Comparative Biochemistry and Physiology, Part A 201 (2016) 132–140

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Regulation of sex steroid production and mRNAs encoding gonadotropin receptors and steroidogenic proteins by gonadotropins, cyclic AMP and insulin-like growth factor-I in ovarian follicles of rainbow trout (*Oncorhynchus mykiss*) at two stages of vitellogenesis

Ikumi Nakamura [a,b], Makoto Kusakabe [a,b], Penny Swanson [c,d], Graham Young [a,d,*]

[a] School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98105, USA
[b] Atmosphere and Ocean Research Institute, University of Tokyo, Chiba 277-8564, Japan
[c] Northwest Fisheries Science Center, NOAA's National Marine Fisheries Service, Seattle, WA 98112, USA
[d] Center for Reproductive Biology, Washington State University, Pullman, WA 99164, USA

ARTICLE INFO

Article history:
Received 5 March 2016
Received in revised form 28 June 2016
Accepted 29 June 2016
Available online 04 July 2016

Keywords:
Ovarian follicle
Gonadotropins
Insulin-like growth factor I
Rainbow trout
Steroidogenesis
Gonadotropin receptors

ABSTRACT

At the completion of vitellogenesis, the steroid biosynthetic pathway in teleost ovarian follicles switches from estradiol-17β (E2) to maturational progestin production, associated with decreased follicle stimulating hormone (Fsh) and increased luteinizing hormone (Lh) signaling. This study compared effects of gonadotropins, human insulin-like growth factor-I (IGF1), and cAMP/protein kinase A signaling (forskolin) on E2 production and levels of mRNAs encoding steroidogenic proteins and gonadotropin receptors using midvitellogenic (MV) and late/postvitellogenic (L/PV) ovarian follicles of rainbow trout. Fsh, Lh and forskolin, but not IGF1, increased testosterone and E2 production in MV and L/PV follicles. Fsh increased steroidogenic acute regulatory protein (*star*; MV), 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (*hsd3b*; MV) and P450 aromatase (*cyp19a1a*; MV) transcript levels. Lh increased *star* mRNA levels (MV, L/PV) but reduced *cyp19a1a* transcripts in L/PV follicles. At both follicle stages, IGF1 reduced levels of *hsd3b* transcripts. In MV follicles, IGF1 decreased P450 side-chain cleavage enzyme (*cyp11a1*) transcripts but increased *cyp19a1a* transcripts. In MV follicles only, forskolin increased *star* and *hsd3b* transcripts. Forskolin reduced MV follicle *cyp11a1* transcripts and reduced *cyp19a1a* transcripts in follicles at both stages. Fsh and Lh reduced *fshr* transcripts in L/PV follicles. Lh also reduced *lhcgr* transcripts (L/PV). IGF1 had no effect on gonadotropin receptor transcripts. Forskolin reduced MV follicle *fshr* transcript levels and reduced *lhcgr* transcripts in L/PV follicles. These results reveal hormone- and stage-specific transcriptional regulation of steroidogenic protein and gonadotropin receptor genes and suggest that the steroidogenic shift at the completion of vitellogenesis involves loss of stimulatory effects of Fsh and Igfs on *cyp19a1a* expression and inhibition of *cyp19a1a* transcription by Lh.

1. Introduction

Development of oocytes of oviparous vertebrates through secondary growth, during which hepatically-derived vitellogenin is taken up by the oocyte and processed (vitellogenesis), and subsequently through maturation/ovulation, is dependent on the somatic cell layers that enclose the oocyte, and together form the ovarian follicle. These somatic cells produce a variety of growth factors, and are also the sites of synthesis of the two major classes of bioactive steroids, estrogens and progestins. In teleost fish, the steroid biosynthetic pathway during vitellogenesis is directed towards production of estradiol-17β (E2), which stimulates hepatic vitellogenin synthesis, while the completion of vitellogenesis is characterized by major changes in the biosynthetic pathway that lead to the production of maturation-inducing steroids (MIS, 17,20β-dihydroxy-4-pregnen-3-one [17,20β-DHP] or 17,20β,21-trihydroxy-4-pregnen-3-one). These MIS act to reinitiate meiosis in the oocyte (reviewed by Young et al., 2005; Nagahama and Yamashita, 2008; Lubzens et al., 2010, 2016; Kagawa, 2013). Two pituitary gonadotropins regulate the ovarian follicle's steroidogenic pathway. Follicle-stimulating hormone (Fsh), whose plasma levels peak during vitellogenesis, promotes E2 production, while luteinizing hormone (Lh), whose levels become maximal during the postvitellogenic period, promotes a change in the biosynthetic pathway resulting in MIS

