Regulation of Ovarian Angiogenesis and Apoptosis by GnRH-I Analogs

FERNANDA PARBORELL,[1] GRISELDA IRUSTA,[1]
ALEJANDRA RODRÍGUEZ CELÍN,[1] AND MARTA TESONE[1,2]*

[1]*Instituto de Biología y Medicina Experimental (IBYME)-CONICET, Departamento de Fisiología, Universidad de Buenos Aires, Buenos Aires, Argentina*
[2]*Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*

ABSTRACT An adequate vascular supply is important to provide endocrine and paracrine signals during follicular development. We evaluated the direct in vivo effects of both the GnRH-agonist Leuprolide acetate (LA) and the GnRH-antagonist Antide (Ant) on the expression of VEGF-A and ANPT-1 and their receptors in ovarian follicles from prepubertal eCG-treated rats. We also examined whether the changes observed in apoptosis by GnRH-I analogs have an effect on the caspase cascade. LA significantly decreased the levels of VEGF-A, its receptor Flk-1, and ANPT-1 when compared to controls, while the co-injection of Ant interfered with this effect. No changes were observed in the levels of Tie-2 after treatment with these analogs. When we measured the follicular content of caspase-3 protein, we observed that LA significantly increased the level of the active form. The co-injection of Ant interfered with this effect and Ant alone significantly decreased caspase-3 cleavage. IHC analyses corroborated these data. Notably, while LA increased caspase-3 activity levels, Ant decreased them when compared to controls. In follicles obtained from LA-treated rats, cleavage of PARP (a substrate of caspase-3) from the intact 113-kDa protein showed a significant enhancement in an 85-kDa fragment. The co-injection of Ant interfered with this effect. Ant alone significantly decreased PARP cleavage as compared to controls. We conclude that the decrease in VEGF-A, its receptor Flk-1/KDR, and ANPT-1 produced by the administration of GnRH-I agonist is one of the mechanisms involved in ovarian cell apoptosis. This suggests an intraovarian role of an endogenous GnRH-like peptide in gonadotropin-induced follicular development. *Mol. Reprod. Dev.*

Key Words: GnRH-I analogs; angiogenesis; apoptosis; ovary

INTRODUCTION

Chronic administration of GnRH-I agonists (GnRH-a) leads to pituitary desensitization, inhibition of gonadotropin and sex steroid production. On the other hand, GnRH-I antagonists (GnRH-ant) produce an immediate and complete suppression of gonadotropins by the competitive blockade of the pituitary GnRH receptors. Besides these effects on the pituitary–gonadal axis, GnRH-I analogs have the potential to modulate the ovarian function through a direct effect on the ovary. Numerous studies have documented a functional ovarian GnRH-I and GnRH receptor system in both the rat and human ovary (Kang et al., 2001). In addition, GnRH-ant have also been demonstrated to directly modulate the ovarian function in the rat (Yano et al., 1997). We have previously demonstrated in prepubertal rats that GnRH-a treatment produces an increase in ovarian follicle DNA fragmentation by interfering with the FSH, cAMP, and/or growth factors pathways (Andreu et al., 1998; Parborell et al., 2001). Moreover, several studies performed in rats have demonstrated the antigonadal effect of GnRH analogs administered either in vivo or in vitro (Hazum and Nimrod, 1982; Muttukrishna et al., 1996; Sridaran et al., 1999a; Yang et al., 2003). We have previously shown an apoptotic follicular effect of GnRH-a in antral follicles (AF), which correlates with an imbalance in the ratio of anti-apoptotic:proapoptotic proteins (Bcl-xL/Bcl-xS) (Parborell et al., 2002). In contrast, a GnRH-I antagonist, Antide (Ant), inhibits apoptosis in AF through a decrease in BAX translocation from the cytosol to the mitochondria and cytochrome C retention (Parborell et al., 2005). These results were obtained in eCG treated prepuberal rats, treatment able to induce multiple and synchronized follicular growth and considered as a valuable model to study the effect of several factors on gonadotropin stimulated follicular development (Tilly et al., 1992a, 1995).

It is accepted that programmed cell death is the consequence of an activation of caspase cascades (Green, 1998; Danial and Korsmeyer, 2004; Kim et al., 2006)

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*Correspondence to: Marta Tesone, Instituto de Biología y Medicina Experimental, Obligado 2490, 1428 Buenos Aires, Argentina.
E-mail: mtesone@dna.uba.ar

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being caspase-3 the principal downstream effector enzyme (Shi, 2004; Fan et al., 2005; Lavrik et al., 2005). A limited number of substrates are cleaved by activated caspase-3 during apoptosis. Among them, PARP (Boone and Tsang, 1998) and actin (Kayalar et al., 1996) are inhibitors of DNase I, which is the endonuclease responsible for ovarian apoptotic DNA fragmentation (Nelipovich et al., 1988; Widlak et al., 2000).

