

## Apoptosis in Bovine Granulosa Cells in Relation to Steroid Synthesis, Cyclic Adenosine 3',5'-Monophosphate Response to Follicle-Stimulating Hormone and Luteinizing Hormone, and Follicular Atresia

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### ABSTRACT

Apoptosis is a process of selective cell deletion implicated as a mechanism underlying the process of ovarian follicular atresia. The aims of this study were 1) to test the hypothesis that granulosa cell death during follicular ( $\geq 4$ -mm diameter) atresia in cows occurs by apoptosis and 2) to define relationships between the occurrence and degree of granulosa cell apoptosis, cAMP response to FSH or LH, extant aromatase activity, and other previously established biochemical and morphometric indices of granulosa cell function and follicular atresia in this species. Granulosa cells and follicular fluid from individual follicles 4–18 mm in diameter were collected from luteal-phase cow ovaries. Follicles were classified by morphometric criteria as “healthy” ( $n = 45$ ) or atretic ( $n = 34$ ). Apoptosis in granulosa cells from each follicle was inferred from detection of internucleosomal DNA cleavage by 3'-end radiolabeling; it was quantitated both subjectively from intensity of oligonucleosome labeling (apoptosis [AP] score = 0, 1, 2, or 3) and objectively by  $\beta$ -counting of low-molecular-weight gel fragments (labeling index; LI). Extant aromatase activity (ng estradiol produced/ $10^6$  cells/3 h) and cAMP response (pmol/ $10^6$  cells) to different doses of FSH or LH (1–10 000 ng/ml) was determined for granulosa cells from most healthy follicles ( $n = 39$ ).

Apoptosis was detected in granulosa cells from all atretic follicles as well as from 76% of healthy follicles, from 80% (16 of 20) of follicles with follicular fluid estradiol to progesterone ratios  $> 1$ , and from 71% (10 of 14) of follicles with extant aromatase activity ( $> 2$  ng/ $10^6$  cells/3 h). For healthy and atretic follicles, degree of DNA fragmentation was inversely related to the number of granulosa cells recovered (as percentage maximum by follicle size). In healthy follicles, FSH stimulated cAMP synthesis in a dose-dependent manner in granulosa cells from all follicles examined ( $\geq 4$  mm), but only 36% of these had appreciable aromatase activity. The cAMP response to FSH (per cell) increased with follicle size from 4–7 mm in diameter and remained high in granulosa cells from follicles  $\geq 8$  mm with aromatase activity; in cells without aromatase activity, cAMP response to FSH decreased with increasing follicle size  $\geq 8$  mm. The cAMP response to LH was generally low or undetectable in granulosa cells from 4–8-mm follicles; it then increased linearly with increasing follicle diameter  $\geq 8$  mm, but to a greater degree in cells with aromatase activity than in cells without. Despite the high incidence of apoptosis in healthy follicles, LI was not related to aromatase activity, cAMP response to FSH or LH, or follicle size and did not influence relationships between these variables. However, both AP score and LI were negatively related to follicular fluid estradiol and positively related to follicular fluid progesterone levels.

In conclusion, these results are consistent with the hypothesis that death of granulosa cells during follicular atresia in cows occurs by apoptosis. However, the results also suggest that granulosa cell apoptosis may occur in healthy follicles during the luteal phase of the estrous cycle and/or very early in the process of atresia, before other morphological or biochemical changes are detected.

### INTRODUCTION

More than 99% of all ovarian follicles in the cow are destined never to ovulate, but undergo atresia at various stages of follicular development [1]. In antral bovine follicles  $> 1$  mm in diameter the earliest and most prominent feature of atresia is death of granulosa cells, leading to almost total destruction of the granulosa cell layer lining the inner follicle wall [2]. Recent biochemical evidence has demonstrated that granulosa cell death during follicular atresia in chicken, pig, and rodent ovaries occurs by apoptosis—an active, intrinsic, genetically governed process of selective cell deletion [3–10]. Apoptosis has also been implicated as the mechanism underlying structural regression of the CL in ewes and cows [11, 12], the degeneration of ovarian surface epithelial cells during the process of ovulation in ewes [13], the loss of primordial germ cells [14],

and atresia of primordial and preantral follicles during neonatal development [15] in the mouse. Thus a common mechanism appears to underly the ultimate fate of both germ cells and somatic (e.g., granulosa) cells within the ovary. From these and more extensive studies on other cell types (particularly hemopoietic and immune cells) it appears that apoptosis is a ubiquitous and highly conserved process that is subject to control by many of the same factors that regulate cell-cycle progression, transformation, and differentiation, the net effect of which determines whether an activated cell divides, differentiates, or dies [15, 16].

A unique biochemical event considered to be characteristic of apoptotic cell death is the internucleosomal cleavage of genomic DNA into fragments, 180–200 bp in size, that separate into a distinctive ladder-like pattern on agarose gel electrophoresis [17]. Detection of this pattern of oligonucleosomes in DNA provides a marker of apoptotic cell death and forms the basis of methods for both qualitative and quantitative analysis of apoptosis in granulosa cells collected from individual ovarian follicles [4, 8].

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Earlier studies in our laboratory suggested that failure to stimulate granulosa cell aromatase activity (the ability to metabolize androgens to estrogen) or a loss of this activity in follicles  $\geq 5$  mm in diameter may be an early event in follicular atresia, preceding any degenerative changes in the theca interna [18, 19]. In ewes, low aromatase activity has been associated with a reduced capacity of granulosa cells to respond to FSH and LH by producing cAMP, which is thought to mediate intracellular actions of these hormones when they interact with specific plasma membrane receptors [20, 21]. Measurement of cAMP production by granulosa cells challenged with FSH or LH in vitro may therefore provide a measure of the responsiveness of granulosa cells to gonadotropin stimulation, but this has not previously been demonstrated in cows.

The aims of the present study were 1) to test the hypothesis that granulosa cell death during follicular ( $\geq 4$ -mm diameter) atresia in cows occurs by apoptosis. 2) to determine whether bovine granulosa cells respond in vitro to FSH or LH by producing cAMP, and 3) to define relationships between the occurrence and degree of granulosa cell apoptosis, cAMP response to FSH or LH, and other previously established biochemical and morphometric indices of granulosa cell function and follicular atresia in this species.

## MATERIALS AND METHODS

### *Recovery of Granulosa Cells and Follicle Classification*

Ovaries were collected postmortem at a local abattoir from mixed-age cows in the luteal stage of the estrous cycle, as assessed from gross morphology of CL [22] and the absence of palpable products of pregnancy, and were transported to the laboratory in chilled Medium A, which is composed of Dulbecco's Modified Eagle Medium (with glucose and L-glutamine, without bicarbonate; Gibco, Grand Island, NY) containing 20 mM Hepes buffer (Sigma, St. Louis, MO), 0.1% (w/v) BSA (reagent grade; Immuno Chemical Products Ltd., Auckland, New Zealand), and 50  $\mu\text{g}/\text{ml}$  gentamicin (Sigma). Individual antral follicles  $\geq 4$  mm in diameter were dissected free from extraneous tissue under a stereomicroscope and their diameters recorded to the nearest 0.5 mm. Tissues were kept in chilled media on ice, except for brief periods while under the microscope, and processed within 2–5 h of slaughter. Follicles were slit open; follicular fluid and granulosa cells were collected separately as previously described; and follicles were classified as healthy (grade 1a) or atretic according to previously established morphometric criteria [18, 19]. "Healthy" follicles had a vascular and pink- or red-colored theca interna, a healthy-looking oocyte, no cellular debris in follicular fluid, and  $>75\%$  of the maximum number of granulosa cells recovered for a given follicle size ( $>75\% G_{\text{max}}$ ). Follicles that did not satisfy any one of these criteria were classified as

atretic. Follicular fluid and cells from each follicle were processed individually.

