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# Expression of urokinase type plasminogen activator receptor (uPAR) in the bovine oviduct: Relationship with uPA effect on oviductal epithelial cells

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ABSTRACT

Urokinase type plasminogen activator (uPA) is an oviductal fluid component whose activity is regulated by binding to urokinase type plasminogen activator receptor (uPAR). In this study uPAR and uPA gene expression in bovine oviduct were evaluated and similar expression patterns for both uPAR and uPA mRNAs were observed during the estrous cycle. Immunolocalization of uPAR at the apical zone of epithelial cells suggests that uPA action would be focalized in the oviductal lumen, triggering intracellular signaling pathways. As uPAR expression was also observed in *in vitro* cultures of oviductal epithelial cells, the effect of uPA was explored using this culture model. Real-time RT-PCR demonstrated that *c-fos* expression in oviductal cell cultures increases under uPA stimulation. These results suggest that uPA/uPAR binding would be involved in signaling pathways that activate transcription factors and would regulate the synthesis of molecules concerned with the arrangement of a particular oviductal microenvironment.



## 1. Introduction

The oviduct plays a pivotal role in mammalian reproduction by providing an optimal environment for sperm capacitation, fertilization and transport of gametes and embryos (Killian, 2004; Mondéjar et al., 2012a). In the oviductal fluid, proteolytic enzymes apparently involved in multiple events that take place in the oviduct have been identified in several mammalian species (Gabler et al., 2001; Jiménez-Díaz et al., 2000; Roldán-Olarte et al., 2012).

The plasminogen activators (PAs)/plasmin system is one of the most broadly expressed systems that catalyze extracellular proteolysis (Martínez-Henández et al., 2011; Ny et al., 2002). PAs can be produced by many cell types in order to convert the widely distributed zymogen plasminogen to plasmin. Plasmin degrades most extracellular proteins either directly or by activating other proteases, thus affecting cell–cell and cell–matrix interactions.

Plasminogen has been found in the oviductal lumen of pigs and cows (Mondéjar et al., 2012b) as well as in the plasma membrane and zona pellucida of hamster oocytes (Jiménez-Díaz et al., 2002), immature porcine oocytes (Roldán-Olarte et al., 2005) and in *in vitro* matured oocytes of pigs and cows (Mondéjar et al., 2012b). The presence of urokinase-type plasminogen activator (uPA) in the oviductal fluid has been studied in different mammalian species such as hamster, (Jiménez-Díaz et al., 2000), bovine (Gabler et al., 2001) and porcine (Roldán-Olarte et al., 2005). uPA activity varies in the oviductal fluid throughout the estrous cycle (Jiménez-Díaz et al., 2000; Roldán-Olarte et al., 2005, 2012).

uPA appears to be involved in the extracellular proteolysis required for cell migration and tissue remodeling. The localization of uPA seems to be controlled through its specific cell-surface receptor (Blasi and Sidenius, 2010). Because it is associated with the external surface of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, uPAR localizes uPA activity to the cell surface (Smith and Marshall, 2010).

uPA binding to uPAR also induces intracellular signaling in a variety of cells (Blasi and Sidenius, 2010; Luft, 2012; Smith and Marshall, 2010; Tang et al., 1998) and initiates the activation of transcription factors, including AP-1 (activator protein 1) (Eferl and Wagner, 2003; Konakova et al., 1998). The AP-1 superfamily includes the Jun and Fos protein families, which are associated to form dimers that bind to regulatory DNA sequences. This interaction would have a positive or negative effect on the expression of specific genes (Cargnello and Roux, 2011; Karin, 1995).

Previously, uPAR expression was identified in pig oviduct (Roldán-Olarte et al., 2012), although no evidence of it was found in bovine oviductal epithelial cells. The hypothesis postulated here is that uPA initiates the transduction of intracellular pathways through binding to its receptor in the bovine oviduct. The objectives of this work were: (i) to study the *uPAR* gene expression of

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oviductal epithelial cells at different stages of the estrous cycle; (ii) to demonstrate the localization of this receptor in the oviduct; (iii) to evaluate uPA action by the analysis of the expression levels of the early gene *c-fos* in bovine oviductal epithelial cell cultures.

