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Hormonal Regulation of Apoptosis in Early Antral Follicles: Follicle-Stimulating Hormone as a Major Survival Factor*

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ABSTRACT

Hormonal regulation of apoptosis has been studied in cultured preovulatory follicles. Because early antral follicles are most vulnerable to undergo atretic degeneration under physiological conditions *in vivo*, the present studies were designed to investigate the hormonal regulation of apoptosis using *in vitro* culture of early antral follicles. Rats were implanted with diethylstilbestrol at 24 days of age to stimulate the development of early antral follicles, and ovaries were collected at day 27 of age. Early antral follicles were dissected and cultured (four per vial) for 24 h with or without hormonal treatments. After culture, DNA was extracted from follicles, and the degree of apoptotic DNA fragmentation was determined using 3′-end labeling and gel electrophoresis. *In situ* analysis of apoptotic DNA fragmentation revealed that granulosa cells in these follicles are the main cell type undergoing apoptosis. Follicles cultured in the absence of hormones showed a 12-fold increase in the level of apoptotic DNA fragmentation which was prevented by treatment with FSH in a dose-dependent manner (60% maximal suppression and apparent ED_{50} of 30 ng/ml). Similarly, treatment with $(Bu)_2$cAMP also suppressed follicle apoptosis. Treatment with LH or human CG, however, minimally suppressed apoptotic DNA fragmentation (35% maximal suppression). Insulin-like growth factor-I (IGF-I) also suppressed apoptosis by 45%. Moreover, the suppressive effect of FSH on apoptosis was partially reversed by coincubation with IGF-binding protein-3, suggesting a potential mediatory role of endogenous IGF-I. However, recombinant bovine GH had no effect on follicle apoptosis despite its ability to stimulate IGF-I messenger RNA (mRNA) levels. Incubation of follicles with epidermal growth factor (EGF) and basic fibroblast growth factor maximally suppressed follicle apoptosis by only 32% and 42%, respectively. Ligand binding analysis indicated the minimal effectiveness of EGF on apoptosis in early antral follicles, as compared with its potent action in preovulatory follicles reported earlier, may be due to a 3.5-fold increase in EGF receptor concentration in the mature follicles. High doses (150 or 500 ng/ml) of interleukin-1β also suppressed apoptosis by 48% whereas treatment with an NO generator, sodium nitroprusside, or a cyclic GMP analog suppressed apoptosis as effectively as that of FSH. Furthermore, treatment with activin resulted in a dose-related suppression of follicle apoptosis, reaching a maximal 40% suppression. In contrast, cotreatment of activin with its binding protein, follistatin, abolished this effect. Collectively, these data demonstrated a stage-dependent difference in the hormonal regulation of follicle apoptosis. Although FSH, LH/human CG, GH, IGF-I, EGF, basic fibroblast growth factor, and interleukin-1β are all effective survival factors for preovulatory follicles, FSH is a major survival factor for early antral follicles, the stage during which a majority of follicles undergo atresia under physiological conditions. (*Endocrinology* **137:** 1447–1456, 1996)

ALTHOUGH the vast majority of ovarian follicles undergo a degenerative process termed atresia, the fundamental mechanism of this atretic process is largely unknown. Recent demonstration of apoptotic cell death during follicle degeneration (1–3) has opened a new avenue by which to study the hormonal regulation of follicle atresia. Based on studies using *in vitro* culture of preovulatory follicles (4–6) and autoradiographic analysis of apoptotic DNA fragmentation (7), it is apparent that redundant endocrine/paracrine/autocrine control mechanisms are involved in the regulation of follicle apoptosis.

Apoptosis of preovulatory follicles cultured *in vitro* is prevented by treatment with gonadotropins (4) and several intraovarian growth factors including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) (8), and insulin-like growth factor-I (IGF-I) (4). In addition, GH also inhibits apoptosis of these cultured follicles via the production of endogenous IGF-I (6). Recently, it has been demonstrated that cytokines can also modulate follicle cell apoptosis. Interleukin-6 is an atretogenic factor in cultured granulosa cells (9), whereas interleukin-1β (IL-1β) acts as a follicle survival factor by stimulating the levels of follicular NO in cultured preovulatory follicles (5).

Earlier studies have shown that atresia occurs at all stages of follicle development (10, 11), but the majority of follicles undergo degeneration at the early antral follicle stage (12, 13), suggesting that the penultimate stage of follicle development is most vulnerable to apoptosis. Detailed analysis of the size distribution of atretic follicles in cycling rats further

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demonstrates that the preponderance of atretic follicles are of the early antral size class (300–350 μm diameter) while preantral and preovulatory follicles rarely undergo atretic degeneration (14). These results suggest that early antral follicles represent a major branching point during follicle development that is susceptible to atresia.

Although *in vivo* studies have demonstrated that apoptotic DNA fragmentation in early antral follicles is induced by withdrawal of estrogen treatment as well as treatment with GnRH and androgen (15, 16), no studies have been reported regarding the hormonal regulation of apoptosis in cultured early antral follicles. To extend studies on the hormonal control of apoptosis in preovulatory follicles, we used an *in vitro* culture model of early antral follicles to study the effect of endocrine and intraovarian factors on follicle apoptosis during this critical stage of follicle development. Our results suggest stage-dependent differences in the hormonal regulation of follicle apoptosis, demonstrating that FSH is a major survival factor for early antral follicles.

Materials and Methods

Hormones and reagents

Human CG (CR-127; 14, 900 IU/mg), purified pituitary human FSH (I-SIAFP-1; 8,466 IU/mg), and ovine LH (LH-S-26; 2, 300 IU/mg) were obtained from the National Hormone and Pituitary Distribution Program, NIDDK, NIH (Baltimore, MD). Human IGF-I and human recombinant IL-1β (2×10^7 U/mg) were purchased from Collaborative Research, Inc. (Waltham, MA) and Genzyme (Cambridge, MA), respectively. Murine EGF, diethylstilbestrol (DES), $(Bu)_2$cAMP, sodium nitroprusside (SNP), and 8-bromoguanosine 3′5′-cyclic monophosphate (8-br-cGMP) were obtained from Sigma Chemical Co. (St. Louis, MO). Porcine IGFBP-3 purified from follicular fluid and recombinant human follistatin (FS) were provided by Dr. N. Ling (Neurocrine, San Diego, CA). Recombinant human bFGF was a gift from Dr. A. Sommer (Synergen, Boulder, CO). Recombinant bovine GH (rbGH) and human activin A were generously provided by Genentech, Inc. (South San Francisco, CA). Antiserum directed against estrogen was from Chemicon (Temecula, CA). Monoclonal antibody to progesterone was kindly provided by Dr. F. Kohen (Weizmann Institute, Rehovot, Israel).

