolated adipocytes were pre-incubated without (basal) or with different concentrations of insulin (0–10 nM) for 30 min.

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[U-^{14}C] D-glucose was then added. Thirty minutes after the addition of radiolabeled glucose, the cell suspension was harvested and adipocytes were separated from the medium. Cell-associated radioactivity was determined and glucose uptake was expressed in amol/min per cell. Cell numbers were determined (Rosenbaum *et al*. 1993) by measuring lipid content of aliquots of cell suspension and measuring sizes of the adipocytes in aliquots of the cell suspension as described above. For each condition, the measurements were done in quadruplicate.

Islet isolation

Islets were harvested from 5-month-old PNA and CON mice on diestrus (determined by vaginal lavage). Noncycling animals are likely to have a very different steroid milieu despite similar vaginal cytology, this is a consequence of the considerable reproductive disruption of the model, and diestrous versus long-term diestrous animals is the most practical comparison to make. Mice were killed with CO_2 and cardiac puncture was performed for blood collection. The pancreas was dissected and islets were isolated by collagenase digestion and Histopaque centrifugation using previously detailed methods (Carter *et al*. 2009). Isolated islets were transferred to a Petri dish containing standard RPMI 1640 (Invitrogen) with 10% fetal bovine serum and penicillin/streptomycin. Islets were incubated in this medium overnight to allow recovery from digestion prior to experiments.

Intracellular calcium imaging of islets

The day following isolation, imaging recordings of intracellular calcium ($[Ca^{2+}]_i$) were made from groups of 10–20 islets from a single mouse ∼18–26 h post isolation; no differences were noted based on time of recording. $[Ca^{2+}]_i$ was measured using the ratiometric dye fura-2 AM and previously described methods (Jahanshahi *et al*. 2009). Briefly, islets were incubated with 1 μM fura-2 AM for 30 min in a modified Kreb's ringer buffer (KRB) solution containing 3 mM glucose, washed, then transferred to a small volume chamber (Warner Instruments, Hamden, CT, USA) mounted on the stage of an upright Olympus BX51WI fluorescence microscope (Olympus, Tokyo, Japan). Islets were perifused using a peristaltic pump (Minipuls 2, Gilson, Middleton, WI, USA) and maintained at 35 °C with an in-line heater (model SF-28 with automatic temperature controller model TC-324B, Warner Instruments). A Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Hamamatsu City, Japan) was used to take sequential images during 340 and 380 nm excitation, and the ratio of emitted light at 510 nm was used to determine the $[Ca^{2+}]_i$. Excitation from a xenon burner was accomplished using a light pipe and filter wheel (Sutter Instrument Company, Novato, CA, USA). Paired images were recorded every 5 s for 15 min. After 5 min in 3 mM glucose, 11 mM glucose was applied for 10 min, during which fluorescence levels were recorded continuously.

Insulin release in vitro

A subset of islets isolated from PNA and CON mice were used for studies of *in vitro* insulin release. Following the overnight culture, islets were incubated at 37 °C and 5% CO_2 for 1 h in standard KRB solution. Islets were then washed and treated for 1 h in KRB supplemented with 3 mM glucose, followed by 1 h in KRB containing 11 mM glucose. Supernatants were collected after each treatment. Insulin concentration in the supernatant was measured by an ELISA insulin assay kit (Mercodia Inc., Winston Salem, NC, USA) according to manufacturer instructions. This assay differed from that used for mouse serum because insulin levels in media differ from those *in vivo*. Intra-assay variation was <10% and inter-assay variation was <5%. *In vitro* insulin release was also assessed in islets isolated from 3-month-old female C57BL/6J mice 3 days post ovariectomy. Islets were harvested and cultured as described above. During the overnight culture, 50 islets per mouse were incubated in DHT, DHT+E_2, or ethanol vehicle (0·0001%). All steroids were used at a final concentration of 10 nM. The following day, islets from each steroid treatment group were incubated in standard KRB solution with steroids omitted to preclude acute effects. Insulin release in 3 and 11 mM glucose was quantified by ELISA as described above.

Endocrine measures

Testosterone was measured in serum using an RIA kit according to the manufacturer's instructions (Siemens Medical Solutions, cat# TKTT2, Los Angeles, CA, USA). Sensitivity averaged 10 ng/dl, and the intra- and inter-assay CV values were 4·4 and 8·1% respectively. An adipokine panel was used to assess insulin, leptin, interleukin 6 (IL6), tumor necrosis factor α (TNFα), plasminogen activator inhibitor 1 (PAI-1; also known as Serpine1), and resistin (mouse serum adipokine kit, Millipore, cat# MADPK-71K-07). Sensitivity was 12 pg/ml for insulin, leptin, PAI-1, and resistin, 2 pg/ml for TNFα, and 5 pg/ml for IL6. Intra- and inter-assay CV values were <10% for all analytes. Adiponectin was measured via RIA (Millipore, cat# MADP-60K); assay sensitivity was 1·3 ng/ml and inter- and intra-assay CV values were <9 and <5% respectively.