* Corresponding author at: School of Aquatic and Fishery Sciences, Box 355020, University of Washington, Seattle, WA 98105, USA.
E-mail address: grahamy@u.washington.edu (G. Young).

http://dx.doi.org/10.1016/j.cbpa.2016.06.035
1095-6433/

production (reviewed by Levavi-Sivan et al., 2010; Lubzens et al., 2010, 2016; Kagawa, 2013).

Associated with the increased ability of the vitellogenic follicle to respond to Fsh and increase E2 production are high levels of follicular Fsh receptors (Fshr) (Kobayashi et al., 2008; Andersson et al., 2009; Guzmán et al., 2014) and high levels of aromatase activity due to increased expression of both the ovarian form of P450 aromatase, encoded by the *cyp19a1a* gene, and of several other genes encoding steroidogenic proteins (Gen et al. 2001; Ijiri et al., 2003, 2006; Montserrat et al., 2004; Nakamura et al., 2005; Guzmán et al., 2014. The switch from E2 to MIS production is characterized by decreased plasma E2 levels and expression of the *cyp19a1a* gene, reduced aromatase activity, increased plasma Lh and follicular Lh receptor (Lhcgr) levels, and an Lh-stimulated increase in 20β-hydroxysteroid dehydrogenase activity, a process that is transcriptionally regulated (Young et al., 1983; Nagahama et al., 1985; Nakamura et al., 2005; Senthilkumaran et al., 2002, 2004; Kobayashi et al., 2008; Andersson et al., 2009; Crespo et al., 2012; Guzmán et al., 2014), that leads to conversion of precursor steroids into MIS (reviewed by Nagahama and Yamashita, 2008; Lubzens et al., 2010, 2016; Kagawa, 2013). Knowledge of what initiates the decline in E2-synthesizing capacity at the completion of vitellogenesis is still limited to a few studies that indirectly or directly implicate Lh (e.g., Crespo et al., 2012).

In vitro studies on species such as zebrafish, medaka and goldfish show that heterologous gonadotropin preparations such as human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG) can affect activity of particular enzymes and/or transcriptionally regulate one or more steroidogenic protein genes (e.g., Nagahama et al., 1991; Ings and Van Der Kraak, 2006), but the precise roles of Lh and Fsh at different stage of ovarian follicle development in these species remain, for the most part, to be clarified. Studies using highly purified homologous Lh and especially Fsh are less common, and with a few exceptions, are confined to various salmonid species at particular stages of development, particularly previtellogenic (Luckenbach et al., 2011) or preovulatory follicles (Planas et al., 1997, 2000; Crespo et al., 2012). Similarly, changes in *fshr* and *lhcgr* transcript levels during ovarian follicle development that are essential to mediate the temporally separate actions of the two gonadotropins have been documented for several species (see Lubzens et al., 2016) but there is limited understanding of how the dynamic expression of these receptors is regulated.

Insulin-like growth factors (Igfs) also appear to participate in the regulation of steroid biosynthesis during vitellogenesis and maturation. In several species, IGF1 and/or IGF2 increase the E2-synthesizing capacity of the follicle (see Reinecke, 2010) and recent work indicates that Igf3, unique to teleosts, regulates transcription of four steroidogenic enzyme genes (Li et al., 2012). In the postvitellogenic follicle, numerous actions of Igfs have been documented, related to increasing responsiveness of the follicle to MIS ("oocyte maturational competence") through increased expression of the membrane progestin receptor (see review by Thomas, 2012). All three Igfs have been shown to promote oocyte maturation alone, but it is unclear if this action is partially mediated through alterations in steroid production.