## 2. Materials and methods

### 2.1. Tissue preparation

#### 2.1.1. Isolation of bovine oviductal epithelial cells

Genital tracts from young beef cows (*Bos taurus*) were collected at a local abattoir within 20 min of death and transported to the laboratory in sodium phosphate buffer (PBS), pH 7.4, at 4 °C for processing within 3 h after collection. The stage of the estrous cycle was defined on the basis of the direct examination of ovarian structures (color, consistency, number and size of follicles and corpora lutea) and of the uterus, as previously described by Ulbrich et al. (2004). These criteria were used to classify the oviducts into three groups: preovulatory stage (Pre-Ov, days 19–21), postovulatory stage (Post-Ov, days 1–5) and mid-luteal stage (Mid-L, days 6–12). The complete oviducts of four animals at each stage of the estrous cycle were rinsed with PBS containing 100 IU/ml of penicillin and 100 mg/ml of streptomycin (Gibco, Life Technology, Burlington, ON, Canada) and disinfected with 70% ethanol. The ampulla and isthmus regions were isolated from each oviduct. Epithelial cells were obtained by opening the oviductal segment longitudinally and scraping the mucosal epithelial layer with a scalpel. The ipsilateral oviducts were used in all cases. These samples were used for the analysis of *uPAR* and *uPA* expression in bovine oviduct throughout the estrous cycle.

#### 2.1.2. Bovine oviductal epithelial cell cultures

Bovine oviducts collected during the Mid-L stage were selected to obtain oviductal epithelial cells. Ipsilateral oviducts obtained from seven animals were used in each experiment. Oviducts were ligated, dissected, and washed in fresh PBS and then dipped in 70% ethanol. After discarding infundibulum and uterotubal junction, oviducts were gently squeezed in a stripping motion with forceps to obtain epithelial cells, as described by Rottmayer et al. (2006). Biological material was collected in culture medium TCM-199 (Gibco, Life Technology, Burlington, ON, Canada) supplemented with 10% bovine fetal serum (Internegocios, Buenos Aires, Argentina), 25 mM Hepes (Gibco, Life Technology, Burlington, ON, Canada), 0.2 mM sodium pyruvate, 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of fungizone (Gibco, Life Technology, Burlington, ON, Canada). The cell suspension was pipetted 15 times through a 21 gauge syringe needle. Three steps of washing were performed, each followed by 25 min sedimentation in culture medium in the cell culture incubator. Cell viability at seeding was analyzed by trypan blue (Sigma) staining and by microscopic observation of beating cilia. Cells were grown in 25 $mm^2$ bottle cultures (Nunclon, Roskilde, Denmark) in 5 ml TCM-199 supplemented as described earlier. Culture took place at 38.5 °C in a humidified atmosphere and 5% $CO_2$. After 48 h, cell cultures were washed twice with PBS. The epithelial nature of the cultured cells was confirmed by immunocytochemical analysis, using monoclonal Anti Pan Cytokeratine clone PCK-26 antibody (Sigma, C1801); more than 90% of the cells were cytokeratin positive.

Twenty milligrams of cell suspension culture was placed in each well of a 24-well culture plate (Nunclon, Roskilde, Denmark) in 500 µl of serum-free medium containing 1% bovine serum albumin (Sigma) for 2 h. Oviductal cells were stimulated with uPA 10 nM (Sigma) at 0, 30 and 45 min in serum-free medium, as described by Dumler et al. (1994). Control samples without uPA stimulation were taken at the same time as samples of induced cultures.

### 2.2. Total RNA isolation

Total RNA was isolated from oviductal cells of ampulla or isthmus corresponding to different stages of the estrous cycle ($n = 4$ oviducts per stage) by using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA). Bovine oviductal epithelial cell cultures ($n = 4$ per treatment) were isolated using RNeasy Micro Kit (Qiagen). The yield of total RNA was determined spectroscopically at 260 nm. RNA quantity and quality were verified after electrophoresis on 1% (w/v) formaldehyde agarose gel containing 0.06 µg/ml Sybr® Safe DNA Gel Stain (Invitrogen, Burlington, ON, Canada).

