

Published in final edited form as:

Can J Physiol Pharmacol. 2008 June ; 86(6): 310–319. doi:10.1139/y08-025.

Role of hypoxia in the regulation of periovulatory endothelin-2 (EDN2) expression in the mouse

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Abstract

Endothelin-2 (EDN2) was recently proposed as a granulosa cell-derived contractile signal that facilitates ovulation (Ko et al., *Endocrinology* 147(4):1770-1779, 2006). Spatially, *Edn2* mRNA expression is restricted to granulosa cells of periovulatory follicles. Temporally, mRNA for this contractile peptide is expressed immediately prior to follicle rupture. The primary objective of this study was to test the hypothesis that hypoxia mediates EDN2 expression in granulosa cells at ovulation and if so, to determine the region within the EDN2 promoter responsible for this effect. To determine the effect of hypoxia on *Edn2* mRNA expression, immature mice were treated with 5 IU PMSG followed 48 h later by 5 IU hCG. Granulosa cells were isolated at 9 h after hCG, cultured under normal or hypoxic conditions and the expression level of mRNA for *Edn2* was compared. *Edn2* mRNA expression was increased when granulosa cells were cultured in a hypoxic environment ($p < 0.05$). Subsequent promoter analysis found that the 5'upstream region of the EDN2 promoter (between -1894 and -1407 bp) was responsible for hypoxia-mediated changes in EDN2 expression. This promoter region contains multiple sites for potential transcriptional regulation including that by hypoxia-inducible factor 1 (HIF-1; ACGTG) at -1297 bp. The second objective of this study was to determine whether the progesterone receptor (PR) or cyclooxygenase-2 (COX-2), two key regulators of periovulatory events, controlled EDN2 expression. To accomplish this, gonadotropin-primed mice were treated with RU-486 or indomethacin and expression of mRNA for *Edn2* was determined in ovaries collected at 12 h after hCG. Treatment with RU-486 or indomethacin did not affect expression of mRNA for *Edn2* ($p > 0.05$). Taken together, it is believed that hypoxia, but not the PR or COX-2, regulate gonadotropin-induced EDN2 expression in the periovulatory follicle.

Keywords

endothelin-2; ovulation; ovary; hypoxia

Introduction

Follicular development and ovulation are dynamic processes. Our understanding of the regulatory mechanisms controlling the growth and final differentiation of a mammalian

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follicle has advanced exponentially since the introduction of modern molecular biology techniques. However, our understanding of even the most fundamental pathways is still not complete. The regulatory mechanism controlling production of endothelin-2 (EDN2) is one of these pathways. Endothelin-2 (EDN2) is a small, 21 amino acid peptide that is produced by granulosa cells at the time of ovulation (Ko et al., 2006). Expression of mRNA for *Edn2*, but not *Edn1* or *Edn3*, is dramatically increased in the periovulatory follicle for only a very brief period of time (1-2 h) around ovulation and blockade of endothelin receptor binding will delay/inhibit the process of follicular rupture (Ko et al., 2006). This rather unique pattern of mRNA expression suggests the involvement of direct signaling pathways to accurately coordinate controlled production of this peptide and we propose hypoxia as one such regulatory signal.

Intra-follicular changes in oxygen concentration are expected when the structure of the growing follicle is taken into consideration. Granulosa cell differentiation, proliferation and the accumulation of follicular fluid are occurring in an avascular compartment, adjacent to a richly vascularized theca externa which is acting under the coordinated action of an array of angiogenic factors (reviewed in (Berkholtz et al., 2006; Fraser, 2006)). Indeed, oxygen partial pressure is reported to decrease with increasing follicular size in women (Fischer et al., 1992) and swine (Basini et al., 2004). Furthermore, hCG regulates expression of hypoxia inducible factor-2 α in human granulosa-lutein cells (Herr et al., 2004) and we have observed increased expression of hypoxia-inducible factor-1-responsive gene in granulosa cells during the periovulatory period in rats (unpublished microarray data). Overall, several lines of evidence are consistent with the induction of hypoxic stress within the periovulatory follicle prior to ovulation.

Hence, we hypothesized that hypoxia is regulating expression of EDN2 in granulosa cells at the time of ovulation. In this manuscript experiments are described that first evaluated whether a standard gonadotropin-primed granulosa cell culture system (Jo and Curry, 2006) was a suitable model to study EDN2 gene regulation *in vitro*. We then modified the protocol for cell collection to more thoroughly test the effect of hypoxia on periovulatory EDN2 gene expression. Following this, we proceeded to evaluate the role of the two most common mediators of periovulatory gene expression (the progesterone receptor and cyclooxygenase-2 (reviewed in (Richards et al., 2002; Sirois et al., 2004)) as potential mediators of EDN2 production. Finally, we used a dual-luciferase reporter assay to identify regions of the EDN2 promoter that may contain important hypoxia-mediated transcriptional elements for future study.

Materials and Methods

Reagents

Pregnant mare's serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), RU-486, indomethacin and all other reagents used for cell culture were purchased from Sigma (St. Louis, MO). Reagents used for real-time PCR were purchased from Applied Biosystems (Foster City, CA). All other specific reagents are listed below.

Animals

Immature female CD-1 mice were purchased from Harlan Inc. (Harlan, IN). Animals were maintained in a 14 h light, 10 h dark cycle and given a continuous supply of chow and water. Follicular development and ovulation were induced by treatment of 23-25 day old mice with 5 IU of PMSG followed 48 hours later by 5 IU hCG. Animals were sacrificed for tissue collection, at the times described for each experiment, by CO₂ inhalation followed by

cervical luxation. The University of Kentucky Animal Care and Use Committee approved all animal procedures.

