REPRODUCTION
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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 (E_2) accelerates oviductal egg transport through intraoviductal 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 E_2 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 E_2 in unmated and mated rats, indicating that an E_2 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 E_2 non-genomic signaling pathway present in unmated rats, but not in mated rats. Mating shuts down this E_2 non-genomic signaling pathway up- and downstream of cAMP production. The *Star* level is increased by E_2 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 E_2-induced gene expression profile in the rat oviduct differs before and after mating, and this difference is probably mediated by an E_2 non-genomic signaling pathway operating on gene expression only in unmated rats.

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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 (E_2) 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). E_2 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).

E_2 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 E_2 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, E_2 is able to activate rapid intracellular signaling pathways (kinase activation, cAMP, and IP_3 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 E_2 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, E_2 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 E_2 to methoxyestradiols (MEs) mediated by the enzyme catechol-*O*-methyltransferase

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(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–IP_3 signaling cascades (Orihuela *et al*. 2006).

After mating, a profound change occurs in the pathways utilized by E_2 to accelerate egg transport. Instead of using the non-genomic pathway (Orihuela *et al*. 2001), it uses genomic pathways (Ríos *et al*. 1997).

The change in pathways utilized by E_2 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 E_2-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 E_2 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 E_2 to accelerate ovum transport after mating, has not been evaluated. Previous reports indicate that E_2 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 E_2 on gene expression in the oviduct of unmated and mated rats. We found that E_2 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 E_2 non-genomic signaling pathway, present only in unmated rats, is responsible for these differences.

Results

Mating changes the transcript profile induced by E_2 in the oviduct

A microarray analysis using oviductal samples of unmated and mated rats treated s.c. with 10 μg E_2 or vehicle was performed to determine whether E_2 elicits different genomic effects before and after mating.

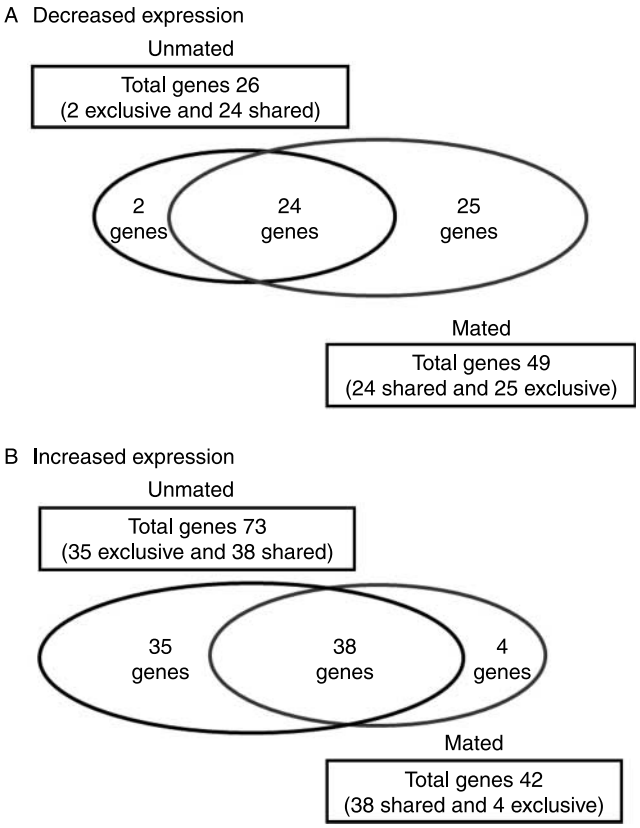

Figure 1 Changes in oviductal transcriptome induced by E_2 before (unmated) and after (mated) mating. Unmated ($N=10$) or mated ($N=10$) rats were injected s.c. with E_2, 10 μg, or vehicle. Three hours later, oviducts were removed to extract their mRNA and perform a microarray analysis. The results obtained 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 E_2 is shown separately.

Figure 1A and B shows the genes that decreased or increased their expression in response to E_2 respectively. The transcript profile induced by E_2 was very different when this hormone was applied before or after mating. Before mating, E_2 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 in response to E_2 before mating, 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 E_2 before mating, whereas only 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

Table 1 Transcripts that decreased their level in the oviduct of unmated and mated rats 3 h after an estradiol (E_2), 10 μg, treatment.