Two experiments were conducted. In experiment 1, both healthy and atretic follicles ranging in size from 4 to 18 mm in diameter ( $n = 30$ ) were collected from the ovaries of 8 cows. Granulosa cells were collected in 1 or 2 ml of  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer (20 mM Tris, 140 mM NaCl, 5 mM KCl, 2 mM EDTA, pH 7.4; TNKE) and snap frozen to  $-70^\circ\text{C}$  within 2–3 min of follicle rupture for subsequent DNA extraction (after a sample had been removed for estimating cell number using a hemocytometer). In experiment 2, only follicles classified as healthy ( $n = 39$ , from 23 cows) were used. In this experiment, granulosa cells were collected from follicles  $\geq 4$  mm in 1 or 2 ml Medium A, without antibiotic, with 0.2 mM 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor; Sigma [Medium B]). A 300–400- $\mu\text{l}$  aliquot of cells from each follicle was pipetted immediately after collection into a 1.5-ml microfuge tube containing 6  $\mu\text{l}$  0.5 M EDTA and snap frozen to  $-70^\circ\text{C}$  for subsequent DNA extraction. A second aliquot was removed for counting in a hemocytometer, and the remaining cells were diluted to 10 ml, washed, and diluted to 200 000 cells per ml in Medium B (at  $4^\circ\text{C}$  or on ice) for determination of extant aromatase activity and cAMP response to FSH and LH.

### *Determination of Granulosa Cell Aromatase Activity and cAMP Response to FSH and LH (Experiment 2)*

For determination of aromatase activity, 0.5-ml amounts of granulosa cell suspensions (prepared from each follicle as described above) were aliquoted in triplicate into 12  $\times$  75-mm plastic test tubes that were semi-immersed in ice water and contained 0.5 ml of either Medium B alone (control) or 0.5 ml of Medium B + testosterone (2  $\mu\text{g}/\text{ml}$ ). Tubes were capped and either snap frozen to  $-70^\circ\text{C}$  (controls) or incubated at  $37^\circ\text{C}$  in a shaking water bath for 3 h and then snap frozen to  $-70^\circ\text{C}$ . Later, all tubes were stored at  $-20^\circ\text{C}$  until assayed for estradiol-17 $\beta$  ( $\text{E}_2$ ). Previous studies have determined the production rate of  $\text{E}_2$  by granulosa cells from healthy bovine follicles under similar conditions to be maximal and not enhanced by provision of additional androgen substrate or culture under perfusion conditions, and also to be linear over a 3-h incubation period [18, 19].

Granulosa cell cAMP response to ovine FSH (1, 10, 100, 1000, and 10 000 ng/ml final concentration, Ovagen, batch #1243; Immuno Chemical Products Ltd., Auckland, New Zealand; biopotency  $7.4 \times \text{NIH-oFSH-S1}$ , 0.013% [w/w] contamination with LH) and bovine LH (1, 10, and 100 ng/ml USDA-bLH-B5, supplied by D.J. Bolt, USDA, Bethesda, MD) was determined. Duplicate or triplicate 0.5-ml aliquots of granulosa cell suspensions (as above) were added to 0.5-ml amounts of the respective doses of each hormone dissolved in Medium B in 12  $\times$  75-mm tubes semi-immersed in ice water. Tubes were capped and incubated at  $37^\circ\text{C}$  in a shaking water bath for 45 min and then heated to  $80^\circ\text{C}$  for 15 min and frozen to  $-20^\circ\text{C}$  until assayed for cAMP. For

controls, additional tubes (in triplicate) containing cells but no hormone were incubated at 37°C for 0 or 45 min and then heated to 80°C for 15 min and frozen as above.

#### *Granulosa Cell DNA Extraction and Quantification*

It was most convenient to work with 300–400- $\mu$ l amounts of granulosa cell suspensions, containing 1–5 million cells, in 1.5-ml microfuge tubes. The method described here is for a 400- $\mu$ l volume. Cells in either TNKE buffer (experiment 1) or Medium B (experiment 2) were thawed in a cold water bath with gentle shaking and then placed on ice. Without delay, 8  $\mu$ l of proteinase K (10 mg/ml; Boehringer Mannheim NZ Ltd., Auckland, New Zealand) and 20  $\mu$ l of SDS (10% w/v) were added while tubes were gently agitated, and samples were incubated at 50°C for 3 h with occasional mixing. Samples were then placed on ice, and 50  $\mu$ l (1/8 vol) of 8 M potassium acetate was added. Samples were vortexed vigorously for 10 sec, incubated on ice for 60 min, and then centrifuged at 5000  $\times$  g for 10 min at 4°C to pellet cellular debris. Supernatants were aspirated using a wide-bore pipette tip and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0); this was followed by a second extraction with an equal volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated by adding 2.5 vol ice-cold 100% ethanol and incubating at –70°C for at least 60 min, and then collected by centrifugation at 15 000  $\times$  g for 30 min at 4°C. Supernatants were discarded and pellets dried under vacuum for 10–15 min; the pellets were then resuspended in 300  $\mu$ l TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). To this mixture, 6  $\mu$ l RNase A (1 mg/ml; Boehringer Mannheim) that had previously been boiled for 5 min to destroy DNase activity was added; samples were incubated at 37°C for a further 60 min before being extracted with phenol/chloroform/isoamyl alcohol and then chloroform/isoamyl alcohol as before. DNA was then precipitated in 2.5 vol of 100% ethanol and 1/10 vol of 3 M sodium acetate and collected as before. It was then washed with 200  $\mu$ l ice-cold 80% ethanol, dried under vacuum for 15 min, and resuspended in 100  $\mu$ l sterile H<sub>2</sub>O by gentle agitation overnight at 4°C. The concentration of DNA recovered was determined by fluorescence assay using 4',6-diamidino-2-phenylindole (DAPI; Sigma) essentially as previously described [23]. The DAPI solution (100 ng/ml) was prepared in TNE buffer (10 mM Tris, 100 mM NaCl, 10 mM EDTA, pH 7.0) that had been filtered through a 0.45- $\mu$ m filter to remove any undissolved or particulate matter. DNA standards were prepared from calf thymus DNA (Boehringer Mannheim) diluted in sterile H<sub>2</sub>O and calibrated by absorption spectroscopy ( $A_{260}$ ). Samples or standards were added (in <1/10th vol, in duplicate) directly to 400  $\mu$ l DAPI solution in microcuvettes, and fluorescence was measured in a Perkin-Elmer LS50 Luminescence Spectrometer with excitation and emission wavelengths of 350 nm and 460 nm, respec-

tively. The assay was conducted at room temperature and had a linear range of 2 to 1000 ng DNA.

