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Induction of Apoptosis in Luteinized Granulosa Cells by the MAP Kinase Kinase (MEK) Inhibitor PD98059[1]

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Our objective is to test the hypothesis that inhibition of mitogen-activated protein (MAP) kinase kinase (MEK) with PD98059 in human luteinized granulosa cells will block epidermal growth (EGF)-stimulated MAP kinase activity and induce apoptosis. Luteinized granulosa cells from human *in vitro* fertilization aspirates were cultured and treated with the following: (1) vehicle; (2) PD98059; (3) EGF; (4) PD98059 + EGF. Treatment with PD98059 suppressed MAP kinase activity, inhibited MAP kinase phosphorylation by Western blot analysis, blocked nuclear translocation of phosphorylated MAP kinase by confocal microscopy, and increased percentages of subdiploid apoptotic nuclei by flow cytometry. Our data are the first evidence that a relationship may exist between the MAP kinase pathway and control of apoptosis in human luteinized granulosa cells. These results support the hypothesis that suppression of the MAP kinase pathway may lead to apoptosis in these cells.

Key Words: **ovary; apoptosis; MAP kinase.**

Peptide growth factors are among the intraovarian signals that contribute to regulation of cell fate during ovarian remodeling (1). Much of the cell deletion that accompanies the periodic cycles of growth and regression in the ovary is due to apoptosis of granulosa cells (2). Growth factors, including the epidermal growth factor (EGF), have been implicated in control of apoptosis and steroidogenesis in cultured granulosa cells (3–7).

EGF transmits intracellular signals through activation of different mitogen activated protein (MAP) kinase cascades (8). We have recently shown that EGF elevates MAP kinase activity and increases the levels of phosphorylated ERK1/ERK2 in cultured human luteinized granulosa cells (9).

MEK is the upstream kinase responsible for the dual phosphorylation of specific threonine and tyrosine residues necessary for the activation of ERK1/ERK2 (10). Intracellular signals initiated by different growth factors, cytokines and integrins can converge on MEK independently of Ras or Raf or through transactivation of EGFR (8). PD98059 inhibits MEK, suppresses the downstream activation of ERK1/ERK2 and effectively blocks transduction through this pathway (11).

PD98059 has been used in a number of studies with other mammalian cells to distinguish between the involvement of different kinase cascades in the induction and prevention of apoptosis (12, 13).

We hypothesize that PD98059, a potent and selective inhibitor of the MAP kinase kinase (MEK), may inhibit the phosphorylation of MAP kinase (ERK1/ERK2) and induce apoptosis in luteinized granulosa cells.

MATERIALS AND METHODS

Isolation of luteinized granulosa cells. Luteinized granulosa cells were isolated from follicular aspirates of women undergoing *in vitro* fertilization procedure, following the method described by Izawa *et al.* (14). The Human Subjects Committee of the University of Minnesota approved the use of these cells for this investigation. Red blood cells were removed from the follicular aspirates by centrifugation through ficoll/hypaque (density 1.07, Gallard-Schlesinger, Carle Place, NY). White blood cells were depleted by treatment with anti-CD45 coated magnetic beads (Dynal, Miami, FL). Luteinized granulosa cells were dispersed by incubation in trypsin (1×, Gibco-BRL, Grand Island, NY). Trypsin digestion was stopped after 3 minutes by addition of six volumes of 10% fetal bovine serum (FBS) (Intergen, Purchase, NY) supplemented with Dulbecco's modified Eagle/ Ham's F12 culture medium, containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin (Sigma, St. Louis, MO). Cells were plated at approximately 2×10^5 cells per 60 mm plastic culture dishes (Nunclon, Denmark).

Treatment of luteinized granulosa cells. Cells were starved overnight in serum-free medium before treatment. Cells were preincubated with PD98059 (100 μM in 1:1 ethanol: dimethyl sulfoxide, Calbiochem, San Diego, CA) prior to EGF treatments (10 ng/ml in distilled water, Gibco BRL). Treatment groups consisted of the fol-

