Available online at www.sciencedirect.com
SCIENCE DIRECT
General and Comparative Endocrinology 147 (2006) 329–335
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Involvement of mitogen-activated protein kinase in 2-hydroxyestradiol-17β-induced oocyte maturation in the catfish *Heteropneustes fossilis* and a note on possible interaction with protein phosphatases

A. Mishra, K.P. Joy *

Department of Zoology, Banaras Hindu University, Varanasi 221005, India

Received 16 December 2005; revised 2 February 2006; accepted 4 February 2006
Available online 22 March 2006

Abstract

Mitogen-activated protein kinase (MAPK) was demonstrated in the postvitellogenic follicles (theca-granulosa and oocyte) of catfish by Western blotting using a polyclonal anti-rabbit serum, which recognized both ERK1 and ERK2. Two distinct protein bands resolved in the 46–48 kDa range of 12% SDS–PAGE were immunoblotted. Incubation of the follicles with 5 μM 2-OHE_2 elicited GVBD significantly in a duration-dependent manner with a concomitant increase in the expression of MAPK (ERK1 and ERK2). Densitometric analysis of the immunoblots showed significant variations in the intensity of staining. The ERK1 expression increased significantly from 6 h onwards but the changes were less pronounced. On the other hand, ERK2 registered a sharp significant increase after 3 h, which paralleled the GVBD response. The MEK inhibitor PD098059 alone did not induce GVBD. Co-incubation of the follicles with 2-OHE_2 and PD098059 significantly inhibited the steroid-induced GVBD at all concentrations. Immunoblot analysis showed that PD098059 inhibited MAPK activity significantly compared to the 2-OHE_2 group. The addition of okadaic acid (OA) in the incubation medium containing both 2-OHE_2 and PD098059 reversed the inhibitory effect of the latter and GVBD was elevated significantly over that of the 2-OHE_2 group but significantly lower than that of the 2-OHE_2 + OA group. The results suggest an involvement of MAPK in meiotic maturation but the site(s) of action: oocyte, follicular envelope or both needs further investigation.

Keywords: 2-Hydroxyestradiol-17β; GVBD; MAPK; Protein phosphatases

1. Introduction

Mitogen-activated protein kinases (MAPK) also called extracellular signal-regulated kinases (ERKs) comprise a family of ubiquitously expressed serine/threonine kinases, which participate in signal transduction pathways controlling development, and cell differentiation, proliferation, and death (Pearson et al., 2001; Roux and Blenis, 2004). They are activated in response to extracellular signals such as hormones, growth factors, cytokines, and stress (Cameron et al., 1996). The Mos/MAPK pathway is generally activated during oocyte maturation in animals but its role in relation to resumption of meiosis is a point of contention. In marine invertebrates, neither maturation-promoting factor (MPF) activation nor stabilization depends on MAPK activity (Eckberg, 1997; Kishimoto, 2004). In *Xenopus*, the presence of an active Mos/MAPK pathway is a prerequisite for MPF activation and stabilization (Kosako et al., 1994; Sagata et al., 1989a,b). In goldfish and amphibians except *Xenopus*, the Mos/MAPK pathway is neither necessary nor sufficient to initiate oocyte maturation (Kajiura-Kobayashi et al., 2000; Yoshida et al., 2000). In frog, MAPK played a role in the stabilization of cdc2 kinase activity by inhibiting cyclin B

* Corresponding author. Fax: +91 0542 2368174.
E-mail address: keerikkattilpailyjoy@yahoo.com (K.P. Joy).

0016-6480/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2006.02.002

degradation and its continuous inhibition during oocyte maturation delayed the MPF activation (Yoshida et al., 2000). While MAPK activity in oocytes is not required for meiotic resumption in mouse, rat, and goat (Dedieu et al., 1996; Sun et al., 1999; Zernicka-Goetz et al., 1997), it is activated before GVBD in equine, porcine, and bovine oocytes (Fan et al., 2002; Fissore et al., 1996; Goudet et al., 1998; Inoue et al., 1998; Zhao et al., 1991). The Mos/MAPK cascade is important to maintain metaphase II arrest during meiosis (Josefsberg et al., 2003). The stage-specific functions of MAPK and MPF in chromosome condensation, GVBD or microtubule assembly, and spindle formation during meiosis were illustrated in *Rana japonica* by Kotani and Yamashita (2002).

MAPK activity in cumulus cells is essential for gonadotropin-induced meiosis resumption in mouse oocytes and the pathways involved are different from those participating in spontaneous or hormone-induced GVBD in vitro (Su et al., 2001, 2002). Gonadotropins (FSH and LH) are potent activators of MAPK in the granulose cells (Cameron et al., 1996) and the enzyme participates in steroidogenic events and cumulus cell expansion (Rennert et al., 1993; Seger et al., 2001; Su et al., 2002).

It has been reported that protein phosphatases (PP1 and PP2A) regulate MAPK activation and microtubule organization (Lu et al., 2001; Sun et al., 1999, 2002; Zernicka-Goetz et al., 1997). Okadaic acid (OA, a specific inhibitor of PP1 and PP2A) stimulated GVBD by promoting MPF and/or MAPK activity (Lu et al., 2001). Further, OA reverses the inhibitory effect of cAMP and PKC activation on meiosis resumption and MAPK phosphorylation (Lu et al., 2001).