#### *DNA 3'-End Labeling and Gel Electrophoresis*

The method used for 3'-end labeling and subsequent purification of DNA fragments was essentially as described previously [8]. Briefly, DNA (500 ng in 29  $\mu$ l sterile water) from granulosa cells collected from individual bovine follicles was radiolabeled with 50  $\mu$ Ci [ $\alpha$ <sup>32</sup>P]-dideoxy ATP (3000 Ci/mmol; Amersham, Buckinghamshire, UK) using 25 U terminal transferase (TdT) in 5 mM cobalt chloride, 200 mM potassium cacodylate, 25 mM Tris-HCl, and 0.25 mg/ml BSA, pH 6.6 (all supplied by Boehringer Mannheim), in a total reaction volume of 50  $\mu$ l in 1.6-ml screw-cap microfuge tubes (Costar, Cambridge, MA). Cobalt chloride, buffer (both 5-strength concentration), radionucleotide, and TdT enzyme were combined immediately before being added to DNA samples as a single aliquot. The labeling reaction was conducted at 37°C for 60 min and stopped by the addition of 5  $\mu$ l 0.25 M EDTA. Labeled DNA was then purified from free nucleotide by two successive ethanol precipitations in the presence of 0.2 vol 10 M ammonium acetate and 50  $\mu$ g yeast tRNA as carrier. After the second ethanol precipitation, DNA was washed with 250  $\mu$ l 80% ethanol, dried in a heating block at 60°C for 10–15 min, resuspended in 40  $\mu$ l TE buffer, and stored at –20°C prior to electrophoresis the next day. Centrifugations during this purification procedure were at 15 000  $\times$  g for 20 min at 4°C.

DNA was size fractionated by electrophoresis in 1.8% (w/v) agarose gels for 90–120 min at 95 V using TBE (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.0) as running buffer and using a 123-bp DNA ladder (Gibco) that had been 3'-end labeled as above as molecular weight  $M_r$  marker. Unlabeled DNA from some follicles was loaded at 2–4  $\mu$ g/lane in loading buffer (5-strength = 50% glycerol, 0.5% SDS, 50 mM EDTA, and 0.1% [w/v] bromophenol blue), stained with ethidium bromide after electrophoresis, and photographed under UV light. For radiolabeled DNA, 10  $\mu$ l of 5-strength loading buffer was added to all samples (in 40  $\mu$ l TE buffer, as above), and duplicate 12.5- $\mu$ l aliquots (containing an estimated 100 ng DNA) were run per lane. After electrophoresis, gels were carefully drained and transferred onto four thicknesses of Whatman (Clifton, NJ) 3MM chromatography paper and dried in a slab gel drier at 50°C for 1 h. Dried gels were sealed in plastic wrap and exposed to Kodak X-Omat AR films (Eastman Kodak Co., Rochester, NY) at –70°C for 30–60 min. The amount of radiolabeled ddATP incorporated into low  $M_r$  (<4.2 kb) DNA fractions was quantitated by cutting individual lanes containing these fractions from dried gels and measuring the radioactivity (cpm) of these immersed in a liquid scintillation cocktail in a  $\beta$ -counter. A labeling index (LI) was calculated as log<sub>10</sub> cpm for each replicate. The coefficient of variation (cv%) for this LI between labeling reactions was 20.8%, between duplicate lanes of the same sample run on different gels

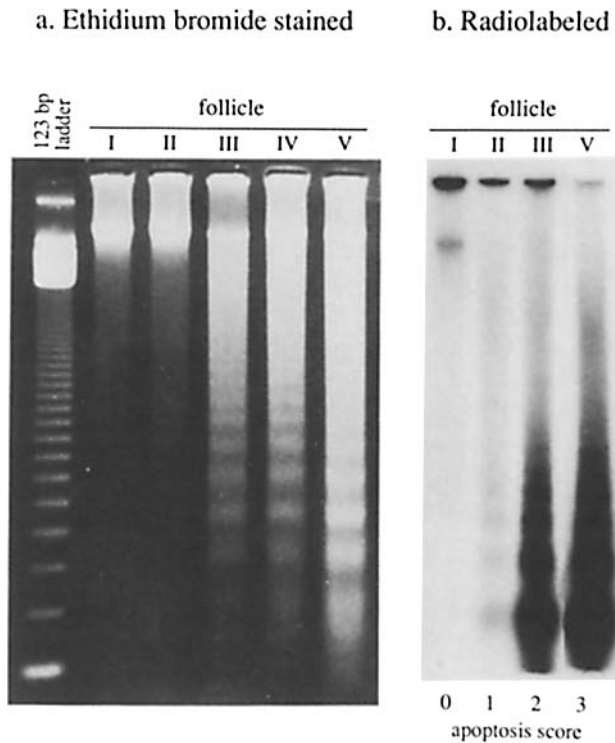


FIG. 1. Oligonucleosome formation in DNA isolated from granulosa cells from individual bovine follicles  $\geq 4$  mm diameter, size fractionated by agarose gel electrophoresis and (a) stained with ethidium bromide (2–4  $\mu$ g DNA/lane) or (b) radiolabeled at 3'-ends and autoradiographed (DNA from the same follicles; 100 ng/lane). Degree of oligonucleosome formation was subjectively classified from intensity of radiolabeling as undetected, slight, moderate, or marked (AP score = 0, 1, 2, 3, respectively).

was 9.3%, and between duplicate lanes run on the same gel was 6.7%.

#### RIA of Steroid Hormones and cAMP

$E_2$  and progesterone ( $P_4$ ) levels in follicular fluid (diluted 10–100-fold in 0.1 M PBS, pH 7.2) and  $E_2$  levels in culture medium (undiluted) were measured directly, without extraction, using previously published RIA procedures [18, 24]. The detection limits for  $E_2$  and  $P_4$  were 5 pg and 20 pg/assay tube, respectively; and intra- and interassay coefficients of variation were  $<10\%$ . Granulosa cell aromatase activity from cell culture samples was expressed as ng  $E_2$  produced per  $10^6$  cells per 3-h culture period.

The cAMP RIA was as previously described [25], except that separation of bound from free fractions was by second-antibody reaction followed by the addition of 2.5 vol 5% (w/v) polyethylene glycol 8000 (Union Carbide Corp., Danbury, CT) just before the final centrifugation. Cell culture samples were thawed and assayed directly, giving a measure of total (both intra- and extracellular) cAMP content as previously determined [25]. The detection limit was 1 fmol/assay tube, and intra- and interassay coefficients of variation were 7.8% and 11.9%. The cAMP results were expressed as pmol/ $10^6$  cells.

#### Statistical Procedures

The General Linear Models procedure of the Statistical Analysis System [26] was used for all analyses of variance, and regression (SAS Type III) sums of squares were used for all hypothesis tests. Linear and quadratic relationships between continuous variables were examined using standard least-squares multiple regression procedures. Where indicated in results, data were transformed to conform with assumptions of analysis of variance that error be normally distributed with constant variance.

Dose-response relationships between FSH or LH and cAMP, and comparison of these between groups classified by follicle size, presence of aromatase activity, and evidence of oligonucleosome formation (as indicated in *Results*), were analyzed by repeated measures analysis of variance using multivariate tests for comparison of dose effects. Near-maximal cAMP response to FSH and LH were used in multiple regression analyses to examine possible relationships between these, follicle size, aromatase activity, and labeling index as continuous variables. Regression coefficients quoted ( $R^2$ ) are adjusted for the number of independent variables included in the model. Logistic regression and analysis of deviance [27] were used to test whether proportions of follicles with follicular fluid  $E_2:P_4$  ratios  $\leq 1$  varied with degree of granulosa cell oligonucleosome formation, classified according to a subjective scoring system (detailed below).

