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Correlation of Mitogen-Activated Protein Kinase Activities with Cell Survival and Apoptosis in Porcine Granulosa Cells

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ABSTRACT—The regulation of granulosa cell survival and death is critical for determining the fate of ovarian follicles. Mitogen-activated protein kinases (MAPKs) play central roles in various cellular responses, but the relationship between MAPK activities and granulosa cell survival as well as death is poorly understood. The present study examines the roles of the extracellular signal-regulated kinase (ERK) and p38 MAPK activities in porcine granulosa cells in response to survival factors and oxidative stress. Cell survival and apoptosis were evaluated by Trypan blue staining, DNA fragmentation, and chromatin staining with Hoechst 33342. Cell survival induced by serum or by follicle-stimulating hormone (FSH) was inhibited when ERK activity was attenuated with PD98059, which led to the induction of apoptosis. The p38 inhibitor SB203580 significantly decreased the cell survival evoked by FSH, but not by serum. Even in the presence of 10% serum, H_2O_2 caused apoptosis, indicating that H_2O_2 may be an atretogenic factor or its mediator. Interestingly, this induction of apoptosis was also prevented by SB203580, suggesting that p38 is involved in an apoptotic pathway induced by H_2O_2 as well as in a survival pathway evoked by FSH in granulosa cells. These results indicate that whereas ERK activity is critical to the survival of granulosa cells, p38 activity contributes to their survival or apoptosis depending on the stimulus.

Keywords: granulosa cell, extracellular signal-regulated kinase, p38, apoptosis, oxidative stress

INTRODUCTION

Over 99% of ovarian follicles in mammals undergo atretic degeneration characterized by granulosa cell apoptosis. The selection of whether granulosa cells undergo apoptosis or survive is a critical process in determining the fate of follicles. A number of endocrine and paracrine factors are known as survival or apoptosis-inducing factors for granulosa cells *in vivo* and *in vitro* (Amsterdam and Selvaraj, 1997; McGee and Hsueh, 2000), but the molecular mechanisms underlying the functions of these factors in these cells are poorly understood.

Mitogen-activated protein kinases (MAPKs) are important enzymes that are involved in key biological functions such as cell proliferation, cell differentiation, cell survival and death (Chang and Karin, 2001; Pearson *et al*., 2001; Wilkinson and Millar, 2000). Mammals express at least four distinctly regulated groups of MAPKs, extracellular signal-regulated kinases (ERK)1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38α/β/γ/δ), and ERK5. Among these MAPKs, the biological roles of p38 and ERK have been extensively studied in various cell types because of the presence of specific inhibitors for p38 and MAP kinase kinase 1/2 (MEK1/2), a direct activator of ERK. The results have shown that ERK acts primarily to positively regulate cell survival and proliferation, whereas p38 has apparently opposite actions, depending on the types of cells and/or stimulation. For example, the activation of p38 and JNK with concurrent inhibition of ERK is critical for the apoptosis of PC12 pheochromocytoma cells induced by withdrawal of nerve growth factor (Xia *et al*., 1995). In contrast, p38β mediates hypertrophic response in rat cardiac myocytes (Wang *et al*., 1998b). These findings suggest that the determination of the survival and apoptosis of granulosa cells is also critically regulated by ERK and p38. However, there are few reports, including ours, indicating the activation of ERK and p38 in these cells (Babu *et al*., 2000; Cameron *et al*., 1996; Das *et al*., 1996: Gebauer *et al*., 1999; Kotani *et al*., 1999; Maizels *et al*., 1998). Moreover, no direct evidence has confirmed the involvement of these two kinases in the

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processes of survival and apoptosis of primary cultured granulosa cells.

The present study examines the roles of ERK and p38 in porcine cultured granulosa cells in response to survival factors and oxidative stress. We focused on follicle-stimulating hormone (FSH) and serum as survival factors. FSH is a well-known critical factor for follicular growth (Chun *et al.*, 1996; Dierich *et al.*, 1998). Serum containing many humoral factors such as growth factors and cytokines is also considered important for folliculogenesis as the influx of serum components is promoted by vascularization for the maintenance and maturation of healthy follicles. On the other hand, we used H_2O_2 to induce apoptosis in granulosa cells because of the following reasons. Firstly, accumulating evidence indicates that H_2O_2 causes apoptosis in many cell types (Mates and Sanchez-Jimenez, 2000). Ovarian corpus luteal cells also undergo apoptosis in response to H_2O_2 (Nakamura and Sakamoto, 2001). Secondly, recent studies have demonstrated that the stimulation of Fas produces reactive oxygen species (ROS) such as H_2O_2 (Jayanthi *et al.*, 1999). Several laboratories have described the expression of Fas and Fas ligand in granulosa cells (Hakuno *et al.*, 1996; Kim *et al.*, 1999). Thirdly, granulosa cell apoptosis may be augmented with a decrease in the number of ovulations in rats in response to heat stress (Shimizu *et al.*, 2000), which is thought to produce ROS due to exaggerated respiration like exercise. Thus, it is very likely that ROS induce granulosa cell apoptosis under physiological and pathological conditions.

