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Direct survival role of vascular endothelial growth factor (VEGF) on rat ovarian follicular cells☆

Griselda Irusta[a,1], Dalhia Abramovich[a,b,1], Fernanda Parborell[a], Marta Tesone[a,b,*]

[a] Instituto de Biología y Medicina Experimental (IByME) – CONICET, Buenos Aires, Argentina
[b] Departamento de Química Biológica, Facultad de Ciencias Exactas, Universidad de Buenos Aires, Buenos Aires, Argentina

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ABSTRACT

The aim of the present work was to analyze the direct effect of VEGF in follicular cell proliferation, apoptosis and activation of the PI3K/AKT and ERK/MEK signaling pathways in early antral follicles or granulosa cells. Antral follicles or granulosa cells were isolated from prepubertal female Sprague Dawley rats treated with DES.VEGF directly stimulates follicular cell proliferation and it also decreases apoptosis by inhibiting caspase 3 activation. In addition, VEGF increases the proliferation and inhibits the apoptosis of isolated granulosa cells in culture. VEGF activates the PI3K/AKT pathway evidenced by an increase in AKT phosphorylation levels and induces the phosphorylation of ERK1/2 in cultured antral follicles. These results demonstrate for the first time that VEGF has a proliferative and cytoprotective role in early antral follicles and in granulosa cells isolated from DES treated prepubertal rats and suggest that PI3K/AKT and ERK/MEK signaling pathways are involved in these processes.

1. Introduction

The female reproductive system is an interesting model for the study of the angiogenesis in adults because it undergoes a number of programmed angiogenic processes coupled with cyclic evolution and decline of ovarian, endometrial, and placental structures. In contrast, the vascular system in other tissues is generally quiescent, with the exception of wound healing and some pathological conditions.

The vascular endothelial growth factor (VEGF) family and its receptors constitute the most important signaling pathway in angiogenesis and have been well characterized by research over the last two decades. Seven family members have been identified, VEGF-A to-E and placental growth factor (PIGF)-1 and -2 all of which signal through three tyrosine kinase receptors, VEGFR-1 to -3. In particular, VEGFA (referred to also as VEGF) is the principal player in angiogenesis and VEGFR2/KDR is the dominant receptor in mediating the proangiogenic functions of VEGF. It has been demonstrated that VEGF, acting through Flk-1/KDR is a crucial physiological component in follicular growth, being necessary for the selection of recruited antral follicles (Zimmermann et al., 2003). In bovine, porcine and murine ovaries, VEGF is expressed during early ovarian follicular development and its expression increases in granulosa and theca cells throughout folliculogenesis (Barboni et al., 2000; Greenaway et al., 2004; Celik-Ozenci et al., 2003). We have recently demonstrated that VEGF protein and its receptor VEGFR2/KDR, are highly expressed in granulosa and theca cells of rat antral follicles (Abramovich et al., 2009).

Several studies have been conducted to study the function of VEGF during follicular development. In mice, inhibition of KDR with a specific antibody inhibits gonadotropin-dependent follicular angiogenesis and blocks the development of mature antral follicles (Zimmermann et al., 2003). Administration of VEGF Trap, a soluble VEGF receptor in the marmoset monkey, produces a decrease in follicular angiogenesis and development, as well as a decrease in FLT1 and KDR receptors (Wulff et al., 2002). Moreover, the administration of an anti-VEGFR2 antibody in the early follicular phase interferes with the normal development of the cohort of recruited antral follicles (Zimmermann et al., 2002). More localized, intrafollicular injection of a VEGF antagonist in rhesus monkeys impairs ovulation and the subsequent development and functional capacity of the corpus luteum (Hazzard et al., 2002). In our laboratory, we have demonstrated that the inhibition of VEGF by intrabursal administration of Trap produces an imbalance in the ratio of antiapoptotic:proapoptotic proteins that leads a larger number of follicles to atresia in the rat ovary (Abramovich et al., 2006; Parborell et al., 2008).

Besides its proangiogenic effects, VEGF has been shown to be a survival factor for different cell types. The best known is its

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* Corresponding author at: Instituto de Biología y Medicina Experimental (IByME), Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina.
Fax: +54 011 4786 2564.
E-mail address: mtesone@dna.uba.ar (M. Tesone).

[1] Irusta G. and Abramovich D. contributed equally to this article.

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mitogenic role in endothelial cells acting through its receptor KDR, which has been demonstrated both *in vitro* and *in vivo* (Gerber et al., 1998a,b). However, this cytokine also exerts a cytoprotective effect in neurons by inhibiting apoptosis, stimulating neurogenesis (Gora-Kupilas and Josko, 2005; Rosenstein and Krum, 2004), producing gliotrophic effects in CNS tissue (Mani et al., 2005) and also acting as a survival factor for myocytes after ischemic injury (Rissanen et al., 2002). Recently, it has been demonstrated that VEGF has a direct role in mouse embryonic eye development and lens differentiation (Saint-Geniez et al., 2009). However, to date, there is little information about the direct survival role of VEGF in rat ovarian cells, especially early antral follicles and granulosa cells in culture. It has been shown that VEGF might act as a survival factor in bovine ovarian follicles acting through its KDR receptor and inhibits follicular atresia by suppressing apoptosis of granulosa cells in the bovine ovary (Greenaway et al., 2004; Kosaka et al., 2007).

