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

Kazuhiro Kawamura [a,c,*], Yinghui Ye [a,d], Cheng Guang Liang [c], Nanami Kawamura [a,b], Maarten Sollewijn Gelpke [e], Rami Rauch [c], Toshinobu Tanaka [a], Aaron J.W. Hsueh [c]

[a] Department of Obstetrics and Gynecology, Akita University School of Medicine, Akita 010-8543, Japan
[b] Dermatology and Plastic Surgery, Akita University School of Medicine, Akita 010-8543, Japan
[c] Divison of Reproductive Biology, Department of Obstetrics and Gynecology, Stanford University School of Medicine, Stanford, CA 94305-5317, USA
[d] Department of Reproductive Endocrinology, Women's Hospital, Zhejiang University School of Medicine, Zhejiang 310-006, China
[e] Molecular Design and Informatics, Schering-Plough Corporation, Oss, P.O. Box 20, 5340 BH, The Netherlands

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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.

* Corresponding author. Hondo 1-1-1, Akita-shi, Akita 010-8543, Japan. Fax: +81 18 884 6447.
E-mail address: kawamura@yf7.so-net.ne.jp (K. Kawamura).

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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

Biochemicals), BQ-788 (Alexis Biochemicals), U0126 (Sigma, St. Louis, MO) or U0124 (Sigma), pertussis toxin (Sigma) or treated with hCG with or without BQ-123 or BQ-788. The vials were flushed at the start of the culture with O_2/N_2 (at a 1: 1 ratio), sealed, and cultured at 37 °C with gentle shaking for 8 h. After culture, COCs were isolated, and, after cumulus cell removal, oocytes were examined for the occurrence of GVBD under a Hoffman modulation contrast microscopy (Nikon Inc., Tokyo, Japan). To examine the role of cumulus cells in the endothelin-1-induced GVBD of oocytes, COCs were obtained by puncturing the largest follicles from preovulatory ovaries, whereas denuded oocytes were separated from cumulus cells by mechanical pipetting. These cells were cultured in minimum essential media (MEM, Invitrogen) without FBS in the presence of 4 mM hypoxanthine (Sigma) with or without 10 ng/ml endothelin-1 for 16 h at 37 °C in 5% CO_2/95% air. Some COCs were also treated with 10 ng/ml endothelin-1 or with 1 μg/ml recombinant follicle-stimulating hormone (FSH) (11,468 IU/mg, Organon) with or without BQ-123 or BQ-788. The dose of FSH was chosen based on previous studies (Park et al., 2004). After culture, the occurrence of GVBD was examined.

In vivo analyses of GVBD

To study the effect of endothelin-1 treatment on oocyte maturation in vivo, intrabursal injection was performed (Kawamura et al., 2004). B6D2F1 mice at 9 weeks of age at the diestrous stage were primed with PMSG (7 IU) and, at 48 h later, lightly anesthetized. One of the ovaries was exteriorized through a small lumbosacral incision. Endothelin-1 [1 or 5 μg per 10 μl of phosphate-buffered saline (PBS)] was injected through a 30-gauge needle threaded into the ovarian bursa via the adjoining fat pad under stereomicroscope (MZ16, Leica, Wetzlar, Germany). The contralateral ovaries were injected with PBS alone and served as negative controls. After injection, the ovary was returned to the abdominal cavity and skin was clipped. After 12 h of treatment, COCs were collected by puncturing the largest ovarian follicles to examine the occurrence of GVBD in oocytes. For positive control, hCG (10 IU) was administrated by i.p. injection and oocytes were retrieved from the oviducts.

Evaluation of first polar body extrusion

For evaluating the transition from metaphase I (MI) to metaphase II (MII) stage oocytes (Kawamura et al., 2005), COCs were obtained from B6D2F1 mouse ovaries at 48 h after PMSG treatment as described above. COCs were washed twice, transferred to MEM supplemented with Earle's salts, 10 μg/ml streptomycin sulfate, 75 μg/ml penicillin G without FBS, and cultured with or without endothelin-1 for 24 h at 37 °C in 5% CO_2/95% air. For positive controls, COCs were treated with 3 ng/ml brain-derived neurotrophic factor (BDNF) (Kawamura et al., 2005). The occurrence of first polar body extrusion in the oocyte was examined after removing cumulus cells by using a small-bore pipette under the Hoffman modulation contrast microscopy.

In vitro maturation, fertilization, and early embryonic development

We performed in vitro maturation of oocytes followed by in vitro fertilization as described (Kawamura et al., 2005). COCs from PMSG-primed B6D2F1 mice were obtained in M2 medium (Sigma) supplemented with 0.1% FBS before culturing in MEM supplemented with Earle's salts, 10 μg/ml streptomycin sulfate, 75 μg/ml penicillin G, and 0.1% fetal bovine serum with or without 10 ng/ml endothelin-1 or 3 ng/ml BDNF at 37 °C in 5% CO_2/95% air. After 16 h of treatment, cumulus cells were removed and the oocytes were examined and classified according to their developmental stages (GV, MI, or MII). MII oocytes were inseminated with sperm from B6D2F1 males and incubated for 6 h at 37 °C in 5% CO_2/95% air. After in vitro fertilization, fertilized oocytes were recovered, washed twice, and cultured in human tubal fluid medium (Chemicon, Temecula, CA). Twenty-two hours after insemination, two-cell-stage embryos were collected and cultured in 50 μl drops of KSOM medium for 5 more days up to the blastocyst stage. Embryonic development was monitored daily under the Hoffman modulation contrast microscopy, and the progression of fertilized eggs to preimplantation embryos was assessed.

