

Gonadotropin-Releasing Hormone Agonist Affects Rat Ovarian Follicle Development by Interfering With FSH and Growth Factors on the Prevention of Apoptosis

F. PARBORELL, L. DAIN, AND M. TESONE*

Instituto de Biología y Medicina Experimental (IBYME) CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina

ABSTRACT Apoptosis is the biological process by which follicular cells are eliminated in atretic follicles. The aim of the present study was to examine the in vitro effect of a GnRH-a (leuprolide acetate, LA) and its interactions with FSH, dibutyl cAMP, and growth factors (IGF-I, EGF, and FGF) on follicular apoptosis in early antral ovarian follicles obtained from prepubertal DES-treated rats. Follicles cultured 24 hr in the absence of hormones showed spontaneous onset of apoptotic DNA fragmentation. The presence of FSH suppressed the spontaneous onset of apoptotic DNA fragmentation (75–85%). Quantitative estimation of DNA cleavage from ovarian follicles revealed no significant changes in DNA fragmentation after in vitro LA treatment (1–100 ng/ml). However, coincubation with LA interfered partially with the effects of FSH on apoptosis suppression. This apoptosis suppression was also obtained by treatment with dibutyl cAMP (80%), and was partially prevented by the presence of LA in the cultures. Follicles were cultured 24 hr with FGF, EGF, or IGF-I, and these factors suppressed DNA fragmentation (70, 60, and 70% respectively), while the presence of LA (100 ng/ml) in the culture medium prevented this effect. In conclusion, we show that the rescue from apoptotic DNA fragmentation produced in early antral follicles by FSH, cAMP, and growth factors, is prevented by coincubation with LA. This GnRH analog would thus interfere in the pathway of FSH, cAMP and/or growth factors by an as yet unknown mechanism. *Mol. Reprod. Dev.* 60: 241–247, 2001. © 2001 Wiley-Liss, Inc.

Key Words: ovary; GnRH; apoptosis; growth factors; early antral follicles

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) and its agonists, have specific effects on extrapituitary tissues such as placenta, breast, and ovary (Fraser et al., 1986; Guerrero et al., 1993; Erickson et al., 1994). A GnRH-like peptide, as well as GnRH receptor and GnRH gene transcription products, have been identified in the ovary (Pipier et al., 1981; Séguin et al., 1982; Birnbau-

mer et al., 1985; Goubau et al., 1992; Peng et al., 1994; Olofsson et al., 1995; Minaetzi et al., 1995; Whitelaw et al., 1995). In addition, several studies performed in rats have demonstrated the antigonadal effect of GnRH analogues administered in vivo or in vitro (Clayton et al., 1979; Jones and Hsueh, 1981; Knecht et al., 1982; Conn et al., 1984; Srisvastava and Sridaran, 1994). These results suggest the putative role of GnRH as an intraovarian hormone.

Apoptosis is the biological process by which follicular cells are eliminated in atretic follicles (Tilly et al., 1991, 1992a,b; Palumbo and Yeh, 1994; Chun et al., 1996; Nahum et al., 1996), and can be observed in the somatic granulosa cells that maintain the oocyte until its release from the follicle during ovulation (Gougeon, 1996; Tilly, 1998). FSH and LH are the primary survival factors for ovarian follicles, and their effects are probably mediated by the production of ovarian growth factors. Evidence to support this observation has been derived from both in vivo and in vitro approaches, and these studies have demonstrated that various growth factors and cytokines (IGF-I, EGF, TGF α , bFGF, KGF, interleukin-I β) prevent apoptosis in antral follicles (Tilly et al., 1992a,b; Chun et al., 1994; Tilly and Tilly, 1995; McGee and Hsueh, 2000). Although atresia occurs at all stages of follicular development, the majority of follicles undergo degeneration, presumably by apoptosis, at the early antral follicle stage (Hirshfield and Midgley, 1978; Hirshfield, 1991; Chun et al., 1996).

