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—Full Paper—

Angiotensin II Secretion by the Bovine Oviduct is Stimulated by Luteinizing Hormone and Ovarian Steroids

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Abstract. Angiotensin II (Ang II), a vasoactive peptide, is secreted by the bovine oviduct and is involved in modulation of local oviductal contraction. Ang II biosynthesis and release during the normal estrous cycle and the effects of luteinizing hormone (LH) and ovarian steroids on biosynthesis and secretion of Ang II were investigated. During the preovulatory period, increases in mRNA expression for Angiotensin converting enzyme 1 (ACE-1) and release of Ang II peptide were detected. Microdialysis of oviductal segments in vitro showed that LH alone significantly increased Ang II release, and combined infusion of LH+E_2+P_4 caused an increase in Ang II release. In cultured oviductal epithelial cells, LH increased Ang II release and ACE-1 mRNA expression, and E_2+P_4 enhanced stimulatory effect of LH on Ang II release and ACE-1 mRNA expression. Thus, it can be concluded that the oviductal Ang II system is upregulated by LH and ovarian steroids during the periovulatory period and may enhance local oviductal contraction. These events could stimulate transport of gametes to the fertilization site.

Key words: Angiotensin II (Ang II), Angiotensin converting enzyme 1 (ACE-1), Fallopian tube, Luteinizing hormone (LH), Ovarian steroids, Uterine tube

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The oviduct controls simultaneous opposite movements of gametes to the fertilization site and passage of the embryo into the uterus at a precise developmental stage. Estrous cycle-dependent oviductal contractile patterns appear to be attributed to these timely movements of gametes and embryos. Studies of spontaneous oviductal contractile patterns in the cow have revealed that the amplitude and frequency of contractions are low during the luteal phase [1], and a gradual increase in contractile amplitude and frequency ouccrs concomitant with a rapid decrease in the progesterone (P_4) level [2]. Both contractile amplitude and frequency reach their maximal values during estrus, and the values quickly diminish over the next 3 days [1, 2].

Angiotensin (Ang) II, the major bioactive peptide of the renin-angiotensin system, is involved in autocrine/paracrine regulation of the oviduct [3]. It is a vasoactive peptide that is converted from Ang I by angiotensin-converting enzyme-1 (ACE-1). Ang I is produced from the precursor angiotensinogen by the enzymatic activity of renin. Expression of Ang II and Ang II receptor (Ang II-R) in the human fallopian tube has been demonstrated [4], and Ang II plays a role in regulating the composition of fallopian tube secretions [5]. We recently reported that Ang II is secreted by the bovine oviduct and stimulates oviductal contractions [6–8]. Indeed, in the bovine oviduct, Ang II stimulates release of prostaglandin E_2 (PGE_2), prostaglandin F2α (PGF2α), and endothelin-1 (ET-1), which are the predominant inducers of contractions [9–11]. Moreover, luteinizing hormone (LH) stimulates e production and release of these hormones by the bovine oviduct during this period, and estradiol-17β (E_2) exerts a synergistical effect on this LH action [11]. Thus, it has been speculated that Ang II production is stimulated by LH and ovarian steroids in the regulation of the peak oviductal contractions responsible for rapid gamete transport to the fertilization site [6].

The Ang II concentration increases dramatically in the ovarian venous plasma after the LH surge, [12] and follicular Ang II rises during the LH surge [12]. Moreover, E_2 increases Ang II production in microvascular endothelial cells from the bovine corpus luteum *in vitro* [13] and induces Ang II-R expression in bovine thecal cells [14]. However, the role of LH and ovarian steroids in regulating the release of Ang II by the bovine oviduct is unknown. Therefore, the objectives of the present study were to determine 1) the mRNA expression for ACE-1 and Ang II release in the bovine oviduct during different phases of the normal estrous cycle, 2) the effect of LH and ovarian steroids on Ang II peptide release and 3) the effect of LH and ovarian steroids on Ang II biosynthesis.

