Regulation of bovine oviductal NO synthesis by follicular steroids and prostaglandins

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Abstract

Nitric oxide (NO) is a regulator of sperm motility, oocyte/embryo survival, and waves of contraction/relaxation in mammalian oviducts. As follicles control oviductal functions by two routes at least, (1) a systemic way via blood vessels before ovulation, (2) a direct way by entering of follicular fluid through fimbria at ovulation, we hypothesized that NO synthesis in the bovine oviduct is regulated by follicular steroids and prostaglandins (PGs). Quantification of mRNA expressions in the ampullary tissues showed that inducible NO synthase (NOS2) mRNA expression was highest on the day of ovulation (day 0). By contrast, NOS2 mRNA expression in the isthmus was highest on days 5–6 and lowest on days 19–21. Endothelial NOS (NOS3) mRNA expressions in either the ampulla or the isthmus did not change during the estrous cycle. PGE2 and PGF2 α increased NOS2 mRNA expressions in cultured ampullary oviductal epithelial cells after 1-h incubation. These increases were suppressed by an antagonist of E-prostanoid receptor type 2, one of the PGE2 receptor. Estradiol-17 β decreased the expression of NOS2 mRNA expression in cultured isthmus epithelial cells 24 h after treatment. This effect was suppressed by an antagonist of estrogen receptor α (ESR1). Expression of ESR1 was highest on days 19–21 in the isthmus tissues. The overall findings indicate region-specific difference of NO synthesis in the oviduct. PGs flowed from ruptured follicle may up-regulate NO synthesis in the oviductal epithelium, whereas circulating E2 seems to inhibit NO synthesis via ESR1 in the isthmus at the follicular stage.

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Introduction

In mammals, the oviduct is an essential organ for the transport of gametes and embryos (Menezes & Guerin 1997, Suarez 2008). Ovulated cumulus–oocyte complexes (COCs) and sperms are transported to the ampulla of the oviduct where fertilization occurs (Ulbrich *et al.* 2010). Early embryos are transported to the uterus within a few days (Freeman *et al.* 1991). The transport of gametes and embryos is induced by alternate waves of contraction and relaxation of the oviductal smooth muscle and the ciliary beating of oviductal epithelial cells (Halbert *et al.* 1976, Hunter 2012). Various substances including nitric oxide (NO), ovarian steroids, and prostaglandins (PGs) regulate the waves of contraction and relaxation (Helm *et al.* 1982, Rosselli *et al.* 1994, Ekerhovd *et al.* 1997, 1999, Al-Alem *et al.* 2007).

Nitric oxide is a physiological mediator of numerous cellular and organ functions (Ignarro *et al.* 2001). It is synthesized from L-arginine by NO synthases (NOSs).

Nitric oxide synthases have three isoforms, neuronal NOS (nNOS), inducible NOS (NOS2), and endothelial NOS (NOS3) (Ulbrich *et al.* 2006). Nitric oxide relaxes the oviductal smooth muscle for gamete/embryo transport (Rosselli *et al.* 1994, Yilmaz *et al.* 2012). In addition, NO has crucial roles in gamete/embryo activity and survivability. NO stimulates the motility of spermatozoa (Miraglia *et al.* 2011), and the inhibition of NO synthesis increases the mortality of early embryos in mice (Gouge *et al.* 1998).

Each oviductal region has various specific functions. The ampulla transports the ovulated oocytes to the site of fertilization and supports fertilization (Hunter 2012). The isthmus promotes embryo development by secreting various molecules and transports embryos to the uterus by smooth muscle activity (Hunter 2012). To perform these functions, the oviductal regions are morphologically different. For example, the proportion of two types of epithelial cells, ciliated cells and secretory

cells, is different among regions of the bovine oviduct (Kölle *et al.* 2009). In the murine oviduct, the ampullary region has high ciliary activity, whereas the isthmus has strong muscular contraction and weak ciliary activity (Noreikat *et al.* 2012). The expressions of genes for endothelins, PG synthases, and sex steroid receptors are also different among regions of the oviduct (Ulbrich *et al.* 2003, Gauvreau *et al.* 2010, Jeoung *et al.* 2010, Saint-Dizier *et al.* 2012). Similarly, *NOS2* mRNA expression at the day of ovulation is higher in the ampulla than in the isthmus of the bovine oviduct (Ulbrich *et al.* 2006). Thus, NO synthesis may be regulated differently in the ampulla and isthmus.

Ovarian steroids are major molecules controlling oviductal functions (Wijayagunawardane *et al.* 1999, Ulbrich *et al.* 2003, Szóstek *et al.* 2011). During the estrous cycle except at ovulation, these steroids participate in oviductal physiological events via systemic way. The highest levels of estrogen in circulation and oviductal tissue at the follicular phase (Wijayagunawardane *et al.* 1998, Acosta *et al.* 2003) support that idea. At ovulation, follicular fluid with COC enters the oviduct through fimbria, the entrance of the oviduct. As follicular fluid contains high concentrations of PGE₂, PGF₂α, and ovarian steroids (Acosta *et al.* 1998, 2000), these molecules are possible to influence oviductal milieu.

