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Gonadotropin-Releasing Hormone Antagonist Antide Inhibits Apoptosis of Preovulatory Follicle Cells in Rat Ovary[1]

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ABSTRACT

Analogs of GnRH, including agonists (GnRH-a) and antagonists (GnRH-ant), have been widely used to inhibit gonadotropin pituitary release. Aside from the effect of GnRH analogs on the pituitary-gonadal axis, studies have shown that GnRH has extrapituitary effects, particularly on rat and human ovaries. In the present study, we evaluated the direct in vivo effects of the GnRH-a, leuprolide acetate (LA), or the GnRH-ant, Antide (Ant), either singly or together, on ovarian follicular development in prepubertal eCG-treated rats. LA significantly decreased ovarian weight, whereas Ant increased ovarian weight compared with controls; however, coinjection of both compounds had no effect. In addition, LA increased the number of preantral follicles (PFs) and atretic follicles, and decreased the number of early antral follicles (EAFs) and preovulatory follicles (POFs). Coinjection of Ant interfered with this LA effect. Ant alone increased the number of POFs compared with that of controls. Analysis of apoptosis has shown that LA increases the percentage of apoptotic cells in PFs, EAFs, and POFs; however, Ant prevented this effect. In addition, Ant alone decreased the percentage of apoptotic cells in EAFs and POFs. Data have shown that Ant per se inhibited BAX translocation from cytosol to mitochondria and retained cytochrome C in the mitochondria, whereas LA induced cytochrome C release. We conclude that Ant inhibits apoptosis in preovulatory follicles through a decrease of BAX translocation to mitochondria, suggesting that GnRH may act as a physiological intraovarian modulator factor that is able to interfere with follicular development through an increase in apoptotic events mediated by an imbalance among the BCL-2 family members.

apoptosis, follicle, gonadotropin-releasing hormone, ovary

INTRODUCTION

In the mammalian ovary, only a small fraction of oocytes ovulate during reproductive life, whereas the majority of ovarian follicles undergo atresia by a hormonally regulated apoptotic mechanism.

Gonadotropin-releasing hormone (GnRH) and its analogs have been widely used to prevent the spontaneous LH surge in assisted reproduction techniques. Suppression of gonadotropin secretion can be achieved with either GnRH-agonist (GnRH-a) or GnRH-antagonist (GnRH-ant). Chronic administration of GnRH-a leads to pituitary desensitization and inhibition of gonadotropin and sex steroid levels by reducing the number of GnRH receptors on the cell membrane. Conversely, GnRH-ant achieves suppression of gonadotropin secretion by the competitive blockade of the GnRH receptors.

Aside from the effects of GnRH analogs on the pituitary-gonadal axis, studies have shown that GnRH and its analogs have extrapituitary effects, particularly on the ovaries of rats and humans [1–4]. However, there is some controversy about the physiological relevance of an endogenous GnRH-like molecule during folliculogenesis.

We have previously demonstrated, in prepubertal rats, that GnRH-a treatment produces an increase in ovarian follicle DNA fragmentation by interfering with the pathways of FSH, cAMP, or growth factors, or a combination of theses [5, 6]. In addition, several studies performed in rats have demonstrated the antigonadal effect of GnRH analogs administered in vivo or in vitro [7–13]. There is also evidence of the presence of GnRH mRNA [14, 15] and GnRH receptor mRNA [3, 13, 16–18] in ovarian cells. These findings support the concept that a GnRH-like peptide may play an autocrine or paracrine regulatory role in the ovary. In addition, in the rat ovary, GnRH has been identified as an atretogenic factor capable of inducing granulosa cell apoptosis [5, 19, 20]. Moreover, Birnbaumer et al. [21] demonstrated that administration of GnRH-ant together with FSH leads to a significant increase in the number of follicles susceptible to ovulation, suggesting that GnRH or GnRH-like substances are present and active under normal physiological conditions.

The molecular mechanism of apoptosis is a matter of an active debate. However, it is accepted that morphological changes observed during programmed cell death are the consequence of an activation of caspase cascades [22]. At least two main signaling pathways have been postulated to participate in this process. The first involves membrane receptors called ''death receptors'' [23, 24], and the second relies on a cell's ability to sense changes in the ratios and numbers between members of the BCL-2 family of proteins. BCL-2 prevents apoptosis induction by a wide range of stimuli, suggesting that different pathways of transduction signals converge at this point [25–27]. Several authors have identified a variety of proteins related to BCL-2, such as BAX, BAK, BID, and the different BCL-X isoforms, that can either promote or prevent apoptosis [28]. Mitochondria play a key role in the apoptotic pathway through the release of several factors, such as cytochrome C, from the intermembrane space to the cytoplasm [29]. It has been suggested that this pathway could be regulated by the relative levels and subcellular distribution of BCL-2 family

