Available online at www.sciencedirect.com
SCIENCE DIRECT
Comparative Biochemistry and Physiology, Part B 144 (2006) 18–28
CBP
www.elsevier.com/locate/cbpb

Development and optimization of a Q-RT PCR method to quantify *CYP19* mRNA expression in testis of male adult *Xenopus laevis*: Comparisons with aromatase enzyme activity

June-Woo Park [a,*], Markus Hecker [a], Margaret B. Murphy [a], Paul D. Jones [a], Keith R. Solomon [b], Glen Van Der Kraak [g], James A. Carr [c], Ernest E. Smith [e], Louis du Preez [f], Ronald J. Kendall [e], John P. Giesy [a,d]

[a] *Department of Zoology, National Food Safety and Toxicology Center, Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, USA*
[b] *Centre for Toxicology and Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1*
[c] *Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409, USA*
[d] *Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong, SAR, China*
[e] *Department of Environmental Toxicology, The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX 79416, USA*
[f] *School of Environmental Sciences and Development, North-West University, Potchefstroom 2520, South Africa*
[g] *Department of Integrative Biology, University of Guelph, Ontario, Canada NIG 2W1*

Received 14 April 2005; received in revised form 10 January 2006; accepted 11 January 2006
Available online 21 February 2006

Abstract

Due to limitations of the currently used enzymatic assays, it is difficult to determine aromatase activity in testicular tissue of amphibians. Quantitative reverse transcription polymerase chain reaction (Q-RT PCR) is a sensitive and reliable technique to detect low amounts of mRNA for specific genes. This study was designed to develop and optimize a SYBR Green I-based Q-RT PCR method to quantify *CYP19* mRNA in testicular tissue from male *Xenopus laevis*. Four quantification methods for measuring *CYP19* mRNA expression were compared. The established test system proved to be highly sensitive (detectable mRNA copies <10), reproducible (interassay CV<5.4%, intraassay CV<0.9%), precise and specific for the *CYP19* gene. To confirm the validity of the applied test system, an ex vivo testicular and ovarian explant study with a known inducer of aromatase, forskolin, was conducted. Forskolin induced *CYP19* gene expression in both ovarian (3.7-fold) and testicular (2.6-fold) explants. Of the four quantification methods, the absolute standard curve and the comparative C_T method appear to be optimal as indicated by their highly significant correlation ($r^2=0.998$, $p<0.001$). In conclusion, we recommend the comparative C_T method over the standard curve method because it is more economical in terms of both cost and labor. Although both aromatase activity and *CYP19* mRNA were clearly detectable in testes of *X. laevis*, both aromatase enzyme activity and *CYP19* gene expression were very low. Also, no significant relationships were found between aromatase enzyme activity and gene expression. This is likely due the fact that the aromatase enzyme may have been dormant at the developmental stage the frogs were in during the experiment.

Keywords: Amphibians; Estrogen synthesis; Frog; Gene quantification; Molecular methods; Optimization

1. Introduction

The cytochrome P450 enzyme aromatase is the key enzyme that catalyzes the conversion of androgens to estrogens and represents the rate-limiting step in estrogen biosynthesis. The protein that catalyzes the aromatization of steroid hormones is encoded by the *CYP19* gene (Thompson and Siiteri, 1974; Simpson et al., 1994). Estrogens, especially estradiol-17β

* Corresponding author. 218C Aquatic Toxicology Laboratory, National Food Safety and Toxicology Center, Michigan State University, East Lansing, MI 48824, USA. Tel.: +1 5174323100x171; fax: +1 5174322310.
E-mail address: parkju11@msu.edu (J.-W. Park).

1096-4959/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2006.01.003

(E2), have been shown to play a key role in ovarian development, reproductive function and sexual differentiation in various amphibian species (Miyashita et al., 2000; Miyata and Kubo, 2000; Kuntz et al., 2003a; Kato et al., 2004). Thus, disruption of either activity or production of this enzyme is likely to result in altered developmental or reproductive biology of organisms. Due to its key function in estrogen biosynthesis and associated reproductive processes, aromatase has been considered as an important endpoint to assess the exposure to compounds that may interact with reproductive endocrinology in vivo and in vitro (Sanderson et al., 2002; Hayes et al., 2002; Rotchell and Ostrander, 2003).

