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ScienceDirect
General and Comparative Endocrinology 155 (2008) 413–421
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Estrogen-2/4-hydroxylase activity is stimulated during germinal vesicle breakdown induced by hCG, IGF-1, GH and insulin in the catfish *Heteropneustes fossilis*

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Received 21 April 2007; revised 5 July 2007; accepted 19 July 2007
Available online 3 August 2007

Abstract

Estrogen-2/4-hydroxylase (EH) activity was measured radiometrically in ovaries of catfish injected with hCG intraperitoneally and in postvitellogenic follicles incubated with different concentrations of hCG, catfish (*Clarias batrachus*) growth hormone (GH), bovine insulin or recombinant human insulin-like growth factor-I (rhIGF-I). The change in enzyme activity was correlated with germinal vesicle breakdown (GVBD), an index of oocyte maturation. A single intraperitoneal injection of hCG (100 IU/fish) stimulated EH activity both at 8 and 16 h prior to stripping of eggs. The activity decreased significantly at 24 h, following ovulation. The follicles incubated with hCG, rhIGF-I, insulin or GH elicited biphasic effects on EH activity. rhIGF-I, insulin and GH increased enzyme activity at the lower or median concentrations. hCG and rhIGF-I stimulated EH activity higher than GH or insulin. All the hormones elicited a dose-dependent increase in GVBD, the effect was greater with rhIGF-I (100 nM) and hCG (5.0 IU/ml). The significance of changes in EH activity (E_2 hydroxylation) and GVBD were discussed.

Keywords: Catecholestrogens; Catfish; Estrogen-2/4-hydroxylase; Growth hormone; hCG; Insulin; Oocyte maturation; Recombinant human insulin-like growth factor-I

1. Introduction

Estrogen hydroxylases (EH) are members of cytochrome P-450 family and catalyze β-nicotinamide adenine dinucleotide phosphate (β-NADPH)-dependent hydroxylation of estrogens to form hydroxyestrogens, which are subsequently methylated by catechol-*O*-methyltransferase (COMT) to form methoxyestrogens. The principal site of these conversions is liver, but other organs like brain, pituitary, ovary, testis, adrenal, kidney, lung and heart are also implicated with catecholestrogen (CE) formation (Ball and Knuppen, 1990; Merriam and Lipsett, 1983). The widespread tissue distribution indicates that CEs are biologically active, contrary to the earlier notion and are involved in tissue-specific functions (Ball and Knuppen, 1990; Merriam and Lipsett, 1983; Spicer and Hammond, 1989). In the ovary of mammals, CEs are implicated in steroidogenesis and other follicular activity (Spicer and Hammond, 1989; Tekpetey and Armstrong, 1994). In teleosts, investigations on CE distribution and function are meager (Butala et al., 2004; Snowberger and Stegeman, 1987; Timmers et al., 1988; Timmers and Lambert, 1989). In recent years, we reported that catfish ovary synthesizes CEs, and hydroxyestrogens are associated with steroidogenesis and oocyte maturation (Mishra and Joy, 2006a, b, c; Senthilkumaran and Joy, 2001).

It is well documented that gonadotropins (FSH and LH) are the primary regulators of steroidogenesis and other activities in gonads of teleosts, as in other vertebrates (Fostier et al., 1983; Jalabert et al., 1991; Nagahama, 1999). A number of recent studies implicate other endocrine and paracrine/autocrine factors in the regulation of gonadal

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doi:10.1016/j.ygcen.2007.07.005

functions (Tyler and Sumpter, 1996). Thus, involvement of growth hormone (GH), insulin, insulin-like growth factor-I and -II (IGF-I and IGF-II) has been demonstrated in follicular development, acquisition of maturational competence and maturation directly or synergistically by influencing steroidogenic activity (Dasgupta et al., 2001; Kagawa et al., 1994; Maestro et al., 1997, 1999; Mukherjee et al., 2006; Negatu et al., 1998; Singh et al., 1988; Srivastava and Van der Kraak, 1994a; Van der Kraak et al., 1990; Weber and Sullivan, 2000, 2005; Weber et al., 2007). The induction of oocyte maturation (GVBD) by these substances suggests that teleosts employ a wide range of maturation-inducing substances (MIS) involving multiple pathways along with progestins such as 17, 20β-dihydroxy-4-pregnen-3-one (17, 20βP) or 17, 20β, 21 trihydroxy-4-pregnen-3-one (20βS) or corticosteroids (Jalabert et al., 1991; Nagahama et al., 1994; Scott and Canario, 1987).

In a pervious study, we characterized CEs in catfish ovary and they showed seasonal variation with peak formation of hydroxyestrogens in spawning phase, and periovulatory changes with the peak level of secretion at 8 h of hCG treatment (Mishra and Joy, 2006a). Further, we also demonstrated periovulatory changes in EH with peak activity at 8 h and COMT at 16 h (Senthilkumaran and Joy, 2001). EH activity also showed seasonal changes in the ovary (our unpublished data). These results clearly show that during the progress of ovarian recrudescence and hormone-induced ovulation, E_2 is hydroxylated to CEs. The hydroxyestrogen 2-hydroxyestradiol (2-OHE_2) has been shown to stimulate oocyte maturation through the production of the MIS, notably 17, 20βP (Mishra and Joy, 2006b, c).

