Periovulatory changes in catfish ovarian oestradiol-17 β , oestrogen-2-hydroxylase and catechol-O-methyltransferase during GnRH analogue-induced ovulation and *in vitro* induction of oocyte maturation by catecholestrogens

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Abstract

In the catfish *Heteropneustes fossilis* and *Clarias batrachus*, ovarian oestrogen-2-hydroxylase (OE-2-H) activity increased significantly at 8 h after the injection of an ovulatory dose (0.15 $\mu\text{g/g}$ body weight) of a mammalian GnRH analogue ([D-Ala⁶-Pro⁹]-LHRH ethylamide) and was restored to the 0 h (control) level after egg-stripping at 16 h. On the other hand, ovarian oestradiol-17 β (OE₂) level and catechol-O-methyltransferase (COMT) activity decreased significantly at 8 h. While the OE₂ level was restored to the 0 h level, COMT activity increased significantly at 16 h. Changes in ovarian OE₂ level and enzymes indicate higher synthesis of 2-hydroxylated catecholestrogens and their degradation during the periovulatory period. Under *in vitro* conditions, the synthetic catecholestrogens (CEs, 2- and 4-hydroxylated oestradiol-17 β and oestrone (OE₁)) induced germinal vesicle break down (GVBD) in a dose- (0.01–10 $\mu\text{g/ml}$) and duration- (1–36 h) dependent manner, the mean values of the responses being in the order 2-OH OE₂ > 4-OH OE₂ >

2-OH OE₁ > 4-OH OE₁. The CE-induced GVBD response (8 h induction) was not blocked by prior and subsequent incubations with steroid synthesis inhibitors (cyanoketone, epostane and aminoglutethimide) up to 36 h, suggesting that *de novo* steroidogenesis is not essential for the response. The percentage of GVBD response to 2-h induction by CEs was significantly inhibited by actinomycin D (a transcriptional inhibitor) and cycloheximide (a translational inhibitor), indicating the involvement of both RNA and protein synthesis. The CE-induced 8-h stimulation of GVBD was mildly blocked by propranolol, the β -adrenergic inhibitor, suggesting the response was partly mediated through a β -adrenergic receptor mechanism. Incubations with phentolamine, an α -adrenergic inhibitor, did not interfere with the CE-induced GVBD response. The results demonstrate CE-related enzymatic changes in teleost (catfish) ovaries and maturation-inducing substance activity of CEs.

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Introduction

The sequential physiological events of vitellogenesis, oocyte final maturation and ovulation during fish oogenesis are under the differential control of regulatory mechanisms involving principally the pituitary gonadotrophin and ovarian steroid regulators (Chieffi & Pierantoni 1987, Ho 1987, Nagahama 1987, Yoshikuni & Nagahama 1991, Kagawa 1994). The vitellogenic phase is initiated and maintained by one of the gonadotrophins (gonadotrophin-I (GTH-I) or follicle-stimulating hormone (FSH)) in the two gonadotrophin model species (Kawauchi *et al.* 1989, Yoshikuni & Nagahama 1991, Swanson & Dittman 1997) through the mediation of ovarian oestrogens, principally oestradiol-17 β (OE₂). Oocyte maturational and ovulatory events are initiated by

the other gonadotrophin (gonadotrophin-II (GTH-II) or luteinizing hormone (LH)) through the mediation of a maturation-inducing hormone or substance (MIH or MIS), the most common being an ovarian progesterone derivative, 17 α ,20 β -dihydroxyprogesterone (17 α ,20 β -DP) as in many salmonids, cyprinids and silurids, or 17 α ,20 β ,21 β -trihydroxyprogesterone (17 α ,20 β ,21 β -TP) as in sciaenid fish (Goetz 1983, Nagahama 1987, 1994, 1997, Scott & Canario 1987, Thomas 1994). MIS activity was also demonstrated for corticosteroids (deoxycorticosterone and cortisol) in several teleosts including catfish (Goswami & Sundararaj 1974, Sundararaj & Goswami 1977, Goetz 1983, Upadhyaya & Haider 1986, Rao & Haider 1992, Haider 1997).

