

Ovarian follicular growth and atresia: The relationship between cell proliferation and survival^{1,2}

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ABSTRACT: Growth factors and steroids play an important role in the regulation of ovarian follicular development. In cattle, two of the earliest detectable differences between the healthy dominant follicle selected for development to the ovulatory stage and subordinate follicles destined to undergo atresia are the greater availability of IGF and the greater capacity to produce estradiol in the dominant follicle. We have shown that IGF-I and estradiol stimulate the proliferation of bovine granulosa cells in vitro and promote granulosa cell survival by increasing resistance to apoptosis. Furthermore, the ability of IGF-I and estradiol to increase resistance to apoptosis is tied to their ability to promote progression through the cell cycle. Blocking the cell cycle at the transition between the first gap phase and the DNA synthesis phase using a specific inhibitor prevented the protective effects of IGF-I and estradiol against apoptosis. Further experiments showed that the protective effect of IGF-I against apoptosis is mediated by the stimulation of phosphatidylinositol 3-kinase and its downstream target, protein kinase B/Akt. Constitutive activation of Akt by the infection of granulosa cells with a recombinant Akt adenovirus protected

against apoptosis, and this effect also depended on cell cycle progression. These experiments show that the protective effect of estradiol and IGF-I against apoptosis depends on unperturbed progression through the cell cycle. Once follicles have developed to the preovulatory stage, the LH surge induces terminal differentiation of granulosa cells and withdrawal from the cell cycle. Bovine granulosa cells withdraw from the cell cycle by 12 h after the LH surge and become resistant to apoptosis, even in the absence of growth factors. Treatment with a progesterone receptor antagonist in vitro caused reentry of granulosa cells into the cell cycle and susceptibility to apoptosis, suggesting that induction of progesterone receptor expression by the LH surge is required for cell cycle withdrawal and resistance to apoptosis. In summary, the susceptibility of granulosa cells to apoptosis depends on the cell cycle. Proliferating granulosa cells in growing follicles depend on growth factors for survival, whereas cells that have terminally differentiated in response to the LH surge are resistant to apoptosis and relatively independent of growth factors for survival.

Key Words: Apoptosis, Atresia, Bovine, Follicle, Ovary, Proliferation

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Introduction

Most follicles that leave the resting stage and begin to grow do not mature fully but instead undergo atresia during the developmental process. Atresia occurs by the programmed cell death, or apoptosis, of the somatic cells of the follicle and of the oocyte (reviewed in Chun and Hsueh, 1998). Apoptosis provides a means for multicellular organisms to eliminate unwanted cells

in response to developmental signals or toxic stimuli. Successful follicle development depends on the presence of survival factors that promote follicle growth and also protect cells from apoptosis. These include factors produced within the ovary as well as the gonadotropins LH and FSH. In the absence of survival factors, endogenous apoptosis pathways within the follicle become activated and lead to follicular atresia. Studies using a variety of cell types have demonstrated interrelationships between the processes of apoptosis and cell proliferation. In this review article, we present evidence for alterations in the susceptibility of granulosa cells to apoptosis during bovine follicle development that are related to the cell cycle of proliferation. In the first part of the review, the role of growth factors and steroids as integrative signals for follicle cell proliferation and survival are described. The effects of IGF and estradiol are highlighted because both are important determi-

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nants of follicle cell survival *in vivo* as well as *in vitro*. In the second part of the review, evidence is presented to show that, following the preovulatory surge of LH, granulosa cells of preovulatory follicles exit the cell cycle and thereby become resistant to apoptosis.

FSH, Estradiol, and IGF Are Follicular Survival Factors

The Role of Gonadotropins

There are two or three waves of follicle development during the bovine estrous cycle. During each wave, a group of three to six follicles simultaneously begin to grow larger than 4 to 5 mm in diameter. Only one of the follicles in the cohort, the dominant follicle, continues to grow and develop to preovulatory status. Growth of the remaining, subordinate follicles ceases, and they begin to undergo atresia. Dominant follicles that develop in the presence of a functional corpus luteum eventually regress because a preovulatory LH surge cannot occur. However, dominant follicles that develop coincident with luteal regression complete the final stages of development and ovulate following exposure to an LH surge. Before each wave of follicle development, a rise in plasma FSH above basal levels occurs. As the follicles develop, FSH declines. If the rise in plasma FSH is experimentally blocked, the wave is prevented or delayed (Kastelic et al., 1990; Turzillo and Fortune, 1993). It is believed that the dominant follicle survives in the face of declining plasma FSH by virtue of its enhanced responsiveness to gonadotropins relative to that of subordinate follicles (reviewed in Ginther et al., 1996, 2001). A number of studies have suggested that IGF and estradiol are critical intrafollicular factors that promote increased responsiveness of the dominant follicle to gonadotropins at the time of follicle selection (Mihm et al., 2000; Austin et al., 2001; Ginther et al., 2003).

The Role of IGF

Studies with genetically engineered mice lacking IGF-I support a critical role of IGF in maintaining gonadotropin responsiveness and follicle development. Mice devoid of IGF-I are sterile, and follicle development is arrested at the small antral stage (Baker et al., 1996). Expression of FSH receptor and aromatase by granulosa cells is deficient, whereas administration of IGF-I restores normal FSH receptor expression (Zhou et al., 1997). Studies *in vitro* also support a role of IGF to augment the actions of FSH. Insulin-like growth factor enhanced FSH-induced aromatase expression and LH receptor expression by murine granulosa cells (reviewed in Adashi et al., 1985). Thus, IGF-I is critical for the development of responsiveness to FSH in granulosa cells.

Studies in domestic species support an important role of IGF in follicle development. Insulin-like growth factor stimulates proliferation, increases estradiol secre-

tion, and increases responsiveness to gonadotropins in cultured bovine or ovine granulosa cells (Monniaux and Pisselet, 1992; Campbell et al., 1995; Glister et al., 2001). Infusion of IGF into ewes stimulated ovarian estradiol secretion (Campbell et al., 1995). A number of studies have revealed an association between increased bioavailability of IGF in follicular fluid and selection of the dominant follicle (reviewed in Poretsky et al., 1999, Fortune et al., 2001, and Ginther et al., 2001). In cattle, concentrations of free IGF-I decreased in the largest subordinate follicle at the time of selection but did not change in the dominant follicle (Beg et al., 2001; Ginther et al., 2003). Dominant and subordinate follicles differ in their complement of IGFBP, a family of proteins that bind IGF-I and IGF-II with affinities equal to or higher than their receptors and thus regulate the bioavailability of IGF (reviewed in Monget et al. 1996). The IGFBP with relatively low molecular weights (BP2, BP4, and BP5) are present in the follicular fluid of subordinate follicles but not dominant follicles in cattle (Echternkamp et al., 1994; de la Sota et al., 1996; Stewart et al., 1996; Mihm et al., 2000; Austin et al., 2001; Beg et al., 2001). The presence of the binding proteins is controlled at the level of synthesis (Armstrong et al., 1998) and by the presence of specific proteases in healthy antral follicles, which break down low-molecular weight IGFBP in cows (Rivera and Fortune, 2001, 2003b; Rivera et al., 2001), sheep (Besnard et al., 1996), pigs (Besnard et al., 1997), humans (Conover et al., 2001), and mice (Hourvitz et al., 2002). Detection of IGFBP-4 protease in dominant follicles (Rivera et al., 2001; Rivera and Fortune, 2003b) and reduced levels of IGFBP-4 in dominant follicles compared with subordinate follicles (Mihm et al., 2000; Austin et al., 2001; Rivera and Fortune, 2003a) are some of the earliest detectable differences at the time of follicle selection in the cow.

