A non-genomic signaling pathway shut down by mating changes the estradiol-induced gene expression profile in the rat oviduct

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Abstract

Estradiol (E2) accelerates oviductal egg transport through intravoductal non-genomic pathways in unmated rats and through genomic pathways in mated rats. This shift in pathways has been designated as intracellular path shifting (IPS), and represents a novel and hitherto unrecognized effect of mating on the female reproductive tract. We had reported previously that IPS involves shutting down the E2 non-genomic pathway up- and downstream of 2-methoxyestradiol. Here, we evaluated whether IPS involves changes in the genomic pathway too. Using microarray analysis, we found that a common group of genes changed its expression in response to E2 in unmated and mated rats, indicating that an E2 genomic signaling pathway is present before and after mating; however, a group of genes decreased its expression only in mated rats and another group of genes increased its expression only in unmated rats. We evaluated the possibility that this difference is a consequence of an E2 non-genomic signaling pathway present in unmated rats, but not in mated rats. Mating shuts down this E2 non-genomic signaling pathway up- and downstream of cAMP production. The Star level is increased by E2 in unmated rats, but not in mated rats. This is blocked by the antagonist of estrogen receptor ICI 182 780, the adenylyl cyclase inhibitor SQ 22536, and the catechol-O-methyltransferase inhibitor, OR 486. These results indicate that the E2-induced gene expression profile in the rat oviduct differs before and after mating, and this difference is probably mediated by an E2 non-genomic signaling pathway operating on gene expression only in unmated rats.


Introduction

The oviduct provides an optimal microenvironment for fertilization and early embryo development (Jansen 1984) and delivers the embryo to the uterus at the right time for implantation (Croxatto 1996). Estradiol (E2) is one of the main regulators of these phenomena modifying expression and secretion of molecules, which assure fertilization and embryo viability (Buhi 2002, Bhatt et al. 2004). E2 determines how long oocytes and/or embryos are retained in the oviduct (Forcelledo et al. 1986, Croxatto et al. 1991), possibly through its action on the activity of muscle and ciliated cells (Villalón & Verdugo 1982, Priyadarsana et al. 2004, Ríos et al. 2007), which provide the driving force for oviductal egg transport (Croxatto 2002).

E2 exerts its effects after binding to estrogen receptors (ERs) which belong to the nuclear receptor superfamily (Cheskis et al. 2007). The molecular mechanisms by which E2 changes the response of target tissues can be through genomic and non-genomic actions. In the genomic actions, estrogens bind to ERs in the nucleus, inducing a conformational change, which results in the regulation of gene transcription. In addition, E2 is able to activate rapid intracellular signaling pathways (kinase activation, cAMP, and IP3 increase). Since the latter actions are not blocked by inhibitors of transcription or translation, they have been defined as non-genomic actions (Bjornstrom & Sjoberg 2005).

A single injection of E2 to unmated or mated rats shortens oviductal transport of eggs from the normal 72–96 h to <24 h (Ortiz et al. 1979). In the absence of mating, E2 uses only intraoviductal non-genomic pathways to accelerate egg transport (Ríos et al. 1997, Orihuela & Croxatto 2001, Orihuela et al. 2001). This non-genomic pathway involves a previous conversion of E2 to methoxyestradiols (MEs) mediated by the enzyme catechol-O-methyltransferase.
(COMT; Parada-Bustamante et al. 2007), ER activation (Orihuela et al. 2003), and successive activation of the cAMP–protein kinase A (PKA) and phospholipase C–IP3 signaling cascades (Orihuela et al. 2006).

After mating, a profound change occurs in the pathways utilized by E2 to accelerate egg transport. Instead of using the non-genomic pathway (Orihuela et al. 2001), it uses genomic pathways (Rios et al. 1997). The change in pathways utilized by E2 to accelerate egg transport, from non-genomic to genomic, has been designated as ‘intracellular path shifting’ (IPS). This IPS caused by mating is a novel example of functional plasticity in well-differentiated cells. Through the effect of protein kinase inhibitors and translation inhibitors on E2-induced accelerated ovum transport, we demonstrated that among mating-associated signals, the mechanical sensory stimulation of the genital area and the presence of spermatozoa in the uterus are able to elicit IPS (Parada-Bustamante et al. 2003, Peñarroja-Matutano et al. 2007); however, the mechanisms by which these signals produce this effect have not been elucidated.

IPS involves, at least, shutdown of the E2 non-genomic signaling pathway, up- and downstream of MEs production, since mating decreases COMT activity and 2ME does not accelerate ovum transport in mated rats (Parada-Bustamante et al. 2007); however, the possibility that IPS involves changes or alterations in the genomic signaling pathway, which is used by E2 to accelerate ovum transport after mating, has not been evaluated. Previous reports indicate that E2 is able to change the expression of different proteins in the rat oviduct (Mathieu et al. 1989, Hermoso et al. 1997, Pérez Martínez et al. 2006); however, to our knowledge, no studies have been performed to determine whether these responses are different before and after mating. Here, we compared the effect of E2 on gene expression in the oviduct of unmated and mated rats. We found that E2 increased the expression of a number of genes common to both conditions, which indicates the presence of a common genomic pathway in unmated and mated rats. Surprisingly, a group of genes decreased its expression in mated rats, but not in unmated rats, and another group of genes increased its expression in unmated rats, but not in mated rats. Therefore, we evaluated whether E2 non-genomic signaling pathway, present only in unmated rats, is responsible for these differences.