Probe set ID affymetrix	Accession number	Gene name	Gene symbol	Order of change	
				Unmated rats	Mated rats
1370019_at	NM_031834	Sulfotransferase family 1A, phenol-preferring, member 1	*Sult1a1*	−2	−2
1368124_at	NM_133578	Dual specificity phosphatase 5	*Dusp5*	−2	−2
1368894_at	NM_053874	CAP, adenylate cyclase-associated protein, 2 (yeast)	*Cap2*	−2	−2
1368718_at	NM_017272	Aldehyde dehydrogenase family 1, subfamily A7	*Aldh1a7*	−2	−2
1371883_at	NM_001007673	Monocyte to macrophage differentiation-associated	*Mmd*	−2	−2
1368128_at	NM_031598	Phospholipase A2, group IIA (platelets, synovial fluid)	*Pla2g2a*	−2	−2
1369670_at	NM_031518	Cd200 antigen	*Cd200*	−2	−2
1368911_at	NM_017099	Potassium inwardly rectifying channel, subfamily J, member 8	*Kcnj8*	−2	−2.1
1368869_at	NM_001033653	A kinase (PRKA) anchor protein (gravin) 12	*Akap12*	−2	−2.1
1370310_at	NM_173094	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2	*Hmgcs2*	−2	−2.3
1386908_at	NM_022278	Glutaredoxin 1 (thioltransferase)	*Glrx1*	−2	−2.5
1369113_at	NM_019282	Gremlin 1 homolog, cysteine knot superfamily (*Xenopus laevis*)	*Grem1*	−2	−2.6
1370228_at	NM_001013110	Transferrin	*Tf*	−2.1	−2
1387658_at	NM_012947	Eukaryotic elongation factor-2 kinase	*Eef2k*	−2.1	−2
1368870_at	NM_013060	Inhibitor of DNA binding 2	*Id2*	−2.1	−2.1
1367631_at	NM_022266	Connective tissue growth factor	*Ctgf*	−2.1	−3.5
1368154_at	NM_017090	Guanylate cyclase 1, soluble, α3	*Gucy1a3*	−2.3	−2.5
1367859_at	NM_013174	Transforming growth factor, β3	*Tgfb3*	−2.3	−2.6
1387850_at	NM_023020	Transmembrane protein with EGF-like and two follistatin-like domains 1	*Tmeff2*	−2.3	−4.9
1387809_at	NM_053703	MAP kinase kinase 6	*Map2k6*	−2.5	−2.1
1387074_at	NM_053453	Regulator of G-protein signaling 2	*Rgs2*	−2.6	−2.6
1368168_at	NM_053380	Solute carrier family 34 (sodium phosphate), member 2	*Slc34a2*	−2.8	−2
1368025_at	NM_080906	DNA-damage-inducible transcript 4	*Ddit4*	−2.8	−2.8
1371731_at	NM_001009617	Mesoderm-specific transcript	*Mest*	−4	−20

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 E_2.

E_2 activates a common genomic pathway in unmated and mated rats

Thirty-eight transcripts increased their expression in unmated and mated rats in response to E_2 (Table 4), indicating that a common signaling pathway activated by E_2 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 E_2 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.

E_2 increases cAMP level in the rat oviduct before, but not after, mating

E_2 increases cAMP levels in the oviduct of unmated rats through a non-genomic signaling pathway (Orihuela *et al.* 2003). Here, we evaluated whether E_2 is able to

Table 2 Transcripts that decreased their level exclusively in the oviduct of unmated rats 3 h after an estradiol (E_2), 10 μg, treatment.

Probe set ID affymetrix	Accession number	Gene name	Gene symbol	Order of change in unmated rats
1370913_at	NM_138881	Radical *S*-adenosyl methionine domain containing 2	*Rsad2*	−2.1
1369153_at	NM_022628	Nephrosis 1 homolog, nephrin (human)	*Nphs1*	−9.8

Table 3 Transcripts that decreased their level exclusively in the oviduct of mated rats 3 h after an estradiol (E_2), 10 μg, treatment.

Probe set ID affymetrix	Accession number	Gene name	Gene symbol	Order of change in mated rats
1368771_at	NM_134378	Sulfatase 1	*Sulf1*	−2
1371179_a_at	XM_001077699	Fibroblast growth factor receptor 2 predicted	*Fgfr2*	−2
1387122_at	NM_012760	Pleiomorphic adenoma gene-like 1	*Plagl1*	−2
1370363_at	NM_133295	Carboxylesterase 3	*Ces3*	−2.1
1369526_at	NM_013084	Acyl-coenzyme A dehydrogenase, short/branched chain	*Acadsb*	−2.3
1368522_at	NM_031340	Timeless homolog (*Drosophila*)	*Timeless*	−2.5
1367598_at	NM_012681	Transthyretin	*Ttr*	−2.5
1387082_at	NM_053348	Fetuin β	*Fetub*	−2.6
1368543_at	NM_053524	NADPH oxidase 4	*Nox4*	−2.8
1371083_at	NM_182474	Serine protease inhibitor	*LOC299282*	−3
1368288_at	NM_012564	Group-specific component	*Gc*	−3
1368161_a_at	NM_012898	α-2-HS-glycoprotein	*Ahsg*	−3.5
1368397_at	NM_001007264	UDP-glucuronosyltransferase 2 family, member 5	*Ugt2b5*	−3.5
1387125_at	NM_053587	S100 calcium-binding protein A9 (calgranulin B)	*S100a9*	−3.5
1370009_at	NM_012501	Apolipoprotein C-III	*Apoc3*	−3.7
1370086_at	NM_012559	Fibrinogen γ polypeptide	*Fgg*	−3.7
1369111_at	NM_012556	Fatty acid-binding protein 1, liver	*Fabp1*	−5.3
1367896_at	NM_019292	Carbonic anhydrase 3	*Ca3*	−6.1
1368627_at	NM_031546	Regucalcin	*Rgn*	−6.5
1367555_at	NM_134326	Albumin	*Alb*	−11.3
1370396_x_at	NM_001034950	Urinary protein 2	*Rup2*	−17.1
1368733_at	NM_012883	Sulfotransferase, estrogen preferring	*Ste*	−22.6
1370593_at	NM_153312	Cytochrome P450, family 3, subfamily a, polypeptide 11	*Cyp3a11*	−24.3
1370349_a_at	XM_001056871	Similar to urinary protein 3 precursor (RUP-3)	*LOC680367*	−24.3
1368048_at	NM_012657	Serine (or cysteine) peptidase inhibitor, clade A, member 3K	*Serpina3k*	−27.9

produce the same response after mating. In unmated rats, E_2 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, E_2 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 E_2 non-genomic signaling pathway in the oviduct, upstream of cAMP generation.