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proteins [30]. The antiapoptotic members (i.e., BCL-2 or BCL-X_L) are mostly associated with the outer membrane of mitochondria and inhibit cytochrome C release, whereas the proapoptotic molecules such as BAX, BAD, or BID are cytosolic proteins. They translocate to the mitochondria and trigger cytochrome C release upon apoptosis induction [31]. Several studies have demonstrated that apoptotic cell death is associated with follicular atresia in chicken, porcine, and rodent ovaries [32–36]. FSH and LH are the primary survival factors for ovarian follicles; the antiapoptotic effects of these gonadotropins are probably mediated by the production of ovarian growth factors. On the other hand, androgens [37], GnRH [20], tumor necrosis factor α [38], and FAS/FAS ligand (FASL) [39–41] are factors that have been involved as potential inducers of granulosa cell apoptosis. In a previous work [19], we described that the apoptotic action of GnRH-a correlates with an imbalance in the ratio of antiapoptotic:proapoptotic proteins (BCL-X_L:BCL-X_S) in rat follicles, suggesting that members of the BCL-2 gene family are involved in GnRH cell death induction in ovarian tissue.

Those observations led us to postulate that an endogenous GnRH ovarian molecule has some inhibitory function during folliculogenesis in the rat, and that this effect could be elucidated by the administration of GnRH analogs to gonadotropin-treated rats. Therefore, the aim of the present study is to examine the in vivo effects of GnRH analogs on follicular development and apoptosis in ovarian follicles obtained from prepubertal eCG-treated rats. In particular, we examine whether the action of GnRH-a leuprolide acetate (LA) and the GnRH-ant antide (Ant) is associated with BAX and cytochrome C subcellular redistribution.

MATERIALS AND METHODS

Hormones and Reagents

The GnRH-a leuprolide acetate (Lupron), was a donation from Abbott Laboratories (Buenos Aires, Argentina). The original ampoule (2.8 mg/5 ml) was dissolved in saline solution. SYNTEX S.A (Buenos Aires) generously provided eCG (Novormon). HEPES, SDS, Antide [*N*-Ac-D-Nal(2)[1], D-pCl-Phe[2], D-Pal(3)[3], Ser[4], Nic-Lys[5], D-Nic-Lys[6], Leu[7], Ipr-Lys[8], Pro[9], D-Ala[10]NH_2; Nal-Lys antagonist] was purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco modified Eagle medium (DMEM, 4.5 g glucose/L), Ham F-12 nutrient mixture (F12), fungizone (250 μg/ml), and gentamicine (10 mg/ml), were from Gibco Laboratories (Grand Island, NY). Polyclonal primary antibodies for BAX (N-20), cytochrome C (H-104), FAS (FL-335), and FASL (Q-20) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit antibody against bovine cytochrome $P450_{scc}$ was a generous gift from Dr. Anita H. Payne (Stanford, CA). Anti-rabbit secondary antibody conjugated with horseradish peroxidase was purchased from Sigma.

In Vivo GnRH Analog Treatment and Superovulation

General care and housing of rats was carried out at the Instituto de Biología y Medicina Experimental in Buenos Aires. Prepubertal rats were from our own colony. Female Sprague-Dawley rats, 23–25 days old, were allowed food and water ad libitum, kept at a room temperature of 21–23°C, on a 12L:12D cycle. GnRH analogs were diluted in saline to the desired concentrations (GnRH-a, 0.5 μg/rat; GnRH-ant, 5 μg/rat) and injected in 0.1 ml of vehicle. Animals were injected s.c. with 0.1 ml eCG (25 IU/rat, control group) to induce multiple follicular growth. They were injected at Time 0 with LA, Ant, or both, and then at 12-h intervals during 48 h. LA, Ant, or both were given at the same dose. Control animals were injected with vehicle only. The last LA and Ant injections were administrated 3 h before rats were killed by cervical dislocation. The ovaries were removed and cleaned of adhering tissue in culture medium for subsequent assays. All animals were treated and cared for in accordance with standard international animal care protocols (e.g., Canadian Council of Animal Care, *Guide to the Care and Use of Experimental Animals*). The experimental protocols were approved by the Animal Experimentation Committee of the IBYME.

Ovarian Morphology and Apoptosis

To evaluate changes in general structure, representative ovaries from the control and treated groups were immediately fixed in 4% neutral buffered formalin for 12 h and then embedded in paraffin. Three-micrometer step sections were mounted at 50-μm intervals onto microscope slides to prevent counting the same follicle twice, according to the method described by Woodruff et al. [42]. Slides were stained with hematoxylin-eosin to count the number of follicles and apoptotic cells per ovary section under a light microscope. The apoptotic cells were counted in 400× microscopic fields of preantral follicles (PFs), early antral follicles (EAFs), and preovulary follicles (POFs). Atresia was defined as the presence of more than 10 pyknotic nuclei per follicle; in the smallest follicles, the criteria for atresia was a degenerate oocyte, precocious antrum formation, or both [5, 43]. The number of preantral, antral, and atretic follicles was determined in ovarian sections obtained from animals (n = 8) after 48 h of vehicle injection or GnRH-a, GnRH-ant, or both. To study ovarian morphology or the number of apoptotic cells, five randomly selected fields were analyzed from each ovarian section (6 sections/ovary, 6–8 ovaries). Apoptotic cells were recognized in hematoxylin-eosin stained tissue sections on the basis of morphological criteria following the procedure described by Goyeneche et al. [44]. The apoptotic index was calculated as the number of death cells from 100 cells per follicle in EAFs and POFs. In PFs, 70 cells were counted per follicle. Apoptotic index is expressed in percentages.

