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In vivo and *in vitro* expression of the plasminogen activators and urokinase type plasminogen activator receptor (u-PAR) in the pig oviduct

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ABSTRACT

Plasminogen activator activities have previously been reported in oviductal fluid. At present the question was whether the source of these activities is molecules come from blood plasma or if these activators are synthesized by the oviduct. Gene expression and protein synthesis of urokinase type (u-PA) and tissue type (t-PA) occur in different regions of the pig oviduct. Their relative concentrations do not vary between the ampulla and isthmus regions and are similar throughout the estrous cycle. However, while relative amounts of t-PA mRNA were not different between the different stages of the estrous cycle, u-PA mRNA was greater after ovulation ($P < 0.05$). Regarding the function of u-PA, its receptor (u-PAR) was distinguished by immunohistochemistry at the apical region of the epithelial cells and was more noticeable in the isthmus. Expression of u-PA, t-PA, u-PAR and PAI-1 genes in primary oviductal epithelial cell cultures was studied under 17-β-estradiol (100 pg/ml) and progesterone (100 ng/ml). u-PA mRNA increased in the presence of progesterone ($P < 0.05$), but not by action of 17-β-estradiol. t-PA, PAI-1 and u-PAR were similar when cultured with the hormones. These results suggest that u-PA could be regulated by progesterone at a transcriptional level, by the balance of their activity for PAI-1 or at the epithelial surface through the binding of u-PAR. In conclusion, plasminogen activation system components might cooperate in the oviductal lumen to control plasmin generation.

1. Introduction

The plasminogen activation system has been implicated in the initiation of proteolytic cascades that mediate processes involved in extracellular matrix (ECM) restructuring (Irigoyen et al., 1999). Plasminogen activators (PAs) are serine proteases that activate the plasminogen, a zymogen present in blood plasma and most other extracellular fluids, to plasmin, a key enzyme with potent serine protease activity with a broad spectrum of substrates present in the ECM (Ny et al., 2002; Martinez-Hernandez et al., 2011). Two types of PAs have been described: the urokinase-type (u-PA), and the tissue-type (t-PA); both are distributed in various mammalian tissues. The biological activities are related with different biological processes: while t-PA is essentially involved in trombolysis, u-PA is implicated in cell migration and tissue remodeling (Kwaan and McMahon, 2009). Activity of both u-PA and t-PA is controlled by specific inhibitors that belong to the serine proteinase inhibitors (serpin) gene superfamily: PA

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inhibitor type 1 (PAI-1), type 2 (PAI-2), type 3 (PAI-3 or protease C inactivator), and protease nexin. In addition, u-PA binds to its receptor (u-PAR), which is anchored at the plasmatic membrane by glycosylphosphatidylinositol. It has been described that the u-PA proteolytic activity can be focalized in their surroundings producing the activation of local intracellular signal pathways (Blasi et al., 1987; Blasi and Sidenius, 2010). PAI-1 inhibits both u-PA and t-PA. PAI-1 binds to u-PA forming a complex that is recognized by u-PAR and permits the internalization of the u-PAR/u-PA/PAI-1 complex. This complex is degraded in lysosomes while u-PAR itself is recycled back from the endocytic compartment to the cell surface (Dass et al., 2008).

Several works support that the plasminogen activation system is involved in mammalian reproduction. The PAs are involved in mammalian gametogenesis (Le Magueresse-Battistoni, 2007; Liu, 2007), ovulation (D'Alessandris et al., 2001; Liu, 2004), fertilization (Huarte et al., 1993), early embryo development and embryo implantation (Aflalo et al., 2007; Papanikolaou et al., 2008).

Given that the mammalian oviduct provides a microenvironment suitable for fertilization, gamete transport and early stages of embryo development, and that there is evidence that in these processes proteolysis plays an important role, knowledge of plasminogen system regulation is highly relevant (Huarte et al., 1993; Buhi, 2002; Aflalo et al., 2007). Research conducted in different species revealed the presence of some plasminogen activation system components in the mammal oviduct (Kouba et al., 2000a; Jiménez Díaz et al., 2000; Gabler et al., 2001). In the pig oviduct, previous results showed that u-PA and t-PA are active in the oviductal fluid and that their activities vary during the estrous cycle, being greater during the post-ovulatory period than other stages (Roldán-Olarte et al., 2005). However, it has not been confirmed whether the source of PAs is the oviductal epithelium itself or if their presence in the oviduct is due to plasma transudation.

The aim of the present research was firstly to demonstrate that the pig oviductal epithelium synthesizes u-PA and t-PA and secondly to study if there are differences in the amounts of mRNA between the different stages of the estrous cycle. As u-PA activity can be focalized by binding to u-PAR, a further objective was to assess if oviductal cells contain receptors for u-PA. Finally, to analyze the possible sexual hormonal regulation in the synthesis of u-PA, t-PA, u-PAR and PAI-1 during the estrous cycle, gene expression by the action of 17-β-estradiol and progesterone was studied on pig oviductal epithelial cells maintained *in vitro*.

2. Materials and methods

2.1. Oviduct collection

A total of 45 Landrace × Yorkshire × Duroc Jersey gilts about 1-year-old and weighing between 85 and 110 kg were obtained from a slaughterhouse in San Miguel de Tucumán (Argentina). Both ovaries and oviducts from each animal were collected within 20 min after slaughter. Oviductal sections for immunohistochemistry were fixed immediately with 4% buffered paraformaldehyde and others were transported to the laboratory in sodium phosphate buffer, pH 7.4 (PBS) at 4 °C for processing within 3 h after collection. Oviducts were isolated from ovaries, uterine horns and broad ligaments (mesometrium). Based on the direct observation of ovarian structures, as previously described by Roldán-Olarte et al. (2005), oviducts were classified into three groups: immediately before ovulation (Pre-Ov), after ovulation (Post-Ov) and in the middle of the luteal phase (Mid-L). Briefly, oviducts corresponding to ovaries with follicles of 9.51 ± 1.01 mm were selected as Pre-Ov; those whose ovaries contained corpora hemorrhagica were classified as Post-Ov and oviducts with an ovary with corpora lutea were classified as Mid-L.