In hypophysectomized, estrogen-treated immature rats, Billig et al. (1994) showed that the in vivo administration of a GnRH agonist (GnRH-a) increases apoptotic cell death in ovarian tissue and granulosa cells, while Papadopoulos et al. (1999) demonstrated

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*Correspondence to: Dr. M. Tesone, Instituto de Biología y Medicina experimental, Obligado 2490 (1428) Buenos Aires, Argentina. E-mail: mtesone@dna.uba.ar

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that administration of a GnRH-a enhances the rate of DNA degradation in the corpora lutea of pregnant rats. In addition to these results, we have demonstrated, using TUNEL assays, that in vivo GnRH-a treatment of eCG + hCG-treated rats produces an increase in the number of apoptotic cells in growing follicles and corpora lutea (Andreu et al., 1998). No studies, however, have been performed on the in vitro effects of GnRH-a on early antral follicle apoptosis and their interactions with FSH and growth factors.

Consequently, the aim of the present study was to examine the in vitro effect of a GnRH-a (leuprolide acetate, LA) and its interactions with FSH, dibutyryl cAMP, and growth factors (IGF-I, EGF, and FGF) on follicular apoptosis in early antral ovarian follicles obtained from prepubertal DES-treated rats.

MATERIALS AND METHODS

Materials

GnRH-a, leuprolide acetate (Lupron, LA), was a donation from Abbott Laboratories (Buenos Aires, Argentina), the original ampoule (2.8 mg/5 ml) was dissolved with saline to obtain appropriate culture concentrations. SYNTEX S.A (Buenos Aires) generously provided equine chorionic gonadotropin (eCG, Novormon). Steroid hormones, HEPES, dibutyryl cAMP (dcAMP), and SDS, were purchased from Sigma Chemical Co. (St Louis, MO). Dulbecco's 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), FGF and IGF1 from Collaborative Research (Bedford, MA), and EGF from Sigma, Ovine FSH (o FSH S17) was kindly provided by The National Hormone and Pituitary Agency, NIADKK-NIH (Bethesda, MD). Growth hormones and FSH were dissolved according provider instructions. All other chemicals were of reagent grade from standard commercial sources.

Animals

Female Sprague-Dawley rats, 23–25 days old, allowed food and water ad libitum and kept in an air-conditioned atmosphere, were injected subcutaneously with diethylstilbestrol (DES: 1 mg/rat dissolved in corn oil) daily for three days to stimulate the development of early antral follicles. Control animals were injected with vehicle. Animals were killed by cervical dislocation, and the ovaries removed for follicle dissection. Experimental protocols were approved by the Animal Experimentation Committee of the IBYME.

Follicle Culture

Early antral follicles (~350 µm in diameter) were dissected from ovaries collected following DES treatment, and cleansed of adhering tissue in culture medium. Ovarian follicles from five animals were pooled for different treatments, and cultures were initiated within 1 hr after ovary removal at 37°C. Eight

follicles per 20 ml glass scintillation vial were incubated under serum-free conditions in 500 µl Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, NY) supplemented with streptomycin and gentamicin, in the presence or absence of different hormones. Follicles were gassed with 95% O₂-5% CO₂ at the start of culture and after 12 hr of culture initiation. Following incubation for 24 hr, follicles were stored at -70°C until DNA isolation.

DNA Isolation and Fragmentation Analysis

Cellular DNA was extracted from follicles as previously described with some modifications (McGee et al., 1997). Briefly, four follicles from each culture were homogenized in a buffer containing 100 mM NaCl, 4 mM EDTA, 50 mM Tris-HCl, and 0.5% SDS, pH 8, and proteinase K (100 µg/ml), and incubated 4 hr at 55°C to facilitate membrane and protein disruption. Following incubation, samples were cooled 30 min on ice in 1 M potassium acetate and 50% chloroform to initiate protein precipitation, and centrifuged 8 min at 5,000g at 4°C. Supernatants were then precipitated 30 min in 2.5 vol ethanol at -70°C and centrifuged 20 min at 5,000g 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 samples were incubated with RNase (10 µg/ml) for 1 hr at 37°C.

Agarose Gel Electrophoresis and Quantitation of DNA Fragmentation

DNA samples (4–6 µg) were electrophoretically separated on 1.9% agarose gels containing ethidium bromide (0.4 µg/ml) in TBE buffer. Within each agarose gel, equivalent amount of DNA was loaded into the wells. To enhance sensitivity, gels were further stained with ethidium bromide for 15 min. 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 Image Quant (Molecular Dynamics) (Tesone et al., 1999).

