Molecular and Cellular Endocrinology 343 (2011) 79–87

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Angiopoietin 1 reduces rat follicular atresia mediated by apoptosis through the PI3K/Akt pathway

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A R T I C L E   I N F O

Article history:
Received 9 December 2010
Received in revised form 5 May 2011
Accepted 9 June 2011
Available online 15 June 2011

Keywords:
Ovary
Follicular development
Angiogenesis
Angiopoietins
Apoptosis

A B S T R A C T

The aim of this study was to determine the effect of the local inhibition of ANGPT1 on steroid production, proliferation and apoptosis of ovarian follicular cells and on the PI3K/AKT pathway. We also examined the effect of ANGPTs on follicular cell apoptosis and proliferation in early antral follicles (EAFs) in culture. Follicular cells expressing PCNA decreased after ANGPT1 Ab treatment. Moreover, ANGPT1 inhibition increased the levels of active caspase 3 and androsterone, but decreased estradiol, AKT phosphorylation and the area of smooth muscle cell actin. In cultured EAFs from prepubertal rats treated with diethylstilbestrol (DES), ANGPT1 increased PCNA and decreased apoptosis while ANGPT2 reversed these effects. These results show that ANGPT1 alters steroidogenesis, reduces ovarian apoptosis, and stimulates cell proliferation in antral follicles. ANGPT1 may exert these roles by regulating ovarian vascular stability and/or by a direct effect on follicular cells, possibly involving the PI3K/AKT pathway.

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1. Introduction

Angiogenesis is a process of vascular growth essential for follicular development and ovulation (Mizunuma et al., 1999). The status of the follicular blood vessel network is important to determine the selection of the dominant follicle/s. Preovulatory follicles have a high degree of vascularity, which results in preferential delivery of nutrients and gonadotropins, compared with the subordinated follicles. The development and the growth of these capillaries are controlled by angiogenic factors produced by granulosa and theca cells. The regression of the theca blood vessel network accompanies the atresia of nonovulatory follicles. It is known that ovarian apoptosis takes place to eliminate follicular cells in atretic follicles (Chun et al., 1996b; Gougeon, 1996; Nahum et al., 1996; Palumbo and Yeh, 1994; Tilly et al., 1991, 1992b; Tilly, 1998). FSH and LH are the primary survival factors for ovarian follicles and these anti-apoptotic effects are probably mediated by the production of ovarian growth factors. Recent findings have suggested that angiogenic factors may play an important role in the modulation of follicular survival (or death) (Abramovich et al., 2006; Parborell et al., 2008).

Numerous inducers of angiogenesis have been identified, including the members of the FGF-2 (Basic Fibroblast Growth Factor) and Vascular Endothelial Growth Factor A (VEGFA) family, angiopoietins, transforming growth factors (TGF) and platelet-derived growth factor (PDGF) (Carmeliet, 2000; Hanahan et al., 1996; Neufeld et al., 1999; Otrock et al., 2007). FGF-2 is a heparin-binding polypeptide that induces proliferation and migration in cultured endothelial cells and neovascularization in vivo (Basilico and Moscatelli, 1992). In addition, FGF induce sprouting of blood vessels in vivo in the chick chorioallantoic membrane and cornea, thus supporting their role in angiogenesis (Folkman, 1982). While VEGFA is the main initiator of angiogenesis, the formation and differentiation of a structurally and functionally mature vascular network probably requires the coordinated action of various factors. These include ANGPT1 and ANGPT2, which act via the tyrosine kinase receptor, TEK (Maisonpierre et al., 1997). Unlike VEGFA, ANGPT1 is unable to stimulate endothelial cell proliferation (Davis et al., 1996), but instead is required for the recruitment of perivascular cells that lead to the maturation and stabilization of newly developed capillaries (Maisonpierre et al., 1997; Suri et al., 1996). ANGPT1 is able to induce the phosphorylation of TEK, which subsequently transduces a biological effect. ANGPT2 binds to TEK with the same affinity as ANPGT1 but does not phosphorylate the receptor, thus acting as a natural antagonist of ANGPT1 (Maisonpierre et al., 1997). Previous studies have demonstrated the expression of *Vegfa, Angpt1* and *Angpt2* mRNAs in the ovary of the rat (Koos, 1995; Maisonpierre et al., 1997; Phillips et al., 1990), the cow (Goede et al., 1998) and the monkey (Hazzard et al., 1999), suggesting a role for these factors in ovarian angiogenesis.

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doi:10.1016/j.mce.2011.06.009

The balance between the ANGPT2:ANGPT1 ratio and VEGFA expression is important for angiogenesis and blood vessel regression (Goede et al., 1998; Hazzard et al., 1999; Wulff et al., 2000). An increase in the ANGPT2:ANGPT1 ratio is associated with destabilization of blood vessels, which is a prerequisite for new blood vessel formation. When VEGF is high, active angiogenesis results in the formation of a new blood vessel network (high ANGPT2:ANGPT1 ratio, high VEGF), whereas a lack of VEGF support results in a regression of blood vessels (high ANGPT2:ANGPT1 ratio, low VEGF).

