Published in final edited form as:

Mol Cell Endocrinol. 2010 February 5; 315(1-2): 277. doi:10.1016/j.mce.2009.09.032.

The Ontogeny of Nuclear Estrogen Receptor Isoform Expression and the Effect of 17 β Estradiol in Embryonic Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract

Ligand bound nuclear estrogen receptor (ER) acts as a transcription factor regulating the expression of estrogen dependent genes. There are four nuclear ER isoforms in rainbow trout (*Oncorhynchus mykiss*). The objective of this study was to measure whole body mRNA levels of the two ER α isoforms ($\alpha 1/\alpha 2$) and the two ER β isoforms ($\beta 1/\beta 2$) in male and female embryos from 50 to 600 degree-days (DD; days post-fertilization x water temperature) and in embryos exposed to vehicle or 17 β -estradiol (E2) for 2 hours at 230, 240 and 250 DD. All four isoforms were detected at every time point in both sexes. Sexual dimorphism was rarely observed; at 50 DD the level of ER $\alpha 2$ mRNA was significantly greater in males than in females and at 100 DD the level of ER $\beta 1$ mRNA was significantly greater in females than in males ($p < 0.05$). Expression profiles of the two ER α isoforms were slightly different from one another, whereas the ER β isoforms exhibited similar expression patterns. The effect of E2 was not different between male and female embryos. The level of ER $\alpha 1$ mRNA increased significantly at 240 DD; a similar but not statistically significant trend was observed at 230 and 250 DD. Despite the critical role of estrogen during sex differentiation in rainbow trout, the receptivity to this hormone as measured by the response in mRNA levels of ER appears to be largely the same between males and females and ER $\alpha 1$ is the only E2 responsive isoform.

Keywords

nuclear estrogen receptor; ontogeny; trout

1. Introduction

Although the developmental fate of the gonad is determined early in rainbow trout (*Oncorhynchus mykiss*) embryonic development, and is considered to be under genetic control with males being the heterogametic (XY) and females the homogametic (XX) sex (Thorgaard, 1977), the master male sex-determining gene, presumably located on the Y chromosome, has not been determined. There is considerable evidence supporting the hypothesis that in the absence of a Y chromosome, early sex determination (i.e. molecular events that fix gonadal

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sex) and subsequent gonadal sex differentiation (i.e. initiation of ovarian development) in female trout are mediated primarily by the estrogen receptor (ER), its native ligand (E2) and cytochrome P450 aromatase (*cyp19a1a*), which catalyzes the irreversible conversion of androgens to estrogens (Guiguen et al., *in press* and references therein). A number of other genes in addition to ER and *cyp19a1a* have also been implicated in ovarian sex differentiation including *foxl2a*, *foxl2b*, *fst*, *bmp4*, *lhr* and *fshb* (Baron et al., 2005; Vizziano-Cantonnet et al., 2008). Because aromatase controls the ratio of androgens to estrogens and treatment with aromatase inhibitors such as fadrozole leads to full or partial masculinization, this enzyme has been proposed as the central mediator of ovarian development in fish (Guiguen et al., 1999; Vizziano et al., 2007).

In fish, E2 is the predominant estrogen and has generally been considered a female specific hormone (Yamamoto, 1969) having been used extensively to sex reverse genotypic males of many species (Devlin and Nagahama, 2002). It has been shown that E2 synthesis precedes and is critical for normal ovarian differentiation in rainbow trout (Guiguen et al., 1999), which is signaled by the appearance of meiotic germ cells between 732 DD (Fitzpatrick et al., 1993) and 750 DD (Takashima et al., 1980). Maternally derived estrogens including E2 are at similar levels in male and female embryos and begin to steadily decrease in both sexes up to the hatching period around 360 DD (Feist and Schreck, 1996). Similar steroid hormone profiles have been observed in coho salmon, *Oncorhynchus kisutch* (Feist et al., 1990) and tilapia, *Oreochromis niloticus* (Rothbard et al., 1987). This early period of embryogenesis is characterized by active steroid metabolism which results in the production of free E2, glucuronide E2 conjugates and other unknown metabolites (Yeoh et al., 1996a; Yeoh et al., 1996b), and by active synthesis of steroidogenic enzymes (Antila, 1984; Yeoh et al., 1996b) suggesting a critical developmental role for estrogen synthesis during this time.