Follicle culture

To stimulate the development of multiple early antral follicles, immature female Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) were implanted with Silastic capsules (15 mm; Dow Corning Co., Midland, MI) containing DES at 24 days of age. Early antral follicles (~350 μm in diameter) were isolated from ovaries collected at 3 days after DES implantation by nonenzymatic dissection using fine forceps and cultured as previously described (4, 5). Four follicles per group were incubated under serum-free conditions in glass vials containing 0.6 ml Eagle's MEM (GIBCO, Grand Island, NY) supplemented with penicillin, streptomycin, L-glutamine, and 0.1% BSA (wt/vol, Fraction V, Sigma, St. Louis, MO) in the absence or presence of different hormones. After culture for 24 h at 37 C under 95% O_2 and 5% CO_2, follicles were snap-frozen, and stored at −70 C until processed for DNA isolation, and culture media were collected for the determination of cAMP and progesterone levels by RIA. To compare hormonal responsiveness between early antral and preovulatory follicles, immature (26-day-old) rats were treated with 10 IU equine (e) CG. Two days after gonadotropin treatment, preovulatory follicles (> 800 μm in diameter) were dissected as previously described (4, 5).

DNA isolation

Follicles from each culture were incubated in 200 μl digestion buffer (pH 8.0; 100 mM NaCl, 50 mM Tris-HCl, 4 mM EDTA, 0.5% SDS) containing 100 μg/ml proteinase K (Boehringer-Mannheim, Indianapolis, IN) at 55 C for 4–5 h. After incubation, samples were cooled on ice for 30–60 min in 1 M potassium acetate and 50% chloroform, centrifuged at 5,000 × *g* for 8 min at 4 C, and then precipitated with ethanol. The precipitate was rinsed with 70% ethanol, air-dried, and resuspended in 50 μl water. DNA content was quantitated by reading the absorbance at 260 nm before RNase treatment (10 μg/ml; Boehringer-Mannheim) at 37 C for 60 min. The yield of DNA from four follicles was about 2.5 μg.

Analysis of DNA fragmentation

DNA from each treatment culture (500 ng) was labeled at 3′-ends with [^{32}P]dideoxy-ATP (3,000 Ci/mmol; Amersham, Arlington Heights, IL) using terminal transferase (Boehringer Mannheim) as previously described (4, 7). Labeled DNA samples were then visualized by autoradiography, and the degree of DNA fragmentation was quantitated by β-counting of low molecular weight DNA fractions (<15 kb).

In situ DNA 3′-end labeling

The extent of DNA fragmentation in follicles was visualized using nonradioactive labeling of DNA 3′-ends as previously described (16) using an ApopTag kit (OnCor, Gaithersburg, MD). Briefly, follicles were fixed in 4% buffered formaldehyde and embedded in paraffin. After deparaffinization and rehydration, follicle sections (5-μm thickness) were treated with 10 μg/ml proteinase K for 10 min at room temperature. Tissues were then treated with 2% H_2O_2 in PBS for 5 min to inactivate endogenous peroxidase, and their DNA was labeled at 3′-ends with digoxigenin-deoxyUTP by incubation with reaction buffer containing terminal deoxynucleotidyl transferase for 60 min at 37 C. The sections were further incubated with anti-digoxigenin antibody conjugated to peroxidase for 30 min at room temperature. Diaminobenzidine, the substrate for peroxidase, was applied to the slides for color reaction. For negative controls, the follicle sections were incubated with reaction mixtures with terminal deoxynucleotidyl transferase omitted. Some sections were stained with hematoxylin and eosin.

Solution hybridization analysis

Follicular IGF-I mRNA levels were quantitated by solution hybridization as previously described (4, 6, 17). After incubation in the absence or presence of rbGH, early antral and preovulatory follicles were homogenized with a Polytron in 1% SDS, 20 mM Tris-HCl (pH 7.5), and 4 mM EDTA. After proteinase K treatment, total nucleic acids were extracted with phenol/chloroform. Samples were hybridized at 70 C for 24 h in 0.06 M NaCl, 20 mM Tris-HCl (pH 7.5), 4 mM EDTA, 0.1% SDS, 10 mM dithiothreitol, and 25% formamide with the ^{35}S-labeled IGF-I antisense RNA probe as described (17). After the addition of 100 μg herring sperm DNA, the samples were treated with RNase A (40 μg/ml) and RNase T_1 (2 μg/ml) (Sigma). RNase treatment of the RNA hybrids results in a 115-bp protected fragment that was precipitated by trichloroacetic acid and collected on glass-fiber filters (GF/C Whatman; Whatman International Ltd., Maidstone, UK) before being counted in a scintillation counter. To determine the level of IGF-I mRNA, the signal was compared with known amounts of sense IGF-I mRNA hybridized in parallel, and the results were expressed as attomoles (10^{-18} mol; amol) IGF-I mRNA per μg DNA.

Determination of EGF binding sites by [^{125}]iodo-EGF

EGF was iodinated by the chloramine-T procedure as previously described (18). All reagents were prepared in 0.05 M phosphate buffer and added to a reaction vial in the following sequence: EGF (5 μg/20 μl), 1 mCi Na ^{125}I (Radiochemical Center, Amersham-Searle, Boston, MA), and chloramine-T (20 μg/10 μl). The reaction was terminated 50 sec later by the addition of sodium metabisulfite (40 μg/10 μl). Purification of the iodinated EGF was performed by gel filtration on a Sephadex G-25

column (1 × 45 cm; Pharmacia Fine Chemicals, Uppsala, Sweden) using a phosphate buffer (pH 7.5) containing sodium chloride (0.075 M). Aliquots of radiolabeled EGF were stored in 0.1% BSA-phosphate buffer. The maximum binding capacity of the labeled EGF was determined by incubation with increasing concentrations of liver membranes. Specific activity of the EGF tracer was about 1×10^5 cpm/ng. The specific binding of EGF was estimated by incubation (16–18 h; 23 C) of follicle homogenates. At the end of the incubation period, the reaction tubes were centrifuged at 1500 × *g* for 30 min at 4 C. Pellets were resuspended with fresh buffer and recentrifuged, and radioactivity in the pellets was determined by counting in a *r*-spectrometer (Nuclear-Chicago, Des Plaines, IL).

