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Ovarian steroidogenesis and the role of sex steroid hormones on ovarian growth and maturation of the Japanese eel☆

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ABSTRACT

Three sex steroid hormones, estradiol-17β (E2), 11-ketotestosterone (11-KT), and 17α,20β-dihydroxy-4-pregnen-3-one (DHP), are well established as primary estrogen, androgen, and progestin, respectively, in teleost fish. Japanese eel, *Anguilla japonica*, would be a suitable candidate to study ovarian steroid physiology of fish because the ovarian growth and steroidogenesis is dormant under laboratory condition but can be induced by administration of exogenous gonadotropic reagents. In this review, we summarized our work on the function and production of sex steroid hormones in the ovary of the Japanese eel during ovarian growth and oocyte maturation artificially induced by treatment with extract of salmon pituitary. *In vitro* and *in vivo* assays suggest that 11-KT and E2 play primary roles in previtellogenic and vitellogenic growth of oocytes, respectively, whereas DHP is essential for induction of final oocyte maturation. We also reviewed the correlation between ovarian steroidogenesis to produce these sex steroid hormones, serum titers and gene expression.

1. Introduction

Sex steroid hormones play important roles in many physiological processes, particularly in the reproduction of vertebrates. In many species of teleost, three sex steroid hormones, i.e., estradiol-17β (E2), 11-ketotestosterone (11-KT), and 17α,20β-dihydroxy-4-pregnen-3-one (DHP), are abundantly produced in gonadal tissues under the control of pituitary gonadotropins (GTH), and are essential for critical steps of gametogenesis [1–3].

Japanese eels (*Anguilla japonica*) caught from the wild have immature gonads, which will not undergo further development under captive conditions. However, previous studies have demonstrated that gonadal development of eels can be easily induced by the administration of gonadotropic reagents, including extract of salmon pituitary (SP) in females [4] and human chorionic gonadotropin in males [5]. Furthermore, relevant *in vitro* culture systems of gonadal tissues to examine steroidogenesis and gametogenesis have been established both in female [6,7] and male eels [3]. Therefore, the Japanese eel provides an excellent model for studying the regulation of steroidogenesis and function of sex steroid hormones in gametogenesis because the gonadotropic effects of exogenous hormones can be readily assessed. Herein, we summarize our study conducted over the last decade on function and production of sex steroid hormones in the eel ovary.

2. Function of sex steroid hormones in the ovary of the Japanese eel

E2, 11-KT, and DHP have been identified as typical sex steroid hormones belonging to each class of sex steroid, estrogen, androgen, and progestin, respectively, in teleosts, including Japanese eel. The functions of these steroid hormones in oogenesis of the Japanese eel have been investigated using *in vitro* and *in vivo* assay systems. A schematic diagram demonstrating their primary roles is summarized in Fig. 1. Our data strongly suggest that these three steroid hormones are involved in different important phases of oocyte development, including previtellogenic and vitellogenic growth and maturation.

2.1. E2

A major estrogen, E2, controls pivotal physiological events in female reproductive cycles in all vertebrates studied to date. A previous study has focused particularly on lower vertebrates including teleost, and has established that one of the primary roles of E2 is to stimulate vitellogenin (Vtg: the precursor of egg yolk protein) expression in the liver, which, in turn, promotes oocyte growth [8]. Furthermore, the changes in Vtg transcript abundance in the Japanese eel liver during maturation artificially induced by SP [9]

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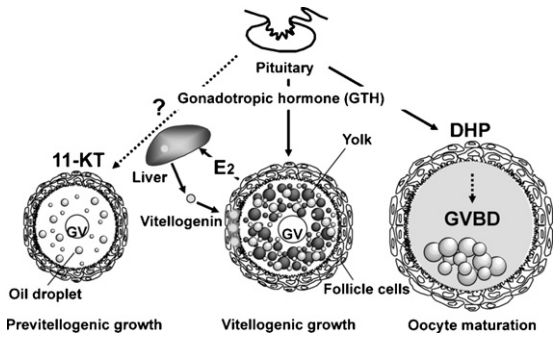