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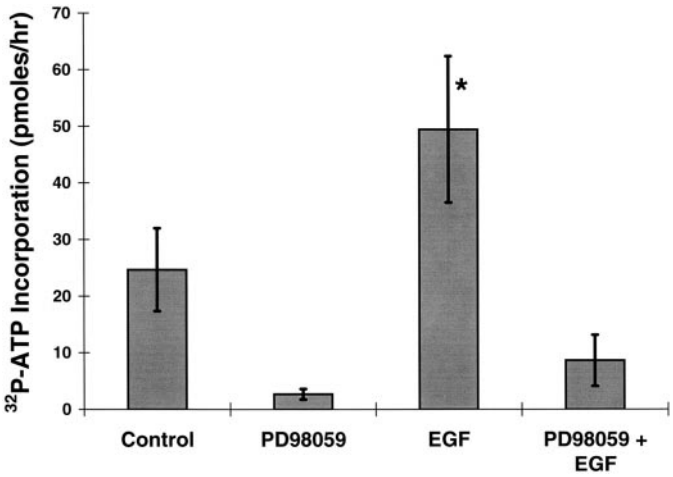

FIG. 1. PD98059 suppresses EGF-stimulated MAP kinase activation in luteinized granulosa cells. MAP kinase activities in whole cell lysates (10 μg total protein) were determined by radiochemical assay of ^{32}-P incorporation into MBP. The mean for EGF (asterisk) differs significantly from means of the other treatments ($p < 0.05$ Student-Neuman-Keuls test). Treatment groups: Controls = vehicle; PD98059 = 100 μM for 60 minutes; EGF = 60 minutes preincubation with vehicle followed by 10 ng/ml EGF for 10 minutes; PD98059 + EGF = 60 minutes preincubation with 100 μM PD98059 followed by 10 minutes treatment with 10 ng/ml EGF. Values represent the mean pmoles/hr ^{32}P incorporation for experiments with $n = 5$ patients. The error bars are ±SEM. Treatment with PD98059 + EGF blocked increases in MAP kinase activity stimulated by EGF (*$p < 0.05$).

lowing: (1) control (vehicle only); (2) PD98059 (100 μM for 60 minutes); (3) EGF (10 ng/ml for 10 minutes + vehicle); (4) PD98059 (100 μM for 60 minutes) followed by EGF (10 ng/ml for 10 minutes).

Treatments were terminated by removal of media followed by 2 washes in cold phosphate-buffered saline (PBS). Luteinized granulosa cells were scraped from plates in ice-cold lysis buffer containing 50 mM Tris-HCl pH 7, 1% TnT X100, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 5 mM Na-pyrophosphate, 0.5 mM Na_3VO_4, 10 mM Na-β-glycerophosphate, 10 mM phenylmethylsulfonyl fluoride with 1 μg/ml protease inhibitors (pepstatin, leupeptin, aprotinin and trypsin inhibitor). Lysates were frozen in liquid nitrogen and stored at −70°C. Total protein was assayed using the bicinchoninic acid method (BCA, Pierce, Rockport, MD).

MAP kinase assay. MAP kinase activity was determined by assay of γ-^{32}P-labelled ATP incorporation into myelin basic protein (MBP) (Upstate Biotechnology Incorporated, Lake Placid, NY). Reaction mixtures (40 μl) contained 10 μg total protein from cell lysates, 500 mM ATP with 5 μCi γ-^{32}P-ATP (NEN, Boston, MA) and 20 μg MBP. Reactions were conducted at 30°C for 15 minutes. The reaction was terminated by transfer of 25 μl of the reaction mix onto phosphocellulose filters to bind phosphoproteins. Heat-treated lysates (5 minutes at 95°C) were used to evaluate the background. Incorporated radioactivity was quantified using liquid scintillation counter (LS2800, Beckman, Irvine, CA).

Western blot analysis. Proteins (25 μg) from whole cell lysates resolved through 12% polyacrylamide minigels (BioRad, Hercules, CA). Proteins were transferred to nitrocellulose filters by electroblotting overnight (14 V at 4°C). After blocking with PBS containing 5% dry milk, filters were incubated with anti-phospho-ERK1/ERK2 antibody (New England Biolabs, Beverly, MA; 1:500 dilution) for 1 hour at room temperature. Filters were washed (3× in PBS/5% dry milk) and incubated for 1 h with second antibody, alkaline phosphatase conjugated anti-rabbit IgG (Sigma, St. Louis, MO). After washing, the levels of MAPK protein were determined using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate method (BioRad). Immunoreactive bands were quantified by digital densitometric imaging (Gel Doc 1000 with Model GS-700 Densitometer and Molecular Analyst Software, BioRad).

