Identification of a Novel Role for Endothelins within the Oviduct

Myoungkun Jeoung, Sungeun Lee, Hee-kyung Hawng, Yong-Pil Cheon, Youn Kyung Jeong, Myung Chan Gye, Marc Iglarz, CheMyong Ko, and Phillip J. Bridges

Division of Clinical and Reproductive Sciences (M.J., S.L., C.K., P.J.B.), University of Kentucky, Lexington Kentucky 40536; School of Biosciences and Chemistry (H.H., Y.C.), Sungshin Women's University, Seoul 136-742, Korea; Department of Life Science (Y.K.J., M.C.G.), Hanyang University, Seoul 133-791, Korea; and Preclinical Development, Actelion Pharmaceuticals Ltd. (M.I.), Allschwil CH-4123, Switzerland

Endothelins were first identified as potent vasoactive peptides; however, diversity in the biological function of these hormones is now evident. We have identified a novel role for endothelins: a requirement for these peptides within the oviduct during fertilization and/or early embryo development. *In vivo*, treatment after ovulation with a dual endothelin receptor antagonist (tezosentan) decreased the number of two-cell embryos that could be collected from within the oviducts. *In vitro* fertilization experiments showed that gamete viability and their ability to fertilize were not affected by treatment with this antagonist, suggesting that the effect observed *in vivo* was mediated by the oviduct itself. Expression of mRNA for all three isoforms of the endothelins and both receptor subtypes was detectable within the oviduct. Expression of mRNA for endothelin-3 was regulated by gonadotropins in epithelial cells of the oviduct and increased specifically within the isthmus of this structure. Immunostaining revealed localization of both endothelin receptors A and B to the columnar epithelial cells within the oviduct, suggestive of a local role for endothelins in the regulation of epithelial function and ultimately oviductal secretions. A microarray analysis revealed three likely endothelin-regulated protein networks for future analysis: the TGFβ, IL-10, and CCAAT/enhancer-binding protein superfamilies. Overall, these results suggest a novel and requisite role for endothelins within the oviduct during fertilization and/or early embryo development. **(*Endocrinology* 151: 2858–2867, 2010)**

The oviduct plays a vital role in the establishment of a pregnancy, providing the microenvironment for fertilization and development of the very early embryo. Briefly, ovulated cumulus-oocyte complexes (COCs) enter the oviduct via the infundibulum and are fertilized within the ampulla, the upper section of the oviduct. The developing embryo stays several days within this tubal structure, traversing the lower and muscular isthmus before entering the uterus in preparation for implantation approximately 4 d after fertilization (1). Endocrine, autocrine, and paracrine factors regulate oviductal function, including control of the secretion of essential embryotrophic factors, together facilitating this tissue's role in the establishment of a new pregnancy (2–4). However, the detailed regulation of oviductal function during the first few days after ovulation is still not well understood.

Considering the essential nature of the oviduct in the establishment of a pregnancy, it could be expected that oviductal dysfunction is a primary contributor to female-based infertility and, hence, the need for women to use assisted reproductive technologies to establish a pregnancy (5). With approximately 140,000 assisted reproductive technology procedures reported to the Centers for Disease Control and Prevention in a recent annual report (5), the significance of understanding oviductal function as a means of improving women's health should be easily

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Abbreviations: C/EBP, CCAAT/enhancer-binding protein; COC, cumulus-oocyte complex; ECE1, endothelin converting enzyme-1; EDN1, endothelin-1; EDNRA, endothelin receptor A; hCG, human chorionic gonadotropin; IVF, *in vitro* fertilization; PMSG, pregnant mare serum gonadotropin.

recognized. Inclusive to this would be an understanding in the etiology of diseases causing tubal blockage that are ultimately responsible for a high proportion of cases of female infertility (6, 7). However, irregularities in the precisely controlled secretion of oviductal fluids cannot be overlooked as another cause of poor fertility. Tubal secretions are known to affect both the ability of the gametes to fertilize (4) and of the embryo to subsequently develop (3).

In this paper, we report a novel requirement for endothelins in the regulation of oviductal function and fertility. Endothelins were first identified as potent vasoactive molecules (8); however, a role in female reproductive function has also proven itself to be significant (9–11). The endothelin family contains three 21-amino-acid peptide ligands [endothelin-1 (EDN1), EDN2, and EDN3] (8, 12–14), and EDN1 is the most well-characterized isoform with functions identified in a multitude of biological systems (15, 16). Studies on EDN2 include roles in the kidney, intestine, ovary, placenta, and uterus (10, 13, 17–19), whereas reports on EDN3 predominantly indicate a neural action for this isoform (20, 21). Endothelins exert their biological response by binding to one of two G protein-coupled receptor subtypes, endothelin receptor A (EDNRA) and EDNRB (22). Although the binding affinity of EDNRB to the three ligand isoforms is similar, the affinity of EDNRA strongly favors EDN1 and EDN2 over EDN3 (23).