**Results**

**Mating changes the transcript profile induced by E2 in the oviduct**

A microarray analysis using oviductal samples of unmated and mated rats treated s.c. with 10 μg E2 or vehicle was performed to determine whether E2 elicits different genomic effects before and after mating.

![Figure 1](https://www.reproduction-online.org/611-644/fig1.png)

**Figure 1** Changes in oviductal transcriptome induced by E2 before (unmated) and after (mated) mating. Unmated (N=10) or mated (N=10) rats were injected s.c. with E2, 10 μg, or vehicle. Three hours later, oviducts were removed to extract their mRNA and perform a microarray analysis. The results obtained represent one affymetrix chip analysis per group performed with a RNA pool obtained from five animals. The number of transcripts that decreased (A) or increased (B) their expression in response to E2 is shown separately.

Figure 1A and B shows the genes that decreased or increased their expression in response to E2 respectively. The transcript profile induced by E2 was very different when this hormone was applied before or after mating. Before mating, E2 decreased the expression of few genes, but increased the expression of a larger number of genes in comparison to the mated rats. A total of 26 known transcripts decreased their expression exclusively in unmated rats, whereas 49 did so after mating. The comparison of transcripts that decreased their level in both conditions indicated that only 2 decreased their expression exclusively in unmated rats, 25 decreased their expression only in mated rats, and 24 decreased their expression in both conditions (Fig. 1A). The complete lists of the corresponding genes are given in Tables 1–3.

A total of 73 known transcripts increased their expression in response to E2 before mating, whereas 42 did so after mating. The comparison of the genes that changed their expression in both conditions indicated that 35 increased their level exclusively in
unmated rats, only 4 did so exclusively in mated rats, and 38 increased their level in both conditions (Fig. 1B). The complete lists of the corresponding genes are given in Tables 4–6. These results indicate that mating has a profound effect on the gene expression profile induced by E2.

**E2 activates a common genomic pathway in unmated and mated rats**

Thirty-eight transcripts increased their expression in unmated and mated rats in response to E2 (Table 4), indicating that a common signaling pathway activated by E2 is present before and after mating. The transcript encoding creatine kinase brain (Ckb) is one of them, and its increase was confirmed by real-time PCR (unmated rats: 51.6 ± 8.4 (N=5) versus 25.7 ± 4.5 (N=5) in the control group and mated rats: 39.2 ± 5.6 (N=5) versus 20.8 ± 1.9 (N=5) in the control group). Since this transcript is regulated by E2 in other organs by mechanisms that involve estrogen response elements (EREs) and Sp1 sites in its promoter (O’Lone et al. 2004), the common signaling pathway would involve, at least, a classical genomic pathway which requires binding of activated ER to these promoter regions.

**E2 increases cAMP level in the rat oviduct before, but not after, mating**

E2 increases cAMP levels in the oviduct of unmated rats but not after mating (Orihuela et al. 2003). Here, we evaluated whether E2 is able to

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### Table 1 Transcripts that decreased their level in the oviduct of unmated and mated rats 3 h after an estradiol (E2), 10 µg treatment.