Immunofluorescent microscopy. Luteinized granulosa cells were cultured in 8-chambered glass slides (Nunclon). The cells were treated with PD98059 and EGF as described above. Following treatment, the cells were fixed in ice-cold methanol:acetone (1:1) for 10 minutes. After washing with 50 mM Tris/150 mM NaCl, pH 7.0 (Tris-NaCl), the cells were blocked for one hour with 5% bovine serum albumin (BSA) in Tris-NaCl. The cells were then incubated with anti-phospho-ERK1/ERK2 antibody (1:500 dilution in Tris-NaCl/5% BSA) at room temperature for 30 minutes. Incubations without primary antibody were used to assess nonspecific binding. After three washes with Tris-NaCl containing 0.05% Tween-20, the cells were incubated for 30 minutes at room temperature with fluoroscein isothiocyanate (FITC) conjugated second antibody (anti-rabbit IgG; 1:1000 dilution). The cells were washed and dehydrated by passage through increasing grades of ethanol (50, 70, 80, 90 and

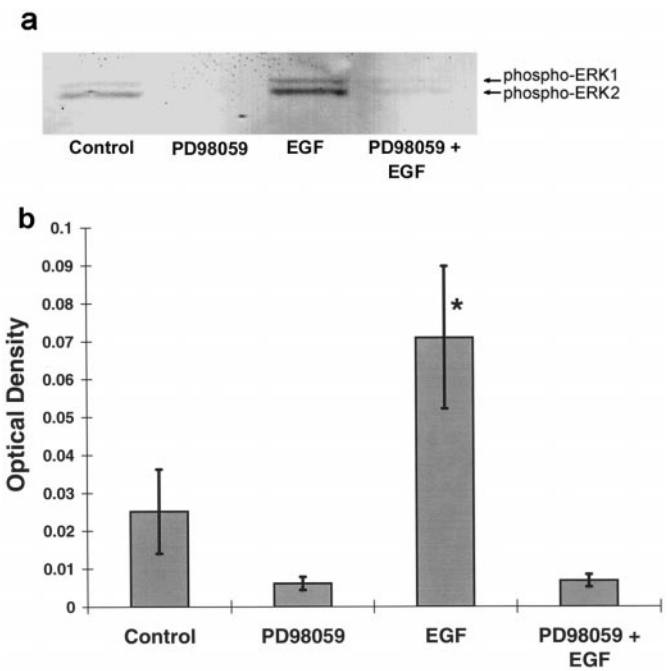

FIG. 2. PD98059 blocks EGF-stimulated phosphorylation of MAP kinase in luteinized granulosa cells. (a) Western blot analysis of cell lysates (25 μg total protein) using polyclonal antibodies anti-phospho-ERK1/ERK2 (anti-phospho-p44/p42 MAPK, New England Biolabs, Beverly, MA). Treatment groups are as described in Fig. 1. Immunoreactive bands for both activated phospho-ERK1/ERK2 are indicated. Only faint bands were visible for PD98059 and PD98059 + EGF treatments. The blot shown is representative of experiments with $n = 5$ patients. (b) Computer imaging densiometry of Western blots described above. The mean for EGF (asterisk) differs significantly from the means of the other treatments ($p < 0.05$ Student-Neuman-Keuls test). Values represent the mean relative optical density units for experiments with $n = 5$ patients. The error bars are ±SEM.

