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The role of the insulin-like growth factor (IGF) system in zebrafish (*Danio rerio*) ovarian development

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ABSTRACT

The presence of an ovarian IGF system in teleosts suggests a distinct role in reproductive physiology. This study investigates the role of the ovarian IGF system in oocyte maturation, the acquisition of maturational competence and steroidogenesis in the zebrafish (*Danio rerio*). Recombinant human IGF-I and IGF-II stimulated germinal vesicle breakdown (GVBD) in early vitellogenic (EV; 0.35–0.44 mm), midvitellogenic (MV; 0.45–0.56 mm) and full grown (FG; 0.57–0.65 mm) follicles incubated *in vitro*. By comparison, the maturation inducing steroid 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) only induced GVBD in MV and FG follicles. Collectively these studies suggest that IGF is involved in oocyte maturation and that follicles become responsive to IGFs at an earlier stage compared to 17,20β-P. IGF-I also increased the responsiveness of the follicle to 17,20β-P, suggesting a role in promoting maturational competence. IGF-I alone and in combination with human chorionic gonadotropin (hCG) stimulated the production of 17,20β-P by ovarian follicles incubated *in vitro*. However, IGF-I had no effect on the production of 17β-estradiol (E_2) or the expression of genes involved in steroidogenesis (20β-hydroxysteroid dehydrogenase; 20β-HSD and P450c17-II). These results provide evidence that the IGF system plays an important role in the promotion of oocyte maturation and ovarian development in the zebrafish.

1. Introduction

Following the follicular growth phase of ovarian development in fish, luteinizing hormone (LH) stimulates follicle cells to become maturationally competent. This involves the ability of the follicle cells to produce maturation inducing steroid (MIS) and for the oocyte to become responsive to MIS. Though many progestins can induce maturation, the MIS in zebrafish has been identified as 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) since it is the most potent inducer of maturation (Selman et al., 1994). In addition to the actions of LH and steroid hormones in the acquisition of maturational competence and oocyte maturation, it is clear that members of the transforming growth factor-β (TGF-β) family including activin, follistatin, TGF-β, epidermal growth factor (EGF) and bone morphogenetic protein-15 (BMP-15) also play a role (Clelland and Peng, 2009). For example, activins and follistatin have been demonstrated to modulate the actions of gonadotropins (human chorionic gonadotropin; hCG) in the acquisition of maturational competence (Pang and Ge, 2002), while BMP-15 inhibits the resumption of meiosis in zebrafish follicles (Clelland et al., 2007). A relatively unstudied family of ovarian local factors in fish is the insulin-like growth factor (IGF) system. Our recent work (Nelson and Van Der Kraak, 2010) demonstrating the presence of a hormonally regulated ovarian IGF system in zebrafish along with evidence from other fish studies suggests that the IGF system may play an important role in ovarian physiology in teleosts.

Several studies have examined the role of IGFs in oocyte maturation in fish. Treatment with IGF-I and IGF-II induced *in vitro* maturation of postvitellogenic follicles in red seabream (*Pagrus major*; Kagawa et al., 1994), common carp (*Cyprinus carpio*; Mukherjee et al., 2006), common mummichog (*Fundulus heteroclitus*; Negatu et al., 1998) and striped bass (*Morone saxatilis*; Weber and Sullivan, 2000). The administration of IGF-I also induced maturational competence in red seabream (Patino and Kagawa, 1999), white bass (*Morone chrysops*; Weber and Sullivan, 2005) and white perch (*Morone americana*; Weber et al., 2007) ovarian follicles. Two events associated with maturational competence, the increase in the number of gap junctions in red seabream ovary (Patino and Kagawa, 1999) and the increase in the number of MIS receptors in spotted seatrout ovaries (*Cynoscion nebulosus*; Thomas et al., 2001) were also stimulated by IGF-I.

