

Production and binding of endothelin-2 (EDN2) in the rat ovary: endothelin receptor subtype A (EDNRA)-mediated contraction

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Abstract. Endothelin-2 (EDN2)-mediated contraction has been proposed as a final mechanical signal facilitating ovulation. The objectives herein were to determine (1) whether ovarian endothelins were increased before ovulation; (2) whether a specific endothelin-converting enzyme (ECE) was mediating their production; (3) which receptor was facilitating ovarian contraction; and (4) whether receptor-specific antagonism affected ovulation. Follicular development was induced in immature rats with 10 IU pregnant mare serum gonadotrophin (PMSG) and the ovulatory cascade was initiated 48 h later with 10 IU human chorionic gonadotrophin (hCG). In Experiment 1, an immunoassay revealed that the ovarian concentration of endothelin peptide was increased 7-fold 12 h after hCG when compared with 48 h after PMSG ($P < 0.05$). In Experiment 2, real-time PCR indicated that mRNA for *Ece1*, but not *Ece2*, was increased in granulosa cells collected 12 h after hCG when compared with those collected before the ovulatory stimulus ($P < 0.05$). In Experiment 3, isometric tension analysis revealed that the contractile effect of EDN2 was mediated by endothelin receptor A (EDNRA), not B (EDNRB). In Experiment 4, no effect was observed on the rate of ovulation when rats were treated with an antagonist specific to EDNRA (BQ123) or EDNRB (BQ788), or when mice were treated with BQ123, BQ788 or BQ123 + BQ788. In conclusion, endothelin peptide is produced before ovulation and the contractile action of EDN2 within the ovary is facilitated via EDNRA. In addition, findings of this study indicate synergistic interactions among contractile factors affect ovulatory outcome, while the role of EDNRB alone in the process of ovulation requires further investigation.

Additional keywords: constriction, ovulation, rupture.

Introduction

The architectural network of smooth muscle cells within the mammalian ovary has been recognised for many years as being responsive to contractile stimuli. The hypothesis that follicular rupture is the result of contraction-induced changes in intra-follicular pressure was one of several proposed to explain the mechanism of ovulation (Espey and Lipner 1963; Coutinho and Maia 1971; Palti and Freund 1972; Coutinho 1974; O'Shea and Phillips 1974; Virutamasen *et al.* 1976; Talbot and Chacon 1982). However, the physiological relevance of ovarian contraction as a facilitator of ovulation remained in question because no definitive hormonal ligand or neurological signal for this mechanical response was identified. With no conclusive ovulatory signal recognised, inconsistency among the reports of changing intra-follicular pressure during the periovulatory interval (Espey and Lipner 1963; Rondell 1964; Schroeder and Talbot 1982) and the knowledge that artificially increasing intra-follicular pressure did not induce ovulation (Rondell 1964), the focus of many

researchers appeared to shift away from this working hypothesis and it became relatively dormant as a field of study.

Recently, interest has returned to the physiology of ovarian contraction and follicular pressure. Using rats as the animal model, Matousek *et al.* (2001) applied new techniques to the paradigm of changing intra-follicular pressure during the ovulatory cascade and we proposed that endothelin-2 (EDN2) was the final contractile signal facilitating ovulation (Ko *et al.* 2006). Evidence suggests that these two seemingly intertwined processes are, however, independent factors within the ovulatory cascade. Changes in intra-follicular pressure are observed as early as midway through the periovulatory cascade (Matousek *et al.* 2001), coincident with increases in factors affecting vascular permeability (Gómez *et al.* 2003; Kitajima *et al.* 2004; Miyabayashi *et al.* 2005) and the array of proteolytic enzymes known to be integral to the breakdown of the extracellular matrix before ovulation (reviewed in Curry and Smith 2006). In contrast, expression of mRNA for *Edn2* is only increased very late in

the periovulatory period, ~12 h after the induction of ovulation with hCG in the rat (Ko *et al.* 2006), well after the first observed changes in intra-follicular pressure. Therefore, we propose that the contractile action of EDN2 would be imposed at the time of ovulation itself, upon an already weakened follicular structure, resulting in no change or an actual decrease in intra-follicular pressure (as the follicle ruptures), consistent with the follicular pressure profiles described over 40 years ago by Rondell (1964).