<table>
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<th>Probe set ID</th>
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<th>Order of change</th>
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<td>1370019_at</td>
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<td>1368124_at</td>
<td>NM_133578</td>
<td>Dual specificity protein phosphatase 5</td>
<td>Dusp5</td>
<td>−2 −2</td>
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<tr>
<td>1368894_at</td>
<td>NM_053874</td>
<td>CAP, adenylyl cyclase-associated protein, 2 (yeast)</td>
<td>Cap2</td>
<td>−2 −2</td>
</tr>
<tr>
<td>1368718_at</td>
<td>NM_017272</td>
<td>Aldehyde dehydrogenase family 1, subfamily A7</td>
<td>Aldh1a7</td>
<td>−2 −2</td>
</tr>
<tr>
<td>1371883_at</td>
<td>NM_001007673</td>
<td>Monocyte to macrophage differentiation-associated</td>
<td>Mmd</td>
<td>−2 −2</td>
</tr>
<tr>
<td>1368128_at</td>
<td>NM_013598</td>
<td>Phospholipase A2, group IIA (platelets, synovial fluid)</td>
<td>Pla2g2a</td>
<td>−2 −2</td>
</tr>
<tr>
<td>1369670_at</td>
<td>NM_031518</td>
<td>Cd200 antigen</td>
<td>Cd200</td>
<td>−2 −2</td>
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<tr>
<td>1368911_at</td>
<td>NM_017090</td>
<td>Potassium inwardly rectifying channel, subfamily J, member 2</td>
<td>Kcnj8</td>
<td>−2 −2.1</td>
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<tr>
<td>1368869_at</td>
<td>NM_001033653</td>
<td>A kinase (PRKA) anchor protein (gravin) 12</td>
<td>Akap12</td>
<td>−2 −2.1</td>
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<td>1370310_at</td>
<td>NM_173094</td>
<td>3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2</td>
<td>Hmgcs2</td>
<td>−2 −2.3</td>
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<td>1389098_at</td>
<td>NM_022278</td>
<td>Glutaredoxin 1 (thioltransferase)</td>
<td>Glrx1</td>
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</tr>
<tr>
<td>1369113_at</td>
<td>NM_019282</td>
<td>Gremlin 1 homolog, cysteine knot superfamily (Xenopus laevis)</td>
<td>Greml1</td>
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<tr>
<td>1370228_at</td>
<td>NM_001013110</td>
<td>Transferrin</td>
<td>Tf</td>
<td>−2.1 −2</td>
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<tr>
<td>1337658_at</td>
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<tr>
<td>1368870_at</td>
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<tr>
<td>1367631_at</td>
<td>NM_002266</td>
<td>Connective tissue growth factor</td>
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<tr>
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<td>Guanylate cyclase 1, soluble, z3</td>
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<tr>
<td>1367859_at</td>
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<td>Transforming growth factor, β3</td>
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<tr>
<td>1387950_at</td>
<td>NM_023020</td>
<td>Transmembrane protein with EGF-like and two follistatin-like domains 1</td>
<td>Tmeff2</td>
<td>−2.3 −4.9</td>
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<td>1387809_at</td>
<td>NM_003703</td>
<td>MAP kinase kinase 6</td>
<td>Map2k6</td>
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<td>1387074_at</td>
<td>NM_003453</td>
<td>Regulator of G-protein signaling 2</td>
<td>Rgs2</td>
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<td>1368168_at</td>
<td>NM_003380</td>
<td>Solute carrier family 34 (sodium phosphate), member 2</td>
<td>Slc34a2</td>
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<tr>
<td>1368025_at</td>
<td>NM_0080906</td>
<td>DNA-damage-inducible transcript 4</td>
<td>Ddit4</td>
<td>−2.8 −2.8</td>
</tr>
<tr>
<td>1371731_at</td>
<td>NM_000109617</td>
<td>Mesoderm-specific transcript</td>
<td>Mest</td>
<td>−4 −20</td>
</tr>
</tbody>
</table>

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### Table 2 Transcripts that decreased their level exclusively in the oviduct of unmated rats 3 h after an estradiol (E2), 10 µg treatment.

<table>
<thead>
<tr>
<th>Probe set ID</th>
<th>Accession number</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Order of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1370913_at</td>
<td>NM_133881</td>
<td>Radical S-adenosyl methionine domain containing 2</td>
<td>Rsad2</td>
<td>−2.1</td>
</tr>
<tr>
<td>1369153_at</td>
<td>NM_022628</td>
<td>Nephrosis 1 homolog, nephrin (human)</td>
<td>Nphs1</td>
<td>−9.8</td>
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</tbody>
</table>

produce the same response after mating. In unmated rats, E2 increased cAMP levels almost twofold (2.76 ± 0.52 pmol/oviduct (N = 3) versus 1.3 ± 0.2 pmol/oviduct (N = 3) in the control group) (Fig. 2), whereas in mated rats, E2 did not change it (1.3 ± 0.2 pmol/oviduct (N = 3) versus 1.2 ± 0.35 (N = 3) pmol/oviduct in the control group) (Fig. 2), indicating that mating shuts down an E2 non-genomic signaling pathway in the oviduct, upstream of cAMP generation.

**Mating shuts down E2 non-genomic signaling pathway downstream of cAMP generation**

Pharmacological treatments that increase cAMP production in the rat oviduct accelerate ovum transport in unmated rats (Orihuela et al. 2003). However, this effect has not been evaluated in mated rats. Twenty-four hours after treatment with forskolin, an average of 40% of oocytes left the oviduct prematurely in unmated rats (6 ± 0.8 (N = 5) versus 10.2 ± 0.8 (N = 5) oviductal oocytes in the control group; Fig. 3A), whereas in mated rats, no effect was observed with this dose (9.6 ± 1.2 (N = 5) versus 10 ± 1.8 (N = 5) oviductal embryos in the control group). To discard whether this effect of forskolin is only due to a differential activation of adenyl cyclase isoforms in unmated and mated rats, the effect of N6,2′,4′,6′-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP), a cAMP analog, was evaluated. In unmated rats, 50% of the oocytes left the oviduct prematurely after treatment with 400 μg of dbcAMP (7 ± 1.1 (N = 5) versus 11.6 ± 0.8 (N = 5) in the control group; Fig. 3B), whereas the same dose had no effect on the number of oviductal embryos in mated rats (8.6 ± 1.0 (N = 5) versus 9.2 ± 1.2 (N = 5) in the control group; Fig. 3B), indicating that mating shuts down an E2 non-genomic signaling pathway used by E2 to accelerate ovum transport, downstream of cAMP generation too.

**E2-induced non-genomic signaling pathway produces changes in gene expression in unmated rats, and mating shuts down this pathway: the case of STAR expression**

E2 increased the level of 35 transcripts exclusively in unmated rats, suggesting that a signaling pathway activated by E2 present before, but not after, mating is responsible for this effect. To characterize this response, a temporal course of Star expression, one of the transcripts that increased its expression in response to E2 only in unmated rats, at RNA and protein levels was performed after E2 treatment.