Statistical analysis

Statistical analysis was carried out by using Mann–Whitney *U* test for paired comparison and the one-way analysis of variance followed

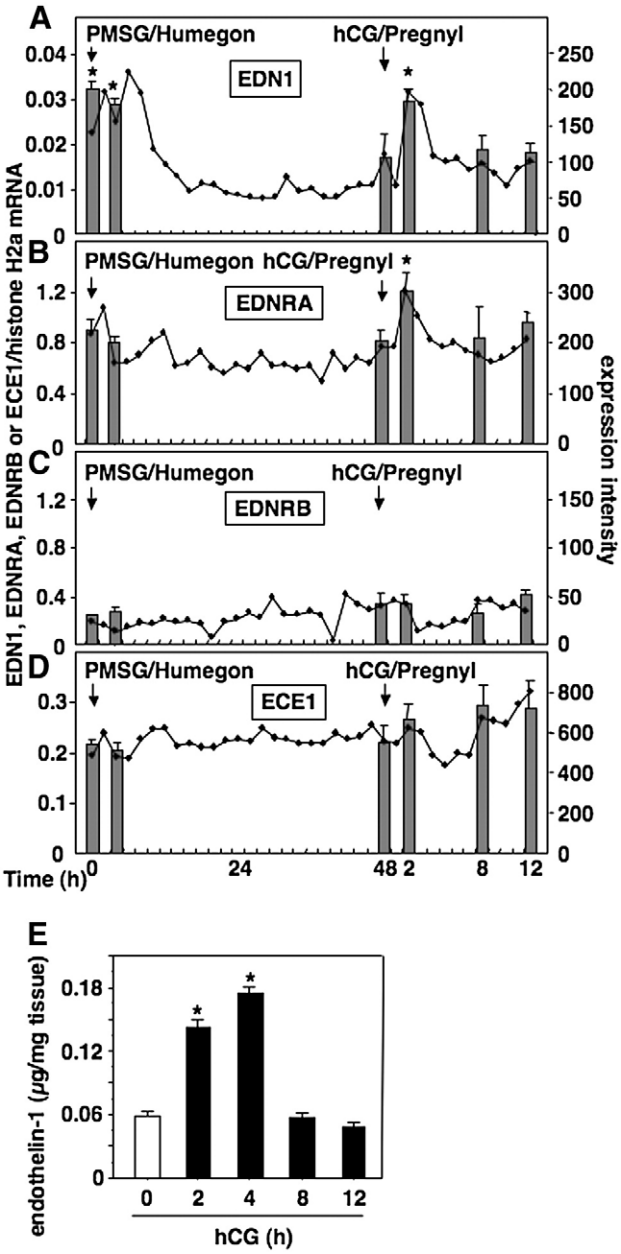

Fig. 1. Gonadotropin stimulation of endothelin-1 (EDN1) and EDNRA expression in mouse ovaries. Gonadotropin regulation of EDN1 (A), EDNRA (B), EDNRB (C), and ECE1 (D) transcripts in ovaries. Line graphs represent DNA microarray data depicting the expression intensity of each transcript (right *y* axis), whereas bar graphs depict quantitative real-time RT-PCR results (left *y* axis; mean±SEM, $n=4$). Values for expression intensity were derived from integration of hybridization signals from multiple probe sets for individual genes. *, $P<0.05$ vs. 48 h of PMSG treatment. (E) hCG stimulation of endothelin-1 peptide levels in ovaries of PMSG-primed mice. Ovarian content of endothelin-1 (μg/mg ovarian wet weight) was measured by EIA (mean±SEM, $n=4$). *, $P<0.05$ vs. 0 h of hCG treatment.

by Fisher's protected least significant difference for multiple group comparison. Results are presented as mean±SEM of at least three separate experiments.

Results

Gonadotropin stimulation of endothelin-1 and EDNRA expressions in preovulatory ovaries

We used DNA microarray analyses to identify ovarian paracrine ligands induced by the LH surge during the preovulatory period. Immature mice were treated with Humegon (containing FSH and LH activities) and Pregnyl (containing LH/hCG activity) to stimulate follicular maturation and ovulation, respectively. As shown in Fig. 1A (line graph), the expression of endothelin-1 mRNA was decreased following treatment with Humegon but treatment with Pregnyl increased endothelin-1 transcript levels, showing a peak at 2 h, followed by a decline. Furthermore, transcript levels for the endothelin-1 receptor, EDNRA were also increased after treatment with Pregnyl (Fig. 1B, line graph), whereas transcript levels for the other endothelin-1 receptor, EDNRB showed minor changes (Fig. 1C, line graph). Although ECE1, known to be essential for endothelin biosynthesis (Schmidt et al., 1994), was expressed throughout the experimental period, treatment with gonadotropins did not regulate its levels (Fig. 1D, line graph). To confirm DNA microarray data, we further performed real-time RT-PCR of ovarian transcripts for these genes in mice treated with PMSG followed by an ovulatory dose of hCG 48 h later (Figs. 1A–D, bar graphs). Similar stimulation of endothelin-1 and EDNRA transcript levels were observed. In addition to demonstrating preovulatory increases in both endothelin-1 and EDNRA transcripts in whole ovaries (Figs. 1A and B), the hCG stimulation of ovarian endothelin-1 peptide levels was detected using EIA (Fig. 1E). Treatment with hCG increased ovarian endothelin-1 antigen levels by >2-fold within 2 h after hormonal treatment, followed by a decline at 8 h.

Localization of endothelin-1 and EDNRA in the ovary and their stimulation by hCG in cumulus and granulosa cells

Using isolated ovarian cells, we examined cell types expressing endothelin-1 and EDNRA in the mouse ovary and the regulation of endothelin-1 and EDNRA transcript levels by hCG treatment based on real-time RT-PCR. As shown in Fig. 2A, the endothelin-1 mRNA was expressed in all cell types tested. In addition, treatment with hCG

