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Cell Cycle Progression and Activation of Akt Kinase Are Required for Insulin-Like Growth Factor I-Mediated Suppression of Apoptosis in Granulosa Cells

CHE-LIN HU, ROBERT G. COWAN, REBECCA M. HARMAN, AND SUSAN M. QUIRK
Department of Animal Science, Cornell University, Ithaca, New York 14853

Ovarian follicle development is dependent on growth factors that stimulate cell proliferation and act as survival factors to prevent apoptosis of follicle cells. We examined the mechanism of the protective effect of IGF-I against Fas ligand-induced apoptosis of granulosa cells and its relationship to cell proliferation. IGF-I activated both the phosphoinositide 3′-OH kinase (PI3K) and the MAPK pathways. Experiments using specific inhibitors of these pathways showed that protection by IGF-I was mediated by the PI3K pathway and not the MAPK pathway. Recombinant adenoviruses were used to test whether the downstream target of PI3K activation, Akt kinase, was required for protection against apoptosis. Expression of dominant negative Akt prevented protection by IGF-I whereas expression of constitutively active Akt (myrAkt) mimicked the effect of IGF-I. Treatment with IGF-I, or expression of myrAkt, increased progression from G_0/G_1 to S phase of the cell cycle whereas expression of dominant negative Akt inhibited G_0/G_1 to S phase progression and prevented the stimulatory effect of IGF-I. We tested whether cell cycle progression was required for protection from apoptosis using the cyclin-dependent kinase-2 inhibitor roscovitine, which blocks cells at the G_1/S transition. Roscovitine prevented the protective effect of IGF-I and myrAkt expression against apoptosis. Therefore, activation of Akt is not sufficient to protect granulosa cells from apoptosis in the absence of cell cycle progression. In summary, IGF-I protects granulosa cells from apoptosis by activation of the PI3K/Akt pathway. This protective effect can occur only when progression from G_1 to S phase of the cell cycle regulated by the PI3K/Akt pathway is unperturbed. (*Molecular Endocrinology* 18: 326–338, 2004)

OVARIAN FOLLICLES CONTINUALLY leave the resting stage and begin to grow, but less than 1% reach the ovulatory stage. Instead, most follicles undergo atresia by programmed cell death (apoptosis) of follicle cells at some point in the developmental process (1, 2). Follicles that are selected for successful development to ovulation are thought to receive precise signals from gonadotropins and locally produced growth factors required for survival. In contrast, follicular atresia is thought to be initiated by inadequate availability of essential survival factors (1, 2). Lack of survival factors triggers cytotoxic responses including signals from death receptors such as the Fas antigen (Fas). Fas is a cell surface receptor which, when stimulated by binding to Fas ligand (FasL), activates cysteine proteases known as caspases, which mediate apoptosis (3). Fas and FasL are expressed by granulosa cells and theca cells and are present at elevated levels in atretic follicles relative to healthy follicles in cattle, rodents, and humans (4–8). Although Fas is expressed in healthy follicles, its activation appears to be inhibited. Granulosa cells cultured in media containing serum or certain growth factors are resistant to induction of apoptosis by addition of FasL, despite expression of Fas (9). When serum is abruptly removed from culture media, expression of Fas and FasL increases and granulosa cells undergo apoptosis that is mediated, at least partially, by Fas/FasL interactions (10). IGF-I is one of the factors identified in serum that inhibits FasL-induced apoptosis of cultured granulosa cells (9). These findings indicate that growth factors like IGF-I, which are present in serum-containing culture media and in follicles *in vivo*, prevent activation of the Fas pathway.

IGF-I is critical for ovarian follicle development. Genetically engineered mice that lack IGF-I are infertile. Follicle development is arrested at the small antral stage, and mature Graafian follicles are not produced (11, 12). In cattle, rodents, and humans, healthy follicles differ from atretic follicles in having reduced levels of inhibitory IGF-binding proteins, and this promotes

Abbreviations: BrdU, Bromodeoxyuridine; cdk, cyclin-dependent kinase; dnAkt, dominant negative Akt; FasL, Fas ligand; GFP, green fluorescent protein; HA, hemagglutinin; ITS, 100 ng/ml insulin, 5 μg/ml transferrin, 20 nM Na-selenite and 0.1% BSA; MEK, MAPK kinase; myrAkt, constitutively active Akt; p-Akt, phosphorylated Akt; p-ERK, phosphorylated ERK; PI, propidium iodide; PI3K, phosphoinositide 3′-OH kinase.

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increased bioavailability of IGF (13–16). IGF-I stimulates two major signal transduction pathways: the phosphoinositide 3′-OH kinase (PI3K) pathway and the MAPK pathway (also known as the ERK pathway) (17). Binding of IGF-I to its receptor initiates events leading to recruitment of PI3K to the inner surface of the plasma membrane where it catalyzes production of 3′-phosphorylated phosphoinositides. These phospholipids bind to Akt kinase, leading to its relocation to the plasma membrane and phosphorylation by regulatory kinases. Akt that has been activated by phosphorylation regulates multiple cellular processes. Activation of the MAPK pathway by IGF-I requires activation of Ras and is mediated by a cascade of successive protein phosphorylation reactions involving Raf, MAPK kinase (MEK-1 and -2) and finally MAPK/ERK-1 and -2. Depending upon the cell type, activation of the PI3K or the MAPK pathway by IGF-I has been reported to stimulate proliferation, differentiation, and/or cell survival (16–19).

IGF-I stimulates proliferation as well as survival in a number of cell types, suggesting potential interactions between these processes. Mounting evidence suggests that regulation of apoptosis and of the cell cycle are tightly linked (20–23). Common control of these two processes could contribute to maintaining the balance between cell proliferation and cell death. Cell cycle progression is mediated by cyclin dependent kinases (cdks) (24). cdks Are activated by binding to specific cyclin proteins that are synthesized periodically during the cell cycle. Progression from G_1 to S phase of the cell cycle requires formation of complexes between cdk4 or cdk6 and D-type cyclins during early to mid-G_1, followed by formation of complexes of cdk2 and cyclin E during late G_1. Accumulation of the D-type cyclins in G_1 is required for cell cycle entry and is regulated by extracellular growth factors. Enzymatic activity of cyclin/cdk complexes is regulated by cdk inhibitors belonging to the INK4 and Cip/Kip families.

In the studies presented here, we examine the mechanism of IGF-I-mediated protection against apoptosis in bovine granulosa cells and its relationship to cell cycle control. We show that IGF-I protects granulosa cells from FasL-induced apoptosis by stimulating the PI3K/Akt pathway. Furthermore, the protective effect of IGF-I is dependent on unperturbed progression through the cell cycle regulated by the PI3K/Akt pathway.

RESULTS

IGF-I Stimulates the PI3K and MAPK Pathways in Bovine Granulosa Cells

When granulosa cells cultured in defined media were treated with IGF-I, both the PI3K pathway and the MAPK pathway were stimulated, as determined by measuring phosphorylated Akt (p-Akt) and total Akt, and phosphorylated ERK (p-ERK) and total ERK, respectively (Fig. 1). Phosphorylation of Akt was detected at $t = 0$ h, before treatment with IGF-I. p-Akt appeared to increase 5 min after the addition of IGF-I, and the increase was statistically significant by 30 min. Pretreatment with the PI3K inhibitor, LY294002, inhibited phosphorylation of Akt by IGF-I, whereas the MEK (MAPK pathway) inhibitor, PD98059, had no significant effect. The two forms of p-ERK, p-ERK-1 (p44) and p-ERK-2 (p42), were detected at $t = 0$ h, before treatment with IGF-I. p-ERK-1/2 increased significantly 10 min after addition of IGF-I and remained elevated at 60 min. Pretreatment with PD98059 inhibited phosphorylation of ERK-1/2 by IGF-I, but treatment with LY294002 had no significant effect.

