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Role of natriuretic peptide receptor 2-mediated signaling in meiotic arrest of zebrafish oocytes and its estrogen regulation through G protein-coupled estrogen receptor (Gper)

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ABSTRACT

Natriuretic peptide type C (NPPC) and its receptor, natriuretic peptide receptor 2 (NPR2), have essential roles in maintaining meiotic arrest of oocytes in several mammalian species. However, it is not known if a similar mechanism exists in non-mammalian vertebrates. Using zebrafish as a model, we show that Nppc is expressed in ovarian follicle cells, whereas Npr2 is mainly detected in oocytes. Treatment of intact and defolliculated oocytes with 100 nM NPPC for 6 h caused a large increase in cGMP concentrations, and a significant decrease in oocyte maturation (OM), an effect that was mimicked by treatment with 8-Br-cGMP. Treatment with E2 and G-1, the specific GPER agonist, also increased cGMP levels. Cyclic AMP levels were also increased by treatments with 8-Br-cGMP, E2 and G1. The estrogen upregulation of cAMP levels was blocked by co-treatment with AG1478, an inhibitor of EGFR activation. Gene expression of *npr2*, but not *nppc*, was significantly upregulated in intact oocytes by 6 h treatments with 20 nM E2 and G-1. Both cilostamide, a phosphodiesterase 3 (PDE3) inhibitor, and rolipram, a PDE4 inhibitor, significantly decreased OM of intact and defolliculated oocytes, and enhanced the inhibitory effects of E2 and G-1 on OM. These findings indicate the presence of a Nppc/Npr2/cGMP pathway maintaining meiotic arrest in zebrafish oocytes that is upregulated by estrogen activation of Gper. Collectively, the results suggest that Nppc through Npr2 cooperates with E2 through Gper in upregulation of cGMP levels to inhibit phosphodiesterase activity resulting in maintenance of oocyte meiotic arrest in zebrafish.

1. Introduction

Natriuretic peptide precursor C (NPPC) is an agonist that selectively activates the type 2 natriuretic receptor (NPR2, also known as NPRB), through which it induces potent natriuretic, diuretic, and vasodilating actions that are important for body fluid homeostasis and blood pressure regulation. The NPPC/NPR2 pathway also has an important reproductive function. NPPC and NPR2 are expressed in mouse mural granulosa cells and cumulus cells, respectively, and NPPC treatment increases cGMP levels in cumulus cells and oocytes, resulting in maintenance of meiotic arrest of mouse oocytes in vitro (Zhang et al., 2010). In a subsequent study, estradiol-17β (E2) treatment was shown to promote cumulus cell expression of NPR2 and maintain meiotic arrest of mouse oocytes (Zhang et al., 2011). Recently, an important role of E2 in regulation of NPPC and NPR2 expression and maintenance of oocyte meiotic arrest has been confirmed by other researchers in several mammalian models (Lee et al., 2013; Liu et al., 2017; Soares et al., 2017; Zhang and Xia, 2012). In contrast, information is currently lacking on the presence of the NPPC/NPR2 signaling pathway in the ovarian follicles of non-mammalian vertebrates such as teleost fishes, its potential regulation by E2, and its role in maintenance of oocyte meiotic arrest. A significant inhibitory effect of E2 on the induction of oocyte maturation in vitro by gonadotropin in salmonid fishes had been observed in early studies (Jalabert, 1975), but the mechanisms involved remained unclear. Extensive evidence has accumulated that the novel membrane estrogen receptor, G protein-coupled estrogen receptor (Gper, formerly known as GPR30), is an intermediary in estrogen maintenance of teleost oocytes (Thomas, 2017). GPER was first identified as a membrane estrogen receptor in mammalian cells (Thomas et al., 2005, Revankar et al., 2005) and subsequently in Atlantic croaker ovaries (Pang et al., 2008). Croaker Gper displays all the binding and signaling characteristics of a specific estrogen membrane receptor coupled to a stimulatory G protein and has been shown to have a critical role in mediating the nongenomic action of estrogen to maintain meiotic arrest through stimulation of cAMP production in croaker oocytes (Pang et al., 2008).

The role of Gper in mediating estrogen upregulation of cAMP production and estrogen inhibition of oocyte maturation (OM) has also

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been demonstrated in zebrafish. Both E2 and G-1, a selective GPER agonist, significantly decreased both spontaneous and maturation-inducing steroid-induced oocyte maturation (Pang and Thomas, 2009), and these effects were significantly attenuated when the oocytes were injected with Gper antisense morpholinos or treated with fish Gper antibodies (Pang et al., 2008, Pang and Thomas, 2010). In contrast, microinjection of zebrafish oocytes with *esr1* antisense morpholinos did not block the inhibitory effects of E2 on OM (Pang and Thomas, 2010). Recently, estrogen inhibition of OM through Gper has also been demonstrated in common carp (Majumder et al., 2015). Estrogen inhibition of OM in zebrafish has been shown to be partly mediated through activation of an Egfr/Mapkinase signaling pathway (Peyton and Thomas, 2011). However, details of the mechanism by which MAPkinase inhibits OM remain unclear.