Despite the evidence that estrogens such as E2 are present from early development through gonadal differentiation and that E2 is critical for ovarian development, they have been detected at similar levels in male and female rainbow trout embryos throughout much of that time. Sexual dimorphism in E2 production *in vitro* from gonadal homogenates has been reported at approximately 903 DD (Fitzpatrick et al., 1993), and a study using whole body steroid extraction detected a slight difference between the sexes around 936 DD (Feist and Schreck, 1996). In coho salmon no sexual dimorphism in E2 production was detected at 1212 DD (Feist et al., 1990). It has also been observed that although there was no sexual dimorphism in ER (ER α 1) mRNA level, female rainbow trout produced a few hundred times more aromatase than males between 550 and 1270 DD (Guiguen et al., 1999).

Early in male embryogenesis gonadal lability has been demonstrated by treating coho salmon (Piferrer and Donaldson, 1989) and rainbow trout (Krisfalusi and Nagler, 2000) with estrogens. In the rainbow trout study male intersex was induced with two 2 hour exposures to 400ug/L E2 as early as 300 DD and in the coho salmon study by one 2 hour exposure 8 days (approximately 405 DD) prior to hatch with the same E2 concentration. Interestingly, ER α 1 mRNA was observed at every stage analyzed, however, the amount of mRNA did not correlate with the period of E2 sensitivity being at the highest level when the male gonad was insensitive to E2 treatment (Krisfalusi and Nagler, 2000). At the time of that study only the ER α 1 isoform had been identified (Pakdel et al., 1989; Pakdel et al., 1990; Pakdel et al., 2000) leaving open the possibility for involvement of the other three ER isoforms in mediating the effects of E2 during early embryogenesis.

The complete nuclear ER family reported in rainbow trout (Nagler et al., 2007) consists of two subtypes (ER α and ER β) each of which has two isoforms (α 1/ α 2 and β 1/ β 2). The mRNAs for the four isoforms have been measured in multiple tissues in juvenile rainbow trout (Nagler et al., 2007) including the ovary and testis and there is evidence that they respond differentially

to E2 treatment in the liver of juvenile male fish (Boyce-Derricott et al., 2009). Currently there is no information concerning the ontogeny of ER isoform mRNA expression in male and female rainbow trout or the effect that exogenous E2 has on their expression during the embryonic period. We hypothesized that 1) the ontogenetic profiles of the four ER isoform mRNAs would be different between males and females, which would provide a functional basis for the obvious and important role of E2 in early sex differentiation in female trout and 2) that treatment with a dose of E2 previously shown to induce intersex in males would elicit the same transcriptional response in both sexes.

2. Materials and Methods

2.1. Animals

All rainbow trout embryos in this study were generated by *in vitro* fertilization using eggs from a commercial fish hatchery (Troutlodge, Inc., Sumner, WA, USA). Three all male (XY) families were generated by fertilizing eggs with all Y bearing sperm from Arlee or Hot Creek males (YY) generated by androgenesis (Parsons and Thorgaard 1985; Young et al. 1996). Two all female (XX) families were generated by fertilizing eggs with all X bearing sperm obtained from sex-reversed Troutlodge females (XX). The resultant embryos were incubated in PVC boxes with mesh bottoms in a Heath incubator (Heath Techna, Kent, WA) at 10°C. The University of Idaho Institutional Animal Care and Use Committee approved all experimental protocols.

2.2. Sample Collection

All embryos were euthanized by a terminal exposure to 0.3 mg/mL tricaine methanesulfonate (Sigma Chemical Co., St. Louis, MO) prior to RNA extraction. Whole embryos (n=6-8 individuals of each sex) were randomly sampled from the families available at 50, 100, 200, 300, 400, 500 and 600 DD. This period encompasses the interval from 50 DD up to the complete absorption of the yolk sac and first acceptance of exogenous food (600 DD). Sex-specific ER isoform expression during the labile molecular sex determination period in response to E2 treatment was assayed approximately 70 DD prior to day of hatching, which in these trout families is at approximately 300 DD. This was done by immersing separate groups of embryos (n=75/day) from each family in E2 (Sigma) or vehicle (dimethyl sulfoxide; DMSO; Sigma) for 2 hours at 230, 240, and 250 DD. Eight milligrams of E2 was dissolved in 1 mL DMSO and 50 μ L of this stock solution was added to 1 liter of dechlorinated tap water to obtain a nominal concentration of 400 μ g/L. This concentration has been shown previously to induce gonadal intersex in male trout (Krisfalusi and Nagler, 2000) and coho salmon (Piferrer and Donaldson, 1989). Following immersion in either E2 or vehicle, embryos (n=10/time point of each sex) were sampled 24 hours post-treatment.