MATERIALS AND METHODS

Reagents

Ovine FSH (NIDDK-oFSH-20) was kindly provided by the National Institutes of Health (Bethesda, MD, USA). The biological potency of this compound was 175-fold greater than that of NIH-FSH-S1. We converted the weight of NIDDK-oFSH-20 into that of NIDDK-oFSH-15, the potency of which is 20-fold greater than that of NIH-FSH-S1. NIDDK-oFSH-15 has been widely applied in many studies of the ovary including ours (Kotani *et al.*, 1999). Fetal bovine serum, L-glutamine, and the antibiotics, penicillin and streptomycin, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). McCoy's 5A medium was obtained from GIBCO Life Technologies. PD98059, an inhibitor of the ERK kinase MEK1, and SB203580, a p38 inhibitor, were obtained from Calbiochem (La Jolla, CA, USA). Anti-phospho-ERK1/2, anti-phospho-p38, anti-phospho-MAPK-activated protein kinase 2 (MAPKAPK2), and anti-cleaved caspase-3 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Hoechst 33342 was from Wako (Osaka, Japan).

Cell culture

Ovaries were obtained from prepubertal pigs slaughtered at a local abattoir. Granulosa cells prepared according to the method of Shen *et al.* (Shen *et al.*, 1998) were plated on dishes 60-mm diameter (4×10^6 cells/dish) and cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin for 24 hr in a CO_2 incubator. After removing dead cells by two washes of phosphate-buffered saline, cells were incubated with or without stimulants and inhibitors in the above medium in the presence or absence of serum for 12 hr to investigate their survival and death. Cells were incubated in medium containing 0.3% serum for 6 hr before adding stimulants to detect MAPK activation.

Stimulation and harvesting of cells

Cells serum-starved for 6 hr were stimulated with various agents for the indicated periods, then harvested in ice-cold lysis buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM Na_3VO_4, 100 mM NaF, 1 mM PMSF, 10 mM sodium pyrophosphate, 5 μg/ml aprotinin, 10 μg/ml leupeptin). After centrifugation of cell lysates at 14,500 rpm for 10 min at 4°C, the supernatant was assayed for protein content and subjected to a Western-blot analysis.

Detection of ERK, p38, caspase-3, and MAPK-activated protein kinase 2

Cell lysates (40 μg of protein) were fractionated by SDS-PAGE on 10% (w/v) acrylamide gels under reducing conditions, then proteins were electrophoretically transferred in a semidry unit to polyvinylidine difluoride (PVDF) membranes as described (Ausubel *et al.*, 1992). The bands of activated ERK1/2, p38, caspase-3, and MAPKAPK2 were detected using antibodies that specifically recognized their activated forms. That is, after blocking by a 1-hr incubation with 5% skim milk for ERK1/2, p38, and caspase-3 and 5% BSA for MAPKAPK2 in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.1% Tween 20 (TBST), the membranes were incubated with the relevant primary antibodies in the same buffer for 1 hr. The membranes were washed in TBST three times and further incubated with horseradish peroxidase-linked second antibodies (1:1000). After three more washes, proteins were visualized using an enhanced chemiluminescence kit (Roche). All immunoblotting procedures proceeded at room temperature. Bound antibodies were stripped for subsequent analysis by boiling for 5 min.

Assessment of cell viability

After culture under various conditions, detached cells were collected, then adsorbed cells were harvested by trypsin treatment. Thereafter, all cells including detached cells were counted by Trypan blue staining. Cell viability is expressed as the percentage of surviving cells compared with the total number of cells.

DNA fragmentation analysis

To detect apoptotic DNA fragmentation, total DNA was extracted from cultured cells (4×10^6 cells/dish) and resolved through 2% (w/v) agarose gels, stained with SYBR Green I nucleic acid gel stain (Wako, Osaka, Japan), and visualized under UV light using LAS1000 (Fuji Film, Tokyo, Japan).

Cytochemical staining

Apoptotic cell death was evaluated after staining with Hoechst 33342 followed by fluorescence microscopy. Cells cultured in wells of collagen-coated 8-well Lab-Tek-II chamber slides (Nunc A/S, Poskilde, Denmark) at a concentration of 2×10^5 cells/well were fixed in 4% (w/v) paraformaldehyde at 4°C for 10 min. After washing with TBS, cells were permealized with 0.3% (v/v) Triton X-100 in TBS for 10 min. After washing with TBS, cells were stained with 1.6 μM Hoechst 33342 for 10 min.

Data analysis

Data are expressed as means±SE of several independent experiments. The effects of various treatments on different culture groups were compared by one-way ANOVA, followed by the post hoc Student-Newman-Keuls test. A difference of $P<0.05$ was considered significant.