2.2. Collection of PAs from a membrane oviductal fraction

Both oviducts from gilts at each stage of the estrous cycle ($n = 3$/stage) were flushed with PBS and divided into ampulla and isthmus. To obtain PAs from these oviductal regions, tissue homogenates were prepared in 4 vol. of 0.1 M Tris–HCl buffer, pH 8.1 and centrifuged for 1 h at 100,000 × *g* (Pereyra-Alfonso et al., 1995). Supernatants were discarded and pellets, corresponding to an oviductal crude membrane fraction (OMF), were suspended in 0.5% Triton X-100, 0.1 M Tris–HCl buffer, pH 8.1, and incubated in an ice bath for 1 h. The Triton-treated OMF was centrifuged at 100,000 × *g* for 1 h at 4 °C to obtain a soluble membrane fraction in the supernatant containing PAs extracted from the OMF with the Triton X-100 treatment (Triton-OMF). One aliquot of each sample was used to determine protein concentration by Lowry's method (Lowry et al., 1951).

2.3. Western blot

The polyclonal antibody to u-PA (rabbit anti-rodent u-PA IgG N° 1190) and the PAM-3 monoclonal antibody against t-PA (N° 373) were from American Diagnostica Inc. (Greenwich, USA). Horseradish peroxidase-linked to anti-rabbit and to anti-mouse immunoglobulins were from Amersham Life Science Inc. (Arlington Heights, IL).

Triton-OMF samples containing 70 μg protein were separated by SDS-PAGE consisting of a 5% (w/v) acrylamide stacking gel and a 12% (w/v) separating gel containing 0.1% SDS. The running buffer was composed of 0.1% SDS, 25 mM Tris and 250 mM glycine (pH 8.3). Electrophoretic fractionation was carried out at a constant current of 15 mA. Proteins were then electroblotted onto an Immobilon p15 membrane (Millipore Corp., Bedford, MA). The filters were blocked with 5% non-fat milk in PBS containing 0.1% Tween-20 (PBS-Tween) for 5 min and then incubated overnight at 4 °C with the primary antibody at a concentration of 1 μg/ml in a blocking solution. After washing in PBS-Tween, the filters were incubated overnight at 4 °C with the respective horseradish peroxidase-conjugated anti-immunoglobulin (1:1000). Following three washes in PBS-Tween, bands were visualized with enhanced chemiluminescence reagent and subsequent exposure to hyperfilm-enhanced chemiluminescence (Amersham Life Science Inc.).

2.4. Reverse transcription-polymerase chain reactions (RT-PCR)

Total RNA was isolated from ampulla or isthmus of oviducts corresponding to different stages of the estrous cycle by using the SV RNA Isolation System kit (Promega). Samples were obtained from four animals of each stage of the estrous cycle studied. The integrity of the RNA samples was checked with agarose gel electrophoresis stained with ethidium bromide and RNA concentration was determined by absorbance at 260 nm. Total RNA (2 μg) from each sample was reversely transcribed using Moloney murine leukemia virus (M-MLV) enzyme (Promega, Madison, WI, USA) and oligo(dT)17 primer. The 25 μl reaction mixture contained 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM of each dNTP, 25 pmol oligo(dT), 10 mM dithiothreitol and 200 units of reverse transcriptase, and 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 PCR with 2.0 U of *Taq* DNA polymerase (Promega) in a 25 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 (see Table 1). PCR conditions were assayed previously to determine the appropriate number of cycles for the amplification of each fragment, as described by Argañaraz et al. (2007). For the amplification of u-PA, u-PAR, t-PA, PAI-1 and β-actin the 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 45 s at the accurate annealing temperature for each pair of primers (u-PA; PAI-1 and β-actin: 55 °C; t-PA: 60 °C and u-PAR: 63 °C), and extension for 50 s at 72 °C; and (3) a final 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 pig cDNA sequences. The GenBank accession numbers, primers sequences and amplification fragment size are shown in Table 1.

PCR products were resolved on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. The nature of the PCR products obtained was confirmed by sequencing. The images of the gels were captured with a video camera connected to a computer. The intensities of the bands were analyzed with the software Molecular Analyst 1.4.1 (Bio Rad) to determine semi-quantitatively the expression levels of the studied genes.

A one-way analysis of variance was used to determine the possible differences between samples in the mRNA levels corresponding at u-PA, t-PA, u-PAR and PAI-1.

2.5. Total RNA from oviductal epithelial cells

To determine if u-PA gene is expressed in the oviductal epithelial cells, both oviducts corresponding to Pre-OV ($n = 4$) and MidD-L ($n = 4$) gilts were separated in ampulla and isthmus regions. Oviductal portions were cut longitudinally and the inner surface was scraped by means of a surgical knife. Every sample was used to obtain total RNA and semi-quantitative RT-PCR reactions were performed, as described above, to analyze the u-PA, u-PAR, t-PA and PAI-1 gene expression.

2.6. u-PAR immunohistochemistry

To determine the u-PAR localization 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 transversally sliced into 5 μm sections and mounted on poly-L-lysine-coated microscope slides. After incubating at 40 °C over night, the sections were deparaffinized and rehydrated. The sections 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. After this step, and without rinsing, the sections were incubated with 8 μg/ml goat anti human u-PAR 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) at a dilution 1:50 during 1 h at room temperature and rinsed again. Then, samples were incubated with extravidin–peroxidase (Sigma, E2886) at a dilution 1:100 during 1 h at room temperature, rinsed and finally sections were developed for 10 min with the substrate, 3,3′diaminobenzidine (Sigma, B8001). The sections processed were counterstained with Mayer's hematoxilin. In negative controls, the primary antibody was omitted. All sections were examined by one operator scoring the relative intensity of the u-PAR immunostaining. Photomicrographs were taken using an Olympus BX40 microscope.

