Tumour necrosis factor- α is the signal induced by mating to shutdown a 2-methoxyestradiol nongenomic action necessary to accelerate oviductal egg transport in the rat

María L Oróstica^{1,2}, Lidia M Zuñiga³, Daniella Utz¹, Alexis Parada-Bustamante³, Luis A Velásquez^{2,4}, Hugo Cardenas^{1,2} and Pedro A Orihuela^{1,2}

¹Laboratorio de Inmunología de la Reproducción, Facultad de Química y Biología, Universidad de Santiago de Chile, Alameda 3363, Casilla 40, Correo 33, Santiago, Chile, ²Centro para el Desarrollo en Nanociencia y Nanotecnología, CEDENNA, Santiago, Chile, ³Millennium Institute for Fundamental and Applied Biology, Santiago, Chile and ⁴Facultad de Medicina, Center for Integrative Medicine and Innovative Science, Universidad Andres Bello, Santiago, Chile

Correspondence should be addressed to P A Orihuela at Laboratorio de Inmunología de la Reproducción, Facultad de Química y Biología, Universidad de Santiago de Chile; Email: pedro.orihuela@usach.cl

Abstract

Mating shut down a 2-methoxyestradiol (2ME) nongenomic action necessary to accelerate egg transport in the rat oviduct. Herein, we investigated whether tumour necrosis factor- α (TNF- α) participates in this mating effect. In unmated and mated rats, we determined the concentration of TNF- α in the oviductal fluid and the level of the mRNA for *Tnf- α* (*Tnf*) and their receptors *Tnfrsf1a* and *Tnfrsf1b* in the oviduct tissues. The distribution of the TNFRSF1A and TNFRSF1B proteins in the oviduct of unmated and mated was also assessed. Finally, we examined whether 2ME accelerates oviductal egg transport in unmated rats that were previously treated with a rat recombinant TNF- α alone or concomitant with a selective inhibitor of the NF- κ B activity. Mating increased TNF- α in the oviductal fluid, but *Tnf* transcript was not detected in the oviduct. The mRNA for TNF- α receptors as well as their distribution was not affected by mating, although they were mainly localized in the endosalpinx. Administration of TNF- α into the oviduct of unmated rats prevented the effect of 2ME on egg transport. However, the NF- κ B activity inhibitor did not revert this effect of TNF- α . These results indicate that mating increased TNF- α in the oviductal fluid, although this not associated with changes in the expression and localization of TNF- α receptors in the oviductal cells. Furthermore, TNF- α mimicked the effect of mating on the 2ME-induced egg transport acceleration, independently of the activation of NF- κ B in the oviduct. We concluded that TNF- α is the signal induced by mating to shut down a 2ME nongenomic action in the rat oviduct.

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Introduction

Mating components include sensory stimulation, seminal plasma and spermatozoa. Either individually or collectively, these constituents impact the female physiology through their interaction with cells composing the female reproductive tract. It is now widely accepted that, independently of its fertilizing role, mating has physiological relevance as it affects at molecular and cellular levels the functioning of reproductive organs near or beyond to the site of insemination (Erskine 1995, Robertson 2005).

The relevance of mating-associated factors on the molecular and cellular changes occurring in the female reproductive organs is clearly illustrated by the fact that mating can regulate the role of oestradiol (E_2) and 2-methoxyestradiol (2ME) on the oviductal egg transport in the rat. In unmated rats, E_2 accelerates ovum transport

via a nongenomic action that requires prior conversion of E_2 to 2ME and successive activation of oestrogen receptors (ERs), cAMP–PKA and PLC–IP3 signalling cascades in the oviduct (Orihuela *et al.* 2001, 2003, 2006, 2009, Parada-Bustamante *et al.* 2003, 2007). This is corroborated by the fact that 2ME accelerates oviductal egg transport in unmated rats, but not in mated rats (Parada-Bustamante *et al.* 2007, 2010). Thus, mating shut down a nongenomic action of 2ME in the oviduct of unmated rats that is necessary to accelerate egg transport. In mated rats, E_2 accelerates embryo transport via a genomic action that does not require conversion of E_2 to 2ME but involve activation of the ER and signalling of connexin 43, s100g and endothelin in the oviduct (Ríos *et al.* 2007, 2011, Parada-Bustamante *et al.* 2012).

