Abstract

Role of cytochrome P450 17α-hydroxylase/c17-20 lyase (P450c17) in the shift in steroidogenesis during oocyte maturation in teleosts is a contentious issue even after identification of a novel type of P450c17 that lacks lyase activity. To understand the role of P450c17 in steroidogenic shift explicitly, a full length cDNA encoding P450c17 from ovary of air-breathing catfish, Clarias gariepinus was cloned. Transient transresection of P450c17 in COS-7 cells converted progesterone to androstenedione through 17α-hydroxyprogesterone and catfish P450c17 was found to express ubiquitously with relatively higher levels in gonads, brain, kidney and gills. P450c17 expression and ratio of lyase to hydroxylase was high in the preparatory and pre-spawning phases of ovary and low in spawning phase. Expression of P450c17 correlated well with testicular recrudescence with a maximum expression in the preparatory and spawning phases. Neither protein expression nor lyase/hydroxylase activity changed significantly during human chorionic gonadotropin-induced oocyte maturation, in vitro and in vivo though mRNA levels increased. These results tend to suggest that the ovarian follicles attains capacity to produce maximum precursor steroid levels before spawning that might contribute to the shift in steroidogenesis.
Introduction

Cytochrome P45017α-hydroxylase/c17-20 lyase (P450c17) is a microsomal enzyme that catalyzes two distinct activities; the 17α-hydroxylase activity converts pregnenolone or progesterone to 17α-hydroxypregnenolone or 17α-hydroxyprogesterone (17α-OHP) and c17-20 lyase activity breaks the C17-20 bond of C21 steroids 17α-hydroxypregnenolone or 17α-OHP to produce dehydroepiandrosterone (DHEA) or androstenedione (AD), respectively (Nakajin et al., 1981). Thus, P450c17 controls an important branch point in steroid hormone biosynthesis leading to the production of three classes of main steroid hormones namely glucocorticoids, mineralocorticoids and precursors of sex steroids (Payne and Hales, 2004).

P450c17 catalyzes two mixed function oxidase reactions utilizing cytochrome P450 oxidoreductase and microsomal electron transfer system. These reactions require NADPH and molecular oxygen (Payne and Hales, 2004). Lyase activity is regulated by a set of modulators in tissue dependent manner. In mammals, modulators of lyase activity include electron-donating redox partners such as P450 reductase (Auchus and Miller, 1999). Cytochrome b5 has been shown to stimulate lyase activity by acting as an allosteric facilitator (Soucy et al., 2003). Moreover, serine/threonine phosphorylation of P450c17 increases the affinity of enzyme for redox partners (Jhang et al., 1995; Miller et al., 1997) and is implicated in adrenarche, polycystic ovarian syndrome and associated insulin resistance in humans (Jhang et al., 1995).
cDNA encoding \textit{P450c17} has been cloned from several higher vertebrates including human (Chung \textit{et al}., 1987), bovine (Zuber \textit{et al}., 1986), pig (Zhang \textit{et al}., 1992), chicken (Ono \textit{et al}., 1988), rat (Fevold \textit{et al}., 1989) and mouse (Youngblood \textit{et al}., 1991). \textit{P450c17} expression is regulated by gonadotropins (Voutilainen \textit{et al}., 1986; Voss and Fortune, 1993), adrenocorticotropic (Zuber \textit{et al}., 1986), growth factor such as transforming growth factor and insulin like growth factor-I (Magoffin and Weithman, 1993; Carr \textit{et al}., 1996), activin, inhibin (Sawetawn \textit{et al}., 1996) and anti-mullerian hormone (Matt Laurich \textit{et al}., 2002). On the other hand, hormonal regulation of \textit{P450c17} in lower vertebrates including teleosts remains to be clarified.

