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Influence of ovarian steroid hormones or platelet-activating factor on mRNA of platelet-activating factor receptor in endometrial explant perfusion cultures from ovariectomized bovine

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Abstract

Platelet-activating factor (PAF) and its receptors are involved in inflammatory-like processes of the uterus associated with increased vascular permeability. PAF is supposed to be influenced by ovarian steroid hormones. The present study was undertaken to examine whether progesterone (P_4), estradiol (E_2) or PAF influence the PAF receptor gene expression in perfused endometrial explants derived from ovariectomized bovine. Furthermore, we identified the cell types in which the PAF receptor gene and protein are expressed. In endometrial explants, applications of 10 nM P_4 or 10 nM P_4 plus 10 nM E_2 for 24 h induced elevated transcript levels of PAF receptor in comparison to the controls or after treatment with 1 nM E_2. When explants were administered 10 nM E_2, a slight decrease in the transcript level was recorded. After treatment of explants with PAF, no significant changes in PAF receptor mRNA expression was observed compared to the control group. We demonstrate that PAF receptor immunoreactivity and mRNA are detected mainly in the luminal epithelium, epithelial cells of the

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superficial glands and to a lesser degree in stroma. Levels of PAF receptor mRNA in bovine endometrial explants were correlated with PAF receptor protein localization assessed by immunohistochemistry. The regulation of PAF receptor by progesterone in bovine endometrial explants suggests that PAF is involved in the physiological process of reproduction.

Keywords: PAF-receptor; Endometrial explant perfusion culture; Ovariectomized bovine

1. Introduction

Platelet-activating factor (PAF) is a chemical substance released from sensitized basophils causing platelet aggregation [1]. This factor is not only a mediator of inflammation and allergy, but has numerous physiological functions, especially in reproduction (for review see: [2]).

In most cells the biological actions of PAF are mediated by a specific PAF receptor (PAF-R). The amino acid sequence of the PAF-R places it in the G-protein-coupled receptor super family. Radioligand binding studies have shown that functional PAF-R is present in the human endometrial cell line HEC-1A [3] and in the oviductal membranes of rabbits during early pregnancy [4]. The cellular localization of PAF-R in bovine endometrial stromal cells was investigated with a polyclonal anti-PAF-R antibody system directed against a peptide antigen corresponding to the N-terminal portion of the receptor. PAF-R was detected mainly in luminal and glandular epithelium of the endometrium, but the staining was markedly increased in the endometrium of pregnant cows on day 20 compared to cyclic animals on the same day [5]. This result was confirmed by immunocytochemical staining measured by flow cytometry in uterine stromal cells [6]. It can be assumed that the regulation of PAF-R expression in these cells during the preimplantative period may be caused by progesterone or PAF. An up-regulation of PAF-R expression following exposure to PAF is observed in human platelets [7], human B-cells [8] and Kupffer cells [9]. Up to date, the biological relevance of the PAF-mediated up-regulation of its own receptor remains to be elucidated. Therefore, an in vitro model was designed to examine the effect of estradiol, progesterone or PAF on levels of PAF-R mRNA or PAF-R gene expression in endometrium derived from uteri of ovariectomized bovine. The animals were ovariectomized to remove the principal source of estrogen and progesterone. Endometrial explants were cultured with steroids or PAF in a perfusion system. The samples collected from the explant cultures were qualitatively and quantitatively analyzed for PAF-R mRNA and qualitatively for PAF-R protein.

2. Material and methods

2.1. Animals

Four German Holstein heifers, approximately 16 months of age, with a mean body mass of 420 kg were used in the study. All animals were ovariectomized (OVX) and given no steroid hormone treatment for several months prior to use. Surgical removal of the ovaries

was carried out by laparotomy via the fornix of the vagina. Plasma progesterone was not detectable (<1 ng/ml) in OVX bovine. Animals were slaughtered by exsanguination. The reproductive tract was removed immediately after slaughtering and transported on ice to the laboratory. The uterus was dissected free of surrounding tissues and washed with 70% ethanol. Then the uterine horns were flushed in Dulbecco's phosphate-buffered saline (PBS) supplemented with 1% antibiotic–antimycotic (ABAM).