The shift in steroidogenesis from the oestrogenic to progesterational phase is indicated by an abrupt decrease in

mRNA transcripts of aromatase, thus arresting the aromatization of testosterone to OE₂ (Nagahama 1994, 1997). However, the fate of OE₂ in the blood during the periovulatory period of different teleosts presents varied patterns, including significant decrease, increase, or no changes (see Joy *et al.* 1998 for references). In *H. fossilis*, during the periovulatory period plasma OE₂ level decreases to the lowest value at 8 h after gonadotrophin-releasing hormone (GnRH) analogue injection and increases after stripping of eggs at 16 h (Joy *et al.* 1998). Since plasma testosterone showed an inverse pattern, we suggested that the OE₂ decrease might have been due to inhibited aromatization. Considering the long half-life of OE₂ (Querat *et al.* 1985), a mere inhibition of its biosynthesis may not be adequate to bring such a drastic change in its titre within 8 h. Furthermore, ovarian OE₂ level never reaches undetectable/low values, unless the ovary undergoes regression (Singh & Singh 1987). Therefore, an active mechanism removing the formed oestrogens may be operating as well. This has prompted us to examine the ovarian OE₂ catabolism. Since oestrogens (OE₂ and oestrone, OE₁) can be hydroxylated at 2- and 4- carbons of ring A by specific hydroxylases (oestrogen-2/4-hydroxylases), ovaries with high concentrations of the steroid can be potential sites of catechol-oestrogen (CE) synthesis (Spicer & Hammond 1989). The hydroxyoestrogens can be subsequently O-methylated by catechol-O-methyltransferase (COMT) to form methoxyoestrogens (Ball *et al.* 1983, Fishman 1983). The presence of these enzymes, therefore, indicates CE synthesis and degradation in a given tissue (COMT can be a marker for catecholamine degradation as well).

In the mammalian ovary, the CEs have been demonstrated as potent autocrine/paracrine regulators of ovarian functions. They stimulate progesterone synthesis, cAMP, β -adrenergic receptors, etc. (Spicer & Hammond 1989). To our knowledge, there are no studies showing ovarian CE metabolism or function in teleosts. In the first part of the present investigation, evidence for CE metabolism in the catfish ovary is presented. In the second part of the study, the significance of CEs in oocyte final maturation is demonstrated by an *in vitro* bioassay system for germinal vesicle break down (GVBD) response.

Materials and Methods

Animals

Sexually mature gravid female catfish (*Heteropneustes fossilis* and *Clarias batrachus*) weighing 90–110 g were collected from local fish markets in Varanasi in the last week of June (late pre-spawning phase) and first week of July (early spawning phase). They were maintained in flow through aquarium under natural photoperiod (average day length 13.2 h) and ambient temperature (average $28 \pm 2^\circ\text{C}$) for 2 weeks. The fish were examined for spontaneous

ovulation by pressing the abdomen towards the ovipore. They were fed daily with goat liver and allowed to feed *ad libitum* till a day before experiments were started.

Chemicals

[D-Ala⁶-Pro⁹]-LHRH ethylamide, DL-adrenaline, oestradiol-17 β (OE₂), 2-hydroxyoestradiol-17 β (2-OH OE₂), 4-hydroxyoestradiol-17 β (4-OH OE₂), 2-hydroxyoestrone (2-OH OE₁), 4-hydroxyoestrone (4-OH OE₁), reduced nicotinamide adenine dinucleotide (NADPH), propranolol, phentolamine, purified COMT, BSA, cycloheximide, actinomycin D, HEPES buffer and aminoglutethimide were obtained from Sigma (St Louis, MO, USA). [¹⁴C]S-adenosylmethionine (SAM, specific activity 43 mCi/mmol) and [2-³H]oestradiol-17 β (specific activity 22.1 Ci/mmol) were purchased from NEN (Boston, MA, USA) and Amersham (Buckinghamshire, UK) respectively. Cyanoketone and epistane were gifts of Sterling-Winthrop Research Institute, New York to Dr S Haider, Department of Zoology, Banaras Hindu University. All other chemicals were of analytical grade and purchased locally.

Dynamics of ovarian oestrogen-2-hydroxylase (OE-2-H) and COMT during periovulatory period

Induction of ovulation Thirty females each of *H. fossilis* and *C. batrachus* were divided into two groups of 15 each. Group 1 was injected with 0.15 $\mu\text{g/g}$ body weight of a mammalian GnRH analogue in the evening (1800 h). Group II fish were injected with an equal volume of the vehicle (0.7% NaCl + 0.25% BSA, fraction V). Five fish each from the two groups were sacrificed by decapitation at 0, 8 and 16 h after the injections. At 16 h, the fish were checked for ovulation by hand stripping before sacrificing.