During final oocyte maturation (FOM), a shift in steroidogenesis from estradiol-17β to 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) is demonstrated for several teleost species (Nagahama, 1997; Joy \textit{et al}., 1998) and reports do exist on high expression of 20β-hydroxysteroid dehydrogenase (Senthilkumaran \textit{et al}., 2004; Chapter 1 & 2), enzyme that produces 17α, 20β-DP. However, it is possible that \textit{P450c17} might play a crucial role in shift in steroidogenesis by controlling the availability of precursor steroids. Though there are reports available on the cloning and expression of \textit{P450c17} from different fish species (Sakai \textit{et al}., 1992; Trant, 1995; Kazeto \textit{et al}., 2000; Halm \textit{et al}., 2003; Wang and Ge, 2004), none of these reports neither recorded changes in expression pattern and protein level/activity during steroidogenic shift nor provided convincing claim of regulation of lyase activity. But very recently, a novel form of \textit{P450c17} that lacks lyase activity has been identified in tilapia (a fortnight breeder) and
medaka (a daily breeder) whose differential expression pattern was proposed to be important during shift in steroidogenesis (Zhou et al., 2007a & b). However, many teleost species that reproduce annually (more importantly fresh water inhabitants) possess only single form of \( P450c17 \) (\( P450c17-I \)) and its role during meiotic maturation is a contentious issue. Against this backdrop, present study is designed to delineate contribution of \( P450c17 \) expression and activity in steroidogenic shift. \( P450c17 \) was cloned and characterized from the ovarian follicles of air-breathing catfish, *Clarias gariepinus*. Expression of \( P450c17 \) during human chorionic gonadotropin (hCG)-induced oocyte maturation *in vitro* and *in vivo* was analyzed by quantitative real-time RT-PCR and Western blot methods. In addition, expression in testicular recrudescence and tissue distribution pattern was analyzed. Results from this study provide interesting information about the role of \( P450c17 \) in steroidogenic shift during FOM.
Materials and methods

Animals and treatments

Adult catfish weighing about 400-500 g were purchased live from local fish markets (Hyderabad, India). Maintenance of animals and hCG-induction was already described in chapter 2.

Molecular cloning of catfish \textit{P450c17}

A set of degenerate primers were designed by aligning the existing sequences of teleost \textit{P450c17} to clone a cDNA fragment from the ovarian follicles of catfish. After obtaining a partial cDNA of \textit{P450c17}, 5' and 3' sequences were cloned following rapid amplification of cDNA ends (RACE) approach using gene specific primers designed from partial cDNA (Table 1). Details of methodology followed for cloning was already described in chapter 2.

Genomic Southern and Northern blot analysis

Southern analysis of genomic DNA prepared from ovarian follicles and Northern blot analysis of total RNA prepared from spawning ovary and tesis was carried out by following methodology described in chapter 2 using a $^{32}$P-labellled 450 bp partial cDNA of \textit{P450c17}. 

\textit{P450c17 in final oocyte maturation}
Production of rabbit anti-catfish P450c17 antiserum

To produce catfish P450c17 antigen, a 1.365 kb NdeI-XhoI fragment of catfish ovarian P450c17 cDNA lacking the region encoding the N-terminal 56 amino acids was inserted into the NdeI and XhoI sites of vector pET28a (Novagen, La Jolla, CA, USA). The expression constructs were verified by restriction analysis and checked for cloning artifacts, if any by nucleotide sequencing. Expression, purification of recombinant P450c17 and production of polyclonal antiserum was carried out following methods described in chapter 2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' – 3'</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>DF1</td>
<td>ASCTGCARMAGAARTAYGG</td>
<td>Degenerate RT-PCR</td>
</tr>
<tr>
<td>DR1</td>
<td>CACYTCYCTGATRGTGGCYTC</td>
<td>Degenerate RT-PCR</td>
</tr>
<tr>
<td>GSP-F1</td>
<td>GACGCTAAGATTTGGAGGACAGG</td>
<td>5' RACE</td>
</tr>
<tr>
<td>GSP-F2</td>
<td>CTGGAAGCCACTATCAGAGTG</td>
<td>5' RACE</td>
</tr>
<tr>
<td>GSP-R1</td>
<td>TAGCAGGACCTCCTTGACATGTTG</td>
<td>3' RACE</td>
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<tr>
<td>GSP-R2</td>
<td>AATGTCTCCGTATTTCTTCTGCAG</td>
<td>3' RACE</td>
</tr>
<tr>
<td>ORF-F</td>
<td>GCTAGCATGGCATGGTTTATTTGTTTG</td>
<td>Cloning</td>
</tr>
<tr>
<td>ORF-R</td>
<td>CTGAGCTAGCTGACTTACACTCTTTG</td>
<td>Cloning</td>
</tr>
<tr>
<td>qRT-F</td>
<td>CCATGGCTCCAGCTCTTTCC</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>qRT-R</td>
<td>CAGTAGACCAACATCCTGAGTGC</td>
<td>Real-time PCR</td>
</tr>
</tbody>
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Table 1. List of primers used for cloning and expression of P450c17.
Functional characterization of catfish \textit{P450c17}