In ovaries there is intense angiogenesis and increased permeability of blood vessels during follicular development, ovulation, and subsequent formation of the corpus luteum. The formation of a dense capillary network in the ovary enables the hormone-producing cells to obtain the oxygen, nutrients, and precursors necessary to synthesize and release different hormones essential for the maintenance of the ovarian function. Nongrowing primordial follicles and preantral follicles (PF) do not present a vascular supply of their own, and thus rely on vessels in the surrounding stroma (Stouffer et al., 2001). However, as the antrum develops in the follicle, the thecal layer acquires a vascular sheath consisting of two capillary networks located in the theca interna and externa, respectively. The acquisition of an adequate vascular supply is possibly a rate-limiting step in the selection and maturation of the dominant follicle destined to ovulate. On the other hand, follicular atresia is associated with inadequate development and/or regression of the thecal vasculature in most species studied (Suzuki et al., 1998; Wulff et al., 2001). Both the ovarian follicle and the corpus luteum have been shown to produce several angiogenic factors such as vascular endothelial growth factor A (VEGF-A) and angiopoietins (ANPT).

VEGF-A exerts its cellular effects through interaction with its tyrosine kinase receptors (RTKs), Flt-1 (VEGF-R1) and Flk-1/KDR (VEGF-R2), which bind the ligand with high affinity (de Vries et al., 1992). Flk-1/KDR is the principal mediator of the angiogenic effects of VEGF, and its importance is highlighted by the failure of Flk-1 null mice to develop organized blood vessels, resulting in lethality between embryonic Day 8.5 and 9.5 (Shalaby et al., 1995). The role of Flt-1 is less apparent. Reports have indicated that Flt-1 has limited signaling activity and may act as a decoy or scavenger receptor (Yang et al., 2002). VEGFR-3 (Flt-4) is a member of the same family of RTKs, and although it is not a receptor for VEGF-A, it binds VEGF-C and VEGF-D (Pajusola et al., 1992).

Expression of VEGF-A in ovarian follicles depends on follicular size. In bovine and porcine follicles, VEGF-A is weakly expressed during early ovarian follicular development and becomes more pronounced in granulosa and theca cells along with dominant follicle development (Barboni et al., 2000; Greenaway et al., 2004). Similar results have been found in the rat ovary, where, in addition, some secondary follicles show extremely strong VEGF-A immunoreactivity in the zona pellucida (Celik-Ozenci et al., 2003).

Endocrine gland-derived VEGF (EG-VEGF) is another pro-angiogenic factor and its expression is restricted to the ovary, testis, adrenal, and placenta and is often complementary to the expression of VEGF, suggesting that these molecules function in a coordinated manner (Ferrara, 2002).

While VEGF-A 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 the ANPT family members, which act via the RTK, Tie-2. Specifically, Ang-1 is the endogenous agonist that enhances the maturation and stability of newly formed blood vessels. Although ANPT-2 binds to Tie-2, it acts mostly as an endogenous antagonist, blocking Ang-1 mediated receptor phosphorylation (Fraser, 2006).

The effect of GnRH-I analogs on ovarian angiogenesis and their regulation is still largely unknown. By using a rat model of ovarian hyperstimulation syndrome (OHSS), Kitajima et al. (2004, 2006) have shown that GnRH-a treatment reduces ovarian vascular permeability and that this effect would be mediated by an increase in the expression of the tight junction protein, claudin-5.

We hypothesized that GnRH-I acts as an intraovarian factor through changes in the ovarian expression of VEGF and ANPT-1, and as a result, causes apoptosis in follicular cells. Consequently, in this study, we examined the effect of in vivo administration of GnRH-I-a and/or GnRH-I-ant on the expression of VEGF and ANPT-1 and their receptors in ovarian follicles from prepubertal eCG-treated rats. In addition, we attempted to elucidate whether the changes observed in apoptosis by GnRH-I-analogs have an effect on the caspase cascade. Thus, we examined the expression of the main apoptotic effector caspase-3 and the proteolytic cleavage of PARP.

MATERIALS AND METHODS

Hormones and Reagents

GnRH-I agonist (Leuprolide acetate, LA) was a donation from Abbott Laboratories (Buenos Aires, Argentina) and the original ampoule (2.8 mg/5 ml) was dissolved in saline solution. SYNTEX S.A (Buenos Aires) generously provided equine chorionic gonadotropin (eCG, Novormon). GnRH-I antagonist (Antide, Ant) was purchased from Sigma Chemical Co. (St. Louis, MO) and the original ampoule (1 mg/ml) was dissolved in saline solution. CHAPS (3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate) was purchased from GE Healthcare (Easton Turnpike Fairfield, CT). Caspase-3 colorimetric substrate (DEVD-pNA, 235400) and caspase-3 inhibitor (253420) were purchased from Biosciences, Inc., Calbiochem (San Diego, CA).

In Vivo GnRH-Analogs Treatment and Superovulation

Female Sprague–Dawley rats, 23–25 days old, were allowed food and water ad libitum, and kept at a room temperature (21–23°C) on a 12L:12D cycle. Prepubertal rats were from our own colony and their general care and

housing was carried out at the IBYME. GnRH-I analogs were diluted in saline to the desired concentrations (LA, 0.5 μg/rat; Ant, 5 μg/rat) and injected in 0.1 ml of vehicle. Animals were injected subcutaneously with 0.1 ml eCG (25 IU/rat, Control group) and then injected with LA (LA group) and/or Ant at time 0 and at 12-hr intervals for 48 hr. Control animals were injected with vehicle only. The last LA and/or Ant injection was administered 3 hr before rats were killed by CO_2 asphyxiation. The ovaries were removed and cleaned off adhering tissue in culture medium for subsequent assays.