Expression of mRNA for *Edn2* was localised to the granulosa cells of periovulatory follicles by *in situ* hybridisation and blockade of the action of EDN2 within the ovary by administration of a dual endothelin receptor antagonist (tezosentan) inhibited or delayed ovulation (Ko *et al.* 2006), providing strong evidence of the importance of this peptide to the process of follicular rupture. However, the increase in levels of mRNA for *Edn2* during the ovulatory cascade were observed 12 h after treatment with hCG, raising the question as to whether translation of mRNA to functional peptide could occur within sufficient time for EDN2 to exert its proposed contractile effect on the periovulatory ovary. Therefore, our first objective was to quantify gonadotrophin-induced production of EDN2 within the ovary before ovulation, to confirm production of the contractile ligand and proposed to facilitate follicular rupture. Expression levels of mRNA encoding endothelin-converting enzymes (ECE) 1 and 2 (*Ece1* and *Ece2*) were then examined to begin to unravel the biochemical pathway involved in EDN2 production at ovulation. Following determination of ligand production and converting enzyme regulation within the ovary before ovulation, our final objectives were to determine which endothelin receptor subtype (EDNRA or EDNRB) facilitated the ovarian contraction induced by EDN2 and whether blockade of that receptor subtype *in vivo* would inhibit ovulation. Determination of EDN2 production before ovulation and identification of its action through a specific receptor subtype will increase our understanding of this final, mechanical component of the ovulatory cascade and provide new insight into the complex mechanism that is ovulation.

Materials and methods

Animals and tissue collection

Immature female Sprague Dawley rats were purchased from Harlan Inc. (Harlan, IN, USA) and maintained under controlled lighting (14 h light: 10 h dark) with continuous access to food and water. At 24–27 days of age, follicular development was induced by treatment of rats with pregnant mare serum gonadotrophin (PMSG; 10 IU, subcutaneous) and 48 h later the ovulatory cascade was initiated by treatment with human chorionic gonadotrophin (hCG; 10 IU, subcutaneous). Using this model, ovulation occurs ~14 h after treatment with hCG. Immature female CD1 mice were also purchased from Harlan Inc. and maintained as described for rats. At 23 days of age, follicular development and ovulation was induced by treatment with PMSG and hCG (5 IU, subcutaneous, 48 h apart) with ovulation expected to occur ~12 h after treatment with hCG. Animals were sacrificed by CO₂ inhalation followed by cervical luxation and the ovaries collected at the times described in each experiment below. Animal use was approved by the University of Kentucky Animal Care and Use Committee.

Immunometric assay

Total ovarian concentrations of endothelins were determined using a modified, commercially available immunometric assay kit (Cayman Chemical, Ann Arbor, MI, USA). Ovaries were collected from rats ($n = 4$ per time point) 48 h after PMSG and at 12 h after hCG. Ovaries were individually homogenised in the assay buffer supplied with the kit and the homogenate centrifuged for 15 min at 2000g at 4°C. The supernatant was then collected, acidified with 4% acetic acid and loaded on activated SPE (C-18) cartridges. The cartridges were washed twice with water and then the bound endothelin was eluted from each cartridge with 4% acetic acid in 86% ethanol. The first 2 mL of the elution was discarded and the following 6 mL collected, dried under vacuum centrifugation and then reconstituted with the assay buffer supplied with the kit. The immunometric assay was then performed on the samples of purified homogenate following the manufacturer's directions and absorbance determined at a wavelength of 405 nm. Because the antibody used in this assay is reported to bind endothelins 1–3 with equal specificity, total endothelin is referred to in the results.

Real-time PCR analysis

Expression of mRNA for endothelin-converting enzyme 1 (*Ece1*) and endothelin-converting enzyme 2 (*Ece2*) in granulosa cells was determined by real-time PCR. Granulosa cells were isolated from ovaries collected before rats were treated with PMSG, 48 h after PMSG and 12 h after hCG ($n = 3$ animals per time point), as described previously (Ko *et al.* 2006). Total RNA was extracted from granulosa cells with TRIzol (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's directions and the integrity verified by visualisation of distinct 18S and 28S rRNA bands after ethidium bromide staining in an agarose gel. Complementary DNA was reverse-transcribed from each sample of total RNA as described previously (Ko *et al.* 2006). Oligonucleotide primers corresponding to cDNA for rat *Ece1* (accession number BC072504; forward 5'-TTT GGG TGG TTG TGA GCA-3', reverse 5'-ACA AGC TCC TTT CTC GAC CA-3'), *Ece2* (accession number NM_001002815; forward 5'-TGA CGC CTC AGA CTG TGA AC-3', reverse 5'-GCC CAG GTT GTT TTC TGT GT-3') and *L32* as an endogenous reference gene (accession number BC061562; forward 5'-GAA GCC CAA GAT CGT CAA AA-3', reverse 5'-AGG ATC TGG CCC TGG CCC TTG AAT CT-3') were designed using PRIMER3 software and the specificity for each primer set was confirmed by both running the PCR products on a 2.0% agarose gel and analysing the melting (dissociation) curve in the MxPro real-time PCR analysing program (Stratagene, La Jolla, CA, USA) after each PCR reaction. The real-time PCR reactions contained 10% of the RT reaction product, 0.4 µM of forward and reverse primers, 0.3 µL of 1 : 10 diluted ROX reference dye (provided with SYBR Green ER qPCR SuperMix Universal kit; Invitrogen), and SYBR Green SuperMix. PCR reactions were performed on Mx3000P QPCR System (Stratagene). The thermal cycling steps included 2 min at 50°C to permit optimal AmpErase uracil-N-glycosylase activity, 10 min at 95°C for initial denaturation, and then each cycle 15 s at 95°C, 30 s at 55°C, and 45 s at 72°C for 40 cycles, followed by 1 min at 95°C, 30 s at 55°C, and then 30 s at 95°C.

for ramp dissociation. The relative amount of each transcript was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) and normalised to the endogenous reference gene *L32*.

