Contents lists available at ScienceDirect

Asian Pacific Journal of Reproduction

Journal homepage: www.elsevier.com/locate/apjr

Document heading 10.1016/S2305-0500(13)60042-1

Differential participation of endothelin receptors in estradiol-induced oviductal egg transport acceleration in unmated and mated rats

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ARTICLE INFO

Article history:
Received 20 December 2011
Received in revised form 8 January 2012
Accepted 6 March 2012
Available online 20 March 2012

Keywords:
Estradiol
Mating
Endothelin receptors
Oviduct
Egg transport

ABSTRACT

Objective: To determine the role of endothelin (EDN) signaling in the estradiol (E_2) nongenomic and genomic mechanism that regulates egg transport in the oviduct. **Methods:** The effect of the selective agonists of ET_A (BQ610) or ET_B (BQ788) on the E_2-induced egg transport acceleration in unmated and mated rats was compared. Then, the level and distribution of ET_A or ET_B in the oviduct of unmated and mated rats following E_2 treatment was analyzed. Finally, the effect of E_2 on the oviductal release of EDN1 in unmated and mated rats was determined. **Results:** The intraoviductal E_2 nongenomic pathway required participation of ET_A and ET_B while the E_2 genomic pathway required only ET_A. Furthermore, expression of ET_A and ET_B was present in the oviduct although their levels were similar between unmated and mated rats and following E_2 treatment. On other hand, both EDN receptors were differentially localized in the oviductal cells where ET_A was mainly localized in the apical zone of epithelial cells and in the stroma and muscle while ET_B was localized exclusively in the apical zone of the epithelium. However, mating and E_2 treatment did not affect the localization of these receptors in the oviductal cells. **Conclusions:** These results indicate that mating influences the role of EDN signaling in the effect of E_2 on egg transport in the rat oviduct. This effect of mating on EDN signaling is not explained by a change in the expression or localization of their receptors along the oviduct. These findings provides the first evidence of an association between mating and EDN in order to modulate a physiological process regulated by E_2 in the mammalian oviduct.

1. Introduction

In the rat, the duration of the passage of eggs or embryos from the oviduct into the uterus is dependent on ovarian hormones and mating-associated signals[1]. A single injection of 17 beta-estradiol (E_2) on day 1 of the cycle (unmated) or pregnancy (mated) shortens oviductal transport of eggs from the normal 72-96 h to less than 24 h[2]. Interestingly, E_2 accelerates egg transport to the uterus through intraoviductal genomic pathways in mated rats and through nongenomic pathways in unmated rats[3,4], and both pathways require activation of estrogen receptors (ER)[4,5]. This change in pathways used by E_2 to accelerate egg transport has been designated as "intracellular path shifting" (IPS). The E_2 nongenomic pathway has been well established and involves conversion of E_2 to 2-methoxyestradiol (2ME) and sequential activation of cAMP-PKA and PLC-IP3 signalling pathways[4,6]. In contrast, the E_2 genomic signaling involves participation of the protein that regulates Ca^{++} storage, s100 g and functional integrity of gap junctions in the oviduct[7,8].

It has been reported that E_2 increases the frequency of pendular motion and the directional velocity of microsphere movement[7,9]. These effects combined are likely to be associated to accelerate embryo transport towards the uterus. In this context, changes in the smooth muscle contraction activity of the oviduct may operate to advance the passage of eggs into the uterus following an E_2 injection. Therefore, elucidate the molecules that regulate the contractile activity of oviductal smooth muscle is crucial to understand how E_2 accelerates egg transport by a nongenomic or a genomic mode.

Endothelins (ET) were identified as potent vasoactive

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molecules, regulating vascular tone and blood pressure[10] and it has been documented that ET induces oviductal contraction in bovine and rat[11,12]. The endothelin system consists of three 21-aminoacid-endothelin isoforms (EDN1, EDN2 and EDN3), which bind to two (ET_A and ET_B) receptors. Furthermore, the expression of ET and their receptors has been reported in the oviduct of several species[11-14]. Although it has been suggested that ET could participate in the regulation of egg transport in the oviduct[11,12], its role in the mechanism by which E_2 accelerates egg transports is unknown. Here, we compared the effect of selective inhibitors of ET_A and ET_B on the E_2-induced egg transport in unmated and mated rats. The changes on the level and distribution of ET_A and ET_B in the oviduct of unmated and mated rats were then determined. In addition, the effects of E_2 on the ET synthesis were determined in the oviduct of unmated and mated rats.