IGF-I Protects Granulosa Cells from FasL-Induced Apoptosis via the PI3K Pathway

Treatment with IGF-I reduced the percentage of cells killed by FasL by 51% ($P < 0.05$, Fig. 2). Pretreatment with the PI3K inhibitor, LY294002, before the addition of IGF-I, completely blocked the protective effect of IGF-I against FasL-induced killing. Pretreatment with the MEK inhibitor, PD98059, did not alter the protective effect of IGF-I. These results indicate that the PI3K pathway, but not the MAPK pathway, is necessary for the protective effect of IGF-I against FasL-induced killing.

To confirm that cells undergo apoptosis in response to FasL, and that this is inhibited by IGF-I, apoptosis was assessed by analysis of cellular DNA content by flow cytometry. Treatment with FasL increased the percentage of cells in the subdiploid apoptotic (A_0) peak, and IGF-I suppressed this effect (Fig. 2, B and C). Pretreatment with LY294002 prevented protection by IGF-I against FasL, but pretreatment with PD98059 had no effect. Pretreatment with either LY294002 or PD98059 did not affect the number of apoptotic cells in cultures not treated with FasL.

As an additional measure of apoptosis, cell lysates were assayed for caspase-3 activity. In both control cultures and cultures pretreated with IGF-I, caspase-3 activity increased significantly 1 h after treatment with FasL and was significantly higher in control *vs.* IGF-I-treated cultures at 2 h (Fig. 3A). Cumulative caspase-3 activity during 24 h after FasL was higher in control cells *vs.* cells treated with IGF-I (Fig. 3B).

Changes in caspase-3 activity were also measured by flow cytometry of cells immunostained for active caspase-3. Expression of active caspase-3 was increased 12 h after FasL, and this effect was reduced by IGF-I (Fig. 3C). Pretreatment with LY294002 prevented the protective effect of IGF-I, but PD98059 had no effect. Therefore, several different assays for cell viability and apoptosis indicate that IGF-I reduces FasL-induced apoptosis through the PI3K pathway.

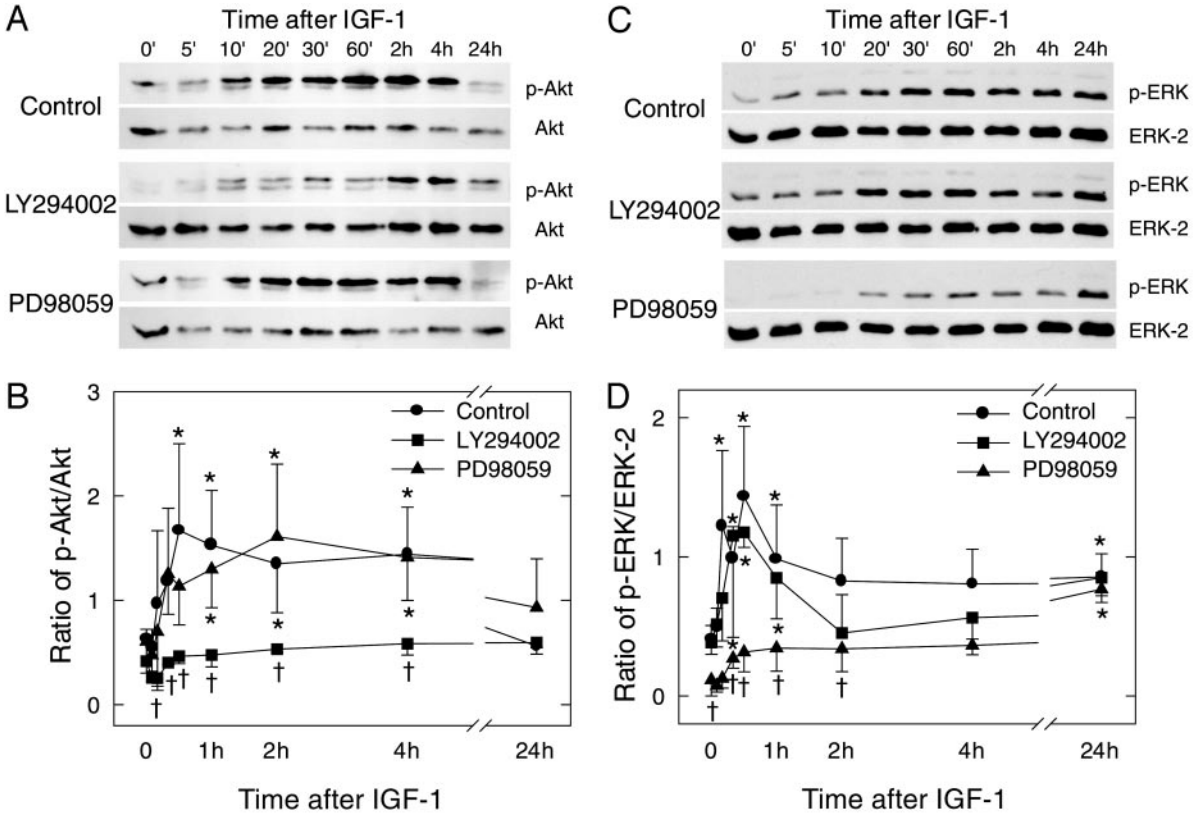

Fig. 1. Activation of Akt and ERK Kinases by IGF-I in Granulosa Cells

Granulosa cells cultured in DMEM-F12-ITS were pretreated with no inhibitor (control), 20 μM LY294002, or 30 μM PD98059 for 30 min. IGF-I (100 ng/ml) was added, and cells were collected for preparation of lysates at 0, 5, 10, 20, 30, and 60 min and 2, 4, and 24 h. p-Akt (activated Akt), total Akt, p-ERK-1/2 (activated ERKs), and total ERK-2 were analyzed by immunoblotting. *Top panels*, Representative immunoblots showing phosphorylation of Akt (A) and ERK-1/2 (C). *Bottom panels*, Activation of Akt and ERK-1/2 by IGF-I represented as a ratio of the signal intensities of p-Akt/total Akt (B) and p-ERK-1/2 /total ERK-2 (D). Data points represent the means ± SEM of results obtained in three experiments using separate granulosa cell preparations. *, $P < 0.05$ *vs.* time 0 within the same treatment. †, $P < 0.05$ *vs.* other treatments at the same time point.

Activation of Akt Is Required for the Protective Effect of IGF-I Against Fas-Mediated Killing

To determine whether Akt is required for the protective effect of IGF-I, granulosa cells were infected with adenoviruses expressing constitutively active Akt (myrAkt) or dominant negative Akt (dnAkt). The myrAkt adenovirus expresses murine Akt that has a c-*src* myristoylation sequence and a hemagglutinin (HA) tag fused to the amino terminus of wild-type murine Akt (25). The dnAkt adenovirus encodes a murine Akt mutant in which the two major phosphorylation sites (Thr^{308} and Ser^{473}) and the phosphate transfer residue in the catalytic site (Lys^{179}) are replaced by Ala. dnAkt is constructed with HA and green fluorescence protein (GFP) tags for detection (26, 27). A null virus that was used as a control adenoviral vector contains no cDNA insert (28). Infection efficiency at 24 h was assessed by analyzing expression of GFP in cells infected with dnAkt. Approximately 90% of granulosa cells infected with 10 multiples of infection of dnAkt adenovirus expressed GFP (Fig. 4A). In subsequent experiments, all adenoviruses were used at 10 multiples of infection. At 48 h after infection, total Akt and HA were elevated in cells infected with myrAkt and dnAkt but were not detected in noninfected cells or cells infected with null adenovirus (Fig. 4B).

