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Acute stimulation of a smooth muscle constrictor by oestradiol-17β via GPER1 in bovine oviducts

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Contents

Oviducts play roles in reproductive processes, including gametes transport, fertilization and early embryo development. Oviductal transport is controlled by various factors such as endothelins (EDNs) and nitric oxide (NO), smooth muscle contracting and relaxing factor, respectively. EDNs and NO production depend on an ovarian steroid hormone, oestradiol-17β (E2) and E2 quickly exerts their biological functions through G protein-coupled oestrogen receptor 1 (GPER1), which mediates rapid intracellular signalling. Because follicular fluid which contains a high concentration of E2 enters the oviduct, we hypothesized that E2 in the follicular fluid participates via GPER1 in producing EDNs and NO. To test this hypothesis, we investigated 1) the expression and localization of GPER1 in bovine oviductal tissues and 2) rapid effects of E2 via GPER1 on EDN1, EDN2 and inducible NO synthase (iNOS) expression in cultured bovine oviductal isthmic epithelial cells. GPER1 was observed in the oviductal epithelium, stroma and smooth muscle, and its expression was highest in the isthmus. Short-term treatments (≤1 hr) of E2 increased *EDN2* mRNA expression in the isthmic epithelial cells, although E2 did not affect *EDN1* and *iNOS* mRNA expressions. Results of GPER1-selective agonist G-1 and GPER1-selective antagonist G-15 treatments revealed acute stimulation by E2, which is mediated via GPER1. The overall findings suggested that E2 in follicular fluid rapidly stimulates EDN2 expression via GPER1 in the isthmic epithelial cells. Follicular fluid may play a role in retention of the ovulated oocyte in the end of ampulla by contracting the isthmus for successful fertilization.

1 | INTRODUCTION

In mammals, oviducts play crucial roles in reproductive processes, including gametes transport, fertilization and early embryo development (Greve & Callesen, 2001; Menezo & Guerin, 1997). Oocyte transport is regulated by oviductal smooth muscle motility and ciliary beating of the oviductal epithelium (Halbert, Tam, & Blandau, 1976). In the murine oviduct, the ampulla has high ciliary activity, whereas the isthmus has strong muscular contraction and weak ciliary activity (Noreikat, Wolff, Kummer, & Kölle, 2012). Because the isthmus has fewer ciliated cells and thick smooth muscle layers (Hunter, 2012; Menezo & Guerin, 1997), smooth muscle activity is more important for regulating oocyte transport. Contraction and relaxation of oviductal smooth muscle depend on various hormones and factors such as nitric oxide (NO) and endothelins (EDNs) (Priyadarsana, Wijayagunawardane, & Miyamoto, 2004; Rosselli, Imthurn, Macas, Keller, & Dubey, 1994).

EDN1 and EDN2 induce oviductal contraction (Al-Alem et al., 2007; Kobayashi, Yoshimoto, Yamamoto, Kimura, & Okuda, 2016; Priyadarsana et al., 2004), while NO induces oviductal relaxation (Rosselli et al., 1994; Yilmaz et al., 2012). Regulation of NO production is mediated by NO synthase (NOS) (Förstermann & Sessa, 2012). EDNs and NOS are transiently expressed during the oestrous cycle in the bovine oviduct. On the day of ovulation, mRNA expressions of *EDN1* and *EDN2* in the bovine oviduct are high (Yamamoto, Kohka, Kobayashi, Woclawek-Potocka, & Okuda, 2016), whereas mRNA expression of inducible NOS (*iNOS*) mRNA expression is low (Ulbrich et al., 2006). These studies indicate that the expressions of EDNs and NOS in the bovine oviduct are controlled by oestradiol-17β (E2), which is a major steroid hormone

produced by ovarian follicles. We previously reported that bovine follicular fluid contains a high concentration of E2 (approximately 250 nmol/L) (Kobayashi, Yamamoto, et al., 2016; Kobayashi, Yoshimoto, et al., 2016). At the time of ovulation, follicular fluid enters oviduct with an oocyte (Ezzati, Djahanbakhch, Arian, & Carr, 2014; Hansen, Srikandakumar, & Downey, 1991). However, it is unclear whether the E2 in follicular fluid has a role in regulating the production of EDNs and iNOS.