Female immune system can recognize and respond to mating-associated signals inducing an inflammatory

response in the genital tract that involves secretion of granulocyte-macrophage colony-stimulating factor, interleukins and chemokines (O'Leary *et al.* 2004, Robertson 2005, Kapelnikov *et al.* 2008). These effects may involve a direct interaction between the cells of the female reproductive tract and the different components of the seminal plasma and/or spermatozoa to modulate the immune system resulting in cellular and molecular changes along the female reproductive tract (reviewed in Sharkey *et al.* (2007)). Tumour necrosis factor- α (TNF- α) is a cytokine associated with immune responses in the female genital tract (Tremellen *et al.* 1998, Maisey *et al.* 2003, Morales *et al.* 2006). Furthermore, transcripts of TNF- α and their receptors are expressed in mice, bovine and human oviduct (Hunt *et al.* 1993, Srivastava *et al.* 1996, Wijayagunawardane *et al.* 2003, Reyes *et al.* 2007). Moreover, there are data showing that TNF- α could participate in reproductive functions including fertilization, embryo development and implantation (Rasmussen *et al.* 1999, Lee *et al.* 2000, Torchinsky *et al.* 2003, Abdo *et al.* 2008, Choo *et al.* 2011). Herein, we determined whether TNF- α is associated with the effect of mating on the nongenomic action of 2ME in the rat oviduct. First, we measured the concentration of TNF- α in the oviductal fluid of unmated and mated rats. We also determined the effect of mating on the levels of mRNA for TNF- α and their receptors *Tnfrsf1a* and *Tnfrsf1b* in the endosalpinx and myosalpinx of the rat oviduct. The tissue distribution of TNFRSF1A and TNFRSF1B was then compared in the oviduct of unmated and mated rats. Finally, we determined whether 2ME accelerates oviductal egg transport in unmated rats previously treated with a local injection of recombinant TNF- α alone or in combination with a selective inhibitor of the NF- κ B activity.

Results

Mating increased the concentration of TNF- α in the oviductal fluid

This experiment was designed to determine whether mating affects the level of TNF- α in the oviductal fluid. At 0000 h of day 1 of cycle, a total of 30 rats were kept isolated (unmated group) or caged with fertile males during 30 min (mated group), and 1, 3 or 6 h later, oviducts were excised and the oviductal fluid was obtained to measure the concentration of TNF- α as described in the Materials and Methods section. Replicas of this experiment are stated in Fig. 1.

Results of this experiment are shown in Fig. 1. In unmated rats, the amount of TNF- α in the oviductal fluid ranged from 0.12 ± 0.04 to 0.18 ± 0.05 pg/ μ g protein. In mated rats, the amount of TNF- α increased five to six times only 3 h after mating (0.72 ± 0.1 pg/ μ g protein) whereas 1 and 6 h post-mating the amount of TNF- α was similar to unmated rats (1 h, 0.19 ± 0.04 pg/ μ g protein and 6 h, 0.18 ± 0.01 pg/ μ g protein).

Figure 1 Levels of TNF- α in the oviductal fluid of unmated or mated rats. Rats at 0000 h of day 1 of the cycle were kept isolated as unmated group or caged with fertile males for 30 min as mated group, and 1, 3 or 6 h later, animals were autopsied to measure the amount of TNF- α in the oviductal fluid. This experiment consisted of five replicates. $a \neq b$, $P < 0.05$. Note a dramatical increase in the amount of TNF- α 3 h after mating.

Mating did not change the level of the transcripts of *Tnf*, *Tnfrsf1a* and *Tnfrsf1b* in the rat oviduct

This experiment determined whether mating changes the level of mRNA for *Tnf-a* (*Tnf*) and their receptors *Tnfrsf1a* and *Tnfrsf1b* in the rat oviduct. A total of 60 rats on day 1 of cycle were kept isolated (unmated group) or caged with fertile males during 30 min (mated group), and 1, 3 or 6 h later, oviducts were excised and separated in endosalpinx and myosalpinx to measure the level of the transcripts as described. Replicas of this experiment are stated in Figs 2 and 3.