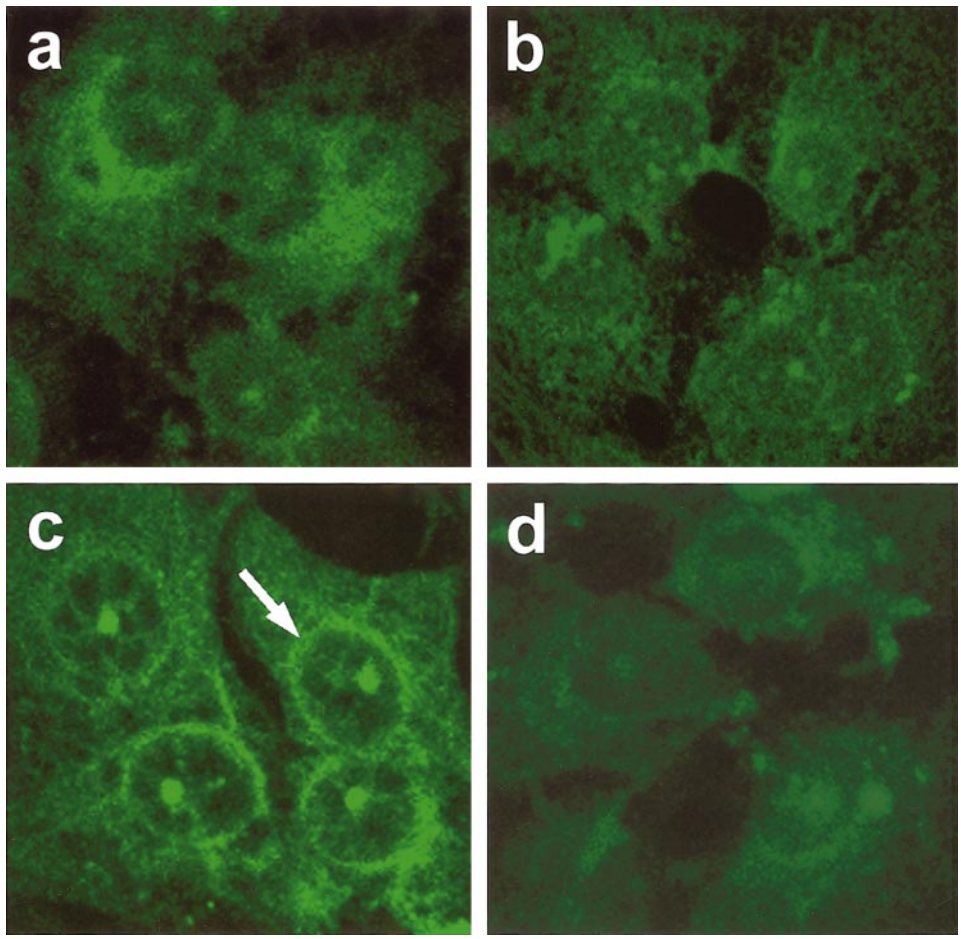

FIG. 3. PD98059 blocks nuclear translocation of activated MAP kinase in luteinized granulosa cells. Immunohistochemical confocal miscroscopy was performed as described under Materials and Methods using anti-phospho-ERK1/ERK2 antibodies. (a) Control cells treated with vehicle only. (b) Cells treated with PD98059 (100 μM) for 60 minutes. (c) Cells treated with EGF (10 ng/ml for 10 minutes). Arrow points to concentrated fluorescent staining surrounding the nuclei. (d) Cells treated with PD98059 (100 μM) for 60 minutes followed by EGF (10 ng/ml) for 10 minutes. Cells were viewed on a Bio-Rad 1000 confocal microscope under a 60× objective. Images were collected and stored using confocal microscope operating system (COMOS) software (Bio-Rad).

100%). Slides were cleared in xylene and mounted with permount. Activated ERK1/ERK2 (phospho-p44/p42 MAPK) exhibiting green fluoresence from FITC was viewed under a confocal microscope (Bio-rad 1000) using a 60× objective.

Analysis of apoptosis. Flow cytometry of isolated nuclei stained with propidium iodide (PI) was used to assess apoptotic fragmentation of DNA in luteinized granulosa cells (15). Cultured cells were treated with PD98059 or EGF for 24 hours as described before. After the treatments, cells were scraped from the plate, centrifuged at 200 × g for 5 minutes, and incubated overnight at 4°C in 500 μl lysis buffer (50 μg/ml propidium iodide (PI), 0.1% sodium citrate, 0.1% Triton X100).

Flow cytometry was performed using a Perkin-Elmer FacsCalibur instrument (Norwalk, CT). Fluorescent events (1.0×10^4) were acquired at low flow setting. Data were recorded and analyzed using Cellquest software (Perkin-Elmer).

Statistical analysis. Data were subjected to analysis of variance (ANOVA) using the CoStat package from CoHort Software (Berkeley, CA). The Student-Neuman-Keuls test of mean separation was used for Post-ANOVA analysis. Statistical significance was at the 5% probability level ($p < 0.05$).

RESULTS

Effects of EGF and PD98059 on MAP kinase activity. MAP kinase activities (mean ± SEM pmoles/hr ^{32}P incorporation) for the different experimental conditions were (1) control = 24.68 ± 7.30; (2) PD98059 = 2.68 ± 0.93; (3) EGF = 49.44 ± 12.91; (4) PD98059 + EGF = 8.67 ± 4.52 (Fig. 1). EGF treatments significantly increased MAP kinase activity. Treatment with PD98059 lowered MAP kinase activity as compared to controls and significantly suppressed the activation induced by EGF.