Enzyme assays The ovaries were removed carefully, placed on ice, weighed (including the stripped eggs) and stored briefly (1–2 days) at -20°C prior to the assay of enzymes and total protein. The ovaries of all the samples were freeze-thawed only once to avoid loss of enzyme activity.

Assay of COMT COMT activity in the ovarian tissue extract (aliquots from 50 mg of ovarian tissue/ml 0.6% KCl solution) was assayed by the radioisotopic method of Parvez & Parvez (1973) as described by Joy & Senthilkumaran (1998). DL-adrenaline and [¹⁴C]SAM were used as substrates and co-substrates respectively. Radioactivity of ¹⁴C-methylated product was counted in a Beckman LS-1801 liquid scintillation counter. The assay was validated using increasing concentrations of the ovarian tissue extract and purified COMT. The resulting plot showed both parallelism and linearity up to 15 mg of ovarian tissue and 500 units of COMT.

Assay of OE-2-H The enzyme was assayed by the radioisometric method of Numazawa *et al.* (1980). The ovaries were homogenized gently on ice in HEPES buffer (0.1 M, pH 7.4; 25 mg of ovarian tissue/ml buffer) using a micropestle and an Eppendorf tube. The supernatant (40 µl (1 mg tissue)) was incubated with [2-³H]OE₂ (0.4 µCi in 0.4 µl) dissolved in propylene glycol (final concentration, 5% v/v), NADPH (2 mM as a co-factor) and HEPES buffer (0.1 M, pH 7.4). The final volume of incubation medium was 50 µl. The incubation was conducted at 26 °C in an Eppendorf tube immersed in a continuous shaking water bath incubator for an hour. The tubes were placed on ice for 5 min to stop the reaction. The volume of the reaction mixture was made up to 1 ml by adding HEPES buffer. The mixture was then extracted with 4 ml dichloromethane to remove any non-converted [2-³H]OE₂ with a prior addition of 40 µg OE₂ (dissolved in propylene glycol, 5% v/v as the carrier). Radioactivity of the tritium label (³H₂O) released into the aqueous fraction was counted in a Beckman LS 1801 counter. Corrections for non-enzymatically released tritium label were made using the radioactivity of ³H in control samples (pre-heated tissue extracts (10–15 min heating at 100 °C)). The assay was validated with increasing concentrations of ovarian tissue extract. The resulting plot showed both parallelism and linearity up to 25 mg of ovarian tissue.

RIA of oestradiol-17β (OE₂)

Ovarian extracts from both control and GnRH analogue treated fish were analysed for OE₂ level, using the RIA protocol described previously (Senthilkumaran & Joy 1994).

Total protein assay

The total protein content in the ovarian extract was assayed by the method of Lowry *et al.* (1951) using BSA as standard.

In vitro bioassay of GVBD

Oocyte incubation The incubation medium was prepared by dissolving 3.74 g NaCl, 0.32 g KCl, 0.16 g CaCl₂, 0.1 g NaH₂PO₄·2H₂O, 0.16 g MgSO₄·7H₂O, 0.8 g glucose and 0.008 g phenol red in 1 litre of triple-distilled water and sterilized (Upadhaya & Haider 1986). The pH was adjusted to 7.5 with 1 M sterilized sodium bicarbonate solution. Penicillin (200 000 U) and streptomycin sulphate (200 mg) 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. Full grown, folliculated postvitellogenic oocytes (henceforth referred to as oocytes) with centrally located germinal vesicles (GV) were only selected for incubations. The incubations were set up in triplicate

for each test in small Petri dishes containing 35–40 oocytes in 3 ml of incubation medium at 21 ± 2 °C. After 36 h of incubation, the oocytes were treated with a clearing solution consisting of 5% formalin and 4% acetic acid to check GVBD and were scored individually. The percentage of GVBD was calculated from the ratio of the number of oocytes that had undergone GVBD to the total number of oocytes incubated.