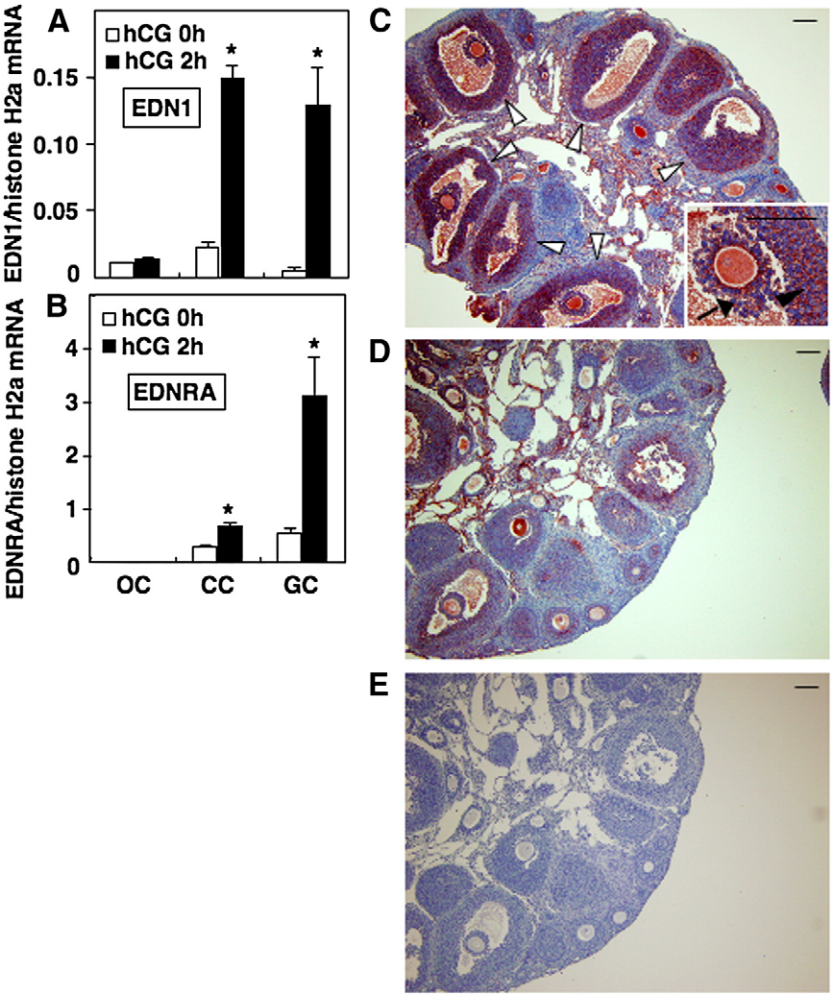

Fig. 2. Localization of EDN1 and EDNRA in mouse ovaries and their stimulation by hCG in cumulus and granulosa cells. Effects of hCG treatment on EDN1 (A) and EDNRA (B) expression in mouse oocytes (OC), cumulus cells (CC), and mural granulosa cells (GC). Transcript levels of EDN1 and EDNRA in isolated ovarian cells were quantified by real-time RT-PCR before and 2 h after hCG treatment (mean±SEM). Five samples prepared from individual animals were used. *, $P<0.05$ vs. 0 h of hCG treatment. (C–E) Immunohistochemical detection of EDNRA in ovaries of PMSG-primed mice 4 h after hCG injection (C, insert: high magnification) and ovaries of PMSG-primed mice before hCG treatment (D). EDNRA was found in cumulus (arrow) and mural granulosa (arrowhead) cells of preovulatory follicles (white arrowheads). EDNRA staining was increased by hCG treatment. (E) Sections with nonimmune IgG and serve as controls. (Scale bars, 100 μm.)