Mating shuts down E_2 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 adenylyl cyclase isoforms in unmated and mated rats, the effect of N^6,2′-O-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 E_2 non-genomic signaling pathway used by E_2 to accelerate ovum transport, downstream of cAMP generation too.

E_2-induced non-genomic signaling pathway produces changes in gene expression in unmated rats, and mating shuts down this pathway: the case of STAR expression

E_2 increased the level of 35 transcripts exclusively in unmated rats, suggesting that a signaling pathway activated by E_2 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 E_2 only in unmated rats, at RNA and protein levels was performed after E_2 treatment.

In unmated rats, E_2 significantly increased *Star* mRNA only 4.5 h after E_2 treatment (61.2±15.2 vs 22.2±6.1

Table 4 Transcripts that increased their level in the oviduct of unmated and mated rats 3 h after an estradiol (E_2), 10 μg, treatment.

Probe set ID affymetrix	Accession number	Gene name	Gene symbol	Order of change	
				Unmated rats	Mated rats
1367894_at	XM_001066529	Similar to insulin-induced gene 1 protein (INSIG-1)	*LOC688922*	+2	+2
1368685_at	NM_031022	Chondroitin sulfate proteoglycan 4	*Cspg4*	+2	+2
1387184_at	NM_024355	Axin2	*Axin2*	+2	+2.1
1368542_at	NM_053583	Zinc finger protein 423	*Zfp423*	+2	+2
1368272_at	NM_012571	Glutamate oxaloacetate transaminase 1	*Got1*	+2	+3
1367802_at	NM_019232	Serum/glucocorticoid-regulated kinase	*Sgk*	+2	+2.3
1370032_at	NM_021594	Solute carrier family 9 (sodium/ hydrogen exchanger), isoform 3 regulator 18	*Slc9a3r1*	+2	+2.8
1368465_at	NM_012892	Amiloride-sensitive cation channel 1, neuronal (degenerin)	*Accn1*	+2	+2
1387908_at	XM_001077321	RAS, dexamethasone-induced 1	*Rasd1*	+2.1	+2
1387391_at	NM_080782	Cyclin-dependent kinase inhibitor 1A	*Cdkn1a*	+2.1	+4.9
1367740_at	M14400	Creatine kinase, brain	*Ckb*	+2.1	+2
1369958_at	NM_022542	Ras homolog gene family, member B	*Rhob*	+2.1	+2
1370074_at	XM_001055943	Tensin	*Tns*	+2.1	+2
1370884_at	M36410	Sepiapterin reductase	*Spr*	+2.1	+2.1
1387091_at	NM_017226	Peptidyl arginine deiminase, type II	*Padi2*	+2.1	+2
1370379_at	NM_138836	Protease, serine, 8 (prostasin)	*Prss8*	+2.1	+2
1388133_at	XM_345860	Cold shock domain containing C2, RNA binding	*Csdc2*	+2.1	+2
1368231_at	NM_017064	Stat5A	*Stat5a*	+2.1	+2
1387332_at	NM_022275	Neuromedin U receptor 2	*Nmur2*	+2.1	+2.1
1387010_s_at	NM_017288	Sodium channel, voltage-gated, type I, β	*Scn1b*	+2.1	+3.7
1368826_at	NM_012531	Catecholamine-*O*-methyltransferase	*Comt*	+2.1	+2
1368223_at	NM_024400	ADAM metallopeptidase with thrombospondin type 1 motif, 1	*Adamts1*	+2.3	+2
1369520_a_at	NM_017253	Branched chain aminotransferase 1, cytosolic	*Bcat1*	+2.3	+2.3
1387099_at	NM_053838	Natriuretic peptide receptor 2	*Npr2*	+2.3	+2
1370173_at	NM_017051	Superoxide dismutase 2, mitochondrial	*Sod2*	+2.3	+2.1
1387563_at	NM_022847	Progesterone receptor	*Pgr*	+2.3	+3.2
1387737_at	NM_134351	Methionine adenosyltransferase II	*Mat2*	+2.3	+2
1387260_at	NM_053713	Kruppel-like factor 4 (gut)	*Klf4*	+2.5	+2
1370333_a_at	NM_178866	Insulin-like growth factor 1	*Igf1*	+2.5	+2
1370225_at	NM_053699	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	*Cited4*	+2.6	+2.3
1367725_at	NM_022602	Serine/threonine-protein kinase pim-3	*Pim3*	+2.6	+2
1368283_at	NM_133606	Enoyl-coenzyme A, hydratase/ 3-hydroxyacyl coenzyme A dehydrogenase	*Ehhadh*	+2.6	+2
1369443_at	NM_133569	Angiopoietin-like 2	*Angptl2*	+3	+2
1370708_a_at	NM_138547	Aldo-keto reductase family 1, member C14	*Akr1c14*	+3	+2.1
1369798_at	NM_012507	ATPase, Na^+/K^+ transporting, β2 polypeptide	*Atp1b2*	+4	+2
1368148_at	NM_012610	Nerve growth factor receptor (TNFR superfamily, member 16)	*Ngfr*	+5.3	+2
1370517_at	NM_153735	Neuronal pentraxin 1	*Nptx1*	+9.2	+3
1368102_at	NM_017081	Hydroxysteroid 11β dehydrogenase 2	*Hsd11b2*	+9.2	+3.5

