

Endothelin as a local regulating factor in the bovine oviduct

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Running head: Endothelin system in the bovine oviduct

Abstract

Endothelin (EDN) is a possible regulating factor of oviductal motility, which is important for the transport of gametes and embryo. To clarify the factors that control the secretion of EDN in the bovine oviduct, the expressions of EDNs, EDN-converting enzymes (ECEs) and EDN receptors (EDNRs) were investigated.

All isoforms of EDN (EDN1-3), ECE (ECE1 and ECE2) and EDNR (EDNRA and EDNRB) were immunolocalized in the epithelial cells of the ampulla and the isthmus. EDNRs were also immunolocalized in the smooth muscle cells. The mRNA expressions of *EDN2* and *ECE2* were higher in the cultured ampullary oviductal epithelial cells than in the isthmus cells. The expressions of *EDN1*, *EDN2* and *ECE2* in the ampullary tissue were highest on the day of ovulation. Estradiol-17 β increased *EDN2* and *ECE1* expressions, while progesterone increased only *ECE1* expression in the cultured ampullary epithelial cells. These results indicate that EDNs are produced by the epithelial cells and their target site is the smooth muscle and the epithelial cells, and suggest that ovarian steroids are regulators of endothelin synthesis in ampullary oviductal epithelial cells.

Introduction

Endothelin (EDN) participates in various biological processes in the reproductive system including luteolysis (Acosta *et al.* 2007), contraction of myometrium (Kozuka *et al.* 1989) and rupture of the follicle (Bridges *et al.* 2011) in several mammalian species, although it was originally found as a vasoactive peptide (Yanagisawa *et al.* 1988). *EDN1*, *EDN2* and *EDN3* encode prepro-EDN1, prepro-EDN2 and prepro-EDN3, respectively (Inoue *et al.* 1989). These prepro-EDNs and “big-EDNs” which are converted from prepro-EDNs are biologically inactive. EDN-converting enzyme (ECE) 1 and ECE2 are required for converting big-EDNs to active EDNs (Turner and Murphy 1996). EDN receptor (EDNR) has two isoforms, EDNRA and EDNRB (Arai *et al.* 1990). The binding affinity of EDNRA to EDN1 and EDN2 is higher than it is to EDN3, while EDNRB has the equal affinities for the three EDNs (Sakurai *et al.* 1990).

For the fertilization to occur and lead to the successful pregnancy establishment, oviductal smooth muscle motility is important for the transport of gametes and embryo. Several factors including prostaglandins (Siemieniuch *et al.* 2009), nitric oxide (Rosselli *et al.* 1994; Szostek *et al.* 2011) and luteinizing hormone (LH) (Wijayagunawardane *et al.* 1999a; Wijayagunawardane *et al.* 1999b; Ziecik . 2007) have been reported to affect oviductal contraction and relaxation. EDN also induces the contraction of oviductal smooth muscle in several species (Priyadarsana *et al.* 2004; Al-Alem *et al.* 2007; Jankovic *et al.* 2009). EDN1 contracts the oviductal smooth muscle in cow (Wijayagunawardane *et al.* 2001), and EDN2 induces the contraction of oviduct via EDNRA in rats (Al-Alem *et al.* 2007). In addition, *EDN1* mRNA is expressed in murine (Jeoung *et al.* 2010), human (Sakamoto *et al.* 2001) and bovine oviducts (Priyadarsana *et al.* 2004). These previous reports imply that the oviduct contains a para- or autocrine EDN system for regulating oviductal motility.

To define the EDN system in the bovine oviduct, the production and the target sites of EDNs were evaluated in the present study. The effects of ovarian steroids on the expressions of *EDNs* and *ECEs* were also investigated *in vitro*.

Materials and Methods

Collection of bovine oviductal tissues

Oviducts from Holstein cows were collected at a local abattoir within 10-20 min after exsanguination.

The stages of the estrous cycle (ovulation day (OV); Days 2-3 after ovulation, early luteal stage (E); Days 5-6, developing luteal stage (D); Days 8-12, mid luteal stage (M); Days 15-17, late luteal stage (L); Days 19-21, follicular stage (F)) were determined based on macroscopic observations of the ovary and the uterus (Okuda *et al.* 1988; Miyamoto *et al.* 2000). Oviductal tissues ipsilateral to the corpus luteum or the dominant follicle were collected at Days 0-3 after ovulation were utilized for immunohistochemistry and cell culture, and oviductal tissues at each stage of the estrous cycle for total RNA extraction.

Immunohistochemistry

The ampullary and isthmic oviductal tissues were fixed with 4% paraformaldehyde phosphate buffer solution and embedded in paraffin. The paraffin-embedded tissues were sectioned (6 μ m) and placed on silane coated slide glasses (S3003, Dako A/S, Glostrup, Denmark). The serial sections were prepared and used for the immunohistochemistry.