In this study, we hypothesized that molecules derived from follicles participate oviductal NO synthesis via two distinct routes: (1) a systemic way via blood vessels during the estrous cycle and (2) a direct way through fimbria only at ovulation. To test the above hypothesis, we determined (1) cyclic changes of NOSs and receptors for estrogen (ESRs), progesterone (PGR), PGE (PTGERs), and PGF (PTGFR) expressions in bovine ampullary and isthmus oviductal tissues during the estrous cycle and (2) the effects of exogenous E₂, P₄, PGE₂, and PGF₂α on NO synthesis in cultured ampullary and isthmus oviductal epithelial cells.

Materials and methods

Experimental design

Expressions of NOSs in oviductal tissues

Changes in mRNA expressions of *NOS2* and *NOS3* in oviductal tissues during the estrous cycle were determined to clarify ovarian stage-specific regulation of NO synthesis in the oviduct. Immunohistochemical investigations of NOS proteins were performed to clarify which types of oviductal cells (epithelial, stromal, or smooth muscle cells) express those proteins.

Expressions of ESRs, PGR, PTGERs, and PTGFR in oviductal tissues

Changes in mRNA expressions of *ESRs*, *PGR*, *PTGERs*, and *PTGFR* and protein distributions in oviductal tissues were

investigated to clarify when the ligands of those receptors affect oviductal physiology and which types of oviductal cells receive those ligands to regulate NO synthesis.

Concentrations of ovarian steroids and prostaglandins (PGs) in follicular fluid immediately before ovulation

To determine appropriate concentrations of estradiol-17β (E₂), progesterone (P₄), PGE₂, and PGF₂α in the following experiments using cultured oviductal epithelial cells, we measured the concentrations of those molecules in follicular fluid obtained from cows that were treated to induce super-ovulation.

Effects of E₂ or P₄ on NOS2 mRNA expression in cultured oviductal epithelial cells

To clarify whether ovarian steroids affect NO synthesis in the oviduct, cultured oviductal epithelial cells isolated from the ampulla and isthmus were incubated with E₂ or P₄. Concentrations of E₂ (0.1, 1, 10 nmol/L) and P₄ (1, 10, 100 nmol/L) were referenced by concentrations in follicular fluid (Table 1) and in circulation (Kaneko 1995). Additionally, antagonist of ESR1 or ESR2 was incubated in combination with E₂ in cultured isthmus epithelial cells to determine which types of estrogen receptors regulate *NOS2* expression.

Effects of PGs on NOS2 mRNA expression in cultured oviductal epithelial cells

To determine the roles of prostaglandins contained in follicular fluid after ovulation, cultured oviductal epithelial cells isolated from the ampulla and isthmus were incubated with PGE₂ or PGF₂α. Concentrations of PGE₂ and PGF₂α (0.01, 0.1, 1 μmol/L) were referenced by concentrations in follicular fluid (Table 1 and previous reports (Acosta *et al.* 1998, 2000)). Additionally, an antagonist of E-prostanoid receptor type 2 (PTGER2) or PTGER4 was added in combination with PGE₂ in cultured ampullary epithelial cells to determine which types of PGE₂ receptors regulate *NOS2* expression. As PTGER1 receptor is not expressed and the expression level of PTGER3 receptor is lower than that of PTGER2 and PTGER4 in the bovine oviduct (Gabler *et al.* 2008), either effects of an antagonist of PTGER1 or PTGER3 on *NOS2* mRNA expression was not investigated in this study.

Differential expression of NOS2 mRNA between the ampulla and isthmus

To investigate the difference of capability for NO synthesis between the ampulla and isthmus, *NOS2* mRNA expressions in oviductal tissues and cultured epithelial cells were compared between the ampulla and isthmus.

Table 1 Concentrations of estradiol-17β (E₂), progesterone (P₄), prostaglandin E₂ (PGE₂), and PGF₂α in follicular fluid obtained from cows that were treated with super-ovulation. Follicular fluid was collected 26–27 h after administering a GnRH analog.

Hormone	Concentration (nmol/L)
E ₂	106.3–409.9
P ₄	771–2763
PGE ₂	5.68–40.1
PGF ₂ α	61.9–835

Collection of bovine oviducts

Oviducts of Holstein cows were collected at a local abattoir within 10–20 min after the exsanguination. The stages of the estrous cycle were determined based on a macroscopic observation of the ovary and the uterus (Okuda *et al.* 1988, Miyamoto *et al.* 2000). After trimming of the oviducts being ipsilateral to the corpus luteum, the ampullary and the isthmus sections were immediately frozen and stored at -80°C until mRNA and protein extraction. Oviductal tissues for immunohistochemistry were fixed in PBS with 10% (v/v) neutral formaldehyde for 24 h and then embedded in paraffin. For cell culture, the oviducts were submerged in ice-cold saline and transported to the laboratory.

