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RESEARCH ARTICLE

Isolation, cloning, sequencing of brain type aromatase and its expression in male and female Wrasse, *Pseudolabrus sieboldi*

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Abstract *Pseudolabrus sieboldi*, wrasse being a diurnal spawner provides a good opportunity to study the endocrine mechanism of estrogen formation in brain and gonads. Moreover, an extremely large amount of E2 was produced in serum and testis of wrasse. It is assumed that the presence of E2 may play a major role in diurnal gametogenesis in male fish. In this study brain type aromatase have been isolated, cloned and sequenced from the brain of wrasse. Further, the expression pattern of brain type aromatase in gonads and adult tissue of male and female fish have been analyzed. In addition, the diurnal expression pattern of brain type aromatase in both male and female fish brain during spawning season have been analyzed.

The P450arom (br) was isolated, cloned and sequenced from both male and female bamboleaf wrasse. The P450arom (br) gene (1877 sequenced nucleotide) contains an ORF of 1470 bp, a 5′-UTR of 18 bp and at least 407 bp in 3′-UTR. The amino acid sequence homology in the coding region of wrasse P450arom (br) is high compared to that of medaka, *Oryzias latipes* (80%), rainbow trout type 2, *Oncorhynchu mykiss* (78.2%), fugu, *Takifugu ribripes* (78%) rainbow trout type 1, (76%), goldfish, *Carassius auratus* (66.8%) and zebrafish, *Danio rerio* (66.2%). Expression study reveals that P450arom (br) mRNA were most abundant in brains of both male and female fish throughout the day during the spawning season. RT-PCR study revealed that P450arom (br) was expressed in skin, anal fin and tail fin of both male and female wrasse. P450arom (br) was not detected at any time of the spawning day in either ovary or testis of wrasse.

Keywords Brain · Aromatase · Wrasse · Diurnal rhythm

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Introduction

In both male and female, estrogens which are synthesized by aromatase, program and coordinate developmental, physiological, and behavioral responses that are essential for reproduction. Conversion of C_{19} androgens to C_{18} estrogens is the rate limiting step in estrogen biosynthesis and it is catalyzed by an aromatase enzyme complex

(E.C.1.6.2.4) comprising NADPH dependent cytochrome P450 reductase and a cytochrome P450aromatase. Although early studies focused on estrogen biosynthesis in placenta and ovaries which are P450aromatase-rich and the major sources of circulating estrogen, it is now understood that smaller amounts of estrogen are formed in brain, fat, bone, gonads and other tissues, where it functions as a paracrine or autocrine factor. P450aromatase was first detected in the rat and human fetal brain by Naftolin et al. (1975) and subsequently identified in the brain of many other vertebrates (Callard 1984).

Based on molecular cloning and characterization of P450aromatase cDNAs from human tissues, the gene encoding aromatase is thought to be a single member of the CYP19 gene family. In contrast, the teleost fish have at least two separate and distinct CYP19 loci named as CYP19A (ovarian type) and CYP19B (brain type) with specific expression domains (Tchoudakova and Callard 1998). This has been demonstrated in goldfish, *Carassius auratus* (Tchoudakova and Callard 1998), zebrafish, *Danio rerio* (Kishida and Callard 2001), fugu, *Takifugu rubripes* (Yamaguchi et al. unpublished data), rainbow trout-br-I, *Oncorhynchus mykiss*, rainbow trout-br-II, (Dalla Valle et al. 2002), medaka, *Oryzias latipes* (Accession No. AAP83449), nile tilapia, *Oreochromis niolotica* (Kwon et al. 2001), mozambique tilapia, *Oreochromis mozambicus* (Cruz and Canario 2000).

Wrasse, *Pseudolabrus sieboldi*, being a diurnal spawner, provides a good opportunity to study the endocrine mechanism of estrogen formation in brain and gonads. It has been shown that an extremely large amount of estradiol-17β (E2) is present in serum and produced the testis of wrasse (Sundaray et al. 2003) and it is assumed that the presence of E2 plays a major role in diurnal gametogenesis in male fish. Ohta and Matsuyama (2002) suggested that testosterone (T) is not synthesized in females and that estrone (E1) is the major source of E2, whereas it is presumed that in males T is the major source of estrogen in testis. However, information on diurnal activities of aromatase in both male and female fish is extremely limited. To clarify the above hypothesis both types of aromatase were isolated, cloned and sequenced from the wrasse. Furthermore, the expression pattern of the brain type of aromatase in gonads and adult tissues of male and female fish was analyzed. In addition, the diurnal expression pattern of the brain type aromatase in brain during spawning season has been analyzed.

