Regulation of apoptosis in the atresia of dominant bovine follicles of the first follicular wave following ovulation

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Abstract

During atresia of bovine follicles, granulosa cells are lost through the controlled form of cell death, apoptosis. The purpose of this study was to characterize the regulation of apoptotic death of granulosa cells in dominant bovine follicles during the first wave of follicular development. Dominant follicles were collected from Holstein heifers on days 4, 6 or 8 of the first follicular wave ($n = 5/\text{day}$). Regulation of apoptosis in granulosa cells was examined by annexin V and propidium iodide staining; measurement of relative levels of mRNA encoding Bcl-2, Bcl-xL and Bax; and activity of caspase-3, -8 and -9. Steady-state levels of mRNA encoding four oxidative stress-response proteins were determined. Compared with day 4, the incidence of apoptotic and nonviable granulosa cells tended to increase on day 6, and numbers of nonviable cells were higher on day 8. The ratios of relative levels of mRNA encoding Bcl-2 to Bax and Bcl-xL to Bax were higher on day 6 than days 4 and 8. Activity of caspases-3 and -9 in granulosa cells did not change among the 3 days, while caspase-8 activity decreased on day 8 compared with days 4 and 6. Amounts of GSHPx, MnSOD and Cu/ZnSOD mRNA in granulosa cells were higher on day 8 than day 6. In theca interna, amounts of Cu/ZnSOD mRNA decreased between days 4 and 6. From the decreased production of estradiol and increased numbers of apoptotic and nonviable granulosa cells, we conclude that atresia of the dominant follicle is initiated between days 4 and 6 of the first follicular wave. However, apoptosis of granulosa cells does not appear to be initiated by changes in expression of oxidative stress-response proteins.

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Introduction

Although the bovine ovary contains approximately 150 000 follicles at birth (Erickson 1966), very few follicles are successfully ovulated and more than 99.9% undergo atresia (Byskov 1978). It has been estimated that the incidence of atresia in bovine follicles is greatest after antrum formation, just before the final stages of follicular development (Fortune 1994). Apoptosis of granulosa cells is an early feature of atresia in bovine follicles (Jolly *et al.* 1994, Van Wezel *et al.* 1999, Yang & Rajamahendran 2000a). However, the mechanisms initiating apoptosis of granulosa cells have not been well characterized.

The Bcl-2 family of proteins includes members which either promote (Bax) or inhibit (Bcl-2, Bcl-xL) apoptosis. These proteins associate to form homo- and/or heterodimers (Mignotte & Vayssiere 1998). A shift in equilibrium toward proapoptotic (Bax) versus antiapoptotic (Bcl-2, Bcl-xL) Bcl-2 proteins makes the mitochondrial membrane permeable, releasing cytochrome c and apoptosis-inducing

factor (AIF) into the cytoplasm (Kluck *et al.* 1997, Yang *et al.* 1997, Amarente-Mendes *et al.* 1998, Jurgenmeier *et al.* 1998). Therefore, the ratio of Bcl-2 and Bcl-xL to Bax expression is important in determining susceptibility to apoptosis (Oltvai *et al.* 1993). Yang *et al.* (2000b) demonstrated that an elevated ratio of Bax to Bcl-2 protein expression occurs during atresia of bovine dominant follicles induced by *in vivo* injection of progesterone. However, the roles of Bcl-2, Bcl-xL and Bax in natural follicular development and atresia remain to be elucidated.

The Bcl-2 proteins affect apoptosis by either promoting or inhibiting cleavage of execution caspases. Caspases are cysteine proteases that cleave their substrate proteins specifically at an aspartate residue. They are constitutively expressed in an inactive proenzyme form and are activated after cleavage at specific aspartate residues. Activation of the execution caspases leads to irreversible progression of the apoptotic cascade (Mignotte *et al.* 1998). To our knowledge, the activity of execution

caspases, particularly caspases-3, -8 and -9, and their role in apoptosis in bovine granulosa cells has not been studied.

In cortical neurons (Geller *et al.* 2001), rat hepatocytes (Shiba & Shimamoto 1999) and mouse fibrosarcoma cells (Kuroda *et al.* 2000), apoptosis can be initiated by reactive oxygen species (ROS), which are byproducts of normal aerobic metabolism (primarily electron transport). Intracellular accumulation of ROS, known as oxidative stress, can damage cells by causing nucleic acid strand breaks, lipid peroxidation, protein degradation and, ultimately, cell death (Yu 1994). It has been suggested that steroidogenically active cells, such as granulosa cells of antral follicles, require high levels of energy production and thus generate large amounts of ROS (Rapoport *et al.* 1995, Tilly 1996). Therefore, it is possible that oxidative stress is involved in the mechanisms that trigger apoptosis in healthy, steroidogenic antral follicles. The primary endogenous defense against oxidative stress is a series of enzymes that work in a cooperative manner to scavenge ROS. Four oxidative stress-response proteins, glutathione peroxidase (GSHPx), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (Cu/ZnSOD) and catalase (CAT), metabolize ROS and thus protect cells from oxidative stress. MnSOD and Cu/ZnSOD, found in the mitochondria and cytosol respectively, catalyze the dismutation of the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) (Yu 1994, McCord & Marecki 1997, Mates 2000). GSHPx, found primarily in the cytosol, and CAT, contained within peroxisomes, are responsible for detoxifying hydrogen peroxide to water (Riley & Behrman 1991, Yu 1994, Mates 2000). *In vitro*, increased expression of oxidative stress-response proteins can prevent apoptosis (Kuroda *et al.* 2000, Laukkanen *et al.* 2001, Pong *et al.* 2001). In the bovine corpus luteum, a decline in expression of oxidative stress-response genes was observed during structural regression (Rueda *et al.* 1995). In the follicle, oxidative stress-response proteins may provide a mechanism to avoid atresia by attenuating or preventing oxidative stress-induced apoptosis. In support of this idea, incubation of rat granulosa cells with SOD or CAT caused a dose-dependent inhibition of apoptosis (Tilly & Tilly 1995). Information on the role of oxidative stress-response proteins in the ruminant follicle is limited. In sheep and goats, an inverse relationship was found between intrafollicular MnSOD activity and concentrations of estradiol-17 β (Singh *et al.* 1998). In a preliminary study in the cow, expression of mRNA encoding MnSOD and Cu/ZnSOD in granulosa cells of dominant follicles was greater during the midluteal phase than the preovulatory phase (Bruemmer *et al.* 1996). These results show that oxidative stress-response genes are expressed in the bovine follicle and may be regulated by endocrine factors.

