General and Comparative Endocrinology 163 (2009) 259–269

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

New evidences for the involvement of 20β-hydroxysteroid dehydrogenase in final oocyte maturation of air-breathing catfish ☆

G. Sreenivasulu, B. Senthilkumaran *

Department of Animal Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500 046, Andhra Pradesh, India

ARTICLE INFO

Article history:
Received 25 January 2009
Revised 15 April 2009
Accepted 20 April 2009
Available online 3 May 2009

Keywords:
20β-HSD
Carbonyl reductase
Final oocyte maturation
Gonadal cycle
GVBD

ABSTRACT

20β-hydroxysteroid dehydrogenase (20β-HSD) synthesizes 17α,20β-dihdroxy-4-pregnen-3-one, the steroid required for resumption of prophase-I arrested oocytes in teleosts. Though *20β-HSD* cDNAs have been cloned from few fish species, its role in final oocyte maturation (FOM) is still questionable. To study the role of *20β-HSD* in FOM more explicitly, we cloned and characterized *20β-HSD* from ovary of air-breathing catfish, *Clarias gariepinus*. Interestingly, *Escherichia coli* expressed recombinant proteins, both full-length and an N-terminal truncated proteins catalyzed the reduction of steroids and xenobiotics, however there was significant difference between them. Semi-quantitative RT-PCR and Western blots demonstrated the presence of 20β-HSD transcript and protein in various tissues with relatively higher level in gonads, gill, kidney and brain. A positive correlation of *20β-HSD* expression was observed in different phases of ovarian cycles. Immunocytochemical/immunofluoroscence analysis with specific antibody identified presence of 20β-HSD in follicular layer of ovary. Real-time RT-PCR and Western blotting showed an induction of *20β-HSD* expression during human chorionic gonadotropin (hCG)-induced oocyte maturation, *in vitro* and *in vivo*. Concomitantly, a rise in 20β-HSD enzyme activity was also noticed. Specific inhibitors of carbonyl reductase inhibited not only recombinant protein catalytic activity but also hCG-induced oocyte maturation in a dose-dependent manner as evidenced by blocking of germinal vesicle break down. These results together provide new evidences for the involvement of *20β-HSD* in the FOM/meiotic maturation.

1. Introduction

Meiotic maturation of prophase-I arrested oocytes is pre-requisite for ovulation and subsequent fertilization to occur in vertebrates (Nagahama and Yamashita, 2008). Studies involving several fish models revealed that oocyte maturation in teleosts are three step induction process involving gonadotropins (GTHs), maturation-inducing hormone (MIH) and maturation-promoting factor (Nagahama and Yamashita, 2008). Different classes of steroid hormones have shown to induce oocyte maturation in vertebrates including teleosts (Goswami and Sundararaj, 1974; Nagahama and Adachi, 1985; Trant and Thomas, 1989; Lutz et al., 2001; Senthilkumaran and Joy, 2001) apart from classical MIH, 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP; Nagahama, 1997). In general, in teleosts, progesterone derived steroids, 17α,20β-DP and 17α,20β-21β-trihydroxy-4-pregnen-3-one have been identified as more potent MIHs (Nagahama and Adachi, 1985; Trant and Thomas, 1989). MIH synthesis occurs in the ovarian follicle layers under the stimulation of GTHs, pre-ovulatory luteinizing hormone (LH) surge being the stimulus for final oocyte maturation (FOM; Nagahama and Yamashita, 2008). A two-cell model has been proposed to explain the production of MIH (Nagahama, 1997). As per this model, the LH surge stimulates the thecal cells to produce 17α-hydroxyprogesterone (17α-OHP) that is acted up on by 20β-hydroxysteroid dehydrogenase (20β-HSD) in granulosa cells to produce MIH via shift in steroidogenesis (Senthilkumaran et al., 2004). Further, studies in some fish species demonstrated the elevation of 20β-HSD enzyme activity in response to GTHs that is emulated by forskolin and dbcAMP (Nagahama, 1997, and references therein; Kazeto et al., 2001). Subsequently transcriptional and translational up-regulation of *20β-HSD* was identified to be involved in the elevation of activity that is observed during FOM (Nagahama, 1997).