2.2. Tissue preparation and explant cultures

The uterine horns were opened and all endometrial tissue was dissected free from the underlying tissue. Pieces were washed with PBS and dried up with filter paper before being weighted in 0.2 g portions. Then they were chopped into pieces of <1 mm^3 using a McIlwain tissue chopper (Dunn Labortechnik, Asbach, Germany). Endometrial explants were transferred to 3.5 ml perfusion chambers and constantly perfused with incubation medium [phenolred-free RPMI 1640 medium containing 0.25% (w/v) BSA, 1% (v/v) ABAM and 2 mM L-glutamine, 25 mM HEPES] at a flow rate of 0.5 ml/min using a commercially available perfusion system (Minucells and Minutissue, Bad Abbach, Germany). A peristaltic pump transported the media, and a thermoplate with a cover lid provided constant temperature of 37 °C.

2.3. Experimental treatments

Endometrial explants were cultured in triplicate in either control media, or media containing 1 or 10 nM estradiol (E_2); 10 nM progesterone (P_4); 10 nM E_2 + 10 nM P_4; 10, 100 or 1000 nM PAF for 24 h. Before the explants were harvested, some explants were removed for in situ hybridization or immunohistochemistry. Remaining explants of each perfusion chamber were separately rinsed twice with ice-cold HBSS on grids and dried up with filter paper and stored at −80 °C until use (quantification of mRNA).

2.4. Relative quantification of bovine PAF-receptor transcripts containing exon 1

Isolation of total RNA was performed with the RNeasy® Mini Kit (QIAGEN, Hilden, Germany) using the QIAshredder procedure (QIAGEN) for homogenization. For removing DNA contamination, the optional DNase I step of the RNeasy® protocol and a final DNase I treatment were included in each RNA preparation using the DNA-free™ protocol (Ambion, Inc., Austin, TX). Amounts of RNA were estimated spectrophotometrically at 260 nm. Quality of total RNA was proven by electrophoresis on denaturing formaldehyde gels. RNA was stored at −80 °C until use.

Reference RNA for establishment the standard curves and for use as a calibrator was isolated for the sake of convenience from one oviduct of a heifer.

cDNA synthesis was performed with 200 units M-MLV reverse transcriptase, RNase H minus, point mutant, 0.5 μg total RNA, 20 pmol random hexamers in the presence of 1 U/μl recombinant RNasin® ribonuclease inhibitor (Promega, Mannheim, Germany). For control of carry over, each RNA sample was run parallel without reverse transcriptase and a water sample with reverse transcriptase.

PCRs for relative quantification of bovine PAFR transcripts containing exon 1 were run with the LightCycler system (Roche Diagnostics, Mannheim, Germany). The LightCycler FastStart DNA Master SYBR Green I Kit was used according to the protocol of the manufacturer (Roche). A final reaction volume of 10 μl was used per capillary. Eight microliters of the master mix including the corresponding primer pair for bovine PAFR transcripts containing exon 1 and the reference mRNA, the following exon 1/3 spanning primers (exon 1: EMBL acc. no. AJ295319; pos. 312, UP 5′-AGCTGCCGATATGCTCAGACCT-3′; exon 3: EMBL acc. no. AJ295321; pos. 651, 5′-AAGGGTACAAGCGGGCAAAGA-3′; amplicon length, 232 bp) or the bovine GAPDH mRNA primers (EMBL acc. no. U85042; pos. 188, UP: 5′-CCATCTTCCAGGAGCGAGATCC-3′; pos. 397, LP:5′-AGGAGGCATTGCTGACAATCTT-3′; amplicon length, 232) were used together with 2 μl of the corresponding "cDNA" dilution at 0.5 μM for each primer and 3 mM Mg^{2+}. The amounts of total RNAs, employed for cDNA synthesis, were used for calculations of the dilutions. Standard curves of target and reference mRNA were established on 40–0.05 ng reference cDNA (four-folds) according to the recommendations of the manufacturer. For analyses of the individual cDNA samples, triplicates for target and reference of calibrator cDNA and also for target and reference of each sample cDNA, respectively, were used at 5 ng cDNA per capillary. Two controls for carry over were included.