In unmated rats, E2 significantly increased Star mRNA only 4.5 h after E2 treatment (61.2 ± 15.2 vs 22.2 ± 6.1
group vehicle, Fig. 4, unmated), whereas its protein level was increased at all times studied, with maximal increases being reached 4.5 and 6 h after E2 treatment (4.0 ± 1.0 and 4.5 ± 1.2 times respectively, Fig. 5, unmated). In mated rats, E2 did not change mRNA at any time studied.

Since mating shuts down E2-induced non-genomic signaling pathway up- and downstream of cAMP generation, the possibility that this pathway is responsible for transcript level changes induced by E2 observed only in unmated rats was evaluated. For this, the E2-induced Star expression increase in unmated rats was studied by blocking the ER and cAMP production, which are the two key components that mediate E2 non-genomic signaling pathway (Orihuela et al. 2003). ICI 182 780, an ER antagonist, and SQ 22536, an adenylyl cyclase inhibitor, or their vehicles were administered.
Table 5 Transcripts that increased their level exclusively in the oviduct of unmated rats 3 h after an estradiol (E2). 10 μg, treatment.

<table>
<thead>
<tr>
<th>Probe set ID</th>
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<td>1371059_at</td>
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<td>Prkar2a</td>
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<tr>
<td>1369177_at</td>
<td>NM_053735</td>
<td>Phosphatidylinositol 4-kinase type 2 α</td>
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Table 6 Transcripts that increased their level exclusively in the oviduct of mated rats 3 h after an estradiol (E2). 10 μg, treatment.

<table>
<thead>
<tr>
<th>Probe set ID</th>
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intradibursally (i.b.) in unmated rats following a s.c. injection with E2 or its vehicle. In rats receiving local treatment with vehicle, E2 increased STAR levels (IC1 experiment: 2.16 ± 0.17 (N = 3), Fig. 6A, and SQ 22536 experiment: 1.81 ± 0.01 (N = 3), Fig. 6B). ICI 182 780 and SQ 22536 completely blocked the increase of STAR induced by E2 (0.65 ± 0.07 (N = 3) and 1.12 ± 0.16 (N = 3), Fig. 6A and B respectively), whereas these drugs when applied alone did not affect STAR expression by themselves (0.8 ± 0.14 (N = 3) and 1.09 ± 0.11 (N = 3), Fig. 6A and B respectively). These results indicate that increased STAR expression induced by E2 in unmated rats requires an active ER and an increase of cAMP levels.

STAR immunoreactivity was detected only in epithelial cells of the ampulla and isthmus (Fig. 7), indicating that at least part of the pathway utilized by E2 to increase STAR expression is present in this cell phenotype.

Altogether these data indicate that some, if not all, genes that increase their expression in the oviductal epithelium in response to E2 exclusively in unmated rats do so, at least in part, in response to the activation of a
local non-genomic signaling pathway that increases cAMP levels. Mating shuts down this pathway, preventing E2 from increasing the expression of these genes in mated rats.

**E2 regulated STAR expression, COMT requirement, and effect of 2ME**

Local formation of MEs mediated by COMT is essential for the intraoviductal non-genomic pathway utilized by E2 to accelerate egg transport in unmated rats (Parada-Bustamante et al. 2007). Here, we evaluated whether OR 486, a COMT inhibitor, blocks the E2-induced increase in STAR levels. OR 486 or its vehicle was administered i.b. in unmated rats after a s.c. injection of E2 or its vehicle. E2 increased STAR levels significantly (2.3 ± 0.6 times over control, Fig. 8), and OR 486 completely blocked this effect (1.1 ± 0.2 times over control, Fig. 8), whereas OR 486 alone had no effect (1.2 ± 0.3 times over control). Moreover, STAR levels increased significantly 1.5 and 3 h after 2ME treatment (2.4 ± 0.6 and 1.6 ± 0.3 times over control respectively, Fig. 9), indicating that local conversion of E2 to ME is an essential component of the non-genomic signaling pathway of E2 that regulates STAR expression in the oviduct of unmated rats.

**Discussion**

Mating has a profound impact on oviductal physiology, changing the pathway by which E2 accelerates ovum transport from non-genomic to genomic (IPS). In this study, the genomic pathways that respond to E2 before and after mating were explored. We found that the E2-induced gene expression profile in the rat oviduct is different depending on whether the animal has mated or not. More genes were increased and fewer genes were decreased by E2 before mating than after mating. Since more information about the mechanisms by which E2 increases gene expression is present in the literature, our analysis was focused on the group of genes that were increased by E2. The fact that 38 genes are increased in unmated and mated rats indicates that a common pathway that is able to increase gene expression is present before and after mating. According to the literature, some of these genes are regulated by E2 in other rat organs such as angiopoietin 2 in the heart, kidney, and lung (Ye et al. 2004), hydroxysteroid 11β dehydrogenase 2 in the kidney (Gómez-Sánchez et al. 2003), insulin-like growth factor 1 (Igf1) in the oviduct (Carlsson et al. 2003), progesterone receptor in the lung (González-Arenas et al. 2003), and Ckb in pituitary gland (Blake et al. 2005). Functional analysis of progesterone receptor promoter, which is one among this group of genes, indicated that it contained imperfect EREs, which are necessary for increasing its levels by E2; moreover, Ckb promoter contains EREs and GC-rich sites, which are used by E2, bound to ER, to increase its levels (Scott et al. 2003). In this work, we confirmed that E2 increased Ckb mRNA levels in unmated and mated rat oviducts.
oviduct, indicating that the common pathway is, at least, a classical genomic pathway which is dependent on direct or indirect ER–DNA interaction.

