Role of the platelet-activating factor and its receptor in the proliferative regulation of bovine ovarian granulosa cells

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Abstract. Proliferation of granulosa cells and their withdrawal from the cell cycle may regulate follicular ovulation. Antagonists of platelet-activating factor (PAF) and its receptor (PAFr) inhibit follicle rupture. *Objectives:* Thus, PAF and PAFr may be involved in proliferative regulation of granulosa cells; however, expression of PAFr in these cells is unknown. *Materials and Methods:* The aim of this study was to investigate the presence of PAFr and the effect of PAF on proliferation of cultured bovine granulosa cells using real-time polymerase chain reaction to assay steady-state level of mRNA, immunocytochemistry to quantify PAFr protein and proliferating cell nuclear antigen protein by flow cytometry. *Results:* We found that granulosa cells express PAFr transcripts and protein. PAF presence did not change the concentration of PAFr mRNA or PAFr protein. Granulosa cells responded to PAF doses of 10 and 50 nM with increasing proportions of cells entering G₀/G₁ phase, as well as a significant expansion of total cell numbers. Rise in G₀/G₁-phase cells was accompanied by a decline in proliferating cell nuclear antigen protein expression, and these effects could be suspended by simultaneous PAFr blockage. The results provide clear evidence for expression of PAFr in bovine granulosa cells and its functional involvement in PAF/PAFr-mediated stimulation of cell recruitment. *Conclusions:* PAF antagonists are suggested to disturb this regulative activity of PAF and to contribute in this way to blockage of ovulation.

INTRODUCTION

Final development of the ovarian follicle is accompanied by rapid population growth of granulosa cells with gonadotropins as major stimulants. However, the response of granulosa cells to gonadotropins changes dramatically during final follicular growth. The precise mechanisms involved in this change remain largely unknown (Gonzalez-Robayna *et al.* 2000). Platelet-activating factor (PAF) may play a role because ovarian follicles produce PAF (Alexander *et al.* 1990) and human follicular fluid contains this phospholipid at low nanomolar levels (Lopez Bernal *et al.* 1992; Narahara *et al.* 1996). Biological effects of PAF are mediated by a specific receptor, PAFr, a seven-transmembrane-spanning G-coupled protein. PAFr is activated by PAF

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in concentrations ranging from picomolar to nanomolar levels and is expressed primarily on cells responsible for inflammatory processes (Terashita *et al.* 1987; Marathe *et al.* 1999). PAFr has also been identified on cells of the oviduct (Tiemann *et al.* 1999) the endometrium (Ahmed *et al.* 1994; Baldi *et al.* 1994; Yang *et al.* 2003), as well as in embryonic cells (Roberts *et al.* 1993; Lee *et al.* 2004).

Binding of PAF and PAF-like phospholipids to PAFr is associated with inositol phospholipid hydrolysis and rapid accumulation of sn-1,2-diacylglycerol and inositol-1,4,5-trisphosphate. This process is accompanied by mobilization of calcium (Izumi & Shimizu 1995; Ogita *et al.* 1997). These second messengers may act on pathways regulating ovarian granulosa cell proliferation, which is important because granulosa cell number has been implicated as a critical factor in ovulation (Robker & Richards 1998). In contrast, differentiation of granulosa cells into the luteal state requires exit from the cell cycle. Granulosa cells of pre-ovulatory follicles cease to divide in response to the pre-ovulatory luteinizing hormone surge. This behaviour has been described as associated with loss of proliferating cell nuclear antigen (Robker & Richards 1998), a sensitive marker of granulosa cell proliferation (Oktay *et al.* 1995). PAF signalling may thus contribute to regulate corresponding pathways; however, expression of PAFr in granulosa cells has not yet been described. Thus, the aim of the present study was to examine the presence of PAFr mRNA and PAFr protein in granulosa cells and to investigate the effect of PAF and PAFr antagonists on proliferation of granulosa cells, from bovine mature follicles.