Experiments *Effects of different concentrations of CEs on GVBD* Thirty-five to 40 oocytes from *H. fossilis* and *C. batrachus* were incubated in triplicate in 3 ml of incubation medium (control) or the medium containing 0.01, 0.05, 0.10, 0.50, 1.0, 2.0, 5.0 and 10.0 µg/ml of 2-OH OE₂, 4-OH OE₂, 2-OH OE₁ and 4-OH OE₁. The CEs were dissolved in 50–100 µl ethanol and diluted with the incubation medium. In the control incubations, the medium contained an equal volume of ethanol. After 36 h, GVBD was scored in all groups. This experiment was repeated with oocytes from three donor fish in the same spawning season and in two consecutive years (data not shown).

Time course effects of CE stimulation of GVBD Thirty-five to 40 oocytes of *H. fossilis* and *C. batrachus* were incubated in triplicate in 3 ml of incubation medium containing 1.0 µg/ml of 2-OH OE₂, 4-OH OE₂, 2-OH OE₁ and 4-OH OE₁ each (dissolved in 50–100 µl of ethanol) for various duration (1, 2, 4, 6, 8, 12, 18, 24 and 36 h). After the end of each pulse interval, the oocytes were transferred to fresh medium without CEs to complete the 36 h interval (except the 36 h group) and GVBD was scored for each group. In the control, oocytes were incubated in the medium containing the same amount of ethanol for 36 h, following which GVBD was scored. This experiment was repeated using oocytes from three donor fish in the same spawning season and in two consecutive years (data not shown).

Effects of steroid synthesis inhibitors (cyanoketone, epostane and aminoglutethimide) on CE-stimulated GVBD Thirty-five to 40 oocytes from five *H. fossilis* and *C. batrachus* each were pre-incubated in triplicate with medium containing 10 µg/ml of cyanoketone, epostane or aminoglutethimide for 2 h, followed by incubation with 1.0 µg/ml each of different CEs in 3 ml of medium for 8 h. The oocytes were then transferred again to the medium containing each of cyanoketone (10.0 µg/ml), epostane (10.0 µg/ml) or aminoglutethimide (10.0 µg/ml) for the remaining 26 h. The inhibitors were dissolved in 50–100 µl ethanol. For controls, oocytes were incubated with each of the steroid synthesis inhibitors or in medium containing equal amounts of ethanol (negative control). A positive control incubation with each CE was run concurrently to monitor the GVBD response. An 8 h stimulation of oocytes was chosen to test the effects of the steroid synthesis inhibitors,

as studies by other workers employed 6–8 h stimulation times to test *de novo* steroidogenic involvement during GVBD bioassay. Furthermore, our pilot studies of pre-incubating oocytes with the inhibitors followed by 2 or 6 h CE stimulation of oocytes and again treated with the inhibitors treatment produced more or less a similar response (data not shown) as observed for 8 h CE-stimulated oocytes. GVBD was scored at the end of 36 h.

Effects of actinomycin D (a transcriptional inhibitor) and cycloheximide (a translational inhibitor) on CE-induced GVBD response About 35–40 oocytes of both catfish species (five donors) were pre-incubated in triplicate with 10.0 µg/ml of actinomycin D or cycloheximide for 2 h, followed by 1.0 µg/ml of each CE for 2 h, and then treated again with actinomycin D (10.0 µg/ml) or cycloheximide (10.0 µg/ml) for the next 2 h. The oocytes were then transferred to the plain medium and incubated for the remaining 30 h. A minimum of 2 h stimulation time by CEs was chosen to show transcriptional or translational involvement during oocyte maturation. For controls, oocytes were incubated in the medium only (negative control), medium containing the inhibitors or medium containing CE (2 h stimulation), followed by the medium only (positive control). GVBD response was scored after 36 h in all cases. The inhibitors were directly dissolved in the incubation medium.

Effects of α - and β -adrenergic inhibitors on CE-stimulated GVBD About 35–40 oocytes of both catfish species (five donors) were pre-incubated in triplicate in the medium containing 10.0 µg/ml of propranolol, a β -adrenergic inhibitor, or phentolamine, an α -adrenergic inhibitor, for 2 h followed by incubation with 1 µg/ml of CEs for 8 h. Subsequently, the oocytes were transferred again to the medium containing 10.0 µg/ml of propranolol or phentolamine for the remaining 26 h. For control, oocytes were incubated with CEs for 8 h followed by the medium only, or in medium containing the drugs only (positive control), or in the medium alone (negative control). After 36 h GVBD was recorded in all cases. The drugs were dissolved in the medium.