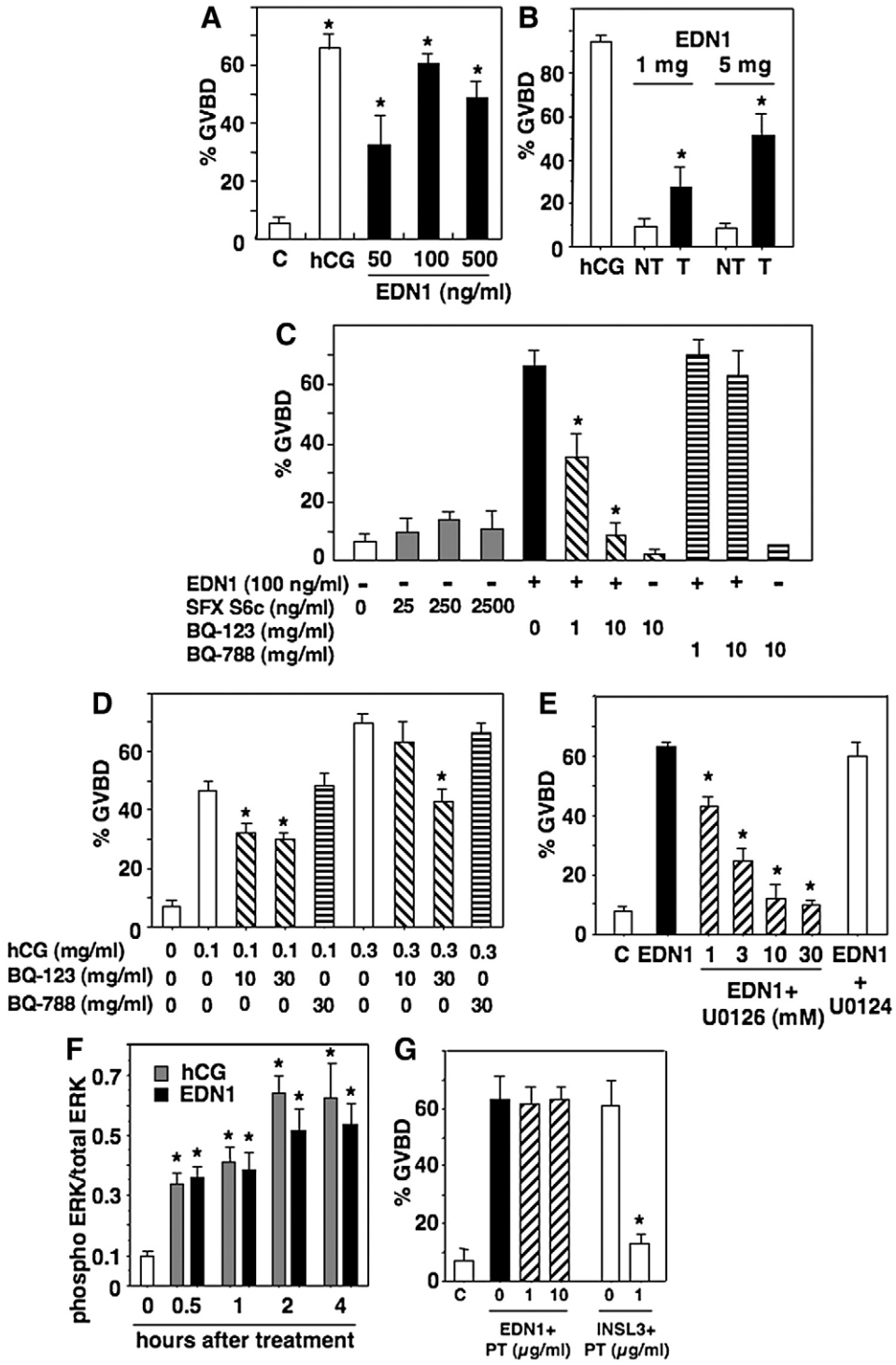

Fig. 3. EDN1 stimulation of meiosis resumption of preovulatory oocytes. (A) In vitro effects of EDN1 on GVBD of oocytes. Preovulatory follicles were cultured without (control, C) or with hCG (1 μg/ml) or different doses of EDN1 for 8 h before evaluation of oocytes undergoing GVBD (n=3, 38–53 follicles per group). *, P<0.05 vs. control. (B) In vivo induction of GVBD after EDN1 treatment. Mice at 2 days after PMSG priming were treated with EDN1 (1 or 5 μg per 10 μl of PBS) via intrabursal injections (treatment, T) or hCG (10 IU) via i.p. injections. After 12 h of treatment, oocytes were retrieved by puncturing of the ovaries to release COCs for assessing morphology (n=4 animals, 33–37 oocytes per group). The contralateral ovaries were injected with PBS alone and served as negative controls (non-treatment, NT). Some PMSG-primed animals were treated with hCG (10 IU) via i.p. injections and oocytes were retrieved from the oviducts. *, P<0.05 vs. non-treatment. (C) Effects of the selective EDNRB agonist, Sarafotoxin S6c (SFX S6c) on GVBD of oocytes and antagonistic effects of the EDNRA antagonist, BQ-123 on EDN1 stimulation of GVBD. Preovulatory follicles were cultured with or without different doses of SFX S6c, or with EDN1 (100 ng/ml) with or without BQ-123 or the EDNRB antagonist, BQ-788 (n≥3, 38–80 follicles per experimental group). *, P<0.05 vs. control or EDN1 alone groups. (D) Partial suppression of the hCG induction of GVBD in cultured preovulatory follicles by the EDNRA antagonist. Preovulatory follicles were cultured with hCG (0.1 or 0.3 μg/ml) with or without different concentrations of BQ-123 or BQ-788 (n≥4, 43–130 follicles per experimental group). *, P<0.05 vs. hCG alone groups. (E) Involvement of the MAPK/ERK pathway in the EDN1 stimulation of GVBD. Preovulatory follicles were cultured with EDN1 (100 ng/ml) with or without different doses of the MEK1/2 inhibitor, U0126 or its inactive analog, U0124 (30 μM). After culture, COCs were isolated and examined for the occurrence of GVBD (n=3, 42–60 follicles per group). *, P<0.05 vs. EDN1 alone groups. (F) Time course of EDN1 induction of ERK1/2 phosphorylation in COCs of preovulatory follicles. Preovulatory follicles were cultured with EDN1 (100 ng/ml) or hCG (1 μg/ml). After culture, COCs were isolated and ELISA was performed to measure levels of phosphorylated and total ERK1/2 (n=3, 30 COCs per group). Results are presented as the ratio of phosphorylated to total ERK1/2. *, P<0.05 vs. 0 h treatment groups. (G) Lack of involvement of the inhibitory G proteins in the EDN1 stimulation of GVBD. Preovulatory follicles were cultured without (controls, C) or with EDN1 (100 ng/ml) or INSL3 (100 nM) in the presence or absence of different doses of pertussis toxin (PT) capable of blocking Gi actions. After culture, COCs were isolated and examined for the occurrence of GVBD (n=3, 42–57 follicles per group). *, P<0.05 vs. INSL3.

stimulated endothelin-1 transcript levels in both cumulus (>6.5-fold) and mural granulosa cells (>22-fold), but not in oocytes (Fig. 2A). Although a clear stimulation of EDNRA transcripts was found in cumulus and mural granulosa cells, levels of this transcript were negligible in oocytes (Fig. 2B). The cell types expressing EDNRA protein in preovulatory ovaries were further determined by using immunohistochemistry. EDNRA staining was found in cumulus and mural granulosa cells of preovulatory follicles at 4 h after hCG treatment (Fig. 2C). Although weak signals were found in granulosa cells of preovulatory follicles in PMSG-primed ovaries before hCG treatment (Fig. 2D), their expressions were increased by hCG treatment (Fig. 2C). Although oocytes were also stained with the EDNRA antibody (Figs. 2C and D), the signals were still present after antigen blocking, suggesting non-specific EDNRA signals in oocytes (data not shown).

Endothelin-1 stimulation of meiosis resumption of preovulatory oocytes

Based on the hCG stimulation of endothelin-1 and EDNRA expression, we tested if endothelin-1 acts as a paracrine factor to regulate oocyte functions. In cultured preovulatory follicles, treatment with endothelin-1, like hCG, induced GVBD in oocytes in a dose-dependent manner (Fig. 3A). To test endothelin-1 actions in vivo, local intrabursal administration of endothelin-1 was performed in the diestrous mice primed with PMSG for 48 h. As shown in Fig. 3B, a dose-dependent induction of oocyte maturation was evident based on percentages of oocytes undergoing GVBD found in treated ovaries as compared with the contralateral untreated ovaries. In contrast, i.p. treatment with hCG induced GVBD in 95% of oocytes retrieved from the oviducts (Fig. 3B). Unlike hCG, treatment with endothelin-1 alone did not lead to follicle rupture (data not shown).