MATERIALS AND METHODS

Cells

Granulosa cells were isolated from bovine follicles > 15 mm in diameter. Morphological assessment and 17β -oestradiol concentration of the follicle fluid (at least 20 ng/mL), exceeding the progesterone level, indicated that follicles were healthy (vascularized, oestrogenic) and were maturing into the final pre-ovulatory stage. Follicles were dissected from ovaries, follicle fluid was aspirated and each follicle was bisected. Mural granulosa cells were scraped from the theca interna, and were collected by centrifugation (60 g, 5 min, 4 °C) as described elsewhere (Porter *et al.* 2001; Löhrlke *et al.* 2005). Cells were re-suspended in ice-cold water for 10 s to lyse erythrocytes, which was terminated by addition of NaCl (to 150 mM) and bovine serum albumin (BSA) (to 1%); cells were collected and re-suspended in cell culture medium (MegaCell, Sigma, St. Louis, MO, USA) supplemented with BSA (1%), insulin (100 ng/mL), L-glutamine (2.5 mM), and an antibiotic mixture (Sigma). PAF and PAFr antagonists (Cellai *et al.* 2006), WEB 2086, 3-[4-(2-chlorophenyl)-9-methyl-6H-thienol[3,2-f](1,2,4)triazolo-[4,3-a][1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone, and PAFA, 1-O-hexadecyl-2-acetyl-sn-glycero-3-phospho (N,N,N-trimethyl) hexanol amine, were added using doses indicated.

Cell culture

Cell density was determined by conductometric counting (Coulter Counter; Coulter Electronics Inc., Hialeah, FL, USA) and cell viability was assessed by trypan blue exclusion, as recommended by the manufacturer (Sigma). In a proportion of experiments, the trypan blue exclusion test, yielding > 95% viable cells, was confirmed by the MTT assay. At the end of culture, medium containing 5 mg/mL MTT was added to each well (to 0.1 mg/mL) and all was incubated for 1 h, at which formazan stain < 0.2 at 550 nm (background reading at 660 nm) was noted after extraction of insoluble formazan with dimethyl sulfoxide. Aliquots were plated at a density of

10^5 cells on 96-well culture plates (Biochrom, Berlin, Germany). Cells were then treated with additives to a constant final volume and concentrations as indicated, and preparations were incubated for 16 h in humidified air-CO₂ (5%) atmosphere. Each experiment was performed using granulosa cells from a single pre-ovulatory follicle and repeats were performed as indicated. Treatments within each experiment were performed in duplicate.

Detection of specific mRNA concentration

Total RNA was isolated by standard procedures (Invitex kit and manufacturer's protocol; Invitex, Berlin, Germany). The reverse transcription (RT) reaction was performed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) using 100 ng total RNA and an oligo (dT)₁₂ primer to synthesize cDNA from poly A-containing mRNA. cDNA was amplified by real-time polymerase chain reaction (PCR) (iCycler, Bio-Rad) using an iQ SYBR Green Supermix according to a previously described method (Löhrke *et al.* 2005). One microlitre of each RT reaction (1/20 of total) was in each 10 µL PCR primed with gene-specific oligonucleotides (Table 1). Primers of PCNA and of S18 (a ribosomal protein used as housekeeping transcript) were designed to span a corresponding intron and to anneal at 60 °C and 70 °C, based on published cDNA and gene sequences; mRNA fragments produced are shown in Table 1. cDNA corresponding to PAFr mRNA fragment was generated by primers based on PAFr gene sequences (Yang *et al.* 2001). Specificity of the products was assessed by melting point analysis and agarose gel electrophoresis, in comparison with an oligonucleotide molecular mass ladder, to confirm that calculated molecular mass of cDNA corresponded to the produced cDNA, as described previously (Löhrke *et al.* 2005). cDNA structure was checked by sequencing. mRNA abundance was calculated using the known concentration of standard oligonucleotides and amplification efficiency displayed by the iCycler (Bio-Rad) as described elsewhere (Löhrke *et al.* 2005).