The experimental protocol consisted of: pre-incubation: 1 cycle, 95 °C, 600 s; amplification: 45 cycles, 95 °C, 15 s; 65 °C, 10 s; 72 °C, 20 s; 84 °C, 5 s with acquisition mode single; melting curve analysis: 1 cycle, 95 °C, 0 s; 70 °C, 30 s; 95 °C, 0 s and a temperature transition rate of 0.1 °C/s with acquisition mode continuous; rotor cooling: 1 cycle, 40 °C, 30 s; all temperature transition rates were at 20 °C/s.

Experimental data were analyzed with the LightCycler software, version 3.5. Data of standard curves and of samples were exported for calculations with the relative quantification software. Fit coefficients of both standard curves were calculated in the dual color mode and saved as a fit coefficient file. This file was used for the calculation of the relative ratio of bPAFR transcripts containing exon 1 to bGAPDH mRNA in each sample to this relative ratio in the calibrator and the final normalized ratios of the individual samples.

The obtained values of the final normalized ratios reflect the transcriptional levels of bPAFR transcripts containing exon 1 of the individual samples to that of the calibrator, which was set equal to one.

2.5. RT-PCR and generation of probes for in situ hybridization

According to the bovine PAF-R gene coding sequence (GenBank acc. no. AF187321) primers were designed to produce the target cDNA template: forward primer PAF-R (5′-GTG GAC TCA GAG TTC CGA TAC AC-3′) and reverse primer PAF-R (5′-GGT CAG CCA TGG TGA GGT TCA C-3′). Amplification products of RT-PCR were separated on a 2% agarose gel. The resulting RT-PCR cDNA product for PAF-R was gel-purified using QIAEX II Extraction kit (Qiagen) and subcloned in pGEM®-T Vector (Promega). The plasmids were transformed in XL1-Blue competent cells (Stratagene, Heidelberg, Germany) and extracted by column purification with QIAprep® Miniprep kit (Qiagen). For transcription of the antisense or sense RNA probes, the plasmid-containing RT-PCR product was linearized with *Not*I [antisense] and *Nco*I [sense] (New England Biolabs, Frankfurt a.M.,

Germany), the transcripts were generated with Dig-RNA-Labeling Mix (Roche) using T7 and SP6 RNA polymerases, respectively. Identity of the probes was verified by sequence analysis in a commercial laboratory (Qiagen).

2.6. In situ hybridization

Sections of 5 μm were deparaffinized, rehydrated and subsequently handled using RNAase-free conditions. After 20 min in 0.2 N HCl the slides were transferred into 2× SSC (sodium saline citrate buffer; 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 20 min at 70 °C. Subsequently, the sections were digested in 20 μg/ml proteinase K (Roth, Karlsruhe, Germany) in phosphate-buffered saline (PBS) for 25 min at 37 °C which was followed by a treatment in 0.2% glycine in PBS and 20% acetic acid in diethylpyrocarbonate (DEPC). Sections were postfixed in 4% paraformaldehyde in PBS for 10 min and prehybridized in 20% glycerol for 30 min. For hybridization, the slides were incubated in a humid chamber containing 50% formamide in 2× SSC for 20 h at 37 °C with hybridization mixture containing the Dig-labeled antisense riboprobe at a dilution of 1:25. One ml of hybridization mix contained 50% formamid, 10% dextran sulfate, 2× SSC, 1× Denhardt's solution, 95 μg salmon testes DNA and 228 μg yeast tRNA. Control sections were incubated with the sense probe. After hybridization, the sections were washed for 3× 10 min in 4× SSC at 37 °C, 15 min in 2× SSC at 60 °C, 15 min in 0.2× SSC at 42 °C, 5 min in 0.1× SSC and 5 min in 2× SSC. After one rinse in 1× TNMT (10× TNMT: 1 M Tris–HCl, 1 NaCl, 0.02 M $MgCl_2$, 0.5% Triton-X-100) the slides were incubated for 60 min with 3% BSA in 1× TNMT. For detection of hybridization, the sections were incubated with sheep-anti-digoxigenin-antibody conjugated with alkaline phosphatase (Roche) for 20 h at 4 °C. Signals were visualized with NBT/BCIP (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate) and the slides were mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) without counterstaining.