Determination of PG concentrations in collected follicular fluid

A Japanese black cow and an Angus cow were utilized for superovulation treatment and collecting follicular fluids. A total of 20 Armour units (AU) of follicle-stimulating hormone (FSH; Antorin R-10; Kyoritsu Seiyaku Corporation, Kanagawa, Japan) were administered twice daily in decreasing doses during 3 days (5, 5, 3, 3, 2 and 2 AU respectively). A PGF 2α analog (cloprostenol, Resipron-C; ASKA Pharmaceutical Co., Tokyo, Japan, 0.5 mg) was administered on the third day of FSH administration. Cows had a controlled internal drug release device (CIDR) (CIDR1900; Pfizer Japan Inc., Tokyo, Japan) inserted for 8–9 days until the injection of the PGF 2α analog and were received estradiol benzoate (Kyoritsu Seiyaku Corporation, 1 mg) 4 days before the first FSH administration. These cows were injected with a GnRH analog (fertirelin acetate; Conceral, Nagase Pharmaceutical, Tokyo, Japan, 100 μg) 24 h after CIDR removal. Transvaginal collection of follicular fluids was carried out at 26–27 h after GnRH administration. Follicular aspiration was conducted using an ultrasound scanner (SSD-900; ALOKA, Tokyo, Japan) and 7.5 MHz convex array transducer (UST-9106P-7.5; ALOKA) attached with a 17-gauge stainless steel needle guide. Follicles over 8 mm in diameter were aspirated by an aspirator (FV4; FHK, Tokyo, Japan) equipped with a disposable aspiration needle (Misawa Medical Industry Co., Ltd. Ibaraki, Japan). Collected follicular fluids were centrifuged (1800 g, 10 min, 4°C) and supernatants were stored at -20°C until measuring concentrations of PGs. All procedures with animal subjects had been reviewed and approved by the Animal Care Committee of the Animal Research Center, Hokkaido Research Organization.

The concentrations of PGE 2 in the collected follicular fluids were determined by enzyme immunoassay as described previously (Tanikawa *et al.* 2005). The PGE 2 standard curve ranged from 0.039 to 10 ng/mL, and the ED 50 of the assay was 0.625 ng/mL. The intra-assay coefficients of variation were on average 2.6%. The range of the concentrations of PGE 2 in follicular fluids was 0.006–0.04 $\mu\text{mol/L}$.

The concentrations of PGF 2α in the collected follicular fluids were determined by enzyme immunoassay as described previously (Uenoyama *et al.* 1997) with our modification using peroxidase-labeled PGF 2α as a tracer (1:25,000 final dilution) and anti-PGF 2α serum (1:100,000 final dilution). The

anti-PGF 2α serum (OK-PGF) was produced by Sigma-Aldrich. Cross-reactivities of the anti-PGF 2α serum were validated by comparing the inhibition of binding of peroxidase-labeled PGF 2α to antiserum: PGF 2α , 100%; PGA 2 , 0.38%; PGD 2 , 2.51%; PGE 1 , 0.53%; PGE 2 , 0.08%; PGF 1α , 5.62%; PGF $2\alpha\beta$, 0.49%; and PGF 2β , 0.58%. The PGF 2α standard curve ranged from 0.016 to 4 ng/mL, and the ED 50 of the assay was 0.25 ng/mL. The intra-assay coefficients of variation were on average 3.9%. The range of the concentrations of PGF 2α in follicular fluids was 0.06–0.83 $\mu\text{mol/L}$. This assay system of PGF 2α has no cross-reaction with cloprostenol, a PGF 2α analog. In this study, cloprostenol was utilized for luteolysis 50–51 h before collection of follicular fluid. The half-life of cloprostenol was approximately 3 h and ^{14}C -labeled cloprostenol was not detected in the ovary 48 h after dose of the PGF 2α analog (Reeves 1978). Thus, we considered the concentration of PGF 2α , which we detected in follicular fluid did not contain exogenous PGF 2α .

Isolation and culture of oviductal cells

Epithelial cells were enzymatically isolated from the ampullary and the isthmus sections of the oviduct at peri-ovulatory phase as described previously (Kobayashi *et al.* 2013). The isolated cells were purified by using two different concentrations of trypsin (Kobayashi *et al.* 2013). All the purified cells were determined to be epithelial cells (no contamination of stromal cells) by immunocytochemistry (Kobayashi *et al.* 2013). The purified cells were seeded onto 48-well plates (677180; Greiner Bio-One, Frickenhausen, Germany) or 25 cm^2 culture flasks (690175; Greiner Bio-One). The plates and flasks for epithelial cells were coated with collagen obtained from mouse tails. The cells were cultured at 38.5°C in a humidified atmosphere of 5% CO_2 in air. The medium was exchanged every 48 h until the cells reached confluency. When the cells reached 90–95% confluency (10–11 days after the isolation of the cells), they were used for experiments.

