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In vitro effects of 2-hydroxyestradiol-17β on ovarian follicular steroid secretion in the catfish *Heteropneustes fossilis* and identification of the receptor and signaling mechanisms

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ABSTRACT

Ovarian pieces containing postvitellogenic follicles were incubated *in vitro* with different concentrations of the catecholestrogen 2-hydroxyestradiol-17β (2-OHE_2) to evaluate its effects on steroid production and germinal vesicle breakdown (GVBD) in the catfish *Heteropneustes fossilis*. The incubation with 2-OHE_2 induced a shift in steroidogenic pattern: the C_{19} and C_{18} steroids testosterone (T) and estradiol-17β (E_2), respectively were significantly decreased with a concomitant significant increase in the C_{21} steroids progesterone (P_4), 17-hydroxyprogesterone (17-OHP), 17,20β-dihydroxy-4-pregnen-3-one (17,20β-DP), 17,20α-dihydroxy-4-pregnen-3-one (17,20α-DP) and cortisol (F). Concomitantly, the catecholestrogen induced dose-dependently GVBD response, the first sign of meiosis resumption. The co- and pre-incubations of the ovarian pieces with 2-OHE_2, and adrenergic (phentolamine, α-blocker and propranolol, β-blocker) or estrogen (tamoxifen) receptor blockers resulted in inhibition of the stimulatory effect of the catecholestrogen on C_{21} steroids and reversed the inhibition of testosterone and E_2. The α-blocker was more effective than the β-blocker. Our results suggest that 2-OHE_2 appears to employ both adrenergic (α-type) and estrogen receptor mechanisms in mediating the effects. The co- or pre-incubation of ovarian pieces with IBMX (a cAMP elevating drug), H89 (a protein kinase A inhibitor), and PD098059 (a MAP kinase kinase inhibitor) significantly inhibited the stimulatory effect of 2-OHE_2 on the C_{21} steroids. The effect of chelerythrine (a protein kinase C inhibitor), on the other hand, varied with the incubation condition. In the co-incubation, the steroids showed varied effects: 17,20β-DP, testosterone and E_2 were elevated, and P_4 and 17-OHP were decreased. In the pre-incubation set up, all the steroids were inhibited except E_2. The inhibition by the blockers was higher in the pre-incubation groups. Taken together, the data suggest the involvement cAMP–protein kinase A, protein kinase C and MAP kinase pathways in the modulation of the steroidogenic activity.

1. Introduction

Catecholestrogens are estrogen metabolites, hydroxylated and methylated in the liver for removal by excretion. These metabolites were earlier thought to be biologically inactive but their extensive distribution in endocrine and neuroendocrine tissues, which are also estrogen targets, has changed this notion and subsequent studies have reported their involvement in diverse biological activities [1,5,24,37,46,53,59]. For instance, in reproductive tissues hydroxyestrogens have been shown to affect embryo implantation, gonadotropin release, parturition, uterine weight, egg transport in cycling rats, ovarian steroidogenesis and angiogenesis [3,36,37,46,51].

In teleost ovary, the occurrence of catecholestrogens (2/4-hydroxyestradiol-17β and 2-methoxyestradiol-17β)/synthesizing enzyme has been reported by us previously [25,43]. The catecholestrogens showed significant seasonal variation with an inverse relationship between E_2 and 2-OHE_2 during ovarian recrudescent phase, and 2-methoxy E_2 appeared late in the spawning phase. Further, 2-OHE_2 showed periovulatory changes with the concentration increasing coincident with final oocyte maturation (FOM) and ovulation after hCG injection. Subsequently, the oocyte maturational activity of 2-OHE_2 was demonstrated, comparable with that of hCG under *in vitro* conditions [26]. During the 2-OHE_2-induced FOM, C_{19} and C_{18} steroids (testosterone and E_2, respectively) were decreased and progestins (progesterone (P_4), 17-hydroxyprogesterone (17-OHP) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-DP)) were stimulated. In many teleosts including the catfish, 17,20β-DP is the major maturation-inducing steroid (MIS) [31,42]. Thus, there is a shift in steroid synthesis pattern at

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the onset of FOM from the estrogenic phase to the progestational phase. Factors underlying the cause of the shift are not fully identified, and have been attributed to a decrease in aromatase expression and activity [35]. The results of our earlier studies show that catecholestrogens may play a critical role in triggering the shift [26].