The multifunctionality of VEGF at the endothelial cellular level results from its ability to initiate a diverse and integrated network of signaling pathways via its main receptor, VEGFR2/KDR. The main pathway through which VEGF promotes cell survival is phosphatidylinositide 3′ OH-kinase (PI3K)-dependent activation of the antiapoptotic kinase, AKT/protein kinase B (Gerber et al., 1998b; Thakker et al., 1999). VEGF also activates the PI3K/AKT pathway in other cell types, such as neurons (Jin et al., 2000) and smooth muscle cells (Banerjee et al., 2008). In addition, VEGF is a strong activator of ERKs (extracellular signal-regulated protein kinases) 1 and 2 via KDR playing a central role in angiogenesis and cell survival (Ilan et al., 1998; Zachary, 2003, 2005; Wang et al., 2009). However, there are no reports about the signaling pathway of VEGF involved in ovarian follicular cells.

We have recently reported that the *in vivo* inhibition of VEGF by TRAP intrabursal administration induces apoptosis and inhibits cellular proliferation in antral follicles from eCG-treated rats, thus regulating follicular growth and development and that the PI3K/AKT signaling pathway is one of the pathways involved in this mechanism. However, we were not able to elucidate whether this *in vivo* effect takes place through either a decrease in blood vessel extension or an inhibition of the interaction of VEGF with its ovarian receptor in follicular cells (Abramovich et al., 2006, 2010).

The goal of this study was to investigate the direct survival role of VEGF in antral follicles and granulosa cells isolated from prepubertal DES-treated rats. In particular, we examined the *in vitro* effect of VEGF on follicular cell proliferation and apoptosis in cultures of early antral follicles and isolated granulosa cells. In addition, we attempted to elucidate whether the PI3K/AKT and ERKs 1 and 2 intracellular pathways are involved in the direct role of VEGF in ovarian cells.

2. Materials and methods

2.1. Reagents

The Phenol red-free DMEM-F12 medium and the antibiotic were products of Life Technologies Inc. (Gaithersburg, MD). Ovine FSH (NIDDK-oFSH-20) was purchased from Dr. A.F. Parlow (National Hormone and Peptide Program, Torrance, CA) and human Recombinant human VEGF from R&D (293-VE). Antibodies against PCNA (sc-7907), Caspase 3 (sc-7148), phospho AKT (sc-7985-R), ERK (sc-154) and phospho ERK (sc-7383) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Actin B (ab-6276) and AKT (9272) antibodies were from Abcam (Cambridge UK), antirabbit and antimouse IgG horseradish peroxidase conjugates Transferrin (T-8158), Bovine Seroalbumin (A-7888) and Ascorbic Acid (A-4544) were obtained from Sigma (St. Louis, MO) and Insulin (Humulin R, HI0219) from Lilly France Laboratory (Fegersheim, France). All other chemicals were of reagent grade and were obtained from standard commercial sources.

2.2. Animals

General care and housing of rats was carried out at the Instituto de Biología y Medicina Experimental (IByME), Buenos Aires, Argentina. Prepubertal female Sprague–Dawley rats (21–23 days) from our colony were allowed food and water ad libitum and kept at room temperature (21–23 °C) on a 12L:12D cycle. The animals were injected subcutaneously with diethylstilbestrol (DES: 1 mg/rat dissolved in corn oil) daily for three days to stimulate the development of early antral follicles. Animals were killed by CO_2 asphyxiation and ovaries were removed and cleaned of adhering tissue in culture medium for subsequent assays. Experimental protocols were approved by the Animal Experimentation Committee of the IBYME (PHS- NIH Approval Statement of Compliance #A5072-01).

2.3. Follicle culture

Early antral follicles (~350 μm in diameter) were dissected from ovaries of animals injected with diethylstilbestrol (DES 1 mg/rat), cleansed of adhering tissue and placed in culture medium either with or without stimulus. Sixty ovarian follicles from 5 animals were pooled for different treatments, and cultures were initiated within 1 h after ovary removal at 37 °C. The follicles were incubated in quadruplicate under serum-free conditions at 37 °C in 350 μl DMEM:F12 (1:1; vol/vol), containing 10 mM HEPES, supplemented with fungizone (250 μg/ml) and gentamicin (10 mg/ml). Several studies have used this culture system to examine the regulation of the ovarian follicular physiology (Cortvrindt et al., 1996; Nayudu and Osborn, 1992; Xu et al., 2006). In addition, this experimental model maintains the structure of the follicle and the interaction between the different follicular cell types. The stimulation of ovarian follicle development by DES treatment in immature rats allows the isolation of follicles that exhibit characteristics of early antral follicles including their size, the small antral cavity, and a thin theca layer (Chun et al., 1996; Andreone et al., 2009). In addition, several studies have used this model to examine the regulation of apoptosis in early antral follicles (Chun et al., 1996; Kaipia et al., 1996; Li et al., 1998; Parborell et al., 2001; Vitale et al., 2002). Follicles were incubated for 24 h in serum-free medium for apoptosis and proliferation studies and for 60 min for intracellular pathways studies. The incubations were performed under different conditions: without stimulus (Basal), with FSH (20 ng/ml) or VEGF (50 ng/ml or 100 ng/ml) with 5% CO_2 as previously described (Parborell et al., 2001; McGee et al., 1997). For apoptotic studies, ten follicles from different ovaries were pooled and cultured in the presence of the same stimulus in 100 μl final volume for 24 h. This incubation in serum-free conditions allows exhibiting the typical apoptotic DNA ladder: presence of internucleosomal fragments of 180-bp multiples. After the corresponding incubation time, the follicles were stored at −70 °C until protein extraction or DNA isolation as previously described (Abramovich et al., 2006).