After deparaffinization with xylene, the tissue sections were incubated with 0.3% H₂O₂ in methanol at room temperature for 30 min to quench nonspecific staining. After washing with phosphate buffer saline (PBS), the sections were subjected to antigen retrieval by heating in 0.01 M sodium citrate buffer (pH 6.0) using microwave at 600 W for 15 min. The sections were then incubated in 10% normal horse serum (MP-7500-50, Vector laboratories, Burlingame, CA, U.S.A.) at room temperature for 30 min. Then they were incubated at 4°C over night with rabbit anti-Endothelin-1 (T4050, Peninsula Laboratories, LLC., San Carlos, CA, U.S.A.), anti-EDN2 rabbit polyclonal (HPA028459, ATLAS Antibodies, Stockholm, Sweden), anti-endothelin-3 rabbit polyclonal (H-023-17, Phoenix Pharmaceuticals, Mannheim, Germany), anti-ECE1 rabbit polyclonal (HPA013616, ATLAS Antibodies), anti-ECE2 rabbit polyclonal (HPA043346, ATLAS Antibodies), anti-endothelin receptor A rabbit polyclonal (NBP1-33614, Novus Biologicals, LLC., Littleton, CO, U.S.A.) and anti-endothelin receptor B rabbit polyclonal (NBP1-31108, Novus Biologicals) at a dilution of in PBS. After incubation with the antibody, sections were treated with ImmPRESS UNIVERSAL Reagent Anti-Mouse/ Rabbit IgG (MP-7500-50, Vector laboratories) at room temperature for 30 min. The reaction products were visualized by treating with 0.05% (w/v) 3,3'-diaminobenzidine tetrachloride (343-00901, Dojindo, Kumamoto, Japan) containing 0.01% H₂O₂. Sections were counterstained with haematoxylin for 30 sec to better visualize the cell types. Specificity of the antibodies

was examined using normal rabbit IgG instead of first antibody.

Total RNA extraction and real-time RT-PCR

Total RNA was extracted from oviductal tissues and cells using TRIsure (BIO-38032, Bioline, London, UK) according to the manufacturer's directions. One μg of each total RNA was reverse transcribed using iScript RT Supermix for RT-qPCR (170-8841, Bio-Rad Laboratories, Hercules, CA, U.S.A.). Quantifications of mRNA expressions were determined by Quantitative RT-PCR using MyiQ (Bio-Rad). The reaction mixture contained 10 μl SoAdvanced SYBR Green Supermix (172-5261B05, Bio-Rad), 1 μM each forward and reverse primer and 2 ng of reverse-transcribed total RNA as described previously (Sakumoto *et al.* 2006). Nuclease-free water was added up to a total volume of 20 μl . Each complementary DNA (cDNA) was analyzed in duplicate. All primers were designed to amplify a product as shown in Table 1, and the specificity of each primer set was confirmed by running the PCR products on a 2.0% agarose gel stained with ethidium bromide. Protocol conditions were consisted of denaturation at 95 °C for 3 min, followed by 45 cycles at 94 °C for 15 sec, 60 °C for 20 sec and 72 °C for 15 sec with a final dissociation (melting) curve analysis. The most stable transcribed housekeeping gene was identified with Normfinder software (download at <http://moma.dk/normfinder-software>). This software calculates the gene expression stability measure (M) and determines the most stable housekeeping gene via a stepwise exclusion or ranking process resulting in the selection of the most stable housekeeping genes for the specific tissue. The M values of *GAPDH*, *β -actin* and *18S rRNA* in the bovine oviduct were 0.017, 0.026 and 0.069, respectively. Thus, *GAPDH* was used as a housekeeping gene for all experiments. Mean \pm standard errors of mean (SEM) of CT values were 27.1 ± 0.21 (*EDN1*), 31.4 ± 0.12 (*EDN2*), 30.6 ± 0.10 (*EDN3*), 28.1 ± 0.12 (*ECE1*) and 30.6 ± 0.10 (*ECE2*) in the tissues (n=30), and 28.0 ± 0.37 (*EDN1*), 27.6 ± 0.81 (*EDN2*), 28.4 ± 0.58 (*EDN3*), 28.1 ± 0.22 (*ECE1*) and 32.8 ± 0.34 (*ECE2*) in the cultured epithelial cells (n=12). To analyze the relative level of expression of each mRNA, the $2^{-\Delta\Delta\text{CT}}$ method was used (Livak and Schmittgen 2001).

Isolation and culture of oviductal epithelial cells

Cells were isolated from 6 oviducts in the present study. Epithelial cells were isolated separately from

