The concentration-dependent effect of progesterone on follicle growth in the mouse ovary

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Abstract. Follicle growth in the mammalian ovary is coordinately controlled by multiple factors to sustain periodic ovulation. In this study, we investigated the role of progesterone on follicle growth in the mouse ovary. As the concentration of progesterone changes during the estrus cycle, we cultured the sliced mouse ovary in a medium containing 10 ng/ml, 100 ng/ml, and 1 μg/ml progesterone. Progesterone promoted the growth of primordial to primary follicles at 100 ng/ml, while it suppressed the growth of secondary follicles at 1 μg/ml. Follicles at other developmental stages in the cultured ovary were unaffected with different concentrations of progesterone. The number of ovulated oocytes increased in the medium containing 100 ng/ml progesterone but decreased in the presence of 1 μg/ml progesterone. Follicles expressed two types of progesterone receptors, progesterone receptor (PGR) and PGR membrane component 1 (PGRMC1). While PGR shows transient expression on granulosa cells of Graafian follicles, PGRMC1 expresses in granulosa cells of developing follicles. These results suggest that progesterone controls the growth of developing follicles through PGRMC1. Our study shows that the effect of progesterone on ovulation and follicle growth in mouse ovary is dependent on the concentration of progesterone and the follicle stage.

Key words: Follicle growth, Ovary culture, Progesterone, Progesterone receptor membrane component 1 (PGRMC1)

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Materials and Methods

Animals

Ovaries were isolated from 4-week-old female ICR mice (Japan SLC, Shizuoka, Japan). Mice were housed in an environmentally controlled room maintained at 23 ± 1°C with a 12-h light/12-h dark cycle. Animal care and experiments were conducted in accordance with the...
with the Guidelines for Animal Experimentation of Aichi Medical University. Experiments in this study were approved by The Animal Care and Use Committee of Aichi Medical University.

**Culture of ovarian tissue**

Ovaries from 4-week-old mice were sliced at a thickness of several hundred micrometers using a microtome blade (Leica Biosystems, Nussloch, Germany) under a stereoscopic microscope (Leica Biosystems). Ovarian tissue slices were placed in a 30-mm cell culture insert (Merck Millipore, Darmstadt, Germany), which was subsequently placed in a 3.5-cm culture dish (AGC Techno Glass, Shizuoka, Japan). Ovarian slices were cultured in the minimum essential medium alpha (MEM-alpha) GlutaMax (Gibco, Carlsbad, CA, USA) supplemented with 5% (v/v) fetal bovine serum (FBS, Gibco), 100 mIU/ml FSH from human pituitary gland (Sigma-Aldrich, St Louis, MO, USA), and 10 mIU/ml LH from equine pituitary gland (Sigma-Aldrich) under conditions of 5% CO2 and 37°C. The cultured ovarian tissue slices were treated with 100 mIU/ml of LH for 12 h every 4 days to reproduce the physiological LH surge. Concentrations of FSH and LH used were based on previous experiments [10, 11]. The effect of P4 on follicle growth was evaluated after adding P4 (10 ng/ml, 100 ng/ml, and 1 μg/ml) (Sigma-Aldrich) to the culture medium, followed by culture for 18 days. In this experiment, one ovary was cut into four slices; two of these slices were treated with dimethyl sulfoxide (DMSO) alone and the other two, with P4 dissolved in DMSO. Four or five ovaries were cultured with each concentration of P4.

**Imaging of cultured ovarian slices**

Cultured ovarian slices were imaged at intervals of 24 h using a confocal microscope (LSM 710, Carl Zeiss Microimaging, Oberkochen, Germany), with a Z-step size of 5 μm and Z-stack thickness approximately 150 μm.