## RESULTS

#### Evidence of Granulosa Cell Apoptosis

Distinct ladder-like patterns of DNA fragmentation characteristics of apoptosis were evident by ethidium bromide staining and/or 3'-end labeling in granulosa cells from all follicles ( $\geq 4$  mm in diameter) that were classified morphometrically as atretic (experiment 1,  $n = 24$ ) (Fig. 1, a and b). Degree of oligonucleosome formation was subjectively classified from intensity of radiolabeling as undetected, slight, moderate, or marked (apoptosis [AP] score = 0, 1, 2, 3, respectively) (Fig. 1b). No evidence of oligonucleosome formation was detected by either method in granulosa cells from 24% of follicles classified morphometrically as "healthy" (experiments 1 and 2 combined,  $n = 45$ ). In the remaining healthy follicles, the AP score ranged from 1 (slight) to 3 (marked), with approximately equal proportions classified in each of these categories. The AP score was strongly associated with level of radioactivity measured in low  $M_r$  gel fragments ( $\log_{10}$  cpm = LI: Spearman rank correlation coefficient = 0.92). Mean LI was significantly higher for granulosa cells from follicles classified morphometrically as atretic than in follicles classified as healthy (combined data,  $F_{(1,67)} = 33.47$ ,  $p < 0.001$ ). The 3'-end labeling method detected oligonucleosomes in some samples that were negative by ethidium bromide staining

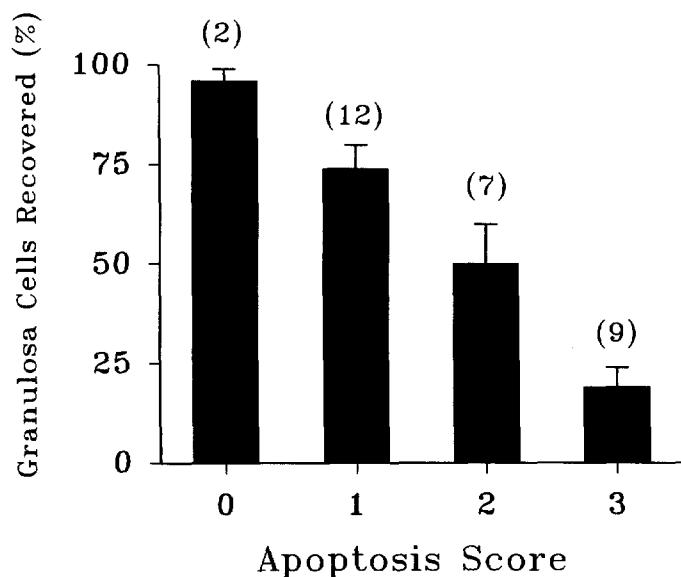


FIG. 2. Percentage of granulosa cells recovered (relative to the maximum recoverable for a given follicle size) from individual healthy or atretic bovine follicles in relation to degree of oligonucleosome formation in granulosa cell DNA, subjectively classified as undetected, slight, moderate, or marked (AP score = 0, 1, 2, 3, respectively). The number of follicles contributing to each mean is shown in parentheses (n).

and required less than 1/20th of the quantity of DNA. Follicles in which oligonucleosomes were not detected were all  $\geq 7$  mm in diameter.

In experiment 1, in which cells were recovered from follicles that ranged from healthy to severely atretic, the degree of DNA fragmentation was inversely related to the percentage of granulosa cells recovered ( $\%G_{max}$ ) from individual follicles when examined both within subjective categories (Fig. 2) and by linear regression of LI ( $\log_{10} \text{ cpm} = 5.35 \pm 0.13 - 0.010 \pm 0.002 \times \%G_{max}$ ,  $R^2 = 0.44$ ). Thus, the loss of granulosa cells associated with the process of ovarian follicular atresia was associated with a progressive increase in evidence of granulosa cell DNA degradation characteristic of apoptotic cell death.

#### Cyclic AMP Response to FSH and LH, and Aromatase Activity

In experiment 2, FSH and LH stimulated cAMP synthesis in a dose-dependent manner in granulosa cells from 100% and 48%, respectively, of individual healthy bovine follicles 4–14 mm in diameter ( $n = 39$ ; Fig. 3). Near-maximal cAMP responses measured were stimulated by 1000 ng/ml FSH or 100 ng/ml LH; these responses ranged from 1.2 to 52.8

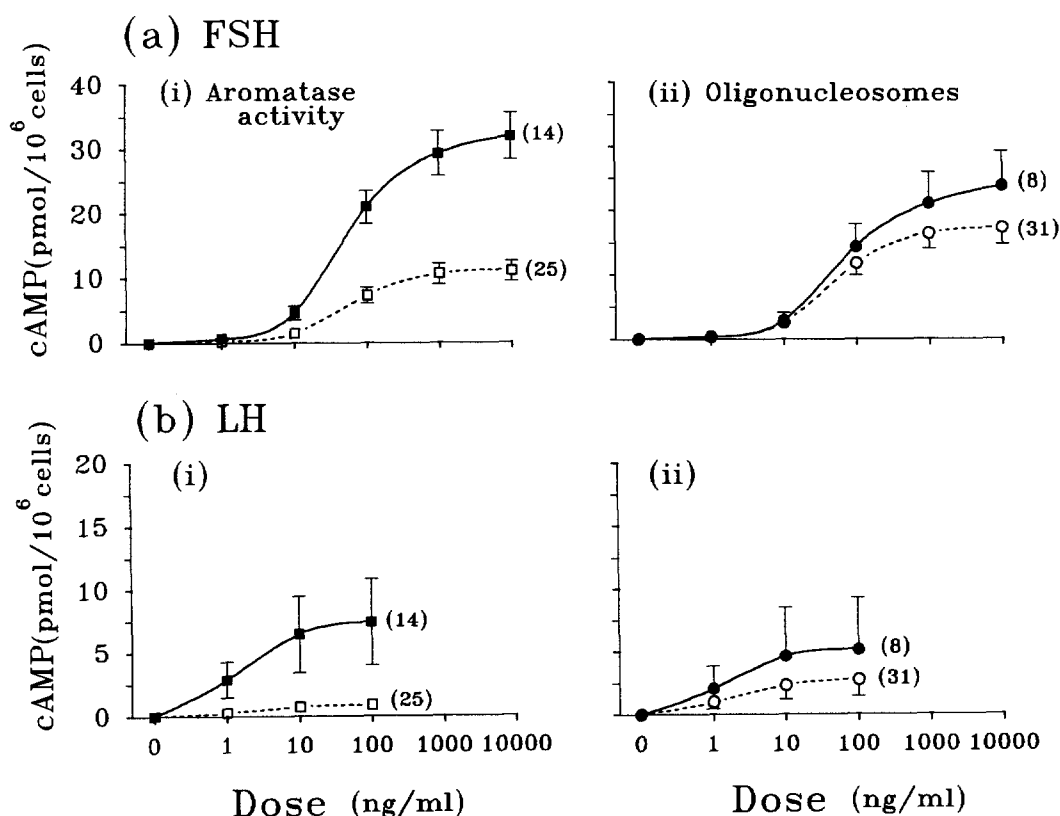


FIG. 3. Granulosa cell cAMP response (mean  $\pm$  SEM) to increasing doses of (a) FSH (Ovagen, biopotency  $7.4 \times \text{NIH-oFSH-S1}$ ), or (b) LH (USDA-bLH-B5), measured in cells from individual "healthy" follicles classified according to (i) aromatase activity (present [solid squares] or absent [open squares]) or (ii) presence of oligonucleosomes (detected [solid circles] or not detected [open circles]) in granulosa cell DNA. The number of follicles contributing to each mean is shown (n). Standard errors not evident are obscured by symbols.