Statistical analysis

Data were expressed as means $\pm$ S.E.M.S. Statistical significance between control and treated (GnRH analogue and various inhibitors) groups was tested by Student's multiple *t*-test. The data of concentration–response and time course studies were analysed by one-way ANOVA, followed by Newman–Keuls' multiple comparison test.

Results

Periovulatory changes in ovarian OE-2-H, COMT and OE₂ level

The administration of 0.15 µg of GnRH analogue per gram body weight of fish induced ovulation in both catfish

Figure 1 Effects of a mammalian GnRH analogue on ovarian OE₂ level and OE-2-H activity (left vertical axis) and COMT activity (right vertical axis) in *H. fossilis* (a) and *C. batrachus* (b). Values are means $\pm$ S.E.M.S. *P<0.01, **P<0.001, Student's multiple *t*-test.

species. Eggs could be stripped out at 16 h after the injection. The fish that received the vehicle did not ovulate upon stripping. In the non-ovulated fish (vehicle control groups), the ovarian activity of OE-2-H and COMT did not vary significantly at any of the intervals (Fig. 1a,b). In the GnRH analogue groups, OE-2-H activity increased significantly at 8 h in both species ($P<0.001$, Student's *t*-test). At 16 h, after egg stripping, the enzyme activity was restored to the control levels. In contrast, COMT activity decreased significantly at 8 h (*H. fossilis*, $P<0.001$ and *C. batrachus*, $P<0.01$) and increased sharply at 16 h. After GnRH analogue treatment, ovarian OE₂ level decreased significantly ($P<0.001$) at 8 h when compared with vehicle control. After egg stripping at 16 h, the OE₂ level was restored to the control range. The activity of enzymes and OE₂ level did not change at 0 h.

In vitro oocyte GVBD response

Effects of different concentrations of CEs In both *H. fossilis* and *C. batrachus*, the incubation of oocytes with different concentrations of CEs (0.01–10 µg/ml) produced overall significant differences on the percentage of GVBD

Figure 2 Effects of different concentrations of CEs (2OH OE₂ and 2OH OE₁, and 4OH OE₂ and 4OH OE₁) on percentage of GVBD (mean $\pm$ S.E.M.) in *H. fossilis* (a) and *C. batrachus* (b). The data show overall significance ($P < 0.001$, one-way ANOVA; F -values for 2OH OE₂, 2OH OE₁, 4OH OE₂ and 4OH OE₁ groups are 201.7, 177.9, 187.9 and 173 in *H. fossilis*, and 200.1, 187.3, 191.7 and 163.9 in *C. batrachus* respectively). The values of all CE-treated groups are significantly higher ($P < 0.05$, Newman–Keuls' test) than control group values. C, control.

response (Fig. 2a,b; $P < 0.001$, one-way ANOVA). The CE-induced GVBD response was significant at all concentrations ($P < 0.05$, Newman–Keuls' test) compared with the control values. A comparison of the mean values of the percentage GVBD response of different CEs at the lowest (0.01 µg/ml) and highest (10 µg/ml) concentrations shows that 2-OH OE₂ is the most, and 4-OH OE₁ the least effective in both species (Student's multiple t -test). The percentage of GVBD is 55 and 96% for *C. batrachus* and 50 and 92% for *H. fossilis* at the respective concentrations of 2-OH OE₂. The responses for 4-OH OE₁ are 35 and 84% for *C. batrachus* and 30 and 80% for *H. fossilis* respectively. The GVBD response in 4-OH OE₂ and 2-OH OE₁ groups is almost in the same range at different concentrations.

Figure 3 Time course effects of CEs on percentage of GVBD (mean $\pm$ S.E.M.) in *H. fossilis* (a) and *C. batrachus* (b). The data show overall significant increases ($P < 0.001$, one-way ANOVA; F -values for 2OH OE₂, 2OH OE₁, 4OH OE₂ and 4OH OE₁ groups are 160.2, 127.9, 138.7 and 110.4 in *H. fossilis*, and 200.0, 162.7, 187.2 and 160.3 in *C. batrachus* respectively). The values of all CE-treated groups are significantly higher ($P < 0.05$, Newman–Keuls' test) than control group values. C, control.