The factors controlling CE synthesis are not adequately investigated in fishes but reports in mammals indicate that EH activity is regulated by gonadotropins, growth hormone, testosterone, 5α-dihydrotestosterone and the haloestrogen 2-bromo estradiol, whereas catecholestrogens and estrogens inhibit it (Brueggemeier, 1983; Brueggemeier and Kimball, 1983; Chakraborty et al., 1988; Li et al., 1986; Mondschein et al., 1987; Quail and Jellinck, 1987).

The objective of the present study was to investigate whether oocyte maturation induced by hormones like hCG, GH, IGF-I and insulin involves stimulation of E_2 hydroxylation. For this, we measured EH activity and correlated it with GVBD response in the postvitellogenic follicles of the catfish *Heteropneustes fossilis*.

2. Materials and methods

2.1. Chemicals

[2, 4 ^{3}H] estradiol-17β (specific activity 30 Ci/mMol), NADPH, 2-[4-(2-hydroxyethyl) 1-piperazinyl] ethane sulphonic acid (Hepes buffer, acid free), bovine serum albumin (BSA), estradiol-17β (E_2) and human recombinant insulin-like growth factor-I (rhIGF-I) were purchased from Sigma Chemical Co., St. Louis, USA. Catfish (*Clarias batrachus*) growth hormone (Lal and Singh, 2005) was a gift of Dr. B. Lal, Department of Zoology, Banaras Hindu University. Human chorionic gonadotropin (hCG, Corion®, IBSA, Switzerland) and bovine insulin (Bovine-Fastact, USV Limited, India) were purchased from a local medical store. 2,5-Diphenyl-1,3-oxazole (POP) and 1,4-bis-[2-(4-methyl-5-phenyl-1,3-Oxazolyl)] benzene (POPOP) (Beckman Instruments Inc., Fullerton, CA, USA), naphthalene (JT Baker Chemical Co., Phillipsburg, NJ), propylene glycol, sucrose, EDTA, sodium carbonate, sodium hydroxide, Folin Ciocalteu reagent, dichloromethane, 1,4-dioxane, methanol, copper sulphate, monobasic sodium phosphate and dibasic sodium phosphate (E. Merck, New Delhi, India) were of analytical grade. Other chemicals were 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 40–60 g (gonadosomatic index = 9.06 ± 0.11) were purchased from a local fish market in and around Varanasi in the early spawning phase (first week of July). They were maintained in aquarium tanks with circulating water under natural conditions and used in various studies 48 h after arrival. During acclimation, the fish were fed minced goat liver daily (*ad libitum*). The experiments were conducted in accordance with the local and national guidelines on animal care and maintenance.

2.3. hCG-induced ovulation and ovarian sampling for EH activity

In early spawning phase (first week of July), sexually mature female catfish (40–60 g) were collected and acclimatized. A few fish were randomly selected and ovaries were dissected out to check the stage of ovarian development. Forty-eight acclimatized fish were divided into two groups of 24 each. The first group was injected with 100 IU/fish of hCG intraperitoneally. The second group was injected with an equal volume of vehicle (0.7% NaCl). Six fish each from the two groups were sacrificed by decapitation at 0, 8 and 16 h after the injection. At 16 h, the remaining fish in the two groups were hand stripped for ovulation and maintained up to 24 h and sacrificed by decapitation. The ovaries were carefully removed on ice, weighed and processed for microsomal preparations.

2.4. Preparation of incubation medium, general procedure and germinal vesicle break down (GVBD) scoring

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. Phenol red (0.4 mg/L) was used as a pH indicator. The pH was adjusted to 7.5 with 1 N sterilized sodium bicarbonate. 200,000 U Penicillin benzoate and 200 mg streptomycin sulphate were added and stored at 4 °C. The ovaries were dissected out under aseptic conditions, cut into small pieces and washed three times with the incubation medium. Rounded, dark green postvitellogenic follicles with centrally located germinal vesicles (GV) were selected for incubation. The incubation was set up in embryo cups containing 50–60 follicles in triplicate from each fish (group size = 3 fish) in 5 ml incubation medium at 23 ± 2 °C. After the incubation, the follicles were treated with a clearing solution (60 ml ethanol, 30 ml formalin and 10 ml glacial acetic acid) to check GVBD and were scored individually. The percentage of GVBD was calculated from the ratio of the number of follicles that had undergone GVBD to the total number incubated. The following experiments were conducted.

2.4.1. Incubation with hCG

hCG was prepared in normal fish saline and diluted to the desired concentration with sterilized medium. Fifty to sixty follicles each were incubated in triplicate in the incubation medium containing the vehicle (control) or the medium containing 0.5, 1.0, 5.0 IU hCG/ml for different duration (0, 8, 16 and 24 h).