E2 increased the expression of some transcripts exclusively in unmated rats, but not in mated rats, indicating that a pathway that is able to increase the expression of these transcripts is present in unmated rats, whereas mating shut it down by unrecognized mechanisms. Previously, we had determined that in IPS, the E2 non-genomic pathway is shut down up- and downstream of 2ME (Parada-Bustamante et al. 2007); here, we show that mating shuts down the non-genomic pathway up- and downstream of cAMP production. The fact that the E2 non-genomic signaling pathway is shut down due to mating, and that some genes, such as inhibin β-A (Ardekani et al. 1998), Smad7 (Bilezikjian et al. 2001), gap junction protein, α1 (Abudara et al. 1999), and Star (Stocco et al. 2005), which increased their expression only in unmated rats are increased by cAMP and PKA activation in other rat tissues, indicates that in unmated rats, E2 increases the expression of these genes activating a non-genomic pathway. After mating, this pathway is shut down and then E2 is unable to increase the expression level of this group of genes. In accordance with this idea, in unmated rats, Star mRNA levels are increased 4.5 h after E2 treatment, whereas in mated rats, Star mRNA levels did not change at any time point studied. Furthermore, the fact that increases of STAR protein induced by E2 require ER activation and cAMP production suggests that this effect is dependent on the intraoviductal E2 non-genomic signaling. Since ICI 182 780 alone did not affect STAR expression, it is

![Figure 4](image1)

**Figure 4** Estradiol increases oviductal Star mRNA levels in unmated rats, but not in mated rats. Star mRNA relative expression obtained through real-time PCR from cDNA of oviductal samples (N=5 animals by treatment) taken from unmated and mated rats 3, 4.5, 6, 12, and 24 h after a s.c. injection of E2, 10 µg, or vehicle. The values were normalized to Gapdh. Each bar represents the mean value obtained from five animals. The asterisk indicates a statistically significant difference between the two bars at 4.5 h (P<0.05).

![Figure 5](image2)

**Figure 5** Estradiol increases oviductal STAR protein levels in unmated rats, but not in mated rats. Densitometric analysis of western blots to detect STAR protein in oviductal samples (20 µg, N=3 animals by treatment) taken 3, 4.5, 6, and 7.5 h after a s.c. injection of E2, 10 µg, or vehicle. The values were normalized to z-tubulin. A representative western blot obtained in this experiment is shown. Numbers inside the bars indicate the number of animals used. Means with different letters are significantly different from each other (P<0.05).
probable that other endogenous signaling pathways are acting to state basal STAR levels in the rat oviduct. In accordance with this idea, previous reports have determined that STAR expression is regulated by several signaling pathways and factors, such as IGF1, arachidonic acid, protein kinase C, and MAP kinases (reviewed in Stocco et al. (2005)).

The fact that Star mRNA levels were increased only in one of the points studied is in agreement with our previous results, which indicated that cAMP levels are increased 3 h after E2 treatment, whereas its levels returned to basal levels at 6 h (P Orihuela 2006, unpublished observations). STAR protein levels were increased slightly, but significantly 3 h after E2 treatment, even though its mRNA levels were increased from 4.5 h onwards after treatment, probably because cAMP–PKA pathway activation increases STAR protein stability (Clark et al. 2001). This also indicates that STAR protein levels are maintained elevated until 7.5 h after E2 treatment. In mated rats, E2 did not change STAR protein levels at any time studied. These results indicate that E2 increases STAR protein levels in unmated rats, activating a non-genomic pathway that requires E2 receptor and adenyl cyclase.

This shutdown of E2 non-genomic signaling pathway in mated rats could be explained by differences in E2 levels or changes in the expression or location of ER induced by mating in the rat oviduct. However, previous reports have shown that plasma E2 levels and oviductal ESR1 (ER-α) and ESR2 (ER-β) mRNA and protein levels did not change in the initial hours after mating (Smith et al. 1975, Orihuela et al. 2004). Another possibility could be the interactions among ERs and proteins that mediate E2 non-genomic signaling pathways in unmated rats, which do not occur in mated rats. For example, it has been found that PELP-1/MNAR is an ER-interacting protein (Brann et al. 2008) which is required for ESR1 interaction with p60 (SRC), which leads to the activation of SRC/MAPK pathway (reviewed in Cheskis et al. (2008)); however, this possible differential interaction in the rat oviduct was not evaluated in this work.

