Life Sciences 130 (2015) 66–72

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Neonatal androgenization-induced early endocrine–metabolic and ovary misprogramming in the female rat

Luisina Ongaro [a], Natalia R. Salvetti [b], Andrés Giovambattista [a], Eduardo Spinedi [c,*], Hugo H. Ortega [b]

[a] Neuroendocrine Unit (IMBICE, CONICET-CICPBA), La Plata, Argentina
[b] Institute of Veterinary Sciences (ICIVET-Litoral), UNL/CONICET, Santa Fe, Argentina
[c] Centre for Experimental & Applied Endocrinology (CENEXA, UNLP-CONICET La Plata), Argentina

ARTICLE INFO

Article history:
Received 24 December 2014
Received in revised form 8 March 2015
Accepted 10 March 2015
Available online 26 March 2015

Keywords:
Growth
Insulin
Adipose tissue
Leptin
Ovary
Steroidogenesis

ABSTRACT

Aim: Androgen excess predisposes the organism to develop metabolic–endocrine and reproductive dysfunctions, among them the development of a phenotype resembling that of human Polycystic Ovary Syndrome (PCOS).
Methods: We analyzed the impact of a single neonatal (5 day-old) testosterone propionate (TP; s.c. 1.25 mg/female pup) dose on: a) several metabolic–endocrine activities and b) ovarian steroidogenic and granulosa cell (GC) functions and also follicular population in juvenile and adult TP and control (CT) rats.
Key findings: Compared to CT rats, TP animals were characterized by: a) accelerated growth, hyperadiposity and hyperleptinemia, b) very early (pre-weaning age) vaginal opening, c) hyperinsulinemia in adult life, d) dysfunctional ovarian steroidogenesis, e) conserved GC functionality in both juveniles (*in vitro*) and adults (*in vivo*), and f) estrous cycles arrested at estrus. Finally, histological studies of the ovaries indicated that in TP (*vs.* CT) rats: i) primary and antral follicle frequencies were 3- and 15-fold higher and lower, respectively, in juveniles and ii) secondary and atretic follicle frequencies were 3- and 5-fold lower and higher, respectively, in adults. Large cystic images without corpus luteum were observed in the ovaries from adult TP rats only.
Significance: Our results strongly suggest that transient neonatal hyperandrogenemia induced early misprogramming of metabolic–endocrine and ovarian (steroidogenesis/folliculogenesis) functions. Conversely, TP rats preserved their ovary GC endocrine function. Our results further support the high risk of developing ovarian hyperstimulation syndrome for infertile women with transient/chronic hyperandrogenemia (PCOS) subjected to assisted reproductive technologies.

1. Introduction

A very common cause of menstrual disturbances, chronic anovulation and hyperandrogenism in pre-menopausal women is Polycystic Ovary Syndrome (PCOS) [9,10]. Although a very large population of women at reproductive age is affected by PCOS, diagnosis of this syndrome is still difficult for physicians [11]. Insulin resistance, with or without compensatory hyperinsulinemia, is frequently associated with PCOS [3,11,13]. Moreover, clinical and experimental studies suggest interaction between insulin and sex hormones in healthy subjects [2]. We previously found that androgenization in normal female rats, at either neonatal [1,19] or early post-pubertal [20] age, developed impaired insulin sensitivity in adulthood. We also observed a severe hypothalamic–pituitary–ovary (HPO) axis dysfunction in neonatally androgenized adult female rats. Indeed, on reaching adulthood, rats developed an altered pulsatile rhythm of gonadotropin secretion (*e.g.* decreased average mean values, decreased FSH peak amplitude values and increased LH:FSH ratio) and impaired *in vivo* LHRH-stimulated LH–FSH release [23]. These abnormalities have been ascribed to a neonatal hypothalamic effect of transient peripheral androgen excess (hypothalamic androgenization), later resulting in infertile individuals [4].