Follicle Isolation and Incubation

Healthy preovulatory follicles (>400 μm in diameter) from 12 ovaries were dissected microscopically using fine needles. The culture was initiated within 1 h of ovary removal. For DNA, four follicles per glass vial were incubated during 24 h under serum-free conditions at 37°C in 500 μl of DMEM:F12 (1:1), containing 10 mM HEPES, supplemented with fungizone (250 μg/ml) and gentamicine (10 mg/ml). Follicles were gassed with 95% O_2-5% CO_2 at the start of culture. This model has the advantage of maintaining the integrity of the follicle. In addition, the incubation in serum-free conditions during 24 h allows it to exhibit the typical apoptotic DNA ladder: presence of internucleosomal fragments of 180 base-pair multiples.

DNA Isolation and Fragmentation Analysis

Cellular DNA was extracted from follicles incubated for 24 h under serum-free conditions in 500 μl of DMEM supplemented with streptomycin and gentamicine in the absence of hormones as previously described [6, 45]. Briefly, 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 of 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 1.9% agarose gels containing ethidium bromide (0.4 μg/ml) in Tris-borate-EDTA buffer. Within each agarose gel, equal amounts of DNA were loaded into each well. To enhance sensitivity, gels were further stained with ethidium bromide for 15 min. DNA was visualized with a UV (302 nm) transilluminator, and photographed with a Polaroid camera system. Densitometric analysis of low-molecular-weight (<15 kilobase) DNA was performed with an Image Scanner (Genius) using the software program Image Quant (Molecular Dynamics). Quantitative results obtained by densitometric analysis of the low-molecular-weight DNA fragments represent the mean ± SEM of three or four independent gel runs.

Preparation of Mitochondria and Cytosolic Fractions

Fresh (t_0), healthy preovulatory follicles were resuspended in ice-cold separation buffer (200 mM mannitol, 50 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM Hepes-KOH pH 7.4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin), and homogenized with an Ultra-Turrax (IKA Werk,

TABLE 1. Effects of GnRH analogs on the ovarian response to gonadotropins.[a]

Analogs	Ovarian weight (mg)	Paired ovaries used (n)	Follicles (n)			
			PF	EAF	POF	ATF
Control	58 ± 3.2	6[b]	3.41 ± 0.35[b]	17.25 ± 1.90[b]	6.58 ± 0.63[b]	1.00 ± 0.24[b]
LA	31 ± 2.4	6[c]	8.08 ± 0.97[c]	8.16 ± 1.43[c]	2.91 ± 0.39[c]	4.66 ± 0.35[c]
Ant	79 ± 1.2	6[d]	3.66 ± 0.84[b]	13.08 ± 2.15[b]	11.75 ± 1.31[d]	1.75 ± 0.39[b]
LA+Ant	56 ± 1.8	6[b]	4.83 ± 0.53[b]	17.86 ± 3.10[b]	14.00 ± 2.60[d]	1.66 ± 0.50[b]

[a] Data are expressed as mean ± SEM.
[b–d] Values with different superscripts are significantly different ($P < 0.05$).

Breisgau) homogenizer. Samples were centrifuged in Eppendorf tubes (at 900 × *g* for 5 min at 4°C) to remove nuclei, followed by centrifugation at 10 000 × *g* (25 min at 4°C) to obtain a membrane pellet enriched in mitochondria. The supernatant was collected and used as the cytosolic fraction. The mitochondrial pellet was resuspended in 20 μl of PBS, 0.2% Triton X-100. The resuspended mitochondrial fraction and the cytosolic fraction were either used immediately or stored at −70°C. Mitochondrial preparation efficiency (mitochondrial fraction/[cytosol + mitochondrial fractions] = 0.80) was estimated by the presence of $P450_{scc}$ enzyme known to be specific for the mitochondrial membrane. Protein concentration was measured by the Bradford assay [46].

Western Blot Analysis

Proteins were resolved on 15% SDS-PAGE and transferred to a nitrocellulose membrane. The blot was preincubated in blocking buffer (5% nonfat dry milk, 0.05% Tween-20 in 20 mM TBS pH 8.0) for 1 h at room temperature and incubated with appropriate primary antibodies (FAS, FASL, BAX, cytochrome C, $P450_{scc}$) in blocking buffer for 1 h at room temperature. Then, it was incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase and finally detected by chemiluminescence and autoradiography using x-ray film. Negative controls were obtained in the absence of the primary antibody.