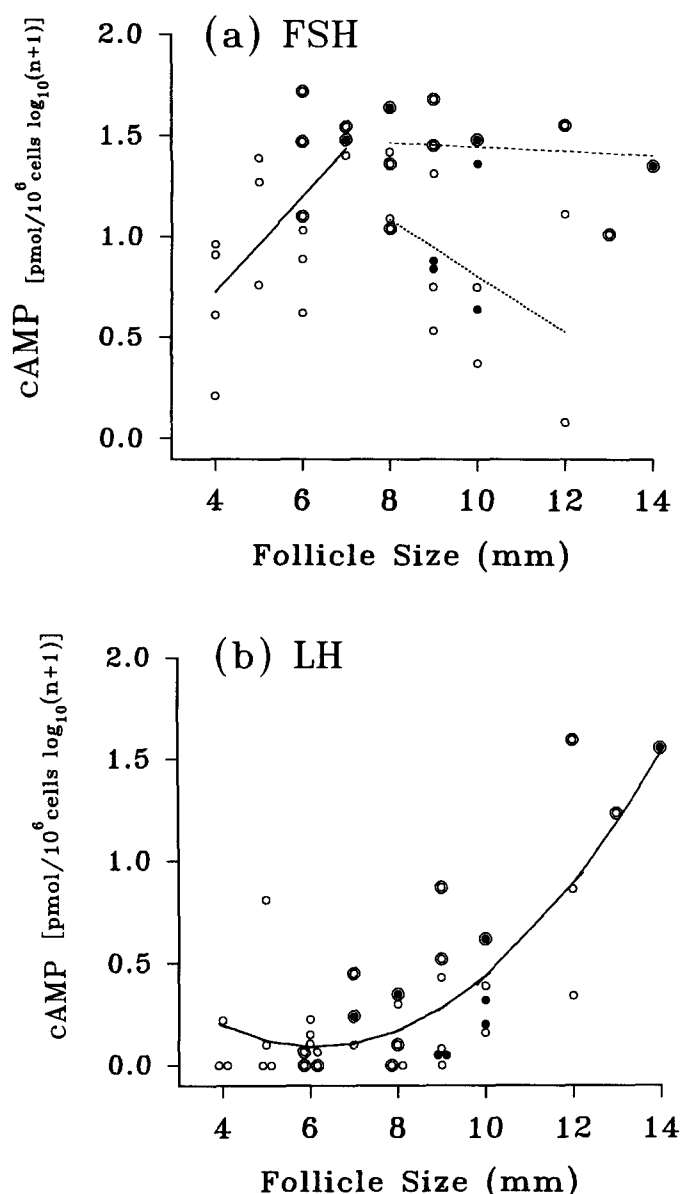


FIG. 4. Near-maximal cAMP response to (a) FSH (1000 ng/ml Ovagen, biopotency  $7.4 \times \text{NIH-}\alpha\text{FSH-S1}$ ) or (b) LH (100 ng/ml USDA-bLH-B5), measured in granulosa cells from individual "healthy" follicles in relation to follicle diameter. Data from each follicle are further classified according to whether cells had aromatase activity  $>2 \text{ ng}/10^6 \text{ cells}/3 \text{ h}$  [large open circles] and evidence of apoptosis (oligonucleosomes detected [small open circles] or not detected [solid circles]). In figure (a), regression lines are for follicles 4–7 mm in diameter (solid line),  $\geq 8 \text{ mm}$  with aromatase (dashed line), or  $\geq 8 \text{ mm}$  without aromatase activity (dotted line).

and from 0.2 to  $38.1 \text{ pmol}/10^6 \text{ cells}$ , respectively, in cells from individual follicles. At these doses, LH stimulated an appreciable cAMP response ( $>2 \text{ pmol}/10^6 \text{ cells}$ ) in granulosa cells from fewer follicles than did FSH (21% vs. 95%, respectively). All follicles with granulosa cells that produced cAMP in response to LH showed a cAMP response to FSH. However, near-maximal responses to LH were generally more than 10-fold lower in magnitude in all except

the six most responsive follicles (which ranged in size from 5 to 14-mm in diameter); in these six follicles, magnitudes of cAMP response to LH and FSH were similar.

Although granulosa cells from the great majority of healthy follicles produced appreciable amounts of cAMP in response to FSH, only 36% showed appreciable aromatase activity ( $>2 \text{ ng}/10^6 \text{ cells}/3 \text{ h}$ ); and in these follicles, both the granulosa cell cAMP response to LH and the presence and degree of oligonucleosome formation were highly variable. Granulosa cells with aromatase activity produced cAMP responses to FSH of greater magnitude ( $F_{(1,37)} = 34.71$ ,  $p < 0.001$ ) but similar  $ED_{50}$  (the dose of FSH required to elicit half-maximal responses within each group, being  $52.6 \pm 5.9$  vs.  $55.7 \pm 1.3 \text{ ng/ml}$ ) compared with cells without appreciable aromatase activity (Fig. 3a). All follicles with aromatase activity had a near-maximal cAMP response to FSH  $>10 \text{ pmol}/10^6 \text{ cells}$ , and all follicles that had a cAMP response to FSH  $\leq 10 \text{ pmol}/10^6 \text{ cells}$  lacked aromatase activity. However, a cAMP response to FSH  $>10 \text{ pmol}/10^6 \text{ cells}$  was also evident in 10 of 25 follicles that lacked aromatase activity.

The cAMP response to LH was also greater overall in cells with aromatase activity than in cells without aromatase activity ( $F_{(1,37)} = 7.82$ ,  $p \leq 0.008$ ; Fig. 3b). Analyses of LH dose response were performed on  $\log_{10}$ -transformed data to control for unequal variance, but the nature of the data precluded meaningful comparison of the  $ED_{50}$ s. A significant cAMP response to LH was detected in 9 of 14 follicles with aromatase activity, and aromatase activity was present in the majority of follicles (6 of 8) that produced appreciable amounts of cAMP in response to LH ( $>2 \text{ pmol}/10^6 \text{ cells}$ ).