Smooth muscle contractility of the oviduct is well documented (24, 25), as is the contractile action of endothelins (8, 26). It is therefore not surprising that a contractile effect of endothelins on this tubal structure has also been revealed (10, 27–29). However, endothelins have a diverse portfolio of actions including acting as effectors of proliferation (30), steroidogenesis (30–32), and apoptosis (33–35), and their precise function within an individual tissue must be evaluated with this divergence in mind. Hence, our primary objective was to test the hypothesis that endothelins are required by the oviduct to facilitate fertilization and/or early embryonic development. To meet this objective, experiments were designed to determine whether endothelins are required for fertilization of the male and female gametes themselves or whether endothelin-regulated fertility was mediated by the oviduct. A thorough analysis of the components of the endothelin system within the oviduct was performed to allow accurate extrapolation of any results. Finally, a microarray approach was used to identify novel biological pathways that were affected by endothelins within this structure. Together, the results of these experiments reveal what appears to be a unique role for endothelins within the oviduct, regulating epithelial cell function and fertility.

Materials and Methods

Animals

Mice were purchased from Charles River [Wilmington, MA; for *in vitro* fertilization (IVF)], Dae Han Biolink Co. (Eumsung, South Korea; for immunohistochemistry), and Harlan Inc. (Harlan, IN; all others). Animal procedures involved in these studies were approved by the Sungshin University, Hanyang University, and the University of Kentucky Animal Care and Use Committees, respectively, according to National Institutes of Health guidelines for the ethical use of animals in research.

Immunohistochemistry

Immature female CD1 mice were injected ip with 5 IU pregnant mare's serum gonadotropin (PMSG) to induce follicular development and, 48 h later, with 5 IU human chorionic gonadotropin (hCG) to induce ovulation. Mice were killed 12, 18, and 24 h later. Ovaries with oviducts attached were retrieved at the time of killing and fixed for 4 h in Bouin's fixative and then embedded in paraffin. Sections were cut to 5 μm, mounted on poly L-lysine-coated glass slides, deparaffinized, and rehydrated. Antigen retrieval was performed by autoclaving for 20 min in 10 mM citrate buffer (pH 6.0). The slides were then incubated for 10 min in the dark in 3.5% H_2O_2 in methanol to remove endogenous peroxidase activity, blocked for 20 min in 1.5% normal goat serum, and incubated overnight at 4 C with a primary antibody against either EDNRA (rabbit polyclonal, catalog no. IMG71259; IMGENEX, San Diego, CA) or EDNRB (rabbit polyclonal, catalog no. ab65972; Abcam, Cambridge, UK) at a dilution of 1:1000. After washing in PBS, the slides were then incubated for 30 min at room temperature in a 1:200 dilution of biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) in blocking solution. Slides were rinsed again, and an avidin-biotin peroxidase complex solution was applied for 30 min at room temperature. After additional rinses in PBS, the coloring reaction was conducted with ImmPACT diaminobenzidine peroxidase substrate for 10 min at room temperature, and nuclei were stained with 20% Harris hematoxylin solution. Sections were rinsed again in PBS and then dehydrated in a graded ethanol series, dipped in xylenes, and permanently mounted. Images were obtained using a digital imaging system (DFC320; Leica Microsystems, Wetzlar, Germany), as described previously (36).

Real-time PCR analysis

Immature female CD1 mice were treated with PMSG and hCG, as described above. Mice were killed for tissue collection at 0 h (before treatment with gonadotropins), PMSG +48 h, hCG +12 h, hCG +18 h, and hCG +24 h. Whole oviducts (at all time points) or separated sections of the ampulla and isthmus (at hCG +12, +18, and +24 h) were collected and pooled from at least three mice per time point, with a total of three independent replicates collected for each tissue type and time. Epithelial cells (at all time points) were collected and pooled from the whole oviducts of four mice per time point, with a total of three independent replicates collected. When present, COCs were removed from within the oviduct. Total RNA was isolated from all tissues using Trizol, and cDNA reverse transcribed from each sample of RNA. The concentration and integrity of each sample of RNA was assessed by UV spectroscopy using an Eppendorf Biophotometer-plus (Eppendorf, Hauppauge, NY) as well as by visual

TABLE 1. Primer sequences employed for real-time PCR measurements

Name	Primer sequence (5′–3′)
EDN1	
Forward	TTC CCG TGA TCT TCT CTC TGC T
Reverse	TCT GCT TGG CAG AAA TTC CA
EDN2	
Forward	GTC GAT GCT CCT GCA ACT C
Reverse	TGG CCT TTC TTG TCA CCT CT
EDN3	
Forward	CTG TGT GCT TGA GAC CTG GA
Reverse	TCC CCA AGG ATC CAC ATT TA
EDNRA	
Forward	CAT AGG ACC TGC ATG CTC AA
Reverse	GCC AGG TTA ATG CCG ATG TA
EDNRB	
Forward	CCT GAT GAC CTG CGA AAT G
Reverse	TGC TTC TCC TCC AAG GAC TG
ECE1	
Forward	ACC CTC GGG GAG AAC ATT
Reverse	GCA GCT CCT TCC CTT TTT CT
ECE2	
Forward	TCC CTG ATT TCA TCC TGG AG
Reverse	GAC GCA GAT TCC CTT CTT TG