### 2.3. Reverse transcription-polymerase chain reactions (RT-PCR)

#### 2.3.1. Determination of uPAR and uPA expression by RT-PCR

Total RNA (1 µg) from each sample of epithelial cells and cultures was reverse transcribed using Moloney murine leukemia virus (M-MLV) enzyme (Promega, Madison, WI, USA), oligo(dT)17 and random primers (Roldán-Olarte et al., 2012). A 25 µl final volume of the reaction mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM of each dNTP, 25 pM oligo(dT) and random primers, 10 mM dithiothreitol and 200 units of reverse transcriptase was incubated at 42 °C for 1 h followed by a reverse transcriptase inactivation at 94 °C for 5 min. Aliquots of 1 µl of the RT reaction were amplified by end-point PCR with 2.0 U of *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada) in a 20 µl reaction volume containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP and 1 µM of each oligonucleotide specific primer for the gene of interest: *uPAR*, *uPA* and *β-actin* (see Table 1). PCR conditions were previously assayed to determine the appropriate number of cycles for the amplification of each fragment, as described by Argañaraz et al. (2007). For the amplification of uPAR, uPA and β-actin PCR cycles consisted of: (1) an initial denaturing step at 94 °C for 1 min; (2) 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at the appropriate annealing temperature for each pair of primers (uPAR and β-actin: 58 °C and uPA: 60 °C), extension for 50 s at 72 °C; (3) a final

**Table 1**
Primers used to amplify specific bovine transcripts from bovine oviductal epithelial culture and oviduct.

| Gene | | Oligonucleotides (5′-3′) (upstream/downstream) | Product size (bp) | GenBank accession number |
|---|---|---|---|---|
| uPAR | Forward | ACCACACCTTCCACTTCCTG | 339 | NM_174423.3 |
| | Reverse | CTGGGTGGTTACAGCCACTT | | |
| uPA | Forward | ATTTGCACGTAGCACCAGGGCC | 498 | NM_174147.2 |
| | Reverse | CCTACAAGTCCCCACAGTCCCG | | |
| β-Actin | Forward | GAGCTACGAGCTTCCTGACG | 245 | NM_173979.3 |
| | Reverse | GGGCAGTGATCTCTTTCTGC | | |
| c-Fos | Forward | CTCTCCTACTACCACTCACC | 154 | NM_182786.2 |
| | Reverse | GTACTAGCCATTGTAGGTCC | | |
| GAPDH | Forward | AGATGGTGAAGGTCGGAGTG | 117 | NM_001034034 |
| | Reverse | GAAGGTCAATGAAGGGGTCA | | |

extension step at 72 °C for 7 min. Each PCR assay was carried out at least twice for each sample. Primer pairs were designed with primer3 software from published *Bos taurus* cDNA sequences. GenBank accession numbers, primer sequences and amplification fragment sizes are shown in Table 1. β-actin mRNA, commonly employed as a standard when comparing samples under different hormonal conditions, was used as a control for RNA quantity and PCR reaction efficiency (Soutar et al., 1997). Non-template controls were included in each experiment. PCR products were resolved on 1.5% agarose gel containing 0.06 μg/ml Sybr® Safe DNA Gel Stain (Invitrogen, Burlington, ON, Canada). The nature of the PCR products obtained was confirmed by sequencing. The gels were photographed with a digital camera (Olympus C-5060). The intensities of the bands were analyzed with the NIH ImageJ software for the semi-quantitative determination of the expression levels of *uPAR* and *uPA*.

#### 2.3.2. c-fos expression by real time RT-PCR

In order to determine *c-fos* expression levels, real time RT-PCR assays were carried out with Fast Plus EvaGreen® qPCR Master Mix (Biotium) and 0.5 μM of each gene-specific primer pairs (Table 1) on a BioRad CFX96 system. Real-time PCR was performed with a total volume of 20 μl, each reaction containing 5 μl of 1/20 dilution of cDNA determined by a standard curve, 5 μl of a 2 μM of primer pairs stock solution (forward and reverse) and 10 μl of Fast Plus EvaGreen® qPCR Master Mix. All primers were designed to amplify a product with a size of 117–154 bp, and the specificity of each primer set was confirmed by running the PCR products on a 1.5% agarose gel. PCR conditions were as follows: 94 °C for 2 min followed by 40 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s using a melting-curve program (increasing the temperature from 65 °C to 95 °C at a rate of 0.5 °C every 10 s) and continuous fluorescence measurement. Sequence-specific products were identified by generating a melting curve, the Ct value represented the number of cycles at which a fluorescent signal rose statistically above background and relative gene expression was quantified (Livak and Schmittgen, 2001). The relative ratios of mRNA levels were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Ct value. Each gene was analyzed in quadruplicate.