RESULTS

Activation of ERK and p38 by FSH, serum, and H_2O_2

We initially examined whether or not FSH, serum, and H_2O_2 regulate the activities of ERK1/2 and p38 in porcine cultured granulosa cells. ERK1/2 and p38 activities were detected by Western blotting using antibodies that specifically recognize the activated forms of these enzymes as shown in Fig. 1. Basal activities of these kinases were decreased by maintaining the cells in medium containing 0.3% serum for 6 hr. Thereafter, the cells were stimulated for the indicated periods. FSH at 200 ng/ml significantly activated both ERK1/2 and p38, which was consistent with findings in rat and porcine granulosa cells (Babu *et al.* 2000; Cameron *et al.* 1996; Das *et al*., 1996: Gebauer *et al*., 1999; Kotani *et al*., 1999; Maizels *et al*., 1998). Serum (10%) and H_2O_2 at 100 μM also increased ERK1/2 and p38 activities within two minutes. Bands of α-tubulin confirmed approximately equal loading for all lanes. Therefore, the activities of ERK1/2 and p38 appear to be involved in the responses of these cells to FSH, serum, and H_2O_2.

Effect of MEK inhibitor and p38 inhibitor on FSH-induced cellular response

To examine the roles of ERK1/2 and p38 in granulosa cells, we used PD98059, which inhibits the ERK1/2 kinase MEK1, and SB203580, which inhibits p38. We initially evaluated the effects of these inhibitors in our experimental conditions. As shown in Fig. 2, 50 μM PD98059 suppressed FSH-, serum-, or H_2O_2-dependent increases in ERK1/2