**Analysis of follicle growth in cultured ovarian tissue slices**

The area of each follicle observed in cultured ovarian tissue slices was measured using ImageJ software (http://rsbweb.nih.gov/ij/). The outline of the follicle in captured images was traced with a tablet pen (Intuos, Wacom, Saitama, Japan); the number of pixels in each follicle was measured and converted into area (μm²). The follicle area was measured at 24-h intervals to track follicle growth in cultured ovarian tissue slices. The development of follicles was classified into three stages — primordial-primary, secondary, and antral — based on the number of granulosa cell layer and measured the area of follicles. Based on these results, we established the criteria for the classification of follicles based on the area. The detailed information is presented in our previous report [11].

**Measurement of 17β-estradiol in the cultured ovary and culture medium**

After sliced ovaries were cultured for 2 days, P4 (100 ng/ml) was added into the culture medium; equal volume of DMSO was used as a control. Following incubation for 12 and 24 h, cultured ovaries and the culture medium were collected. The right and left ovary from the same mouse was used as the control and test (P4-treated), respectively. Proteins from the ovary tissue were extracted using Tissue Extraction Reagent I (Invitrogen, Carlsbad, CA, USA) containing protease inhibitor cocktail (Clontech, Mountain View, CA, USA). The concentration of 17β-estradiol in the ovary extract and culture medium was measured using 17β-estradiol high-sensitivity enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Science, Farmingdale, NY, USA) following the manufacturer’s protocol. Three ovaries were cultured in each condition and used in this experiment.

**Staining of ovarian sections for PGRMC1**

Ovaries of 4-week-old female mice were fixed with SUPER FIX (KURABO, Osaka, Japan) and embedded in paraffin. The embedded ovary was sliced into 6-μm thick serial sections. Endogenous peroxidase activity was blocked by treatment with 0.6% hydrogen peroxide/methanol at room temperature for 40 min. Sections were treated with anti-PGRMC1 antibody (2 μg/ml; Abcam, Cambridge, UK) or rabbit IgG (2 μg/ml; Vector Laboratories, Burlingame, CA, USA) and subjected to immunoperoxidase staining using the VECTASTAIN ABC Kit (Vector Laboratories) according to the standard protocol. All sections were counterstained with hematoxylin (MUTO PURE CHEMICALS, Tokyo, Japan).

**Measurement of progesterone in serum of mice**

The estrus cycle of female mice was checked by vaginal smear method at 0900 h and 50 μl blood at proestrus, estrus, metestrus, diestrus, and pregnant day 7 was collected from the caudal vein of the mouse at 1700 h. Blood serum was collected by centrifugation at 25,000 × g for 10 min at 4°C. The concentration of P4 in the serum of three mice at each stage was measured using Progesterone ELISA kit (Enzo Life Science) following the manufacturer’s protocol.

**Statistical analyses**

Statistical analyses were performed using the software R (http://www.r-project.org/). First, the normality of all data was evaluated using the Shapiro-Wilk normality test (P < 0.05). We then analyzed the normally distributed data using the unpaired t-test/one-way analysis of variance (Figs. 2, 3, 4 and 6) and non-normally distributed data using the Mann-Whitney U test (Fig. 2). The normality of the data for follicular area differed between the culture days, the test chosen (unpaired t-test/one-way ANOVA, or Mann-Whitney U test) (Fig. 2).