2.7. PAF receptor immunohistochemistry

For structural and immunohistochemical analysis, sections of 5 μm-thick were used. The presence of immunoreactive PAF-R protein in histological sections of endometrial explants was assessed as described by [5]. Endometrial explants were fixed in 4% paraformaldehyde. After fixation, tissue was dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin for sectioning; 5 μm sections were cut and mounted on poly-L-lysine slides. For immunohistochemical analysis, sections were deparaffinized and dehydrated. Tissue sections were boiled in 0.01 M sodium citrate for 20 min, incubated in 0.3% H_2O_2, and blocked with 20% normal horse serum. Tissues were incubated with rabbit polyclonal anti-PAF-R antibody at 1:200 dilution in Tris-buffered saline (TBS: 2.5% BSA + 0.05% Tween) for 60 min at room temperature. Subsequently, the sections were incubated with anti-rabbit IgG biotinylated secondary antibody (Chemicon, Hofheim, Germany) at 1:1000 dilution in TBS (0.5% BSA + 0.05% Tween) for 60 min at room temperature. Localization of PAF-R was detected using streptavidin-immunoperoxidase (Chemicon) at 1:1000 dilution for rabbit IgG at room temperature for 60 min. After a 10 min wash in TBS, localization of the primary antibody was visualized with diaminobenzidine for 3–5 min, producing

a brown-colored stain. Negative controls included omission of the primary antibody or its replacement by normal rabbit serum. Sections were mounted in glycerin jelly and examined using a Zeiss microscope.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to distinguish differences among the treatments, followed by Newman–Keul's test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. PAF-receptor transcript quantification

To quantify changes in PAF receptor gene expression in endometrial explants treated with sex steroids, variations in transcript levels were determined using the relative quantification by real-time PCR (Light Cycler®) and normalization of the amount of PAF-R-PCR product for the PAF-R to that of bovine GAPDH. The transcript levels were significantly up-regulated ($p < 0.05$) in endometrial explants of ovariectomized bovine treated with P_4 alone or with P_4 plus E_2. In contrast, when bovine explants were treated with 1 nM estradiol alone, the level of PAF-R transcripts containing exon 1 was in the range of that seen in the control group. When explants were administered 10 nM E_2, a slight decrease ($p > 0.05$) in the transcript level was recorded (Fig. 1A). With respect to PAF applications, mRNA expression of PAF-R did not change significantly (Fig. 1B).

3.2. Localization of PAF receptor mRNA

In situ hybridization using a Dig-labeled antisense riboprobe demonstrated changes in the expression of PAF-R mRNA in endometrial explants cultured with sex steroid hormones like those obtained with LightCycler. The specificity of the signal was confirmed by the use of sense probe (Fig. 2B). Thus, in the control group, PAF-R mRNA was expressed in the luminal epithelium, epithelial cells of glands and stroma using in situ hybridization (Fig. 2A). Similar to the control group, at 1 nM E_2 PAF-R mRNA expression was in the same range (Fig. 2C). Compared to that, the transcript level was lowest in the 10 nM E_2 group (Fig. 2D). The degree of hybridization was consistently higher in the glands of groups treated with 10 nM P_4 (Fig. 2E) and 10 nM E_2 plus 10 nM P_4 (Fig. 2F) compared with the glands from other groups, whereas the PAF-R mRNA expression was approximately in the same range after culture of explants with different concentrations of PAF (data not shown).

3.3. Localization of PAF receptor protein

Finally, we observed a correlation of PAF receptor mRNA levels in bovine endometrial explants with PAF-receptor protein localization assessed by immunohistochemistry. Fig. 4