Quantification for Western Blot Assay

The loading protein ranged from 20 to 40 μg for both antibodies. 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). Data are expressed as mean (cytosol or mitochondria fraction)/[cytosol + mitochondrial fractions]) for both proteins analyzed (BAX and cytochrome C).

The proper loading was evaluated by staining the membranes with Ponceau-S. As an internal control, the density of each protein was normalized to the density of a band that was observed in the protein transference pattern in all Ponceau-S stained membranes. This band was selected because it was unchanged under the different treatments (data not shown).

Data Analysis

Data are expressed as the mean ± SEM of triplicate incubations of at least three experiments using six animals per group. Representative gels are shown in the figures. Statistical analyses were performed using one-way analysis of variance followed by either the Tukey or Newman-Keuls Multiple test. Values of $P < 0.05$ were considered significant.

RESULTS

Morphological Studies

Prepubertal rats were superovulated with eCG (control group, C) and treated with LA (LA group; 1 μg rat day during 48 h), or Ant (Ant group; 10 μg rat day during 48 h), or both (LA + Ant group). When the GnRH-agonist, LA, was injected alone, the ovarian weight decreased by 0.5-fold, whereas injection of GnRH-Ant increased ovarian weight by 0.4-fold compared with controls. Coinjection of both GnRH substances did not show differences from that of controls (Table 1).

Histological ovarian slides were stained with hematoxylin-eosin to determine the number of different follicle stages and the percentage of apoptotic cells (Fig. 1). Table 1 shows that injection of LA significantly increased the number of PFs and atretic follicles (ATFs). However, this treatment decreased the number of EAFs and POFs. The coinjection of GnRH-Ant interfered with the inhibitory effect of LA. Thus, the results show no change in the number of PFs, EAFs, and ATFs after cotreatment with the GnRH analogs. However, in this group, an increase in the number of POFs compared with that of the control group was observed. Surprisingly, the treatment with GnRH-Ant alone did significantly increase the number of POFs compared with that of the control group.

Morphological and Biochemical Studies on Ovarian Apoptosis

To elucidate whether the changes observed in ovarian follicles content were mediated by a consequence of programmed cell death, the percentage of apoptotic cells in each follicular stage was determined (Table 2). LA increased the percentage of apoptotic cells in PFs, EAFs, and POFs. By contrast, GnRH-Ant treatment prevented the effect observed with LA. Unexpectedly, treatment with GnRH-Ant alone produced a decrease in the percentage of apoptotic cells in EAFs and POFs compared with that of the control group (Table 2).

These results were partially confirmed by DNA fragmentation analysis. Preovulatory follicles cultured in serum-free medium showed spontaneous onset of apoptotic DNA fragmentation (Fig. 2, lane 2). An increase in the spontaneous onset of apoptotic DNA fragmentation was observed in follicles obtained from LA-treated rats, with a 2.6-fold increase compared with that of controls (Fig. 2, lane 3). However, the treatment of superovulated rats with the GnRH-ant, Antide (5 μg/injection every 12 h), did not change the level of ovarian DNA fragmentation (Fig. 2, lane 4). On the other hand, coincubation with the GnRH-ant reverted the stimulation of apoptosis by LA, because quantitative estimation of DNA cleavage from ovarian follicles revealed no changes in DNA fragmentation after in vivo LA + Ant treatment when compared with control cultured follicles (Fig. 2, lane 5). DNA fragmentation was minimal in freshly isolated preovulatory follicles, showing an increase in those follicles obtained from LA-treated rats (data not shown).

Protein Levels of FAS and FASL in Antral Follicles

Studies from a number of laboratories have suggested that FAS and FASL may be central to the regulation of cell death in the ovary [39–41]. In addition, it was described