E2 increased STAR levels only in the epithelial cells, indicating that E2-activated non-genomic pathway is present only in these cells. This is in agreement with our previous results using primary cell cultures from rat oviduct, where E2 increased cAMP levels in epithelial cells, but not in muscle cells (P Orihuela 2007, unpublished observations); however, we do not discount that other components of the non-genomic pathway are present in other cell types, since IP3 levels are increased 1 and 6 h after E2 treatment in the whole rat oviduct (Orihuela et al. 2006).

cAMP regulates STAR expression in other tissues and organs through PKA activation and subsequent transcription factor phosphorylation such as GATA4 and CREB (Stocco et al. 2005). The effectors that are downstream of cAMP production, responsible for E2-induced STAR increases in the rat oviduct, were not studied in this work. To our knowledge, this is the first time that STAR expression is reported in the oviduct, and that its expression is increased by E2; a previous work reported that STAR expression is decreased in interstitial and theca ovarian cells in newborn rats treated with E2 benzoate (Ikeda et al. 2001). STAR is a key protein in steroidogenesis, because it mediates cholesterol entry from external to internal mitochondrial membranes (Stocco & Clark 1996). The function of STAR in the oviduct was not explored in this work; however, since E2-induced acceleration transport is not blocked by transcription and translation inhibitors in unmated rats,
the increase of its expression would not mediate this phenomenon.

The $E_2$ non-genomic pathway in the oviduct involves conversion of $E_2$ to MEs mediated by COMT (Parada-Bustamante et al. 2007). In accordance with this, a COMT inhibitor blocked $E_2$-induced STAR increased expression and 2ME increased STAR protein expression. The fact that STAR levels were increased only 3 h after 2ME treatment, whereas they were increased 3, 4.5, 6, and 7.5 h after $E_2$ treatment can be explained by a fast 2ME metabolization. cAMP levels are increased in the rat oviduct only 3 h after $E_2$ treatment (Orihuela et al. 2006); this latency time can represent the time required by $E_2$ to be metabolized to 2ME. We hypothesize that 2ME treatment would increase cAMP faster than $E_2$, but this was not tested in this work.

The physiological relevance of preventing increased expression of a group of genes by $E_2$ in the rat oviduct by mating-associated signals was not explored in this work. We postulate that failure of the mechanism that is responsible for the shutdown of this pathway would affect normal embryo development. In order to corroborate this idea, it is necessary to prevent IPS and then to determine whether in this condition there are alterations in reproductive phenomena such as embryo development and implantation; however, this was not explored in this work.

In summary, these results indicate that $E_2$-induced gene expression profile in the rat oviduct differs before and after mating, and this difference is possibly mediated by the effects of $E_2$ non-genomic signaling pathway on gene expression operating only in unmated rats; the early events induced by mating responsible for this phenomenon are still unknown.

Materials and Methods

Animals

Locally bred Sprague–Dawley rats were used. The animals were kept under controlled temperature (21–24°C), and lights were kept on from 0700 to 2100 h. Water and pelleted rat chow were supplied ad libitum. Females weighing 200–220 g were selected from those that had at least two regular cycles of 4 days immediately before the experiments were started. Daily vaginal smears, which were taken between 0800 and 0900 h, were used to verify cycle regularity (Turner 1961). To obtain unmated and mated rats, females in the evening of proestrus were either kept isolated or caged with fertile males. The next morning, isolated rats that presented cornified cells in the vaginal smear, a cell phenotype associated with ovulation (estrus day), were designated as unmated rats, and those caged with fertile males that presented cornified cells and spermatozoa in the vaginal smear were designated as mated rats. The care and manipulation of the animals were carried out in accordance with the ethical guidelines of Pontificia Universidad Católica de Chile and Universidad de Santiago de Chile.

Treatments

All treatments described below were administered at 1200 h in unmated and mated rats.

Figure 7 STAR is expressed in epithelial cells in unmated rat oviduct. Representative photomicrographs obtained from unmated rat oviducts 4.5 h after injecting $E_2$, 10 μg, s.c. ($N=3$) to detect STAR expression (green). Nuclei were stained with propidium iodide (red). Arrows point to immunoreactivity obtained only in the epithelial cells. The specificity of immunoreactivity was assessed by incubating samples with preimmune serum.

Figure 8 OR 486 blocks increased expression of STAR levels in the oviduct in response to $E_2$ in unmated rats. Densitometric analysis of western blots to detect STAR protein in oviductal samples (20 μg, $N=3$ animals by treatment) of unmated rats 4.5 h after injecting $E_2$, 10 μg, or vehicle s.c. and concomitantly the COMT inhibitor, OR 486, or vehicle given i.b. The values were normalized to $\alpha$-tubulin. A representative western blot is shown. Numbers inside the bars indicate the number of animals used. Means with different letters are significantly different from each other ($P<0.05$).
Effect of mating on E2 pathways in the oviduct

Figure 9 2-Methoxyestradiol increases oviductal STAR levels in unmated rats. Densitometric analysis of western blot to detect STAR protein in oviductal samples (20 μg, N = 3 animals per treatment) from unmated rats taken 1.5 and 3 h after a s.c. injection of 2-methoxyestradiol, 100 μg, or vehicle. The values were normalized to α-tubulin. A representative western blot is shown. Numbers inside the bars indicate the number of animals used. 

Systemic administration of E2 or 2ME

Unmated and mated rats were injected s.c. with 10 μg of E2 as a single dose in an injection volume of 0.1 ml of propylene glycol. Other rats received a single s.c. injection of 100 μg of 2ME (Steraloids, Newport, RI, USA) in an injection volume of 0.1 ml of propylene glycol. Control rats received propylene glycol alone.