cDNAs encoding *20β-HSD* have been cloned from rainbow trout (Guan et al., 1999), ayu (Tanaka et al., 2002), the Nile tilapia (Senthilkumaran et al., 2002) and zebrafish (Wang and Ge, 2002) ovaries. Surprisingly, cDNA cloning studies have demonstrated a striking similarity of fish *20β-HSD* to that of mammalian carbonyl reductase1 belonging to short chain alcohol dehydrogenase/reductase (SDR) superfamily (Tanaka et al., 1991). Consistent with mammalian carbonyl reductases (CRs), piscine counterparts showed NADPH-dependent CR activity on a wide range of carbonyl

☆ *Note:* Catfish ovarian *20β-HSD* nucleotide sequence has been submitted to GenBank under the Accession No. FJ031008.

* Corresponding author. Fax: +91 40 23010307/23010120.
E-mail addresses: bsksl@uohyd.ernet.in, senthilkumaranb@yahoo.com (B. Senthilkumaran).

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doi:10.1016/j.ygcen.2009.04.022

compounds including several xenobiotics, endogenous prostaglandins and steroids. A relatively higher Km value for prostaglandins and steroids raises skepticism on the involvement of 20β-HSD in metabolism of steroids, *in vivo* (Wermuth, 1981; Inazu et al., 1992). Nevertheless, there are studies to support its involvement in the production of MIH (Tanaka et al., 2002), localization of expression in ovarian follicles (Wang and Ge, 2002). However, its role in FOM is still ambiguous.

In light of the above cited literature that *20β-HSD* is involved in the gametogenesis and given the importance of this enzyme in different functions, to our knowledge there are no studies on the expression pattern of both transcript, protein and enzyme activity of *20β-HSD* during FOM. Immunolocalization, regulation of *20β-HSD* expression and interaction with other autocrine/paracrine factors is also not known. Air-breathing catfish, *Clarias gariepinus* is selected as experimental model that shows annual breeding pattern characterized by an extended period of oocyte growth culminating in a single spawning event. The synchronous development of ovarian follicles in this species facilitates the correlation of expression of steroidogenic enzyme genes with gametogenesis. Further, induced-breeding in this fish facilitate for natural collection of *in vivo* matured oocytes from live fish at different time-points. We used this strategy to perform both mRNA and protein expression patterns of *20β-HSD* during human chorionic gonadotropin (hCG)-induced oocyte maturation *in vitro* and *in vivo*. To enable this, we cloned and characterized *20β-HSD* from catfish ovary. Present study also utilized chemical inhibitors and immunolocalization to delineate the importance of CR-like *20β-HSD* in FOM. Taken together, we attempted to provide new evidences for the involvement of *20β-HSD* in FOM.

2. Materials and methods

2.1. Animals

Air-breathing catfish, *C. gariepinus* (400–500 g) caught from local (Hyderabad, India) fresh water ponds were purchased 2–3 weeks before experimentation. Fish were acclimated for 2–3 weeks by maintaining in aquarium tanks filled with filtered tap water under normal photoperiod and ambient temperature (26 ± 2 °C). Catfish were fed with minced goat liver *ad libitum* during acclimation and experimentation. Four broadly distinguishable phases, namely preparatory, pre-spawning, spawning and post-spawning/regressed/resting were seen in both sexes of wild caught and laboratory-reared catfish (Swapna et al., 2006).

2.2. RT-PCR amplification of partial cDNA homologous to 20β-HSD

Degenerate primers were designed by aligning the existing sequences of vertebrate CRs to clone *20β-HSD* cDNA fragment from the ovarian follicles of catfish. Using (Sense: 5′-CAG AGT GGT GAA TGT VTC HAG C-3′, Antisense: 5′-CCT GCC ATG TCR GTK CKG ACC-3′) these degenerate primers, a cDNA fragment homologous to *20β-HSD* was amplified by RT-PCR and cloned in pGEM-T Easy vector (Promega, Madison, WI, USA).