The Role of Estradiol

Secretion of estradiol is the hallmark of successful ovulatory follicles. In addition to its role in triggering the preovulatory surge of gonadotropins, estradiol is an important intraovarian growth, differentiation, and survival factor (reviewed in Rosenfeld et al., 2001). Estradiol stimulates the proliferation of granulosa cells and protects against apoptosis (reviewed below). Estradiol modulates granulosa cell differentiation by enhancing the ability of FSH to induce expression of LH receptors (reviewed in Richards, 1980). Acquisition of LH receptors by granulosa cells around the time of follicular selection in cattle promotes follicular growth and differentiation (reviewed in Ginther et al., 1996). Estradiol was also shown to increase secretion of IGF-I by porcine granulosa cells (Hsu and Hammond, 1987). An early detectable difference between the dominant follicle and the subordinate follicles at the time of selection in cattle is the increased capability of granulosa cells to synthesize estradiol (Evans and Fortune, 1997). When small

samples of follicular fluid were obtained from bovine follicles *in vivo* and their subsequent fate as dominant or subordinate follicles followed over time, it was found that dominant follicles had higher estradiol and lower IGFBP-4 levels than subordinate follicles before a detectable difference in follicle size had occurred (Mihm et al., 2000). Several studies have associated follicle selection with increases in intrafollicular estradiol and increased bioavailability of IGF (Mihm et al., 2000; Austin et al., 2001; Ginther et al., 2003; Rivera and Fortune, 2003a,b).

Apoptosis and Survival Pathways

Major Pathways That Regulate Apoptosis

Mammalian cells are endowed with the pathways to carry out apoptosis, but under favorable conditions apoptosis is prevented by intricately controlled survival pathways. Descriptions of several major pathways that regulate apoptosis are summarized here for background (reviewed in Hengartner, 2000). The primary effectors of apoptosis are a class of cysteine proteases, known as *caspases*, that reside in the cell in inactive form until a trigger for apoptosis is received. The mitochondrion serves as a center for integration of signals for apoptosis vs. survival. Interaction among pro- and antiapoptotic members of the bcl-2 family of proteins in the mitochondrion determines whether pathways for apoptosis are activated or suppressed. A partial list of members of the Bcl-2 family includes proapoptotic proteins: Bax, Bad, Bim, Bid, Bok, and Bcl-x-short and anti-apoptotic proteins: Bcl-2, Bcl-x-long, and Bcl-w. Extracellular signals and internal signals, such as DNA damage, initiate apoptosis by causing the release of cytochrome c from the mitochondria into the cytoplasm. Cytochrome c binds to apoptosis-activating factor (Apaf)-1 and then to procaspase-9, forming a complex known as the *apoptosome*. Active caspase-9 within the apoptosome activates downstream caspases, including caspase-3. Coordinated cleavage of important cellular substrates by caspases eventually kills the cell. Smac-diablo and apoptosis-inducing factor (**AIF**) are additional proapoptotic proteins released from the mitochondria, whereas inhibitor of apoptosis proteins (**IAP**) in the cytoplasm can prevent caspase activation and are themselves inhibited by Smac/Diablo.

Important pathways for induction of apoptosis in mammalian cells are mediated through cell-surface receptors known as *death receptors*. Most of these proteins are members of the tumor necrosis factor receptor family, and one of the best-studied members is the Fas antigen (Fas, CD95; reviewed in Nagata, 1997). Binding of Fas ligand (**FasL**) to Fas stimulates protein-protein interactions at the cytoplasmic "death domain" of Fas. These lead to activation of caspase-8 and subsequent activation of downstream caspases. Whereas this path to apoptosis apparently bypasses cytochrome c, Fas ligation also triggers mitochondrial pathways for cell

death through caspase-8-induced activation of proapoptotic Bid (reviewed in Hengartner, 2000).

Apoptosis of Cells in the Ovarian Follicle

Many of the pro- and antiapoptotic signaling pathways described above have been characterized in ovarian follicle cells of a variety of species. The expression of pro- and antiapoptotic members of the bcl-2 family of proteins and their association with follicular health or atresia have been described, and studies using genetically modified mice have provided evidence *in vivo* for the roles of bcl-2 family proteins in regulation of follicle cell apoptosis (reviewed in Tilly, 2001; Johnson, 2003). The importance of caspases in the apoptosis of ovarian germ cells and somatic cells has been demonstrated (reviewed in Johnson and Bridgham, 2002) and the expression of IAP family members in follicle cells and their role in regulating apoptosis have been described (reviewed in Johnson, 2003). Evidence for a functional Fas-mediated pathway for apoptosis in the ovarian follicle has emerged from studies in cattle (Vickers et al., 2000; Porter et al., 2000, 2001a; Hu et al., 2001), rodents (Hakuno et al., 1996; Sakamaki et al., 1997; Kim et al., 1998, 1999; Quirk et al., 1998), humans (Quirk et al., 1995; Kondo et al., 1996), and chickens (Bridgham and Johnson, 2001).

The Modulation of Ovarian Cell Apoptosis by Cell Survival Pathways

Studies from our laboratory have shown that granulosa cells possess endogenous pathways to trigger apoptosis that are inhibited in the presence of survival factors. For example, bovine granulosa cells express Fas but are resistant to killing by exogenous FasL *in vitro* when serum is present in the culture medium (Porter et al., 2000; Quirk et al., 2000; Vickers et al., 2000). In contrast, granulosa cells cultured in defined media containing insulin (100 ng/mL), transferrin, and selenium are viable but undergo apoptosis in response to treatment with FasL (Quirk et al., 2000). These findings suggest that serum contains factors that suppress FasL-induced apoptosis. When serum is abruptly removed from cultures of bovine granulosa cells, the cells spontaneously die by apoptosis over 24 h of culture and this is associated with increased expression of Fas and FasL protein (Hu et al., 2001). Furthermore, serum withdrawal-induced death is at least partially mediated by the endogenous Fas pathway because it was inhibited by addition of reagents that specifically blocked Fas/FasL interactions (Hu et al., 2001). In order for follicle development to succeed, proapoptotic pathways within the granulosa cell must be suppressed. Evidence presented below indicates that factors critical for follicle development *in vivo* suppress Fas-mediated apoptosis of bovine granulosa cells.