### 2.4. uPAR immunohistochemistry

To determine uPAR location in the oviductal tissue, segments of 10 mm from ampulla and isthmus of Pre-Ov ($n = 3$), Post-Ov ($n = 3$) and Mid-L ($n = 3$) stages were fixed in 4% formaldehyde/PBS (pH 7.4) and embedded in paraffin. Paraffin wax-embedded blocks of oviduct segments were processed as described by Roldán-Olarte et al. (2012). The pieces were rinsed with PBS, pH 7.4, and endogenous peroxide activity was eliminated by incubation with 3% (v/v) $H_2O_2$ in PBS, pH 7.4, for 1 h. All sections were rinsed in PBS and incubated in the blocking solution (BSA 1 mg/ml in PBS, pH 7.4) for 60 min at room temperature. Then, without rinsing, the sections were incubated with 8 μg/ml goat anti-human uPAR polyclonal antibody (R&D System, AF807) in the blocking solution at 4 °C overnight. The sections were rinsed three times in PBS. After rinsing, they were incubated with rabbit anti-goat IgG (whole molecule) biotin conjugated antibody (Sigma B7024, St. Louis, MO, USA) at a dilution of 1:50 for 1 h at room temperature and rinsed again. Then, samples were incubated with extravidin–peroxidase (Sigma E2886, St. Louis, MO, USA) at a dilution of 1:100 for 1 h at room temperature and rinsed. Finally, sections were developed for 10 min with the substrate, 3, 3′diaminobenzidine (Sigma B8001, St. Louis, MO, USA). Processed samples were counterstained with Mayer's hematoxylin. Negative controls were carried out by omitting the primary antibody treatment and adding 3% (vol/vol) preimmune goat serum to assess the level of nonspecific background labeling. One operator scoring the relative intensity of the uPAR immunostaining examined all sections. Photomicrographs were taken with an Olympus BX40 microscope. The same procedure was followed to detect uPAR in epithelial cell cultures.

### 2.5. Statistical studies

The statistical analysis for uPAR and uPA mRNA levels was performed on values from densitometry analyses normalized to corresponding β-actin mRNA. All data were normally distributed and underwent equal variance testing. Model parameters included oviduct sections (ampulla and isthmus) and the different stages of the estrous cycle (Pre-Ov, Post-Ov and Mid-L). c-fos mRNA levels were analyzed in uPA induction treatments at different times. Multiple means for uPAR, uPA and c-fos mRNAs were compared using ANOVA, and when a significant effect was obtained the difference between means was determined by *t*-test. A *P* value of less than 0.05 ($P < 0.05$) was considered statistically significant.

## 3. Results

### 3.1. uPAR and uPA expression in oviductal epithelial cells

The expression of uPAR and its ligand, uPA, in bovine oviductal epithelial cells was analyzed by semiquantitative RT-PCR on cDNA samples obtained from ampulla and isthmus during Pre-Ov, Post-Ov and Mid-L stages. Both uPAR and uPA mRNAs were detected in all the samples assayed (Fig. 1). uPAR and uPA showed a low mRNA expression level during the Post-Ov stage compared with the Pre-Ov and Mid-L stages of the bovine estrous cycle. These differences were significant in the ampulla for uPAR and for uPA mRNA between the Post-Ov and the Mid-L stage ($P < 0.05$) (Figs. 1A–C). Also, a considerable difference in *uPAR* expression was observed between ampulla and isthmus during the Pre-Ov stage.

### 3.2. uPAR immunolocalization in the bovine oviduct

Immunohistochemical analysis using anti-human uPAR polyclonal antibody revealed the localization and distribution pattern of uPAR in the bovine oviduct. Positive immunostaining of uPAR at the apical region of epithelial cells from ampulla (Figs. 2A, A1) and isthmus (Figs. 2C, C1) was observed. The most intense signal was detected during the Mid-L stage of the estrous cycle (Fig. 2). No signal was detected in controls (Figs. 2B, B1 and 2D, D1).

### 3.3. Expression of uPAR in epithelial cell cultures

In order to evaluate if uPAR is expressed in oviductal cell cultures, immunohistochemical and RT-PCR analyses were performed in a bovine oviduct epithelial cell suspension primary culture system. The anti-human uPAR polyclonal antibody revealed the presence of the protein in epithelial cells (Fig. 3A). RT-PCR assays also confirmed *uPAR* expression in the oviductal epithelial cells kept in *in vitro* conditions (Fig. 3C).