the ampullary and isthmic sections of the oviduct by perfusion with 20 ml Hank's balanced salt solution (HBSS) as described previously (Kobayashi *et al.*, 2013). HBSS contained 0.25% (wt/vol) bovine trypsin (>7500 BAEE units/mg solid; T9201, Sigma-Aldrich, St. Louis, MO, USA), 0.02% (wt/vol) EDTA2Na (Sigma-Aldrich), 0.1% (wt/vol) BSA (10735-086001, Roche, Mannheim, Germany), 100 IU/ml penicillin (611400, Meiji Seika Pharma, Tokyo, Japan) and 100 μ g/ml streptomycin (6161400, Meiji Seika Pharma). HBSS was delivered at flow rate of 10 ml/min, 38 C, for 30 min. The dissociated epithelial cells were filtered through metal meshes (150 μ m and 77 μ m) to remove undissociated tissue fragments. The filtrates were washed by centrifugation (180 \times g for 10 min at 4 C) with Tris-buffered ammonium chloride (pH 7.5) to remove hemocytes, followed by washing with Dulbecco's Modified Eagle's Medium (DMEM; D1152, Sigma-Aldrich) supplemented with 0.1% (wt/vol) BSA, 100 IU/ml penicillin and 100 μ g/ml streptomycin. After the washing, the final pellets were resuspended by DF (DMEM/Ham's F-12; 1:1 (vol/vol) (12400-024, Life Technologies, Carlsbad, CA, USA) supplemented with 10% (vol/vol) bovine calf serum (16170-078, Life Technologies), 20 mg/ml gentamicin (G1397, Life Technologies) and 2 mg/ml amphotericin B (A9528, Sigma-Aldrich)). The cells were seeded at a density of 1.0×10^5 viable cells/ml to 25-cm² culture flasks (690160, Greiner Bio-One, Frickenhausen, Germany) and cultured at 38.5 C in a humidified atmosphere of 5% CO₂ in air. The stromal and epithelial cell culture media were changed every 48 h for 5-10 days until the cells reached 80-90% confluence. The epithelial cells were trypsinized using 0.02% porcine trypsin and 0.02% bovine trypsin for purification as described previously (Kobayashi *et al.*, 2013). The purified epithelial cells were seeded to 24-well plates (662160, Greiner) at a density of 1.0×10^5 viable cells/ml and incubated at 38.5 C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every 48 h until the cells reached 90-95% confluence.

Experimental design

Experiment 1: Immunolocalization of EDNs, ECEs and EDNRs in the bovine oviduct

The localization of each factor was detected by immunohistochemistry using the ampullary and the isthmic segment of oviduct collected at Day 0-3 after ovulation to define production and target sites of EDNs.

Experiment 2: EDN and ECE mRNA expressions in the epithelial cells of ampulla and isthmus

Since the epithelial cells immediately after isolation were not pure, we used cultured epithelial cells to analyze mRNA expressions of *EDNs* and *ECEs* in the epithelial cells. The cultured ampullary and isthmic oviductal epithelial cells were isolated from 6 cows. After the cells reached 90-95% confluence (< 7 days after seeding), they were washed by PBS twice followed by culture in fresh DF (DMEM/Ham's F-12; 1:1 (vol/vol) (D2906-10, Sigma-Aldrich)) supplemented with 0.1% BSA (15408, Roche), 500 μ M ascorbic acid (013-12061, Wako Pure Chemical Industries), 5 μ g/ml holo-transferrin (T3400, Sigma-Aldrich), 5 μ g/ml sodium selenite (S5261, Sigma-Aldrich) and 2 μ g/ml insulin (I4011, Sigma-Aldrich) for 4 h incubation. Total RNA was extracted from the cells cultured in 3 wells per oviduct and the expressions of *EDNs* and *ECEs* were measured by real-time RT-PCR.

Experiment 3: EDN mRNA expressions in oviductal tissues

Oviductal tissues were collected from 5 cows at each stage of estrous cycle. The expressions of *EDNs* and *ECEs* were measured by real-time RT-PCR.

Experiment 4: Effects of estradiol-17 β (E2) and progesterone (P4) on EDN and ECE mRNA expressions in ampullary oviductal epithelial cell

Since stage-specific differences between the expressions of *EDNs* and *ECEs* were not observed in the isthmic tissue in Experiment 3, only ampullary cells were investigated in Experiment 4. Cultured ampullary oviductal epithelial cells isolated from 6 cows were utilized. After the cells reached 90-95% confluence (< 7 days after seeding), the cells were treated with E2 (0.1, 1, 10 nM) and P4 (1, 10, 100 nM) in fresh DF as described in Experiment 2. Each treatment was performed in triplicate. After 4 h incubation, total RNA was extracted from the cells. The expressions of *EDNs*, *ECEs* and *EDNRs* were measured by real-time RT-PCR.

Statistical analyses

Outliers were detected by Grubbs' test and removed from each group in all experiments. The statistical significance of differences was assessed by t-test for two groups, and by one-way analysis of

variance followed by Tukey's multiple comparisons test for more than three groups. P values less than 0.05 were considered to be statistically significant.

Results

Experiment 1: Immunolocalization of EDNs, ECEs and EDNRs in the bovine oviduct

Distinct immunolocalization of EDN1, EDN2 and EDN3 were observed in the luminal epithelial layer as well as in the smooth muscle layer of both the ampullary and the isthmic sections (Figure 1C-H). On the other hand, immunoreactions of ECE1 and ECE2 were observed clearly in the epithelial layer, but faintly in the smooth muscle layer in the ampulla and isthmus (Figure 1I-L). Both EDNRA and EDNRB were localized in the smooth muscle layer as well as in the epithelium in both the ampulla and isthmus (Figure 2).

Experiment 2: EDN and ECE mRNA expressions in cultured epithelial cells of ampulla and isthmus

The mRNA expressions of all *EDN* and *ECE* isoforms were detected in cultured epithelial cells in both ampulla and isthmus of the bovine oviduct (Figure 3). The mRNA expressions of *EDN2* (F= 168.1, DF=10, P= 0.032; Figure 3B) and *ECE2* (F= 9.5, DF=11, P= 0.047; Figure 3B) were significantly higher in the ampullary cells than in the isthmic cells.