2.4. Western blots

The follicles were resuspended in lysis buffer (20 mM Tris–HCl pH 8, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) supplemented with protease inhibitors (0.5 mM PMSF, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM N-*p*-tosyl-lysine chloromethyl ketone and 0.025 mM L-1-tosylamide-2-phenylethylchloromethyl ketone) and phosphatase inhibitors (25 mM sodium fluoride; 0.2 mM sodium orthovanadate and 10 mM β-glycerophosphate) and homogenized with an Ultra-Turrax (IKA Werk, Breisgau) homogenizer. Samples were centrifuged at 4 °C for 10 min at 10,000 × *g* and the resulting pellets were discarded. Protein concentration in the supernatant was measured by the Bradford assay. After boiling for 5 min, 40 μg of protein was applied to a 15% SDS-polyacrylamide gel and electrophoresis was performed at 25 mA for 1.5 h. The resolved proteins were transferred for 2 h onto nitrocellulose membranes. The blot was preincubated in blocking buffer (5% nonfat milk, 0.05% Tween-20 in 20 mM TBS pH 8.0) for 1 h at room temperature and incubated with appropriate primary antibodies (PCNA, caspase-3, AKT and p-AKT, ERK and p-ERK) in blocking buffer over night at 4 °C. It was then incubated with antirabbit or antimouse secondary antibodies conjugated with horseradish peroxidase (1:1000 and 1:10,000 respectively) and finally detected by chemiluminescence and autoradiography using X-ray film. Protein loading was normalized by reprobing the same blots with antibody against actin B band.

2.5. DNA isolation and fragmentation analysis

Ten follicles from each culture were homogenized in a buffer containing 100 mM NaCl, 4 mM EDTA, 50 mM Tris–HCl, 0.5% SDS, pH 8 and proteinase K (100 μg/ml) at 55 °C for 4 h to facilitate membrane and protein disruption. After incubation, samples were cooled for 30 min on ice in 1 M potassium acetate and 50% chloroform to initiate protein precipitation and centrifuged at 9000 × *g* for 8 min at 4 °C. Supernatants were then precipitated for 30 min in 2.5 volumes ethanol at −70 °C and centrifuged for 20 min at 5000 × *g* at 4 °C. Finally, samples were extracted in 70% ethanol and resuspended in water. DNA content was measured by reading the absorbance at 260 nm, and incubated for 1 h with RNase (10 μg/ml) at 37 °C. DNA samples (4 μg) were electrophoretically separated on 1.7% agarose gels containing ethidium bromide (0.4 μg/ml) in TBE buffer. Within each agarose gel, equal amounts of DNA were loaded into each well. DNA was visualized in an UV (302 nm) transilluminator, and photographed with a Polaroid camera system. Densitometric analysis of low molecular weight (<15 kb) DNA was performed with an Image Scanner (Genius) using the software program Scion Image for Windows (Scion Corporation, Worman's Mill, CT). Quantitative results obtained by densitometric analysis of the low molecular weight DNA fragments represent the mean ± SEM of three independent gel runs.

2.6. Granulosa cell isolation and culture

Granulosa cells were isolated from ovaries of DES-treated rats as described previously (Bley et al., 1992). Cells were seeded onto 96-well plastic plates or 8-well LabTek plates (Nunc, Denmark) precoated with rat tail collagen. Initial plating density was 1×10^5 viable cells/well. Cells were kept at 37 °C with 5% CO_2 in DMEM:F12 medium with BSA 3 mg/ml; insulin 5 μg/ml; transferrin 10 μg/ml and ascorbic acid 50 μg/ml. After 3 h, the medium was changed to remove non-attached cells and replaced with fresh enriched medium. Cells were kept for another 24 h and the enriched medium was then removed and replaced with DMEM:F12 containing FSH (20 ng/ml) and E_2 (50 ng/ml) either with or without VEGF (50 or 100 ng/ml). No stimulus was considered as basal.

2.7. Proliferation assays

Tritiated thymidine (0.4 μCi/well, final specific activity 1.2 Ci/mmol, Perkin Elmer NE027E) was added to the culture 24 h after stimulus. After additional 24 h, cells (100,000/well) were harvested in hollow glass fibers using a multiwell harvester (Vitale et al., 2006; Bley et al., 1992). Excess ^{3}H-thymidine was removed washing with 10 vol. of distilled water followed by 1 vol. of ethanol. Filters were allowed to dry and then transferred to vials, and radioactivity was counted in a scintillation counter (efficiency 66.29%). Each experiment contained six to eight replicates per treatment, and each experiment was carried out three times with different pools of granulosa cells. Each pool of granulosa cells was obtained from six rats.

2.8. TUNEL

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL, Apoptag plus peroxidase in situ Apoptosis detection kit; Chemicon International, Inc.) was used to identify granulosa apoptotic nuclei following the manufacturer's instructions. Eight-chamber plastic Lab-Tek slides (Nunc, Denmark) previously coated with rat tail collagen were plated at 3–3.5 $\times 10^4$ cells per well in 0.4 ml DMEM/F12 with 2.2 g/l $NaHCO_3$ culture medium supplemented with BSA 3 mg/ml; insulin 5 μg/ml; transferrin 10 μg/ml and ascorbic acid 50 μg/ml for 24 h. After 24 h of culture, the enriched medium was removed and replaced with DMEM:F12 containing FSH (20 ng/ml) and E_2 (50 ng/ml) either with or without VEGF (50 or 100 ng/ml). No stimulus was considered as basal. After another 24 h, the cells were fixed in 1% paraformaldehyde and permeabilized with EtOH/acetic acid 2:1. Endogenous peroxidase was quenched with 3% hydrogen peroxide in PBS and the cells were incubated with the TdT enzyme at 37 °C for 1 h. After incubating the cells for 30 min with the anti-digoxygenin peroxidase-conjugated antibody, the apoptotic cells were visualized as positively immunostained after the addition of the peroxidase substrate.