COS-7 cells were grown in DMEM medium supplemented with 10% fetal bovine serum. Cells were transfected using Tfx20 (Promega) with either pcDNA3.1 (Mock) or pcDNA3.1 containing cDNA encoding catfish \textit{P450c17}. After 24 hours, fresh medium was added and 50,000cpm/well of $^3\text{H}$ progesterone (Amersham) was added. Cells were then incubated at 37°C and medium was collected at the specified times. Steroids in the medium were extracted twice with 3ml of diethyl ether, separated on thin layer chromatography (TLC) plates using benzene:acetone (4:1 v/v) solvent system and analyzed using phosphorimager. The signals were identified based on the Rf values of standards.

Real-time RT-PCR

Expression of \textit{P450c17} was analyzed by real-time RT-PCR as described in chapter 2 using \textit{P450c17} primer pair listed in table 1. Transcript abundance of \textit{P450c17} was normalized to that of $\beta$-actin and reported as fold change in abundance relative to the values obtained for spawning phase using the formula $2^{\text{\Delta\DeltaCT}}$.

Western blot analysis

Analysis of P450c17 protein levels following hCG-induced oocyte maturation, \textit{in vitro} and \textit{in vivo} as well as in different stages of ovarian cycle was done by Western blot using anti-cfP450c17 polyclonal antiserum.
Enzyme assay

17α-hydroxylase and C17-20 lyase activities of catfish ovarian P450c17 were assayed as described by Zhang et al. (1995). Ovarian follicle microsomes were prepared by homogenizing 500 mg of tissue in 3 ml of 0.25 M sucrose/5mM EDTA pH 7.4, clearing debris at 9000Xg for 20 min, and centrifuging at 105,000Xg for 1 hour. The crude microsomal pellet was washed in 0.1 M K. PO₄ pH7.4/0.1 mM EDTA; microsomes were harvested at 105,000Xg for 1 hour, resuspended in 200 µl of 0.1 K. PO₄ buffer/0.1mM EDTA/20% v/v glycerol. P450 activity was measured by incubating 100 µg of microsomal protein and 1 nmol of [³H] progesterone in 0.2 ml of 100 mM K. PO₄ (pH7.4), 1 mM MgCl₂, 0.4 mM NADP⁺, 5 mM glucose-6-phosphate and 0.2 unit of glucose-6-phosphate dehydrogenase at 37°C for 1 hour. Steroids were extracted with three volumes of diethyl ether and analyzed by TLC in benzene/acetone (4:1 v/v) solvent system. Reaction products were identified by Rf values of the respective standards, eluted in alcohol and the radioactivity was measured using liquid scintillation counter.