2.3. RNA Extraction and Preparation of cDNA

Total RNA was obtained by homogenizing whole embryos in 1mL of TRIzol Reagent (Invitrogen, California, USA) with a Teflon pestle according to the manufacturer's protocol. The quantity and quality of the total RNA was determined using spectrophotometric readings at 260 and 280 nm. Four hundred nanograms of total RNA was reverse transcribed using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). The resulting cDNA was diluted and stored at -20°C until use in real time quantitative PCR.

2.3. Real Time Quantitative PCR

The mRNAs for the four ER subtypes (ER α 1, ER α 2, ER β 1, ER β 2) were quantified in whole embryos using primers and protocols as described in Nagler et al., (2007) and Boyce-Derricott et al., (2009). Enhanced green fluorescent protein (eGFP) cRNA generated by *in vitro*

transcription was used as an internal standard to account for amplification differences between samples because an endogenous gene could not be found that consistently amplified across time points and tissues. Generation of the eGFP plasmid and application of the *in vitro* transcription protocol were as described in Gilsbach et al., (2006) and Nagler et al., (2007). Messenger RNA levels for the four ER's and the eGFP internal standard were determined using absolute quantities calculated from standard curves as described in Nagler et al., (2007).

2.4. Statistical Analysis

All statistical analyses were conducted with the SAS analysis package (SAS Institute Inc., Cary, North Carolina). Quantitative data were analyzed by least-squares ANOVA using the general linear model procedure and where necessary were log₁₀ transformed to meet the assumptions of the parametric tests. Data presented in figures were back transformed and are presented as least-squares means with asymmetric SEs for treatment groups. In experiment 2 data were analyzed by 2-way ANOVA with sex and treatment as main effects. Overall significant effects were then tested with a Tukey's post-hoc multiple range test. Significance was set at $P < 0.05$.

3. Results

3.1. Ontogeny of ER Isoform Expression

The mRNAs corresponding to the four ER isoforms were detected at every developmental stage in both sexes (Fig. 1). For all ERs the lowest mRNA levels were observed at 50 DD and they gradually increased as development proceeded before dropping back to lower levels at 600 DD. Least-squares ANOVA detected a significant effect of sex and DD on the relative levels of the four ER isoform mRNAs, and there was a significant interaction between sex and DD for ER α 2, ER β 1 and ER β 2. The mean level of ER α 1, ER β 1 and ER β 2 mRNAs were higher in females than males. In contrast, the mean level of ER α 2 mRNA was generally higher in males than in females due to the large and significant difference in mRNA level at 50 DD (Fig. 1b). There was also a significant difference in the amount of ER β 1 mRNA at 100 DD between males and females (Fig. 1c). Although there were subtle but not significant sex-specific differences for all four isoforms, there was a large difference in the general pattern of expression between the two α subtypes (Fig. 1a and b). Whereas the ontogenetic pattern appears to be the same for the β subtypes (Fig. 1c and d), ER α 1 gradually increased up to 200 DD and then decreased at 500 DD (Fig. 1a); alternatively, ER α 2 showed a sharp rise in mRNA level between 50 and 100 DD with a decrease back to initial levels by 600 DD (Fig. 1b). The absolute amounts of the ER α subtype mRNAs were approximately double that of the ER β subtypes.