The number of apoptotic cells was determined by counting 200 cells per well (2 wells/treatment) and the percentage of apoptotic cells for each condition was determined from a total of five experiments (Total cell number counted for each condition: 2000).

2.9. Data analysis

Statistical analysis was carried out using GraphPad Prism software, Inc. (CA, USA). All follicular incubations were performed in triplicate using follicular or granulosa cell pools. Data were expressed as means ± SE of pooled results obtained from at least four independent experiments ($n = 4$). Representative gels and images are shown in the figures. Statistical analysis was performed using one-way ANOVA for experiments including one variable. When values were significant, ANOVA analysis was followed by either Tukey's or Bonferroni's multiple comparison test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. VEGF stimulates cell proliferation of antral follicles in culture

The effect of VEGF on follicular cell proliferation was studied by examining the expression of the PCNA (Proliferating cell nuclear antigen), which is expressed in the G1 and S phases of the cell cycle (Maga and Hubscher, 2003). The incubation of isolated early antral follicles with 100 ng/ml of VEGF significantly increased the follicular PCNA protein content compared with the other conditions (Basal: 0.38 ± 0.09; VEGF 100: 1.04 ± 0.22, $p < 0.05$, $n = 5$). We did not detect stimulation of the proliferation when follicles were incubated in the presence of either FSH or 50 ng/ml of VEGF (Fig. 1).

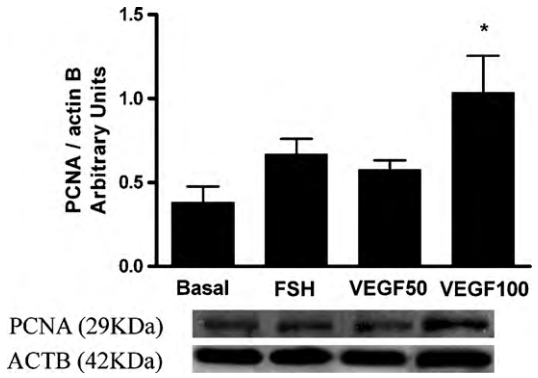

Fig. 1. Direct effect of VEGF on follicular PCNA protein expression. Immunoblot analysis was performed for PCNA in protein extracts of isolated early antral follicles obtained from DES-treated rats and cultured under different conditions. Follicles were incubated during 24 h with no stimulus (Basal), with FSH 20 ng/ml, VEGF 50 ng/ml or VEGF 100 ng/ml. Graph of densitometry measurements shows data from follicular incubations performed in triplicates using follicular pools. Data were expressed as means ± SE of pooled results obtained from five independent experiments ($n = 5$). The asterisk indicates significant differences between different conditions with $p < 0.05$. Representative immunoblot bands are shown for each culture condition.

3.2. VEGF inhibits apoptosis and caspase 3 activation in cells of antral follicles in vitro

The final step of the apoptotic process is the cellular DNA fragmentation in internucleosomal fragments multiple of 180-bp resulting in a typical apoptotic pattern observed in agarose gels. DNA isolated from follicles after 24 h of culture exhibit the typical apoptotic DNA degradation pattern due to a spontaneous-onset apoptosis (Zeleznik et al., 1989; Parborell et al., 2001). In a previous study, we demonstrated that the inhibition of VEGF by the intrabursal administration of TRAP, leads a large number of follicles to atresia (Abramovich et al., 2006). Because of this evidence, we next determined whether VEGF is able to directly regulate follicular atresia. Both concentrations of VEGF (50 ng/ml and 100 ng/ml) significantly decreased apoptotic DNA fragmentation (Basal: 216.7 ± 17.5; VEGF 50: 139.5 ± 12.4; $p < 0.05$ vs Basal; VEGF 100: 118.0 ± 24.7; $p < 0.01$ vs Basal, $n = 5$), being this effect more significant in the presence of the highest concentration of this cytokine ($p < 0.01$ vs basal conditions) (Fig. 2A). FSH also decreased spontaneous apoptotic DNA fragmentation as we have previously described (FSH: 107.1 ± 7.2; $p < 0.01$ vs Basal, $n = 5$) (Parborell et al., 2001).

As we observed that VEGF decreases apoptosis in follicular cells, we next investigated whether this inhibition is accompanied with a decrease in caspase 3 activation. To this end, we performed western blot of the active fragment of caspase 3 in follicular protein extracts after the culture. VEGF diminished the p17 active fragment of caspase 3 when used in a final concentration of 50 ng/ml, as compared to basal conditions (Basal: 0.58 ± 0.05; VEGF 50: 0.26 ± 0.04, $p < 0.05$, $n = 5$). The incubation with FSH had the same effect (FSH: 0.27 ± 0.05, $p < 0.05$ vs Basal, $n = 5$). Unexpectedly, we could not detect differences between follicles incubated in the absence of stimulus and those stimulated with VEGF 100 ng/ml (VEGF 100: 0.47 ± 0.09) (Fig. 2B), suggesting a biphasic effect of VEGF as it was described in other systems (Zhu et al., 2002; Meng et al., 2006; von Degenfeld et al., 2006).