Estrous cycle monitoring

Estrous cycles were monitored by vaginal lavage. Cycle stage was classified as estrus (primarily cornified cells), diestrus (primarily leukocytes), or proestrus (primarily nucleated cells).

Statistical analysis

GraphPad Prism software (La Jolla, CA, USA) was used for all analyses unless otherwise indicated. For GTT and insulin tolerance test (ITT), glucose values from PNA and CON mice were compared at each time point using a two-tailed Student's

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t-test. Area under the curve (AUC) for the GTT was calculated using Igor Pro software (Wavemetrics, Lake Oswego, OR, USA); AUC across age was compared in PNA versus CON mice using a repeated-measures ANOVA and Fisher's protected least significant differences *post hoc* test. Two-tailed Student's *t*-test or Mann–Whitney test was used to compare body mass and fat pad mass, fat percentages, fat cell sizes, adipokines, HOMA indices, and glucose uptake in adipocytes. For islet calcium imaging studies, calcium measurements from 10–20 islets per mouse were averaged, and AUC was calculated using Igor Pro. Insulin secretion in 3 and 11 mM glucose from control, DHT, and DHT+E_2 groups was compared by two-way ANOVA. For all statistical tests, significance was set at $P<0{\cdot}05$. Parametric or nonparametric comparisons were used as dictated by data distribution.

Results

PNA mice do not have altered body composition but do have enlarged visceral adipocytes

As adiposity contributes to metabolic disease, we assessed body mass and composition in PNA ($n=8$) and CON ($n=9$) mice. Body mass at age 3, 4, and 5 months did not differ between groups (all comparisons at $P>0{\cdot}8$); representative data from age 5 months are shown (Fig. 1A). Body fat percentages as measured by DEXA also showed no difference in total body fat or abdominal fat at these ages ($P>0{\cdot}4$, Fig. 1B). Since DEXA cannot differentiate visceral and subcutaneous fat compartments, we could not exclude the possibility that PNA mice have changes in fat distribution. To better assess visceral adiposity, parametrial fat pads were weighed in a subsequent group of PNA mice and further analyzed for adipocyte size. Fat pad weights were similar ($n=8$ CON, $n=11$ PNA, $P>0{\cdot}5$, Fig. 2A), indicating no increase in visceral adiposity in PNA mice, consistent with the DEXA measurements. However, PNA mice had larger visceral adipocytes ($2592\pm150\ \mu m^2$ CON, $n=10$, $3230\pm211\ \mu m^2$ PNA, $n=12$, $P<0{\cdot}05$, Fig. 2B–D), indicating that despite similar total amounts of parametrial fat in these mice, prenatal androgenization induced changes in adipocyte differentiation and/or function leading to larger fat cells. Adipokine assays showed no differences between PNA and CON mice in the fed or fasted state, except for a strong trend (two-tailed $P=0{\cdot}08$) for reduced adiponectin in fed PNA mice (Table 1). Fasting decreased insulin, leptin, PAI-1, IL6, and resistin in CON mice ($P<0{\cdot}05$). In PNA mice, insulin was reduced and IL6 and resistin showed a tendency ($P<0{\cdot}06$) to be lower in fasted animals. Of note, however, leptin was not reduced by fasting in PNA mice. Glucose uptake assays in isolated adipocytes showed no change in insulin sensitivity or maximal insulin-stimulated glucose uptake, but higher basal glucose uptake in PNA adipocytes (basal: $4{\cdot}0\pm0{\cdot}5$ amol/glc per min per cell CON, $6{\cdot}4\pm1{\cdot}0$ PNA, $n=5$ each, $P<0{\cdot}05$; at 0·1 nM insulin: $17{\cdot}4\pm2{\cdot}4$ CON, $20{\cdot}6\pm3{\cdot}3$ PNA, $n=5$ each, $P>0{\cdot}4$; at 1 nM insulin: $27{\cdot}1\pm3{\cdot}3$ CON, $n=5$, $26{\cdot}9\pm5{\cdot}7$ PNA, $n=4$, $P>0{\cdot}9$; Fig. 2E).