Data analysis

All the data were expressed as mean ±SEM. Significant among groups was tested by ANOVA followed by Student’s-Newmann-Keuls’ test using Sigmastat 3.1 software. Differences among groups were considered at P<0.05.
Results

Molecular cloning of catfish ovarian *P450c17*

Using a set of degenerate primers, a partial cDNA of 911 bp was isolated by RT-PCR and sequence identity was confirmed by BLAST search. Full-length cDNA of *P450c17* was then obtained through 5' and 3' RACE approaches using gene specific primers designed from partial cDNA fragment (Fig 1). The cloned full length cDNA encoding *P450c17* from the ovary of air-breathing catfish was 2071 bp in length with a 463 bp 3' untranslated region (UTR) and 66 bp 5' UTR. The 3' UTR has one polyadenylation signal. The open reading frame was 1542 bp long encoding a putative enzyme of 514 amino acids (Fig 2). ClustalW multiple alignment demonstrated the presence of signature domains including heme binding region, Ozol’s tridecapeptide and Ono sequence were well conserved in catfish *P450c17* and all these signature domains showed high homology to that of *P450c17-I* of other teleosts (Fig. 3). In contrast, catfish *P450c17* exhibited considerable difference in amino acid sequence in Ono sequence and Ozol’s tridecapeptide of *P450c17-II* while heme binding region seems to be more or less similar in both forms of *P450c17* (Fig.3). Phylogenetic analysis revealed that catfish *P450c17* has about 60-87% homology with other teleost *P450c17-I*. In contrast it exhibited only about 41-43% homology to that of teleost *P450c17-II* (Fig. 4).
Genomic Southern and Northern blot analyses

Southern analysis of genomic DNA prepared from ovarian follicles identified single band in all the restriction digests when probed with a partial cDNA fragment that possess heme binding region (Fig. 5A). Northern blot analysis identified single transcript of about ~2.1 kb both in testis and ovary (Fig. 5B).

Functional characterization in COS-7 cells

A cDNA corresponding to the open reading frame of catfish P450c17 was obtained by PCR and cloned in to mammalian expression vector pcDNA3.1. COS-7 cells transfected with P450c17 expression construct was able to convert progesterone to AD through the intermediate 17α- OHP demonstrating that the cDNA product was indeed functional possessing both hydroxylase and lyase activity (Fig. 6). Similar results were obtained with HEK293 cells after P450c17 expression (data not shown).

Tissue distribution

Using RT-PCR analysis, P450c17 expression was detected in several tissues other than gonads including brain, gill, liver, intestine, kidney, heart and muscle. The expression was relatively higher in gonads, brain, kidney and spleen (Fig. 7).
Stage dependent expression of \textit{P450c17} in ovary and testis

\textit{P450c17} transcript level found to be high during expressed the preparatory and pre-spawning phases while it was low level in spawning and regressed phases (Fig. 8A). Western blot analysis of protein expression in these phases of ovarian follicles was in accordance with mRNA levels (Fig. 9A). Consistent with expression, the ratio of lyase to hydroxylase activity was high in preparatory and pre-spawning phases while it was low in spawning and regressed phases (Fig. 9B). Cf-P450c17 antiserum characterization details were presented in figures 10 & 11. Real-time RT-PCR analysis demonstrated that the expression of \textit{P450c17} was high in preparatory and spawning phases of testicular cycle while it was low in pre-spawning and regressed phases (Fig. 8B).

\textit{P450c17} expression and activity during hCG-induced oocyte maturation, \textit{in vitro} and \textit{in vivo}

