NOTE *Toxicology*

Comparison of *Aryl Hydrocarbon Receptor* Gene Expression in Laser Dissected Granulosa Cell Layers of Immature Rat Ovaries

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ABSTRACT. In order to understand ovarian toxicity of aryl hydrocarbon receptor (AhR) agonists, *in situ* gene expression of the *AhR* was examined during follicle development in immature rats. *In situ* hybridization on frozen sections of ovaries from 24-day-old Sprague-Dawley rats showed that the *AhR* mRNA was localized in the granulosa cells and occasionally in the theca cells of the follicles irrespective of the developmental stage. *In situ* gene quantification on granulosa cell layers collected by laser microdissection further revealed that the granulosa cells expressed less *AhR* mRNA according to development of belonging follicles, but more *β-subunit of inhibin A* mRNA, a quality control gene. These results may help to elucidate vulnerable developmental stages of follicles to toxicities of the AhR agonists.

KEY WORDS: aryl hydrocarbon receptor (AhR), β-subunit of inhibin A, follicular development, laser microdissection, ovary.

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Aryl hydrocarbon receptor **(**AhR) is a ligand-activated transcription factor that mediates toxicity of AhR agonists, such as the polycyclic aromatic hydrocarbons (PAHs) [2, 6, 12], and is expressed in mammalian ovaries [2]. Treatment with AhR agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [16] and 3,3',4,4',5-pentachlorobiphenyl (PCB 126) [17] increases *cytochrome P450 (CYP) 1A1* mRNA, a phase I enzyme, in rat ovaries, indicating that AhR signaling cascades are activated. Ligand-activated AhR regulates the ovarian atresia in primordial and primary follicles via activation of the Bax signaling pathway [9, 10], and ovotoxicity induced by PAH is prevented by the concomitant treatment with AhR antagonists, thus indicating that AhR is the key mediator of ovotoxicity [11]. Although AhR has been confirmed in oocytes, granulosa and theca cells of growing follicles in the rat by its mRNA and protein expression levels [4, 13], little has been studied precise expression level of the AhR in the follicles at each developmental stage. Therefore, the present study was designed to examine *in situ* expression of the *AhR* mRNA by *in situ* hybridization (ISH) and gene quantification of laser-dissected granulosa cell layers from healthy follicles at different developmental stages. The mRNA encoding the β-subunit of inhibin A was also quantified to confirm the quality of the dissected specimens, since striking and dynamic changes of the *β-subunit of inhibin A* mRNA have been detected in rats during developmental maturation of the follicles [18], and increased inhibin A secretion occurs with the advancing estrous cycle up to the LH surge [8].

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In this study, three females born in our animal facility from pregnant Sprague-Dawley rats (Charles River Japan, Yokohama, Japan) were used after weaning on postnatal day (PND) 21 in accordance with the guidelines approved by the Animal Research Committee of Azabu University. On PND 24, the rats were sacrificed by decapitation, and their ovaries were collected, frozen in liquid nitrogen and stored at –80°C until analyses.

For the ISH and the *in situ* gene quantification, frozen sections of 8-μm and 10-μm thickness were made, respectively, from each ovary using a cryostat. After thawing, the frozen sections for ISH were processed as previously described [14]. The anti-sense or sense digoxigenin-labeled RNA probe was generated by transcription with digoxigenin-labeled deoxy-UTP (Roche, Mannheim, Germany) and the appropriate T7 or SP6 polymerase (Roche) from sequence-confirmed amplicons (418–1069, GenBank ID: U04860) cloned in PGEM-T Easy vectors (Promega, Madison, WI, U.S.A.), and was hybridized at 60°C overnight. After washing under an appropriate condition [14], positive signals were detected using DIG Nucleic Acid Detection Kit (Roche) and visualized by nitroblue tetrazolium chloride. Duplicate sections were hybridized with each sense and anti-sense probes in a single experiment, and were confirmed no staining in the cells hybridized with sense probe (data not shown).

As shown in Fig. 1, irrespective of the developmental stage of the follicles, *AhR* mRNA was localized in granulosa cells and occasionally in theca cells. Intensities of the positive signals in the granulosa cells were similar among the follicles, but those in the theca cells might be less abundant as compared with those in the granulosa cells. Oocytes in the specimen were not evaluated because of their defective morphology in this study.