RIAs

Concentrations of progesterone in culture media were determined by RIA as previously described (19). Medium cAMP levels were determined by specific RIA (20).

Data analysis

Each experiment was repeated at least three times, and the number of independent cultures is shown in each figure legend. Representative autoradiograms are presented to demonstrate apoptotic DNA fragmentation. Quantitative data represent the mean ± SEM of at least three cultures expressed as percent change as compared with control samples incubated without hormone treatment. Statistical differences were assessed by ANOVA followed by Student's *t* test, and $P < 0.05$ was considered to be significant.

Results

In situ apoptosis of DNA fragmentation in cultured early antral follicles

To study cell types undergoing apoptotic DNA fragmentation, *in situ* 3′-end labeling of DNA with digoxigenin-dUTP was performed on sections of cultured follicles. As shown in Fig. 1A, histological staining of follicle sections revealed a small follicular antrum, several layers of theca cells (Tc) surrounding multiple layers of granulosa cells (Gc), and an oocyte (Oo). Freshly prepared follicles before incubation did not show detectable levels of DNA labeling (Fig. 1B). In contrast, follicles cultured for 24 h in the absence of hormones showed heavily stained DNA fragmentation signal (Fig. 1C), confined mainly to the granulosa cells (Fig. 1E). Lower apoptotic signal was detected in follicles cultured in the presence of FSH (Fig. 1D). Negative control sections incubated with reaction mixtures without terminal transferase did not show specific signals (Fig. 1F).

Effects of treatment with gonadotropins on DNA fragmentation in cultured early antral follicles

To evaluate the role of gonadotropins as survival factors at the penultimate stage of follicle growth, early antral follicles isolated from ovaries of DES-implanted immature rats were incubated in serum-free medium for 24 h in the absence or presence of increasing doses of gonadotropins or an analog of their second messenger, $(Bu)_2$cAMP.

Analysis of DNA fragmentation in follicles cultured in the absence of hormones (24 h) revealed a spontaneous increase of apoptosis (Fig. 2), consistent with studies on preovulatory follicles (4). Incubation of follicles with increasing doses of FSH caused a dose-dependent suppression of apoptotic DNA fragmentation (Fig. 2A). Quantification of the low molecular weight DNA (Fig. 2B) showed that 100 or 300 ng/ml FSH suppressed DNA fragmentation by 60% ($P < 0.01$). This suppression of apoptosis was mimicked by treatment with an analog of the second messenger for FSH, $(Bu)_2$cAMP (5 mM). In contrast, treatment with LH or hCG was less effective than FSH (Fig. 2A). Quantitative analysis indicated that treatment with 60 ng/ml LH or 200 ng/ml hCG suppressed DNA fragmentation only by 20% (Fig. 2B). Inclusion of higher doses of LH (200–600 ng/ml) in the culture medium suppressed follicle apoptosis by 30–35% ($P < 0.05$).

Effect of treatment with IGF-I and GH on DNA fragmentation in cultured early antral follicles

IGF-I, an intraovarian growth factor, and GH have been shown to suppress apoptosis in cultured preovulatory follicles (4, 6). Early antral follicles were cultured in the absence or presence of IGF-I or GH. Treatment with 200 or 600 ng/ml IGF-I suppressed follicle apoptosis as revealed by autoradiographic analysis (Fig. 3A). Quantification of low molecular weight DNA (Fig. 3B) demonstrated a maximal suppression of 45% ($P < 0.05$) by IGF-I as compared with follicles cultured without hormones (24 h). In contrast, treatment with 500 or 1500 ng/ml rbGH, known to stimulate IGF-I production in preovulatory follicles (6), did not inhibit DNA fragmentation ($P > 0.05$; Fig. 3). However, solution hybridization analysis indicated that increased levels of IGF-I mRNA transcripts could be found in early antral as well as preovulatory follicles cultured in the presence of 500 ng/ml rbGH ($P < 0.05$; Table 1).

To test if the suppressive action of FSH is mediated through the endogenous production of IGF-I, the effect of cotreatment with IGFBP-3 on FSH suppression of apoptotic DNA fragmentation was investigated. Addition of 10 μg/ml IGFBP-3 resulted in 40% reversal ($P < 0.05$) of the suppressive effect of FSH on follicle apoptosis (Fig. 3B). Treatment with IGFBP-3 alone had no effect on follicle DNA fragmentation ($P > 0.05$).

Effects of treatment with EGF and bFGF on DNA fragmentation in cultured early antral follicles

Since EGF and bFGF have been shown to inhibit apoptosis in cultured preovulatory follicles and in cultured granulosa cells derived from these follicles (8), we studied the role of these growth factors in regulating apoptosis of early antral follicles. Incubation of follicles with 200 or 500 ng/ml EGF maximally suppressed apoptosis by only 32% ($P < 0.05$; Fig. 4). Likewise, treatment with 200 or 600 ng/ml bFGF resulted in 35–42% ($P < 0.05$) suppression of apoptosis (Fig. 4).

To examine whether the differential responses to EGF between preovulatory and early antral follicles are due to variations in the number of EGF receptors, the concentrations of EGF binding sites were investigated. As shown in Table 2, preovulatory follicles exhibited 3.5-fold higher EGF-binding sites than early antral follicles ($P < 0.05$).