Cell survival in the face of apoptotic stimuli is mediated by receptors for growth factors, which utilize the

phosphoinositide 3'-OH kinase (**PI3K**) pathway and the mitogen-activated protein kinase (**MAPK**) pathway (reviewed in Hancock, 1999 and Vincent and Feldman, 2002). Depending on the cell type, such factors as epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, and IGF activate one or both pathways. In the PI3K pathway, binding of growth factor to its receptor initiates the recruitment of PI3K to the inner surface of the plasma membrane. Phosphoinositide 3'-OH kinase catalyzes production of 3'-phosphorylated phosphoinositides. These bind to protein kinase-B (also known as Akt), leading to its relocation to the plasma membrane, phosphorylation, and activation by regulatory kinases (Datta et al., 1999; Vincent and Feldman, 2002). Activation of Akt has been associated with numerous antiapoptotic effects in mammalian cells. Akt phosphorylates and inactivates apoptosis-inducing caspase-9 and Bad, a proapoptotic bcl-2 family member, and Akt phosphorylates the forkhead transcription factors, which modulate apoptosis as well as cell proliferation. It activates the transcription factor $\text{NF}\kappa\beta$, which in turn induces the expression of antiapoptotic genes. The Akt also stimulates expression of the antiapoptotic protein FLICE-inhibitory protein (**FLIP**). Activation of the MAPK pathway through growth factor receptors requires activation of Ras and a series of protein phosphorylation reactions involving Raf and MAPK. Activated MAPK phosphorylates substrates including transcription factors and signal transduction molecules and thereby increases or decreases their activity (reviewed in Hancock, 1999). The relative importance of the MAPK and PI3K pathways in protecting various types of cells from apoptosis has been determined in studies using specific inhibitors of the pathways. In some cell types, both pathways protect against apoptosis whereas in others one or the other pathway is utilized exclusively (Gallaher et al., 2001).

Regulation of Cell Proliferation and Survival Are Linked

Evidence has accumulated for an intimate relationship between the cell cycle of proliferation and the susceptibility of cells to apoptosis (reviewed in Meikrantz and Schlegel, 1995, King and Cidlowski, 1998, Guo and Hay, 1999, Schutte and Ramaekers, 2000). In the developing embryo and in adult tissues, apoptosis occurs at the highest frequency in proliferating rather than quiescent tissues. In contrast, cells that have exited the cell cycle upon terminal differentiation are resistant to apoptosis, a phenomenon that has been observed in myocytes, monocytes, and neuronal cells (Poluha et al., 1996; Wang and Walsh, 1996; Asada et al., 1999). These relationships appear valid in the ovary. Granulosa cells from bovine preovulatory follicles that have exited the cell cycle in response to the LH surge are resistant to apoptosis (described below). Little apoptosis is observed in small, slow-growing follicles. The occurrence of atresia is low in bovine antral follicles from 0.13 to 0.28

mm in diameter (1.6%) and from 0.29 to 0.67 mm in diameter (6.6%) (Lussier et al., 1987). However, the proportion of follicles undergoing atresia increased dramatically with follicular diameter: 40% atresia in follicles from 0.68 to 1.52 mm in diameter, 30% in follicles from 1.53 to 3.67 mm, and 67% in follicles from 3.68 to 8.57 mm. This increased rate of atresia was associated with an increase in the mitotic index and a decrease in the doubling time of granulosa cells. In the rat, the highest frequency of atresia occurs in follicles of 200 to 400 μm in diameter, a group of follicles that have relatively rapid granulosa cell proliferation in comparison to smaller follicles (Hirshfield and Midgley, 1978). In the mouse, little atresia is observed in small- and medium-size follicle classes. As follicles move into the large size classes, both growth rate and frequency of atresia increase (Pedersen, 1970). Therefore, studies of follicle growth and atresia in several species indicate that granulosa cells that are dividing rapidly appear to be most susceptible to apoptosis.

Mechanisms regulating cell cycle progression have been reviewed (Sherr and Roberts, 1999) and are briefly described here for background. Cell cycle progression is mediated by a family of cyclin-dependent kinases (cdk) that are sequentially activated by binding to specific cyclin proteins synthesized periodically during the cell cycle. Expression of D-type cyclins is required for cell cycle entry and is increased by extracellular growth factors and mitogens. Progression through the cell cycle from the first gap phase (**G1**) to the DNA synthesis (**S**) phase requires the formation of complexes of cdk4 or cdk6 with D-type cyclins during early- to mid-G1 phase, followed by the formation of complexes of cdk2 with cyclin E during late-G1 phase. The transition from G1 to S is known as the *restriction point*, after which cells are committed to cell division. Just before the G1/S transition, cyclin/cdk complexes induce phosphorylation of the retinoblastoma protein, resulting in loss of its ability to inhibit cell cycle progression. Formation of additional complexes between various cdk and their cyclin partners are required for progression through S, the second gap (**G2**), and mitotic (**M**) phases. Enzymatic activity of cyclin/cdk complexes is regulated by phosphorylation of catalytic subunits as well as by two families of structurally distinct cdk inhibitors that bind to the cyclin/cdk complex, the INK4 and CIP/KIP families.

Numerous factors suggest coordinated control of the cell cycle and apoptosis in the ovarian follicle. In follicles at early stages of atresia, most granulosa cells are morphologically normal and some granulosa cells continue to synthesize DNA (Hirshfield and Midgley, 1978). Cells with features characteristic of apoptosis appear gradually (Hirshfield and Midgley, 1978), suggesting differences within the cell population in the susceptibility to apoptosis. A possible explanation for this heterogeneity is that the initiation of apoptosis may be dependent on the stage of the cell cycle and reflects the asynchronous nature of cell proliferation in the follicle. Granulosa cells subjected to removal of serum from culture me-

dium also undergo apoptosis gradually rather than synchronously (Hu et al., 2001). A number of cell types appear susceptible to apoptosis at the G1-to-S phase transition (reviewed in Meikrantz and Schlegel, 1995). For example, lymphocytes undergoing activation-induced cell death, a process mediated by Fas, undergo apoptosis at the G1-to-S transition (Lissy et al., 1998). In addition, treatment of T cells with agents that block the cell cycle in early G1 induces resistance to apoptosis, whereas blocking at the G1/S transition induces sensitivity to apoptosis (Meikrantz and Schlegel, 1995).

Effects of IGF on Cell Survival and Proliferation

Insulin-like growth factor is well recognized for its ability to increase proliferation as well as survival in a number of cell types. Insulin-like growth factor suppressed spontaneous apoptosis of bovine (Yang and Rajamahendran, 2000) and pig (Guthrie et al., 1998) granulosa cells in response to removal of serum from the culture medium. Insulin-like growth factor was identified as at least one of the components of serum-containing culture medium that prevented FasL-induced apoptosis of bovine granulosa cells (Quirk et al., 2000). The protective effect of IGF against FasL-induced apoptosis in vitro is consistent with the presumptive protective effect of IGF in vivo. During the first wave of follicle development of the bovine estrous cycle, the expression of Fas and FasL are elevated in subordinate follicles compared to dominant follicles (Porter et al., 2000, 2001a). Granulosa cells from subordinate follicles are more susceptible to apoptosis induced by treatment with exogenous FasL in vitro than granulosa cells from dominant follicles (Porter et al., 2000). It is possible that an increased availability of survival factors such as IGF in the dominant bovine follicle in vivo inhibits the expression of Fas and FasL and prevents activation of the Fas pathway. The effect of growth factors such as IGF-I to protect cells from apoptosis appears to be correlated with their ability to stimulate progression through the cell cycle. Insulin-like growth factor-I, basic fibroblast growth factor, and epidermal growth factor decreased FasL-induced apoptosis of cultured bovine granulosa cells and simultaneously increased cell proliferation. In contrast, platelet-derived growth factor, hepatocyte growth factor, and keratinocyte growth factor had no protective effect against apoptosis and did not alter proliferation (Quirk et al., 2000). These results suggest that the proliferative and protective effects of growth factors may be linked.