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 E_2 treatment (4.0±1.0 and 4.5±1.2 times respectively, Fig. 5, unmated). In mated rats, E_2 did not change mRNA (Fig. 4, mated) and protein (Fig. 5, mated) levels of STAR at any time studied.

Since mating shuts down E_2-induced non-genomic signaling pathway up- and downstream of cAMP generation, the possibility that this pathway is responsible for transcript level changes induced by E_2 observed only in unmated rats was evaluated. For this, the E_2-induced *Star* expression increase in unmated rats was studied by blocking the ER and cAMP production, which are the two key components that mediate E_2 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 (E_2), 10 μg, treatment.

Probe set ID affymetrix	Accession number	Gene name	Gene symbol	Order of change in unmated rats
1369554_at	NM_053553	Synaptogyrin 2	*Syngr2*	+2
1387629_at	NM_022261	B-box and SPRY domain containing	*Bspry*	+2
1370153_at	NM_019216	Growth differentiation factor 15	*Gdf15*	+2
1371059_at	NM_019264	Protein kinase, cAMP-dependent, regulatory, type 2 α	*Prkar2a*	+2
1369177_at	NM_053735	Phosphatidylinositol 4-kinase type 2 α	*Pi4k2a*	+2
1370212_at	NM_053310	Homer homolog 3 (*Drosophila*)	*Homer3*	+2
1368845_at	NM_024000	caM kinase-like vesicle-associated	*Camkv*	+2
1368147_at	NM_053769	Dual specificity phosphatase 1	*Dusp1*	+2
1368449_at	NM_133567	Centaurin α 1	*Centa1*	+2
1387894_at	NM_144730	GATA binding protein 4	*Gata4*	+2
1369012_at	NM_017128	Inhibin β-A	*Inhba*	+2
1387353_at	NM_017093	Thymoma viral proto-oncogene 2	*Akt2*	+2
1387876_at	NM_022380	Stat5B	*Stat5b*	+2.1
1386909_a_at	NM_031353	Voltage-dependent anion channel 1	*Vdac1*	+2.1
1369650_at	NM_053306	p21 (CDKN1A)-activated kinase 2	*Pak2*	+2.1
1368931_at	NM_031238	SH3-domain GRB2-like 3	*Sh3gl3*	+2.1
1387133_at	NM_053988	Calbindin 2	*Calb2*	+2.3
1387844_at	NM_032613	LIM and SH3 protein 1	*Lasp1*	+2.3
1370780_at	NM_145094	RAB31, member RAS oncogene family	*Rab31*	+2.3
1368884_at	NM_022587	Ectonucleoside triphosphate diphosphohydrolase 1	*Entpd1*	+2.3
1369640_at	NM_012567	Gap junction membrane channel protein α1	*Gja1*	+2.5
1369278_at	NM_031034	Guanine nucleotide binding protein, α12	*Gna12*	+2.5
1383096_at	XM_343513	Amyloid β (A4) precursor-like protein 2	*Aplp2*	+2.5
1387707_at	NM_017102	Solute carrier family 2 (facilitated glucose transporter), member 3	*Slc2a3*	+2.5
1387810_at	NM_057152	Kelch-like ECH-associated protein 1	*Keap1*	+2.5
1370488_a_at	NM_019140	Protein tyrosine phosphatase, receptor type, D	*Ptprd*	+2.6
1387420_at	NM_031818	Chloride intracellular channel 4	*Clic4*	+2.8
1387858_at	NM_080907	Protein phosphatase 4, regulatory subunit 1	*Ppp4r1*	+2.8
1370344_at	NM_153629	Heat shock protein 4	*Hspa4*	+3
1368406_at	NM_031558	Star	*Star*	+3.2
1370714_a_at	NM_147205	β-Galactoside-α–2,6 sialyltransferase 1	*St6gal1*	+3.2
1369703_at	NM_023090	Endothelial PAS domain protein 1	*Epas1*	+3.2
1368897_at	NM_030858	MAD homolog 7 (*Drosophila*)	*Smad7*	+3.5
1387833_at	NM_130424	Transmembrane protease, serine 2	*Tmprss2*	+6.5
1388249_at	XM_001079347	Rap guanine nucleotide exchange factor (GEF) 1	*Rapgef1*	+10.6

intrabursally (i.b.) in unmated rats following a s.c. injection with E_2 or its vehicle. In rats receiving local treatment with vehicle, E_2 increased STAR levels (ICI 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 E_2 (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 E_2 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 E_2 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 E_2 exclusively in unmated rats do so, at least in part, in response to the activation of a

Table 6 Transcripts that increased their level exclusively in the oviduct of mated rats 3 h after an estradiol (E_2), 10 μg, treatment.