The bovine estrous cycle is characterized by two or three waves of follicular development (Savio *et al.* 1988, Sirois & Fortune 1988, Ginther *et al.* 1989). Previous

studies have characterized the onset of atresia in nonovulatory bovine follicles during the first wave following ovulation. Xu *et al.* (1995a) found that all dominant follicles collected on days 4 and 6 of the wave appeared morphologically healthy, and only 1 of 4 follicles collected on day 8 showed signs of advanced atresia. Not until day 10 did all dominant follicles appear to be in advanced stage of atresia, as indicated by destruction of follicular structure, degeneration of granulosa cells and/or decrease in the number of granulosa cells. The authors concluded that dominant follicles of the first follicular wave are morphologically healthy until day 8 of the follicular wave and maintain their diameter until day 10. This characterization of the atretic process during the first follicular wave in cattle provides an excellent framework to study atresia of dominant follicles, but did not address apoptosis or expression of genes that regulate apoptotic cell death. The purpose of this study was to investigate possible mechanisms that lead to atresia in dominant bovine follicles during the first wave of follicular development, namely, changes in expression of genes and activity of enzymes that regulate apoptotic death of granulosa cells. We hypothesized that apoptosis of granulosa cells would be associated with increased expression of Bax relative to antiapoptotic Bcl-2 genes, increased caspase activity and decreased expression of oxidative stress-response proteins.

Materials and Methods

Animals

All animal protocols were approved by the University of Arizona Use and Care Committee and were within guidelines established by the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (Federation of Animal Science Societies 1999). Sexually mature Holstein heifers were housed in dry lots, fed high-quality alfalfa hay and flaked corn with mineral supplement, and provided with constant access to water and shade. Experiments were conducted during October, November and December.

Ultrasonography and collection of follicles

Heifers were observed twice daily for estrous behavior. Beginning on the day of estrus, blood samples were collected by coccygeal venipuncture, and ultrasonographic examinations of ovaries were performed daily as previously described (Turzillo & Fortune 1990) using a real-time B-mode linear array ultrasound scanner equipped with a 7.5 MHz intrarectal probe (Aloka SSD-550V Zug, Switzerland). Examinations were recorded on videotape (digital Handycam, Hi8 Recording Tape, Sony Electronics, Park Ridge, NJ, USA). Ovulation was identified by the disappearance of a large follicle preceded by estrus. After ovulation, diameters and positions of all follicles of ≥ 4 mm in diameter were analyzed. Day 1 of the follicular

wave was defined as the day that two or more follicles of ≥ 4 mm in diameter were first observed. These follicles continued to grow until one follicle deviated from the cohort to become dominant while other follicles in the wave regressed.

The ovary bearing the dominant follicle was surgically removed via flank incision on day 4, 6 or 8 of the first follicular wave ($n = 5$ per day). These days were established by previous work (Xu *et al.* 1995a, 1995b) showing that functional regression of the dominant follicle occurs between days 4 and 8 of the first wave and is marked by decreases in both steroidogenic capacity and expression of genes encoding key steroidogenic enzymes. Ovaries were placed in dissection medium (1×MEM with Earle's salts and 25 mM HEPES, without L-glutamine; Life Technologies, Rockville, MD, USA), and transported immediately on ice to the laboratory. The dominant follicle was dissected from the ovarian stroma. Follicular diameter was measured with calipers, and follicular fluid was aspirated and stored at -20°C without centrifugation. The collapsed follicle was cut into four pieces. Theca interna, along with the basement membrane and granulosa cells, was separated from the theca externa and remaining stroma with fine forceps. With an angled, finely pulled Pasteur pipette, granulosa cells were scraped from the basement membrane and theca interna. Granulosa cells were collected in dissection medium, centrifuged for 15 min at 800 *g*, resuspended in 1 ml fresh dissection medium and counted with a hemocytometer. A small portion of granulosa cells from each follicle (100 000) was set aside for annexin and propidium iodide (PI) staining while the remaining cells were repelleted and stored, along with theca interna, at -80°C .