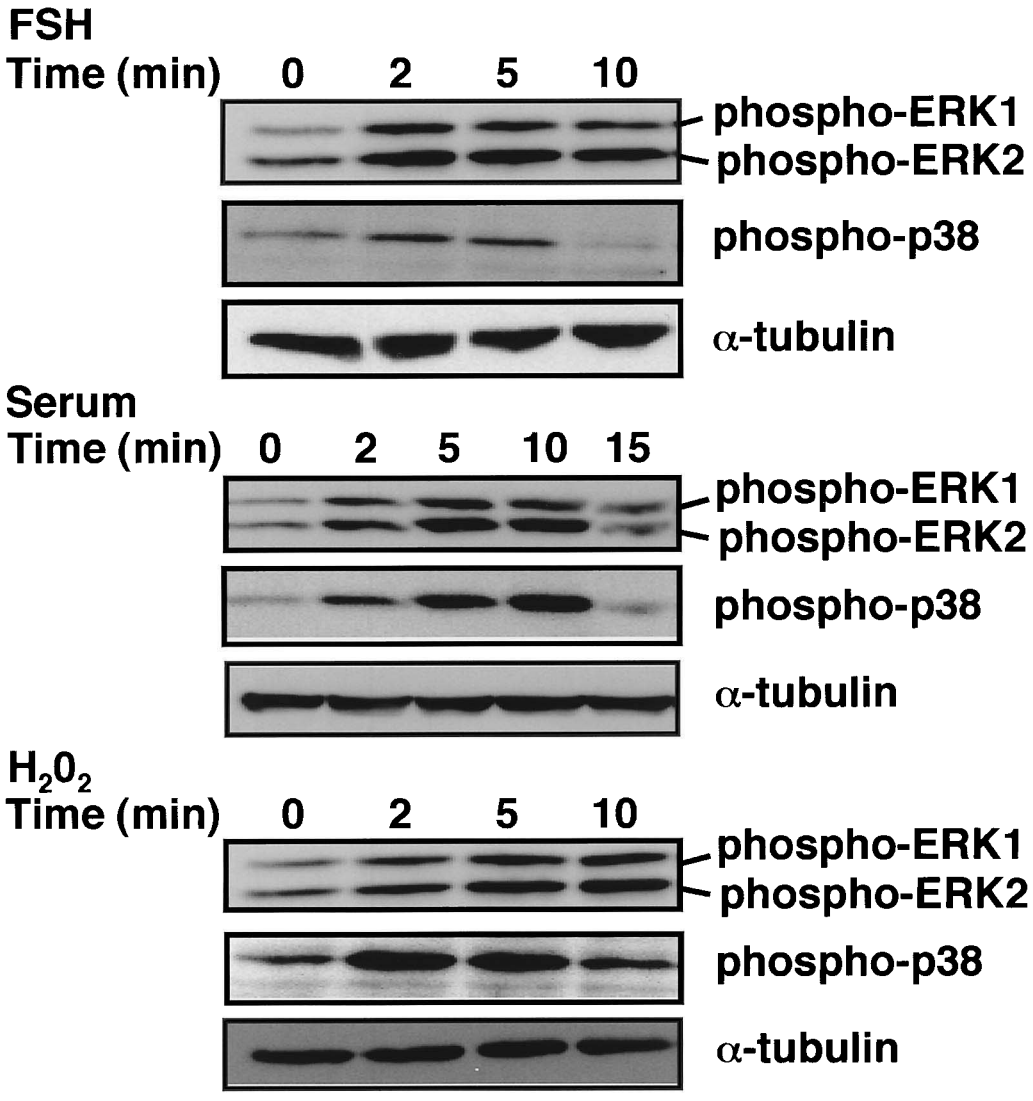

Fig. 1. Activation of ERK1/2 and p38 in response to FSH, serum, and H_2O_2. Cells cultured in medium containing 0.3% serum for 6 hr were stimulated with 200 ng/ml FSH, 10% serum, or 100 μM H_2O_2 for the indicated periods. Thereafter, cell lysates (40 μg of protein) were loaded on 10% SDS-PAGE and immunoblotted using phospho-specific anti-ERK1/2, phospho-specific anti-p38, and anti-α-tubulin antibodies as described in "Materials and Methods". The bands of α-tubulin are indicated as internal control. The same membrane was reprobed in each panel. Data represent three separate experiments. Similar results were obtained in two other experiments.

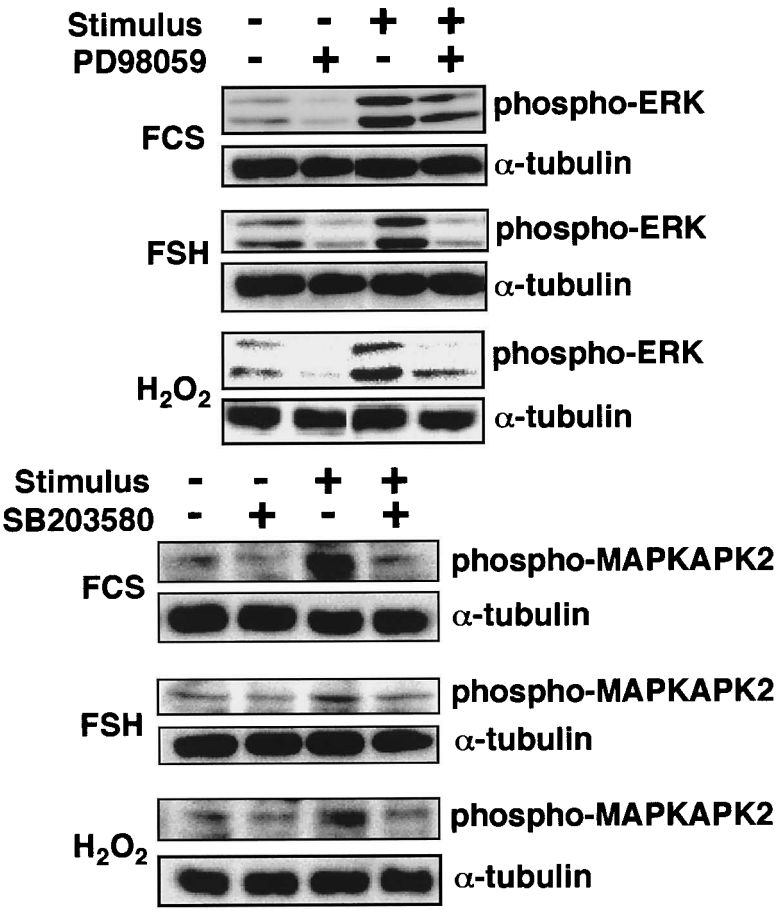

Fig. 2. Effectiveness of PD98059 and SB203580.Cells cultured in medium containing 0.3% serum for 6 hr were stimulated with 200 ng/ml FSH, 10% serum, or 100 μM H_2O_2 in the presence and absence of 50 μM PD98059 or 20 μM SB203580 for 5 min. The inhibitors were added to the medium 30 min prior to the stimulation. Thereafter, the ERK1/2 and MAPKAPK2 activities were assessed by Western blotting using phospho-specific ERK1/2 and phospho-specific MAPKAPK2 antibodies. The same membrane was stripped and reprobed with an anti-α-tubulin antibody. Data represent two separate experiments that yielded similar results.

activities (upper panel). As for SB203580, we assessed the activation of the main p38 substrate MAPKAPK2, using an antibody that specifically recognizes its phosphorylated, activated form. Expectedly, SB203580 at 20 μM significantly inhibited FSH-, serum-, or H_2O_2-induced increase in the phosphorylation level of MAPKAPK2 (lower panel). Thus, PD98059 and SB203580 effectively blocked the ERK1/2 and p38 pathways, respectively, in our experimental conditions.

We examined the roles of ERK1/2 and p38 in FSH-stimulated granulosa cells incubated for 12 hr in the presence or absence of FSH with or without these MAPK cascade inhibitors, using Trypan blue exclusion to measure survival. Whereas only 15% of cells were viable in its absence, FSH at 200 ng/ml increased cell survival to 65% as shown in Fig. 3A. The MEK1 inhibitor PD98059 at 50 μM or the p38 inhibitor SB203580 at 20 μM reduced this value to approximately 25% in the presence of FSH. Neither PD98059 nor SB203580 significantly affected the viability of unstimulated cells (data not shown). Together with the finding that FSH activates ERK1/2 and p38, these data demonstrate that both MAPKs are critically implicated in FSH-induced granulosa cell survival.

