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The dual role of cGMP in oocyte maturation of zebrafish

Jianzhen Li*, Wenni Zhou, Yamei Wang, Caiyan Niu

College of Life Sciences, Northwest Normal University, Lanzhou, Gansu, 730070, China

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ABSTRACT

The roles of cyclic guanosine monophosphate (cGMP) signaling in oocyte maturation attracts much attention in mammals, but its roles in fish are still largely unknown. Using zebrafish as a model, we demonstrated for the first time in fish that cGMP is involved in oocyte maturation, and its functional model in oocyte maturation is different from that of mammals. The intracellular cGMP could be regulated by nitric oxide (NO), we found that all three NO synthase enzymes and four soluble guanylyl cyclases (sGC) are expressed in the zebrafish ovary. Intriguingly, either the activation or inhibition of the NO/sGC/cGMP pathway in fully grown follicles could lead to oocyte maturation. During oocyte maturation, cGMP levels increased in the follicular cell layer but decreased in oocytes, while NO levels increased in follicular cells but remained constant in oocytes. Based on these findings in zebrafish, we propose a hypothetical model on the dual role of cGMP in oocyte maturation: in follicular cells the LH signal could increase the level of NO and cGMP which induces oocyte maturation, while in the oocyte the decreased cGMP level can also induce oocyte maturation. These findings help us to understand the molecular mechanism of fish oocyte maturation.

1. Introduction

Ovary development is under precise regulation by the interactions of various hormones and local factors. Two development arrests occur at the prophase I and metaphase II stages during oocyte development. The resumption of the meiotic cell cycle from the prophase I to metaphase II stage is called oocyte maturation, which occurs before the ovulation of fertilizable oocytes [1,2]. Luteinizing hormone (LH) secreted from the pituitary triggers the resumption of oocyte maturation. In recent years, the involvement of cyclic guanosine monophosphate (cGMP) in oocyte maturation has attracted much attention [3]. cGMP levels in both somatic and intraoocyte compartments were decreased by LH treatment or during spontaneous oocyte maturation, suggesting a functional relationship between cGMP levels and oocyte maturation [3,4]. Many functional studies have demonstrated the role of cGMP in oocyte maturation [5–8]. Thus, the production of cGMP in the somatic cells is a critical component required to maintain prophase I arrest. The cGMP in the granulosa cells could be regulated by the guanylate cyclase activators (such as nitric oxide [NO] and natriuretic peptide [NP]) [8–10]. Many studies have indicated the involvement of the NO systems in mammalian oocyte maturation by regulating the cGMP [3,11,12]. All of these findings support that cGMP signaling is important for regulating oocyte maturation, however, most information comes from studies in mammals. The aim of this study was to reveal the role of cGMP signaling in fish oocyte maturation using the zebrafish as a model.

2. Materials and methods

2.1. Animals

Adult female zebrafish (*Danio rerio*) were purchased from a local market. All fish were maintained under a 14-h light/10-h dark cycle in circulating freshwater aquaria at 26–28 °C. Fish were fed twice daily with newly hatched brine shrimp (Brine Shrimp Direct, USA). Fish experiments were conducted in accordance to the regulations of the Animal Experimentation Ethics Committee of The Northwest Normal University, Lanzhou, China.

2.2. Chemicals and reagents

AR grade chemicals were obtained from Sigma-Aldrich (USA) and culture media from Gibco (USA). The 8-Bromo-cGMP (8-Br-cGMP), Human chorionic gonadotropin (hCG), and aminoguanidine

* Corresponding author. College of Life Sciences, Northwest Normal University, Anning, Lanzhou, China.
E-mail address: lijz1983@126.com (J. Li).

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(AG) were obtained from Sigma-Aldrich (USA). L-NAME, Vinyl-L-NIO, NOC-5, S-nitroso-N-acetylpenicillamine (SNAP), YC-1 and dibromo-cGMP (db-cGMP) was purchased from Cayman (USA). PTIO was purchased from Wako (Japan). 1H- [1,2,4]VOxadiazolo [4,3-a] quinoxalin-1-one (ODQ) was purchased from Aladdin (China).

2.3. RNA isolation and RT-PCR

Total RNA samples were isolated from ovarian follicles of zebrafish using TRIzol Reagent (Invitrogen, USA). The amount and purity of the RNA were determined on a NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific, USA). RT-PCR was carried out as previously described [13]. The primers used in this study were listed in Supplementary Table-1.

2.4. Immunostaining

The adult zebrafish ovary was collected and fixed overnight in 4% paraformaldehyde (containing 5% sucrose, pH7.4) at 4 °C. Frozen sections were cut in 7 μm thickness on a freezing microtome (Leica CM1520, Germany). The immunostaining was performed as described at [13]. A polyclonal sheep anti-cGMP antibody (1:200 dilution) was the gift from the Prof. Harry W.M. Steinbusch, Maastricht University Medical Center, Maastricht, The Netherlands.