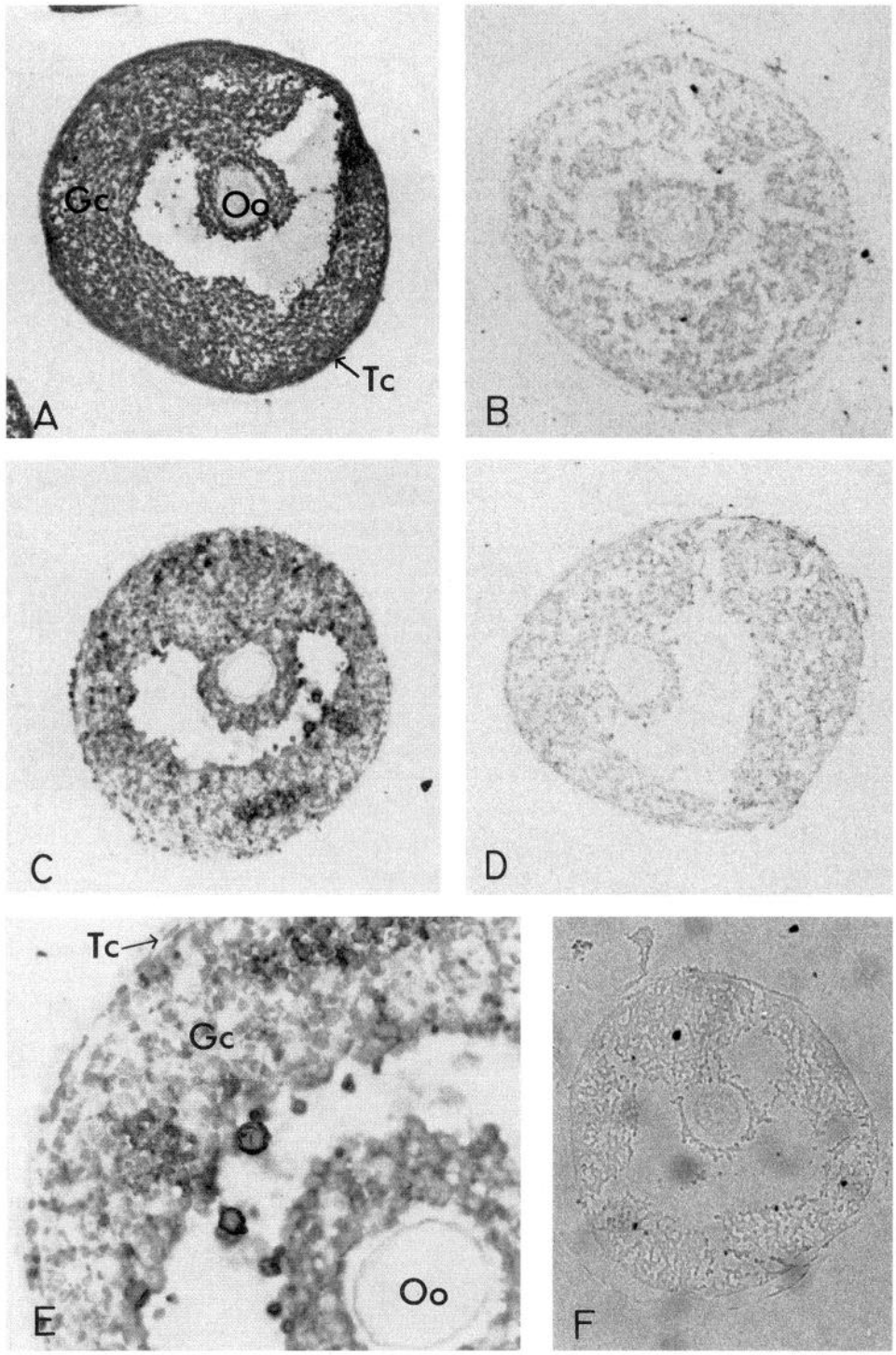

FIG. 1. *In situ* detection of DNA fragmentation in early antral follicles cultured *in vitro*. Early antral follicles isolated from ovaries of DES-implanted immature rats were cultured in the absence or presence of FSH (100 ng/ml). Follicles are fixed before incubation for histological staining with hematoxylin and eosin (A). Several layers of theca cells (Tc) surround multiple layers of granulosa cells and oocyte. B-F, Some of the follicle sections before or at 24 h after culture were used for *in situ* analysis of DNA fragmentation by labeling with digoxigenin-dUTP followed by incubation with anti-digoxigenin antibody conjugated to peroxidase, as described in *Materials and Methods*. B, Follicles at 0 h of culture. C, Follicles cultured in the absence of hormones for 24 h. D, Follicles treated with FSH. E, Higher magnification for follicles in panel C showing positive staining in granulosa cells. F, Negative control section incubated without the terminal transferase. Oo; oocyte, Gc; granulosa cells, Tc; theca cells. Panels A, B, C, D, F, ×40; E, ×400.

Effects of activation of IL-1β/NO/cGMP pathway on DNA fragmentation in cultured early antral follicles

Because IL-1β, NO, and cGMP have been shown to suppress DNA fragmentation in preovulatory follicles (5), we determined whether this pathway is also operating in early antral follicles. Follicles cultured in the presence of 50 ng/ml IL-1β, a dose that markedly suppresses preovulatory follicle apoptosis (5), showed negligible changes in DNA fragmentation of early antral follicles (Fig. 5A). At higher doses (150 or 500 ng/ml), IL-1β treatment caused a maximal suppression of follicle apoptosis by 37–48% ($P < 0.05$; Fig. 5B). Treatment with 0.5 mM SNP, an NO generator, suppressed DNA fragmentation by 60%, comparable with that induced by FSH. Moreover, inclusion of 5 mM bromo-cGMP, a second messenger for NO, in the incubation medium also resulted in the suppression of follicle apoptosis by 53% ($P < 0.05$; Fig. 5B).