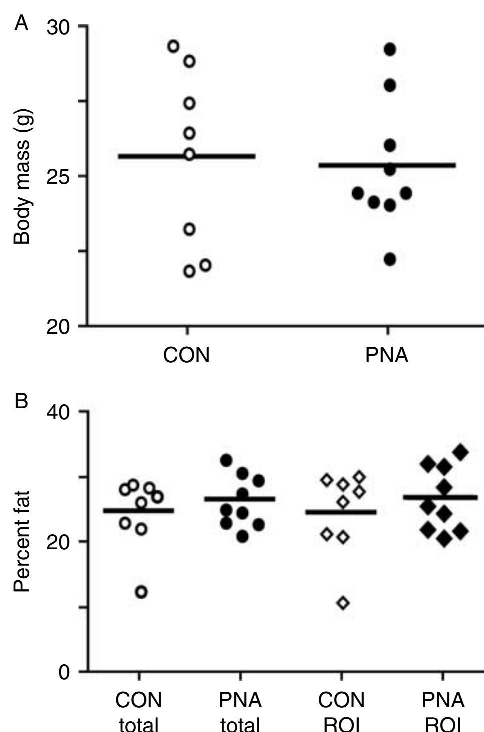

Figure 1 PNA does not alter body mass or composition in PNA mice at 5 months of age. (A) Body mass in CON (open circles, $n=8$) and PNA mice (closed circles, $n=9$) ($P>0{\cdot}8$). (B) Total body fat or abdominal fat (region of interest, ROI; subcutaneous and visceral fat combined) in CON and PNA ($P>0{\cdot}4$).

PNA mice exhibit impaired glucose tolerance

To test whether prenatal androgenization alters glucose disposal in PNA mice, GTTs were performed. PNA mice exhibited impaired glucose tolerance (IGT) at all ages studied, with the exception of 2 months, when glucose tolerance transiently worsened in controls ($n=9$ CON, $n=7$ PNA, $P<0{\cdot}05$ at age 1, 3–6 months at 45–120 min time points). Figure 3A shows representative average glucose curves at age 5 months; Fig. 3B shows the average area under the glucose curve at each age studied (repeated-measures ANOVA, $P<0{\cdot}05$). Fasting glucose was significantly higher in PNA mice (glucose: CON $68{\cdot}7\pm4{\cdot}2$ mg/dl, $n=9$, PNA $86{\cdot}7\pm4{\cdot}9$ mg/dl, $n=7$, $P<0{\cdot}02$).

PNA mice exhibit normal peripheral insulin sensitivity

Glucose intolerance occurs due to the failure of insulin target tissues to adequately dispose of circulating glucose. This can be a consequence of impaired insulin secretion and/or impaired insulin action. To assess the latter, we performed

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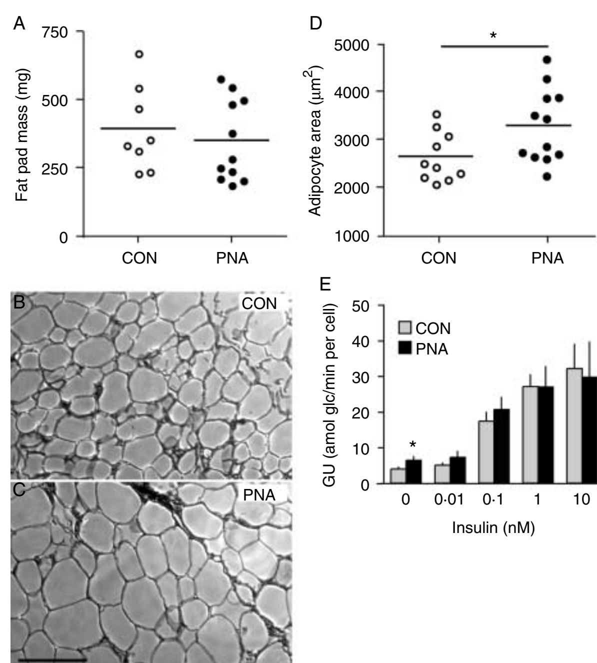

Figure 2 PNA does not alter fat pad mass but increases adipocyte size. (A) Fat pad mass in CON (open circles) and PNA mice (closed circles) (n=8 CON, n=11 PNA, $P>0{\cdot}5$). (B and C) Representative photomicrographs of adipose tissue from control (CON) (B) and PNA (C) mice. Scale bar represents 200 μM. (D) Mean adipocyte size (n=10 CON, n=12 PNA). (E) Mean±S.E.M. glucose uptake (GU) into CON (gray bars) and PNA (black bars) adipocytes in response to varying concentrations of insulin (n=2–5 assays per insulin concentration). Basal uptake was higher in PNA adipocytes, but insulin sensitivity was similar. *$P<0{\cdot}05$.