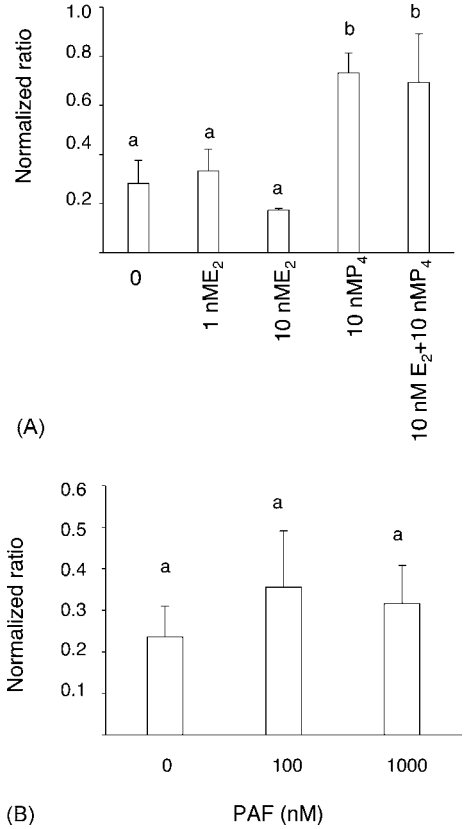

Fig. 1. Levels of bovine PAF-receptor (PAF-R) transcripts containing exon 1 in the endometrial explants derived from OVX-bovine ($n = 4$) cultured in serum-free RPMI medium supplemented (A) without or with 1 nM estradiol, 10 nM estradiol, 10 nM progesterone or 10 nM estradiol plus 10 nM progesterone and (B) without or with 100 nM PAF, 1000 nM PAF. Transcriptional levels are indicated as normalized ratios, which are calculated from the relative ratio of bovine transcripts containing exon 1 to GAPDH mRNA in each sample divided by this ratio of a calibrator, which was set equal to one. Triplicate or duplicate determinations per experiment [OVX-bovine: A ($n = 2$); B ($n = 2$)]. ANOVA on ranks, Newman–Keul's ONE WAY, mean ± S.D. Means with different letters are significantly different ($p < 0.05$).

shows representative pictures of the specific labeling for PAF-R in the glandular epithelium of the endometrium in ovariectomized heifers. In general, PAF-R-specific staining was mainly observed in the cytoplasm of glandular epithelial cells, with the highest intensity toward the luminal side. Additionally, a punctate staining pattern was discernible in the stroma associated with walls of small blood vessels. Different staining patterns for PAF-R were observed between the groups. In the control group, immunohistochemical analysis showed a labeling for PAF-R in glandular epithelium of the endometrium. Staining was most intense at the apical border of the epithelial cells. Stromal cells showed a distinctly weaker immunostaining (Fig. 3A). After treatment of explants with 1 nM E_2 (Fig. 3C) the

