The susceptibility of granulosa cells to apoptosis is influenced by oestradiol and the cell cycle

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Abstract

Experiments were conducted to test whether oestradiol (E2) protects granulosa cells from Fas ligand (FasL)-induced apoptosis and whether protection involves modulation of the cell cycle of proliferation. Treatment of cultured bovine granulosa cells with E2 decreased susceptibility to FasL-induced apoptosis. The effects of E2 were mediated through oestrogen receptor and were not mediated by stimulation of IGF production. E2 also increased the percentage of cells progressing from G1 to S phase of the cell cycle, and increased expression of cyclin D2 protein and the cell proliferation marker Ki67. Progression from G1 to S phase of the cell cycle was necessary for the protective effect of E2; blocking progression from G1 to S phase with the cdk2 inhibitor roscovitine, or blocking cells in S phase with hydroxyurea, prevented

protection by E2. The stages of the cell cycle during which granulosa cells are susceptible to apoptosis were assessed. First, treatment with the G1 phase blocker, mimosine, protected cells from FasL-induced apoptosis, indicating that cells in G0 or early- to mid-G1 phase are relatively resistant to apoptosis. Secondly, examination of recent DNA synthesis by cells that became apoptotic indicated that apoptosis did not occur in S, G2 or M phases. Taken together, the experiments indicate that cells may be most susceptible to apoptosis at the transition from G1 to S phase. E2 stimulates transition from G1 to S phase and protects against apoptosis only when cell cycle progression is unperturbed.

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Introduction

Most ovarian follicles fail to develop fully but instead undergo degeneration by apoptosis of follicle cells. Select follicles that evade atresia and reach ovulatory status are thought to be supported by survival pathways stimulated by gonadotrophins and growth factors (Chun & Hsueh 1998, Johnson 2003). A pathway that mediates apoptosis in numerous cell types including ovarian cells is the Fas pathway. Fas is a cell surface receptor that triggers apoptosis in sensitive cells in response to binding Fas ligand (FasL) (Hengartner 2000). Granulosa and theca cells express both Fas and FasL, and expression is elevated in atretic compared with healthy follicles (Porter *et al.* 2000, Vickers *et al.* 2000). Bovine granulosa cells from atretic subordinate follicles are more sensitive to FasL-induced apoptosis than cells from healthy dominant follicles (Porter *et al.* 2000). Removal of serum from the media of cultured bovine granulosa cells increases expression of Fas and FasL, and induces apoptosis that is at least partially mediated by endogenous Fas/FasL interactions (Hu *et al.* 2001). Induction of apoptosis by addition of exogenous FasL to cultured granulosa cells is inhibited by serum and a number of growth factors (Quirk *et al.* 2000).

Growth factors that suppressed FasL-induced apoptosis also increased proliferation of granulosa cells (Quirk *et al.* 2000). This association suggested that the ability of growth factors to inhibit apoptosis might be dependent upon their effects on the cell cycle of proliferation. In rodents and cattle, the highest frequency of atresia occurs in size classes of follicles in which granulosa cells are proliferating rapidly (Pedersen 1970, Hirshfield & Midgley 1978, Lussier *et al.* 1987). Follicles are believed to become increasingly dependent upon gonadotrophins and other growth factors for viability during stages of follicle development when rapid granulosa cell proliferation occurs (reviewed in Hirshfield (1991)). The concept that growth factors may promote the survival of granulosa cells by maintaining progression through the cell cycle is supported by increasing evidence that the susceptibility of cells to apoptosis varies with stage of the cell cycle (Meikrantz & Schlegel 1995, King & Cidlowski 1998, Guo & Hay 1999, Schutte & Ramaekers 2000). We have demonstrated that the ability of insulin-like growth factor-I (IGF-I) to suppress FasL-induced apoptosis of bovine granulosa cells is mediated by the PI3K/Akt pathway and is dependent on unperturbed progression through the cell cycle (Hu *et al.* 2004).

Oestrogen is known to increase proliferation of granulosa cells and to regulate the differentiation of follicle cells (Rao *et al.* 1978, Rosenfeld *et al.* 2001, Drummond *et al.* 2002, Couse *et al.* 2005). Oestrogen is also thought to be a survival factor in granulosa cells, based primarily on the finding that treatment of immature hypophysectomized rats with implants of diethylstilbestrol (DES) was followed by implant withdrawal-induced apoptosis of granulosa cells in multilayered preantral follicles (Billig *et al.* 1993). The mechanism for this effect of oestrogen in promoting granulosa cell viability was not determined. In cattle, follicles that are selected for continued growth and development to the ovulatory stage have increased capacity to secrete oestradiol (E2) relative to follicles destined to undergo atresia (Evans & Fortune 1997, Mihm *et al.* 2000). Thus, elevated E2 correlates with follicle survival. We hypothesize that the mechanism by which E2 and other growth factors protect against apoptosis is associated with their involvement in promoting progression through the cell cycle. The current study provides evidence in support of this concept. We demonstrate that E2 protects bovine granulosa cells from FasL-induced apoptosis *in vitro* and that this effect is dependent on progression through the cell cycle. Furthermore, we show that granulosa cells are most susceptible to apoptosis at the G1 to S phase transition of the cell cycle.