Local administration of drugs

Unmated and mated rats were injected in the ovarian bursa (i.b.) with one of the drugs described below. Control rats received the appropriate vehicle only. Forskolin (7β-acetoxy-8, 13-epoxy-1,6β,9-trihydroxy-labd-14-ene-11-one; Sigma Chemical) and dbcAMP (Sigma Chemical) were used to determine whether E2 non-genomic signaling pathway is functional downstream of cAMP generation in mated rats using acceleration of ovum transport as end point response. Forskolin, 20 μg, dissolved in 4 μl of 25% ethanol and dbcAMP, 400 μg, dissolved in saline were injected i.b. in unmated and mated rats. Previously, we had determined that this dose of forskolin applied i.b. accelerates ovum transport in unmated rats (Orihuela et al. 2003). ICI 182 780 (kindly donated by W Elger, Entech, Jena, Germany) was used to block the ER, and to determine whether ER is required for local conversion of E2 to MEs is important to increase STAR protein level in unmated rats. OR 486, 125 μg, dissolved in 4 μl of 25% ethanol was injected i.b. in unmated rats immediately before injecting 10 μg of E2 s.c.

Once oviducts were removed, all manipulations were done on an ice-cooled plate.

Measurement of cAMP levels

Three hours after E2 or vehicle injection, unmated (N = 6) or mated (N = 6) rats were killed and their oviducts were flushed individually. Oviducts in groups of two (one rat) were homogenized in 0.5 ml of ice-cold 10% (v/v) trichloroacetic acid and centrifuged for 15 min at 2200 g at 4 °C. The pellet was discarded, and the supernatant was washed four times with five volumes of water-saturated diethyl ether. The upper layer was discarded after each wash. Following the last wash, the aqueous extract was dried under a stream of nitrogen at 60 °C. Levels of cAMP in dried extracts were determined using Biotrak cAMP enzyme immunoassay system (catalog no. RPN 225; Amersham Pharmacia Biotech, Piscataway, NJ, USA). This kit is based on the competition between unlabeled cAMP and a fixed quantity of peroxidase-labeled cAMP for a limited number of binding sites on a cAMP-specific antibody. This allows for the construction of a standard curve and the measurement of cAMP levels in unknown samples. Color was developed with 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide as a substrate. Optical density was read at 630 nm with a microplate reader (BIO-TEK Instruments, Winooski, VT, USA). Previously, we had determined that E2 increases cAMP levels at this time in the oviduct of unmated rats through a non-genomic signaling pathway (Orihuela et al. 2006; P Orihuela 2006, unpublished observations).

Animal surgery

Intrabursal administration, which minimizes the dose needed to affect the oviduct, avoiding systemic effects, was performed on unmated and mated rats at 1200 h as described by Orihuela & Croxatto (2001). Briefly, the oviduct and ovary were exposed through flank incisions made under anesthesia, and using a surgical microscope (OPMI 6-SDFC; Zeiss, Oberkochen, Germany), the drugs or vehicle alone was injected into the periovarian sac using a Hamilton syringe (Hamilton Co., Reno, NV, USA), and the injection site in the bursa was immediately sealed with an electric coagulator (Codman CMC-1; Codman and Shurtleff Inc., Randolph, MA, USA). The organs were returned to the peritoneal cavity, and the muscles and skin were sutured. Since ovulation was completed at this time point, this treatment did not affect the number of oocytes that ovulated. Furthermore, we had previously demonstrated that drugs that are administered i.b. act locally on the oviduct (Orihuela & Croxatto 2001, Orihuela et al. 2006).
Assessment of egg transport

Animals were killed 24 h after treatment, 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. We had previously determined that the recovery of eggs using this method is close to 100% by comparing the average number of eggs obtained from oviducts with this technique with the number of implanted embryos on day 12 of pregnancy (Ortiz et al. 1979). 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 E2 corresponds to premature transport to the uterus (Ortiz et al. 1979). Thus, we refer to this phenomenon as E2-induced acceleration of oviductal transport.

Oviduct collection and microarray analysis

Unmated (N=10) and mated (N=10) rats were injected s.c. with 10 μg of E2 (N=5) or vehicle (N=5) at noon, and 3 h later, they were killed and their oviducts were collected and flushed. Total oviductal RNA was isolated from each rat using Trizol reagent (Invitrogen), and equivalent quantities were mixed to generate four pools: unmated group treated with E2 (unmated group E2), unmated group treated with vehicle (unmated group vehicle), mated group treated with E2 (mated group E2), and mated group treated with vehicle (mated group vehicle). RNA probes prepared from each group were hybridized by Genome Explorations Inc. to the Rat Genome 230 2.0 chips (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s instructions. The transcriptome profile of the genes that were increased or decreased by E2 in the unmated group was compared to that of those that were increased or decreased by E2 in the mated group.