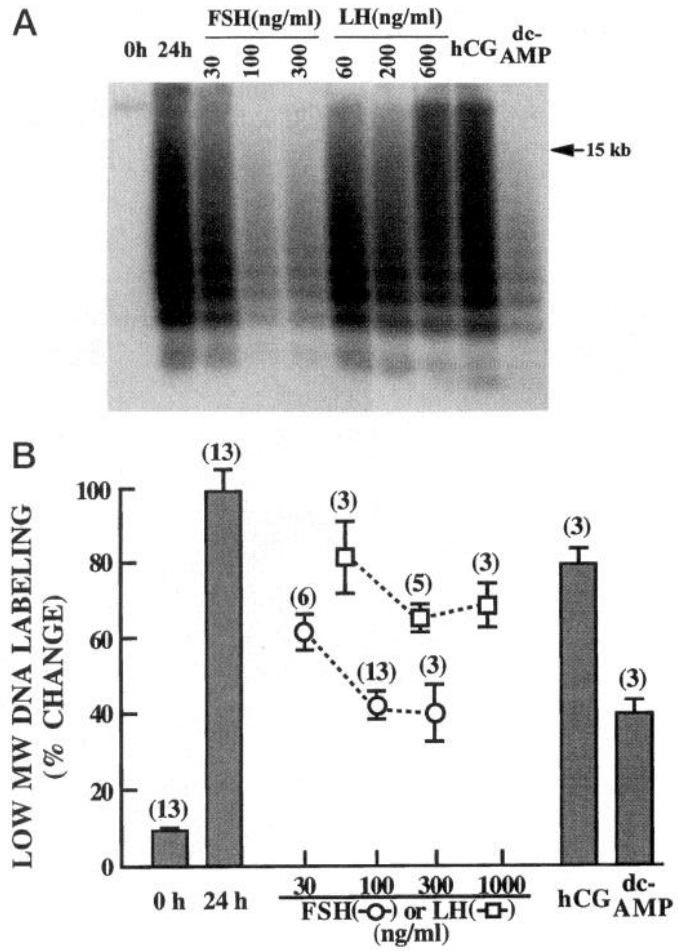

FIG. 2. Gonadotropin-induced suppression of apoptosis in cultured early antral follicles. Early antral follicles were isolated from ovaries of DES-implanted immature rats and incubated under serum-free conditions (four per culture) for 24 h in the absence (24 h) or presence of increasing doses of FSH (30–300 ng/ml) or LH (60–600 ng/ml). Some cultures were treated with 200 ng/ml hCG or 5 mM $(Bu)_2$cAMP (dcAMP). After incubation, follicle DNA was extracted, labeled at 3′-ends with [^{32}P]dideoxy-ATP, and fractionated through 2% agarose gels (250 ng/lane). DNA fragmentation was then visualized by autoradiography (A) and quantitated by β-counting of low molecular weight (<15 kb) DNA fragments (B). *Data points* reflect the mean ± SEM of 3–13 cultures as indicated in *parentheses*. Time zero represents DNA prepared from early antral follicles before incubation.

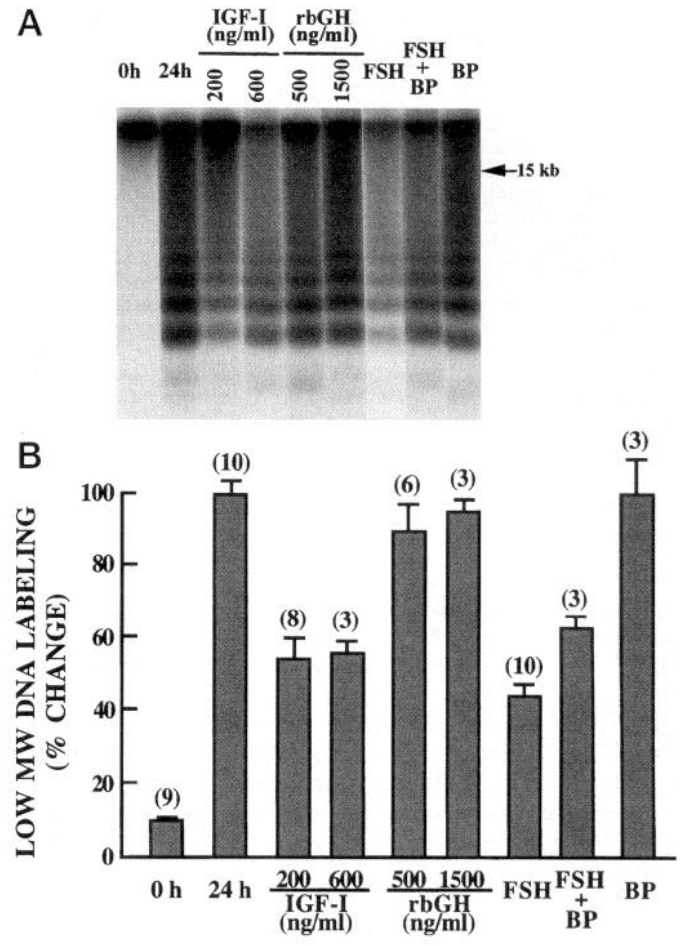

FIG. 3. Suppression of apoptosis by IGF-I and GH in cultured early antral follicles. Early antral follicles obtained from DES-treated ovaries were incubated for 24 h in the absence (24 h) or presence of IGF-I (200 or 600 ng/ml) or recombinant bovine GH (rbGH; 500 or 1500 ng/ml). In some cultures, follicles were incubated with FSH (100 ng/ml), IGFBP-3 (BP; 10 μg/ml) or a combination of FSH and IGFBP-3. Autoradiogram (A) and quantitative estimation of DNA fragmentation (B) were analyzed as described in Fig. 2. *Data points* reflect the mean ± SEM of 3–10 cultures as indicated in *parentheses*. Time zero represents DNA prepared from early antral follicles before incubation.

Activin suppression of apoptosis in cultured early antral follicles

Recent studies indicated the important role of activin in folliculogenesis *in vitro* (21). To evaluate the role of activin in follicle apoptosis, early antral follicles were cultured in the absence or presence of increasing doses (6–600 ng/ml) of activin with or without follistatin (FS; 10 μg/ml), an activin-binding protein. Incubation of follicles for 24 h with activin caused a dose-dependent reduction in the extent of apoptotic DNA fragmentation (Fig. 6A), reaching maximal suppression by 40% at 200–600 ng/ml activin ($P < 0.05$; Fig. 6B). However, no additive effect of activin and FSH on the suppression of follicle apoptosis was observed. The coincubation of FS with activin (200 ng/ml) abolished the suppressive effect of activin ($P < 0.05$) while incubation of follicles with FS alone did not alter follicle apoptosis ($P > 0.05$; Fig. 6).

Production of cAMP and progesterone by cultured early antral follicles

cAMP and progesterone production by early antral follicles during the 24 h of culture with different hormones was analyzed and is shown in Table 3. Treatment with 200 ng/ml FSH or LH resulted in 10- to 12-fold increases in both cAMP and progesterone production as compared with untreated cultures (control). In contrast, treatment with 200 ng/ml IGF-I or 500 ng/ml rbGH had no significant effect on cAMP or progesterone levels. Incubation of follicles with 150 ng/ml IL-1β or 200 ng/ml EGF significantly increased progesterone production but had no effect on cAMP production. Interestingly, treatment with bFGF (200 ng/ml) resulted in 3-fold increases in cAMP and progesterone levels.