Materials and Methods

Materials

Culture media, fetal bovine serum (FBS), BSA, penicillin, streptomycin and fungizone were obtained from Life Technologies, Inc. Sodium pyruvate, L-glutamine, E2 and bromodeoxyuridine (BrdU) were obtained from Sigma. ICI 182,780 (Fulvestrant) was obtained from AstraZeneca. Tissue culture plates were obtained from Corning-Costar (Cambridge, MA, USA), and slide wells were obtained from Nalge Nunc International (Naperville, IL, USA). Soluble recombinant human FasL was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Cell cycle inhibitors (roscovitine, hydroxyurea and mimosine) were from Calbiochem (San Diego, CA, USA). Rabbit anti-human cyclin D2 (sc-181) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-human Ki67 was from Novocastra Laboratories (Newcastle upon Tyne, UK). Mouse anti-human IGF-I receptor (clone 33255-111) was from R&D Systems (Minneapolis, MN, USA), and mouse anti-BrdU (clone 3D4) was from BD Biosciences (San Diego, CA, USA). Alexa 488-conjugated goat anti-rabbit IgG, Alexa 488-conjugated goat anti-mouse IgG and propidium iodide (PI) were from Molecular Probes (Eugene, OR, USA).

Figure 1 Treatment with E2 protects granulosa cells from FasL-induced cell death. Granulosa cells were cultured for 2 days in media containing FBS and 0–1000 ng/ml E2. Media were changed to defined media containing the same concentrations of E2 ($t=0$ h), and 0 or 100 ng/ml FasL were added to all treatments at 6 h. Numbers of viable cells were determined at 24 h by cell counts following staining with trypan blue. The percentage of cell death in response to FasL was calculated by comparing the number of viable cells in cultures treated with or without FasL. Bars with different superscripts are significantly different ($P<0.05$).

Cell isolation and culture

Freshly excised cow ovaries were obtained from an abattoir, transported in saline at 4 °C (approximately 1.5 h) and processed immediately. Granulosa cells were collected by aspiration of 2–4 mm follicles and cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 containing 10% FBS and supplemented with 1 mM pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone. Follicles in this size range contain proliferative granulosa cells. In some experiments, 0–1000 ng/ml E2 were added to the media at the time of plating. A dose of 1000 ng/ml E2 was used in most experiments because it was determined to be a maximally effective dose in initial dose–response studies (Fig. 1). The concentration of E2 in follicular fluid varies from approximately 0.5–20 ng/ml in bovine follicles of 0.5–4.5 mm diameter (Austin *et al.* 2001) to 1 µg/ml in healthy preovulatory follicles >15 mm diameter (Fortune *et al.* 1988). Cells were plated (day 0) at 5×10^4 cells/well in 96-well plates for cell viability assays, at 1×10^6 cells/well in 35 mm dishes for flow cytometric analyses or at 2×10^5 cells/well in 8-well slide wells for immunocytochemistry. On day 1, media were replaced with the same media. On day 2, media were changed to DMEM-F12 supplemented as above but without serum and containing 100 ng/ml insulin, 5 µg/ml transferrin, 20 nM sodium selenite and 0.1% BSA (ITS). Treatments were applied at this time as described below. The concentration of insulin in ITS is significantly lower than in commercial preparations of ITS

(1 µg/ml or greater). In preliminary experiments, a dose of 100 ng/ml insulin was found to be sufficient to maintain viability of granulosa cells but not to block apoptosis in response to treatment with FasL.

Assay of granulosa cell susceptibility to apoptosis

On day 2, culture media were changed to ITS, and treatments were applied as described in the Results. Within each replicate of an experiment, each treatment was tested in eight wells. After 6 or 8 h, depending on the experiment, FasL was added at a final concentration of 0 ng/ml to four wells and 100 ng/ml to four wells. After 24 h, cells were trypsinized, collected and stained with trypan blue, and live cells were counted in a haemocytometer. The percentage of granulosa cells killed by FasL was calculated for each treatment within an experimental replicate by comparing the mean number of live cells present in the four FasL-treated wells vs the mean number of cells in the four wells receiving no FasL. Each experiment was repeated five times using separate granulosa cell preparations. In some experiments, flow cytometry of cells stained for DNA content with PI was used to determine the percentage of apoptotic cells (described below).

Immunocytochemical assessment of proliferation in cultured granulosa cells

The effects of the various treatments on the proliferation of granulosa cells was examined by determining the percentage of cells expressing the cell proliferation marker, Ki67, and the cell cycle regulator, cyclin D2. Granulosa cells plated in eight-well slide-well chambers were treated on day 2 with 0 or 1000 ng/ml E2. Twenty-four hours later, the cells were fixed in cold acetone for 2 min, and stained for Ki67 or cyclin D2 using similar protocols. Fixed cells were incubated with primary antibody (1 µg/ml mouse anti-human Ki67 or 0.5 µg/ml rabbit anti-human cyclin D2) in PBS–2% BSA for 1 h at 37 °C, rinsed, and incubated with secondary antibody (2 µg/ml goat anti-mouse IgG–Alexa 488 for Ki67 or 1 µg/ml goat anti-rabbit IgG–Alexa 488 for cyclin D2) for 1 h at 37 °C. After rinsing, cells were counterstained with 1 µg/ml PI. Stained cells were examined under epifluorescent illumination, and coincident images of Alexa 488 and PI fluorescence obtained. The filters used were: for Alexa-488, excitation 460–500 nm and emission 500–540 nm; for PI, excitation 536–556 nm and emission >590 nm. The number of cells with distinct nuclear Alexa 488 fluorescence and the number of cells with PI fluorescence were used to calculate the percentage of cells expressing Ki67 or cyclin D2. In each experiment, images were obtained from four randomly chosen fields for each treatment, and experiments were repeated 5 times using separate granulosa cell preparations. Cell counts of Alexa

488-positive cells and total cells were determined by two observers without knowledge of treatment.

Cell cycle analysis

The effects of treatments on the distribution of cells in the cell cycle were determined by flow cytometry of cells stained for DNA content using PI (Hu *et al.* 2004). Granulosa cells cultured in six-well culture plates were collected by trypsinization, fixed in 80% ethanol and stored at 4 °C until staining for flow cytometry. Cells (5×10^5) were stained with 5 µg/ml PI in 0.01 M PBS containing 0.01% Triton X-100 and 30 µg/ml DNase-free RNase A. Cells (10 000 per sample) were analyzed on a FACScan flow cytometer (Becton, Dickinson and Co, NJ, USA). Data were gated for single cells and DNA content assigned to G0/G1, S or G2/M phases based on the method of Ormerod (1994) using WinMDI software (The Scripps Research Institute, La Jolla, CA, USA).