Materials and Methods

Animals and sample collection

Whole reproductive tracts were collected from non-pregnant Holstein cows at a local slaughterhouse within 20 min of slaughter. The phase of the estrous cycle was identified according to previous reports [15, 16] based on the morphology of the corpus luteum (CL), uterine fluid and cervical mucus characteristics, as well as the luteal progesterone (P_4) concentration. Oviducts were separated from the utero-tubal junction, and the surrounding connective tissues were trimmed. They were immediately transported to the laboratory in ice-cold tissue culture medium 199 (M 199; Sigma

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Table 1. Primers used to amplify specific bovine transcripts

Gene	Primer	Sequence (Position of cDNA)	Fragment size
ACE-1	Forward	5'-GGTCCATCCTCCCCTACTTC-3' (60-79)	176
	Reverse	5'-GCGTGCAGGTTCAGGTAGA-3' (215–239)	
β-actin	Forward	5'-CCAAGGCCAACCGTGAGAAGAT-3' (374–395)	256
	Reverse	5'-CCACGTTCCGTGAGGATCTTCA-3' (608–629)	

Chemical, St. Louis, MO, USA).

RNA extraction from oviducts

Total RNA was extracted from oviducts during different cycle phases (n=5–6 for each phase) according to a previously established method [17]. Briefly, tissue samples were homogenized in a denaturing solution, and total RNA was extracted from cell lysates/tissue homogenates with phenol-chloroform [18]. The extracted total RNA was further purified, treated with DNase using an SV Total RNA Extraction Kit (Promega Corporation, Madison, WI, USA) and stored in *RNA* storage solution (Ambion, Austin, Texas, USA) at –80 C until used for *cDNA* synthesis.

ACE-1mRNA quantification

The total RNA was quantified, and its purity was assessed by spectrophotometry. First strand cDNA synthesis was then performed according to a commercial protocol described in the SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The first cocktail was prepared using 2 μg of total *RNA*, 150 ng random primer (Invitrogen) 0.83 mM PCR Nucleotide Mix (dNTP; Roche Diagnostic, Indianapolis, IN, USA) and H_2O, making a final volume of 12 μl/tube, and was subsequently incubated at 65 C for 5 min in a thermal cycler (Mastercycler®, Eppendorf, Netheler Hinz GmbH, Hamburg, Germany). The samples were kept on ice, and 7 μl of the second cocktail 0.01M DTT (Invitrogen), 60 units of Ribonuclease inhibitor (RNasin®, Promega) and First Strand Buffer (Invitrogen) were added. Then, the samples were again incubated for 2 min at 42 C, and 200 units of SuperScript™ II Reverse Transcriptase in 1 μl/tube were added. The thermal cycle program was performed at 25 C for 10 min, 42 C for 50 min and finally 75 C for 15 min. The synthesized *cDNA* was stored at –30 C.

A real-time RT-PCR system (LightCycler; Roche Diagnostics, Mannheim, Germany) and a LightCycler-FastStart DNA Master SYBR Green 1 kit (Roche) with commercially synthesized primers (Hokkaido System Science, Sapporo, Japan; Table 1) were used to amplify each specific bovine transcript. Each capillary (Roche) contained 2 μl of cDNA, 2 μl of LightCycler-FastStart Reaction Mix SYBR Green 1 with LightCycler-Fast Start Enzyme, 2.5 mM $MgCl_2$, 0.5 μM each of forward and reverse primers, and 14.8 μl of H_2O, making a final volume of 20 μl/capillary. A negative control, which was prepared by replacing the cDNA with PCR-grade water in the above mixture, was included in each run. The capillaries were centrifuged in a LightCycler Carousal Centrifuge (Roche) before amplification. The amplification was performed with an initial denaturing at 95 C for 10 min, followed by 40 cycles of denaturing at 95 C for 10 sec, annealing at 65 C for 10 sec and the extension at 72 C for 20 sec. Finally, cooling was performed at 30 C. The melting curve was obtained from 70 to 95 C at the rate of 0.1 C/sec.