2.7. Pig oviductal epithelial cells culture

Pig oviducts collected during the Pre-Ov stage were selected to obtain oviductal epithelial cells. The process of obtaining and culturing cells was based on that described by Thomas et al. (1995) and Ijaz et al. (1994). Immediately after slaughter, oviducts were immersed in a flask containing sterile PBS with 100.000 IU/l penicillin, 100 mg/l streptomycin and 250 μg/l Fungizone (GIBCO BRL) and transported on ice to the laboratory. Then they were washed twice with sterile PBS supplemented with antibiotics and dissected in a Petri dish, to remove the ovary and other tissue. For each experiment six oviducts obtained from three Pre-Ov gilts were used. After washing in sterile PBS, the oviducts were filled with 1 mg/ml collagenase type I (GIBCO BRL) in medium D-MEM + HAM-F12. Both ends of the oviducts were clamped and incubated at 38.5 °C during 60 min. After incubation the ends of the oviducts were cut off and the cells were separated by compressing the oviducts with tweezers. The cells were recovered in a sterile Petri dish and disaggregated mechanically with a pipette. Cells were collected in a 50 ml sterile conical tube with 15 ml medium D-MEM + HAM-F12 and were washed twice at room temperature. The cells were diluted

Table 1
Primers used to amplify specific porcine transcripts.

Gene		Oligonucleotides (5′-3′) (upstream/downstream)	Product size (bp)	GenBank accession number
u-PA	Forward	**GTCACCACCAAAATGCTGTG**	434	X02724
	Reverse	**CCCAGCACCCAGACTTGTAT**		
u-PAR	Forward	**TAGCTGCCGAGGCCCCATGAA**	211	XM_003127198.1
	Reverse	**CCCAGTGCGGGGCCTGACAT**		
t-PA	Forward	**AGGAGGCCTCTATGCTGACA**	544	AF364605
	Reverse	**GGCACACAGCATATTGTTGG**		
PAI-1	Forward	**GAGGCCATGCAGTTCAAGAT**	406	Y11347
	Reverse	**TGCCATCAGACTTGTGGAAG**		
β-Actin	Forward	**CGTGGGCCGCCCTAGGCACCA**	242	AF054837
	Reverse	**TTGGCCTTAGGGTTCAGGGGGG**		

in culture medium (medium 199, Gibco) with 10% fetal bovine serum (FBS, GIBCO BRL), 0.2 mM pyruvic acid and 50 μg/ml gentamycin sulfate. An aliquot of the cellular suspension was stained with Tripan Blue and then it was employed to determine viability and concentration of the cells in a hemocytometer. Viability of the flushed cells was confirmed by observation of beating cilia under a microscope. The cells (1×10^5/ml) were incubated at 38.5 °C, 5% CO_2, and 100% humidity; in a Petri dish of 60 mm of diameter (Nunc) containing 2 ml pre-equilibrated medium 199. After 2 days in culture, medium was removed to eliminate cellular detritus, and replaced with fresh medium. Cell adhesion was observed in a reverse microscope. Every other day, the culture cells were observed to check absence of contamination, cilia movement and growth of the cells. When the cells formed a monolayer with 80–90% confluence, cell cultures were used for hormone treatment.

Verification of the epithelial nature of the cultured cells was performed by immunocytochemical analysis, using cytokeratin as an epithelial specific marker. Monoclonal Anti Pan Cytokeratine clone PCK-26 antibody (Sigma, C1801) showed a positive staining of >90% of the cells in the primary cell cultures. Oviductal cells growing in cover-slides immersed in the Petri dish of the culture were employed after 5 to 7 days. The cells were washed in PBS before fixing in a methanol:acetone (7:3, v/v) during 7 min and then they were incubated with blocking solution (BSA 1% in PBS) during 20 min. 50 μl of monoclonal antibody anticytokeratine (dilution 1:20, v/v) in PBS plus TritonX-100 5%) were added to the cells and they were incubated at 37 °C during 2 h. The samples were washed with PBS and the biotin-goat antimouse IgG secondary antibody (Sigma, B9904) was added (1:300, v/v in PBS plus Triton X-100 0.5% and incubated 2 h at 37 °C in a humidifier chamber). A negative control without primary antibody was performed to evaluate the secondary antibody specificity. Cells were washed in PBS three times. Finally, 500 μl of extravidin–FITC (1:200, v/v) were added to the samples and incubated during 1 h at room temperature in a dark chamber. The samples were evaluated in an epifluorescence microscope (Olympus BX40).

2.7.1. Hormonal stimulation of the oviductal cell cultures

The effect of sexual steroidal hormones on u-PA, t-PA, PAI-1 and u-PAR gene expression was assayed in three different experiments, which consisted of cells cultured for 7 days that were then stimulated with 100 pg/ml 17-β-estradiol (Sigma, E-1024) or 100 ng/ml progesterone (Sigma, P-0130) for 24 h. The hormone concentrations were selected according to the maximum hormonal concentrations of E_2 and P_4 reported in sows by Mariscal et al. (1998) during the estrus and diestrus, respectively. E_2 and P_4 concentrations employed by other authors in porcine primary cell cultures (Sirotkin and Nitray, 1993; Cox et al., 2005), were also considered. Cells cultured without hormones were employed as control. Culture media were replaced by medium supplemented with hormones and cultured during 24 h. After that, cells were used for RNA extraction with the SV RNA Isolation System kit (Promega).After determining the RNA concentration from the pig epithelial cell cultures, reverse transcription reaction and PCR amplifications were conducted as described above. To achieve the best comparative amplification of u-PA, u-PAR, t-PA and PAI-1, the cycle numbers consisted of 20 instead of 30 cycles. β-Actin mRNA was used as control for RNA quantity and PCR reaction efficiency, due to the fact that β-actin is commonly used as a standard when comparing samples under different hormonal conditions, as it is constitutively expressed (Soutar et al., 1997).