an ITT in a group of 3-month-old mice. No differences were found between PNA and CON mice (n=10 per group, all comparisons at $P>0{\cdot}2$, Fig. 3C). Fed basal glucose levels were also not different (CON 164·8±11·8 mg/dl, PNA 162·7 ±10·1 mg/dl, n=10 per group, $P>0{\cdot}8$). Additionally, fasting insulin and glucose values were used to determine homeostasis model assessment of insulin resistance (HOMA-IR) as a surrogate measure of insulin resistance. This index was developed using human data but subsequently has been validated in rodents (Cacho *et al*. 2008, Mather 2009). No difference was found between groups (0·78±0·10 CON versus 0·86±0·18 PNA, $P>0{\cdot}6$).

PNA mice have an early form of islet dysfunction

The presence of apparently normal insulin sensitivity in PNA mice suggested glucose intolerance might have originated in an insulin secretion defect at the level of the pancreatic β cell. To assess pancreatic islet function, we used the ratiometric fluorescent probe fura-2 AM to measure glucose-stimulated calcium (GSCa) in islets from PNA and CON mice. GSCa is a measure of islet glucose sensitivity that allows high frequency sampling, which captures the dynamics of the biphasic response (Jahanshahi *et al*. 2009) and approximates that of glucose-stimulated insulin secretion (Henquin *et al*. 2006). $[Ca^{2+}]_i$ was monitored in islets during perifusion with 3 mM glucose and following a switch to 11 mM glucose. Islets from PNA (n=6) and CON (n=8) mice had similar calcium levels in 3 mM glucose ($P>0{\cdot}4$), but the rise in intracellular calcium following a switch to 11 mM glucose was blunted in islets from PNA mice ($P<0{\cdot}05$, Fig. 4).

The limited number of islets precluded performing parallel insulin secretion studies in islets from all mice. Insulin secretion was measured in islets from two mice per group. While this sample size was insufficient to perform statistical comparisons, there was good agreement between calcium responses and insulin release in high glucose, consistent with observations in the literature (Deering *et al*. 2009, Evans-Molina *et al*. 2009) (3 mM: CON 2·1±0·06 pg/ml, PNA 5·7±1·5 pg/ml; 11 mM: CON 33·0±2·1 pg/ml, PNA 20·8±4·2 pg/ml).

Insulin release from isolated islets

Steroids can exert organizational effects, which are mediated by developmental programing and persist in the absence of hormone, as well as activational effects, which require the immediate presence of hormone (Arnold & Breedlove 1985). To probe the activational role of androgens in adult islet

Table 1 Adipokine levels in plasma from control (CON) and prenatally androgenized (PNA) mice under fed and fasted conditions (n=10–16 per group)

	CON fed	**PNA fed**	**CON fasted**	**PNA fasted**
Analyte				
Insulin (pg/ml)	1071±178*	1331±306*	146±22·5	107±16·5
Leptin (pg/ml)	2476±350*	1849±435	926±192	1468±366
TNFα (pg/ml)	4·7±0·4	5·3±0·5	6·4±2·0	4·1±0·5
PAI-1 (pg/ml)	8361±1049*	6858±1377	3635±635	4246±664
IL6 (pg/ml)	17·7±2·2*	24·6±5·4†	9·2±1·7	5·3±0·8
Resistin (pg/ml)	1758±160*	1497±144†	891±95	1073±159
Adiponectin (mg/ml)	16·2±0·7‡	14·0±1·0	–	–

*$P<0{\cdot}05$ versus fasted. †$P<0{\cdot}06$ versus fasted. ‡$P=0{\cdot}08$ versus PNA.