Data Analysis

All experiments were repeated at least three times, and a representative gel is shown in the figures. Quantitative results obtained by densitometric analysis of the low molecular weight DNA fragments represent the mean ± SEM of combined data from replicate experiments. Statistical comparisons were performed using one-way ANOVA followed by Scheffe's multiple range test. Values of $P < 0.05$ were considered significant.

RESULTS

To study the in vitro effects of LA on follicular apoptosis, early antral follicles obtained from ovaries of rats primed 3 days with DES were cultured 24 hr in

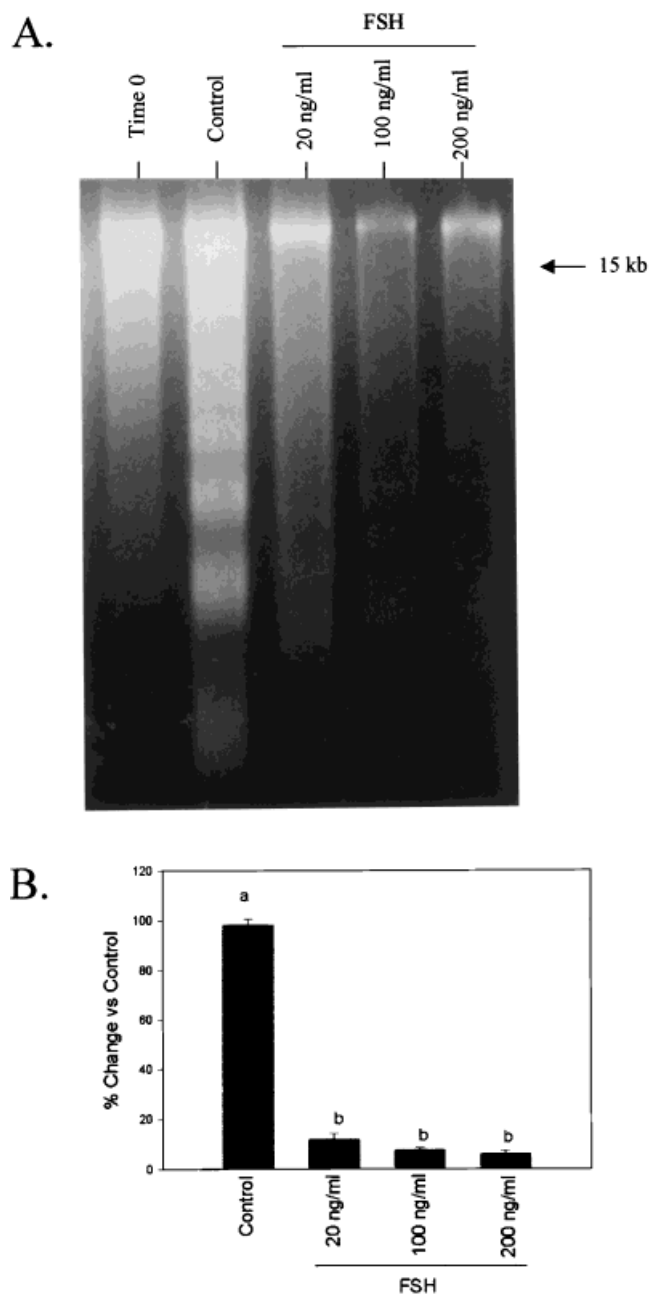


Fig. 1. Suppressive effect of FSH on DNA fragmentation. **A:** Agarose gel showing DNA fragmentation. **B:** Quantitative estimation of DNA cleavage. Early antral follicles were cultured 24 hr in serum-free medium in the presence or absence of FSH (20–200 ng/ml). DNA extracted from follicles in each culture was analyzed by ethidium bromide staining, as described in Materials and Methods. Low molecular weight DNA (<15 kb) from the gel was examined to determine apoptotic DNA fragmentation. Results obtained by densitometric analysis of the gel are expressed as percent changes compared to 24 hr culture in serum free medium, arbitrarily designated 100%. Data points represent the mean \pm SEM of three or four independently run gels. a vs. b: $P < 0.05$.