Recently, concern was raised about the potential of triazine herbicides to interact with the endocrine system of male frogs by inducing aromatase resulting in an increase of endogenous estrogen production and subsequently causing feminization or demasculinization of males (Hayes et al., 2002). Although studies by Sanderson et al. (2002) and Roberge et al. (2004) have found that high concentrations of triazine herbicides can induce aromatase in mammalian cells in culture, to date there have been no reports of this mechanism of action being observed in vivo in amphibians. This may be due to the fact that testicular aromatase enzyme activities are often low and are thus difficult to detect because they are near the detection limits of the commonly used enzymatic assays (Hecker et al., 2004). Therefore, to increase our ability to determine possible changes in aromatase activity in the testis, a more sensitive test system is needed that allows for detecting even subtle changes. One way to examine the potential for such subtle effects on the expression of aromatase activity is by measuring the changes in the expression of *CYP19* mRNA. Quantitative (real-time) reverse transcriptase polymerase chain reaction (Q-RT PCR) is a sensitive and flexible technique that can detect small quantities of mRNA in small amounts of tissue (Bustin, 2000, 2002). This technique, which amplifies the number of copies of mRNA many times, can theoretically measure as little as a single molecule of the target mRNA (Linz et al., 1990; Bej et al., 1991).

There have been few studies analyzing *CYP19* gene profiles in the African clawed frog (*Xenopus laevis*) or in amphibians in general (Miyashita et al., 2000; Akatsuka et al., 2004; Kuntz et al., 2004). None of above studies, however, have focused on adult males and, to our knowledge, Q-RT PCR methods using reliable quantification methods have not yet been applied to quantify the gene expression levels of *CYP19* in testes of *X. laevis*. It is known that *CYP19* is differentially expressed based on the sex or life-stage in most vertebrate species (Miyashita et al., 2000; Liu et al., 2004; Sakata et al., 2005; Forlano and Bass, 2004) and that one cannot simply extrapolate between sexes, especially with regard to effects of chemical exposure. Therefore, the objective of this study was to develop and optimize a Q-RT PCR procedure to measure the expression level of *CYP19* in testicular tissue of male *X. laevis.* To facilitate accurate quantification, a cDNA standard was produced that could be used for the determination of absolute copy numbers of *CYP19* mRNA in addition to the relative quantification determined by comparison to the expression of housekeeping genes. Furthermore, we compared *CYP19* gene expression in males with aromatase enzyme activities to establish a link between expression and function of gonadal aromatase in male *X. laevis*.

2. Materials and methods

2.1. Animals

Adult male *X. laevis*, 30–50 g, were purchased from Xenopus Express (Plant City, FL, USA). Each frog was treated with 0.06% NaCl upon their arrival at the laboratory to reduce the risk of possible infections. Frogs were acclimated for several weeks at the Michigan State University's Aquatic Toxicology Laboratory before the experiment was initiated. During acclimation, animals were held in 600-L fiberglass tanks under flow-through conditions. The photoperiod was 12:12-h light/dark. Frogs were fed Nasco frog brittle (Nasco, Fort Atkinson, WI, USA) three times per week ad libitum.

2.2. Isolation of total RNA and first-strand cDNA synthesis

Total RNA was isolated from gonad tissues of 14 male *X. laevis* using the SV Total RNA Isolation System (Promega, Madison, WI, USA) following the manufacturer's specifications with minor modifications to maximize the efficiency of total RNA isolation. Briefly, tissues were homogenized using a Kontes pestle and lysed in microcentrifuge tubes with guanidine thiocyanate and β-mercaptoethanol mixture. After centrifugation to remove precipitated proteins and cellular debris, nucleic acids were precipitated with ethanol and bound to a glass fiber membrane. All samples were treated with RNase-free DNase I at room temperature for 15 min to remove the chromosomal DNA. RNA integrity was checked by denaturing agarose gel electrophoresis (not shown) and 260:280 nm absorbance ratio (2.33 ± 1.03) using a DU530 UV/VIS spectrophotometer (Beckman Coulter, Inc., CA, USA). Concentrations of total RNA were determined using the RiboGreen™ RNA quantitation reagent (Molecular Probes, Inc., OR, USA) in a TD700 laboratory fluorometer (Turner BioSystems, Sunnyvale, CA, USA). Purified RNA was stored at −80 °C until further analysis.

A sample containing 500 ng of total RNA was used to synthesize single-strand cDNA in accordance with the manufacturer's directions (SuperScript™ First-Strand Synthesis System for RT PCR, Invitrogen, CA, USA). Briefly, prior to reverse transcription, total RNA was treated with DNAse I to remove potential chromosomal DNA. Then, 1.25 μL of $_{12-18}$Oligo(dT) (0.5 μg/μL) and 10 mM dNTP mix were added to the total RNA, and incubated at 65 °C for 5 min. The reaction was stopped by chilling the test solution on ice. Reaction mixture (10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT and recombinant ribonuclease inhibitor) was added to the RNA/primer mixture and incubated at 42 °C for 2 min. SuperScript II reverse transcriptase (1.25 μL of 50 U M-MLV) was added and the reaction mixture was incubated at 42 °C for 50 min, followed by a second incubation at 70 °C for 15 min. To confirm complete removal of possible genomic contamination, a negative control (sample without reverse transcriptase) was run in parallel in the Q-RT PCR system, which resulted in no