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Aromatase Inhibitor and 17α-Methyltestosterone Cause Sex-Reversal From Genetical Females to Phenotypic Males and Suppression of P450 Aromatase Gene Expression in Japanese Flounder (*Paralichthys olivaceus*)

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ABSTRACT The sex of Japanese flounder (*Paralichthys olivaceus*) is easily altered by water temperature or sex steroid hormone treatment during the period of sex determination. We have previously shown that rearing the genetically female larvae at high water temperature caused the suppression of P450 aromatase (P450arom) gene expression in the gonad and phenotypic sex-reversal of the individuals to males (Kitano et al. 1999. J Mol Endocrinol 23:167–176). In the present study, we show that treatment of genetically female larvae with fadrozole (aromatase inhibitor) or 17α-methyltestosterone induces sex-reversal as well as suppression of P450arom gene expression. The effect of fadrozole was counteracted by co-administration of estradiol-17β. Effective periods for fadrozole treatment to induce sex-reversal were similar to those for high water temperature treatment. RT-PCR did not detect P450arom mRNA in gonad of the sex-reversed, phenotypic males. These results indicate that sex-reversal of the genetically female larvae by aromatase inhibitor (or 17α-methyltestosterone) may be due to the suppression of P450arom gene expression and the resultant decrease in the amount of estrogen. *Mol. Reprod. Dev. 56:1–5, 2000.* © 2000 Wiley-Liss, Inc.

Key Words: fish, P450 aromatase; aromatase inhibitor; 7α-methyltestosterone; sex reversal

INTRODUCTION

Sex determination in some reptiles, amphibians, and fishes is known to be influenced greatly by environmental factors (Adkins-Regan, 1987). In Japanese flounder (*Paralichthys olivaceus*), a teleost fish that has a XX (female)/XY (male) sex determination mechanism, genetic females can be sex-reversed to phenotypic males by rearing the larvae at high water temperature or through treatment with sex steroid hormones (Tanaka, 1988; Tabata, 1991) during the period of sex determination. It is possible to obtain all XX larvae by mating normal females with sex-reversed, meiotic gynogenetic males. Thus, both males and females with the XX karyotype can be produced by rearing the XX larvae at a high (27°C) and normal (18°C) water temperatures, respectively. Considering these factors, the flounder provides an excellent model to elucidate the roles of steroids and aromatase in the sex determination mechanism of fishes.

Cytochrome P450 aromatase (P450arom) is the steroidogenic enzyme responsible for the conversion of androgen to estrogen. Estradiol-17β, produced by P450arom, is thought to be essential for ovarian development (Wallace, 1985). Previously, we demonstrated that rearing genetically female flounder larvae at high water temperature caused suppression of P450arom gene expression in the gonad and resulted in sex-reversed males (Kitano et al., 1999).

In the present study, we show that treatment of genetically female larvae with aromatase inhibitor or 17α-methyltestosterone causes sex-reversal to phenotypic males and suppresses P450arom gene expression.

MATERIALS AND METHODS

Animals

All genetically-female, Japanese flounder (*Paralichthys olivaceus*) broods were produced artificially by mating normal females with sex-reversed, meiotic gyno-

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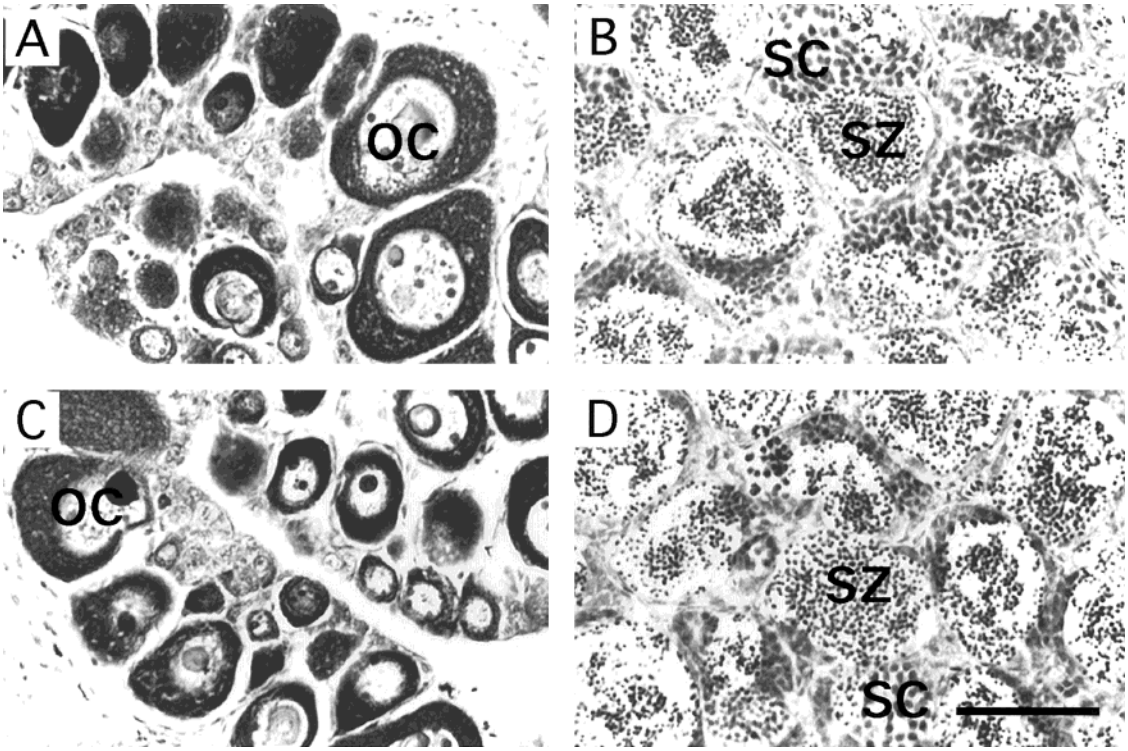