3.3. VEGF stimulates granulosa cell proliferation

To further investigate whether this cytoprotective effect of VEGF is due, in part, to a direct action of the cytokine in granulosa cells, we isolated granulosa cells and we cultured them

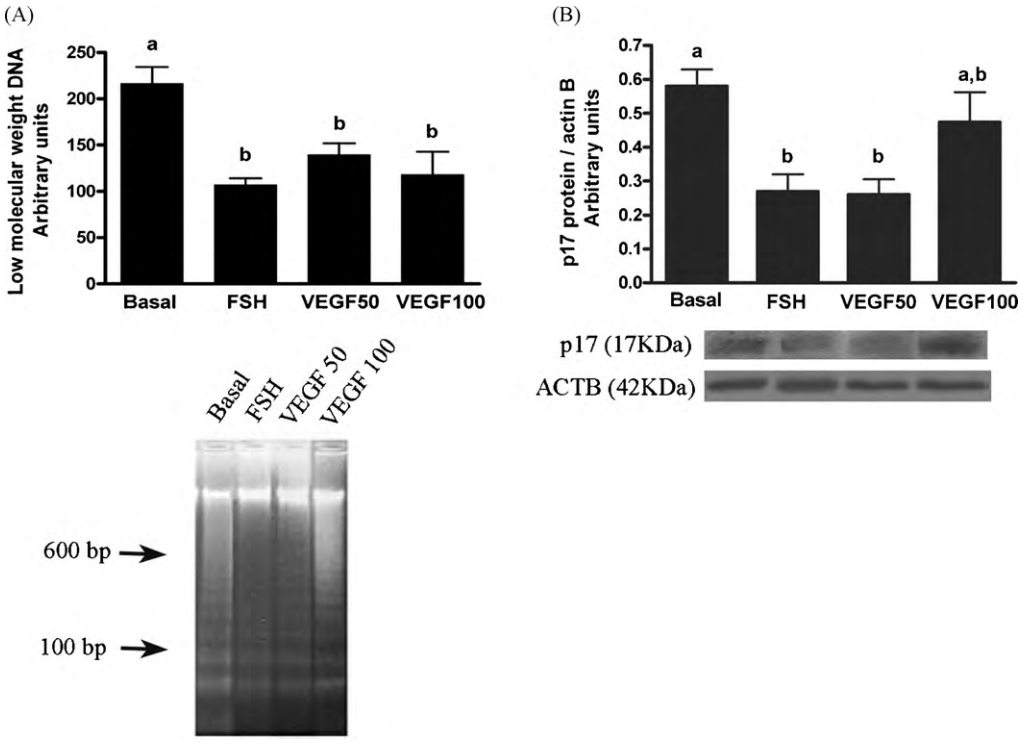

Fig. 2. Direct effect of VEGF on apoptotic DNA fragmentation and caspase 3 activation in antral follicles. (A) Apoptosis was evaluated by analysis of DNA fragmentation pattern in agarose gels of early antral follicles isolated and incubated with different stimulus during 24 h (FSH, VEGF 50 ng/ml and VEGF 100 ng/ml). FSH and VEGF100 vs Basal, $p < 0.01$; VEGF50 vs Basal, $p < 0.05$. Bars represent mean ± SEM of different experiments. Lower panel shows a representative gel of the apoptotic DNA fragmentation pattern. (B) Densitometric quantification of caspase 3 active fragment in antral follicles in culture. Caspase 3 protein was visualized using an anti-caspase 3 antibody which recognizes the cleaved form of this protein. Data were expressed as means ± SE of pooled results obtained from five independent experiments ($n = 5$). Different letters in the densitometric quantification of the p17 band indicate a significant difference between the follicles incubated in Basal conditions and those incubated in the presence of FSH or VEGF 50 ng/ml ($p < 0.05$ vs Basal).

under different conditions, as described in the materials and methods section. We first analyzed the proliferation index of the cells using the ^{3}H-Thymidine incorporation assay. In agreement with that previously described by our laboratory and other authors (Vitale et al., 2002; Bussmann et al., 2006), the stimulation with both FSH and estradiol increased the thymidine incorporation in this system (Basal: 1.51 ± 0.23; FSH + E_2: 4.17 ± 0.21 (×10^3 cpm); $p < 0.01$, $n = 4$). Interestingly, this effect on granulosa cell proliferation was intensified with the addition of VEGF 50 ng/ml (FSH + E_2 + VEGF 50: 5.54 ± 0.29 (×10^3 cpm) vs FSH + E_2 alone; $p < 0.01$) and of VEGF 100 ng/ml (FSH + E_2 + VEGF 100: 6.04 ± 0.19 (×10^3 cpm) vs FSH + E_2 alone; $p < 0.001$, $n = 4$) (Fig. 3A).