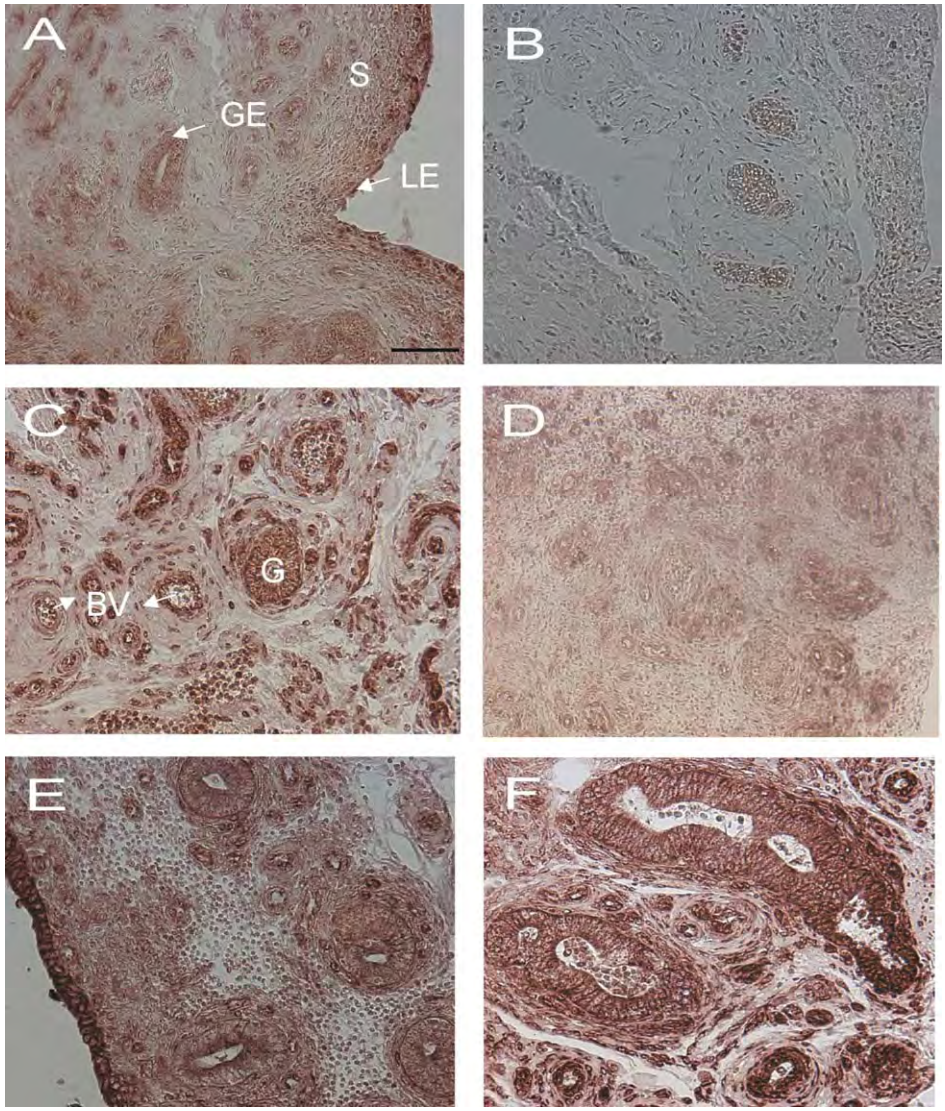

Fig. 2. Representative microphotograph of paraffin sections of perfused endometrial explants from ovariectomized bovine treated with steroids. In situ localization of platelet-activating factor receptor (PAF-R) mRNA expression in cultured endometrial explants from ovariectomized heifers after in situ hydridization with with a Dig-labeled PAF-R antisense RNA probe (A, C–F) and corresponding sense section (B). (A) without steroids, (C) 1 nM estradiol, (D) 10 nM estradiol, (E) 10 nM progesterone, (F) 10 nM estradiol plus 10 nM progesterone. Glandular epithelium (GE), luminal epithelium (LE), gland (G), stroma (S), blood vessel (BV). Scale bar represents 20 μm.

