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Effects of RU486 on cyclooxygenase-2 gene expression, prostaglandin F2α synthesis and ovulation in *Xenopus laevis*

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ABSTRACT

RU486 is a synthetic analog of progesterone and functions as a progesterone receptor antagonist. It binds to the progesterone receptor to prevent progesterone from occupying its receptor in many cellular systems. Early studies from our laboratory have shown that in *Xenopus laevis* ovarian follicles progesterone stimulates the expression of cyclooxygenase-2 (COX-2) gene which leads to a rapid increase in the production of prostaglandin F2α (PGF2α) and subsequent ovulation. In this study, we examined the effect of RU486 on the synthesis of COX-2 mRNA, production of PGF2α and ovulation in *X. laevis*. Ovarian tissue fragments were primed with human chorionic gonadotropin (hCG) and then incubated with progesterone (P4) alone or in the presence of varying concentrations of RU486 over a period of 12 h. After the incubation ovulated oocytes were counted, COX-2 expression and synthesis of PGF2α were measured. Results demonstrated that RU486 attenuated the expression of COX-2 gene, reduced the synthesis of PGF2α, and inhibited ovulation in a dose-dependent manner. This finding suggests that progesterone receptor is an important regulator in the progesterone–cyclooxygenase–prostaglandin-mediated ovulation in amphibians.

1. Introduction

Full-grown *Xenopus* oocytes are known to be physiologically arrested at late prophase of meiosis I until exposed to progesterone (P4). P4 is the natural maturation-inducing substance in ovarian follicles of amphibian species, induces oocyte maturation, leading to subsequent ovulation (Liu and Patiño, 1993; Tian et al., 2000; Liu et al., 2005). P4 is also the relevant steroid controlling oocyte maturation, through interactions with the progesterone receptor (PR) (Bayaa et al., 2000; Tian et al., 2000; Ben-Yehoshua et al., 2007). Two forms of classical progesterone receptors XPR-1 and XPR-2 have been cloned in *Xenopus laevis* and they are differentially expressed in the follicle cells and the oocytes (Liu et al., 2005). RU486, also known as mifepristone, is a synthetic analog of P4 with a high affinity for the PR. In many cellular systems, it functions as a P4 antagonist by binding to the PR and preventing P4 from occupying its receptor (Moguilewsky and Philibert, 1984). During ovulation, the classical PR is competed by RU486 and inhibits ovulation of an oocyte. Thus, the gene transcription induced normally by P4 is blocked because RU486 binds to the same region of PR as P4 itself (Moguilewsky and Philibert, 1984). Preovulatory administration of RU486 has been shown to interfere with the functioning of luteinizing hormone (LH)/human chorionic gonadotropin (hCG), it reduces P4 production (Dimattina et al., 1986), delays follicular maturation and inhibits ovulation (Liu et al., 1987). Thus, RU486 is considered as an inhibitor of oocyte maturation and ovulation in mammals (Lipner and Greep, 1971; Snyder et al., 1984; Brannstrom and Janson, 1989; Lutz et al., 2001). RU486 has also been reported to affect prostaglandin F2α (PGF2α) production by inhibiting hCG-induced cyclooxygenases-2 (COX-2) expression in human granulosa luteal cells (Tsai et al., 2008). In amphibians, full-grown follicles are known to undergo maturation, followed by ovulation, in response to P4, produced by follicle cells. The stimulation of gonadotropin LH, along with P4 production leads to the expression of COX-2, the enzyme that catalyzes the production of prostaglandins (PGs) from arachidonic acid, and these PGs are the key mediators of several female reproductive functions including ovulation, fertilization, luteolysis, implantation and parturition (Williams et al., 1999; Sales and Jabbour, 2003), but the effect of the RU486 on ovulation is still not clear in *Xenopus*. As both RU486 and P4 have been reported to bind at the same region of ligand binding domain of PR (Bayaa et al., 2000; Tian et al., 2000) one of the aims of this study was to study the effect of RU486 on ovulation in *X. laevis*.

Prostaglandins are key mediators of several female reproductive functions including ovulation, fertilization, luteolysis, implantation and parturition (Williams et al., 1999; Sales and Jabbour, 2003).

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Arachidonic acid is converted into prostaglandin (PGH_2), which is then converted into primary prostaglandins (PGs) including PGF2α (Smith et al., 1998; Arosh et al., 2003). Cyclooxygenases (COXs) are known to catalyze the conversion of arachidonate to prostaglandins and two different COX isozymes, a constitutive form (COX-1) and inducible form (COX-2), have been identified in many species (DeWitt and Smith, 1988; Yokoyama et al., 1988; Funk et al., 1991; Hla and Neilson, 1992; Smith and Dewitt, 1996; Grosser et al., 2002; Sirois et al., 2004). In rat preovulatory follicles, COX-2 mRNA and its protein synthesis are selectively induced in granulosa cells just prior to ovulation (Sirois et al., 1992, 2004). Moreover, COX-2-deficient female mice show multiple reproductive dysfunctions, including ovulatory failures (Lim et al., 1997; Davis et al., 1999; Richards, 2005). In some teleost species, ovarian PG synthesis increases dramatically during spontaneous or artificially-induced ovulation and indomethacin, a non-selective COX inhibitor effectively blocks the ovulation *in vivo* and *in vitro* (Goetz and Theofan, 1979; Cetta and Goetz, 1982; Pankhurst, 1985; Goetz et al., 1991; Chang et al., 1997; Kagawa et al., 2003; Patiño et al., 2003). A very recent study has explored the potential for ovarian-derived PGs to be involved in the regulation of oocyte maturation and ovulation in zebrafish, and their experiments indicate that the arachidonic acid pathway in zebrafish ovaries is involved in the regulation of oocyte maturation and ovulation and a non-selective inhibitor of COX disrupts these processes (Lister and Van Der Kraak, 2008). COX-1 and COX-2 genes from *X. laevis* have been recently cloned in our laboratory (Sena and Liu, 2008). In *Xenopus* ovarian follicles PGF2α is synthesized during the periovulatory period, similar to mammals and PGF2α synthesis is regulated by *de novo* transcription of COX-2 but not COX-1 (Sena and Liu, 2008). As the gonadotropin induced expression of mRNA for COX-2 and production of prostaglandins are regulated by P4 acting though the PR, in this study, our aim was to explore the effect of RU486, a PR antagonist, on the expression of COX-2 in *X. laevis* and also on prostaglandin synthesis. A better understanding of this mechanism will enable us to further understand the regulation of the COX-2 gene expression, and ultimately use the new knowledge in facilitation of the development of agents for inhibition of ovulation (contraceptive) or induction of ovulation in anovulatory patients.

