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ARTICLES

Apoptosis of Ovarian Granulosa Cells: Correlation With the Reduced Activity of ERK-Signaling Module

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Abstract Apoptosis of the ovarian granulosa cells plays a crucial role in the determination of the number of follicles destined to ovulate in each reproductive cycle. While the activation of specific apoptotic pathway or the inactivation of cell survival pathway can initiate apoptosis, the signaling mechanism(s) involved in initiating the onset of apoptosis in granulosa cells is not fully understood. In the present study, using granulosa cells derived from eCG-primed immature rats, we investigated the temporal signaling events involved in the onset of apoptosis in the granulosa cells. The administration of 15 IU of eCG to 21-day-old immature female rats stimulate the growth and development of ovarian follicles until 72 h, after which the granulosa cells of the ovarian follicles undergo apoptosis due to the waning levels of tropic hormonal support. An analysis of the signaling events leading to apoptosis indicates that the DNA fragmentation can be seen in these cells from 96 h. A small increase in the levels of the pro-apoptotic factor Bax can be seen from 96 h while an increase in the activity of JNK can be seen from 108 h onwards. By contrast, a reduction in ERK signaling can be seen by 48 h. Similar reduction in Raf-1 kinase activity can be discerned from 48 h onwards. A concomitant decrease in the phosphorylated form of Bad can also be detected. These findings taken together, suggest that the loss of tropic hormone support is translated into the attenuation of Raf-1-MEK-ERK signaling pathway and this reduction along with a reduction in the levels of phosphorylated form of Bad triggers the onset of apoptosis in the ovarian granulosa cells. J. Cell. Biochem. 75:547–554, 1999.

Key words: apoptosis; granulosa cells; ERK; Raf; JNK; p38MAPK; Bcl-2; Bax; Bad

Apoptosis or programmed cell death determines the life span of eukaryotic cells [Nagata, 1997; Jarpe et al., 1998]. Cells initiate apoptosis in response to various apoptotic stimuli [Jarpe et al., 1998; Ashkenazi and Dixit, 1998]. Apoptosis has been also known to play a critical role in cell differentiation and developmental programs [Sanders and Wride, 1995]. However, the precise temporal and molecular events involved in these developmentally regulated apoptotic pathways have remained largely unknown. To define the molecular and temporal sequence of apoptotic events that occur during normal differentiative and developmental programs in higher vertebrates, we investigated the apoptotic pathways involved in the regulation of growth and apoptosis of rat ovarian follicles.

Apoptosis plays a major role in regulating the reproductive cycle of mammalian ovaries [Hsueh et al., 1996; Kaipia and Hsueh, 1997]. During each reproductive cycle, gonadotropic hormones stimulate the growth of numerous immature ovarian follicles. However, excepting a selected few (the number of which is more or less specific to each species), most of these follicles undergo apoptosis known as follicular atresia. Recent studies have indicated that the follicular atresia is mediated through the initiation of apoptosis of the follicular granulosa cells [Hughes and Gorospe, 1991; Hakuno et al., 1996; Tilly et al., 1995; Johnson et al., 1997;

Abbreviations used: eCG, equine chorionic gonadotropin; ERK, Extracellular-signal regulated kinase; JNK, Jun N-terminal kinase; MAPK, mitogen activated protein kinase; MEK, MAPK/ERK-kinase; MEKK, MEK-kinase.

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Maravei et al., 1997]. It is significant to note that over the course of the reproductive life span, more than 90% of ovarian follicles undergo follicular apoptosis. In humans, less than 400 of the more than 400,000 follicles found at puberty will eventually ovulate whereas the overwhelming majority of follicles undergo apoptosis [Hsueh et al., 1996; Kaipia and Hsueh, 1997]. Although follicular apoptosis plays a critical role during the recruitment of follicles for ovulation, the exact mechanism of this process is unknown other than the morphological changes that have been observed during this process [Dhanasekaran and Moudgal, 1989a; Hsueh et al., 1996; Kaipia and Hsueh, 1997]. This is in part, due to difficulties involved in studying the individual follicles, each of which may have unique developmental fate. This is further complicated by the availability of only a limited number of cells that can be obtained from these follicles for in-depth biochemical analysis. Using a model system based on the granulosa cells from the equine chorionic gonadotropin (eCG)-primed immature rats, here we present our studies defining a possible role played by extracellular-signal regulated kinase (ERKs) in the regulation of ovarian follicular growth and apoptosis.