Fig. 1. A schematic diagram of the primary roles of E2, 11-KT, and DHP in oocyte development of the Japanese eel. These sex steroid hormones differentially regulate oocyte development; 11-KT and E2 control previtellogenic and vitellogenic growth of oocyte while DHP plays a pivotal role in final oocyte maturation.

were demonstrated to correlate well with the serum levels of E2 [6] and Vtg [10]. These findings suggest that SP-induced E2 regulates Vtg production at the transcriptional level. In fact, E2-induced Vtg expression in the liver of the Japanese eel has also been demonstrated both *in vitro* [11] and *in vivo* [12]. We have also confirmed that E2 upregulated hepatic Vtg expression *in vitro*, and this E2 action was enhanced by growth hormone, in part via elevation of gene expression of estrogen receptor α subtype (Kazeto et al., unpublished).

While many studies strongly suggest that E2 functions as a natural inducer of ovarian differentiation in fish [13], there is currently no evidence that E2 plays a critical role in natural ovarian differentiation of the Japanese eel, largely because almost all elvers of eel differentiate into males under captive conditions [14]. However, E2 would likely associate with ovarian differentiation in the Japanese eel since oral administration of E2-formulated diet has been shown to result in feminization at a high rate [14].

2.2. 11-KT

Androgens are generally considered to play a central role in regulating testis differentiation, spermatogenesis, development of secondary sexual characters and sexual maturation in male teleosts [15]. 11-KT was originally identified as a unique androgen in teleost [16] and has been verified as the most potent androgen in the male [3,15]. However, it was reported in several species of fish, including those of the Anguillidae family, that 11-KT was also identified as the major androgen in the blood of females, in addition to males [17]. Furthermore, *in vivo* studies showed that treatment of immature female *Anguilla* spp. with 11-KT resulted in induction of reminiscent changes in the developmental transformation known as "silvering" (i.e., morphological change in the pectoral fin, larger size of eyes, and increased mass of ovary) [18]. In addition, it was reported that 11-KT induced ovarian growth accompanied by enlargement of previtellogenic oocyte showing increased number of oil droplets [18,19]. Similarly, *in vitro* culture has also demonstrated that 11-KT directly promoted late perinucleolar oocyte growth, and thus increased diameters of ovarian follicles in short-finned eel, *Anguilla australis* [20]. Induction of oil droplet accumulation in the oocytes has also been shown to require 11-KT and very low-density lipoprotein in the Japanese eel [21]. Recent research also demonstrated that transcripts of two subtypes of androgen receptor (ARα and ARβ) dominantly localized in ovarian follicle cells, and that the transcript abundance of ARs was high in ovaries during the developmental period when oocytes actively accumulate oil droplets [22]. These findings strongly suggest that 11-KT plays an essential role in controlling growth of the previtellogenic oocyte via ARs localized in the follicular cells in the Japanese eel.

2.3. DHP

It has been demonstrated in teleosts that final oocyte maturation (FOM) is induced by maturation-inducing steroid (MIS) produced in ovarian follicle layers [23]. Progestin, typically either DHP [2] or 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) [24], has been identified as a MIS. DHP was reported as a potent inducer of FOM both *in vitro* [25] and *in vivo*, in the Japanese eel [26]. Furthermore, examination of the effectiveness of various steroids to induce FOM revealed that DHP is the most potent inducer of FOM, followed by 17α-hydroxyprogesterone (OHP: precursor of DHP) and 20β-S [27]. Moreover, DHP was shown to be the most predominant product among steroid metabolites converted from pregnenolone by ovarian follicles at the maturational stage and showed the highest maturation-inducing activity, whereas trace amounts of putative 20β-S were detected [27]. Collectively, these findings indicate that DHP act as a MIS in the Japanese eel.