Probe set ID affymetrix	Accession number	Gene name	Gene symbol	Order of change in mated rats
1368339_at	NM_012521	S100 calcium-binding protein G	*S100g*	+2.5
1368342_at	NM_031544	Adenosine monophosphate deaminase 3	*Ampd3*	+2.8
1368290_at	NM_031327	Cysteine-rich protein 61	*Cyr61*	+4
1371193_at	XM_001065494	Tumor necrosis factor α-induced protein 6	*Tnfaip6*	+10

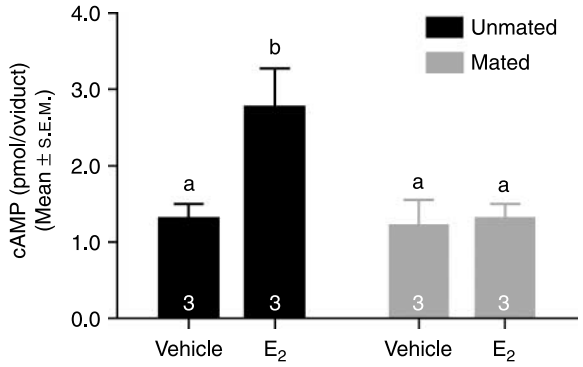

Figure 2 Effect of estradiol on oviductal cAMP level before (unmated) and after (mated) mating. cAMP level in the oviduct of unmated and mated rats ($N=3$ animals by treatment) 3 h after a s.c. injection of E_2, 10 µg, or vehicle. Numbers inside the bars indicate the number of animals used. Means with different letters are significantly different from each other ($P<0.05$).

local non-genomic signaling pathway that increases cAMP levels. Mating shuts down this pathway, preventing E_2 from increasing the expression of these genes in mated rats.

E_2 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 E_2 to accelerate egg transport in unmated rats (Parada-Bustamante *et al.* 2007). Here, we evaluated whether OR 486, a COMT inhibitor, blocks the E_2-induced increase in STAR levels. OR 486 or its vehicle was administered i.b. in unmated rats after a s.c. injection of E_2 or its vehicle. E_2 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 E_2 to ME is an essential component of the non-genomic signaling pathway of E_2 that regulates STAR expression in the oviduct of unmated rats.

Discussion

Mating has a profound impact on oviductal physiology, changing the pathway by which E_2 accelerates ovum transport from non-genomic to genomic (IPS). In this study, the genomic pathways that respond to E_2 before and after mating were explored. We found that the E_2-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 E_2 before mating than after mating. Since more information about the mechanisms by which E_2 increases gene expression is present in the literature, our analysis was focused on the group of genes that were increased by E_2. 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 E_2 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.* 1993), 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 E_2; moreover, *Ckb* promoter contains EREs and GC-rich sites, which are used by E_2, bound to ER, to increase its levels (Scott *et al.* 2003). In this work, we confirmed that E_2 increased *Ckb* mRNA levels in unmated and mated rat

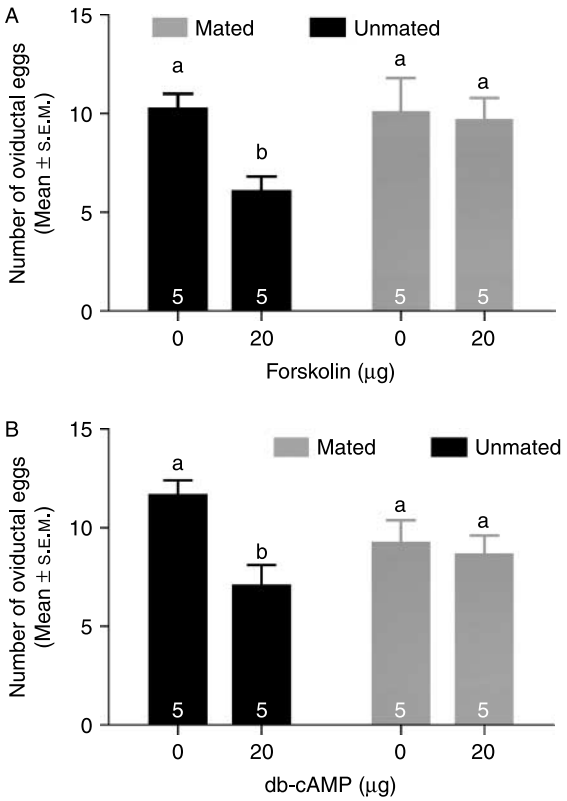

Figure 3 Forskolin and dibutyryl-cAMP accelerate ovum transport in unmated rats, but not in mated rats. Number of oocytes or embryos recovered from the oviduct of unmated and mated rats ($N=5$ animals by treatment) 24 h after being injected with forskolin, 20 µg, or vehicle i.b. (A) or with dbcAMP, 4 or 400 µg, or vehicle i.b. (B). Numbers inside the bars indicate the number of animals used. Means with different letters are significantly different from each other ($P<0.05$).