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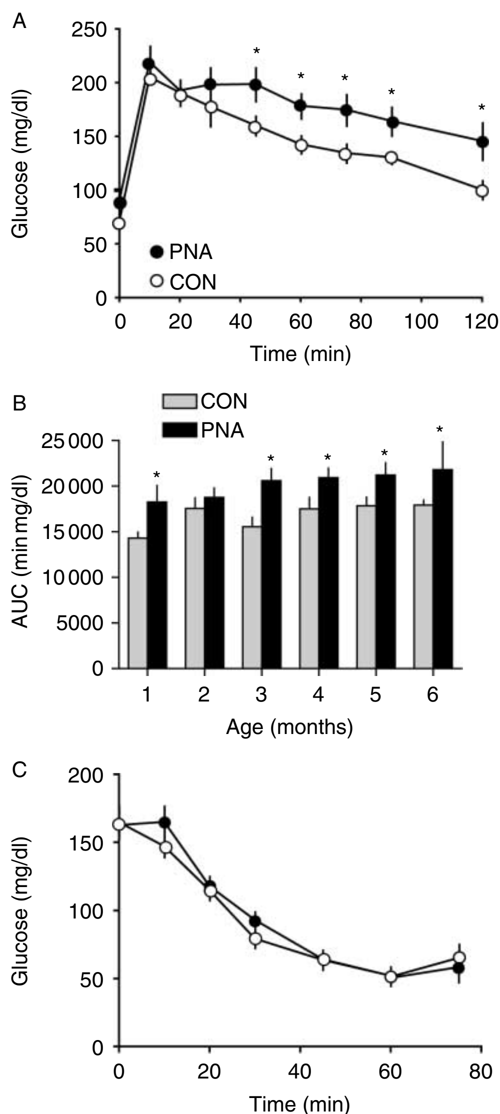

Figure 3 PNA mice exhibit impaired glucose tolerance but not insulin resistance. (A) Glucose tolerance was impaired in 5-month-old PNA (closed circles) compared to CON (open circles) mice. (B) Area under the curve (AUC) in CON (gray bars) and PNA (black bars) mice examined at different ages illustrates that glucose intolerance develops by 1 month of age. (C) PNA mice are not insulin resistant, based on insulin tolerance testing (n=10 per group, P>0·2). *P<0·05.

function, we performed an *in vitro* study of islet insulin secretion after incubation with different steroids. Pancreatic islets express steroid receptors, including receptors for androgen (Winborn *et al.* 1987, Díaz-Sánchez *et al.* 1995), but few studies have examined the direct roles of androgens in the islet. Islets were harvested from OVX mice 3 days post surgery. OVX mice were used to control for effects of intrinsic steroids and estrous cycles. Isolated islets were incubated overnight in 10 nM DHT, DHT+E_2, or ethanol vehicle. Insulin secretion was measured in 3 and 11 mM glucose. Neither DHT nor the combination of DHT+E_2 had an effect on insulin secretion in 3 mM glucose (P>0·05, n=12 per group, Fig. 5). However, DHT and the combination of DHT+E_2 significantly inhibited insulin secretion in 11 mM glucose (P<0·01 and P<0·05 respectively). This blunting of the islet response to high glucose was similar to that observed in PNA mice.

Reproductive measures

The lack of a difference in glucose tolerance at 2 months was due to an increase in glucose levels in CON mice (P<0·05 by paired *t*-test), rather than an improvement in glucose tolerance in PNA mice. Interestingly, 2 months is around the age of final sexual maturation in mice (Gore *et al.* 1999). Given that puberty is a period of relative insulin resistance (Amiel *et al.* 1986), we speculated that the increase in glucose levels in CON mice may be due to pubertal changes, and PNA mice may have experienced puberty earlier or later than CON mice. To address this question, vaginal opening was monitored in a subsequent group of mice and found to occur earlier in PNA mice (CON 34·4±1·3 days, n=18, PNA 29·5±1·7 days, n=16, P<0·05). Body mass at the time of vaginal opening was significantly lower in PNA mice (CON 14·9±0·3 g, PNA 12·7±0·5 g, P<0·001), suggesting that increased body mass was not the cause of early vaginal opening.

Serum testosterone was assayed in blood samples taken at euthanasia on the day of islet harvest. In contrast to our previous report (Sullivan & Moenter 2004), PNA mice in this study did not exhibit elevated testosterone levels at 5 months of age (CON 10·5±3·6 ng/dl, n=9, PNA 11·9±3·0 ng/dl, n=8, P>0·7). However, testosterone levels measured in a different group of mice from this cohort of PNA animals at 8 months of age revealed significantly higher levels than CON mice (CON 17·7±2·7 ng/dl, n=7, PNA 28·9±1·9 ng/dl, n=5, P<0·01). Thus, as the mice continue to age, differences in androgen levels may become apparent. In this report, metabolic studies were performed up to age 6 months.