Flow cytometric detection of BrdU incorporation in apoptotic cells

Incorporation of BrdU was used in conjunction with PI binding to DNA to examine whether cells residing in S phase, or which had recently passed through S phase, were susceptible to apoptosis. On day 2, granulosa cells in six-well plates were treated at 0 h with 0 or 10 µM BrdU in DMEM–F12–ITS and at 6 h with 0 or 100 ng/ml FasL. At 24 h cells were trypsinized, resuspended in DMEM–F12 and fixed in 80% ethanol. Detection of BrdU was performed as previously described (Wilson 1994) with minor modifications (Hu *et al.* 2004). Briefly, cells were pretreated with 100 µg/ml RNase A for 20 min, rinsed in PBS, and treated with 0.1 M Na-citrate in 0.5% Triton X-100 in PBS for 10 min on ice. Cells were rinsed in 0.01 M Tris buffer containing 10 mM MgCl₂, DNA was partially digested by addition of 30 U/ml BamHI for 30 min, and cells were rinsed in Tris buffer. Cells were incubated with 2 µg/ml mouse anti-BrdU in PBS–0.5% BSA–0.5% Tween 20 for 2 h at room temperature and then with 0.5 µg/ml goat anti-mouse IgG–Alexa 488 in the same buffer for 2 h at room temperature. The cells were counterstained with PI to measure the DNA content, and 20 000 cells were analyzed for both BrdU and PI fluorescence on a FACScan flow cytometer. Events were gated for single cells based on PI fluorescence. Within each experimental replicate, a fluorescent threshold for identification of positive cells was established based on negative control cells that received no BrdU and were processed as described above. The threshold was chosen such that >95% of the cells that did not receive BrdU were negative. Cells were assigned to A0 (apoptotic cells that appeared to have sub-diploid content of DNA), G0/G1, S or G2 M phases based on the method of Ormerod (1994) using WinMDI software (The Scripps Research Institute).

In areas where A0 or S phases overlap with cells in G0/G1, and where S phase overlaps with G2/M, cells were assigned to G0/G1 or G2/M respectively, so that cells identified as A0 or S phase contained no cells in other phases. Cells within each phase were then separated into those containing BrdU (i.e. having made new DNA in the last 24 h of culture) and those not containing BrdU (i.e. quiescent cells).

Statistical analysis

Most experiments were analyzed by one-way ANOVA using a randomized complete block design with experimental replicates as blocks. All treatments were applied to each replicate. All data were subjected to, and passed, tests for normality and equality of variance prior to ANOVA. The Student–Newman–Keuls procedure was used for comparison of means when overall significance was observed (Ott & Longnecker 2001). The percentage of cells staining positively for Ki67 and the percentage of cells staining positively for cyclin D2 were analyzed by paired, two-tailed *t*-tests. Each experimental replicate included both treatments (control and E2) and results from each replicate were paired in the analyses.

Results

E2 protects cells from FasL-induced cell death by interaction with oestrogen receptor (ER)

Initial experiments were performed to test whether E2 protects granulosa cells from cell death induced by treatment with FasL. Granulosa cells were plated (day 0) and cultured for 2 days in media containing FBS and doses of E2 from 63 to 1000 ng/ml. On day 2, media were changed to defined media with the same concentrations of E2, and the cells were treated with FasL 6 h later. Cell viability was determined at 24 h. The percentage of cell death in response to FasL was calculated by comparing the number of cells present in wells treated with FasL to the number of cells in wells treated identically but without FasL (Fig. 1). Treatment with E2 caused a dose-dependent decrease in FasL-induced cell death. At the highest dose of E2, the percentage of cell death was reduced by 47%.

To determine whether the observed effect of E2 is mediated through ER, the ability of the specific ER antagonist, ICI 182,780, to block the effect of E2 was tested. The treatment protocol was designed to allow pretreatment with ICI 182,780 before addition of E2 to cultures. Cells were cultured in media containing FBS in the absence of E2 for 2 days and were then changed to defined media. After 3.5 h, cells were treated with 0 or 20 μ M (12.1 μ g/ml) ICI 182,780 and 0.5 h later with 0 or 1000 ng/ml E2 (*t*=0 h). FasL was added to the appropriate cultures 4 h after E2, and cells were examined at 24 h

Figure 2 Protection by E2 against FasL-induced cell death is mediated by ER. Granulosa cells in defined media were pretreated with 20 μ M ICI 182,780, an ER antagonist, and treated 0.5 h later with 0 or 1000 ng/ml E2 (*t*=0 h). At 4 h cells were treated with 0 or 100 ng/ml FasL. Numbers of viable cells were determined at 24 h by cell counts following staining with trypan blue. The percentage of cell death in response to FasL was calculated by comparing the number of viable cells in cultures treated with or without FasL. Bars represent the means $\pm$ S.E.M. of results obtained in five experiments using separate granulosa cell preparations. Bars with different superscripts are significantly different (*P*<0.05).

for viability. Treatment with E2 protected cells from FasL-induced cell death but pretreatment with ICI 182,780 blocked this effect (Fig. 2).

The number of apoptotic cells in granulosa cell cultures was assessed by analysis of cellular DNA content by flow cytometry of cells stained with PI. The peak of cells with apparent sub-diploid content of DNA (A0 peak) represents apoptotic cells present in the culture 24 h after treatment with FasL. Treatment with FasL increased the percentage of apoptotic cells from non-detectable to 14%, and E2 suppressed this effect (Fig. 3). Pretreatment with ICI 182,780 abolished the protective effect of E2. Note that the A0 peak does not represent the cumulative population of cells undergoing apoptosis over 24 h of culture because many cells in advanced stages of apoptosis are lost by degradation prior to flow cytometry.