Fig. 1. Histological images of the gonads from the flounder at 10 months of age, which were provided with the artificial diet mixed with none (**A**), fadrozole (100 µg/g diet) (**B**), a combination of fadrozole (100 µg/g diet) and estradiol-17β (1 µg/g diet) (**C**), and 17α-methyltestosterone (10 µg/g diet) (**D**) from day 30 to 100 after hatching. In A and C, the gonads had differentiated to typical ovaries in which fully developed oocytes (OC) were observed. In B and D, the gonads had differentiated to typical testes filling with seminiferous tubules containing many spermatocytes (SC) or spermatozoa (SZ). Scale bar = 50 µm.

genetic males as described previously (Kitano et al., 1999).

Experimental Treatments

Fadrozole (CGS16949A; Ciba-Geigy Ltd.), 17α-methyltestosterone (Nakarai Tesque Inc.), or estradiol-17β (Sigma Chemical Co.) was dissolved in ethanol and mixed with an artificial diet (Love Larva, Maruha). Treatment with the aromatase inhibitor and steroids was carried out by providing the larvae with the diet containing 1, 10, or 100 µg fadrozole/g diet, 10 µg 17α-methyltestosterone/g diet, or a combination of 100 µg fadrozole and 1 µg estradiol-17β/g diet for various time periods at normal water temperature (18°C) (Tanaka, 1988; Tabata, 1991). The amount of the diet provided to 100 larvae in a 500-liter tank were 1, 2, 5, and 7 g/diet/day during 30–45, 46–58, 59–83, and 84–105 days after hatching, respectively. High water temperature treatment was performed by rearing the genetically female larvae at 27°C for various time periods. Control of sea water temperature at 18°C or 27°C was carried out by heating the natural sea water in the tanks using a Hot-Water Heater (MFV-P; Maeda Inc.). In order to determine the phenotypic sex by histological observation according to the previous criteria (Yamamoto, 1995), the gonads of the individuals were fixed in Bouin's solution, embedded in paraffin, and sectioned at 5 µm. Sections were stained with hematoxylin and eosin.

Detection of P450arom mRNA

Total RNA was extracted using ISOGEN (Nippongene) from individual gonads of juveniles at 100 days after hatching. P450arom mRNA in each RNA sample was amplified by P450arom-specific reverse transcription-polymerase chain reaction (RT-PCR) (Kitano et al., 1999). The products were electrophoresed on a 2% agarose gel. Relative quantities were determined by Southern blotting with labeled P450arom or EF-1α cDNA (normalization control) as described previously.