### 3.4. c-fos gene expression in bovine oviductal epithelial cell cultures under uPA stimulation

Real time RT-PCR analysis demonstrated the expression of *c-fos* in bovine oviductal epithelial cell cultures. Stimulation with uPA 10 nM at different incubation times (0, 30 and 45 min) showed a time-dependent increase in c-fos mRNA. Significant differences were found between mRNA levels at 45 min and those at 0 and 30 min after stimulation with uPA. Cell cultures incubated in the presence of 1% bovine serum albumin instead of uPA did not show changes in *c-fos* expression at different incubation times (Fig. 4).

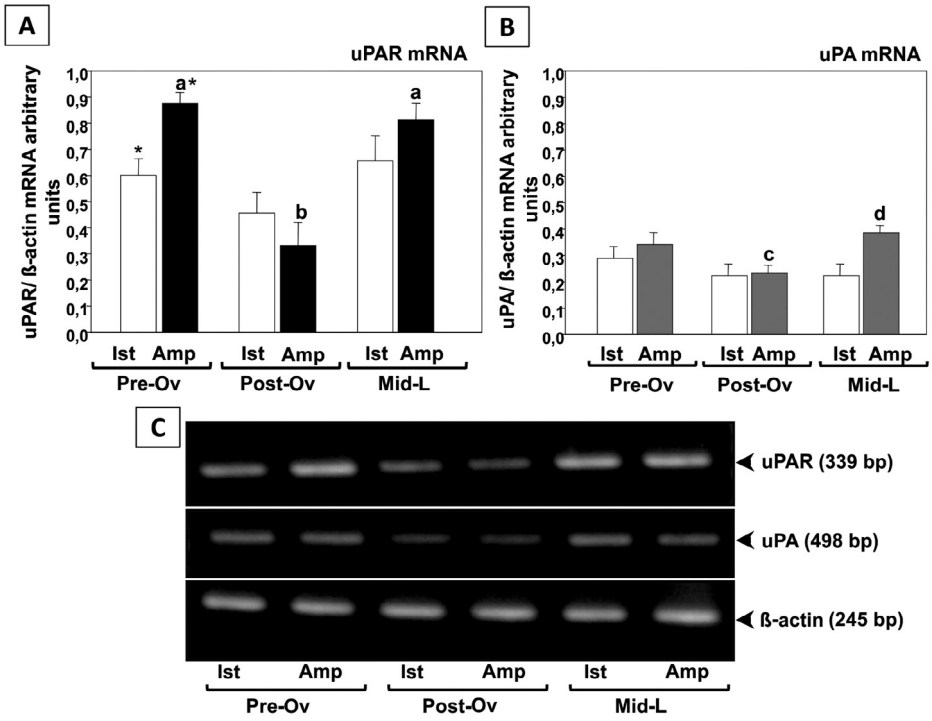


**Fig. 1.** Comparison of relative density of uPAR/β-actin (A) and uPA/β-actin (B) RT-PCR products in bovine oviductal cells. Values are mean ± SE ($n = 4$). Means with different indices (a, b, c, d) between ampulla cells from stages of the estrous cycle are significantly different for $P < 0.05$. Significant differences were found between oviductal cells from ampulla and isthmus in uPAR expression (see *). Arbitrary units were determined by relating the band density to the corresponding β-actin band for each sample. (C) Specific RT-PCR products for uPAR (339 bp), uPA (498 bp) and β-actin (245 bp) in ampulla (Amp) and isthmus (Ist) bovine oviductal cells in different stages of the estrous cycle separated by agarose gel electrophoresis. Pre-Ov: preovulatory stage, Post-Ov: postovulatory stage, Mid-L: mid-luteal stage.