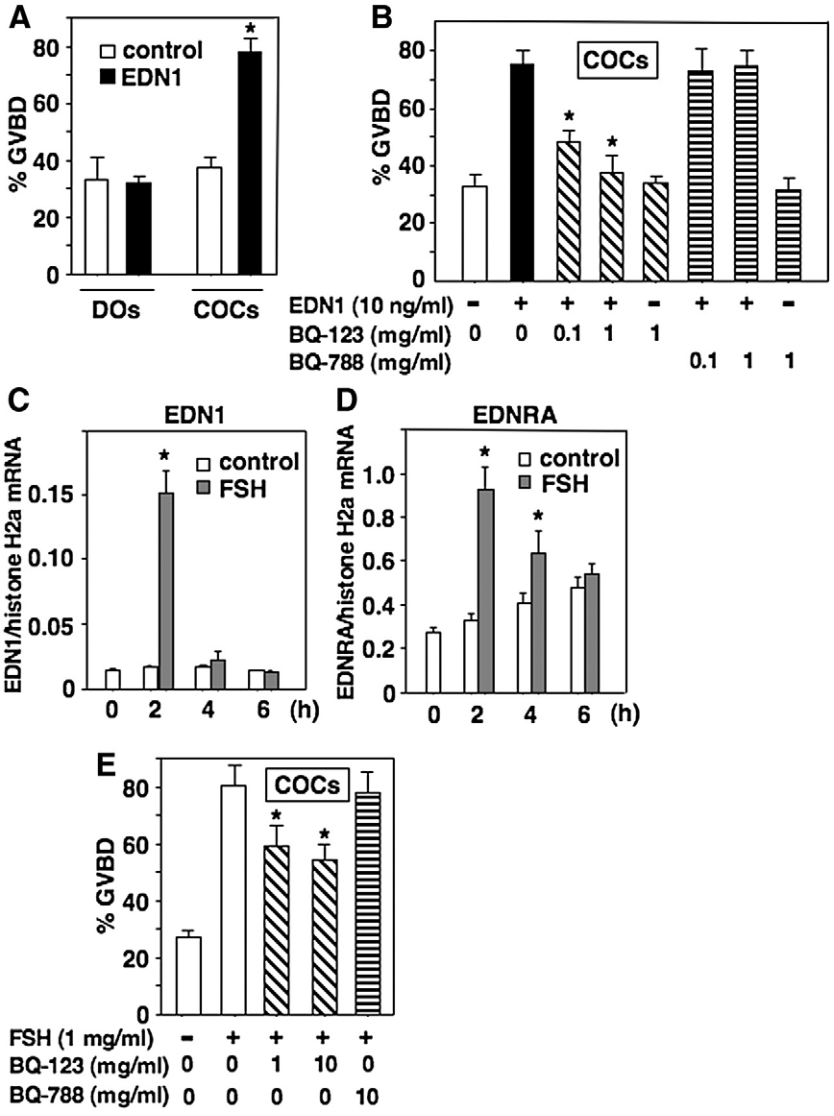

Fig. 4. EDN1 stimulation of GVBD by cultured COCs. (A) Effects of EDN1 treatment on GVBD by cultured denuded oocytes (DOs) and COCs. Denuded oocytes or COCs isolated from preovulatory follicles were cultured in the presence of hypoxanthine (4 mM) without (controls, C) or with EDN1 (10 ng/ml) for 16 h. After culture, the proportion of GVBD was determined ($n \geq 4$, 67–150 oocytes per group). *, $P<0.05$ vs. control. (B) Antagonistic effects of BQ-123 on the EDN1 stimulation of GVBD by cultured COCs. COCs were cultured with EDN1 (10 ng/ml) with or without BQ-123 or BQ-788 in the presence of hypoxanthine (4 mM) ($n \geq 4$, 45–101 follicles per experimental group). *, $P<0.05$ vs. EDN1 alone groups. (C and D) FSH stimulation of EDN1 and EDNRA mRNA levels by cumulus cells. COCs were cultured without (control) or with FSH (1 μg/ml) for 2, 4, and 6 h before quantification of EDN1 (C) and EDNRA (D) transcript levels by real-time RT-PCR (mean±SEM, $n=3$). Three samples in each time point prepared from 10 COCs were used. *, $P<0.05$ vs. control. (E) Partial suppression of the FSH induction of GVBD in cultured COCs by BQ-123. COCs were cultured with FSH (1 μg/ml) with or without BQ-123 or BQ-788 ($n \geq 4$, 42–69 oocytes per experimental group). *, $P<0.05$ vs. FSH alone groups.

Endothelin-1 binds both EDNRA and EDNRB (Arai et al., 1990; Sakurai et al., 1990). Although no EDNRA agonist is available, treatment of cultured preovulatory follicles with a selective EDNRB agonist, Sarafotoxin S6c (Williams et al., 1991) did not induce GVBD (Fig. 3C). Of interest, the stimulatory effect of endothelin-1 on GVBD was blocked by cotreatment with an EDNRA antagonist, BQ-123 (Douglas et al., 1994), but not an EDNRB antagonist, BQ-788 (Ishikawa et al., 1994) (Fig. 3C). In contrast, treatment with these antagonists alone was ineffective (Fig. 3C). These data suggest that the ability of endothelin-1 to induce GVBD is mediated through EDNRA. Unlike hCG, treatment with endothelin-1 did not induce cumulus cell expansion (data not shown). Using the EDNRA antagonist, we further examined whether the suppression of endothelin-1/EDNRA signaling inhibits hCG-induced GVBD in oocytes of cultured preovulatory follicles. As shown in Fig. 3D, cotreatment with the EDNRA antagonist, BQ-123 blocked by the hCG stimulation of GVBD in a dose-dependent manner. At 0.1 and 0.3 μg/ml of hCG, the antagonist at 30 ng/ml suppressed the hCG actions by 36 and 38%, respectively (Fig. 3D). We further analyzed the roles of the mitogen-activated protein kinase (MAPK)/ERK signaling pathway as downstream mediators of the endothelin-1/EDNRA-induced GVBD. The ability of endothelin-1 to stimulate GVBD was suppressed by cotreatment with a mitogen-activated protein kinase kinases 1/2 (MEK1/2) inhibitor, U0126, but not with its inactive analog, U0124 in a dose-dependent manner (Fig. 3E). Phosphorylation of ERK1/2 in COCs was induced by treatment with either endothelin-1 or hCG within 30 min, with peak levels found at 2 h after culture (Fig. 3F), consistent with previous studies showing ERK1/2 phosphorylation in cumulus cells after hCG stimulation (Su et al., 2002). Because EDNRA belongs to the seven-transmembrane G-coupled receptor superfamily, we examined possible involvement of the inhibitory G proteins to decrease cellular cAMP levels during endothelin-1 induction of GVBD in oocytes. Pretreatment with an inhibitor of inhibitory G protein, pertussis toxin, did not affect endothelin-1 actions but was effective in blocking the INSL3 stimulation of GVBD (Fig. 3G), consistent with an earlier report (Kawamura et al., 2004).