All animals were treated and cared for in accordance with standard international animal care protocols (Canadian council of Animal care (Eds.), guide to the Care and Use of Experimental A, Ottawa, Ontario, 1984). The experimental protocols were approved by the Animal Experimentation Committee of the IBYME.

Isolation of Ovaries and Follicles

One ovary from each rat (n = 5) was used in immunohistochemistry studies. The individual ovarian follicles were dissected using fine needles from the collateral ovary under a stereoscopic microscope as previously described in our laboratory (Parborell et al., 2005). In brief, healthy AF (>400 μm in diameter) from five ovaries were pooled in triplicate and used for Western blot. Each experiment was carried out three times with five rats per group.

Western Blot Analysis

Pooled follicles obtained from the control, LA, Ant and LA + Ant groups were lysed for 20 min at 4°C in lysis buffer (20 mM Tris-Cl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) supplemented with protease inhibitors (0.5 mM phenylmethylsulphonyl fluoride (PMSF); 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone (ZPCK); 0.025 mM N′-p-tosyl-lysine chloromethyl ketone (TLCK); 0.025 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK). The lysate was centrifuged at 4°C for 10 min at 10,000*g* and the pellet was discarded. Protein concentrations in the supernatant were measured by the Bradford assay (Bio-Rad, CA). After boiling for 5 min in sample buffer (6% SDS, 15% β-mercaptoethanol, 60% glycerol, 0.006% bromophenol blue, 0.18 M Tris, pH 6.8), 40–60 μg of protein from each sample was applied to a 15% SDS–polyacrylamide gel, and electrophoresis was performed at 25 mA for 1.5 hr. The separated proteins were transferred onto nitrocellulose membranes (Amersham Biosciences, UK, England) in transfer buffer (20% methanol (vol/vol), 0.19 M glycine, 0.025 M Tris-base, pH 8.3) at 4°C for 2 hr at 80 V. Blots were blocked for 1 hr in TBS (4 mM TRIS-Cl, pH 7.5, 100 mM NaCl) containing low-fat powdered milk (2%) and Tween 20 (0.2%) at room temperature. The blot was incubated in blocking buffer overnight at 4°C with appropriate primary antibodies: VEGF in a 1/100 dilution (Rabbit polyclonal, Santa Cruz sc-507, CA), ANPT-1 in a 1/100 dilution (Goat polyclonal, Santa Cruz sc-9360, CA), Flk-1 in a 1/100 dilution (Mouse monoclonal, Santa Cruz sc-6251, CA), Tie-2 in a 1/100 dilution (Rabbit polyclonal, Santa Cruz sc-9026, CA), Caspase-3 in a 1/500 dilution (Rabbit polyclonal, Santa Cruz sc-7148, CA) and PARP in a 1/200 dilution (Rabbit polyclonal, Santa Cruz sc-7150, CA). Then, the blot was incubated with anti-rabbit or anti-goat secondary antibodies conjugated with horseradish peroxidase (1/1,000) (Sigma Chemical Co.) for 1 hr at room temperature. Finally, the membranes were incubated with Amersham chemiluminescence reagent for 1 min and exposed to X-ray film for 1 min in a dark room. Negative controls were obtained in the absence of the primary antibody.

Quantification for Western Blot Assay

In each experiment, equal amounts of protein were loaded for all samples, and all groups in one experiment were loaded on the same gel. For quantification, a screening was performed on blots with x-ray film using different times of exposure to optimize the signal. The levels of protein were compared in extracts from the different groups, and analyzed by densitometric studies. Optical density data are expressed as arbitrary units ±SEM (n = 3). The density in each band was normalized to the density of the actin B band that was used as an internal control.

Caspase-3 Activity Assay

Caspase-3 activity was measured by colorimetric assay based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrates DEVD-pNA. Follicles (50 per sample) were homogenized in lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA) containing protease inhibitors (PMSF, SIGMA). The lysates were clarified by centrifugation and the supernatants were used for the assays. Protein concentrations were determined by the Bradford assay (Bio-Rad). Enzymatic reactions were carried out in assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol) containing 50 μg of protein and 200 μM DEVD-pNA. Each sample was divided into three parts: one of them included 100 nM Ac-DEVD-CHO in addition to the extract and substrate, in order to inhibit caspase activity, and two of them included the extract and substrate without the inhibitor. The reaction mixtures were incubated at 37°C for 3 hr and absorbance was read every 10 min. Negative controls (blank, without sample) were subtracted from all samples. Following the manufacturer's suggestion, results were selected at 90 min. Baseline values of negative controls and samples with inhibitor did not increase during the 90-min interval.