The effects of E2 are mediated by oestrogen receptors. Three isoforms of oestrogen receptors have been identified: oestrogen receptor α (ERα), ERβ and G protein-coupled oestrogen receptor 1 (GPER1) (Eyster, 2016). Although ERα and ERβ, which are nuclear receptors, induce slow transcriptional regulation (Nilsson et al., 2001), GPER1, which is a seven-transmembrane receptor, induces rapid intracellular signalling (Maiti et al., 2011; Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005). In the bovine oviduct, ERα and ERβ expression and localization has been observed (Ulbrich, Kettler, & Einspanier, 2003), but GPER1 expression and localization in the bovine oviduct is not known. GPER1 is thought to have a role in oocyte transport in the bovine oviduct, because intracellular signalling of E2 controls the movement of the oocyte through oviductal contraction and ciliary beating in cycling rats (Orihuela, Parada-Bustamante, Cortés, Gatica, & Croxatto, 2003).

Here, we hypothesized that E2 in the follicular fluid participates via GPER1 in producing EDNs and NO, that is, the factors that regulate contraction and relaxation of smooth muscle in the bovine oviduct. To test this hypothesis, we investigated (i) the expression and localization of GPER1 in the oviductal tissues and (ii) rapid effects of E2 via GPER1 on EDN1, EDN2 and iNOS expression in cultured bovine oviductal epithelial cells.

2 | MATERIALS AND METHODS

2.1 | Collection of bovine oviducts

Oviducts of Holstein cows were collected from a local abattoir (Okayama meat centre, Okayama, Japan). Approval to obtain specimens was granted by Okayama prefectural government office. The stages of the oestrous cycle were determined based on macroscopic observation of the ovary and uterus (Ireland, Murphee, & Coulson, 1980; Miyamoto, Skarzynski, & Okuda, 2000; Okuda, Kito, Sumi, & Sato, 1988). For collection of oviductal samples of days 0-3 after ovulation, the bovine oviduct which has follicles with a diameter of <1 cm were selected (Ireland et al., 1980). The infundibular, ampullary, ampullary–isthmic junctional and isthmic sections of oviducts ipsilateral to the corpus haemorragicum, which were collected on days 0–3 after ovulation, were utilized for mRNA and protein extraction and immunohistochemistry. For cell culture, isthmic sections of the oviduct were collected on days 0–3 or days 18–21 after ovulation.

2.2 | Isolation and culture of oviductal epithelial cells

The methods of isolation and culture of oviductal epithelial cells were described previously (Kobayashi, Wakamiya, Kohka, Yamamoto, & Okuda, 2013). Epithelial cells isolated separately from isthmic sections of the oviduct were seeded to 24-well plates and incubated until the cells reached 90%–95% confluence. The cells were then incubated with E2 (E8875, Sigma-Aldrich, St. Louis, MO, USA; 10 and 100 nmol/L) in phenol-red free DMEM/F-12 Ham (D2906, Sigma-Aldrich) supplemented with 500 μM ascorbic acid (013-12061, Wako Pure Chemical Industries, Osaka, Japan), 5 μg/ml holo-transferrin (T4132, Sigma-Aldrich), 5 ng/ml sodium selenite (S5261, Sigma-Aldrich), 2 μg/ml insulin (I4011, Sigma-Aldrich), 0.1% (w/v) bovine serum albumin (A7888, Sigma-Aldrich) and 20 mg/ml gentamicin (G1397, Sigma-Aldrich) for 0.5 and 1 hr at 38.5°C. To examine effects of GPER1 on the bovine oviductal isthmic epithelial cells, isthmic epithelial cells were incubated with the GPER1-selective agonist G-1 (10 or 100 nmol/L; 10008933, Cayman, Ann Arbor, MI, USA) for 0.5 and 1 hr, or with the GPER1-selective antagonist G-15 (20 nmol/L; 14673, Cayman) co-incubated with E2 (100 nmol/L) for 1 hr. G-15 was pre-incubated with the cells for 1 hr before incubation with E2. The doses of E2 was based on the E2 concentration in follicular fluid (Kobayashi, Yamamoto, et al., 2016; Kobayashi, Yoshimoto, et al., 2016), and that of G-1 and G-15 were based on the concentrations in previous reports (Bologa et al., 2006; Dennis et al., 2009).

2.3 | Total RNA extraction and cDNA synthesis

Total RNA was extracted from oviductal tissues and oviduct epithelial cells using TRIzol (15596-018, Life Technologies, Grand Island, NY, USA) according to the manufacturer's directions. Using iScript RT Supermix for RT-qPCR (170-8841, Bio-Rad Laboratories, Hercules, CA, USA), 1 μg of each total RNA was reverse transcribed.