Hormone assays

Concentrations of progesterone were measured in serum by the Coat-A-Count Progesterone radioimmunoassay (Diagnostic Products, Los Angeles, CA, USA), according to the manufacturer's recommendations, as previously validated in our laboratory (Sanders *et al.* 2002). The progesterone antiserum exhibits extremely low cross-reactivity with related steroids (androstenedione and estradiol, not detectable; pregnenolone and testosterone, 0.1%). Serum was not extracted before assay. Sensitivity of the assay, calculated as two standard deviations below the mean c.p.m. at maximum binding, was 0.02 ng/ml. The intra-assay CV was 15.2%.

Concentrations of estradiol-17 β (estradiol) were measured in follicular fluid by the Double Antibody Estradiol RIA (Diagnostic Products), according to the manufacturer's recommendations, as previously validated in our laboratory (Sanders *et al.* 2002). The estradiol antiserum exhibits very low cross-reactivity with related steroids (androstenedione, 0.004%; estriol, 0.235%; progesterone and testosterone, not detectable). Follicular fluid was not extracted prior to assay. Sensitivity of the assay, calculated

as two standard deviations below the mean CPM at maximum binding, was 1.5 pg/ml. The intra- and inter-assay CV were 5.91 and 9.5% respectively.

Annexin V and propidium iodide (PI) staining

Early-stage apoptosis of granulosa cells was assessed by staining with annexin V with the Vybrant Apoptosis Assay Kit no. 2 (Molecular Probes, Eugene, OR, USA), according to the manufacturer's recommendations with modifications. Annexin V binds phosphatidylserine residues, which are translocated from the inner to the outer leaflet of the plasma membrane early in apoptosis (van Engeland *et al.* 1998). To distinguish intact, apoptotic cells from cells lacking intact plasma membranes (nonviable), PI is used to stain double-stranded nucleic acids. Granulosa cells (approximately 100 000) were washed in cold PBS, centrifuged at 800 *g* for 15 min, and resuspended in 100 μl 1× annexin-binding buffer. Alexa Fluor 488 annexin V (5 μl) and PI (1 μl of 100 $\mu\text{g}/\text{ml}$ working solution) were added, and cells were incubated at room temperature for 15 min. Cells were then spread on a glass slide, cover-slipped, and viewed with an Olympus BX50WI fluorescence microscope with a FITC filter. At least 800 granulosa cells per follicle were observed. Numbers of cells that stained for annexin V exclusively or annexin V and PI were recorded.

Quantitative RT-PCR

Fluorescent, real-time quantitative RT-PCR was used to determine differences in mRNA expression of Bax, Bcl-xL and Bcl-2. Total RNA was extracted from granulosa cells and theca interna by the guanidinium isothiocyanate-phenol-chloroform extraction procedure (Chomczynski & Sacchi 1987) with TRIzol Reagent (Life Technologies). Optical densities at 260 and 280 nm were measured to determine the quantity and purity of RNA samples. An amount of 2 μg total RNA was subjected to reverse transcription with SuperScript III RNase Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol, using 3 μg random hexamers. Primers (Table 1) for quantitative RT-PCR were made with PrimerQuest software (Integrated DNA Technologies, Coralville, IA, USA) according to the manufacturer's restrictions.

Each PCR reaction (total volume of 10 μl) consisted of 16 ng reverse transcription product, 0.4 μl of 25 μM MgCl_2 , 1.0 μl of each 5.0 μM forward and reverse primer,

Table 1 Quantitative RT-PCR primers.

Bax	Forward: TTGCTGATGGAAGCTACAACCTG Reverse: CCTTGGTGCACAGGGCCTTGAG
Bcl-xL	Forward: CACTGTGCGTGGAAACCGTA Reverse: AAAGTGTCCCAGCCGCC
Bcl-2	Forward: CGCATCGTGGCCTTCTTTGAGTT Reverse: GCCGGTTCAGGTACTCAGTCAT
18S	Forward: CCTCCGCGAGGATCCATTG Reverse: CGCTCCCAAGATCCAACCTAC

5.0 µl of SYBR Green Master Mix (Qiagen), and Rnase-free water. Thermal cycling conditions were as follows: 15 min at 95 °C to activate Hotstart Taq, 40 cycles of 15 s at 95 °C for denaturing, 15 s at 58 °C for annealing, and 20 s at 72 °C for extension, followed by a ramp from 72 °C to 99 °C over 15 min to determine the melting curve.

Ribosomal 18S rRNA was used as a housekeeping gene to normalize samples for variation in RNA loading. To verify that 18S rRNA was a valid housekeeping gene, the ability to detect fold changes in amounts of RNA loaded in a parallel fashion with the target probe (Bax, Bcl-2, or Bcl-xL) was tested. Decreasing amounts of 18S rRNA (500, 100, 50, 10, 5 or 1 pg) demonstrated parallel fold changes in amplification compared with decreasing amounts of each target (Bax, Bcl-2 or Bcl-xL) mRNA (500, 100, 50, 10, 5 or 1 ng).

