Molecular and Cellular Endocrinology xxx (2017) 1–8

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Ovulation is associated with the LH-dependent induction of *pla2g4aa* in zebrafish

Haipei Tang, Le Wang, Yu Chen, Jianan He, Ling Qu, Yin Guo, Yun Liu, Xiaochun Liu*, Haoran Lin

State Key Laboratory of Biocontrol, Institute of Aquatic Economic Animals and Guangdong Province Key Laboratory for Aquatic Economic Animals, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China

ARTICLE INFO

Article history:
Received 20 September 2017
Received in revised form
17 December 2017
Accepted 7 January 2018
Available online xxx

Keywords:
pla2g4aa
LH
PG
Ovulation
Zebrafish

ABSTRACT

The effects of the preovulatory luteinizing hormone (LH) surge on the ovulatory process are mediated by prostaglandins (PGs), the synthesis of which involves prostaglandin synthetase and cytosolic phospholipase A2 (cPLA2). In our previous study, we systematically investigated the function of prostaglandin endoperoxide synthase (*ptgs*) genes on ovulation in zebrafish. However, the role of cPLA2 in ovulation was not determined in zebrafish. In this study, we investigated the function of *cpla2α* in PGs production and ovulation in periovulatory follicles. Our data showed that the expression of *pla2g4aa* increased during zebrafish folliculogenesis and the follicular layer was the primary region with expression of *pla2g4aa.* In addition, the expression of *pla2g4aa* was regulated by LH *in vitro* and *in vivo*. Furthermore, injection of AACOCF3, a specific inhibitor of cPLA2, significantly reduced ovarian PGs level and blocked hCG-induced ovulation. Collectively, these findings suggest that *pla2g4aa* is related to the ovulation process in zebrafish.

1. Introduction

In vertebrates, the ovulatory process begins when the endogenous luteinizing hormone (LH) surge stimulates the luteinizing hormone receptor (LHR), which is distributed on the surface of granulosa cells and theca cells of preovulatory follicles (Robker et al., 2000; Diouf et al., 2006). It is well established that gonadotropins induce ovarian follicular production of prostaglandins (PGs), which are essential for ovulation to occur (Matsumoto et al., 2001; Espey, 2006; Murdoch et al., 1993). During the 1970s, the importance of PGs and their connection to ovulation was increasingly demonstrated. In mammals, the ovulatory surge of LH acts on large periovulatory follicles to stimulate granulosa cell expression of PG-synthesizing enzymes and increase follicular fluid PG concentration, and the resulting elevated follicular concentrations of PGs are necessary for ovulation to occur (Wong and Richards, 1991; Sirois and Dore, 1997; Duffy and Stouffer, 2001).

PGs are synthesized through the concerted action of a series of enzymes, including PG endoperoxide synthase (PTGS) and specific PG synthase enzymes, on intracellular arachidonic acid (AA) (Joyce et al., 2001; Diouf et al., 2006), beginning with the cleavage of AA from membrane phospholipids by phospholipase A2 (PLA2). In mammals, many forms of PLA2 have been identified to date; however, under physiological conditions, cytosolic PLA2 (cPLA2) is believed to contribute most significantly to the production of AA (Murakami and Kudo, 2002; Kudo and Murakami, 2002). Intracellular AA concentrations are determined largely by the activity of cytosolic phospholipase A2 enzymes (Kurusu et al., 2012). AA is later converted to PGH_2 by the activity of PG endoperoxide synthase (also known as cyclooxygenase, COX) (Vane et al., 1998). PGH_2 is the common precursor for the production of PGE_2 and $PGF_{2\alpha}$, and specific PG synthases convert PGH_2 into these bioactive PGs (Murakami et al., 2002; Watanabe, 2002). Therefore, local concentrations of PGs are regulated by the availability of AA and cell-specific expression of these enzymes.

The expression of phospholipase A2 group IVA (PLA2G4A, also known as cytosolic PLA2 alpha, cPLA2α) can induce the release of AA (Monget and Bondy, 2000). *PLA2G4A* expression is important for ovulation, as injection of the specific cPLA2 inhibitor arachidonyl trifluoromethyl ketone (ATK, AACOCF3) significantly decreases intraovarian bursal ovulation and total ovarian PGE_2 synthesis in

* Corresponding author. School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China.
E-mail address: lsslxc@mail.sysu.edu.cn (X. Liu).

https://doi.org/10.1016/j.mce.2018.01.002
0303-7207/

rats (Kurusu et al., 1998). Mice lacking the *cPla2* gene were generated on the C57BL/6J background, and the females were observed to have reproductive defects, including reduced litter sizes and delayed parturition, which usually results in loss of pregnancy or stillborn pups (Sapirstein and Bonventre, 2000; MacPhee et al., 1995; Kennedy et al., 1995). Subsequent studies with *Pla2g4a*$^{-/-}$ mice found that the primary reproductive defect was in the embryo implantation process (Song et al., 2002; Brown et al., 2009). These data demonstrate an important role for *cPLA2* activity in ovulation and PG synthesis.

In zebrafish, *cpla2α* and *ptgs2* are two genes critical for the successful ovulation of mature ova (Lister and Van Der Kraak, 2009). In a previous study, we systematically investigated the function of three *ptgs* genes on ovulation in zebrafish. Recently, two *cpla2α* genes (*pla2g4aa* and *pla2g4ab*) have been identified in zebrafish (Naini et al., 2016). The *cpla2α* gene is involved in prostaglandin synthesis in the ovary in zebrafish (Lister and Van Der Kraak, 2008; Lister and Van Der Kraak, 2009; Knight and Van Der Kraak, 2015). However, the definitive role of the two *cpla2α* genes, as well as the molecular mechanism underlying the *cpla2α*-mediated pro-ovulatory activity of LH, remains poorly understood. In this study, we further investigated the expression and regulation of *cpla2α* in the ovary to determine the mechanism underlying the LH-induced expression of *cpla2α* in preovulatory follicles that are destined to ovulate. We provide *in vitro* and *in vivo* evidence that *pla2g4aa* is required for LH-induced ovulation in zebrafish.