To investigate whether this reduction in cell viability was due to apoptosis, DNA prepared from cells was resolved by electrophoresis to detect DNA fragmentation, a biochemical characteristic of apoptosis. Fig. 3B shows that oligonucleosomal length DNA fragments formed an obvious ladder in the absence of FSH, whereas the fragmentation was moderately suppressed by FSH. Either PD98059 or SB203580 augmented DNA degradation in medium containing FSH. Apoptosis was also morphologically detected by staining the cells with Hoechst 33342, which binds to chromatin. Fig. 3C indicates the morphological changes typical of apoptosis, namely chromatin condensation and nuclear cleavage, in cells without FSH (panel a) and in those cultured with either PD98059 (panel c) or SB203580 (panel d) in the presence of FSH. In contrast, most of cells cultured with FSH alone (panel b) exhibited the normal shape of living cells. These findings agreed well with the data shown in Fig. 3A, and further suggested that ERK1/2 and p38 activities prevent granulosa cells from undergoing apoptosis and mediate the sur-

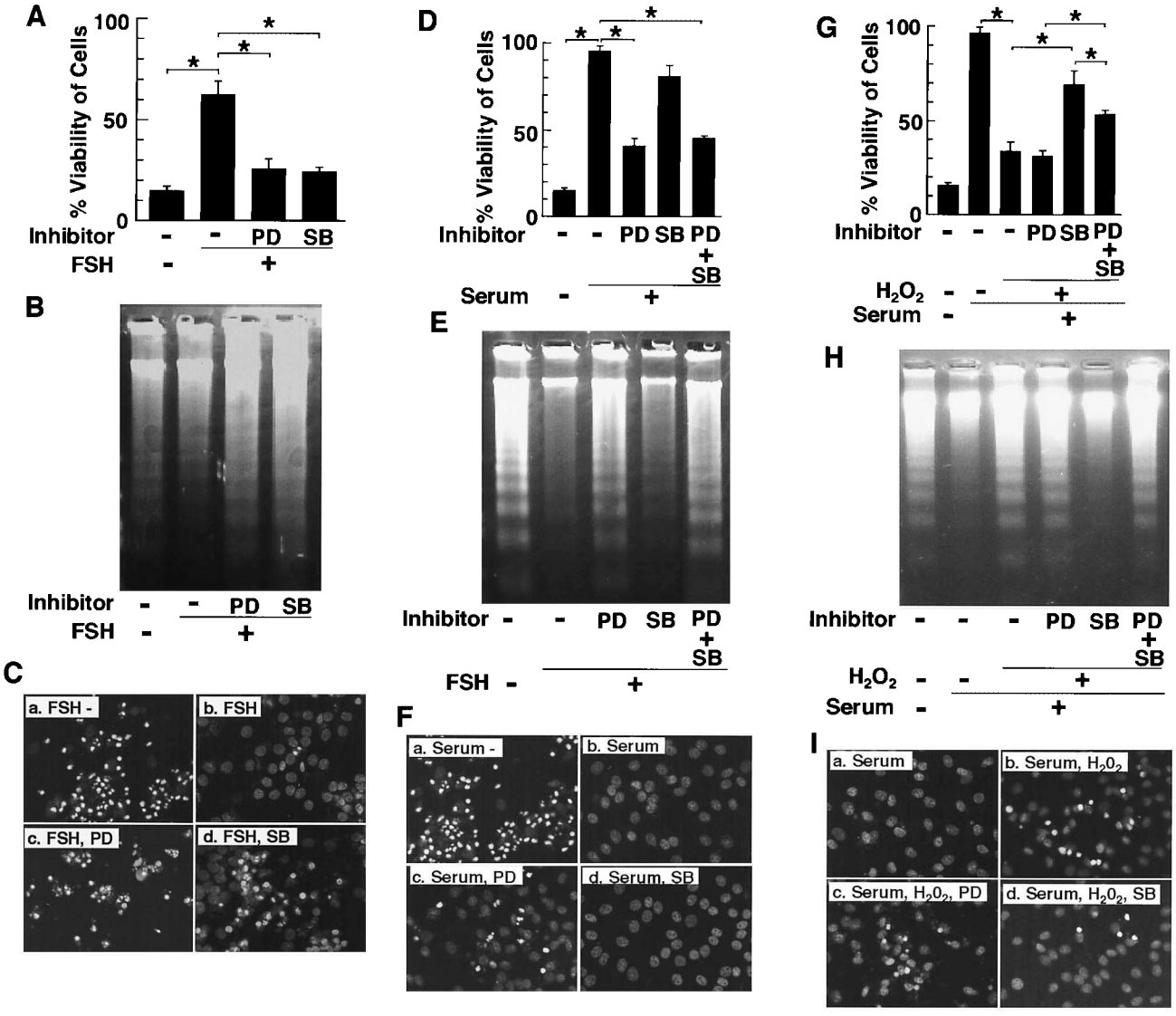

Fig. 3. Effect of PD98059 and SB203580 on FSH- or serum-dependent survival and H_2O_2-induced apoptosis of granulosa cells. Cells were cultured in the presence and absence of FSH (A–C) or serum (D–F) with and without PD98059 (PD) or SB203580 (SB) for 12 hr. Cells were also incubated in the presence and absence of 100 μM H_2O_2 with and without indicated reagents for 12 hr (G–I). Concentrations of FSH, serum, PD98059, and SB203580 were 200 ng/ml, 10%, 50 μM, and 20 μM, respectively. A, D, and G indicate cell viability. Both detached and adsorbed cells on dishes were collected and counted, respectively, by staining with Trypan blue. Cell viability is expressed as percentage of surviving, among total cells. Data are the means ± SE (vertical bars) of three independent experiments, each performed in duplicate. * indicates probability < 0.05. B, E, and H indicate DNA fragmentation. DNA fragmentation was analyzed as described in "Materials and Methods". C, F, and I show cytochemical staining of cells with Hoechst 33342.

vival promoted by FSH.

Effect of MEK inhibitor and p38 inhibitor on serum-induced cellular response

The effect of serum on cell viability and the effect of the MAPK cascade inhibitors on the serum-induced cellular response were investigated under conditions identical to those in which FSH promoted survival. As shown in Fig. 3D, serum increased cell viability from only 15% in its absence to over 95%. PD98059 at 50 μM diminished this value to 42%. In contrast, 20 μM SB203580 had little effect. We consider that the viability of SB203580-treated cells was underestimated. This is because cell attachment became