FasL induced significant killing in cells infected with null virus, and killing was reduced by 44% when the cells were pretreated with IGF-I ($P < 0.05$, Fig. 5). In cells infected with myrAkt, FasL-induced killing was reduced by 59% compared with null-infected cells ($P < 0.05$), and pretreatment with IGF-I did not further reduce killing. In cultures infected with dnAkt, 38% more cells were killed by FasL than in null-infected cells ($P < 0.05$), and pretreatment with IGF-I conferred no protection from FasL-induced killing. These results show that activation of Akt is necessary for the protective effect of IGF-I against FasL-induced killing.

Regulation of the Cell Cycle by IGF-I Is Mediated by Both PI3K/Akt and MAPK Pathways

In addition to promoting cell survival, IGF-I stimulates proliferation in many cell types (29, 30). Therefore, the ability of IGF-I and its signaling pathways to stimulate

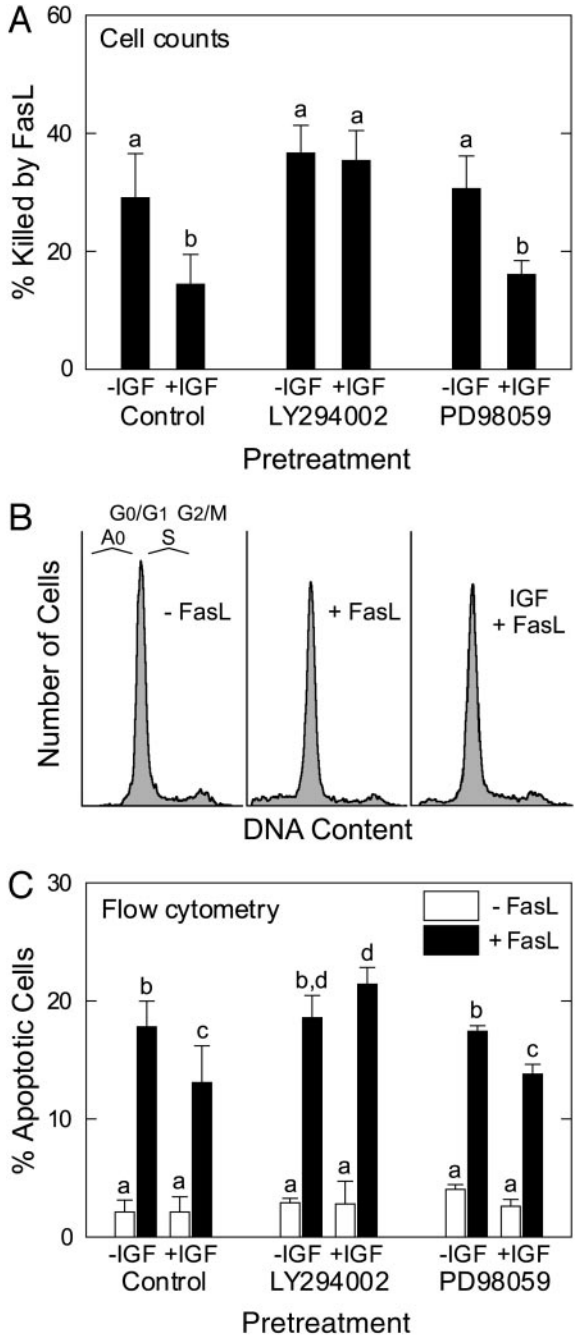

Fig. 2. Effect of the PI3K Inhibitor, LY294002, and the MAPK Pathway Inhibitor, PD98059, on IGF-I-Mediated Protection from FasL-Induced Apoptosis

Granulosa cells cultured in DMEM-F12-ITS were pretreated with no inhibitor (control), 20 μM LY294002, or 30 μM PD98059 at t = −0.5 h and treated with 0 or 100 ng/ml IGF-I at t = 0 h. At t = 4 h, 0 or 100 ng/ml FasL were added. A, Assay of cell viability. Numbers of viable cells were determined at t = 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. B, The distribution of cells in various stages of the cell cycle (G_0/G_1, S, G_2/M) and the presence of apoptotic cells with subdiploid content of DNA (A_0) was determined at t = 24 h by flow cytometry of PI-stained cells. Representative analyses are shown. C, The percentage of cells in the A_0 peak in response to treatments. *Bars* represent the means ± SEM of results obtained in three experiments using separate granulosa cell preparations. Within each panel, *bars with no common superscripts* are significantly different ($P < 0.05$).

granulosa cell proliferation was examined. Incorporation of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) was used to assess replication of DNA during the S phase of the cell cycle. Treatment of cells with IGF-I increased the percent of cells incorporating BrdU over 24 h by 37% *vs.* controls (the percentage of cells incorporating BrdU was 28.5 ± 5.5% in cultures without IGF-I *vs.* 39.0 ± 4.1% in cultures treated with IGF-I; $P < 0.05$), indicating that IGF-I increased passage of cells through S phase. The distribution of cells in various stages of the cell cycle was determined by flow cytometric analysis of cellular DNA content using propidium iodide (PI) staining of DNA. Treatment with IGF-I increased progression from G_0/G_1 to S phase, as evidenced by a 31% increase in the percent of cells in S phase and a decrease in the percent of cells in G_0/G_1 phases ($P < 0.05$; Table 1). The percent of cells in G_2/M phases was not affected. Inhibition of the PI3K pathway with LY294002 decreased progression from G_0/G_1 to S phase: the percent of cells in G_0/G_1 was increased, and the percent of cells in S was decreased compared with control cultures ($P < 0.05$; Table 1). In the presence of LY294002, the IGF-I-mediated increase in progression from G_0/G_1 to S phase was blocked. Inhibition of the MAPK pathway with PD98059 resulted in no changes in cell cycle distribution in the absence of IGF-I. However, treatment with PD98059 blocked the ability of IGF-I to promote cell cycle progression (Table 1). These results show that a functional PI3K pathway is required for progression from G_1 to S phase in basal media, and that both the PI3K and MAPK pathways are involved in increased cell cycle progression induced by IGF-I.

Further experiments showed that activation of Akt is required for cell cycle progression. Constitutively active myrAkt exhibited the same ability as IGF-I to increase cell cycle progression. Compared with cells infected with null adenovirus, myrAkt increased the percent of cells in S phase and decreased the percent in G_0/G_1 phases ($P < 0.05$; Table 2). In cells expressing myrAkt, IGF-I did not further increase the percent of cells in S phase. Expression of dnAkt reduced cell cycle progression *vs.* null-infected cells, as shown by a reduced percent of cells in S phase. In cells expressing dnAkt, the ability of IGF-I to promote cell cycle progression was abolished. Therefore, stimulation of cell cycle progression by IGF-I requires activation of Akt.

