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Neonatal exposure to estradiol valerate reprograms the rat ovary androgen receptor and anti-Müllerian hormone to a polycystic ovary phenotype

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ABSTRACT

To understand the impact of exposure to steroids in the early step of ovary development (a stage occurring *in uterus* in humans), we studied neonatal exposure to estradiol valerate (EV) in rats regarding polycystic ovary (PCO) development as well as expression of androgen receptor (Ar) and anti-Müllerian hormone (AMH), a marker of ovarian follicular development. Rats exposed to one dose of EV (10 mg/kg, sc) during their first 12 h of life were euthanized at 2, 30 and 60 days of age. Gene array and real-time PCR studies showed *Ar* and *AMH* up regulation in the ovary at 2 days of age and persisted at 60 days of age, when a PCO phenotype was evident with increased levels of Ar and AMH proteins. The single neonatal exposure in rats suggests participation of EV in developing PCO syndrome. Its persistence also suggests that estradiol reprograms ovarian function and disease during adulthood.

1. Introduction

In mammals, ovarian folliculogenesis starts with the formation of primordial follicles, following oocyte nest breakdown, allowing the oocytes to be surrounded by a layer of somatic cells and form individual primordial follicles in a process known as follicular assembly and undergo the initial transition from the primordial to primary follicular stage [1–3]. These processes are controlled by multiple regulatory factors among which estradiol is one of the most important [4,5], making this process vulnerable to hormonal changes induced by external factors. Environmental endocrine disruptors mimicking estrogenic compounds cause detrimental effects on these early ovarian processes. Compared with ovarian development in humans, the ovarian primary follicle population in the rat is not reached until shortly after birth because is an immature mammal. Thus, the neonatal rat is a good model to study early ovarian development without the contribution of the multitude of factors regulating pregnancy during the last trimester of pregnancy in humans. [2,3].

One of the most frequent ovarian pathologies in women during their reproductive years is the polycystic ovary syndrome (PCOS). In humans, PCOS is a complex endocrine disorder characterized by hyperandrogenism, ovulatory/menstrual irregularity, and polycystic ovaries [6]. Interestingly, women with PCOS exhibit a significant increase in androgen concentrations during pregnancy [7]. An important proportion of the first-degree female relatives of women with PCOS have been shown to be at risk for developing PCOS [8], suggesting a reprogramming factor that affects key hormones or receptors controlling ovary function and, hence, follicular development. In fact, compared with control prepubertal girls, daughters of PCOS women exhibit higher levels of anti-Müllerian hormone (AMH), a marker of growing follicles, beginning at the peripubertal stage [9,10]. It has been proposed that this inheritance is not the result of a genetic condition but is due to fetal programming [6,11].

A form of PCO resembling some aspects of human PCOS can be induced in rats by a single injection of estradiol valerate. This model has been widely used to study PCO and the metabolic effects of hormone expression that resemble human PCOS such as oligo/anovulation, follicular cysts, and insulin resistance [12–23].

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Although the steroid-induced PCO model in rats lacks some characteristics of women with PCOS, such as high androgen plasma levels, the ovary presents a high androgen and estradiol secretory and biosynthetic capacity [15,24]. Brawer et al. [19,20] demonstrated that administering estradiol to adult rats induces the polycystic condition 60 days after hormone exposure. Subsequently, Rosa-e-Silva et al. [15] and Sotomayor-Zárate et al. [16] verified that the same occurred in rats that were injected with EV at 14 days of age and in the first day after birth, respectively. In addition, our laboratory demonstrated a temporal window in which the administration of EV induces irreversible damage to folliculogenesis, ovulation, and the reproductive physiology, distinguishing the neonatal stage as a vulnerable period [13]. Regarding the fetal exposure to steroids as a factor to modify ovary function, it has also been reported that the exposure of pregnant sheep to testosterone or the nonaromatizable androgen dihydrotestosterone (DHT) produced differential effects on the reproductive function of progeny. Increased numbers of growing follicles and reduced numbers of primordial follicles were found in 10-month-old prenatal testosterone-treated but not DHT-treated female sheep, suggesting that increased follicular recruitment of prenatal testosterone-treated female sheep is facilitated by androgenic programming but that postpubertal follicular growth, antral follicular disruptions, and follicular depletion are mediated largely via estrogenic reprogramming [25,26].

These results suggest that the exposure of rats to EV during the neonatal period acts to determine ovarian function during development in a similar way as that occurs in humans and sheep, in which ovary development occurs *in uterus* during development for both mammals. Thus, we analyzed whether neonatal exposure to estradiol changes the genes that regulate ovarian morphology and function and whether these changes persist during development.

