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Co-administration of insulin with a gonadotropin partly improves ovulatory responses of estrogen-deficient mice

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ABSTRACT

Administration of 17-βestradiol (E2) with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) can induce ovulation in estrogen-deficient (ArKO) mice; nevertheless, ovulatory efficiency and rate are low. In this study, effects of insulin on the ovulatory responses were investigated. In ArKO ovary, hCG signal was found to be transmitted in an uncoordinated manner when phosphorylation levels of signaling molecules are examined. Co-administration of insulin with hCG improved the transmission of hCG signal as well as the ovulatory efficiency in ArKO mice. It also improved the ovulatory rate but far below the wild-type rate. Gene expression analysis demonstrated that *Cyp11a1* and *Cyp17a1* mRNAs were significantly induced 4 h after PMSG administration in the wild-type ovary, but not in ArKO ovary. Collectively, these results suggest that insulin improves ovulatory responses of ArKO mice, but it fails to ameliorate follicular dysfunctions caused possibly by an inappropriate intraovarian milieu during follicular maturation.

1. Introduction

Ovarian follicles, each of which is composed of an oocyte and two types of somatic cell, named granulosa and thecal cells, are the functional compartment for female reproduction. The ovary harbors follicles at various developmental stages, including primordial, primary, preantral and antral follicles. Among antral follicles, only selected follicles attain the preovulatory stage, whereas the remainder undergo atresia, which is a hormonally controlled process (Adhikari and Liu, 2009; Barnett et al., 2006; Lintern-Moore and Moore, 1979; McGee and Hsueh, 2000). During transition from the preantral to the antral follicular stage, the regulatory mode of follicular development and differentiation shifts from a gonadotropin-independent to -dependent fashion. Follicle-stimulating hormone (FSH), one of the pituitary gonadotropins, is known to induce granulosa cell proliferation and differentiation (Richards, 1994). Recent study further indicated that FSH acts as a coordinator to ensure the synchronous development of oocytes, granulosa and thecal cells of the antral follicles (Demeestere et al., 2012). LH action is also essential for follicular development from the antral stage onward (Zhang et al., 2001). In particular, LH is a critical mediator of events at the periovulatory stage. An abrupt increase in circulating levels of LH, called LH surge, is a vital stimulus to trigger a series of events leading to meiotic resumption of oocyte, cumulus expansion, follicle rupture (ovulation), and terminal differentiation of the granulosa and thecal cells to luteal cells (Richards et al., 1998). Thus, successful ovulation is the result of orchestrated regulation of multiple signaling cascades elicited by pituitary FSH and LH (Espey and Richards, 2002).

The classical signaling pathway triggered by the stimuli of FSH and LH after binding to respective G-protein-coupled receptors, FSH receptor and luteinizing hormone receptor (LHCGR), is an adenylyl cyclase/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway that phosphorylates and activates a transcription factor, cAMP-response element-binding protein (CREB), resulting in transcriptional regulation of a number of target genes of the gonadotropins (Hunzicker-Dunn and Maizels, 2006; Richards, 2001). Although the cAMP/PKA pathway is critical and regulates many ovarian functions, other signaling pathways were documented to be activated by the gonadotropins, which lead to the phosphorylation of thymoma viral proto-oncogene (AKT, also known as protein kinase B), p38MAPK and extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Fan et al., 2009; Gonzalez-Robayna et al., 2000; Hunzicker-Dunn and Maizels, 2006; Richards, 2001; Wayne et al., 2007; Zeleznik et al., 2003). Thus, perturbation of phosphorylation events of these signaling molecules leads to the impairment of ovarian physiology (Fan et al., 2009; Zeleznik et al., 2003).