3. Results

3.1. mRNA of u-PA and t-PA in oviductal regions during sexual cycle

Amplification fragments of 434 bp and 544 bp corresponding to u-PA and t-PA mRNAs respectively were detected in all samples (Fig. 1A). Semi-quantitative RT-PCR analysis showed significant differences between different phases of the estrous cycle. The relative amounts of u-PA were greater after ovulation in both ampulla and isthmus oviductal regions (Fig. 1B; $P < 0.05$). No significant differences were detected, however, in the relative amounts of t-PA between samples corresponding to Pre-Ov, Post-Ov or Mid-L phases of the estrous cycle in either ampulla or isthmus regions (Fig. 1B, $P > 0.05$).

3.2. u-PA and t-PA protein in oviductal regions during sexual cycle

By Western blot, a peptide of 50 kDa was specifically recognized by the rabbit anti-rodent u-PA antibody, in

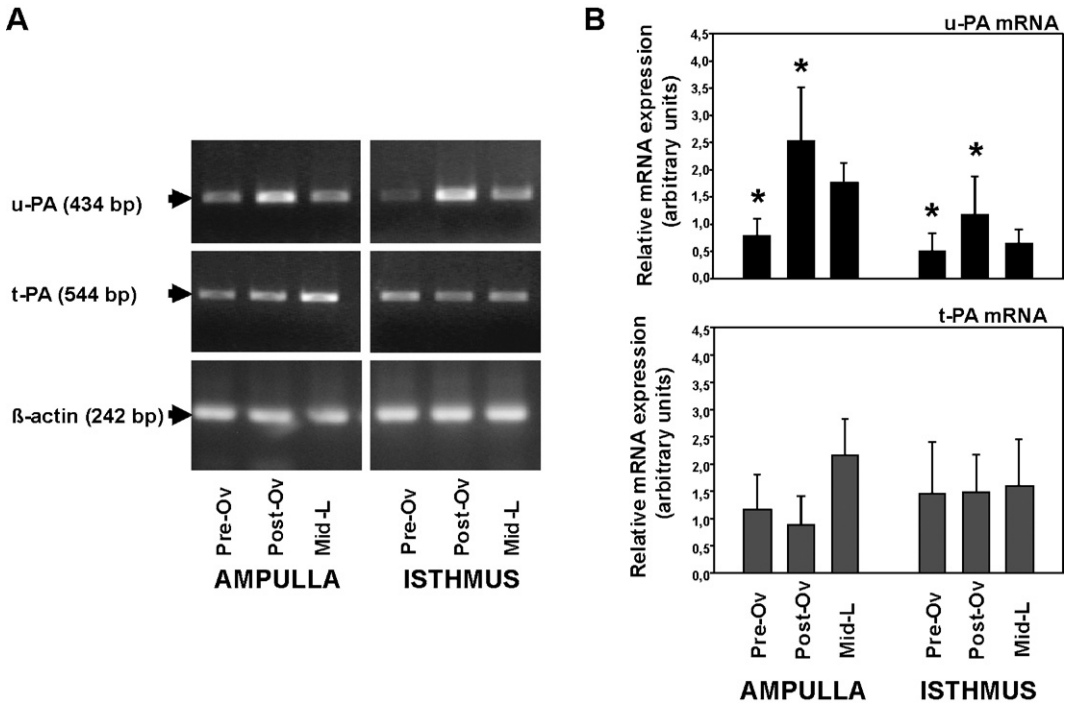

Fig. 1. (A) Semi-quantitative RT-PCR analysis of u-PA and t-PA mRNA in ampulla and isthmus pig oviductal regions in different stages of the estrous cycle. Electrophoresis on 1.5% agarose gel showing bands of 434 bp (u-PA), 544 bp (t-PA) and 242 bp of β-actin. Pre-Ov: preovulatory stage. Post-Ov: postovulatory stage. Mid-L: mid-luteal stage. (B) Histogram of semi-quantitative RT-PCR results. Band densities representing relative amounts of u-PA and t-PA mRNA in pig oviducts were normalized to β-actin. Arbitrary units were determined relating the band density with the corresponding β-actin band for each sample. Values are mean ± SD ($n = 4$). Significant differences (see asterisk) were found between Pre-Ov and Post-Ov ampulla and isthmus for u-PA mRNA ($P < 0.05$).

both ampulla and isthmus in all the estrous cycle stages tested (Fig. 2); the molecular weight of the specific protein detected is consistent with the MW reported previously for the pig u-PA molecule by Sudol and Reich (1984).

PAM-3 monoclonal antibody against human t-PA, which is known to have cross reactivity with the pig t-PA, was used for recognizing t-PA in the oviduct Triton-OMF fraction. A 64 kDa protein (Fig. 2) was detected. It is likely that this molecule would be a single chain t-PA according to the MW reported previously (Wallen et al., 1982).

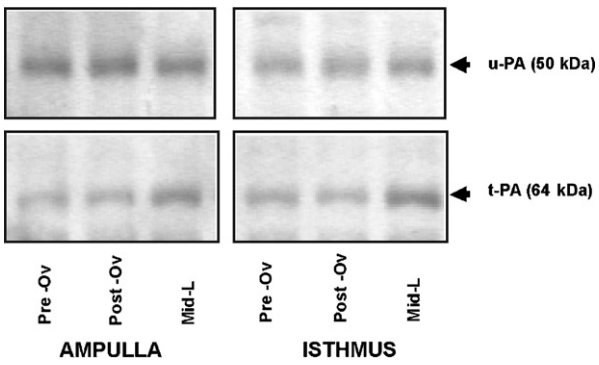

Fig. 2. Western blot of plasminogen activators in ampulla and isthmus Triton-OMF during the estrous cycle. Pre-Ov: preovulatory stage. Post-Ov: postovulatory stage. Mid-L: mid-luteal stage. u-PA detection with rabbit anti-rodent u-PA IgG. t-PA detection with monoclonal anti-human t-PA.

