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# Paracrine regulation of the resumption of oocyte meiosis by endothelin-1

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ABSTRACT

Mammalian oocytes remain dormant in the diplotene stage of prophase I until the resumption of meiosis characterized by germinal vesicle breakdown (GVBD) following the preovulatory gonadotropin stimulation. Based on genome-wide analysis of peri-ovulatory DNA microarray to identify paracrine hormone-receptor pairs, we found increases in ovarian transcripts for endothelin-1 and endothelin receptor type A (EDNRA) in response to the preovulatory luteinizing hormone (LH)/human chorionic gonadotropin (hCG) stimulation. Immunohistochemical analyses demonstrated localization of EDNRA in granulosa and cumulus cells. In cultured preovulatory follicles, treatment with endothelin-1 promoted oocyte GVBD. The stimulatory effect of endothelin-1 was blocked by cotreatment with antagonists for the type A, but not related type B, receptor. The stimulatory effect of hCG on GVBD was partially blocked by the same antagonist. The endothelin-1 promotion of GVBD was found to be mediated by the MAPK/ERK pathway but not by the inhibitory G protein. Studies using cumulus–oocyte complexes and denuded oocytes demonstrated that the endothelin-1 actions are mediated by cumulus cells. Furthermore, intrabursal administration with endothelin-1 induced oocyte GVBD in preovulatory follicles. Our findings demonstrate a paracrine role of endothelin-1 in the induction of the resumption of meiosis and provide further understanding on the molecular mechanisms underlying the nuclear maturation of oocytes induced by the preovulatory LH surge.



## Introduction

In mammalian developing follicles, primary oocytes enter meiosis but are arrested at the diplotene stage of prophase I. The oocytes stay in this dormant state for months and years until they are about to be ovulated. In response to the preovulatory luteinizing hormone (LH) increase, the large nucleus of the oocytes (called the germinal vesicle, GV) in preovulatory follicles undergo GV breakdown (GVBD), followed by first polar body extrusion. Although the preovulatory surge of LH is the primary event responsible for the induction of maturation of the oocyte, LH and its surrogate human chorionic gonadotropin (hCG) do not act directly on the oocyte due to the absence of functional LH receptors in germ cells. Instead, actions of LH/hCG are mediated either by paracrine factors secreted by LH-responsive somatic cells (theca and mature granulosa cells) or by the transport of cellular messengers from granulosa/cumulus cells to oocytes through intercellular tight junctions (Gilula et al., 1978). We reported the ability of thecal cell-derived insulin-like 3 (INSL3) to activate its receptor, leucine-rich repeat-containing G protein-coupled receptor 8, in the preovulatory oocytes, leading to decreases in intra-oocyte cAMP levels and subsequent GVBD (Kawamura et al., 2004). Furthermore, several granulosa cell-derived epidermal growth factor (EGF)-like ligands (epiregulin, amphiregulin, and betacellulin) were also found to be important for GVBD of the preovulatory oocyte by acting at the cumulus cells (Park et al., 2004).

Endothelin-1 is a 21-amino acid multifunctional peptide. In addition to its potent vasoconstrictor actions (Levin, 1995), Endothelin-1 is also important in renal, pulmonary, and reproductive physiology (Boiti et al., 2005; Kon and Badr, 1991; Levin, 1995, 1996; Meidan and Levy, 2002; Noll et al., 1996; Otani et al., 1996). Using genome-wide analysis of DNA microarray datasets from periovulatory ovaries, we found major increases in the expression of endothelin-1 and one of its receptors endothelin receptor type A (EDNRA) and demonstrated the ability of endothelin-1 to promote GVBD of preovulatory oocytes by using in vitro and in vivo models.