Despite the absence of elevated testosterone, the primary reproductive phenotype of disrupted estrous cycles was apparent in PNA compared to CON mice. Cycle duration, defined as the estrus-to-estrus interval, was significantly lengthened (6·6+0·4 days CON, 15·7+2·6 days PNA, n=10 each, P=0·002) with the percent of time in estrus significantly decreased (15·2+1·6% CON, 4·8+1·6% PNA, P=0·0002). PNA mice exhibited prolonged periods in which leukocytes were the primary cell in the vaginal lavage. This finding differs somewhat from the original report of this model, in which similar disruptions were observed in cyclicity but prolonged periods of cornified cells were observed. We believe the difference may be attributed to a switch from phytoestrogen-containing to reduced-phytoestrogen chow. The observations of cyclicity for the three rounds of prenatal androgenization used for this study, as well as ongoing studies in the laboratory, are consistent with prolonged diestrus.

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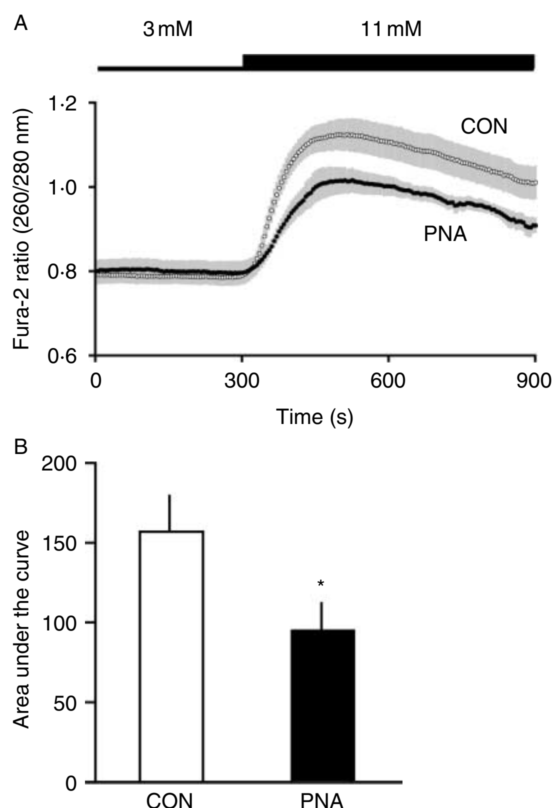

Figure 4 PNA mice have impaired pancreatic response to elevated glucose. (A) Mean ± S.E.M. ratios (n=8 CON, n=6 PNA) of fura-2 fluorescence at 260 and 280 nm excitation, demonstrating the impaired response of islets from PNA mice on an increase in glucose concentration from 3 to 11 mM. Shaded area indicates S.E.M. (B) Area under the curve was lower in PNA mice. *$P<0·05$.

PNA mice exhibit normal birth weight

Some of the metabolic effects observed in this model coincide with those induced by intrauterine growth restriction, which has been demonstrated in sheep PNA with testosterone (Steckler *et al.* 2005). Birth weight was assessed in subsequent litters of PNA mice but was not altered by prenatal treatment (CON 1·4 ± 0·03 g, n=33, PNA 1·4 ± 0·03 g, n=29, $P>0·7$).

Discussion

Prenatal androgenization of female mammals has profound lasting effects on reproductive function in adulthood, and may underlie the fertility and metabolic disorder PCOS. In this study, we assessed whether the same prenatal DHT treatment that caused reproductive abnormalities in female mice (Sullivan & Moenter 2004) could also induce metabolic dysfunction. PNA mice exhibited glucose intolerance that was present before puberty and persisted into adulthood. IGT was not associated with increased adiposity or peripheral insulin resistance; however, pancreatic islet function was altered in PNA mice and may be a causative factor in glucose intolerance.

Glucose tolerance was studied across postnatal development since increasing adiposity with age, or changes in circulating hormones following puberty, might influence the phenotype. Of interest, PNA mice exhibited glucose intolerance at the earliest age studied, 4 weeks, and the difference relative to controls remained stable throughout the study, except at the 2-month time point. A difference in the timing of puberty may account for the disparity at 2 months. PNA mice underwent vaginal opening earlier than CON mice; thus, we may have missed the window of pubertal insulin resistance in this group. Alternatively, the already impaired glucose handling in PNA mice may have masked the effects of pubertal insulin resistance. The finding of earlier puberty in PNA mice is supported by work in sheep showing pubertal advancement following prenatal androgen exposure in females (Wood *et al.* 1991, Jackson *et al.* 2008), and recent studies implicating androgens in the timing of puberty (Brill & Moenter 2009). Further, the observation of IGT in PNA mice at only 1 month of age corresponds with the appearance of some aspects of PCOS in adolescents (McCartney *et al.* 2006).