Protective effect of E2 is not mediated by IGF

Experiments were performed to test whether the protective effect of E2 against FasL-induced apoptosis is mediated through induction of IGF secretion by granulosa cells and subsequent effects through IGFR. We have previously shown that treatment of granulosa cells with IGF-I protects cells from FasL-induced apoptosis (Hu *et al.* 2004). Effects mediated through IGFR were assessed using an anti-IGFR antibody that blocks the binding of ligand to the receptor. Cells were cultured in media containing FBS for 2 days. Media were changed to

Figure 3 E2 reduces apoptosis in granulosa cells treated with FasL. Granulosa cells in defined media were pretreated with 20 μ M ICI 182,780 and treated 0.5 h later with 0 or 1000 ng/ml E2 ($t=0$ h). At 4 h cells were treated with 0 or 100 ng/ml FasL. At 24 h, cells were fixed, stained with PI and analyzed by flow cytometry to determine the proportion of apoptotic cells. (A) Percentage of cells having apparent sub-diploid content of DNA (A0) characteristic of apoptosis. In cultures not treated with FasL (not shown) the percentage of A0 cells was less than 2%. Bars represent the means $\pm$ S.E.M. of results obtained in five experiments using separate granulosa cell preparations. Bars with different superscripts are significantly different ($P<0.05$). (B) Representative histograms of cell number vs DNA content (PI fluorescence), used to determine the number of cells in different phases of the cell cycle or in the A0 region (shaded grey).

Figure 4 Protection by E2 against FasL-induced cell death does not require IGF signalling. Granulosa cells in defined media were pretreated with 5 μ g/ml antibody against IGFR (anti-IGFR) and treated 0.5 h later with 0 or 1000 ng/ml E2, or 200 ng/ml IGF-I ($t=0$ h). At 4 h, cells were treated with 0 or 100 ng/ml FasL. Numbers of viable cells were determined at 24 h by cell counts following staining with trypan blue. The percentage of cell death in response to FasL was calculated by comparing the number of viable cells in cultures treated with or without FasL. Bars represent the means $\pm$ S.E.M. of results obtained in five experiments using separate granulosa cell preparations. Bars with different superscripts are significantly different ($P<0.05$).

defined media and 4 h later cells were treated with 5 μ g/ml anti-IGFR antibody or non-specific IgG as control. Cells were treated 0.5 h later with 0 or 1000 ng/ml E2, or with 0 or 100 ng/ml IGF-I ($t=0$ h). Cultures were treated 4 h later with 0 or 100 ng/ml FasL and viability was determined at 24 h. Treatment with anti-IGFR antibody had no effect on the ability of E2 to suppress FasL-induced cell death (Fig. 4). In contrast, anti-IGFR antibody blocked interaction of IGF-I with receptor since it prevented the protective effect of IGF-I against FasL-induced cell death (Fig. 4). These results show that the protective effect of E2 is not mediated through IGF.

E2 promotes cell cycle progression

Several markers of cell cycle progression were examined in granulosa cells cultured in the presence or absence of E2. Ki67, a nuclear protein that is expressed in proliferating cells during S, G2 and M phases and, in some cells, during G1 phase (Endl & Gerdes 2000, Scholzen & Gerdes 2000), was expressed in $19.4 \pm 2.4\%$ of cells treated with E2 but in only $14.0 \pm 1.4\%$ of cells not treated with E2 ($P<0.05$; Fig. 5A). Cyclin D2, a D-type cyclin associated with entry of granulosa cells into the cell cycle (Sicinski *et al.* 1996), was expressed in $24.8 \pm 4.4\%$ of cells treated with E2 but in only $12.1 \pm 3.0\%$ of cells not treated with E2 ($P<0.05$; Fig. 5B). Granulosa cells

Figure 5 Treatment with E2 increases the percentage of granulosa cells progressing through the cell cycle. Cells were cultured for 2 days in media containing FBS and 0 (CON) or 1000 ng/ml E2. Media were changed to defined media containing the same concentration of E2 ($t=0$ h), and cells were fixed at 24 h. Expression of Ki67 (A) and cyclin D2 (B) were detected by immunocytochemistry and cell nuclei were counterstained with PI. Images of fluorescence in representative fields are shown. Immunocytochemistry was performed on five separate granulosa cell preparations.

stained for DNA content using PI were analyzed by flow cytometry to determine the percentages of cells in various stages of the cell cycle. Treatment of cells with E2 increased the percentage of cells in S phase and

Figure 6 The effect of E2 in increasing progression through the cell cycle is mediated by ER. Granulosa cells in defined media were pretreated with 20 μ M ICI 182,780, an ER antagonist, and treated 0.5 h later with 0 or 1000 ng/ml E2 ($t=0$ h). At 4 h, cells were treated with 0 or 100 ng/ml FasL. The proportion of cells in various stages of the cell cycle (G0/G1, S and G2/M) was determined at 24 h by flow cytometry of PI-stained cells. Bars represent the means $\pm$ S.E.M. of results obtained in five experiments using separate granulosa cell preparations. Within each stage of the cell cycle, bars with different superscripts are significantly different ($P<0.05$).

decreased the percentage of cells in G0/G1 phases (Fig. 6), indicating that E2 increased progression from G1 to S phase. Pretreatment of cells with ICI 182,780 prevented E2-induced changes in the cell cycle (Fig. 6). Relatively small differences among treatment groups in the percentages of cells positive for markers of proliferation at a single time point represent a larger percentage of cells affected by treatments over a 24 h period of culture.

