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Effects of androgen on immunohistochemical localization of androgen receptor and Connexin 43 in mouse ovary

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ABSTRACT

Androgens have essential roles in the regulation of follicular development and female fertility. Androgen excess is the leading defect in polycystic ovary syndrome (PCOS) patients and involved in the ovarian dysfunction. The aim of this study was to elucidate the regarding regulatory role of androgen in the follicular development of female mouse. Immunohistochemical staining and Western blot analyses were performed to detect androgen receptor (AR) and Connexin 43 (Cx43) expression in ovaries from both control and testosterone-treated group mice. In this study, localizations of AR and Cx43 were dramatically altered in testosterone-treated mouse ovaries. In addition, AR expression was significantly increased, whereas Cx43 expression was markedly decreased after testosterone treatment. Alterations of AR and Cx43 expression by testosterone with concomitant reduction of MII oocytes. Overall, these results suggest the involvement of androgen in the regulation of AR and Cx43 localizations in mouse ovary. Alterations of AR and Cx43 expression by testosterone may affect normal folliculogenesis. Together these findings will enable us to begin understanding the important roles of AR and Cx43 actions in the regulation of follicular development, as well as providing insights into the role of AR and Cx43 actions in the androgen-associated reproductive diseases such as PCOS.

1. Introduction

The major stages of ovarian folliculogenesis were formation of the primordial follicle; recruitment into the growing pool to form a primary, secondary, and tertiary follicle; and lastly ovulation and subsequent formation of a corpus luteum (Edson et al., 2009). Autocrine, paracrine and endocrine hormonal regulation were essential for ovarian folliculogenesis (Hsueh et al., 2015). Recently, there had been numerous interest toward the role of androgens in the regulation of follicular development and female fertility. Although the pivotal role of androgens in male reproductive function and the obligatory role of androgens as an estradiol precursor in females were well understood, the direct involvement of androgens in female reproductive physiology remained controversial (Walters et al., 2008). However, there was a strong pathological association of hyperandrogenism with polycystic ovary syndrome (PCOS), and androgens were implicated in the origins of premature ovarian failure (Walters et al., 2010).

Androgens activated androgen receptor (AR), a key transcription factor mediating androgen-induced signaling as well as a member of the nuclear receptor superfamily encoded by X chromosomal gene (Lubahn et al., 1988; Bennett et al., 2010). AR expression in all mammalian ovaries strongly supported a universal role for AR-mediated androgen actions in influencing ovarian follicular development (Walters, 2015). Further evidence of direct role for AR-mediated actions in ovarian function came from some studies had been reported to associate with follicle growth and development (Lebbe and Woodruff, 2013). Notably, AR had been suggested to regulate signaling pathways occurring in granulosa cells, and granulosa cells-specific AR appeared to promote preantral follicle growth and prevent follicular atresia, it was essential for normal follicular development and fertility (Sen and Hammes, 2010; Duggavathi and Murphy, 2009). Recent reports have documented that AR was widely believed to be an important regulator of granulosa cells biology, high expression of AR in granulosa cells was primarily considered to associate with PCOS (Yang et al., 2014; Wang et al., 2015). While androgen excess enhanced follicular development and dysfunction formation of antral follicles leading to PCOS, low androgen levels may be associated with abnormalities of follicular growth (Prizant et al., 2014). Androgens had traditionally been considered detrimental to ovarian function and were often associated with infertility. Hyperandrogenism overrided the

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follicular–stromal dialog, resulting in follicular arrest and disturbed ovulation (Walters, 2015). At present, there is imminent concern that regarding mechanisms on unregulated follicle growth seen in diseases of excess androgens such as PCOS (Qiao and Feng, 2011; Sirmans and Pate, 2013).

Gap junctions represent plasma membrane channels that connect adjacent cells in most mammalian tissues and mediate electrical and metabolic cell–cell coupling (Kumar and Gilula, 1996). It has been documented that gap junctions built of Connexin 43 (Cx43) were necessary for ovarian follicle development and oocyte growth (Gershon et al., 2008). The level of Cx43 protein expression increased during follicular development and decreased with follicular atresia. Oocytes from Cx43-null mice failed to reach meiotic maturation, resulting in infertility (Ackert et al., 2001). In addition, based on targeted genetic deletion of Cx43 gene, this connexin also played an essential role in germ cell proliferation, differentiation, survival and apoptosis in male reproductive system (Chevallier et al., 2013). Thus, Cx43 was required during folliculogenesis as well as spermatogenesis, and might be regulated by androgens.

Although it was reported that the Cx43 expression in human and rat granulosa cells was reduced by high levels of androgen, limited knowledge existed regarding report of hyperandrogenism on Cx43 expression in mouse ovary (Lee et al., 2014; Wu et al., 2010). We therefore hypothesized that androgen regulated the distribution and expression of Cx43 in mouse ovary, then further affected follicular development and ovulation. Moreover, to answer the question whether androgen signaling was altered in mouse ovary, the expression of AR was evaluated. Concurrently, to the best of our knowledge there have been no reports to depict the specific effect of androgen on localization and expression of AR in mouse ovary. To this end, the aims of the present study, utilizing the administration of androgen, were to investigate immunolocalization and expression of AR and Cx43 protein levels during normal ovarian folliculogenesis as well as following testosterone treatment in mice, and to elucidate the underlying relationship between alterations of both proteins in ovary and abnormal follicular development.