serum-free medium. As shown in Figure 1, follicles cultured in the absence of hormones showed spontaneous onset of apoptotic DNA fragmentation. In contrast, DNA fragmentation was minimal in freshly

isolated early antral follicles (no culture, time zero). The presence of FSH (20–200 ng/ml) in the incubation medium suppressed the spontaneous onset of apoptotic DNA fragmentation (24 hr follicle culture, 75–85%, $P < 0.05$). Quantitative estimation of DNA cleavage from ovarian follicles revealed no significant changes in DNA fragmentation after in vitro LA treatment (1–100 ng/ml) when compared to untreated controls (Fig. 2), while coincubation with LA (100 ng/ml) interfered partially with the effect of FSH (200 ng/ml) on apoptosis suppression (Fig. 3). Lower LA concentrations produced a less pronounced effect (data not shown). Suppression of apoptosis was also obtained by treatment with an analog of the second messenger for FSH, dibutyryl cAMP (1 mM, 80% of apoptosis inhibition, $P < 0.05$), and could be partially prevented by the presence of LA in the cultures (Fig. 4).

The in vitro effect of growth factors on apoptotic DNA fragmentation in the presence or absence of LA, was also evaluated. Follicles were cultured 24 hr with increasing concentrations of EGF, FGF, or IGF-I, all factors which suppress DNA fragmentation, and a maximal inhibitory response (70, 60, and 70%, respectively) was obtained at 1 μ g/ml (data not shown). The presence of LA (100 ng/ml) in the culture medium prevented this effect (Fig. 5).

DISCUSSION

Follicular atresia is an important and selective degenerative process of ovarian follicular growth and development in mammals. Follicles are most susceptible to atresia at the early antral stage (Hirshfield and Midgley, 1978; Hirshfield, 1991; Chun et al., 1996). The present study investigates the in vitro effects of a GnRH-a (LA), and its interaction with FSH and growth factors, on apoptotic DNA fragmentation in early antral follicles isolated from DES-primed rats. Serum-free culture of follicles is a model currently used to investigate the hormonal factors and second messenger pathways which control apoptosis and follicle atresia. In addition, this system has the advantage of conserving the integrity of the follicle to be studied.

DNA isolated from follicles incubated 24 hr in serum-free medium exhibited the typical apoptotic DNA degradation pattern (presence of internucleosomal fragments of 180-bp multiples). The causes of spontaneous-onset apoptosis which occurs during follicle culture might be the high levels of endonucleases and cations such as calcium and magnesium, since it was suggested that high concentrations of these factors might activate the endonucleases and result in apoptotic DNA fragmentation (Zeleznick et al., 1989).

The incubation of follicles with increasing doses of FSH suppressed apoptotic DNA fragmentation. We have found that the anti-apoptotic effect of FSH was partially suppressed by the addition of LA to the incubation medium, indicating that LA has a negative effect on FSH-prevented follicular apoptosis. Furthermore, an analog of cAMP, which acts as a second messenger to gonadotropins, also inhibited the frag-