Isometric tension measurement

The receptor subtype mediating EDN2-induced ovarian contraction was determined by isometric tension analysis. Ovaries were collected 10 h after hCG and maintained in Krebs's solution throughout the duration of each recording. Each ovary was cut into 2–3 sections and, using silk threads, individual sections were then tied to the arms of a force transducer, as described previously (Guo *et al.* 2003; Ko *et al.* 2006). Sections were allowed to equilibrate then treated with K^+ to induce depolarisation and establish a baseline response. In the first experiment, the contractile response to treatment with PBS (vehicle) or 50 nM EDN2 (American Peptide Co., Sunnyvale, CA, USA) was determined after the sections were pre-treated for 15 min with 3 μ M of the specific EDNRA or EDNRB antagonists, BQ123 and BQ788, respectively (American Peptide Co.). In the second experiment, the ability of 10 μ M BQ123 or BQ788 to overcome EDN2-induced ovarian contraction was determined. Antagonists were administered 10 to 15 min after contraction was induced with EDN2 and doses were chosen based on other studies (Tirapelli *et al.* 2006; Chester *et al.* 2007) and our own preliminary experiments (data not shown). Each treatment was replicated with 4–5 different ovarian sections. Isometric tension was recorded and the results expressed relative to K^+ -induced depolarisation (first experiment) or EDN2-induced contraction (second experiment), as described further in the results.

Ovulation assay: rats

The effect of EDNRA and EDNRB inactivation on ovulation rate was examined by intra-peritoneal (i.p.) and intra-ovarian (i.o.) administration of BQ123 and BQ788 *in vivo*. Immature rats were treated with 10 IU PMSG to induce follicular development and 48 h later with 10 IU hCG to induce the ovulatory cascade. At 9–10 h after hCG, injections of 10 mg per kg body weight BQ123, BQ788 or ~450 μ L vehicle (PBS) were administered by i.p. injection or 10 μ L of a 1 mM solution of BQ123, BQ788 or 10 μ L vehicle (PBS) was injected into the stroma of the right ovary (i.o.; Ko *et al.* 2006). A dose of 10 mg per kg body weight was chosen for i.p. treatments based upon the use of these antagonists in mice (Palanisamy *et al.* 2006), whereas 10 μ L of a 1 mM solution of each antagonist was chosen for i.o. treatments to give an estimated total ovarian concentration ~10-fold higher than that used for the isometric tension experiments *in vitro*. Animals ($n = 5–7$ per treatment) were sacrificed 18–20 h after hCG and cumulus–oocyte complexes (COCs) dissected from each oviduct and counted, as described previously (Al-Alem *et al.* 2007). The results are expressed as the mean rate of ovulation per rat $\pm$ s.e.m. (intra-peritoneal injections) or the mean rate of ovulation per ovary $\pm$ s.e.m. (intra-ovarian injections).

Ovulation assay: mice

The effect of inactivation of EDNRA, EDNRB and both receptors on ovulation rate was then reexamined in mice

by administration of BQ123, BQ788 or BQ123 plus BQ788 *in vivo*. Immature CD1 mice were treated with 5 IU PMSG to induce follicular development and 48 h later with 5 IU hCG to induce the ovulatory cascade. At 9 h after hCG, injections of 10 mg per kg body weight BQ123, BQ788 or vehicle (Experiment 1) or 10 mg per kg body weight BQ123 plus BQ788 or vehicle (Experiment 2) were administered by i.p. injection. Animals ($n = 4–5$ per treatment) were sacrificed 18 h after hCG and cumulus–oocyte complexes dissected from each oviduct and counted. For each *in vivo* treatment, the results are expressed as the mean rate of ovulation per mouse $\pm$ s.e.m.

Statistical analysis

The Student's *t*-test was used to determine differences in the isometric tension and immunometric assay analysis. One-way ANOVA using SigmaStat 3.5 (Systat Software Inc., Point Richmond, CA, USA) was used to determine differences in the real-time PCR analysis and the ovulation assays. If differences were detected, Tukey's test was used to determine which means differed.