Materials and methods

Total RNA was isolated from frozen brains using ISOGEN-LS reagent. Poly $(A)^+$ RNA was purified from total RNA using Oligotex dT30 super. Total RNA from brain tissues was reverse transcribed using oligo (dt) primers and superscript III ($RNaseH^-$) reverse transcriptase. Aliquot of the reaction was PCR amplified with one pair of oligonucleotide primers for P450aromatase (br) to get the internal fragment. Subsequently, the rapid amplification of cDNA ends (RACE) method was utilized to obtain full length of cDNA encoding wrasse P450aromatase (br). The purified amplicon was ligated into pGEM-T vector and sequenced. Diurnal variation of mRNA transcripts of P450aromatase (br) in both male and female brain were studied by Northern blot and RT-PCR analysis at 3:00 h intervals throughout the day. Phylogenetic analysis was done by multiple alignments of deduced amino acid sequences using the Neighbor-Joining method within Clustal W program.

Results

P450aromatase (br) cDNA isolation

The first clone obtained by degenerated PCR is 435 bp long. RACE-PCR was performed to obtain a 5′ RACE fragment of 885 bp and a 3′ RACE fragment of 557 bp. The P450aromatase (br) (1,877 sequenced nucleotide) contains an ORF of 1,470 bp, a 5′-UTR of 18 bp and at least 407 bp in 3′-UTR.

Amino acid similarity with other P450aromatase brain type

The amino acid sequence homology in the coding region of wrasse P450aromatase (br) is high compared to that of medaka (80%) (Accession No. AAP83449), rainbow trout type 2 (78.2%), (Dalla Valle et al. 2002), fugu (78%) (Yamaguchi et al. unpublished data), rainbow trout type-I (76%),

goldfish (66.8%) and zebrafish (66.2%) (Kishida and Callard 2001).

Northern blot analysis

Two transcripts of approximately 3.8 and 4.4 kb (Fig. 1) were detected by using Alkphos labeled P450aromatase (br) cDNA. Duplicate blot was developed and hybridized with a cDNA probe for β-actin as control for loading variations.

Tissue-specific expression in adults

Abundant expression was detected in both male and female brain at all sampling times throughout the day. Diurnal transcripts were not observed in either ovary or testis at any time throughout the day. Further, adult tissues of male (Fig. 2) and female (Fig. 3) fish (pituitary, liver, skin, anal fin and tail fin, respectively) expressed the P450aromatase (br) transcript. However, expression was not detected in the following adult tissues: gill, heart, spleen, kidney and muscle of both male and female.

Phylogenetic analysis

This analysis clearly segregated a brain P450aromataseatase branch, an ovarian P450aromatase branch and stingray P450aromatase form.

Discussion

Unlike human and mammalian species, in which the gene encoding aromatase is present as a single-copy gene, there is increasing evidence that, in teleost fish, at least two CYP19 loci encode distinct P450aromatase isozymes that are differentially expressed in brain and ovary (Tchoudakova and Callard 1998). In this study, the presence of a second CYP19 gene encoding P450aromatase (br) cDNA expressed preferentially in brain was determined in the wrasse.

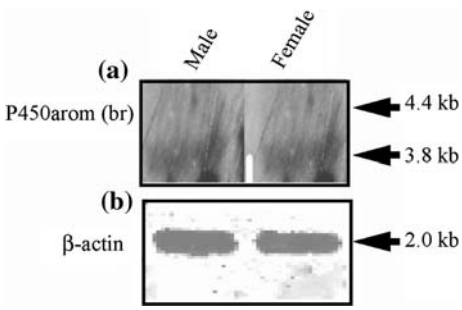

Fig. 1 Northern hybridization of brain poly $(A)^+$ RNA (5 μg) from wrasse male and female brain during spawning season (**a**) Northern hybridization of brain poly $(A)^+$RNA (5 μg) from male and female brain using β-actin as probe (**b**)

In fish, P450aromatase (br) has been cloned from several fish species. The amino acid sequence homology in the encoding region of wrasse P450aromatase (br) is high compared to that of medaka (80%), rainbow trout type 2 (78.2%), fugu (78%) rainbow trout type 1 (76%), goldfish (66.8%) and zebrafish (66.2%), Nile tilapia (81.6%), Mozambique tilapia (82.1%).

As expected, P450aromatase (br) mRNA were most abundant in brain of both male and female fish throughout the day during spawning season. This result suggests that P450aromatase (br) has diurnal activities and may play a role in fish behavior and reproduction. There is no evidence of aromatase in organizing brain sex or triggering sex behavior in any fishes. However, immuno localization study of P450aromatase in neurons of the telencephalon, midbrain and hindbrain suggested that the neurons may be involved in transmission and processing of olfactory, visual, auditory and vibratory information (Lahbib-Mansais et al. 1997). Hence, the existence of mRNA transcript throughout the day may suggests that P450aromatase (br) may help the fish in sensitivity or responsiveness to environmental cues important for behavior and reproduction as the same hypothesis has been proposed for goldfish (Gelinas et al. 1998). However, P450aromatase (br) was not detected at any time of the spawning day in either ovary or testis of bamboo leaf wrasse. Tchoudakova and Callard (1998) and Kishida and Callard (2001) reported that in goldfish and zebrafish P450aromatase (br) was not expressed in ovary. In contrast, P450aromatase (br) expression has been detected in rainbow trout ovary (Dalla Valle et al. 2002) and in ovary and testis of Nile tilapia (Kwon et al. 2001). P450aromatase (br) was detected in both male and female fish pituitary in wrasse. Recent reports on trout (Menuet et al. 2003) suggest that high messenger and protein levels of P450aromatase was observed in pituitary. However, at present time, the identity of pituitary cells expressing aromatase is still unknown.