For quantification of each target gene, a DNA standard was constructed by amplifying a fragment of *DNA* (approximately 700 bp), which contained the target sequence for quantitative PCR (100 to 150 bp). The PCR products were subjected to electrophoresis; the target band was cut out and purified using a DNA purification kit (SUPRECTM-01; TaKaRa Bio., Otsu, Japan). Three to five stepwise-diluted DNA standards were included in each PCR run. Quantification of mRNA expression was performed using LightCycler Software (Version 3.5; Roche). The values were normalized using β-actin as the internal standard and the delta Ct method [21]. The mRNA expression for ACE-1 in the oviducts collected during different phases of the estrous cycle was expressed as a percentage of the values obtained for oviducts during the luteal phase, and the mRNA expression values for ACE-1 in cultured oviductal epithelial cells (OECs) were expressed as a percentage of the control values.

Collection of Ang II by microdialysis

The *in vitro* microdialysis system for the oviduct has been described previously [6, 11]. The lumen of each 10-cm-long segment of oviduct collected during different cycle phases (n=5–6 for each phase) was implanted with microdialysis system and incubated in M199 with 0.5% BSA (Sigma) at 38 C. Ringer's solution was continuously perfused through the microdialysis system, and perfusates were collected in 4-h fractions for 16 h. The collected perfusates were stored at –30 C until the Ang II measurements.

Effect of LH and ovarian steroids on Ang II release

An *in vitro* microdialysis system was implanted in the lumen of each oviduct collected during different phases of the estrous cycle as described above. Control (Ringer's solution only), LH (1 μg/ml; USDA-bLH-B-6, USDA Animal Hormone Program, Beltsville, MD, USA), E_2 (100 ng/ml; Sigma) and a cocktail of LH+E_2+P_4 (100 ng/ml; Sigma) were infused at 4–8 h of incubation. Perfusates werecollected in 4-h fractions and stored at –30 C.

Culture of OECs

Isolation and culture of OECs was based on the method described previously [9, 10]. Briefly, the surrounding connective tissues were trimmed from the oviduct and washed with Hank's balanced salt solution (HBSS) the oviductal lumen was flushed with 10 ml HBSS and the OECs were mechanically dislodged while flushing with 15 ml HBSS. The collected sheets of OECs were washed twice with HBSS by centrifugation at 300 g for 10

min at 4 C. The cell pellet was suspended in 4 ml HBSS, layered over 5 ml Percoll and centrifuged at 900 *g* for 20 min at 4 C. The cells at the interface between the HBSS and Percoll were collected, washed twice with HBSS, and cultured overnight in M199 with 5% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD, USA) at 38.5 C in 5% CO_2 and 95% air. The OECs still in the suspension were washed twice with HBSS, trypsinized (0.05% trypsin; Amresco, Salon, Ohio, USA), plated in 6-well culture dishes (Nalge Nunc International, DK-4000 Roskilde, Denmark) and incubated at 38.5 C in 5% CO_2 and 95% air.

After monolayer formation, cells were again trypsinized and plated in 6-well culture dishes (Nalge Nunc International, DK-4000 Roskilde, Denmark) at a density of 3×10^4/ml and cultured until the monolayer covered 70–80% of bottom of the culture plate. Monlayers were washed twice with M199 supplemented with 1% BSA (Sigma Chemical, St. Louis, MO, USA), and incubated 24 h in the presence or absence of LH (10 ng/ml), E_2 (1 ng/ml) or LH+E_2+P_4 (1 ng/ml) at 38.5 C in 5% CO_2 and 95% air. The concentrations of E_2 and P_4 used in this study were chosen based on their levels in the bovine oviduct during the normal estrous cycle [16]. All 6 wells in a single culture dish were used for one treatment. The medium was then collected from each well of the 6-well culture dishes and pooled. The cells in all 6 wells of a plate were trypsinized, washed twice with 15 ml 0.01 M PBS (pH=7.4; Sigma) and re-suspended in 6 ml PBS. A 50-μl aliquot of the cell suspension was used to evaluate the cell concentration. The remaining cells were again separated by centrifugation (300 *g* for 10 min at 4 C), lysed with SV RNA Lysis Buffer (Promega) and stored at –80 C until RNA extraction. Total RNA was extracted using the SV Total *RNA* Isolation System (Promega Corporation,) according to the manufacture's protocol, and was stored in *RNA* storage solution at –30 C until used for *cDNA* synthesis. The experiment was repeated 5 times using different sources of OECs.