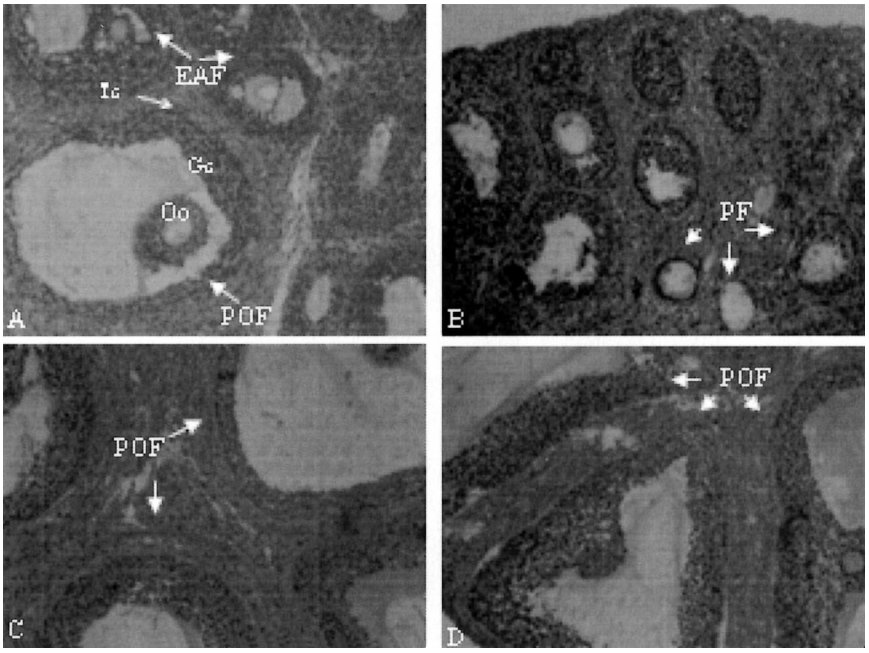

FIG. 1. Representative photomicrographs of ovarian sections from gonadotropin-stimulated rats treated with vehicle (control) or GnRH analogs (LA, 0.5 μg/rat; Ant, 10 μg/rat) during 2 days. **A**–**D**) Sections stained with hematoxylin-eosin. Note the presence of numerous PFs in the LA group (**B**), and POFs in the Ant group (**C**) and the LA + Ant group (**D**), in comparison with control group (**A**). EAF; early antral follicle, Oo; oocyte, Gc; granulosa cells, Tc; theca cells. Magnification ×100.

that GnRH controls FAS and FASL expression in other cell types [47, 48]. Therefore, we decided to evaluate the protein expression of these cytokines. The follicular contents of FASL and FAS were evaluated by Western blotting (Fig. 3, A and B). No changes in the levels of FASL and FAS were observed when we compared fresh preovulatory follicles obtained from controls with those from the LA, Ant, or LA + Ant groups.

Effect of Analogs of GnRH on Subcellular Distribution of BAX and Cytochrome C

In previous works from our laboratory we showed that the apoptotic action of GnRH-a was correlated with an imbalance in the ratio of antiapoptotic:proapoptotic proteins (BCL-X_L:BCL-X_S), reducing the stability of the BCL-X_L protein [19]. In this study we have determined the in vivo effect of GnRH analogs on the subcellular distribution of BAX and cytochrome C. Mitochondrial and cytosolic fractions were prepared from isolated preovulatory follicles from the control, LA, Ant, and LA + Ant groups, and the levels of these proteins were determined by Western blot analysis. Mitochondrial preparation efficiency (80%) was estimated by the presence of $P450_{scc}$ enzyme, known to be specific for the mitochondrial membrane (Fig. 4C).

In the control group, BAX protein was mainly detected in the mitochondria (Fig. 4A). Similar results were observed in the LA group. Conversely, BAX protein was retained in cytosol when the animals were treated only with Ant. This effect was partially blocked when LA was coinjected with Ant. The analysis of cytochrome C distribution showed that this protein is localized mainly in mitochondria of samples belonging to animals injected with Ant or LA + Ant (Fig. 4B). We also noted that, despite the mitochondrial localization of BAX, the distribution of cytochrome C was similar in both fractions in the control group (Fig. 4B).

TABLE 2. In vivo effect of GnRH analog treatment on the percentage of apoptotic ovarian cells.[a]

	Follicles		
Analogs	PF	EAF	POF
Control	8.4 ± 0.67[b]	10.5 ± 0.71[b]	11.3 ± 0.52[b]
LA	43.6 ± 5.41[c]	31.2 ± 2.42[c]	27.3 ± 2.53[c]
Ant	6.3 ± 0.53[b]	5.7 ± 0.60[d]	4.6 ± 0.74[d]
LA+Ant	3.4 ± 0.64[b]	4.9 ± 0.80[d]	5.4 ± 0.84[d]

[a] Five randomly-selected fields from each ovarian section were analyzed (6 sections/ovary, 6–8 ovaries/group). Data are expressed as mean ± SEM. [b–d] Values with different letters in each follicular stage are significantly different ($P < 0.05$).

DISCUSSION

Several studies performed in rats have demonstrated the in vivo or in vitro antigonadal effects of GnRH analogs [7, 49]. In previous studies, we have demonstrated that GnRH-a treatment produces a failure in the steroidogenic luteal capability and an increase in the apoptotic process in the ovary, eliciting consequently an interference in the follicular recruitment, growth, and luteinization induced by gonadotropins [5, 50]. These observations led us to study the in vivo effect of GnRH analogs on follicular development, atresia, and programmed cell death. The results presented in this work, assessed by the determination of ovarian weight and the number of follicles at different stages, confirm the inhibitory effect of the GnRH-agonist, LA, on ovarian development previously observed by our laboratory [5, 19]. Particularly interesting is that coinjection of the GnRH-antagonist, Ant, reverts the inhibitory effect of LA, suggesting that LA action is mainly mediated by an ovarian GnRH receptor. Of interest, the injection of Ant alone increased the number of preovulatory follicles, reflecting the augmentation also observed in ovarian weight, suggesting that this compound improves follicular development.