In a previous report (Abramovich et al., 2009) we have demonstrated by immunohistochemical studies and Western immunoblot that the expression of ANGPT1 and ANGPT2 increases during follicular development and is mainly localized in theca cells of follicles both in immature, gonadotropin-stimulated and adult rats. In addition, in that study we showed that TEK immunoreactivity in theca cells increases gradually throughout follicular development. However, we found that TEK receptor immunostaining in granulosa cells is absent in all follicular stages. The co-expression of ANGPTs and TEK receptor in theca cells implies that the ANGPTs system may exert both autocrine and paracrine actions. In addition, we have established that inhibition of ANGPT1 by intrabursal administration of ANGPT1 antibody causes an imbalance in the ratio of antiapoptotic:proapoptotic proteins, leading a larger number of follicles to atresia (Abramovich et al., 2006; Parborell et al., 2008). However, the molecular pathway involved in the ANGPT1 survival role in ovarian cells is poorly understood.

A variety of studies have demonstrated that the ANGPTs/TEK signaling system plays a strong anti-apoptotic action in endothelial cells (Kwak et al., 1999; Papapetropoulos et al., 1999), neurons (Valable et al., 2003), synovial cells (Hashiramoto et al., 2007), and cardiomyocytes (Wang et al., 2007). However, it is not known whether ANGPT1 also has a similar direct anti-apoptotic effect on rat ovarian follicular cells.

In this study, experiments were designed to determine the effect of the local inhibition of ANGPT1 on steroid production, proliferation and apoptosis in ovarian follicular cells from prepubertal eCG-treated rats and whether the PI3K/AKT signaling transduction pathway is involved in these processes. In addition, we also examined the *in vitro* effect of ANGPT1 and ANGPT2 on follicular cell apoptosis and proliferation in cultures of early antral follicles (EAFs).

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). Recombinant human Angiopoietin 1 (923-AN) and Recombinant human Angiopoietin 2 (623-AN) were purchased from R&D Systems, Inc. Antibodies against PCNA (sc-7907), Caspase 3 (sc-7148, which recognizes procaspase 3 and its cleaved forms), phospho AKT (sc-7985-R) (Ser^{473}), BAD (FL-335) and phospho BAD (Q-20) (Ser^{136}) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against actin B (ab-6276) and alpha smooth muscle actin (ab18147) were from Abcam antibodies (Cambridge, UK). Antibody against AKT (9272) was from Cell Signaling Technology, Inc. Antirabbit IgG horseradish peroxidase conjugates were obtained from Sigma (St. Louis, MO). All other chemicals were of reagent grade and were obtained from standard commercial sources. Purified goat immunoglobulin G (IgG; sc-2028; Santa Cruz Biotechnology) was obtained from normal goat sera.

Polyclonal ANGPT1/4 antibody (sc-9360; Santa Cruz Biotechnology, Santa Cruz, CA) was raised in goats immunized against a peptide mapping at the c-terminus of the mature chain of ANGPT4 of human origin and detects ANGPT1 of mice, rats and humans (Parborell et al., 2008). It is worth mentioning that ANGPT3 (mouse) and ANGPT4 (human) are interspecies orthologs (Lee et al., 2004). ANGPT4 and ANGPT3 share only 54% overall amino acid identity, and 65% identity within their fibrinogen-like domain (Lee et al., 2004; Valenzuela et al., 1999). In addition, ANGPT4 is present only in human tissues. Therefore, according to these data, we consider that our results obtained using an ANGPT1/4 polyclonal antibody in a rat model, are due to a block in ANGPT1 action.

2.2. In vivo treatment and superovulation

General care and housing of rats was carried out at the Instituto de Biología y Medicina Experimental (IByME) in Buenos Aires. Prepubertal rats were from our colony. Immature female Sprague–Dawley rats (21–23 days) were anesthetized intraperitoneal with ketamine HCl (80 mg/kg; Holliday-Scott S.A., Buenos Aires) and xylazine (4 mg/kg; König Laboratories, Buenos Aires). The ovaries were exteriorized through an incision made in the dorsal lumbar region. Rats then received 10 ng of antibody (Ab) for Angiopoietin 1/4 in 5 μl of saline under the bursa of one ovary (ANGPT1 Ab ovary), as previously described (Parborell et al., 2008). The contralateral ovary was injected with the same volume and concentration of normal goat IgG (Control ovary). After injection, ovaries were replaced and the incision closed with skin adhesive. Rats were then injected s.c. with 0.1 ml eCG (25 IU/rat) and killed 48 h after surgery by CO_2 asphyxiation. For AKT phosphorylation studies, animals were killed 4 or 8 h after surgery. The ovaries were removed and cleaned of adhering tissue in culture medium. Some ovaries were frozen in dry ice and stored at −80 °C until processed for Western blot. The other ovaries were placed in 10% neutral buffered formalin for 12 h and processed for immunohistochemistry.

Experimental protocols were approved by the Animal Experimentation Committee of the IBYME (PHS-NIH Approval Statement of Compliance # A5072–01).

2.3. Western blots

The individual antral follicles (300–450 μm in diameter) from in vivo ANGPT-1-treated rats (6–8 ovaries/group) were dissected from the ovaries under a stereoscopic microscope as previously described (Parborell et al., 2005; Tilly et al., 1992a). A pool of isolated follicles from each ovary was frozen and the results obtained from each pool were considered as a single datum.

