Comparative Biochemistry and Physiology, Part A 161 (2012) 250–258

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Ontogenic and sexually dimorphic expression of *cyp19* isoforms in the rainbowfish, *Melanotaenia fluviatilis* (Castelnau 1878)

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ARTICLE INFO

Article history:
Received 11 September 2011
Received in revised form 5 November 2011
Accepted 6 November 2011
Available online 17 November 2011

Keywords:
Aromatase
Maternal inheritance
Australian native species

ABSTRACT

To investigate the role of cytochrome P450 aromatase, we isolated *cyp19* isoforms in the Murray River rainbowfish, *M. fluviatilis*. The cloned cDNA for *cyp19a1a* and *cyp19a1b* had an open reading frame (ORF) of 492 and 499 amino acid residues, with shared identity of up to 83% and 87% with the corresponding homologues of other teleosts respectively. In contrast, the *cyp19a1a* and *cyp19a1b* of the Murray River rainbowfish had a shared identity of only 61%. Not surprisingly, the phylogenetic analysis clustered the *M. fluviatilis cyp19 isoforms* with the corresponding isoforms of other teleosts, suggesting a shared evolutionary ancestry of the respective isoforms. We also studied the expression of *cyp19* isoforms during ontogeny and in adult fish using quantitative Real-Time PCR (qPCR). Results suggest that uniquely only *cyp19a1b* transcripts are maternally inherited, suggesting its role in early development and growth in the species. In contrast to reports in many teleosts, the *cyp19a1a* was exclusively expressed in the ovarian tissue and completely absent in other tissues examined, including testis. The *cyp19a1b* like in most teleosts was predominantly expressed in the brain of both males and females with low level of expression in other tissues including gonads of both sexes.

1. Introduction

Aromatase is a key steroidogenic enzyme that converts androgens to estrogens by aromatization of C19 androgens to C18 estrogens (Blázquez and Piferrer, 2004). This enzyme is membrane-bound and is located in the endoplasmic reticulum of estrogen-producing cells of ovaries, placenta, testes, adipose and brain tissues (Sundaray et al., 2005). It is expressed in different key tissues including brain, gonads, retina, spleen, kidney, liver and other tissues and is essential for gonad development and other physiological processes including growth, neurogenesis and reproductive behavior. Although, several putative multiple functions for both ovarian and brain aromatases of teleosts have been proposed (Barney et al., 2008; Penman and Piferrer, 2008; Guigen et al., 2010), a number these functions as well as the regulatory mechanisms largely remain unresolved.

In mammals, except in porcines, there is a single *cyp19* gene which is expressed in different tissues. In contrast, two structurally and functionally different *cyp19* isoforms have been found in teleosts which are products of different *cyp19* gene loci, one preferentially expressed in the ovary and the other in the brain, designated *cyp19a1a* (*CYP19a/P450AromA/CYP19A1*) and *cyp19a1b* (*CYP19b/P450AromB/CYP19A2*) respectively (Tchoudakova and Callard, 1998; Blázquez and Piferrer, 2004; Chang et al., 2005; Guigen et al., 2010). The cDNAs encoding P450Arom isoforms have been isolated from several (over twenty) teleost species including the Japanese medaka (Fukuda et al., 1996), goldfish (Gelinas et al., 1998; Halm et al., 2001), zebrafish (Chiang et al., 2001), pejerrey (Strobl-Mazzulla et al., 2005) and common carp (Barney et al., 2008). This gene is duplicated in all the investigated teleosts except the Japanese eel, *Anguilla japonica* (Jeng et al., 2005).

Strikingly teleosts are peculiar in that their aromatase activity is 100–1000 times higher than that of mammals and other vertebrates (Pasmanik and Callard, 1985). The significance of elevated aromatase is not yet clear at the biological level even though several hypotheses on neuroprotection (Garcia-Segura et al., 2001) and neurogenesis (Callard et al., 2001) have been proposed. Among *cyp19* genes, *cyp19a1a* is predominantly expressed in the ovary and believed to play a role in sex differentiation and ovarian development; whereas *cyp19a1b* is thought to be involved in neural development in brain, retina and pituitary as well as play a key role in sexual behavior (Kishida and Callard, 2001).

