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Fadrozole and finasteride exposures modulate sex steroid- and thyroid hormone-related gene expression in *Silurana* (*Xenopus*) *tropicalis* early larval development

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ABSTRACT

Steroidogenic enzymes and their steroid products play critical roles during gonadal differentiation in amphibians; however their roles during embryogenesis remain unclear. The objective of this study was to investigate the expression and activity of aromatase (cyp19; estrogen synthase) and 5β-reductase (srd5beta; 5β-dihydrotestosterone synthase) during amphibian embryogenesis. Expression and activity profiles of cyp19 and srd5beta were first established during *Silurana* (*Xenopus*) *tropicalis* embryogenesis from Nieuwkoop–Faber (NF) stage 2 (2-cell stage; 1 h post-fertilization) to NF stage 46 (beginning of feeding; 72 h post-fertilization). Exposures to fadrozole (an aromatase inhibitor; 0.5, 1.0 and 2.0 μM) and finasteride (a putative 5-reductase inhibitor; 25, 50 and 100 μM) were designed to assess the consequences of inhibiting these enzymes on gene expression in early amphibian larval development. Exposed embryos showed changes in both enzyme activities and sex steroid- and thyroid hormone-related gene expression. Fadrozole treatment inhibited cyp19 activity and increased androgen receptor and thyroid hormone receptor (α and β) mRNAs. Finasteride treatment inhibited srd5beta (activity and mRNA), decreased *cyp19* mRNA and activity levels and increased estrogen receptor α mRNA. Both treatments altered the expression of deiodinases (thyroid hormone metabolizing enzymes). We conclude that cyp19 and srd5beta are active in early embryogenesis and larval development in *Silurana tropicalis* and their inhibition affected transcription of genes associated with the thyroid and reproductive axes.

1. Introduction

Sex steroids play a critical role during gonadal differentiation in amphibians (Hayes, 1998). In the frog *Silurana* (*Xenopus*) *tropicalis*, the first histological signs of gonadal differentiation are observed after the beginning of feeding, during tadpole development at Nieuwkoop–Faber (NF; Nieuwkoop and Faber, 1994) stage 48 (~8 days post-fertilization) (El Jamil et al., 2008). Studies have reported the presence of sex steroid hormones in vertebrate embryos (fish, Iwamatsu et al., 2005; frog, Bogi et al., 2002; bird, Carere and Balthazart, 2007; mammal, Goldman-Johnson et al., 2008). Sex steroid synthesis involves the actions of many enzymes such as aromatase (cyp19) and members of 5-reductase (srd5) family, although their presence and functionality during embryogenesis remains unclear.

In vertebrates, cyp19, a cytochrome P450 enzyme converts testosterone (T) into estradiol (E2) and androstenedione into estrone (E1; Lephart, 1996; Simpson et al., 1994), and its presence is an indicator of active estrogen synthesis. Estradiol is a critical hormone in female and male reproductive development and function (reviewed in Hayes, 1998). Testosterone metabolism also includes the reduction of T to either 5α-dihydrotestosterone (5α-DHT) by 5α-reductases (srd5alpha type 1, 2 and 3) or to 5β-dihydrotestosterone (5β-DHT) by 5β-reductase (srd5beta; Hutchison and Steimer, 1984). The steroid 5α-DHT is a potent androgen most often associated with male sexual development (Russell and Wilson, 1994). In humans, srd5alpha enzymes are also involved to some extent in pseudohermaphroditism, prostate cancer, polycystic ovarian syndrome and hirsutism (Andersson et al., 1991; Goodarzi et al., 2006; Thomas et al., 2009). In marked contrast, the reproductive and/or developmental functions of srd5beta (a member of the

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aldo–keto reductase superfamily) are largely unexplored because of the long held view that 5β-DHT is biologically inactive (Kokontis and Liao, 1999). However, there are numerous reports to the contrary (reviewed in Langlois et al., 2009). For example sexual behavior studies indicate important roles of srd5beta, including the regulation of steroid production by T inactivation and/or direct inhibition of cyp19 by 5β-reduced metabolites of T in the brain of birds (Hutchison and Steimer, 1981; Steimer and Hutchison, 1981). Furthermore, exposure to 5β-DHT inhibits mammary development in mice (Yanai et al., 1977). Most recently, it has been demonstrated that 5β-DHT induces vasorelaxation in thoracic aorta (Montano et al., 2008) and vas deferens (Lafayette et al., 2008) in the rat.

In addition, thyroid hormones (THs) can also interact with the reproductive axis in amphibians. The two forms of THs, triiodothyronine (T3) and thyroxine (T4) mediate their physiological effects by binding to nuclear thyroid hormone receptors (tr) encoded by two genes *tralpha* and *trbeta* (Tsai and O'Malley, 1994). The metabolism of THs is regulated by three types of deiodinases (type I, II and III). Deiodinase type I (dio1) catalyzes outer-ring deiodination to produce T3 from T4 and also inner-ring deiodination to produce rT3 (reverses-T3, inactive) from T4. Type II deiodinase (dio2) exclusively activates THs by catalyzing the conversion from T4 to T3. In contrast, type III deiodinase (dio3) inactivates THs by inner-ring deiodination of T4 and T3 to produce rT3 and T2 (diiodothyronine), respectively (reviewed in Galton, 2005). During gonadal differentiation, THs regulate the expression of sex steroid-related genes in the Leopard frog tadpole brain (Hogan et al., 2007) and chronic inhibition of TH synthesis produces female-biased sex ratios in *Xenopus laevis* (Goleman et al., 2002). Thyroid hormone-related genes, for example, *tr* and *dio* are expressed and functional during amphibian embryogenesis (Havis et al., 2006; Morvan Dubois et al., 2006), therefore, an interaction between the thyroid and reproductive axes might also be present in early amphibian development.