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-phenyl–ethylchloromethyl ketone) and phosphatase inhibitors (25 mM sodium fluoride, 0.2 mM sodium orthovanadate and 10 mM glycerophosphate) and homogenized with an Ultra-Turrax (IKA Werk, Breisgau) homogenizer. Samples were centrifuged at 4 °C for 10 min at 10,000g 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 12% 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 1:500, caspase 3

1:200, AKT 1:5000 and pAKT 1:400, BAD 1:200 and pBAD 1:400, actin B 1:4000) in blocking buffer overnight at 4 °C. The blot was then incubated with anti-rabbit or anti-mouse 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 the actin B band.

2.4. Immunohistochemistry

Ovaries from both the control and the ANGPT1 Ab group were immediately fixed in 4% neutral buffered formalin for 12 h and then embedded in paraffin. Five-micron step sections were mounted at 50-μm intervals onto microscope slides to prevent counting the same follicle twice, according to Woodruff et al. (1990). Tissue sections were deparaffinized in xylene and rehydrated by graduated ethanol washes. Endogenous peroxidase activity was blocked with hydrogen peroxide in PBS solution and nonspecific binding was blocked with 2% BSA overnight at 4 °C. Sections were incubated with rabbit polyclonal anti-PCNA (1:100) or mouse monoclonal anti-alpha smooth muscle actin (1:100) overnight at 4 °C. After washing, the slides were incubated with biotinylated anti-rabbit or anti-mouse IgG and after 30 min with avidin–biotinylated horseradish peroxidase Complex (Vectastain ABC system from Vector Laboratories, Burlingame, CA). Protein expression was visualized with diamino/benzoate staining. The reaction was stopped with distilled water, stained with hematoxylin and dehydrated before mounting with mounting medium (Canada Balsam Synthetic, Biopack, Argentina). For the negative controls, the primary antibody was omitted.

To study the percentage of periendothelial cell area, six randomly selected fields were analyzed from each ovarian section (a total of 30 sections were analyzed: six sections/ovary, five ovaries from five different rats). The percentage of periendothelial cell area was quantified using the Image J program (NIH) (Rasband, 1997–2011). A proliferation index (PCNA-positive cells expressed as a percentage of the total number of cells) was calculated in the thecal and granulosa compartments for each early antral follicle. Six antral follicles for each ovary were counted (six follicles/ovary; five ovaries/group).

2.5. Steroid assay

Steroid follicular contents were examined after acetone extraction as previously described (Irusta et al., 2003). Briefly, each ovary was homogenized in acetone. Labeled steroids were added as internal standards and the percentage of recovery was between 60% and 80%. Following centrifugation, the samples were evaporated to dryness. Distilled water was added and samples were extracted twice with diethyl ether. The phase was transferred and evaporated to dryness and the remaining residue dissolved in methanol. After adding distilled water, the samples were subjected to a solvent partition. The upper layer was discarded and the lower phase evaporated.

Samples were stored in buffer and steroid levels were measured by RIA. Progesterone and estradiol were measured using specific antibodies supplied by Dr. G.D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO). Androsterone was determined by RIA using an antibody supplied by Dr. G. Barbe (Department of Physiology, University of Western Ontario, London, Canada). Under these conditions, the intraassay and interassay variations were 8.0% and 14.2% for progesterone, 7.2% and 12.5% for estradiol and 8.1% and 14.5% for androsterone, respectively.

2.6. Follicle culture

For *in vitro* experiments, prepubertal rats were injected subcutaneously with diethylstilbestrol (DES: 1 mg/rat dissolved in corn oil) daily for 3 days to stimulate the development of early antral follicles. The animals were sacrificed 24 h after the last injection with DES. The ovaries were obtained as described in Section 2.2. Early antral follicles (~350 μm in diameter) were dissected under a stereoscopic microscope from ovaries of animals injected with DES, cleaned of adhering tissue and placed in culture medium either with or without stimulus.

For Western blot studies, 60 ovarian follicles from five animals were pooled for different treatments, and cultures were initiated within 1 h after ovary removal at 37 °C. The follicles were incubated in sextuplicate 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., 1996a; Nayudu and Osborn, 1992; Xu et al., 2006). 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 (Andreone et al., 2009; Chun et al., 1996a). In addition, several studies have used this model to examine the regulation of apoptosis in early antral follicles (Chun et al., 1996a; Kaipia et al., 1996; Li et al., 1998; Parborell et al., 2001; Vitale et al., 2002). Follicles were incubated for 16 or 24 h in serum-free medium. The incubations were performed with 5% CO_2 under different conditions: without stimulus (Basal), with ANGPT 1 (100 ng/ml), with ANGPT 2 (100 ng/ml) or with both ANGPTs.

For apoptotic studies, 10 follicles from different ovaries were pooled and cultured in the presence of the same stimuli in 100 μl final volume for 16 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 DNA isolation as previously described (Abramovich et al., 2006). In brief, the 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. Samples were incubated for 1 h with RNase (10 μg/ml) at 37 °C and DNA content was measured by reading the absorbance at 260 nm. 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).