Akt was reported to down-regulate expression of the cdk inhibitor $p27^{Kip1}$ (31). Increased expression of $p27^{Kip1}$ prevents progression from G_0/G_1 to S phase

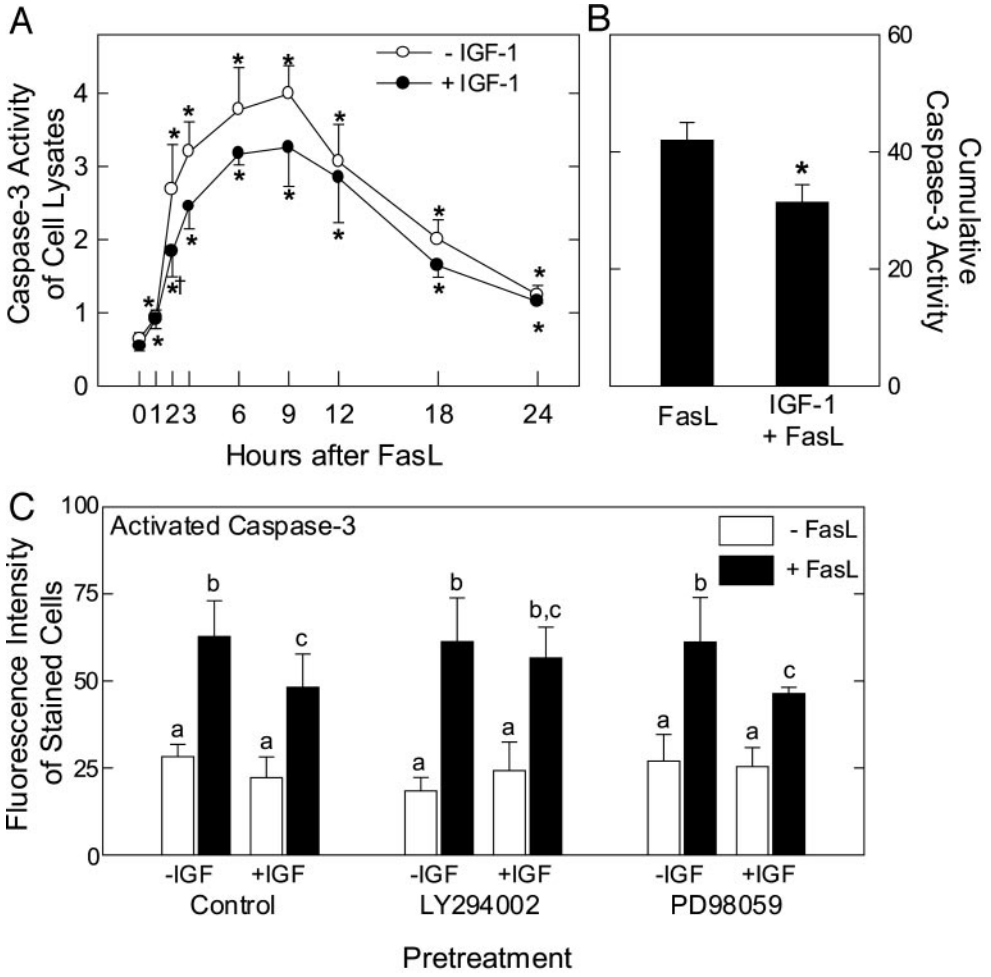

Fig. 3. Effect of IGF-I on FasL-Induced Caspase-3 Activation in Cultured Granulosa Cells

Granulosa cells in DMEM-F12-ITS were treated with 0 or 100 ng/ml IGF-I at t = 0 h and 0 or 100 ng/ml FasL at t = 4 h. A, Caspase-3 activity was assayed in lysates prepared from cells collected at 0, 1, 2, 3, 6, 9, 12, 18, and 24 h after treatment with FasL and is expressed as picomoles of substrate cleaved/μg total protein·h. Data points represent the mean ± SEM of five experiments using separate granulosa cell preparations. *, $P < 0.05$ *vs.* time 0 within the same treatment. †, $P < 0.05$ *vs.* − IGF-I at the same time point. B, Cumulative caspase-3 activity over 24 h determined by calculating the area under the curves shown in panel A. *, $P < 0.05$. C, Detection of activated caspase-3 at 12 h after treatment with FasL by immunofluorescent staining of cells with an antibody recognizing active caspase-3 followed by flow cytometry to quantify fluorescence intensity. *Bars* represent the means ± SEM of results obtained in three experiments using separate granulosa cell preparations. *Bars with no common superscripts* are significantly different ($P < 0.05$).

by inhibiting cdk2. Expression of $p27^{Kip1}$ increased 2.6 ± 0.9-fold in granulosa cells expressing dnAkt *vs.* cells infected with null adenovirus ($P < 0.05$; Fig. 4C). Therefore, at least one mechanism for the stimulation of cell cycle progression, inhibition of $p27^{Kip1}$, is regulated by Akt in granulosa cells.

Inhibition of Cell Cycle Progression Prevents the Protective Effect of IGF-I Against FasL-Induced Apoptosis

Experiments were performed to test whether cell cycle progression was required for the protective effect of IGF-I against FasL. Cells were treated with roscovitine, an inhibitor of cdk2 activity that blocks transition from G_1 to S phase and G_2 to M phase, or with hydroxyurea, which blocks DNA synthesis by inhibition of ribonucleotide reductase and causes cells to remain in S phase. In control cultures, treatment of granulosa cells with IGF-I decreased FasL-induced killing by 31% (Fig. 6). However, in the presence of roscovitine, IGF-I had no protective effect against killing. Cell cycle analysis confirmed that roscovitine inhibited the cell cycle as expected: in cells treated with roscovitine, the percent of cells in G_0/G_1 phases was increased, and the percent in S phase was 34% lower compared with control cultures ($P < 0.05$; Table 3). Treatment with roscovitine prevented the effect of IGF-I to increase progression from G_0/G_1 to S phase. These results are consistent with cell cycle blockage by roscovitine at

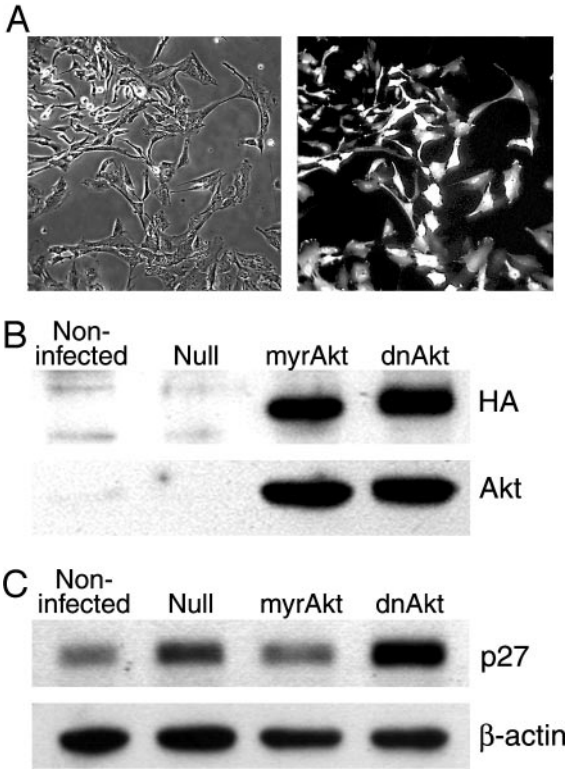

Fig. 4. Adenoviral Expression of myrAkt and dnAkt in Granulosa Cells and Effects on $p27^{kip}$ Expression

Cells were infected with null, myrAkt (with HA tag), or dnAkt (with HA and GFP tags) adenoviruses or were uninfected. A, Expression of GFP in granulosa cells infected with dnAkt adenovirus was examined microscopically at 24 h to assess infection efficiency. Phase contrast (*left panel*) and fluorescent (*right panel*) images are shown (magnification = ×260). B, Immunoblotting for HA and total Akt in lysates from noninfected and adenovirus-infected cells. Media were changed to DMEM-F12-ITS at 24 h after infection with adenoviruses, and expression of HA and Akt proteins was assayed in lysates collected at 48 h. C, Immunoblotting for p27kip1 expression in lysates of noninfected and adenovirus-infected cells at 48 h. Blots were probed with anti-$p27^{Kip1}$ antibody, stripped, and then probed with anti-β-actin antibody as an internal loading control. The blots shown are representative of results obtained using three separate granulosa cell preparations.