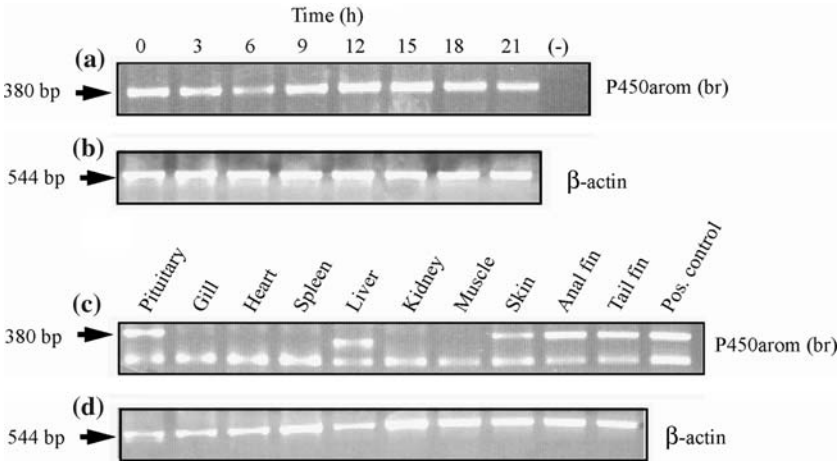

Fig. 2 Tissue-specific expression of the wrasse P450aromatase (br) in brain, and adult male tissue was determined by RT-PCR. (**a**) Brain at 8 different times, (**c**) male adult tissue. (**b**) & (**d**) β-Actin amplification from same tissue, (−) negative control

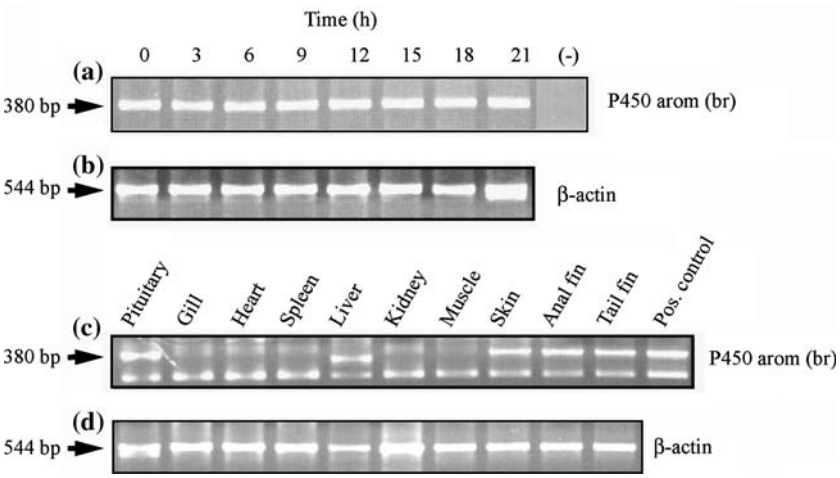

Fig. 3 Tissue-specific expression of the wrasse P450aromatase (br) in brain and adult female tissues was determined by RT-PCR. (**a**) Brain at 8 different times, (**c**) female adult tissue. (**b**) & (**d**) β-Actin amplification from same tissue, (−) negative control

RT-PCR study revealed that P450aromatase (br) was expressed in skin, anal fin and tail fin of both male and female bamboo leaf wrasse. In addition, P450aromatase (ov) mRNA expression was also found in the above tissues of both male and female fish (Sundaray, unpublished data). To our knowledge, aromatase transcripts of both P450aromatase (br) and (ov) isoforms have never been demonstrated in the skin and fins of any teleost species. However, physiological role of any estrogen formed in skin and fins remains unknown.

P450aromatase (br) expression was not found in the spleen of both male and female fish. A strong RT-PCR amplification of P450aromatase (br) was observed in the liver of both male and female fish. The liver is generally considered as a non-steroidogenic tissue in vertebrates, although aromatase mRNA was detected in human fetal and adult liver (reviewed by Conley and Hinshelwood 2001). Until to date, in fish P450aromatase (br) aromatase mRNA expression has not been observed in the liver of goldfish (Gelinas et al. 1998), zebrafish (Kishida and Callard 2001), Nile tilapia (Kwon et al. 2001) and rainbow trout (Dalla Valle et al. 2002). To our knowledge, this is the first report of P450aromatase (br) expression in the liver of any fish. Finally, the presence of a second P450aromatase has demonstrated in this fish and confirmed that bamboo leaf wrasse contains at least two separate and distinct P450 aromatase genes.

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