2. Materials and methods

2.1. Animals

Locally bred Sprague-Dawley rats were used. Animals were kept under controlled temperature (21-24 °C) and lights were on from 07:00 to 21:00. Water and pelleted rat chow was supplied *ad libitum*. Daily vaginal smears were used to verify cycle regularity[15]. Females weighing 200-220 g were selected from among those having 4-day estrous cycles. Females in proestrus were either kept isolated or caged with fertile males. The following day (estrus) was designated as day 1 of the cycle (C1) in the first instance and day 1 of pregnancy (P1) in the second, provided spermatozoa were found in the vaginal smear of the later. The protocols on animal manipulation have been approved by the Ethical Committees of our National Fund of Science (CONICYT-FONDECYT 1080523).

2.2. Treatments

2.2.1. Local administration of drugs

Rats on C1 or P1 were injected in the ovarian bursa (*i.b.*) with one of the drugs described below. Control rats received the appropriate vehicle only. BQ 610 and BQ 788 (Calbiochem, La Jolla, CA) were used to selectively inhibit to the ET_A[16] and ET_B[17], respectively. BQ 610, 4 μg and BQ 788, 0.4 μg dissolved in 4 μL of DMSO/H_2O 1:1 000 solution were injected *i.b.* in unmated and mated rats.

2.2.2. Systemic administration of E_2

Unmated and mated rats were injected subcutaneously (*s.c.*) with 1 μg of E_2 dissolved in 0.1 mL of propylene glycol. Control rats received the vehicle alone.

2.3. Animal surgery

Intraoviductal administration of drugs was done in the morning of C1 or P1 using a surgical microscope (OPMI 6-SDFC; Zeiss, Oberkochen, Germany) as previously described[3]. Since ovulation was completed at this time point, this treatment did not affect the number of oocytes that ovulated.

2.4. Assessment of egg transport

Animals were euthanized 24 h after different treatments, and their oviducts were flushed individually with saline. Each flushing was examined under low-power magnification (25×). The number of eggs in both oviducts was recorded as a single datum. Attempts to recover eggs from the uterus and vagina with or without placing ligatures in the uterine horns have shown that the reduction in the number of oviductal oocytes following treatment with E_2 corresponds to premature transport to the uterus[2]. Thus, we refer to it as E_2-induced acceleration of oviductal transport.

2.5. Western blot

Oviducts from rats on C1 (n=3) or P1 (n=3) were treated *s.c.* with E_2 and autopsied 8 h later. Total proteins were isolated, resolved by electrophoresis and electroblotted onto nitrocellulose membranes as previously described[3]. Nitrocellulose blots were blocked by incubation overnight at 4 °C in TTBS [100 mmol/L Tris/HCl pH 7.5, 150 mmol/L NaCl and 0.05% (v/v) Tween 20] containing 50 g/L nonfat dry milk. Afterward, blots were incubated for 1 h with a rabbit anti-ET_A or anti-ET_B (Chemicon International, Temecula, CA) or with a mouse anti-rat beta-actin (clone JLA20, Calbiochem, La Jolla, CA) as load control because expression level does not change in the rat oviduct after E_2 treatment[7] in 1:2 500 or 1:5 000 dilutions, respectively. Blots were rinsed 5 times for 5 min each in TBS (100 mmol/L Tris/HCl pH 7.5, and 150 mmol/L NaCl) and were incubated for 2 h in TTBS containing 1:5 000 dilution of goat anti-rabbit or anti-mouse IgG horseradish peroxidase conjugate (Chemicon International, Temecula, CA). The horseradish peroxidase activity was detected by enhanced chemiluminescence using Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA). Negative controls consisting of oviductal samples without anti-ET_A, anti-ET_B or anti-beta-actin were included.

2.6. Densitometry of the western blot

Immunoblots were scanned using an Epson model Expression 636 scanner and each band density was quantitatively analysed with the NIH Image 1.61 software. The intensity of bands was calculated as pixel^2[4].