Caspase-3 Immunohistochemistry (IHC)

Ovaries were fixed in 10% neutral buffered formalin for 1 week. Then, tissues were dehydrated in a series of ethanol solutions (50%, 70%, and 100%) and paraffin-embedded. Four-micrometer sections were deparaffinized with xylene and hydrated with a graded series of

ethanol. Endogenous peroxidase was then quenched with 25-min incubation in 3% H_2O_2. Sections were incubated in phosphate-buffered saline (PBS) prior to antigen retrieval in 0.01 M citrate. Sections were placed in a blocking buffer (2% BSA in PBS) for 20 min, and then incubated with the primary antibody in 1% BSA PBS buffer for 1 hr at room temperature and overnight at 4°C. Concentration caspase-3 antibody was 1:200 (Rabbit polyclonal, sc-7148, Santa Cruz, CA). According to the manufacturer, this antibody recognizes both the inactive and the active form of the caspase. Primary antibody was detected using a biotinylated anti-rabbit IgG secondary antibody (1:400; Vector Laboratories, Burlingame, CA) and the Vector ABC Kit, visualized with DAB (diaminobenzidine) with a metal enhanced substrate as described by the manufacturer (1718096, Roche, Penzberg, Germany), and counterstained with hematoxylin. As blocking peptide was not available for the antibody used, negative controls included ovarian sections incubated without the primary antibody.

Data Analysis

Data are expressed as the mean ± SEM of at least three experiments, using five animals per group. Representative gels are shown in figures. Statistical analyses were performed using one-way ANOVA followed by the Tukey test. Values of $P < 0.05$ were considered significant.

RESULTS

In Vivo Effect of GnRH-Analogs on Protein Expression of VEGF and ANPT1

To elucidate the effect of GnRH-analogs on protein expression of angiogenic factors, we measured the follicular content of VEGF and ANPT-1 protein by Western blotting from follicles isolated 48 hr after treatments (Fig. 1). In the LA group, the levels of VEGF and ANPT-1 significantly decreased when compared to the control group ($P < 0.05$). In addition, the co-injection of Ant interfered with this LA effect and Ant per se did not show any significant change.

In Vivo Effect of GnRH-Analogs on Protein Expression of Flk-1 and Tie-2

Flk-1/KDR and Tie-2 protein levels were measured by Western blot in follicles from analog-treated rats (Fig. 2A). In the LA group, AF exhibited Flk-1/KDR protein levels significantly lower than those of the control ($P < 0.05$). On the other hand, the co-injection with Ant reversed the stimulation of apoptosis by LA. Moreover, Ant per se did not show any significant change. No changes were observed in the levels of Tie-2 after treatment with analogs (Fig. 2B).

Effect of GnRH Analog Treatment on Caspase-3 Protein Content, Enzyme Activity Levels, and PARP Cleavage of Antral Follicles

In previous studies, we have demonstrated that LA causes an increase both in the percentage of apoptotic cells of growing follicles and in the spontaneous DNA fragmentation of preovulatory follicles cultured in serum-free medium. The co-injection of Ant reverses the apoptotic effect of LA, and Ant alone increases the number of preovulatory follicles as compared with that of the control group (Parborell et al., 2005).

In order to investigate whether the changes in the follicular contents of VEGF-A and ANPT-1 observed