Granulosa cell culture

Ovaries were collected from gonadotropin-primed mice and granulosa cells isolated by follicular puncture, as described previously (Ko et al., 1999). Briefly, granulosa cells were collected in cold serum-free 4F medium consisting of 15 mM HEPES (pH 7.4), 50% DMEM and 50% Ham's F12 with bovine transferrin (5 µg/ml), human insulin (2 mg/ml), hydrocortisone (40 ng/ml) and antibiotics. Cells were washed three times in 4F medium then plated on serum-coated six-well plates at a density of approximately 1×10^6 cells per well and incubated at 37°C in a humidified atmosphere using modular incubation chambers under either normal (20% O₂, 5% CO₂, 75% N₂) or hypoxic (0.1% O₂, 5% CO₂ with N₂ base) conditions. To examine the induction of mRNA for *Edn2* by treatment with an ovulatory dose of hCG *in vivo* versus *in vitro*, granulosa cells were collected from ovaries of gonadotropin-primed mice sacrificed at 48 h after PMSG and cultured with or without 5 IU hCG under normal oxygen conditions for 0, 6 or 12 h. Granulosa cells collected from ovaries obtained at 12 h after hCG served as *in vivo* controls. To examine the effect of hypoxia on expression of mRNA for *Edn2*, granulosa cells were collected from gonadotropin-primed mice sacrificed at 9 h after treatment with hCG and cultured under normal or hypoxic condition for 0, 3, 6 or 9 h. All cultures were performed in duplicate and replicated 3 times on different days.

In vivo experiment

The progesterone receptor (PR) and cyclooxygenase-2 (COX-2), two well defined mediators of periovulatory events within the ovary, are required for ovulation (Dinchuk et al., 1995; Lydon et al., 1996; Lim et al., 1997). Recently, it was proposed that expression of EDN2 in granulosa cells was regulated by the progesterone receptor (Palanisamy et al., 2006). To test this hypothesis and further explore the regulatory pathway of EDN2 gene expression, expression of mRNA for *Edn2* was determined in ovaries collected from mice treated with the PR antagonist RU-486 or the cyclooxygenase inhibitor, indomethacin. Gonadotropin-primed mice (n = 6-8 per treatment) were left untreated (as a control for the vehicle), or treated with vehicle alone (DMSO), with 10 mg/kg body weight RU-486 i.p. at 47 h after PMSG or with 10 mg/kg body weight indomethacin at 49 h after PMSG (1 h after hCG). Ovulation was induced in all mice by the administration of hCG at 48 h after PMSG. At 12 h after hCG, mice were anaesthetized and one ovary / mouse collected and snap frozen on dry ice for later RNA extraction and measurement of *Edn2* mRNA by real-time PCR. Mice were sacrificed at 20 h after hCG and ovulation rate of the contralateral ovary determined, as described previously (Ko et al., 2006; Al-Alem et al., 2007). Due to the inconsistent effect of treatment with either DMSO, RU-486 or indomethacin on ovulation rate (Fig. 4A), it was determined that the best approach to test the hypothesis that EDN2 gene expression was regulated by the PR and/or COX-2 was to measure mRNA for *Edn2* in a selected group of these ovaries (discussed later). Thus, mRNA for *Edn2* was measured in ovaries collected at 12 h after hCG when the contralateral ovary was shown to ovulate normally (DMSO) or when ovulation rate was low (RU-486 and indomethacin).

Real-time PCR analysis

To determine the level of expression of mRNA for *Edn2* in mice during follicular development, ovulation and luteal development, real-time PCR analysis was performed using ovaries collected at 48 h after PMSG administration and at 6 h, 12 h or 24 h after hCG injection (n = 3 animals/time point). To determine the effect of hypoxia or hCG on EDN2 gene expression *in vitro*, real-time PCR was performed using granulosa cells collected at the completion of the aforementioned cultures. In each case, total RNA was extracted from

ovaries or granulosa cells with TRIzol (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's directions and the integrity of RNA verified by visualization of distinct 18S and 28S rRNA bands after ethidium bromide staining in an agarose gel. cDNA was reverse transcribed from each sample of total RNA as described previously (Ko et al., 2006) and real-time PCR performed using the Applied Biosystems 7300 detection system (Applied Biosystems, Foster City, CA). Expression levels of mRNA for *Edn2* were determined relative to GAPDH as an endogenous control. cDNA for EDN2 and GAPDH was amplified using TaqMan® Universal PCR Master Mix (Applied Biosystems) and the following EDN2 gene-specific primers and probes: *Edn2*-F 5'-CTC CTG GCT TGA CAA GGA ATG-3'; *Edn2*-R 5'-GCT GTC TGT CCC GCA GTG TT-3'; *Edn2*-Probe 5'-/56-FAM/TCT GCC ACC TGG ACA TCA TCT GGG T/36-TAMSp/-3' and GAPDH primer and probe were used from TaqMan® Rodent GAPDH Control Reagents (Applied Biosystems). cDNA generated from the appropriate cells at each treatment or time point was included in each PCR reaction and the analysis was replicated 2-3 times on different days. DEPC-treated water was used to replace cDNA and act as a negative control for each analysis. The relative amount of transcript was calculated by the $\Delta\Delta\text{CT}$ method (Livak and Schmittgen, 2001) and normalized to GAPDH.