2.7. Immunohistochemistry

Oviducts from rats on C1 (n=3) or P1 (n =3) were treated *s.c.* with E_2 and autopsied 6 h later, and then fixed in cold 4% (v/v) paraformaldehyde in PBS pH 7.4-7.6 for 2 h, and

then a sequential transfer to 100 g/L sucrose in PBS for 60 min at 4 °C and 300 g/L sucrose in PBS at 4 °C overnight was done. Cryostat sections, 4-6 μm thick, were placed onto gelatin-coated slides and were blocking with PBS containing 10 g/L BSA for 120 min, and then incubated with anti-ET_A or anti-ET_B antibody 1:50 in PBS containing 10 g/L BSA in a humidified chamber overnight. Three PBS rinses were followed by 60 min incubation at room temperature with secondary antibody biotin-conjugated anti rabbit IgG (Biosource, Nivelles, Belgium) diluted in PBS containing 10 g/L BSA. After three PBS rinses, the slides were incubated with Alexafluor 546-conjugated goat anti rabbit IgG (In vitrogen, Carlstad, CA) diluted 1:1 000 in PBS containing 10 g/L BSA. Samples were subsequently washed with PBS, counterstained with 1 μg/mL propidium iodide and mounted in DABCO (Sigma). As negative controls the primary antibody was replaced by preimmune serum. The resulting staining was evaluated on a Zeiss confocal laser-scanning microscope.

2.8. Measurement of EDN1 levels

Thirty minutes after E_2 or vehicle injection, rats on C1 (n=5) or P1 (n=5) were autopsied and their oviducts were flushed individually. Oviducts were transferred to 0.5 mL of prewarmed minimum essential medium and incubated for 8 h at 37 °C on a rocking platform in an atmosphere of 5% (v/v) CO_2 and 100% relative humidity. At the end of incubation, culture medium was recovered and centrifuged for 10 min at 0.1 g at 4 °C and the supernatant stored at -20 °C until use. Levels of EDN1 were determined in 100 μL of the supernatant using an enzyme immunoassay system (catalog no. RPN 228; Amersham Pharmacia Biotech, Buckinghamshire, England). The assay is based on a two-site immunoenzymetric 'sandwich' format. Standards and samples are incubated in microtitre wells precoated with anti-EDN1 antibody. Any EDN1 present will be bound to the wells, other components of the sample being removed by washing and aspiration. EDN1 bound to the wells is detected using Horseradish peroxidase labelled Fab' fragment of EDN1 antibody conjugate. The amount of peroxidase bound to each well is determined by the addition of TMB substrate. The reaction is stopped by addition of an acid solution, and the resultant color read at 450 nm in a microplate spectrophotometer. The concentration of EDN1 in a sample is determined by interpolation from a standard curve with a microplate reader (BIO-TEK Instruments, Winooski, VT). EDN concentration was normalized to total soluble proteins and is presented as pg/mg protein.

2.9. Statistical analysis

The results are presented as mean±SEM. Overall analysis was carried out using the Kruskal-Wallis test, followed by the Mann-Whitney test for pairwise comparisons when overall significance was detected. The actual N value in experiments to determine the effects of drugs on oviductal egg transport is the total number of rats used in each experimental group.

3. Results

3.1. Effect of selective blockers of ET_A and ET_B on the E_2-induced egg transport acceleration

Here we determined whether *i.b.* administration of BQ 610 or BQ 788 affect the oviductal egg transport in unmated and mated rats. Rats on C1 or P1 were injected *s.c.* with 1 μg of E_2 and 6 h later BQ 610 4 μg or BQ 788 0.4 μg was injected *i.b.* This dose of E_2 accelerates oviductal transport consistently in mated and unmated rats. The mean number of eggs recovered from the oviducts of the control group was 7.8±2.6 and 10.8±0.5 in unmated and mated rats, respectively. As expected, E_2 significantly reduced the number of oviductal eggs in unmated rats (1.4±1.1) and in mated rats (6.2±1.0) (P<0.05). Local administration of BQ 610 blocked the effect of E_2 in unmated and in mated rats, 6.1±1.1 and 11.5±0.3 respectively (P<0.05). On the other hand, BQ 788 administration blocked the effect of E_2 on egg transport in unmated (6.7±1.5) (P<0.05), but not in mated rats (6.4±1.3).