Our experiments provided evidence that the widely used glyceraldehyde-3-phosphate dehydrogenase standard for mRNA quantification itself is subject to alterations due to PAF and PAF antagonists. We therefore employed ribosomal S18 mRNA for internal standardization, which performed more stably under PAF and PAF antagonist treatment.

Cell cycle analysis

Cultured cells were fixed in ethanol (70%) and were stored at -20 °C until required for staining. After removing ethanol, RNA was digested (37 °C, 30 min) by RNase (type II-A, Sigma), 1 mg/mL in HEPES buffered saline (HBS) (5 mM HEPES, pH 7.3, 150 mM NaCl). DNA was stained (37 °C, 30 min) with propidium iodide (70 µM) in HBS. Red fluorescence was recorded by flow cytometry. Under these staining conditions, relative fluorescence intensity (flow cytometric channel) corresponds to cellular DNA content. Single cells were gated and DNA content assigned to G₀/G₁, S or G₂/M phases was quantified (Ormerod 1994; Lange *et al.* 2004), using computer-aided multicycle program (Phoenix, San Diego, CA, USA). To distinguish proliferative response from cell aggregation, fitting and correction for the effects of cellular or nuclear aggregation was performed as described previously (Rabinovitch 1994) because microscopic inspection indicated some formation of clumps of cultured cells.

Flow cytometry

Cellular fluorescence was analysed by an argon laser-equipped flow cytometer (Beckmann Coulter, EPICS-XL, Krefeld, Germany) after the method described previously (Löhrke *et al.* 1996, 1997). Fluorescence was excited at 488 nm and 5000 particles were counted. Standard Fluorospheres (9.8 µm diameter, Beckman Coulter) were used as controls in determining cell counts per time (seconds) required for counting 5000 particles. Recorded seconds paralleled particle

Table 1. Sequences of primer sets used for amplification of specific cDNA

Transcript	Forward primer sequence	Reverse primer sequence	Product size (bp)	Access number
PAFR	77–100 5'-AAATAATTCCTTTCGTGTGGACTC-3'	313–290 5'-TCACCCTGGTTGTAGTAGTAGACG-3'	237	GI_1192303
S18	293–316 5'-CTTAAACAGACAGAAGGACGTGAA-3'	533–510 5'-CCACACATTATTTCTTCTTGGACA-3'	218	GI_74268022
PCNA	442–465 5'-CAAATCAGGAAAAGGTTTCAGACT-3'	635–612 5'-AGAAAATTTCACTCCATCTTTTGC-3'	194	GI_77735938

Primers were constructed according to data from the gene bank, and bp, PAFr, S18 and PCNA denote the base pairs, genes encoding platelet-activating factor receptor, ribosomal protein S18 and proliferating cell nuclear antigen, respectively.

concentration and were used to calculate concentration of counts in the specimen with unknown particle density (gating single cells).

PAF receptor protein assay

Granulosa expression of PAFr protein was detected and quantified by immunocytochemistry and by flow cytometry. Following culture, cells were harvested, fixed in ice-cold methanol overnight, collected (300 g, 10 min, 4 °C), re-suspended in phosphate-buffered saline containing BSA (1%) and ethylenediaminetetraacetic acid (0.2 mM), and were incubated (1 h) with rabbit anti-PAFr antibody (12 µg/mL) (Cayman, Hornby, ON, Canada). Excess antibodies were removed by washing, bound antibodies were rendered fluorescent by fluorescein thiocyanate (FITC)-conjugated antirabbit F(ab')₂ fragment (2.5 µg/mL) (Sigma). A control of non-specific fluorescence was obtained by omitting the specific antibody. Additionally, specific immunoreaction was controlled by using PAFr blocking peptide (Cayman) to block protein-antibody complex formation. The peptide corresponds to human leucocyte PAF receptor amino acids 1–17, bearing one epitope (Yang *et al.* 2001). Fluorescence of single cells was measured by flow cytometry and concentration of PAFr protein was determined as described previously (Löhrke *et al.* 1998). Briefly, calibration of fluorescence intensity (corresponding to flow cytometric channel number) was accomplished using standard beads (26p, FCSC) with a known concentration of fluorescent molecules. Concentration was expressed as molecular equivalents of soluble FITC (MESF; Schwartz & Fernandez-Repollet 1994). Fluorescence intensity of granulosa cells was converted into concentration with the aid of the linear calibration function (Fig. 2c) using the data provided by the manufacturers, that both the rabbit anti-PAFr antibody and the fluorescent antirabbit F(ab')₂ fragment recognized a single epitope, the FITC molecules averaged 4 ± 1 a F(ab')₂ molecule, and supposing that dissociation rate was negligible during the immunoreaction.