Results

Quantification of endothelin peptide in the ovary

Microarray analysis previously revealed that expression of mRNA for *Edn2*, but not *Edn1*, was dramatically increased by hCG in the ovaries of PMSG-primed immature rats (Ko *et al.* 2006). This dramatic induction of mRNA for *Edn2* in granulosa cells 12 h after hCG was confirmed by real-time PCR analysis (Fig. 1a, adapted from Al-Alem *et al.* 2007) and is now quantified at the protein level (Fig. 1b). Since no currently available assay system can fully distinguish EDN2 from EDN1 peptide, an immunoassay 100% inclusive to all the isoforms was utilised. The concentration of endothelin peptide present in the ovaries of rats 48 h after treatment with PMSG was low and likely reflects basal production of EDN1. In contrast, the total ovarian concentration of endothelins 12 h after treatment with hCG was 7-fold higher ($P < 0.05$). Because only mRNA for *Edn2* is induced by hCG, this simultaneous increase in total endothelins is likely to represent EDN2 and indicates functional translation of mRNA to peptide before ovulation.

Expression of mRNA for endothelin-converting enzymes

Real-time PCR analysis indicated that expression of mRNA for *Ece1*, but not *Ece2*, was increased in granulosa cells before ovulation (Fig. 2). Expression of mRNA for *Ece1* did not differ among granulosa cells from untreated ovaries and those collected 48 h after treatment with PMSG. However, in comparison to these earlier time points, mRNA for *Ece1* increased 2-fold in granulosa cells from ovaries collected 12 h after hCG (Fig. 2a, $P < 0.05$). In contrast, expression of mRNA for *Ece2* in granulosa cells from ovaries collected 48 h after PMSG and 12 h after hCG decreased when compared with untreated (0 h) controls (Fig. 2b, $P < 0.05$). Analysis of mRNA levels alone would suggest regulation by *Ece1*, but not *Ece2*, in the overall production of EDN2 by granulosa cells before ovulation.

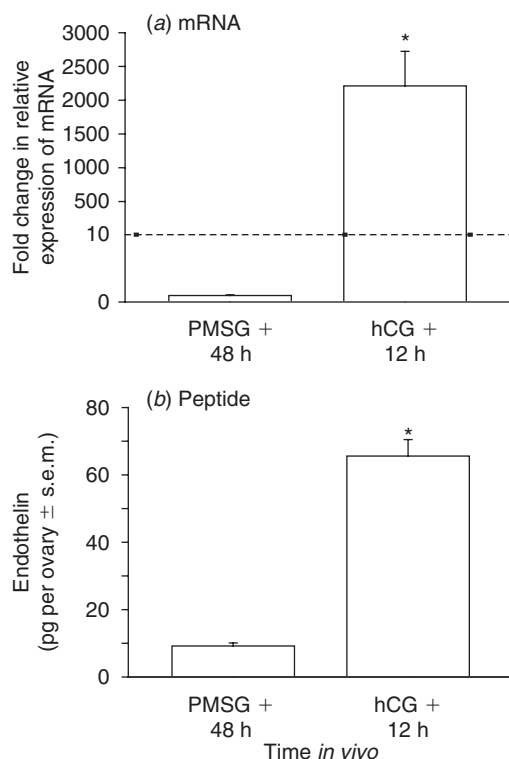


Fig. 1. Expression of *Edn2* mRNA and peptide during gonadotrophin-primed follicular development and ovulation. Immature rats were treated with PMSG to induce follicular development and with hCG 48 h later to synchronise ovulation. Animals were sacrificed 48 h after PMSG and 12 h after hCG. (a) Real-time PCR analysis of the expression of mRNA for *Edn2* in granulosa cells ($n = 3$ animals per group). (b) Immunoassay determination of total ovarian concentrations of endothelin peptide ($n = 4$ per animals per group). Data are the means $\pm$ s.e.m. Within each panel, an asterisk denotes a significant difference ($P < 0.05$).

Endothelin receptor-specific contraction of the ovary

Treatment of sections of ovarian tissue with PBS does not induce any contractile response, whereas treatment with 50 nM EDN2 induces a sustained contractile response (Ko *et al.* 2006). Hence, receptor subtype-specific antagonists were utilised to determine whether EDNRA, EDNRB or both mediated the contractile effect of EDN2 on the ovary. In the first isometric tension experiment, sections of the ovary were pretreated for 15 min with 3 μ M of the EDNRA or EDNRB antagonists and the contraction induced by 50 nM EDN2 determined. EDN2 induced a sustained contraction of sections of ovarian tissue in the presence of the EDNRB antagonist (BQ788), but not in the presence of BQ123, the EDNRA antagonist (Fig. 3, $P < 0.05$). In the second isometric tension experiment, sections of the ovary were induced to contract with 50 nM EDN2 and the ability of the antagonists to overcome EDN2-induced contraction was determined. Treatment with 10 μ M BQ123, but not 10 μ M BQ788 successfully reduced the contraction induced by EDN2 (Fig. 4, $P < 0.05$). Taken together, the results demonstrate that EDNRA mediates EDN2-induced ovarian contraction.