Protection from apoptosis by E2 requires cell cycle progression

The results presented above demonstrate two effects of E2 on granulosa cells: protection against FasL-induced apoptosis and promotion of cell cycle progression. Experiments were performed to test whether cell cycle progression was necessary for the protective effect of E2 against apoptosis. The effects of the drugs roscovitine (which inhibits cyclin-dependent kinase 2 and blocks cells at the G1/S interface) and hydroxyurea (HU; which blocks cells in S phase) were determined. Granulosa cells were cultured for 2 days in media containing FBS and 0 or 1000 ng/ml E2.

Figure 7 Protection by E2 against FasL-induced cell death is prevented by blocking at the G1/S interface. Granulosa cells were cultured for 2 days in media containing FBS and 0 or 1000 ng/ml E2. Media were changed to defined media containing the same concentration of E2 and 0 or 20 μ M roscovitine, which blocks cells at the G1/S interface ($t=0$ h). (A) Percentage of cell death in response to FasL. At 6 h, 0 or 100 ng/ml FasL were added to all treatments. Numbers of viable cells were determined at 24 h by cell counts following staining with trypan blue. The percentage of cell death in response to FasL was calculated by comparing the number of viable cells in cultures treated with or without FasL. (B) Cell cycle stages in cells treated with or without E2 or roscovitine. Cells were collected at 24 h, stained with PI, and DNA content determined by flow cytometry. Bars represent the means $\pm$ S.E.M. of results obtained in five experiments using separate granulosa cell preparations. Within each panel, and for a given stage of the cell cycle, bars with different superscripts are significantly different ($P<0.05$).

Media were then changed to the defined media with or without E2, and with 0 or 20 μ M roscovitine or with 0 or 25 μ g/ml HU ($t=0$ h). Cultures were treated 6 h later with 0 or 100 ng/ml FasL, and cell viability assays and cell cycle analysis were done at 24 h. As expected, treatment with E2 decreased the percentage of cell death in response to FasL (Fig. 7A). Treatment with roscovitine alone had no effect on FasL-induced cell death but treatment with both roscovitine and E2 prevented the protective effect of E2 (Fig. 7A). Cell cycle analysis confirmed that treatment with roscovitine inhibited cell cycle progression, as evidenced by an increase in the percentage of cells in G0/G1 phases and a decrease in the percentage of cells in S phase (Fig. 7B). Thus, the protective effect of E2 was abolished when progression from G1 to S phase was suppressed. HU had no effect alone but prevented the protective effect of E2 against FasL-induced cell death (Fig. 8A). Analysis of the cell cycle showed that HU treatment alone and in the presence of E2 blocked cells in S phase (Fig. 8B). Examination of cell cycle histograms indicates that most of these cells were in early-S phase, suggesting that synthesis of DNA was reduced relative to that normally observed in S phase (data not shown).

Granulosa cells in G0 and early- to mid-G1 phases are relatively resistant to FasL-induced apoptosis

Studies in a variety of cell types, including studies in our laboratory with bovine granulosa cells, have suggested that quiescent cells are relatively resistant to apoptosis (Quirk *et al.* 2004). In contrast, proliferative granulosa cells may be susceptible to apoptosis unless protected by growth factors such as IGF-I (Hu *et al.* 2004). We therefore compared the effects of E2 (which promotes G1 to S phase progression) and mimosine (which blocks cells in early- to mid-G1 phase) on FasL-induced cell death. Granulosa cells were cultured for 2 days in media containing FBS and 0 or 1000 ng/ml E2. Media were then changed to defined media with or without E2, and containing 0 or 1000 μ M mimosine ($t=0$ h). Cultures were treated 6 h later with 0 or 100 ng/ml FasL, and cell viability and distribution in the cell cycle were determined at 24 h. Treatment with either mimosine or E2 was equally protective against FasL-induced cell death (Fig. 9A). Co-treatment with mimosine and E2 did not further reduce cell death in response to FasL (Fig. 9A). Analysis of the cell cycle by flow cytometry confirmed that mimosine had the expected effect of blocking cells in G1 phase; mimosine increased the percentage of cells in G0/G1 and decreased the percentage of cells in S and G2/M phases. E2 increased progression from G0/G1 to S phase and this effect was blocked by co-treatment with mimosine (Fig. 9B). These results show that treatment with mimosine or E2 protects cells against apoptosis. The protective effect of mimosine is associated with blockage

Figure 8 Protection by E2 against FasL-induced cell death is prevented by blocking in S phase. Granulosa cells were cultured for 2 days in media containing FBS and 0 or 1000 ng/ml E2. Media were changed to defined media containing the same concentration of E2 and 0 or 25 µg/ml HU, which blocks cells in S phase ($t=0$ h). (A) Percentage of cell death in response to FasL. At 6 h, 0 or 100 ng/ml FasL were added to all treatments. Numbers of viable cells were determined at 24 h by cell counts following staining with trypan blue. The percentage of cell death in response to FasL was calculated by comparing the number of viable cells in cultures treated with or without FasL. (B) Cell cycle stages in cells treated with or without E2 or HU. Cells were collected at 24 h, stained with PI, and DNA content determined by flow cytometry. Bars represent the means $\pm$ S.E.M. of results obtained in five experiments using separate granulosa cell preparations. Within each panel, and for a given stage of the cell cycle, bars with different superscripts are significantly different ($P<0.05$).