RESULTS

When genetically female larvae were provided with control diet and reared at normal water temperature (18°C), all of them developed into normal females in which gonads differentiated to ovaries (Figs. 1A, 2). In contrast, treatment with fadrozole or 17α-methyltestosterone during the period from days 30–100 after hatching (the critical period of sex determination and differentiation in flounder (Yamamoto, 1995, 1999) caused their masculinization: Their gonads differentiated to testes (Fig. 1B and D; Fig. 2). The efficiency of masculinization by fadrozole was dose-dependent: One hundred micrograms per gram of diet was required to

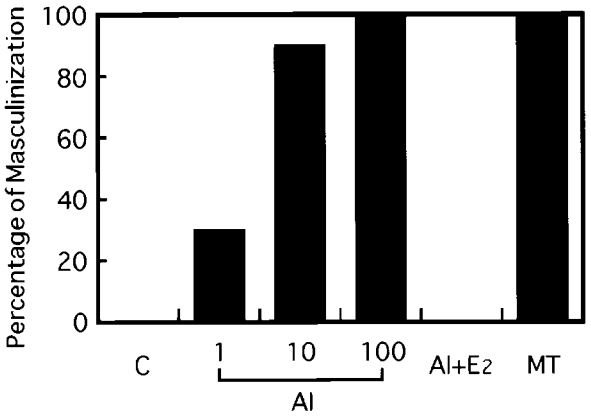

Fig. 2. Masculinization efficiency by treatment of the genetically female larvae with 1 μg/g diet (1, n = 20), 10 μg/g diet (10, n = 20), 100 μg/g diet (100, n = 24) fadrozole (AI), 17α-methyltestosterone (MT, n = 20), or a combination of fadrozole and estradiol-17β (AI + E_2, n = 20) from days 30–100 after hatching. C, no treatment (n = 48). Phenotypic sex was determined at 10 months of age by histological observation of the gonad.

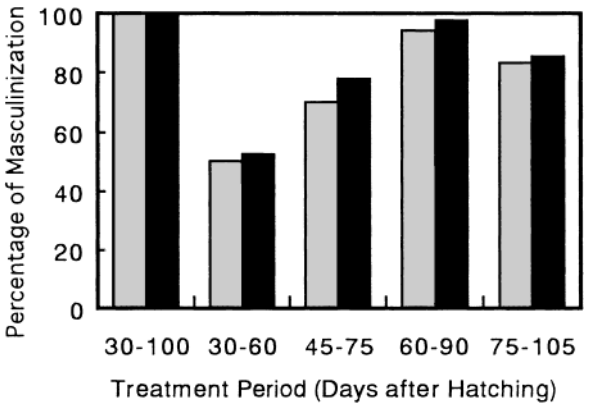

Fig. 3. Masculinization efficiency by treatment of the genetically female larvae with fadrozole (100 μg/g diet, AI, dotted column) or high water temperature (27°C, HT, closed column) during days 30–100 (AI, n = 24; HT, n = 31), 30–60 (AI, n = 20; HT, n = 21), 45–75 (AI, n = 20; HT, n = 41), 60–90 (AI, n = 34; HT, n = 38), or 75–105 (AI, n = 34; HT, n = 48) after hatching. Phenotypic sex was determined at 10 months of age by histological observation of the gonad.

cause complete masculinization. The masculinization of the larvae by fadrozole, however, was counteracted by co-administration of estradiol-17β (1 μg/g diet) during the fadrozole treatment (Figs. 1C, 2). We did not observe ovotestis in any samples examined.

Effective periods for fadrozole treatment in masculinization were examined during development (Fig. 3). Treatment of the larvae with fadrozole from days 30–100 after hatching resulted in 100% masculinization. However, treatment during days 30–60 caused only 50% of masculinization. A greater percentage of masculinization was obtained when they were treated with fadrozole from days 45–75 (70%) or days 75–105 (83.3%) after hatching. The highest percentage (94.1%) of masculinization was observed when treated from days 60–90.

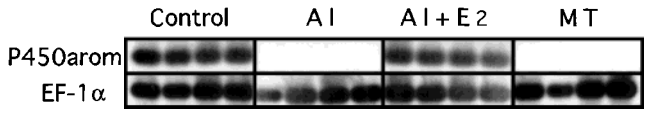

Fig. 4. Expression of P450arom mRNA in the gonad of genetically female juveniles that were treated without (control) or with 100 μg/g diet of fadrozole (AI), 10 μg/g diet of 17α-methyltestosterone (MT), or a combination of 100 μg/g diet of fadrozole and 1 μg/g diet of estradiol-17β (AI + E_2) from 30 to 100 days after hatching. To compare the amount of P450arom mRNA quantitatively among total RNA samples extracted from the gonads of individuals immediately after treatment, EF-1α served as an internal control. Fragments specific to P450arom and EF-1α cDNAs amplified by RT-PCR are predicted to be 344 bp and 577 bp in length, respectively. The products were electrophoresed on a 2% agarose gel. Relative quantities were determined by Southern blotting with labeled P450arom or EF-1α cDNA (normalization control).