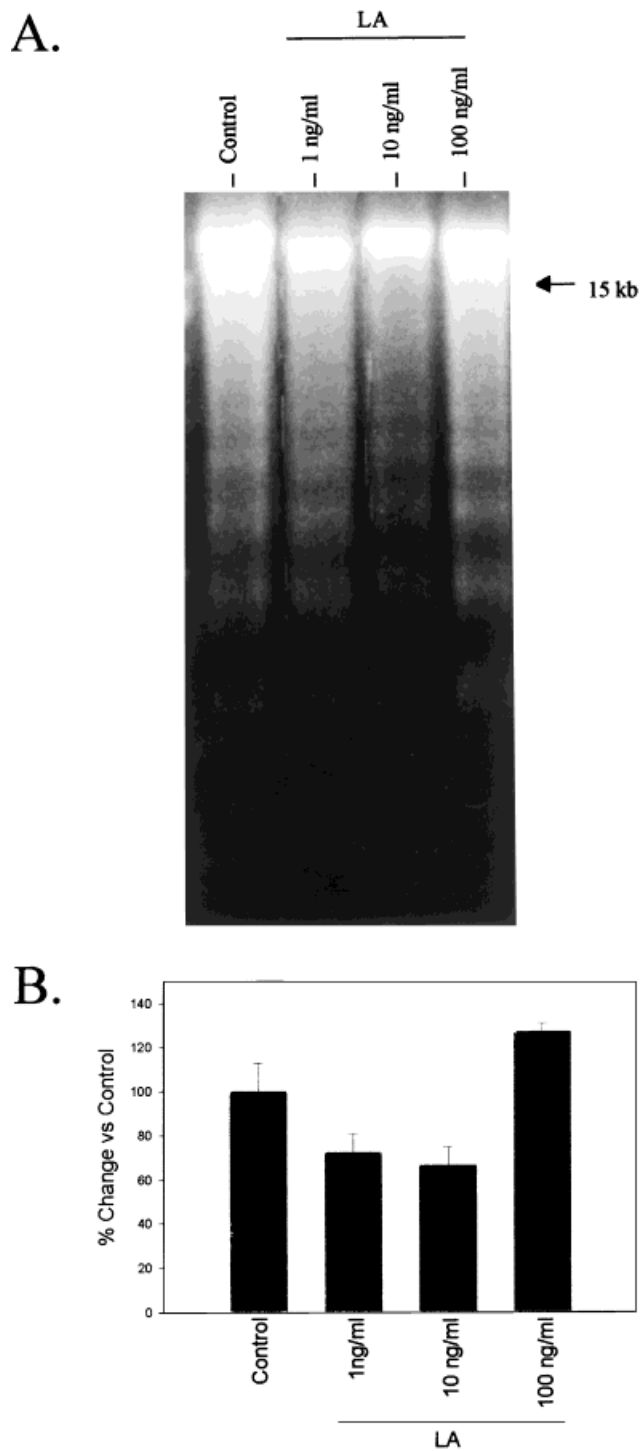


Fig. 2. Effect of LA on DNA fragmentation in early antral follicles. **A:** Agarose gel showing DNA fragmentation. **B:** Quantitative estimation of DNA cleavage. Early antral follicles were cultured 24 hr in serum-free medium in the presence or absence of LA (1–100 ng/ml). DNA extracted from follicles in each culture was analyzed by ethidium bromide staining, as described in Materials and Methods. Low molecular weight DNA (<15 kb) from the gel was examined to determine analysis of the gel are expressed as percent changes compared to 24 hr culture in serum-free medium, arbitrarily designated 100%. Data points represent the mean \pm SEM of three or four independently run gels.