Northern blot analysis

Northern blot analyses were carried out as described previously (Gieske et al., 2005). Briefly, total RNA was extracted from ovaries using TRIzol reagent according to the manufacturer's protocol and quantified by spectrophotometry. Ten micrograms of total RNA were separated by electrophoresis, capillary transferred to a nylon membrane (0.2- μm pore size, Nytran N; Schleicher & Schuell Inc., Keene, NH) and fixed to the membrane by UV cross-linking (UV crosslinker, UVP CL-1000; VWR, West Chester, PA). Plasmids containing partial cDNA for the gene of interest were digested from an appropriate restriction enzyme and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). A [^{32}P]-dCTP-labeled cDNA probe was synthesized using the Prime-It II random-primer labeling kit (Stratagene, La Jolla, CA). Random oligonucleotide primers were annealed to 25 ng of template DNA, followed by incubation with [α - ^{32}P]-dCTP (10 mCi/ml) and unlabeled deoxynucleotides in the presence of Exo(2) Klenow enzyme. Unincorporated nucleotides were removed with mini Quick Spin RNA columns (Roche, IN, USA). The membrane was prehybridized in UltraHyb hybridization buffer (Ambion Inc., Austin, TX) for 30 min at 68°C, and probe was added to a concentration of 10⁶ cpm/ml and then hybridized at 68°C for 16 h. Blots were washed twice (5 min each) with shaking in 2 \times SSC, 0.1% SDS at room temperature, then washed at high stringency (0.1 \times SSC, 0.1% SDS) twice for 30min each at 68°C. The membrane was exposed to film (Kodak Biomax XAR) for 48 h at -80°C.

5' RACE PCR

To determine the transcription initiation site of the 5'-end of the EDN2 gene, GeneRacer (Invitrogen, Carlsbad, CA) was used according to the manufacturer's protocol. In this procedure, total RNA (1 μg) is treated with calf intestinal phosphatase to remove 5' phosphates. The sample is then treated with tobacco acid pyrophosphatase to remove the 5' cap from the full-length mRNA while leaving a 5' phosphate required for ligation. An RNA oligonucleotide of known sequence is ligated to the 5' ends of decapped mRNA with T4 RNA ligase, which provides a known priming site for the GeneRacer PCR primers. A reverse transcription reaction is then performed, using the 5' oligomer-ligated mRNA as template for the GeneRacer oligo(dT) primer and avian myeloblastosis virus reverse transcriptase. A touchdown PCR amplification of the resulting reverse transcription is then performed with proofreading Platinum *Pfx* DNA polymerase, the GeneRacer 5' primer (specific to the GeneRacer RNA oligomer derived sequences), and a gene specific primer

(EDN2sp1; CGC AGT GTT CAC CCA GAT GAT G). After successful amplification is confirmed by agarose gel electrophoresis, a second round of nested PCR amplification is performed as above, except that the GeneRacer 5' nested primer is used in place of the GeneRacer 5' primer and a gene specific primer (EDN2sp2; CAG AAG TAC ACA CAT TCC TTG TCA AG). The amplified PCR products are then isolated from an agarose gel by excising the band, loaded into a SNAP column and microfuged. They are then cloned into plasmid vector pCR4-TOPO and transformed into competent One Shot TOP10 cells with a TOPO TA Cloning kit (part of the GeneRacer kit package). The clones were sequenced commercially (UK-AGTC, Lexington, KY) and sequences were analyzed to determine the transcription initiation site and promoter region of the endothelin gene. The nucleotide sequence of the endothelin promoter region was obtained from GenBank and used to find putative transcription factor binding sites using TESS (Transcription Element Search System; <http://www.cbil.upenn.edu/tess>).

Plasmid constructs

The mouse genomic 5'-flanking region of *Edn2* mRNA was characterized by 5'-RACE PCR as described above. Promoter regions of different sizes (+117 bp to -413 bp, -945 bp, -1407 bp and -1894 bp) were amplified from PCR using specific primers (Table 1). The PCR was conducted at 94°C for 5 min followed by 40 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 5 min. The amplified PCR fragments were then subcloned into a pCRII-TOPO vector (Invitrogen) and cloned PCR sequences were verified by nucleotide sequencing. Each promoter fragment was excised with Mlu I and Nco I digestion (Promega, Madison WI, USA) and ligated into the equivalent site of the pGL3-basic vector (Promega) to form the END2 promoter-luciferase reporter constructs. The resulting constructs were confirmed by restriction enzyme digestion and nucleotide sequencing.

Reporter gene assays

Granulosa cells were isolated from ovaries collected from gonadotropin-primed mice sacrificed at 9 h after hCG and incubated under normal oxygen conditions at 37°C in 1.5 ml F12-DMEM medium without serum and ampicillin for preparation of the constructs to transfect. The cells were then transfected with lipofectimine 2000 (Invitrogen) in 6-well plates. Each well contained 1×10^6 cells, 5 μ g pGL3-EDN2 promoter, 1 μ g of the internal control vector pRL-TK (Promega), 10 μ l lipofectimine 2000, and 500 ml Opti-MEM I (Invitrogen) medium without serum and antibiotics. The cells were then incubated for 24 h under normal or hypoxic conditions, as described above. At the completion of the transfection, the dual-luciferase reporter assay was performed. The activity of firefly luciferase in pGL3 and Renilla luciferase in pRL-TK were determined following the dual-luciferase reporter assay protocol (Promega). The cells were rinsed with PBS and cell lysates were prepared by manually scraping the cells from the culture plates in the presence of $1 \times$ passive lysis buffer (PLB). Cell lysate (20 μ l) was transferred into luminometer tubes containing 100 μ l Luciferase Assay Reagent II (LAR II). Firefly luciferase activity (M1) was determined first, and following the addition of 100 μ l of Stop&Glo Reagent, Renilla luciferase activity (M2) was recorded. All transfections were performed in duplicate and each experiment was conducted on at least three separate occasions. Data are presented as relative luciferase activity of firefly (M1)/Renilla luciferase (M2).