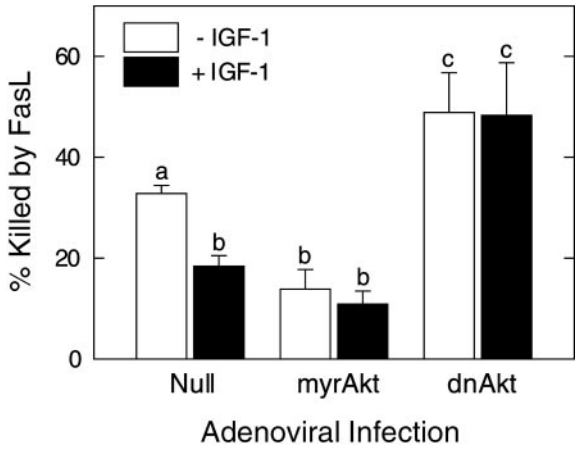

Fig. 5. Effect of myrAkt and dnAkt Expression on IGF-I-Mediated Protection from FasL-Induced Apoptosis

Granulosa cells were infected with adenoviruses for 24 h. Media were changed to DMEM-F12-ITS, and 0 or 100 ng/ml IGF-I were added (t = 0 h). At t = 4 h, 0 or 100 ng/ml FasL were added. The number of viable cells was determined at t = 24 h, and the percentage of cells killed by FasL was calculated by comparing the number of viable cells in cultures treated with or without FasL. *Bars* represent the means ± SEM of results obtained in three experiments using separate granulosa cell preparations. *Bars with no common superscripts* are significantly different ($P < 0.05$).

the G_1/S transition and show that IGF-I does not overcome the blockage. In cells pretreated with hydroxyurea, IGF-I decreased FasL-induced killing by 21% ($P < 0.05$; Fig. 6). The percent of hydroxyurea-treated cells in G_0/G_1 phases and in G_2/M phases was less than controls, whereas the percent of cells in S phase was 54% higher ($P < 0.05$; Table 3), consistent with cell cycle blockage in S phase. Treatment of cells with hydroxyurea plus IGF-I resulted in even more cells in S phase ($P < 0.05$ *vs.* hydroxyurea alone), suggesting that IGF-I increased progression from G_0/G_1 to S phase but did not overcome the blockage in S phase by hydroxyurea. These data show that when cells were blocked at the G_1/S transition, IGF-I did not provide protection from apoptosis, but that when cells were blocked in S phase, some protection by IGF-I was maintained.

We next tested whether the protective effect of Akt against FasL-induced killing required progression from G_0/G_1 to S phase. Expression of myrAkt protected granulosa cells from FasL-induced killing by 45% ($P < 0.05$; Fig. 7). Addition of roscovitine blocked the protection by myrAkt and increased FasL-induced killing by 150% ($P < 0.05$) compared with myrAkt-infected cells alone. Cell cycle analysis showed that the percent of cells in S phase was increased in cells expressing myrAkt but that this effect was inhibited by roscovitine ($P < 0.05$; Table 4). These results suggest that the protective effect of Akt against FasL-induced apoptosis requires active cdk2 and/or cell cycle progression from G_1 to S phase.

DISCUSSION

IGF-I is critical for ovarian follicular development. In genetically engineered mice lacking IGF-I, development of ovarian follicles is blocked at the early antral stage, and treatment with exogenous gonadotropins fails to induce ovulation (11, 12). In cattle, a reduction in levels of inhibitory IGF binding proteins leads to increased bioavailability of IGF to cells within a single dominant follicle, and this is associated with its selection for development to the preovulatory stage. In contrast, increased concentrations of IGF binding pro-

Table 1. Effect of the PI3K Inhibitor (LY294002) or MAPK Pathway Inhibitor (PD98059) on Cell Cycle Distribution of Granulosa Cells Treated with or without IGF-I

	Pretreatment					
	None		LY294002		PD98059	
	−IGF-I	+IGF-I	−IGF-I	+IGF-I	−IGF-I	+IGF-I
G_0/G_1 (%)	78.5 ± 0.2^a	74.5 ± 1.9^b	87.2 ± 0.5^c	87.1 ± 0.9^c	78.5 ± 0.9^a	79.8 ± 0.5^a
S (%)	13.0 ± 0.7^a	17.0 ± 2.8^b	5.7 ± 0.1^c	6.9 ± 0.9^c	12.9 ± 1.2^a	12.0 ± 1.4^a
G_2/M (%)	8.5 ± 0.6^a	8.6 ± 1.2^a	7.2 ± 0.4^{ab}	6.0 ± 0.8^b	8.7 ± 0.9^a	8.2 ± 0.9^a

Granulosa cells in DMEM-F12-ITS were pretreated with no inhibitor, 20 μM LY294002, or 30 μM PD98059 at t = −0.5 h, and 0 or 100 ng/ml IGF-I was added at t = 0 h. Cells were collected at t = 24 h for analysis of DNA content by flow cytometry. Data represent the means ± SEM of three separate granulosa cell preparations. In each cell cycle phase, *numbers with no common superscripts* are significantly different ($P < 0.05$).

Table 2. Effect of Expression of myrAkt and dnAkt on Cell Cycle Distribution of Granulosa Cells Treated with or without IGF-I

	Adenovirus					
	Null		myrAkt		dnAkt	
	−IGF-I	+IGF-I	−IGF-I	+IGF-I	−IGF-I	+IGF-I
G_0/G_1 (%)	82.4 ± 0.5^{ab}	80.8 ± 2.2^{ac}	79.7 ± 1.0^c	80.0 ± 1.2^c	82.6 ± 0.5^b	81.7 ± 0.3^{ab}
S (%)	8.1 ± 1.0^a	10.5 ± 1.1^b	10.9 ± 1.5^b	10.8 ± 1.0^b	5.8 ± 0.6^c	7.4 ± 1.1^{ac}
G_2/M (%)	9.5 ± 0.5^{ab}	8.7 ± 1.0^a	9.5 ± 0.9^{ab}	9.2 ± 0.5^{ab}	11.6 ± 0.3^c	10.9 ± 1.4^{bc}

Granulosa cells were infected with adenoviruses for 24 h. Media were changed to DMEM-F12-ITS, and cells were treated with 0 or 100 ng/ml IGF-I (t = 0 h). Cells were collected at t = 24 h for analysis of DNA content by flow cytometry. Data represent the means ± SEM of three separate granulosa cell preparations. In each cell cycle phase, *numbers with no common superscripts* are significantly different ($P < 0.05$).

teins, and the resulting reduced bioavailability of IGF, are associated with follicular atresia (13–15). IGF-I promotes survival and proliferation in a variety of cell types, including granulosa cells (9, 16, 32–34). A potential connection between the protective and mitogenic effects of IGF-I has not been defined. In this study, a relationship between the protective effect of IGF-I against apoptosis and cell cycle progression in granulosa cells was demonstrated, and the pathways mediating this effect were determined.

IGF-I stimulated both the PI3K and MAPK pathways in granulosa cells, as evidenced by increased phosphorylation of Akt and ERK-1/2, respectively. Whereas the PI3K inhibitor, LY294002, prevented the protective effect of IGF-I against FasL-induced apoptosis, the MEK inhibitor, PD98059, had no effect. Therefore, the protective effect of IGF-I is mediated by the PI3K pathway and not by the MAPK pathway. These results are consistent with reports that survival of granulosa cells in serum-free culture medium in response to IGF-I is associated with increased phosphorylation of Akt (35) and increased Akt kinase activity (36). In the current study, a dominant negative form of Akt prevented the protective effect of IGF-I whereas constitutively active myrAkt mimicked the action of IGF-I and protected cells from killing by FasL. These results are the first to demonstrate that activation of Akt kinase, downstream of PI3K, is required for protection of granulosa cells by IGF-I. The results are consistent with observations in fibroblasts and neuronal cells in which IGF-I stimulated both the PI3K and MAPK pathways, but only the PI3K pathway was required for protection against apoptosis (37–39). In contrast, in some cell types, IGF-I-mediated protection from apoptosis appears to occur through both the PI3K and MAPK pathways (40, 41). Our findings in granulosa cells are similar to results obtained using endothelial cells and various cell lines in which activation of the PI3K/Akt pathway inhibited FasL-induced apoptosis (42–44). The fact that Fas-mediated apoptosis is impaired in phosphatase and tensin homologue (PTEN)-mutant mice, in which phosphorylation of Akt is abnormally elevated, also supports a role for the PI3K/Akt pathway in regulating susceptibility to FasL-induced apoptosis (45).