Female reproductive function is also dependent on sex-steroid hormones produced by ovarian follicular cells (Drummond and

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Findlay, 1999). FSH induces E2 production in granulosa cells. The synthesized E2 plays critical roles in an autocrine or paracrine manner for granulosa cells to grow and differentiate. Vital roles of E2 in ovarian physiology have been confirmed by analyses of genetically inactivated mouse models of aromatase (Fisher et al., 1998; Toda et al., 2001), an enzyme responsible for the conversion of androgens to estrogens (Simpson et al., 2002). Although follicles at all stages of maturation were observed (Britt et al., 2002), all trials to induce ovulation in ArKO mice with exogenous gonadotropins were unsuccessful (Fisher et al., 1998; Huynh et al., 2004; Toda et al., 2001) until a recent report demonstrating that the administration of appropriate doses of E2 and gonadotropins at certain time points could induce ovulation in immature ArKO females (Toda et al., 2012). Nevertheless, high doses of gonadotropins were absolutely required to transduce the ovulatory signals into ovaries of ArKO mice. Furthermore, around 70% of mice treated for ovulatory induction actually showed ovulation and the numbers of the cumulus-oocyte complexes released into the oviduct per mouse were significantly fewer in ArKO mice than those in wild-type (WT) mice induced ovulation. Clarifying the molecular basis of these modest ovulatory phenotypes of ArKO mice advances our understanding of the roles of estrogens in the ovulatory process and might provide new therapeutic options for the treatment of female infertility. Thus, in the present study, we examined the ability of ArKO ovaries to transmit ovulatory signals by analyzing phosphorylation levels of signaling molecules such as CREB, p38MAPK and ERK1/2 after gonadotropin administration. We also analyzed the effects of insulin on the ovulatory responses and phosphorylation levels in ArKO mice, as insulin was shown to play important roles in ovarian physiology; stimulation of progesterone synthesis in porcine ovarian granulosa cells (Romero et al., 1993), amplification of LH-stimulated steroidogenesis in human theca cells (Nestler et al., 1998) and rat theca cells (Carvalho et al., 2003; Kayampilly et al., 2010), enhancement of LH effects in porcine granulosa cells (Sekar et al., 2000), and female infertility due to deletion of insulin receptor substrate-2 (Burks et al., 2000). Furthermore, quantitative RT-PCR (qRT-PCR) analysis was carried out on ovarian RNAs in order to clarify the differences of physiological conditions of ovaries between WT and ArKO mice.

2. Materials and methods

2.1. Animals

Animals were treated according to institutional animal regulations. In the present study, mice at 4 weeks of age were used, because we would like to avoid unnecessary complications derived from estrus cycles in WT mice and obese and hemorrhagic phenotypes of ArKO females (Toda et al., 2001). They were maintained on a 12-h light/dark cycle at 22–25 °C and given water and rodent chow (CE-2, Oriental Yeast Ltd., Tokyo, Japan). ArKO mice were generated using an aromatase disruption vector by replacing an 87-bp fragment located within exon 9 of the *Cyp191a* gene with a neomycin resistance gene derived from pMC1-neo (Toda et al., 2001). E14-1 embryonic stem cells targeted with the *Cyp191a* gene were injected into blastocysts of C57BL/6J mice to generate chimeric mice. The chimeric mice were bred with C57BL/6J mice to generate a mouse line with a C57BL/6J genetic background (Toda et al., 2008). The heterozygous offspring were mated to obtain ArKO mice. WT mice were injected intraperitoneally (i.p.) with 5 international units (IU) of pregnant mare serum gonadotropin (PMSG) (Serotropin®, ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) followed 48 hours later by 5 IU of human chorionic gonadotropin (hCG) (Wako Pure Chemical Industries, Osaka, Japan) to induce ovulatory responses. ArKO mice received subcutaneous injection of E2 (Sigma-Aldrich Corp., Tokyo, Japan) (18 mg/kg body weight), which was dissolved in sesame oil (Nacalai Tesque, Kyoto, Japan) at a concentration of 1.87 mg/ml, on day 1. They were then injected with E2 and 25 IU of PMSG on day 4, E2 on day 5 and 25 IU of hCG on day 6 (48 h after PMSG injection) (see Table 3). Some groups of mice received insulin (0.75 IU/kg body weight) together with hCG, a dose used for the insulin tolerance test for ArKO mice (Toda et al., 2010). Mice were injected between 1400 and 1600 on the day. The numbers of released cumulus-oocyte complexes (COCs) were counted by oviductal inspection 15 h after hCG injection. To investigate the signaling responses of ovaries to hCG, the ovaries were collected at 0, 20, 60 or 120 min after hCG injection for WT mice and at 0 or 60 min after hCG injection for ArKO mice, and snap-frozen in liquid nitrogen. For immunohistochemistry, ovaries were collected at 0 or 60 min after hCG injection and immediately fixed in 10% (v/v) buffered formaldehyde. To investigate mRNA expression, the ovaries were collected before hCG injection or 4 h after the hCG administration to PMSG-primed mice (see Table 3, Groups 1, 2, and 3, n = 5). Ovaries were also collected from WT mice without treatment, or 4 h or 48 h after 5 IU of PMSG injection or from ArKO mice without treatment, or 4 h or 48 h after E2 plus 25 IU of PMSG injection. The ovaries were soaked in RNA*later*® (Ambion Inc., Austin, USA) and stored at –20 °C until use.