There was significant increase in the expression of \textit{P450c17} by two hours after treatment with hCG both \textit{in vitro} and \textit{in vivo} compared to saline-treated controls (Fig. 12). On the contrary, Western blot analysis revealed no changes in protein levels of P450c17 (Fig. 13). Concomitantly, no significant difference was noticed between the ratio of lyase to hydroxylase activity (Fig. 14).
Fig. 1. 1% Agarose gels showing RT-PCR products using degenerate primers for amplification of partial $P450c17$ cDNA fragment, 3' and 5' RACE products using gene specific primers to obtain $P450c17$ full-length cDNA. Marker in all the gels used was 1 kb ladder.
Fig. 2. Nucleotide (blue) and deduced amino acid (red) sequence of catfish ovarin P450c17. UTRs are shown in black letters and polyadenylation signal is shown in boldface letters with underline.
Fig. 3. Alignment of deduced amino acid sequence of catfish P450c17 with those of P450c17-I and P450c17-II of other telesots by ClustalW multiple alignment. Conserved domains are shown in rectangles. I; putative membrane spanning region, II; the P450c17 specific Ono sequence, III; Ozols tridecapeptide regions, IV; the heme binding region. (Accession no.: Trout NM_001124747; Eel AY498619; Tilapia-I AB292401; Tilapia-II EF423917; Zebrafish-I AY281362; Zebrafish-II EF624003; Medaka-I NM_001105094; Medaka-II EF429318).
Fig. 4. Phylogenetic tree showing the evolutionary relationship of catfish P450c17. (Please refer fig. 2 for accession numbers of teleost P450c17s. Accession no.: Rhesus monkey NM_001040232; Pig NM_214428; Goat AF251387; Chimpanzee NM_001009052; Baboon AF297650; Sheep AF251388; Chicken M21406; Human M14564; Mouse NM_007809; Rat M31861; Frog AF325435).
Fig. 5. Genomic Southern analysis of catfish ovarian follicles probed with a partial cDNA lacking sites for the enzymes used in digestion (A). 1kb DNA ladder was used to identify the size of signals. Northern blot analysis of 25 µg of total RNA from catfish ovary and testis (B). RNA ladder was used to identify the size of bands.
**P450c17 in final oocyte maturation**

**Fig. 6.** Autoradiogram showing the 17α-hydroxylase and C17-20 lyase activities of catfish P450c17 transiently expressed in COS-7 cells. Lanes 1. Standards, 2. pcDNA3.1, 3. pcDNA3.1-P450c17.

**Fig. 7.** RT-PCR analysis of spatial expression pattern of catfish P450c17 in different tissues. Plasmid clone containing catfish P450c17 was used as positive control (+ve ctl). Negative control (-ve ctl) contains no cDNA template. Ant. kidney, Anterior kidney; Post. kidney, Posterior kidney. Marker used was 100 bp ladder.
Fig. 8. Real-time RT-PCR analysis of P450c17 expression in catfish ovarian (A) and testicular (B) cycles. P, Preparatory; PS, pre-spawning; S-spawning; R, regressed (* indicates the significance, n=3, P<0.05, ANOVA).
Fig. 9. Representative Western blot showing P450c17 expression in different stages of ovary (A). Ponceau S staining was used to depict equal loading (lower panel). Lyase to hydroxylase ratio in different stages of ovarian cycle (B). P, Preparatory; PS, Pre-spawning; S, Spawning; R, Regressed (* indicates the significance n=3, $P<0.05$, ANOVA).
Fig. 10. A 10% SDS-PAGE showing the expression of catfish recombinant P450c17 in *E. coli* BL21 (A). Lane 1, protein marker, lane 2, un-induced and lanes 3, 4 are induced with IPTG at 2 and 4 hrs respectively. Western blot analysis of recombinant P450c17 with mouse anti-His antibody (B).

Fig. 11. Western blot analysis of catfish recombinant P450c17 with Pre-immune serum (A) and rabbit anti-cfP450c17 (B).
Fig. 12. Real-time RT-PCR analysis of \textit{P450c17} expression during hCG-induced oocyte maturation \textit{in vitro} (A) and \textit{in vivo} (B). * Indicates the significance (n=3, \( P<0.05 \), ANOVA).
Fig. 13. Western blot analysis (representative, n=3) of P450c17 during hCG-induced oocyte maturation \textit{in vitro} (A) and \textit{in vivo} (B). Ponceau S staining was used to depict equal loading (lower panel).

Fig. 14. Changes in lyase to hydroxylase ratio during hCG-induced oocyte maturation \textit{in vitro} (A) and \textit{in vivo} (B).
Discussion