Taking into account that follicular atresia is mediated by apoptosis, we have analyzed the effect of both GnRH analogs on programmed cell death. Actually, injection of LA caused an increase in the percentage of apoptotic cells in growing follicles and in the spontaneous DNA fragmentation of preovulatory follicles cultured in serum-free medium. Ovarian follicles cultured in these conditions is a model currently used to investigate the pathways that control

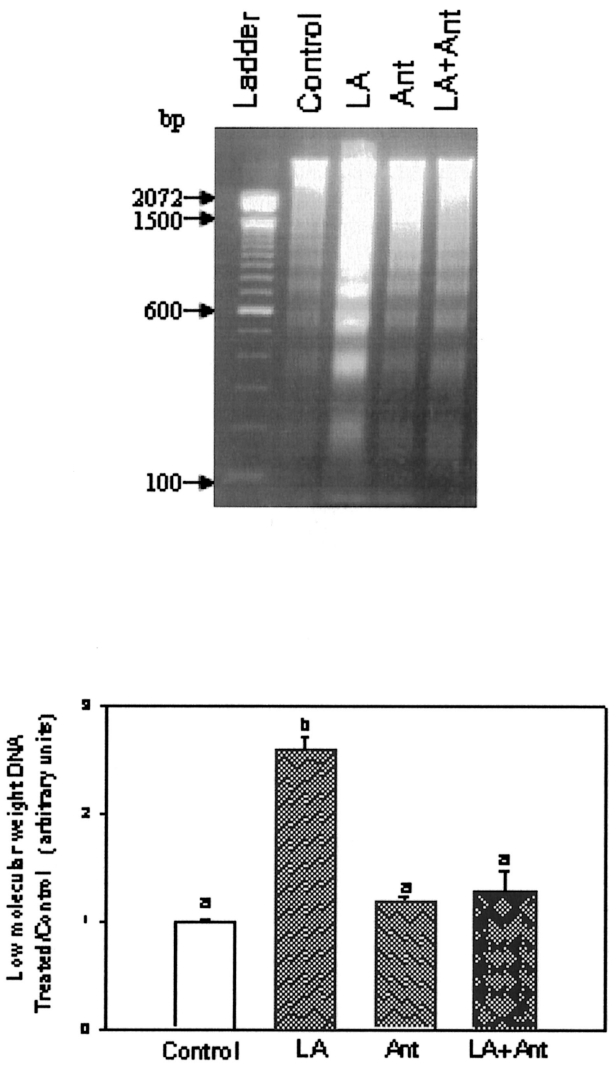

FIG. 2. In vivo effect of treatment with GnRH analogs on ovarian internucleosomal DNA fragmentation. **Upper**) Representative agarose gel showing DNA fragmentation and quantitative estimation of DNA cleavage. Prepubertal rats were superovulated with eCG and were injected every 12 h for 48 h with LA (0.5 μg/rat), Ant (10 μg/rat), or both. Control animals were treated with vehicle alone. Three hours after the last injection, preovulatory follicles were isolated and cultured for 24 h in serum-free medium. Four micrograms of follicular DNA extracted from each culture was analyzed by ethidium bromide staining, as described in *Materials and Methods.* Low-molecular-weight DNA (<1.5 kilobase) from the gel was examined to calculate apoptotic DNA fragmentation. Values obtained by densitometric analysis of the gel were expressed as fold increases compared with follicles collected after the 24-h incubation without GnRH analog treatment. **Lower**) Quantitative estimation of DNA cleavage. Data points represent the mean ± SEM of three to four independent gel runs. Bars with different letters are significantly different ($P < 0.05$).