tight by SB203580 treatment and this tight attachment prevented complete removal of cells from culture dishes for counting. Thus, we concluded that SB203580 had no effect on serum-dependent cell survival. Both PD98059 and SB203580 had no additional effect (46% viability) compared with that of PD98059 alone (42% viability). The viability of cells incubated with PD98059 alone or PD98059 and SB203580 in medium containing serum did not fall to that in serum-free medium (15%). Together, these data suggested that ERK1/2, but not p38, are implicated in serum-induced granulosa cell survival and that an ERK1/2-independent survival path-

way(s) also functions.

We confirmed biochemically and morphologically that the reduced cell survival was due to apoptosis. Fig. 3E shows that serum suppressed DNA fragmentation. PD98059 alone and together with SB203580 largely blocked this effect of serum. In contrast, SB203580 was without effect. Fig. 3F indicates the results of the morphological analysis. PD98059 (panel c), but not SB203580 (panel d) caused chromatin condensation and nuclear cleavage in serum-containing medium. Serum alone caused no such morphological changes (panel b). Therefore, these biochemical and morphological data are in close agreement with the findings obtained by Trypan blue staining in Fig. 3D.

Effect of MEK inhibitor and p38 inhibitor on H_2O_2-induced cellular response

We investigated the effect of H_2O_2 on serum-dependent granulosa cell survival and the effect of the MAPK cascade inhibitors on H_2O_2-elicited response under identical conditions in which FSH and serum exerted effects. As shown in Fig. 3G, cell viability in serum-containing medium (96%) was reduced to 33% by adding 100 μM H_2O_2. PD98059 at 50 μM had no significant effect on this H_2O_2-induced reduction in cell survival, but 20 μM SB203580 recovered the viability to 70%. The mixture of both inhibitors also increased cell viability to 52%, a value that lay in the middle of those caused by the presence (70%) and absence of SB203580 (33%). Therefore, H_2O_2 appears to inhibit granulosa cell survival even in the presence of serum in a p38-dependent manner.

Figs. 3H and 3I biochemically and morphologically supported the data of Fig. 3G. In Fig. 3H, H_2O_2 dramatically caused DNA fragmentation despite the presence of 10% serum. SB203580, but not PD98059, effectively blocked this DNA fragmentation. Fig. 3I shows the typical morphological characteristics of apoptosis in many cells treated with H_2O_2 (panel b). The numbers of these apoptotic cells were reduced by SB203580 (panel d), but not PD98059 (panel c). Together, these data indicated that H_2O_2 induces granulosa cell apoptosis and that the activity of p38, but not of ERK, is critically involved in this process.