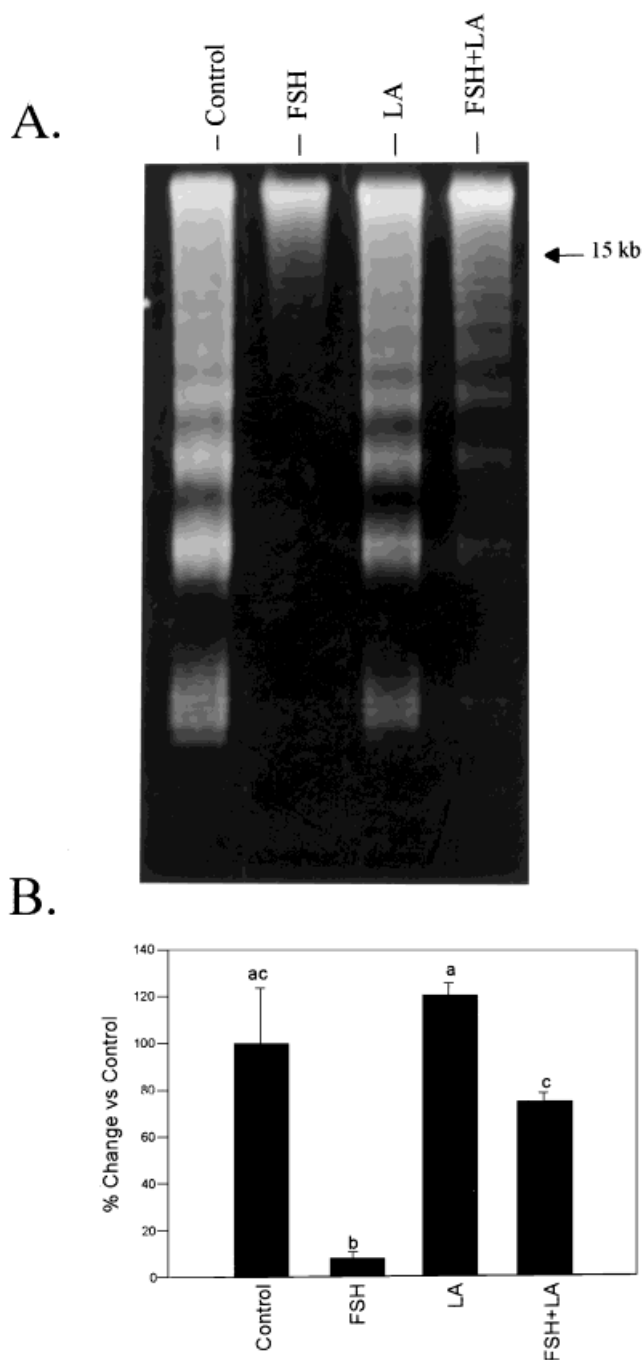


Fig. 3. In vitro effect of LA on the FSH inhibition of spontaneous apoptosis DNA fragmentation. **A:** Agarose gel showing DNA fragmentation. **B:** Quantitative estimation of DNA cleavage. Early antral follicles were cultured 24 hr in serum-free medium in the presence or absence of FSH (200 ng/ml) and/or LA (100 ng/ml). DNA extracted from follicles in each culture was analyzed by ethidium bromide staining, as described in Materials and Methods. Low molecular weight DNA (<15 kb) from the gel was examined to determine apoptotic DNA fragmentation. Results obtained by densitometric analysis of the gel were expressed as percent changes compared to 24 hr culture in serum-free medium, arbitrarily designated 100%. Data points represent the mean \pm SEM of three or four independently run gels. Values displaying different letters are significantly different ($P < 0.05$).