Due to the expression of EDNRA transcripts in cumulus cells, but not in oocytes, we assessed the importance of cumulus cells in mediating endothelin-1 actions using cultured COCs and denuded oocytes. Because oocytes obtained from preovulatory follicles underwent spontaneous GVBD, we cultured COCs and denuded oocytes in the presence of hypoxanthine to inhibit the spontaneous GVBD. In cumulus-enclosed oocytes, treatment with endothelin-1 overcame the inhibitory effect of hypoxanthine on oocyte GVBD; however, similar treatment was ineffective in denuded oocytes (Fig. 4A). In the COCs model, the stimulatory effect of endothelin-1 on GVBD was also blocked by cotreatment with the EDNRA antagonist, BQ-123 but not the EDNRB antagonist, BQ-788 (Fig. 4B). Because COCs express few or no LH receptors in mice (Wang and Greenwald, 1993), COCs were treated with FSH to induce GVBD. Treatment with FSH increased transcript levels of both endothelin-1 and EDNRA in COCs (Figs. 4C and D), although the levels of endothelin-1 and EDNRA in the ovary at 2 h after PMSG treatment were unchanged (Figs. 1A–D). Because the present in vitro FSH treatment was performed using COCs obtained from preovulatory follicles at 48 h after PMSG priming whereas ovaries used in the DNA microarray were obtained from ovaries of immature mice with no preovulatory follicles, these two findings cannot be directly compared. Similar to the partial suppression of hCG-induced GVBD of oocytes in cultured preovulatory follicles by the EDNRA antagonist, BQ-123, cotreatment with BQ-123 blocked by 32% the FSH stimulation of GVBD in COCs (Fig. 4E).

Lack of effects of endothelin-1 on the extrusion of first polar body and cytoplasmic maturation of preovulatory oocytes

We further tested the ability of endothelin-1 to promote further development of oocytes. Because oocytes showed high spontaneous maturation rates (>80%) when COCs were cultured in serum-containing media (data not shown), we used a low concentration of serum (0.1% FBS) to minimize effects of uncharacterized serum factors on oocyte maturation (Kawamura et al., 2005, 2008). Treatment of COCs with endothelin-1, unlike BDNF, did not affect the transition of oocytes from MI to MII stage of development (Fig. 5A). The role of endothelin-1 in conditioning the oocytes for subsequent fertilization and progression to blastocysts was also evaluated in vitro (Fig. 5B). COCs obtained from mice primed for 48 h with PMSG were cultured with or without endothelin-1. To avoid hardening of the zona pellucida that is unfavorable for in vitro fertilization, 0.1% FBS was included for all cultures. These MII oocytes were then fertilized in vitro without further treatment with endothelin-1. As shown in Fig. 5B, pretreatment with endothelin-1, unlike BDNF, did not increase the proportions of MII oocytes that developed into the two-cell or blastocyst-stage embryos.

Discussion

The present data suggested that endothelin-1 is a novel paracrine factor secreted by ovarian somatic cells of preovulatory follicles to

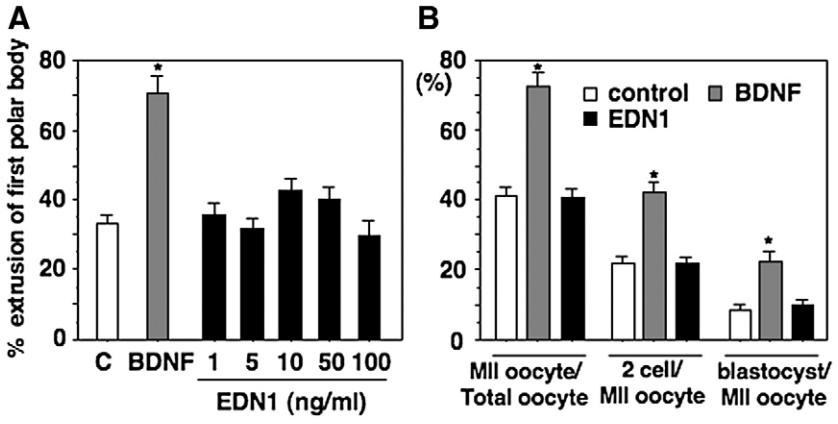

Fig. 5. Lack of effects of EDN1 on the extrusion of first polar body and cytoplasmic maturation of preovulatory oocytes. (A) Effects of EDN1 treatment on first polar body extrusion by cultured COCs. COCs isolated from preovulatory follicles were cultured without (controls, C) or with EDN1 or BDNF (3 ng/ml) for 24 h. After culture, the percentage of oocytes showing first polar body extrusion was determined ($n \geq 3$, 47–150 oocytes per group). (B) Effect of EDN1 in the conditioning of oocytes for the development into preimplantation embryos. COCs obtained from preovulatory follicles were cultured without (control) or with EDN1 (10 ng/ml) or BDNF (3 ng/ml) for 16 h. After progression into the MII stage, oocytes were inseminated and cultured for 5 more days without hormones. The percentage of MII oocytes developing into 2-cell-stage or blastocyst-stage embryos were evaluated ($n \geq 3$, 102–168 oocytes per group).

regulate oocyte maturation. Because endothelin-1 could only induce GVBD in cultured COCs but not denuded oocytes, endothelin-1 likely acts on cumulus cells to regulate the communication between cumulus cells and oocytes. Since only INSL3 (Kawamura et al., 2004) and EGF-like ligands (Park et al., 2004) have been found as ovarian paracrine factors induced by the preovulatory LH surge and capable of promoting GVBD, the present findings of a potent action of endothelin-1 in the nuclear maturation of the oocyte provide another intraovarian mechanism underlying meiotic resumption.

Endothelin-1 belongs to a structurally homologous peptide family that includes endothelin-2 and endothelin-3. Endothelin peptides bind two G protein-coupled receptors, EDNRA and EDNRB. The EDNRA receptor has a high specificity for endothelin-1 (endothelin-1>endothelin-2>endothelin-3), whereas EDNRB binds all three ligands with similar affinity (Arai et al., 1990; Sakurai et al., 1990). The present use of specific antagonists and agonists for these receptors indicated the essential role of EDNRA in mediating endothelin-1 actions. The importance of the endothelin-1/EDNRA signaling system in meiosis resumption could not be investigated in endothelin-1 or EDNRA null mice, because these animals die shortly after birth (Clouthier et al., 1998; Kurihara et al., 1994). It is interesting to note that increases in endothelin-1 transcripts and proteins were evident within 2 h after hCG treatment, before the induction of GVBD that was usually found at 4–5 h after hCG treatment in the present model. In contrast, a major increase in endothelin-2 levels was found only at 12 h after hCG treatment in the preovulatory rat ovary and endothelin-2 has been found as a paracrine factor important for follicle rupture by disrupting somatic cell organization which takes place at 12–14 h after hCG administration (Ko et al., 2006). Because endothelin-1 induces meiotic resumption through the EDNRA and endothelin-2 regulates follicle rupture by acting through EDNRB (Palanisamy et al., 2006), it is apparent that the two endothelin peptides regulate different ovulation-related processes in a receptor-, time- and cell type-specific manner.