In the present study, we used hCG-induced oocyte maturation both in vitro and in vivo to delineate the role of P450c17 in steroidogenic shift. To achieve this, a full-length cDNA encoding P450c17 was isolated from catfish ovarian follicles. The catfish P450c17 has high homology to that of P450c17-I cDNA sequences reported in other teleosts and their counterparts in higher vertebrates. The close relationship of P450c17s among these species is likely to be common. In general, P450 enzymes exist as single gene in multiple species mediating multiple enzymatic steps (Miller, 2002) and multiple forms of P450c17 have not been found in mammals and other lower vertebrates except in few teleost species in which a second form of P450c17 that lacks the lyase activity (Zhou et al., 2007a & b). However, since we used partial cDNA fragment spanning the common heme binding region as probe in genomic Southern analysis, our identification of single copy gene for P450c17 seems to be reasonable. COS-7 cells transfected with catfish P450c17 cDNA converted exogenous progesterone to 17α-OHP and AD that is in accordance with previous reports in which only single form of P450c17 was identified. Consistent with cDNA cloning and genomic Southern analysis, a single transcript was detected both in ovary and testis.

The observed positive correlation of P450c17 expression in catfish gonadal cycle shares similarity with other teleosts like trout (Sakai et al., 1992), eel (Kazeto et al., 2000) and channel catfish (Kumar et al., 2000). However, there was no obvious association of P450c17 expression with ovarian development in fathead minnow (Halm et al., 2003)
while the expression of \textit{P450c17} was high throughout all the developmental stages of ovarian follicles without any significant difference among the developmental stages in zebrafish (Wang and Ge, 2004). The discrepancy in expression pattern of \textit{P450c17} among these species could be attributed to the sensitivity of the techniques used or the varied pattern of reproductive cycles of zebrafish and fathead minnow compared to eel, trout and catfish. On the other hand, in tilapia \textit{P450c17-I} expression peaks about midvitellogenic stage and thereby decreases with maturational stage while \textit{P450c17-II} expression was maximum during maturation. Consistent with mRNA, protein levels and ratio of lyase to hydroxylase activity in different stages of follicle development (present study) corroborates to \textit{P450c17} expression in eel and channel catfish (Kazeto \textit{et al.}, 2000; Kumar \textit{et al.}, 2000). To our knowledge, present study was first of its kind to correlate expression analysis to enzyme activity for \textit{P450c17}. The higher expression of \textit{P450c17} during early and mid stages of follicle development is presumed to be important for production of higher levels of $\Delta^4$ steroids and is supported by the correlation between expression and enzyme activity (Kumar \textit{et al.}, 2000). Intriguingly, a negative correlation was identified with testicular development in fathead minnow (Halm \textit{et al.}, 2003) while we found a maximum expression of \textit{P450c17} in preparatory and spawning phases of catfish testis. The expression pattern of \textit{P450c17} in catfish testis matches with levels of $\Delta^4$ steroids in preparatory and $17\alpha$, $20\beta$-DP levels in spawning phase.