Estimation of the concentrations of the steroids administrated in the microdialysis and cell culture experiments

The concentrations of the different substances used in this study were chosen based on their concentrations measured in the bovine oviduct during the periovulatory stage of the normal estrous cycle [16]. The P_4 dose was the expected basal physiological level during the periovulatory period. Diffusion through the MDS capillary membrane was estimated as 1% for steroids. Thus, the steroid doses were made to be 100 times higher than the concentrations estimated at the site of action [11].

Measurements of Ang II

Ang II in the microdialysis fractions and medium from cell culture was purified using Sep-Pak C_{18} cartridges as described previously [19] and re-suspended in assay buffer for the peptide EIA (42 mM Na_2HPO_4, 8 mM KH_2PO_4, 20 mM NaCl, 4.8 mM EDTA and 0.05% BSA, pH=7.5) to be 40-fold concentrated from the original concentration. The recovery rate, estimated by adding 3 different concentrations of Ang II (1,10 or 100 pg/ml) to the medium prior to extraction, was 90%. The EIAs for the Ang II assay were performed according to Hayashi and Miyamoto [20]. The intra- and interassay coefficients of variation (CV) were 2.67 and 7.99%; the ED_{50} of the assay was 150 pg/ml; and the range of the standard curve for this assay was 2.4–5000 pg/ml.

Experimental design and statistical analysis

All experiments were carried out under standard laboratory conditions with a Complete Randomized Block (CRD) design keeping control for every treatment. Data on release of Ang II into the culture medium and the expressions of mRNA for ACE-1 were compared with the control (without treatments) values using ANOVA followed by the Tukey-Kramer test for mean separation. Effects between treatments, cyclic phase and time after incubation in microdialysis fractions were analyzed using ANOVA. The effects of treatments against untreated controls were compared using Dunnett's Multiple Range Test, and effects between treatments as well as cyclic phases were compared using the Tukey-Kramer test. The data were subjected to arcsine transformation before statistical analysis. Probabilities less than 0.05 ($P<0.05$) were considered to be significant

Results

The average time taken by OECs to form a monolayer was 5 days. Cell viability was estimated using Trypan-blue staining and confirmed to be more than 90% at each time of plating as well as at the end of the experiment. The average cell concentration at the end of the experiment was 8×10^4 cells/ ml.

Expression of mRNA for ACE-1 and Ang II release in the bovine oviduct varied with the estrous cycle

Expression of mRNA for ACE-1 was greater ($P<0.001$) during the postovulatory phase than during the luteal and follicular phases of the estrous cycle, which did not differ. The basal release of Ang II during the follicular and post-ovulation phases was about 2-fold greater ($P<0.05$) than that during the luteal phase of the estrous cycle (Fig. 1).

Effect of LH and ovarian steroids on Ang II release in microdialysis

The basal releases of Ang II in the microdialysis for the follicular, postovulatory and luteal phases were 2.3 ± 0.2, 2.5 ± 0.4 and 1.2 ± 0.4 pg/ml Ringer's solution, respectively. The basal releases during the follicular and postovulatory phases were significantly higher ($P<0.01$) than that during the luteal phase (n=5–6).

For oviducts collected during the follicular and postovulatory phases, LH alone ($P<0.05$), and combined infusion of LH+E_2+P_4 ($P<0.05$–0.01) caused an increase in the amount of Ang II collected by microdialysis when compared with the untreated control at the same time point. However, infusion of E_2 did not show any significant effect on Ang II release (Fig. 2).