Statistical analysis

Data are presented as means \pm SEM. Data sets were first tested for homogeneity of variance. If heterogeneity was detected, data were log-(base 10) transformed before statistical analysis. Statistical analysis was performed using commercially available software from SPSS (Version 12.0, SPSS Inc.) and statistical significance was defined as a P-value of less

than 0.05. If differences were detected, Tukey's test was used to determine which means differed. Non-transformed data are depicted in all the figures.

Results

Expression of mRNA for *Edn2* in the mouse ovary

Real-time PCR was utilized to determine the relative level of expression of mRNA for *Edn2* in the mouse ovary during the ovulatory cascade (Fig. 1A). Expression of mRNA for *Edn2* in ovaries obtained at 48 h after treatment with PMSG and at 6 h after subsequent treatment with hCG was low. A dramatic increase in expression of mRNA for *Edn2* was observed at 12 h after hCG ($p < 0.05$), with expression of mRNA returning to basal levels by the final collection point, 24 h after hCG. This temporal expression pattern was confirmed by Northern blot analysis, which showed a dramatic increase in *Edn2* mRNA expression at 12 h after hCG (Fig. 1B). Overall, the pattern of expression of mRNA for *Edn2* in the mouse ovary during the ovulatory cascade is consistent with our microarray analysis (data not shown) and with results previously described for the rat (Ko et al., 2006; Al-Alem et al., 2007).

Induction of mRNA for *Edn2* by hCG: *in vivo* vs. *in vitro*

To determine whether the dramatic induction of mRNA for *Edn2* observed in ovaries collected at 12 h after hCG *in vivo* could be mimicked by the addition of hCG *in vitro*, granulosa cells were isolated from ovaries collected from mice at 48 h after treatment with PMSG and incubated for 0, 6 and 12 h with or without the addition of hCG (5 IU/ml). Total RNA isolated from granulosa cells collected at the end of each culture was assessed relative to RNA extracted from granulosa cells obtained at 12 h after hCG *in vivo*. Real-time PCR analysis revealed that expression of mRNA for *Edn2* was increased by hCG at 12 h *in vitro* ($p < 0.05$, Fig. 2), however the level of induction of mRNA was low when compared to the *in vivo* control, bringing into question on the validity of using hCG *in vitro* to examine the regulatory mechanism of EDN2 expression.

Effect of hypoxia on expression of mRNA for *Edn2*

To determine whether expression of mRNA for *Edn2* by granulosa cells was affected by hypoxia, modular incubation chambers were utilized to control O₂ levels within each culture. Granulosa cells were isolated from ovaries collected from gonadotropin-primed mice sacrificed at 9 h after hCG and cultured under normal (20% O₂, 5% CO₂, 75% N₂) or hypoxic (0.1% O₂, 5% CO₂ with a N₂ base) conditions for 0, 3, 6 and 9 h. Maximal expression of *Edn2* was observed after 3 h in culture, under both normal and hypoxic conditions (Fig. 3). At both 3 and 6 h of culture, expression of mRNA for *Edn2* was higher in the granulosa cells that were incubated under hypoxic conditions ($p < 0.05$), suggesting modulation of EDN2 expression by O₂ concentration.

Effect of PR or COX-2 on expression of mRNA for *Edn2*

To determine whether the PR and/or COX-2 play a role in the regulation of EDN2 gene expression in the ovary, we proposed to measure gene expression after treatment of mice with RU-486 (a PR antagonist) or indomethacin (a COX inhibitor) *in vivo*. As a control for the effectiveness of the treatments, one ovary was collected at 12 h after hCG for RNA extraction, and the other left *in situ* for determination of ovulation rate. Inconsistency was observed in the rate of ovulation after treatment with either the vehicle or antagonists (Fig. 4A). Therefore, mRNA for *Edn2* was measured in a subset of the mice, those with the expected antagonist-induced reduction in ovulation rate. Real-time PCR analysis revealed no effect of treatment with RU-486 or indomethacin on the expression of mRNA for *Edn2* ($p >$

0.05, Fig. 4B). Considering the conclusions drawn by others (that PR regulates EDN2 expression, (Palanisamy et al., 2006)) with the results reported herein, it is suggested that the potential regulation of EDN2 expression by either the PR or COX-2 is investigated further.

Promoter analysis

To identify the promoter region of the EDN2 gene, the transcription initiation site was identified by 5' RACE PCR using total RNA isolated from ovaries collected 12 h after hCG. The 5' RACE PCR amplified a major band and some minor secondary bands (Fig. 5A). Subsequent cloning and sequencing of the bands identified a single transcription initiation site at the location of 87 bp upstream of the translation initiation codon of the EDN2 gene (Fig. 5B). To determine the proximal region of EDN2 promoter that is responsible for the response to hypoxia, a promoter-reporter assay was performed using constructs in which four different sizes of the EDN2 promoter fragments were flanked by firefly luciferase (Fig. 6). Granulosa cells collected at 9 h after hCG were transfected with the constructs, cultured under normal or hypoxic condition for 24 h, and the promoter activity was measured by the dual luciferase assay. In the granulosa cells that were transfected with either -1894 or -1407 bp construct, hypoxia increased EDN2 promoter activity ($p < 0.05$). No significant difference, however, was induced by hypoxia when granulosa cells were transfected with either the -945 or -413 bp constructs. The 1894 bp of EDN2 promoter was analyzed using a filter-string based search supported by TESS program to identify any potential regulatory elements. A putative hypoxia-inducible factor 1 (HIF-1) binding site at -1297 bp (Fig. 5B) was found that is identical with the hypoxia response element (HRE) sequence (ACGTG) described in the zFIGFBP-1 gene promoter of zebrafish (Kajimura et al., 2006) and in the vascular endothelial growth factor (VEGF) gene promoter of human (Liu et al., 1995). Whether this element is functional bears further investigation.