Proliferating cell nuclear antigen protein assay

This procedure followed a standard technique (Viergutz *et al.* 2000). Briefly, cultured granulosa cells were fixed in ice-cold ethanol (70%), were washed and then incubated with purified monoclonal IgG_{2a} mouse antibody (5 µg/mL, PC10, DAKO, Carpinteria, CA, USA) conjugated with FITC for 1 h at room temperature in the dark. A negative control was obtained using non-specific mouse IgG_{2a}-FITC (5 µg/mL, DAKO). Excessive antibody was washed off, cells were re-suspended in HBS and cellular fluorescence was analysed by flow cytometry gating single cells.

Statistical analysis

ANOVA or ANOVA on ranks and analysis of linear regression were performed using the Jandel Scientific Statistical program package (San Rafael, CA, USA). Results were expressed as mean $\pm$ SEM or, when a normality test failed, as median $\pm$ confidence limits, using the Kruskal–Wallis or Dunnett's method for testing statistical significance of difference. Difference between groups or between treatment and control were considered significant at a value of $P < 0.05$.

RESULTS

Expression of PAFr and PCNA mRNA

Products obtained by RT-PCR corresponded with the calculated base number of the sequence produced by primers shown in Table 1. These results were confirmed by sequencing the products, yielding an identity of 99–100% homology. The steady-state concentration of PAFr mRNA was

Table 2. Response of proliferating cell nuclear antigen (PCNA) mRNA and immunoreactive PCNA protein in cultured (16 h) granulosa cells, to treatment with the platelet-activating factor (PAF) and PAFA, a PAF antagonist, relative to the untreated control

Treatment			PCNA mRNA pg/pg S18, fold control				PCNA protein channel, fold control			
			Median	25%	75%	<i>P</i>	Median	25%	75%	<i>P</i>
PAF	10 nM	(4)	0.95	0.82	1.30	n.s.	0.70	0.63	0.97	0.030
PAF	50 nM	(5)	1.06	0.96	1.54	n.s.	0.80	0.69	0.98	0.041
PAF	250 nM	(5)	0.72	0.65	0.96	0.028	1.13	0.95	1.39	n.s.
PAF	50 nM	(4)	0.79	0.68	1.07	n.s.	1.10	0.99	1.43	n.s.
PAFA	1 µM									

Number in parentheses indicates number of independent experiments (number of follicles providing the cells). Median and *P* represent values from ANOVA on ranks and tests using the Kruskal–Wallis method, n.s. denotes non-significant. Treatment with the solvent for PAFA, dimethyl sulfoxide to 0.001%, did not change the result.

0.95 ± 0.17 pg per microgram total RNA or 0.0021 ± 0.0010 pg per picogram S18 mRNA control. The mean amount of PCNA mRNA was 0.005 ± 0.002 pg per picogram S18 mRNA. Treatment with PAF with a dose up to 50 nM did not modulate concentration of mRNA for either PAFr (data not shown) or PCNA (Table 2), while 250 nM of PAF decreased PCNA mRNA level (Table 2). In contrast, a 5-fold rise in PAFr mRNA levels in response to the PAFr antagonist 1-O-hexadecyl-2-acetyl-sn-glycero-3-phospho (N,N,N-trimethyl) hexanol amine (PAFA, 1 µM) was observed in three cultures prepared from different pre-ovulatory follicles (data not shown).