Cell treatment

Oviductal epithelial cells that reached confluency were incubated with estradiol-17 β (E 2 , 0.1, 1, and 10 nmol/L, E8875; Sigma-Aldrich), progesterone (P 4 , 1, 10, and 100 nmol/L, P8783; Sigma-Aldrich), prostaglandin E 2 (PGE 2 , 0.01, 0.1, and 1 $\mu\text{mol/L}$, 14010; Cayman Chemical), and PGF 2α (0.01, 0.1, and 1 $\mu\text{mol/L}$; 16010, Cayman Chemical) in phenol red-free DMEM/F-12 Ham (D2906; Sigma-Aldrich) supplemented with 500 $\mu\text{mol/L}$ ascorbic acid (013-12061; Wako Pure Chemical Industries), 5 $\mu\text{g/mL}$ holo-transferrin (T4132; Sigma-Aldrich), 5 ng/mL sodium selenite (S5261; Sigma-Aldrich), 2 $\mu\text{g/mL}$ insulin (I4011; Sigma-Aldrich), 0.1% (w/v) bovine serum albumin (A7888; Sigma-Aldrich), and 20 mg/mL gentamicin (G1397; Sigma-Aldrich) for 1, 4, and 24 h at 38.5°C . Selective antagonists of estrogen receptors (ESRs) were co-incubated with E 2 (10 nmol/L). MPP dihydrochloride (100 nmol/L, M7068; Sigma-Aldrich) for ESR1 and PHTPP (100 nmol/L, 2662; R&D Systems) for ESR2 were used as selective antagonists. MPP dihydrochloride has been demonstrated to be a selective antagonist for ESR1 (Sun *et al.* 2002) and PHTPP

Figure 1 (A) Cyclic changes of *NOS2* and *NOS3* mRNA expressions in tissues collected from the ampulla and isthmus of the bovine oviduct (mean $\pm$ S.E.M., $n=5$ oviducts). Significance of differences was assessed by the analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons. Different superscript letters indicate significant differences ($P<0.05$). (B) Immunohistochemical analysis of NOS protein distributions in the ampulla and isthmus of the oviduct obtained from cows at the day of ovulation (L: lumen, E: epithelium, ST: stroma, SM: smooth muscle). Green color (Alexa 488) indicates each target protein (*NOS2* or *NOS3*), and blue color (DAPI) indicates nuclei of the cells. Scale is the same in all the photomicrographs.

is known to display 36-fold selectivity for *ESR2* than *ESR1* (Compton *et al.* 2004). Antagonists of *PTGER2* or *PTGER4* were incubated in combination with *PGE2* (1 μ mol/L) in cultured ampullary epithelial cells. AH6809 and AH23848 are selective antagonists for *PTGER2* and *PTGER4*, respectively (Coleman *et al.* 1994, Woodward *et al.* 1995). All the antagonists were pre-incubated with the cells for 1 h before incubation with each ligand. After incubations, supernatants were collected for measuring NO concentration and the cells were collected to measure DNA content for standardizing the NO concentration. Total RNA of the cells was extracted from another culture plate for the determination of mRNA expressions.

Total RNA extraction and quantitative RT-PCR

Total RNA was extracted from oviductal tissues and cells using TRIre according to the manufacturer's directions. Using iScript RT Supermix for RT-qPCR (170-8841; Bio-Rad Laboratories), 1 μ g of each total RNA was reverse transcribed. Quantifications of mRNA expressions were determined by Quantitative RT-PCR using MyiQ (Bio-Rad Laboratories) and SoobAdvanced SYBR Green

Figure 2 (A) Cyclic changes of estrogen receptor α (*ESR1*), *ESR2*, and progesterone receptor (*PGR*) mRNA expressions in tissues collected from the ampulla and isthmus of the bovine oviduct (mean $\pm$ S.E.M., $n=5$ oviducts). Significance of differences was assessed by the analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons. Different superscript letters indicate significant differences ($P<0.05$). (B) Immunohistochemical analysis of *ESR1* and *PGR* protein distributions in the ampulla and isthmus of the oviduct obtained from cows at follicular stage (L: lumen, E: epithelium, ST: stroma, SM: smooth muscle). Green color (Alexa 488) indicates each target protein (*ESR1* or *PGR*), and blue color (DAPI) indicates nuclei of the cells. Scale is the same in all the photomicrographs.