3. Ovarian steroidogenesis and changes in serum titer of sex steroid hormones during ovarian growth and oocyte maturation

Sex steroid hormones are synthesized from cholesterol with the aid of a variety of steroidogenic enzymes; therefore, the ability to produce sex steroid hormones is regulated by the activity of these enzymes, which is primarily controlled via the regulation of steroidogenic enzyme expression. We assessed the *in vitro* ability of eel ovaries to produce sex steroid hormones using two primary methods; one is radioimmunoassay (RIA) to determine the concentration of steroid hormones in culture media after incubation of ovarian follicles with radioinert steroid substrates and another is thin-layer chromatography (TLC) for analysis of steroid metabolites generated by conversion of radiolabeled steroid substrates. Furthermore, in order to gain a deeper understanding of eel ovarian steroidogenesis, we also paid considerable attention to isolation and characterization of cDNAs encoding steroidogenic enzymes, i.e., cytochrome P450 cholesterol side-chain cleavage [28], 3β-hydroxysteroid dehydrogenase (3β-HSD) [29], cytochrome P450 17α-hydroxylase/C17-20 lyase (P450c17) [30], 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) [31], cytochrome P450 aromatase (P450arom) [32], cytochrome P450 11β-hydroxylase (P45011β) [33] and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) [34]. We discuss here on steroidogenic activity, expression of the steroidogenic enzymes in the eel ovary, and serum titer of sex steroid hormones during ovarian growth and oocyte maturation induced artificially by SP.

3.1. E2

TLC analysis demonstrated that androstenedione (AD) was highly produced as the most predominant androgen product when ovarian follicles at the vitellogenic and migratory nucleus stages were incubated with radiolabeled pregnenolone (P5), however estrogens including estrone (E1) and E2 were not detected [35]. Therefore, aromatase activity that converts aromatizable androgens, AD and testosterone (T), into estrogens, E1 and E2, in eel follicles seems to be thoroughly quite low during artificial ovarian growth. However, aromatase activity was detected when a more sensitive method, RIA, was employed to assess E2 production from radioinert P5, OHP, or T, which indicates that E2 was produced in low levels during vitellogenesis, followed by increased production at the migratory nucleus stage. This developmental change in E2 productivity in the ovary of cultivated eels positively correlates with those changes previously demonstrated in the serum

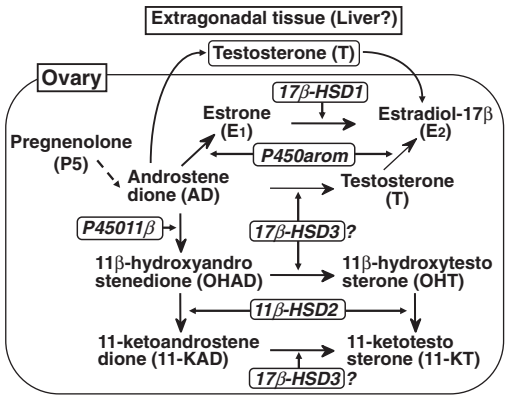

Fig. 2. Major steroidogenic pathway depicting the production of E2 and 11-KT in the Japanese eel. The font size of arrows indicates preferential usage of steroidogenic pathway.

titer of E2 [6] and in the transcript abundance of ovarian P450arom [32], which suggests that ovarian gene expression of P450arom is a major limiting factor for E2 production in the Japanese eel. For further understanding of the mechanism of E2 production in this species, elucidation of the gene regulation of ovarian P450arom would be indispensable. In other species of teleost, multiple factors, e.g., gonadotropins [36,37], insulin-like growth factor-I [38,39] and cortisol [40], has been recently demonstrated to be putative regulators of ovarian P450arom.