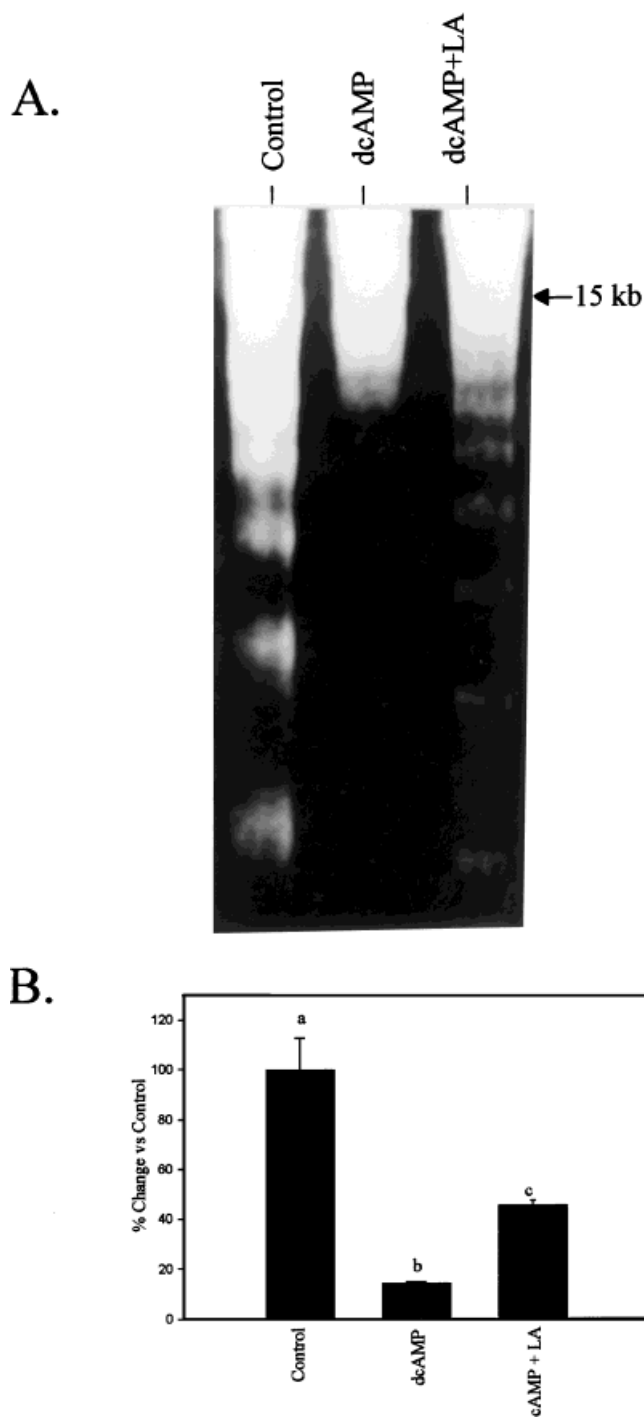


Fig. 4. Effect of LA on the dcAMP inhibition of apoptotic DNA fragmentation. **A:** Agarose gel showing DNA fragmentation. **B:** Quantitative estimation of DNA cleavage. Early antral follicles were cultured 24 hr in serum-free medium in the presence or absence of dcAMP (1 mM) and/or LA (100 ng/ml). DNA extracted from follicles in each culture was analyzed by ethidium bromide staining, as described in Materials and Methods. Low molecular weight DNA (<15 kb) from the gel was examined to determine apoptotic DNA fragmentation. Results obtained by densitometric analysis of the gel are expressed as percent changes compared to 24 hr culture in serum-free medium, arbitrarily designated 100%. Data points represent the mean \pm SEM of three or four independently run gels. Values displaying different letters are significantly different ($P < 0.05$).

mentation of DNA, and this effect was also partially prevented by LA. Treatment of follicles with EGF, IGF-1, or FGF, suppressed the spontaneous onset of DNA fragmentation, corroborating earlier data (Chun et al., 1996). Similar to the results obtained for FSH and the cAMP analog, coincubation with LA suppressed the inhibition of apoptosis produced by growth factors, though in these experiments LA was able to prevent all of the growth factor-induced inhibition of apoptosis. Taking these differences into account, we suggest a complex signal transduction pathway for FSH in the apoptosis prevention action, where FSH employs a cell survival mechanism that includes other elements than those used by growth factors.

Despite the fact that several studies have demonstrated that some of the ovarian inhibitory effects of *in vivo* GnRH-a treatment are mediated by apoptosis (Billig et al., 1994; Papadopoulos et al., 1999), this is the first demonstration that, in early antral follicles, LA *in vitro* treatment interferes with the FSH and growth factor-mediated prevention of apoptosis. In this way, we consider that this paper is an important contribution to the study of the direct effect of GnRH on the ovarian function.

Considering that FSH and growth factors utilize different second messenger systems (activation of protein kinase A or intrinsic tyrosine kinase activity), the existence of overlapping hormonal cascades controlling follicular development has been proposed (McGee and Hsueh, 2000). In addition, our findings, describing GnRH-mediated inhibition on FSH and growth factors apoptosis prevention, suggest that the GnRH effect is distal in the cascade of events leading to apoptosis. The mechanism of action of this peptide includes, as in pituitary cells, activation of GnRH receptors in ovarian cells, and association with G protein-mediated activation of phospholipase C (Leung and Steele, 1992; Stojilkovic et al., 1994). Members of the *bcl-2* (B-cell lymphoma/leukemia-2 protein) gene family have been described as main participants in the cascade of events that activate or inhibit apoptosis (Boise et al., 1993; Johnson et al., 1996). The *bcl-2* related proteins can be separated into anti- and pro-apoptotic members, and the balance between these counteracting proteins presumably determines cell fate (White, 1996). Through hetero- and homodimeric interactions, proteins encoded by various members of this gene family are thought to be primary determinants of the susceptibility of a cell to an apoptotic stimulus. One of these proteins, termed bax, counters the effects of *bcl-2* on cell survival. Tilly et al. (1995) postulate that in granulosa cells the ratio of *bcl-2* to bax expression is determinant of cell fate, and demonstrate that the inhibition of granulosa cell apoptosis and follicular atresia mediated by gonadotropin treatment is related to the ability of gonadotropins to reduce the expression of bax in granulosa cells, producing as a consequence a change in the ratio between bax and the constitutive levels of *bcl-2* and *bcl-x*long. In this regard, Papadopoulos et al. (1999) correlate the apoptosis