Experiment 3: EDN mRNA expressions in oviductal tissues

The mRNA expression levels of *EDNs* and *ECEs* varied among the estrous stages were shown in only the ampullary tissue but not in the isthmic tissue. *EDN1* expression was significantly higher at the day of ovulation than at and the follicular stage in the ampullary tissues (F= 2.7, DF= 21, P=0.037; Figure 4A). *EDN2* expressions at the day of ovulation were significantly higher than at the early stage (P=0.049), the luteal stage (P=0.022) and the follicular stage (P=0.008) (F=4.2, DF=24; Figure 4B). *ECE2* expressions were significantly higher at the day of ovulation than the follicular stage (F=3.2, DF=21, P=0.013; Figure 4E).

Experiment 4: Effects of estradiol-17 β (E2) and progesterone (P4) on EDN and ECE mRNA expressions in

ampullary oviductal epithelial cell

E2 increased both *EDNs* and *ECE* in the cultured ampullary epithelial cells. *ECE1* (F= 3.4, DF= 5, P= 0.03) and *EDN2* (F= 3.3, DF= 5, P= 0.045) expressions were significantly stimulated by 1 nM and 10 nM E2 treatment, respectively (Figure 5). E2 treatment (1 nM) tended to increase *EDN1* expression, although the increase was not significant (F= 3, DF= 5, P= 0.06; Figure 5). On the other hand, P4 increased only *ECE1* expression, which was significantly stimulated by 10 nM (F=3.6, DF= 5, P= 0.027; Figure 6) P4 treatment.

Discussion

The present study demonstrated the presence of all isoforms of EDN, ECE and EDNR in the bovine oviduct. This is the first report of EDN2 and EDN3 expressions in the bovine oviduct. EDNs and ECEs were localized in epithelial cells of both the ampulla and isthmus. Since ECEs convert big-EDNs to bioactive EDNs, the main production site of bioactive EDNs seems to be epithelial cell in the bovine oviduct. Despite the presence of immunoreactive EDNs, the immunoreactions of ECEs were faint in the smooth muscle layer. Therefore, the smooth muscle layer seems to produce less EDNs than the epithelial layer. Since both immunoreactive EDNs and EDNRs were observed, the bovine oviduct may possess a local EDN system in which EDNs produced by the epithelial cells act on oviductal smooth muscle via EDNR. EDNs produced by the endothelial cells are known to affect the vascular smooth muscle cells via EDNRs resulting in vasoconstriction (Kawanabe and Nauli 2011). In addition to its vasoactive effect, EDN1 stimulates the contraction of the bovine oviduct (Priyadarsana *et al.* 2004) and EDN2 also induces oviductal contraction in human and rat (Al-Alem *et al.* 2007; Jankovic *et al.* 2009). EDNRA activation induces the contraction of smooth muscle in several tissues (Al-Alem *et al.* 2007; Kawanabe and Nauli 2011), while EDNRB activation induces vasodilation by increasing endothelial NO production (Tsukahara *et al.* 1994; Tykocki *et al.* 2009). Therefore, EDNRA and EDNRB might have different roles in the bovine oviduct, although both seem to be involved in oviductal motility. Additionally, EDNRA has higher affinities for EDN1 and EDN2 than EDN3, whereas EDNRB has equal affinities for the three EDNs (Sakurai *et al.* 1990). The oviductal contraction caused by EDN1 (Priyadarsana *et al.* 2004) and EDN2 (Al-Alem *et al.* 2007; Jankovic *et al.* 2009) might be mainly due to EDNRA activation in the smooth

muscle layer. Although it remains uncertain which EDN and EDNR isoforms are dominant in the bovine oviduct, our results suggest the presence of a paracrine EDN system for controlling oviductal motility.

Our results indicate that the syntheses of EDNs and ECEs are regulated by ovarian steroids during the estrous cycle in the bovine oviduct. In cultured ampullary epithelial cells, E2 increased *EDN2* and *ECE1* expressions, while P4 stimulated only the expressions of *ECE1* in the present study. Ovarian steroids regulate the secretory functions of oviductal epithelial cells (Eberhardt *et al.* 1999; Wijayagunawardane *et al.* 1999a; Wijayagunawardane *et al.* 1999b). Co-treatment of cultured bovine oviductal epithelial cells with E2 and low dose P4 increased EDN1 production but treatment with P4 alone had no effect (Wijayagunawardane *et al.* 1999a). These findings suggest that P4 supports the effect of E2 on EDN production by stimulating the syntheses of ECEs. Co-treatment of cultured bovine oviductal epithelial cells with high E2 and low P4 level mimicked the hormonal condition at peri-ovulation. This finding, together with the finding that the mRNA expressions of *EDNRs* in the bovine oviduct are high after ovulation (Priyadarsana *et al.* 2004) suggest that EDN system is regulated to be most active around the ovulation. Interestingly, in contrast to our results, E2 treatment decreased *ECE1* expression in rat vessels (Rodrigo *et al.* 2003) and *ECE2* expression in rat brain (Jayaraman *et al.* 2012). The effects of E2 on *ECE* expressions seem to be specific to each organ. Since *ECEs* are key enzymes for producing bioactive EDNs, further studies are required to determine the roles of EDNs *in vivo*.