Phosphorylation of MAP kinase. Distinct immunoreactive bands corresponding to activated ERK1 (phospho-p44 MAPK) and activated ERK2 (phospho-p42 MAPK) were observed in Western blots (Fig. 2a). Only faint bands were visible from treatments with PD98059 alone or in combination with EGF. No other

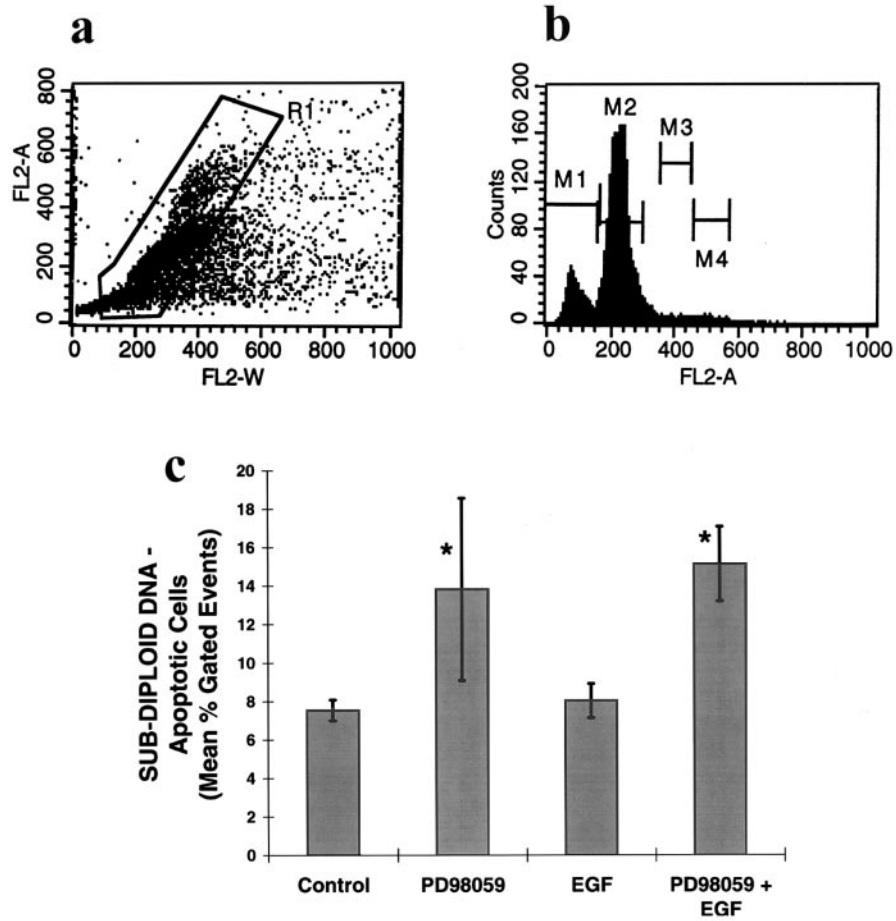

FIG. 4. Flow cytometry of PD98059 induced apoptotic subdiploid DNA in luteinized granulosa cells. Nuclei from human luteinized granulosa cells were isolated, stained with PI, and analyzed for subdiploid DNA content by flow cytometry as described under Materials and Methods. Treatment groups: Control = vehicle only, PD98059 = 100 μM PD98059 for 24 hours; EGF = 10 ng/ml EGF for 24 minutes; PD98059 + EGF = 100 μM PD98059 + 10 ng/ml EGF for 24 hours. (a) Scatterplot showing the accumulation of fluorescent events (PI-stained nuclei) detected from luteinized granulosa cells treated with PD98059. (b) Histogram analysis of the scatterplot shown. M = Marker; M1 = subdiploid DNA (fragmented or condensed chromatin); M2 = diploid DNA (Gap0/Gap1 (G0/G1) cell cycle phase); M3 = >diploid DNA (Synthesis (S) cell cycle phase); M3 = 2× diploid DNA (Gap2/Mitosis (G2/M) cell cycle phase). *X* axis = increasing DNA content. *Y* axis = accumulated fluorescent event counts (PI-stained nuclei). (c) Quantification of subdiploid DNA (M1). Values represent the mean percentage (%) of nuclei containing subdiploid DNA content for experiments with $n = 8$ patients. The error bars are ±SEM. The means for PD98059 and PD98059 + EGF (asterisks) are significantly greater ($p < 0.05$ Student-Neuman-Keuls test) than those of the other treatments.