MATERIALS AND METHODS

Granulosa Cell Isolation

The experiments described in this paper were performed in adherence with the National Institutes of Health guidelines on the use of experimental animals. Approval was obtained from the Temple University Animal Care and Use Committee prior to the initiation of this experiment. Immature (21-day-old) female Sprague-Dawley rats, obtained from Charles-River (Wilmington, MA) were housed in environmentally controlled rooms with food and water ad libitum. The rats were kept under observation for a few days and this gave them time to acclimatize to their new surroundings and handling procedures. Forty-seven rats (TRT) received 15 IU (in 100 µl of saline) of eCG (Professional Compounding Centers of America, Inc. Houston, TX) and 15 rats (CONT) received 100 µl of saline. The dose of eCG used has been shown to stimulate the growth and development of ovarian follicles for 2–3 days, after which the follicles undergo atresia because of the waning levels of tropic support caused by metabolism of gonadotropin. The injections were given subcutaneously. Twelve and three CONT animals were sacrificed at time 0 and at 144 h, respectively. TRT animals were sacrificed at the following time periods: at 6 h = 6, at 12 h = 6, at 24 h = 6, at 48 h = 4, at 72 h = 4, at 96 h = 4, at 108 h = 6, at 120 h = 6, and at 144 h = 5. Animals were killed by cervical dislocation. Ovaries were excised, cleaned of surrounding connective tissue, and granulosa cells were obtained by a non-enzymatic procedure [Dhanasekaran and Moudgal, 1989b]. The granulosa cells were snap-frozen and kept frozen until used for the enzyme studies.

Preparation of Cell Lysates for Immunoblots

The cell lysates for immunoblots were prepared following the previously published procedures [Dhanasekaran et al., 1994; Vara Prasad et al., 1995]. The cells were harvested, washed twice in PBS and lysed with 0.5 ml of RIPA buffer (10 mM $NaPO_4$, pH 7.0, 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 1% NP40, 0.1% SDS, 50 mM NaF, 200 mM Na_3VO_4, 0.1% β-mercaptoethanol, 1 mM PMSF, 4 µg/ml aprotinin, and 2 µg/ml leupeptin). The soluble proteins were cleared by centrifugation at 15,000*g* for 10 min at 4°C. The aliquots of the lysate were snap-frozen in liquid nitrogen and stored at −80°C.

Western Blot Analysis

The granulosa cells were lysed in RIPA buffer following the previously published procedures [Dhanasekaran et al., 1994; Vara Prasad et al., 1995]. The lysate (50 µg) was resolved by SDS-PAGE and electroblotted onto PVDF membranes (Millipore, Bedford, MA) in 10 mM CAPS buffer containing 10% methanol using a Trans-Blot apparatus (Hoefer), and probed with specific antibodies according to the published procedures [Dhanasekaran et al., 1994]. The monoclonal antibodies specific to the phosphorylated kinases (Phospho-P38MAPK # 9211, Phospho-p44/42 MAPK # 9105, and P-SAPK/JNK # 9251) were obtained from New England Biolabs whereas the other antibodies (ERK1 # sc-19 , ERK2 # sc-154, JNK1 # sc-474 , JNK2 # sc-827, p38MAPK # sc535, AKT 1 # sc1618, Bcl-2 # sc492G, Bad # sc-942, and Bax # sc-526) were rabbit polyclonal antibodies obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All these antibodies can identify the respective murine antigens. The immunoblots are de-

veloped with horse radish peroxidase coupled rabbit antibodies using NEN chemiluminescence methods [Dhanasekaran et al., 1994].