Quantification of gene expression was made by setting an arbitrary threshold in the geometric portion of the RT-PCR amplification plot after examining the log view. Relative quantification of Bax, Bcl-2 or Bcl-xL mRNA expression was done by the comparative cycle threshold (Ct) method (ABI Prism Sequence Detection System User 1997, Livak & Schmittgen 2001). Briefly, ΔC_t was determined by subtracting 18S Ct from the target unknown (Bax, Bcl-2 or Bcl-xL) Ct value. For each target mRNA, $\Delta\Delta C_t$ was determined by subtracting the highest ΔC_t from all other ΔC_t values within each experiment. Fold changes in mRNA expression of Bax, Bcl-2 or Bcl-xL were then calculated as $2^{-\Delta\Delta C_t}$. Each sample was run in triplicate. Data were expressed as ratio of relative expression of Bcl-2:Bax or Bcl-xL:Bax.

Template generation and cRNA probe synthesis

Complementary DNA encoding bovine GSHPx and bovine MnSOD were kindly provided by Dr Bo Rueda at Massachusetts General Hospital. Both of these cDNAs were generated by RT-PCR using RNA derived from bovine corpus luteum. The 456 bp GSHPx cDNA (corresponding to bp 166–622 of GenBank accession no. X13684) and the 394 bp MnSOD cDNA (corresponding to bp 200–594 of GenBank accession no. L22092) were ligated into the pGEM 4Z Vector (Promega). A 255 bp cDNA encoding Cu/ZnSOD and a 159 bp cDNA encoding CAT were generated in our laboratory by RT-PCR using bovine liver RNA. The Cu/ZnSOD cDNA (corresponding to bp 166–369 of GenBank accession no. X54799) and the CAT cDNA (corresponding to bp 154–240 of human cDNA, GenBank accession no. XM_030347) were ligated into the pGEM-T Easy Vector (Promega). A linear antisense template encoding 18S ribosomal RNA (pTRI RNA 18S) was purchased from Ambion (Austin, TX, USA). The identity and orientation of all cDNAs were verified by dideoxy sequencing (Sanger *et al.* 1977).

To provide a shorter transcription product that could be used simultaneously with other cRNA probes for ribonuclease protection assay, the template for MnSOD was

linearized at sites within the cDNA insert with PstI. All other cDNAs were linearized at PstI sites within the vector. Antisense (32 P) UTP-labeled cRNA probes were transcribed from linearized cDNA templates with T7 polymerase and the MAXiscript *In Vitro* Transcription Kit (Ambion), according to the manufacturer's recommendations. Because of the high abundance of 18S ribosomal RNA, the 18S antisense riboprobe was generated at 1/10 the specific activity of the other riboprobes. Antisense cRNA probes were purified on a denaturing acrylamide gel and used for hybridization within 1 day.

Ribonuclease protection assays

Ribonuclease protection assays were carried out using the RPA III Ribonuclease Protection Assay Kit (Ambion), according to the manufacturer's recommendations. Assays for mRNA encoding GSHPx, MnSOD, CuZnSOD, CAT and 18S ribosomal RNA were performed with 5 µg RNA isolated from granulosa cells or theca interna. All hybridizations were carried out at 50 °C for 15 h, followed by incubation with a 1:50 dilution of RNase A/TI cocktail (Ambion) and resolution of protected fragments on a 5% acrylamide/8M urea gel. After electrophoresis at 260 V for 2 h, gels were transferred to filter paper, covered with plastic wrap and placed in an InstantImager Electronic Autoradiography System (Packard Instruments, Meriden, CT, USA) for 15 min to quantify size and abundance of beta emission, as counts per minute (c.p.m.), of all protected fragments. Gels were also exposed with one intensifying screen overnight to Hyperfilm MP autoradiography film (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at –80 °C.

Western analysis

After RNA isolation, DNA was precipitated from the interphase and organic phase with ethanol. After sedimentation of the DNA by centrifugation, protein was isolated from the phenol-ethanol supernatant (Chromczynski 1993). Protein was quantified by the Bradford method with BSA as the protein standard (Bradford 1976). Granulosa cell (30 µg) and theca interna (25 µg) protein were added to an equal volume of SDS sample buffer (1.25 M Tris–base, 30% glycerol, 0.2% SDS, 0.02% 2-ME and 0.0001% bromophenol blue, pH 6.8). Samples were heated for 5 min at 100 °C before electrophoresis through a 15% acrylamide gel for 45 min at 150 V. Proteins were transferred at 90 mA overnight to nitrocellulose membranes at 4 °C. Ponceau S staining was used to confirm consistent transfer of proteins. After three washes in TTBS (Tween-Tris buffered Saline: 0.1% Tween-20 in 100 mM Tris-ClpH 7.5 0.9% NaCl), membranes were incubated for 1 h with 5% nonfat dry milk (NFDM) in TTBS. One of four primary antibodies was then applied. Sheep anti-bovine GSHPx (Biogenesis, Kingston, NH, USA), rabbit anti-human MnSOD (StressGen Biotechnologies, Victoria, BC, Canada),

rabbit antibovine Cu/ZnSOD, and rabbit antibovine CAT (both from Chemicon International, Temecula, CA, USA) were diluted 1:1000 in TTBS containing 5% NFDM and incubated for 1 h with the nitrocellulose membranes. The membranes were washed three times in TTBS and incubated for 1 h with incubation with anti-sheep or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Chemicon) and detected by chemiluminescence and autoradiography with radiographic film.