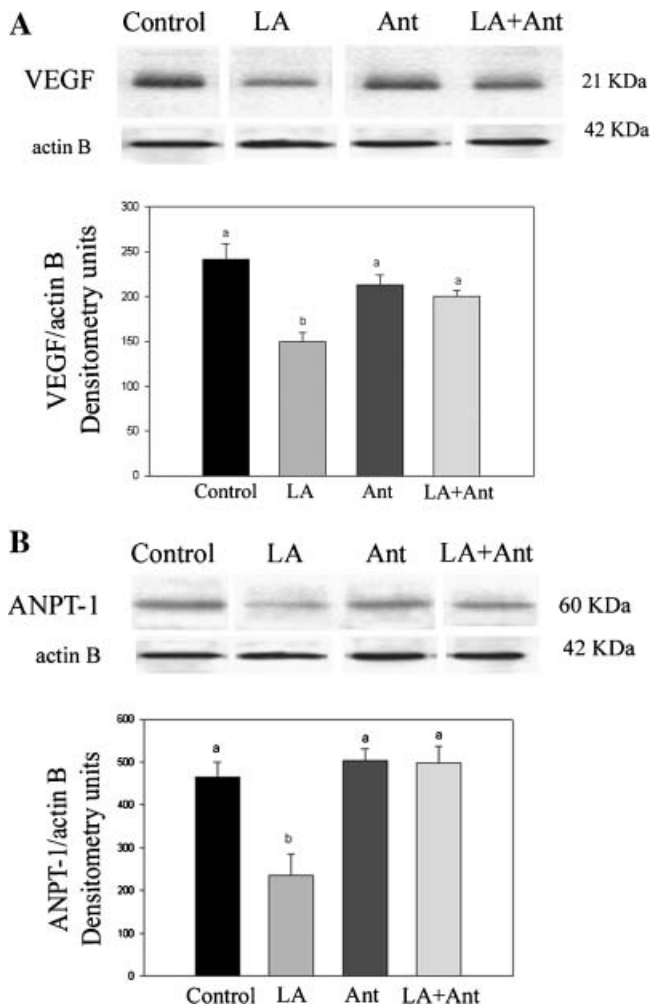

Fig. 1. In vivo effect of GnRH-I analog treatment on VEGF and ANPT-1 protein content in antral follicles. **A**: **Upper panel**, representative immunoblot of VEGF protein content in antral follicles from Control, LA-, Ant-, and LA + Ant-treated rats. **Lower panel**, densitometric quantification of VEGF content. Bars represent mean ± SEM normalized to actin B. Prepubertal rats were superovulated with eCG and were injected every 12 hr for 48 hr with LA (0.5 μg/rat) and/or Ant (5 μg/rat). Control animals were treated with vehicle alone. Antral follicles were isolated 3 hr after the last injection. After homogenization, proteins were extracted and subjected to 15% SDS–polyacrylamide gel electrophoresis (40–60 μg), and transferred onto nitrocellulose membranes. VEGF protein was visualized using a VEGF antibody. Bars with different letters are significantly different ($P < 0.01$). **B**: **Upper panel**, representative immunoblot of ANPT-1 protein content in antral follicles from Control, LA-, Ant-, and LA+Ant-treated rats. **Lower panel**, densitometric quantification of ANPT-1 content. ANPT-1 protein was visualized by using an ANPT-1 antibody. Data indicate mean ± SEM normalized to actin B of three experiments and are presented in arbitrary units. Bars with different letters are significantly different ($P < 0.05$).

In order to investigate whether the changes in the follicular contents of VEGF-A and ANPT-1 observed

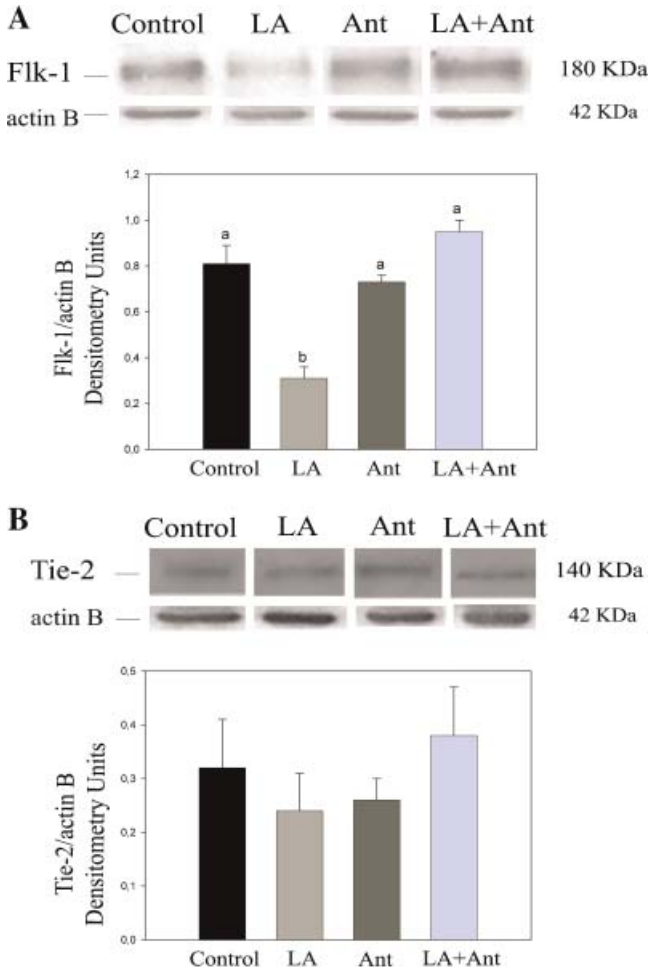

Fig. 2. In vivo effect of GnRH-I analog treatment on Flk-1 and Tie-2 protein content in antral follicles. **A**: **Upper panel**, representative immunoblot of Flk-1 protein content in antral follicles from Control, LA-, Ant-, and LA + Ant-treated rats. **Lower panel**, densitometric quantification of Flk-1 content. Flk-1 protein was visualized using an anti-Flk-1 antibody. **B**: **Upper panel**, representative immunoblot of Tie-2 protein content in antral follicles from control, LA-, Ant-, and LA + Ant-treated rats. **Lower panel**, densitometric quantification of Tie-2 content. Tie-2 protein was visualized using an anti-Tie-2 antibody. Data indicate mean ± SEM normalized to actin B of three experiments and are presented in arbitrary units. Bars with different letters are significantly different ($P < 0.01$). See details in Figure 1. [See color version online at www.interscience.wiley.com.]

after GnRH-I analogs treatment is associated to changes in the caspase cascade, we measured the follicular content of caspase-3 protein by Western blotting (Fig. 3A). The LA group showed a significant increase in the level of the active form (17 kDa) when compared to the control group ($P < 0.05$). The co-injection of Ant interfered with this LA effect and Ant alone significantly decreased caspase-3 cleavage ($P < 0.05$). None of the treatments changed the procaspase-3 levels (33 kDa). It is worth noting that the procaspase-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 20 kDa fragment was also observed in Figure 3A.

To determine whether the treatment with GnRH-I analogs has an effect on caspase-3 activity, we measured the cleavage of DEVD-pNA, a colorimetric substrate specific for caspase-3, and we also analyzed the cleavage of PARP by Western blot (Rosen and Casciola-Rosen, 1997; Nicholson, 1999). As shown in Figure 3B, caspase-3 activity levels in the LA group were 1.8-fold greater ($P < 0.05$) compared to those in the control group. The co-injection of Ant interfered with this LA effect and moreover, activity levels for caspase-3 in the Ant group were 0.5-fold lower than those in the control group. The activity measured in the follicle lysates was specific for caspase-3 because the addition of the caspase-3 inhibitor DEVD-CHO completely abolished this activity (data not shown). When analyzing the cleavage of PARP (Fig. 3C), we observed that LA produced a significant enhancement in the cleaved PARP fragment as compared to the control group ($P < 0.05$). Conversely, Ant alone significantly decreased PARP cleavage as compared to the control group and, once again, the co-injection of Ant reversed the apoptotic effect of LA ($P < 0.05$). Of note is that each of the different treatments did not seem to significantly change the level of uncleaved PARP.