Developing ovarian follicles produced AD from P5 in high abundance as described above, however TLC failed to detect a significant amount of T [35]. 17β-HSD type 3 (17β-HSD3) is known as an androgenic, reductive 17β-HSD catalyzing AD to T and is predominantly expressed in mammalian testis [41]. Therefore, eel ovary does not appear to express 17β-HSD3 on a high level. Furthermore, previous studies also demonstrated low T production (less than 10 ng/ml in most cases) by the follicles in the presence of a large amount of OHP (1000 ng/ml) throughout development [6]. These results suggest that reductive 17β-HSD activity against AD is fairly low, even though T production tended to increase in the ovarian follicles at the maturational stage [6]. Furthermore, recombinant eel P450arom has been shown to catalyze the conversion of AD to E1 [32] and 17β-HSD1, the gene responsible for reduction of E1 to E2, is highly expressed in the developing ovary [31]. These findings indicate that, in eel ovary, a major steroidogenic pathway producing E2 from AD via E1 exists (Fig. 2).

Although AD was produced significantly more than T in ovaries in our studies, the serum level of T was consistently higher than that of AD [42,43]. Similarly, it was reported in one study that testis of male African catfish produced 11β-hydroxyandrostenedione (OHAD) as a predominant androgen, but 11-KT as their primary plasma androgen [44]; the investigators concluded that the high level of circulating 11-KT in the male catfish was produced from OHAD of testicular origin by extratesticular sites, especially liver. These findings indicate that the high reductive 17β-HSD activity, which converts OHAD to 11β-hydroxytestosterone (OHT) and oxidative 11β-HSD activity converting OHT to 11-KT, for which 11β-HSD2 is responsible [34], exist in the catfish hepatic tissues. In fact, it was recently demonstrated that recombinant protein of the catfish 11β-HSD2 could catalyze the conversion of OHT to 11KT, and the gene prominently expressed in the liver [45]. Fourteen types of 17β-HSD with different catalytic properties, substrate specificity, and tissue-distribution have been cloned and characterized in mammals [46]. In particular, 17β-HSD type 5 (17β-HSD5) is known as an enzyme that preferentially catalyzes AD to T and is dominantly expressed in the liver [47,48]. These findings suggest that extragonadal sites (e.g., liver) which express T-synthesizing 17β-HSD, like 17β-HSD5, are likely sources of serum T, and that E2 is produced in ovaries from the bulk of circulating T, in addition to ovarian synthesis from AD via E1 in the Japanese eel (Fig. 2). Gaining a better understanding of the regulation of E2 synthesis is reliant on studies on extragonadal metabolism of androgens and estrogens, and molecular characterization of multiple forms of 17β-HSD in fish, although such reports [31,44,49–52] have been restricted to date.