3.4. VEGF inhibits granulosa cell apoptosis

Given the inhibition of follicular apoptosis by VEGF previously observed in follicles, we extended the study to determine whether apoptosis occurred in isolated granulosa cells, by using the TUNEL assay. The incubation of granulosa cells in the presence of FSH and estradiol decreased the percentage of apoptotic cells compared to basal conditions (Basal: 68.36 ± 0.87%; FSH + E_2:

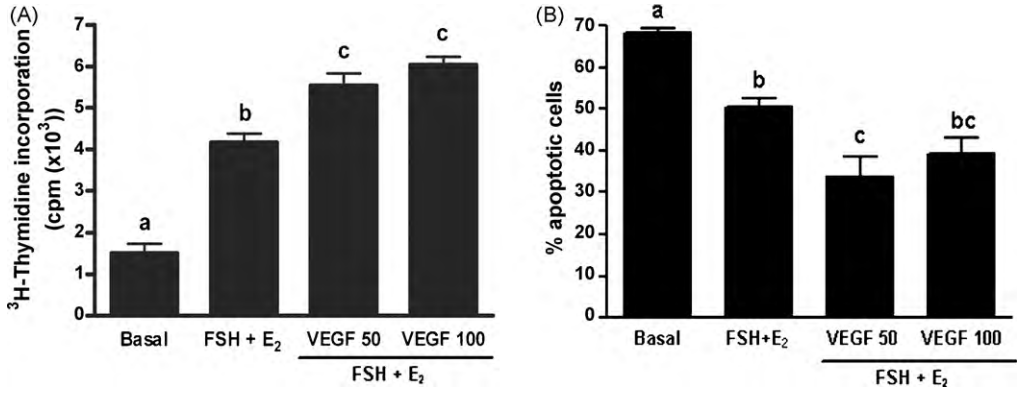

Fig. 3. Direct effect of VEGF on granulosa cell proliferation and apoptosis. (A) Granulosa cells (100,000/well) isolated form early antral follicles were incubated in absence of stimulus (Basal), in the presence of FSH + E_2 (stimulated) and simulated plus VEGF 50 ng/ml or VEGF 100 ng/ml. VEGF 50 ng/ml and VEGF 100 ng/ml $p < 0.01$ and $p < 0.001$ vs stimulated condition, respectively as analyzed by ANOVA. (B) TUNEL apoptosis kit was used to detect apoptotic cells after culture in different conditions as described in Materials and Methods. The bar graph shows the percentage of apoptotic cells and different letters indicate significant differences between incubation conditions ($p < 0.05$ Basal or VEGF50 vs stimulated condition). Data were expressed as means ± SE of pooled results obtained from four independent experiments ($n = 4$).

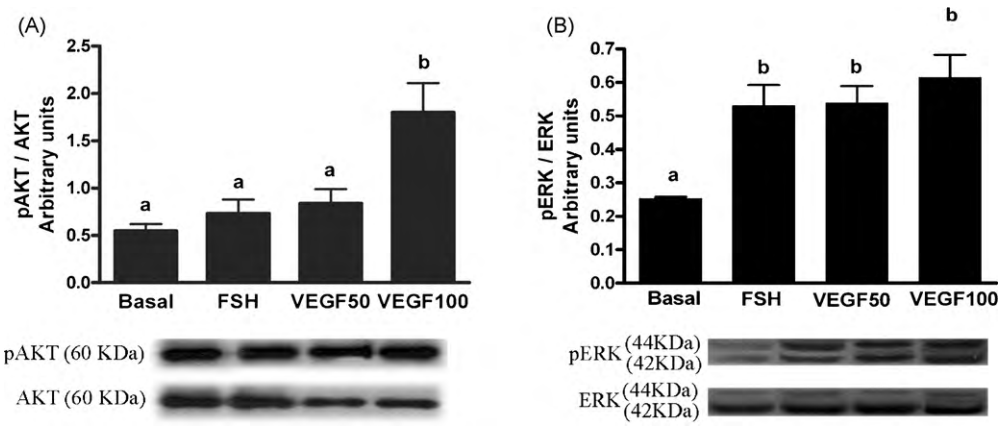

Fig. 4. Direct effect of VEGF on AKT and ERK phosphorylation in antral follicles. Densitometric quantification of pAKT (A) and pERK (B) in protein extracts of early antral follicles incubated in different conditions for 60 min. Bands represent mean ±SEM of different experiments. Each protein was normalized to Actin B (a vs b, $p < 0.001$ for pAKT protein and a vs b, $p < 0.05$ for pERK protein, ANOVA). Data were expressed as means ± SE of pooled results obtained from five independent experiments ($n = 5$). Lower panels indicate representative immunoblots of each condition.

50.40 ± 2.29%; $p < 0.05$, $n = 4$), as previously described (Vitale et al., 2002; Bussmann et al., 2006). In addition, VEGF 50 ng/ml intensified this effect opposed to VEGF 100 ng/ml which showed no significant difference vs VEGF 50 ng/ml or stimulated conditions (FSH + E_2 + VEGF 50: 33.80 ± 4.82 vs FSH + E_2 alone, $p < 0.05$, $n = 4$) (Fig. 3B).

3.5. VEGF activation of AKT and ERK intracellular pathways

In light of these results, we sought to find out the signaling mechanisms by which VEGF regulates follicular survival and apoptosis in these follicular cells. Therefore, we examined whether VEGF actions occur through the phosphatidylinositide 3′ OH-kinase (PI3K)/AKT pathway in the follicular compartment. To test this, the follicles were incubated for 1 h in basal conditions or in the presence of FSH, VEGF 50ng/ml or VEGF 100ng/ml. We performed western blot of both the total (AKT) and the phosphorylated form of AKT (pAKT) protein after the stimulations. As shown in Fig. 4A, VEGF significantly increased AKT phosphorylation when the follicles were stimulated with 100 ng/ml of the cytokine, as compared to the other conditions ($p < 0.001$). We could not detect any effect in the presence of VEGF 50ng/ml at this time point (Basal: 0.44 ± 0.06; FSH: 0.35 ± 0.08; VEGF 50: 0.44 ± 0.08; VEGF 100: 1.51 ± 0.39, $n = 5$).