Expression of *Tnf* mRNA was not detected in the endosalpinx and myosalpinx of unmated and mated rats (data not shown). As a proof of the efficiency of the real-time-PCR for *Tnf*, we used the RNA from liver samples of unmated rats. A band of 180 bp was detected corresponding to *Tnf* as verified after sequencing it (data not shown). On the other hand, the mRNA levels of *Tnfrsf1a* and *Tnfrsf1b* were not different between the endosalpinx (*Tnfrsf1a*, 30 ± 0.03 – 42 ± 0.042 and *Tnfrsf1b*, 0.12 ± 0.03 – 0.22 ± 0.08) and myosalpinx (*Tnfrsf1a*, 22 ± 4.8 – 30 ± 5.6 and *Tnfrsf1b*, 0.32 ± 0.08 – 0.61 ± 0.24) of unmated and mated rats. However, it was clear that the transcript of *Tnfrsf1a* was more highly expressed than *Tnfrsf1b* in the oviducts of unmated and mated rats (Figs 2 and 3).

Mating did not change the tissue distribution of TNF- α receptors in the rat oviduct

Here, we investigated the tissue distribution of TNFRSF1A and TNFRSF1B in the oviduct of unmated and mated rats. A total of 12 rats on day 1 of cycle were

Figure 2 mRNA levels of *Tnfrsf1a* in the oviduct of unmated or mated rats. Rats at 0000 h of day 1 of the cycle were kept isolated as unmated group or caged with fertile males for 30 min as mated group, and 1, 3 or 6 h later, the endosalpinx and myosalpinx layers were separated from the oviducts to determine the *Tnfrsf1a* mRNA relative expression obtained through real-time PCR. The values were normalized to *Gapdh*. This experiment consisted of five replicates. No differences were found between treatment groups.

kept isolated or caged with fertile males during 30 min, and 1 or 3 h later, oviducts were excised and processed by immunohistochemistry as described. Replicas of this experiment are stated in Figs 4 and 5. Immunoreactivity of TNFRSF1A and TNFRSF1B was detected in the endosalpinx and myosalpinx with no differences in their distribution between the oviducts of unmated and mated (Figs 4 and 5).

TNF- α blocks the 2ME-induced egg transport acceleration in unmated rats

As mating increases the level of TNF- α in the oviductal fluid, here we determined whether administration of TNF- α into the oviduct of unmated rats can block the effect of 2ME on the egg transport. A total of 60 animals on day 1 of cycle were injected with saline into one ovarian bursa and with TNF- α 0.16, 0.33 or 3.3 ng/ μ l in the contralateral bursa, and 12 h later, animals were s.c. injected with 2ME 100 μ g or propylene glycol as vehicle. Twenty-four hours after 2ME or propylene glycol injections, egg transport was assessed as described in

the Materials and Methods section. Replicas of this experiment are stated in Fig. 6. In order to confirm that intrabursal injection of TNF- α was effective, another 18 animals on day 1 of cycle were injected with saline or TNF- α 0.16 ng/ μ l, and 0.5, 1 or 3 h later, oviducts were excised and NF- κ Bp50 immunoreactivity was assessed as described in the Materials and Methods section.

The results are shown in Fig. 6. In rats s.c. treated with propylene glycol (vehicle), the number of eggs recovered from the oviducts treated with saline (vehicle) was 5.2 ± 0.6 – 5.7 ± 1.0 while in the contralateral oviducts treated with TNF- α ranged from 5.1 ± 0.7 to 6.8 ± 0.7 . Treatment with 2ME reduced the number of eggs in the oviducts treated with saline (1.9 ± 0.8 – 2.8 ± 0.9) while TNF- α partially blocked the 2ME-induced egg transport acceleration in the contralateral oviducts treated with 0.16 ng/ μ l (4.3 ± 0.8) and 0.33 ng/ μ l (4.1 ± 0.9), but not in the oviducts treated with 3.3 ng/ μ l (2.4 ± 1.1). On the other hand, Fig. 7 shows that NF- κ Bp50 immunoreactivity was mainly detected in the cytoplasm of epithelial cells of the control group while TNF- α 0.16 ng/ μ l induced transient translocation of NF- κ Bp50 at 0.5 h of treatment,

Figure 3 mRNA levels of *Tnfrsf1b* in the oviduct of unmated or mated rats. Rats at 0000 h of day 1 of the cycle were kept isolated as unmated group or caged with fertile males for 30 min as mated group, and 1, 3 or 6 h later, the endosalpinx and myosalpinx layers were separated from the oviducts to determine the *Tnfrsf1b* mRNA relative expression obtained through real-time PCR. The values were normalized to *Gapdh*. This experiment consisted of five replicates. No differences were found between treatment groups.