Discussion

The primary goal of the experiments described herein was to test the hypothesis that hypoxia mediates the induction of EDN2 by periovulatory granulosa cells. Promoter analysis could then be utilized to identify regions on the EDN2 promoter bearing important transcription sites for future investigation. However, experiments were also conducted to determine EDN2 gene expression profiles in the mouse (our previous studies utilized rats (Ko et al., 2006; Al-Alem et al., 2007)), to evaluate gonadotropin-treatment of granulosa cells as an *in vitro* model for the examination of the regulatory mechanism of EDN2 production and to test whether the PR and/or COX-2 played a role in the regulation of EDN2 expression. Overall, a new model for the production of EDN2 by granulosa cells is presented, with hypoxia but not PR or COX-2 regulating this final signal of the ovulatory cascade.

Expression of mRNA for *Edn2* is increased in a very transient manner, immediately prior to the time of follicular rupture (Ko et al., 2006). EDN2 is a contractile peptide and we previously hypothesized that production of EDN2 by granulosa cells of the periovulatory follicle was inducing contraction of the smooth muscle layers within the ovary, facilitating expulsion of the cumulus-oocyte complex through an already weakened follicular structure (Ko et al., 2006). The regulatory pathway for EDN2 production and ovulation has not been closely examined and the results generated herein suggest that care must be taken when examining the mechanism of EDN2 production within the ovary. Although treatment of granulosa cells collected from PMSG-primed mice with hCG did increase expression of mRNA for *Edn2*, suggesting potential use as an *in vitro* model, the magnitude of the induction observed *in vitro* was not comparable to that observed when the granulosa cells were exposed to hCG *in vivo*. Perhaps similar in context to the preference of using hCG versus forskolin as an "ovulatory" treatment of granulosa cells, arguments could be made for or against whether the difference in the magnitude of gene expression when hCG is used *in*

vivo versus *in vitro* should dictate exclusion of this commonly used culture model (treatment of granulosa cells with hCG *in vitro*) from future studies examining the regulation of periovulatory EDN2 expression. As such, the results are presented for the interpretation of others pursuing this area of reproductive biology.

The results generated suggest that EDN2 is not under the regulation of PR or COX-2. Levels of mRNA for *Edn2* at 12 h after hCG did not differ when mice were previously treated with a PR antagonist or a COX inhibitor. However, this result must also be interpreted with an open mind. Palanisamy et al. (2006) reported that PR was driving EDN2 gene expression in mice. We used an alternate approach to test their results, however inconsistency in the effect of treatment with both the vehicle and the inhibitors led us to evaluate *Edn2* expression in only a subset of the mice we injected and our results should be examined with this in mind. Interestingly, and similar to our observations using mice, when injecting these compounds into immature gonadotropin-primed rats, RU-486 appears not as effective as indomethacin in the expected reduction of ovulation (Gaytan et al., 2004). Perhaps pertinent to hypoxia and ovulation, DMSO (the vehicle used to administer both RU-486 and indomethacin) is a known hydroxyl radical scavenger (Panganamala et al., 1976; Del Maestro et al., 1980). Whether this may have influenced our results, or the anesthesia of mice at the time of ovulation, warrants further investigation. Due to the inconsistency we observed in ovulation, we measured the level of expression of mRNA in mice with normal ovulation in response to treatment with DMSO and low ovulation after treatment with RU486 and indomethacin. Levels of mRNA for *Edn2* between these treatment groups did not differ, indicating that these treatments (which effectively reduced ovulation) did not affect *Edn2* mRNA expression. Overall this would suggest that PR and/or COX-2 are not involved in the regulation of EDN2 gene expression in mice.

It appears that hypoxia is a regulator of EDN2 expression at ovulation in the mouse. When granulosa cells were collected at 9 h after hCG and cultured under normal or hypoxic conditions, expression of mRNA for *Edn2* was increased in the hypoxic treatment group. Coupled with reports of decreasing oxygen tension with advancing follicular development (Fischer et al., 1992; Basini et al., 2004), and hCG regulation of hypoxia-related genes in granulosa cells *in vitro* (Herr et al., 2004) and *in vivo* (unpublished microarray data), it appears that hypoxia may be a physiological regulator of this final contractile mediator of ovulation. Results of the dual luciferase assay suggest that a relatively complex regulatory mechanism is in effect. As expected, analysis of the longest construct (-1894 bp) revealed increased luciferase (EDN2 promoter) activity under hypoxic conditions. While the next largest construct (-1407 bp) also revealed hypoxia-induced promoter activity, analysis of third construct (-945 bp) revealed no difference. Interestingly, under normal conditions EDN2 promoter activity appeared to increase with decreasing promoter length (-1894 to -945 bp) suggesting that inhibitory transcriptional elements are present between -1894 to -1407 and -1407 to -945 bp. Among the regulatory elements searched by the TESS program, a PU.1 binding site at -1982 bp is a potential inhibitory element. PU.1 functions as a negative regulator in the expression of the *flt3* gene (Inomata M et al., 2006) and *c-myc* gene by forming a complex with mSin3A and HDAC1 *in vivo* (Kihara-Negishi et al., 2001). In contrast, when the -945 and -413 bp constructs are compared, no effect of hypoxia on promoter expression is observed. However, promoter activity appears to be increased in the larger of these two constructs, suggesting the presence of a stimulatory transcriptional element in this section of the EDN2 gene promoter. Overall, the results suggest that both inhibitory and stimulatory transcriptional elements are present on the EDN2 promoter with hypoxia-induced elements located from -1894 to -945 bp upstream of the transcription initiation site.