The near-maximal cAMP responses to FSH and LH (both  $\log_{10} [n + 1]$ ) were used in multiple regression analyses to examine possible relationships between these, follicle size, aromatase activity, and LI as continuous variables. On a per-cell basis, the near-maximal cAMP response to FSH increased with increasing follicle size up to 7 mm in diameter ( $p \leq 0.014$ ); this response remained high in granulosa cells from larger follicles with aromatase activity, but decreased with increasing follicle size in cells without aromatase activity (difference in regression slopes due to the presence of aromatase,  $t_{(1,21)} = 4.30$ ,  $p < 0.001$ ; Fig. 4a). In contrast, the near-maximal cAMP response to LH was generally low or undetectable in granulosa cells from 4.8-mm follicles; it then increased linearly with increasing follicle diameter  $\geq 8 \text{ mm}$  (Fig. 4b), but to a greater degree in cells with, than in cells without, appreciable aromatase activity (difference in regression slopes,  $t_{(1,21)} = 4.83$ ,  $p < 0.001$ ).

When data were log transformed, aromatase activity was positively, but weakly, correlated with cAMP responses to both FSH ( $R^2 = 0.34$ ,  $p < 0.001$ ) and LH ( $R^2 = 0.37$ ,  $p < 0.001$ ) when examined separately; however, aromatase activity increased more predictably as a linear function of these two variables together ( $R^2 = 0.56$ ; comparison of regres-

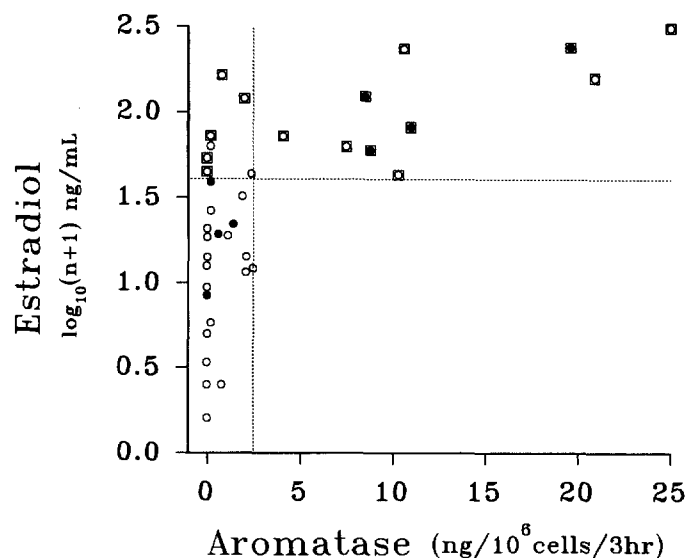


FIG. 5. Follicular fluid estradiol concentrations in relation to granulosa cell aromatase activity in individual "healthy" follicles 4–14 mm in diameter. Data from each follicle are further classified on the basis of evidence of granulosa cell apoptosis (oligonucleosomes detected [open circles] or not detected [solid circles]) and whether follicular fluid estradiol to progesterone ratios were  $>1$  (open squares). Grid lines correspond to 40 ng/ml estradiol, and 2.5 ng/ $10^6$  cells/3 h aromatase activity.

sion model sums of squares,  $F_{[1,37]} > 16.7$ ,  $p < 0.001$ ). This relationship was independent of follicle size.

#### Follicular Fluid Estradiol and $P_4$ Levels

Follicular fluid  $E_2$  levels increased with increasing follicle size in follicles classified as healthy ( $p < 0.001$ ) or atretic ( $p \leq 0.045$ ), but were 10-fold higher on average in follicles that were classified as healthy ( $p < 0.001$ ; data not shown). In contrast, follicular fluid  $P_4$  levels did not vary with follicle size, but were higher in follicles classified as atretic than in follicles classified as healthy ( $p \leq 0.026$ ), being 81 (58, 113) vs. 52 (41, 65) ng/ml, respectively (geometric means and 95% confidence intervals). Follicular fluid  $E_2$  levels also increased linearly with granulosa cell aromatase activity ( $R^2 = 0.67$ ,  $p < 0.001$ ). All follicles with aromatase activity  $> 2.5$  ng/ $10^6$  cells/3 h had follicular  $E_2$  levels  $> 40$  ng/ml,  $P_4$  levels  $< 80$  ng/ml, and  $E_2:P_4$  ratios  $> 1$  (Fig. 5).

#### Relationships between Granulosa Cell Function and Evidence of Apoptosis

In addition to being detected in granulosa cells from all follicles classified as atretic and from 76% of follicles classified as healthy, oligonucleosomes were evident in granulosa cells from 80% (16 of 20) of follicles with follicular fluid  $E_2:P_4$  ratios  $> 1$ , and from 71% (10 of 14) of follicles with aromatase activity. However, no linear or quadratic relationships were evident between the degree of DNA fragmentation (LI) and either the cAMP response (near maximal) to FSH or LH or the aromatase activity in granulosa

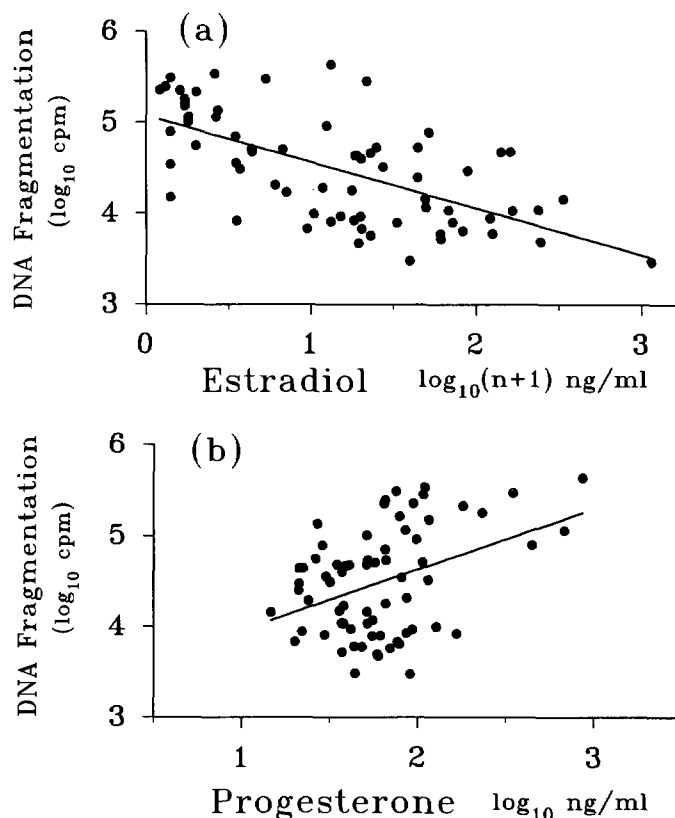


FIG. 6. DNA fragmentation (LI) indicative of apoptosis in granulosa cells from individual "healthy" or atretic bovine follicles 4–18 mm in diameter in relation to (a) estradiol ( $R^2 = 0.40$ ,  $p < 0.001$ ) and (b) progesterone concentrations ( $R^2 = 0.14$ ,  $p < 0.001$ ) in follicular fluid.

cells collected from individual healthy follicles. Similarly, the dose-response relationships between FSH or LH and cAMP were not significantly affected by the degree of oligonucleosome formation evident in granulosa cell DNA (undetected vs. slight, moderate, or marked; FSH,  $F_{[4,34]} = 1.61$ ,  $p \leq 0.195$ ; LH,  $F_{[2,36]} = 0.49$ ,  $p \leq 0.615$ ; Fig. 3, a[ii] and b[ii]). Moreover, none of the relationships described above between the cAMP response to either FSH or LH and aromatase activity or follicle size were influenced by the degree of DNA fragmentation evident in cells from these same follicles.