Both cyp19 and srd5 are involved in the final biosynthesis step of estrogen and DHT, respectively; therefore they are good candidate enzymes to study these steroidogenic pathways during early amphibian development. We hypothesized that cyp19 and srd5 are present and active during early anuran development and that their inhibition will affect the transcription of sex steroid- and thyroid hormone-related genes. The objectives of this study were to first establish the ontogenic expression of *cyp19* and *srd5* during embryogenesis and second to investigate the effects of inhibiting cyp19 or srd5 on both sex steroid- and thyroid hormone-related gene expression. The amphibian model *S.* (*Xenopus*) *tropicalis* was selected because of the ease of studying embryonic development and for its available genomic sequence information (Amaya et al., 1998; Hirsch et al., 2002).

2. Materials and methods

2.1. Breeding and maintenance of S. (Xenopus) tropicalis

The scientific name of our model species changed from *Xenopus tropicalis* to *Silurana tropicalis* which explains the double genus notation (Cannatella and Trueb, 1988). From now on *S. tropicalis* will be used to refer to this species. Frogs were reared in dechlorinated and aerated water from the University of Ottawa Animal Care Facility (Ontario, Canada). Fertilized eggs were obtained from eight pairs of frogs by injecting human chorionic gonadotropin hormone (hCG; Sigma) into the dorsal lymph sac of adult *S. tropicalis*. Both males and females received a priming injection of 12.5 IU hCG followed by a boosting injection of 100 IU hCG after 20 h. Staging was determined by following the Nieuwkoop and Faber developmental table (NF; Nieuwkoop and Faber, 1994). A 12:12 h light:dark cycle was maintained with the light cycle occurring from 7 am to 7 pm at 24–25 °C. The care and treatment of animals used in this study were in accordance with the guidelines of the Animal Care Committee, University of Ottawa and the Canadian Council on Animal Care following standard protocols.

2.2. Tissue collection for developmental profiles

Eggs and larvae were raised in Petri dishes containing modified Ringer's solution (0.1 M NaCl, 1.8 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 300 mg/L $NaHCO_3$; 1:9 v/v) and 0.04 mg/L of the antibiotic gentamycin (Sandoz Canada, Inc.). Pooled embryos (before hatching; NF 2–34) and larvae (NF 41 and 46) were used to ensure a sufficient amount of RNA for gene expression (20–25 per pool; n = 6–8 pools) and protein for cyp19 (40–50 per pool; n = 7–8 pools) and srd5beta activity (20–50 per pool; n = 6 pools). For gene expression profiles, whole embryos and larvae were sampled at NF 2, 7, 16, 21, 26–27, 34, 41 and 46, which corresponds to 1, 4.5, 13, 15, 20, 36, 44 and 72 h post-fertilization (hpf) under our husbandry conditions. Embryos were frozen immediately in dry ice and stored at −80 °C. Larvae at NF 41 and NF 46 were anaesthetized by immersion in 3-aminobenzoic acid ethyl ester (MS-222; 0.01%; Sigma) before freezing. For enzyme activity analyses, whole embryos and larvae were also collected at NF 7, 21, 34 and 46. Embryos were frozen immediately in dry ice and stored at −80 °C.

2.3. Fadrozole and finasteride exposure

Eggs were allowed to develop to NF 6, at which point they were collected and dejellied with 2% (w/v) L-cysteine (pH 8.0; Sigma). The eggs were washed three times with modified Ringer's solution (1:9 v/v) following cysteine treatment and placed in Petri dishes (containing modified Ringer's solution and antibiotic, as described above) at a density of 50 eggs per dish. Embryos were exposed from NF 12 to NF 46 (8–72 hpf) to nominal concentrations of fadrozole (0.5, 1.0, 2.0 μM; Novartis Pharma AG, Basel, Switzerland) dissolved in water and finasteride (25, 50, 100 μM; Sigma Canada Ltd., Oakville, ON) delivered in ethanol (0.05%). Embryos were also exposed to water and ethanol (0.05%) controls. Throughout the exposure, the medium and antibiotic were refreshed daily. Whole NF 46 larvae were sampled from each treatment for gene expression (10 per pool; n = 5–8 pools), cyp19 (40 per pool; n = 5 pools) and srd5beta activity assays (20 per pool; n = 5–6 pools).

2.4. RNA isolation and cDNA synthesis

Homogenization of the samples was achieved using an MM301 Mixer Mill (Retsch, Newton, PA, USA) at 20 Hz for 4 min. For the developmental profile samples, total RNA was obtained using the Qiagen RNeasy Micro Kit (including the RNase-free DNase treatment), whereas for the fadrozole and finasteride exposure, RNA was extracted using the Qiagen RNeasy Mini Kit as described by the manufacturer (Qiagen, Mississauga, ON, Canada). Isolated RNA was resuspended in RNase-free water and stored at −80 °C. Concentrations of RNA were determined using the NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Inc.). Total cDNA was prepared from 2 μg of total RNA and 0.2 μg random hexamer primers (Invitrogen) using Superscript II reverse transcriptase as described by the manufacturer (Invitrogen). The cDNA products were diluted 80-fold prior to PCR amplification.

2.5. Real-time RT-PCR

Specific primer sets (Table 1) were designed for simplex real-time RT-PCR for *cyp19*, *srd5alpha1*, *srd5alpha2*, *srd5alpha3*, *srd5beta*, androgen receptor (*ar*), *tralpha*, *trbeta*, *dio1*, *dio2* and *dio3*. Primers