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The maturation-inducing hormone 17α,20β-dihydroxy-4-pregnen-3-one regulates gene expression of *inhibin* β_A and *bambi* (bone morphogenetic protein and activin-membrane-bound inhibitor) in the rainbow trout ovary ☆

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ABSTRACT

Transforming growth factor-beta (TGFβ) superfamily members are important paracrine and autocrine regulators of ovarian development and steroidogenesis in mammals and birds, but their reproductive roles in fish are not well understood. The activin system, Tgfb, and bone morphogenetic protein 15 (Bmp15) participate in the regulation of follicle maturation in some fish species. In addition, transcript levels of TGFβ superfamily members and their inhibitor, *bambi* (*bmp* and activin-membrane-bound inhibitor), change in the rainbow trout (*Oncorhynchus mykiss*) ovary during reproductive development including the transition from vitellogenesis to follicle maturation. The objective of the present study was to determine if the maturation-inducing hormone (MIH) in trout, 17α,20β-dihydroxy-4-pregnen-3-one, regulates gene expression of TGFβ superfamily members and their inhibitors. Transcript levels of *inhibin* β_A subunit (*inhba*) were increased and *bambi* decreased in isolated follicles incubated overnight without hormones compared to abundance in freshly excised tissues from the same fish, suggesting systemic factors influenced transcript abundance. Incubation with MIH decreased *inhba* and increased *bambi* expression in a dose-dependant manner and MIH was the most potent steroid examined. The transcripts' responses to incubation with and without MIH were observed in maturationally competent follicles, which are follicles competent to resume meiosis in response to MIH, and incompetent follicles, although the responses to MIH were greater in competent follicles. In summary, MIH regulates *inhba* and *bambi* expression in a stage specific manner supporting a role for MIH regulation of the TGFβ superfamily system and participation of the TGFβ superfamily system in the regulation of follicle maturation in rainbow trout.

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1. Introduction

Ovarian follicle maturation in teleost fishes and amphibians consist of two stages. During the first stage the follicle switches from production of estradiol-17β (E2) to the progestogen maturation-inducing hormone, (MIH), which has been identified as 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) in salmonids (Nagahama, 1987). The oocyte also attains oocyte maturational competence (OMC), which is the ability of the oocyte to respond to the MIH. During the second stage the oocyte is released from meiotic arrest in response to the M-phase promoting factor (MPF) which is activated in response to the MIH (see reviews, Patiño and Sullivan, 2002; Nagahama and Yamashita, 2008). Follicle maturation is generally considered to be under the sequential control of gonadotropins (Gths), the MIH, and then the MPF (Nagahama and Yamashita, 2008).

Evidence also suggests growth factors including insulin-like growth factors 1 (Igf1) and 2 (Igf2) as well as members of the transforming growth factor-β (TGFβ) superfamily are involved in the regulation of follicle maturation in fish. Insulin-like growth factors have been shown to affect follicle maturation in a wide diversity of fish species with very disparate actions even among fish within the same genus (e.g., Kagawa et al., 1994; Negatu et al., 1998; Weber and Sullivan, 2000; Mukherjee et al., 2006; Weber et al., 2007). Most TGFβ superfamily members and their innate inhibitors have received little attention but the limited data support some have actions in regulating follicle maturation in fish, and like the Igfs, their actions are species specific. An activin system that affects follicle maturation has been partially characterized in zebrafish (see review, Ge, 2005), and members of the TGFβ

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superfamily system including activins A and B, inhibin, Tgfb, bone morphogenetic protein (Bmp) 15, and also follistatin have been shown to affect oocyte maturation in several fish species (Calp et al., 2003; Kohli et al., 2003; Clelland et al., 2006; Petrino et al., 2007). It is also worth noting that cross-talk between the IGF and the TGFβ superfamily signaling pathways has been established in mammalian systems (Danielpour and Song, 2006; Guo and Wang, 2009).

Most of what is known of the roles of TGFβ superfamily members and their regulatory systems in rainbow trout reproduction comes from observed changes in transcript expression. Tada and colleagues (1998, 2002) first described the localization of *inhibin* β_A (*inhba*), *inhibin* β_B (*inhbb*) and *inhibin* α (*inha*) subunit transcripts to the theca cell layer in maturation-phase follicles. Bobe and colleagues (2004) first suggested TGFβ superfamily members were involved in the regulation of OMC or resumption of meiosis in trout after demonstrating changes in transcript abundance in deyolked follicles during in vivo acquisition of OMC and resumption of meiosis. Specifically, *inhba* and *bmp 7* (*bmp7*) displayed a progressive rise from low competence through maturation, whereas *bmp 4* (*bmp4*) and *inha* sharply increased in abundance when closely approaching or at maturation. Lankford and Weber (2010) observed a decrease in *growth differentiation factor 9* (*gdf9*) transcript abundance in late vitellogenic follicle-enclosed oocytes compared with competent follicles, and an increase in expression of the TGFβ superfamily inhibitor, *bambi* (bone morphogenetic protein and activin-membrane-bound inhibitor; aka *nma*), in competent follicles. Transcripts for *bambi* were concentrated in the somatic follicle cells. BAMBI functions as a type I pseudoreceptor that can interact with and inactivate most type-II receptors thereby inhibiting signal transduction of most TGFβ superfamily members (Onichtchouk et al., 1999; Tsang et al., 2000; Sekiya et al., 2004). BAMBI has been shown to function as part of a negative feedback system for TGFβ superfamily ligands (Onichtchouk et al., 1999; Tsang et al., 2000; Sekiya et al., 2004).