In the present study, we tested the hypothesis that the Nppc/Npr2 signaling pathway is present in ovarian follicles of a teleost fish, zebrafish, and that it participates in the maintenance of zebrafish oocyte meiotic arrest. We also investigated a possible mechanism of Nppc/Npr2 inhibition of OM through upregulation of cGMP. A second hypothesis that the expression and functions of Nppc and Npr2 are regulated by estrogens in zebrafish ovarian follicles was also tested. In addition, the mechanism of Mapkinase inhibition of OM through Gper/Egfr was further explored. Finally, potential interactions between the Nppc/Npr2 and E2/Gper inhibitory mechanisms maintaining meiotic arrest were investigated. The results suggest inhibition of zebrafish OM is coordinately controlled through Nppc/Npr2/cGMP and E2/Gper/Mapkinase pathways.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated. The GPER specific agonist G-1 was purchased from Calbiochem (Billerica, MA); Cilostamide and rolipram were purchased from Enzo Life Sciences (Farmingdale, NY). Polyclonal antibodies against NPPC and NPR2 were purchased from Santa Cruz Biotechnologies (Dallas, TX) and OriGene (Rockville, MD), respectively, and both antibodies (goat and rabbit) were generated against the antigen peptides that match human, mouse and rat sequences and are 100% identical to those of zebrafish.

2.2. Animals

Mature zebrafish (*Danio rerio*) were purchased from a commercial supplier and stocked in the ratio of 5 females to 1 male in aquaria tanks (~20 L) maintained at 25–27 °C with a 14-h light:10-h dark photoperiod. The fish were fed twice a day with commercial tropical fish food and supplemented with live brine shrimp once or twice a week.

2.3. Zebrafish oocyte maturation in vitro bioassay

Zebrafish were maintained and sacrificed following procedures approved by the University of Texas at Austin Animal Care and Use Committee. Fish ovarian follicles were isolated, defolliculated, and incubated following procedures established previously (Pang and Thomas, 2009, 2010). The follicles were either treated with 100 µg/ml collagenase for 30 min to remove the follicle cells (defolliculated oocytes, DFOs), or directly used for incubation (follicle-enclosed oocytes, FEOs). Successful removal of follicle cells was confirmed through microscopic observation for lacking of DAPI-stained follicle cells around oocytes.

2.4. Immunohistochemistry analysis of Nppc and Npr2 in zebrafish ovaries

Cryosections of ovarian tissues and immunocytochemistry of zebrafish ovarian cryosections were performed following procedures published previously (Pang and Thomas, 2010). The polyclonal antibodies raised against NPPC (goat) and NPR2 (rabbit) (both were validated with Western blot assays) were added to the blocking solution (2% BSA in PBS, 1:500) and incubated with the tissue sections at 4 °C overnight. The sections were then incubated with Alexafluor 488- and Alexafluor 647-conjugated secondary antibodies (1:2000. Invitrogen, Carlsbad, CA) in 2% BSA for 1 h at room temperature. The sections were wet-mounted with Antifade Embedding Reagent with DAPI (Invitrogen) under a cover glass and examined with a Nikon Eclipse TE2000U inverted fluorescent microscope and the images were recorded and processed with the Nikon Nis-Elements Ar Imaging system.

2.5. Preparation of zebrafish ovarian tissue lysate

Ovarian follicles (~40 follicles) were homogenized in 50 µl ice-cold RIPA buffer containing protease inhibitor cocktail (Thermo Scientific), then the mixture was incubated on a shaker at 4 °C for 30 min followed by centrifugation at 10,000 × *g*. The supernatant was collected and the protein concentration was adjusted to 1–2 mg/ml. The lysates were either used for Western blot analysis immediately or stored at −80 °C for future use.

2.6. RT-PCR

The RT-PCR method used in this study was published previously (Pang and Thomas, 2009, 2010) and the sequences of the primers used and the sizes of amplicons are listed in Table 1.

2.7. Quantitative real-time RT-PCR

QRT-PCR assays performed in the present study were based on the procedures published earlier (Pang and Thomas, 2010) using *nppc* and *npr2*, as well as zebrafish *β-actin* primers as a reference for loading controls. The efficiencies of amplification for the two genes were calculated by the equation EFF = 10(−1/slope) − 1. Standard curves using serially diluted template were run for NPPC, NPR2 and β-actin. The primers had sufficient amplification efficiency (> 98%), and the QRT-PCR results were considered comparable. A no template control was included in each experiment and the ΔCt value of each RNA sample

Table 1
Target genes and sequences of primers used in RT-PCR and QPCR assays.

Name	Access No.	Sequence (sense)	Sequence (antisense)	Amplicon
NPPC	NM_001161341.1	CCACATCCCTTCGCATTAGT	CTGCAACCTTTAGCCCAGAG	269 bp
NPR2	XM_009301825.3	GTGGTCCCCAACAGAGAAGA	CTTCCTGCACGCTTGTGATA	335 bp
PDE3a	XM_009299997.3	CCGCTTCTGTGTCAGATCAA	TGGGATGTCCCTGTATCCAT	200 bp
PDE3b	XM_686791.5	GTGTGCATTAAGCTGGCAGA	GTCGGATCCTTCCTCATCAA	270 bp
PDE4a	XM_695720.4	GAAGACAACCGGGACTGGTA	TCGTCTTCGTCCTGACCTCT	293 bp
PDE4b	XM_703863.5	ATCAAAGGGGAAGCCAAAGT	TTTTACAGTGGGCCAAAAGG	203 bp
18s rRNA	KY486501	GGTGAAATTCTTGGACCGGC	CAAAGACTCGTGGTTTCCCG	200 bp
β-actin	AF057040	GAACGACCAACCTAAACTCTC	GAGGAGGGCAAAGTGGTAA	192 bp