2.4 | Quantitative PCR (Real-time PCR)

mRNA expression quantification was determined by quantitative RT-PCR using MyiQ (170-9740, Bio-Rad Laboratories) and SsoAdvanced SYBR Green Supermix (1725261B10, Bio-Rad Laboratories) starting with 4 ng of cDNA as described previously (Sakumoto, Komatsu, Kasuya, Saito, & Okuda, 2006). Protocol conditions consisted of denaturation at 95°C for 30 s, followed by 45 cycles at 95°C for 6 s, 60°C for 6 s and 72°C for 6 s with a final dissociation (melting) curve analysis. To standardize the relative mRNA expression level of each gene, three potential housekeeping genes, β-actin (ACTB), 18S rRNA (RNA18S1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were initially tested. NormFinder plug-in for Microsoft Excel (MOMA, Aarhus, Denmark) was used to select appropriate housekeeping genes based on a variance estimation approach (Andersen, Jensen, & Ørntoft, 2004). The GAPDH mRNA expression was most stable among the three genes; therefore, GAPDH was selected as the internal control in our experiments. To analyse the relative mRNA expression level of each gene, the $2^{-\Delta\Delta CT}$ method was used (Livak & Schmittgen, 2001). All the primer sequences are listed in Table 1.

TABLE 1 Primer sequences for quantitative PCR

Gene	Primer Sequence	Accession number	Product size
18SrRNA	F: 5′-TCGCGGAAGGATTTAAAGTG-3′ R: 5′-AAACGGCTACCACATCCAAG-3′	AY779625	141 bp
ACTB	F: 5′-CAGCAAGCAGGAGTACGATG-3′ R: 5′-AGCCATGCCAATCTCATCTC-3′	AY141970	137 bp
EDN1	F: 5′-TCTGGACATCATCTGGGT-3′ R: 5′-TTTGGTTGTTCCAGGCTTTC-3′	NM_181010.2	227 bp
EDN2	F: 5′-AGCGGCTGAGGGTTATTTCT-3′ R: 5′-ATGCCCCTCTCCTCCTGTAT-3′	NM_175714.2	165 bp
GAPDH	F: 5′-CACCCTCAAGATTGTCAGCA-3′ R: 5′-GGTCATAAGTCCCTCCACGA-3′	NM_001034034.2	103 bp
iNOS	F: 5′-TACCCTCAGTTCTGCGCTTT-3′ R: 5′-GGGATCTCAATGTGGTGCTT-3′	AF340236	107 bp

2.5 | Western blotting

Oviductal tissues were homogenized and sonicated in homogenizing buffer (300 mmol/L sucrose, 25 mmol/L Tris-HCl, 2 mmol/L EDTA2Na, pH 7.4) with a proteinase inhibitor (11697498001, Roche Diagnostics, Basel, Switzerland). The samples (30 μg protein/lane) were separated on SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membrane was washed in TBS-T (0.1% [v/v] of Tween 20 in TBS [25 mmol/L Tris-HCl, 137 mmol/L NaCl, pH 7.5]), incubated in blocking buffer (5% [w/v] non-fat dry milk in TBS-T) for 1 hr at room temperature, incubated with GPER1 antibody diluted at 1:2000 (SAB2700363, Sigma-Aldrich) or β-actin antibody diluted at 1:20,000 (A2228, Sigma–Aldrich) overnight at 4°C, washed with TBS-T, incubated with anti-rabbit-IgG, HRP-linked whole antibody produced by donkey (NA934, GE healthcare, Milwaukee, WI, USA) at 1:10,000 dilution or anti-mouse IgG HRP-linked whole antibody produced by sheep (NA931, GE healthcare) at 1:40,000 dilution and for 60 min at room temperature and washed with TBS-T. The signal was detected by chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate (P36599, Merck Millipore, Darmstadt, Germany). The expression of β-actin protein was used as an internal control.