2. Materials and methods

2.1. Animals

Six-week-old ICR female mice were purchased from Peking University Health Science Center (Beijing, China). The mice were bred in a room with 12 h/12 h light–dark cycles and given food and water *ad libitum*. The animals were euthanized by decapitation before tissue removal within 24 h of capture and the ovarian tissues were obtained. One part of the samples were immediately fixed in 4% paraformaldehyde in 0.05 M PBS (pH 7.4) for histological and immunohistochemical observations; the other part of the samples were immediately frozen in liquid nitrogen and stored at −80 °C for Western blot analysis.

2.2. Testosterone injection and superovulation in mice

The control group mice were injected intraperitoneally with bean oil (Sigma–Aldrich, St. Louis, MO, USA), and the testosterone group mice were injected intraperitoneally with 1.3 mg/kg testosterone (Testosterone Undecanoate Injection, Zhejiang Xian Ju Pharmaceutical CO., LTD). The duration was 7 days of both groups. Then all of the mice were induced to superovulate by administrating pregnant mare's serum gonadotropin (PMSG) (Serum Gonadotropin for Injection, Chifeng BO EN Pharmaceutical CO., LTD) 10 IU followed 48 h later by human chorionic gonadotropin (hCG) (Chorionic Gonadotropin for Injection, Ningbo Pharmaceutical CO., LTD) 10 IU. The MII oocytes were obtained from the oviductal ampullae of superovulated mice killed 14–16 h after hCG injection. The mice ovaries proteins were used to detect the expression of AR and Cx43 by Western blot analysis. The dose conversion and injection frequency of the testosterone we used in mouse were referred to drug instruction and previous publication (Yang et al., 2014; Gu et al., 2009).

2.3. Histology

Ovarian samples were dehydrated in an ethanol series and embedded in paraffin wax. Serial sections (4–6 μm) were mounted on slides coated with poly-L-lysine (Sigma–Aldrich), and stained with hematoxylin–eosin (HE) for observation of general histology.

2.4. Immunohistochemistry (IHC)

All slides were formalin-fixed and paraffin-embedded. Deparaffinization and hydration were performed followed by removal of endogenous peroxidase activity using 0.3% hydrogen peroxide for 30 min. Antigen retrieval was performed by microwaving the sample in 10 mmol/L sodium citrate buffer (pH 6.0) for 20 min. Anti-AR rabbit monoclonal antibody (1:100 dilution; Epitomics), anti-Cx43 rabbit polyclonal antibody (1:1000 dilution; Sigma–Aldrich) 2 μg/mL were used for experiments. The primary antibodies were incubated at 4 °C overnight. Then, PV9000 two-step plus Poly-HRP Anti-rabbit IgG Detection System (Zhong Shan Jin Qiao, China) was used. The streptavidin–biotin–peroxidase method was used for detection and diaminobenzidine was used as a substrate (ChemMate Detection Kit, Dako, Denmark). At last, hematoxylin was used for counterstaining. Negative controls were performed by substituting the primary antibody with nonimmune rabbit immunoglobulin G (IgG, Sigma–Aldrich).

2.5. Western blot analysis

The mouse ovaries lysates were extracted using the PBSTDS lysis buffer containing a 10% protease inhibitor cocktail, incubated on ice for 30 min, and then centrifuged at 12000 rpm for 15 min at 4 °C. Gel electrophoresis was performed on 8% SDS-PAGE gel, and electroblotted onto PVDF membrane. Transferred membrane was blocked for 1 h at room temperature with 5% non-fat milk in Tris-Buffered Saline Tween and incubated with primary antibody overnight at 4 °C, AR (1:3000 dilution; Epitomics), Cx43 (1:2000 dilution; Sigma–Aldrich). After washing, membrane was incubated with its corresponding secondary antibody for 1 h at 4 °C. To assess the amount of AR and Cx43 protein loaded, the membranes were treated with stripping buffer (Applygen Technologies, Inc., China) for 30 min at room temperature and reprobed β-actin (1:2000 dilution; Santa Cruz Biotechnology). Immobilized antibodies were detected by enhanced chemiluminescence (Pierce Chemical Co.), with the relative level in each sample normalized to β-actin. Densitometric analysis was performed with ImageJ software (National Institutes of Health). Data shown were representative of at least three independent experiments with similar results.

2.6. Statistical analysis

Statistical analysis was performed using SPSS (version 16.0; SPSS, Inc.). Differences between control and testosterone-treated groups were assessed using Student's *t* test. A value of $P < 0.05$ was considered to be statistically significant. The values were expressed as mean ± standard deviation (SD).