3.2. Transcriptional Response of ER Isoform mRNAs to E2 Treatment

Least-squares ANOVA detected a significant effect of sex, treatment and DD as well as a significant interaction between sex and DD on the relative levels of ER α 1 mRNA (Fig. 2). The mean level of ER α 1 mRNA was higher in males than females. Females showed a consistent rise in mRNA level from 230 to 250 DD, whereas the level in males dropped at 240 DD. Although treatment with E2 elicited the same general response for ER α 1 at all three developmental time points (Fig. 2a, b, c) only at 240 DD did E2 significantly increase the relative amount of ER α 1 mRNA (Fig. 2b). There was a significant effect of sex and a significant interaction between sex and DD on the amount of ER α 2 mRNA. The mean level of ER α 2 mRNA was higher in females, which increased as development proceeded. In males ER α 2 mRNA level dropped at 250 DD resulting in a significant difference in the baseline level of ER α 2 mRNA between males and females (Fig. 2f). There was a significant effect of DD and a significant interaction between sex and DD for ER β 1 (Fig. 3). The baseline level of ER β 1 mRNA in males and females was lower at 240 DD than at 230 and 250 DD (Fig. 3b). There was a significant effect of sex and DD and a significant interaction between sex and DD on

the amount of ER β 2 mRNA. The mean level of ER β 2 mRNA was higher in males than females. Males at 250 DD had a higher baseline level of ER β 2 mRNA than males at 230 and 240 DD (Fig. 3f). There was no difference in the amounts of ER α 2, ER β 1, and ER β 2 mRNAs between the vehicle and E2 treated groups on all days.

4. Discussion

This is the first ontogenetic expression analysis of a complete family of nuclear ER isoform mRNAs during the period of early embryonic development in fish in which it is thought the molecular sex of the gonad is determined. The initial hypothesis that there would be extensive sexual dimorphism in the expression patterns of the four ER mRNAs was not supported. The ER mRNAs were detected at every developmental stage between 50 and 600 DD. With only two exceptions (ER α 2 at 50 DD and ER β 1 at 100 DD), there were no significant differences between males and females although the general trend was that males had lower amounts of ER mRNA than did females (Fig. 1). This result is similar to a previous study of ER α 1 mRNA expression during a different developmental period that found no difference between male and female trout from 550 to 1270 DD (Guiguen et al. 1999). In both sexes in the present study the two ER α isoforms displayed divergent patterns of expression with ER α 1 increasing slowly and leveling off by 200 DD whereas ER α 2 increased quickly by 100 DD and then dropped off to initial levels by 600 DD (Fig. 1a and b). The two ER β isoforms displayed highly similar expression patterns and overall were measured in lower amounts than the ER α isoforms (Fig. 1c and d).

In contrast, the second hypothesis that E2 treatment would elicit the same transcriptional response for the four ER isoforms in males and females was supported. Both sexes treated with E2 just prior to hatching at 230, 240 and 250 DD all responded in a similar way. The amount of ER α 1 mRNA increased in the presence of E2 whereas the level of the other three isoforms did not change (Fig. 2a, b, c). As in a previous study of the rainbow trout nuclear ER family in the juvenile male liver (Boyce-Derricott et al., 2009), ER α 1 appears to be the only isoform mRNA that increases due to E2 treatment. The increase seen in the amount of this isoform was observed in both sexes and although the difference in mRNA amount between the vehicle and E2 treated groups was only statistically significant at 240 DD, the trend was similar at the other two time points.

Other studies of ER isoforms in fish have detected some sex-related differences in expression; however, these analyses were conducted either after morphological signs of sex differentiation had begun (Filby and Tyler, 2005; Blázquez, 2008), in adult fish (Davis et al., 2008; Lynn et al., 2008) or during testis recrudescence (Zhu et al., 2008). In fathead minnow (*Pimephales promelas*) the three ER isoforms (*esr1*, *esr2a*, *esr2b*) were detected prior to hatch (125 DD) with no sexual dimorphism (Filby and Tyler, 2005). Later during sexual differentiation, *esr2a* (ER β 2) was the dominant isoform in the ovary; however, sexual dimorphism was only detected in the gonadal levels of *esr1* (ER α 1). In tilapia, levels of ER α and ER β were higher in ovaries than in testis (Davis et al., 2008), and in yellow perch (*Perca flavescens*), *esr1* and *esr2a* were strongly expressed in ovary but were not detected in testis (Lynn et al., 2008). In contrast during sexual differentiation in sea bass, (*Dicentrarchus labrax*), there were higher levels of *erb1* (ER β 1) and *erb2* (ER β 2) in testis than ovary (Davis, 2008). In males of the cyprinid, *Spinibarbus denticulatus*, the two ER α isoforms and the two ER β isoforms were positively and negatively correlated with testis recrudescence, respectively, while there was no information concerning ER expression in females of this species (Zhu et al., 2008). Clearly there is evidence for divergent functional roles of ER in fish sexual differentiation in males and females; however, there is much less information available for the earliest developmental time points corresponding to sex determination despite the fact that the gonad is sensitive to sex steroid treatments during that period.