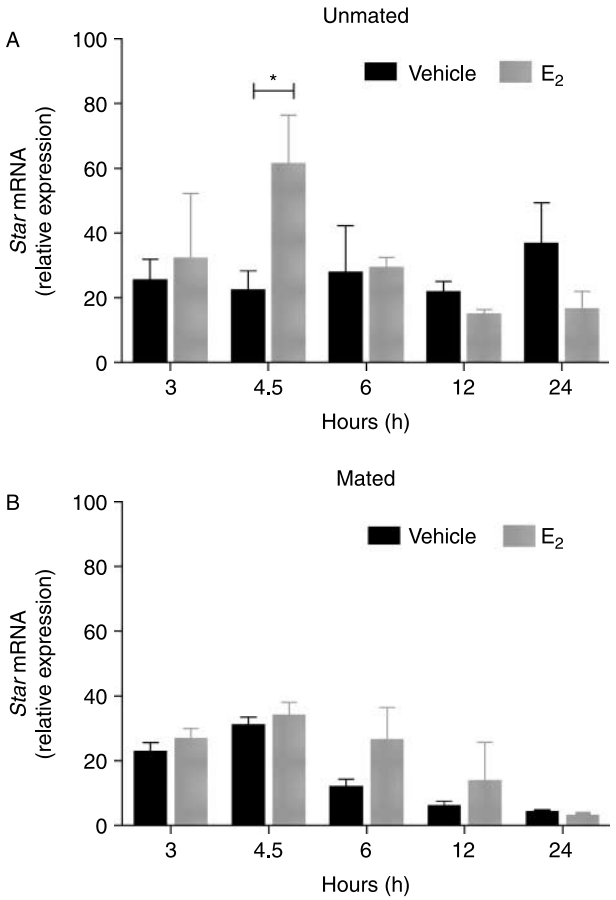

Figure 4 Estradiol increases oviductal *Star* mRNA levels in unmated rats, but not in mated rats. *Star* mRNA relative expression obtained through real-time PCR from cDNA of oviductal samples ($N=5$ animals by treatment) taken from unmated and mated rats 3, 4.5, 6, 12, and 24 h after a s.c. injection of E_2, 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 ($P<0.05$).

oviduct, indicating that the common pathway is, at least, a classical genomic pathway which is dependent on direct or indirect ER–DNA interaction.

E_2 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 E_2 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 E_2 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, E_2 increases the expression of these genes activating a non-genomic pathway. After mating, this pathway is shut down and then E_2 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 E_2 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 E_2 require ER activation and cAMP production suggests that this effect is dependent on the intraoviductal E_2 non-genomic signaling. Since ICI 182 780 alone did not affect STAR expression, it is

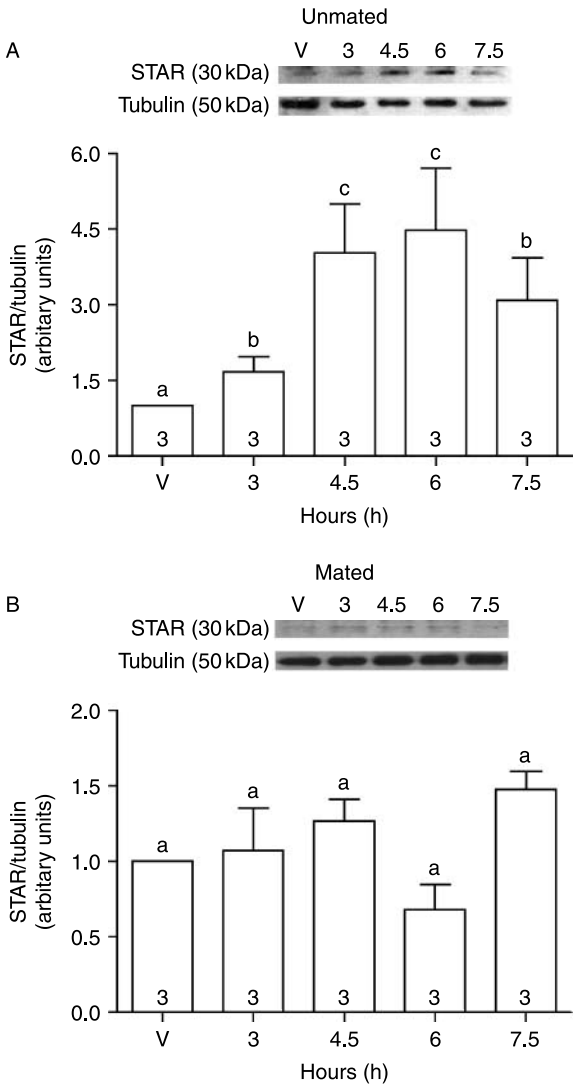

Figure 5 Estradiol increases oviductal STAR protein levels in unmated rats, but not in mated rats. Densitometric analysis of western blots to detect STAR protein in oviductal samples (20 μg, $N=3$ animals by treatment) taken 3, 4.5, 6, and 7.5 h after a s.c. injection of E_2, 10 μg, or vehicle. The values were normalized to α-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 ($P<0.05$).