It has been shown that IGF has varying effects on ovarian steroidogenesis in fish. In white perch ovarian follicles IGF-I decreased MIS production, but upregulated 17β-estradiol (E_2) production (Weber et al., 2007). The release of E_2 and MIS was increased in striped bass ovaries after IGF-I treatment while testosterone production was decreased (Weber and Sullivan, 2000). Treatment with

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IGF-I promoted E_2 production by stimulating aromatase activity in red seabream (Kagawa et al., 2003). Administration of IGF-I inhibited testosterone and MIS production in coho salmon theca cells during the preovulatory period, but stimulated the production of E_2 and MIS in the granulosa cells prior to germinal vesicle breakdown (GVBD) (Maestro et al., 1997). In mummichog ovary, IGF-I had no effect on steroid production at any stage of follicle examined (Negatu et al., 1998). In addition, IGF-I did not affect basal or 25-hydroxycholesterol stimulated testosterone production in goldfish vitellogenic follicles (Srivastava and Van Der Kraak, 1994).

The purpose of this study was to investigate the role of the IGF system in oocyte maturation and steroidogenesis in the zebrafish. The first objective was to determine if recombinant human IGF-I and IGF-II induced oocyte maturation *in vitro* alone or in combination with gonadotropin, 17,20β-P or E_2. The second objective was to examine whether IGF-I or IGF-II induced maturational competence by assessing the ability of the follicle to respond to 17,20β-P. The final objective was to examine whether IGF-I promoted steroidogenesis by ovarian follicles incubated *in vitro* by measuring the release of 17,20β-P and E_2 to the incubation media. Changes in the expression of genes associated with oocyte maturation and steroidogenesis were also measured. These genes included bone morphogenetic protein-15 (BMP-15; *bmp15*) which inhibits follicle maturation (Clelland et al., 2007), as well as 20β-hydroxysteroid dehydrogenase (20β-HSD; *hsd20b*) and P450c17-II (*cyp17a2*) which are involved in the synthesis of 17,20β-P (Nagahama and Yamashita, 2008). The effect of IGF-I on the expression of the follicle stimulating hormone receptor (FSHr; *fshr)* and luteinizing hormone receptor (LHr; *lhcgr*) was measured. Finally, the expression of the membrane progestin receptor α (mPRα; *mpra*) which mediates the effects of 17,20β-P on oocyte maturation was also examined.

2. Materials and methods

2.1. Animals

Adult zebrafish were obtained from DAP International (Etobicoke, ON) and held at the Hagen Aqualab (University of Guelph). Fish were housed within a controlled environmental chamber in an Aquatic Habitat unit (A-HAB; Aquatic Eco-Systems, Apopka, FL) supplied with 28 °C re-circulated well water. The photoperiod was 12 h light, 12 h dark. Fish were fed twice daily to satiation with a diet of commercial salmon fry formulation (Martin Mills, Elmira, ON) supplemented occasionally with frozen blood worms (Oregon Desert Brine Shrimp Co., Lakeview, OR).