However, LI was linearly and negatively related to the level of  $E_2$  measured in follicular fluid ( $\log_{10}$ ) (Fig. 6a). This relationship was similar in the two experiments (combined data,  $R^2 = 0.40$ ,  $p < 0.001$ ). An independent but positive linear relationship was also evident between degree of DNA fragmentation and follicular fluid  $P_4$  levels ( $\log_{10}$ ), though this relationship was weak (combined data;  $R^2 = 0.14$ ,  $p < 0.001$ ; Fig. 6b). When considered in relation to AP score, mean follicular fluid  $E_2$  levels decreased ( $F_{[3,65]} = 6.90$ ,  $p < 0.001$ ) and  $P_4$  levels increased ( $F_{[3,65]} = 5.89$ ,  $p \leq 0.001$ ) with increasing evidence of oligonucleosome formation (data not shown).

When considered in relation to  $E_2:P_4$  ratios, LI was four-fold higher in follicles with  $E_2:P_4$  ratios  $\leq 1$  than in those with  $E_2:P_4$  ratios  $> 1$ , being 42 660 (29 520, 61 640) vs. 11 750 (7360, 18 760) (geometric means with 95% confidence intervals;  $F_{1,68} = 35.28, p < 0.001$ ). Moreover, the proportion of follicles with  $E_2:P_4$  ratios  $\leq 1$  increased linearly with increasing AP score, being 0.55, 0.60, 0.75, and 0.92 for scores 0, 1, 2, and 3, respectively (analyzed by logistic regression, change in deviance = 7.4, 1 degree of freedom,  $p \leq 0.007$ ).

## DISCUSSION

The results of the present study are consistent with the hypothesis that granulosa cell death during ovarian follicular atresia in cows occurs by apoptosis. However, our results suggest that apoptotic death of granulosa cells in the cow may also occur in healthy follicles during the luteal phase of the estrous cycle in cows and/or occur early in the atresia process before other morphological or biochemical signs of degeneration or dysfunction are evident.

Oligonucleosome formation considered to be characteristic of apoptotic cell death [16, 17] was detected in granulosa cell DNA isolated from all follicles classified as atretic, and was increasingly evident in degree with degeneration of the granulosa cell layer, reflected by decreases in the percentage of granulosa cells recovered from follicles ( $\%G_{max}$ ). Oligonucleosomes were evident in granulosa cells from follicles that ranged in diameter from 4 to 18 mm, in which the earliest and most prominent feature of atresia described in cows is degeneration of the granulosa cell layer [2]. This result provides strong evidence that granulosa cell death during atresia in the cow occurs by apoptosis, consistent with morphological [28–30] and similar biochemical evidence [3–6, 8, 10] in other species. A recent preliminary report has also described oligonucleosome formation in DNA isolated from whole bovine follicles classified histologically as atretic, after their loss of functional dominance (as indicated by detection of a new “wave” of smaller follicle growth by ultrasonography), but not in DNA isolated from preovulatory follicles collected 40 h after prostaglandin-induced luteolysis that were estrogen active and confirmed histologically to be nonatretic [31].

The presence of oligonucleosomes in DNA from granulosa cells of “healthy” follicles in our study is a novel finding. In all previous reports cited, the presence of oligonucleosomes has been confined solely to DNA isolated from follicles considered to be atretic. Aside from differences between studies in the criteria used to classify follicles, our study appears to be the first to have examined large numbers of follicles collected from cows during the luteal phase of the estrous cycle—a period during which regular patterns of large antral follicle growth and regression (without ovulation) occur (reviewed in [32]). In the only other study on cows reported to date, oligonucleosomes were not detected in dominant preovulatory follicles recovered during

a prostaglandin-induced follicular phase [31]; but the endocrine environment governing follicular growth and maturation at this stage of the estrous cycle differs markedly from that of the luteal phase. Our results are consistent with the hypothesis that granulosa cell apoptosis may occur to a limited extent at times during the normal development of antral follicles during the luteal phase of the estrous cycle in cows. An alternative, but not necessarily exclusive, hypothesis that may also explain this result is that apoptotic death of granulosa cells is a very early event in the process of atresia and is detectable before other morphological or biochemical signs of degeneration or dysfunction appear.

The radiolabeling method used in our study is sufficiently sensitive to detect oligonucleosomes in DNA isolated from as few as 10 000 cells [4, 8]. This represents from 0.25% to 0.025% of the estimated number of granulosa cells ( $G_{max}$ ) recoverable from healthy bovine follicles from 4 to 18 mm in diameter, respectively [18]. Thus detection of oligonucleosomes by this method may in some cases reflect a very low prevalence of apoptotic cell death. Although the precise relationship between the prevalence of pyknosis (identified by conventional histological staining) and apoptosis in granulosa cells is not known, descriptions of nuclear pyknosis and subsequent karyorrhexis leading to the formation of “atretic bodies” [28] are remarkably similar to descriptions of cellular and nuclear changes that occur during apoptotic cell death [17, 33]. In histological definitions of atresia applied in studies of sheep and cattle ovaries, the presence of some pyknotic cells in an otherwise intact granulosa cell layer is widely accepted in follicles considered to be nonatretic [28, 34–37]. Indeed, in a semiquantitative study on growth rates of bovine follicles, Lussier et al. [36] reported a mean prevalence of pyknotic cells of 0.13–0.67% in granulosa cell layers considered to be intact and normal in nonatretic antral follicles. In another series of studies on the development of large antral follicles during the follicular phase of the cycle in cows, 30–60% of estrogen-active (presumed preovulatory) follicles present after prostaglandin-induced or spontaneous luteolysis had some pyknotic cells present in the granulosa layer [38, 39]. Those authors argued that the mere presence of pyknotic cells in large bovine follicles did not necessarily imply that they were atretic. The actual relationship between the prevalence of pyknotic cells in the granulosa cell layer of large bovine follicles and their ability to maintain functional dominance and ovulate an oocyte capable of fertilization and normal conceptus development (sure signs of “health”) is not known in the cow. Thus, the notion that apoptotic cell death may occur, though to a limited extent, during normal medium- to large-sized antral follicle development in this species is tenable in the light of results from conventional histological studies, and this hypothesis warrants further testing.

The absence of detectable oligonucleosome formation evident in 24% of morphometrically healthy follicles in our

study, and in preovulatory follicles during the follicular phase of the estrous cycle [31], may reflect a variable incidence of apoptotic cell death at different (and as yet undefined) stages of follicular development or different phases of the estrous cycle. This may in turn reflect changes in the endocrine environment and/or in the sensitivity of individual follicles to these changes at critical stages of their development, as well as the rapid time course (2–6 h) of the cell death process and clearance of cell fragments by phagocytosis [16].