3.3. u-PAR and PAI-1 in ampulla and isthmus

Knowing that u-PA activity is regulated by the specific binding to its receptor, the relative amounts of u-PAR mRNA were evaluated. The presence of u-PAR transcripts indicated the occurrence of a single band of 211 bp which match to the amplified u-PAR fragment generated by the specific primers used in the assays (Fig. 3A). Relative amounts of mRNA were not different during the estrous cycle neither in ampulla nor isthmus.The u-PA activity can also be controlled by its inhibitor, PAI-1. Specific primers designed from PAI-1 porcine cDNA, amplified a 406 bp cDNA fragment in all the samples (Fig. 3A). The relationship between the relative amounts of mRNA for PAI-1 was greater during Mid-L phase either in ampulla or isthmus ($P < 0.05$).

3.4. Immunolocalization of u-PAR in pig oviduct

Taking into account that u-PAR is present in the pig oviduct; the next aim was to evaluate the u-PAR protein distribution throughout the oviduct. Immunoreactive u-PAR was detected in ampulla and isthmus segments regardless of the stage of estrous cycle. No differences in staining intensity could be detected between phases. Representative data of the immunohistochemical localization in both ampulla and isthmus regions are shown in Fig. 4. The u-PAR was localized in the epithelium and smooth muscle cells, while only background staining could be

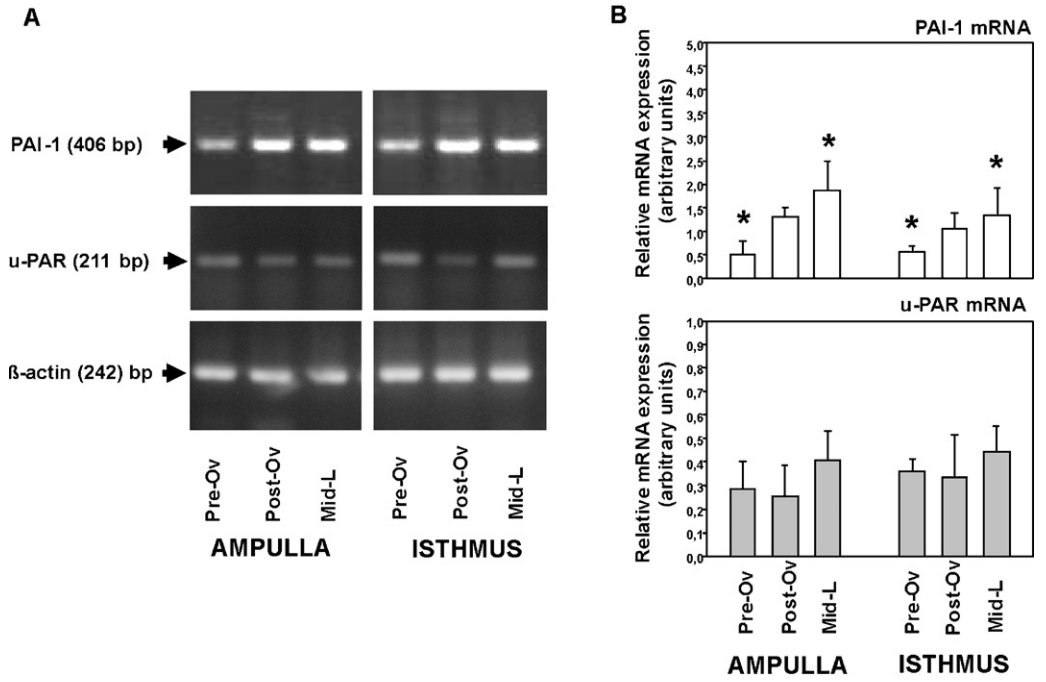

Fig. 3. (A) Semi-quantitative RT-PCR analysis of PAI-1 and u-PAR mRNA in ampulla and isthmus pig oviductal regions in different stages of the estrous cycle. Electrophoresis on 1.5% agarose gel showing bands of 406 bp (PAI-1), 211 bp (u-PAR) and 242 bp of β-actin. Pre-Ov: preovulatory stage. Post-Ov: postovulatory stage. Mid-L: mid-luteal stage. (B) Histogram of semi-quantitative RT-PCR results. Band densities representing PAI-1 and u-PAR mRNA levels in pig oviducts were normalized to β-actin. Arbitrary units were determined relating the band density with the corresponding β-actin band for each sample. Values are mean ± SD ($n = 4$). Significant differences (see asterisk) were found between Pre-Ov and Mid-L ampulla and isthmus for PAI-1 mRNA ($P < 0.05$).

identified in the stromal tissue. Isthmus epithelial cells are probably the target of u-PA because u-PAR was noticeably observed at the apical region of the mucosa cells in this segment (Fig. 4D1, arrow).

3.5. Expression of genes and correlation to amounts of plasmin

The presence of mRNA corresponding to all of the above mentioned genes proves that all these genes are expressed in the oviductal epithelium (Fig. 5A). Nevertheless, no significant differences were found neither between samples of different oviductal regions nor between stages of the estrous cycle analyzed (Fig. 5B).

3.6. Effects of 17-β-estradiol and progesterone on the expression of genes

The expression of *u-PA*, *u-PAR*, *t-PA* and *PAI-1* was confirmed in pig oviductal epithelial cell cultures under *in vitro* conditions (Fig. 6A). The relative mRNA amount of each gene is shown in Fig. 6B. A significant increase in the relative amounts of u-PA mRNA was observed when cell cultures were incubated with 100 ng/ml progesterone ($P < 0.05$). On the contrary, there were no differences in relative amounts of u-PAR, t-PA and PAI-1 in the culture cells under hormone stimulation in comparison to the cells cultured without hormones (Fig. 6B).