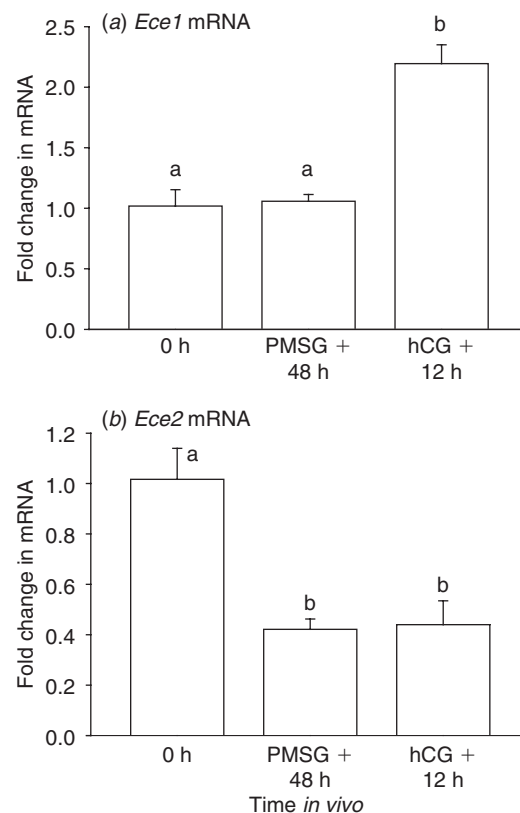


Fig. 2. Expression of mRNA for *Ece1* and *Ece2* during gonadotrophin-primed follicular development and ovulation. Animals were sacrificed before treatment with PMSG, 48 h after PMSG and 12 h after hCG ($n = 3$ per group). (a) Real-time PCR analysis of the expression of mRNA for *Ece1* in granulosa cells. (b) Real-time PCR analysis of the expression of mRNA for *Ece2* in granulosa cells. Data are the means $\pm$ s.e.m. Within a panel, values with different superscripts differ ($P < 0.05$).

Effect of receptor-specific antagonists on ovulation rate

Because the isometric tension analysis revealed that EDN2 mediated ovarian contraction via EDNRA, we tested the hypothesis that EDN2 binding to EDNRA was required for ovulation. However, no effect of treatment with either the EDNRA antagonist (BQ123) or the EDNRB antagonist (BQ788) was observed on ovulation rate in rats by either i.p. or i.o. injection ($P > 0.05$, Fig. 5a, b). This was an unexpected finding given that (1) intra-ovarian treatment of rats with the dual endothelin receptor antagonist tezosentan decreases ovulation rate (Ko *et al.* 2006) and (2) intra-peritoneal treatment of mice with BQ788, but not JKC-301, is also reported to reduce the rate of ovulation (Palanisamy *et al.* 2006). We therefore sought to reexamine the effectiveness of using peptide-based receptor-specific antagonists on the rate of ovulation using mice as the experimental model. First, the effect of treatment with each antagonist alone was determined. No effect of treatment with either BQ123 or BQ788 was observed on the ovulation rate of gonadotrophin-primed CD1 mice ($P > 0.05$, Fig. 6a). This was an unexpected finding and suggested that either inactivation of both endothelin

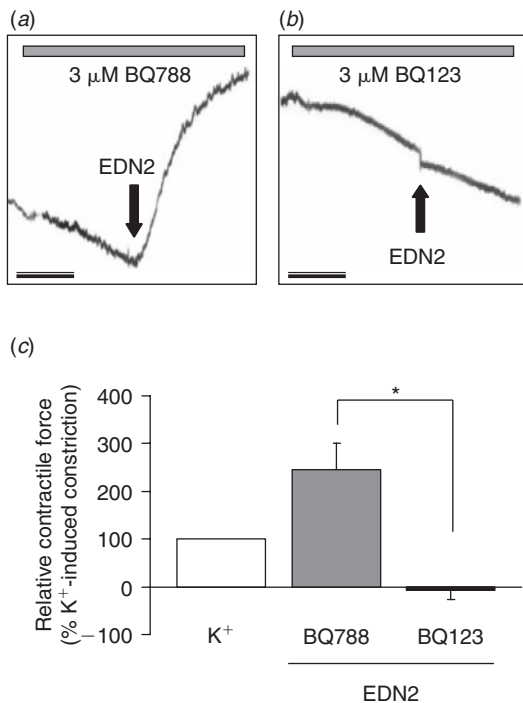


Fig. 3. Effect of pretreatment with BQ123 and BQ788 on EDN2-induced contraction of the ovary. Immature rats were treated with PMSG to induce follicular development and with hCG 48 h later to synchronise ovulation. Animals were sacrificed 10 h after hCG for tissue collection. (a, b) Representative isometric tension recordings illustrating the contractile effect of 50 nM EDN2 after pretreatment of ovarian sections with 3 μM BQ788 and BQ123. The bar in the lower left corner of each panel indicates a 10-min recording. (c) Relative contractile force of ovarian sections is expressed as a percentage of K⁺-induced depolarisation. Data are the means $\pm$ s.e.m. of 4–5 isometric recordings per treatment group. No effect of PBS (vehicle) was observed. Treatment effects are indicated by an asterisk ($P < 0.05$).

receptors was required to inhibit or delay ovulation or that these peptide-based antagonists do not retain sufficient bioactivity *in vivo* to effect ovulation. A second experiment was therefore performed to determine the rate of ovulation after treatment of mice with BQ123 plus BQ788. There was no effect on the rate of ovulation when mice were treated with BQ123 plus BQ788 ($P > 0.05$, Fig. 6b), suggesting that these peptide-based antagonists do not retain sufficient bioactivity *in vivo* to affect ovulation.