Our data demonstrates that endothelin-1, like EGF-like ligands (Park et al., 2004), acts through cumulus cells to regulate oocyte maturation, whereas INSL3 directly suppresses intra-oocyte cAMP levels (Kawamura et al., 2004). The cellular mechanisms underlying cumulus-oocyte communication during meiotic resumption are only partially known. It is possible that, after the LH surge, an inhibitory influence of the follicular environment is decreased. This could occur as a consequence of the breakdown in gap junction communication between the cumulus cells and oocytes (Gilula et al., 1978), leading to the interruption of the transfer of inhibitory molecules (such as cAMP) to the oocyte. But a role for positive stimuli has also been suggested (Kawamura et al., 2004; Park et al., 2004). Following gonadotropin stimulation, overriding signals, such as endothelin-1 or EGF-like ligands, could be induced in cumulus or granulosa cells to promote the nuclear maturation of oocytes. It is becoming clear that a combination of both inhibitory and stimulatory factors plays redundant roles to insure the prolonged meiotic arrest before ovulation and the successful oocyte maturation during the ovulatory process induced by the LH surge.

It is believed that intra-oocyte cAMP is critical in the control of meiosis progression, with high levels of cAMP inhibiting the resumption of meiosis (Eppig and Downs, 1984). Intra-oocyte cAMP may be produced either endogenously (Horner et al., 2003) or transferred to the oocyte via gap junctions by surrounding somatic cells (Dekel et al., 1981). Also, purines present in the follicular fluid, such as hypoxanthine, are believed to contribute to the meiotic block by causing accumulation of cAMP within the oocyte through the repression of cAMP phosphodiesterase activity (Downs et al., 1985, 1989). Although the preovulatory LH surge has been proposed to disrupt the transfer of cAMP from cumulus cells to oocytes (Dekel et al., 1981), intra-oocyte cAMP levels in the present COC model are maintained in the presence of hypoxanthine. We found that treatment with endothelin-1, similar to FSH (Eppig and Downs, 1987), overcame the inhibitory effect of hypoxanthine on oocyte GVBD in cultured COC. However, unlike INSL3 (Kawamura et al., 2004), endothelin-1 stimulation of oocyte GVBD is not mediated by the inhibitory G proteins. Because our data showed that endothelin-1 acts on cumulus cells to indirectly regulate oocyte GVBD, these results suggest that the stimulatory effect of endothelin-1 on oocyte GVBD is not mediated by G proteins in cumulus cells. Accumulating evidences indicate that activation of MAPK in cumulus cells, but not oocytes, is important for gonadotropin-induced meiotic resumption in mammals (Liang et al., 2007). We also found the involvement of the MAPK pathway in the endothelin-1 mediation of oocyte maturation. Gonadotropin regulation of oocyte GVBD has been attributed to the breakdown of gap junctions between cumulus cells and oocyte (Dekel et al., 1981), increases in oocyte phosphodiesterase activity by hypoxanthine and/or the secondary actions of uncharacterized inductive factors released by cumulus cells. Future studies are required to determine the exact mechanisms underlying endothelin-1 induced GVBD.

The important roles of ovarian paracrine factors in the regulation of oocyte functions are becoming clear. Genome-wide analyses based on our DNA microarray datasets indicated that a limited number of ligands are induced by the preovulatory LH surge to promote oocyte maturation. In addition to endothelin-1, EGF-like ligands (Park et al., 2004) and INSL3 (Kawamura et al., 2004) were found to mediate the actions of LH in controlling GVBD of the oocyte. Although no study is available regarding the ability of EGF ligands to directly induce GVBD in vivo, intrabursal treatment with endothelin-1 and INSL3 (Kawamura et al., 2004) induced premature oocyte maturation without the LH surge. Elucidation of the potentially overlaying mechanisms underlying these ovarian paracrine signaling systems could provide better strategies for in vitro maturation of oocytes and allow the formulation of new contraceptive strategies.

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References

Arai, H., Hori, S., Aramori, I., Ohkubo, H., Nakanishi, S., 1990. Cloning and expression of a cDNA encoding an endothelin receptor. Nature 348, 730–732.
Boiti, C., Guelfi, G., Brecchia, G., Dall, Aglio, C., Ceccarelli, P., Maranesi, M., Mariottini, C., Zampini, D., Gobbetti, A., Zerani, M., 2005. Role of the endothelin-1 system in the luteolytic process of pseudopregnant rabbits. Endocrinology 146, 1293–1300.
Clouthier, D.E., Hosoda, K., Richardson, J.A., Williams, S.C., Yanagisawa, H., Kuwaki, T., Kumada, M., Hammer, R.E., Yanagisawa, M., 1998. Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. Development 125, 813–824.
Dekel, N., Lawrence, T.S., Gilula, N.B., Beers, W.H., 1981. Modulation of cell-to-cell communication in the cumulus–oocyte complex and the regulation of oocyte maturation by LH. Dev. Biol. 86, 356–362.
Douglas, S.A., Meek, T.D., Ohlstein, E.H., 1994. Novel receptor antagonists welcome a new era in endothelin biology. Trends Pharmacol. Sci. 15, 313–316.
Downs, S.M., Coleman, D.L., Ward-Bailey, P.F., Eppig, J.J., 1985. Hypoxanthine is the principal inhibitor of murine oocyte maturation in a low molecular weight fraction of porcine follicular fluid. Proc. Natl. Acad. Sci. U. S. A. 82, 454–458.
Downs, S.M., Daniel, S.A., Bornslaeger, E.A., Hoppe, P.C., Eppig, J.J., 1989. Maintenance of meiotic arrest in mouse oocytes by purines: modulation of cAMP levels and cAMP phosphodiesterase activity. Gamete Res. 23, 323–334.
Eppig, J.J., Downs, S.M., 1984. Chemical signals that regulate mammalian oocyte maturation. Biol. Reprod. 30, 1–11.
Eppig, J.J., Downs, S.M., 1987. The effect of hypoxanthine on mouse oocyte growth and development in vitro: maintenance of meiotic arrest and gonadotropin-induced oocyte maturation. Dev. Biol. 119, 313–321.
Gilula, N.B., Epstein, M.L., Beers, W.H., 1978. Cell-to-cell communication and ovulation. A study of the cumulus–oocyte complex. J. Cell Biol. 78, 58–75.
Horner, K., Livera, G., Hinckley, M., Trinh, K., Storm, D., Conti, M., 2003. Rodent oocytes express an active adenylyl cyclase required for meiotic arrest. Dev. Biol. 258, 385–396.