Figure 4 TNFRSF1A is expressed in the endosalpinx and myosalpinx of the oviduct of unimpaired or mated rats. Representative photomicrographs from rat oviducts processed by confocal microscopy. Rats at 0000 h of day 1 of the cycle were kept isolated as unimpaired group (A and B) or caged with fertile males for 30 min as mated group (E and D), and 1 or 3 h later, oviducts were excised to detect expression of TNFRSF1A (green). Nuclei were stained with propidium iodide (red). Arrows point to immunoreactivity obtained in the endosalpinx and myosalpinx with no differences between all groups. This experiment consisted of three replicates. Controls of the immunoreactivity were incubated with preimmune serum (C and F). e, endosalpinx; m, myosalpinx; l, lumen.

suggesting that intrabursal treatment with TNF- α was effective to activate its canonical signalling pathway in the oviductal epithelial cells.

A selective inhibitor of NF- κ B did not revert the effect of TNF- α on the 2ME-induced egg transport acceleration in unimpaired rats

This experiment was done to establish whether inhibition of the NF- κ B signalling cascade in the oviduct blocks the effect of TNF- α on the egg transport

acceleration following treatment with 2ME. A total of 20 animals on day 1 of cycle were divided into the following treatment groups: i) saline plus propylene glycol; ii) saline plus 2ME 100 μ g; TNF- α 0.16 ng/ μ l plus 2ME and iii) TNF- α plus pyrrolidine dithiocarbamate (PDCT) 75 μ g/ μ l plus 2ME. Propylene glycol or 2ME was injected 12 h after intrabursal injection of TNF- α or PDCT. Twenty-four hours after 2ME injection, egg transport was assessed as described in the Materials and Methods section. Replicas of this experiment are stated in Fig. 8.

Figure 5 TNFRSF1B is expressed in the endosalpinx and myosalpinx of the oviduct of unimpaired or mated rats. Representative photomicrographs from rat oviducts processed by confocal microscopy. Rats at 0000 h of day 1 of the cycle were kept isolated as unimpaired group (A and B) or caged with fertile males for 30 min as mated group (E and D), and 1 or 3 h later, oviducts were excised to detect expression of TNFRSF1B (green). Nuclei were stained with propidium iodide (red). Arrows point to immunoreactivity obtained in the endosalpinx and myosalpinx with no differences between all groups. This experiment consisted of three replicates. Incubating samples with preimmune serum assessed the specificity of immunoreactivity. (negative controls, C and F). e, endosalpinx; m, myosalpinx; l, lumen.

Figure 6 TNF- α blocks the 2ME-induced egg transport acceleration in unmated rats. Number of oocytes recovered from the oviducts of unmated rats on day 2 of the oestrous cycle. At 0060 h of the day 1 of the cycle, rats were injected with saline (vehicle) into one ovarian bursa and with TNF- α 0.16, 0.33 or 3.3 ng/ μ l in the contralateral bursa, and 12 h later, animals were s.c. injected with 2ME 100 μ g or propylene glycol (vehicle). Twenty-four hours after 2ME treatment, egg transport was assessed. This experiment consisted of six replicates. $a \neq b \neq c$, $P < 0.05$.

The results are shown in Fig. 8. The number of eggs recovered from the oviduct of the control group (saline plus propylene glycol) was 10.2 ± 1.1 , while in the group treated with 2ME (saline plus 2ME) was 3.2 ± 0.8 . As previously shown, local administration of TNF- α (TNF- α plus 2ME) blocked the 2ME-induced egg transport acceleration (10.2 ± 1.3) while PDCT (TNF- α plus PDCT plus 2ME, 7.7 ± 1.4) did not revert this effect of TNF- α .

Discussion

The presence of cytokines has been demonstrated in the mammalian oviduct (Fahey *et al.* 2005, Jiwakanon *et al.* 2010), an important site of gamete transport,

fertilization, early embryo development and egg transport (Croxatto 1996). In contrast to other species (Hunt *et al.* 1993, Srivastava *et al.* 1996, Wijayagunawardane *et al.* 2003, Reyes *et al.* 2007), we did not find expression of TNF- α mRNA in the endosalpinx and myosalpinx of the rat oviduct, indicating that the regulation of the transcription of the TNF- α mRNA in the mammalian oviduct could be species specific. However, the number of species studied in this regard is too small to formulate a phylogenetic based algorithm describing species specificity in the presence of the transcript for TNF- α in the mammalian oviduct.