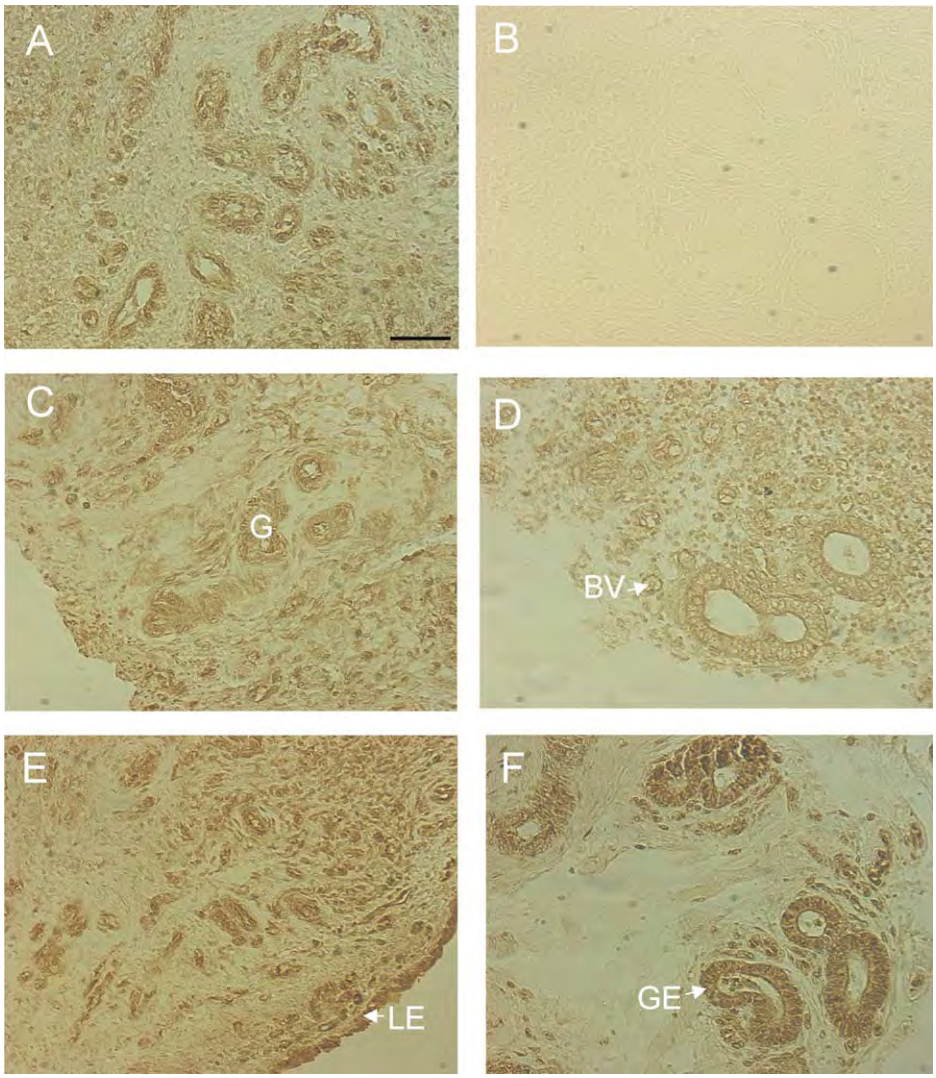

Fig. 3. Representative photomicrograph of paraffin sections of perfused endometrial explants from ovariectomized bovine. Platelet-activating factor receptor (PAF-R) immunohistochemistry (brown staining). (A) without steroids, (C) 1 nM estradiol, (D) 10 nM estradiol, (E) 10 nM progesterone, (F) 10 nM estradiol plus 10 nM progesterone, (B) non-immune serum displayed no immunostaining. Glandular epithelium (GE), luminal epithelium (LE), gland (G), blood vessel (BV). Scale bar represents 20 μm.

staining of glandular and stromal cells was approximately in the range of that of the controls, while a reduction of staining was observed when the explants were incubated with 10 nM E_2 (Fig. 3D). After incubation with 10 nM P_4 (Fig. 3E) or combination of 10 nM E_2 + 10 nM P_4 (Fig. 3F) a stronger cytoplasmic staining was seen in the glandular epithelium compared

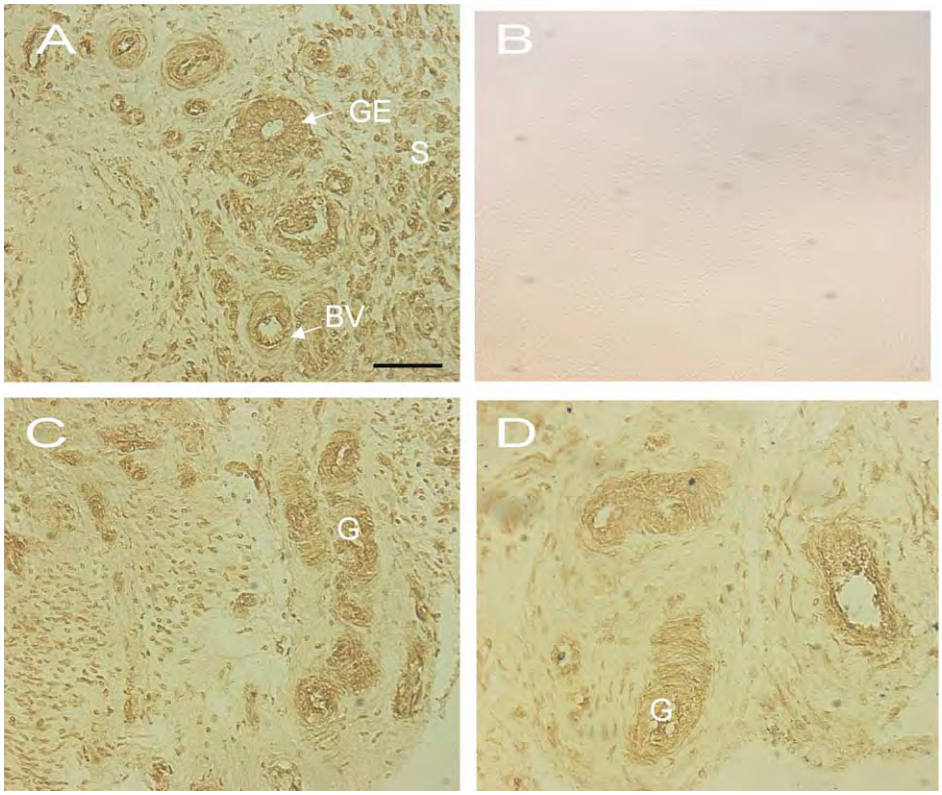

Fig. 4. Platelet-activating factor receptor (PAF-R) immunohistochemistry (brown staining) of cultured endometrial explants from ovariectomized bovine during various treatments. (A) 0 nM PAF, (C) 100 nM PAF, (D) 1000 nM PAF, (B) non-immune serum displayed no immunostaining. Glandular epithelium (GE), luminal epithelium (LE), gland (G), stroma (S), blood vessel (BV). Scale bar represents 20 μm.

to that of the controls. Due to identical results concerning immunolocalization, the luminal images are not shown. No staining was observed when the primary antibody was replaced by normal rabbit serum (Fig. 3B).