2. Materials and methods

2.1. Frogs

The adult female *X. laevis* were purchased from NASCO (Modesto, CA), and three frogs were maintained in a 10-gallon aquarium at 22 °C water temperature and 12L/12D photoperiod. The frogs were fed frog brittles (NASCO) thrice a week. Prior to tissue collection, frogs were deeply anesthetized with 0.2% tricaine methanesulfonate and spinally transected. Ovaries were surgically removed and used in the following experiments. All procedures were reviewed and approved by the Animal Care and Use Committee of Eastern New Mexico University.

2.2. Reagents

PGF2α EIA kit was obtained from Cayman Chemical Co. (Ann Arbor, MI). RU486 (mifepristone), human chorionic gonadotropin (hCG) and progesterone (P4) and all other chemicals were obtained from Sigma (St. Louis, MO).

2.3. Ovarian tissue culture

Ovarian tissues were placed in modified Barth's solution (MBS: 88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.41 mM $CaCl_2 \cdot 2H_2O$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES, pH 7.6) supplemented with streptomycin sulfate (30 mg/l) and penicillin (30 mg/l). Subsequently, tissues were cut into small pieces (approximately 100 mg of ovarian fragment containing full-grown ovarian follicles) and washed with MBS for 30 min under gentle agitation to remove the yolk components from damaged oocytes. To examine the effects of RU486 on COX mRNA levels and PG synthesis, ovarian fragments were first primed with hCG at a dose of 25 IU/ml for 30 min at 22 °C under gentle agitation. The ovarian tissue was then washed and individual pieces were incubated in 2 ml each of MBS (negative control), MBS with 10 μM P4 (positive control), MBS with P4 and varying concentrations of RU486 (10, 50, and 100 μM). P4 (10 mM stock solution) and RU486 (10 mM stock solution) were prepared in ethanol (100%). At time 0, 6 and 12 h, tissue samples and incubated media were collected serially for subsequent analysis. During the incubation period, conditions of ovarian fragments were inspected under a dissecting microscope to monitor maturational and ovulatory progression at the morphological level. PGF2α concentrations in the cultured media were measured by prostaglandin enzyme immunoassay (EIA). Each individual experiment was conducted with ovarian tissue fragments from a single donor frog, and triplicate samples per treatment per incubation time were used. The experiment was repeated four times with a different donor frog.

2.4. Bioassay with RU486

For this study, ovarian tissues were prepared as per the method described in Section 2.3. The total number of oocytes, as well as the number of ovulated oocytes was counted in each well at 0, 6, and 12 h of incubation. To study the effect of RU486 on ovulation, the percentage of ovulation was calculated in all samples.

2.5. Real-time PCR

Xenopus laevis COX-2 gene expression was measured in ovarian follicles at 0, 6 and 12 h via real-time PCR using the MyIQ real-time PCR detection system (Bio-Rad Laboratories Inc., Richmond, CA). Initially, using full-length COX-2 sequences, forward and reverse PCR primers were designed for COX-2 (Forward: 5′- ATG TTC AGT TTG CTG TCG GGC AAG -3′, Reverse: 5′-ACT CGA TTG TGC TCC CGT AAC CAT-3′). *X. laevis* β-actin was used as an internal control (Forward: 5′-ACA GCT TCA CCA CCA CAG CCG A-3′, Reverse: 5′-AGA AGA GGC AGC TGT GGC CAT CTC-3′). Total RNA was extracted from ovarian follicles using the RNeasy Lipid Tissue mini kit (QIAGEN) as specified by the manufacturer's instructions. On-column DNA digestion was performed using the RNase-free DNase set (QIAGEN) as specified by the manufacturer. cDNA was synthesized from total RNA (3 μg/60 μl reaction/sample) using the iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., Richmond, CA). Real-time PCR for COX-2 was performed with the iQ SYBR Green Supermix (Bio-Rad Laboratories Inc., Richmond, CA). All PCR reactions had a final volume of 25 μl, with 2 μg of total cDNA per sample. PCR conditions were 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s for 45 cycles with a 3 min initial 95 °C denaturation step. For gene expression studies, data was analyzed using the Δ CT method in which all expression was measured relative to negative control (ovarian tissue suspended in MBS without any P4 or RU486) at that hour (0, 6 or 12 h). For real-time PCR experiments the ovarian tissue samples used were from three donor frogs, and triplicate samples per frog per incubation time were used.

2.6. Prostaglandins enzyme immunoassay (EIA)

PGF2α concentrations in culture media were measured in 0, 6 and 12 h samples using EIA kit (Cayman Chemical, Ann Arbor,