An interesting finding is that in culture cell system, the PAI-1 gene expression was greater than t-PA and u-PA under the same experimental conditions and basal amounts of u-PAR mRNA. These results were not consistent with those obtained with fresh epithelial cells (see Fig. 5).

4. Discussion

In a previous research, it has been demonstrated that the pig oviductal fluid has plasminogen dependent activity of u-PA and t-PA (Roldán-Olarte et al., 2005). Also, PA activity was present in the membrane fractions of the ampulla and isthmus oviductal regions in samples corresponding to different stages of the estrous cycle. In the present study, by detecting mRNAs and proteins of u-PA and t-PA in the pig oviduct, it is demonstrated that the PAs are synthesized and secreted by the oviductal cells.

The semi-quantitative RT-PCR assays made it possible to distinguish differences in the expression of the gene encoding u-PA. Relative amounts of mRNA for u-PA were greater during Post-Ov than in Pre-Ov stage in both the ampulla and isthmus. Tsantarliotou et al. (2005) also detected u-PA mRNA in the pig oviduct by RT-PCR and reported a greater amount of u-PA mRNA in the isthmus than in the ampulla; however, no variations in relative amounts of the mRNA were detected during the estrous cycle probably due to the lack of a quantitative RT-PCR analysis. The present results are consistent with the previous reports: increases in amounts of u-PA mRNA during the Post-Ov

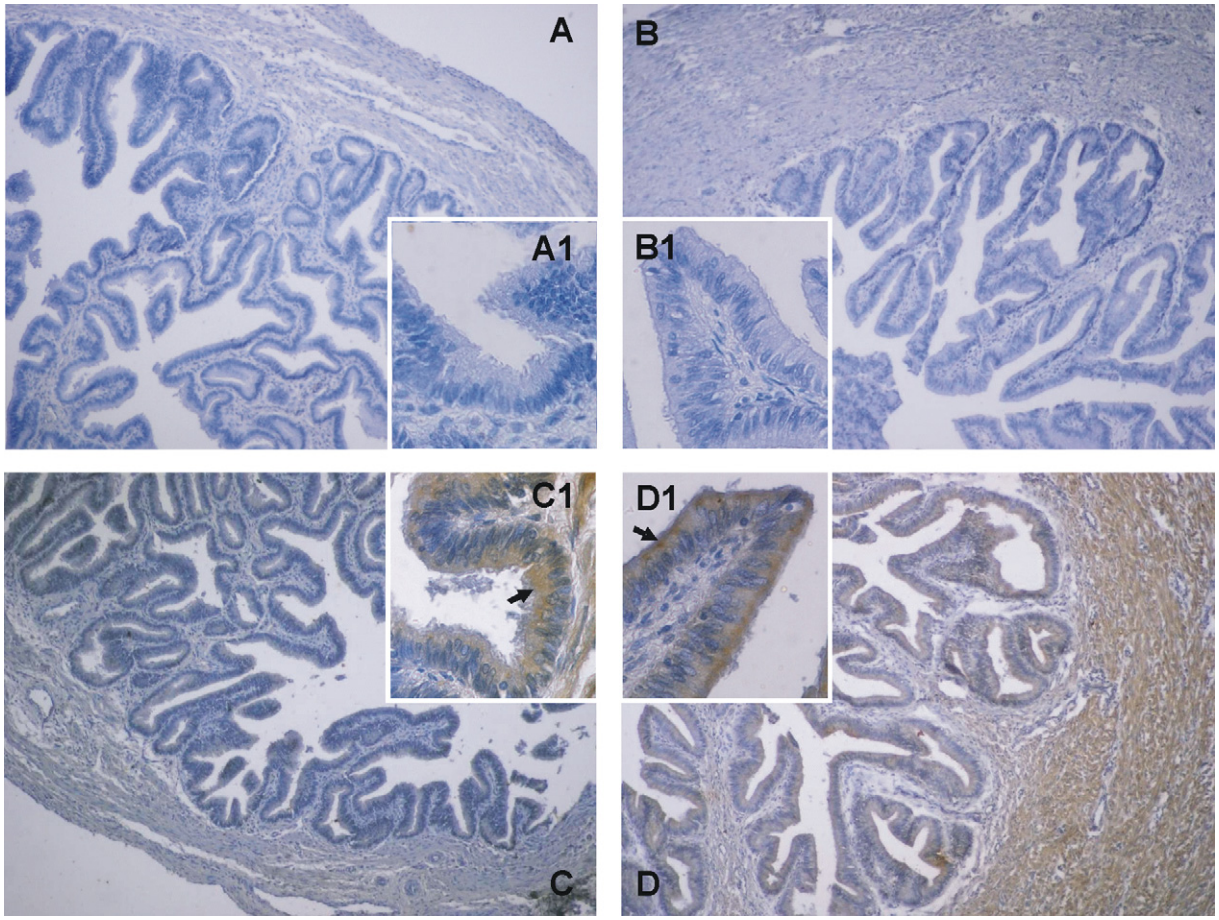

Fig. 4. Urokinase-type plasminogen activator receptor (u-PAR) immunolocalization in pig oviductal sections from ampulla (C and C1) and isthmus (D and D1). Arrows indicate the positive localization in the epithelial cells in both ampulla and isthmus oviductal regions. A, A1, B and B1: Primary antibody (goat polyclonal anti u-PAR) omitted. Magnification: A–D, ×100; A1–D1, ×400.

stage was in agreement with the increase in u-PA activity and relative concentration of this protein in oviductal fluid during this stage of the estrous cycle (Roldán-Olarte et al., 2005). It is believed that u-PA is synthesized and secreted into the oviductal lumen, mainly during the Post-Ov stage, when gametes and early embryos go through the oviduct.Previously, t-PA activity has been measured in the pig oviductal fluid (Roldán-Olarte et al., 2005). In the present study, the presence of t-PA in the Triton-OMF fraction from ampulla and isthmus by Western blot assessment was demonstrated. RT-PCR assays indicate that the pig oviductal epithelial cells synthesize t-PA even though the relative amounts of mRNA for t-PA did not vary during the estrous cycle.