Expression of PAFr and PCNA protein

Cellular concentrations of PAFr and PCNA protein were determined by flow cytometry as shown in Figs 1 and 2. The number of immunoreactive PAFr sites per cell was calculated using calibrated fluorescent beads as reference (Fig. 1). The regression line was used to determine cellular PCNA and PAFr protein levels. Basal concentration of PAFr protein averaged 38.455 ± 3.712 immunoreactive sites per granulosa cell ($n = 5$ independent experiments). This number did not significantly change in response to PAF treatment (data not shown). In contrast, PCNA protein levels decreased with PAF in doses up to 50 nM, a response that could be suspended by PAFr blockage (Table 2). Exposure to 250 nM PAF did not affect PCNA protein level (Table 2).

Modulation of cell cycle distribution by PAF

Effects of PAF on the granulosa cell cycle was analysed by DNA flow cytometry (Fig. 3). A corresponding flow cytometry histogram is shown in Fig. 3, demonstrating the portion of $G_0 + G_1$ cells assayed with a coefficient of variation of $2.1 \pm 1.0\%$, indicating sufficiently exact data. This result was supported by the position of G_2/M phase cells (flow cytometric channel number) that corresponded to twice the channel number of G_0/G_1 phase cells in gating single cells (Fig. 3). Granulosa cells responded to PAF doses of 10 and 50 nM with increasing proportions of cells in G_0/G_1 phase; S and G_2/M fractions simultaneously declined (Table 3). Lack of sub G_0/G_1 DNA signal at any PAF level did not provide evidence of an immediate apoptotic reaction. Pre-treatment with PAFr antagonists abolished the G_0/G_1 response to PAF (Table 3), indicative of PAFr-mediated effects.

Figure 1. Detection of PAF receptor protein in granulosa cells. Immunoreaction was performed in cultured (16 h) cells and fluorescence (emission of FITC at 525 nm) was measured by flow cytometry. Representative results are shown (a), demonstrating unspecific fluorescence (I) and immunoreactive protein (II) (b), demonstrating calibration *via* fluorescent beads with a size similar to granulosa cells (c), demonstrating expression of number of fluorescent molecules in terms of MESF (molecular equivalents of soluble fluorescence, FITC). Corresponding fluorescence intensities (FI, mean channel) were (I) 8474 and 0.58, (II) 40 337 and 1.90, (III) 118 800 and 4.48, (IV) 353 127 and 12.10. Analysis of linear regression (c) resulted in the equation ($r^2 = 0.999$) number of molecules (MESF) = $30\,218 (\pm 514) \times \text{FI}$ (mean channel) with confidence limits as shown by dashed lines.

Effects of PAF on cell proliferation

Table 4 demonstrates that exposure of granulosa cells to 10 nM PAF recruited the cellular reproduction rate. Notably, this effect was accompanied with a significant decline in internal PCNA protein content (Table 2). Again, cell population growth stimulation due to PAF treatment could be suspended by simultaneous PAFr blockage.

Figure 2. Flow cytometry histogram, demonstrating immunofluorescent PCNA protein in cultured (16 h) granulosa cells. Representative result shows basal immunoreactive PCNA level. (I) Denotes unspecific fluorescence, (II) immunoreactive protein. Fluorescence was from FITC-conjugated monoclonal IgG_{2a} against PCNA. Staining and flow cytometry was performed according to the technique described in the Materials and Methods sections. Increasing channel number was proportional to an increase in fluorescence intensity of the granulosa cells.