Immunecomplex Raf-1 Assay

Cell extracts were prepared as described previously [Vara Prasad et al., 1995]. Briefly, Raf-1 in 100 µg of cell lysate protein was immunoprecipitated by the Raf-1 antibodies (1 µg, sc-227, Santa Cruz Biotechnology, Inc.) for 1 h followed by an additional incubation with 20 µl of protein-A Sepharose (Pharmacia) for 1 hour. The in vitro kinase assay was carried out with using the Raf-1 immunecomplex bound to protein-A Sepharose beads. The beads were resuspended in 30 µl of kinase buffer containing 10 µM [γ-^{32}P]-ATP (5,000 cpm/pmol) and the kinase assay was carried out for 20 min at 30°C. The reaction was stopped by the addition of Laemmli's sample buffer followed by the boiling of the samples for 3 min. The proteins were resolved on 12% SDS PAGE; gel was dried and an autoradiogram was developed.

Analysis of DNA Fragmentation

Granulosa cell DNA was extracted following the previously published procedures [Sentman et al., 1991; Hughes and Gorospe, 1991]. The cells were collected by centrifugation and the cell pellets were resuspended in 0.5 ml of lysis buffer containing 50 mM Tris (pH 8.0). 10 mM EDTA, and 0.5% Triton X-100 along with proteinase K. The cells were incubated for 3 h at 55°C. The cell lysates were extracted three times with phenol-chloroform (1:1) and the DNA in the aqueous phase was precipitated with ethanol. After drying, the DNA was resuspended in 200 µl of TE (10 mM Tris, pH 8.0, and 0.1 mM EDTA) containing 10 µg/ml of RNAse A and incubated for 1 h at 37°C. The quantity and the purity of the DNA preparations were determined by monitoring the optical density of each DNA sample at 260 vs. 280 NM. The extent of DNA fragmentation was determined using the previously published methods [Tilly and Hsueh, 1993]. Briefly, after the quantification of the DNA, the samples of DNA (500 ng/sample) were labeled with [α^{32}P]-deoxy ATP (3,000 Ci/mmol, NEN at their 3′ end using terminal transferase (NEB, Beverly, MA) reaction. The DNA samples were resolved on 1.5% agarose gel. The resolved DNA was blotted on to a Zeta-Probe GT blotting membrane (Bio-Rad, Hercules, CA) using the alkaline capillary blotting transfer method as described by the manufacturer. The blot was subjected to autoradiography.

RESULTS

Validation of the Animal Model System

It has previously been established that the administration of 15 IU of eCG to 21-day-old immature female rats stimulates the growth and development of ovarian follicles by 72 h. In the absence of further hormonal support—because of the waning levels of tropic support caused by metabolism of gonadotropin—the follicles undergo apoptosis from then onwards [Dhanasekaran and Moudgal, 1989a,b]. Due to the lower yield of granulosa cells and hence the DNA, DNA fragmentation analysis rather that DNA-ladder formation has been used to monitor the apoptosis of the granulosa cells [Hughes and Gorospe, 1991; Tilly and Hsueh, 1993]. Using similar methods, we characterized this model system by monitoring the presence of low molecular weight DNA fragments associated with apoptosis. As shown in Figure 1, a progressive increase in the low molecular weight DNA can be seen from 96 h to 120 h correlating well with the previous results [Dhanasekaran and Moudgal, 1989a,b; Hughes and Gorospe, 1991] thus validating the model system.

Temporal Changes in JNK Activity During Granulosa Cell Growth and Apoptosis

Previous results using the eCG-primed immature rat model system have shown that the injection of 15 IU eCG results in the initial proliferative phase of the granulosa cells followed by a progressive apoptotic phase from 72 h [Dhanasekaran and Moudgal, 1989a,b; Hughes and Gorospe, 1991]. Studies from several laboratories have indicated that the sustained activation of stress activated protein kinases, Jun N-terminal kinases, and p38 MAPK are involved in promoting cell apoptosis [Fanger et al., 1997; Dhanasekaran and Reddy, 1998]. Hence we tested whether the activation of any of these kinases can be correlated with the onset of granulosa cell apoptosis. Using the phosphospecific antibodies to JNK or p38MAPK, we monitored whether the activation of either or both of these kinases can be correlated with the onset of granulosa cell apoptosis. As shown in Figure 2, our results clearly indicated that an increased activation of JNK can be seen only from 108 h onwards (Fig. 2A). Western blot