TABLE 1. Effect of treatment with rbGH on IGF-I mRNA levels in cultured early antral and preovulatory follicles

Treatment	N	IGF-I mRNA (amol/μg DNA)
Early antral follicle		
Control	5	7.77 ± 1.61
rbGH (500 ng/ml)	5	17.54 ± 2.36[a]
Preovulatory follicle		
Control	3	8.54 ± 0.73
rbGH (500 ng/ml)	4	13.00 ± 1.36[a]

Early antral follicles from DES-implanted immature rats or preovulatory follicles from eCG-primed immature rats (4, 5) were cultured in serum-free medium for 24 h in the absence (Control) or presence of rbGH. The levels of IGF-I mRNA transcripts were determined by a solution hybridization assay as described in *Materials and Methods*. N represents the number of independent follicle cultures analyzed. Data are presented as the mean ± SEM.

[a] $P < 0.05$ compared to corresponding control group without rbGH treatment.

Discussion

Atresia, the degeneration of ovarian follicles, occurs at all stages of follicle growth and development (10, 11). However, the time during which follicles become most susceptible to atresia is at the early antral stage (12- 14). Using cultured preovulatory follicles, we have reported the regulation of follicle apoptosis by gonadotropins and several intraovarian regulators (4- 6). However, considering the massive atresia of follicles at the penultimate stage of follicle growth under physiological conditions, studies of apoptosis in early antral follicles are important. In the present study, we used *in vitro* cultures of early antral follicles to examine stage-dependent differences in the hormonal regulation of follicle apoptosis. Unlike preovulatory follicles, our results demonstrate differential potencies of different hormones on apoptosis suppression, with FSH as a major survival factor in early antral follicles.

As verified by histological sections in the present study, follicles obtained from ovaries of DES-treated immature rats exhibited characteristics of early antral follicles including their size, small antral cavity, and a thin theca layer (12, 13). Moreover, *in situ* DNA fragmentation analysis revealed the granulosa cells as a major cell type undergoing apoptosis consistent with previous *in vivo* studies using similar *in situ* analysis (15, 16, 22).

Physiological levels of FSH are capable of stimulating early antral follicles to complete their final differentiation, reaching the preovulatory stage (1, 13). In the present study, FSH treatment suppresses apoptosis of early antral follicles as it does in preovulatory follicles (4), underscoring the role of FSH as a follicle survival factor. Likewise, an analog for cAMP, a second messenger for gonadotropins (23, 24), also inhibited apoptosis. In contrast to their suppressive effect on apoptosis of preovulatory follicles, treatment with LH/hCG, however, had a marginal effect on the suppression of apoptosis in early antral follicles. These findings reflect the fact that LH/hCG receptors are restricted predominantly to theca cells of small antral follicles whereas only preovulatory follicles possess LH/hCG receptors in both the granulosa and theca cells (25–27). FSH receptors, on the other hand, are confined to the granulosa cells of healthy developing follicles (26). The marked stimulation of cAMP and progesterone levels by FSH and LH further confirm the presence of receptors for FSH and LH in these early antral follicles.

In addition to gonadotropins, the development of ovarian follicles is controlled by locally produced intraovarian reg-

TABLE 2. Determination of EGF-binding sites in early antral and preovulatory follicles

	N	EGF-binding sites (pg EGF bound/μg DNA)
Early antral follicle	9	0.68 ± 0.02
Preovulatory follicle	9	2.35 ± 0.09[a]

Early antral follicles from DES-implanted immature rats or preovulatory follicles from eCG-primed immature rats (4, 5) were isolated for the assay of EGF-binding sites as described in *Materials and Methods*. Replicate tubes of increasing numbers of follicles were incubated with a saturating dose (3 ng/ml) of [I^{125}]EGF with or without 100-fold excess of unlabeled EGF. At the end of incubation, specific binding per DNA was determined. Data are presented as the mean ± SEM.

[a] $P < 0.05$ compared to early antral follicle.

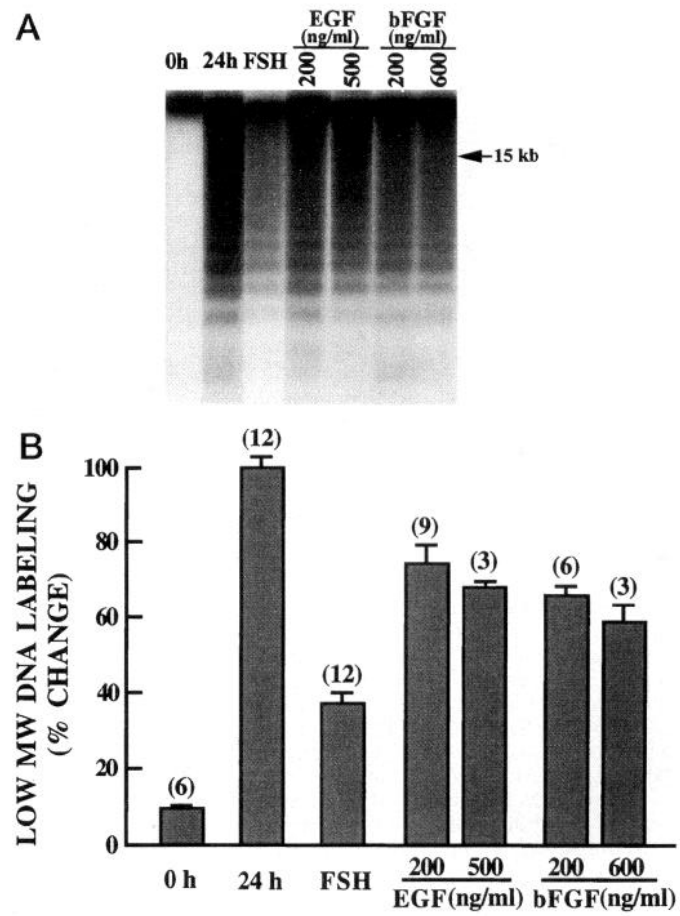

FIG. 4. Suppression of apoptosis by EGF and bFGF in cultured early antral follicles. Early antral follicles obtained from DES-treated ovaries were incubated for 24 h in the absence (24 h) or presence of either EGF (200 or 500 ng/ml), or bFGF (200 or 600 ng/ml). Autoradiogram (A) and quantitative estimation of DNA fragmentation (B) were analyzed as described in Fig. 2. *Data points* reflect the mean ± SEM of 3–12 cultures as indicated in *parentheses*. Time zero represents DNA prepared from early antral follicles before incubation.