Previous studies in our laboratory showed that growth factors, including IGF-I, epidermal growth factor, and basic fibroblast factor, which effectively suppressed FasL-induced apoptosis, also appeared to increase proliferation of bovine granulosa cells *in vitro* (9). These results suggested the possibility that progression through the cell cycle alters susceptibility to apoptosis. In the current study, analysis of the distribution of granulosa cells in various stages of the cell cycle by flow cytometry showed that IGF-I increased progression from G_0/G_1 to S phase. Furthermore, analysis of BrdU incorporation into DNA showed that treatment with IGF-I increased synthesis of DNA. The

protective effect of IGF-I against FasL-induced apoptosis was prevented when progression from G_1 to S phase of the cell cycle through the PI3K/Akt pathway was perturbed. Blocking the PI3K/Akt pathway by treatment with LY294002 or expression of dnAkt inhibited G_1 to S phase progression in basal media (containing 100 ng/ml insulin) and prevented the increase in G_1 to S phase progression in response to IGF-I. Expression of constitutively active myrAkt increased G_1 to S phase progression. These results indicate that the PI3K/Akt pathway regulates cell cycle progression as well as survival. The fact that treatment with the cdk2 inhibitor, roscovitine, inhibited G_1 to S phase progression and prevented the protective effects of IGF-I and myrAkt against FasL-induced apoptosis indicates that cell cycle progression through the PI3K/Akt pathway is necessary for protection from apoptosis. Treatment with the MAPK pathway inhibitor, PD98059, prevented the increase in G_1 to S phase progression in response to IGF-I but did not alter G_1 to S phase progression in basal media or the extent of FasL-induced killing. These results indicate that stimulation of the MAPK pathway by IGF-I and the associated increase in G_1 to S phase progression is not sufficient for protection against apoptosis.

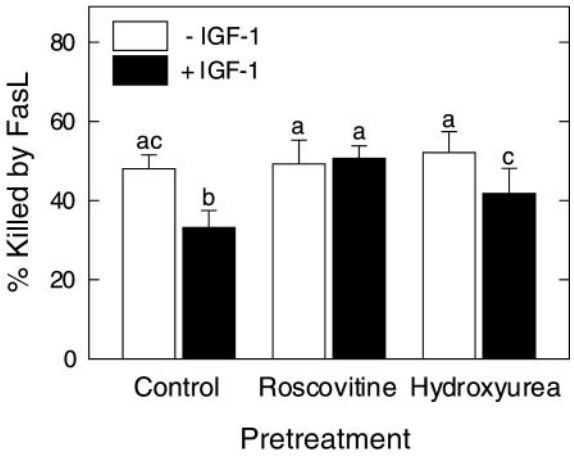

Fig. 6. Effect of Cell Cycle Inhibitors, Roscovitine and Hydroxyurea, on IGF-I-Mediated Protection from FasL-Induced Apoptosis

Granulosa cells cultured in DMEM-F12-ITS were pretreated with no inhibitor, 20 μM roscovitine, or 25 μg/ml hydroxyurea at t = −0.5 h, and 0 or 100 ng/ml IGF-I were added at t = 0 h. At t = 4 h, 0 or 100 ng/ml FasL were added. The number of viable cells was determined at t = 24 h, and the percentage of cells killed by FasL was calculated by comparing the number of viable cells in cultures treated with or without FasL. *Bars* represent the means ± SEM of results obtained in three experiments using separate granulosa cell preparations. *Bars with no common superscripts* are significantly different ($P < 0.05$).

A number of substrates of activated Akt that regulate cell survival and proliferation have been identified. Proteins that are phosphorylated and inactivated by Akt include the apoptosis-inducing protease, caspase-9, and the proapoptotic Bcl-2 family member, Bad (46, 47). Akt also phosphorylates the transcription factor, forkhead, causing it to be localized in the cytoplasm. In the absence of active Akt, nonphosphorylated forkhead localizes in the nucleus where it can either induce transcription of FasL and thereby promote apoptosis, or stimulate transcription of $p27^{Kip1}$ and induce withdrawal from the cell cycle (31, 48, 49). Another mechanism by which Akt is thought to prevent apoptosis is by activating the transcription factor nuclear factor-κB (NFκB), which in turn induces the expression of antiapoptotic genes (50, 51). Furthermore, Akt stimulates the expression of the antiapoptotic protein, FADD-like ICE (FLICE)-inhibitory protein (FLIP), which inhibits activation of caspase-8 (42, 52). Akt is thought to modulate the cell cycle through its effects on several cell cycle-regulatory proteins. Akt inactivates forkhead transcription factors and thus prevents transcriptional activation of the cdk inhibitor $p27^{Kip1}$ (31, 49). Akt also phosphorylates and inactivates glycogen synthase kinase-3β (GSK3). Active glycogen synthase kinase-3β phosphorylates cyclin D1, leading to its degradation (53). Akt thus promotes cell cycle progression by increasing levels of cyclin D1 and decreasing levels of $p27^{Kip1}$. In the

Table 3. Effect of Cell Cycle Inhibitors, Roscovitine and Hydroxyurea, on Cell Cycle Distribution of Granulosa Cells Treated with or without IGF-I

	Pretreatment					
	None		Roscovitine		Hydroxyurea	
	−IGF-I	+IGF-I	−IGF-I	+IGF-I	−IGF-I	+IGF-I
G_0/G_1 (%)	78.5 ± 0.2^a	74.5 ± 1.9^b	84.5 ± 1.3^c	84.9 ± 0.7^c	74.9 ± 1.4^b	72.3 ± 0.7^b
S (%)	13.0 ± 0.7^a	17.0 ± 2.8^b	8.6 ± 1.3^c	7.4 ± 1.0^c	20.0 ± 0.6^d	22.9 ± 0.9^c
G_2/M (%)	8.5 ± 0.6^a	8.6 ± 1.2^a	6.9 ± 0.6^b	7.8 ± 0.4^{ab}	5.1 ± 1.0^c	4.8 ± 0.3^c

Granulosa cells cultured in DMEM-F12-ITS were pretreated with no inhibitor, 20 μM= roscovitine, or 25 μg/ml hydroxyurea at t = −0.5 h and treated with 0 or 100 ng/ml IGF-I at t = 0 h. Cells were collected at t = 24 h for analysis of DNA content by flow cytometry. Data represent the means ± SEM of three separate granulosa cell preparations. In each cell cycle phase, *numbers with no common superscripts* are significantly different ($P < 0.05$).

current study, expression of dnAkt increased levels of $p27^{Kip1}$, suppressed G_1 to S phase progression, and prevented the effect of IGF-I to protect against FasL-induced apoptosis. Our results demonstrate that activation of Akt in granulosa cells is a key step in a pathway that regulates cell cycle progression as well as survival.