Real-time PCR

In order to determine the response of Star and Ckb RNA expression in the oviduct to E2, unmated and mated rats were injected s.c. with 10 μg of E2 or vehicle, and they were killed at various time intervals and their oviducts were collected and flushed. Total oviductal RNA was isolated from each rat using RNAseolv (Omega Bio-Tek, Norcross, GA, USA), and 1 μg of total RNA of each sample (two oviducts from one rat) was treated with DNase I (amplification grade; Invitrogen). The single-strand cDNA was synthesized by reverse transcription using the SuperScript III Reverse Transcriptase First Strand System for RT-PCR (Invitrogen) according to the manufacturer’s protocol. The Light Cycler instrument (Roche Diagnostics) was used to quantify the relative gene expression of Star and Ckb in the oviducts of unmated and mated rats treated with E2 or vehicle; Gapdh was chosen as the housekeeping gene for loading control. The SYBR Green I double-strand DNA binding dye from QuantiTec Real-Time RT-PCR kit (Qiagen) was the reagent of choice for these assays. The following primers were used: for Star, sense 5’-CTG CTA GAC CAG CCC ATG GAC-3’ and antisense 5’-TTG TTT CTA CAT TTT GTG TCT-3’; for Ckb, sense 5’-AAG CTG GCA GTA GAA GCC CT-3’ and antisense 5’-TTG AAG AGG AAG TGG TC-3’; and for Gapdh, sense 5’-ACC ACA GTC CAT GCC ATC AC-3’ and antisense 5’-TCC ACC ACC CTG TTG CTG TA-3’. All real-time PCR assays were performed in duplicate. The thermal cycling conditions included an initial activation step at 95 °C for 25 min, followed by 40 cycles of 95 °C for 15 s, 59 °C for 30 s, and 72 °C for 30 s, with an ultimate melting cycle (95–60 °C). In order to verify the specificity of each product, amplified products were subjected to melting curve analysis as well as to electrophoresis, and product sequencing was performed using an ABI Prism 310 sequencer. The expression of Star was determined using the equation: Y=2^−ΔCp, where Y is the relative expression, Cp (crossing point) is the cycle in the amplification reaction in which fluorescence begins to expand exponentially above the background baseline, and −ΔCp is the result of subtracting the Cp value of Star from the Cp value of Gapdh for each sample. To simplify the presentation of the data, the relative expression values were multiplied by 10^3 (Livak & Schmittgen 2001).

Immunoblotting

Oviducts obtained from unmated and mated rats (N=3 animals for each described experiment) were flushed, and their total proteins were isolated as described by Irusta et al. (2003). Briefly, oviducts were lysed in lysis buffer (20/ml3 Tris–HCl, pH 8.0, 137/ml3 NaCl, 1% Nonidet P-40, and 10% glycerol) supplemented with a protease inhibitor cocktail (Complete; Roche). The lysate was centrifuged at 4 °C for 10 min at 10 000 g, and the pellet was discarded. Protein concentrations in the supernatant were measured by the Bradford assay (Bio-Rad). After boiling for 5 min, proteins (20 μg) were separated on 15% SDS-PAGE slab gels in a Mini PROTEAN electrophoretic chamber (Bio-Rad). Proteins resolved in the gels were electroblotted onto nitrocellulose membranes (Bio-Rad). The membranes were blocked for 3 h in TTBS (100/ml3 Tris–HCl (pH 7.5), 150/ml3 NaCl, 0.05% (v/v) Tween-20) that contained 5% non-fat dry milk, and were incubated overnight with rabbit anti-STAR (kindly donated by Dr Douglas Stocco, Texas University) or mouse anti-α-tubulin antibody ((T5168) Sigma Chemical) at 1:1500 or 1:5000 antibodies were detected using the ECL Western Blotting Detection System (Amersham Pharmacia Biotech, Amersham, UK). The bands were visualized using the Bio Imaging System (Bio-Rad). Relative protein levels were estimated by densitometry. The bands were quantified by scanning the blots with a digital densitometer (Alpha Innotech Corporation, San Leandro, CA) and the ratio was calculated by dividing the optical density of the band of interest by the optical density of β-actin. The results are expressed as the percentage of the value obtained in the control treatment group.

Immunohistochemistry

Oviducts from unrestrained rats treated s.c. with E2 (N=3 animals) and sacrificed 4.5 h later were fixed in cold 4% paraformaldehyde in PBS, pH 7.4–7.6, for 2 h, and then a sequential transfer to 10% w/v sucrose in PBS for 60 min at 4 °C and 30% w/v sucrose in PBS at 4 °C overnight was done. Cryostat sections, 4–6 μm thick, were placed onto gelatin-coated slides and were blocked with 1% PBS–BSA for 120 min,
and then incubated with 1:100 anti-STAR antibody in 1% PBS–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 (Bio-source, Nivelles, Belgium) diluted in 1% PBS–BSA. After three PBS rinses, the slides were incubated with avidin–FITC (Sigma) that was diluted 1:5000 for 60 min at room temperature. Samples were subsequently washed with PBS, counterstained with 1 μg/ml propidium iodide, and mounted in DABCO (Sigma). As a negative control, the primary antibody was replaced by preimmune serum. The resulting staining was evaluated using a Zeiss confocal laser scanning microscope.

Statistical analyses

The results are presented as mean ± S.E.M. 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 the experiments that were performed to determine the effects of drugs on oviductal egg transport is the total number of rats used in each experimental group.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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