2. Materials and methods

2.1. Animals and experimental design

Sprague- Dawley rats weighing 250–300 g were maintained at 20 °C with a 12-h light and 12-h dark cycle. Water and food were available *ad libitum*. The estrous cycling activity of the rats was monitored with daily vaginal smears. On an afternoon of proestrus, rats were mated, and pregnancy was confirmed the following morning by checking for a vaginal plug. From the pups obtained from these pregnancies, 36 females were randomly assigned to the control group (n = 18) or estradiol valerate (EV) group (n = 18). A single dose of estradiol valerate (EV; Sigma, St. Louis, MO, USA) was administered during the first 12 h after birth. The administered dose was 10 mg/kg of body weight dissolved in 50 μL of sesame oil as the vehicle (Sigma, St. Louis, MO, USA) as previously described [16]. The controls were injected with 50 μL of the vehicle.

Females rats were euthanized by decapitation at 2 (24 h after EV administration), 30 or 60 days of age (n = 6 for EV-treated and control rats per age group). These ages were used because they represent different stages of reproductive development in rats, and, hence, different stages of follicular development. The ovaries were isolated, the left ovary was frozen at −80 °C for RNA extraction (2, 30 and 60 days of age) and the right ovary was fixed in Bouin solution for immunohistochemistry (60 days of age). Trunk blood was collected, and plasma was obtained for estradiol measurements.

For the 60 days of age rats, estrous cyclicity was also monitored by analysis of vaginal lavage fluid taken at 10 AM daily to verify the stage of the estrous cycle. Because rats treated with EV at different ages are in persistent estrous [14–16,20], control rats were also euthanized during the estrous stage of the cycle. All experimental procedures were approved by the Bioethics Committee of the Faculty of Chemistry and Pharmaceutical Sciences at the University of Chile and complied with national guidelines (CONICYT Guide for the Care and Use of Laboratory Animals).

2.2. Determination of the clearance rate of plasma estradiol valerate and measurements of the plasma levels of estradiol

Another group of 18 control female pups and 23 EV female pups were used to determine the half-life and plasma levels of estradiol. We injected neonatal female rats during the first 12 h after birth with one dose of EV, 10 mg/kg of body weight, in 50 μL of sesame oil as the vehicle. The rats were sacrificed at 2.5 days of age (n = 4 control rats, n = 6 EV rats), at 3.5 days of age (n = 4 controls rats, n = 5 EV rats), at 4.5 days of age (n = 5 controls rats, n = 5 EV rats) and at 15 days of age (n = 5 controls rats, n = 7 EV rats).

At the time of sacrifice, trunk blood was collected from the rats of both groups, and plasma was isolated and used to determine the levels of estradiol using the ELISA kit 11-ESTHU-E01 (ALPCO Diagnostics, Salem, NH, USA). Each sample was run in duplicate. The kit sensitivity was 10 pg/mL with a cross reactivity of less than 1.6% with other steroidal hormones. The intra- and inter-assay variability was less than 10%.

Data from the plasma of the rats of the EV group were used to calculate the EV clearance half-life ($t_{½}$), which was calculated by plotting the ln of the plasma concentrations of estradiol versus time.

2.3. PCR array

To analyze the general modifications in gene expression, a quantitative RT-PCR-based array assay (PCR array) was performed containing 84 genes. To analyze the short-term effects of EV on gene expression, the ovaries from 2 days of age rats were analyzed with a PCR-array kit for nuclear receptors and coregulators (SABiosciences, Frederick, MD, USA) according to the manufacturer's instructions. For the long-term effects, a PCR-array kit for growth factors was used with ovaries from 60 days of age rats. Total RNA extraction was performed using RNeasy columns (Qiagen, Hilden, Germany). cDNA was synthesized using the RT^{2}First Strand kit (SABiosciences). The cDNA from the ovaries of 2 days old rat was synthesized from 1 μg of pooled total RNA from 6 ovaries of different rats. For the 60 days of age rats, one ovary was used and pooled from 6 rats. For both ages, the PCR array was repeated twice. The PCR array protocol was performed using an IQ5 real-time thermocycler (BioRad). The results were analyzed using the virtual platform from SABiosciences. It was considered a cut-off threshold when the determined mRNA was upregulated or downregulated at least 2-fold compared with the control value.

2.4. Real-time PCR

Real-time PCR was performed using single ovary samples, each age group comprised 6 controls and 6 EV female rats. Total RNA extraction was performed using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions, which included digestion of DNA by DNAse in the column.

The primers were designed using the Primer Select program (DNASTAR Inc., USA) and the mRNA sequence provided by the PubMed database as the template. The primer specificity was evaluated *in silico* using Primer-BLAST from PubMed (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to determine whether there were amplification byproducts in the rat genome. Table 1 shows the sequences of the designed primers for real-time PCR and GenBank accession number of each gene used as a reference. The PCR reaction mix contained 10 μL of Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Inc., California, USA); 0.25 μM of each 18S primer, 0.15 μM of each *Amh* primer, or 0.1 μM of each *Ar*

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