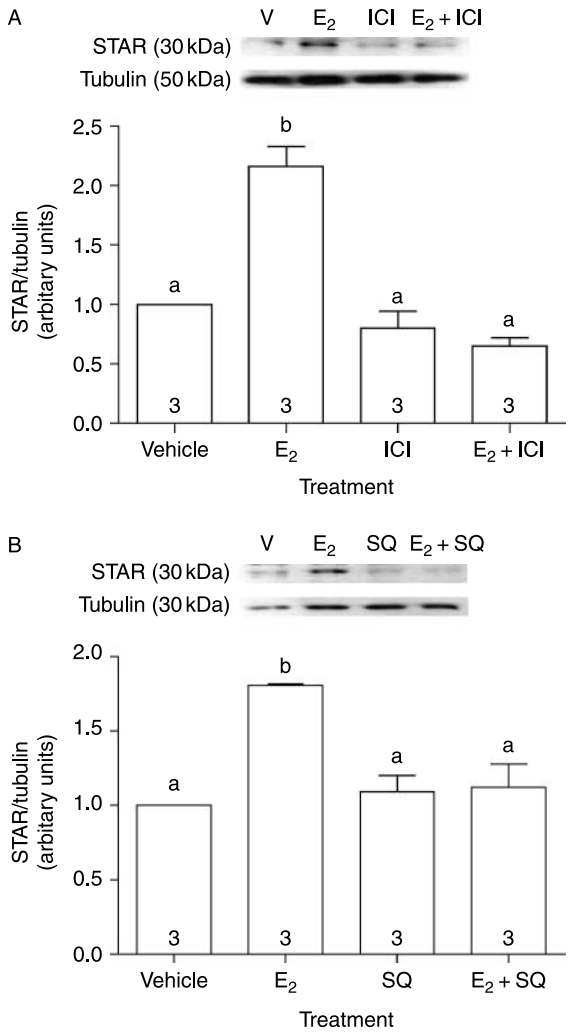

Figure 6 Increased expression of STAR levels in the oviduct in response to E_2 requires estrogen receptor and cAMP. Densitometric analysis of western blots to detect STAR protein in oviductal samples (20 µg, $N=3$ animals by treatment) of unmated rats 4.5 h after injecting E_2, 10 µg, or vehicle s.c. and concomitantly the estrogen receptor antagonist, ICI 182 780 (A), the adenylyl cyclase inhibitor, SQ 22536 (B), or their vehicle given i.b. The values were normalized to α-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 ($P<0.05$).

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 E_2 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 E_2 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 E_2 treatment. In mated rats, E_2 did not change STAR protein levels at any time studied. These results indicate that E_2 increases STAR protein levels in unmated rats, activating a non-genomic pathway that requires E_2 receptor and adenylyl cyclase.

This shutdown of E_2 non-genomic signaling pathway in mated rats could be explained by differences in E_2 levels or changes in the expression or location of ER induced by mating in the rat oviduct. However, previous reports have shown that plasma E_2 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 E_2 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.

E_2 increased STAR levels only in the epithelial cells, indicating that E_2-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 E_2 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 E_2 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 E_2-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 E_2; a previous work reported that STAR expression is decreased in interstitial and theca ovarian cells in newborn rats treated with E_2 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 E_2-induced acceleration transport is not blocked by transcription and translation inhibitors in unmated rats,

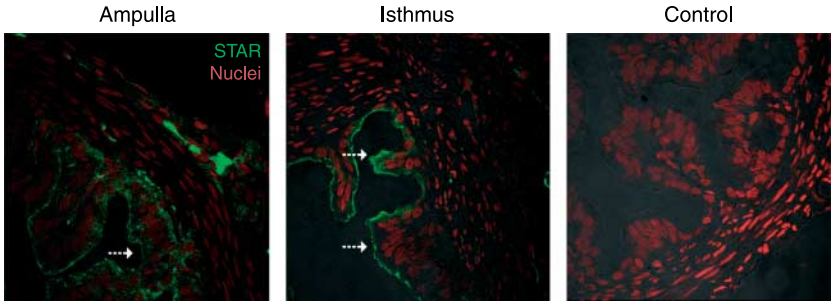

Figure 7 STAR is expressed in epithelial cells in unmated rat oviduct. Representative photomicrographs obtained from unmated rat oviducts 4.5 h after injecting E_2, 10 μg, s.c. ($N=3$) to detect STAR expression (green). Nuclei were stained with propidium iodide (red). Arrows point to immunoreactivity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

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 hypothetize 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.

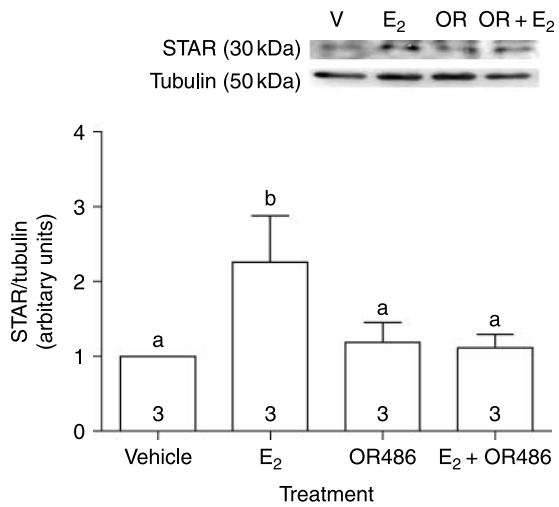

Figure 8 OR 486 blocks increased expression of STAR levels in the oviduct in response to E_2 in unmated rats. Densitometric analysis of western blots to detect STAR protein in oviductal samples (20 μg, $N=3$ animals by treatment) of unmated rats 4.5 h after injecting E_2, 10 μg, or vehicle s.c. and concomitantly the COMT inhibitor, OR 486, or vehicle given i.b. The values were normalized to α-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 ($P<0.05$).