Figure 9 Granulosa cells are relatively resistant to FasL-induced cell death in G0 and early-G1 phases. Granulosa cells were cultured for 2 days in media containing FBS and 0 or 1000 ng/ml E2. Media were changed to defined media containing the same concentration of E2 and 0 or 1000 µM mimosine (Mim), which blocks cells in G1 ($t=0$ h). (A) Percentage of cell death in response to FasL. At 6 h, 0 or 100 ng/ml FasL were added to all treatments. Numbers of viable cells were determined at 24 h by cell counts following staining with trypan blue. The percentage of cell death in response to FasL was calculated by comparing the number of viable cells in cultures treated with or without FasL. (B) Cell cycle stages in cells treated with or without mimosine. Cells were collected at 24 h, stained with PI, and DNA content determined by flow cytometry. Bars represent the means $\pm$ S.E.M. of results obtained in five experiments using separate granulosa cell preparations. Within each panel, and for a given stage of the cell cycle, bars with different superscripts are significantly different ($P<0.05$).

of cells in early- to mid-G1 phase. The protective effect of E2 is associated with increased progression from G1 to S phase.

Granulosa cells are susceptible to FasL during progression from G1 to S phase

The data presented above indicate that cells are relatively resistant to apoptosis in G0 and early-G1 phases of the cell cycle. Experiments using roscovitine suggest that cells progressing through the G1 to S phase transition may be more susceptible to apoptosis than quiescent cells. The final experiment used a combination of cell labelling for DNA synthesis and flow cytometry to determine whether cells undergo apoptosis during the G1 to S transition or in later stages (S, G2 or M). Granulosa cells that had entered or passed through S phase were labelled by culturing in the presence of the thymidine analogue BrdU, and FasL was added to cultures 6 h later. At 24 h cells were stained with both an anti-BrdU antibody and PI, and flow cytometry was performed to determine the percentages of BrdU-labelled cells in various stages of the cell cycle and in the A0 region of apoptotic cells. The rationale was that if FasL-induced apoptosis occurs during the G1 to S phase transition, apoptotic cells would not have synthesized DNA during the culture period and would not be labelled with BrdU. If FasL-induced apoptosis occurs during S, G2 or M phase, a relatively high percentage of apoptotic cells would be labelled with BrdU. Overall, $32 \pm 5\%$ of cells had incorporated BrdU during the treatment period. As shown in Fig. 10A, most of the cells in S and G2/M phases had incorporated BrdU, although about 40% of the cells in S and G2/M phases were unlabelled. The majority of cells in S phase that did not incorporate BrdU were in early-S phase and had levels of BrdU-associated fluorescence just below the threshold for positive cells (marked with a dashed line in panels in Fig. 10B). Among G2/M phase cells that did not incorporate BrdU, approximately half were just below the threshold. The remainder, which had low fluorescence, probably include the small number of cell doublets which escape gating for single cells and are thus negative for BrdU. Cells in G0/G1 that were positively stained for BrdU (25%) probably represent cells that had incorporated BrdU during S phase and re-entered G0/G1 during the 24 h of culture. The percentage of cells in the A0 peak positively staining for BrdU was 17%, significantly lower than the percentage of BrdU-labelled cells in each phase of the cell cycle ($P < 0.05$; Fig. 10A). The data indicate that cells that became apoptotic had not entered into or passed through S phase, as evidenced by their predominant lack of BrdU labelling. Since cells in G0 and early G1 were shown above to be resistant to apoptosis (Fig. 9), and cells marked for passage through S, G2 or M phases are not among the apoptotic cells, it can be concluded that apoptosis occurs predominantly during the G1 to S transition.

Figure 10 FasL-induced apoptosis of granulosa cells occurs predominantly at the G1 to S phase transition. Granulosa cells in defined media were treated with 0 or 10 μM BrdU ($t=0$ h), and with 0 or 100 ng/ml FasL at 6 h. BrdU is incorporated into cellular DNA during S phase. At 24 h, cells that had passed through S phase in the presence of BrdU were detected by immunofluorescence using an anti-BrdU antibody. Cells were co-stained with PI and analyzed by flow cytometry. Cells were categorized as apoptotic (A0) or in G0/G1, S or G2/M phases based on PI fluorescence. In cultures receiving BrdU, the percentage of cells positively labelled with BrdU in each stage was determined. (A) Percentage of cells in A0 or in each stage of the cell cycle that incorporated BrdU in cultures of granulosa cells treated with FasL. Bars represent the means $\pm$ S.E.M. of results obtained in five experiments using separate granulosa cell preparations. Bars with different superscripts are significantly different ($P < 0.05$). (B) Representative dot plots of BrdU incorporation vs DNA content (PI fluorescence). Each dot represents a single cell. The left panel shows the pattern of fluorescence in cells not treated with BrdU. The fluorescent threshold, above which cells are considered positively stained for BrdU, is indicated by the dashed line. The center panel shows one of the samples used to generate the data presented in panel A. Note that cells in the A0 region are predominantly below the threshold for BrdU fluorescence. The right panel shows a control sample not treated with FasL, with few cells in the A0 region.

Discussion

The major findings of this study are that the mechanism whereby E2 protects granulosa cells from apoptosis is dependent on progression through the cell cycle and that granulosa cells are most susceptible to apoptosis at the G1

to S transition of the cell cycle. The data show for the first time that E2 protects granulosa cells against apoptosis induced by treatment with FasL. Furthermore, this effect is mediated through ER and is dependent upon progression through the cell cycle. Fas and FasL are expressed in granulosa and theca cells at higher levels in atretic vs healthy follicles in a number of species (Hakuno *et al.* 1996, Kondo *et al.* 1996, Kim *et al.* 1998, Porter *et al.* 2000, Bridgham & Johnson 2001) and cells from healthy bovine follicles are more resistant to FasL-induced apoptosis than cells from atretic follicles (Porter *et al.* 2000). A number of growth factors – including IGF-I, fibroblast growth factor (FGF) and epidermal growth factor (EGF) – as well as serum, suppress FasL-induced apoptosis of granulosa cells *in vitro* (Quirk *et al.* 2000, Hu *et al.* 2001). Interestingly, growth factors that protected against FasL-induced apoptosis also increased cell proliferation (Quirk *et al.* 2000, Hu *et al.* 2004). This suggested the possibility that the protective effect of mitogens against apoptosis may be dependent upon their effects on the cell cycle. The current study focused on E2 because of its importance in follicular development and granulosa cell proliferation.