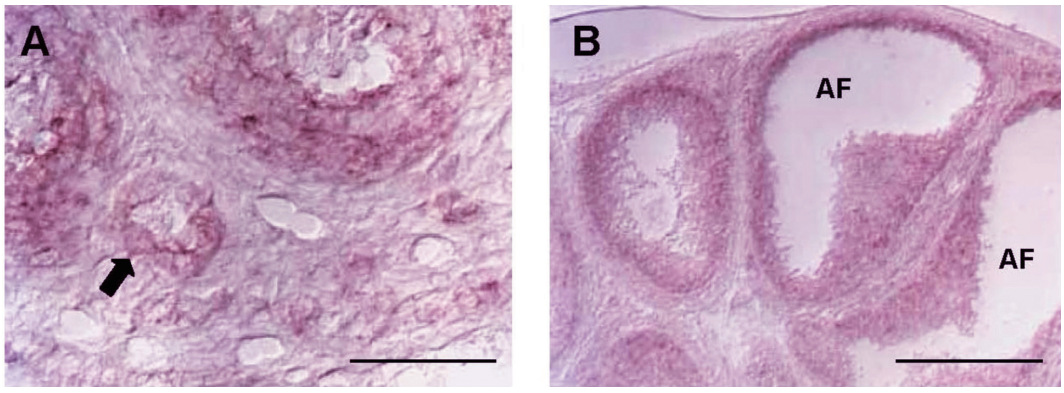

Fig. 1. *In situ* hybridization of *AhR* mRNA in immature rat ovaries obtained at 24 days of age. Photographs were visualized using a phase-contrast microscope. *AhR* mRNA is expressed in granulosa cells and theca cells of growing follicles from preantral follicles (A, arrow) to antral follicles (B, AF). Purple red fields indicate *AhR* mRNA-expressing cells. Bars=100 μm (A) or 200 μm (B).

In situ gene quantification was performed on laser dissected granulosa cell layers from the frozen sections. At least six serial frozen sections were cut from each ovary, and the first 2 sections were used for morphological evaluation of the follicles, i.e., the maximum diameter, which was determined when the oocyte nucleolus was observed and morphology of the follicles was determined from the first section that was stained with hematoxylin and eosin (H-E); the expression of inhibin-α was determined from the second section by immunohistochemistry of inhibin-α [14]. Among the follicles on these two sections, those of which granulosa cells expressed inhibin-α but exhibited no nuclear pyknosis were classified from their maximum diameter into preantral follicles (100–200 μm maximum diameter), early antral follicles (>300 μm maximum diameter with scattered cavity), and antral follicles (>300 μm maximum diameter with single cavity). Then, 60 healthy preantral, 20 early antral and 10 antral follicles in each ovary were selected for dissection.

Laser microdissection (LMD) was performed using at least four sections as previously described [14]. The dissected granulosa cell layers from each ovary were collected into tubes with an appropriate volume of buffer RLT (RNeasy Mini Kit, Qiagen, Valencia, CA, U.S.A.). Total RNA was extracted from the dissected granulosa cell layers, and the amounts of mRNAs encoding *AhR* and *β-subunit of inhibin A* were quantified using real-time PCR (PRISM 7700 Sequence Detector, Applied Biosystems, Foster City, CA, U.S.A.), as described previously [14]. The specific TaqMan probe (Applied Biosystems) and primer pairs for *AhR* (GenBank ID: U04860) and *β-subunit of inhibin A* (GenBank ID: NM_017128) were designed using Primer Express software (Applied Biosystems) based on their sequences. The *AhR* TaqMan probe was 5'-CTGCTCAAGTCTGCCGAGTAGGCTTCA-3'. The *AhR* forward primer sequence was 5'-GCAGTCTGAAGGTGGCCAAT-3', and its reverse primer sequence was 5'-GCAAGTTTCAGAGTCCGAGCA-3'. The *β-subunit of inhibin A* TaqMan probe was 5'-CTTGGGCACTCACCCTCACAATAGTTGG-3'. The *β-subunit of inhibin A* forward primer sequence was 5'-CCCAGAGGTGCCTGCTATGT-3', and its reverse primer sequence was 5'-CATTGCTCCCTCTGGCTATCA-3'. For analysis of rodent *glyceraldehyde-3-phosphatase dehydrogenase (GAPDH)*, TaqMan Rodent GAPDH Control Reagents (Applied Biosystems) were used. All PCR amplifications were carried out in duplicate for each sample, and the mean gene expression values were calculated as a ratio to *GAPDH*, and expressed as values relative to the data of preantral follicles. In this study, a rat ovarian cDNA preparation of an arbitrary unit was employed as the assay standard. The levels of each mRNA and their estimated crossing points in each sample were determined relative to the standard preparation using the Sequence Detection System Software (Applied Biosystems). For PCR, each of the standard cDNA pools were diluted 1:10, $1:10^2$, $1:10^3$, $1:10^4$ and $1:10^5$ in sterile DW and then added to the individual tubes.