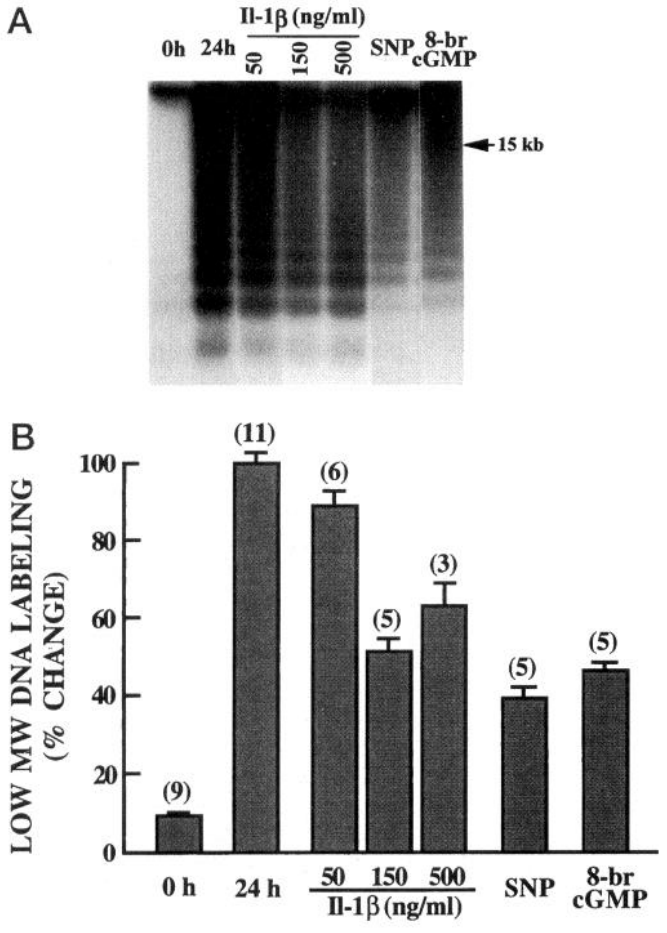

FIG. 5. Suppression of apoptosis by IL-1β, an NO generator (SNP), and a cGMP analog in cultured early antral follicles. Early antral follicles were cultured for 24 h in serum-free medium in the absence (24 h) or presence of IL-1β (50, 150, or 500 ng/ml), SNP (0.5 mM), or 8-bromo cGMP (8-br-cGMP; 5 mM). Autoradiogram (A) and quantitative estimation of DNA fragmentation (B) were analyzed as described in Fig. 2. *Data points* reflect the mean ± SEM of 3–11 cultures as indicated in *parentheses*. Time zero represents DNA prepared from early antral follicles before incubation.

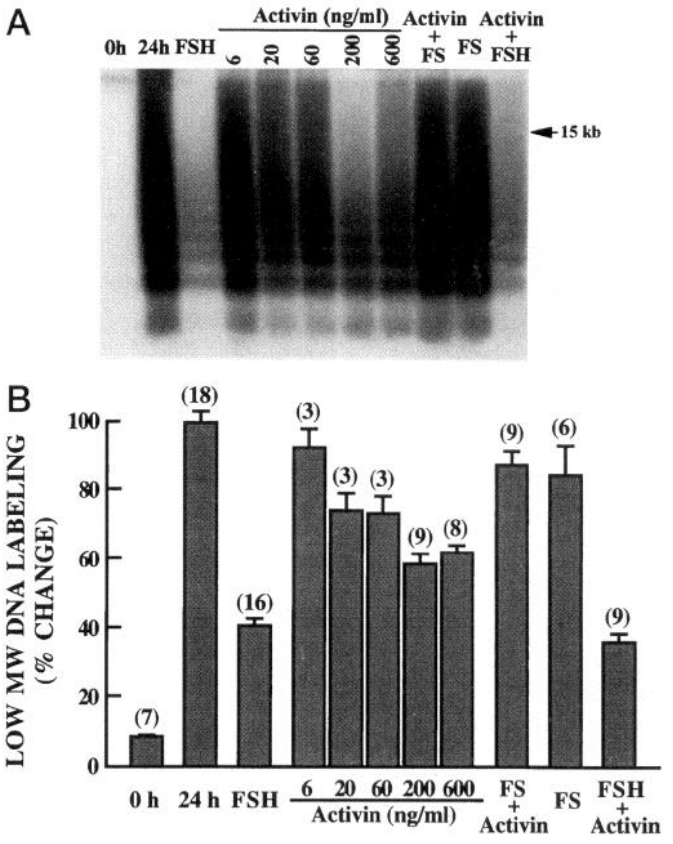

FIG. 6. Suppression of apoptosis by activin in cultured early antral follicles. Early antral follicles were cultured for 24 h in serum-free medium in the absence (24 h) or presence of increasing doses of activin (6–600 ng/ml). Some cultures were treated with 10 μg/ml follistatin (FS) alone, activin (200 ng/ml) plus FS, or activin (200 ng/ml) plus FSH (100 ng/ml). Autoradiogram (A) and quantitative estimation of DNA fragmentation (B) were analyzed as described in Fig. 2. *Data points* reflect the mean ± SEM of 3–18 cultures as indicated in *parentheses*. Time zero represents DNA prepared from early antral follicles before incubation.

ulators such as growth factors and cytokines that exert paracrine/autocrine actions (28, 29). Several growth factors including IGF-I, EGF, and bFGF have been shown to suppress follicle apoptosis as effectively as gonadotropins in cultured preovulatory follicles (4, 8). In the present study, we demonstrated that these local growth factors are less potent than FSH to inhibit the apoptosis of early antral follicles. The present observations imply that follicles become more responsive to these growth factors possibly through the acquisition of increasing numbers of receptors during the final stage of maturation.

The levels of IGF-I receptor are higher in granulosa cells from large than small antral follicles in rat (30), human (31), and bovine ovaries (32) and are up-regulated by gonadotropins. The interactions of IGF-I with its receptors are further modulated by the locally produced IGF binding proteins (IGFBPs) that have been shown to neutralize IGF-I action in the ovary (33). Our previous study demonstrate that IGFBP-3 partially blocks the suppressive action of gonadotropins on preovulatory follicle apoptosis (4). In the present study, the suppressive action of FSH on apoptosis of early antral follicles was also partially reversed by IGFBP-3, suggesting that part of the FSH action is mediated by endogenously produced IGF-I. Indeed, it has been shown that IGF-I production is increased by gonadotropins and estrogen (4, 34).