2.7. Data analysis

Data are expressed as the mean ± SEM of four or five experiments. Quantitative results obtained by densitometric analysis of the low molecular weight DNA fragments represent the mean ± SEM of three independent gel runs. For quantification of active caspase 3, AKT phosphorylation and PCNA protein levels, three

independent measurements were performed within each group. Representative gels are shown in the figures. Statistical analysis was performed using paired or unpaired Student's *t*-test or one-way ANOVA followed by either Tukey's or Bonferroni's multiple comparison test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. In vivo ANGPT1 inhibition reduces follicular cell proliferation

The effect of in vivo ANGPT1 inhibition on follicular cell proliferation was studied by examining the expression of the proliferating cell nuclear antigen (PCNA), which is expressed in the G1 and S phases of the cell cycle (Maga and Hubscher, 2003).

PCNA levels from ovarian follicles measured by Western blot showed a significant decrease after 48 h of ANGPT1 Ab treatment (28%; $p < 0.05$; $n = 5$) (Fig. 1A).

To further analyze the cellular type affected by ANGPT1 inhibition, we performed immunohistochemistry on ovarian sections from control and treated ovaries (Fig. 1B). ANGPT1 Ab treatment showed a reduced frequency of cell proliferation in the thecal (Tc) and granulosa (Gc) layer compared to the control group. The proliferation index (PCNA-positive cells expressed as a percentage of the total number of cells) was calculated in the thecal and granulosa compartments. The percentage of Tc and Gc expressing PCNA protein significantly decreased after ANGPT1 Ab treatment (Tc: 31%, Gc: 33%, $n = 5$, $p < 0.01$, respectively) (Fig. 1C).

3.2. In vivo ANGPT1 inhibition increases caspase 3 cleavage

In a previous study, we demonstrated that ANGPT1 inhibition by the intrabursal administration of a neutralizing antibody leads a large number of follicles to atresia (Parborell et al., 2008). To analyze whether caspase 3 is involved in this effect, we performed a Western blot using an antibody that recognizes procaspase 3 and the active fragment of caspase 3, p17. As shown in Fig. 2, ANGPT1 inhibition increased the levels of p17 when compared to control (30%, $n = 5$; $p < 0.05$). It is worth noting that the procaspasa 3 is first cleaved to produce the p11 subunit and the 20-kDa (p20) peptide and subsequently, the p20 peptide is cleaved to generate the mature p17 subunit (active form) (Yacobi et al. 2004). Accordingly this p20 kDa fragment was also observed in Fig. 2.

3.3. In vivo inhibition of ANGPT1 decreases phosphorylation of AKT

To determine whether ANGPT1 is involved in the phosphorylation of the AKT protein, we performed a Western blot of the phosphorylated form of AKT (pAKT) in ovarian follicles at 4 or 8 h after ANGPT1 inhibition. Administration of the neutralizing

