Molecular and Cellular Endocrinology 327 (2010) 56–64

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

The lack of estrogen and excess luteinizing hormone are responsible for the female ArKO mouse phenotype☆

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ARTICLE INFO

Article history:
Received 13 January 2010
Received in revised form 4 May 2010
Accepted 7 May 2010

Keywords:
ArKO mice
Estrogen
Gonadotrophins
Testosterone
Ovary
Follicles

ABSTRACT

It remains to be established as to whether the absence of estrogen (direct) or the elevated levels of gonadotrophins and androgens (indirect) are responsible for the ArKO (aromatase knockout) ovarian phenotype. The aim of this study was to determine the effects of E_2 (17β-estradiol) replacement, acyline (GnRH antagonist) and flutamide (anti-androgen) treatment on the ovarian phenotype of ArKO mice. E_2 replacement and acyline treatment but not flutamide treatment, reduced serum gonadotrophin levels of ArKO mice to within normal ranges. E_2 replacement improved uterine and ovarian follicular phenotypes and reduced the number of Sertoli-like filled cords by 62%. Acyline treatment reduced the number of hemorrhagic cysts and the number of Sertoli-like filled cords within ArKO ovaries. The data indicate that the absence of estrogen in concert with elevated levels of circulating gonadotrophins, principally LH, is responsible for the abnormal reproductive phenotype of the female ArKO mouse.

1. Introduction

Steroids (androgen and estrogen) and gonadotrophins (follicle stimulating hormone (FSH) and luteinizing hormone (LH)), are essential for the maintenance of the cyclical pattern of ovarian folliculogenesis and female fertility. In mice, the roles of steroidogenic and gonadotrophic hormones in reproductive development and function have been studied by specific hormone alterations and the generation of receptor deficient models (Findlay et al., 2001; Drummond et al., 2002; Britt and Findlay, 2002; Abel et al., 2003; Cheng et al., 2002; Couse et al., 2003; Fisher et al., 1998; Krege et al., 1998; Kumar et al., 1997; Risma et al., 1995, 1997; Lubahn et al., 1993; Walters et al., 2007). However, the precise role of each hormone in various ovarian functions has not been fully elucidated, although it is clear that alterations in the actions of these hormones can alter fertility (Abel et al., 2003; Risma et al., 1995, 1997; Nilson et al., 2000; Britt et al., 2000, 2001; Toda et al., 2001).

Estrogen has been known for a long time to influence fertility (Hisaw, 1947). It modulates steroidogenesis (Roberts and Skinner, 1990), promotes granulosa cell proliferation (Robker and Richards, 1998a,b), facilitates the responsiveness of follicles to gonadotrophins (Robker and Richards, 1998a,b; Richards et al., 1979; Roy and Albee, 2000) and maintains ovarian follicular development in general (Drummond and Findlay, 1999). The aromatase knockout mouse model which lacks estrogen, offers important insights into the role of estrogen in ovarian function. Female ArKO mice are infertile (failure to ovulate); they have elevated levels of circulating gonadotrophins and testosterone, severely underdeveloped uteri (Fisher et al., 1998; Britt et al., 2000, 2001), increased adiposity (Jones et al., 2001, 2000), and apparent sex-reversal of the ovarian somatic cells; that is granulosa cells become Sertoli-like cells (Britt et al., 2002, 2004). E_2 (17β-estradiol) replacement in ArKO mice partially restored folliculogenesis, suppressed serum gonadotrophins to within the normal range and increased uterine weight (Britt et al., 2002, 2004) suggesting that the ovarian phenotype in the ArKO mouse is due to the lack of estrogen alone. However, these results do not dismiss the possibility that the ArKO ovarian phenotype is not due entirely to the lack of E but could also involve elevated gonadotrophins or testosterone.

We hypothesised that it is the absence of estrogen (direct) and not the elevated levels of gonadotrophins and androgens (indirect) which is responsible for the ArKO ovarian phenotype.

☆ The financial support of the National Health and Medical Research Council of Australia (Regkeys 241000, 494802, 198705 and 441101) is acknowledged.
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0303-7207/$ – see front matter.
doi:10.1016/j.mce.2010.05.003

In order to test this, we compared the effects of E_2 replacement, acyline (GnRH antagonist to lower gonadotrophins) or flutamide (anti-androgen) treatment on reproductive parameters of the female ArKO mouse compared to wildtype (WT) and placebo treated mice. Ovarian function was evaluated by quantification of antral follicles, hemorrhagic cysts and corpora lutea (Risma et al., 1995). To assess the extent of formation of Sertoli-like cells, a Sertoli cell specific protein, Sry-like HMG box protein 9 (Sox 9) was immunolocalised to the ovarian granulosa/Sertoli-like cells and was semi-quantified. Estrous cycle lengths and serum hormones were also measured. We conclude that the ovarian phenotype of the ArKO female mouse is due to the absence of estrogen and to the elevated circulating levels of gonadotrophins, possibly LH, but not to elevated levels of testosterone.

2. Materials and methods

2.1. Animals

WT and ArKO (Fisher et al., 1998) mice on a J129/C57B6 background were maintained under specific pathogen-free (SPF) conditions, on a 12L:12D regimen and fed *ad libitum* a soy free mouse chow (Glen Forrest Stockfeeders, Western Australia). All animal procedures were approved by a Monash University Animal Ethics Committee and were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2. Treatments

WT and ArKO mice 16 weeks of age ($n = 8$ per group) were used after confirming that the ArKO ovarian phenotype was characterised by the presence of containing Sertoli-like cells, elevated levels of FSH, LH and testosterone and an absence of corpora lutea (Risma et al., 1995) as described previously (Fisher et al., 1998; Britt et al., 2000, 2001, 2002).