It is also possible that the high prevalence of follicles in which granulosa cell apoptosis was evident in our study could be attributed in part to postmortem change during the time that elapsed from slaughter to the collection and freezing of cells (2–5 h). However, it is unlikely that postmortem change was a major factor for the following reasons. The effect of time postovariectomy on prevalence of oligonucleosome formation in granulosa cells collected from large healthy (preovulatory) follicles has been examined previously in cows through use of methods similar to those employed in our study; no oligonucleosome formation was evident in cells collected up to 5 h postovariectomy [31]. In another study in which spontaneous onset of apoptosis was demonstrated in freshly harvested rat granulosa cells in serum-free culture, oligonucleosome formation (again detected by the same radiolabeling method that we used) was not clearly evident until 16 h after cell collection [5]. Moreover, previous experience in our laboratory has shown that the quality of granulosa cell messenger RNA and its ability to hybridize with cDNA probes in Northern blots does not differ between ovaries processed immediately after ovariectomy and those processed 2–5 h after slaughter, but does deteriorate with time if there is any delay between the time granulosa cells are dissociated after follicle rupture and the time cells are frozen (P. Greenwood, K. McNatty, D. Heath, and S. Lun, unpublished data). In our study, cells were collected within 2–5 h of slaughter, during which time tissues were maintained at 4°C or on ice (from the time of ovary collection); and once follicles were ruptured, granulosa cells were collected and snap frozen to –70°C immediately. Further studies using tissues processed immediately after ovary removal, and in situ labeling of fragmented DNA [7], may clarify this point.

Although it is tenable that granulosa cell apoptosis may occur at times during normal antral follicle development, our data are also consistent with an alternative hypothesis, that apoptotic death of granulosa cells occurs very early in the atresia process. These hypotheses are not necessarily exclusive if the distinction between “healthy” and atretic follicles involves variation in the incidence of granulosa cell death, for instance in relation to stage of follicular development and/or the presence of a CL—something that has not been defined in the cow. It is possible (even likely) that some follicles classified morphometrically as healthy in our study were in fact already in the early stages of atre-

sia. However, it is interesting that oligonucleosomes were evident in a similarly high percentage (71–80%) of follicles whether they were classified morphometrically as healthy or biochemically as estrogen active (having follicular fluid  $E_2:P_4$  ratios >1 or appreciable granulosa cell aromatase activity). Moreover, among the follicles having granulosa cells in which oligonucleosomes were not detected, the number that had appreciable aromatase activity and follicular fluid  $E_2:P_2$  ratios >1 was equal to the number that lacked aromatase activity and had follicular fluid  $E_2:P_4$  ratios  $\leq 1$ .

The relationships evident in our study between degree of granulosa cell apoptosis and follicular fluid  $E_2$  and  $P_4$  levels in healthy and atretic follicles were not particularly strong; these relationships may simply reflect mutually related changes that occur during the process of atresia. Atresia of bovine follicles has been commonly associated with low levels of  $E_2$  and high levels of  $P_4$  in follicular fluid [19, 35, 38, 39]. Similarly, granulosa cells from atretic bovine follicles were reported to produce low amounts of  $E_2$  and high amounts of  $P_4$  relative to cells from “healthy” follicles in culture [40]. Our data are consistent with the notion that failure to acquire or maintain aromatase activity and the expression of a more natural tendency to synthesize  $P_4$  are common events during follicular atresia in cows, in association with an increasing prevalence of apoptotic granulosa cell death.

Our data also demonstrate the ability of bovine granulosa cells to produce cAMP in response to both FSH and LH stimulation, presumably reflecting the presence of specific plasma membrane receptors for these hormones and their functional coupling to the adenylate cyclase/protein kinase-A second messenger system. All follicles ( $\geq 4$  mm) responded in a dose-dependent manner to FSH; this is consistent with data from other species demonstrating the presence of FSH receptors and their coupling to adenylate cyclase in granulosa cells throughout most of the course of follicular development [30]. Our findings are also consistent with earlier studies in sheep suggesting that aromatase activity of granulosa cells is regulated at least in part by the capacity of cells to produce cAMP in response to FSH [20]. Also consistent with that study was our finding that granulosa cells from some follicles had low aromatase activity but were still able to produce substantial amounts ( $>10$  pmol/ $10^6$  cells) of cAMP when stimulated with FSH, suggesting that in these cases although FSH receptors were coupled to adenylate cyclase, the adenylate cyclase was uncoupled from the aromatase enzyme system.

An interesting result in our study was that the magnitude of the cAMP response to near-maximal doses of FSH varied with both follicle size and the presence or absence of aromatase activity. On a per-cell basis, cAMP response to FSH increased with follicle size up to 7 mm in diameter; cAMP response remained high in granulosa cells from larger follicles that had aromatase activity, but decreased with increasing follicle size in cells without aromatase activity. An

increase in granulosa cell cAMP response to FSH with increasing follicle diameter (2 mm to preovulatory size) has previously been reported in sheep [20]. The maintenance or decline in cAMP response (depending on the presence of aromatase activity) that was evident in follicles  $\geq 8$  mm in our study is also consistent with earlier reports that  $^{125}\text{I}$ -oFSH binding to bovine granulosa cells decreased with increasing follicle size ( $\geq 8$  mm) and was higher in cells from estrogen-active than from estrogen-inactive follicles [38, 41].

In contrast to the FSH response, the cAMP response to LH was generally low or not detected in granulosa cells from 4–8-mm follicles; it then increased linearly with increasing follicle diameter  $\geq 8$  mm, but to a greater degree in cells with appreciable aromatase activity than in cells without. This increase in cAMP response with increasing follicle diameter  $\geq 8$  mm parallels previously reported increases in specific binding of  $^{125}\text{I}$ -hCG to bovine granulosa cells [38, 41]. A similarly low prevalence of the cAMP response compared to that of FSH and a similar apparent threshold effect of follicle size on cAMP responsiveness to LH have also been previously reported in ewes [20]. The acquisition of LH receptors by granulosa cells is thought to be a critical event during the final phase of follicular growth and differentiation (reviewed for the ewe, cow, and sow in [1]). However, the precise stage of follicular development at which granulosa cells develop functional LH receptors in cows is not known. In our study, dose-responsive increases in cAMP were stimulated by LH in granulosa cells from "healthy" follicles as small as 4.5 mm in diameter and from 9 of 29 (31%) follicles  $< 10$  mm, suggesting the presence of functionally coupled LH receptors on granulosa cells at an earlier stage of follicular development in cows than previously thought [42]. Such a maturational event may play an important role in the mechanism(s) underlying the selection and maintenance of dominant follicles in this species.

The ability of follicles to grow larger than 4.5 mm in diameter in cows, and in particular, the ability of one follicle to continue to grow and become "dominant" when all other follicles  $\geq 4$  mm regress in temporal association with declining FSH levels, may be due in part to maturational changes in the capacity of granulosa cells to respond to FSH and LH by producing cAMP and to the functional coupling of this response to the aromatase enzyme complex. The positive relationships that were evident between cAMP responses to both FSH and LH and follicle size, and the influence that aromatase activity had on these in our study, suggest that such maturational changes may indeed occur in bovine follicles between 4 and 8 mm in diameter. Although no quantitative relationship was evident between the capacity of granulosa cells to produce cAMP in response to FSH or LH and the degree of DNA fragmentation in our study, preliminary studies by others have revealed that both FSH and LH are effective inhibitors of apoptosis in cultured rat follicles and that this effect is probably mediated by the adenylate cyclase-cAMP pathway as the immediate second

messenger (see [15]). Whereas the precise role of gonadotropins on large antral follicle growth and development in the cow is becoming better defined [32, 43–45], associated intracellular mechanisms transducing these signals and governing follicle cell proliferation, differentiation, or death largely remain to be determined.

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