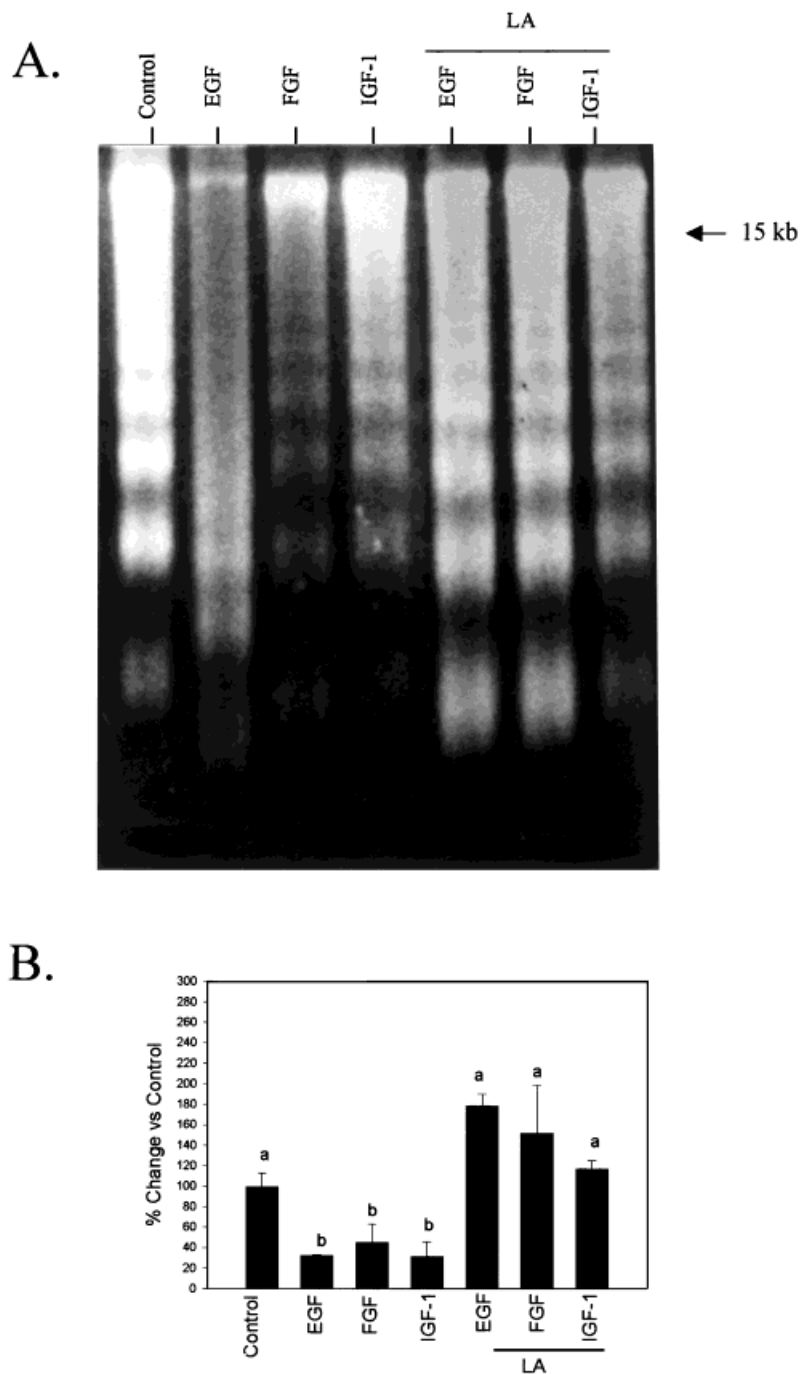


Fig. 5. Suppressive effect of LA on growth factors rescue of apoptotic DNA fragmentation in early antral follicles. **A:** Agarose gel showing DNA fragmentation. **B:** Quantitative estimation of DNA cleavage. Early antral follicles were cultured 24 hr in serum-free medium with growth factors (EGF: 1 µg/ml, IGF-1: 1 µg/ml and FGF: 1 µg/ml) in the presence or absence of LA (100 ng/ml). DNA

extracted from follicles in each culture was analyzed by ethidium bromide staining, as described in Materials and Methods. Results obtained by densitometric analysis of the gel are expressed as percent changes compared to 24 hr culture in serum-free medium, arbitrarily designated 100% (control). Data points represent the mean ± SEM of three or four independently run gels. a vs. b: *P* < 0.05.

produced by a GnRH-a in corpora lutea from pregnant rats, with changes in the expression of some bcl-2 gene family members. In our experimental model, an important as yet unresolved point, is whether LA modifies the expression of the above mentioned genes, and interfering with the FSH or growth factor-mediated antiapoptotic effects. Experiments are cur-

rently in progress in our laboratory to answer these questions. In conclusion, we show that the rescue from apoptotic DNA fragmentation in early antral follicles by FSH, cAMP and growth factors is prevented by coincubation with LA. This GnRH analog would interfere with the pathway of FSH, cAMP and/or growth factors action by an as yet unknown mechanism.

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