Caspase-3 activation in H_2O_2-induced cellular response

Since a central step in the execution of apoptosis is the activation of a class of cysteine proteases, termed caspases (Nakamura and Sakamoto, 2001; Pearson *et al.*, 2001), we examined whether H_2O_2 caused caspase activation in granulosa cells. Especially, we focused on caspase-3 because this enzyme is involved in both mitochondoria-dependent and -independent apoptotic pathways. The activation of caspase-3 was assessed by Western blot with an antibody specifically recognizing its cleaved, activated form. Fig. 4 shows an intense band of activated caspase-3 in the absence of serum that was almost totally absent in the presence of serum. H_2O_2 generated the band in cells incubated in medium containing serum. The density of this band was decreased by SB203580, but not by PD98059. These data

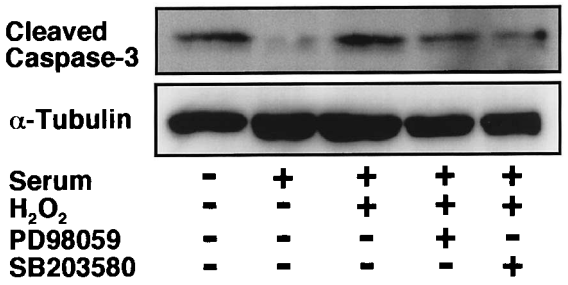

Fig. 4. Correlation of caspase-3 activity with progression of apoptosis. Cells were cultured in the presence or absence of 10% serum with or without indicated reagents for 12 hr. Concentrations of H_2O_2, PD98059, and SB203580 were 100 μM, 50 μM, and 20 μM, respectively. Thereafter, cell lysates were prepared and activated caspase-3 was detected using an anti-cleaved caspase-3 antibody as described in "Materials and Methods". Bands of α-tubulin were shown as internal controls in the same membrane. A representative data is shown from three separate experiments.

are consistent with the results of Fig. 3G.

DISCUSSION

The present study generated three key findings as follows. First, the FSH-induced survival of granulosa cells is predominantly dependent on both the ERK and p38 pathways. Second, ERK, but not p38, is involved in the serum-dependent survival of these cells. Third, H_2O_2 induces granulosa cell apoptosis that is mediated by the p38 pathway. These findings strongly suggest that ERK and p38 activities are critical for determining the survival or apoptosis of granulosa cells, which is closely associated with the fate of ovarian follicles.

Several reports indicated that FSH elicits ERK activation in rat and porcine primary cultured granulosa cells (Cameron *et al.*, 1996; Das *et al.*, 1996; Kotani *et al.*, 1999). However, none of them showed direct evidence for the involvement of ERK in regulating granulosa cell survival or apoptosis. Gebauer *et al.* (1999), using a model system based on granulosa cells from the equine chorionic gonadotropin-primed immature rats, have recently revealed that activities of ERK and the MEK kinase Raf-1 were reduced with a concomitant decrease in the phosphorylation level of the pro-apoptotic factor Bad prior to the onset of granulosa cell apoptosis. This finding suggests that the attenuation of Raf-1-MEK-ERK pathway may trigger the onset of apoptosis by decreasing phosphorylated Bad in these cells, which agrees well with our finding that ERK plays an important role in FSH- and serum-induced granulosa cell survival. Babu *et al.* (2000) have demonstrated that FSH activated ERK and the inhibition of ERK activity with PD98059 markedly reduced FSH-induced DNA synthesis in immortalized granulosa cells overexpressing the recombinant novel growth factor type 1 receptor for FSH. Although these results must be confirmed in primary cultured cells, they suggest the contribution of ERK activity for FSH-elicited proliferation of granulosa cells. More recently, Seger *et al.* (2001) have indi-

cated that ERK down-regulated gonadotropin-stimulated progesterone production in granulosa-derived cell lines expressing recombinant gonadotropin receptors. Therefore, ERK appears to be involved in not only survival but also in other critical processes closely related to folliculogenesis such as proliferation and steroidogenesis in these cells.

In addition to the significance of ERK, several reports have demonstrated the involvement of phopshatidylinositol 3-kinase (PI3K)/Akt pathway in granulosa cell survival (Asselin *et al*., 2001; Johnson *et al*., 2001; Westfall *et al*., 2000). Insulin-like growth factor (IGF-I) activated the PI3K/Akt pathway and inhibition of this pathway induced apoptosis in porcine and hen granulosa cells (Johnson *et al*., 2001; Westfall *et al*., 2000). In contrast, apoptosis induced by the PI3K inhibitor LY294002 was blocked by leutinizing hormone or 8-bromo cAMP (Johnson *et al*., 2001), suggesting that the PI3K/Akt pathway is not implicated in the cAMP-dependent survival of these cells. Consistent with this data, FSH, which induces cAMP production, did not activate Akt under our experimental conditions (data not shown). Thus, involvement of the PI3K/Akt pathway in granulosa cell survival may be dependent on the stimulus. On the other hand, inhibitors of the ERK pathway did not affect the survival of hen granulosa cells evoked by IGF-I and TGF-α whereas these growth factors activated ERK (Johnson *et al*., 2001). The significance of ERK activities found in the present study is not in agreement with these phenomena. One explanation for this discrepancy may be that receptor tyrosine kinases for growth factors promote granulosa cell survival through an ERK-independent pathway(s).