In summary, the experiments described herein identified hypoxia as a novel mediator of periovulatory EDN2 production, with promoter analysis identifying likely sites of transcriptional regulation for future investigation. Results are also presented that indicate care should be taken with both *in vivo* and *in vitro* models when studying the regulatory pathway of this final contractile signal of ovulation.

Acknowledgments

Supported by grant RO1HD052694 (C.K.) and P20 RR15592 (P.B. and C.K.) from the National Institutes of Health.

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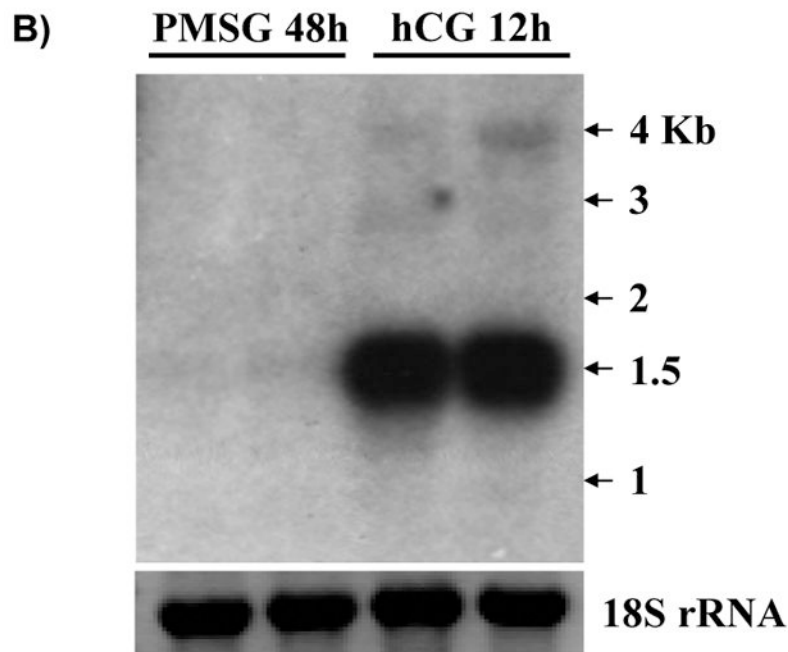
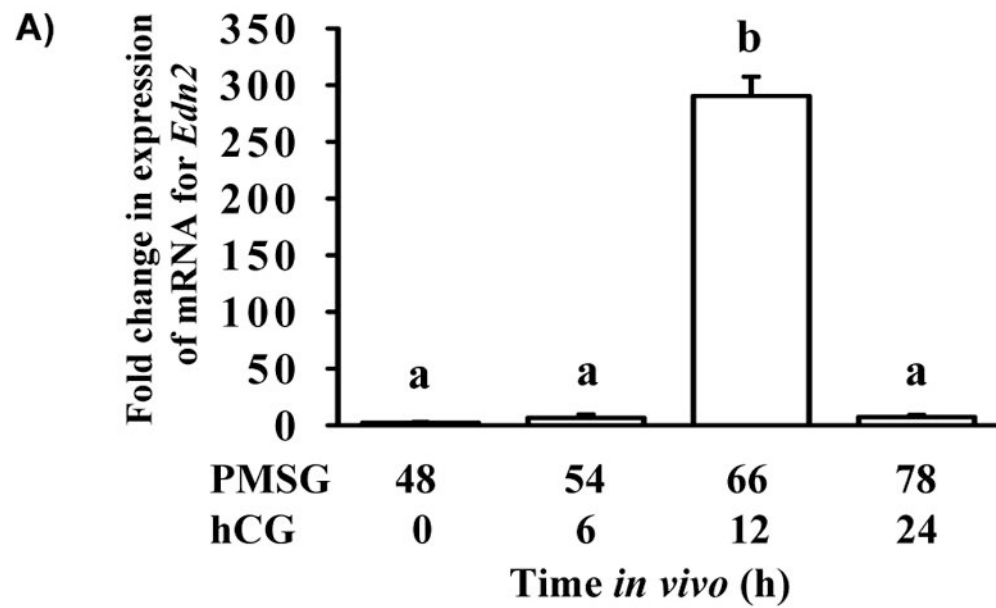


Figure 1.

Expression of mRNA for *Edn2* in the mouse ovary during gonadotropin-primed follicular development and ovulation. Immature mice were treated with 5 IU PMSG to induce follicular development and with 5 IU hCG 48 h later to synchronize ovulation. A) Animals were sacrificed for tissue collection at 48 h after PMSG and at 6, 12 and 24 h after hCG ($n = 3$ per time point). Real-time PCR was performed to determine expression of mRNA for *Edn2*. Expression of mRNA for GAPDH was used to normalize data. Expression of mRNA for *Edn2* was increased at 12 h after hCG in comparison to all other time points examined ($p < 0.05$). B) Animals were sacrificed for tissue collection at 48 h after PMSG and at 12 h

after hCG (n = 2 per time point). Northern analysis was used to determine expression and transcript size of mRNA for *Edn2*. Expression of 18S rRNA was used as a loading control.