Figure 3. Flow cytometry DNA histograms of granulosa cells after culture (16 h) without and with platelet-activating factor (PAF), demonstrating sub G₀/G₁ DNA is not induced by PAF. Cell cycle data from this experiment comprise part of the results shown in Table 3. Correction for small effects of aggregation (triplets, <2%) was accomplished by gating single cells as described in the Materials and Methods section.

DISCUSSION

Using bovine pre-ovulatory follicles, this is the first study demonstrating expression of PAFr in granulosa cells. PAFr transcripts were found at concentrations corresponding to roughly 0.1% of ribosomal S18 protein control mRNA. Data on follicle cells are lacking, but PAFr has recently been identified in bovine endometrial explants (Tiemann *et al.* 2005). PAFr mRNA was found at a concentration of about 25% glyceraldehyde-3-phosphate dehydrogenase mRNA equivalent. Endometrial expression of PAFr mRNA did not respond to PAF, a result that could be confirmed

Table 3. Cell cycle distribution of granulosa cells after culture (16 h) without (control) and with platelet-activating factor (PAF) and PAF antagonists (WEB 2086 and PAFA)

			G ₀ /G ₁ (%)			S (%)			G ₂ /M (%)		
			Mean	SEM	<i>P</i>	Mean	SEM	<i>P</i>	Mean	SEM	<i>P</i>
Control		(5)	88.0	1.8		2.2	0.48		9.8	1.6	
PAF	10 nM	(4)	90.3	1.5	0.03	1.6	0.58	n.s.	8.1	1.3	0.04
PAF	50 nM	(5)	91.0	1.7	0.04	1.2	0.43	0.04	7.8	1.6	0.04
PAF	250 nM	(5)	89.0	1.6	n.s.	2.8	0.41	0.04	8.2	1.4	0.05
PAF	50 nM	(4)	86.4	1.6	n.s.	2.6	0.41	n.s.	11.0	1.2	0.04
WEB	1 μM										
PAF	50 nM	(4)	87.4	1.4	n.s.	1.8	0.50	n.s.	10.8	1.7	n.s.
PAFA	1 μM										

Number in parentheses stands for independent experiments (number of follicles providing the cells). Means and SEM are calculated using a flow cytometric single data set without normalization to control, while *P* denotes data from tests using the ratio treatment/control to correct for individual basal variation and ANOVA on ranks and Dunnett's method to test differences versus control; n.s. denotes non-significant. DNA content of cells was assayed by flow cytometry of propidium iodide-stained cells subsequently to RNA digestion.

Table 4. Exposure to platelet-activating factor (PAF) and a PAF antagonist (PAFA) changes number of cultured granulosa cells relative to the untreated control

Treatment			Cell number, fold control			
			Median	25%	75%	P
PAF	10 nM	(4)	1.24	1.15	1.50	0.028
PAF	50 nM	(5)	1.05	1.01	1.40	0.020
PAF	250 nM	(5)	1.15	1.10	1.30	0.002
PAF	50 nM	(4)	1.27	0.98	1.40	n.s.
PAFA	1 µM					

Cells were counted after incubation for 16 h without and with PAF in doses indicated, and after pre-treatment with PAFA. Cell response to the solvent of PAFA, dimethyl sulfoxide to 0.001%, did not differ from the untreated control. Number in parentheses indicates independent experiments (number of follicles providing granulosa cells). Median and *P*-values denote results from ANOVA on ranks and Kruskal–Wallis tests; n.s. denotes non-significant.

by the present study for bovine granulosa cells, despite the difference in mRNA quantification procedures.

Platelet-activating factor receptor protein expression was identified by flow cytometry and concentrations were calculated by using calibrated fluorescent beads as reference. PAFr protein content, as determined in the present study on bovine granulosa cells, corresponded to that reported for human B lymphocytes (Zhuang *et al.* 2000) but it amounted to only around 40% of the value obtained for human peripheral monocytes (Thivierge *et al.* 1993). Certainly, such variability may reflect tissue- and species-specific differences in regulation of PAFr expression (Mutoh *et al.* 1994). In human B lymphocytes, surface PAFr expression correlates with PAFr mRNA content (Zhuang *et al.* 2000), and increasing PAFr transcript concentrations

were reported to correlate with an analogous increase in PAFr protein expression (Yang *et al.* 2003).