In contrast to ERK, the implication of p38 in FSH-induced cell rounding/aggregation was its only known function in granulosa cells (Maizels *et al*., 1998). Thus, the present study is the first report to describe the relationship of the p38 pathway with granulosa cell survival and apoptosis. In result, interestingly, p38 was found to mediate apoptosis as well as survival in these cells. Although the mechanisms remain unclear, p38 apparently has opposite actions, depending on the types of cells and/or the stimuli. Wang *et al.* (1998b) have recently demonstrated that p38β mediated the hypertrophic response, whereas p38α contributed to an apoptotic process in rat cardiac muscle cells. Juo *et al.* (1997) have also indicated that p38α was implicated as part of the Fas-induced apoptotic pathway involving caspases in T lymphocytes. These reports raise the notion that in granulosa cells, p38α and β may be respectively involved in H_2O_2-induced apoptosis and FSH-elicited survival. However, the activities of p38α and β cannot be separately detected, because SB203580 inhibits the activities of both of them, and p38 isoform-specific antibodies are not yet available. We plan to examine the relation between p38 isoforms and cellular responses of the survival or apoptosis, by over-expressing dominant negative types of p38α and β using adenovirus vectors. The usefulness of this method has been substantiated for identifying differences in the roles of p38α and β in cardiac myocytes (Wang *et al*., 1998b) and indicated in the high efficient introduction of recombinant genes by adenovirus vectors into granulosa cells (Somers *et al*., 1999).

Reactive oxygen species (ROS) stimulate growth-related pathways such as cell proliferation and hypertrophy in some cells, and contribute to apoptotic processes in others. The present study found that H_2O_2 causes granulosa cell apoptosis even in the presence of 10% serum, suggesting that H_2O_2 may be an atretogenic factor or its mediator. It is known that granulosa cells undergo apoptosis, at least in part, via the Fas-Fas ligand pathway (Hakuno *et al*., 1996; Kim *et al*., 1999) and that in this death signaling pathway, H_2O_2 is produced as a functional mediator (Jayanthi *et al*., 1999). Therefore, H_2O_2 may act as an intracellular mediator for Fas function in granulosa cells under normal physiological conditions. Another possibility is that H_2O_2 itself may initially cause the apoptosis of granulosa cells as an atretogenic factor. Environmental heat stress decreases fertility of cows and goats in a subtropical climate. Shimizu *et al.* (2000) have suggested that heat stress attenuated the number of ovulations by enhancing granulosa cell apoptosis in the rat ovary. These findings raise the notion that exaggerated respiration due to heat stress may produce ROS such as H_2O_2 leading to granulosa cell apoptosis. Although the production of ROS in heat-stressed animals is not supported by direct evidence, ROS may function as an atretogenic factor under specific conditions such as excessive heat or demanding exercise.

In our study, H_2O_2 stimulated both ERK and p38 activation, resulting in the induction of apoptosis. Under many conditions of stress leading to apoptosis, ERK activity is suppressed with a concomitant increase in the activities of the stress-activated MAP kinases, JNK and p38, suggesting that the balance between stress-activated MAP kinase and ERK activities is a key factor in regulating apoptosis. Thus, our finding that H_2O_2 stimulated both ERK and p38 activities does not support this notion. However, other groups have reported data similar to ours with respect to apoptosis caused by H_2O_2 (Guyton *et al*., 1996; Wang *et al*., 1998a). In these reports, H_2O_2 induced apoptosis accompanied by activation of ERK, JNK, and p38, and this apoptosis was augmented or diminished by inhibiting or enhancing ERK activity, respectively. Therefore, ERK is considered to play a critical role in cell survival following oxidant injury. Consistent with this idea, we found that PD98059 leading to ERK inhibition enhanced the reduced viability of granulosa cells treated with H_2O_2 in the presence of SB203580 (Fig. 3G).

Although many factors downstream of p38 lead to cell survival (Chang and Karin, 2001; Pearson *et al*., 2001; Wilkinson and Millar, 2000), the small heat shock protein Hsp27 may be involved in the process of p38-mediated granulosa cell survival dependent on FSH. Recently, Bruey *et al.* (2000) have revealed that Hsp27 binds to cytochrome c released from the mitochondoria into the cytosol and blocks the cytochrome-c-mediated interaction of Apaf-1 with procaspase-9, which prevents the mitochondrial pathway of

caspase-dependent cell death in the leukaemic cell line U937. Since ROS such as H_2O_2 generated in the mitochondria have been proposed as early events in the induction of apoptosis, H_2O_2 may liberate cytochrome c from the mitochondria in granulosa cells. In fact, FSH induces Hsp27 phosphorylation through p38 activation, leading to cell rounding/aggregation in these cells (Maizels *et al*., 1998). Therefore, p38 activation may prevent H_2O_2-caused apoptosis in granulosa cells as it does in U937 cells (Bruey *et al*., 2000).

The MAP kinase cascade plays central roles in various cellular responses. A vast number of extracellular stimuli are integrated into this kinase cascade and in reverse, the cascade affects many of the signaling pathways induced by extracellular stimuli. Therefore, our findings regarding the correlation of ERK and p38 activities with granulosa cell survival and apoptosis will provide a basis for understanding the roles and molecular mechanisms of various extracellular stimuli controlling the fate of follicles.

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