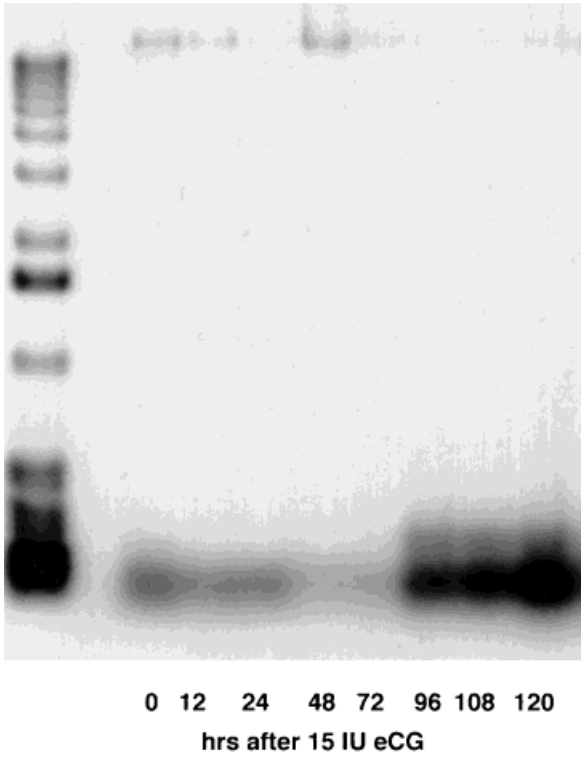

Fig. 1. Electrophoretic analysis of low-molecular weight DNA fragments in granulosa cells. Granulosa cells were obtained from ovaries (n = 3) at different time points after injection of 15 IU of eCG. Low molecular weight DNA (500 ng) was prepared from the granulosa cells and 3′-end-labeled with ^{32}P-dideoxy-ATP (3,000 Ci/mmol) using the terminal transferase reaction (NEB) as described under Materials and Methods. The DNA was resolved on 1.5% agarose gel and analyzed by autoradiography. **Lane 1** represents molecular weight markers.

analysis using antibodies against non-phosphorylated kinases showed no change in their levels of expression over the time (data not shown). Although the initial progressive decrease in JNK activity can be correlated with the proliferative phase of the granulosa cells. An increase in JNK activity can be seen only at 108 h suggesting that the activation of JNK is not the primary signaling event that transmits the apoptotic signal to the granulosa cells. Although the activation of p38MAPK showed a biphasic response, the finding that there is no significant increase in its activity by 72 h that can be correlated with the initiation of apoptosis in the granulosa cells (Fig. 2B).

Temporal Changes in the Activity of ERK-Signaling Module During Granulosa Cell Growth and Apoptosis

In some physiological contexts, it has been shown that the apoptotic signals are transmitted via a reduction in the anti-apoptotic factors [Johnson et al., 1997; Fanger et al., 1997]. These studies have also indicated that a reduction in the ERK signaling module activates the apoptotic pathway in diverse cell types. Therefore, we examined whether the reduction in the activity of ERK plays a critical role in transmitting the apoptotic signals to the granulosa cells. Our results indicate that the ERK activity which increases during the proliferative phase (0–24 h), shows a sharp decrease by 48 h onwards (Fig. 3A). By 72 h the activities of ERK are much less than that of the control levels. Since the morphological studies have indicated that these cells are well into apoptosis by 72 h, these results clearly suggest that the reduction in the ERK activity seen as early as 48 h, is involved in transmitting the apoptotic signals. However, an increase in ERK activity can be seen after 96 h. Monitoring the levels of expressions using ERK-1 and ERK-2 antibodies indicated that there is no change in their levels during these time-points (data not shown). The significance of the activation of ERK during the later time period (96–120 h) is presently not known.