During the 4–8 h of incubation in both the follicular and post ovulation phases, infusion of LH alone and combined infusion of LH+E_2+P_4 were shown to have a significantly higher effect ($P<0.05$) than infusion of E_2 alone. During 8–12 h of incubation, only combined infusion of LH+E_2+P_4 was shown to produce a significant elevation ($P<0.05$) of Ang II release. Moreover, none of the treatments used stimulated Ang II release during 12–16 h of

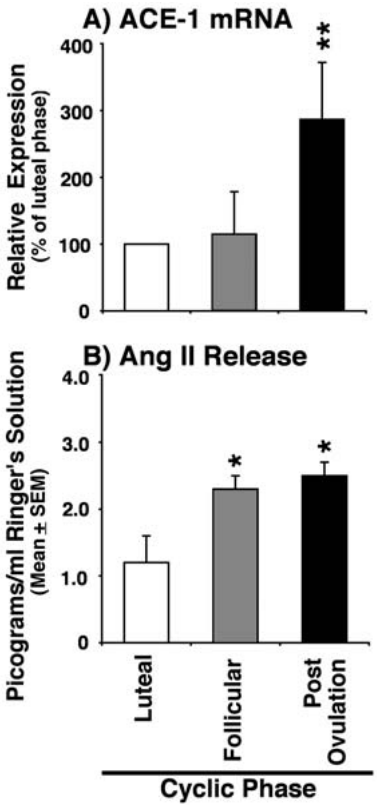

Fig. 1. A) Angiotensin II converting enzyme-1 (ACE-1) mRNA expression and B) angiotensin II (Ang II) release in the oviducts collected during different phases of the estrous cycle (n=4–5). *P<0.05 and **P<0.01 *vs*. values obtained for the luteal phase.