Discussion

The experiments described herein indicate that EDN2, the contractile ligand previously proposed as a mechanical signal that would induce ovulation (Ko *et al.* 2006), is produced by the periovulatory ovary before follicular rupture. Furthermore, the contractile response of EDN2 is mediated by binding to the EDNRA, not the EDNRB, subtype. When considered with the report that EDN2 also affects oviductal contractility via the same receptor subtype (Al-Alem *et al.* 2007) it appears that production and binding of EDN2 to EDNRA may play an integral

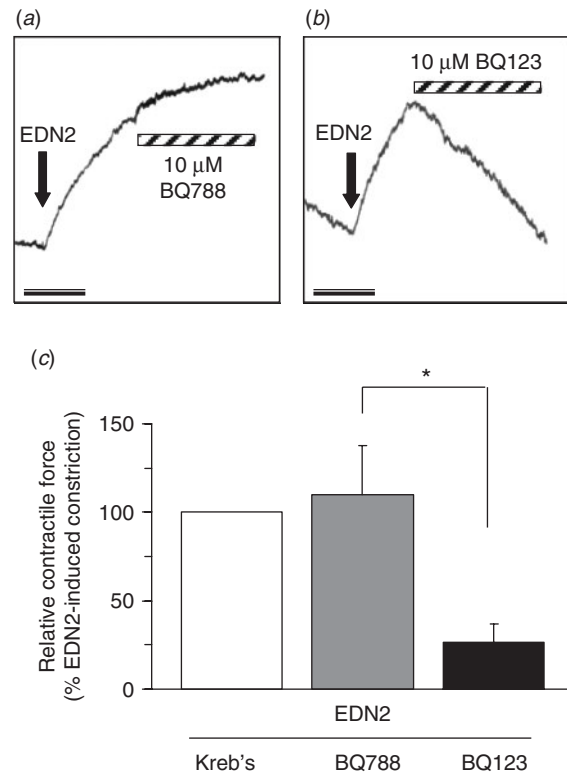


Fig. 4. Effect of post-treatment with BQ123 and BQ788 on EDN2-induced contraction of the ovary. Immature rats were treated with PMSG to induce follicular development and with hCG 48 h later to synchronise ovulation. Animals were sacrificed 10 h after hCG for tissue collection. (a, b) Representative isometric tension recordings illustrating the effect of treatment with 10 μM BQ788 and BQ123 on ovarian sections previously induced to contract by treatment with 50 nM EDN2. The bar in the lower left corner of each panel indicates a 10-min recording. (c) Relative contractile force of ovarian sections is expressed as a percentage of EDN2-induced contraction. Data are the means $\pm$ s.e.m. of 4–5 isometric recordings per treatment group. Treatment effects are indicated by an asterisk ($P < 0.05$).

role in both facilitating oocyte release and subsequent transport of the cumulus–oocyte complex within the oviduct.

Consistent with the profile of expression of mRNA for *Edn2*, total ovarian concentrations of endothelin peptide were increased in ovaries collected 12 h after hCG. Although this assay was not able to distinguish among EDN1, EDN2 and EDN3 peptides, mRNA for *Edn1* remains at a low basal level throughout the ovulatory cascade and mRNA for *Edn3* is not detectable within the ovary (Ko *et al.* 2006). Therefore, it is highly likely that the increased endothelin measured by immunometric assay 12 h after hCG represents production of EDN2 by granulosa cells of the periovulatory ovary. The low level of total endothelin that was detected in ovaries collected 48 h after PMSG is also consistent with the production and function of EDN1 described during earlier stages of follicular development (Tedeschi *et al.* 1992; Acosta *et al.* 1999; Flores 2000).