Because of the structural and the functional differences between the ampulla and the isthmus, each EDN might have segment-specific actions in the oviduct. The expressions of *EDN2* and *ECE2* in cultured epithelial cells were higher in the ampulla than in the isthmus in the present study. In addition, differences of *EDN* and *ECE* mRNA expressions among the estrous stages were observed in only the ampulla. The present results demonstrate that immunoreactive EDNRs localize in not only the smooth muscle but also the epithelial cells of the bovine oviduct. EDNR localizations in epithelial cells suggest that EDNs play some roles in epithelial cell function besides the regulation of smooth muscle motility. Although the structures of the three EDNs differ by only several amino acids, each EDN has unique roles in several organs (Ling *et al.* 2013). EDN1 in many kinds of cancer cells affect cellular proliferation and EDN2 is involved in immunity (Ling *et al.* 2013). EDN3 also has been suggested to be involved in fertilization and early embryonic development in the murine oviduct (Jeoung *et al.* 2010). Further studies are needed to

understand the roles of EDNs in the bovine oviduct.

The overall findings of this study indicate that the EDN is produced by oviductal epithelial cells and EDNRs localize in the smooth muscle layer, suggesting that the EDN system works to regulate oviductal motility for the transport of gametes and the embryo in the bovine oviduct. In addition, ovarian steroids may control EDN production through the endocrine system during the estrous cycle.

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Figure legends

Figure 1. Micrographs of immunohistochemical staining of endothelin (EDN) 1 (C, D), EDN2 (E, F), EDN3 (G, H), endothelin converting enzyme (ECE) 1 (I, J), ECE2 (K, L) and negative control (NC; A, B). Serial sections were utilized for the experiments. Left column: Ampullary part, Right column: Isthmic part. Open arrowhead: Smooth muscle layer, close arrowhead: Epithelial layer. Each scale bar indicates 100 μ m.

Figure 2. Micrographs of immunohistochemical staining of endothelin receptor (EDNR) A (C, D), EDNRB (E, F) and negative control (NC; A, B). Serial sections were utilized for the experiments. Left column: Ampullary part, Right column: Isthmic part. Open arrowhead: Smooth muscle layer, close arrowhead: Epithelial layer. Each scale bar indicates 100 μ m.

Figure 3. Relative expressions of endothelin (*EDN*) 1 (A), *EDN*2 (B), *EDN*3 (C), EDN converting enzyme (*ECE*) 1 (D) and *ECE*2 (E) mRNA in cultured epithelial cells collected from the ampullary and the isthmic oviduct (mean \pm SEM, n= 6 oviducts). The statistical significance of differences was assessed by t-test. Different superscript letters indicate significant difference (P<0.05).

Figure 4. Relative expressions of endothelin (*EDN*) 1 (A), *EDN*2 (B), *EDN*3 (C), EDN converting enzyme (*ECE*) 1 (D) and *ECE*2 (E) mRNA in bovine oviduct throughout the estrous cycle (ovulation day: OV, Days 2-3 after ovulation: E, Days 5-6: D, Days 8-12: M, Days 15-17: L, Days 19-21: F, mean \pm SEM, n= 5 oviducts per stage). Open bar: Ampullary part, closed bar: Isthmic part. The statistical significance of differences was assessed by one-way analysis of variance followed by Tukey's multiple comparison test. Different superscript letters indicate significant difference (P<0.05).

Figure 5. Effects of estradiol-17 β (E2; 0.1, 1, 10 nM) on mRNA expression of endothelin (*EDN*) 1 (A), *EDN*2 (B), *EDN*3 (C), EDN converting enzyme (*ECE*) 1 (D) and *ECE*2 (E) in the cultured epithelial cells of bovine ampullary oviduct. Results were shown as mean \pm SEM, n= 6 oviducts. Significant differences were determined by one-way analysis of variance followed by Tukey's multiple comparison test.

Figure 6. Effects of progesterone (P4; 1, 10, 100 nM) on mRNA expression of endothelin (*EDN*) 1 (A), *EDN*2 (B), *EDN*3 (C), EDN converting enzyme (*ECE*) 1 (D) and *ECE*2 (E) in the cultured epithelial cells of bovine ampullary oviduct. Results were shown as mean \pm SEM, n= 6 oviducts. Significant differences were determined by one-way analysis of variance followed by Tukey's multiple comparison test.

Table 1. Primer sequences for real-time PCR

Gene	Primer sequence	Accession number	Product size (bp)
<i>EDN1</i>	F: 5'-TCTGGACATCATCTGGGTCA-3' R: 5'-TTTGGTTGTTCCAGGCTTTC-3'	NM_181010.2	227
<i>EDN2</i>	F: 5'-AGCGGCTGAGGGTTATTTCT-3' R: 5'-ATGCCCTCTCCTCCTGTAT-3'	NM_175714.2	165
<i>EDN3</i>	F: 5'-GGGTGTTAGCCTTGACCAA-3' R: 5'-CCACCGAAAGGAGTTGATGT-3'	NM_001101979.1	170
<i>ECE1</i>	F: 5'-TGTGGCGGCTGGATCAAAGC-3' R: 5'-AGGTGCTTGATGATGGCTTG-3'	NM_181009.2	101
<i>ECE2</i>	F: 5'-GCTGCTGGAGACCCTCTATG-3' R: 5'-CTGATCATCCCCTCTGCAAT-3'	NM_177956.2	159
<i>GAPDH</i>	F: 5'-CACCCCTCAAGATTGTCAGCA-3' R: 5'-GGTCATAAGTCCCTCCACGA-3'	BC102589	103