ERK1 and ERK2 are activated by the upstream dual specificity kinases MEK1 and MEK2 through the phosphorylations of specific Tyr- and Thr-residues [Dhanasekaran and Reddy, 1998]. The MEKs in turn, are activated through phosphorylation through the ser/thr kinase Raf. In addition to its activation of MEKs, Raf is known to be involved in the regulation of apoptosis through its interaction with the pro- as well as anti-apoptotic factors such as Bcl-2 and Bad [Zha et al., 1996; Wang et al., 1996]. Therefore, we investigated the activation profile of Raf-1 in these granulosa cells by monitoring their autophosphorylation. Similar to the activation profiles of ERKs, Raf1 activity showed an initial increase followed by a gradual decrease, correlating well with the pattern of apoptosis in the granulosa cells. Significantly, the reduction in Raf-1 activity from the control (0 h levels) can be seen from as early as 24 h time point (Fig. 3B).

Changes in Pro- and Anti-Apoptotic Proteins During Granulosa Cell Growth and Apoptosis

The Bcl-2-family of proteins has been identified to play a major role in the activation of apoptotic pathways. The observation that Bcl-2 is involved in targeting Raf-1 to mitochondria and the observation that Raf-1 can phosphorylate Bad in vitro suggested the possibility that

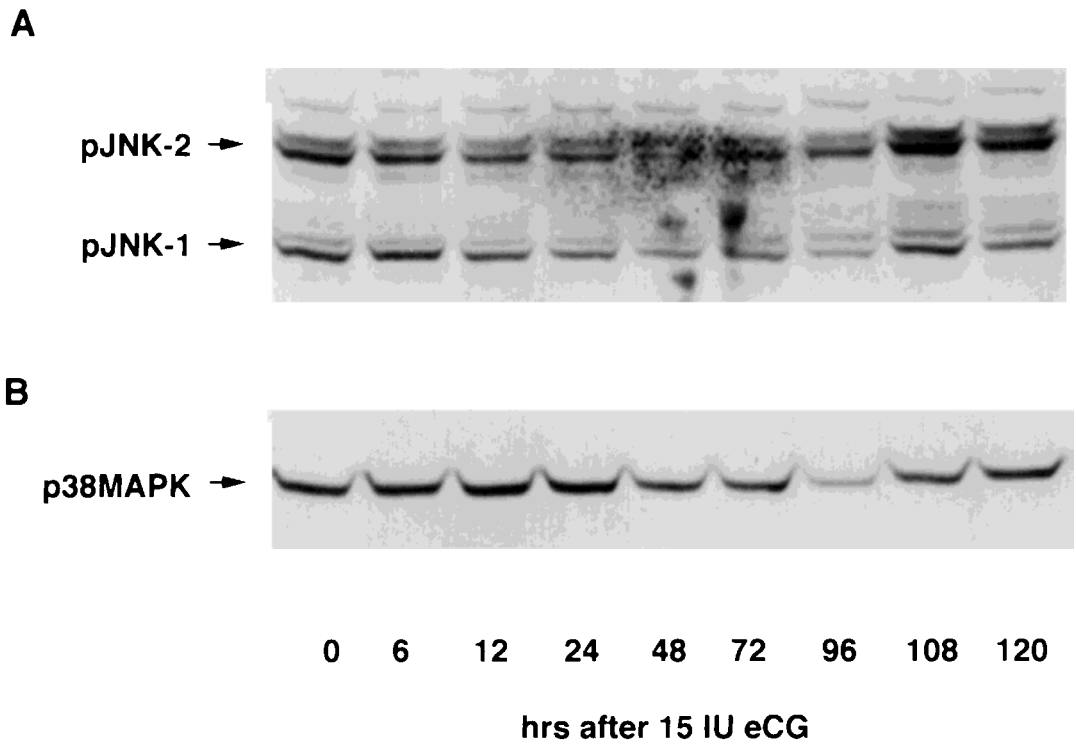

Fig. 2. Immunoblot analyses of activated JNK and p38MAPK. Protein lysates (100 μg/lane) of ovarian granulosa cells from control and eCG-treated animals (n = 3) at different time points after the injection of saline or 15 IU of eCG were resolved on SDS PAGE. Immunoblot analyses were performed using phospho-specific antibodies to phosphorylated JNKs (**A**) or phosphorylated p38 MAPK (**B**) as described under Materials and Methods.