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## Materials and methods

### *DNA microarray analyses*

Female B6D2F1 mice ($n=108$) were injected at 21 days of age with Humegon (7.5 IU per animal, Organon, Oss, Netherlands) to stimulate follicular growth. Forty-eight hours later, some animals were treated i.p. with Pregnyl (5 IU per animal, Organon) to induce ovulation. Ovaries were dissected from animals killed bi-hourly after Humegon treatment (three mice per group) and hourly after Pregnyl treatment (one mouse per group) for RNA extraction (TRIzol, Invitrogen, Carlsbad, CA). Aliquots of 6 μg of total RNA at 1 μg/μl for one-chipset hybridization were stored at −80 °C. Samples were hybridized to the Affymetrix mouse MGU74v2 arrays A, B, and C according to standard Affymetrix protocols. The pooled follicular phase samples were hybridized in duplicate, and the post Pregnyl samples were single determinations (Kawamura et al., 2005).

### *Animals*

Ovarian samples were obtained from female B6D2F1 mice at 25 days of age (CLEA Japan, Tokyo, Japan) during treatment with a single i.p. injection of 7 IU of pregnant mare serum gonadotropin (PMSG) (Calbiochem, Cambridge, MA) followed at 48 h later with 10 IU of hCG (ASKA Pharmaceutical, Tokyo, Japan) administrated i.p. to stimulate follicle maturation and ovulation, respectively. The care and use of animals was approved by the Animal Research Committee, Akita University School of Medicine and Stanford University School of Medicine.

### *Real-time RT-PCR*

For quantitative real-time RT-PCR, GV stage oocytes, cumulus cells, and mural granulosa cells were collected from ovaries of PMSG-treated immature mice at 48 h after treatment (Kawamura et al., 2005, 2004). Cumulus oocyte complexes (COCs) were obtained by puncturing the largest follicles of preovulatory ovaries, and denuded oocytes were separated from cumulus cells by mechanical pipetting. Granulosa cells were obtained separately by puncturing preovulatory follicles, followed by the removal of COCs.

Quantitative real-time RT-PCR of transcript levels in ovarian cells and whole ovaries was performed using a SmartCycler (Takara, Tokyo, Japan) as described (Kawamura et al., 2007, 2005). The primers and hybridization probes for real-time PCR of endothelin-1, EDNRA, endothelin receptor type B (EDNRB), endothelin converting enzyme-1 (ECE1), and histone H2a are as follows: endothelin-1: sense 5′-AGGTTCTTCCAGGTCCAAGC-3′, antisense 5′-GGTGAGCGCACTGACATCTA-3′, probe 5′-6-carboxy-fluorescein (FAM)-CCAATAAGGCCACAGACCAGGC-6-carboxy-tetramethyl-rhodamine (TAMRA)-3′; EDNRA: sense 5′-GAGGCGTAATGGCTGACAAT-3′, antisense 5′-GTGGTGCCCAGAAAGTTGAT-3′, and probe 5′-FAM-CAGCGCTAATCTAAGCAGCCACATG-TAMRA-3′; EDNRB: sense 5′-CAGGAAGAAGAGCGGTATGC-3′, antisense 5′-CCAACAGAGAGCAAACACGA-3′, probe 5′-FAM-AAGTGGCCAAGACAGTCTTCTGCCT-TAMRA-3′; ECE1: sense 5′-AAGAACGGAGCTGAGCAGAC-3′, antisense 5′-GGACAGAGCACCAGACCTGT-3′, probe 5′-FAM-CCAGCAACCAGCTCTTCTTCCTAGG-TAMRA-3′; histone H2a: sense 5′-ACGAGGAGCTCAACAAGCTG-3′, antisense 5′-TATGGTGGCTCTCCGTCTTC-3′, probe 5′-FAM-AACATCCAGGCCGTGCTGCT-TAMRA-3′. To determine the absolute copy number of target transcripts, cloned plasmid cDNAs for individual gene were used to generate a calibration curve. Purified plasmid cDNA templates were measured, and copy numbers were calculated based on absorbance at 260 nm. A calibration curve was created by plotting the threshold cycle against the known copy number for each plasmid template diluted in log steps from $10^5$ to $10^1$ copies. Each run included standards of diluted plasmids to generate a calibration curve, a negative control without a template, and samples with unknown mRNA concentrations.