Ishikawa, K., Ihara, M., Noguchi, K., Mase, T., Mino, N., Saeki, T., Fukuroda, T., Fukami, T., Ozaki, S., Nagase, T., et al., 1994. Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. Proc. Natl. Acad. Sci. U. S. A. 91, 4892–4896.

Kawamura, K., Kawamura, N., Fukuda, J., Kumagai, J., Hsueh, A.J., Tanaka, T., 2007. Regulation of preimplantation embryo development by brain-derived neurotrophic factor. Dev. Biol. 311, 147–158.

Kawamura, K., Kawamura, N., Mulders, S.M., Sollewijn Gelpke, M.D., Hsueh, A.J., 2005. Ovarian brain-derived neurotrophic factor (BDNF) promotes the development of oocytes into preimplantation embryos. Proc. Natl. Acad. Sci. U. S. A. 102, 9206–9211.

Kawamura, K., Kumagai, J., Sudo, S., Chun, S.Y., Pisarska, M., Morita, H., Toppari, J., Fu, P., Wade, J.D., Bathgate, R.A., Hsueh, A.J., 2004. Paracrine regulation of mammalian oocyte maturation and male germ cell survival. Proc. Natl. Acad. Sci. U. S. A. 101, 7323–7328.

Kawamura, K., Ye, Y., Kawamura, N., Jing, L., Groenen, P., Gelpke, M.S., Rauch, R., Hsueh, A.J., Tanaka, T., 2008. Completion of Meiosis I of preovulatory oocytes and facilitation of preimplantation embryo development by glial cell line-derived neurotrophic factor. Dev. Biol. 315, 189–202.

Ko, C., Gieske, M.C., Al-Alem, L., Hahn, Y., Su, W., Gong, M.C., Iglarz, M., Koo, Y., 2006. Endothelin-2 in ovarian follicle rupture. Endocrinology 147, 1770–1779.

Kon, V., Badr, K.F., 1991. Biological actions and pathophysiologic significance of endothelin in the kidney. Kidney Int. 40, 1–12.

Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W.H., Kamada, N., et al., 1994. Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. Nature 368, 703–710.

Levin, E.R., 1995. Endothelins. N. Engl. J. Med. 333, 356–363.

Levin, E.R., 1996. Editorial: Endothelin-1, prostaglandin F 2 alpha, and the corpus luteum—the crisis of lysis. Endocrinology 137, 5189–5190.

Liang, C.G., Su, Y.Q., Fan, H.Y., Schatten, H., Sun, Q.Y., 2007. Mechanisms regulating oocyte meiotic resumption: roles of mitogen-activated protein kinase. Mol. Endocrinol. 21, 2037–2055.

Meidan, R., Levy, N., 2002. Endothelin-1 receptors and biosynthesis in the corpus luteum: molecular and physiological implications. Domest. Anim. Endocrinol. 23, 287–298.

Noll, G., Wenzel, R.R., Luscher, T.F., 1996. Endothelin and endothelin antagonists: potential role in cardiovascular and renal disease. Mol. Cell. Biochem. 157, 259–267.

Otani, H., Yamoto, M., Fujinaga, H., Nakano, R., 1996. Presence and localization of endothelin receptor in the rat ovary and its regulation by pituitary gonadotropins. Eur. J. Endocrinol. 135, 449–454.

Palanisamy, G.S., Cheon, Y.P., Kim, J., Kannan, A., Li, Q., Sato, M., Mantena, S.R., Sitruk-Ware, R.L., Bagchi, M.K., Bagchi, I.C., 2006. A novel pathway involving progesterone receptor, endothelin-2, and endothelin receptor B controls ovulation in mice. Mol. Endocrinol. 20, 2784–2795.

Park, J.Y., Su, Y.Q., Ariga, M., Law, E., Jin, S.L., Conti, M., 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 303, 682–684.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., Masaki, T., 1990. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature 348, 732–735.

Schmidt, M., Kroger, B., Jacob, E., Seulberger, H., Subkowski, T., Otter, R., Meyer, T., Schmalzing, G., Hillen, H., 1994. Molecular characterization of human and bovine endothelin converting enzyme (ECE-1). FEBS Lett. 356, 238–243.

Su, Y.Q., Wigglesworth, K., Pendola, F.L., O, Brien, M.J., Eppig, J.J., 2002. Mitogen-activated protein kinase activity in cumulus cells is essential for gonadotropin-induced oocyte meiotic resumption and cumulus expansion in the mouse. Endocrinology 143, 2221–2232.

Tsafriri, A., Chun, S.Y., Zhang, R., Hsueh, A.J., Conti, M., 1996. Oocyte maturation involves compartmentalization and opposing changes of cAMP levels in follicular somatic and germ cells: studies using selective phosphodiesterase inhibitors. Dev. Biol. 178, 393–402.

Wang, X.N., Greenwald, G.S., 1993. Hypophysectomy of the cyclic mouse. II. Effects of follicle-stimulating hormone (FSH) and luteinizing hormone on folliculogenesis, FSH and human chorionic gonadotropin receptors, and steroidogenesis. Biol. Reprod. 48, 595–605.

Williams Jr., D.L., Jones, K.L., Pettibone, D.J., Lis, E.V., Clineschmidt, B.V., 1991. Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. Biochem. Biophys. Res. Commun. 175, 556–661.