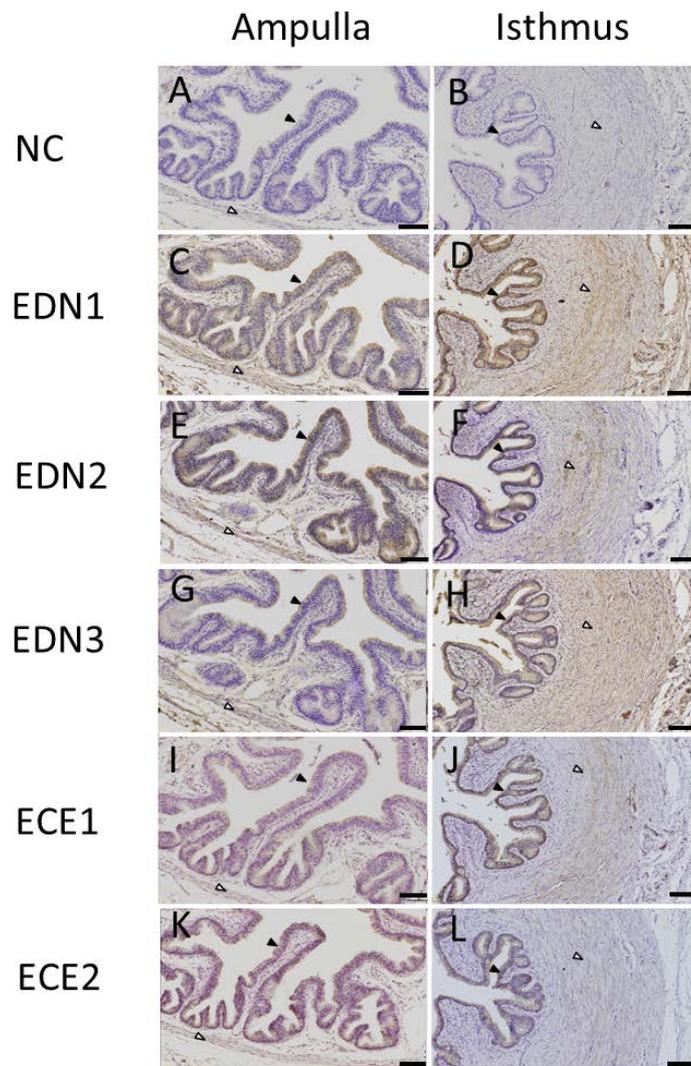


Figure 1

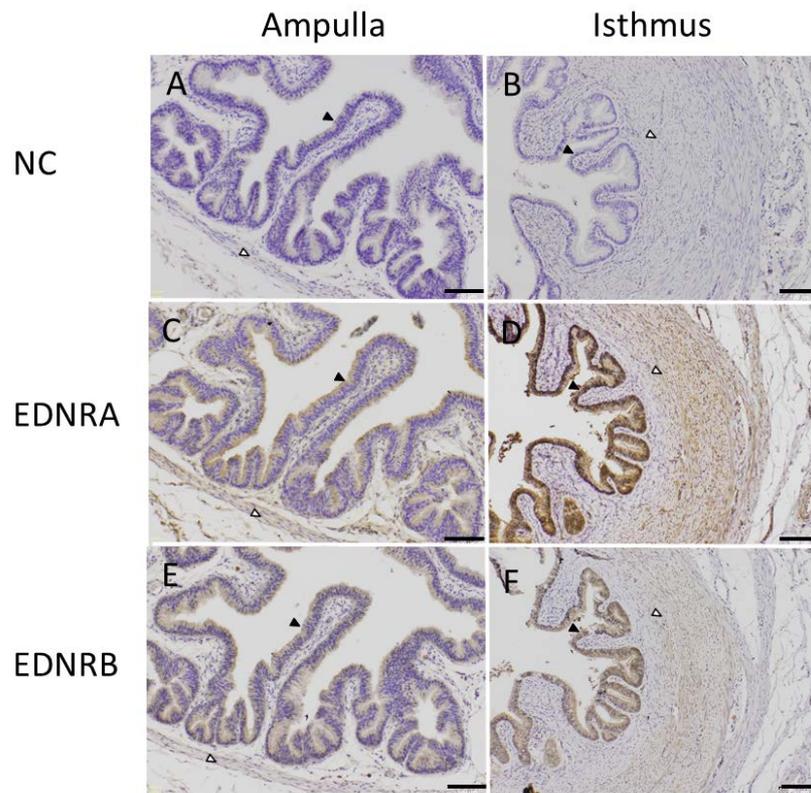


Figure 2