distinction of 18S and 28S rRNA bands after ethidium bromide staining in an agarose gel. Real-time PCR was then performed to determine expression levels of mRNA for EDN1, EDN2, EDN3, endothelin converting enzyme-1 (ECE1), ECE2, EDNRA, and/or EDNRB using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) and gene-specific primer pairs (Table 1) on a Bio-Rad IQ5 system under similar cycling conditions, as described previously (10). Real-time PCR was performed with a total volume of 25 μl per reaction, with each reaction containing 5 μl cDNA, 1 μl of a 10 μM stock of each primer (forward and reverse), 12.5 μl 2× SYBR Green PCR Master Mix, and 5.5 μl diethylpyrocarbonate-treated water. All primers were designed to amplify a product with a size of 195–205 bases, and the specificity of each primer set was confirmed by running the PCR products on a 1.5% agarose gel. Protocol conditions consisted of denaturation at 95 C for 30 sec, followed by 40 cycles at 94 C for 30 sec, 55 C for 30 sec, and 72 C for 45 sec with a final dissociation (melting) curve analysis. The relative level of expression of each mRNA was standardized against ribosomal protein L19 as a housekeeping gene and analyzed by the $2^{-\Delta\Delta}$CT method (37).

Embryo collection analysis

Immature female CD1 mice were treated with PMSG and hCG, as described above. At the time of hCG administration, females were allocated to pairs, and each pair of females was introduced into the cage of a male of confirmed fertility. Two females were placed with each male to minimize any individual effect of male fertility on the results. At hCG +14 h (after ovulation), one female of each pair was injected with 20 mg/kg body weight (ip) of the dual endothelin receptor antagonist tezosentan (Actelion Pharmaceuticals, Switzerland) and the other with an equal volume of vehicle as a control (PBS, 30 μl). Tezosentan was administered at hCG +14 h because earlier treatment with this antagonist affects ovulation (38) and therefore the number of oocytes that can be fertilized. At hCG +24 h, males were removed and copulation plugs confirmed, and each female received a second dose of the same treatment. The dose of tezosentan was based upon our previous use (10, 38) with the second treatment administered to ensure maintained blockade of endothelin receptors. At hCG +48 h, the female mice were killed, the oviducts were flushed, and the number of two-cell embryos within the oviducts of each mouse was counted. The results reflect the mean ± SEM of four replicated pairs.

IVF analysis

Six-week-old female CD1 mice were treated with PMSG and hCG, as described above. Females were killed at hCG +9 h (before ovulation), and COCs were collected by follicular puncture of the whole ovaries. Male mice were killed at 8.5 h after the females had been injected with hCG, and the cauda epididymis and vas deferens were isolated for sperm collection. To determine whether endothelin receptor activation is required for fertilization of the female gamete, COCs were maintained for 1 h in medium with or without tezosentan (0, 10, or 100 μg/ml). COCs were then washed several times in plain medium, and the rate of IVF was determined following a well-established protocol (39). Similarly, a requirement for endothelin receptor activation on fertility of the male gamete was evaluated by additionally maintaining sperm in medium containing tezosentan (0, 10, or 100 μg/ml) before IVF. In each case, sperm were incubated for 1.5 h at 37 C in Biggers-Whitten-Whittingham medium to allow capacitation (with or without tezosentan), and then the rate of IVF was determined by the addition of sperm ($1–2 \times 10^6$/ml) to individual drops of medium containing COCs. Oocytes were then transferred to fresh medium after 4 h, and the rate of IVF was determined by classification under an inverted microscope (Olympus ×70) after 48 h culture. The results reflect the average of seven biological replicates for each treatment group.

Microarray analysis

Immature female CD1 mice were treated with PMSG and hCG, as described above. At hCG +9 h, mice were treated with tezosentan (20 mg/kg body weight) or PBS as a vehicle control. Tezosentan was administered at hCG +9 h to ensure blockade of endothelin receptors before the increase in ovarian EDN2 that occurs at the time of ovulation (38). Mice were killed for tissue collection 9 h later (hCG +18 h). Whole oviducts were collected and pooled from at least four mice per treatment, with a total of two independent replicates (microarray chips) collected for each treatment. When present, COCs were removed from within the oviducts before processing. Total RNA was extracted from all tissues using Trizol and purified with RNeasy (QIAGEN, Valencia, CA) according to the manufacturer's directions. The integrity of total RNA was verified by visualizing the intact and distinct 28S and 18S rRNA bands on a 1.5% agarose gel. Microarray hybridization was then performed using the Affymetrix Mouse 430-2.0 whole genome arrays (Affymetrix; DNA Microarray Core Facility, University of Kentucky, Lexington, KY), as described previously (40). The signal intensity on each chip was normalized using a quantile normalization algorithm following the Affymetrix GeneChip protocol. The signal for each gene (probe set ID) was normalized to the median signal value for that gene across all the chips among treatment groups. The dataset was then analyzed to identify endothelin-regulated biological pathways. Briefly, the level of expression of each gene was compared between the two treatment groups (PBS *vs.* tezosentan),