these proteins may be involved in transducing the reduction in Raf-MEK-ERK pathway into apoptotic signals [Zha et al., 1996; Wang et al., 1996]. In addition, it has been suggested that the gonadotropin-regulated changes in the ratio of anti-apoptotic factor, Bcl-2 vs. apoptotic factor, Bax play a determining role in committing the follicular granulosa cells to apoptosis [Tilly et al., 1995; Johnson et al., 1997]. To investigate whether such modulation of the levels of Bcl-2 or Bax is involved in transmitting the apoptotic signals to granulosa cells, we analyzed the changes in the levels of Bcl-2 and Bax during proliferative as well as the apoptotic phases of the granulosa cells. Our results indicate that the levels of Bax remain unchanged until 72 h (Fig. 2). However, a small increase can be seen from 96 h onwards. In contrast, Bcl-2 levels showed little or no change until 96 h, but exhibited a reduction from 108 h onwards.

It has been recently shown that the pro-apoptotic factor, Bad, can be phosphorylated at Ser-112 and Ser-136 and the phosphorylation of Bad results in the loss of its pro-apoptotic activity [Zha et al., 1996; Wang et al., 1996]. Based on the observation that Raf-1 can phosphorylate Bad, it has been proposed that Raf-1 can transmit cell survival signals by phosphorylating Bad. By the same token, a reduction in Raf-1 activity can lead to a decrease in the levels of phospho-Bad, thus unleashing its pro-apoptotic activity. Therefore, we investigated whether such a reduction in the levels of phospho-Bad plays a role in the apoptosis of the granulosa cells. Since phosphorylated Bad shows a reduced mobility in SDS-PAGE compared to the non-phosphorylated Bad, we examined the mobility shift of Bad in SDS-PAGE during apoptosis. The results indicated that the levels of phospho-BAD showed a sharp decline from 72–96 h onwards and the levels remained low from then onwards. By contrast, the levels of non-phosphorylated Bad showed only minor changes at the different time points tested.

DISCUSSION

Understanding the mechanism of ovarian granulosa cell apoptosis is of great clinical importance since an aberrant signaling in this pathway is likely to be involved in granulosa cell tumors and cystic ovaries. Using different