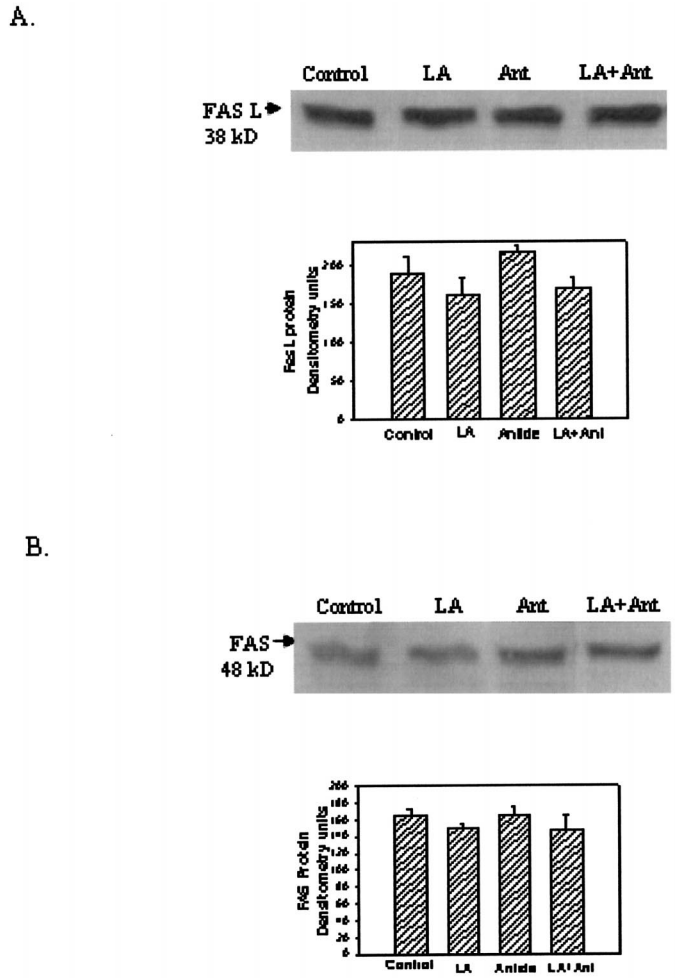

FIG. 3. Effect of GnRH analogs on FAS/FASL protein content in preovulatory follicles. **A**) Upper panel: representative immunoblot of FASL protein content in preovulatory follicles from control rats, and those treated with LA, Ant, or both. After homogenization, proteins were extracted and subjected to 15% SDS-PAGE, and transferred onto nitrocellulose membranes. FASL protein was visualized by using an anti-FASL antibody. Lower panel: Densitometric quantification of FAS content. Bars represent mean ± SEM of four experiments. **B**) Upper panel: representative immunoblot of FAS protein content in preovulatory follicles from control rats, and those treated with LA, Ant, or both. FAS protein was visualized by using an anti-FAS antibody. Lower panel: densitometric quantification of FAS content. Bars represent mean ± SEM of four experiments.

apoptosis and follicular atresia [19, 32, 34]. Our results show that DNA isolated after the incubation exhibited the typical apoptotic DNA fragmentation pattern, and demonstrated that in vivo LA treatment sensitizes granulosa cells to undergo apoptosis. Once again, the coinjection of Ant reverted the apoptotic effect of LA. Besides, we detected a decrease in the percentage of apoptotic cells measured in EAFs and POFs when compared with the control group. However, the level of ovarian DNA fragmentation did not significantly change after Ant treatment. It is important to highlight that morphological criteria to detect apoptotic cells are more sensitive than biochemical analysis of DNA fragmentation when few apoptotic cells are assessed.

Taking into account all these results, we suggest that Antide would be able to block specific ovarian GnRH receptors, avoiding the binding of GnRH or GnRH-like substances synthesized in the ovary and, consequently, improving follicular development. This is in agreement with previous findings [21, 51] that reported evidence in support of ovarian GnRH or GnRH-like material being also an atretogenic signal.

Although several intracellular molecules, including BCL-2 [36, 52], BCL-X [19], BAX [36], caspases [53, 54], and inhibitor-of-apoptosis proteins [55] have been implicated as being directly involved in the regulation of ovarian apoptosis, several studies have suggested that FAS antigen and FAS ligand may be central in the induction of follicular atresia. In the present study, the FAS/FASL protein levels were not affected by GnRH analog treatment. These results suggest that another pathway, related to mitochondria pro-