MacLusky et al. [21] stated that catecholestrogens possess unique biochemical properties since the hydroxyl group at C-2 or C-4 of the ring A structure alters its properties as an estrogen and makes it superficially resembling catecholamine neurotransmitters. This property of both a steroid and catechol renders the molecules to interact with both estrogen and catecholamine binding sites (receptors). However, binding and receptor studies in mammalian models have shown that the interaction between estrogen and catecholamine receptors are varied and complex depending on the tissues and species, chemical nature of the catecholestrogen used, parameters of the study, and a number of other variables. The interactions may involve classical estrogen receptors (ERα and ERβ) only, both estrogen and adrenergic (α-adrenoceptor and β-adrenoceptor) receptors, the α-type or β-type adrenergic receptor, or novel putative catecholestrogen binding sites located in cytosol or membranes that are intracellular transcription factors potentially related to ERs [5,8,11,22,37,45,51,55]. Cell signaling pathway(s) mediating the responses of catecholestrogens is not adequately investigated. 2-OHE_2 was shown to enhance cAMP production stimulated by gonadotropin or epinephrine [46] and thereby enhanced hormone action (progesterone secretion). Catecholestrogens significantly induced β-adrenoceptor activity, which is a known stimulator of cAMP [45]. In contrast, 2-OHE_2 has been shown to inhibit soluble adenylyl cyclase activity [48]. Mishra and Joy [28] demonstrated that follicular cAMP was inhibited during the 2-OHE_2-induced GVBD response. Further, these workers have shown that mitogen-activated protein kinase (MAP kinase) and protein kinase C (PKC) pathways were stimulated by 2-OHE_2 [29,30]. Inhibition of protein phosphatases by okadaic acid stimulated the 2-OHE_2-induced GVBD. These results suggest that catecholestrogens may use different cell signaling pathways, which may point to a non-genomic action [1].

The objective of the present study was to identify the receptor and signaling mechanisms during 2-OHE_2-induced steroid secretion and FOM in the catfish. The concentration of 2-OHE_2 used in our previous studies was relatively high and, therefore, we tested lower concentrations of the hydroxyestrogen for its efficacy in this study. Furthermore, the effects of inhibitors of signaling pathways on GVBD were demonstrated but the changes in steroid profiles were not reported. The nature of the receptors involved in the mediation of the hydroxyestrogen is also not known. Therefore, this investigation was conducted to fill those gaps in our understanding of the hydroxyestrogen's involvement in FOM. For this postvitellogenic follicles were incubated with 2-OHE_2 (concentration- and time-dependent studies). Secondly, the tissues were incubated with a selected concentration of the catecholestrogen in the presence or absence of adrenergic or estrogen receptor blockers, and inhibitors of the signaling (cAMP–protein kinase A (PKA), PKC and MAP kinase) pathways. Ovarian C_{21} and C_{19}–C_{18} steroids were measured by HPLC/ELISA and GVBD was monitored as a sign of resumption of meiotic maturation.