Supermix (1725261B10; Bio-Rad Laboratories) starting with 4 ng reverse-transcribed total RNA as described previously (Sakumoto *et al.* 2006). All primers were designed to amplify specific products for *NOS2* (forward: 5'-TAC CCT CAG TTC TGC GCT TT-3'; reverse: 5'-GGG ATC TCA ATG TGG TGC TT-3'), *NOS3* (forward: 5'-AGG CTC TCA CCT TCT TCC TG-3'; reverse: 5'-AAC CAC TTC CAC TCC TCG TA-3'), *ESR1* (forward: 5'-CAG GCA CAT GAG CAA CAA AG-3'; reverse: 5'-TCC AGC AGC AGG TCG TAG AG-3'), and *ESR2* (forward: 5'-CTG AAG CAT GAA CTC CAG CAC-3'; reverse: 5'-CAG GAA GGA CCA CAT AGC AGA-3'). The specificity of each primer set was confirmed by running the PCR products on a 2.0% agarose gel. Protocol conditions were 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 level of expression of each mRNA, three potential housekeeping genes, β -actin (*ACTB*; forward: 5'-CAG CAA GCA GGA GTA CGA TG-3'; reverse: 5'-AGC CAT GCC AAT CTC ATC TC-3'), 18S ribosomal RNA (*18S rRNA*; forward: 5'-TCG CGG AAG GAT TTA AAG TG-3'; reverse: 5'-AAA CGG CTA CCA CAT CCA AG-3'), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH* forward: 5'-CAC CCT CAA GAT TGT CAG CA-3';

Figure 3 (A) Cyclic changes of E-prostanoid receptor 2 (*PTGER2*), *PTGER4*, and F-prostanoid receptor (*PTGFR*) mRNA expressions in tissues collected from the ampulla and isthmus of the bovine oviduct (mean $\pm$ S.E.M., $n=5$ oviducts). Significance of differences was assessed by the analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons. There were no significant differences ($P > 0.05$). (B) Immunohistochemical analysis of PTGER and PTGFR protein distributions in the ampulla and isthmus of the oviduct obtained from cows at the day of ovulation (L: lumen, E: epithelium, ST: stroma, SM: smooth muscle). Green color (Alexa 488) indicates each target protein (*PTGER2*, *PTGER4*, or *PTGFR*), and blue color (DAPI) indicates nuclei of the cells. Scale is the same in all the photomicrographs.

reverse: 5'-GGT CAT AAG TCC CTC CAC GA-3') were initially tested. *GAPDH* was found to be the most stable of the three genes by Normfinder software (<http://moma.dk/normfinder-software>), and so *GAPDH* transcripts were selected as the internal control in our experiments. Amplification efficiencies of all the primers were checked by the determination of Ct values for a dilution series of the target template. Efficiencies of all the primers were 95–100%. The relative level of expression of each mRNA was measured using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

Immunohistochemistry

Sections (6 μ m) were deparaffinized and rehydrated in a graded series of ethanol and washed in tap water. Antigens were retrieved by using microwave in Tris–EDTA buffer (pH 9.0, for NOS2, NOS3, and PGR) or in 0.01 mo/L citrate buffer (pH 6.0, the other antigens) for 15 min at 600W. Nonspecific binding was blocked in 2.5% horse serum (S-2012; Vector Laboratories) for 20 min at room temperature. The sections were incubated with specific primary antibodies for NOS2 (160862;

Figure 4 (A) Effects of estradiol-17 β (E2) or progesterone (P4) on *NOS2* mRNA expression in the oviductal epithelial cells isolated from the ampulla (black bar) and isthmus (gray bar) of the oviduct (mean $\pm$ S.E.M., $n=8$ oviducts, $n=12$ only in the incubation with P4 for 24 h). Significance of differences was assessed by the analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons. Different superscript letters indicate significant differences ($P < 0.05$). (B) Effects of selective antagonists of each ESR on *NOS2* mRNA expression in isthmus oviductal epithelial cells (mean $\pm$ S.E.M., $n=8$ oviducts). MPP dihydrochloride (ESR1 antagonist) or PHTPP (ESR2 antagonist) was co-incubated with E2 (10 nmol/L) for 24 h. Significance of differences was assessed by the analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons. Different superscript letters indicate significant differences ($P < 0.05$).

Cayman Chemical), NOS3 (160880; Cayman Chemical), ESR1 (SAB2100712; Sigma-Aldrich), PGR (SAB4502185; Sigma-Aldrich), *PTGER2* (ab167171; Abcam), *PTGER4* (ab133170; Abcam), or *PTGFR* (101802; Cayman Chemical) overnight at 4°C, washed with PBS three times, and incubated with secondary antibody for rabbit-IgG conjugated with Alexa 488 (21206; Life Technologies) for 1 h at room temperature, washed with PBS three times, covered with ProLong Gold Antifade

Figure 5 (A) Effects of prostaglandin (PG) E2 and PGF2α on NOS2 mRNA expression in ampullary and isthmus oviductal epithelial cells (mean $\pm$ S.E.M., $n=5$ oviducts). Significance of differences was assessed by the analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons. Different superscript letters indicate significant differences ($P<0.05$). (B) Effects of selective antagonists of each PTGER on NOS2 mRNA expression in isthmus oviductal epithelial cells (mean $\pm$ S.E.M., $n=7$ oviducts). AH6809 (PTGER2 antagonist) or AH23848 (PTGER4 antagonist) was co-incubated with PGE2 (1 μ mol/L) for 1 h. Significance of differences was assessed by the analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons. Different superscript letters indicate significant differences ($P<0.05$).