Treatment of granulosa cells with E2 increased progression from G1 to S phase of the cell cycle and this effect was mediated through ER. This was demonstrated by a decrease in the percentage of cells in G0/G1 and an increase in the percentage of cells in S phase in response to treatment with E2; however, this response was not observed with E2 in the presence of the ER antagonist ICI 162,780. E2 also increased the percentage of cells staining positively for the cell proliferation marker Ki67, and for cyclin D2, a cyclin critical for entry of cells into the cell cycle and progression through G1 phase (Sherr & Roberts 1999). Cyclin D2 is the critical D-type cyclin required for proliferation of granulosa cells (Sicinski *et al.* 1996). Our findings with bovine granulosa cells are consistent with the previous observations that treatment of immature, hypophysectomized rats with E2 promoted the proliferation of granulosa cells (Rao *et al.* 1978) and increased levels of cyclin E protein (Robker & Richards 1998a) and that E2 increased cyclin D2 mRNA levels in cultured granulosa cells from immature rats by an ER-mediated mechanism (Robker & Richards 1998b). The ER antagonist used in our study inhibits actions of E2 through both ER α and ER β . The data presented, therefore, do not distinguish between an effect of E2 through ER α or ER β . It is possible, however, that effects may be mediated through ER β since it is the predominant form of ER in granulosa cells of the cow and rodents (Rosenfeld *et al.* 1999; reviewed in Couse *et al.* (2005)). Furthermore, the phenotypes of mice null for ER β or ER α indicate that ER β is more critical for intraovarian effects than ER α (Couse *et al.* 2005).

The importance of cell cycle progression for the protective effect of E2 against FasL-induced apoptosis was

tested using drugs that prevent cell cycle progression. Treatment of granulosa cells with roscovitine blocked progression from G0/G1 to S phase and prevented the protective effect of E2 against FasL-induced apoptosis. Treatment with HU blocked cells in S phase and this also prevented protection by E2. These results indicate that protection by E2 is dependent upon uninterrupted progression through G1 and S phases of the cell cycle. We previously reported that treatment with IGF-I protects bovine granulosa cells from FasL-induced apoptosis (Quirk *et al.* 2000, Hu *et al.* 2004). The mechanism of action of IGF-I has similarities to what we have observed for E2. Protection by IGF-I is associated with increased progression from G1 to S phase and is dependent upon uninterrupted progression through the cell cycle (Hu *et al.* 2004). Thus, two different agonists, E2 and IGF-I, which are known to play critical roles in folliculogenesis, protect against apoptosis by mechanisms that involve modulation of the cell cycle. These observations may be relevant to mechanisms that promote follicle development *in vivo* since selection of the healthy dominant follicle in cattle is associated with increased intrafollicular E2 and increased bioavailability of IGF (Mihm *et al.* 2000, Austin *et al.* 2001, Rivera *et al.* 2001). Although the effect of E2 to protect against FasL-induced apoptosis has similarities to what was observed for IGF-I, the effect of E2 is not mediated by stimulation of IGF production by granulosa cells (Hsu & Hammond 1987). This was demonstrated by failure of an anti-IGFR antibody, which effectively blocked protection by IGF-I, to block protection by E2.

Granulosa cells appear to be relatively resistant to apoptosis in G0/early-G1 phases of the cell cycle. Treatment with the drug mimosine blocked cells in G1 phase of the cell cycle and this was associated with increased resistance to FasL-induced apoptosis. Treatment with both mimosine and E2 did not further suppress FasL-induced apoptosis. This suggests that protection by E2 and mimosine are mediated differently. While mimosine blocked cells in G1 phase of the cell cycle, E2 promoted progression from G1 to S phase. Thus, the protective effect of mimosine is associated with blocking cells in G0 or early- to mid-G1 phase while the protective effect of E2 is associated with increased progression from G1 to S phase. The finding that granulosa cells in G0/G1 are resistant to apoptosis is consistent with our previous study which demonstrated that granulosa cells from bovine preovulatory follicles that have been exposed to a luteinizing hormone (LH) surge *in vivo* are resistant to apoptosis and that this effect is dependent upon their withdrawal from the cell cycle (Porter *et al.* 2001, Quirk *et al.* 2004). Interestingly, in GDF-9 null mice, the defect in proliferation of granulosa cells and subsequent formation of luteinized nests of cells is associated with lack of granulosa cell apoptosis (Elvin *et al.* 1999). Studies on neuroblastoma cells (Poluha *et al.* 1996) and myoblasts (Wang & Walsh 1996) have also shown that terminal differentiation and

associated withdrawal from the cell cycle promote resistance to apoptosis. In addition, members of the bcl-2 family of proteins, which are involved in supporting cell survival, are reported to prevent quiescent cells from entering the cell cycle (Schutte & Ramaekers 2000).