2.3. Estrogen

Pellets containing either 0.05 mg of 17β-estradiol (E_2) or placebo (Innovative Research of America, Sarasota, FL) were placed subcutaneously for 21 days in 16-week-old WT and ArKO mice as previously described (Jones et al., 2000; Britt et al., 2002).

2.4. Acyline

The GnRH antagonist, acyline, was obtained from the National Institutes of Health (Bethesda, MD) and freshly prepared in 5% mannitol immediately before use. Subcutaneous (sc) injection of either 1.5 mg/kg/week of acyline (Porter et al., 2006) or placebo (5% mannitol) were administered weekly for 21 days to 16-week-old WT and ArKO mice.

2.5. Flutamide

Sc pellets containing either 3 mg of flutamide or placebo (Innovative Research of America, Sarasota, FL) were implanted for 21 days in 16-week-old WT and ArKO mice as described previously (Cheng et al., 2002).

2.6. Tissue collections and processing

Vaginal smears collected daily during the treatment period were stained using Diff Quick Stain (Lab Aids, Narrabeen, Australia). Animals were killed at the end of the treatment period. The abdomen was opened and approximately 1 mL of blood was collected from the inferior vena cava. Blood samples were centrifuged (10 min, 3000 rpm) at room temperature and the serum stored at −20 °C for subsequent hormone analysis. Both ovaries and uterine horns from each animal were collected and weighed. One ovary was immersion-fixed in 10% formalin. Fixed tissue was processed through a graded series of alcohols and embedded in paraffin wax. Serial 5 μm sections were cut, and every 10th section was stained with haematoxylin and eosin. The intervening sections were used for immunohistochemistry.

2.7. Immunohistochemical localisation of Sox9

Sox 9 immunolocalisation was used to identify and quantify Sertoli-like filled cord structures in the ovary ($n = 4$ mice/group). Every 10th section of each ovary was stained with Sox9 according to a method modified from Ikeda et al. (2008). Tissue sections (5 μm) were deparaffinized in histolene and put through a graded series of descending concentrations of ethanol. Antigen retrieval was performed by microwaving slides for 10 min in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxide in MilliQ water for 5 min, after which the slides were washed twice in Tris buffered saline (TBS). The sections were incubated in a blocking solution, 5% normal goat serum in a humidified chamber at room temperature for 30 min and subsequently incubated with rabbit anti-Sox9 antibody (sc-20095; Santa Cruz; 1:100) overnight at 4 °C. After washing the slides three times in TBS, sections were incubated with rabbit anti-goat serum (1:500) for 1 h, followed by staining using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Negative control sections received normal rabbit serum. Immature mouse testis was used as a positive control for the Sox 9 antibody. All slides were masked prior to the study and analysed blind.

2.8. Quantification of corpora lutea, antral follicles, hemorrhagic cysts and Sertoli-like filled cords

All counting was performed using a 10× objective on an Olympus BX-50 microscope (Tokyo, Japan). Estimates of numbers of antral follicle, hemorrhagic cysts, CL and Sertoli-like filled cords per group were analysed using analySIS Professional Imaging software, version 5.0. Every 10th section was used and follicles with a visible oocyte nucleus, CL and Sertoli-like filled cords were counted only if they appeared in one section but not in the consecutive section. In order to minimize the structures not to be counted more than once, all pictures taken during quantification were spread on computer monitor to validate the quantification.

2.9. Hormone assays

Established RIAs were used to measure LH and FSH levels in serum using reagents supplied by the NIADDK (Britt et al., 2000). The lower limits of detection were 1.13 ng/ml (FSH) and 0.10 ng/ml (LH). The intra-assay CVs for FSH and LH assays were 8.0% and 7.3%, respectively, calculated using a pool of normal mouse serum.

Serum E_2 and testosterone were measured in single assays using commercially available kits and protocols from Diagnostic Systems Laboratories (E_2, DSL-4800 ultra-sensitive; T, DSL-4100; Webster, TX). The lower limits of detection were 5 pg/ml (E_2) and 45 pg/ml (T). The intra-assay CVs for E_2 and T assays were 4.9% and 6.7%, respectively, calculated using a pool of normal mouse serum.

2.10. Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using SigmaStat statistical software version 2.1 (Jandel Corporation, San Rafael, CA, USA). Data were subjected to a one-way ANOVA, and significance was determined using a Tukey's post hoc test. Data was log transformed in the case of unequal variance. Significance ($P < 0.05$) was determined using the Student–Newman–Keuls test.

3. Results

3.1. Pattern of estrous cycles

Placebo-treated ArKO mice are acyclic as described previously (Britt et al., 2000) with vaginal smears showing persistent diestrus. The placebo-treated WT mice have normal estrous cycles. Vaginal smears from WT and ArKO mice treated with E_2 indicated persistent estrus; acyline- and flutamide-treated ArKO mice did not cycle and were in constant diestrus or early estrus. Acyline-treated WT mice were also in constant diestrus or early estrus, whereas flutamide-treated WT mice were in constant post-estrus.

3.2. Body (Table 1) and reproductive organ (Fig. 1) weights

ArKO mice were heavier than their WT counterparts ($P = 0.015$). E_2 treatment had no significant effect on WT body weight but significantly decreased the body weight of ArKO mice ($P = 0.028$). There were no significant effects on body weight in the acyline and flutamide treatment groups compared to their respective placebo groups.

All placebo-treated ArKO mice had decreased ovarian weights compared with placebo-treated WT animals ($P = 0.001$). E_2 and flutamide treatment had no significant effect on ovarian weight in either WT or ArKO animals. Acyline treatment however, decreased ovarian weight in both WT ($P < 0.001$) and