PNA mice exhibited elevated fasting glucose levels in the presence of normal fasting insulin. Impaired fasting glucose is associated with hepatic insulin resistance (Bock *et al.* 2007), which is characterized by a failure of insulin to suppress gluconeogenesis under fasting conditions. Typically, insulin would also be elevated in this situation, but this assumes normal pancreatic β cell compensation. Another possibility is that PNA mice have a higher stress response to fasting and handling, leading to acutely elevated glucose. However, studies of steroid programing of the hypothalamic–pituitary–adrenal axis indicate that adult stress responses are blunted by developmental androgen exposure (McCormick *et al.* 1998). DHT administered close to parturition as in our study could potentially change maternal nurturing

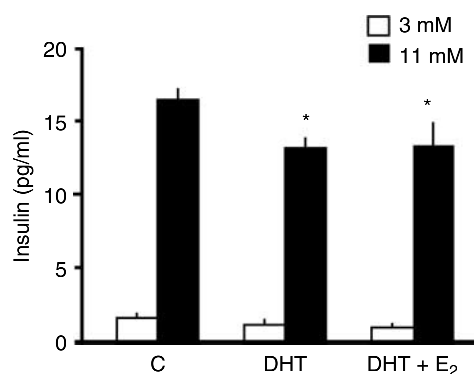

Figure 5 Steroids can act directly at the islet to alter insulin release in response to a glucose challenge. DHT or DHT in combination with estradiol (DHT + E_2) impairs the release of insulin in response to a high-glucose challenge (n=12 mice). *$P<0·05$ versus control (C, ethanol vehicle) in 11 mM glucose.

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behavior, which could also affect offspring stress responses (Francis *et al*. 1999).

Isolated islet studies were performed because the limited blood volume of mice makes sequential measurements of insulin secretion *in vivo* difficult to perform. $[Ca^{2+}]_i$ is closely coupled to insulin release in the β cell, permitting $[Ca^{2+}]_i$ changes to be monitored as a surrogate for insulin secretion amid changing glucose concentrations (Jahanshahi *et al*. 2009). Islets are a heterogeneous tissue comprising α, β, δ, pancreatic polypeptide and ε cells (Brissova & Powers 2008), but β cells comprise 65–80% of the islet mass; thus glucose-stimulated $[Ca^{2+}]_i$ variations primarily reflect changes in this cell type. IGT in PNA mice did not progress to frank diabetes. Nevertheless, the observed defects in islet function are similar to the early islet dysfunction in type 2 diabetes mellitus (Jahanshahi *et al*. 2009). Hallmarks of pending islet failure include elevated basal calcium, loss of oscillatory activity, and failure to generate an appropriate rise in calcium (and thus insulin secretion) on high-glucose stimulation (Evans-Molina *et al*. 2009, Jahanshahi *et al*. 2009). Type 2 diabetes occurs in the context of peripheral insulin resistance, when pancreatic compensation to increase insulin production is no longer adequate (Kahn 2001). Thus, impaired pancreatic islet function in PNA mice may predispose them to develop type 2 diabetes in the presence of other risk factors, such as obesity. Similarly, women with PCOS have impaired β cell function and are at increased risk for diabetes (Dunaif & Finegood 1996, Legro *et al*. 1999).

An additional tissue-specific abnormality was identified, with PNA mice exhibiting increased visceral adipocyte size. Although enlarged adipocytes are often observed with increased fat mass, total fat pad mass was unchanged in PNA mice, suggesting that adipocyte number may be reduced. This idea is speculative; however, reports in the literature suggest that androgens can indeed alter adipocyte differentiation and size. DHT reduces omental adipocyte differentiation in tissue culture, and both DHT and testosterone inhibit differentiation of pluripotent cells into the adipogenic lineage (Singh *et al*. 2003, Blouin *et al*. 2009). Androgen receptor knockout mice have smaller adipocytes than wild-type controls, suggesting androgen receptor activation increases adipocyte size (Yeh *et al*. 2002). It must be emphasized that the change in adipocyte size observed in this study was small and did not result in associated changes in adipocyte insulin sensitivity (Lundgren *et al*. 2007) or changes in circulating levels of the majority of adipokines measured (Scherer & Trujillo 2006, Skurk *et al*. 2007). In PNA mice, there was a strong trend for decreased adiponectin in the fed state, which is in agreement with reduced adiponectin levels in women with PCOS (Wickham *et al*. 2010). Further, leptin levels were not reduced by fasting in PNA mice, suggesting altered regulation of this hormone, which is permissive for fertility (Moschos *et al*. 2002). Differences in other adipokines, or in adipocyte insulin sensitivity, could appear under conditions of metabolic stress, such as diet-induced obesity. This is an interesting area for future studies.