We studied a potential connection between the protective and mitogenic effects of IGF-I and the intracellular pathways mediating these effects because of the recognized importance of IGF in follicle development. The potential roles of the PI3K and MAPK pathways in mediating the protective effect of IGF-I were examined because each of these pathways has been shown to have effects on cell survival as well as proliferation in a number of cell types (reviewed in Gallaher et al. 2001). In

our experiments, granulosa cells from 2- to 4-mm bovine follicles were plated in media containing 10% FBS and after 24 h were cultured in defined media containing insulin (100 ng/mL), transferrin, and selenium (ITS). Cells were then treated with various combinations of PI3K or MAPK pathway inhibitors, IGF-I, and FasL. Insulin-like growth factor-I activated both the PI3K and MAPK pathways in granulosa cells as demonstrated by phosphorylation of critical intermediates in these pathways (Akt kinase and ERK-1/2 kinases, respectively). Furthermore, inhibitors specific for each pathway prevented the responses (Hu et al., 2004). Treatment with FasL induced apoptosis of granulosa cells and co-treatment with IGF-I suppressed this effect (Figure 1, panel A). When granulosa cells were treated with the PI3K inhibitor, LY294002, before treatment with IGF-I and FasL, the protective effect of IGF-I against FasL-induced apoptosis was blocked. In contrast, treatment of granulosa cells with the MAPK pathway inhibitor, PD98059, before treatment with IGF-I and FasL did not alter the ability of IGF-I to protect cells against apoptosis. These results indicate that protection by IGF-I was mediated through the PI3K pathway and not the MAPK pathway (Figure 1, panel A; Hu et al., 2004). In these experiments, cell viability was assessed by counting trypan blue-stained cells because this method provides a quantitative measure of the percentage of cells susceptible to apoptosis. Additional assays to assess apoptosis (caspase-3 activity assays and determination of the percentage of cells with sub-diploid content of DNA by flow cytometry) confirmed the results of cell viability assays (data not shown). Subsequent experiments tested whether activation of Akt, a downstream substrate of PI3K, was necessary for the protective effect of IGF-I against FasL-induced apoptosis. Infection of granulosa cells with a recombinant adenovirus expressing a dominant negative form of Akt (dnAkt) prevented the protective effect of IGF-I against apoptosis. In contrast, infection with a recombinant adenovirus expressing a constitutively active form of Akt (myrAkt) mimicked the protective effect of IGF-I (Figure 1, panel B; Hu et al., 2004). These results demonstrate that the effect of IGF-I to protect granulosa cells against FasL-induced apoptosis is mediated through the PI3K/Akt pathway.

Experiments were performed to determine the roles of the PI3K and MAPK pathways in IGF-I-stimulated cell cycle progression in order to test for a potential link between cell proliferation and survival (Hu et al., 2004). The percentage of cells in various stages of the cell cycle were determined by flow cytometry of cells stained for DNA content using propidium iodide. Treatment with IGF-I decreased the percentage of cells in G0/G1 phases from 78.0 ± 0.2 to 74.5 ± 1.9 and increased the percentage of cells in S phase from $13.0 \pm 0.7\%$ to $17.0 \pm 2.8\%$ (mean \pm SE, ($P < 0.05$), indicating an increase in G0/G1-to-S phase progression (Hu et al., 2004). Insulin-like growth factor-I also increased DNA synthesis as demonstrated by a 37% increase in the incorporation

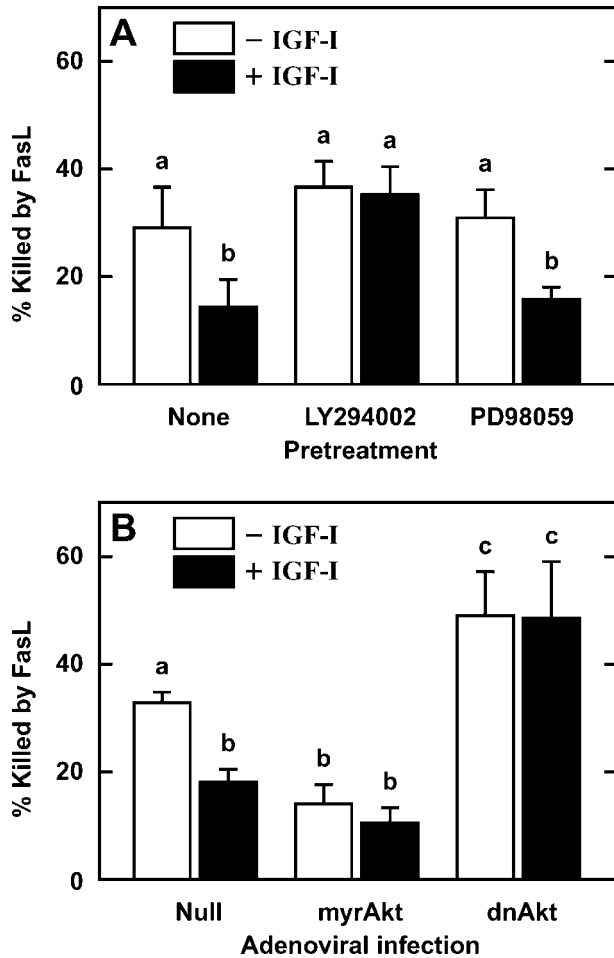


Figure 1. The protective effect of IGF-I against Fas ligand (FasL)-induced apoptosis is mediated by the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. **A)** Granulosa cells in defined media (1×10^5 cells per well in 96-well plates) were pretreated with no inhibitor, a PI3K inhibitor, LY294002 (20 μ M), or a mitogen-activated protein kinase (MAPK) pathway inhibitor, PD98059 (30 μ M), at 0.5 h and with 0 or 100 ng/mL IGF-I at 0 h. At 4 h, 0 or 100 ng/mL of FasL was added. The number of viable cells was determined at 24 h by staining with trypan blue and cell counts. The percentage of cells killed by FasL was calculated by comparing the number of viable cells in cultures treated with or without FasL. The protective effect of IGF-I against FasL-induced killing was blocked by LY294002, but not by PD98059, indicating that the protective effect of IGF-I is mediated through the PI3K pathway. **B)** Granulosa cells (1×10^5 cells per well in 96-well plates) were infected with a control adenovirus (null) or adenoviruses expressing constitutively active Akt (myrAkt) or dominant negative Akt (dnAkt) for 24 h. Cells were treated in defined media with 0 or 100 ng/mL IGF-I at 0 h, and with 0 or 100 ng/mL FasL at 4 h. The number of viable cells was determined at 24 h and the percentage of cells killed by FasL was calculated as described above. Expression of myrAkt mimicked the protective effect of IGF-I against FasL-induced killing. In contrast, dnAkt enhanced killing by FasL and prevented the protective effect of IGF-I. These results indicate that the protective effect of IGF-I against apoptosis requires activation of Akt. Bars represent the means \pm SE of results obtained in three experiments using separate granulosa cell preparations. Within each panel, bars with no common superscripts are different ($P < 0.05$). Data are from Hu et al. (2004).

over 24 h of the thymidine analog, bromodeoxyuridine (BrdU), into DNA. The expression of myrAkt also increased progression from G0/G1-to-S phase of the cell cycle. In contrast, expression of dnAkt or treatment with the PI3K inhibitor LY294002 inhibited G0/G1-to-S phase progression (Hu et al., 2004). These results show that treatments that increased G0/G1-to-S phase progression protected cells from FasL-induced apoptosis. In contrast, treatments that inhibited G0/G1-to-S phase progression regulated by the PI3K/Akt pathway prevented protection by IGF-I. In order to determine whether cell cycle progression was required for the protective effect of IGF-I against FasL-induced apoptosis, cells were treated with roscovitine, an inhibitor of cdk2 activity that blocks transition from phases G1 to S and G2 to M. Treatment with roscovitine inhibited G0/G1-to-S phase progression and prevented the protective effect of IGF-I (Figure 2, panel A; Hu et al., 2004). Therefore, the protective effect of IGF-I, mediated through the PI3K/Akt pathway, occurred only when progression through the G0/G1-to-S phase transition was unperturbed.