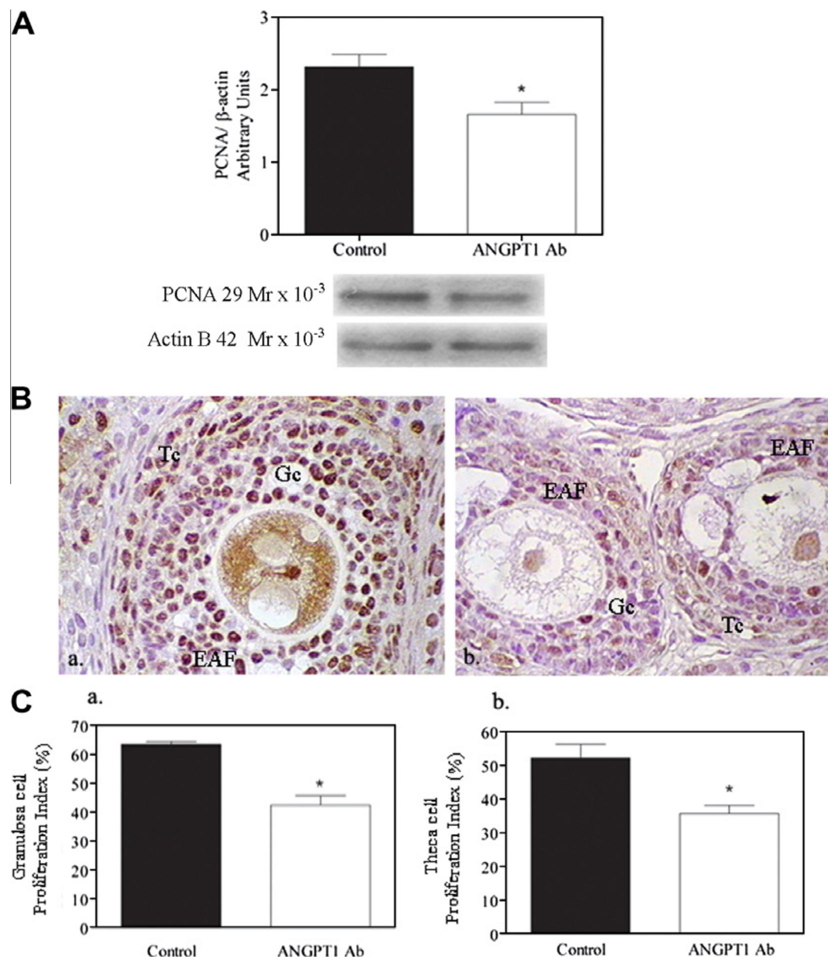

Fig. 1. Effect of ANGPT1 antibody treatment on PCNA levels in follicular cells. (A) Upper panel: Densitometric quantification of PCNA in control and ANGPT1 Ab-treated ovaries 48 h after injection ($n = 8$). Lower panel: Representative immunoblots of PCNA content in antral follicles from control and ANGPT1 Ab-treated ovaries. $^*p < 0.05$; Paired Student's *t*-test. (B) Representative fields of ovarian sections immunostained for PCNA (400×) (a) Control ovary; (b) ANGPT1 Ab-treated ovary. EAF: early antral follicle; Gc: granulosa cell; Tc: theca cell. (C) PCNA expression analysis. (a) Granulosa cells; (b) theca cells. The proliferation index was determined by counting labeled cells at 400× in randomly selected fields of antral follicles.

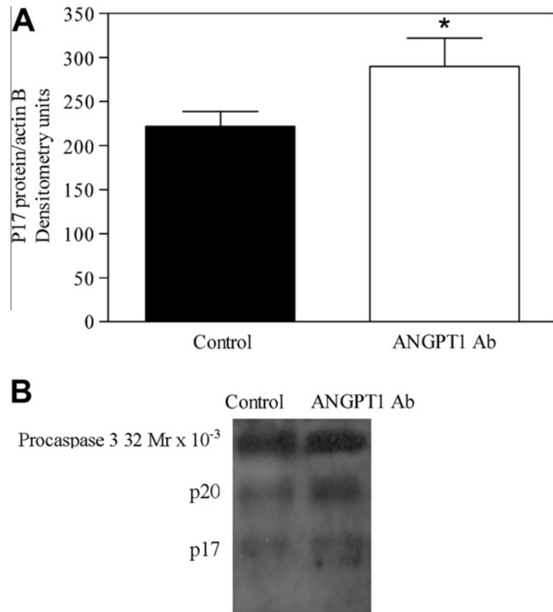

Fig. 2. Effect of ANGPT1 Ab-treatment on cleavage of caspase 3 protein in antral follicles. (A) Densitometric quantification of the caspase 3 active fragment p17 follicular content ($n = 8$; $^{*}p < 0.05$; Paired Studenťs t-Test). (B) Representative immunoblot of caspase 3 protein content in antral follicles from control and ANGPT1 Ab-treated ovaries.

Ab significantly decreased AKT phosphorylation 8 h after surgery (23%, $p < 0.05$, $n = 8$) (Fig. 3B). No significant differences were observed between groups at 4 h after surgery (Fig. 3A).

To establish a relationship between the antiapoptotic effect observed after ANGPT1 inhibition and the PI3K/AKT intracellular pathway, we examined phosphorylation of BAD protein, a pivotal downstream target of pAKT. No significant differences were observed in pBAD protein between groups at the times analyzed (data not shown).

3.4. In vivo inhibition of ANGPT1 decreases vascular stability

To determine the effect of ANGPT1 inhibition on follicular vessel stability, blood vessels were immunostained with smooth muscle cell actin (SMCA) antibody, a periendothelial cell marker, and the percentage of immunostained area was quantified. Fig. 4A shows representative fields of ovarian sections stained to SMCA. The positive area for SMCA was significantly decreased after 48 h of ANGPT1 inhibition compared to the control ovary (Fig 4B) (30%, $p < 0.05$, $n = 5$).

3.5. ANGPT1 regulates steroid content in the ovary

To further analyze the effect of ANGPT1 in the ovary, we evaluated the content of steroids after ANGPT1 inhibition. To this end, we extracted steroids from control and 48 h-ANGPT1 Ab-treated ovaries and measured them by RIA. In vivo inhibition of ANGPT1 decreased estradiol content (control: 0.40 ± 0.09 ng/mg ovary; ANGPT1 Ab: 0.26 ± 0.06 ng/mg ovary; $n = 7$; $p < 0.05$), increased androsterone content (control: 2.02 ± 0.39 ng/mg ovary; ANGPT1 Ab: 3.08 ± 0.54 ng/mg ovary; $n = 7$; $p < 0.05$), and showed no effects on progesterone content (control: 0.16 ± 0.39 ng/mg ovary; ANGPT1 Ab: 0.18 ± 0.04 ng/mg ovary; $n = 7$; $p > 0.05$).

3.6. ANGPT1 and ANGPT2 regulate proliferation and apoptosis in follicles in culture

To analyze a possible direct effect of ANGPTs on follicular cells, we isolated and cultured early antral follicles from prepubertal rats treated with DES. Stimulation of follicles during 24 h with ANGPT1 caused an increase in the levels of PCNA protein compared to basal conditions (47%, $n = 8$, $p < 0.01$). ANGPT2 alone had no effect on PCNA levels; however, when follicles were cultured in the presence of both ANGPTs, ANGPT2 reversed ANGPT1 stimulation of proliferation (26%, $n = 8$, $p < 0.05$) (Fig. 5A).