Reagent with DAPI (36935; Life Technologies), and observed under a fluorescence microscope (FSX100; Olympus).

Statistical analysis

All experimental data are expressed as the mean $\pm$ S.E.M. The statistical significance of differences was assessed by analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons using GraphPad Prism (GraphPad Software). P values less than 0.05 were considered to be statistically significant.

Figure 6 Differential expression of NOS2 mRNA between the ampulla and isthmus in oviductal tissues (A, $n=6$, mean $\pm$ S.E.M.) and cultured epithelial cells (B, $n=5$, mean $\pm$ S.E.M.). Significance of differences was assessed by the analysis of variance (ANOVA) followed by Student's t -test. Asterisks indicate significant differences ($P<0.05$).

Results

Expressions of NOSs in oviductal tissues

NOS2 mRNA expression in the ampulla was highest at the day of ovulation (day 0) (Fig. 1A, $P<0.05$). In the isthmus, NOS2 mRNA expression was highest at days 5–6 and gradually decreased toward the follicular phase. NOS2 expression in the isthmus was lowest at days 19–21 (Fig. 1A, $P<0.05$). However, NOS3 expressions in both segments did not significantly change during the estrous cycle. NOS2 and NOS3 proteins were immunohistochemically detected in epithelial, stromal, and smooth muscle layers of the ampullary region (Fig. 1B). In the isthmus, NOS3 was expressed in the epithelium, stroma, and smooth muscle. NOS2 was clearly detected in epithelium, but only weakly detected in stroma and smooth muscle. Activity of NO synthesis by NOS2 is well known to be extremely higher than by NOS3 (Moncada & Higgs 1993). As NOS3 expression did not change during the estrous cycle in this study, only NOS2 expression was investigated in the following experiments.

Expressions of ESRs, PGR, PTGERs, and PTGFR

ESR1 mRNA expression was highest at days 19–21 (follicular phase) in isthmus oviductal tissues, whereas the expression of ESR1 mRNA did not change in ampullary tissues during estrous cycle (Fig. 2A). The mRNAs of other steroid and PG receptors were stably

Figure 7 Proposed model to illustrate region-specific regulations of nitric oxide (NO) synthesis in the ampulla and isthmus of the oviduct. Before ovulation, high level of estradiol-17 β (E2) released from follicles systemically suppressed NO production by isthmus oviductal epithelial cells via estrogen receptor α (ESR1). Because NO stimulates the motility of spermatozoa (Miraglia *et al.* 2011), suppressing NO by E2 in the isthmus may contribute to decreasing the motility of spermatozoa and promoting their binding to epithelial cilia until they become hyperactivated. However, prostaglandin (PG), E2, and PGF2 α derived from follicular fluid directly stimulate NO production by ampullary oviductal epithelial cells immediately after ovulation via E-prostanoid receptor type 2 (PTGER2) and F-prostanoid receptor (PTGFR) respectively. Because NO contributes to the survival of oocyte and embryos (Manser *et al.* 2004, Goud *et al.* 2005), PGs may play roles in maintaining the optimal concentrations of NO in the ampulla for the survival of oocyte and embryos.

expressed in both ampullary and isthmus tissues during the estrous cycle (Figs 2A and 3A). ESR1 and PGR proteins were localized in epithelial cells of the ampulla and isthmus of the oviduct (Fig. 2B). PGR protein was detected in smooth muscle layer of the isthmus region (Fig. 2B). PTGER2, PTGER4, and PTGFR proteins were distributed in the epithelium of both regions (Fig. 3B). PTGER2 and PTGFR were also detected in the stromal and smooth muscle layer (Fig. 3B).

Concentrations of ovarian steroids and PGs in follicular fluid immediately before ovulation

Concentrations of E2, P4, PGE2, and PGF2 α in follicular fluid obtained from cows that were treated to induce super-ovulation were determined. Ranges of concentrations of E2, P4, PGE2, and PGF2 α were 106.3–409.9 nmol/L, 771.0–2763 nmol/L, 5.68–40.1 nmol/L, and 61.9–835 nmol/L respectively (Table 1).