The evident regulation of TGFβ superfamily system transcripts during late stage ovarian development supports a functional role in the regulation of follicle maturation in trout. Still in question are the actions of the TGFβ system in follicle maturation and what factors regulate the intraovarian TGFβ superfamily system during this phase of reproduction. Effects of TGFβ superfamily members on steroid production in mammals and avian species, including the shift from E2 to progesterone production, have been characterized (Onagbesan et al., 2003, 2009; Spicer et al., 2006; Elis et al., 2007; Miyoshi et al., 2007). Conversely, little research has been conducted on the effect of sex steroids on the intraovarian TGFβ superfamily system. DNA microarray-based investigations identified responses of TGFβ superfamily members to in vivo androgen treatments during sex differentiation in rainbow trout (Baron et al., 2007, 2008), providing evidence of sex steroid regulation of TGFβ superfamily gene expression in this species. Progestogen actions on the intraovarian TGFβ superfamily system, however, have not been investigated in any vertebrate. Furthermore, the regulation of the TGFβ system by primary regulators of ovarian development, GTHs and sex steroids, and the IGF system, has received little attention. Nonetheless, Gths have been shown to increase *inhba*, *follistatin* (*fst)*, and *activin receptor-II*, and decrease *inhbb* expression in zebrafish follicle cells (Pang and Ge, 2002b; Wang and Ge, 2003a,b; DiMuccio et al., 2005) and the possibility that steroids mediated or participated in these responses must be considered. The objective of the present study was to investigate the regulation of gene expression of the intraovarian TGFβ superfamily system during follicle maturation in the rainbow trout, with an emphasis on the response to MIH.

2. Materials and methods

2.1. Chemicals

The steroids 17,20βP, 17-αhydroxyprogesterone (17OHP), progesterone (P4), and cholesterol (Chol) were purchased from Steraloids, Inc. (Wilton, NH). The steroids testosterone (T), E2, 11-deoxycortisol (S), and cortisol (F) were purchased from Sigma–Aldrich Co. (St. Louis, MO). The recombinant human-IGF1 (rh-IGF1) was purchased from the National Hormone and Peptide Program, Harbor-UCLA (Torrance, CA). The steroids were dissolved in absolute ethanol and the rh-IGF1 was dissolved in 10 mM HCl, before dilution in culture medium. Solvents for these chemicals never exceeded 0.1% (v/v) of the culture medium.

2.2. Fish care and tissue sampling

The rainbow trout used in these studies were either from stocks maintained at the USDA National Center for Cool and Cold Water Aquaculture (NCCCWA) in Kearneysville, West Virginia, USA; obtained from Troutlodge, Inc. (Sumner, Washington, USA) as eyed eggs and reared at NCCCWA; or obtained as adults from the White Sulphur Springs National Fish Hatchery (USFWS), White Sulphur Springs, West Virginia, USA. All fish were reared and maintained in continuous-flow ground water with an ambient temperature of 13 ± 1 °C and dissolved oxygen content near air saturation while at NCCCWA. Photoperiod was maintained with artificial lighting that was adjusted weekly to simulate the ambient photoperiod. Fish were fed Zeigler Gold food (Zeigler Bros. Inc., Gardners, Pennsylvania) at 1% body weight d^{-1}. Fish care and experimentation followed the guidelines outlined by the USDA and the NCCCWA Animal Care and Use Committee, which are in line with the National Research Council publication *Guide for Care and Use of Laboratory Animals*.

2.3. In vitro bioassay

Incubations of isolated oocytes in intact follicles (follicles) and subsequent evaluation of oocyte maturational stages followed methods previously described for this species (Jalabert and Fostier, 1984; Bobe et al., 2003). Ovaries were excised from maturing virgin rainbow trout following anesthetization by immersion in MS-222 (150 mg L^{-1}) and decapitation, and placed into sterile ice-cold trout mineral medium (TMM; Bobe et al., 2003). Fish were chosen based on migration of the oocyte germinal vesicle (GV). Follicles were incubated in Falcon 12- or 24-well culture plates (Becton Dickinson, Franklin Lakes, NJ) in an incubator at 10 °C, under air, on an orbital oscillator at 80 rpm. Tissues were pre-incubated for 2 h in control medium before the medium was replaced with treatment medium containing hormones to be tested. At termination, follicles for mRNA quantification were flash frozen in liquid nitrogen and stored at −80 °C for RNA isolation. Follicles to be scored for yolk-vesicle fusion or GV breakdown (GVBD) were fixed and cleared in Davidson's solution (2 parts 37% formaldehyde:3 parts 95% EtOH:1 part acetic acid:3 parts H_2O). Follicles completing GVBD within 96 h of incubation in 290 nM MIH were considered maturationally competent. Details on numbers of fish, in vitro treatment replicates, and duration of incubations are included in the results sections specific to those experiments.

2.4. Total RNA isolation and quantitative RT-PCR techniques

For each sample five follicles were homogenized in Tri Reagent (Sigma, St. Louis, MO) using a MM300 multi-tube homogenizer (Retsch Inc., Haan, Germany) and total RNA was isolated according