In addition, we evaluated the direct effect of ANGPTs on apoptosis in follicles in culture. As shown in Fig. 5B, follicles cultured in serum-free medium exhibited a typical apoptotic DNA fragmentation pattern (basal) FSH is a cytoprotective agent for follicular cells. Interestingly, 16 h of ANGPT1 incubation mimicked this FSH antiapoptotic effect on follicles in culture ($n = 4$, $p < 0.05$). ANGPT2 had no effect on follicular cell apoptosis compared to basal conditions, but was able to reverse the cytoprotective effect of ANGPT1 when follicles were cultured in the presence of both ANGPTs (Fig. 5B).

4. Discussion

This study is the first to demonstrate that the inhibition of ANGPT1 using a neutralizing antibody locally injected under the bursa of the ovary inhibits cell proliferation, induces apoptosis and affects steroidogenesis of follicular cells from antral follicles in eCG-treated rats. In addition, we showed that AKT phosphorylation may be involved in ANGPT1 action in the ovary.

In this report, we demonstrated that ANGPT1 inhibition by neutralizing antibody causes a decrease in follicular cell proliferation in both theca and granulosa compartments. In this regard, unlike VEGFA, the ANGPTs-receptor system does not promote proliferation in endothelial cells. However, this is the first study to demon-

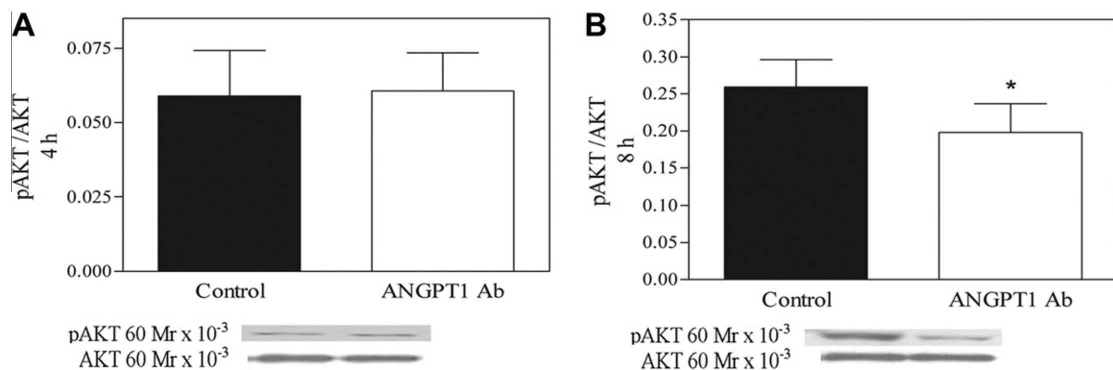

Fig. 3. Effect of ANGPT1 Ab treatment on phosphorylaion of AKT protein in antral follicles. Upper panels: Densitometric quantification of pAKT/AKT in control and ANGPT1 Ab-treated ovaries 4 h (A) and 8 h (B) after injection ($n = 8$; $^{*}p < 0.05$; Paired Student's t-test). Lower panels: Representative immunoblots of AKT and pAKT content in antral follicles from control and ANGPT1 Ab-treated ovaries.

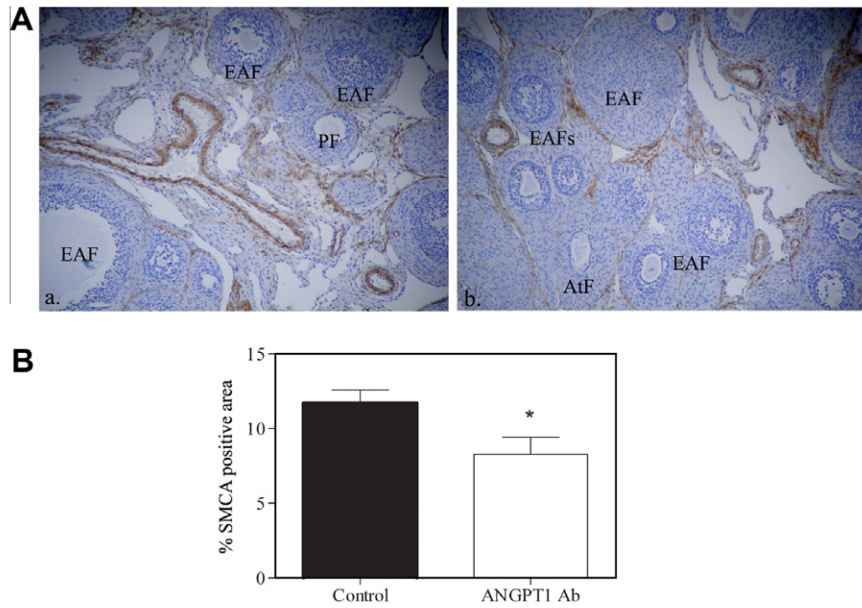

Fig. 4. Effect of ANGPT1 Ab treatment on blood vessel stability in antral follicles. (A) Representative fields of ovarian sections immunostained for SMCA (100×). (a) Control ovary; (b) ANGPT1 Ab-treated ovary. EAF: early antral follicle; AtF: atretic follicle. PF: preantral follicle. (B) SMCA expression was determined by counting labeled areas at 400× in randomly selected fields of antral follicles.