Regarding the relation between early hyperandrogenemia and adipose tissue function, we previously established that 100 day-old neonatal androgenized female rats are heavier and display greater adiposity, hyperleptinemia, impaired peripheral insulin sensitivity, and increased risk of developing dyslipidemia and cardiovascular risk [1]. In this regard, although physiological peripheral leptin levels are needed for normal HPO axis function [21], chronic high plasma leptin concentrations (leptin resistance) are critical for consequent ovary dysfunction (*e.g.* anovulation) [32]. Nevertheless, it is accepted that follicle oocytes and, granulosa and theca cells express androgen receptor, a gonadotropin-regulated element [28] that must be fine-tuned by appropriate androgen stimulation to assure normal follicle development and ovulation [28]. Tyndall et al. [24] recently investigated the effects of fetal and post-natal treatment with multiple high doses of androgen in female rats. These authors found that fetal injury (transient hyperandrogenemia in dams) failed to alter rat ovary folliculogenesis

* Corresponding author at: CENEXA (UNLP-CONICET), Argentina.
E-mail address: spinedi@cenexa.org (E. Spinedi).

http://dx.doi.org/10.1016/j.lfs.2015.03.008
0024-3205/

or fertility. The authors also claimed that multiple doses of androgen administered at any post-natal age (before 25 days of age) induced an incomplete PCOS rat phenotype [24].

In the present study, we aimed to investigate in the female rat whether a single, early post-natal (day 5), testosterone propionate (TP; 1.25 mg/pup, s.c.) treatment would be able to change the programming of metabolic–endocrine and reproductive functions. With this aim, we evaluated: a) several peripheral biomarkers and b) ovarian functionality (some steps of the steroidogenic process, folliculogenesis and granulosa cell endocrine function) in TP and their littermate control (CT) female rats when reaching juvenile and adult ages.

2. Materials and methods

2.1. Experimental animals

We used the classical Barraclough's animal model [4], previously validated in our laboratory [1]. Briefly, 5 day-old Sprague–Dawley female rat pups (10.65 ± 0.27 g BW; n = 80) were s.c. injected with 50 μL of sterile corn oil either alone (CT group; n = 35/40) or containing 1.25 mg of TP (Organon Lab., Argentina, n = 35/40). The vaginal opening was checked between 7 and 45 days of age. After weaning (21 days of age), rats were housed individually. Body weight (BW) and food intake were recorded daily (between 07:30 and 08:30 h) up to the corresponding experimental day (juvenile and adult ages, 30 and 60 days of age, respectively). Animals were kept in a light- (lights on from 07:00 to 19:00 h) and temperature (22 °C)-controlled room throughout the experiment. Rats were fed a commercial rat diet (Ganave Lab., Argentina) and provided with tap water *ad libitum* throughout the entire housing period. The experiments were approved by our Institutional Animal Care Committee; international regulations concerning ethical use of animals were strictly followed.

2.2. Experimental designs

Experiment 1. Rats from both groups were killed (between 08:00 and 09:00 h) at 30 days of age (juvenile age) in non-fasting conditions. Immediately after killing trunk blood was collected (into EDTA 10%–sodium fluoride coated plastic tubes), parametrial adipose tissue (PMAT) and ovaries were dissected, weighed, and either used for (see below) histological analyses or kept frozen (at −80 °C) until further assays.