Time course effects of CEs on GVBD The incubation of oocytes with 1 µg/ml concentration of CEs produced overall significant increases in the percentage of GVBD in both species (Fig. 3a,b; $P < 0.001$, one-way ANOVA). The percentage of GVBD increased significantly at all intervals ($P < 0.05$, Newman–Keuls' test). The highest response was in the 2-OH OE₂ groups and the lowest in the 4-OH OE₁ groups.

Effects of steroid synthesis inhibitors on CE-stimulated GVBD The incubation of catfish oocytes with cyanoketone, epostane or aminoglutethimide (10 µg/ml) for 2 h, followed by stimulation with 1 µg/ml of various CEs for 8 h, and subsequent incubation with the inhibitors (10 µg/ml) up to a total of 36 h did not inhibit the GVBD response due to the CEs (Fig. 4; $P < 0.001$, Student's multiple t -test). The inhibitors alone did not

Figure 4 Effects of the steroid-synthesizing inhibitors, cyanoketone (CK), epostane (EP) and aminoglutethimide (GT) on CE₂ and 2OE₁, and 4OE₂ and 4OE₁ on GVBD in *H. fossilis* and *C. batrachus* (mean $\pm$ S.E.M.). C, control. The inhibitors did not significantly affect the GVBD responses in the CE-treated groups (Student's multiple *t*-test).

elicit any significant response compared with the control groups.

Effects of actinomycin D and cycloheximide on CE-induced GVBD The incubation of catfish oocytes first with actinomycin D or cycloheximide for 2 h, followed by various CE₂ (1 μ g/ml) for 2 h and then again with actinomycin D or cycloheximide for the next 2 h, and finally in plain medium up to a total of 36 h significantly inhibited GVBD response in comparison with the respective CE groups (Fig. 5; $P < 0.001$, Student's multiple *t*-test). The inhibition was greater in the actinomycin D (*C. batrachus*, 66–72%; *H. fossilis*, 60–65%) than

Figure 5 Effects of transcriptional inhibitor, actinomycin D (ACD) and translational inhibitor, cycloheximide (CYH) on CE-induced GVBD in *H. fossilis* and *C. batrachus* (mean $\pm$ S.E.M.). C, control. The GVBD response was significantly inhibited compared with respective control groups ($*P < 0.001$, Student's multiple *t*-test).

Figure 6 Effects of α -adrenergic inhibitor, phentolamine (PH) and β -adrenergic inhibitor, propranolol (PR) on CE-induced GVBD in *H. fossilis* and *C. batrachus* (mean $\pm$ S.E.M.). Only the β -adrenergic inhibitor decreased GVBD response significantly ($*P < 0.001$, Student's multiple *t*-test).

cycloheximide (*C. batrachus*, 49–53%; *H. fossilis*, 36–55%) groups. The inhibitors alone did not induce any significant effect.

Effects of phentolamine and propranolol on CE-induced GVBD The incubation of catfish oocytes with 10 μ g/ml of phentolamine, an α -adrenergic inhibitor for 2 h, followed by stimulation with various CE₂ (1 μ g/ml) for 8 h, and then incubated again with 10 μ g/ml of phentolamine for 26 h did not inhibit the CE-induced GVBD response (Fig. 6). However, incubation of the oocytes with propranolol (10 μ g/ml, a β -adrenergic blocker) under similar incubation conditions inhibited significantly the GVBD response: *C. batrachus*, 25–31%, *H. fossilis*, 18–32% ($P < 0.001$, Student's multiple *t*-test). The inhibitors alone did not produce any significant effect.