**Results**

The effect of progesterone on follicle growth in the ovary

We determined the effect of P4 on follicle growth in the ovary by adding P4 to the culture medium at 10 ng/ml, 100 ng/ml, and 1 μg/ml concentrations (Fig. 1). These P4 concentrations reproduced the change in blood P4 concentration during estrus cycle. We analyzed the effect of each P4 concentration by tracking the growth of each follicle in the cultured ovary during 18 days in culture. We measured the follicle area at 24-h intervals as the index of follicle growth (Fig. 2). Based on the area of each follicle on culture day 1, the growth of follicle was divided into three stages as follows: primordial-primary follicle, < 3000 μm²; secondary follicle, 3000–10000 μm²; antral...
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Details of follicle classification were described in our previous report [11]. At 10 ng/ml, P₄ showed no effect on follicle growth (Fig. 2A, D, and G). While P₄ promoted growth of primordial-primary follicles at 100 ng/ml (Fig. 2B), it suppressed the growth of secondary follicles at 1 μg/ml (Fig. 2F). At 100 ng/ml and 1 μg/ml, P₄ exhibited no effect on other follicle stages (Fig. 2C, E, H and I). Significant differences were observed in the growth of secondary and antral follicles between 10 and 100 ng/ml P₄ at some time points (Fig. 2D, E and G). At these time points, some of the follicles ovulated or degenerated and hence were excluded from the measurement, thereby decreasing the average follicle area. Therefore, these significant differences are not contributed by the effect of P₄ on follicle growth. In addition, we cultured ovarian tissues in presence of 10 μg/ml P₄, but its effect on the follicle growth and ovulation was same as seen with 1 μg/ml P₄ (data not shown). These results indicate that the effect of P₄ on follicle growth is dependent on its concentration.

The effect of progesterone on ovulation

During the 18-day culture period, we counted the number of follicle, > 10000 μm² (Fig. 2). Images of the cultured ovaries. Images of cultured ovaries of culture day 1 and culture day 14 are presented. Except the cultured ovary containing 1 μg/ml P₄, each cultured ovary ovulated some oocytes before culture day 14. Scale bar: 200 μm.
ovulated oocytes in the culture medium at each P4 concentration (Fig. 3). At 100 ng/ml, P4 significantly increased the average number of ovulated oocytes (control, 6 oocytes; 100 ng/ml P4, 10.8 oocytes). On the other hand, P4 treatment at 1 μg/ml concentration tended to decrease the number of ovulated oocytes (control, 6 oocytes; 1 μg/ml P4, 2.8 oocytes; no significant difference, P = 0.12). At 10 ng/ml, P4 showed no effect on ovulation (control, 6.3 oocytes; 10 ng/ml P4, 5.5 oocytes).

The effect of progesterone on the concentration of 17β-estradiol in the cultured tissue and culture medium

Progesterone is converted to 17β-estradiol in follicles. Thus, the growth of follicles and process of ovulation may be promoted by P4 either directly or through 17β-estradiol. We measured the concentration of 17β-estradiol in the cultured ovary and culture medium with ELISA. The concentration of 17β-estradiol recorded was as follows: cultured ovary of a 12-h culture, P4(−): 255.5 ± 6.3 pg/ml, P4(+): 252 ± 13.0 pg/ml; culture medium of a 12-h culture, P4(−): 30.0 ± 7.0 pg/ml, P4(+): 27.3 ± 3.8 pg/ml; cultured ovary of a 24-h culture, P4(−): 276.8 ± 33.8 pg/ml, P4(+): 283.1 ± 7.1 pg/ml; and culture medium of a 24-h culture, P4(−): 33.9 ± 5.1 pg/ml, P4(+): 38.3 ± 17.0 pg/ml (Fig. 4). P4 exhibited no effect on the concentration of 17β-estradiol in the cultured ovary and culture medium, indicative of its direct action on the follicle growth.

The expression of PGRMC1 in follicles

Progesterone receptors are expressed in Graafian follicles only during the periovulatory period. We, therefore, evaluated the expression of PGRMC1 in follicles (Fig. 5). The expression of PGRMC1 was detected in granulosa cells of some primordial follicles (Fig. 5A) and all of the primary, secondary, and antral follicles (Fig. 5C and E). The level of expression increased with the growth of follicles. Staining with rabbit IgG revealed weak signals in granulosa cells of secondary and antral follicles (Fig. 5D and F); however, these signals were much weaker than those recorded with anti-PGRMC1 antibody (Fig. 5C–F). As shown in Figs. 5C and D, the expression of PGRMC1 was detected in granulosa cells of secondary and antral follicles. In addition, the expression of PGRMC1 was detected in ovarian surface cells and some stromal cells (Fig. 5A).