This study focused on how gonadotropins and IGF1 direct the steroidogenic pathway to E2 production during vitellogenesis and how they may participate in reducing E2 synthesis during postvitellogenic development. We examined the stage-and gonadotropin-specific regulation of expression of four steroidogenic proteins and of two gonadotropin receptors in the ovarian follicles of rainbow trout. We determined the effects of Fsh and Lh on midvitellogenic (MV) follicles and on follicles that were just completing vitellogenesis (late/postvitellogenic, L/PV), when we expected the follicles to lose sensitivity to Fsh and display increased responsiveness to Lh, because of changes in expression of their cognate receptors. We also employed forskolin, which increases adenylyl cyclase activity, to examine the effects of elevated cAMP levels to determine if any identified gonadotropic regulation of expression of target genes involved intracellular cAMP/protein kinase A (cAMP/PKA) signaling, and how that signaling might differ with stage of ovarian follicle development.

2. Materials and methods

2.1. Animals and tissues

Two-year-old virgin female rainbow trout (560–800 g body weight, 33–40 cm fork length) with MV or L/PV ovaries (classification based on follicle diameter and maximum size of follicles achieved in this stock of fish, see Discussion) were sampled from a breeding stock held in flow-through fresh water under ambient conditions. Trout were euthanized in 300 mg/l MS222 (tricaine methanesulfonate, Sigma–Aldrich, St. Louis, MO) buffered with sodium bicarbonate. Ovaries were dissected out and kept in trout Ringer's solution (Kagawa et al., 1983) for further in vitro studies. Follicles were also fixed in Bouin's fixative, processed, embedded in paraffin and sectioned using standard histological procedure to determine the morphological characteristics of each stage. All procedures involving animals were approved by the Institutional Animal Care and Use Committee (protocol 4078–01).

2.2. Reagents

Coho salmon Fsh and Lh were purified as described previously (Swanson et al., 1991). Forskolin was obtained from Sigma-Aldrich. Recombinant human IGF1 was purchased from GroPeP Pty Ltd. (Adelaide, SA, Australia).

2.3. In vitro culture of ovarian follicles

Follicles from each of three females at each stage were isolated from ovarian fragments held in ice-cold trout Ringer's solution. Diameters of 20 intact follicles were measured under a dissecting microscope. From the ovary of each female, MV follicles (10 follicles/well/1 ml Ringer's) or L/PV follicles (10 follicles/well/2 ml Ringer's) were incubated in the presence or absence of Fsh, Lh, human IGF1 (all at 100 ng/ml) or forskolin (10 μM) in trout Ringer's solution at 12 °C for 18 h with gentle shaking (100 rpm); thus, there were three independent replicates per treatment. Concentrations used were based on those used on previous studies (Maestro et al., 1997; Planas et al., 1997, 2000; Luckenbach et al., 2011). After the incubation, tissue samples were frozen using liquid nitrogen and stored −80 °C until RNA extraction. Samples of incubation media were removed and stored at −20 °C.

2.4. Expression of genes encoding steroidogenic proteins and gonadotropin receptors

Total RNA was isolated from the ovarian follicles using Trizol reagent as described by the manufacturer (Invitrogen, Carlsbad, CA). Total RNA concentrations in extracts were determined using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE). Each RNA sample (200 ng) was incubated with 0.2 units of DNase I Amp Grade (Invitrogen) at room temperature for 15 min to eliminate genomic DNA contamination. Subsequently, DNase I was inactivated by heat denaturation at 65 °C for 10 min. Single strand cDNAs were then synthesized using High-Capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR with TaqMan probes (Applied Biosystems) was used to quantify mRNA using the ABI PRISM 7300 sequence detection system (Applied Biosystems). Primer pairs and probes used for the detection of transcript for steroidogenic acute regulatory protein (*star*), P450 cholesterol side-chain cleavage enzyme (*cyp11a1*), 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (*hsd3b*) and *cyp19a1a* were identical to those used in previous studies (Kusakabe et al., 2006; Nakamura et al., 2009). Each PCR reaction contained 25 μl PCR mixture made