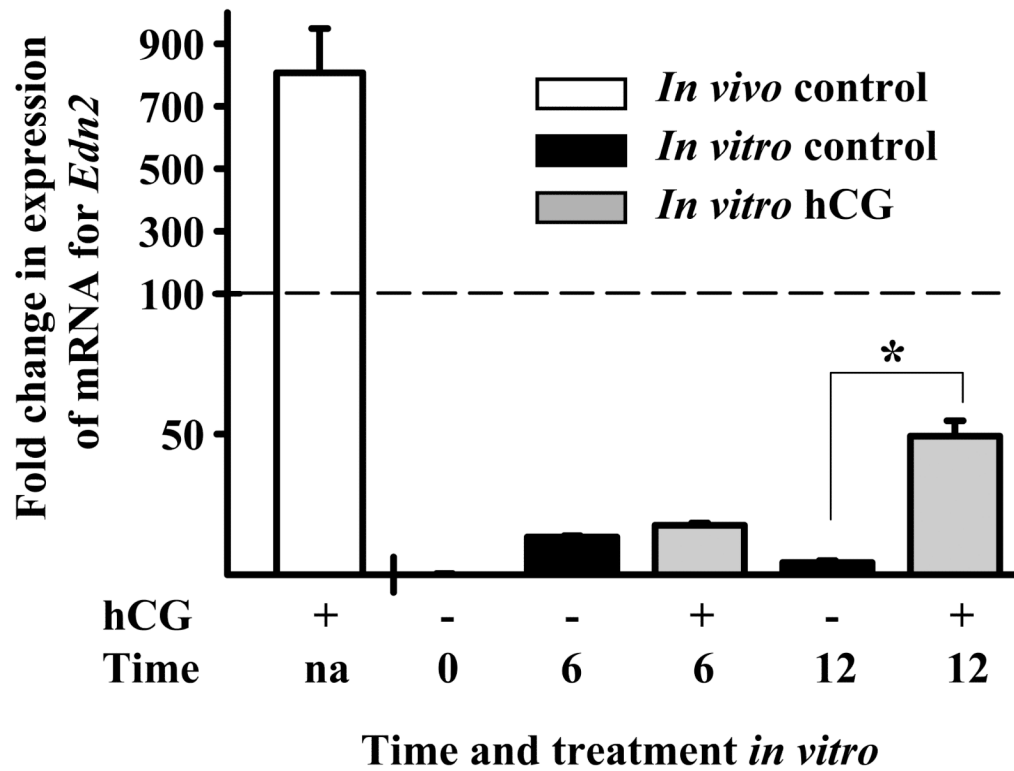


Figure 2.

Induction of mRNA for *Edn2* by hCG *in vivo* vs. *in vitro*. Real-time PCR analysis of the induction of mRNA for *Edn2* in ovaries of PMSG/hCG-primed mice sacrificed at 12 h after treatment with hCG (*in vivo* control) and in granulosa cells collected from PMSG-primed mice sacrificed at 48 h after treatment with PMSG and cultured for 0, 6 and 12 h with 0 or 5 IU hCG. Expression of mRNA for *Edn2* was increased in hCG-treated granulosa cells after 12 h in culture ($p < 0.05$). However expression of mRNA for *Edn2* after treatment of granulosa cells with hCG for 12 h *in vitro* was low compared to the *in vivo* control.

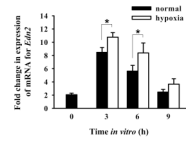


Figure 3.

Effect of hypoxia on expression of mRNA for *Edn2*. Immature mice were treated with PMSG and 48 h later with hCG. Granulosa cells were obtained from ovaries at 9 h after hCG and cultured under normal or hypoxic condition for 0, 3, 6 or 9 h. Real-time PCR was performed to determine expression of mRNA for *Edn2* in granulosa cells collected at the end of each culture. Expression of mRNA for GAPDH was used to normalize data. Expression of mRNA for *Edn2* was increased when granulosa cells were cultured under a hypoxic environment for 3 and 6 h ($p < 0.05$).