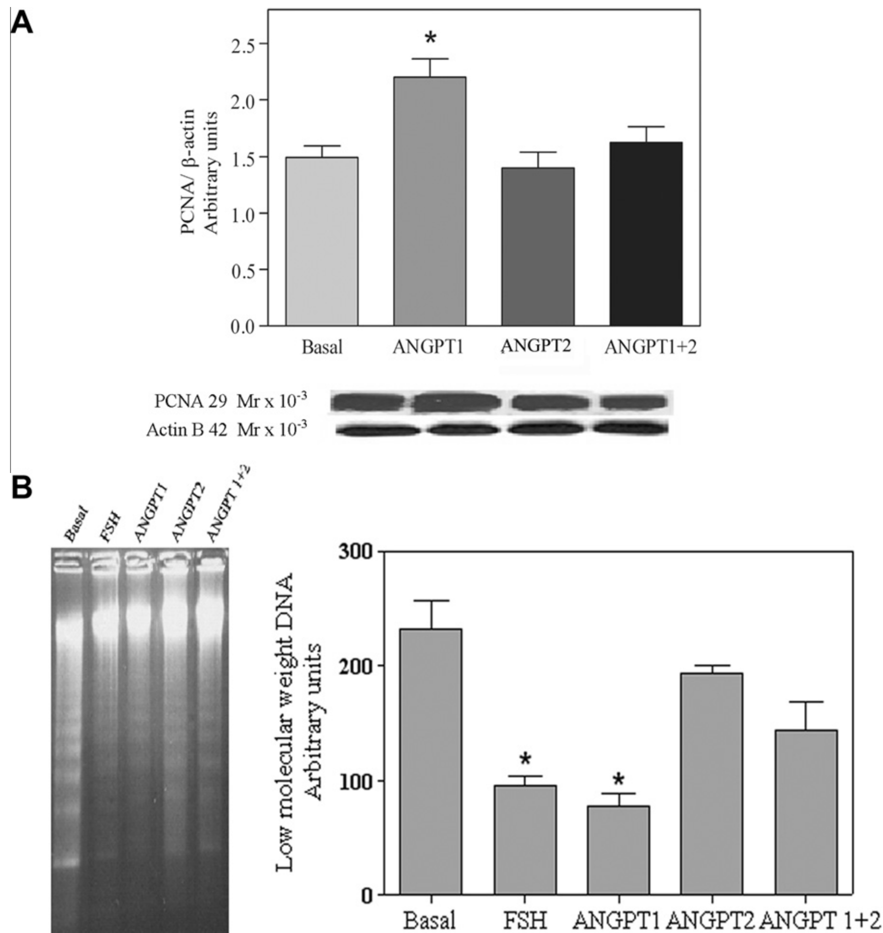

Fig. 5. Direct effect of ANGPT1 on apoptosis and PCNA levels in antral follicles in culture. (A) Upper panel: Densitometric quantification of PCNA in antral follicles in culture ($^{*}p < 0.05$, one-way ANOVA followed by Tukey's multiple comparison test). Lower panel: Representative immunoblots of PCNA content in antral follicles in culture. (B) Left panel: agarose gel showing DNA fragmentation. Right panel: Quantitative estimation of DNA cleavage. Low-molecular-weight DNA (<15 kb) from the gel was examined to calculate apoptotic DNA fragmentation ($^{*}p < 0.05$, one-way ANOVA followed by Tukey's multiple comparison test).

strate that ANGPT1 is necessary for proliferation of granulosa and theca cells in antral follicles from eCG-treated rats.

ANGPT1, together with the TEK receptor, promotes endothelial cell survival during vascular development, stimulates branching as well as maturation of embryonic blood vessels, and maintains vascular integrity in mature animals (Harfouche et al., 2002; Sato et al., 1995; Suri et al., 1996). Also, the ANGPT1/TEK system plays an important role as an antiapoptotic system to maintain a dedifferentiated phenotype of other cell types, such as neurons and hematopoietic cells (Arai et al., 2004; Valable et al., 2003). It has been well demonstrated that in order to complete the apoptotic process, ovarian cells must express and activate caspase-3 (Boone and Tsang, 1998). Previously, we have shown by terminal dUTP nick end labeling (TUNEL) studies that ANGPT1 neutralizing antibody treatment causes an increase in the number of apoptotic granulosa cells in antral follicles and in the spontaneous DNA fragmentation of antral follicles cultured in serum-free medium (Parborell et al., 2008). In the present study, we evaluated the effect of ANGPT1 inhibition in the activation of caspase 3, the main effector caspase. We specifically analyzed the levels of p17, the active fragment of this proapoptotic protein. ANGPT1 antibody treatment increased p17 levels, thus suggesting that caspase-3 is involved in the effect of ANGPT1 in follicular cell apoptosis. These results, together with our previous studies (Abramovich et al., 2006; Parborell et al., 2008), indicate that angiogenic factors have an important role in follicular selection, dominance and atresia mediated by apoptosis.