3.2. Effect of E_2 on the levels and distribution of ET_A and ET_B in the oviduct of unmated and mated rats

This experiment was designed to establish the level and distribution of ET_A and ET_B following E_2 treatment. Rats injected with 1 μg of E_2 or vehicle on C1 (n=5) or P1 (n=5) were autopsied 6 h after treatment and the oviducts were collected to measure the level of EDN receptors by western blot. Figure 1 shows a representative western blot of ET_A and ET_B in the rat oviduct. The densitometric analysis shows that E_2 did not change the levels of ET_A and ET_B in the oviduct of unmated and mated rats. Other rats on C1 (n=3) or P1 (n=3) were treated with E_2 1 μg and 6 h after treatment the oviducts were excised and processed to determine the distribution of oviductal ET_A and ET_B by confocal microscopy.

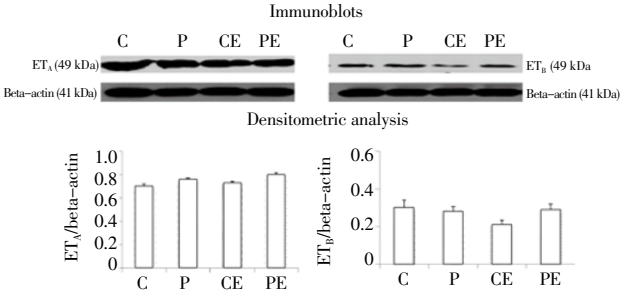

Figure 1. Representative immunoblots of ET_A and ET_B in the oviducts of rats on day 1 of cycle (C) or pregnancy (P) treated with E2 1 μg (CE or PE).
Densitometric analyses show no differences between different treatment groups. This experiment consisted of three replicates (n=3).

In the oviduct of unmated and mated rats, ET_A was mainly localised in the apical zone of epithelial cells and in the stroma and muscle while ET_B was localized exclusively in the apical zone of the epithelium. Treatment with E_2 did not change the localization of both receptors along of the rat oviduct (Figure 2).

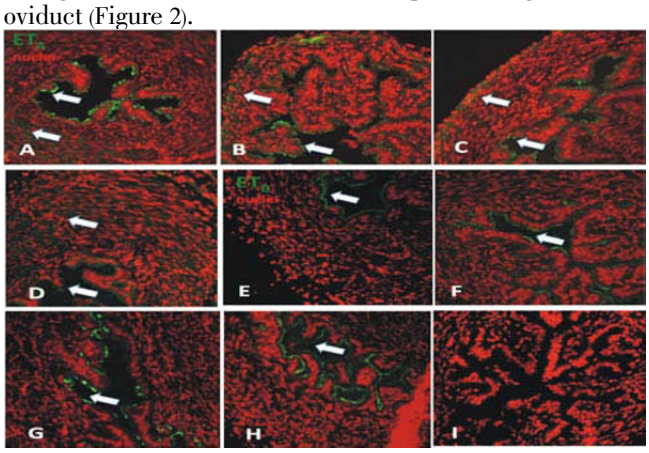

Figure 2. Representative photomicrographs of rats oviducts on day 1 of cycle or pregnancy processed by immunofluorescence for ET_A (A-D) or ET_B (E-H), 6 h after E_2 1 μg (C, D, G and H) or vehicle (A, B, E and F).
Arrows show positive immunoreactivity for ET_A or ET_B (green) whereas no marcation was recognized in the negative control (I). This experiment consisted of three replicates (n=3).

3.3. Effect of E_2 on the release of EDN1 in the rat oviduct

The mean number of the concentration of EDN1 in the culture medium of oviducts of unmated and mated rats was (443.54±29.48) and (368.36±56.51) pg/mg protein, respectively. In unmated rats treated with E_2, EDN1 concentration was decreased [(316.97±16.58) pg/mg protein] while in mated rats EDN1 concentration was similar [(275.58±63.45) pg/mg protein] to the control group.

4. Discussion

Mating changes the mechanism of action by which E_2 regulates oviductal egg transport, from a nongenomic to a genomic mode. This changes in pathways has been denominated IPS and reflects a functional plasticity in well-differentiated cells. Now we report that EDN system participates in the nongenomic and genomic mechanism by which E_2 accelerates oviductal egg transport in the rat, although mating influences the EDN signalling pathway that responds to E_2 in the oviduct. Thus, the nongenomic pathway by which E_2 accelerates egg transport operates through activation of either EDN receptors ET_A and ET_B while the genomic pathway only operates through ET_A. Interestingly, we have also observed that mating suppress the requirement for estrogen receptor-beta (ESR2) in the E_2 genomic pathway that accelerates egg transport[5]. Therefore, based in this study and previous results[3,8,18] we can postulate that mating exerts a profound influence at cellular and molecular level on the oviduct in order to regulate a complex physiological process (ovum transport) accomplished by diverse cell phenotypes.