Our results provide substantial evidence of PAFr-mediated influence of PAF on cell cycle progression and exit from the proliferation cycle. Notably, such effects are important for final development of granulosa cells during pre-ovulatory maturation of the follicle (Robker & Richards 1998). PAF treatment at physiological concentrations resulted in a shift of the cell cycle phase distribution pattern with decreasing proportions of proliferating cells in S and G₂/M phase in favour of increasing numbers of cells entering the G₀/G₁ compartment. Responses observed to PAF treatment were rather moderate that may be due to typically high proportions of resting cells in pre-ovulatory granulosa cell populations (Robker & Richards 1998).

However, that PAF induced exit of cells from the proliferation cycle is also supported by the concurrent decrease of PCNA protein content. In contrast to the clear response of PCNA protein levels to PAF treatment, PCNA mRNA amounts remained rather constant. This feature is not completely unexpected, as an internal PCNA mRNA pool is detectable even in serum-starved cells and cells in G₁ phase of the cell cycle (Tommasi & Pfeifer 1999). As the half-life of PCNA is about 20 h, substantial amounts of the protein are still detectable after the cell cycle has been completed. This is particularly true for the current experiments that were performed on granulosa cells cultured for 16 h.

Correspondent to the effect on cell cycle progression, PAF treatment also resulted in a considerable increase in cell recruitment. These seemingly synchronized processes could not be induced by non-physiologically high PAF concentrations. It would be speculative to guess at reasons for this cellular behaviour. However, lack of a sub-G₁ peak in corresponding DNA histograms did not point towards immediate apoptotic effects of a PAF overdose. That PAF generates different proliferative patterns in bovine granulosa cells depending on dose may suggest dissimilar mechanisms of PAF action. Evidence has been seen in other cell types that high PAF doses influence cellular behaviour even independent of a PAF receptor (Brewer *et al.* 2002). However, the functional involvement of PAFr-mediated PAF action on cell cycle progression and granulosa cell differentiation is further substantiated by inhibition of these processes by silencing PAFr signalling, using PAF antagonists and PAF at a level that approximates data from the follicle fluid (Lopez Bernal *et al.* 1992; Narahara *et al.* 1996) and from follicular production of PAF *in vitro* (Alexander *et al.* 1990).

In the rat, PAF has reversed inhibition of follicle rupture induced by PAFr antagonists. Moreover, PAF reversed partially the ovulatory blockade by eicosanoid synthesis inhibitor (Abisogun *et al.* 1989; Li *et al.* 1991), suggesting a role of PAFr in ovulatory regulation. In turn, PAFr knockout mice generated by targeted gene disruption, do not show gross morphological abnormalities in any organ system (Ishii & Shimizu 2000). However, gonadotropin-induced activation of genes, encoding cyclo-oxygenase and the progesterone receptor (essential for ovulation), occurs normally in follicles that fail to ovulate due to reduced number of granulosa cells (Lydon *et al.* 1995; Robker & Richards 1998). These observations implicate granulosa cell number as a critical factor in ovulation. In consideration of our results, PAFr antagonist may disturb *in vivo* proliferative regulation of granulosa cells, that is, withdrawal from their cell cycle that is associated with resistance to apoptosis (Quirk *et al.* 2004), and as a corollary to this, inhibits ovulation.

Taking together, our findings provide evidence for expression of PAFr at the levels of transcript and protein. Exposure of PAFr-bearing granulosa cells to PAF modifies their proliferative regulation. PAFr antagonists reverse or attenuate effects of PAF on the cell cycle, cell number and PCNA expression. These data suggest that overlapping but not identical pathways are activated by PAF to promote cell cycle progress subsequent withdrawal from cell cycle.

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