and differentially expressed genes were extracted. However, marginally expressed genes had a large impact on the differential expression classifications, so to eliminate this potential problem, we excluded all marginally expressed genes with an intensity of less than 200 from the analysis. Differentially expressed genes were then selected by fold change, with only genes with at least a 1.5-fold treatment effect carried further in the analysis. Overall, 942 genes were selected with an intensity of more than 200 and a fold change of more than 1.5 between the two treatments (PBS *vs.* tezosentan). Selected genes were then subjected to an enrichment analysis using Pathways Studio 6 Software (Ariadne, MD) to indicate potential protein networks affected by treatment. The pattern of gene expression for a random selection of genes that were identified as being differentially expressed by the microarray analysis was then confirmed by real-time PCR analysis, as described above. The selected genes, expression levels, and primer pairs are described in Supplemental Figs. 1–3 and Supplemental Tables 1–4 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Statistical analysis

Datasets were first tested for normality and homogeneity of variance. When appropriate, data were transformed before statistical analysis. Nontransformed data are depicted in all the figures. One-way ANOVA using SigmaStat 3.5 (Systat Software, Inc., Point Richmond, CA) was used to determine differences in expression of mRNA levels and the effect of treatment on rate of IVF. If differences were detected, Tukey's test was used to determine which means differed. The Student's *t* test was used to determine the effect of treatment in the embryo collection analysis.

Results

Expressions of EDNRA and EDNRB in the oviduct

The oviduct is the site of fertilization and very early embryonic development. Studies suggest the involvement of EDNRA and/or EDNRB in mediating the action of endothelins within this structure (10, 28), and we hypothesized a requirement for these peptides in fertility. However, no clear analysis of the spatial and temporal expression of both receptor subtypes within this tissue around the time of ovulation and fertilization is available. Hence, real-time PCR and immunohistochemistry were used to determine the relative level of expression and location of endothelin receptors (mRNA and protein) within the oviduct. Expression of mRNA for both EDNRA and EDNRB was detectable in oviducts at all times examined (Fig. 1) and was not affected by treatment with tezosentan in the microarray analysis. Expression of mRNA for EDNRA in whole oviducts was not affected by treatment with gonadotropins ($P > 0.05$; Fig. 1A). In contrast, expression of mRNA for EDNRB in whole oviducts was increased by gonadotropins ($P < 0.05$), with maximal levels observed at 12 h after hCG was injected to induce ovulation. Consistent with expression of mRNA in whole oviducts, no differences in levels of mRNA for either subtype were observed in the ampulla at 12–24 h after hCG ($P > 0.05$; Fig. 1B), when both receptors were shown to be localized to the epithelial cells adjacent to the ampullary lumen (Fig. 2). Interestingly, expression of EDNRA appeared relatively homogeneous among the epithelial cells of the ampulla, whereas some heterogeneity was observed in the expression of EDNRB. Within the isthmus of the oviduct, expression of mRNA for EDNRA was increased at 24 h after hCG, in contrast to the expression of mRNA for EDNRB, which showed a concurrent decrease in expression at this same time of collection ($P < 0.05$). Immunohistochemistry revealed that both subtypes were strongly expressed within the columnar epithelial cells of the isthmus; however, minor smooth muscle or stromal expression of both receptors can be observed (especially for EDNRB), consistent with reports of endothelin-induced oviductal contraction (10, 27–29). Because no dramatic differences in the temporal expression or location of endothelin receptors were revealed by immunohistochemical analysis of sections from oviducts collected at hCG +12, +18, or

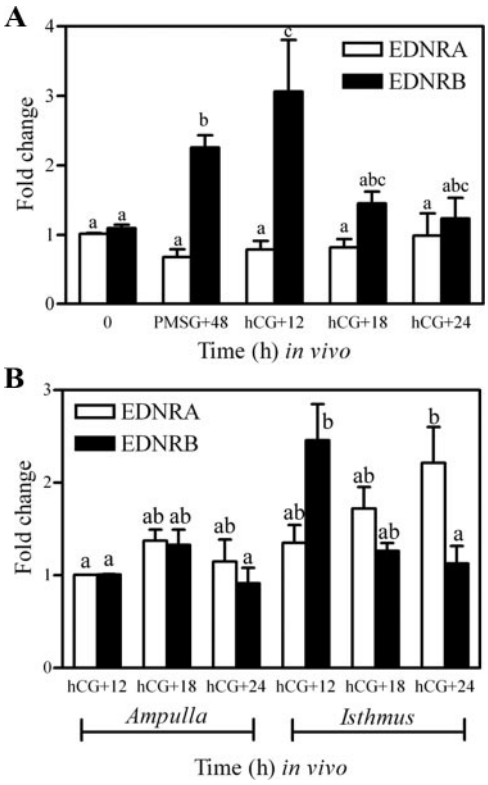

FIG. 1. Expression of mRNA for EDNRA and EDNRB in whole oviducts (A) and isolated sections of ampulla and isthmus (B) collected from gonadotropin-primed mice. Immature mice were treated with 5 IU PMSG to induce follicular development and with 5 IU hCG 48 h later to synchronize ovulation. Tissues were collected at the times described in each *panel*. Data are the means ± SEM of triplicate replicates from each of three samples per time point. Levels of mRNA were obtained by real-time PCR and are expressed as fold changes. For each mRNA within a panel, values with different *superscript letters* differ ($P < 0.05$).