2. Materials and methods

2.1. Chemicals

1,3,5 (10) Estratriene-3,17β-diol (estradiol-17β, E_2), 1,3,5 (10) estratriene-3,16α,17β-diol (estriol), 17β-hydroxy-4-androsten-3-one (testosterone, T), 11β,17,21,trihydroxy-4-pregnene-3,20 dione (cortisol, F), 4-pregnene-3,20-dione (progesterone, P_4), 17-hydroxyprogesterone (17-OHP), 17,20β-dihydroxy-4-pregnen-3-one (17,20β-DP), 17,20α-dihydroxy-4-pregnen-3-one (17,20α-DP) (steroid nomenclature after Kime [20]), tamoxifen, 3-isobutyl-1-methylxanthine (IBMX), H89 dihydrochloride hydrate (H89), chelerythrine, PD098059, phentolamine hydrochloride (PA), and propranolol (PP) were purchased from Sigma Chemical Co., St. Louis, USA. 1,3,5 (10) Estratriene-2,3,17β-triol (2-hydroxyestradiol-17β, 2-OHE_2) was purchased from Steraloids Inc., Newport, R.I., USA. Progesterone, 17α-hydroxyprogesterone, cortisol, testosterone and estradiol-17β ELISA kits were purchased from Dia Metra, Italy. Monobasic sodium phosphate, dibasic sodium phosphate, potassium chloride, sodium potassium tartrate, sodium carbonate, sodium hydroxide, Folin Ciocalteu reagent, copper sulfate, acetonitrile (HPLC grade), and diethyl ether were purchased from E. Merck, New Delhi, India. Degassed and filtered nanopure water (Bernstead, USA) was used throughout chromatography. Other chemicals were of analytical grade and procured locally.

2.2. Animal collection and acclimatization

Heteropneustes fossilis is a freshwater, air-breathing catfish whose reproductive cycle can be divided into resting (November–January), preparatory (February–April), prespawning (May–June), spawning (July–August) and postspawning (September–October) phases. Adult female catfish weighing 30–50 g were collected in June (late prespawning phase–vitellogenic phase, gonadosomatic index = 9.06 ± 0.11%) from a local fish market in Varanasi in 2006 and 2008. They were maintained in aquarium tanks under natural conditions and used in various experiments 48 h after arrival. During acclimation, the fish were fed minced goat liver daily (*ad libitum*). The experiments were conducted in accordance to local/national guidelines for experimentation in animals and all care was taken to prevent cruelty of any kind.

2.3. In vitro effects of 2-OHE_2 on ovarian steroids

In late prespawning phase (June 2006), acclimatized gravid female *H. fossilis* were sacrificed by decapitation and ovaries were transferred to a sterile petri dish containing fresh cooled incubation medium. About 300 mg ovarian pieces chiefly containing round, intact, dark green postvitellogenic follicles were incubated in triplicate (group size = 3) with 6 ml incubation medium containing 100 pM, 1, 10, 100 nM or 1 μM of 2-OHE_2 for 0, 8, 16 and 24 h. The steroid was dissolved in a small amount of ethanol. At every 4 h, the medium was collected, and replenished with fresh medium containing the required concentrations of the steroid. As control, the ovarian pieces were incubated with plain medium containing the vehicle. The incubation medium was prepared by dissolving 3.74 g NaCl, 0.32 g KCl, 0.16 g $CaCl_2$, 0.1 g $NaH_2PO_4{\cdot}H_2O$, 0.16 g $MgSO_4{\cdot}7H_2O$ and 0.8 g glucose in 1 l of triple distilled water and sterilized. The pH was adjusted to 7.5 with 1 N sterilized sodium bicarbonate. Penicillin benzoate (200,000 U) and streptomycin sulfate (200 mg) were added and stored at 4 °C. Fresh medium was prepared every week. Percentage of germinal vesicle breakdown (GVBD) was scored. The tissues and incubation medium were collected (group-wise) for steroid assay.

2.4. In vitro effects of α- and β-adrenergic receptor blockers and an estrogen receptor blocker on 2-OHE_2-induced steroid production

2.4.1. Co-incubation study

In late prespawning phase (2008), about 300 mg ovary pieces in triplicate from each fish ($n = 3$) were incubated with 5 ml medium with or without 1 μM each of phentolamine (an α-adrenoceptor