2.3. Rapid amplification of cDNA ends (RACE)

The 5′ and 3′ end sequences of catfish *20β-HSD* cDNA were cloned by RNA-ligase mediated RACE system (Invitrogen, Carlsbad, CA, USA). Preparation of 5′ and 3′ cDNA (RACE) templates and RT-PCR were done using gene specific primers (5′-RACE GSP: 5′-CAC TCC GGA AGC GAG CCT GGA GGT C-3′; Nested GSP: 5′-GGA ACT CAT TGC ACA AGT CTC TGG T-3′; 3′-RACE GSP: 5′-CCT GTG CAA TGC CTG CTG TCC AGG A-3′, Nested GSP: 5′-GGA TGG GTCA GCA CCG ACA TGG CAG A-3′) designed from partial cDNA fragment as per the manufacturer's protocol and different sizes of RACE products were cloned into pGEM-T Easy vector and subsequently sequenced.

2.4. Genomic Southern analysis

Genomic DNA was prepared from ovarian follicles of catfish using genomic DNA preparation kit (Bangalore Genei, Bangalore, India), digested separately with *BamH*I, *Hind*III, *Pst*I, *Kpn*I, and *EcoR*I, electrophoresed on 0.8% agarose gel and transferred on to Hybond-N$^+$ nylon (Amersham) membrane by capillary transfer. Membrane was hybridized with radiolabeled cDNA of catfish ovarian *20β-HSD* and after high stringency washes, signals were detected using phosphorimager (Amersham).

2.5. Northern blot analysis

Total RNAs from different stages of ovary of catfish were prepared using TRI-reagent (Sigma) and 25 μg of total RNA were separated on a 1% denaturing formaldehyde-agarose gel and transferred onto a nylon membrane (Hybond$^+$, Amersham). The membrane was hybridized under high stringency conditions with partial cDNA fragment (313 bp) labeled with ^{32}P-dCTP by random primer labeling kit (Perkin Elmer). After overnight hybridization, the membrane was washed with 2 × SSC, 1 × SSC, 0.1 × SSC containing 0.1% SDS at 60 °C each for 10–15 min. The signals were detected using phosphorimager (Amersham).

2.6. Expression of catfish recombinant 20β-HSD cDNA in Escherichia coli

Primers were designed to introduce *Nhe*I at 5′ end and *Hind*III site at 3′ end of catfish *20β-HSD* cDNA open reading frame (ORF). An N-terminal truncated construct was also created through the deletion of fourteen amino acid residues using a primer with *Nhe*I site. The PCR amplified products (both full-length and N-terminal truncated) were digested with restriction enzymes, cloned into expression vector pET28a$^+$ (Novagen, La Jolla, CA, USA) and subsequently introduced into *E. coli* BL21 strain. Expression of recombinant 20β-HSD was carried out by inducing the cultures with 0.1 mM IPTG at an O.D. of 0.4. One liter culture was harvested, suspended in 50 mM Tris–Cl buffer, pH 8.0, and homogenized by sonication. Clear lysate was obtained by centrifuging at 15,000 rpm for 30 min. Recombinant proteins were purified by Ni-NTA (Qiagen) affinity chromatography as per manufacturer's instructions.

2.7. Production of rabbit anti-catfish 20β-HSD antiserum

E. coli expressed catfish recombinant 20β-HSD protein was purified as mentioned above and further purification was carried out to remove contaminants if any, by separating purified fractions on a 12% SDS–polyacrylamide gels followed by elution from the gels. Protein eluted from the gels was used as an antigen, mixed with Fruend's complete adjuvant and injected subcutaneously to 3 month old male rabbits. Rabbits were housed and handled as per Institutional Animal Ethic Committee guidelines. Following primary dose, booster doses were given with Fruend's incomplete adjuvant and antiserum was collected from marginal ear vein. Subsequently, IgG fraction was purified using Protein-A CL-agarose column (Bangalore Genei) and antibody was characterized by Western blot method.

2.8. Enzymatic characterization of recombinant 20β-HSD

Purified recombinant 20β-HSD proteins were incubated in100 mM phosphate buffer, pH 7.4, with the substrate [^{3}H]-17α-