In teleosts, brain is the primary organ expressing aromatase with both isoforms exhibiting subtle species and or sex-specific differences in their spatial and temporal expression patterns. For example, the expression of *cyp19a1a* is strictly restricted to ovaries in some species,

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doi:10.1016/j.cbpa.2011.11.006

while in others albeit dominant in ovary, is also expressed in other tissues, including testes at low levels (See Table 1 for summary). Similar observations have been reported for *cyp19a1b* (Table 1), including within organ and between sex differences in the common carp (Barney et al., 2008). Further, cellular examination of *cyp19a1b* shows its expression to be restricted to radial glial cells in the brain of trout (Menuet et al., 2003) and zebrafish (Goto-Kazeto et al., 2004; Pellegrini, et al., 2005). However, there was a striking difference in that the *cyp19a1b* transcripts appear to be exported into the extensions of the radial glial cells in zebrafish but not in trout (Menuet et al., 2003). Significance of such within organ, between species and or sex specific differences, as well as mechanisms of their regulation remains poorly understood. In this context, the inherent diversity of teleost species combined with functional specialization of the two isoforms provides unique opportunities to dissect the cellular and molecular basis of the diverse roles of estrogen in vertebrates at large.

In the present study, aromatase gene expression in the Murray River rainbowfish was investigated. This species has been utilized in laboratory experiments for various reasons including its ease of maintenance and short life cycle enabling the study of all life stages quickly (Pollino et al., 2007). The purpose of this study was to isolate the cDNA encoding the aromatase isoforms and to characterize their expression during ontogeny and adulthood.

2. Material and methods

2.1. Animals

Murray River rainbowfish were purchased from a commercial aquarium (Aquarium Industries, Melbourne, Australia) and reared at 25 ± 1 °C in 16:8 h light:dark regime in flow-through aquaria with carbon filtered aerated water. Throughout the maintenance, water quality parameters including temperature, dissolved oxygen, pH and conductivity were monitored and the fish were fed commercial fish pellets (Tetra color™, Blacksburg, VA, USA) twice daily. Reproductively active (spawning) male and female fish were anesthetized in AQUI-S (Lower Hutt, New Zealand) and decapitated. Samples of brain, gonad, liver, spleen, eyes and body tissues were collected, weighed and stored in RNA*later* (Sigma-Aldrich Pty. Ltd.) according to the manufacturer's instruction for RNA extraction. Muscle tissues were used for genomic DNA extraction. All procedures were conducted under the Royal Melbourne Institute of Technology, Animal Ethics Committee (RMIT AEC) approved project number 0732.

2.2. Cloning, sequencing and sequence analysis of *M. fluviatilis cyp19a1a and cyp19a1b* cDNA

Genomic DNA (gDNA) was extracted from muscle tissue using Cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987), quantified by a spectrophotometer and used as template in a standard PCR reaction. To facilitate cloning the aromatase isoforms, partial genomic regions were amplified by a simple and rapid method previously developed for *Gambusia holbrooki* (Patil et al., unpublished) using degenerate primers corresponding to highly conserved amino acid sequences amongst teleosts. Primers sets gArB980Fdeg, gArB1250Rdeg and gArO315Fdeg, gArO1400Rdeg (Table 2) were used to amplify the partial genomic fragments of brain and ovarian aromatase genes respectively. The PCR cocktail contained 0.4 μM of each primer, 0.125 mM dNTPs, 2.5 mM $MgCl_2$, 1X AmpliTaq Gold® buffer and 0.625 units AmpliTaq Gold® polymerase (Applied Biosystems Pty. Ltd. Australia). Cycling conditions were: 94 °C for 10 min then 30 cycles (94 °C, 30s/60 °C, 30s/72 °C, 1 min), followed by 72 °C for 2 min. Amplified products were cloned using a PCR®2.1TOPO® vector (Invitrogen Australia Pty. Ltd.) according to the manufacturer's instructions, resulting clones were sequenced using M13 forward and M13 reverse primers and ABI BigDye terminator v3.1 reaction mix. Reactions were then analyzed on ABI3730xl DNA analyzer and the respective identity confirmed using an NCBI blast search.