We have reported that the hydroxylated metabolites of estrogens 2- and 4-hydroxyestrogens induce oocyte maturation in the catfish and that formation of catecholestrogens is a means of removal of estrogens, which inhibit meiosis (Mishra and Joy, 2006a,b; Senthilkumaran and Joy, 2001). In the present study, effects of 2-OHE_2 on MAPK expression and oocyte maturation were investigated. Further, it was shown that the 2-hydroxyestrogen 2-OHE_2 stimulated progestin pathway (progesterone, 17-hydroxyprogesterone and 17,20β-dihydroxy-4-prognen-3-one; 17,20β-DP) to mediate the maturational activity (Mishra and Joy, 2006c). In addition, evidence for a possible interaction between the OA-sensitive protein phosphatases and MAPK was also presented.

2. Materials and methods

2.1. Chemicals

2-Hydroxyestradiol-17β (2-OHE_2), PD098059 [MEK (MAPK kinase) inhibitor], okadaic acid, anti-MAPK (rabbit polyclonal, Product No. M 5670), and anti-α-tubulin (mouse monoclonal, Product No. T 5168) were purchased from Sigma Chemical Company, St. Louis, MO, USA. HRP-conjugated anti-rabbit and anti-mouse antibodies raised in goat were purchased from Bangalore Genei, Bangalore, India. All other chemicals were of analytical grade and purchased locally.

2.2. Animal collection and maintenance

The experiments were performed in accordance with local/national guidelines for experimentation in animals and all care was taken to prevent cruelty of any kind.

Gravid female *Heteropneustes fossilis* (30–40 g) were obtained from a local fish market in the prespawning phase (June) of the reproductive cycle. They were maintained in the laboratory under normal photoperiod (13.0 L:11.0 D) and temperature (25 ± 2 °C) until used for experiments. The fish were fed goat liver daily ad libitum. A few fish were randomly checked for spontaneous ovulation and sacrificed to determine the maturation of ovary. The fish containing ovaries filled with dark green postvitellogenic follicles were used in the study.

2.3. Preparation of incubation medium and test compounds

The incubation medium was prepared as follows: NaCl 3.74, KCl 0.32, $CaCl_2$ 0.16, $NaH_2PO_4 \cdot 2H_2O$ 0.10, $MgSO_4 \cdot 7H_2O$ 0.16, glucose 0.40, and phenol red 0.008 in gram were dissolved in 1 L of triple distilled water. The pH was adjusted to 7.5 with 1 N sodium bicarbonate and autoclaved. Penicillin (2,00,000 U) and streptomycin sulphate (200 mg) were added and filtered. The medium was stored at 4 °C and prepared fresh every week.

Stock solutions of PD098059 in DMSO, and 2-OHE_2 and OA in ethanol were prepared separately on the day of the experiment and kept at 0 °C. Just before the incubation, they were diluted with the incubation medium to give different working concentrations.

2.4. Collection, selection, and incubation of postvitellogenic follicles

All instruments and glassware were sterilized. The acclimatized gravid female *H. fossilis* were sacrificed by decapitation and ovaries were transferred to a petri dish containing fresh cooled incubation medium. Rounded dark green postvitellogenic follicles were separated from each other with the help of a fine brush and watchmaker's forceps. For maturation studies, batches of about 30–40 follicles were incubated in embryo cups containing 3 ml of the incubation medium or the medium containing various test compounds at 25 ± 2 °C. As controls, the follicles were incubated in plain medium (control) or the medium containing vehicle (vehicle control). At the end of the incubations, the follicles were cleared in a clearing solution (ethanol/acetic acid/formalin; 6:1:3) and examined under a stereobinocular for determining germinal vesicle breakdown (GVBD), as an index of oocyte maturation.

2.5. Experiments

2.5.1. Effect of 2-OHE_2 on oocyte maturation

About 30–40 follicles in triplicate (group size = 3 fish) were incubated in the medium containing 5 μM of 2-OHE_2 for 3, 6, 12, 24, and 30 h. We selected the 5 μM concentration based on our earlier concentration–response study (Mishra and Joy, 2006b). As controls, the follicles were incubated in the incubation medium alone or in the medium containing the same volume of vehicle. At the end of each interval, the incubation was continued further in fresh medium to complete the 30 h incubation duration (except the 30 h group). The follicles were processed for calculating the percentage of GVBD.

For MAPK expression, about 120 follicles each from 5 fish were incubated for different time intervals (3, 6, 12, and 30 h) in the medium containing 5 μM 2-OHE_2 or vehicle control, as described above. The follicles were harvested groupwise and stored at −20 °C till the assay.

2.5.2. Effect of PD098059 on 2-OHE_2-induced oocyte maturation

About 30–40 follicles were incubated in triplicate (group size = 3 fish) with 5 μM 2-OHE_2 alone or in combination with PD098059 (10, 50, 100, and 1000 μM) for 24 h. Control groups were maintained concurrently. After the completion of the incubations, the follicles were cleared and scored for GVBD.