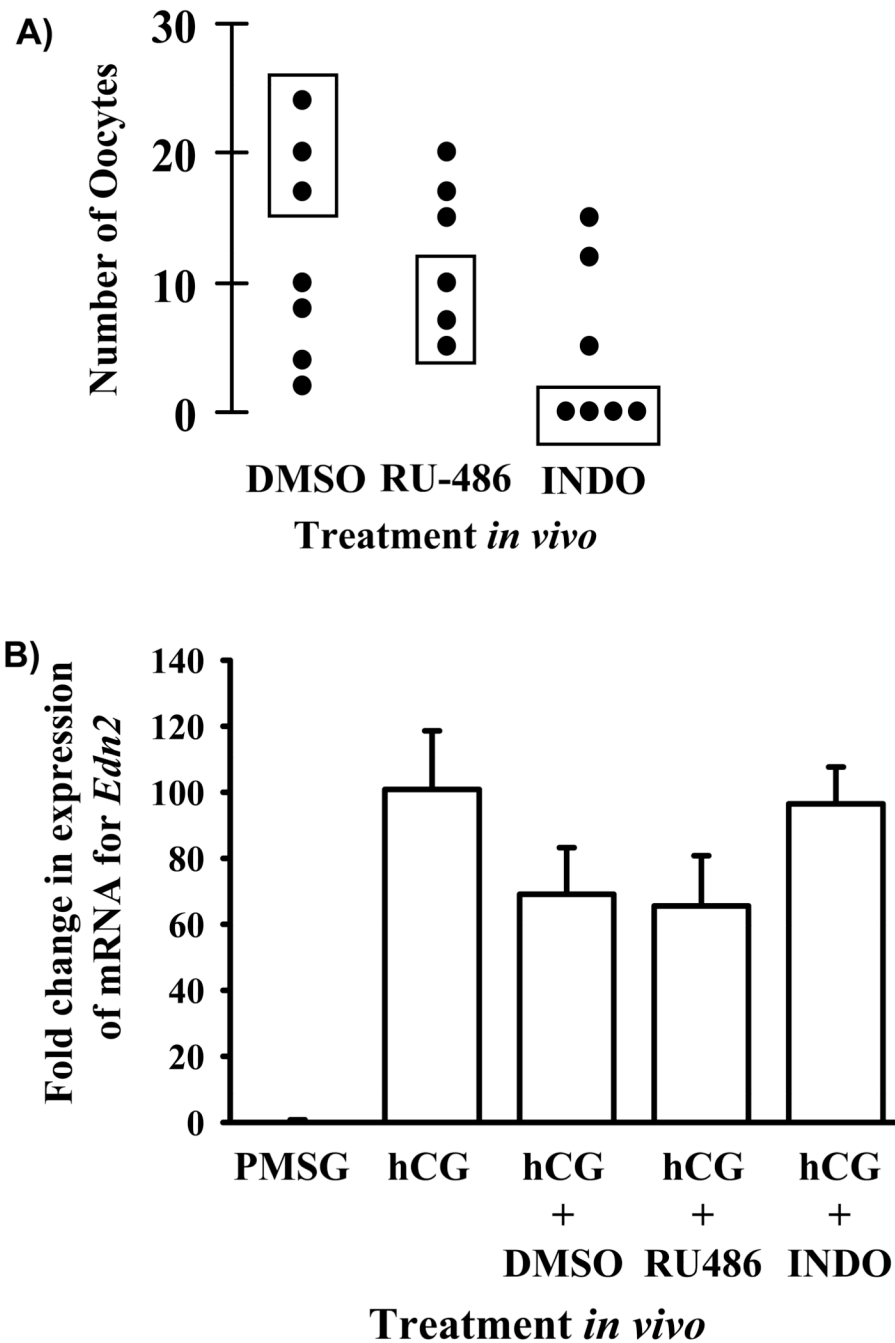


Figure 4. Effect of PR or COX-2 on *Edn2* expression. A) Ovulation rate in response to treatment with vehicle, RU-486 or indomethacin. Immature mice were treated with PMSG and 48 h later with hCG. Vehicle (DMSO), RU-486 (10 mg/kg body weight) or indomethacin (10 mg/kg body weight) were administered at PMSG + 48 h, 47 h and 49 h, respectively. One ovary per mouse was collected at 12 h after hCG for RNA extraction. The rate of ovulation for the contralateral ovary was determined at 20 h after hCG. Filled circles represent individual mice. Ovaries collected at 12 h after hCG from the mice represented by filled circles enclosed in boxes were utilized for the determination of *Edn2* mRNA expression. B) Real-time PCR analysis of *Edn2* mRNA expression, relative to GAPDH. No effect of treatment

with vehicle, RU-486 or indomethacin on the expression of mRNA for *Edn2* was detected ($p > 0.05$).

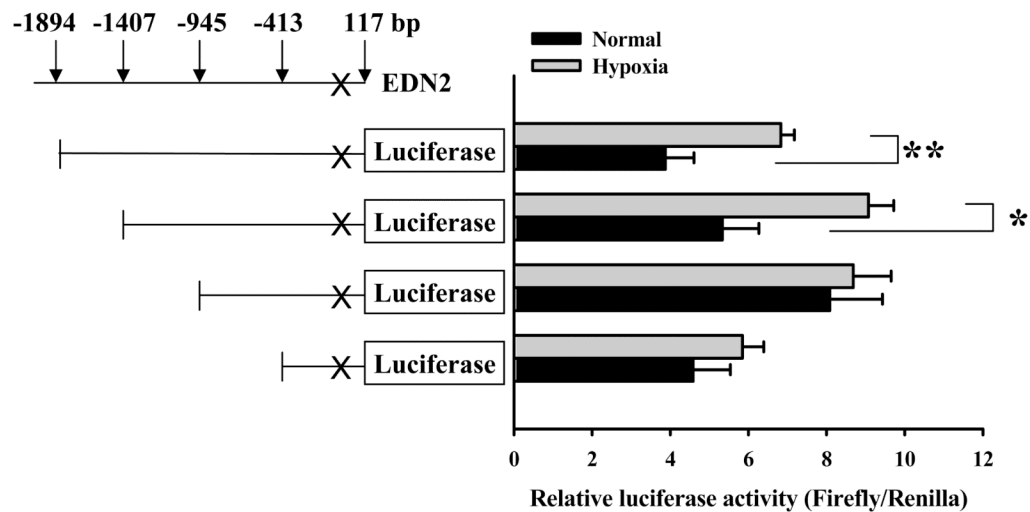


Figure 6.

Dual luciferase assay of the EDN2 promoter. Promoter constructs of different sizes (+117 to -413, -945, -1407 and -1894 bp) that were flanked by firefly luciferase were transfected using granulosa cells collected at 9 h after hCG and cultured under normal or hypoxic conditions for 24 h. Hypoxia increased EDN2 promoter activity in the granulosa cells that were transfected with either the -1894 or -1407 bp constructs ($p < 0.05$), but not the -945 or -413 bp constructs ($p > 0.05$).

Table 1
Sequences of oligonucleotides used to create EDN2 promoter constructs

Gene Name	Primer Sequence	Length (bp)
EDN2 P1	5'-CGCGT(Mlu1)CATATGCCAGCTATGTGGCTGA-3'	1979
EDN2 P2	5'-ACGCGT(Mlu1)CCTTCTACTTTGCCTTCCTCCT-3'	1494
EDN2 P3	5'-ACGCGT(Mlu1)CAGCCTCTGGCCTCTCCA-3'	1032
EDN2 P4	5'-ACGCGT(Mlu1)CTTGCTTCACCGTGGGCAG-3'	500
EDN2 PR	5'-AGCAGCAGCGGCAGAGTGCCATGG(Nco1)-3'	