2.6 | Immunohistochemistry

Sections (6 μm) were deparaffinized, rehydrated in a graded series of ethanol, washed in tap water, microwaved in Tris–EDTA buffer

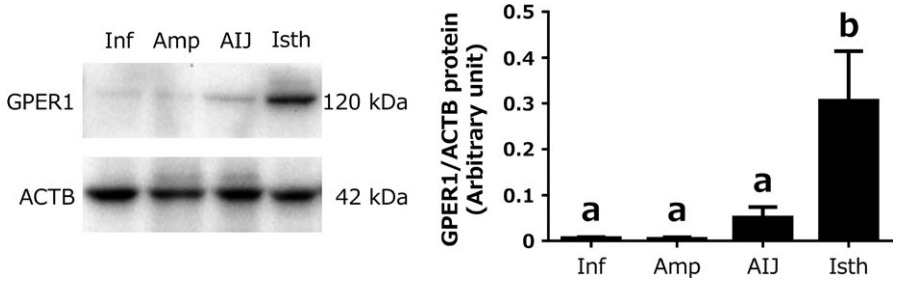

FIGURE 1 GPER1 expression in each region of the bovine oviducts on days 0–3 after ovulation. Inf; infundibulum, Amp; ampulla, AIJ; ampullary–isthmic junction, Isth; isthmus (mean ± *SEM*, *n* = 4). Representative bands and quantification of GPER1 in bovine oviducts are shown. ACTB served as an internal control. Different superscript letters indicate significant differences between regions of the oviducts, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p < .05$)

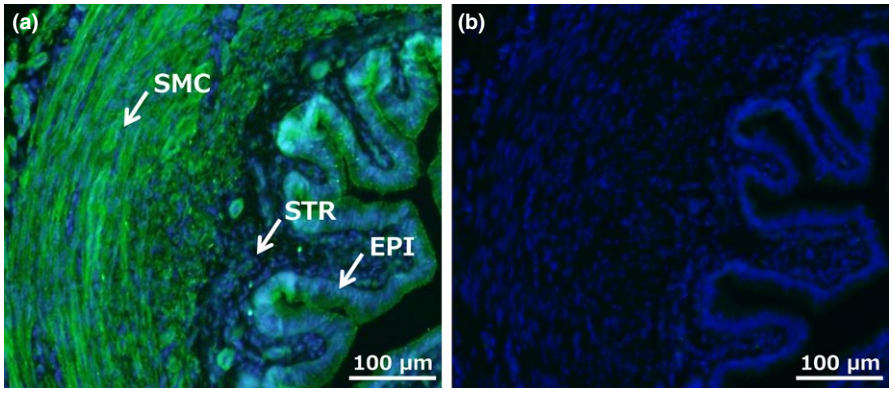

FIGURE 2 Immunohistochemistry staining micrographs of (a) GPER1 and (b) the negative control in the isthmic region of bovine oviducts (*n* = 3). Serial sections were utilized to analyse expression. EPI, epithelial layer; STR, stromal layer; SMC, smooth muscle layer. Scale bar = 100 μm

for 15 min at 600 W to retrieve antigens, incubated with 10% (v/v) normal horse serum for blocking, incubated with GPER1 antibody at 1:100 dilution with PBS, washed with PBS three times, incubated with secondary antibody for rabbit-IgG conjugated with Alexa 488 (21206; Life Technologies) for 1 hr at room temperature, washed with PBS three times, covered with ProLong Gold Antifade Reagent with DAPI (36935; Life Technologies) and observed under a fluorescence microscope.

2.7 | Statistical analysis

All experimental data are shown as the mean ± *SEM*. Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The normal distribution was tested by the Kolmogorov–Smirnov method. Statistical significant differences were assessed by analysis of variance (ANOVA) followed by Tukey's test or Dunnet's test for multiple comparisons. *p* values less than .05 were considered statistically significant.

3 | RESULTS

3.1 | Expression and localization of GPER1 in the bovine oviducts

GPER1 expression was observed in each region of the oviductal tissues obtained on days 0–3 after ovulation. The expression was higher in the isthmus than the other parts of the oviducts (Figure 1: $p < .05$). Immunoreactive GPER1 was predominantly observed in epithelial and smooth muscle tissues, but was slightly observed in the stromal cells of the oviduct (Figure 2).