Discussion

This study presents evidence for dynamics of OE₂ and CE-related enzymes in teleost (catfish) ovaries during GnRH analogue-induced ovulation. In a previous study with similar experimental approach, we reported peri-ovulatory changes in plasma levels of GTH-II, OE₂, testosterone, progesterone and cortisol after GnRH analogue treatment that led to ovulation in the catfish *H. fossilis* (Joy *et al.* 1998). The peak rise of ovarian OE-2-H coincided with the GTH-II and testosterone peak rises, and OE₂ decline at 8 h after the injection. This indicates that GTH-II seems to stimulate the enzyme activity as has been reported by Chakraborty *et al.* (1988) in rat ovary which resulted in decreased OE₂ level. OE-2-H activity is also stimulated by testosterone (Poth *et al.* 1983, Li *et al.* 1986, Quail & Jellinck 1987). The rise in plasma

testosterone level reported by us (Joy *et al.* 1998) may suggest a similar rise of the steroid in the ovary and the high ovarian testosterone concentration may facilitate OE-2-H activity in the catfish. After stripping the ovulated eggs (16 h after the injection), OE-2-H returned to the 0 h level to coincide with the fall in plasma GTH-II and testosterone (Joy *et al.* 1998), and the rise in both plasma and ovarian OE₂. In contrast, ovarian COMT decreased significantly at 8 h and increased sharply after egg stripping at 16 h, suggesting methylation of CEs. Since CEs have a short half-life (1 h for 2-OH OE₂, see Spicer & Hammond 1989), the decrease in endogenous COMT activity at 8 h is an endogenous mechanism to prolong the half-life of CEs (Spicer *et al.* 1987) for physiological activity during the periovulatory period (8 h). This interval coincides with the oocyte maturational process since eggs can be stripped from this time onwards. The high activity of COMT at 16 h suggests degradation of CEs, a terminal inactivation step (Ball *et al.* 1983, Fishman 1983). The regulation of ovarian COMT activity is not clearly understood. In rats, ovarian COMT activity is significantly lower during oestrus (ovulation) than during dioestrus. COMT activity is increased after progesterone plus OE₂ treatment in rabbit uterus (Spicer & Hammond 1989). In the catfish ovary, increased level of COMT was found coinciding with the rise in OE₂ level at 16 h, suggesting OE₂ modulation of COMT activity. In the catfish, ovariectomy and/or OE₂ replacement modulate hypothalamic COMT activity (Joy & Senthilkumaran 1998). The patterns of periovulatory changes in OE₂ and CE-related enzymes strongly indicate enhanced OE₂ catabolism towards CE formation and degradation in the catfish ovary. Since oestrogens have little or even an inhibitory role on oocyte final (meiotic) maturation in teleosts including the catfish (Sundararaj & Goswami 1977, Nagahama 1987), their removal by converting into CE may be an important physiological step during the periovulatory period. A direct demonstration of CEs and their metabolites remains. Since CEs have very short half-lives and are very labile (Spicer & Hammond 1989), measurement of CEs requires sensitive HPLC with electrochemical detector system, at present a constraint for us.

The physiological significance of CEs in ovarian function is not known in teleosts. In mammals, CEs have been demonstrated to be paracrine/autocrine regulators of ovarian physiology (Hudson *et al.* 1987, Spicer & Hammond 1989, Tekpetey & Armstrong 1994). The pattern of enzyme changes during the periovulatory period prompted us to study the direct effect, if any, of synthetic CEs on catfish oocyte final (meiotic) maturation. The results show that all the CEs tested induced GVBD and that this could be positively correlated with both CE concentration and incubation time. To our knowledge, this is the first report on CEs eliciting a MIS-like response in any teleost species or vertebrate. Out of the four CEs tested, 2-OH OE₂ appears to be more effective than

others in inducing GVBD, judging from the mean percentage of GVBD at the lowest and highest concentrations tested. The *C. batrachus* oocytes showed a higher response than the *H. fossilis* oocytes. The MIS property of the CEs seems to be associated with the hydroxylations at 2- or 4- position of the ring A, as their parent steroids exhibit only negligible MIS activity in catfish (Sundararaj & Goswami 1977, Upadhyaya & Haider 1986, Rao & Haider 1992). The structure–activity relationship needs to be examined in detail in future studies.