The role of Akt in promoting survival has been associated with its ability to phosphorylate substrates with known functions in cell survival or apoptotic pathways, such as Bad and caspase-9 (reviewed above). In addition to its effects on cell survival, the PI3K/Akt pathway stimulates proliferation in some cells. Kinase Akt is known to modulate the cell cycle by phosphorylating and thereby altering the activities of forkhead transcription factors and glycogen synthase kinase 3- β , which in turn alter the activities of several cell cycle regulatory proteins (Diehl et al., 1998; Medema et al., 2000; Nakamura et al., 2000). We tested whether cell cycle progression was required for the protective effect of constitutively active myrAkt by experimentally blocking cell cycle progression using roscovitine. Treatment of granulosa cells with roscovitine prevented the effect of myrAkt to increase G0/G1-to-S phase progression and to protect cells against FasL-induced apoptosis (Figure 2, panel B; Hu et al., 2004). Thus, activation of Akt is not sufficient to protect granulosa cells from apoptosis in the absence of cell cycle progression. These studies demonstrate that the ability of the PI3K/Akt pathway to promote cell survival is linked to maintenance of cell cycle progression.

Effects of Estradiol on Cell Survival and Proliferation

Estradiol modulates ovarian cell proliferation, differentiation, and survival (reviewed in Palter et al., 2001). Treatment of immature hypophysectomized rats with diethylstilbestrol (DES) implants stimulated the development of large numbers of healthy, multilayered preantral follicles (Billig et al., 1993). Subsequent removal of the DES implants resulted in apoptosis within the granulosa cell layer. Therefore, DES promoted the proliferation of granulosa cells, and continuous exposure

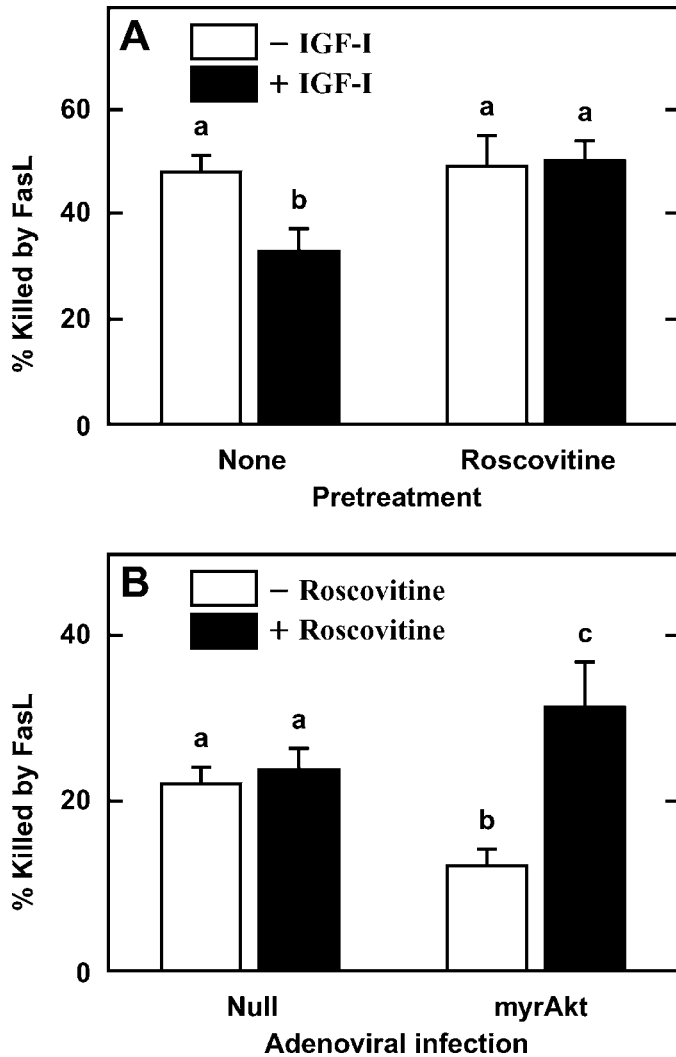


Figure 2. Cell cycle progression through the Gap 1/DNA synthesis (G1/S) phase transition is required for the protective effect of IGF-I against Fas ligand (FasL)-induced apoptosis. A) Granulosa cells in defined media were pretreated at 0.5 h with roscovitine (20 μ M), a cyclin dependent kinase (cdk) 2 inhibitor which blocks cells at the G1/S transition, and with 0 or 100 ng/mL IGF-I at 0 h. At 4 h, 0 or 100 ng/mL FasL was added. The number of viable cells was determined at 24 h and the percentage of cells killed by FasL was calculated (as described in the caption to Figure 1). Inhibition of cell cycle progression with roscovitine blocked the protective effect of IGF-I, suggesting that unperturbed progression through G1/S is required for protection. B) Granulosa cells were infected with a control adenovirus (null) or an adenovirus expressing constitutively active myrAkt for 24 h. Cells in defined media were pretreated at 0 h with 20 μ M roscovitine. At 4 h, 0 or 100 ng/mL FasL was added, and the number of viable cells was determined at 24 h. Inhibition of cell cycle progression with roscovitine blocked the protective effect of myrAkt. Therefore, activation of Akt is not sufficient to protect cells against apoptosis when cell cycle progression is blocked. Bars represent the means \pm SE of results obtained in three experiments using separate granulosa cell preparations. Within each panel, bars with no common superscripts are different ($P < 0.05$). Data are from Hu et al. (2004).

to DES was required for cell survival. Estradiol acted in synergy with FSH to increase the number of LH receptors on granulosa cells of immature, hypophysectomized rats, suggesting that estradiol promotes follicle development and survival by increasing responsiveness to LH (reviewed in Richards, 1980). Estradiol increased expression of cyclin D2 in rat granulosa cells (Robker and Richards, 1998b). A critical role for cyclin D2 in follicle development was demonstrated by a targeted deletion of cyclin D2 in mice, which blocked granulosa cell proliferation (Sicinski et al., 1996).