Caspase-3 Immunochemistry

To determine which ovarian cell type was involved in caspase-3, we examined the localization of this caspase in ovarian sections from GnRH analogs-treated rats. IHC analyses showed that theca cells from PF exhibited a moderate signal for caspase-3 only in both the control and LA groups (Fig. 4A–C; Table 1). In contrast, theca cells from AF showed immunoreactivity in all groups, being more intense in the LA group (Fig. 4A–H; Table 1). Furthermore, the staining was more evident in the nuclei of the theca cells from the LA group (Fig. 4C,D). In the Ant group, immunostaining for caspase-3 was weak in the theca cells from the AF (Fig. 4E,F; Table 1). On the other hand, granulosa cells of PF and AF exhibited either a weak or an absent caspase-3 immunostaining in all treatments (Fig. 4A–H; Table 1).

DISCUSSION

The present study is the first to evaluate the expression of angiogenic factors, VEGF-A and ANPT-1, and the main apoptotic effector caspase-3 in AF from GnRH-I analog-prepubertal-eCG-treated-rats. GnRH-I agonist (LA) treatment resulted in statistically significant reductions in VEGF-A, its receptor Flk-1/KDR and ANPT-1 expression in AF from these animals. Moreover, a GnRH-I antagonist (Ant) reversed the action of the agonist, supporting the hypothesis that LA local effects would be exerted through the action of this agonist on ovarian GnRH receptors. Thus, our results suggest that LA would affect the degree of vascularity of AF, resulting in a decreased delivery of hormones, growth factors and other nutrients to these kinds of follicles. Accordingly, there is a close relationship between the status of the follicular blood vessel network and follicular function. Specific inhibition of the VEGF pathway in vivo, with resultant effects on the follicular function,