Normal embryo transport along the mammalian oviduct results from the coordinated activity of smooth muscle, ciliated and secretory cells and is considered to be a stochastic process[19,20]. Further investigation has revealed that the pendular motion of eggs throughout the oviduct is due to phasic muscle contractions[21]. It has been shown that ovarian hormones, nitric oxide (NO), protaglandins, cytokines and EDN modulate smooth muscle motility and ciliary beats[12] suggesting that these molecules may regulate the gamete and embryo transport along the oviduct. Our findings reinforce this notion because inhibition of endothelin receptors blocked the E_2-induced egg transport acceleration providing evidence of a direct association between smooth muscle motility and egg movement in the rat oviduct. In this context, Perez-Martinez *et al.* have provided data that in the rat, nitric oxide synthase inhibitors accelerates oviductal egg transport concomitant with an increase in the frequency of contractions of the oviductal smooth muscle[22]. Thus, the coordinated waves of contraction and relaxion responsible for the gamete and embryo transport throughout the entire rat oviduct involve activation of the signaling pathways of EDN and NO.

The role of EDN receptors ET_A and ET_B on the accelerated egg transport was dependent whether the female was unmated or mated suggesting that mating-associated factors regulates the EDN signaling in the oviductal cells. Mating provides to the female reproductive with sensory stimulation, seminal fluid and sperm cells, and we have shown that the sensory stimulation with a rod glass or intrauterine insemination with epidydimal spermatozoa can independently induce IPS[23,24] so that any of these two factors could regulate EDN signaling in the oviduct, but this remains to be determined. On other hand, we found that protein levels of ET_A and ET_B were detectable in the rat oviduct and that these levels were not altered following E_2 treatment in unmated and mated rats. Furthermore, cellular distribution of ET_A and ET_B was similar between oviducts of unmated and mated rats and it was not affected by E_2. Thus, the silencing of ET_B signaling in the E_2-induced egg transport acceleration induced by mating could not be explained by a change in the expression or localization of both EDN receptors in the oviductal cells following of mating or E_2 treatment.

In the oviduct of unmated and mated rats, ET_A was mainly localized in the apical zone of epithelial cells and in the stroma and muscle layers while ET_B was localized exclusively in the apical zone of the epithelium showing a differential localization of both receptors, independently of mating. This localization of EDN receptors is compatible with a functional role of EDN in the oviduct because it has been found presence of EDN1 and EDN2 in the oviductal fluid and in the epithelial cells of the oviduct of several species[11,13,25]. The presence of ET_A in the stroma and smooth muscle could indicate a direct effect of this receptor on the oviductal contractility while ET_A and ET_B localized in the epithelial cells could be in relation to the role of EDN on the fertilization and embryo development[12]. In contrast to our findings, Al-Alem *et al.* reported expression of ET_A only in the luminal epithelium of the ampulla and isthmus segments while ET_B was weakly expressed along the oviduct[11]. In this study, immature rats were treated with gonatrophins to induce the endogenous luteneizing hormone surge so that this treatment could have affected the distribution of ET_A and ET_B in the oviduct.

Since mating and E_2 treatment did not change the level and distribution of EDN receptors in the rat oviduct we move us to explore whether E_2 could regulate the release of EDN1

in the oviduct of mated and unmated rats. According with previous observations[14,26], we found that E_2 decreased the secretion of EDN1 in the rat oviduct. However, this effect of E_2 was reverted by mating suggesting that control in the oviductal EDN1 release may be the mechanism by which mating shut down the ET_B signaling in the E_2-induced egg transport acceleration. Probably, EDN1 has a higher affinity to ET_B than others EDN isoforms in the rat oviduct, but this need to be proved.

In summary, we conclude that the E_2 nongenomic pathway that accelerates egg transport requires activation of ET_A and ET_B in the oviduct while the E_2 genomic pathway only requires oviductal ET_A activation suggesting that mating influence the role of EDN signaling in the effect of E_2 on egg transport. This effect of mating on EDN signaling is not explained by a change in the expression or localization of their receptors along the oviduct. These findings provides the first evidence of an association between mating and EDN in order to modulate a physiological process regulated by E_2 in the mammalian oviduct.

Conflict of interest statement

We declare that we have no conflict of interest.

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