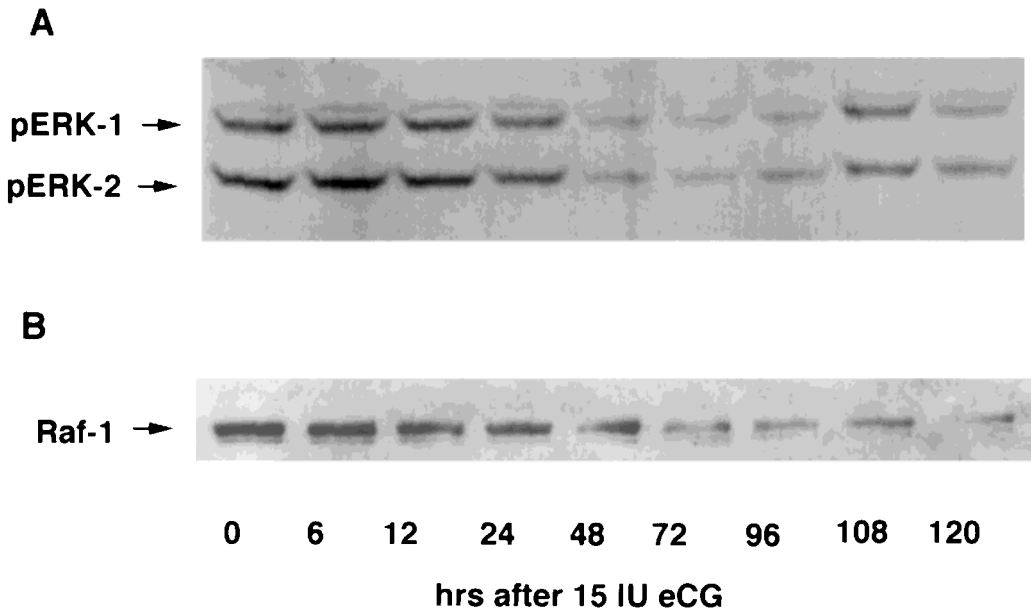

Fig. 3. Activity profiles of ERK1, ERK2, and Raf-1. Protein lysates (100 μg/lane) of ovarian granulosa cells from control and eCG-treated animals (n = 3) at different time points after the injection of saline or 15 IU of eCG were resolved on SDS PAGE. Immunoblot analyses were performed using phospho-specific ERK 1/2-antibodies (**A**). The autophosphorylating activity of Raf-1 was monitored using an immunecomplex kinase assay as described under Materials and Methods (**B**).

models systems several laboratories have investigated the mechanisms underlying granulosa cell apoptosis. Many of these findings have defined the effects of ensuing apoptosis rather than the cause. Nevertheless, these studies have indicated that the granulosa cell apoptosis involves an increase in Bcl-2 along with a decrease in Bax messages [Tilly et al., 1995]. Using the model system described here, previously it has been shown that the loss of gonadotropin precedes the morphological changes associated with the granulosa cell apoptosis. Further analysis of this system indicated that the withdrawal of tropic hormone support leads to the loss of their receptors. The loss of the receptors in turn results in the acute "shutting off" of the signaling machinery, which leads to apoptosis. Thus essentially the granulosa cell apoptosis is analogous to apoptosis activated by growth factor or serum withdrawal seen in cultured cells. The observation that the administration of gonadotropin during the initial apoptotic phase can rescue these granulosa cells is consistent with this view [Dhanasekaran and Moudgal, 1989b].

It has been observed that the serum withdrawal stress often involved the activation of SAPKs, JNK, and/or p38MAPK. Recently, it has been shown that in Rat1 and PC12 cells, apoptosis induced by the withdrawal of, tropic growth factors involves a rapid increase in p38MARK [Kummer et al., 1997]. Surprisingly, apoptosis induced by a similar "withdrawal" of tropic hormonal support—as a function of normal metabolic clearance of eCG in this system—as seen here in the granulosa cells appears not to involve either JNKs or p38MAPK. Rather, a decrease in the activities of ERK-signaling pathway correlates well with the observed apoptotic events. An analysis of the activity of Akt-1, a major survival pathway known to down-regulate cellular apoptotic pathways [Kauffmann-Zeh et al., 1997; Cardone et al., 1998], did not show any change over the time period of investigation (data not shown). Previously, it has been shown that the administration of 15 IU of eCG results in the stimulation of the tropic responses of granulosa cells until 48–72 h followed by a sharp decrease in these responses from then onwards. This has been correlated with the loss of the gonadotropin receptors from 24–48 h onwards [Dhanasekaran and Moudgal, 1989b]. In this context our observation that the ERK activity shows a decrease from 24–48 h similar to that of Raf-1 activity is of great significance. Since the morphological studies have indicated that these cells are well into apoptosis by 72 h, these results sug-