To prevent the unrestrained activity of proteolytic enzymes of plasminogen/plasmin system there are two main regulation mechanisms: (i) the secretion as inactive precursors of proteases and (ii) inactivation by forming complexes with specific inhibitors. The presence of some inhibitors of PAs, such as PAI-1 (Kouba et al., 2000a,b), PAI-2 and protease nexin-1 (PN-1) (Blasi et al., 1987) have been described in mammalian oviducts. PAI-3 or protein C inhibitor (PC I), serpin that inhibits acrosin, has been characterized in the mouse reproductive tract (Geiger et al., 1996). *In vitro* fertilization experiments in the presence of this inhibitor indicate that PC-1 inhibits the interaction of sperm with mouse oocytes and decreases fertilization rates (Geiger et al., 1996). Consistent with Kouba et al. (2000b), there was a presence of PAI-1 transcripts in the present study in the pig oviduct, where the greatest relative amounts of mRNA were determined in ampulla and isthmus at the Mid-L stage of the estrous cycle (Fig. 3). PAI-1 could regulate the activity of u-PA and t-PA present in the oviductal fluid. The inhibitory activity of these compounds could be significant in the isthmus, avoiding damage to the embryos or their ectopic implantation in oviducts due to an exacerbated invasive activity in this organ.

The u-PA receptor (u-PAR) is also a key molecule for regulating the activity of u-PA, because it focuses the activity of this protease at the cell surface (Smith and Marshall, 2010). Through its receptor, u-PA is able to send intracellular signaling cascades into the cells of the oviduct, as takes place in other cell types (Dumler et al., 1998; Tang et al., 1998; Katz and Streuli, 2007). In the present study, u-PAR was present in the epithelial cells of the oviduct in both ampulla and isthmus regions. The inmunolabeling

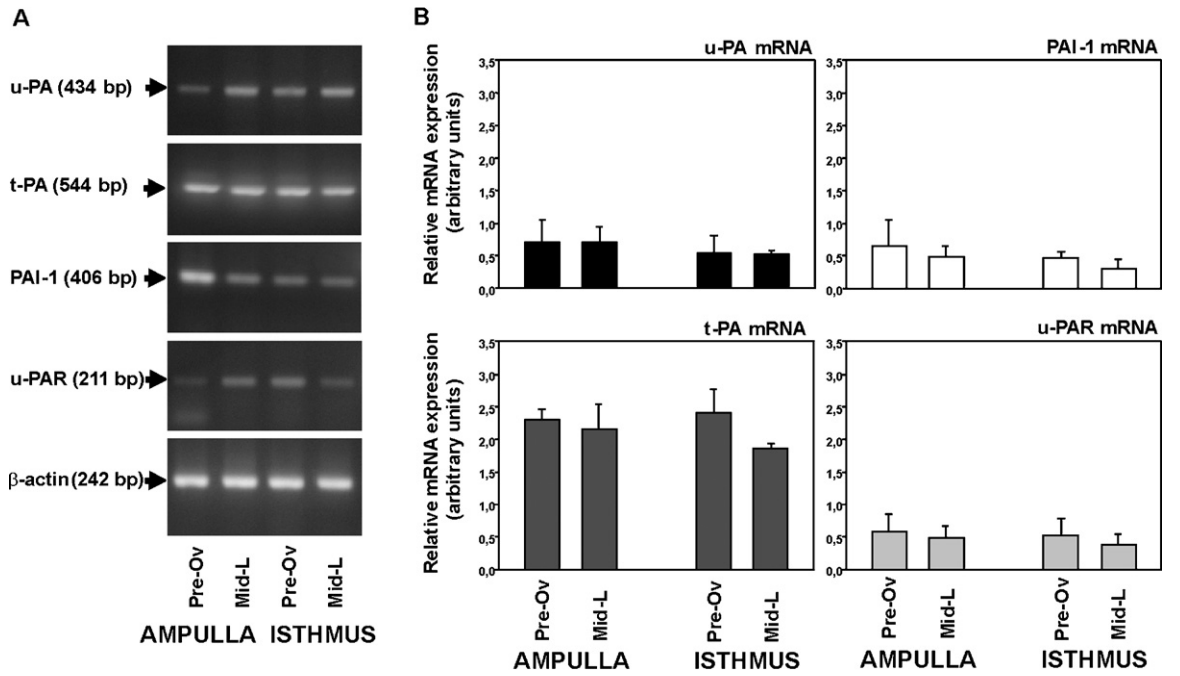

Fig. 5. (A) Semi-quantitative RT-PCR analysis of u-PA, u-PAR, t-PA and PAI-1 mRNA in pig oviductal epithelial cells obtained from ampulla and isthmus. Electrophoresis on 1.5% agarose gel showing bands of 434 bp (u-PA), 544 bp (t-PA), 406 bp (PAI-1), 211 bp (u-PAR) and 242 bp of β-actin. Pre-Ov: preovulatory stage. Post-Ov: postovulatory stage. Mid-L: mid-luteal stage. (B) Histogram of semi-quantitative RT-PCR results. Bars represent relative u-PA, t-PA, PAI-1 and u-PAR mRNA in oviductal epithelial cells from ampulla or isthmus regions of pig oviducts. Arbitrary units were determined by relating the band density with the corresponding β-actin band for each sample. Values are mean ± SD ($n = 4$). No significant differences were found between stages of the estrous cycle studied for the mRNA analyzed.