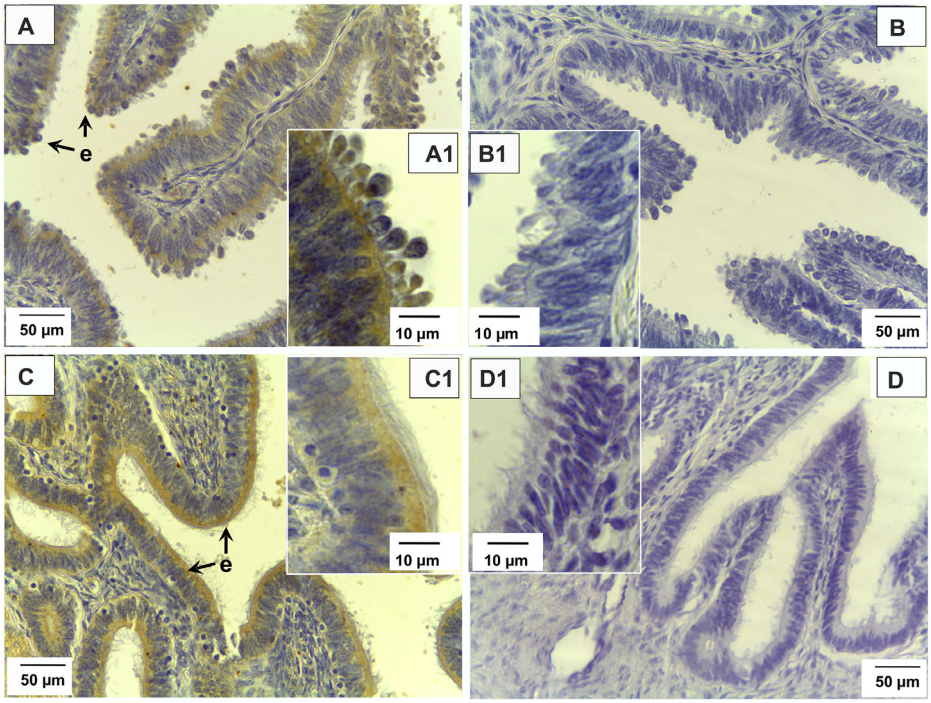


**Fig. 2.** Immunohistochemical localization of uPAR in oviductal epithelium of the ampulla (A, A1) and isthmus (C, C1), during the Mid-L stage of the estrous cycle. Arrows indicate positive localization in the epithelial cells in both ampulla and isthmus oviductal regions. B, B1 and D, D1: Preimmune goat serum instead of the primary antibody; e: epithelium.

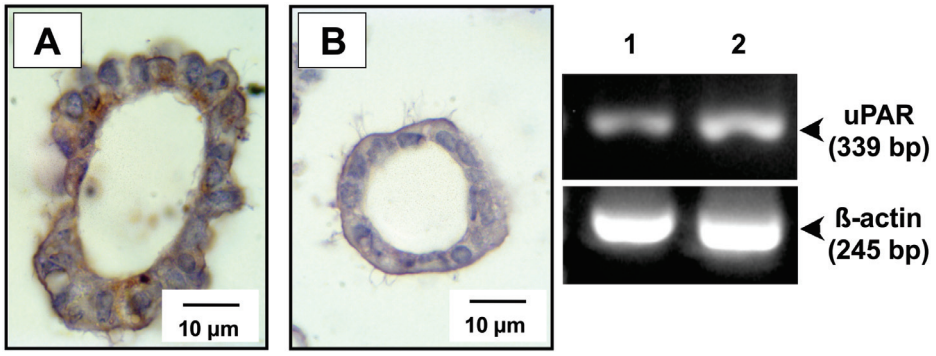


**Fig. 3.** (A) Immunohistochemical localization of uPAR in oviductal epithelial cultures. (B) Preimmune goat serum instead of the primary antibody. (C) Specific RT-PCR products for uPAR and β-actin in *in vivo* (1) and *in vitro* (2) bovine oviductal cell conditions separated by agarose gel electrophoresis.

## 4. Discussion

Several lines of evidence support the involvement of the plasminogen activation system in mammalian reproduction (Martínez-Hernández et al., 2011; Mondéjar et al., 2012b; Roldán-Olarte et al., 2012). uPA expression has been reported in cow (Gabler et al., 2001) and sow oviducts (Roldán-Olarte et al., 2012). The present study demonstrates that bovine oviductal epithelial cells express the uPA receptor. The expression of both uPA and uPAR in the oviduct during the normal estrous cycle was also compared. Results showed the expression of uPA and uPAR in the epithelia of ampulla and isthmus oviducts during the Pre-Ov, Post-Ov and Mid-L stages. Lowest expression levels were detected during the Post-Ov stage of the estrous cycle, suggesting that high expression levels are maintained during Mid-L and Pre-Ov and diminish after ovulation. These results agree with the ones reported by Gabler et al. (2001), who found that uPA expression is low in oviductal cells obtained from day 1 to 5 of the estrous cycle. These variations in the expression levels detected during the estrous cycle are probably influenced by sexual hormones as reported by other authors (Casslén et al., 1995; Roldán-Olarte et al., 2012).