3.2 | Effect of E2 on EDN1, EDN2 and iNOS mRNA expression in cultured isthmus epithelial cells of bovine oviducts

EDN2 mRNA expression in isthmic epithelial cells was stimulated by 10 nmol/L E2 incubation for 1 hr and 100 nmol/L E2 incubation for 0.5 and 1 hr (Figure 3c,d: $p < .05$). The expression was increased in

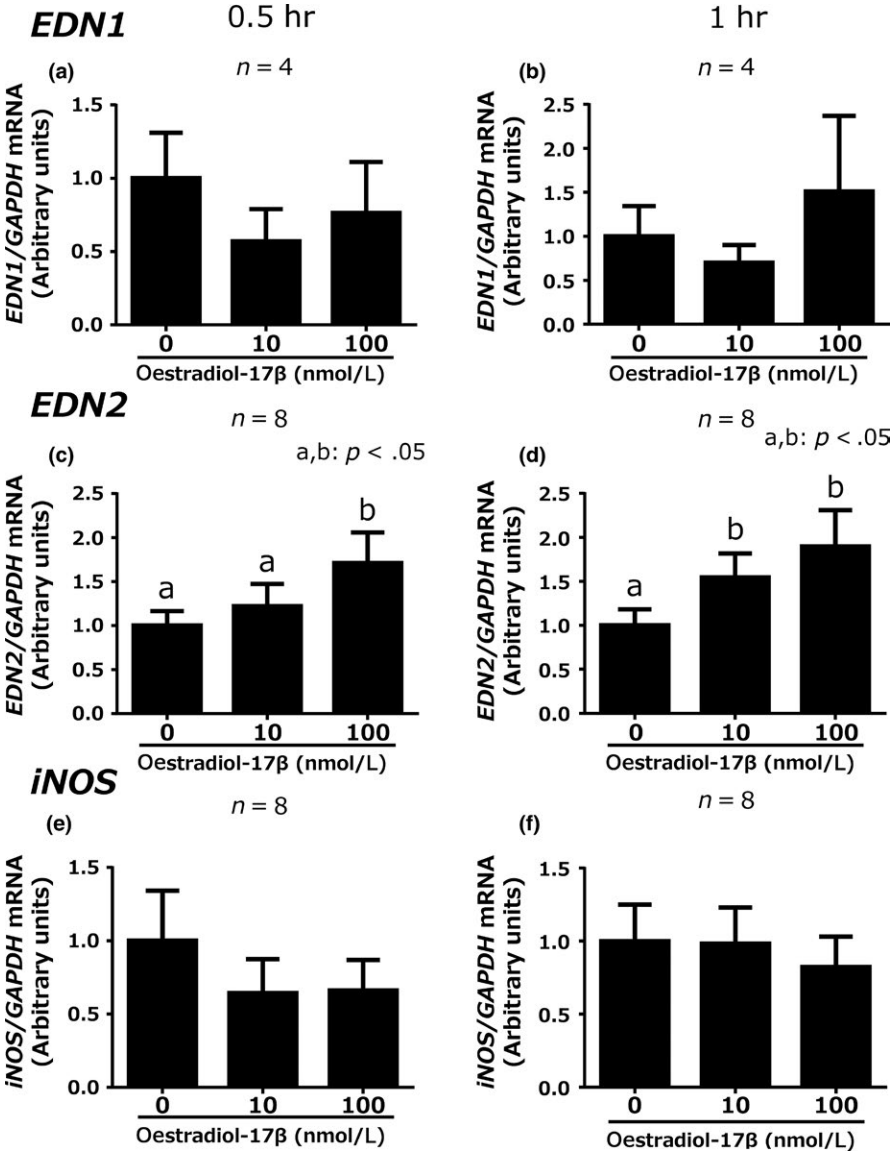

FIGURE 3 Effect of E2 on mRNA expression of (a and b) *EDN1*, (c and d) *EDN2* and (e and f) *iNOS* in cultured isthmus epithelial cells obtained from the isthmus of bovine oviducts (mean ± *SEM*). The cells were incubated for 0.5 (a, c and e) or 1 (b, d and f) hr with or without E2 (10 and 100 nmol/L). Different superscripts indicate significant differences between groups, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p < .05$)