### *Immunohistochemistry*

To localize EDNRA, ovaries were obtained from PMSG-primed mice before and at 4 h after hCG injection. After fixation with 20% formalin neutral buffer solution for 24 h at room temperature, tissues were embedded in paraffin and sectioned at 3-μm intervals before deparaffinization and dehydration. Endogenous peroxidase activities were quenched with 1% periodic acid for 30 min. After blocking with 5% normal goat serum (Dako, Carpinteria, CA) for 30 min slides were incubated with rabbit anti-EDNRA polyclonal antibodies (NLS4073; Novus Biologicals, Littleton, CO) at 1:100 dilution overnight at 4 °C and washed three washes in Tris-buffered saline (TBS). Slides were then incubated with biotinylated anti-rabbit secondary antibodies (Dako) for 30 min at room temperature. After three washes, bound antibodies were visualized using a Histostain SP kit (Zymed Laboratories, San Francisco, CA). For negative controls, the primary antibody was replaced by nonimmune rabbit IgG (Dako). For antigen blocking experiments, the primary antibody was adsorbed with 10 μg/ml of the synthetic immunogen peptide (Novus Biologicals).

### *Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA)*

For EIA measurement of the endothelin-1 peptide, mouse ovaries were homogenized in a buffer containing 137 mM NaCl, 20 mM Tris–HCl, 1% Nonidet P40, 10% glycerol, and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) before centrifugation at 8000×*g* for 5 min at 4 °C. The supernatant was removed and stored at −80 °C until use. After determination of protein levels using the DC Protein Assay kit (Bio-Rad, Hercules, CA), samples were adjusted to 1 M acetic acid–20 mM HCl and then acetone was added to a final concentration of 66%. Precipitated proteins were removed by centrifugation for 30 min at 3000×*g*. The extracted peptide fractions were lyophilized using a lyophilizer (Labconco, Kansas City, MO). Levels of endothelin-1 in reconstituted samples were quantified using the EIA kit for endothelin-1 (Peninsula laboratories, San Carlos, CA) according to the manufacturer's instructions. The results were normalized by protein concentrations and expressed as μg of endothelin-1 per mg protein.

To measure phosphorylation of extracellularly regulated kinase 1/2 (ERK1/2) in COCs, preovulatory follicles were treated with or without 100 ng/ml endothelin-1 or 1 μg/ml hCG in Leibovitz's L-15 medium without fetal bovine serum (FBS) for 0.5, 1, 2, and 4 h. After culture, COCs were isolated, and proteins from 30 COCs were extracted in a buffer containing 150 mM NaCl, 20 mM Tris–HCl, 1 mM ethylene diamine tetraacetate, 1 mM ethylene glycol-bis (2-aminoethyl)-*N*, *N*,*N*′,*N*′-tetraacetic acid, 1% Triton-X 100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, and 1 μg/ml leupeptin, before centrifugation at 8000×*g* for 10 min at 4°C. The supernatant was removed and stored at −80 °C until use. Levels of phosphorylated and total ERK1/2 in the same sample were quantified using the PathScan Phospho-p44/42 MAPK Sandwich ELISA kit (Cell Signaling Technology, Beverly, MA) and ERK1/2 ELISA kit (Calbiochem, Cambridge, MA) according to the manufacturer's instructions, respectively. The levels of phosphorylated ERK1/2 were normalized to total ERK1/2 levels.

### *Evaluation of GVBD*

Preovulatory follicles were excised from mouse ovaries at 48 h after PMSG treatment and cultured to examine GVBD of oocytes (Kawamura et al., 2004; Tsafriri et al., 1996). Follicles (20–30 per vial) were treated with or without endothelin-1 (AnaSpec, San Jose, CA), Sarafotoxin S6c (Alexis Biochemicals, San Diego, CA) or 1 μg/ml hCG (American Pharmaceutical Partners, Inc., Schaumburg, IL) in Leibovitz's L-15 medium (Invitrogen) without FBS. Some follicles were also treated with 100 ng/ml endothelin-1 with or without BQ-123 (Alexis