Table 1
Summary of *cyp19a1a* and *cyp19a1b* expression in brain and gonads of teleosts published as of September 2011.[a]

Species	*cyp19a1a*				*cyp19a1b*				References
	♂		♀		♂		♀		
	Brain	Testis	Brain	Ovary	Brain	Testis	Brain	Ovary	
Melanotaenia fluviatils	−	−	−	+*	+	+	+	+	This study
Odontesthes bonariensis	−	−	−	+*	+	−	+	+	Strobl -Mazzulla et al. (2005); Karube et al. (2007)
Oryzias latipes	+	+	+	+	+	+	+	+	Fukuda et al. (1996); Patil and Gunasekera (2008)
Dicentrarchus labrax (Juveniles)	−	+	−	+	+	−	+	−	Blázquez and Piferrer (2004)
Dicentrarchus labrax (Adults)	+	+	+	+	+	+	+	+	Dalla Valle et al. (2002a)
Oreochromis niloticus	−	−	−	+*	+	+	+	+	Chang et al. (2005)
Trimma okinawae	+	+	+	+	+	+	+	+	Kobayashi et al. (2004)
Danio rerio	+	+	+	+	+	+	+	+	Trant et al. (2001); Kishida and Callard (2001)
Cyprinus carpio	+	+	+	+	+	+	+	+	Barney et al. (2008)
Carassius auratus	+	−	+	−	+	−	+	+	Tchoudakova and Callard (1998)
Oncorhynchus mykiss	NA	NA	+	+	NA	NA	+	+	Dalla Valle et al. (2002b)
Fundulus heteroclitus	−	−	−	+*	+	−	+	+	Greytak et al. (2005)
Ictalurus punctatus	NA	NA	NA	NA	+	+	+	+	Kazeto and Trant (2005)
Pimephales promelas	+	+	+	+	NA	NA	+	+	Halm et al. (2001); Villeneuve et al. (2006)
Pagrus major	NA	NA	NA	+	NA	NA	NA	NA	Gen et al. (2001)
Paralichthys olivaceus	+	+	+	+	NA	NA	NA	NA	Kitano et al. (1999)
Paralichthys lethostigma	+	+	+	+	NA	NA	NA	NA	Luckenbach et al. (2005)
Hippoglossus hippoglossus	+	−	+	+	+	+	+	+	Van Nes et al. (2005)
Sparus aurata	+	+	+	+	NA	NA	NA	NA	Wong et al. (2006)
Perca flavescens	+	−	+	+	NA	NA	NA	NA	Lynn et al. (2008)
Monopterus albus	+	−	+	+	NA	NA	NA	NA	Yu et al. (2008)
Anguilla anguilla	+	+	+	+	NA	NA	NA	NA	Tzchori et al. (2004)
Protogynous wrasse	+	+	+	+	+	+	+	+	Choi et al. (2005)
Cynoglossus semilaevis	−	+	−	+	NA	NA	NA	NA	Deng et al. (2009)
Amphiprion clarkii	+	+	+	+	NA	NA	NA	NA	Kobayashi et al. (2010)
Gobiocypris rarus	+	+	+	+	+	+	+	+	Wang et al. (2010)

+: Expression, −: No expression, *: Restricted expression to either ovary or brain, NA: Not analyzed
[a] Only those species with known duplicated *cyp19* are presented.