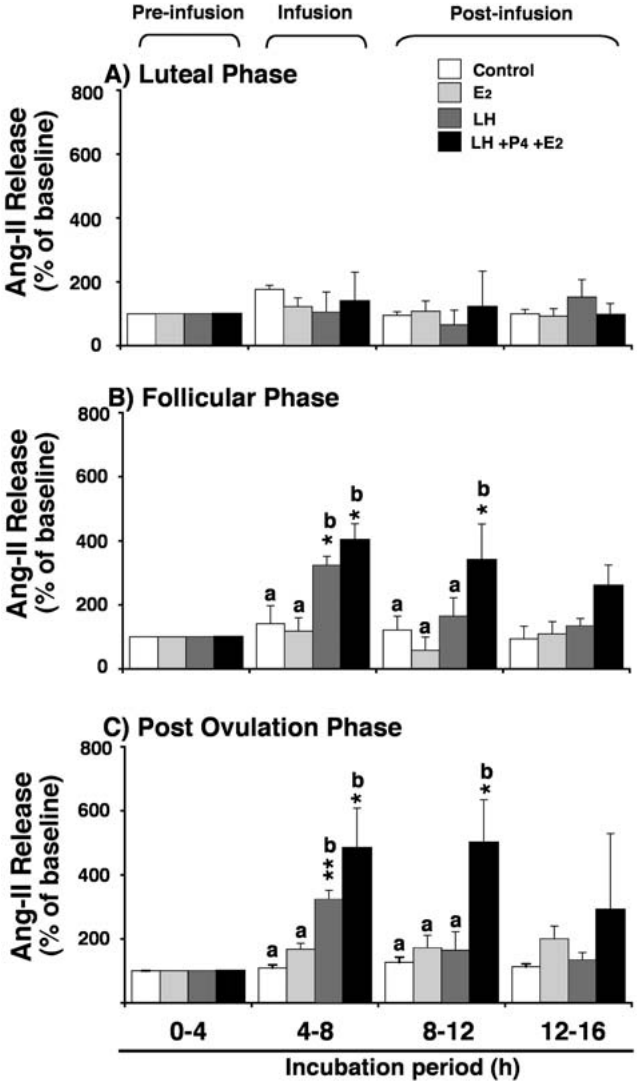

Fig. 2. Effect of the infusion of LH (1 μg/ml), E_2 (100 ng/ml) or P_4 (100 ng/ml) on the release of Ang II in microdialyzed bovine oviducts during the A) luteal phase, B) follicular phase and C) postovulatory phase of the estrous cycle (n=4–5 cows; mean ± SEM), *P<0.05, ** P<0.01, *** P<0.001 *vs*. the values at the same point in the control group. [a,b] P<0.05 *vs*. values at the same time point between treatments.

incubation in any cyclic phase (Fig. 2).

None of the treatments stimulated Ang II release during the luteal phase. The stimulatory effects of the treatments between the follicular and post-ovulation phases were not significant (Fig. 2).

Effect of LH and ovarian steroids on Ang II biosynthesis and release in the cultured OECs

Treatments with LH and LH+P_4+E_2, but not E_2 alone, increased (P<0.05) Ang II release and ACE-1 mRNA expression in the OECs (Fig. 3).

Discussion

Our results provide the first direct evidence that expression of ACE-1 and release of Ang II in the oviduct is higher during the periovulatory period of the estrus cycle. Furthermore, we showed that LH upregulates mRNA expression for ACE-1 and release of Ang II in the bovine oviduct while ovarian steroids synergistically enhance the stimulatory effect of LH on Ang II synthesis and release. Thus, an active oviductal Ang II system is regulated by major endocrine factors for estrus and ovulation during the periovulatory period. As a result, Ang II may enhance local oviductal contraction and promote rapid transport of gametes to the fertilization site.

In this study, two *in vitro* systems were utilized to investigate the effect of LH and ovarian steroids on Ang II biosynthesis and release. The microdialysis allows cell-to-cell integrity and cell-to-cell communication to be maintained and enables observations of real-time changes in the local release of various substances [11]. On the other hand, isolation and culture of OECs provides a simple, abundant source of cells for testing the effects of specific treatments [22]. However, monolayers of bovine OECs will not fully substitute for a fully differentiated oviductal epithelium in contact with underlying connective tissue [23]. In the present study, the

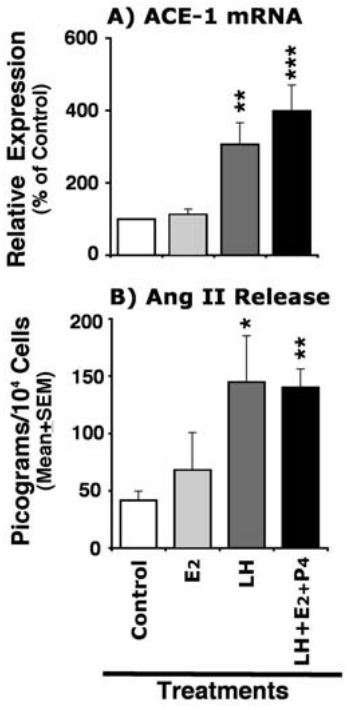

Fig. 3. Effect of LH (10 ng/ml), E_2 (1 ng/ml) or P_4 (1 ng/ml) on A) ACE-1 mRNA expression and B) Ang II release (mean ± SEM) from OEC monolayers after 24 h of incubation (n=4–5). *P<0.05, **P<0.01, ***P<0.001 *vs*. controls.

stimulatory effects of LH and ovarian steroids on Ang II release were observed in the microdialysis and OEC culture experiments. Thus, intact histoarchitecture of the oviduct may not be an essential component for the function of OECs and OECs can, at least in part, independently react to various factors.