In addition, effective periods of high water temperature treatment in masculinization were examined during development. The efficiency of masculinization of larvae was highest when treated from days 60–90 (97.4%), and the lowest when treated from days 30–60 (52.4%). Consequently, effective periods of fadrozole treatment to induce sex-reversal correlate well with those of high water temperature treatment.

To elucidate whether sex-reversal by fadrozole or 17α-methyltestosterone is accompanied by inactivation of the P450arom gene, P450arom mRNA expression was examined by RT-PCR in juveniles at 100 days of age after hatching, which were treated with fadrozole or 17α-methyltestosterone from days 30–100 after hatching (Fig. 4). P450arom mRNA was barely expressed in the gonad of phenotypic males produced by treatment with fadrozole or 17α-methyltestosterone; however, it was highly expressed in the gonads of phenotypic females produced by treatment with fadrozole + estradiol-17β or controls. Extremely low level expression of P450arom mRNA was also confirmed in the gonads of the sex-reversed males at 10 months of age (data not shown). These results indicate that sex-reversal by fadrozole or 17α-methyltestosterone is accompanied by suppression of P450arom gene expression.

DISCUSSION

In the present study, we demonstrated that both fadrozole and 17α-methyltestosterone induced sex reversal of genetically female flounder into males and the suppression of P450arom gene expression in the gonad when the larvae were treated during a period of sex determination and differentiation. Fadrozole is a nonsteroidal, competitive inhibitor for aromatase in mammals (Steele et al., 1987). The efficacy of this compound in reducing estrogen biosynthesis has been demonstrated in both in vivo and in vitro studies on mammals (Steele et al., 1987) and fish (Afonso et al., 1997, 1999), and in in vivo studies on chicken (Elbrecht and Smith, 1992). This compound has also been shown to induce the sex-reversal of genetic females to phenotypic males in chinook salmon (Piferrer et al., 1994), newt (Chardard

and Dournon, 1999), reptiles (Dorizzi et al., 1994; Rhen and Lang, 1994; Wibbels and Crews, 1994), and chicken (Elbrecht and Smith, 1992), and the suppression of P450arom gene expression in chicken (Elbrecht and Smith, 1992). Therefore, masculinization of genetically female larvae and the suppression of P450arom gene expression in the gonad by fadrozole treatment in the present studies could be ascribable to the reduction of the P450arom activity, concomitantly with the decrease of estrogen content.

Masculinization efficiency by a short treatment period with fadrozole was in good accordance with that of high water temperature. Our previous results (Kitano et al., 1999) showed that rearing larvae at high water temperature caused sex-reversal to males as well as suppression of P450arom gene expression, suggesting that P450arom gene activation is thermo-sensitive. Taken together, the period sensitive to aromatase inhibitor corresponds to the critical period for P450arom gene activation, suggesting that inhibition of P450arom activity results in suppression of P450arom gene expression as demonstrated by the results of RT-PCR.

One question arises from our current results: Why does inhibition of P450arom activity by fadrozole treatment result in suppression of P450arom mRNA transcription? In the developing gonad of genetic females, P450arom mRNA must be transcribed according to the developmental programs and used as a template for biosynthesis of P450arom. It is highly probable that inhibition of P450arom activity by fadrozole results in suppression of estrogen biosynthesis in the gonad. Therefore, the estrogen seems to regulate the P450arom gene expression directly or indirectly. This speculation is supported by the results that the suppression of P450arom gene expression and the masculinization of the larvae by fadrozole were counteracted by co-administration of estradiol-17β during the fadrozole treatment. Tanaka et al. (1995) showed that the promoter region of the medaka P450arom gene contains the latter half of the estrogen responsive element (ERE), suggesting that P450arom gene expression is regulated by a positive feedback mechanism. More recently, we also observed the presence of a latter half of ERE in the promoter region of the flounder P450arom gene (data not shown). If this element is functional, the binding of estrogen-estrogen receptor complex to this element in the genome may be one of the regulatory mechanisms for maintaining the high level expression of the P450arom gene in the gonad.

17α-methyltestosterone is a well known steroid hormone for inducing sex-reversal of genetic females to phenotypic males (Hunter and Donaldson, 1983; Yamazaki, 1983). However, the mechanism of 17α-methyltestosterone action has never been clarified. To the best of our knowledge, our results are the first demonstration that excess amounts of 17α-methyltestosterone induce the suppression of P450arom gene expression. Nevertheless, further investigations are needed to determine if 17α-methyltestosterone directly regulates the expression of the P450arom gene. Future efforts to elucidate sex determination and differentiation mechanisms will focus on the regulatory mechanisms of P450arom gene expression by temperature and sex steroid hormones.

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