Effects of estradiol-17 β (E2) or progesterone (P4) on NOS2 mRNA expression

To determine the regulation of NO synthesis in the oviduct, we investigated the effects of ovarian steroid hormones (E2 or P4) on NOS2 mRNA expression in cultured oviductal epithelial cells. Estradiol-17 β suppressed NOS2 mRNA expression and NO production in isthmus epithelial cells after 24-h incubation, but not in ampullary epithelial cells (Fig. 4A). Progesterone stimulated NOS2 mRNA expression after 24-h incubation but not after 1- or 4-h incubations. By contrast, P4 did not affect NOS2

expression in epithelial cells obtained from the isthmus (Fig. 4A). An ESR1 antagonist blocked the inhibiting effect of E2 on NOS2 expression, whereas an ESR2 antagonist did not (Fig. 4B).

Effects of PGs on NOS2 mRNA expression in cultured oviductal epithelial cells

NOS2 mRNA expression was increased by PGE2 and PGF2 α after 1-h incubation, whereas NOS2 mRNA expression was decreased after 24-h incubation in ampullary epithelial cells (Fig. 5). However, NOS2 mRNA expression was not affected by PGs in isthmus epithelial cells. A PTGER2 antagonist decreased NOS2 mRNA expression stimulated by PGE2 after 1-h incubation, whereas a PTGER4 antagonist did not (Fig. 5B).

Differential expression of NOS2 mRNA between the ampulla and isthmus

In both oviductal tissues and cultured epithelial cells, NOS2 mRNA expression was higher in the ampulla than in the isthmus (Fig. 6).

Discussion

Nitric oxide (NO), a molecule that has been identified as an endothelium-derived relaxing factor, participates in various physiological events such as the immune response, neurotransmission, and cell proliferation (Bredt & Snyder 1994, Ignarro *et al.* 2001). In the oviduct, NO is also known to relax smooth muscle (Rosselli *et al.* 1994), and its synthesis is regulated by ovarian hormones during the estrous cycle

(Lapointe *et al.* 2006, Ulbrich *et al.* 2006). The present results suggest that prostaglandins (PGs) in follicular fluid as well as estradiol-17 β in blood stream regulate NO synthesis in oviductal epithelial cells. A model of this regulatory system is shown in Fig. 7. The oviduct is not only the site where an ovulated oocyte and sperm meet, but it also provides a microenvironment for early embryonic development in the first days of pregnancy (Ulbrich *et al.* 2010, Ezzati *et al.* 2014). The present finding that NO synthesis in the ampulla and isthmus was regulated by specific molecules (PGs and E2 respectively) may reflect the idea that the oviduct has region-specific functions. For example, the ampulla serves to transport the oocyte and to provide an environment for fertilization, whereas the isthmus serves to transport spermatozoa and the embryo and to provide an environment for embryo development.

Estradiol-17 β concentrations in the oviduct are highest immediately before ovulation (Wijayagunawardane *et al.* 1998), indicating that a large amount of E2 is transported to the oviduct at the pre-ovulatory stage. In addition, our result showed that ESR1 was highly expressed at the follicular stage in the isthmus (Fig. 2A). These results, together with our finding that E2 decreased NO synthesis in cultured isthmic epithelial cells (Fig. 4A), suggest that the E2 transported to the isthmus of the oviduct suppresses NO synthesis via ESR1 at the follicular stage. Spermatozoa entering the oviduct bind to cilia of the oviductal epithelium, which temporarily stops their migration toward the oocyte (Kölle *et al.* 2009). Because NO is reported to stimulate the motility of spermatozoa (Miraglia *et al.* 2011), suppression of NO synthesis by E₂ in the isthmus may decrease the motility of spermatozoa, contributing to their binding to epithelial cilia until they become hyperactivated. We showed that the effects of E2 on NO synthesis were different between ampullary and isthmic epithelial cells. The suppressing effect of E2 on NOS2 mRNA expression was observed only in isthmic epithelial cells despite less expression of ESR1 protein in isthmic tissues than in ampullary tissues (Ulbrich *et al.* 2003). These different effects of E2 may be involved in the specific characters of epithelial cells in each region of the oviduct. Although oviductal epithelium is composed by two epithelial cells, ciliated and secretory, the isthmic secretory cells interestingly have short kinocilia on the cell surface, but not ampullary secretory cells (Kölle *et al.* 2009). These differences imply that the secretory cells play specific roles in each region of the oviduct. In fact, NOS2 mRNA expressions in the ampullary tissues and epithelial cells were higher than in the isthmic tissues and epithelial cells respectively (Fig. 6). In addition, partial differences of NO synthases in cyclic epithelium of the oviduct (Ulbrich *et al.* 2006) and whole layers containing stroma and smooth muscle of the oviduct (Fig. 1) suggest layer-specific control of NO synthesis in the oviductal milieu. Further studies are

needed to clarify the region-specific roles of epithelial cells in the oviduct.