There is evidence for a relationship between stage of the cell cycle and the susceptibility to apoptosis (20–23). In general, apoptosis is detected *in vivo* at the highest frequency in proliferating rather than quiescent tissues. Certain cell types appear to be most susceptible to undergoing apoptosis during the transition from G_1 to S phase (20). For example, in lymphocytes undergoing activation-induced cell death, a process mediated by Fas/FasL interactions, cells undergo apoptosis at the G_1 to S transition (54). In contrast, cells that are arrested in early G_1 or G_0 of the cell cycle may be resistant to apoptosis. This includes cell types such as myocytes, in which withdrawal from the cell cycle upon terminal differentiation is associated with resistance to apoptosis (55). Furthermore, treatment of T cells with agents that block the cell cycle in early G_1 induces resistance to apoptosis, whereas blocking at the G_1/S transition induces sensitivity to apoptosis (20). The Bcl-2 protein family, which is well recognized for its control over apoptosis, also modulates the cell cycle. Overexpression of Bcl-2 protects against apoptosis and also prevents quiescent cells from entering the cell cycle (56, 57). The results of the current study provide further evidence for a relationship between the cell cycle and susceptibility to apoptosis.

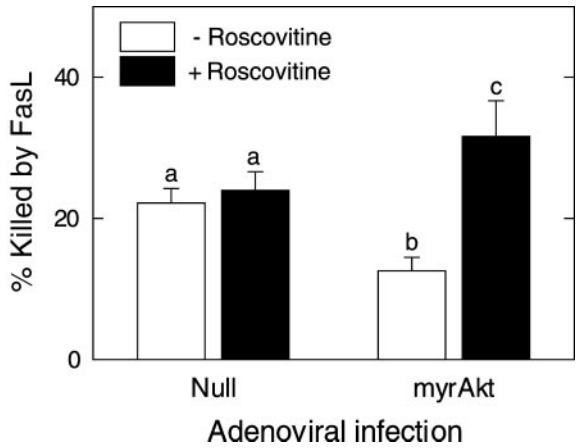

Fig. 7. Effect of Roscovitine on myrAkt-Mediated Protection from FasL-Induced Apoptosis

Granulosa cells were infected with adenoviruses for 24 h. Media were changed to DMEM-F12-ITS, and cells were treated with 0 or 20 μM roscovitine (t = 0 h). At t = 4 h, 0 or 100 ng/ml FasL were added. The number of viable cells was determined at t = 24 h, and the percentage of cells killed by FasL was calculated by comparing the number of viable cells in cultures treated with or without FasL. *Bars* represent the means ± SEM of results obtained in three experiments using separate granulosa cell preparations. *Bars with no common superscripts* are significantly different ($P < 0.05$).

The ability of IGF-I to prevent Fas-mediated apoptosis may play a critical role in promoting follicle development. Expressions of Fas and FasL are low in healthy follicles and are elevated in atretic follicles in cows, rodents, and humans (4–8). Granulosa cells from atretic bovine follicles are more susceptible to FasL-induced apoptosis *in vitro* than cells from healthy follicles (4). These findings indicate that the Fas pathway becomes activated in follicles undergoing atresia but is suppressed in healthy follicles. It is likely that the greater bioavailability of IGF-I within healthy follicles, compared with atretic follicles (16), contributes to inhibition of the Fas-mediated death pathway. Support for a role of IGF-I in suppressing Fas-mediated apoptosis has been provided by experiments with cultured bovine granulosa cells. Withdrawal of serum from granulosa cell cultures stimulates expression of Fas and FasL and induces apoptosis through Fas/FasL interaction (10). IGF-I was identified as at least one of the factors present in serum that prevents FasL-induced apoptosis of bovine granulosa cells (9). The ability of locally produced growth factors such as IGF-I to suppress expression of Fas and FasL, and to render granulosa cells resistant to Fas-mediated apoptosis, is likely to be important for promotion of follicle development and survival.

Table 4. Effect of the cdk2 Inhibitor, Roscovitine, on Cell Cycle Distribution of Granulosa Cells Expressing myrAkt

	Adenovirus			
	Null		myrAkt	
	−Roscovitine	+Roscovitine	−Roscovitine	+Roscovitine
G_0/G_1 (%)	78.7 ± 2.2[a]	83.6 ± 1.9[b]	75.4 ± 2.2[a]	84.2 ± 0.5[b]
S (%)	13.0 ± 2.1[a]	5.8 ± 0.9[b]	16.4 ± 0.9[c]	5.6 ± 0.2[b]
G_2/M (%)	8.3 ± 0.2[a]	10.7 ± 1.3[a]	8.2 ± 1.4[a]	10.2 ± 0.6[a]

Granulosa cells were infected with adenoviruses for 24 h. Media were changed to DMEM-F12-ITS, and cells were treated with 0 or 20 μM roscovitine (t = 0 h). Cells were collected at t = 24 h for analysis of DNA content by flow cytometry. Data represent the means ± SEM of three separate granulosa cell preparations. In each cell cycle phase, *numbers with no common superscripts* are significantly different ($P < 0.05$).

In summary, the effect of IGF-I to protect granulosa cells from apoptosis is mediated through the PI3K/Akt pathway and requires that PI3K/Akt-mediated progression from G_1 to S phase of the cell cycle is not perturbed. Furthermore, the ability of activated Akt to protect cells against apoptosis is prevented when cell cycle progression is blocked. These results suggest that effects of activated Akt on substrates known to be involved in antiapoptotic pathways (such as Bad and forkhead transcription factors) are not adequate to protect cells against apoptosis when cell cycle progression is prevented. An interdependency between cell proliferation and survival is relevant to the physiology of ovarian follicle development. Granulosa cells are dependent upon growth factors for survival during the period of proliferation in the developing follicle. Stimulation of the PI3K/Akt pathway by IGF-I may promote cell survival by suppression of apoptotic pathways and by concomitantly maintaining appropriate progression through the cell cycle.

MATERIALS AND METHODS

Materials

All culture media and additives including recombinant human IGF-I were obtained from Life Technologies, Inc. (Gaithersburg, MD). Sodium pyruvate and L-glutamine were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture plates were obtained from Corning-Costar (Cambridge, MA). Soluble recombinant human FasL was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Kinase inhibitors (LY294002 and PD98059) and cell cycle inhibitors (roscovitine and hydroxyurea) were from Calbiochem (San Diego, CA). Rabbit antimouse p-Akt (Ser^{473}, no. 9271) and total Akt antibodies (no. 9272) were obtained from Cell Signaling Technology (Beverly, MA). Mouse antihuman p-ERK (Tyr^{204}, sc-7383), polyclonal goat antirat ERK-2 (sc-154-G), rabbit antiinfluenza HA (sc-805), rabbit antihuman $p27^{Kip1}$ (sc-528), horseradish peroxidase-conjugated goat antimouse IgG, and horseradish peroxidase-conjugated donkey antigoat IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-β-actin (clone AC-15) and BrdU were purchased from Sigma Chemical Co. Monoclonal mouse anti-BrdU (clone 3D4) and rabbit antiactive human caspase-3 (no. 557035) were obtained from BD Biosciences (San Diego, CA). Horseradish peroxidase-conjugated goat antirabbit IgG was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Alexa 488-conjugated goat antimouse IgG, Alexa 488-conjugated goat antirabbit IgG, and PI were from Molecular Probes (Eugene, OR). Recombinant adenoviruses containing Akt kinase mutants were obtained from Dr. K. Walsh (St. Elizabeth Medical Center of Boston, Boston, MA), and null adenovirus was obtained from Dr. A. Quaroni (Cornell University, Ithaca, NY).