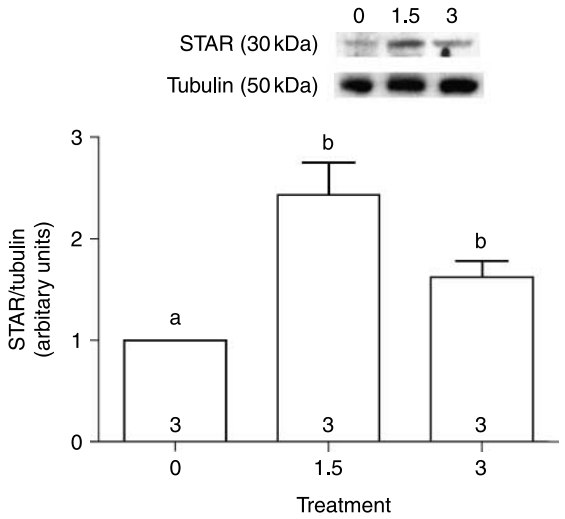

Figure 9 2-Methoxyestradiol increases oviductal STAR levels in unmated rats. Densitometric analysis of western blots to detect STAR protein in oviductal samples (20 μg, $N=3$ animals by treatment) from unmated rats taken 1.5 and 3 h after a s.c. injection of 2-methoxyestradiol, 100 μg, or vehicle. The values were normalized to α-tubulin. A representative western blot is shown. Numbers inside the bars indicate the number of animals used. Means with different letters are significantly different from each other ($P<0.05$).

Systemic administration of E_2 or 2ME

Unmated and mated rats were injected s.c. with 10 μg of E_2 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 E_2 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 increased expression of STAR induced by E_2. ICI 182 780, 25 μg, dissolved in 4 μl of 25% DMSO was injected i.b. in unmated rats immediately before injecting 10 μg of E_2 s.c. This dose of ICI 182 780 completely blocks the effect of E_2 on egg transport (Orihuela *et al.* 2003). SQ 22536, an adenylyl cyclase inhibitor, was used to determine whether the E_2 non-genomic signaling pathway is responsible for increasing STAR protein level in unmated rats. SQ 22536, 30 μg, dissolved in saline was injected i.b. in unmated rats immediately before injecting 10 μg of E_2 s.c. OR 486 (Tocris, Langford, UK) was used to inhibit COMT activity in the oviduct, and to determine whether local conversion of E_2 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 E_2 s.c.

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

Measurement of cAMP levels

Three hours after E_2 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 E_2 increases cAMP levels at this time in the oviduct of unmated rats through a non-genomic signaling pathway (Orihuela *et al.* 2006; and 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 Shurleff 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 E_2 corresponds to premature transport to the uterus (Ortiz *et al.* 1979). Thus, we refer to this phenomenon as E_2-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 E_2 ($N=5$) 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 E_2 (unmated group E_2), unmated group treated with vehicle (unmated group vehicle), mated group treated with E_2 (mated group E_2), 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 GeneChip System; Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. The transcriptome profile of the genes that were increased or decreased by E_2 in the unmated group was compared to that of those that were increased or decreased by E_2 in the mated group.

Real-time PCR

In order to determine the response of *Star* and *Ckb* RNA expression in the oviduct to E_2, unmated and mated rats were injected s.c. with 10 μg of E_2 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 RNAsolv (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 E_2 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′-TGA TTT CCT TGA CAT TTG GGT TCC-3′; for *Ckb*, sense 5′-AAG CTG GCA GTA GAA GCC CT-3′ and antisense 5′-TTG TCG 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: $Y = 2^{-\Delta C_p}$, where Y is the relative expression, C_p (crossing point) is the cycle in the amplification reaction in which fluorescence begins to expand exponentially above the background baseline, and $-\Delta C_p$ is the result of subtracting the C_p value of *Star* from the C_p 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/ml^3 Tris–HCl, pH 8.0, 137/ml^3 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/ml^3 Tris–HCl (pH 7.5), 150/ml^3 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 dilution respectively. The blots were rinsed five times for 5 min each in TBS (100/ml^3 Tris–HCl, pH 7.5, and 150/ml^3 NaCl) and were incubated for 1 h in TTBS that contained HRP-conjugated goat anti-rabbit or anti-mouse IgG (1:5000 dilution; Chemicon, Billerica, MA, USA). HRP activity was detected by ECL using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA). Oviductal samples without anti-STAR or anti-tubulin antibody were included as negative controls.

Immunohistochemistry

Oviducts from unmated rats treated s.c. with E_2 ($N=3$ 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 (Biosource, 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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