3.2. 11-KT

Although it has been reported that high levels of 11-KT circulate in the female of other Anguillidae, including short-finned (*A. australis*) and long-finned eel (*Anguilla dieffenbachii*) [17], the exact location and mechanism of 11-KT production in female eels remains unclear. Since the ovary was identified as a site for 11-KT production in females of other fish species [53–55], we first examined whether the ovary of the Japanese eel could also produce 11-KT. RIA analysis confirmed that ovarian follicles at the migratory nucleus stage produced 11-KT when incubated with AD [56]. To gain a deeper understanding of ovarian 11-KT production, we also investigated the steroidogenic pathway that produces 11-KT in the ovarian follicles of Japanese eels. OHAD was not detected by TLC when ovarian follicles in pre and early vitellogenic stages were incubated with radiolabeled AD; however, follicles in the mid-vitellogenic stage produced OHAD as a major steroid metabolite. Thereafter, the converting rate, i.e., 11β-hydroxylase activity, did not significantly change during ovarian growth induced by SP treatment, which coincides with ovarian gene expression of P45011β [56]. In contrast, in ovarian follicles throughout development, T failed to be converted into OHT. We also confirmed that follicles converted OHAD to both OHT and 11-ketoandrostenedione (11-KAD) and that these 11-oxygenated androgens were converted into 11-KT. Therefore, we conclude that 11-KT is produced from OHAD originated from AD, via either OHT or 11-KAD (Fig. 2), which would be also supported by a finding that zebrafish and human forms of 17β-HSD3 could convert OHAD and 11-KAD to OHT and 11-KT, respectively, as well as AD to T [57]. The conversion rate from OHAD to 11-KT gradually increased with consecutive stages of ovarian growth [19,56], which is in agreement with the changes in serum 11-KT levels of female Japanese eel during artificial induction of maturation [43]. These findings suggest that ovarian tissues of the advancing developmental stages could be an important source for circulating 11-KT, although we cannot rule out the possibility that extraovarian sources produce a significant portion of serum 11-KT, since extragonadal tissue (e.g., blood cell) has been demonstrated as a site for its production in several fish species [49]. Therefore, different tissues from female Japanese eels at the migratory nucleus stage were incubated with AD (100 ng/ml) and 11-KT concentrations in the media were determined by RIA. Activity to produce 11-KT from AD was highest in head kidney (4.45 ± 0.6 ng/mg protein), followed by ovary (0.61 ± 0.03 ng/mg protein) and forebrain (0.60 ± 0.08 ng/mg-protein). However, the amount of ovarian tissue was much greater than other tissues; therefore total amount of 11-KT produced by ovary was much higher (over 100 times) than those by other tissues [19,56].

3.3. DHP

Both RIA and TLC analyses demonstrated the ability of the ovaries to produce DHP at high levels from either P5 or OHP in conjunction with ovarian growth induced by SP, although ovarian follicles in the previtellogenic stage (before SP treatment) showed DHP synthesis at a low level [6,26,35]. Therefore, we conclude that the enzymatic activity of 20β-hydroxysteroid dehydrogenase (20β-HSD), an enzyme responsible for converting OHP into DHP,

is drastically enhanced. The amount of DHP produced from P5 by ovarian follicles at the various developmental stages was comparable with that from OHP [6]. In addition, P5-induced OHP production increased with ovarian growth [26], which indicated that the activity of 3β-HSD and/or 17α-hydroxylase were also possibly elevated. Transcript abundance of P450c17, which possesses activities of two enzymes: 17α-hydroxylase and C17-20 lyase, was greatly increased with ovarian growth [30], whereas gene expression of 3β-HSD was found at a high level even prior to SP treatment and did not change thereafter [29]. These findings strongly suggest that the heightened production of OHP from P5 was due to 17α-hydroxylase activation. Recombinant eel P450c17 expressed in COS-7 (a mammalian cell line) cells showed high activity both of 17α-hydroxylase and C17-20 lyase, therefore these enzymatic activities presumably increased during the transition from the vitellogenic to the maturational stage because gene expression of P450c17 was elevated during this period [30]. However, there is also strong evidence to suggest that a shift in the ovarian steroidogenic pathway from production of androgens (mainly AD) to synthesis of 17α-hydroxylated progestins, OHP and DHP, occurs at this phase [35], which indicates C17-20 lyase activity was reduced in spite of upregulation of P450c17 gene. Considering together with these findings, we proposed the existence of a mechanism that suppresses C17-20 lyase activity at the post-transcriptional level in the Japanese eel ovary [30]. Recently, a novel P450c17, named P450c17-II, was cloned and characterized from multiple species of teleost, including tilapia, medaka, and zebrafish [58]. Unlike the original P450c17, which is now also designated as P450c17-I, P450c17-II forms of tilapia and medaka possess only 17α-hydroxylase activity; studies have also demonstrated that P450c17-II is predominantly expressed in the ovary at the maturational phase and produces DHP [58,59]. It remains to be elucidated whether the P450c17-II gene exists and plays important roles in ovarian DHP production in the Japanese eel.