Typical to the synthesis of many peptide hormones, EDN2 is derived from selective proteolysis of larger proteins. Biologically inactive ‘big endothelins’ are cleaved from a precursor

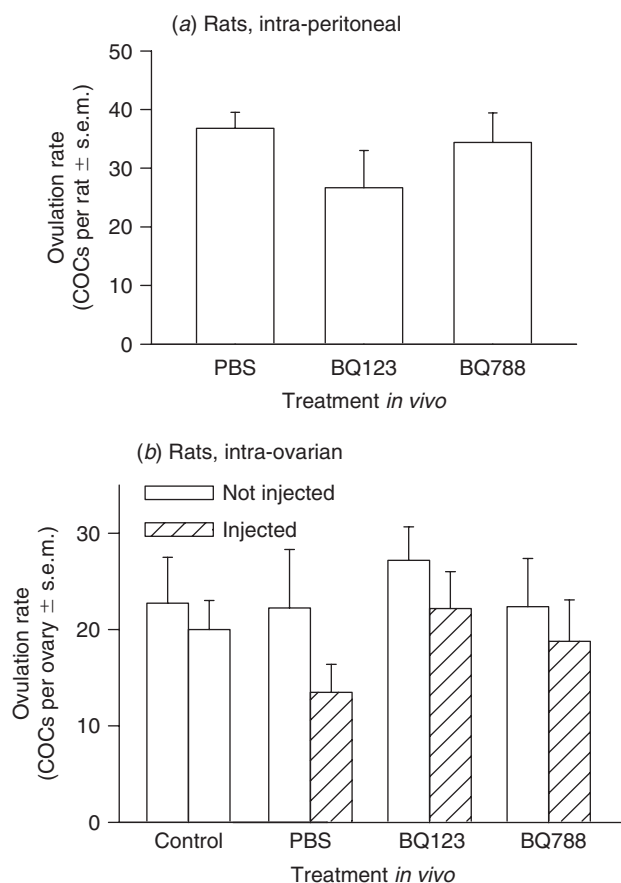


Fig. 5. Effect of BQ123 and BQ788 on ovulation rate in rats. Immature rats were treated with PMSG to induce follicular development and with hCG 48 h later to synchronise ovulation. (a) At 9–10 h after hCG, rats were treated with PBS (vehicle) or with 10 mg per kg body weight BQ123 or BQ788 by intra-peritoneal injection. (b) At 9–10 h after hCG, rats were left untreated (control) or the right ovary was injected with PBS (vehicle) or 10 μ L of a 1 mM solution of BQ123 or BQ788 (intra-ovarian injection). At 18–20 h after hCG, animals were sacrificed and the number of ovulated oocytes within each oviduct counted. Data are the mean rate of ovulation $\pm$ s.e.m. of 4–7 animals per treatment group. No effect of treatment was detected ($P > 0.05$).

protein and then converted to active endothelins by hydrolysis catalysed by endothelin-converting enzymes (reviewed in Turner and Murphy 1996). Immunoreactive ECE1 is detected in luteinising granulosa cells collected 36 h after the administration of hCG in women previously treated with human menopausal gonadotrophin for *in vitro* fertilisation purposes (Yoshioka *et al.* 1998) and we observed regulation of mRNA for *Ece1* in granulosa cells collected from pre- v. periovulatory follicles. Although modest, the increase in expression of mRNA for *Ece1* 12 h after treatment with hCG would suggest regulation of this converting enzyme as part of the mechanism to increase total EDN2 production at ovulation; however, alternative roles should be considered. ECE1 is reported to hydrolyse peptides including bradykinin, neurotensin and substance P (Hoang and Turner 1997; Johnson *et al.* 1999), which are all reported to affect ovarian function or ovulation itself (Alexander *et al.* 1989; Hellberg

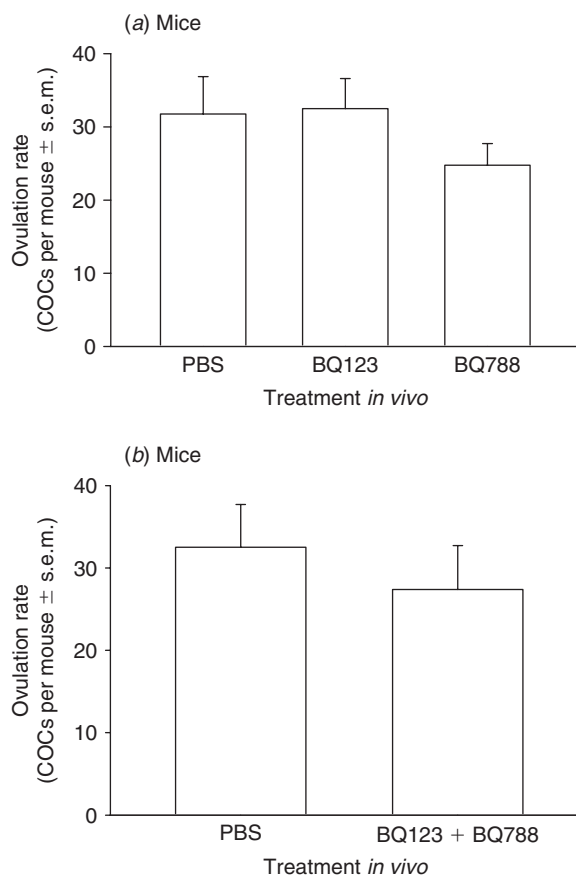


Fig. 6. Effect of BQ123 and BQ788 on ovulation rate in mice. Immature mice were treated with PMSG to induce follicular development and with hCG 48 h later to synchronise ovulation. (a) At 9 h after hCG, mice were treated with PBS (vehicle) or with 10 mg per kg body weight BQ123 or BQ788 by intra-peritoneal injection. (b) At 9 h after hCG, mice were treated with PBS (vehicle) or with 10 mg per kg body weight BQ123 plus BQ788 by intra-peritoneal injection. At 18 h after hCG, animals were sacrificed and the number of ovulated oocytes within each oviduct counted. Data are the mean rate of ovulation $\pm$ s.e.m. of 4–5 animals per treatment group. No effect of treatment was detected ($P > 0.05$).