The protective effect of E2 is associated with increased progression from G1 to S phase and can be prevented by drugs that interfere with G1 to S phase progression. These findings suggest that granulosa cells may be most susceptible to apoptosis, and dependent upon growth factors for survival, at the transition from G1 to S phase of the cell cycle. We tested this hypothesis by labelling granulosa cells synthesizing DNA in S phase, by incorporation of the thymidine analogue BrdU, and determining the fate of cells after treatment with FasL. Analysis of the DNA content of cells by flow cytometry showed that most apoptotic cells, identified by sub-diploid content of DNA, had not incorporated BrdU. The percentage of apoptotic cells that had incorporated BrdU was lower than the percentage of cells incorporating BrdU in any of the cell cycle stages, including G0/G1. This indicates that most of the cells that were susceptible to FasL-induced apoptosis during the 24 h culture period had not synthesized DNA during S phase of the cell cycle or reached G2/M phase. Taken together with our finding that blockage of granulosa cells in early- to mid-G1 phase of the cell cycle using mimosine protected against FasL-induced cell death, the results suggest that cells may undergo apoptosis predominantly at the G1 to S phase transition. Our data show that cells are most sensitive to apoptosis at the G1/S interface, E2 increases the passage of cells from G1 through the G1/S interface to S phase, and yet E2 protects cells from apoptosis. Two different mechanisms can be proposed to account for this. One is that E2 specifically protects cells as they move from G1 to S phase. The second possibility is that cells that enter the cell cycle but do not have critical support, whether from E2 or from growth factors, will remain at the G1/S interface and succumb to apoptosis. Cells that do have support will proceed rapidly into S phase, thus limiting their susceptibility to apoptosis. The relationship between the cell cycle and susceptibility to apoptosis in granulosa cells is similar to what has been reported for T cells: agents that block the cell cycle in early G1 induce resistance of T cells to apoptosis while agents that block at the G1 to S phase transition increase apoptosis (Meikrantz & Schlegel 1995, Lissy *et al.* 1998).

In the current study, serum-containing media were used for the first 2 days of culture to promote cell attachment and viability. Media were then changed to serum-free media supplemented with ITS to study susceptibility to FasL-induced apoptosis. While bovine granulosa cells are resistant to FasL-induced apoptosis in the presence of serum, shifting cells to a more defined media containing 100 ng/ml insulin generates cultures in which at least a portion of the cells undergo apoptosis in response to treatment with FasL (Quirk *et al.* 2000). These con-

ditions were used in our previous studies to examine the effect of various growth factors on susceptibility to FasL-induced apoptosis (Quirk *et al.* 2000, Hu *et al.* 2004). A concern is the tendency of granulosa cells to differentiate toward a luteal phenotype when cultured in the presence of serum. When granulosa cells luteinize after exposure to the LH surge *in vivo*, they exit the cell cycle (demonstrated in cows (Quirk *et al.* 2004), primates (Chaffin *et al.* 2001) and rodents (Robker & Richards 1998a, 1998b)). In cattle, granulosa cells of preovulatory follicles exit the cell cycle within 12 h after the LH surge, as demonstrated by examination of cell proliferation markers, cell cycle profiles and expression of cell cycle regulatory proteins (Quirk *et al.* 2004). In the current experiments, granulosa cells isolated from 2–4 mm follicles and initially cultured in serum-containing media continued to proliferate; the cells expressed Ki67 and cyclin D2, incorporated BrdU into DNA and were observed in S and G2/M phases of the cell cycle. Therefore, under the culture conditions used, granulosa cells continue to proliferate, a characteristic of non-luteinized granulosa cells. This facilitated the study of interactions between the cell cycle in proliferating cells and their susceptibility to apoptosis.

A previous study showed that treatment of immature hypophysectomized rats with implants of DES followed by implant withdrawal induced a wave of apoptosis in the granulosa cells of multilayered preantral follicles (Billig *et al.* 1993). The mechanism for the protective effect of DES was not determined. In this animal model, DES is known to promote proliferation of granulosa cells leading to development of follicles with multiple layers of granulosa cells and the absence of an antral cavity. Withdrawal of DES support, therefore, probably removes oestrogenic effects on cell proliferation as well as other possible effects on cell survival pathways. A possible role of the transcription factor, nuclear factor (NF) κ B, in mediating the anti-apoptotic effect of E2 in bovine granulosa cells has been suggested (Valdez & Turzillo 2005). Elevated production of E2 by bovine granulosa cells *in vitro* was associated with higher levels of NF κ B activity and fewer cells in early apoptosis. In addition, levels of active NF κ B in the nucleus of granulosa cells of dominant follicles were highest in follicles with elevated concentrations of E2 in follicular fluid (Valdez & Turzillo 2005). The association between NF κ B and cell survival in bovine granulosa cells is consistent with studies on rodent granulosa cells that suggested a pro-survival role of NF κ B (Xiao *et al.* 2001, Wang *et al.* 2002, Xiao *et al.* 2002). In addition to promoting cell survival, NF κ B stimulates cell proliferation in a manner similar to E2 by increasing expression of cyclin D (Guttridge *et al.* 1999, Joyce *et al.* 2001). It is therefore possible that like E2, NF κ B may promote resistance to apoptosis by effects on the cell cycle.

In summary, protection by E2 against apoptosis requires progression through the cell cycle. Granulosa cells may be most susceptible to apoptosis at the transition from G1 to

S phase of the cell cycle and relatively resistant in the quiescent state. Our findings with cells *in vitro* are consistent with events *in vivo*. In cattle and rodents, the frequency of atresia is highest in follicle classes in which cell proliferation is greatest (Pedersen 1970, Hirshfield & Midgley 1978, Lussier *et al.* 1987). Follicles within these classes are dependent upon gonadotrophins and growth factors for continued growth and survival. It is noteworthy that during the normal process of follicular atresia, granulosa cells do not undergo apoptosis synchronously (Hirshfield & Midgley 1978), suggesting differences in the susceptibility of cells within a follicle to apoptosis. Initiation of apoptosis may be dependent upon stage of the cell cycle and may reflect the asynchronous proliferation of cells in the follicle. Failure of granulosa cells to receive signals for continued proliferation and the absence of signals to induce terminal differentiation and associated withdrawal from the cell cycle probably trigger apoptosis. The effects of E2 and growth factors such as IGF-I (Hu *et al.* 2004) in protecting against apoptosis are linked to their effects in promoting granulosa cell proliferation. Fas and other pathways for apoptosis are functional in numerous cell types and are triggered by adverse conditions that are cell-type specific. It seems likely that sensitivity to Fas-mediated apoptosis in additional cell types may be dependent upon stage of the cell cycle.

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