Consistent with previous reports in mammals, birds and fishes, we could also detect \textit{P450c17} transcript in many tissues of catfish. \textit{P450c17} was originally thought to be present exclusively in gonads and adrenals, later on mRNA, protein and/or activity were found in several other tissues including the brain (Yu \textit{et al.}, 2002; Halm \textit{et al.}, 2003;), gastrointestinal tract, (Dalla Valle \textit{et al.}, 1995) and liver (Katagiri \textit{et al.}, 1998). In agreement with its widespread localization, \textit{P450c17} is a potent oxidant and catalytic reactions other than hydroxylation and lyase have been suggested (Lieberman and Warne, 2001). Ying Liu \textit{et al.} (2005) reported that \textit{P450c17} also functions as squalene monooxygenase involved in cholesterol biosynthesis. The 17α-hydroxylase activity of P450c17 is necessary for synthesis of cortisol in adrenal while the lyase activity becomes important for production of sex steroids in gonads (Payne and Hales, 2004). The mechanism behind organ-specific differential actions of P450c17 was attributed to post-translational modification regulations such as abundance of electron donating partner P450 oxidoreductase, cytochrome b5 and Ser/Thr phosphorylation in mammals (Zhang \textit{et al.}, 1995). Alternatively, the presence of P450c17 isoenzymes has been proposed to explain differential actions of P450c17. In teleost ovary, lyase activity is required for the production of estrogens during growth phase while hydroxylase activity is necessary for the production of 17α, 20β-DP during maturational phase (Nagahama, 1997). Until recently (Zhou \textit{et al.}, 2007a & b), only one form of \textit{P450c17} that is highly homologous to mammals has been found in few fish species and it has been thought that the similar mechanism found in mammalian
adrenals were responsible for these differential actions of P450c17 (Kazeto et al., 2000; Wang and Ge, 2004). In the present study neither protein levels nor enzyme activity showed significant changes to hCG both in vitro and in vivo although mRNA levels increased. The significance of P450c17 mRNA rise after treatment with hCG is not clear at present. But the increase in P450c17 mRNA levels could be attributed to the increase in intracellular cAMP levels with hCG as observed in rat Leydig cells (Payne, 1990). It is also plausible that the increase in P450c17 transcripts during hCG-induced oocyte maturation might have higher input to steady levels of P450c17 protein. This contention needs further evaluation. Similarly in eel, P450c17 mRNA levels increased gradually throughout the artificial induction of gonadal development (Kazeto et al., 2000). In contrast, the cultured zebrafish ovarian follicles did not respond to either hCG or activin (Wang and Ge, 2004). In the case of tilapia and medaka, differential expression patterns of two forms of P450c17 are presumed to be important during FOM (Zhou et al., 2007a & b). All the previous reports, including the recent identification of P450c17-II (Zhou et al., 2007a & b), did not study enzyme activity and regulation of lyase activity in detail. Based on the reports in mammals (Miller et al., 1997), it seems to be compulsory to gain knowledge both at the level of mRNA as well as protein and enzyme activity owing to the complexity of regulation of these enzymes. However, this second form of P450c17 is unique to only two teleost species and it is hypothesized that this gene might have been evolved from the fish specific genome duplication (Hoegg et al., 2004; Mayer and Van de Peer, 2005). Hence differential actions of P450c17 with
presence of multiple forms can not be generalized to all the teleost species. Though present study categorically demonstrated the \textit{P450c17} expression and activity during oocyte maturation, regulation of lyase activity in telesots possessing single form of \textit{P450c17} continues to be an issue which needs to be resolved. Further analysis assessing the regulation of lyase activity with reference to P450 oxidoreductase and cytochrome b5 is of worthy that might provide interesting clues about the regulation of P450c17 in telesots possessing single form of \textit{P450c17}.