Obtained data was analyzed using JMP Statistical Analysis Software (SAS Institute). Initially, all the data were analyzed using a one-way analysis of variance (ANOVA). Then, significant differences among groups were analyzed by Turkey-Kramer's HSD test. A P value of less than 0.05 was considered to be statistically significant.

As shown in Fig. 2, the *β-subunit of inhibin A* remarkably increased in the granulosa cell along with the development of the follicles, whereby the granulosa cell in the antral follicle was estimated to express more than 10 times the amount of mRNA in the preantral follicles. Therefore, granulosa cells collected for the present quantification was validated to be assessable.

Although ISH showed no remarkable differences in signal intensity of *AhR* mRNA among the follicles at different developmental stages, gene quantification from granulosa cell layers revealed that less *AhR* mRNA was expressed in the granulosa cell layers from follicles at the more advanced developmental stages. The amount of mRNA in the granu-

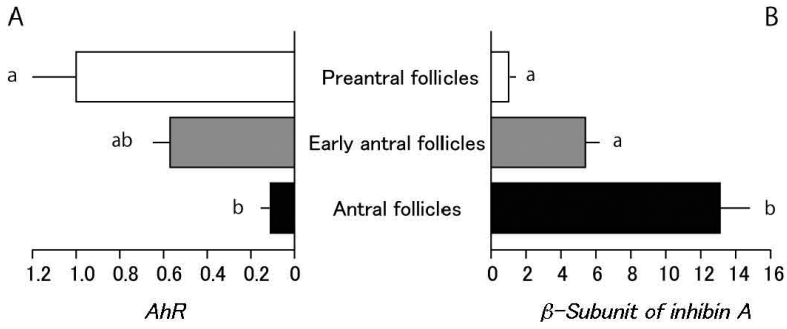

Fig. 2. The amounts of mRNAs encoding *AhR* (A) and *β-subunit of inhibin A* (B) in granulosa cell layers dissected from preantral (open column), early antral (shaded column) and antral (closed column) follicles in immature rat ovaries at 24 days of age. The data are normalized for *GAPDH*, and expressed as values relative to the data of preantral follicles. Each column and bar represents the mean value of three ovaries ± SE, respectively. Different letters next to each column indicate significant differences ($P<0.05$) among the groups.

losa cell layers from the antral follicles was estimated to be about 10 times smaller than that from the preantral follicles, and significant differences were observed between those from antral and early antral follicles and early antral and preantral follicles (Fig. 2A). Since LMD permits direct quantification of target tissues, the amounts quantified in the present study represent the amounts in the granulosa cell. Therefore, the present results indicate that expression of *AhR* mRNA in the granulosa cell decreases along with follicular development, and this is the first report to indicate the quantity of *AhR* mRNA in granulosa cells *in situ*.

Rationale for the decrease in the *AhR* mRNA is not explained from the present study, however, previous *in vitro* study has been shown that expression of the AhR protein and mRNA in the granulosa cells is decreased under the control of both FSH and estradiol [3]. During the transition from the preantral to the antral stage, the follicles acquire the ability to secrete more estradiol-17β depending on FSH, and we have demonstrated in immature rat ovaries that *P450 aromatase* mRNA is expressed more in the granulosa cell layers from antral follicles than those from preantral follicles when dissected by LMD [14]. Increased intrafollicular estradiol could reduce *AhR* mRNA in the granulosa cells of antral follicles. Interestingly, it has been found that the AhR could activate *aromatase* gene transcription cooperating with Ad4BP/SF-1 [1]. Furthermore, recent study on *AhR* knockout mouse indicates involvement of the AhR in estradiol biosynthesis and follicle growth during the prepubertal period [5]. Taken together these and the present results, the AhR may involve in activation of estradiol synthesis in the preantral follicle, but consequently increased intrafollicular estradiol may decrease the AhR expression during the transition to the antral follicles.

It has been reported that basal levels of *AhR* mRNA expression determines the sensitivity of the exposure to PAHs [7]. The present results obtained by the *in situ* gene quantification on the immature rat ovaries suggest that preantral follicles may be more sensitive to exposure to PAHs than antral follicles, as indicated by *in vivo* study [15].

In conclusion, expression of *AhR* mRNA in the granulosa cell of immature rat ovaries is decreased by the transition of follicles from the preantral to the antral stage. These results may help to elucidate vulnerable developmental stages of follicles to toxicities of the AhR agonists.

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