et al. 1991; Debeljuk 2006). Hence, future studies are needed to delineate the precise role(s) of this converting enzyme within the periovulatory follicle during the ovulatory cascade.

Two G-protein-coupled receptors (EDNRA and EDNRB) are believed to mediate the many actions of endothelins (Masaki *et al.* 1999). In the rat ovary, gene expression profiles for *Ednra* and *Ednrb* generated after microarray analysis were inconclusive in suggesting which receptor subtype facilitated ovarian contraction, as was immunohistochemical staining (Ko *et al.* 2006). In contrast, functional isometric analysis clearly indicated that the contractile effect of EDN2 within the ovary was mediated by EDNRA, which is consistent with EDNRA-mediated contraction of the oviduct (Al-Alem *et al.* 2007) as well as of smooth muscle cells in the vasculature (Masaki *et al.* 1999). However, the results herein suggest that (1) the role of each receptor subtype in the process of ovulation and (2) the effectiveness of these commonly used peptide-based antagonists on analysis of

the ovulatory cascade, need to be re-evaluated. Furthermore, the role of alternative contractile factors such as prostaglandins (Priddy and Killick 1993), histamines (Schmidt *et al.* 1987) and bradykinin (Smith and Perks 1984) should be considered inclusive to the process of ovulation.

Palanisamy *et al.* (2006) reported that a dose of 10 mg per kg bodyweight of the EDNRB antagonist, BQ788, administered intra-peritoneally to immature gonadotrophin-primed mice resulted in a dramatic decrease in ovulation. They treated mice with BQ788 at 4, 6 or 8 h after hCG and reported increasing effectiveness of BQ788 in the ability to inhibit ovulation the closer the antagonist was administered towards the time of ovulation. Greater than 85% inhibition of ovulation was reported when BQ788 was administered at hCG + 8 h. In contrast, the isometric tension analysis led us to hypothesise that treatment with the EDNRB antagonist BQ123, but not BQ788, would inhibit EDN2-induced ovarian contraction and reduce or delay ovulation. We used the same dose (by bodyweight) as Palanisamy *et al.* (2006) when administering the antagonists by intra-peritoneal injection and a dose of 10 μ L of 1 mM antagonists ($\sim 90 \mu$ g per kg bodyweight) when administering the antagonists directly into the ovarian stroma. We chose to use this alternative technique as it (1) reduced the potential for non-specific effects of treatment with these vaso-regulatory antagonists and (2) was successful in reducing the rate of ovulation when tezosentan was administered in a previous experiment (Ko *et al.* 2006). We observed no effect of treatment with either antagonist via either route of administration on the rate of ovulation in rats. We then sought to test the possibility that the reported EDNRB dependence in ovulation was specific to mice and treated gonadotrophin-primed mice with 10 mg per kg bodyweight BQ788 or BQ123. Again, neither antagonist affected the rate of ovulation. Because tezosentan, a dual-receptor antagonist, was successful in reducing or delaying the rate of ovulation (Ko *et al.* 2006), we then sought to test the possibility that endothelin-mediated ovulation was dependent upon signalling through both EDNRB and EDNRA. Mice were treated with 10 mg per kg bodyweight BQ788 plus BQ123. Again, no effect on the rate of ovulation was observed. Because there are a multitude of proteolytic enzymes present within the ovary during the periovulatory period (Chun *et al.* 1992; Liu *et al.* 1997; Hasan *et al.* 2002), it is likely that the lack of any effect of these antagonists on oocyte release is due to rapid degradation of these peptide-based antagonists *in vivo*. However, given the results reported herein, the requirement for EDNRB-mediated signalling in the process of ovulation needs to be re-evaluated.

In conclusion, endothelin peptide is produced before ovulation and the total production of this peptide may be regulated by the coordinate action of endothelin-converting enzymes. The contractile response to EDN2 within the rat ovary appears to be mediated via EDNRB in a receptor subtype-specific manner. These results are consistent with the hypothesis that EDN2 acts as a final mechanical signal to facilitate rupture of the periovulatory follicle. However, our inability to affect the rate of ovulation using either antagonist in both rats and mice suggests that these pathways and antagonists need to be examined in more detail. Overall, contraction as a final, requisite mediator of ovulation bears further investigation in relation to both

assisted reproductive technologies and the possible development of strategies to overcome anovulation-based ovarian diseases.

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