Although mRNA for TNF- α is not expressed in the rat oviductal cells, we have found that TNF- α protein was dramatically increased in the oviductal fluid at 3 h after

Figure 7 TNF- α induces translocation of NF- κ Bp50 from the cytoplasm into the nucleus of oviductal cells in unmated rats. Representative photomicrographs obtained from unmated rat oviducts 0.5, 1 or 3 h after injecting TNF- α 0.16 ng/ μ l or saline (V) intrabursal. ($n=3$) to detect expression of NF- κ Bp50 (green). Nuclei were stained with propidium iodide (red). Note strong immunoreactivity of NF- κ Bp50 (arrowheads) in the nucleus of cells of the endosalpinx only 0.5 h after TNF- α injection. Incubating samples with preimmune serum assessed the specificity of immunoreactivity (negative control). E, endosalpinx; M, myosalpinx; L, lumen.

Figure 8 Inhibition of the NF- κ B activity in the oviduct did not revert the effect of TNF- α on the 2ME-induced egg transport acceleration in unmated rats. Number of oocytes recovered from the oviducts of unmated rats following intrabursal injections of TNF- α 0.16 ng/ μ l and PDCT 7.5 μ g/ μ l or saline (V) and a s.c. injection of 2ME 100 μ g or propylene glycol (V) 12 h later. Twenty-four hours after 2ME treatment, egg transport was assessed. This experiment consisted of five replicates. $a \neq b$, $P < 0.05$.

mating. How mating increase this cytokine in the oviductal fluid? We can speculate that some of the factors provided by mating such as the spermatozooids or sensory stimulation can interact on the uterine or vagina cells increasing the TNF- α release in these organs with the subsequent transport of TNF- α into the oviduct. Vascular connections between uterus and oviduct that facilitate the transport of several molecules along the reproductive tract may be responsible to transport TNF- α into the oviductal fluid (Bulletti *et al.* 1997). The fact that TNF- α levels were increased only at 3 h after mating can be explained by a rapid TNF- α degradation in the oviductal fluid and also indicate that mating has a time of latency of 3 h to exert its effects on the oviduct. On the other hand, TNF- α receptors were expressed in the oviduct, although mating did change neither the TNF- α receptor transcript levels nor the localization of these receptors in the rat oviduct. The presence of both TNF- α receptors in the endosalpinx demonstrate a compatible localization with the increased level of TNF- α in the oviductal fluid and reinforce our hypothesis of a role of TNF- α in the effect of mating on the oviductal cells.

With the purpose of demonstrating that the increased amount of TNF- α in the oviductal fluid observed after mating is associated with the silencing of the 2ME nongenomic action that accelerates oviductal egg transport, we determined whether 2ME accelerates egg transport in unmated rats previously treated with an intrabursal injection of TNF- α . Our results showing that 2ME did not accelerate oviduct egg transport in unmated rats indicate that effectively TNF- α mimicked the effect of mating on the silencing of this 2ME nongenomic effect as previous works of our group have clearly established that 2ME does not accelerate egg transport in mated rats

(Parada-Bustamante *et al.* 2007, 2010). This suggests that TNF- α could be a key factor by which mating regulates the intraoviductal signalling of E₂ and 2ME relevant to control egg transport. On the other hand, the effect of exogenous TNF- α occurred at small concentrations similar to the detected in the oviductal fluid, suggesting that TNF- α uses a receptor with high affinity to block the effect of 2ME on egg transport. As both TNF- α receptors are expressed in the rat oviduct, the identity of the receptor that participates in this effect of TNF- α remains to be determined.

The TNF- α canonical signalling pathway involves activation of the transcription factor NF- κ B on their target cells (reviewed in Heissmeyer *et al.* (1999) and Aggarwal (2003)). In this context, we have also found translocation of the p50 subunit of NF- κ B from the cytoplasm into the nucleus in oviductal epithelial cells from rats treated with TNF- α showing that the canonical pathway of TNF- α is active in the rat oviduct. However, inhibition of the NF- κ B activity in the oviduct by administration of PDCT did not revert the effect of TNF- α on the 2ME-induced egg transport acceleration. Thus, TNF- α acts independently of the activation of NF- κ B to shut down the nongenomic action of 2ME in the rat oviduct. As PDCT prevents activation of NF- κ B by a mechanism that involves inhibition of I- κ B degradation (Liu & Malik 1999), we cannot determine which NF- κ B subunit is effectively inhibited. It has been shown that NF- κ B does not account for all the intracellular targets of TNF- α (MacEwan 2002, Aggarwal 2003). For example, TNF- α receptors can activate the Janus kinase or p38 pathways, as well as caspase signalling (Aggarwal 2003). Furthermore, TNF- α stimulates adenylyl cyclase activity in human myometrial cells (Gogarten *et al.* 2003), and in Jurkat lymphoblastic leukemia cells, TNF- α induced ceramide production that leads to enhance the growth of this cell line but is unable to activate NF- κ B (Dbaiibo *et al.* 1993). Further studies are needed to determine whether some of these alternative pathways participate in the effect of TNF- α on the silencing of the nongenomic action of 2ME in the rat oviduct.

