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Involvement of estradiol-17β and its membrane receptor, G protein coupled receptor 30 (GPR30) in regulation of oocyte maturation in zebrafish, *Danio rario* ☆

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ABSTRACT

The orphan G protein coupled receptor, GPR30, has the characteristics of a high affinity, specific estrogen membrane receptor on Atlantic croaker oocytes and mediates estrogen inhibition of oocyte maturation in this perciform fish. In order to determine the broad applicability of these findings to other teleosts, similar experiments were conducted in a cyprinid fish, zebrafish, in the present study. GPR30 mRNA expression was detected in zebrafish oocytes but not in the ovarian follicular cells. Both spontaneous and 17, 20β-dihyroxy-4-pregnen-3-one (DHP)-induced maturation of follicle-enclosed zebrafish oocytes was significantly decreased when they were incubated with either estradiol-17β, or the GPR30 agonists, ICI 182 780 and tamoxifen, or with the GPR30 specific agonist G-1. On the other hand spontaneous oocyte maturation increased two-fold when zebrafish ovarian follicles were incubated with an aromatase inhibitor, ATD. Moreover, the stimulatory effects of ATD on germinal vesicle breakdown (GVBD) were partially reversed by co-treatment with 100 nM of E2 or G-1. These results suggest that endogenous estrogens acting through GPR30 are involved in maintaining meiotic arrest of zebrafish oocytes.

1. Introduction

Recent studies show that the orphan G protein coupled receptor, GPR30, originally cloned from human and rat tissues (O'Dowd et al., 1998), is involved in estrogen signaling in breast cancer cells (Filardo et al., 2000; Filardo et al., 2002) and may function as a membrane estrogen receptor (Filardo and Thomas, 2005). Studies in our laboratory, subsequently confirmed by another group, showed that recombinant human GPR30 displays high affinity, specific estrogen binding typical of an estrogen membrane receptor (Revankar et al., 2005; Thomas et al., 2005). Moreover, we showed that estrogen binding to GPR30 caused activation of a stimulatory G protein resulting in increased cAMP production (Thomas et al., 2005). Subsequently we cloned and characterized GPR30 from a representative of the distantly-related teleost fishes, Atlantic croaker, to test the hypothesis that estrogen receptor binding and signal transduction is a fundamental and shared function of the GPR30 protein in the vertebrates. Atlantic croaker GPR30 had similar estrogen receptor binding and signaling characteristics as human GPR30 and showed high expression in croaker oocytes (Pang et al., 2008). In addition preliminary evidence was obtained for an involvement of GPR30 in maintaining oocyte meiotic arrest in croaker oocytes (Pang et al., 2008). Furthermore, GPR30 was also identified in zebrafish oocytes and microinjection of antisense mopholino oligo nucleotides to the GPR30 mRNA sequence into zebrafish oocytes resulted in an increase in oocyte maturation (Pang et al., 2008), indicating that GPR30 also may play a role in controlling oocyte maturation in this fish species.

To further examine the roles of estrogens acting through GPR30 in the regulation of meiotic arrest in zebrafish, in the present study the effects of inhibiting endogenous estrogen production with an aromatase inhibitor as well as incubation with GPR30 agonists on oocyte maturation were investigated in this second fish model belonging to a different teleost Superorder, Ostariophysi.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) or Steraloids Inc. (Newport, RI) unless otherwise stated. G-1, the GPR30 specific agonist, was purchased from EMD Chemicals (San Diego, CA). The nuclear estrogen receptor antagonist, ICI 182 780 was purchased from Tocris Biosciences (Ellisville, MO).

2.2. Animals

Mature zebrafish (*Danio rerio*) were purchased from a local pet store and maintained at a ratio of 1 male to 5 females in 50 L aquaria at 26 °C on a 14-h light:10-h dark photoperiod. The fish were

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fed twice a day with commercial tropical fish food and frozen brine shrimp and given a supplement of live brine shrimp twice a week.

2.3. Isolation of zebrafish ovarian follicles and GVBD bioassay

Ovarian follicles were isolated and incubated according to procedures described previously (Pang and Ge, 2002). Briefly, gravid female zebrafish were anaesthetized in 0.01% tricaine methanesulfonate solution for 2 min and humanely killed by severing the spinal cord using a protocol approved by the University of Texas at Austin Animal Care and Use Committee. The ovaries were then removed, placed in a 35-mm culture dish and washed several times with 60% Leibovitz L-15 medium. The follicles were carefully separated with the aid of a fine scalpel blade without damaging the ovarian membrane and their diameters measured with an ocular micrometer under a dissecting microscope. Intact vitellogenic and full-grown follicles 0.50–0.55 mm in diameter were selected, pooled, and randomly distributed in wells of a 24-well plate (Falcon) for the in vitro GVBD experiments (30–40 follicles/1 ml medium/well). The follicles were incubated for 6–16 h in the presence or absence of 17, 20β-dihydroxy-4-pregnen-3-one (DHP), estrogens, and the aromatase inhibitor, 1,4,6-Androstatrien-3,17-dione (ATD). The follicles were scored at the end of incubation for GVBD, an easily identifiable marker for oocyte maturation. All experiments were repeated three times to confirm the results.