Although the expression of inhibitors of plasminogen activators has not been studied in this work, it is important to note that several authors demonstrated the presence of PA inhibitors in bovine and porcine oviduct that would regulate the proteolytic activity of uPA (Gabler et al., 2001; Kouba et al., 2000; Roldán-Olarte et al., 2012). Similar observations were reported by Mullins et al. (1980) and Fazleabas et al. (1983) in porcine endometrium, where the release of inhibitors of the plasminogen activator system would prevent a proteolytic cascade of reactions initiated by blastocyst PAs that might otherwise damage the uterine epithelium.

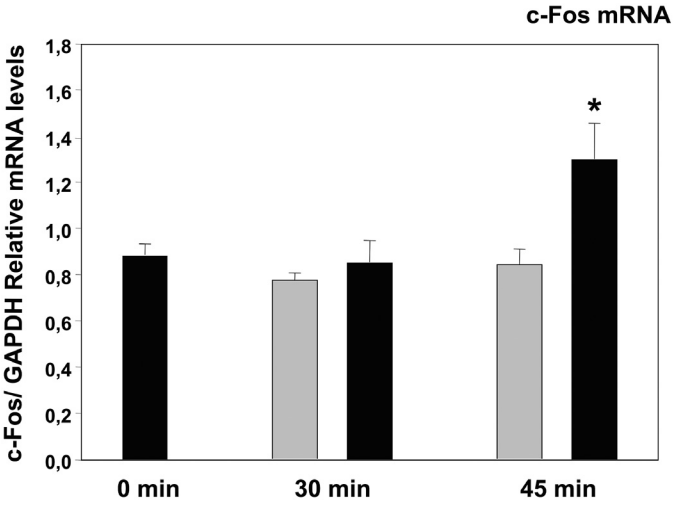


**Fig. 4.** Expression of c-fos mRNA in bovine oviductal epithelial cell cultures. Bars represent cultures with 1% bovine serum albumin (gray bars) and stimulated with uPA 10 nM (black bars) at different times. Values are mean ± SE ($n = 4$). * indicates significant differences between treatments ($P < 0.05$).

The results of the present work would point to the possible existence of a concerted regulatory action in the oviduct at the moment of fertilization and during the first stage of embryo development. This action would protect gametes and embryos against localized proteolytic degradation by diminishing uPA and uPAR mRNA during the Post-Ov stage; further studies are needed to confirm this possibility.

The receptor protein was detected in the apical zone of the mucosa cells from the ampulla and isthmus oviductal regions in different stages of the estrous cycle. Its location in the apical zone of the cells indicates that uPA from the oviductal fluid can act on epithelial cells through its receptor, allowing the generation of plasmin at focalized sites. Binding of uPA to its receptor could also initiate intracellular signaling pathways not yet explored in the oviduct. This study verifies that oviductal epithelial cells maintain uPAR expression after 48 h of culture. Then, this culture model was used to investigate the activation of *c-fos* expression in oviductal epithelial cell cultures by uPA stimulation. According to Dumler et al. (1994) and Tang et al. (1998), the addition of 10 nM uPA in the incubation medium increased c-fos mRNA levels and reached a maximum at 45 min. This effect was not observed when culture cells were incubated in a medium supplemented with bovine serum albumin instead of uPA, indicating that uPA is able to activate *c-fos* expression.

This work provides the first evidence of the occurrence of the uPA receptor in bovine oviductal epithelial cells showing that uPA can act through its specific receptor. Since uPA increases c-fos mRNA levels in oviductal cells and uPAR gene expression is up regulated by AP-1 (Chauhan and Boyd, 2012), we propose that uPA binding to uPAR could be involved in triggering the MEK-ERK signaling pathway that activates transcription factors, favoring the auto-regulation of the uPA/uPAR system (Smith and Marshall, 2010). Moreover, in the oviductal lumen the binding of uPA to uPAR could participate in the regulation of plasmin generation in the neighborhood of oviductal epithelial cells. Plasmin would probably be implicated in the proteolytic activation of extracellular matrix components such as growth factors and matrix metalloproteases present in the oviductal fluid. In conclusion, regulation of the early gene *c-fos* by uPA may be involved in the synthesis of molecules concerned with the arrangement of a particular oviductal microenvironment after ovulation that is necessary for fertilization and early embryo development.

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