Our data show that inhibition of ANGPT1 action results in an increase in follicular androsterone levels and a decrease in estradiol. These findings suggest that intraovarian antibody against ANGPT1 interferes with the rat follicular development mediated in part by an imbalance in the ratio of androgen/estrogen steroids since it has been described that an androgenic environment induces follicular atresia (Billig et al., 1994). It is worth mentioning that estradiol synthesis occurs in granulosa cells in follicles, but we have shown that the ANGPT receptor, TEK, is detected only in theca cells from rat follicles by immunohistochemistry technique (Abramovich et al., 2009). These results indicate that ANGPT1 exerts this effect in a paracrine manner on granulosa cells mediated by the TEK receptor in theca cells. ANGPT1 would act on its receptor TEK in theca cells, producing the release of an unknown factor. Consequently, this factor would operate in a paracrine way in the adjacent granulosa cells through a mechanism not yet explained, thus decreasing apoptosis and increasing cell proliferation and estradiol levels. In addition, these results are consistent with previous data observed by Hayashi et al., who showed that ANGPT1 increases the secretion of estradiol from bovine preovulatory follicles *in vitro* using a microdialysis system implanted in the theca layer (Hayashi et al., 2003).

Several studies have reported that ANGPT1 inhibits endothelial cell apoptosis through several pathways, including PI3K/AKT activation and upregulation of survivin protein (Harfouche et al., 2002; Papapetropoulos et al., 2000). Several researchers have focused on the role of this pathway in explaining the antiapoptotic effects of ANGPT1 (Fujikawa et al., 1999; Kim et al., 2000; Papapetropoulos et al., 2000). It is well known that PI3K/AKT is a pathway involved in cell survival and proliferation (Testa and Tsichlis, 2005). We demonstrated that inhibition of ANGPT1 significantly decreased the phosphorylation of AKT at Ser^{473} at 8 h after intrabursa injection of ANGPT1 antibody. These results suggest that ANGPT1 may exert its survival role and promote proliferation in ovarian follicular cells involving this pathway. One of AKT mechanisms to promote cell survival is the inhibition of the proapoptotic member of the BCL2 family, BAD. This protein binds to the prosurvival molecule $BCL2L1_L$, also known as $BCLX_L$, and inhibits its function. However, we did not find significant differences in the phosphorylation of BAD (Ser^{136}) either at 4 or 8 h after surgery. We cannot discard phosphorylation of BAD at different time points not evaluated in the present work. In our laboratory, further studies are currently under way to elucidate this mechanism.

Angiopoietins act on vascular endothelial cells via a tyrosine kinase receptor, TEK, and contribute to blood vessel stabilization not only through an interaction with perivascular cells (pericytes) but also through an interaction between endothelial cells (Papapetropoulos et al., 1999). Previously, we and other authors have shown the expression of angiopoietins and its receptor TEK in the follicle and corpus luteum in rats, cows, monkeys and humans, suggesting the involvement of these factors in the regulation of follicle and corpus luteum function via stabilization of blood vessels in these compartments (Abramovich et al., 2009; Hazzard et al., 2000; Tanaka et al., 2004; Wulff et al., 2000). In this work, we demonstrated that the intrabursal administration of ANGPT1 antibody decreases the stabilization of blood vessels, measured as the percentage of periendothelial cell area, in the ovaries from eCG-treated rats. However, we cannot discard a direct effect of ANGPT1 on follicular cells.

A variety of culture systems have been used in rodent species to explore the autocrine/paracrine effects of different factors on ovarian follicular physiology (Cortvrindt et al., 1996b; Eppig and Wigglesworth, 1995; Nayudu and Osborn, 1992; Xu et al., 2006). Early antral is the stage when follicles become most susceptible to atresia and FSH is the main survival factor (Chun et al., 1996a). We and other authors have used this model to examine the regulation of apoptosis in EAFs (Chun et al., 1996a; Kaipia et al., 1996; Li et al., 1998; Parborell et al., 2001; Vitale et al., 2002). This experimental model allows the study of the endocrine follicular activity in a system that maintains the structure of the follicle and the interaction between the different follicular cell types. In addition, in a recent study we have demonstrated that EAFs obtained from DES-treated rats are physiologically similar to EAFs from cycling rats (Abramovich et al., 2009). The proliferative and apoptotic capacity in response to exogenous angiopoietins in isolated EAFs in culture has not yet been fully examined. So, we here demonstrate for the first time that ANGPT1 decreases the spontaneous apoptotic DNA fragmentation in EAF culture. Moreover, this effect was reversed when follicles were incubated with both ANGPTs (ANGPT1 and ANGPT2), suggesting an antagonic effect of ANGPT2 on ANGPT1 action. In addition, we showed that, in EAF culture, ANGPT1 increases the protein levels of PCNA and that that effect was also reversed when follicles were incubated with both ANGPTs. All these *in vitro* results are consistent with the results obtained in our in vivo studies using an ANGPT1 neutralizing antibody.

5. Conclusion

In conclusion, we demonstrated that ANGPT1 alters steroidogenesis, reduces ovarian apoptosis and stimulates cell proliferation in antral follicles from eCG-treated rats. ANGPT1 may exert these proliferative and anti-apoptotic roles by regulating ovarian vascular stability and/or by a direct effect on follicular cells. In addition, we showed that the PI3K/AKT pathway is involved in these ANGPT1 local effects.

Grant support

This study was supported by UBA (EX237), ANPCyT (PICT 5-26047), CONICET (PIP5471) and Roemmers Foundation.

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