As we have previously demonstrated that the silencing of the intraoviductal 2ME nongenomic effect induced by mating involves inhibition of the catechol-O-methyltransferase (COMT) activity in the oviduct (Parada-Bustamante *et al.* 2007), it is probable that TNF- α downregulate the COMT activity in the oviduct. According to this, Salama *et al.* (2009) have reported that in human endometrial cells, TNF- α increases the local biosynthesis of oestrogens while decreases the gene expression of *Comt* as a mechanism to prevent deleterious effects of cytokines in endometrium such as inflammation or endometrial cancer. Contrary to Salama *et al.* (2009), *Comt* expression was upregulated by TNF- α in human myometrial cells in preparation for uterine contractions, labour and delivery (Wentz *et al.* 2006). Thus, oestrogen metabolism dependent on TNF- α is

influenced by the physiological context that occurs in the female reproductive tract.

There are scarce studies describing the role of TNF- α in the oviduct beyond inflammatory process. TNF- α has been associated with the cell and tissue damage observed in gonococcal salpingitis (McGee *et al.* 1992, Morales *et al.* 2006). This damage is correlated with increased TNF- α release by epithelium cells of the human oviduct induced by gonococcal infection (McGee *et al.* 1992). On the other hand, TNF- α stimulated secretion of the vascular endothelial growth factor by cultured human oviductal epithelial cells and stromal fibroblasts, which could be relevant to regulate oviductal fluid secretion necessary for preimplantation embryo development (Nasu *et al.* 2007). Moreover, preimplantation bovine embryos express and release TNF- α in order to regulate the oviductal motility necessary for the transport of the embryo into the uterus at the optimal time for implantation (reviewed in Wijayagunawardane & Miyamoto (2004)). Here, we describe a new physiological function of TNF- α in the mammalian oviduct associated with a change in the intraoviductal 2ME signalling induced by mating.

In summary, these results indicate that mating increases the TNF- α release into the oviductal fluid, although this is not associated with changes in the expression and localization of TNF- α receptors in the rat oviduct. Furthermore, TNF- α mimics the effect of mating on the 2ME-induced egg transport acceleration in unmated rats. It is concluded that TNF- α could be the signal by which mating shut down the intraoviductal 2ME nongenomic action that accelerates egg transport. These findings provide new evidence to understand the mechanism underlying the silencing of the 2ME nongenomic effect induced by mating in the oviduct and illustrate a new physiological role of TNF- α in the female reproductive tract.

Materials and Methods

Animals

Locally bred Sprague Dawley rats weighing 200–260 g were used. Animals were kept under controlled temperature (21–24 °C), and lights were on from 0700 to 2100 h. Water and pelleted rat chows were supplied *ad libitum*. The phases of the oestrous cycle were determined by daily vaginal smears (Turner 1961), and all females were used after showing two consecutive 4-day cycles. To obtain unmated and mated rats, females at 0000 h of the day 1 of cycle were kept either isolated or caged with fertile males during 30 min. Then, isolated rats that presented cornified cells in the vaginal smear, a cell phenotype associated with ovulation, 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 done in accordance with the ethical guidelines of the Universidad de Santiago de Chile.

ELISA measurement of TNF- α levels

At 0000 h of the day 1 of cycle, animals were kept isolated as unmated group or caged with fertile males for 30 min (mated group), and 1, 3 or 6 h later, oviducts were excised and flushed with 50 μ l saline solution in order to obtain the oviductal fluid. Levels of TNF- α were determined using a Rat TNF- α ELISA Ready-SET-Go (catalog no. 88-7340, eBioscience, San Diego, CA, USA). Protein concentration was assessed according Bradford (1976) using BSA as standard.

