Journal of Steroid Biochemistry & Molecular Biology 112 (2008) 5–12

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

Immunohistochemical detection and biological activities of CYP17 (P450c17) in the indifferent gonad of the frog *Rana rugosa*

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ARTICLE INFO

Article history:
Received 15 January 2008
Received in revised form 25 April 2008
Accepted 2 July 2008

Keywords:
CYP17
Immunohistochemistry
Enzymatic activity
Real-time RT-PCR
FISH
Gonad
Sex differentiation
Frog

ABSTRACT

Sex steroids play a crucial role in the gonad differentiation in various species of vertebrates. However, little is known regarding the localization and biological activity of steroid-metabolizing enzymes during gonadal sex differentiation in amphibians. In the present study, we showed by real-time RT-PCR analysis that the expression of *CYP17*, one of the key steroidogenic enzymes, was higher in the indifferent gonad during sex differentiation in male than in female tadpoles of *Rana rugosa* but that there was no difference detected in the *3βHSD* mRNA level between the male and female gonads. We next examined the localization of CYP17, 3βHSD and 17βHSD in the indifferent and differentiating gonads by using three kinds of antibodies specific for CYP17, 3βHSD and 17βHSD, respectively. Positive signals for CYP17, 3βHSD and 17βHSD were observed in somatic cells of the indifferent gonad of males and in the interstitial cell of the testis. The enzymatic activity of CYP17 was also examined in the gonad during sex differentiation in this species. [^{3}H]Progesterone (Prog) was converted to [^{3}H]androstenedione (AE) in the indifferent gonad in males and females, but the rate of its conversion was higher in males than in females. Moreover, fluorescence *in situ* hybridization (FISH) analysis revealed that the *CYP17* gene was located on the q arm of chromosome 9, indicating that *CYP17* was autosomal in *R. rugosa*. Taken together, the results demonstrate that the CYP17 protein is synthesized in somatic cells of the indifferent gonad during gonadal sex differentiation in *R. rugosa* and that it is more active in converting Prog to AE in males than in females. The data suggest that *CYP17* may be involved in testicular formation during sex differentiation in this species.

1. Introduction

Gonadal formation is very sensitive to sex steroids in some species of amphibians [1,2] as it is in fish [3], reptiles [4] and birds [5]. Sex steroids can induce sex-reversal either from male to female or from female to male in certain species of amphibians [6]. Interestingly, the Japanese wrinkled frog *Rana rugosa* has two sex-determining systems [7,8]. *R. rugosa* living in the Western and Southeastern parts of Japan have the XY system, whereas those found in the Northeastern part of Japan have the ZW one. The sex of *R. rugosa* can be reversed artificially from female to male or male to female by sex steroids [7,9,10]. However, the mechanism by which sex steroids regulate the differentiation of the gonads still remains to be elucidated.

Sex steroids are produced from cholesterol *via* a series of enzyme-catalyzed reactions [11,12]. First, cholesterol is converted into pregnenolone by CYP11A1 (cytochrome P450 side-chain cleavage enzyme). Pregnenolone is then converted into progesterone (Prog) by 3βHSD (3β-hydroxysteroid dehydrogenase) or into dehydorepiandrosterone (DHEA) by CYP17 (cytochrome P450 17α-hydroxylase/C_{17-20} lyase). Next, Prog is converted into androstenedione (AE) by CYP17, whereas DHEA is converted into AE by 3βHSD. Then, AE is converted into testosterone by the action of 17βHSD (17β-hydroxysteroid dehydrogenase). Finally, testosterone is converted into estradiol (E_2) by CYP19 (cytochrome P450 aromatase). Thus, five steroid-metabolizing enzymes (CYP11A1, 3βHSD, CYP17, 17βHSD and

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doi:10.1016/j.jsbmb.2008.07.002

CYP19) are essential for sex-steroid production in vertebrate gonads.

The expression patterns of the genes encoding steroid-metabolizing enzymes in the gonad of vertebrates have been previously described. In the rainbow trout, CYP11A1, 3βHSD and CYP17 are localized in Leydig cells of the testis [13]. *CYP17* is expressed in the ovary of the rice-field eel [14], rainbow trout [15] and medaka [16]. In reptiles, the enzymatic activity of CYP19 is higher in the gonad of the turtle *Dermochelys coriacea* at the female-than at the male-producing temperature [17], but its activity is the same at either temperature in the turtle *Trachemys scripta* [18]. In chickens, *CYP17* and *CYP19* are expressed in the gonads after sex determination [19]; and their expression remains high in the ovary, but low in the testis [19,20]. In *Xenopus*, *Rana* and *Bufo*, the activity of various steroid-metabolizing enzymes has been detected in the testis and ovary [21–23]. In the urodele *Necturus maculosus*, CYP17 and CYP19 enzymes are active in the testis [24]. Thus, although the steroid biosynthesis pathways in the testis and ovary of various vertebrate species have been thoroughly characterized, the localization and enzymatic activity of CYP17 have not been investigated in the gonads during gonadal sex differentiation in amphibians. Here we report the localization and biological activity of CYP17 in the indifferent gonad during sex differentiation in the frog *R. rugosa*. We also show by FISH that *CYP17* is an autosomal gene in this species.

2. Materials and methods

2.1. Animals

R. rugosa with the ZW-type sex chromosome were used for all experiments. They were captured in 2007 in the Kita-Uonuma region of Niigata Prefecture, which is situated in the northeast part of Japan and then kept at 22 °C in laboratories and fed small crickets twice a week. Unfertilized eggs were obtained and artificially inseminated as described elsewhere [9,10]. Fertilized eggs were allowed to grow to tadpoles of various stages for use in the experiments. Embryos and tadpoles were staged according to Shumway [25] and Taylor and Kollros [26]. The sex of tadpoles was determined according to Sakisaka et al. [27]. Animal manipulations were approved by the Ethics Committee of Waseda University.