Analyses of these early developmental time points have all been limited to measurements of ER mRNA levels. The precise measurement of ER protein levels when the appropriate tools are available (e.g., using isoform specific antibodies) will be more informative. Whole-body measurements of ER mRNA may also lack explanatory power due to the ubiquitous distribution of these isoforms. In rainbow trout ERs have been identified in every tissue (n=12) examined (Nagler et al., 2007). The relatively larger amount of ER mRNA in the liver for example may mask subtle differences in gonadal levels of these isoforms. With the development of germ line-specific transgenic rainbow trout (Yoshizaki et al., 2000) it has become easier to isolate gonadal tissue at early developmental stages (Cavileer et al., 2009), which may facilitate fine scale analyses of gene expression at the level of mRNA and protein.

The interpretation of the results presented in this study is challenging when considered in the context of normal ovarian development and male sex reversal. On the one hand there is overwhelming evidence that E2 is a potent endocrine signal critical for the fixation, subsequent ovarian development and maintenance of ovarian function, and on the other hand there does not appear to be a large difference in the E2 profile or receptivity (i.e. the level of ER mRNAs) between males and females during embryogenesis. Thus it may be reasonable to hypothesize that there is a mechanism of intra-gonadal estrogen regulation resulting in a differential hormonal milieu between males and females during the very early period of development after fertilization. It has been suggested that enzymes responsible for the hydrolysis of conjugated steroids might be involved in the local regulation of biologically active steroid metabolites (Guiguen et al., *in press*); however, there is very little data to support this. There is some evidence from the stinging catfish (*Heteropneustes fossilis*) that ovarian catecholestrogens modulate aromatase activity (Mishra and Joy, 2006; Chourasia and Joy, 2008). The recent finding of a new ovarian sex hormone binding globulin (*shbgb*) in trout, which binds E2 and testosterone, has led to the hypothesis that it may provide a means of protecting E2 and its precursor from metabolic processes during vitellogenesis (Bobe et al., 2008). Whether or not this gene is expressed during the sex determination period and is sexually dimorphic remains to be seen but it would provide a mechanism by which the products of estrogen metabolism and steroidogenic enzyme synthesis could exert divergent effects on male and female gonads.

Acknowledgments

This research was partially supported by National Institute of Environmental Health Sciences Grant ESO12446. The all male families were created using YY sperm generated by androgenesis that was generously supplied by Dr. Gary Thorgaard (Washington State University, Pullman, WA).

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Fig. 1.

The mean whole body levels of ERα1 (a), ERα2 (b), ERβ1 (c), and ERβ2 (d) mRNAs from 50 to 600 degree-days (DD) of development in female (closed circles) and male (open boxes) rainbow trout. The mRNA level is expressed relative to an internal GFP mRNA control. Error bars represent least square means (± SE). (*p<0.05).

Fig. 2.

The effect of 17β -estradiol treatment on whole body ER α 1 (a, b, c) and ER α 2 (d, e, f) mRNA levels in male (closed bars) and female (open bars) rainbow trout at 230, 240, and 250 degree-days (DD) of development. Level of mRNA is expressed relative to internal GFP mRNA control. Error bars represent least square means ($\pm$ SE). Letters represent statistical difference between the groups according to Tukey's test ($p < 0.05$).

Fig. 3.

The effect of 17β -estradiol treatment on whole body ER β 1 (a, b, c) and ER β 2 (d, e, f) mRNA levels in male (closed bars) and female (open bars) rainbow trout at 230, 240, and 250 degree-days (DD) of development. Level of mRNA is expressed relative to internal GFP mRNA control. Error bars represent least square means ($\pm$ SE). Letters represent statistical difference between the groups according to Tukey's test ($p < 0.05$).