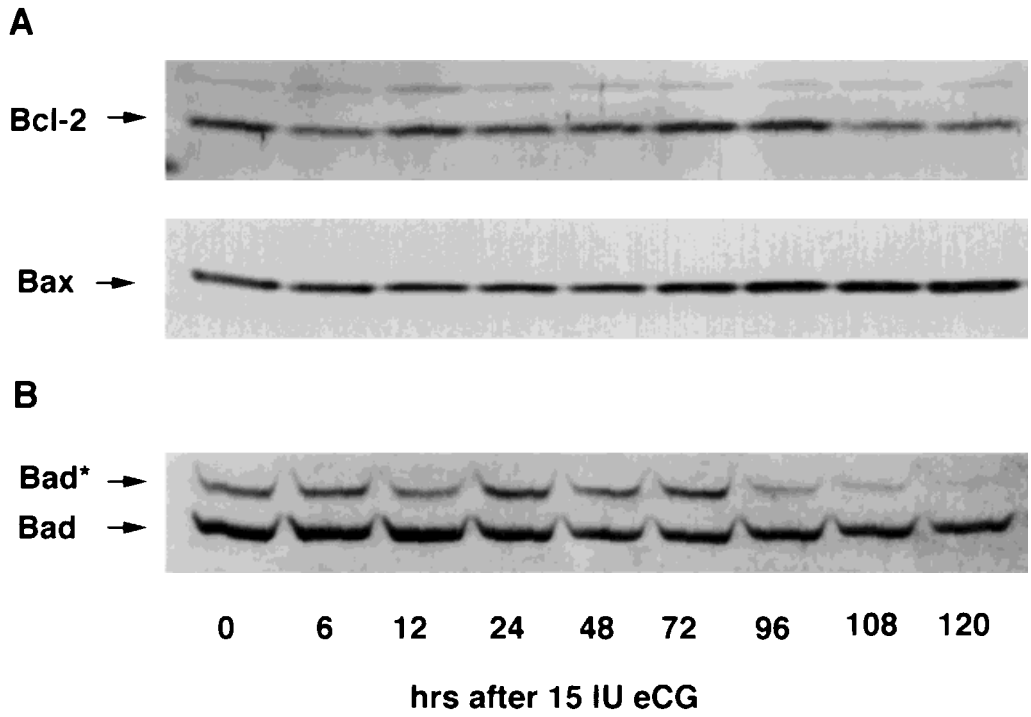

Fig. 4. Immunoblot Analyses of Bcl-2, Bax, and Bad. Protein lysates (100 μg/lane) of ovarian granulosa cells from control and eCG-treated animals (n = 3) at different time points after the injection of saline or 15 IU of eCG were resolved on SDS PAGE. Immunoblot analyses were performed using the respective Bacl-2, Bax, or Bad-specific antibodies.

gest that the weakening of Raf-1/MEK/ERK signaling, seen as early as 24–48 h, is involved in triggering apoptosis. Although an increase in ERK activity is seen from 96 h, this appears to be Raf-1 independent. It is likely that an upstream signaling event that gets activated during apoptosis (such as MEKK) can co-incidentally activate ERKs. In fact, MEKK has been shown to activate ERK rather weakly in different cell types [Dhanasekaran and Reddy, 1998]. Our observation that Raf-1 is not activated during these time periods also supports this view. Since the granulosa cells are actively in the process of apoptosis by this time point, the significance of the activation of ERK during the later time period (96–120 h) is presently not understood. Perhaps, the activation of ERK at this later time point is involved in the regulation of a process other than cell proliferation and survival. It is also possible that in the presence of the activated stress kinases such as JNK and p38 MAPK as well as other death proteins such as Bax during this period, the increased ERK-activity fails to have a protective effect on the granulosa cells that are already committed to apoptosis. A marked decrease in the levels of phosphorylated Bad observed from 72 h onwards, suggests the interesting possibility that the weakening of ERK-signaling is translated into apoptotic signals through the consequent increase in the levels of non-phosphorylated Bad. Perhaps the lag period between the weakening of ERK-signaling and the reduction in phosphorylated-Bad may be indicative of the additional intermediary signaling molecules.

Our results presented here, for the first time identify the decreased activity of the ERK-signaling module and the concomitant decrease in the phosphorylation of Bad as the earliest signaling events involved in the onset of apoptosis in ovarian granulosa cells. In light of the recent observations that the anti-apoptotic factor Bcl-2 binds to Raf-1 and possibly targets it to mitochondria where activated Raf-1 protects the cells from apoptosis by phosphorylating Bad [Zha et al., 1996; Wang et al., 1996], a reduction in Raf followed by a reduction in ERK activities could initiate the onset of apoptotic events by more than one mechanism. The role of Raf in phosphorylating Bad in the granulosa cells is presently being investigated in the laboratory.

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