2.5. Isolation of ovarian follicles and ovarian follicle incubations

The staging system that we have adopted is based on definition of Selman [14]. The ovaries were dissected out from 15 to 20 female zebrafish after anesthetization and decapitation, and placed in a 100-mm culture dish containing 60% Leibovitz L-15 medium as previously described [15]. After treatment, follicles that underwent GVBD were identified by their ooplasmic clearing (due to proteolytic cleavage of vitellogenin). Similar to others' studies [16,17], the percentage of spontaneous oocyte maturation in our study is highly variable among different batch of experiments, which might be due to the ovary condition of zebrafish. Thus each experiment was repeated at least three times.

2.6. Primary culture of ovarian follicular cells

Primary culture of zebrafish ovarian follicular cells was performed according to an established protocol [18]. Briefly, follicles of the vitellogenic stage from 15 to 25 females were carefully selected and cultured in 25 cm^2 flask for 6 days in M199 medium plus 10% fetal bovine serum (FBS) under the conditions of 28 °C and 5% CO_2. The cells were sub-cultured in 24-well plates at a density of 100,000 cells per well for 24 h before hormone and drug treatment.

2.7. Intraperitoneal injection into adult zebrafish

The procedure of Kinkel et al. [19] was followed with minor modifications. Briefly, after fasting and anesthetization, zebrafish were quickly placed on an agar gel plate. Using a microinjection system (WPI, USA), 3 μl hCG (5 IU/μl) was carefully injected into the midline between the pelvic fins. After injection, the fish were immediately transferred back to the aquarium for recovery.

2.8. Separation of follicular cells and oocyte from ovarian follicles

The follicular cell layer was carefully peeled off from the follicle with fine forceps without damaging the oocyte inside. The isolated follicular cell layers and the denuded but intact oocytes were pooled and subjected to NO and cGMP assessment separately. The denuded oocytes were also stained by propidium iodide (PI) to demonstrate complete removal of the follicular cell layer. Observation was made using a fluorescent microscope (Leica, Germany).

2.9. cGMP ELISA assay

Cyclic GMP concentrations were determined using the cGMP enzyme immunoassay (EIA) kit (Cayman, USA). About 20 intact follicles, 40 denuded oocytes and follicular cells separated from 80 follicles were collected and washed three times with PBS, and the PBS was aspirated. Samples and cGMP standards were quantified as described in the cGMP EIA kit. The approximate cGMP concentration in the follicle and oocyte was calculated based on an estimated average diameter of 0.69 mm, thus 172 nl calculated for a sphere of this diameter. Since the follicular cells surrounding the oocyte is very thin in zebrafish, the volume of mechanically dissected denuded oocyte is regarded as same volume of intact follicles. For these reasons, the absolute cGMP concentrations presented in the figures and text should be considered as approximate values. It should also be noted that, for the assessment of cGMP concentration in follicular cells and primary cultured follicular cells, the relative level of cGMP was compared.

2.10. Measurement of nitric oxide

Concentration of nitric oxide by cells was measured using nitric oxide assessment kit (Nanjing Jiancheng, China). About 20 intact follicles, 20 denuded oocyte or follicular cell separated from 80 follicles were homogenized in PBS using 1 ml syringe on the ice. The protein concentration of these samples was determined by BCA protein concentration determination kit (Biosharp, China). The determination of NO level followed the manufacturer's instructions. Absorbance of each sample was measured at 550 nm using the ultraviolet spectrophotometer (Agilent, USA). Concentrations of nitric oxide in the samples were determined using a calibration curve generated with standard $NaNO_2$ solutions (0.1–100 μM).

2.11. Statistical analysis

All data were expressed as mean values ± S.E.M. Statistical analysis was performed using ANOVA, followed by the Bonferroni post-test for comparisons of multiple groups. The paired Student t-test was used for comparison of data derived from two groups. Values with $P < 0.05$ were considered statistically significant.

3. Results

1 Activation of NO/sGC/cGMP signaling could stimulate oocyte maturation in zebrafish

To examine whether the nitric oxide (NO) system has functions in the ovary, three types of NOS (*nos1*, *nos2a* and *nos2b*) were identified in the zebrafish. As the classic NO sensor, there are four types of soluble guanylate cyclase (sGCs, including *gucy1a2*, *gucy1a3*, *gucy1b2* and *gucy1b3*) identified in zebrafish. The results showed that transcripts of all three NOS and four sGC could be detected in the zebrafish ovary (Fig. 1A). To analyze the involvement of NO/sGC/cGMP signaling in oocyte maturation, a series of pharmacological drugs were used to activate NO/sGC/cGMP. These include two NO donors (SNAP and NOC-5), two sGC activators (protoporphyrin IX and YC-1), and two membrane-permeable cGMP analogs (8-Br-cGMP and db-cGMP). We found that the oocyte maturation could be significantly stimulated by treatment with all these activators in a dose-dependent manner (Fig. 1B, C and

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