Treatments

Local administration of drugs

Unmated rats were injected in the ovarian bursa (intrabursal) with the drug described below. Control rats received the appropriate vehicle only. Rat recombinant TNF- α (BD Biosciences, San Jose, CA, USA) was injected into each ovarian bursa at a concentration of 0.16, 0.33 or 3.3 ng/ μ l in saline solution. The selective inhibitor of the NF- κ B activity PDCT, Sigma Chemical Co.) was used at a concentration of 75 μ g/ μ l dissolved in saline solution (Liu & Malik 1999). All treatments were given at 0600 h of day 1 of cycle (oestrus day).

Systemic administration of 2ME

Unmated rats were injected s.c. with 100 μ g 2ME (Steraloids, Newport, RI, USA) as a single dose in an injection volume of 0.1 ml propylene glycol. Control rats received propylene glycol alone. All treatments were given 12 h after local administration of drugs.

Animal surgery

Intrabursal administration of drugs, which minimizes the dose needed to affect the oviduct without systemic effects, was performed at 0600 h of day 1 of cycle (oestrus day) as described previously (Orihuela *et al.* 2001). At this time, ovulation has already taken place, so this treatment cannot affect the number of oocytes ovulated.

Assessment of egg transport

Egg transport was evaluated as previously published (Orihuela *et al.* 2001, 2003, 2006). Twenty-four hours after 2ME or vehicle treatment, animals were killed and their oviducts were flushed individually with saline. Flushing was examined under low-power magnification (25 $\times$), and the number of eggs found was recorded.

Real-time PCR

Oviducts were dissected in endosalpinx and myosalpinx and RNA was isolated using TRIzol Reagent (Invitrogen). Total RNA 1 μ g of each sample was treated with DNase I Amplification grade (Invitrogen). The single-strand cDNA was synthesized by RT 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 GmbH) was used to quantify the relative gene expression of *Tnf*, *Tnfrsf1a* or *Tnfrsf1b* in the oviduct; *Gapdh* was chosen as the housekeeping gene for load control because we have previously demonstrated that mating did not affect its expression (Ríos *et al.* 2007). Primers for *Tnf* were 5'-AGCTGTCTTCAGGCCAACAT-3' (sense) and 5'-ACAGC-CTGGTCACCAAATCA-3' (antisense), *Tnfrsf1a* 5'-ACCAAGTCCACAAAGGAAC-3' (sense) and 5'-CTGGAGGTAGGCACAGCTTC-3' (antisense), *Tnfrsf1b* 5'-ATGGTGCCTCATCTGCC-3' (sense) 5'-GGACCTGCTCATCCTTTG-3' (antisense) and for *Gapdh* were 5'-CTTCTATTCCTGCTCGTGG-3' (sense) 5'-GGTATGAAATGGCAAATCGG-3' (antisense). Furthermore, liver samples of unmated rats were included as positive controls. The SYBR Green I double-strand DNA binding dye (Roche Diagnostics) was the reagent of choice for these assays. The thermal cycling conditions included an initial activation step at 95 °C for 25 min, followed by 40 cycles of denaturizing and annealing–amplification (95 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s) and finally one cycle of melting (95–60 °C). To verify specificity of the product, amplified products were subject to melting curve analysis as well as electrophoresis, and product sequencing was performed to confirm identity as described by Muscillo *et al.* (2001). The expression of transcripts was determined using a method previously reported (Livak & Schmittgen 2001, Parada-Bustamante *et al.* 2010).

Immunofluorescence

Oviducts 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 anti-TNFRSF1A (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TNFRSF1B (Santa Cruz Biotechnology) or anti-NF-κBp50 (Santa Cruz Biotechnology) antibody 1:50 in 1% PBS–BSA in a humidified chamber overnight. Three PBS rinses were followed by 60-min incubation at room temperature with secondary antibody Alexa fluor 546-conjugated goat anti-rabbit IgG (Invitrogen) diluted in 1% PBS–BSA. Samples were subsequently washed with PBS, counterstained with 1 µg/ml propidium iodide and mounted in DABCO (Sigma). As negative controls, the primary antibody was replaced by preimmune serum. The resulting staining was evaluated using a Zeiss confocal laser-scanning microscope.

Statistical analysis

The results are presented as mean ± s.e.m. Overall analysis was done by Kruskal–Wallis test followed by Mann–Whitney *U* test for pair-wise 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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