The production of rat ovarian IGF-I also appears to be GH dependent (6, 35), and the expression of GH receptor mRNA has been observed in the granulosa cells of healthy follicles in rat and human ovaries (36, 37). We have previously shown that GH suppresses preovulatory follicle apoptosis by increasing the production of follicular IGF-I (6). In the present study, we further analyzed the effect of GH on IGF-I mRNA transcripts in early antral follicles. Although GH was unable to suppress apoptosis in these follicles, it markedly increased IGF-I mRNA levels in early antral follicles, ruling out the absence of GH receptors. The present findings suggest that pathways important for GH suppression of apoptosis in preovulatory follicles is not functional in early antral follicles. Also, local IGF-I in-

TABLE 3. Production of cAMP and progesterone by cultured early antral follicles

Treatment	N	cAMP (fmol/follicle)	Progesterone (pg/follicle)
Control	16	39 ± 6	23 ± 4
FSH (200 ng/ml)	12	4162 ± 475[a]	245 ± 40[a]
LH (200 ng/ml)	5	5334 ± 225[a]	184 ± 10[a]
IGF-I (200 ng/ml)	3	39 ± 3	28 ± 12
rbGH (500 ng/ml)	3		21 ± 7
Il-1β (150 ng/ml)	5	41 ± 8	205 ± 11[a]
EGF (200 ng/ml)	5	41 ± 3	238 ± 32[a]
bFGF (200 ng/ml)	3	131 ± 9[a]	110 ± 17[a]

Early antral follicles were cultured in serum-free medium for 24 h in the absence (Control) or presence of different hormones. Levels of cAMP and progesterone in culture medium were determined by RIA. Values reflect the mean ± SEM of several independent follicle cultures as indicated by N.

[a] $P < 0.05$ compared with corresponding control value.

duced by GH is insufficient to suppress follicle apoptosis in early antral follicles.

EGF and bFGF also play an important role in the growth and differentiation of granulosa cells. Parallel with the expression of EGF, the number of EGF receptors in the granulosa and theca interna cells is higher in mature preovulatory follicles than in small antral follicles of rat and human ovaries (38, 39). Moreover, functional receptors for EGF as well as its ligand have been shown to be up-regulated by gonadotropins (38, 40). In the present study, we demonstrated that the number of EGF receptors in preovulatory follicles is much higher than that of early antral follicles. These findings may explain the higher potency of EGF on the suppression of apoptosis in preovulatory follicles (8) as compared with early antral follicles. Although changes in the expression of FGF receptors have not been demonstrated during follicle maturation, the expression of FGF receptor mRNA has been detected in developing antral follicles with higher levels in the theca cells than in the granulosa cells (41).

Activins, originally recognized for their ability to release FSH from pituitary, are dimeric glycoprotein growth factors structurally related to proteins of the transforming growth factor-β family (42, 43). Activin subunits have been found in the granulosa cells of growing follicles, and their expression increases with follicle diameter (43, 44). It thus becomes apparent that locally produced activins may play autocrine and paracrine roles in ovarian folliculogenesis (45). In undifferentiated granulosa cells, activin has been shown to enhance granulosa cell differentiation including increases in FSH receptor number, FSH-induced aromatase activity, and FSH-induced LH receptors (42, 45). Recent studies have demonstrated that activin, in the presence of FSH, causes primary follicles to develop into large preovulatory follicles *in vitro* (21). The actions of activin have been shown to be modulated by the activin-binding protein, FS, produced by granulosa cells of growing follicles in the rat ovary (45, 46). In the present study, for the first time, we demonstrate the role of activin in follicle cell survival by suppressing DNA fragmentation of early antral follicles. Furthermore, FS abolished the suppressive effect of activin on follicle apoptosis, implying that the action of activin is specific. Activin type II receptor mRNA has been found in the granulosa cells of antral follicles in the rat ovary (47). These receptors may mediate the action of activin on the suppression of follicle apoptosis.

In addition to growth factors, locally produced cytokines have also been implicated as ovarian paracrine and autocrine regulators (29). Among a growing number of cytokines, the IL-1 family has been shown to play an important role in the regulation of follicular function. In cultured preovulatory follicles, IL-1β acts as a survival factor, and its action is mediated via follicular NO and cGMP generation (5). In the present study of early antral follicles, IL-1β also suppressed follicle apoptosis, but with a 3-fold higher dose as compared with preovulatory follicles. The lower potency of IL-1β to suppress apoptosis in early antral follicles may be due to the fact that these follicles contain less differentiated theca cells than preovulatory follicles, because IL-1β is believed to exert its action through theca cell receptors (48). In contrast, a NO generator (SNP) and an analog of its second messenger, cGMP, could suppress apoptosis of early antral follicles as effective as that of preovulatory follicles. These results indicate an important role of the NO/cGMP pathway in the suppression of follicle apoptosis independent of the stage of antral follicle growth.

Our observed stimulation of cAMP and progesterone production by early antral follicles treated with FSH and LH underscores the important role of the cAMP/protein kinase A pathway in gonadotropin action (24). The lack of effect of IGF-I and GH on these functional parameters is consistent with their action through tyrosine kinase activation. Consistent with earlier findings in preovulatory follicles (5), IL-1β treatment increased progesterone production by early antral follicles. However, unlike preovulatory follicles, IL-1β did not stimulate cAMP production. Furthermore, EGF has been shown to stimulate the biosynthesis of progesterone in cultured granulosa cells without altering cAMP levels (28). The present observations in early antral follicles are consistent with these findings. In contrast, bFGF treatment increased the production of both cAMP and progesterone in early antral follicles, a finding that agrees with earlier studies using cultured granulosa cells (49, 50).

In summary, unlike preovulatory follicles in which gonadotropins, several growth factors, and IL-1β are equally potent in the suppression of apoptosis, the present studies demonstrate differential effectiveness of these hormones to inhibit apoptosis of early antral follicles with FSH > IGF-I = IL-1β > LH = EGF = bFGF = activin ≫> GH. In conclusion, the hormonal control of follicle apoptosis is stage-dependent, and FSH appears to be the major survival factor for early antral follicles, a critical stage during which a majority of follicles undergo atresia.

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