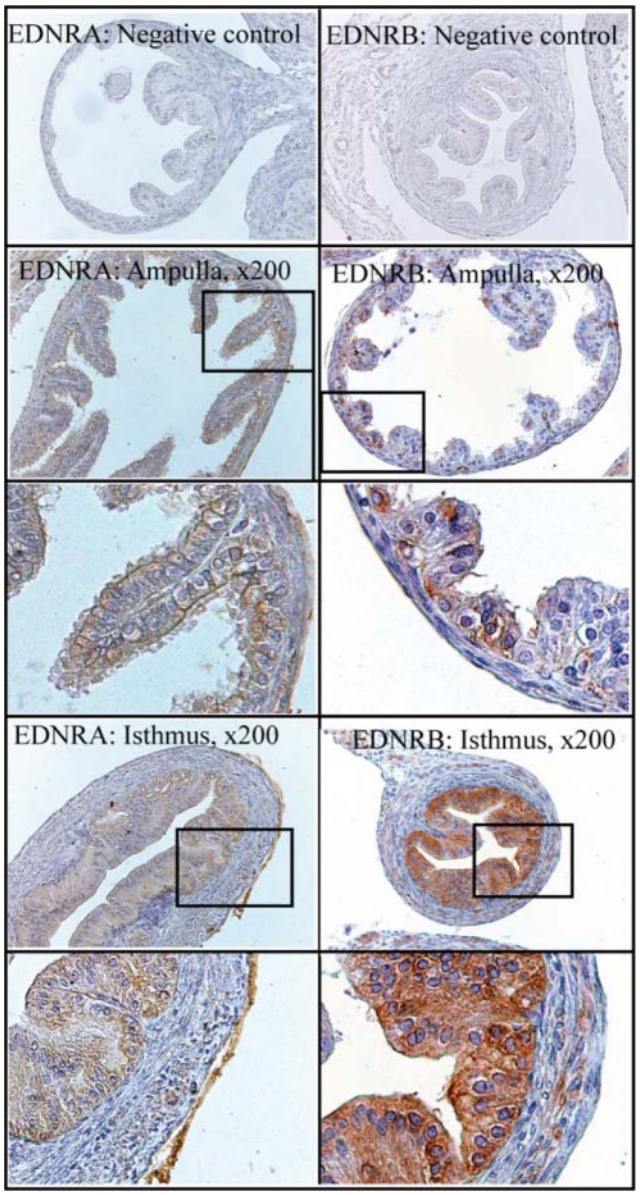

FIG. 2. Immunohistochemical localization of EDNRA and EDNRB in the ampulla and isthmus of the mouse oviduct. Immature mice were treated with 5 IU PMSG to induce follicular development and with 5 IU hCG 48 h later to synchronize ovulation. Tissues were collected at hCG +18 h, fixed in Bouin's fixative, and embedded in paraffin. A higher magnification of the *boxed* section is presented for each receptor subtype and section of the oviduct.

+24 h, immunohistochemical expression at hCG +18 h alone is presented. Note that the intensity of staining cannot be used to compare levels of protein between the two receptor subtypes.

Requirement for endothelins in early embryonic development, *in vivo*

Real-time PCR and immunohistochemistry revealed abundant expression of both receptor subtypes in the oviduct, especially within the secretory epithelial cells lining the oviductal lumen. We hypothesized that endothelin receptor-mediated actions would be required to fulfill the oviduct's physiological goal of facilitating fertilization and early embryonic development and used an *in vivo* fertility analysis to test this hypothesis. Mice were primed with gonadotropins, bred, and treated with the dual endothelin receptor antagonist tezosentan or PBS as a vehicle control. Treatment of mice with tezosentan had a dramatic effect on the number of two-cell embryos that were retrieved from within the oviducts (Fig. 3). Tezosentan induced a decrease in the number of two-cell embryos to only 21% of vehicle-treated controls (17.8 ± 4.1 *vs.* 3.8 ± 2.3, $P < 0.05$), suggesting that endothelins play a vital role in the establishment of a new pregnancy.

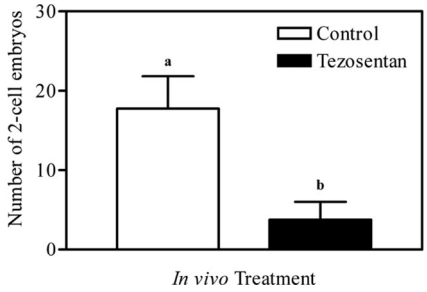

FIG. 3. Number of two-cell embryos collected from the oviducts of mice treated with vehicle (PBS, control) or tezosentan. Immature mice were treated with 5 IU PMSG to induce follicular development and with 5 IU hCG 48 h later to synchronize ovulation. At the time of hCG treatment, gonadotropin-primed females were bred with fertile males. Females were treated with PBS or tezosentan at hCG +14 h and hCG +24 h. Mice were killed at hCG +48 h and the number of two-cell embryos counted after flushing the oviducts. Treatment with tezosentan decreased the number of two-cell embryos that were retrieved from within the oviducts at the time of killing (a *vs.* b, $P < 0.05$).