2.2. Analysis of the CYP17 and 3βHSD expression by real-time RT-PCR

The expression of *CYP17* and *3βHSD* in the indifferent gonad of males and females was analyzed by real-time RT-PCR analysis by using a LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche, Indianapolis, IN, USA). Total RNA was isolated three times independently from the gonad/mesonephros complexes of tadpoles at 1, 2 and 4 weeks after they had reached stage 25, and also at stages I, III and V [9,10]. Then, the RNA was used as an initial template for RT-PCR. The reaction consisted of 4 min at 94 °C, followed by 33 cycles for *CYP17* and *3βHSD* and 15 cycles for *GA3PDH* of 94 °C (30 s), 65 °C (30 s) and 72 °C (30 s).

Real-time RT-PCR analysis was performed to examine *CYP17* and *3βHSD* expression in the indifferent gonad before, during, and after sex differentiation. The reaction mixture contained 4 μl of master mix, 4 μl of a 2.5 μM concentration of each forward and backward primer, 1 μl of each template cDNA and 11 μl of water in a final volume of 20 μl. The template cDNAs used were those of *3βHSD* (GenBank Accession No. AB284117) and *CYP17* (GenBank Accession No. AB284119). *GA3PDH* (GenBank Accession No. AB284116) cDNA was also used to standardize the level of *CYP17* and *3βHSD* mRNAs. The reaction consisted of 10 min at 95 °C, followed by 55 cycles of 95 °C (10 s), 65 °C (10 s) and 72 °C (10 s), and then 95 °C (1 s) and 65 °C (15 s) to 95 °C (0.1 °C/s), ending with a step at 40 °C. The increase in the fluorescence-signal of SYBR Green I was automatically detected during the 72 °C phase of the reaction by LightCycler 1.5 (Roche, Indianapolis, IN, USA). The cycle threshold (C_t) of the target genes was measured by using the second derivative maximum method in LightCycler Software Version 3.5. The concentration of target genes was calculated from target genes C_t values by constructing a standard curve of 10-fold serial dilutions of known concentrations. Primers used for real-time RT-PCR analysis were as follows:

GA3PDH	Forward	5′-GAAGTGAAGGCTGACGGAGGA-3′
	Backward	5′-CGCCTTGTCATAGCTTTCATGGT-3′
3βHSD	Forward	5′-GACTCAATGCTCCAACCTTCACAG-3′
	Backward	5′-GGACCTCTGGCAGGTCCTCA-3′
CYP17	Forward	5′-CGCTGTGTATGTTCGGTGAAGG-3′
	Backward	5′-GGTCTCGAGCTGCCACTGACT-3′

2.3. Measurement of biological activity of CYP17

The biological activity of CYP17 was determined by measuring the conversion of [1,2, 6, 7-^{3}H]Prog [specific activity, 4255 GBq/mmol (37 GBq = 1 Ci); PerkinElmer, Waltham, MA, USA] to [^{3}H]AE in gonadal homogenates as described elsewhere [28]. In brief, homogenates containing 180 μg of the gonad/mesonephros complexes from male or female tadpoles (50 complexes/homogenate) were incubated for 3 h at 25 °C in 500 μl of PBS (10 mM Na_2HPO_4/10 mM NaH_2PO_4/140 mM NaCl at pH 7.5) containing [^{3}H]Prog (1×10^6 cpm), 0.24 mM NADPH, and 4% propylene glycol. Homogenates of adult testis (180 μg) were also incubated for up to 5 h under the same conditions as mentioned above. After incubation, steroids were extracted with ethyl acetate and subjected to HPLC analysis by using a reversed-phase column, LiChrospher 100 RP-18 (4.0 mm × 250 mm; Kanto, Tokyo, Japan). The column was eluted with a 30-min linear gradient of 40–70% acetonitrile (ACN) at a flow rate of 0.7 ml/min, followed by an isocratic elution with 70% ACN. The effluent was collected from 5 to 40 min and fractions (30 s) were counted in a liquid scintillation counter (model RLC-701; Aloka, Tokyo, Japan). For confirmation of the involvement of the CYP17 enzyme in the formation of radioactive 17α-dihydroprogesterone (DHP), incubations were performed in the presence of ketoconazole (an inhibitor of CYP enzymes; Sigma–Aldrich, St. Louis, MO, USA) at the final concentration of 10^{-4} M according to Tsutsui et al. [29] and then subjected to HPLC analysis. To assess the formation of 5α- and 5β-DHP from [^{3}H]Prog, we performed HPLC analysis, followed by TLC with chloroform/ethyl acetate (4:1), as described elsewhere [28,29]. Reference standards of [^{3}H]Prog and [^{3}H]AE were chromatographed under conditions similar to those used for the tissue extracts and were detected by using a liquid scintillation counter (model RLC-701; Aloka, Tokyo, Japan). The amount of protein was determined by using a BCA protein assay kit according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

2.4. Cell culture and chromosome preparation

Two females were used for cell culture and chromosome preparation. After adult heart, lung and kidney tissues had been dissected, they were minced and put into cell culture. Fibroblast cells were cultured in 50% Leibovitz's L-15 medium (Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum (Invitrogen), 26 mM HEPES buffer solution (Invitrogen), 100 μg/ml kanamycin,