In conclusion, a single form of \textit{P450c17} that is homologous to \textit{P450c17-I} has been cloned from the ovarian follicles of catfish. \textit{P450c17} mRNA, protein levels and activity was found to be high during preparatory and pre-spawning stages of follicle development while testis has maximum expression during preparatory and spawning stages. During hCG-induced oocyte maturation both \textit{in vitro} and \textit{in vivo}, neither protein level nor ratio of lyase/hydroxylase changed significantly although there was an increase in mRNA levels by 2 hr after induction with hCG. Taken together, it seems that \textit{P450c17} potentiates during preparatory/pre-spawning phases which might exert influence on the shift in steroidogenesis during ovarian and testicular recrudescence.
References


Abstract

Complementary DNAs encoding steroidogenic acute regulatory protein (StAR) have been isolated from different fish species, yet the relevance of StAR during gonadal cycle and more importantly in final oocyte maturation has not been assessed so far. A cDNA encoding StAR was isolated from the ovarian follicles of air-breathing catfish, Clarias gariepinus. Catfish StAR exhibited 55 to 72% identity at nucleotide level with other vertebrate orthologs. RT-PCR analysis of tissue distribution pattern demonstrated the presence of StAR mRNA in various tissues including gonads, head kidney, liver, brain and intestine of catfish. Real-time RT-PCR analysis revealed high expression of StAR mRNA in the pre-spawning phase of ovary while it was low in preparatory, spawning and regressed phases. In testis, maximum expression was noticed during the preparatory phase. During human chorionic gonadotropin (hCG)-induced oocyte maturation, both in vitro and in vivo, StAR mRNA levels were augmented by 2 hrs and then declined gradually to reach basal levels by 12 hrs as that of saline-treated controls. Taken together, high level of expression during hCG-induced oocyte maturation vis-à-vis in spawning suggests a role for StAR, in addition to the steroidogenic enzyme genes in final oocyte maturation.
Introduction
Steroid hormones play a crucial role in the regulation of growth, development, differentiation, reproduction and several other functions in vertebrates. Production of different classes of steroids occurs from a common precursor, cholesterol and involves a battery of oxidative enzymes (Payne and Hales, 2004). The first committed step in steroid hormone biosynthesis is the conversion of cholesterol to pregnenolone, which occurs in mitochondria by the action of P450 side chain cleavage enzyme (P450scc). However, cholesterol cannot cross the mitochondria from cytoplasm and is delivered by a sterol transfer protein, steroidogenic acute regulatory protein (StAR; Stocco, 2000). Now it has been accepted that the true rate limiting step in steroidogenesis is the delivery of cholesterol across mitochondrial membrane. This is an important target for acute steroidogenesis by tropic hormones (Stocco, 2001), few other mediators and some endocrine disruptors (Walsh and Stocco, 2000) as well. Therefore, StAR is indispensable for mediating cholesterol transfer vis-à-vis steroidogenesis. Perhaps the most compelling evidence arose from the identification of mutations in StAR gene during congenital adrenal hyperplasia (CAH), a condition in which cholesterol and cholesterol esters accumulate and the newborn is unable to synthesize adequate levels of steroid hormones (Lin et al., 1995). This is further evidenced by StAR knockout mice which showed phenotypic mirrors of human lipoid CAH (Caron et al., 1997).
A cDNA encoding a 30-kDa mouse StAR was first characterized by Clark et al. (1994). StAR is believed to transfer cholesterol across mitochondria either by forming a
transport tunnel (Tsujishita and Hurley, 2000) or by a cavity (Mathieu et al., 2003). It is plausible that StAR interacts with contact sites where the inner and outer mitochondrial membranes are in close proximity (Thomson, 2003). Further, studies have shown that StAR is rapidly synthesized in response to stimulation of several hormones such as luteinizing hormone (LH), adrenocorticotropic hormone (ACTH; Clark et al., 1995) typically with activation of the cAMP second messenger system. Although StAR appears to be critical for steroidogenesis in the adrenal and gonads, some of the tissues that do not express StAR, including placenta, synthesize large amounts of pregnenolone suggesting for the existence of StAR-independent mechanisms for movement of cholesterol to P450scc enzyme (Stocco, 2001).

Complementary DNA-encoding proteins with high homology to StAR of mammals were cloned from zebrafish, rainbow trout, eel, cod and stingray (Bauer et al., 2000; Kusakabe et al., 2002; Li et al., 2003; Goetz et al., 2004, Nunez et al., 2005). In teleosts, besides correlating increase in StAR mRNA to acute interrenal or gonadal steroid production, very little is know about the dynamics of StAR transcripts in relation to gonadal cycle and more importantly final oocyte maturation. A shift in steroidogenesis from estradiol-17β (E₂) to 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) is important for final oocyte maturation that is associated with pre-ovulatory LH surge (Nagahma, 1997; Senthilkumaran et al., 2004; Nagahama and Yamashita, 2008). This steroidogenic shift is governed by the down regulation of ovarian P450 aromatase and up-regulation of 20β-hydroxysteroid dehydrogenase, the enzymes that
produce $E_2$ and 17α, 20β-DP, respectively (Yoshiura et al., 2003; Senthilkumaran et al., 2004; chapter 1 & 2). However, involvement of StAR during shift in steroidogenesis would be possible, owing to the reason that StAR is rapidly synthesized in response to trophic hormone stimulation. In the present study, a cDNA encoding StAR was isolated from ovarian follicles of the air-breathing catfish, Clarias gariepinus. We then analyzed StAR transcript abundance during human chorionic gonadotropin (hCG)-induced oocyte maturation, in vitro and in vivo by real-time RT-PCR. To complement our results, expression of StAR was also analyzed during different stages of gonadal cycle.