Experiment 2. Individually caged rats (CT and TP) were injected (in the morning of days 27, 28 and 29 of age) s.c. daily with 1 mg of xenoestrogen [diethylstilbestrol (DES) dissolved in 100 μL of sterile corn oil] to stimulate the development of early antral follicles. Animals were killed the next morning (08:00–09:00 h; 30 days of age) and ovaries were immediately removed for granulosa cell (GC) isolation and culture. Briefly, GCs from DES-treated rats were isolated by ovary puncture (30-gauge needle) as described elsewhere [26] and incubated in Dulbecco Modified Eagle medium (DMEM, 4.5 g glucose/L)-Ham F12 (1:1, Gibco, Gaithersburg, MD, USA), EGTA (6.8 mM), and HEPES (10 mM; 15 min at 37 °C), then washed and incubated in DMEM-F12 (1:1), sucrose (0.5 M), and HEPES (10 mM; 5 min at 37 °C). After incubation, the medium was diluted with 2 volumes of DMEM-F12 and HEPES (10 mM), and ovaries were allowed to sediment. GCs were obtained by pressing ovaries between two pieces of nylon mesh (Nytex 50, Geneva, Switzerland). To eliminate contaminating theca/interstitial cells, the crude GC suspension was layered over a 40% Percoll solution in saline solution and centrifuged at 400 ×*g* for 20 min. The purified GC layer was aspirated from the top of the Percoll solution and resuspended in DMEM-F12 (1:1) containing sodium bicarbonate (2.2 g/L; pH: 7.4). Cells were seeded on P12 multiwell plastic plates (Nunc, Roskilde, Denmark) precoated with collagen at a density of 3.5×10^5 viable cells per well. Cells were kept at 37 °C in a 5% CO_2 atmosphere. After 3 h, media were changed to remove nonattached cells and replaced by fresh media containing antibiotics, delta-4-androstenedione (10^{-7} M) and 100 μL of fresh medium either alone or containing hCG (0.05–0.5 IU/mL). Cells were then cultured for 48 h. At the end of this period, media were removed and kept frozen (−20 °C) until measurement of 17β-estradiol (E2) concentrations (see below).

Experiment 3. Vaginal smears of rats from both groups were examined daily for three consecutive estrous cycles before experimentation [1]. Throughout this time period, samples obtained from TP rats indicated that they had attained constant estrus. Rats from both groups were then killed (between 08:00 and 09:00 h) on day 60 of age (adult age), at proestrous stage (CT) or arrested estrous stage (TP), in non-fasting condition. Immediately after killing, trunk blood was collected (into EDTA 10%–sodium fluoride coated plastic tubes) and ovaries were dissected and either used for histological analyses or kept frozen (at −80 °C).

Experiment 4. Vaginal smears of rats from both groups were examined daily for three consecutive estrous cycles before experimentation. On the morning (07:00–08:00 h) of the experimental day (age 60–61 days), CT (at the proestrus stage) and TP (at the arrested estrus stage) rats were i.v. implanted, under light phenobarbital anesthesia, to inhibit endogenous LH surge [30], with an indwelling catheter kept permeable by administering a small volume (100 μL) of vehicle (10 IU heparin/mL of sterile saline solution, containing 10 mg/mL bovine serum albumin). After recovery (10:00–11:00 h), rats were i.v. bled (a small volume, 200 μL) before (sample time zero) and 1 and 2 h after i.v. injection of 5 mIU/kg hCG (dissolved in 50 μL of vehicle, see above). Samples were rapidly centrifuged (at 4 °C, 15 min, 2600 ×*g*) and plasma samples kept frozen (−20 °C) until measurement of E2 concentrations.

2.3. Peripheral metabolite measurements

Circulating levels of leptin, insulin, total testosterone and corticosterone (Cort) were determined by specific radioimmunoassays (RIAs) as described earlier [1]. Plasma concentrations of 17-hydroxyprogesterone (17OHP4) were determined by a commercial RIA kit (Immunotech, France). Peripheral concentrations of triglyceride and glucose were determined by commercial enzymatic-colorimetric assays (Weiner Lab., Argentina). Finally, plasma and medium concentrations of E2 were determined by a specific RIA published and described in detail earlier [1]; the intra-assay coefficient of variation ranged between 4 and 8%, whereas the inter-assay coefficient of variation ranged between 9 and 12%.

2.4. Ovarian histology

For histological studies, five ovaries from each group were removed and immediately fixed in 4% paraformaldehyde (in 0.2 M pH 7.4 phosphate buffer), at 4 °C for a maximum of 3 days. Tissues were then washed with 0.01 M PBS, immersed in 70% ethanol for 24 h, embedded