Enzyme assays

Follicles were collected and granulosa cells isolated as described above on days 4, 6 or 8 of the first follicular wave ($n = 5$ per day). Granulosa cells were isolated and divided for isolation of functional protein by one of two methods. For GSHPx, SOD and CAT enzyme activity assays, cells were resuspended in 200 μ l buffer (PBS, 100 mM EDTA, pH 7.4) on ice and sonicated at 40% power for 5 s. Cell lysates were centrifuged (10 000 g , 10 min, 4°C) three times, retaining the supernatant each time, and stored at -80°C . For caspase activity assays, protein was isolated with reagents in the BD ApoAlert Caspase Assay Plate kit (BD Biosciences Clontech, Palo Alto, CA, USA), according to the manufacturer's recommendations. Briefly, granulosa cells were resuspended in ice-cold 1 $\times$ cell lysis buffer, at a concentration of 2×10^5 cells per 50 μ l buffer, and incubated on ice for 10 min. Cells were centrifuged at 12 000 g for 5 min at 4°C, and the supernatant was transferred to a new tube and stored at -80°C . Protein isolated by both methods was quantitated by the bicinchoninate (BCA) method, using a protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

GSHPx activity in granulosa cells was determined by the Glutathione Peroxidase Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). This assay measures GSHPx indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione, produced by reduction of hydroperoxide by GSHPx, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP^+ is accompanied by a decrease in absorbance at 340 nm. When GSHPx activity is limiting, the rate of decrease in the A_{340} is directly proportional to the GSHPx activity of the sample. Each sample was assayed in triplicate, according to the manufacturer's recommendations. Briefly, 10 μ g lysate in 20 μ l sample buffer were added to 100 μ l assay buffer and 50 μ l cosubstrate mixture in a 96-well microplate. Reactions were initiated by the addition of 20 μ l cumene hydroperoxide, and the absorbance was read immediately at 340 nm in a microplate reader. The absorbance was read every minute for 9 min and the change in absorbance (ΔA_{340}) per minute determined. The rate of $\Delta A_{340}/\text{min}$ of the background (determined by reading the absorbance of assay buffer, cosubstrate mixture and cumene hydroperoxide) was subtracted from the rate of each sample. One unit of

GSHPx activity is defined as the amount of enzyme that will cause oxidation of 1.0 nmol NADPH to NADP^+ per minute at 25°C , with the NADPH extinction coefficient being $0.00373 \mu\text{M}^{-1}$. Therefore, the following equation was used to determine GSHPx activity:

$$\text{GSHPx activity} = \frac{\Delta A_{340}/\text{min}}{0.00373 \mu\text{M}^{-1}} \times \frac{0.19 \text{ ml}}{0.02 \text{ ml}} \times \frac{\text{sample}}{\text{dilution}} = \text{nmol/min/ml}$$

Total SOD activity of granulosa cells was determined by the SOD Assay Kit-WST, according to the manufacturer's recommendations (Dojindo Molecular Technologies, Gaithersburg, MD, USA). This assay relies on WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon reduction with O_2^- , a reaction that is inhibited by SOD. Standards for the inhibition curve were obtained by diluting SOD (Sigma) to 200, 100, 50, 20, 10, 5, 1, 0.1, 0.05, 0.01 and 0.001 U/ml. All standards, samples (5 μ g) and controls (B1, B2 and B3) were assayed in triplicate. In a 96-well microplate, 20 μ l sample solution or water (B1 and B3) were mixed with 200 μ l WST working solution. For B2 and B3, 20 μ l dilution buffer were added. B2 also received 20 μ l pooled granulosa cell lysate (5 μ g). Enzyme working solution (20 μ l) was added to each standard and sample well, along with B1 wells. The plate was incubated at 37°C for 20 min and the absorbance read at 450 nm with a microplate reader. SOD activity (inhibition rate%) was determined by the following equation:

$$\text{SOD activity (inhibition rate \%)} = \frac{[(A_{B1} - A_{B3}) - (A_{\text{sample}} - A_{B2})]}{(A_{B1} - A_{B3})} \times 100$$

CAT activity of granulosa cells was determined by the Amplex Red CAT Assay Kit (Molecular Probes, Eugene, OR, USA), according to the manufacturer's recommendations. In the assay, CAT reacts with H_2O_2 to produce water and oxygen. The Amplex Red reagent reacts with any unreacted H_2O_2 in the presence of horseradish peroxidase to produce the fluorescent oxidation product, resorufin. A standard curve was prepared by diluting the provided CAT to 1000, 500, 250, 125, 62.5 and 0 mU/ml. Samples (500 ng) were diluted in 1 $\times$ reaction buffer to 25 μ l. Samples, standards, and controls were pipetted in triplicate into separate wells of a 96-well microplate. A solution of 40 μM H_2O_2 was added (25 μ l) to each well. After 30 min at 25°C , 50 μ l Amplex Red/HRP working solution was added to each well. The reaction was carried out for 30 min at 37°C and the plate read on a fluorescence microplate reader with excitation of 530 nm and emission detection at 590 nm. The change in fluorescence was reported by subtracting the sample value from that of a no-CAT control.

Assays for the activity of caspase-3, -8 and -9 of granulosa cells were carried out with the BD ApoAlert Caspase Assay Plate kit (BD Biosciences Clontech, Palo Alto, CA, USA), according to the manufacturer's recommendations. The included microplate contains fluorogenic substrates specific for the caspase-3, -8 or -9 immobilized in the wells. When cell lysate is applied to the wells, each caspase cleaves its substrate, and a fluorescent product is released. To perform the assay, 50 µl 2×reaction buffer/DTT mix were added to each well and incubated at 37°C for 5 min. Granulosa cell lysate (50 µl) was added and the plate was incubated at 37°C for 2 h. The plate was analyzed in a fluorescent plate reader with an excitation of 380 nm and emission detection at 460 nm. All samples were analyzed in triplicate.