We conducted experiments to determine whether estradiol protects granulosa cells from FasL-induced apoptosis and, if so, whether protection is dependent on progression through the cell cycle (Quirk et al., 2001). Bovine granulosa cells were cultured as described in the previous section. Treatment with estradiol decreased FasL-induced apoptosis by 20 to 50% at doses ranging from 31 to 1,000 ng/mL. Estradiol also increased progression through the cell cycle. Estradiol increased progression from G0/G1 to S phase (indicated by a decrease in the percentage of cells in G0/G1 phases from 75.3 ± 2.4 to $70.4 \pm 3.6\%$ and an increase in the percentage of cells in S phase from 15.5 ± 2.3 to $19.6 \pm 2.6\%$; mean \pm SE, ($P < 0.05$). Estradiol also increased the percentage of cells expressing immunoreactive cyclin D2 protein (from 12 ± 3 to $25 \pm 4\%$) and increased the percentage of cells synthesizing DNA (increased the incorporation of BrdU into DNA over 24 h by 24%). The effects of estradiol to protect against apoptosis and to promote progression through the cell cycle were mediated through the estrogen receptor (ER) since both effects were blocked by the specific ER antagonist ICI 182,780. The protective effect of estradiol was not mediated by increasing expression of IGF by granulosa cells, because an anti-IGF receptor antibody, which effectively blocked the protective effect of IGF-I against apoptosis, did not prevent the protective effect of estradiol. Treatment with the cdk2 inhibitor, roscovitine, inhibited G0/G1 to S progression and completely blocked the protective effect of estradiol against FasL-induced apoptosis. Therefore, as observed in experiments with IGF-I, the protective effect of estradiol against FasL-induced apoptosis occurs only when progression through the cell cycle is not perturbed.

Susceptibility of Granulosa Cells to Apoptosis at the G1/S Transition

The fact that IGF-I and estradiol increase G1-to-S phase progression and that cell cycle progression is required for the protective effect of each against apoptosis suggests that cells may be particularly susceptible to undergoing apoptosis at the G1/S transition. We performed a series of experiments to determine whether there is a phase of the cell cycle when granulosa cells are particularly susceptible to apoptosis. In the first experiment, the effects of mimosine, a drug that blocks cell cycle progression during G1 phase, was tested. The

effect of mimosine differs from the effect of roscovitine in that mimosine blocks cells during early G1 phase whereas roscovitine blocks cells at the G1-to-S phase transition by inhibiting cdk2 activity. Granulosa cells were treated with or without mimosine and 6 h later with or without FasL. In control media, $22 \pm 3\%$ of cells were killed by FasL over 24 h, but only $10 \pm 2\%$ of cells treated with mimosine were killed by FasL. Flow cytometric analysis of cellular DNA content showed that mimosine blocked cells in G1 as expected (the percentage of cells in G0/G1, S, and G2/M phases were 75 ± 2 , 18 ± 2 , and 6.7 ± 1 in controls and 88 ± 1 , 9 ± 1 , and 3.3 ± 0.3 in mimosine-treated cells). These results indicate that resting (G0) granulosa cells and those in early G1 are relatively resistant to apoptosis. Therefore, the effect of blocking cells in early-G1 phase of the cell cycle is to protect against apoptosis whereas the effect of blocking at the G1-to-S transition is to increase susceptibility to apoptosis.

We further defined the stages of the cell cycle during which cells are killed by FasL by marking cells that passed into or through S phase by their incorporation of the thymidine analog BrdU into DNA. Analog BrdU was detected by flow cytometry using an anti-BrdU antibody, and cells were simultaneously stained for DNA content using propidium iodide. This allowed calculation of the percentage of cells in various stages of the cell cycle that had incorporated BrdU. In addition, the percentage of cells that were apoptotic could be determined by quantifying the percentage of cells with subdiploid content of DNA. Bovine granulosa cells were treated with BrdU and 6 h later with FasL. Cells were fixed for flow cytometry 12 h after FasL. The majority of subdiploid, apoptotic cells had not incorporated BrdU; only $17 \pm 4\%$ were positive for BrdU. In contrast, $25 \pm 5\%$ of G0/G1 phase cells, $57 \pm 5\%$ of S phase cells, and $61 \pm 4\%$ of G2/M phase cells had incorporated BrdU (mean \pm SE, all ($P < 0.05$) vs. apoptotic cells). Among all nonapoptotic cells, $35 \pm 5\%$ of cells had incorporated BrdU ($P < 0.05$ vs. apoptotic cells). The relative lack of BrdU incorporation by apoptotic cells indicates that cells in S, G2, and M phases (the majority of which incorporate BrdU) are resistant to apoptosis. Since experiments using mimosine (described above) showed that cells in G0 and G1 phases are resistant to apoptosis, we can conclude that cells are most susceptible to apoptosis in response to FasL when they are at the G1/S transition (Figure 3). Susceptibility to apoptosis at the G1/S transition is consistent with the effects IGF-I and estradiol, which increase G1-to-S progression and protect against apoptosis, and with the effect of roscovitine, which blocks cells at the G1/S transition and prevents protection by IGF-I and estradiol. The fact that IGF-I and estradiol promote survival of proliferating granulosa cells in vitro is consistent with their increased availability in dominant follicles compared with subordinate follicles at the time of follicle selection and may explain their critical role to support follicle development in vivo.

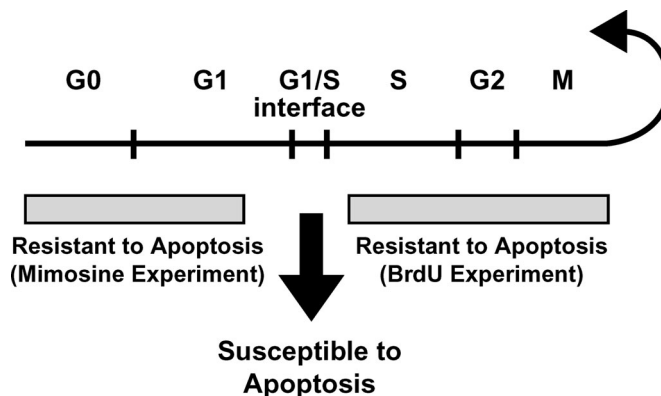


Figure 3. Granulosa cells are susceptible to apoptosis at the Gap 1/DNA synthesis (G1/S) phase transition of the cell cycle. Experiments in which granulosa cells were pretreated with mimosine, to block cells in the resting (G0) or G1 phase of the cycle, showed that cells were relatively resistant to Fas ligand (FasL)-induced apoptosis during the G0 and G1 phases. Experiments in which granulosa cells were cultured with bromodeoxyuridine (BrdU) to label cells that had synthesized DNA during S phase and then treated with FasL showed that very few apoptotic cells had incorporated BrdU. This indicates that cells in middle to late S, Gap 2 (G2), and mitotic (M) phases of the cycle were relatively resistant to apoptosis. By the process of elimination, cells seem to be susceptible to apoptosis at the G1/S transition. See the text for details of the experiments.