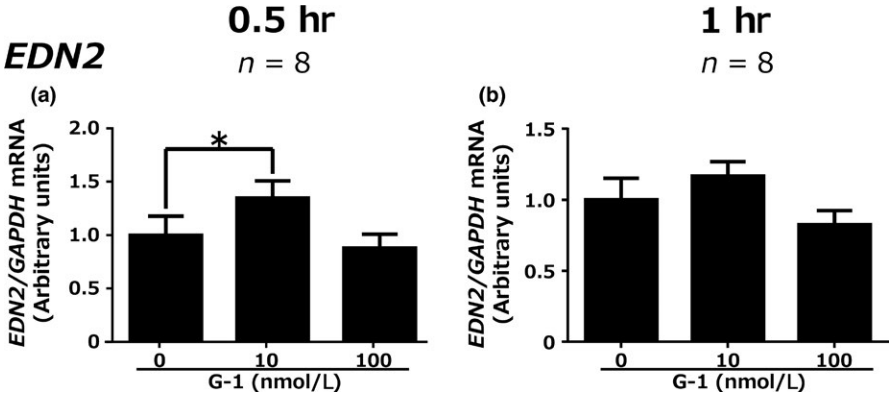

FIGURE 4 Effect of GPER1-selective agonist (G-1; 10, 100 nmol/L) on *EDN2* mRNA expression in cultured isthmic epithelial cells obtained from the isthmus of bovine oviducts (mean ± *SEM*, *n* = 8). The cells were incubated for 0.5 (a) or 1 (b) hr. The asterisks indicate significant differences between the control group and treatment groups, as determined by one-way ANOVA followed by Dunnet's multiple comparison test ($p < .05$)

a dose-dependent manner of E2 (Figure 3c,d). Expression of EDN1 and iNOS mRNA was not affected by E2 in the isthmic epithelial cells (Figure 3a,b,e,f).

3.3 | Effects of GPER1-selective agonist and antagonist on EDN2 mRNA expression in cultured isthmic epithelial cells of bovine oviducts

To examine whether EDN2 mRNA expression stimulated by E2 is mediated through GPER1 in bovine oviductal isthmic epithelial cells, GPER1-selective agonist G-1 and GPER1-selective antagonist G-15 were used. EDN2 mRNA expression in isthmic epithelial cells was increased by 0.5 hr incubation with 10 nmol/L G-1 ($p < .05$), but not by 1 hr incubation with G-1 (Figure 4). EDN2 mRNA expression in the cells was increased by 100 nmol/L of E2, and the expression stimulated by E2 was significantly inhibited by 20 nmol/L treatment of G-15 (Figure 5: $p < .05$).

4 | DISCUSSION

Our present study showed that GPER1 is expressed on the oviductal epithelium (Figure 2), and E2 rapidly stimulates *EDN2* mRNA expression mediated by GPER1 in the isthmus (Figures 3–5). It was previously determined that EDN2 promotes oviductal contraction in humans and rats (Al-Alem et al., 2007; Jankovic, Jankovic, Lukic, Canovic, & Folic, 2010). Based on our findings, a high concentration of E2 contained in follicular fluid seems to contract the isthmus by promoting EDN2 secretion mediated by GPER1 in the isthmic epithelial cells. EDNs bind to two types of receptor, type A (EDNRA) and type B (EDNRB). EDN1 and EDN2 bind to both EDNRA and EDNRB, while EDN3 only binds to EDNRB (Bridges, Cho, & Ko, 2011). EDNRA (but not EDNRB) is expressed in bovine oviductal smooth muscle cells (Kobayashi, Yamamoto, et al., 2016; Kobayashi, Yoshimoto, et al., 2016). In various tissues, EDNRA is involved in contraction of smooth muscle (Al-Alem et al., 2007; Rosselli et al., 1994; Sakamoto et al., 1999). This finding, together with the stimulatory effect of E2 on EDN2 expression in oviductal isthmic epithelial cells (Figure 3), suggests that EDN2, which is increased by E2 in isthmic epithelial cells, binds to EDNRA on smooth muscle cells, which induces contraction of the oviductal isthmus.

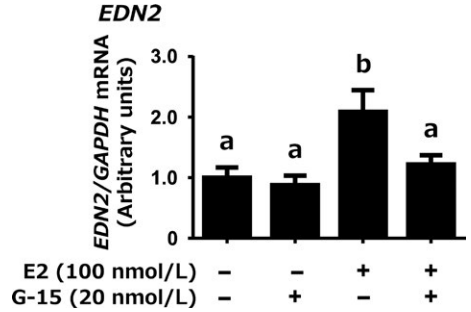

FIGURE 5 Effect of GPER1-selective antagonist (G-15, 20 nmol/L) incubated with or without oestradiol-17β (100 nmol/L) on *EDN2* mRNA expression in cultured epithelial cells obtained from the isthmus of bovine oviducts (mean ± *SEM*, *n* = 12). The cells were pre-incubated with 20 nmol/L G-15 for 1 hr followed by incubation with 20 nmol/L G-15 and 100 nmol/L E2 for 1 hr. Different superscript letters indicate significant differences between groups, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p < .05$)