Statistical analyses

One-way analysis of variance (ANOVA) using the general linear models (GLM) procedure of SAS (SAS 1987) was used to analyze differences among groups. Means were separated by least significant differences. When necessary, data were log-transformed to achieve normality and homogeneity of variances. Values of $P \leq 0.05$ were considered significant.

Results

Follicular characteristics

In all heifers, circulating concentrations of progesterone were undetectable on the day of estrus and increased to levels of 2.4 ± 2.0 , 5.2 ± 1.5 , and 7.7 ± 2.0 ng/ml on days 4, 6 and 8 of the first follicular wave respectively, indicating normal luteal function after ovulation. The first follicular wave was initiated 1.3 ± 0.1 (range = 1–2) days after ovulation, and in each heifer a single follicle deviated from the recruited cohort to become the dominant follicle. Follicular diameter after dissection remained constant among dominant follicles collected on days 4, 6 or 8, while the number of granulosa cells recovered tended to decrease ($P = 0.07$) between days 6 and 8 (Table 2). Follicles collected on all days appeared morphologically healthy, as indicated by color of the inner follicular wall (pink), extensive vascularization, intact basement membrane and clarity of follicular fluid. Concentrations of

estradiol in follicular fluid were higher ($P < 0.01$) on day 4 than days 6 and 8 (Table 2).

Annexin V and PI staining

The percentage of granulosa cells in early stages of apoptosis (stained with annexin V alone) tended to increase ($P = 0.06$) between days 4 and 6 (Fig. 1). The percentage of nonviable granulosa cells (stained for both annexin V and PI) in follicles collected on day 4 was lower ($P \leq 0.05$) than those collected on days 6 and 8.

Bcl-2 family mRNA

The ratios of relative levels of mRNA encoding Bcl-2 to Bax and Bcl-xL to Bax were higher ($P < 0.05$) on day 6 than days 4 and 8 of the first follicular wave in granulosa cells (Fig. 2).

Caspase-3, -8 and -9 activity

The activity of caspase-3 and -9 did not change among granulosa cells collected on days 4, 6 or 8 of the first follicular wave (Fig. 3). However, caspase-8 activity in granulosa cells decreased ($P < 0.05$) on day 8 compared with days 4 and 6.

Levels of mRNA encoding oxidative stress-response proteins

In granulosa cells, relative levels of mRNA encoding GSHPx and MnSOD were higher ($P < 0.01$) in follicles collected on day 8 than those collected on days 4 or 6 (Fig. 4). Amounts of mRNA encoding Cu/ZnSOD were higher ($P < 0.05$) on day 8 than day 6. Levels of mRNA encoding CAT in granulosa cells were similar across all 3 days.

In theca interna, relative levels of mRNA encoding GSHPx and MnSOD were similar on days 4, 6 and 8 (Fig. 5). Thecal expression of mRNA encoding Cu/ZnSOD decreased ($P < 0.02$) between days 4 and 6 and remained low on day 8. Levels of mRNA encoding CAT in theca interna were similar on days 4, 6 and 8.

Levels of oxidative stress-response proteins

In granulosa cells, relative amounts of GSHPx, MnSOD, Cu/ZnSOD and CAT protein did not change among

Table 2 Characteristics of dominant follicles collected during the first follicular wave.^a

Day of wave	Follicular diameter (mm)	Number of granulosa cells ($\times 10^6$)	Follicular fluid estradiol (ng/ml)
4	13.0 ± 0.2^b	22.1 ± 2.5^d	299 ± 52^b
6	13.0 ± 0.7^b	30.2 ± 0.2^e	44 ± 7^c
8	13.6 ± 0.4^b	19.7 ± 4.2^e	48 ± 9^c

^a Data are means $\pm$ S.E.M.

^{b,c} Values within each column without common superscripts differ ($P < 0.05$).

^{d,e} Values within each column with no common superscripts tend to differ ($P = 0.07$).

Figure 1 Percentage of granulosa cells in dominant follicles collected on day 4, 6 or 8 of the first follicular wave, stained with either annexin V alone (indicative of early apoptosis) or annexin V and PI (indicative of nonviability). Data are mean $\pm$ S.E.M. Letters a and b indicate a trend to differ among days within staining category ($P=0.06$). Letters c and d indicate significant differences among days within staining category ($P \leq 0.05$).

follicles collected on day 4, 6 or 8 of the first follicular wave (data not shown). Similarly, relative expression of these proteins did not change in theca interna (data not shown).

Activity of oxidative stress-response proteins

In granulosa cells, activity of GSHPx and CAT did not change among follicles collected on day 4, 6 or 8 of the first follicular wave (data not shown). Activity of SOD in granulosa cells was higher ($P=0.05$) on day 6 than day 4 of the first follicular wave (data not shown).