In addition to the PI3K/AKT signaling pathway, it has been demonstrated that VEGF induces activation of the MEK/ERK pathway via its receptor VEGFR2 in different cell types (Ilan et al., 1998; Zachary, 2003, 2005; Wang et al., 2009). Because the activation of this pathway is involved in the survival role of VEGF, we tested the levels of phospho-ERK in follicles in culture by western blot analysis. After 1 h of incubation with FSH or VEGF 50 ng/ml, the levels of phospho-ERK were significantly increased compared to basal conditions (Basal: 0.25 ± 0.005; FSH: 0.53 ± 0.06; VEGF 50: 0.54 ± 0.05; $p < 0.05$, $n = 5$). This effect was more evident when the follicles were incubated in the presence of VEGF 100 ng/ml (VEGF 100: 0.61 ± 0.07; $p < 0.01$, $n = 5$) (Fig. 4B).

4. Discussion

In the present study, we demonstrated for the first time that VEGF has a proliferative and cytoprotective role in early antral follicles and in granulosa cells isolated from prepubertal rats treated with DES. Although the best known effects of VEGF are on endothelial cells, in the last years there has been a growing body of evidence about the survival role of VEGF in nonvascular cells, such as neuronal cells and myocytes (Rissanen et al., 2002; Zachary, 2005, 2003). So far, there have been few reports about the survival role of VEGF in nonvascular tissues in the ovary (Greenaway et al., 2004; Kosaka et al., 2007).

To analyze the effect of VEGF on follicular cells, the first model we used was an *in vitro* early antral follicle culture system, which preserves the structure of the follicle and allows studying the interaction between the different follicular cells. Using this approach, we demonstrated that VEGF is able to stimulate follicular cell proliferation of rat ovaries when added to the culture medium. This result is consistent with our previous work where, by inhibiting VEGF *in vivo* locally in the ovary we demonstrated that VEGF blockage decreases granulosa and theca cell proliferation in the rat (Abramovich et al., 2010). This is also in agreement with other investigators, who have shown a decrease in theca cell proliferation after inhibition of VEGF in the primate ovary (Wulff et al., 2002). In addition, it has been recently shown that VEGF improves follicular ultrastructural integrity and promotes follicular growth in the goat ovary (Bruno et al., 2009). These results suggest that VEGF interacts with its receptor present in granulosa and/or theca cells and acts as a mitogenic factor in an autocrine or paracrine way in the developing follicle. Despite the fact that early antral follicles have a poorly developed vascular sheet, we cannot discard a possible effect of VEGF on endothelial cells.

On the other hand, we analyzed the effect of VEGF on cultured follicle apoptosis. DNA isolated from follicles cultured 24 h in serum-free medium exhibited the typical apoptotic DNA degradation pattern (Zeleznik et al., 1989; Parborell et al., 2001). Incubation of follicles with FSH, a very well-known survival factor for ovarian follicles decreases apoptotic DNA fragmentation (Parborell et al., 2001; Chun et al., 1996). Our results demonstrated that the addition of VEGF to the culture medium decreased the spontaneous DNA fragmentation pattern of apoptosis as well as FSH. Moreover, VEGF also inhibited caspase 3 activation in early antral follicles in serum-free conditions. There are several studies in different cell types such as embryonic neurons that show an apoptotic rescue role of VEGF interfering with the activation of caspase 3 and thus suppressing the apoptosis of the cells (Jin et al., 2001). In this study we showed that VEGF is acting as a survival factor for follicular cells through a caspase-dependent mechanism.

To further analyze the effect of VEGF on follicular cells, we studied the possible action of this factor directly on granulosa cells. It is known that granulosa cells of follicles from rats and other species express KDR differentially during follicular development.

Greenaway et al. (2004) demonstrated the coexpression of VEGF and its receptor, Flk-1/KDR, in bovine granulosa cells of healthy follicles. In agreement with that work, we have recently demonstrated that VEGF and its receptor, Flk-1/KDR, increase their expression throughout folliculogenesis in the rat (Abramovich et al., 2009). In order to study a possible direct effect of VEGF on granulosa cells, we isolated and cultured these cells from prepubertal rats treated with diethylestilbestrol. When cells were stimulated with FSH and estradiol we observed an increase in follicular proliferation. This effect has been previously described by us (Vitale et al., 2002) and others (Bussmann et al., 2006). When cells were stimulated with FSH, estradiol and VEGF, we observed a higher increase in the tritiated thymidine incorporation. VEGF alone had no effect on the proliferation of the cells when compared with basal condition. These results demonstrate that VEGF has a synergistic effect with FSH and estradiol on granulosa cell proliferation and that VEGF is acting directly on this cell type. VEGF also stimulates cultured granulosa cell proliferation of medium and large porcine follicles (Grasselli et al., 2002) when added alone to the culture medium. Our present study demonstrated that, in rat granulosa cells obtained from early antral follicles, VEGF alone has no effect on cellular proliferation but enhances the FSH and estradiol ability to promote cellular growth. The differences observed between the effect of FSH or VEGF on early antral follicles or granulosa cell proliferation could be due to the accessibility to the FSH receptors in granulosa cells or the interaction with other factors present in the entire follicle.