Different regions of the oviduct have different structures and functions. In the murine oviduct, the ampulla has high ciliary activity, whereas the isthmus has strong muscular contraction and weak ciliary activity in the murine oviduct (Noreikat et al., 2012). In the rabbit oviduct, the ampulla transports ovulated oocytes at a speed of about 7.4 mm/min in the rabbit oviduct (Boling & Blandau, 1971; Bourdage & Halbert, 1988). Subsequently, the oocyte is retained in the end of the ampulla by contraction of the isthmus (Menezo & Guerin, 1997). As oocyte transport in the ampulla is rapid, acute contraction of the isthmus is essential for retention of the oocyte at the fertilization site. In this study, *EDN2* mRNA expression was upregulated by 0.5 and 1 hr after treatment of E2 in the isthmus (Figure 2), but not in the ampulla (unpublished data). This rapid and local regulation of EDN2 expression by GPER1 would be attributed to the acute contraction of the isthmus. As follicular fluid including a high concentration of E2 enters with an oocyte (Ezzati et al., 2014; Kobayashi, Yamamoto, et al., 2016; Kobayashi, Yoshimoto, et al., 2016), follicular fluid may contribute to retention of the oocyte within the ampulla.

Because *EDN1* mRNA expression is highest and *iNOS* mRNA expression is lowest on the day of ovulation in the bovine oviductal isthmus (Ulbrich et al., 2006; Yamamoto et al., 2016), expression of EDN1

and iNOS is likely under the control of E2. However, short-term (0.5 and 1 hr) E2 treatment did not affect *EDN1* and *iNOS* mRNA expression in isthmic epithelial cells (Figure 3). Thus, expression of EDN1 and iNOS seems not to be mediated by GPER1 in the isthmus. In bovine oviductal isthmic epithelial cells, long-term (24 hr) E2 treatment increases *EDN1* mRNA expression (Yamamoto et al., 2016). Moreover, E2 suppresses *iNOS* mRNA expression via ERα in bovine oviductal isthmic epithelial cells (Kobayashi, Yamamoto, et al., 2016; Kobayashi, Yoshimoto, et al., 2016). Together, it is considered that stimulation of EDN1 expression and suppression of iNOS expression in the isthmus are mediated by nuclear oestrogen receptors.

Expression patterns of EDNs in the oviduct through the oestrous cycle are differed among species. In bovine oviduct, expression of *EDN1* and *EDN2* mRNA is highest on ovulation day (Yamamoto et al., 2016). Wijayagunawardane et al. (1999) reported that EDN1 protein is increased by 24 and 48 hr treatment of E2 in bovine. Moreover, we showed that E2 stimulates *EDN2* mRNA expression in the isthmic epithelial cells (Figure 2). In mouse, *EDN2* mRNA was changed through the oestrous cycle, but *EDN1* mRNA was not changed (Jeoung et al., 2010). In rat, EDN1 and EDN2 were also not changed through the oestrous cycle (Al-Alem et al., 2007). Accordingly, the pattern of EDNs expression during oestrous cycle differs depending on species, that is, regulatory mechanisms of EDNs production by sex steroids may be species specific.

In the present study, GPER1 was localized not only on epithelial cells, but also on smooth muscle cells in the bovine oviduct (Figure 2). The GPER1-selective agonist G-1 was found to stimulate smooth muscle contraction in rat myometrium (Tica et al., 2011), whereas G-1 induces relaxation in human vascular smooth muscle (Holm et al., 2013). Those studies revealed that the roles of GPER1 in smooth muscle cells depend on the cell type. Consequently, GPER1 in oviductal smooth muscle may directly mediate contraction or relaxation. Further studies on the direct effects of E2 on smooth muscle via GPER1 are needed.

The overall findings suggest that the high concentration of E2 in follicular fluid quickly stimulates EDN2 secretion via GPER1 in the bovine oviductal isthmic epithelium. E2 in follicular fluid may play a role in retention of the ovulated oocyte by contracting the isthmus for successful fertilization.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

TN and YK designed the study. TN, YK and KK analysed the data. TN, KK and KO drafted the manuscript. TN, KK and KO reviewed and edited the manuscript.

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