Discussion

Each wave of follicular growth in cattle is characterized by recruitment of a cohort of follicles, from which a single follicle is selected to become morphologically and steroidogenically dominant. If luteolysis does not occur during the growth phase of the dominant follicle, it undergoes atresia and a new follicular wave appears. Bao & Garverick (1998) suggested that functional atresia of the dominant follicle in cattle is initiated between days 4 and 6 of

Figure 2 Relative expression of Bcl-xL:Bax and Bcl-2:Bax mRNA in granulosa cells from dominant follicles collected on day 4, 6 or 8 of the first follicular wave. Data are mean $\pm$ S.E.M. Letters indicate significant differences among days ($P<0.05$).

Figure 3 Caspase activity of granulosa cells of dominant follicles collected on day 4, 6 or 8 of the first follicular wave. Data are mean $\pm$ S.E.M. Letters indicate significant differences among days for each caspase ($P<0.05$).

the first follicular wave. This conclusion was based on a marked decrease in concentrations of estradiol in follicular fluid and reduced expression of mRNA encoding FSH receptor and several steroidogenic enzymes (Xu *et al.* 1995a,b). We also observed a dramatic reduction in follicular fluid concentrations of estradiol between days 4 and 6 of the wave. It is important to note that these alterations in steroid production and gene expression occurred before decreased follicular diameter or consistent loss of morphologic integrity (e.g., degeneration of basement membrane) was evident (Xu *et al.* 1995a,b). To our knowledge, this is the first study to characterize the temporal pattern of apoptosis in granulosa cells of the dominant follicle during the first follicular wave. Externalization of phosphatidylserine residues, a marker for early apoptosis, tended to increase between days 4 and 6. The percentage of nonviable granulosa cells increased between days 4 and 6, and remained over 20% on day 8. The percentages of nonviable granulosa cells were higher than expected. It is possible that brief ischemia induced during surgical removal of the ovary and/or manipulation of the granulosa

Figure 4 Amounts of GSHPx, MnSOD, Cu/ZnSOD and CAT mRNA in granulosa cells of dominant follicles collected on day 4, 6 or 8 of the first follicular wave. Data are mean c.p.m. $\pm$ S.E.M. Letters indicate significant differences among days for each oxidative stress response gene ($P<0.05$).

Figure 5 Amounts of GSHPx, MnSOD, Cu/ZnSOD and CAT mRNA in theca interna of dominant follicles collected on day 4, 6 or 8 of the first follicular wave. Data are mean c.p.m. $\pm$ S.E.M. Letters indicate significant differences among days for each oxidative stress-response gene ($P < 0.02$).

cells *in vitro* induced a secondary necrosis in granulosa cells collected on all 3 days. The greater incidence of apoptosis and/or death in granulosa cells on day 6 accompanied by dramatically reduced estradiol production leads us to conclude that atresia of the dominant follicle is probably initiated between days 4 and 6 of the first follicular wave, as previously suggested by Bao & Garverick (1998). However, it is important to note that in the event of early luteal regression induced by prostaglandin (PG) $F_{2\alpha}$ injection, some dominant follicles are capable of ovulating 2 days after reaching maximal diameter (Fortune *et al.* 1991). Therefore, it appears that in some cases, dominant follicles can be 'rescued' from atresia. The molecular and cellular characteristics that distinguish these follicles from those at similar stage of development that fail to ovulate after luteolysis are not known.

The Bcl-2 family of proteins includes members which either promote (Bax) or inhibit (Bcl-2, Bcl-xL) apoptosis. Overexpression of Bax accelerates apoptotic death response to death signals (Oltvai *et al.* 1993). In addition, Bax can heterodimerize with Bcl-2 or Bcl-xL and prevent their effects on cellular survival (Oltvai *et al.* 1993). In the current study, the ratio of relative levels of mRNA encoding Bcl-2 to Bax and Bcl-xL to Bax increased slightly on day 6 compared with day 4, and then decreased on day 8 compared with day 6. These results do not support the hypothesis that the Bcl-2 family of proteins is involved in the activation of apoptosis during atresia of dominant bovine follicles. However, recent evidence supports the concept that the translocation of Bax, Bcl-2 and Bcl-xL between the mitochondria and the cytosol is as critical as changes in their expression (Pawlowski & Kraft 2000, Hu *et al.* 2001). A shift of Bcl-2 and Bcl-xL protein to the mitochondria, where they can heterodimerize with mitochondria-associated Bax, allows them potentially to block Bax-induced apoptotic events. Future studies that address the intracellular translocation of these proteins are needed to elucidate further the role of the Bcl-2 family in bovine granulosa cell apoptosis.