The change in the concentration of P4 in mouse blood during estrus cycle and pregnancy

In the cultured ovary, the effect of P4 was concentration dependent. We investigated the change in the concentration of P4 in the mouse blood by measuring the serum concentration of P4 at each estrus stage and pregnant day 7. The concentration of P4 detected at each stage was as follows: proestrus, 8.7 ± 1.7 ng/ml; estrus, 10.1 ± 2.3 ng/ml; metestrus, undetected; diestrus, undetected; and pregnant day 7, 10.2 ± 1.8 ng/ml (Fig. 6). The level of P4 at proestrus, estrus, and pregnant day 7 was almost similar. On the other hand, P4 was undetected at metestrus and diestrus, owing to its low concentration. Blood was collected from same mice at proestrus and estrus; blood collection at estrus was 24 h following blood collection at proestrus. These results indicate that the concentration of P4 in blood of the mouse varies during estrus cycle and pregnancy.

Discussion

In this study, we evaluated the effect of P4 on follicle growth and ovulation in the mouse ovary and found that the P4 effect differed between follicle stages in a concentration-dependent manner (Figs. 1, 2 and 3). In the ovary, P4 is produced in both follicular and CL cells [12, 30]. The concentration of P4 in blood regularly changed based on the dynamics of ovarian tissue, follicular development, ovulation, follicle atresia, as well as the formation and atresia of CL.
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follicle growth [36]. Therefore, the effect of P4 on follicle growth may not be exerted directly through blood but through follicles and CLs in the ovary. The follicle environment in the ovary is important for the regulation of follicle growth because P4 production is higher in CLs than in follicles and P2 production in follicles varies with the follicle stage [12, 30]. Therefore, the localization and developmental stage of follicles may determine the growth speed of each follicle. In any case, P3 is one of the key factors controlling follicle growth based on the physiological condition of the ovary.

P4 is metabolized to estrogens and androgens in follicles of rodents [37–39]. Estrogens have some roles in the follicle development [40, 41]. We evaluated whether P3 was converted into 17β-estradiol in the cultured ovary and studied its ability to bind to receptors on follicles. The treatment with P3 failed to alter the concentration of 17β-estradiol (Fig. 4), indicating the direct effect of P4 on follicle growth through its receptor.

P4 transduces signal to granulosa cells through PGR. However, PGR is not expressed in granulosa cells of developing follicles [42–44]. A recent study revealed high-level expression of PGRMC1 in granulosa cells of developing follicles [26]. Studies have shown that P4 suppresses the proliferation and apoptosis of cultured granulosa cells [17, 34, 45], consistent with our observation that P4 suppressed the growth of secondary follicles at high concentration. However, this is contradictory to our observation that P4 promoted the growth of primordial-primary follicle at 100 ng/ml (Fig. 2B and F). The contrasting result may be attributed to two factors. First, the effect of P4 varied between the granulosa cells from the primordial-primary and secondary-antral follicles. Second, the interaction between follicles in the ovary may alter the effect of P4. The growth of follicles must be coordinated within the ovary; the interaction between follicles is important in controlling the growth of each follicle. Secondary or antral follicles may influence the effect of P4 on primordial-primary follicles. In this case, the actual in vivo effect of P4 on follicular growth at each stage is not observed in cultured granulosa cells. We observed the expression of PGRMC1 only in a few primordial follicles (Fig. 5A). Therefore, P3 may mainly promote the growth of primary follicles (Fig. 2B).

Our results show that the P3 concentration in blood or ovary controls the follicular growth at each stage. The concentration-dependent effect of P4 is important for sustaining long-term periodic ovulation. The P2-PGRMC1 pathway is thought to be involved in premature ovarian failure [35]. Thus, it is important to understand the regulatory mechanism of follicle growth through the P2-PGRMC1 pathway not only in the cultured granulosa cells but also in the ovary containing follicles at various developmental stages, CLs, and other many tissues. Culture methods that mimic the in vivo conditions may help us study the effect of factors controlling the follicle growth in the ovary. Our culture method is useful to reveal the regulatory mechanism of the follicle growth in the ovary.

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