The high concentrations of PGE2 and PGF2 α in the follicular fluids by injection of gonadotropin-releasing hormone are consistent with the previous finding that PGE2 and PGF2 α concentrations increase immediately before ovulation, and with the idea that PGs induce follicular rupture (Acosta *et al.* 1998). After the rupture, the oocyte and follicular fluid containing high concentrations of PGs enters the oviduct. There is evidence that PGs affect NO synthesis in other tissues. For example, PGE2 appears to activate NO synthesis in mouse aortic epithelium (Hristovska *et al.* 2007), and PGF2 α increases NO production in bovine luteal endothelial cells (Lee *et al.* 2009). PGE2 and PGF2 α bind to G protein-coupled receptors (GPCRs), named E-prostanoid receptors or F-prostanoid receptor (Gabler *et al.* 2008, Zhang *et al.* 2010), inducing rapid signal transductions (Hristovska *et al.* 2007, Siemieniuch *et al.* 2009). Therefore, our finding that NOS2 mRNA expression in ampullary epithelial cells increased after 1-h incubation with PGs at concentrations similar to that in follicular fluid (Fig. 5A) suggests that PGs in follicular fluid directly stimulate NO synthesis in the ampulla, thus controlling the oviductal microenvironment immediately after ovulation. Inhibition of NO synthesis suppresses development of pre-implantation murine embryos *in vitro* (Manser *et al.* 2004). NO also delays oocyte aging, suggesting that it has a role in maintaining the quality of oocytes (Goud *et al.* 2005). Therefore, immediately after ovulation, follicular fluid might have a role in maintaining the optimal concentrations of NO in the oviductal cavity for survival of oocytes and development of embryos. The findings that an NO donor activates ciliary beat frequency in rat oviductal ciliated cells (Chiu *et al.* 2010) implies that PGs in follicular fluid promote oocyte transport to the site of fertilization via stimulating NO production immediately after ovulation. In contrast to exposing ampullary oviductal epithelial cells to PGs for 1 h, exposing them to PGs for 24 h decreased NOS2 mRNA expression (Fig. 5A). This down-regulation of NOS2 may be caused by other molecules that are secreted by PG stimulation. In our unpublished data, a NO donor (NONOate) inhibited NOS2 mRNA expression in ampullary oviductal epithelial cells. Thus, NOS2 expression might be decreased by NO stimulated by PGs. In contrast to PGs, P4 increased NOS2 expression after 24-h stimulation in cultured ampullary cells (Fig. 4). Because NO can be involved in both cellular survival and death (Manser *et al.* 2004, Gotoh & Mori 2006), NO concentration in the oviductal cavity is possible to be strictly controlled by various molecules derived from inside and outside of the oviduct.

The isthmus is an crucial region of the oviduct for the transport and development of embryos (Ulbrich *et al.* 2010). A thick smooth muscle layer surrounding

isthmus mucosa contributes to the waves of contraction and relaxation (Menezes & Guerin 1997, Hunter 2012), whereas effective transport driven by ciliary beating is not observed in this region (Noreikat *et al.* 2012). Various molecules regulate the motility of oviductal smooth muscle. Contraction is induced by PGF₂ α , endothelins, angiotensin II, and E2 (Helm *et al.* 1982, Wijayagunawardane *et al.* 2001a,b, Siemieniuch *et al.* 2009), whereas relaxation is induced by NO, PGE₂, luteinizing hormone, and P4 (Helm *et al.* 1982, Rosselli *et al.* 1994, Gawronska *et al.* 1999, Siemieniuch *et al.* 2009). Some of these molecules are secreted by the oviductal epithelium (Rosselli *et al.* 1994, Wijayagunawardane *et al.* 2009, Szóstek *et al.* 2011), contributing to activate the motility of the oviductal smooth muscle around the time of ovulation. In cattle, the embryo reaches the uterus around 4 days after fertilization (Freeman *et al.* 1991). Utero–tubal junction is known to strictly contract immediately before the embryo passes through (Croxatto 2002). Thus, the finding in this study that *NOS2* expression was highest at days 5–6 after ovulation implies that a large amount of NO produced by isthmus epithelial cells relaxes the isthmus and utero–tubal junction and allows the embryo to enter the uterus. This is supported by the findings that *NOS2* mRNA expression in the isthmus oviduct were highest at day 3.5 of the estrous cycle (Ulbrich *et al.* 2006). Inhibition of NO synthesis kills morula embryos *in vitro* (Gouge *et al.* 1998). As the embryo is considered to reach the uterus at the morula or young blastocyst stage (Hunter 2012), a large amount of NO produced by isthmus epithelial cells may contribute to embryonic development several days after fertilization.

In conclusion, our results indicate that the regulatory mechanisms of NO synthesis are different between the ampulla and isthmus of the oviduct. Our results also suggest that PGs in follicular fluid regulate NO synthesis in the ampullary oviductal epithelium. The different regulations of NO synthesis in the ampulla and isthmus might reflect the different roles of these regions during the first days of pregnancy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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