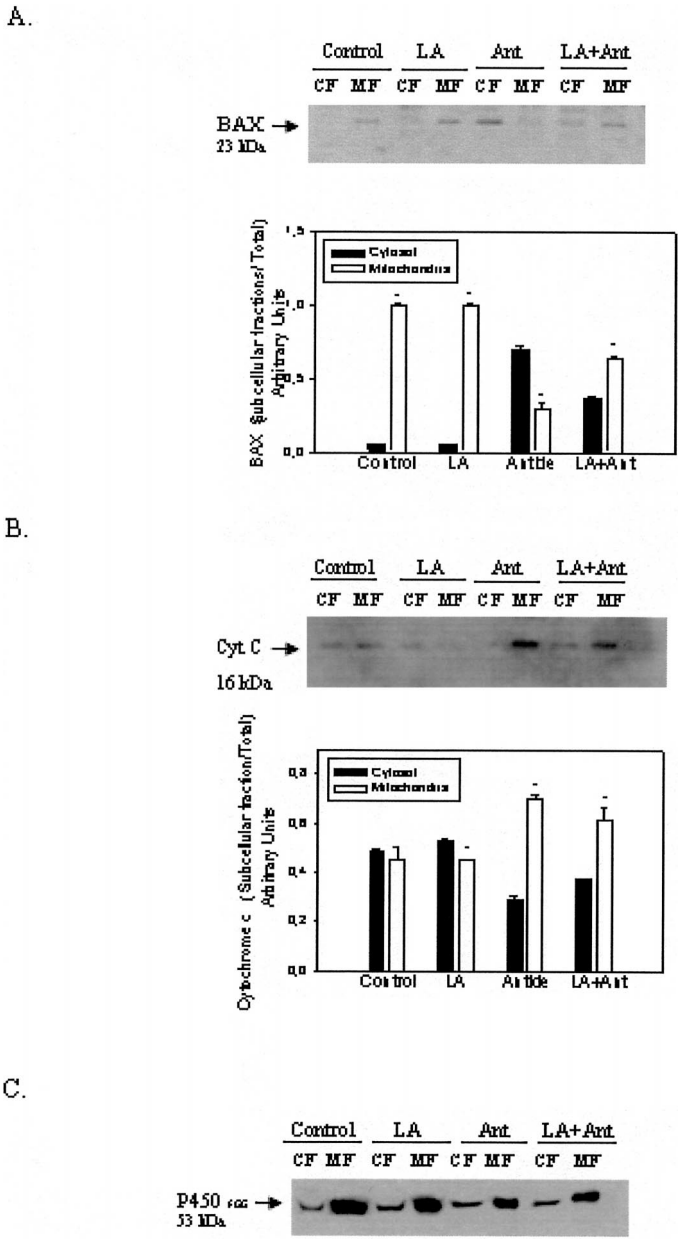

FIG. 4. GnRH analog-induced alteration in subcellular localization of BAX and cytochrome C. Cytosolic and mitochondrial fractions were prepared from isolated preovulatory follicles from prepubertal superovulated rats and treated with vehicle alone (control group, C) or with LA (1 μg rat day during 48 h; LA group), Ant (10 μg rat day during 48 h; Ant group), or both LA and Ant (LA + Ant group). **A**) Upper panel: representative immunoblot of BAX in cytosolic and mitochondrial fractions. Fractions were analyzed by Western blot analysis by using primary antibody against BAX as described in *Materials and Methods.* **B**) Lower panel: densitometric quantification of BAX content. Bars represent mean ± SEM of three experiments. $*P < 0.05$ comparing cytosol vs. mitochondria fraction contents in each treatment. **B**) Upper panel: representative immunoblot of cytochrome C in cytosolic and mitochondrial fractions. Fractions were analyzed by Western blot analysis using primary antibody against cytochrome C as described in *Materials and Methods.* Lower panel: densitometric quantification of cytochrome C content. Bars represent mean ± SEM of three experiments. $*P < 0.05$ comparing cytosol vs. mitochondria fraction contents in each treatment. **C**) Representative immunoblot of $P450_{scc}$ in cytosolic and mitochondrial fractions. Fractions were analyzed by Western blot analysis using primary antibody against $P450_{scc}$ as described in *Materials and Methods.*

tein regulation, could be involved in GnRH-mediated follicular atresia.

The regulation of mitochondrial membrane integrity and the release of cytochrome C from mitochondria are key processes during apoptosis and are controlled by the BCL-2 family [56, 57]. In previous studies we showed that the apoptotic action of GnRH-agonist was correlated with an imbalance in the ratio of antiapoptotic:proapoptotic proteins ($BCL\text{-}X_L$:$BCL\text{-}X_S$), reducing the stability of the $BCL\text{-}X_L$ protein [19]. However, we never detected changes in the levels of the other members (BAX and BCL-2). One of the intriguing aspects of the BCL-2 members is their subcellular localization and the control of their translocation during the apoptotic pathway. In particular, the proapoptotic proteins, such as BAX, located in cytoplasm of healthy cells, are redistributed to mitochondria upon apoptotic stimuli. After translocation to mitochondria, BAX induces cytochrome C release either by forming a pore through oligomerization or by opening a channel called voltage-dependent anion channel via direct interaction [30, 58].

In accordance with the previous results [19] described in our experimental model, antral follicles obtained from LA-treated rats showed no changes in total BAX protein levels. This report shows that Ant alone fails to influence total BAX expression. However, upon analyzing the subcellular distribution of BAX following treatment with GnRH analogs, we observed that Ant injected alone completely blocked BAX translocation to mitochondria. This effect was not observed in specimens coinjected with LA. Our results thus demonstrated that Ant inhibits BAX translocation from cytosol to mitochondria. Conversely, Ant alone also retains cytochrome C in mitochondria, and this effect was also inhibited by LA. It is likely that the inhibition of BAX translocation and, as a consequence, cytochrome C release, may be involved in the promotion of follicle development mediated by Antide. Moreover, LA and Ant affect follicular apoptosis in an opposite way; the molecular mechanisms involved on those effects may not be the same. In fact, the GnRH agonist LA did not affect the subcellular distribution of BAX, but induced cytochrome C release. These results correlate with our previous data [19] demonstrating that the GnRH agonist reduced the stability of the $BCL\text{-}X_L$ protein, resulting in an imbalance in favor of the levels of the proapoptotic proteins. In this sense, the LA effect on cytochrome C release may be mediated mainly by this mechanism. On the other hand, Ant treatment fails to affect the levels of BCL-2 family members. However, it inhibits the distribution of BAX and, as a consequence, the release of cytochrome C. These results and those from our previous work, in which we demonstrated that LA decreases the levels of the antiapoptotic protein $BCL\text{-}X_L$, suggest that not only translocation of apoptotic proteins, but also an imbalance in the levels among the BCL-2 family members, are necessary to induce apoptosis.

In summary, the GnRH antagonist Antide inhibits cell death in preovulatory follicles through a decrease of BAX translocation to mitochondria. Finally, our findings seem to suggest that GnRH acts as an intraovarian factor modulator that is able to interfere with the follicular development induced by gonadotropins. GnRH may increase apoptotic events mediated by an imbalance among the BCL-2 family members.

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