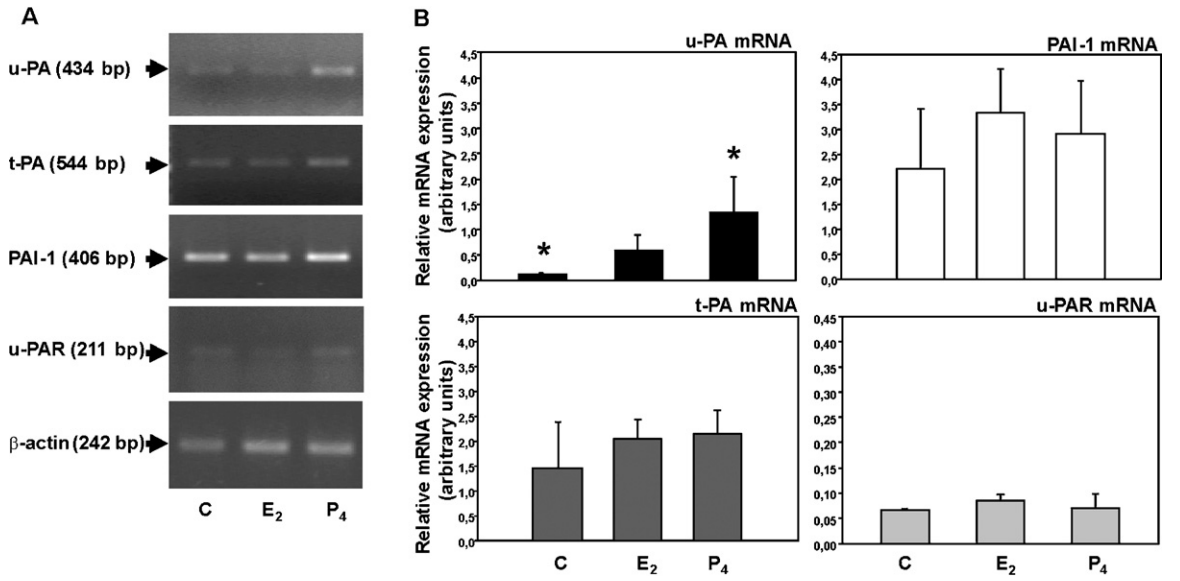

Fig. 6. (A) Semiquantitative RT-PCR analysis of u-PA, t-PA, PAI-1 and u-PAR mRNA in pig epithelial oviductal primary culture cells. (A) Amplification products separated by 1.5% agarose gel electrophoresis. C: control. E_2: cells under 17-β-estradiol stimulation. P_4: cells under progesterone stimulation. The figure shows a representative assay of three different experiments. (B) Histogram of semi-quantitative RT-PCR results. Bars represent the u-PA, t-PA, PAI-1 and u-PAR mRNA in pig epithelial oviductal cells *in vitro* conditions. Arbitrary units were determined by relating the band density with the corresponding β-actin band for each sample. Values are mean ± SD ($n = 3$). Significant differences were found under P_4 stimulation for u-PA mRNA ($P < 0.05$).

were localized at the apical region of the epithelia cells. We predicted that u-PA of oviductal fluid could bind to epithelial cells u-PAR and localize the plasmin generation in the epithelium surface surroundings. The u-PAR can also bind the u-PA/PAI-1 complex and, therefore, is able to remove u-PA from oviductal fluid by a receptor-mediated endocytosis mechanism described for other experimental models (Cubellis et al., 1990; Czekay et al., 2011). The *u-PA, u-PAR* and *PAI-1* genes are expressed in both *in vivo* isolated epithelial cells and in cultured cells. The immunodetection of u-PAR in the oviductal smooth muscular cells suggest that it could be involved in processes such as angiogenesis (Blasi and Sidenius, 2010) or contraction/relaxation of smooth muscule cells (Haj-Yehia et al., 2000).

The role of u-PA in the oviduct might be involved in the proteolytic degradation of ECM proteins and also in the release of certain growth factors such as FGFs and VEGF (Plouët et al., 1997; Saksela and Rifkin, 1988, 1990), which have been found in mammalian oviducts (Buhi and Alvarez, 2003; Killian, 2004). The activity of these growth factors may be important in early embryonic development, a process that occurs in the lumen of the oviduct. We suggest that the functions of u-PA oviductal lumen could be involved in the activation of (i) plasminogen present in the oviductal fluid for generating plasmin, which may act on several target proteins; (ii) growth factors such as VEGF (Plouët et al., 1997) that may influence early embryo development, and (iii) certain members of metalloproteases, present in the oviductal fluid (Gabler et al., 2001).

The finding that u-PA was in greater relative amounts in cell cultures stimulated by progesterone is in agreement with the results obtained by RT-PCR from oviducts during the estrous cycle, as greater relative amounts of u-PA were found during Post-Ov stages, where progesterone was also greater. The genes encoding t-PA, PAI-1 and u-PAR did not differ in relative expression under the action of the hormones used in these assays. An interesting finding is that in culture cell system, PAI-1 gene expression was greater than t-PA and u-PA under the same experimental conditions and there was a small relative amount of u-PAR mRNA. These results are not consistent with the results obtained with fresh epithelial cells (see Fig. 5). The cause of these observations is not clear, but it could be due to the conditions of the *in vitro* assays that differ to the *in vivo* environment, where some regulatory components could be present in the pig oviductal cells and be responsible for the regulation of PAI-1 and u-PAR gene transcription.

The results of the present work lead us to propose that u-PA activity might be regulated in the oviduct at three levels: (i) at the epithelial cells in transcription, probably by the action of progesterone, (ii) outside the cells in the oviductal fluid by the balance of the activity for its specific inhibitor, PAI-1, (iii) at the epithelial surface through the binding of u-PAR.

In conclusion, the presence of components of the plasminogen activation system in the pig oviduct could act as regulators of the plasmin proteolytic action within the oviductal lumen; u-PAR localized in the epithelial surface could regulate the generation of plasmin close to the surface of oviductal cells, gametes or preimplantation embryos.

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