2.4. Preparation of ovarian follicle cell and denuded oocyte fractions

Isolated ovarian follicles were washed several times with 5 ml 60% L-15 medium followed by a 30 min treatment at room temperature with 0.1 mg/ml collagenase (Sigma). The ovarian preparation was subsequently forced through a narrow pipette (~1 mm diameter) 10–20 times to break up the tissue. The resulting mixture of follicle cells and denuded oocytes was transferred to a new tube and allowed to settle for 1 min. The supernatant containing the follicle cells was then transferred to 1.5 ml tubes and washed 2 times with L-15 medium by centrifugation (1000 rpm, 2 min). The effectiveness of the separation procedure was confirmed by examination of the denuded oocytes and follicle cell suspensions under a microscope, followed by DAPI staining of the oocytes to confirm they were completely denuded of follicle cells. The denuded oocyte and follicle cell fractions were either used for extraction of total RNA immediately, or stored at −80 °C for later analysis.

2.5. RT-PCR

Total RNA was extracted with Tri-reagent (Sigma–Aldrich) from zebrafish oocytes and ovarian follicle cells according to the protocol provided by the manufacturer. The integrity of mRNA was confirmed by electrophoresis on a denaturing agarose gel followed by staining with ethidium bromide. Total RNA (1–3 μg) was reverse transcribed into cDNA at 50 °C for 1 h in 10 μl reaction solution containing 1× First Strand Buffer, 10 mM dithiothreitol, 0.5 mM each dNTP, 0.5 μg oligo-dT, and 100 U SuperScript III (Invitrogen). Each PCR was carried out in 20 μl reaction mix diluted from 2× PCR master mix (Promega) with 0.5 μl of the RT product. The zebrafish GPR30 primers, designed according to the sequence of zebrafish GPR30 (GenBank Accession No. XM_688551), were: sense, 5′-CAT CGG CCT GTT TCT CTC AT; antisense, 5′-GTA GCA CAG GCC GAT AAT, with an expected PCR product size of 534 base pairs. After an initial denaturation for 4 min at 94 °C, the cycling reaction was performed on a thermal cycler (Eppendorf) with a profile of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C for 35 cycles, followed by a 10-min extension at 72 °C. The PCR mix (10 μl) was electrophoresed on an agarose gel containing ethidium bromide to visualize the products. β-actin gene (primers: sense, 5′-GAG CAG GAG ATG GGA ACC; antisense, 5′-GAT GGA GTT GAA GGT GGT CT, with an expected PCR product size of 176 base pairs) was chosen for an internal control to normalize the mRNA concentration in the RT reaction.

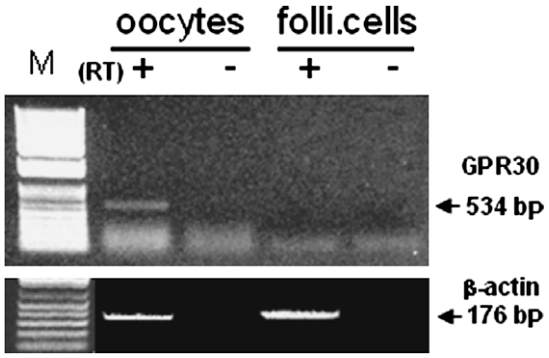

Fig. 1. RT-PCR detection of GPR30 mRNA in oocytes and ovarian follicle cells. RT +, −: PCR with or without reverse transcript of total RNA samples. M, DNA marker.

3. Results

3.1. Detection of GPR30 mRNA in zebrafish oocytes and ovarian follicle cells

GPR30 mRNA was detected in zebrafish oocytes but not in the surrounding follicle cells after 35 PCR cycles (Fig. 1). The β-actin RT-PCR confirmed the presence of equivalent amounts of mRNA in the oocyte and follicle cell preparations.

3.2. Effects of E2, ICI 182 780 and tamoxifen on DHP–induction of GVBD

DHP (10 nM) significantly increased the percentage of fully-grown oocytes (500–550 μm in diameter) that had completed GVBD (63.9% ± 2.5%) compared to vehicle treated controls (34.2% ± 5.8%) after 16 h incubation. Co-treatment with E2 (20 nM), ICI 182 780, or tamoxifen significantly attenuated the GVBD in response to DHP and the oocytes that completed GVBD (35.8% ± 8.7%, 30.50% ± 11.1% and 26.90% ± 4.27%, respectively) were not significantly different than that of the vehicle-treated controls (Fig. 2).

3.3. Effects of the GPR30 specific agonist G-1 on DHP induction of GVBD

G-1 (20 nM) exhibited a significant inhibitory effect on DHP-induced oocyte maturation (GVBD G-1: 37.7% ± 4.5%, DHP: 56.1% ± 3.5%), similar to that observed with E2 and the other GPR30 agonists. The percent of oocytes that completed GVBD in the G-1 group was low (5.4% ± 2.8%) but was not significantly different from controls (9.0% ± 2.20%) (Fig. 3).

3.4. Effects of the aromatase inhibitor ATD on oocyte maturation

Ovarian follicles were treated with the aromatase inhibitor, ATD, to determine whether endogenous estrogens are produced by ovarian follicles in sufficient amounts to influence oocyte maturation. Treatment with 10 μg/ml of ATD increased the percent of oocytes that completed GVBD fivefold over control levels GVBD to (ATD: 65.9% ± 4.5%, compared to controls: 12.3% ± 7.1%, $P < 0.05$). Co-treatment with 20 nM E2 or G-1 reversed the effects of ATD on GVBD (42.5% ± 6.6% and 47.3% ± 7.8%, respectively, $P < 0.05$) (Fig. 4).