The activation of execution caspases, such as caspase-3, -8 and -9, indicates the 'point of no return' in the apoptotic pathway. These proteins either directly or indirectly cleave a broad array of proteins necessary for cell survival, such as those involved in DNA maintenance and repair and organization of intermediate filaments (Tewari *et al.* 1995, Caulin *et al.* 1997). In the current study, an increase in caspase activity was not observed in granulosa cells of dominant follicles during the period encompassing days 4, 6 and 8 of the first follicular wave. Instead, the activity of caspase 8 decreased on day 8 compared with days 4 and 6. Activation of the Fas pathway, which has been shown to mediate bovine granulosa cell apoptosis (Porter *et al.* 2000), leads to cleavage and activation of caspase 8 (Ashkenazi & Dixit 2000, 1998). Changes in activation of the Fas pathway could explain the reduction in caspase 8 activity we observed on day 8. Caspases activate endonucleases which cleave DNA at specific internucleosomal intervals, giving DNA a characteristic ladder appearance when run on an agarose gel (Liu *et al.* 1997). This internucleosomal DNA fragmentation increased in granulosa cells during atresia of bovine dominant follicles induced by progesterone treatment (Yang & Rajamahendran 2000b). Most likely, the activation of execution caspases occurs later (day 9 or 10) during the wave than the time points examined in this study. The fact that dominant follicles of the first wave are capable of ovulating early during the plateau phase of growth supports the idea that the granulosa cells have not been committed to the apoptotic pathway at this point (Fortune *et al.* 1991). However, the tendency for translocation of phosphatidylserine residues provides evidence that the apoptotic pathway has been initiated on day 6 of the first follicular wave. Therefore, it appears that this period encompasses a critical turning point in the developmental pathway of the nonovulatory dominant follicle when a signal (or series of signals) is delivered that attenuates production of estradiol, initiates apoptosis in granulosa cells, and directs the follicle down an atretic pathway.

A number of mechanisms have been proposed to induce apoptosis in granulosa cells. These include binding of ligands, such as tumor necrosis factor- α and fas ligand, to their respective receptors (Kaipia *et al.* 1996, Porter *et al.* 2000), inhibition of cell-cell contact (Trollice *et al.* 1996), presence or absence of specific growth factors (Quirk *et al.* 2000), and altered levels of hormones such as estrogens and androgens (Billig *et al.* 1993). In addition to these mechanisms, follicular apoptosis may be induced by oxidative stress (Tilly & Tilly 1995). To begin to address the role of oxidative stress in bovine follicular atresia, we examined the temporal relationship between apoptosis in granulosa cells and expression of proteins responsible for preventing oxidative stress. In contrast to our original hypothesis that decreased expression of genes encoding oxidative stress-response proteins would lead to accumulation of ROS and thus trigger apoptosis of granulosa cells, we observed an increase in expression of genes encoding

oxidative stress-response proteins on day 8 of the follicular wave. However, these levels of mRNA did not translate into increased expression of the corresponding proteins or greater enzyme activity. Because this increase in gene expression occurred after elevated numbers of apoptotic and nonviable granulosa cells were observed on day 6, our results do not support the hypothesis that apoptosis is initiated by oxidative stress in the bovine follicle. This is in contrast to previous observations in cultured rat granulosa cells in which the addition of SOD inhibited spontaneous apoptosis (Tilly & Tilly 1995). The unexpected increase in expression of three oxidative stress-response genes in nonovulatory bovine follicles on day 8 of the wave leads us to consider the possibility that these enzymes may play a physiologic role in later stages of atresia. While our study focused on measurement of oxidative stress-response proteins, measurement of additional endpoints, such as intrafollicular levels of ROS or evidence of oxidative stress-induced cellular damage (such as lipid peroxidation), would further delineate the relationship between oxidative stress and apoptosis in bovine follicles.

In contrast to the consistent pattern of increased oxidative stress-response gene expression observed in granulosa cells, the only corresponding change observed in theca interna was a decrease in amounts of Cu/ZnSOD mRNA on day 6 of the follicular wave. Although apoptosis is rarely observed in theca interna cells (Palumbo & Yeh 1994), we cannot rule out the possibility that this disruption of the oxidative stress-response system in the thecal compartment may influence the atretic process.

Our data demonstrate that changes in expression of oxidative stress-response genes are associated with atresia of dominant follicles, but the factors regulating these changes remain to be elucidated. Previous studies have implicated estrogen as an inhibitor of apoptosis (Billig *et al.* 1993, Pelzer *et al.* 2000), and we observed a general inverse relationship between concentrations of estradiol in follicular fluid and incidence of apoptotic and nonviable granulosa cells. The drop in estrogen production during follicular atresia may also play a role in regulating expression of oxidative stress-response proteins. In sheep and goats, follicular MnSOD activity and estradiol content were inversely related (Singh *et al.* 1998). In our study, decreased concentrations of estradiol in follicular fluid on day 6 of the follicular wave preceded increased levels of mRNA encoding GSHPx, MnSOD and Cu/ZnSOD on day 8. These results provide evidence that estradiol may inhibit expression of oxidative stress-response genes in the bovine follicle. In cardiac myocytes, inhibition of apoptosis by estradiol is associated with reduced activity of nuclear factor κ B (NF- κ B), a known stimulator of MnSOD gene transcription (Pelzer *et al.* 2000). Activation of NF- κ B is associated with apoptotic cell death in a variety of cell types (May & Ghosh 1998). Therefore, reduced exposure of granulosa cells to estradiol during follicular atresia may stimulate NF- κ B activity, resulting in increased

granulosa cell apoptosis and increased expression of oxidative stress-response genes.

In summary, decreased production of estradiol is accompanied by increased numbers of nonviable granulosa cells in dominant follicles between days 4 and 6 of the first follicular wave in cattle. Also associated with the dominant follicle's descent into atresia are alterations in the relative expression of genes encoding the Bcl-2 family of proteins and an increase in expression of mRNA encoding oxidative stress-response proteins in granulosa cells. The results of this study expand our knowledge of the mechanisms involved in atresia of bovine dominant follicles, provide the groundwork needed to identify molecular factors that regulate expression of genes involved in apoptosis, and determine the contribution of these genes to atresia of the dominant follicle.

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