Requirement for endothelins in fertilization, *in vitro*

To determine whether the dramatic reduction in the number of two-cell embryos collected after treatment with tezosentan *in vivo* was due to a direct inhibitory or potentially toxic effect of that antagonist on the male or female gamete, the same receptor antagonist was used in a series of experiments using IVF, thus removing the oviduct as a site of endothelin-mediated action. Treatment of COCs with tezosentan had no effect on the rate of IVF ($P > 0.05$; Fig. 4A), nor did treatment of sperm ($P > 0.05$; Fig. 4B). These results indicate that the effect observed after treatment of the antagonist *in vivo* is not due to a direct inhibitory or toxic effect on the viability and ability of gametes to fertilize but rather an indirect effect mediated at the level of the oviduct. It is important to note that the higher dose of tezosentan used *in vitro* (100 μg/ml) is quite comparable to the dose used *in vivo*. Twenty milligrams per kilogram body weight (*in vivo*) reflects a total dose of 400 μg injected into a 20-g mouse (*vs.* 100 μg/ml culture medium). Overall, these results suggest that the requirement for endothelins in the processes of

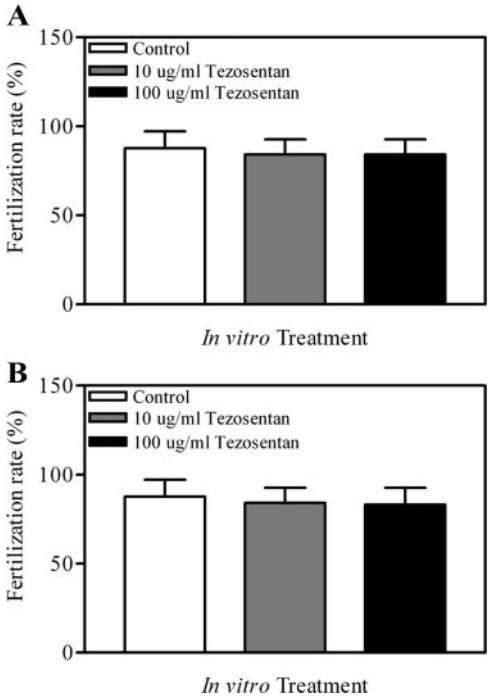

FIG. 4. Rate of IVF after treatment of incubation media containing COCs (A) or COCs plus sperm (B) with vehicle (PBS) or 10 or 100 μg/ml tezosentan. COCs were collected from 6-wk-old gonadotropin-primed females; sperm was collected from males of proven fertility. No effect of treatment with tezosentan on the rate of IVF was detected ($P > 0.05$).

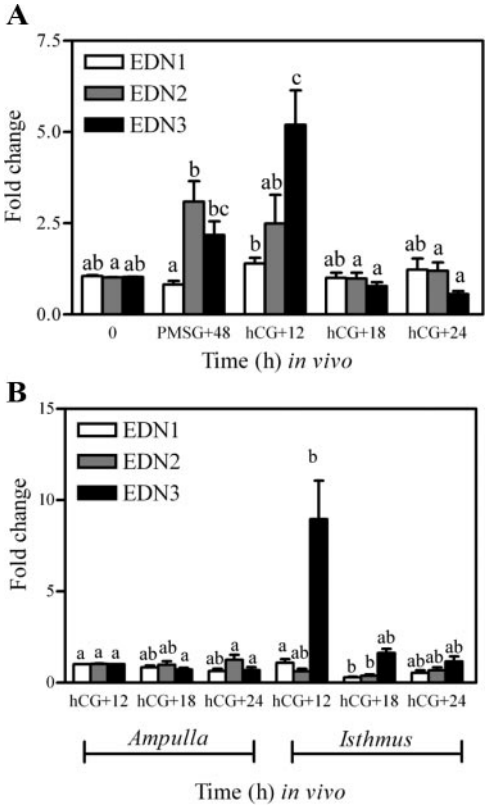

FIG. 5. Expression of mRNA for EDN1, EDN2, and EDN3 in whole oviducts (A) and isolated sections of ampulla and isthmus (B) collected from gonadotropin-primed mice. Immature mice were treated with 5 IU PMSG to induce follicular development and with 5 IU hCG 48 h later to synchronize ovulation. Tissues were collected at the times described in each panel. Data are the means ± SEM of triplicate replicates from each of three samples per time point. Levels of mRNA were obtained by real-time PCR and are expressed as fold changes. For each mRNA within a panel, values with *different superscript letters* differ ($P < 0.05$).

fertilization and/or early embryonic development is mediated by the oviduct itself.