Terminal Differentiation, Exit from the Cell Cycle, and Resistance to Apoptosis

Proliferating cells tend to be susceptible to apoptosis and highly dependent on growth factors for survival, but cells that have exited the cell cycle through differentiation are resistant to apoptosis (reviewed above). Once a follicle leaves the resting stage, its growth is continuous until it either undergoes atresia, owing to a lack of the appropriate survival factors, or, in the case of a preovulatory follicle, is stimulated by the LH surge to undergo the final stages of maturation, or “terminal differentiation.” Studies in rodents and primates have shown that granulosa cells withdraw from the cell cycle following the LH surge (Robker and Richards, 1998a; Chaffin et al., 2001). We found that granulosa cells from bovine preovulatory follicles that have been exposed to an LH surge in vivo are resistant to apoptosis in vitro (Porter et al., 2000, 2001b). In light of our findings that granulosa cells blocked relatively early in G0/G1 phases of the cell cycle using mimosine are resistant to apoptosis (described above), we tested the hypothesis that bovine granulosa cells become resistant to apoptosis after the LH surge in association with their withdrawal from the cell cycle.

The following model was used to generate preovulatory follicles at known intervals after an LH surge.

Cows were injected with PGF_{2 α} on d 6 of the estrous cycle in order to induce luteolysis and promote the development of the dominant follicle of the first wave to the preovulatory stage. At 36 h after the PGF_{2 α} injection, cows were injected with GnRH to induce a preovulatory LH surge, and preovulatory follicles were isolated at various times after GnRH injection. This protocol results in stimulation of an LH surge within 2 h after injection of GnRH.

Granulosa cells isolated at 0 h were sensitive to FasL-induced apoptosis *in vitro* while granulosa cells isolated at 14 h after GnRH injection were completely resistant. Whereas granulosa cells isolated at 0 h responded to removal of serum from the culture medium with 50% cell death within 24 h, cells isolated 14 h after GnRH did not undergo apoptosis during culture. Interestingly, theca cells differed from granulosa cells in that they were sensitive to FasL-induced apoptosis at both 0 and 14 h after GnRH (Porter et al., 2001b). Further studies showed that granulosa cells are susceptible to FasL-induced apoptosis at 0, 6, and 10 h after GnRH but become resistant at 14 h (Figure 4, panel A; Quirk et al., 2002). Analysis of changes in the cell cycle after injection of GnRH was consistent with the possibility that withdrawal from the cell cycle promoted resistance to apoptosis. Flow cytometric analysis of freshly isolated granulosa cells showed that within 6 h after GnRH the percentage of cells in G₀/G₁ increased and the percentage of cells in S phase decreased. These changes became increasingly more pronounced at 10 and 14 h after GnRH (Figure 4, panel B; Quirk et al., 2002). As another measure of proliferation, sections of follicle wall were stained for Ki67, a protein that is expressed in cells that are progressing through the cell cycle. The staining decreased in the granulosa cell layer between 10 and 14 h after GnRH but did not change in the theca cell layer (Figure 4, panel C; Quirk et al., 2002). Additional evidence for exit of granulosa cells but not theca cells from the cell cycle 14 h after GnRH is the fact that levels of the cdk inhibitor p27^{kip1} increased between 10 and 14 h in granulosa cells but remained constant between 0 and 14 h in theca cells (data not shown). Therefore, induction of the LH surge by GnRH initiates withdrawal of granulosa cells from the cell cycle within 6 h and this affects increasingly greater numbers of cells over time. By 14 h after GnRH, when granulosa cells have become resistant to apoptosis, most cells have exited the cell cycle.

The progesterone receptor (PR) is a potential mediator of changes in granulosa cell proliferation and survival induced by the LH surge. Expression of PR is low to undetectable in preovulatory follicles before the LH surge but is induced in granulosa cells after the LH surge in cows (Cassar et al., 2002; Jo et al., 2002), primates (Chaffin et al., 1999), and rats (Natraj and Richards, 1993). The PR could potentially alter cell proliferation and survival in the follicle; it decreases the proliferation of human granulosa cells (Chaffkin et al., 1992) and promotes the survival of rat granulosa cells

(reviewed in Peluso [1997]) and bovine luteal cells (Rueda et al., 2000). Furthermore, PR appears to mediate the resistance to the apoptosis of granulosa cells from rat and human preovulatory follicles that have been exposed to the LH surge (Svensson et al., 2000, 2001). We found that PR in bovine follicles is undetectable by immunohistochemistry at 0 and 6 h, becomes detectable in granulosa cells at 10 h, and continues to be expressed 14 h after GnRH (data not shown; Quirk et al., 2002). Staining for PR in the theca yields undetectable results at all times (Quirk et al., 2002), a finding consistent with studies in rats in which the expression of PR was detected in granulosa cells but not theca cells after the LH surge (Robker et al., 2000). In order to determine whether PR mediates the resistance of bovine granulosa cells to apoptosis after the LH surge, granulosa cells isolated at various times after injection of GnRH were treated with or without the PR antagonist, RU486, *in vitro* and susceptibility to FasL-induced killing was tested. The antagonist RU486 had no effect on FasL-induced apoptosis in cells isolated at 0, 6, or 10 h after GnRH but reversed resistance to apoptosis in cells isolated at 14 h (data not shown; Quirk et al., 2002). These results suggest that the resistance of granulosa cells to apoptosis at 14 h is mediated through PR. It was of interest to test whether the effect of PR to promote resistance to apoptosis involved PR-induced exit from the cell cycle. Granulosa cells isolated at 14 h after GnRH were treated with or without RU486 *in vitro* and proliferation was measured using both Ki67 staining and BrdU incorporation. The effect of treatment with FasL on the number of labeled, proliferating cells was tested. Antagonist RU486 increased the percentage of cells staining positively for Ki67 and BrdU and treatment with FasL reduced the number of labeled cells (data not shown; Quirk et al., 2002). Therefore, antagonizing PR with RU486 induced cell cycle reentry and susceptibility to FasL-induced apoptosis. We next tested whether reentry of cells into the cell cycle in response to RU486 was necessary to promote susceptibility of cells to FasL-induced killing. When granulosa cells isolated 14 h after GnRH were treated with RU486 and also treated with the G₁ blocker mimosine, the effect of RU486 to promote susceptibility to apoptosis was lost (data not shown; Quirk et al., 2002). These results support the concept that PR-mediated resistance of granulosa cells to apoptosis requires withdrawal from the cell cycle.

Implications

Survival factors for growing follicles *in vivo* include insulin-like growth factor and estradiol, which are available at elevated levels in the dominant relative to the subordinate follicles at the time of follicle selection. Insulin-like growth factor and estradiol protect cells from apoptosis *in vitro*, an effect dependent on unperurbed progression through the cell cycle. Because granulosa cells seem to undergo apoptosis predominantly