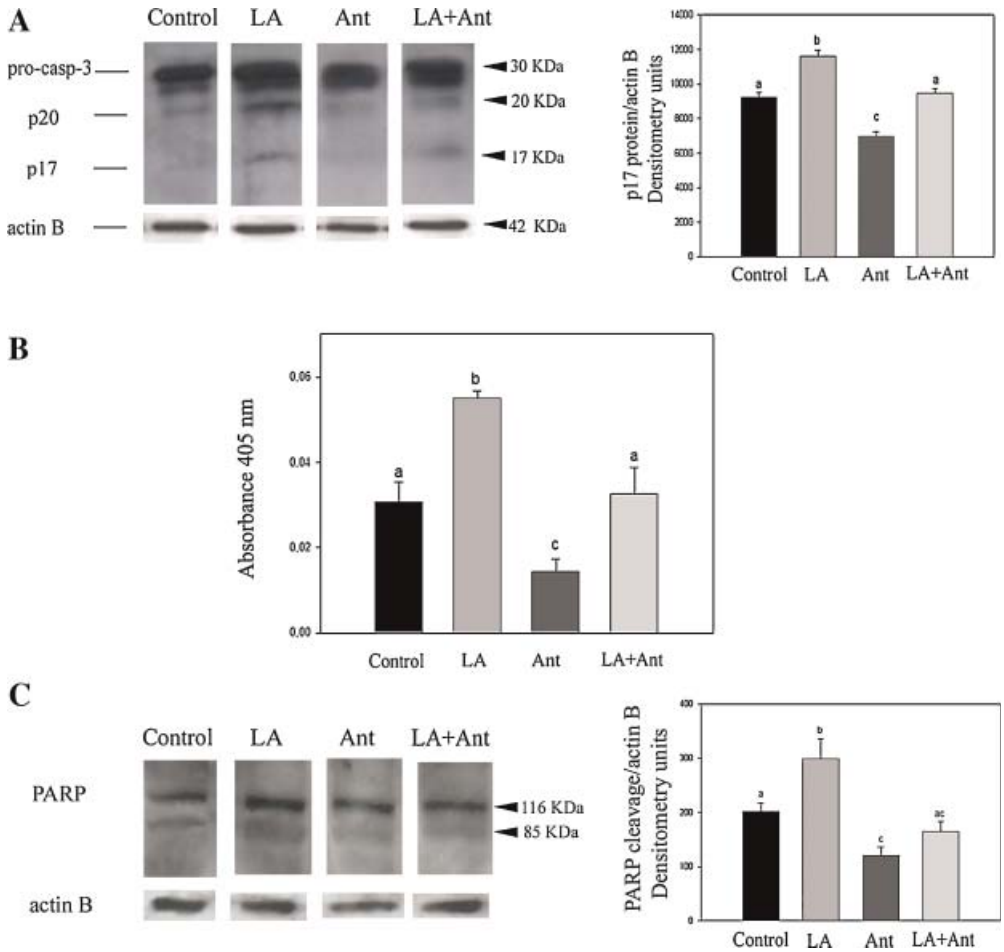

Fig. 3. Effect of GnRH analog treatment on caspase-3 protein content, enzyme activity levels and PARP cleavage of antral follicles from stimulated rats. **A**: **Upper panel**, representative immunoblot of caspase-3 protein content in antral follicles from Control, LA-, Ant-, and LA + Ant-treated rats. **Lower panel**, densitometric quantification of caspase-3 content. Caspase-3 protein was visualized using an anti-caspase-3 antibody. Data indicate mean ± SEM normalized to actin B of three experiments and are presented in arbitrary units. Bars with different letters are significantly different ($P < 0.05$). **B**: Caspase-3 activity was assessed using the peptide colorimetric substrate DEVD-pNA. Bars with different letters represent a significant difference ($P < 0.05$) in the activity between groups. **C**: **Upper panel**, representative immunoblot of PARP cleavage in antral follicles from control, LA-, Ant-, and LA+Ant-treated rats. Data indicate mean ± SEM normalized to actin B of three experiments and are presented in arbitrary units. Bars with different letters are significantly different ($P < 0.05$). **Lower panel**, densitometric quantification of PARP cleavage. PARP cleavage was visualized by using an anti-PARP antibody. See details in Figure 1. [See color version online at www.interscience.wiley.com.]

has been achieved using different approaches (Hazzard et al., 2000; Zimmermann et al., 2001; Ferrara, 2002; Mattot et al., 2002). More recently, we have demonstrated that the local inhibition of VEGF-A activity appears to produce an increase in ovarian apoptosis through an imbalance among the BCL2 family members, thus leading to a larger number of follicles to atresia (Abramovich et al., 2006).

Previously, we have demonstrated that LA produces follicular apoptosis, which correlates with an imbalance in the ratio of antiapoptotic:proapoptotic proteins (BCL-X_L/BCL-X_S) (Parborell et al., 2002). Moreover, we have shown that Ant per se inhibits BAX translocation from the cytosol to mitochondria and retains cytochrome C in the mitochondria, while LA induces cytochrome C release (Parborell et al., 2005). In order to study whether the decrease in VEGF and ANPT-1 expression in LA-treated rats is correlated with apoptotic follicular changes, we measured the follicular content of caspase-3 protein by Western blot. Caspase-3, which plays a central role in the execution of the apoptotic program in cells, exists as a proenzyme that is cleaved and activated in response to a variety of apoptotic stimuli (Nicholson, 1999). Here, we showed that LA treatment increased the active form of caspase-3 (17 kDa) and the intensity of this protein in theca cells of AF when compared to the control group. Interestingly, this staining was localized in the nucleus of this type of cells, in agreement with the translocation of cleaved caspase-3 once the apoptosis process is achieved. The localization of caspase-3 in IHC experiments does not distinguish between the proenzyme and activated fragments of the enzyme. However, Flaws et al. (1995) were able to prevent apoptosis in ovarian cells using antibodies of caspase-3, suggesting that this enzyme is activated during follicular cell apoptosis. In the present study, theca cells showed very