Expression of mRNA for ECE1, ECE2, EDN1, EDN2, and EDN3 in the oviduct

The results above indicated a requirement of endothelin receptor activation for fertilization and/or early embryonic development at the level of the oviduct. Both receptor subtypes are predominantly expressed in the secretory luminal epithelial cells of the oviduct; however, the endothelin isoform mediating this effect is unknown. To begin to elucidate the mechanism whereby endothelins are regulating fertilization and/or early embryonic development, identification of the converting enzymes and isoforms present within the oviduct and their spatial and temporal distribution was needed. Hence, real-time PCR was used to determine the expression of mRNA for ECE1, ECE2, EDN1, EDN2, and EDN3 in whole oviducts as well as sections of isolated ampulla and isthmus. In whole oviducts, no difference was detected in the temporal expression of mRNA for ECE1 ($P > 0.05$); however, levels of mRNA for ECE2 were lower at hCG +18 h when compared with 0 h and PMSG +48 h ($P < 0.05$). Consistent with these findings, no differences were detected in expression of mRNA for ECE1 and ECE2 in isolated sections of ampulla and isthmus collected at hCG +12, +18, and +24 h ($P > 0.05$). Unexpectedly, gonadotropin-induced expression of mRNA for EDN3 was the most readily detected endothelin subtype mRNA observed in the whole oviducts (Fig. 5). Expression of mRNA for EDN3 was increased by treatment with gonadotropins, with maximal expression found in oviducts collected at hCG +12 h ($P < 0.05$; Fig. 5A). Levels of mRNA for this isoform decreased by hCG +18 h ($P < 0.05$) and remained low thereafter. Further analysis of mRNA for EDN3 in 1) spatially distinct sections of the oviduct (Fig. 5B) and 2) isolated epithelial cells collected from whole oviducts (Fig. 6) suggested that the increase in mRNA observed at hCG +12 h was specific to epithelial cells of the isthmus. In contrast to the temporal and spatial regulation of mRNA for EDN3, expression of mRNA for EDN1 and EDN2 remained relatively stable with only minor changes in expression of these mRNA observed. Overall, determination that mRNA for EDN3 was abundant and increased within the epithelial cells around the time of ovulation (hCG +12 h) is a very unique finding. Given the expression of EDNRA and EDNRB within the luminal epithelium of the oviduct,

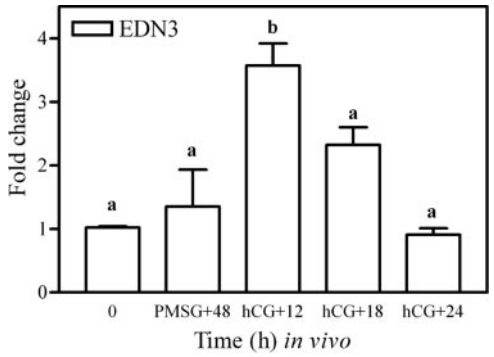

FIG. 6. Expression of mRNA for EDN3 in isolated epithelial cells collected from the whole oviducts of gonadotropin-primed mice. Immature mice were treated with 5 IU PMSG to induce follicular development and with 5 IU hCG 48 h later to synchronize ovulation. Tissues were collected at the times indicated and epithelial cells pooled from four mice for each sample. Data are the means ± SEM of duplicate replicates from each of three samples per time point. Levels of mRNA were obtained by real-time PCR and are expressed as fold changes. Values with *different superscript letters* differ ($P < 0.05$).

with spatial and temporal differences in expression of mRNA for the three endothelin isoforms, it appears that multiple isoform/receptor-specific pathways are involved in affecting oviductal function and fertility.

Analysis of endothelin-regulated pathways in the oviduct

Endothelin receptor antagonism resulted in a decrease in the number of developing two-cell embryos, and mRNAs encoding all three of the ligands were expressed in the oviduct. To elucidate the biological pathways governed by these peptides, a microarray analysis was performed using a similar experimental paradigm as that used for the *in vivo* fertility analysis, and genes were extracted and sorted to build a database of endothelin-regulated biological pathways. Using the extraction guidelines described in *Materials and Methods*, treatment with tezosentan resulted in the regulation of 14 major protein networks (Table 2). Three of these 14 protein networks were suggested by 26–28 reference genes that are shown in Supplemental Figs. 1–3. Overall, the pathway analysis indicated that the TGFβ, IL-10, and CCAAT/enhancer-binding protein (C/EBP) families are under the regulation of endothelins in the oviduct.

TABLE 2. Endothelin-regulated pathways identified by microarray analysis

Name	Number of regulated genes	*P* value
TGF family	28	0.025
IL10	28	0.027
CEBPA	26	0.043
Histone	13	0.039
PRKCA	10	0.038
ACTB	8	0.040
IL3	7	0.047
MYOD1	7	0.033
Notch	7	0.029
LEF	7	0.017
RUNX1T1	7	0.001
NPPA	6	0.037
TERT	5	0.049
SMAD6	5	0.037