It has been reported that the ACE-1 in an ejaculate can modulate generation of Ang II in the female tract to affect smooth muscle tonus of the uterus and the oviduct, which could facilitate sperm transport [3]. Higher concentrations of ACE-1are found in the epithelial cells of the oviducts of non-pregnant sheep [24], and ACE-1 activity in the sow oviduct peaks at estrus and metestrus [25]. Our results show that ACE-1 mRNA expression is greater during the postovulatory phase and that Ang II release by oviductal tissues is greater in the follicular and postovulatory phases than the luteal phase of the estrous cycle. Thus, these results emphasize the importance of the Ang II system during the periovulatory period when both the LH and E_2 levels are enhanced. The lack of significant elevation of the ACE-1 mRNA level when Ang II release is increased during the follicular phase is difficult to explain; however, it has been reported that E_2 induces hepatic angiotensinogen synthesis and release [26, 27] and that the plasma rennin concentration is higher during estrus [28] in rats. Thus, the elevated precursor levels in the oviducts coincident with the elevated systemic E_2 levels during the follicular phase could be attributed to increased Ang II release without significant rise in the ACE-1 mRNA level.

Previously, we have shown that LH activates a system of various vasoactive peptides in the bovine oviduct, including ET-1 [8], vascular endothelial growth factor (VEGF) [29] and tumor necrosis factor α (TNFα) [7, 8]. In the present study, we also observed that LH upregulates ACE-1 mRNA expression in cultured OECs and that LH stimulates the release of Ang II in both microdialysis and cell culture. Furthermore, ovarian steroids synergistically enhanced the above effects of LH on ACE-1 mRNA expression and Ang II release. Moreover, a higher expression of mRNA for ACE-1 was observed only during the postovulatory phase of the estrous cycle, which is after exposure of the oviduct to the preovulatory LH surge. These observations provide evidence that the oviductal Ang II system is activated after the LH surge, and this activation may be mediated through upregulation of oviductal ACE-1. Therefore, the preovulatory LH surge can be considered as the crucial factor, which activates oviductal muscle contraction.

On the other hand, human fallopian tubes at the secretory phase contain more LH/human chorionic gonadotrophin (hCG)-receptors (R) than at the proliferative phase [30], and this variation in LH-R levels with reproductive stage suggests that the LH-Rs are regulated by other reproductive hormones [31]. It has been reported that E_2 promotes the synthesis of LH/hCG-Rs in the pig myometrium [32], and combined administration of E_2 and P_4 increases LH/hCG-R capacity, more than E_2 alone [33]. Thus, the synergistic effect of ovarian steroids on LH action in the stimulation of biosynthesis and release of Ang II during the periovulatory period may be partly through activation of LH-Rs in the oviduct.

It has been reported that prostaglandins (PGs) are involved in oviductal contraction, since E-series relax while F-series contract the smooth muscle [34]. Additionally, ET-1 is produced by bovine OECs and is involved in oviduct muscle contractility [35]. We recently reported that PGE_2, $PGF_{2\alpha}$, ET-1 and Ang II directly increase the amplitude of muscular contractions in the bovine oviduct *in vitro* [6, 11]. On the other hand, LH in combination with a basal level of P_4 and a high concentration of E_2 has a maximal stimulatory effect on oviductal release that is similar to the effect of contraction-related-substances such as PGE_2, $PGF_{2\alpha}$ and ET-1 [6, 9–11]. Moreover, our recent reports revealed that TNF_α [7, 8], VEGF [29] and atrial natriuretic peptide (ANP) [6] stimulates the production and release of PGE_2, $PGF_{2\alpha}$ and ET-1in the bovine oviduct [7, 8, 11, 29]. Also, Ang II stimulate PGE_2, $PGF_{2\alpha}$ and ET-1 release [6]. Thus, Ang II appears to be a local mediator for TNF_α, VEGF and ANP to stimulate PGE_2, $PGF_{2\alpha}$ and ET-1 release from the oviduct, in addition to acting directly on oviductal smooth muscle [11].

In summary, our data suggest that the preovulatory LH-surge, together with increasing E_2 release from the Graaffian follicle and P_4 from the regressing CL, upregulates the oviductal Ang II system. Thus, the elevated Ang II interact with other contraction-related substances to activate oviduct contraction for rapid transport of gametes to the fertilization site.

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