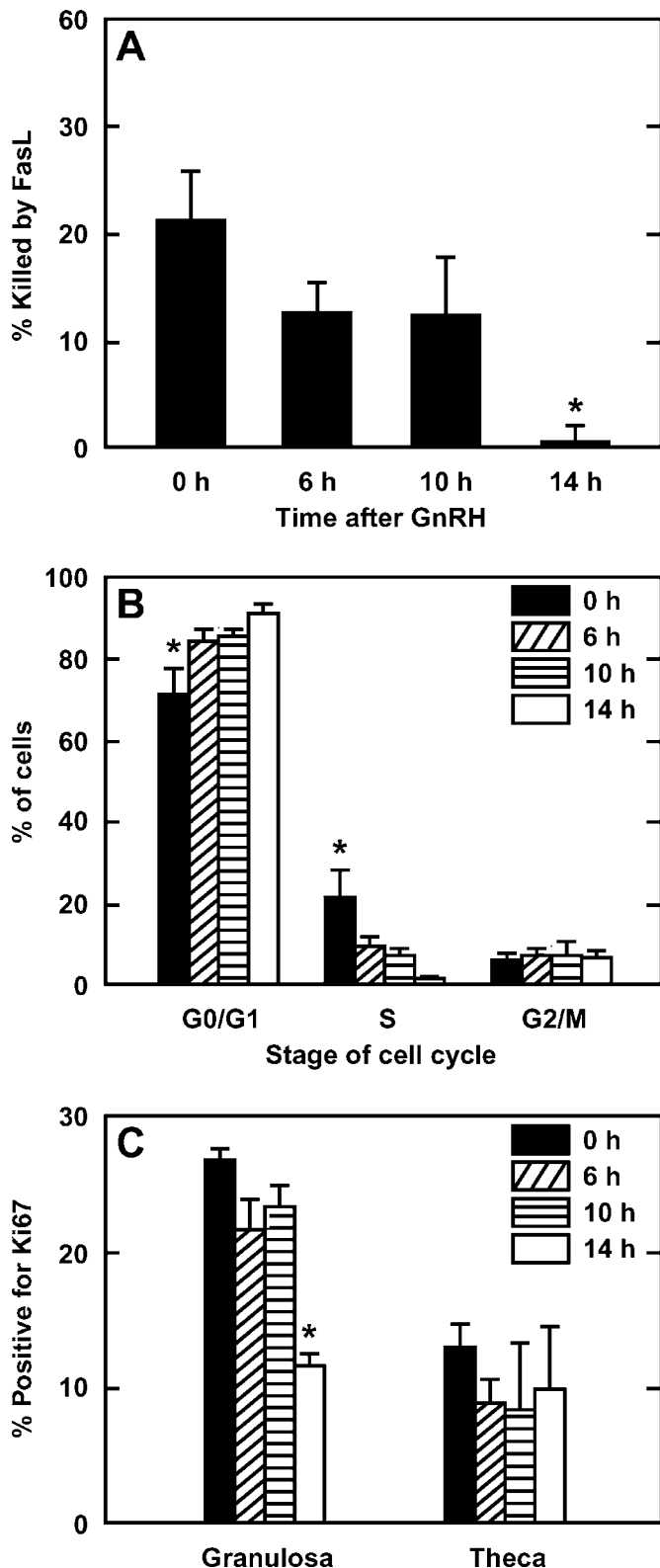


Figure 4. Resistance of granulosa cells of preovulatory follicles to apoptosis after exposure to a luteinizing hormone (LH) surge is associated with withdrawal from the cell cycle. Preovulatory follicles were obtained at 0, 6, 10, and 14 h after injection of cows with gonadotropin-releasing hormone (GnRH). A) Fas ligand (FasL)-induced apoptosis of cultured granulosa cells. Granulosa cells from preovulatory follicles were cultured for 24 h (5×10^4 cells per well in 96-well plates) and then treated with 0 or 100 ng/mL FasL in defined media. The number of viable cells was determined 24 h later, and the percentage of cells killed by FasL was calculated as described in the caption to Figure 1. The results show that granulosa cells from preovulatory follicles become resistant to FasL-induced killing by 14 h after injection of GnRH. B) Percentage of granulosa cells from preovulatory follicles in various stages of the cell cycle. The DNA content of isolated granulosa cells was determined by flow cytometric analysis of propidium iodide staining. The results show that within 6 h after GnRH injection, the percentage of granulosa cells in the resting (G0)/Gap 1 (G1) phases increased, whereas the percentage in the DNA synthesis (S) phase decreased ($P < 0.05$). C) Proliferative index of granulosa and theca cells in sections of follicle wall stained for the proliferation marker Ki67. The results show that following treatment with GnRH, the percentage of cells staining positively for Ki67 decreased in the granulosa cell layer by 14 h ($P < 0.05$) but did not change in the theca cell layer. Data are from Quirk et al. (2002).

at the G1 to S transition of the cell cycle and insulin-like growth factor and estradiol increase G1 to S transition, it is likely that the protective effect of these factors stems from their ability to facilitate G1 to S transition

(Figure 5). Exit from the cell cycle by granulosa cells of preovulatory follicles in response to the LH surge induces resistance to apoptosis, a property that may be important for long-term survival in the corpus luteum

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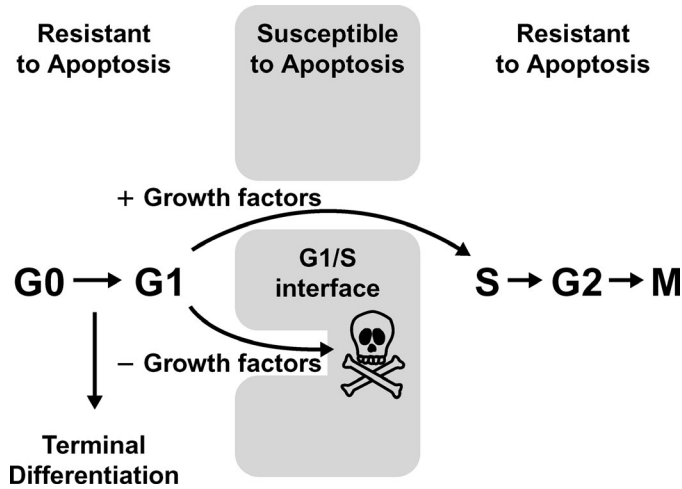


Figure 5. Model depicting the relationship between the stage of the cell cycle and susceptibility to apoptosis. Granulosa cells undergo apoptosis predominantly at the transition from the Gap 1 (G1) phase to the DNA synthesis (S) phase. The effect of growth factors such as IGF-I and estradiol to protect against apoptosis *in vitro* is associated with their ability to promote G1-to-S progression and is prevented when that progression is blocked. G0 = resting phase; G1 = Gap 1 phase; S = DNA synthesis phase; G2 = Gap 2 phase; M = mitotic phase.

(Figure 6). Future studies should investigate mechanisms for the interactions between pathways for cell proliferation and apoptosis.

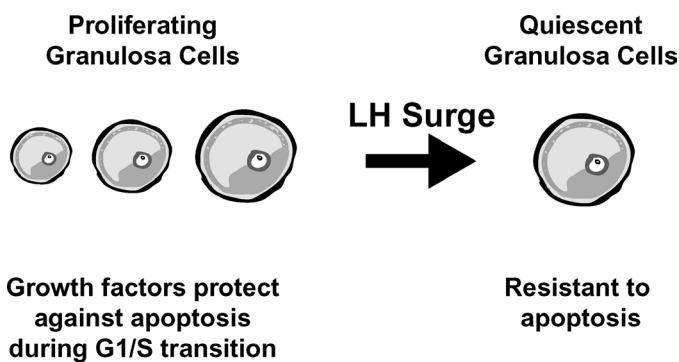


Figure 6. Model depicting the relationship between follicle growth and differentiation and the susceptibility of granulosa cells to apoptosis. Granulosa cells in growing follicles require growth factors to promote proliferation as well as survival, and these effects are intimately tied. Granulosa cells from preovulatory follicles that have exited the cell cycle after the luteinizing hormone (LH) surge are resistant to apoptosis, an effect that may be important for their long-term survival in the corpus luteum.

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