2. Materials and methods

2.1. Chemicals and hormones

Human chorionic gonadotropin (hCG), Forskolin, H-89, IBMX, PMA, U0126, AACOCF3 and DMSO were purchased from Sigma-Aldrich. hCG was first dissolved in water, whereas Forskolin, H-89, IBMX, PMA, U0126 and AACOCF3 were first dissolved in dimethylsulfoxide (DMSO) and were diluted to the desired concentrations in water or medium before use.

2.2. Zebrafish husbandry

Wild-type and *npr* mutant zebrafish (established in our previous study) (Tang et al., 2016) were reared in the laboratory of the Sun Yat-Sen University following the protocols described in Westerfield, (1993). Briefly, fish were maintained in flow-through aquaria under an artificial photoperiod of 14 h light (9:00–23:00): 10 h dark (23:00–9:00) at 28 ± 1 °C. The larvae and adult zebrafish were fed twice a day with brine shrimp (hatched from eggs in 20 mL in 4 L salt water). All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of the Sun Yat-Sen University.

2.3. Isolation of ovarian follicles

The follicle stage system we adopted in this study is based on recent studies (Tang et al., 2016; Li et al., 2015). The ovaries were carefully dissected from 15 to 20 female zebrafish (wild-type or *npr* mutant fish) after anaesthetisation and decapitation and placed in a 100 mm culture dish containing 60% Leibovitz L-15 medium. Follicles of different stages were manually isolated with fine forceps and grouped into six developmental stages, including primary growth (PG), previtellogenic (PV), early vitellogenic (EV), mid-vitellogenic (MV), full grown (FG) and mature (M stage), based on both diameter and morphology. Follicles of different stages were transferred in 24-well culture plates (30–40 follicles/well) and incubated with or without drug treatments in 60% Leibovitz L-15 medium under the condition of 28 °C. Each group had 4 replicate wells. We performed this experiment twice using different batches of zebrafish to confirm the results.

2.4. Removal of follicular layer from ovarian follicles

Follicular layer (including theca cell and granulosa cell layers) were removed from the ovarian follicles by enzymatic digestion according to previous reports (Li et al., 2015; Das et al., 2013; Peyton and Thomas, 2011). Intact follicles were incubated in 60% L-15 medium containing collagenase (100 μg/mL) for 1 h at room temperature with mild agitation and repeated (30 times) gentle pipetting of the follicles through a narrow pipette (1 mm in diameter) during incubation. Denuded oocytes were collected under an inverted tissue culture microscope and washed thoroughly with fresh medium. Removal of the follicular layer was confirmed by staining the denuded oocytes with propidium iodide (PI; 1 mg/ml) and observation under an up-right fluorescent microscope.

2.5. Primary cultures of ovarian follicular cells

Primary culture of zebrafish ovarian follicular cells was performed according to an established protocol (Pang and Ge, 2002; Tang et al., 2016). Briefly, follicles of vitellogenic stage from 15 to 25 females were carefully selected and washed with M199. The follicles were later cultured in a 25 cm^2 flask for 6 days in M199 medium with 10% foetal bovine serum under the condition of 28 °C and 5% CO_2. The medium was changed on the third day. After 6 days, the cells were subcultured in 24-well plates at a density of 100 000 cells per well for 24 h. Next, cells were incubated for 2 h with or without hCG, Forskolin, H89, IBMX, PMA, and U0126 or solvent control DMSO in triplicate wells per treatment in M199 medium plus 10% foetal bovine serum under the condition of 28 °C and 5% CO2. We performed this experiment three times using different batches of zebrafish to confirm the results.

2.6. Induction of ovulation in vivo

hCG-induced ovulation *in vivo* was performed according to our previous reports (Tang et al., 2016). Briefly, hCG (Sigma-Aldrich) was dissolved in sterile distilled saline solution (NaCl 0.7%) at a concentration of 10 IU/μl. A cPLA2 enzyme inhibitor (AACOCF3) dissolved in DMSO at a concentration of 10 mM and later dissolved in sterile distilled saline solution to 100 μM. Prior to drug treatment, adult female fish were anaesthetised. Fish were intraperitoneally injected with a volume of 5 μl/g body weight. After injection, the fish were reared individually in the tank. At least 5 fish were used in each group. Follicular layer or denuded oocytes obtained from more than 50 follicles were collected (4 replicates were used in each group) to examine the mRNA levels of *cpla2α* every 0.5 h interval. Three hours later, the ovaries were carefully dissected out of the body cavity. All FG stage follicles, M stage follicles or oocytes were manually isolated according to their size and morphology. The ovulation rate was defined as the percentage of oocytes at a given time (Ogiwara et al., 2005; Tokumoto et al., 2011). At least 50 FG stage follicles, M stage follicles or oocytes from each replicate were scored for percentage ovulation. We performed this experiment three times using different batches of zebrafish to confirm the results.

2.7. RNA isolation and RT-PCR

Total RNA was extracted from the ovarian follicle samples, cultured follicular cells of zebrafish using TRIzol reagent

Please cite this article in press as: Tang, H., et al., Ovulation is associated with the LH-dependent induction of *pla2g4aa* in zebrafish, Molecular and Cellular Endocrinology (2017), https://doi.org/10.1016/j.mce.2018.01.002