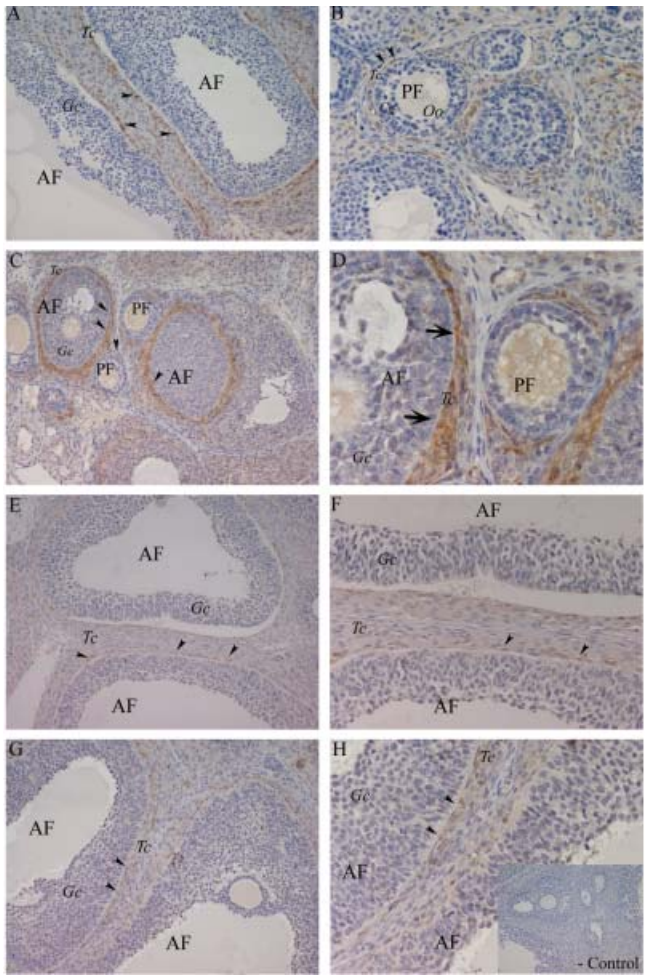

Fig. 4. Immunohistochemical localization of caspase-3 in follicles of different stages of development in stimulated rats treated with either vehicle (control) or GnRH analogs (LA and Ant) for 2 days. Positive immunostaining (brown) was mainly observed in theca cells (*Tc*) of PF and AF (arrowhead) in control group (**A**,**B**), LA group (**C**,**D**), Ant group (**E**,**F**), and LA + Ant group (**G**,**H**). Immunostaining for caspase-3 was either absent or weak in granulosa cells (*Gc*) of PF and AF. In *Tc*, an intense staining was found not only in the cytoplasm but also in the nucleus (arrow) (D). PF, preantral follicle; AF, antral follicle; *Oo*, oocyte. A,E,G: ×100. B,C,F,H: ×400. D: ×600. To perform this study, five randomly selected fields were analyzed from each ovarian section (six sections/ovary, five ovaries).

TABLE 1. Immunohistochemistry of Caspase-3 at Different Stages of Follicles

	PF		AF	
Treatments	Gc	Tc	Gc	Tc
Control	−	+	−	++
LA	−	++	−/+	+++
Antide	−	−	−	+
LA + Ant	−	−	−/+	+

Signal intensity: −, absence; −/+, weak; +, moderate; ++, intense; +++, very intense.

intense immunoreactivity for caspase-3 in LA antral follicles. The role of caspase-3 in theca cells is not clear, since this cell type does not typically undergo apoptosis (Boone and Tsang, 1998). Few studies have been performed to clarify the function of caspases in theca cells. Boone and Tsang (1998) have suggested the presence of a sterol regulatory element binding protein (SREBP) which acts as a substrate of caspase-3. In theca cells the activation of caspase-3 and cleavage of SREBP might account for the hypertrophy and accumulation of lipid droplets, leading to large amounts of androgens and consequently inducing apoptosis in granulosa cells. However, Yacobi et al. (2004) have demonstrated that gonadotropins induce an increase in caspase-3 and -7 activity in intact rat AF, and that this enhancement occurs mainly in theca cells and is accompanied by an increase in the apoptosis of these cells.

In order to examine the effect of caspase-3 changes induced by GnRH-I analogs, we evaluated the cleavage of PARP, a substrate of caspase-3 (Rosen and Casciola-Rosen, 1997). In LA follicles, cleavage of PARP from the intact 113-kDa protein showed a significant enhancement in an 85-kDa fragment. The co-injection of Ant interfered with this LA effect. In addition, Ant alone significantly decreased PARP cleavage as compared to the control group. Similar results were obtained when the activity of caspase-3 was determined using the specific caspase-3 colorimetric substrate. These data expanded our results obtained from the Western blot, IHC experiments and activity of caspase-3 observed in this study. Furthermore, this is in agreement with our previous findings (Parborell et al., 2002, 2005), where we provided evidence in support of the apoptotic effect of LA and the anti-apoptotic effect of Ant in ovarian follicles. In those reports, we observed that LA treatment of eCG-superovulated-rats increases the number of atretic and preantral follicles, while it decreases the number of AF. In addition, LA reduces the stability of the antiapoptotic protein Bcl-xL while, in contrast, Ant treatment increases the number of preovulatory follicles and inhibits apoptosis in these follicles through a decrease in the translocation of the proapoptotic protein Bax to the mitochondria.

There are several lines of evidence indicating that GnRH-like peptides play autocrine or paracrine regulatory roles in the ovary. It has been reported that an intrinsic GnRH system, with endogenous GnRH-I, GnRH receptors and a biological response exists in rodent (Aten et al., 1986; Oikawa et al., 1990; Goubau et al., 1992) and human ovaries (Hsueh and Erickson, 1979; Fraser et al., 1986; Clayton et al., 1992; Kaiser et al., 1992; Peng et al., 1994; Kang et al., 2000; Cheng and Leung, 2005). It is well documented that GnRH possesses antigonadotropic effects in the rat ovary, downregulating the expression of FSH and LH receptors (Tilly et al., 1992b; Piquette et al., 1991), inhibiting gonadotropin-stimulated cAMP production (Knecht et al., 1985; Orly et al., 1994), and suppressing steroidogenic enzymes (Hsueh and Schaeffer, 1985; Sridaran et al., 1999b). In addition, we demonstrated that GnRH-I treatment interferes with follicular recruitment, growth,

and luteinization induced by gonadotropins (Hsueh and Erickson, 1979; Guerrero et al., 1993; Andreu et al., 1998). This is in agreement with previous results that show that ovarian GnRH-I or GnRH-like substances are also atretogenic signals (Birnbaumer et al., 1985; Aten et al., 1987; Parborell et al., 2005).

Considering our results on the activity of caspase-3 and the cleavage of PARP, we suggest that Ant is able to block specific ovarian GnRH-I receptors, by preventing the binding of GnRH-I or GnRH-like substances synthesized in the ovary to its receptors.

In conclusion, these results indicate that the decrease in VEGF-A, its receptor Flk-1/KDR, and ANPT-1 produced by the administration of GnRH-I agonist is one of the mechanisms involved in the apoptosis of ovarian cells. This suggests an intraovarian role of an endogenous GnRH-like peptide in follicular development induced by gonadotropins.

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