Discussion

The oviduct provides the microenvironment for fertilization and subsequent development of the early embryo. We now provide evidence that endothelins are required by the oviduct to facilitate the initiation of a pregnancy. Treatment of mice with an endothelin receptor antagonist *in vivo* resulted in a dramatic decrease in the number of two-cell embryos that could be collected from within the animals' oviducts. Subsequent *in vitro* experiments using the same receptor antagonist as well as immunolocalization studies were consistent in their indication that the effect observed was mediated by the oviduct and not the gametes themselves. Furthermore, both endothelin receptor subtypes (EDNRA and EDNRB) were highly expressed in the luminal epithelium of the oviduct, suggestive of requisite endothelin-regulated function of the epithelial cells. Regulated expression of mRNA for EDN3 within the epithelial cells of the oviduct (likely the epithelial cells of the isthmus) indicated a novel and local role for the least understood of the endothelin isoforms; however, with all three isoforms and both receptors expressed within this structure, multiple ligand- and receptor-specific biological pathways are expected to be involved in endothelin-regulated function in the oviduct.

Considering that many proteins such as growth factors, enzymes, protease inhibitors, and cytokines have been identified within oviductal fluid (41), it was not unexpected that receptor localization indicated potential endothelin-regulated epithelial cell functions. This is especially pertinent when considering that epithelial cell secretions have proven crucial for fertilization and early embryonic development (42–44). Our observation that mRNA for EDN3 was highest in epithelial cells around the time of ovulation is also consistent with the cyclic changes in the secretory activity of the oviduct as a whole. Maximal numbers of the secretory cells are observed around the time of ovulation (45, 46), and endothelins are known mediators of cell secretion in other tissues (47). However, it should be emphasized that a role for EDN3 in the regulation of epithelial cell secretion would be distinct from the known contractile role of these peptides (10, 27–29), which is likely mediated through stromal expression of the two endothelin receptor subtypes. Previous reports have

localized EDN3 to the pituitary gland, brain, and intestinal epithelial cells (48–52), and the literature suggests that this isoform functions in roles other than the typical endothelin-induced contractility (20, 48, 50, 53). EDN3 is required for the development of neurons and melanocytes (50) with both the EDN3 and EDNRB knockouts sharing a similar Hirschsprung's disease-like phenotype (50). Determination of a gonadotropin-induced increase in mRNA for EDN3 in epithelial cells and the isthmus of the oviduct was therefore an exciting finding; however, the mechanism involved in endothelins as facilitators of fertility is far from understood, especially when considering potential differences in the affinity of each receptor subtype for its available ligands (23).

Results of the isoform and receptor analysis suggest the likelihood that multiple endothelin-regulated pathways will be unraveled within the oviduct. With that in mind, it appeared that a microarray analysis was the most appropriate approach to take with the objective of determining the mechanisms whereby endothelins are regulating oviductal function and fertility. Overall, the TGFβ, IL-10, and C/EBP families appeared to be key candidate pathways for further study. However, caution in interpretation is warranted because these biological pathways are drawn from microarray studies and computer-generated literature searches. Of these three candidate pathways, endothelin regulation of the TGFβ superfamily was not a surprising finding given that TGFβ is a known regulator of reproductive functions, including of those within the oviduct (54–59). In fact, TGFβ1, TGFβ2, and TGFβ receptors are expressed in the oocyte, embryo, and oviduct of the mouse (59–61), and analysis of human oviductal fluid has suggested a role for the TGFβ family in gamete maturation and early embryo development (60, 61). Given that the expression level of TGFβ2 in the oviduct is most abundant before fertilization, declining rapidly thereafter (61) (59), it appears likely that infertility due to treatment with tezosentan in the studies described herein is mediated in part through this network.

The gene pathway analysis also indicated that the IL-10 and C/EBP families were regulated by tezosentan. IL-10 has several reported immunosuppressive effects, including suppression in production of proinflammatory cytokines such as IL-1, IL-6, IL-8, TNFα (62, 63), and prostaglandins (62). This is suggestive of an endothelin-regulated immune response within the oviduct in which cytokines like IL-10, secreted from epithelial cells of the oviduct, may change the microenvironment faced by the early developing embryo, *i.e.* regulate immunological factors involved in early embryonic development. Less specific than the regulation of TGFβ and IL-10 is endothelin regulation of C/EBPs. These transcription factors are known regulators of cell differentiation, proliferation, apoptosis, and gene expression in several organs (64–67). In reproductive function, C/EBPβ is a downstream mediator of ERK1/2, which is activated by gonadotropins and plays an important role in fertilization, embryo development (68), and implantation (69, 70).

In conclusion, antagonism of endothelin receptors decreased the number of two-cell embryos developing within the oviduct. The effect was not mediated directly by the gametes but by the oviduct itself. Gonadotropin-induced expression for mRNA for EDN3 within the epithelial cells of the isthmus of the oviduct suggests a specific role for this isoform during the processes of fertilization and/or early embryonic development. However, multiple receptor- and ligand-specific pathways were likely disrupted by the antagonist. Overall, the results indicate that endothelins play an important role within the oviduct in the initiation of a pregnancy.

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Address all correspondence and requests for reprints to: Phillip Bridges, Division of Clinical and Reproductive Sciences, University of Kentucky, Lexington, Kentucky 40536. E-mail: pbrid2@email.uky.edu.

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