Treatment of endometrial explants with 100, 1000 nM PAF did not show significant differences in the pattern of staining. PAF-R immunoreactivity was detected in glandular and luminal epithelial cells (Fig. 4).

4. Discussion

In this study we used a perfusion culture system for tissue preservation, because the continuous supply of nutrients was improved by medium perfusion. The permanent replacement of the medium has more benefits to offer. Metabolites, paracrine factors, and

secretory products are drained with the medium flow and thus do not accumulate to reach unphysiological concentrations in the culture chamber [10].

Several studies have been conducted to examine the expression or localization of PAF-R in reproductive tissues of different species [11–14]. In the present study, immunohistochemistry revealed a specific staining for PAF-R mainly in glandular and luminal epithelia of the endometrium, while stromal cells showed a distinctly weaker immunoreaction. This result is in agreement with the data reported for human glandular epithelium [15] and glandular epithelial cells of pregnant rabbit endometrium [16].

Ovariectomy was employed to suppress steroid levels in the heifers, because we aimed to investigate whether sex steroid hormones or PAF is sufficient as well as necessary to sustain expression and regulation of PAF-R gene in perfused bovine endometrial explants. In this investigation, we demonstrate that PAF-R transcripts are expressed in bovine endometrial explants depending on the presence of sex steroid hormones. We found that P_4 stimulated expression of the PAF-R gene when using relative quantification. Similar changes were observed in both, intensity of hybridization signal and immunostaining. This fact supports our previous results indicating a significant increase in PAF-R immunocytochemical staining of endometrial stromal cells measured by flow cytometry on day 20 of pregnancy (P_4 is high) compared to day 20 of the estrous cycle [6], and it can be suggested that the expression of the PAF-R is modulated by progesterone. In explants derived from ovariectomized bovine of the present study, which were synchronously treated with estradiol and progesterone, the level of endometrial PAF-R mRNA expression was in the same range from that observed with progesterone treatment alone. Thus, concomitant administration of estradiol plus progesterone did not further increase the level of endometrial PAF-R mRNA observed with progesterone alone, indicating that estradiol did not influence PAF-R transcripts. In contrast to the present results, Sato et al. [11] demonstrated that estradiol up-regulates the PAF-evoked intracellular Ca^{2+} release in cultured human endometrial cells. Similarly, Ahmed at al. [13] reported that PAF-R immunoreactivity and mRNA were predominantly detected in human endometrial tissue from the proliferative phase of the cycle. The discrepancy between both the results can probably be explained by differences in hormonal conditions due to cycle status before tissue preparation. Additionally, a species-specific regulation of the PAF-R gene by steroid hormones cannot be excluded, which was postulated by Yang et al. [14] and attributed to the appearance of different transcript levels.

Furthermore, we investigated whether PAF could regulate its own receptor in bovine endometrial tissue. The up-regulation of PAF-R expression following exposure to PAF was reported for human platelets [17], B-cells [18], Kupffer cells [9] and monocytes [19]. In human monocytes, 10 nM to 1 μM PAF induced a two-fold increase in PAF-R mRNA expression. Contrary to that in the present study, we did not find a significant increase of PAF receptor gene under the influence of exogenous PAF in endometrial explants. Our result corresponds with that reported by Chami et al. [20], where the authors found that the ovine uterus only responds to PAF if it is estrogen and/or progesterone-primed in vivo. Thus, we may conclude that steroid hormones and/or other factors cooperate to maintain the PAF receptor.

The fact that progesterone regulates the expression of PAF-receptor mRNA in uterine endometrial cells derived from ovarectomized bovine suggests that PAF is involved in the physiological process of reproduction.

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