Activational effects of androgens in islets were assessed because PNA mice previously exhibited elevated androgens (Sullivan & Moenter 2004), a cardinal feature of PCOS. The androgen receptor (Li *et al*. 2008) and synthesis enzyme cytochrome P450(17α) (Ogishima *et al*. 2008) are expressed in β cells, but their roles in this tissue are unclear. One study showed that micromolar concentrations of testosterone increase insulin transcription and secretion in the rat *in vivo* and in islets *in vitro* (Morimoto *et al*. 2001), but the possibility that effects were mediated by aromatization to E_2 or by nongenomic pathways were not examined. We found that overnight treatment with nanomolar concentrations of DHT impaired high-glucose-stimulated insulin release in islets from OVX mice. This effect was most likely genomic, as steroids were not present when insulin release was measured. E_2 had no added effect. The reduction in glucose-stimulated insulin secretion following islet exposure to DHT *in vitro* was similar to the decrement in GSCa flux in islets from PNA mice. Since we did not observe elevated circulating androgens at 5 months of age when islets were isolated, islet dysfunction could not be attributed to activational effects of androgens. However, the effects of elevated androgen in adult life may be additive to those induced by prenatal androgen. PCOS is thought to involve elevated androgen prenatally and in adulthood, potentially generating a double insult on pancreatic function.

The absence of peripheral insulin resistance in PNA mice differs from previously published studies of PNA monkeys, sheep, and rats (Bruns *et al*. 2004, Recabarren *et al*. 2005, Demissie *et al*. 2008). In these models, insulin resistance was accompanied by an increase in total and/or visceral adiposity; the association between these conditions is well recognized (Kahn *et al*. 2006). PNA mice did not exhibit changes in body mass or composition, which may correlate with the absence of insulin resistance. An important distinction that may account for these differences is the androgen used. This study used DHT to examine primarily androgen receptor-mediated effects, whereas other studies used testosterone, which can be aromatized to E_2. This suggests a possible role of excess prenatal estrogen, or estrogen in combination with androgen, in programing obesity and peripheral insulin resistance. Prenatal exposure to estrogenic substances such as bisphenol A and diethylstilbestrol increases postnatal weight and adiposity (Miyawaki *et al*. 2007, Newbold *et al*. 2009). Estrogens may also potentiate the effects of androgens by upregulating the androgen receptor in specific tissues, including adipocytes and brain (McAbee & Doncarlos 1999, Shao *et al*. 2007). Further, intrauterine growth restriction and its metabolic sequelae have been shown to be an effect of prenatal testosterone, but not DHT exposure (Steckler *et al*. 2005, Carlsen *et al*. 2006). In addition to the type of steroid treatment, other differences that may account for phenotypic discrepancies include the difference in species and variations in the timing of androgen administration. Sexual differentiation occurs during gestation in primates and sheep, whereas in rodents, it is incomplete at birth (MacLusky & Naftolin 1981, Jackson *et al*. 2009). Thus, androgen

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exposure late in gestation in mice may be comparable to an early treatment in other species. Nevertheless, the developmental timeline in rats is similar to that in mice, and the treatment period in the PNA rat (days 16–19) overlaps ours. Hence, the steroid may be most important in establishing differences among models.

In this study, we have shown that prenatal androgens program long-term alterations in metabolic function in female mice. These findings have implications for gestational androgen exposure that originates from endogenous or exogenous sources. The absence of changes in circulating glucose, insulin, or the majority of adipokines assessed under fed conditions suggests that the previously observed reproductive dysfunction in this model was likely caused by androgen programing of the reproductive axis, and was not secondary to metabolic changes. Further, as the previously reported effects of prenatal androgenization to induce adiposity and insulin resistance were absent from PNA mice, it appears that metabolic programing by testosterone may be dependent on aromatization to E_2 in addition to androgen receptor-mediated effects. The impairments observed in glucose tolerance and pancreatic islet function, as well as increased adipocyte size, may predispose PNA mice to develop diabetes in the presence of aggravating factors. This suggests a two-hit hypothesis, in which prenatal androgen programing sets the stage for metabolic dysfunction, and weight gain and insulin resistance secondary to prenatal estrogens or postnatal weight management drive the progression to diabetes.

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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Author contribution statement

CSN performed islet calcium imaging and insulin secretion studies. SRK performed glucose uptake assays in adipocytes. All other experiments were designed and carried out by AVR under the supervision of her graduate advisor SMM. The manuscript was written by AVR.

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