2.2. Immunoblot analyses

The ovaries from each mouse were sonicated to prepare homogenates in 50 µl of homogenization buffer consisting of 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 0.1% (w/v) SDS, a protease inhibitor cocktail (Roche Diagnostics GmbH, Germany) and a phosphatase inhibitor cocktail (Pierce, Rockford, IL, USA). Protein concentrations of the ovarian homogenates were quantified using a bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA).

The samples of ovarian homogenates (12 µg of protein/lane) were separated on 10% (w/v) SDS-PAGE and transferred to polyvinylidene difluoride membrane filters (Bio-Rad Laboratories, Inc., CA, USA). The membrane filters were blocked with 5% (w/v) bovine serum albumin solution (Wako Pure Chemical Industries, Osaka, Japan) in 20 mM Tris-HCl (pH 7.6), 0.14 M NaCl and 0.05% (w/w) TWEEN®20 (Sigma-Aldrich, St. Louis, MO, USA) (TBS-T) for 2 h. The membranes were then incubated with primary antibodies in Immunoshot® reagent 1 (Cosmo Bio Co., Ltd., Tokyo) overnight at 4 °C. After washing with TBS-T, they were reacted with anti-rabbit horseradish peroxidase-conjugated IgG as the secondary antibody for 1 h at 25 °C, washed with TBS-T and incubated with Luminata™ Crescendo Western HRP Substrate (Millipore Corporation, MA, USA). The immunoreacted bands were visualized with luminescent image analyzer, ImageQuant™ LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and quantified by Image Gauge Ver. 3.2 (Fujifilm Corp. Tokyo), by which the immunoreacted bands were expressed as numbers with arbitrary units. Using these numbers with arbitrary units, ratios of phosphorylated forms to non-phosphorylated forms of the signaling molecules were calculated. Fold enhancement in the phosphorylation level of each signaling molecule was obtained by dividing the ratio after the hCG treatment by the ratio before hCG treatment. Means of the ratio of CREB, p38MAPK, ERK1 and ERK2 before hCG treatment in WT mice (Table 3, Group 1) were respectively 0.21 ± 0.03, 0.41 ± 0.04, 0.17 ± 0.04 and 0.48 ± 0.07 (n = 7, three independent experiments), those before hCG treatment in ArKO mice (Groups 2 to 4) were respectively 0.38 ± 0.05, 0.54 ± 0.07, 0.29 ± 0.04 and 0.42 ± 0.04 (n = 7, three independent experiments) and those before hCG treatment in ArKO mice (Group 5) were respectively 0.19 ± 0.04, 0.53 ± 0.02, 0.20 ± 0.04 and 0.41 ± 0.09 (n = 5, three independent experiments). These ratios were displayed as 1 in Figs 1 to 3.