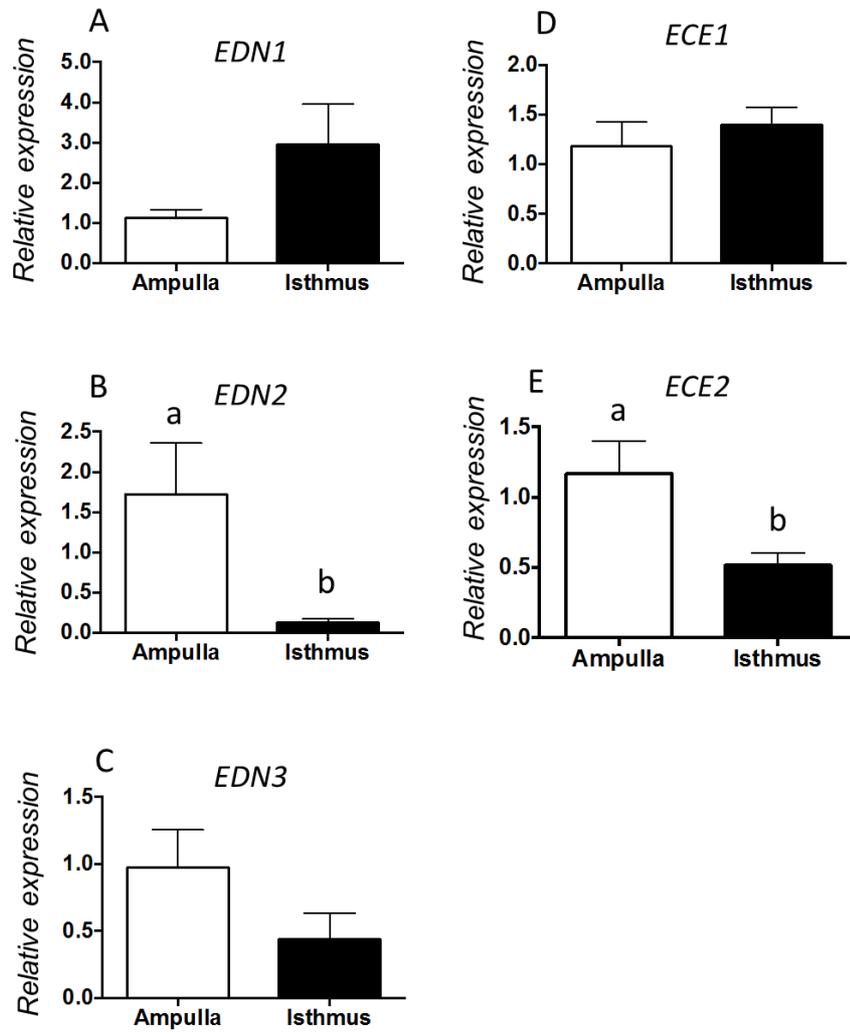


Figure 3

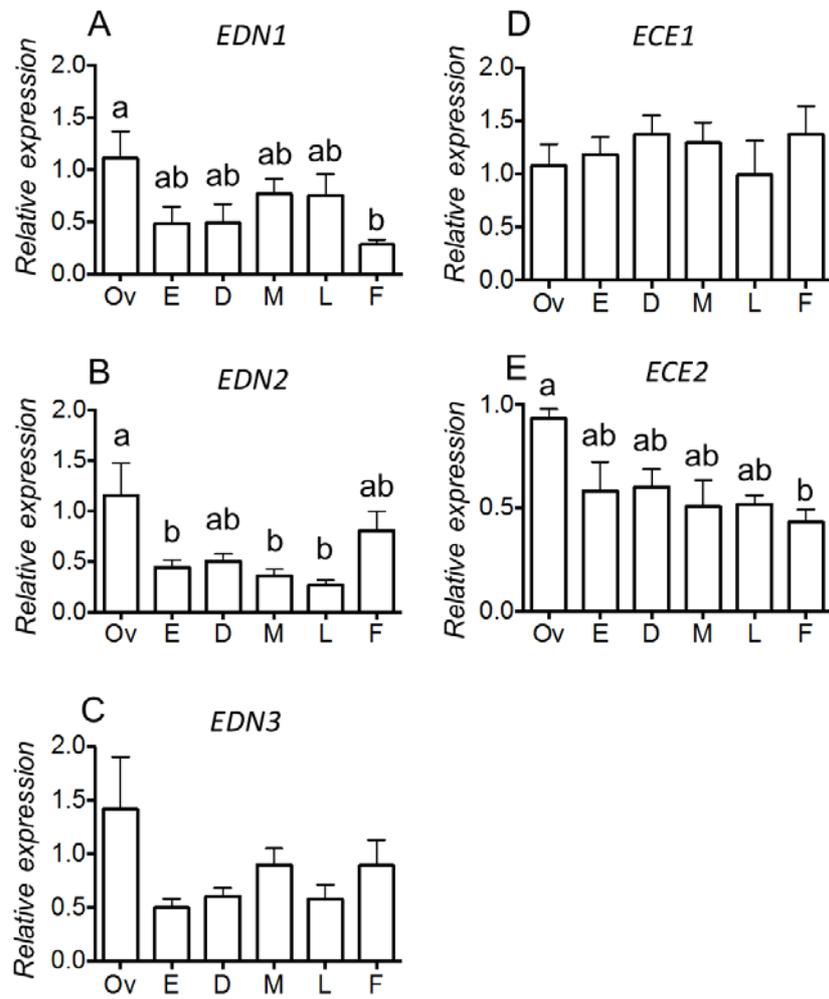


Figure 4