Since it is well known that ovarian CEs are potent stimulators of progesterone synthesis (Spicer & Hammond 1989, Tekpetey & Armstrong 1994), we examined whether *de novo* steroidogenesis is essential for the CE-induced GVBD response. When the oocytes were pre-incubated with cyanoketone and epostane (inhibitors of 3 β -hydroxysteroid dehydrogenase, 3 β -HSD), and aminoglutethimide (a P450 antagonist) for 2 h, and then stimulated with different CEs for 8 h, and subsequently incubated again with steroidogenic inhibitors, the GVBD response was not affected, indicating that it was independent of *de novo* steroidogenesis by ovarian follicles. It has been demonstrated that the 3 β -HSD blockers cyanoketone and epostane do not block progesterone- or 17 α ,20 β -DP-induced oocyte maturation but block gonadotrophin/pituitary extract-induced maturation in teleosts, including catfish (Nagahama 1987, 1994, 1997, Rao & Haider 1992). In the present study, we also used the P450 antagonist, aminoglutethimide, that has a broad action at several steroid conversion steps and obtained similar results. These results show that, like 17 α ,20 β -DP, CEs stimulate oocytes directly to initiate maturational process.

The CE-induced GVBD response was inhibited to varying degrees by actinomycin D (a transcriptional inhibitor) and cycloheximide (a translational inhibitor) indicating simultaneous RNA and protein synthesis during the maturation of the oocytes. The pattern of the inhibitory response, however, showed differences with regard to the inhibitors, CEs and species. GVBD response was more significantly inhibited by actinomycin D (*C. batrachus*: 66–72%, *H. fossilis*: 60–65%) than cycloheximide (*C. batrachus*: 49–53%, *H. fossilis*: 36–55%). The strong inhibitory response of actinomycin D on CE-induced GVBD response is similar to that observed in gonadotrophin-induced GVBD but different from other steroid- (e.g. deoxycorticosterone, 17 α ,20 β -DP) and insulin-like growth factor-I-induced oocyte maturation (Sundararaj & Goswami 1977, Goetz 1983, Nagahama 1987, 1994, Jalabert *et al.* 1991, Kagawa *et al.* 1994). In the latter case, only translational activity (protein synthesis) was involved during the GVBD response. While the significance of translational inhibition by cycloheximide has been related to the synthesis of oocyte cytoplasmic maturation promoting factors (MPF) (Nagahama 1990, 1997, Kagawa *et al.* 1994), that of transcriptional inhibition is not clear. It may

be related to the MIH production as in the case of gonadotrophin-induced oocyte maturation (Nagahama 1990, 1997; Kagawa *et al.* 1994) or to the synthesis of MPF itself. The involvement of transcriptional activity during the CE-induced GVBD response suggests that the CEs may interact with nuclear receptors, similar to the parent oestrogens, as reported in mammals (MacLusky *et al.* 1983, Spicer & Hammond 1989).

Because of the catechol structure of the ring A, the CEs can bind to catecholamine (adrenergic) receptors on the cell surface (Paden *et al.* 1983, Spicer & Hammond 1989). In the present study, propranolol, the β -adrenoceptor antagonist, could significantly inhibit the CE-induced GVBD response but phentolamine, an α -adrenoceptor antagonist, did not. This indicates that CEs may act on β -adrenergic receptors on the oocyte membrane to mediate GVBD response. This fact is also strengthened by the results of a preliminary study (our unpublished data) that noradrenaline (1 $\mu\text{g}/\text{ml}$ for 6 h), but not dopamine and adrenaline, induced GVBD in *H. fossilis* ($44.0 \pm 1.25\%$) and *C. batrachus* ($40.7 \pm 1.32\%$). Considering the bifunctional potentials of the CEs, they may employ both mechanisms (steroid and adrenergic receptors), the elucidation of which remains.

Since folliculated oocytes (oocytes surrounded by a single granulosa cell layer, basement membrane and thecal cells) were used in the incubations, the GVBD agonists might have acted through the follicle cells or gained direct access on oolemma through gap junctions (between granulosa cells or between granulosa cell and oolemma). OE-2-H activity and CE synthesis occur mainly in the granulosa in mammalian ovarian follicles (Spicer & Hammond 1989). This may be true for fish ovary too, in which case CEs can act directly on oolemma to effect GVBD response. These aspects are to be investigated in further studies.

In conclusion, the pattern of changes in ovarian OE₂, OE-2-H and COMT points to stimulation of CE synthesis and degradation during GnRH-induced ovulation. Synthetic CEs induced GVBD that is concentration- and duration-dependent. The GVBD response is independent of *de novo* steroidogenesis and involves both transcriptional and translational processes. The CEs may engage both oestrogen and β -adrenergic receptors during the GVBD response.

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