Cell Isolation, Preparation, and Culture

Freshly excised cow ovaries were obtained from an abattoir, transported in saline at 4 C (~1.5 h), and processed immediately. Granulosa cells were collected by aspiration of 2- to 4-mm follicles and cultured in DMEM-F12 (supplemented with 1 mM pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone) containing 10% fetal bovine serum. Cells were plated (d 0) at 1×10^5 cells per well in 96-well plates for cell viability assays, at $1.5–2 \times 10^6$ cells per well in 35-mm dishes for immunoblotting and flow cytometric analyses, and at 3×10^5 cells per well in 24-well plates for assays of caspase-3 activity. In all experiments except those involving adenoviral infection (described below), media were changed on d 1 to serum-free DMEM-F12 supplemented as above and containing 100 ng/ml insulin, 5 μg/ml transferrin, 20 nM Na-selenite and 0.1% BSA (ITS). The concentration of insulin in ITS was experimentally determined to be the minimum required to prevent the cells from undergoing spontaneous apoptosis (data not shown). Insulin concentrations in commercial preparations of ITS are much higher. On d 2, cells were treated with 0 or 100 ng/ml IGF-I ($t = 0$ h) and, where appropriate, with 0 or 100 ng/ml FasL at $t = 4$ h. In some experiments, parallel cultures were pretreated 0.5 h before IGF-I with specific second messenger or cell cycle inhibitors. The inhibitors used were: LY294002 (20 μM), a PI3K inhibitor; PD98059 (30 μM), a MEK (MAPK kinase) inhibitor; roscovitine (20 μM), a cdk2 inhibitor which blocks cells at the G_1/S interface; and hydroxyurea (25 μg/ml), a ribonucleotide reductase inhibitor that blocks cells in S phase. Cell viability assays and flow cytometric analyses were performed 24 h after treatment. Cells were collected for immunoblotting or assay of caspase-3 activity at various times from 0–24 h after treatment.

Expression of Recombinant Akt Proteins by Adenoviral Infection

On d 2 after plating, bovine granulosa cells at approximately 90% confluency were infected with recombinant adenoviruses (myrAkt, dnAkt, and null) in DMEM-F12 containing 10% fetal bovine serum. On d 3, media were changed to DMEM-F12-ITS, and various treatments were applied. On d 4, cell viability assays were performed and cells were collected for immunoblotting or cell cycle analysis.

Assays for Cell Viability and Activated Caspase-3

The number of viable cells attached to each culture dish at the end of the culture period was determined. Cells were trypsinized, collected, and stained with trypan blue, and live cells were counted in a hemacytometer. The percent of granulosa cells killed by FasL was calculated by comparing the number of live cells present in FasL-treated cultures *vs.* the number present in control cultures that received the same pretreatment without addition of FasL. Treatments within each experiment were done in quadruplicate wells of 96-well culture plates. This assay provides the best quantitative measure of the percentage of granulosa cells susceptible to FasL-induced apoptosis. Assays for caspase-3 activity and cellular DNA content (described below) were used to confirm that cell death occurred by apoptosis.

Caspase-3 activity in cell lysates was determined by measuring the amount of fluorescent-labeled caspase-3-specific substrate (R110-DEVD) cleaved per hour using the EnzChek caspase-3 assay kit (Molecular Probes, Eugene, OR). Activity is expressed as picomoles substrate cleaved/μg protein per h.

Levels of immunoreactive active caspase-3 were determined by flow cytometric analysis of cells obtained 12 h after treatment with 0 or 100 ng/ml FasL. Cells were trypsinized, collected, and fixed in 80% ethanol. Cells were incubated with 1 μg/ml antiactive caspase-3 antibody in PBS containing 1% calf serum, 0.1% saponin, and 0.1% sodium azide at room temperature for 2 h. Cells were rinsed, and 0.5 μg/ml Alexa 488-conjugated goat antirabbit IgG was used to detect the primary antibody. Log fluorescence of Alexa 488 was detected in 10,000 cells on a FACScan flow cytometer (Becton Dickinson and Co., Franklin Lakes, NJ). Events were gated for single cells based on side scatter *vs.* forward scatter, and the mean fluorescent intensity of single cells was determined for each sample.

Immunoblotting

Cells from duplicate wells were scraped into ice-cold HEPES-buffered saline (10 mM HEPES, 150 mM NaCl), pelleted, and suspended in RIPA buffer (1.0% Nonidet P-40, 0.05% Na-deoxycholate, 0.1% SDS in PBS) containing freshly added proteinase inhibitors (100 μg/ml phenylmethylsulfonyl fluoride and 3 mg/ml aprotinin). Cells were sonicated for 10 sec, and lysates were frozen until further analysis. Protein content of cell lysates was determined using the DC Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA).

Protein lysates (~60–120 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in Tris-buffered saline (TBST; 20 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% nonfat milk for 30 min at room temperature. Membranes were incubated at 4 C overnight in TBST-5% BSA containing antibodies to p-Akt, p-ERK, or HA (dilution 1:1000) or $p27^{Kip1}$ (dilution 1:500). Membranes were washed, incubated with horseradish peroxidase-conjugated secondary antibodies in TBST-5% nonfat milk for 30 min at room temperature, and washed. A chemiluminescent signal was generated using Western Blot Chemiluminescence Reagent (NEN Life Science Products, Boston, MA), and membranes were exposed to x-ray film (Eastman Kodak, Rochester, NY). After detection of p-Akt, p-ERK, and $p27^{Kip1}$, membranes were stripped and then reprobed with antibodies for total Akt or ERK-2 (dilution 1:1000) or β-actin (dilution 1:5000), respectively. Signals were quantified by densitometry of digitized images using Scion Image Software (Scion Corp., Frederick, MD), and ratios of p-kinases to total kinases and $p27^{Kip1}$ to β-actin were calculated.

Measurement of Proliferation by BrdU Incorporation and Detection

Granulosa cells in DMEM-F12-ITS were treated on d 2 of culture with 0 or 10 μM BrdU and 0 or 100 ng/ml IGF-I. After 24 h, cells were trypsinized, resuspended in DMEM-F12, and fixed in 80% ethanol. Detection of BrdU was performed as previously described with minor modifications (58). 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 $MgCl_2$, DNA was partially digested by addition of 30 U/ml *Bam*HI for 30 min, and cells were rinsed in Tris buffer. Cells were stained with 2 μg/ml anti-BrdU antibody in PBS containing 0.5% BSA and 0.5% Tween 20 at room temperature for 2 h and rinsed, and 0.5 μg/ml Alexa 488-conjugated goat antimouse IgG was used to detect the BrdU antibody. 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 (Becton Dickinson and Co.). 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.

Flow Cytometric Determination of Cellular DNA Content: Analysis of Apoptosis and the Cell Cycle

Cellular DNA content was determined by flow cytometric measurement of PI binding. Adherent granulosa cells in six-well plates were trypsinized, resuspended in DMEM-F12, and fixed in 80% ethanol. Cells 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 by flow cytometry. Data were gated for single cells. In experiments to determine the distribution of cells in various stages of the cell cycle, DNA content was assigned to G_0/G_1, S or G_2M phases based on the method of Ormerod (59) using WinMDI software (The Scripps Research Institute, La Jolla, CA). Cell cycle data presented in Tables 1 and 3 were performed and statistically analyzed as a single experiment but are presented separately for clarity. In experiments to measure the effects of various treatments on FasL-induced apoptosis, the percentage of cells with less than diploid content of DNA (A_0 peak) was determined.

Statistical Analysis

All experiments were repeated using at least three separate preparations of granulosa cells. Caspase-3 activity in cell lysates and Akt and ERK immunoblotting densities were analyzed by two-way ANOVA using a randomized complete block design. Linear contrast was performed to compare individual means when overall significance was observed (60). All other data were analyzed by one-way ANOVA using a randomized complete block design, and Duncan's new multiple range test was used for comparison of means when overall significance was observed (61).

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Address all correspondence and requests for reprints to: Susan M. Quirk, Department of Animal Science, Morrison Hall, Cornell University, Ithaca, New York 14853. E-mail: smq1@cornell.edu.

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