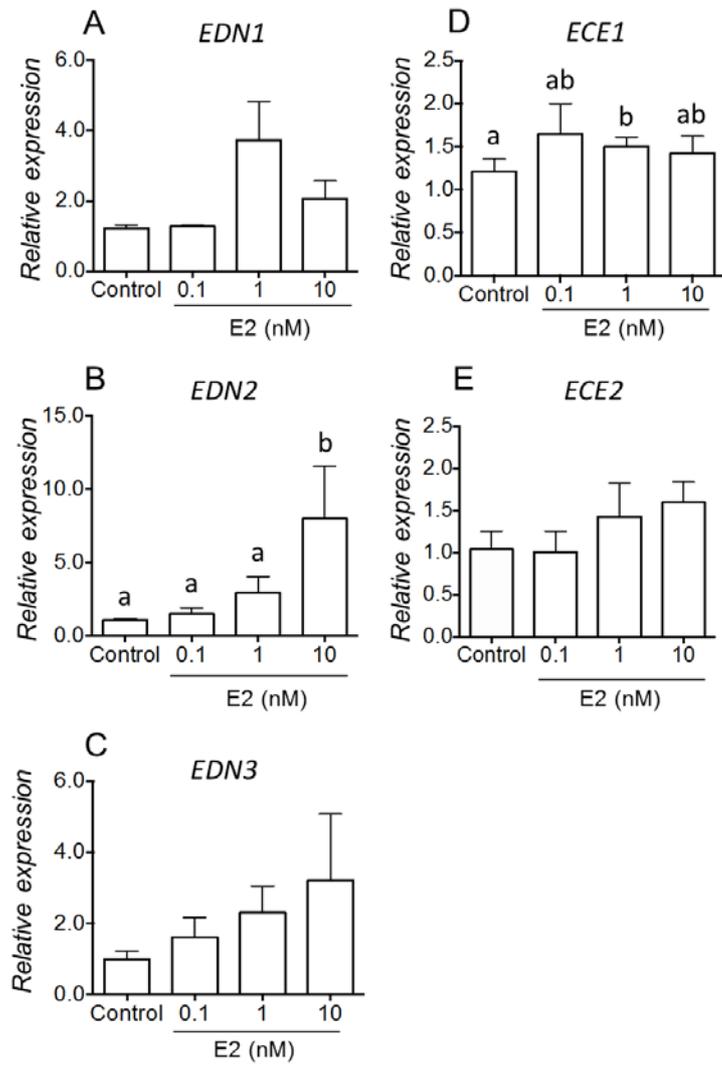


Figure 5

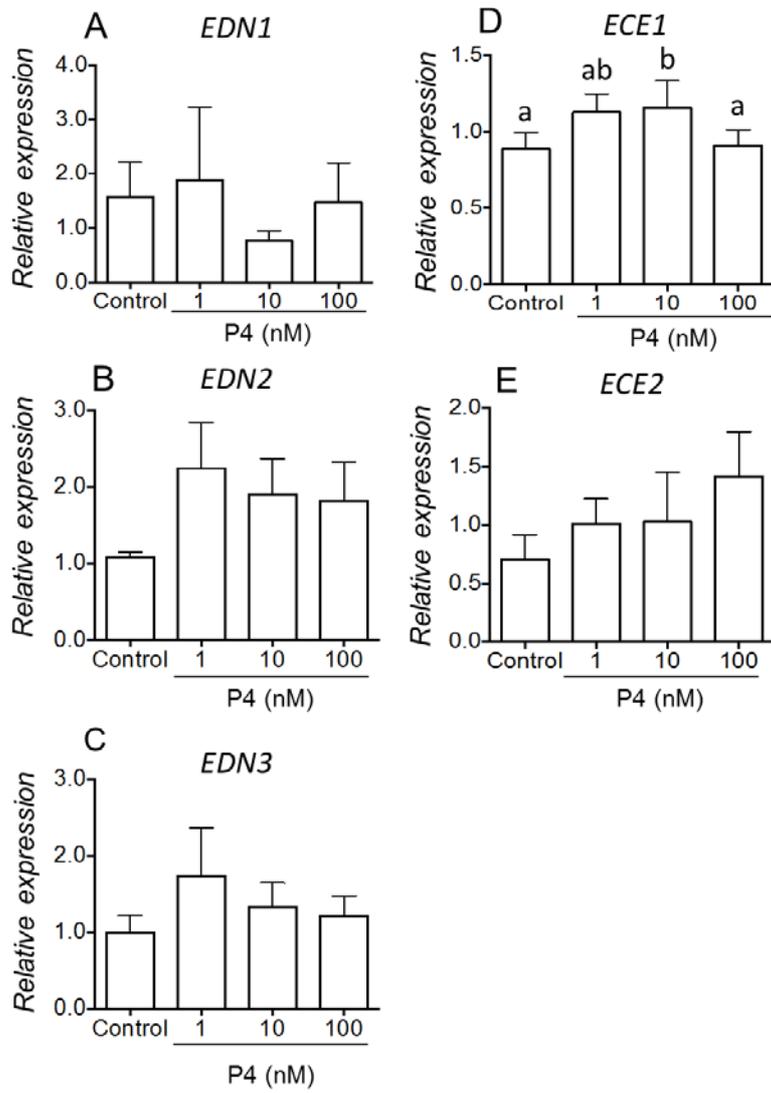


Figure 6