VEGF has been widely shown to be a survival factor for different species and cell types, by acting directly through its receptor KDR present in endothelial and nonendothelial cells, such as neurons, myocytes, endothelial cells and ovarian cells from apoptosis (Gerber et al., 1998a; Gora-Kupilas and Josko, 2005; Rosenstein and Krum, 2004; Rissanen et al., 2002; Greenaway et al., 2004; Kosaka et al., 2007). In the present study, by using the TUNEL technique, we showed that VEGF alone had no effect on granulosa cell apoptosis but that this factor intensifies the cytoprotective effect of FSH and estradiol in granulosa cells in culture. We conclude that rather than being a proliferative cytokine in follicular cells, VEGF also possesses a protective role in early antral follicles, preventing granulosa cell apoptosis and consequently, follicular atresia. These actions take place in a synergistic way with FSH and estradiol in rat granulosa cells obtained from early antral follicles. It is worth pointing out that we have previously obtained similar results when VEGF was inhibited *in vivo* during follicular development, which led to an increase in the number of granulosa apoptotic cells, an increase in spontaneous DNA fragmentation and an increase in the activation of caspase 3 (Abramovich et al., 2006, 2010). However, with that experimental design, we were not able to discriminate whether the effects observed were mediated by a decrease in the vascular supply to the follicles or by a direct action of VEGF with its receptor localized in follicular cells. The present study demonstrated that VEGF acts directly on follicular cells synergistically with FSH and estradiol by preventing apoptosis and stimulating proliferation, thus promoting follicular development and selection of the follicle to ovulate. Thus, increased VEGF action in concert with other growth factors might be part of the mechanism that allows selected follicles to grow while other follicles in the same FSH environment become atretic.

VEGF activates the PI3K/AKT pathway in different cell types, such as endothelial cells (Fujio and Walsh, 1999; Gerber et al., 1998b), neurons (Jin et al., 2000) and smooth muscle cells (Banerjee et al., 2008). We have recently reported that the *in vivo* inhibition of VEGF during follicular development in rats, leads to a decrease in AKT phosphorylation (Abramovich et al., 2010). In the present work, we tested the hypothesis that VEGF exerts its direct functions on follicular cells through an activation of the PI3K/AKT pathway. In effect, we observed that VEGF increases the phosphorylation of Akt at the serine 473 when added to the culture medium of the early antral follicles incubated with different stimuli. In contrast, we found no activation of Akt under FSH action. However, there is evidence that FSH stimulates PI3K/AKT signal transduction in many cells, including Sertoli cells, oocytes (Alam et al., 2004; Carvalho et al., 2003; Meroni et al., 2004) and granulosa cells (Gonzalez-Robayna et al., 2000; Kayampilly and Menon, 2009). This discrepancy could be due to the cell type studied and the interval of time of exposure to FSH analyzed in each case.

Besides the well-known activation of the PI3K/Akt pathway by VEGF, there are also several lines of evidence on the stimulation of extracellular-regulated-kinase 1/2 (ERK1/2) signaling by this cytokine (Swendeman et al., 2008). ERK is predominantly activated by growth factors or mitogens leading to cell differentiation, growth and survival. VEGF stimulates DNA synthesis and proliferation *via* the Flk-1/KDR receptor and ERK1/2 in endothelial cells (Parenti et al., 1998; Pedram et al., 1998) and neuronal cell types (Zachary, 2005). Interestingly, in our experimental model, we observed that both FSH and VEGF increase the phosphorylated state of ERK1/2 in culture follicles. We speculate that VEGF exerts its local survival action in follicular cells by activating both signaling mechanisms, the PI3K/AKT and ERK intracellular pathways. However, we cannot discard that other pathways could also be involved in this VEGF action in the rat ovary.

In the present work, we have observed some differences in the effect of VEGF depending on the concentration used in some apoptotic parameters analyzed. This can be due to a biphasic effect of VEGF in our experimental models. It is worth to note that other authors have observed biphasic effects of VEGF on cell differentiation and phosphorylation of intracellular mediators in different cell types (Zhu et al., 2002; Meng et al., 2006; von Degenfeld et al., 2006). In fact, Meng et al. (2006) demonstrated in the adult mouse neural progenitor cells that a high dose of exogenous VEGF significantly downregulates the expression of endogenous KDR and FLT1, whereas a low dose of VEGF significantly upregulates these VEGF receptors. Biphasic effects have also been reported for other growth factors like Transforming Growth Factor β (TGFβ) (Pepper et al., 1993).

Taken together our results suggest that more than being a factor that increases vascular permeability and growth; VEGF plays an important role in preventing apoptosis and stimulating proliferation of nonvascular follicular cell thus preventing the follicle from undergoing atresia. In this regard, VEGF is not only a crucial factor for the development of follicle vasculature but also has a direct effect stimulating follicular growth. To our knowledge, this is the first report about the local action of VEGF as a proliferative and cytoprotective agent against apoptosis in rat ovarian cells. We also demonstrate the intracellular pathways likely involved in this VEGF novel role. Moreover, we show that some of the VEGF functions are due to its direct action on granulosa cells.

In conclusion, VEGF stimulates proliferation and inhibits apoptosis of follicular cells of rat early antral ovarian follicles, particularly granulosa cell type. It also inhibits caspase 3 activation. In addition, we show that the PI3K/AKT and ERK/MEK pathways are involved in these VEGF local effects. Our novel results demonstrate a new role for VEGF in early antral follicles mediated by the PI3K/AKT and ERK/MEK pathways, besides its classical and well-known proangiogenic function.

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