No DHP was detected in serum during the artificial induction of ovarian maturation, in spite of increased enzymatic activity that converts P5 into DHP [6]. DHP was highly produced *in vitro* when ovarian follicles were incubated with P5 or OHP (100 – 1000 ng/ml), whereas it was consistently produced at very low levels in the absence of exogenous steroid. Therefore, the limiting factor for DHP production *in vivo* is likely the supply of its precursors, especially OHP, since the serum level of OHP was much lower than that of P5 [43]. Recently, we captured female Japanese eels, presumably immediately after spontaneous spawning, in their spawning area within the West Mariana Ridge in the western North Pacific and determined that these eels had relatively high serum titers of DHP (around 1 ng/ml) (Kazeto et al., unpublished). This indicates that DHP is produced during spontaneous oocyte maturation and ovulation *in vivo*.

Although 20β-HSD is the most important steroidogenic enzyme for production of DHP in teleosts, the cDNA encoding this enzyme has remained to be identified. An orthologous enzyme was first purified from a cytosolic fraction of pig testis [60], and a cDNA encoding this enzyme was subsequently cloned and identified as a monomeric carbonyl reductase (CR) [61], which was established as a cytosolic oxidoreductase with broad substrate specificity for carbonyl compounds [62]. Thereafter, CR cDNAs were cloned from salmonids [63,64] and tilapia [65] and their related proteins were determined to exhibit 20 β-HSD activity. Furthermore, upregulation of CR gene during final oocyte maturation has been demonstrated in some fish species [64–66]. These findings suggest that CR functions as 20β-HSD and is responsible for DHP production in fish ovary. However, it was demonstrated in eel ovary that 20β-HSD activity is located in the membrane-bound subcellular (mitochondrial and microsome) fractions [26], which implies that subcellular localization of 20β-HSD appears to differ from that of monomeric CR. However, it cannot be ruled out that CR is possibly a membrane-bound protein in the Japanese eel ovary, unlike in mammals. To interpret this contradictory finding, we recently isolated eel CR cDNA and investigated the subcellular localization of the CR protein. Western blot analyses using an antibody against recombinant eel CR expressed in *Escherichia coli* revealed that CR protein existed in the ovarian cytosolic fraction, but not the membrane fractions. These findings suggest that cytosolic CR would not be a membrane-bound 20β-HSD responsible for a crucial role in DHP production for oocyte maturation, at least in the Japanese eel (Kazeto et al., unpublished).

4. Conclusion and perspectives

The series of studies discussed herein revealed many major details of steroidogenesis in female Japanese eel, including the changes in serum titers of steroids, steroidogenic ability, and expression levels of steroidogenic enzyme genes in the ovary, throughout the artificially induced vitellogenic growth and final maturation. However, further molecular studies, i.e., molecular cloning and expression analysis at both transcriptional and translational level, exploring 17β-HSD, 20β-HSD, and P450c17-II, are needed to gain a deeper understanding of the control mechanism. In spite of our observations, it is unclear how the results from the present study carried out on eels with SP-induced sexual maturation will perpetuate in the naturally maturing eel in the wild. Capturing maturing eels in the wild and carrying out additional studies would enable us to understand steroidogenesis in the natural situation. In addition, the endocrine component in SP that controls the changes during steroidogenesis in ovaries has not been studied up to now. Our recent technical advancement in mass-production of bio-active recombinant proteins of two GTH (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]) will help to reveal the regulatory mechanism of steroidogenesis in detail. However, the regulatory mechanism of steroidogenesis is still deeply complicated and is controlled by a variety of unexamined factors in addition to GTHs, such as peptide hormones and growth differentiation factors. The fact that eels do not activate steroidogenesis spontaneously without being stimulated by exogenous hormones indicates that this species is an excellent model for investigating ovarian steroidogenesis during sexual maturation. The molecular mechanism underlying the control of steroidogenesis in the eel should be illustrated in the future.

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