immunoreactive bands were detected. Relative optical densities of the imuunoreactive bands as determined from computer densiometry were (1) control = 0.025 ± 0.011; (2) PD98059 = 0.006 ± 0.0017; (3) EGF = 0.071 ± 0.019; (4) PD98059 + EGF = 0.0068 ± 0.0016 (Fig. 2b). The levels of activated ERK1/ERK2 (phospho-p44/p42 MAPK) were significantly increased by EGF treatments. PD98059 abolished the increases in levels of phosphorylated MAP kinase in EGF-treated cells.

Immunocytochemical localization of activated MAP kinases. Representative imaging of immunofluorescent-labeled cells from the four treatment groups is shown in Fig. 3. Green fluorescence, indicative of phosphorylated MAP kinase, was observed in the cytoplasm and nuclei of control and EGF-treated cells. Treatments with EGF enhanced the concentration of fluorescent staining at the nuclei of luteinized granulosa cells (arrow, Fig. 3c). In contrast, PD98059 alone or in combination with EGF, reduced the levels of cytoplasmic and nuclear fluorescence (Figs. 3b and 3d). No signal was observed in the negative controls (not shown).

Induction of subdiploid apoptotic DNA fragmentation. A representative scatterplot and histogram showing flow cytometric analysis of DNA content in PI-stained nuclei is shown in Figs. 4a and 4b. The percentages of apoptotic nuclei for the specific treatment groups were (1) control = 7.55 ± 0.055%; (2) PD98059 = 13.83 ± 1.73%, (3) EGF = 8.04 ± 0.89%; (4) PD98059 + EGF = 15.15 ± 1.94%. Significantly in-

creased percentages of subdiploid DNA were detected in cells treated with PD98059 and PD98059 plus EGF (Fig. 4c).

DISCUSSION

Activation of the MAP kinase pathway in different cell types is involved in proliferation, differentiation and apoptosis (16, 17, 18). Recent biochemical and genetic analysis suggest that MAP kinases may potentiate cell survival in *D. melanogastor* and determine cell fate in *C. elegans* (19, 20). In mammalian cells, the MAP kinase pathway can prevent (21–26) or induce (27–29) apoptosis depending on the type of cell and the extracellular stimuli that initiate the pathway. We found, from the experiments reported herein, that EGF increases MAP kinase activity above control levels in cultured luteinized granulosa cells, consistent with our previous results and with those of others (9, 30). We now report that MEK inhibition abolishes the effect of EGF treatments. Treatments with PD98059 in combination with EGF resulted in decreased MAP kinase activity as measured by radiochemical assay and by Western blot analysis. PD98059 also decreased the concentration of activated MAP kinase at the nuclei of treated cells. This suggests that PD98059 suppresses EGF-stimulated MAP kinase activity in human luteinized granulosa cells. Since treatment with PD98059 alone and in combination with EGF increased the subdiploid DNA levels, we conclude that an active MAP kinase pathway is required for survival of human luteinized granulosa cells.

Our preliminary results demonstrating that EGF treatments can attenuate apoptosis caused by tyrphostin 51, an inhibitor of EGF receptor autophosphorylation, also support a direct role for EGF-mediated survival of granulosa cells (9). However, we have not observed a decline in the rate of apoptosis in cultured luteinized granulosa cell controls as a result of EGF treatments. This may reflect differences due to the species of origin, the developmental stage or the culture conditions of the cells examined. Taken together, these results suggest that apoptotic mechanisms in granulosa cells are related to but independent of the EGF-mediated MAP kinase pathway. Further studies are necessary to define the extent to which specific growth factors may utilize the MAP kinase pathways to regulate cell survival during follicular development.

The functional capacity and eventual fate of granulosa cells are influenced by a complex array of signals including the pituitary gonadotropins, steroids, prostaglandins, and peptide hormones (16). Apoptosis of granulosa cells during follicular atresia and corpus luteum regression is a crucial aspect of ovarian physiology (31–34). Our results suggest that suppression of the MAP kinase pathway may lead to apoptosis in these cells. Since abnormal regulation of granulosa cell apoptosis may contribute to the pathophysiology of some ovarian dysfunctions, a better understanding of the control of apoptosis in these cells could enable development of more effective treatment modalities for ovarian disorders (35, 36).

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