2.2. Experiments

A series of studies examined the effects of IGFs, gonadotropin and steroids on the *in vitro* induction of oocyte maturation in zebrafish ovarian follicles. In this commonly used bioassay, follicles undergoing GVBD are detected as they change from an opaque to translucent appearance (Lessman, 2009). Ovarian follicles were isolated and sorted as described in Nelson and Van Der Kraak (2010). Most experiments were conducted with midvitellogenic (MV) follicles (0.45–0.56 mm in diameter) and full grown (FG) immature follicles (0.57–0.65 mm in diameter). The MV follicles were chosen because they respond poorly to 17,20β-P as they are developing competence for maturation whereas FG follicles have already acquired maturational competence. In one of the experiments, early vitellogenic follicles (EV; 0.35–0.44 mm in diameter) were also tested to determine at what stage follicles begin to mature in response to hormonal treatment. Using fine forceps, follicles from 15–20 females were separated and pooled in 60% Lebovitz's L-15 medium with phenol red (Invitrogen, Carlsbad, CA) including 200 μg/L streptomycin and 200 IU/L penicillin (Invitrogen, Carlsbad, CA). Forty follicles were randomly distributed to each well of a 24-well polystyrene tissue culture plate (Corning Inc., Corning, NY) containing 1 ml of 60% Lebovitz's L-15 medium. Follicles were treated with test compounds and then incubated in the dark at 28 °C for various times up to 24 h and then scored for treatment effects on GVBD. Recombinant human insulin-like growth factor I and II (rh-IGF-I, rh-IGF-II) and hCG were obtained from Sigma–Aldrich, St. Louis, MO and were dissolved in 60% Lebovitz's L-15 medium. The steroids 17,20-β-P and E_2 were obtained from Sigma–Aldrich, St. Louis, MO and were dissolved in ethanol. Control wells received the same volume of ethanol. All treatments were performed in quadruplicate wells and each experiment was repeated three times with ovarian tissue from separate groups of fish.

An experiment tested whether IGF-I, IGF-II or hCG induced induce maturational competence by testing the ability of the follicles to mature in response to 17,20-β-P. Both MV and FG follicles were pre-treated with 100 nM rh-IGF-I, 100 nM rh-IGF-II, 20 IU/ml hCG or control medium and then incubated in the dark at 28 °C for 6 h. Media were replaced and follicles were subsequently treated with either 10 ng/ml 17,20-β-P (in EtOH) or an equivalent amount of EtOH (0.01% of final well volume) and incubated for a further 12 h.

An experiment was conducted to determine if IGF-I would induce steroid production either alone or in combination with hCG. Whole ovary pieces were weighed and randomly distributed to wells of a 24-well culture plate containing 1 ml 60% Lebovitz's medium. Each well contained 20 ± 2 mg ovary tissue pooled from 3 to 4 fish. Media were replaced and ovaries were treated with control media, rh-IGF-I (100 nM), hCG (20 IU/ml) or the combination of rh-IGF-I and hCG. Tissue was incubated for 6 h in the dark at 28 °C before media samples were snap frozen on dry ice and stored at −80 °C prior to measurement of 17,20β-P and E_2 content.

Further experiments examined whether IGF-I affected expression of selected genes involved in maturation. Both MV and FG follicles were treated with 100 nM rh-IGF-I and incubated for 3 or 18 h. Follicles were collected and snap frozen until RNA extraction and gene expression analysis using RT-qPCR. Genes of interest included *bmp15*, *hsd20b*, *cyp17a2*, *fshr*, *lhcgr* and *mpra*. All treatments were performed in quadruplicate and the experiment was repeated to confirm the results.

2.3. RT-qPCR

Follicle samples from IGF-I *in vitro* experiments were extracted, quantified and reverse transcribed as described previously in Nelson and Van Der Kraak (2010). Briefly, total RNA was extracted from follicle samples using a guanidine thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). Samples of RNA were reconstituted in sterile water and quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Each reverse transcription reaction used 2 μg of RNA and was carried out using M-MLV reverse transcriptase (200 U, Invitrogen). Gene expression was measured using a SYBR green method of qPCR previously described by Ings and Van Der Kraak (2006). Each reaction contained 5 μl diluted first strand cDNA, forward and reverse primers (0.05 μM; Sigma), and SYBR Green PCR Master Mix ® (Applied Biosystems, Foster City, CA) to a final volume of 25 μl. Using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA), samples were incubated at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A four point standard curve was run for each gene for each experiment to quantify gene expression. Sequences for *hsd20b*, *lhcgr*, *fshr* and elongation factor 1α (EF1α; *ef1a*) were based on previous studies (Lister et al., 2009; Lister and Van Der Kraak, 2009). Primers for *cyp17a2*, *bmp15* and *mpra* were designed using Primer Express software v. 2.0 (Applied Biosystems; Forster