EGF-like growth factors as LH mediators in the human corpus luteum

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BACKGROUND: This study aims to investigate the role of epidermal growth factor-like ligands, amphiregulin (Ar) and epiregulin (Ep), in regulation of apoptosis in luteinized human granulosa cells.

METHODS: Luteinized human granulosa cells were obtained from women undergoing IVF treatment. Ar and Ep mRNA levels were measured by real-time RT–PCR. The rate of apoptosis was measured by TUNEL. Progesterone levels were measured using radioimmunoassay. Ar- and Ep-induced activation of signaling cascades and Ar protein levels were detected by western blotting.

RESULTS: LH stimulation of luteinized human granulosa cells induced biosynthesis of Ar and Ep mRNA in a time-dependent manner. The blockade of MEK (by U0126) reduced the expression of LH-induced Ar and Ep biosynthesis. Incubation of the cells with Ar and Ep completely abolished the increase in apoptosis rate induced by serum starvation, and concomitantly caused a pronounced increase in progesterone production. Stimulation of the cells with Ar and Ep also activated the ERK and AKT signaling cascades. Finally, we demonstrated that the pro-survival effect of Ar and Ep is partially dependent on their ability to induce progesterone production.

CONCLUSIONS: Ar and Ep serve as pro-survival LH mediators in the human corpus luteum.

Key words: amphiregulin / epiregulin / luteinized granulosa cells / apoptosis / corpus luteum

Introduction

The corpus luteum plays a central role in the regulation of the menstrual cycle and in the maintenance of pregnancy before the luteo-placental shift. This function is carried out largely by progesterone, which is the main steroid synthesized by this transient endocrine gland. If the oocyte is not fertilized, the corpus luteum regresses, allowing a new cycle to begin. The major event that causes the regression of the corpus luteum is death by apoptosis of luteal cells (reviewed in Stocco et al., 2007). Indeed, characteristic morphological features such as apoptotic bodies have been clearly demonstrated in luteal cells of regressing human corpus luteum (Morales et al., 2000). Although it is known that LH is the primary luteotropin, the structural development, integrity and control of the lifespan of the corpus luteum rely on other factors or intrinsic mechanisms (Stouffer, 1996). Accumulating evidence shows that ovarian-derived local growth factors are involved in these processes (Pan et al., 2002; Ben-Ami et al., 2006a). Indeed, apoptosis in the ovary can be triggered not only by death signals but also by deprivation of local growth factors (Hsu and Hsuen, 2000).

Epidermal growth factor (EGF) is a member of a large group of closely related proteins that include amphiregulin (Ar), epiregulin (Ep), betacellulin, epigen, neuregulins and heparin-binding EGF-like growth factor. EGF-like growth factors, Ar and Ep, have drawn considerable attention in recent years, reflecting the discovery of their crucial role in...
reproduction. LH stimulation of pre-ovulatory mural granulosa cells as well as cumulus–oocyte complexes was found to induce Ar and Ep expression (Motola et al., 2008). Incubation of germinal vesicle stage mouse follicles with Ar and Ep was found to recapitulate the morphological and biochemical events triggered by LH, including cumulus expansion and oocyte maturation, suggesting that these EGF-like growth factors serve as LH-mediators in the pre-ovulatory follicle (Park et al., 2004). Interestingly, EGF receptor (EGFR) protein expression was evident not only in pre-ovulatory follicles, but also throughout the luteal phase (Fuji-naga et al., 1992; Assarsson et al., 1995; Tamura et al., 1995; Garnett et al., 2002; Akayama et al., 2005), suggesting that these growth factors might also affect the corpus luteum. However, although Ar and Ep are known to play a central role in periovulatory processes, the expression of these factors and their possible role in the corpus luteum is not known.

In contrast to Ar and Ep, the role of EGF in the corpus luteum has been considerably investigated. EGF was found to inhibit granulosa cell apoptosis in rats (Luciano et al., 1994); whereas EGFR inhibition was found to induce apoptosis in human luteinized granulosa cells (Khan et al., 2005). Likewise, it was found that the soluble form of HB-EGF inhibit apoptosis of luteinized human granulosa cells (Pan et al., 2002). In addition, there is cross-talk between EGF and other signaling systems, including insulin and insulin-like growth factor, which are both known to serve as survival factors in the corpus luteum (Roudabush et al., 2000; Adams et al., 2004).

Through binding to cell surface receptors, EGFs activate an extensive network of signal transduction pathways that include activation of the Ras/ERK and PI3K/AKT pathways (reviewed in Henson and Gibson, 2006). Interestingly, inhibition of these signal transduction pathways was found to induce apoptosis in luteinized granulosa cells. Attenuation of Raf-MEK-ERK signaling pathways was found to be associated with the onset of apoptosis in granulosa cells (Gebauer et al., 1999), whereas inhibition of MEK was found to induce apoptosis in luteinized human granulosa cells (Oliver et al., 1999). Additionally, it was shown that inhibition of the AKT pathway induces apoptosis in granulosa cells (Johnson et al., 2001). Taken that EGF induces a potent pro-survival effect on luteinized granulosa cells in vitro, it is puzzling that we could neither detect the expression of EGF in quiescent luteinized human granulosa cells nor following LH stimulation. These data suggest that EGF is not part of the physiologic survival factors repertoire of the human corpus luteum (Ben-Ami et al., 2006). Nevertheless, we demonstrated a dramatic up-regulation of the EGF-like factor genes, Ar and Ep, for the first time in luteinized human granulosa cell cultures in response to LH stimulation, by using DNA microarray techniques (Rimon et al., 2004). These data were later supported by RT–PCR techniques (Freimann et al., 2004, 2005).

That LH induces up-regulation of Ar and Ep gene expression in human luteinized granulosa cells means that these EGF-like growth factors are synthesized not only in pre-ovulatory granulosa cells, but also in the corpus luteum in response to LH stimulation. Since Ar and Ep are members of the EGFs family, we hypothesized that they serve as pro-survival, downstream mediators of LH in the human corpus luteum.

Materials and Methods

Cultures of primary human granulosa cells

Primary granulosa cells were obtained from women, aged 22–38 years, undergoing IVF treatment in Assaf Harofeh Medical Centre, because of female factor infertility. The patients had given consent and the local ethics committee approved the project. Patients were treated according to the long protocol guidelines, i.e. received a GnRH agonist at the mid-luteal phase, followed by FSH or human menopausal gonadotrophin and eventually by the administration of hCG. Granulosa cells were isolated from aspirated follicular fluid after oocyte retrieval. The follicular fluid was centrifuged at 300g for 5 min at room temperature. The resulting pellets were resuspended in 10 mL Tris, 0.84% NH₄Cl, pH 7.4, to cause the lysis of blood cells (15 min shaking at 37°C). Several washings in phosphate-buffered saline (PBS) eliminated debris. Cells were plated in Dulbecco’s modified Eagle’s medium (DMEM/Ham F12 1:1), supplemented with penicillin (100 IU/mL), streptomycin (100 mg/mL) and 10% fetal calf serum (FCS). Cells were cultured for an additional 6 days in medium containing 10% FCS and washed every 24 h with PBS in a hormone-free medium as described previously (Breckwoldt et al., 1996; Sasson and Amsterdam, 2002).

Antibodies

Goat polyclonal anti-human ampliregulin antibody was obtained from Santa Cruz (CA, USA). Mouse monoclonal anti-diphospho-ERK1/2 (anti-pERK) antibody, anti-human β-tubulin, rabbit polyclonal anti-general ERK (anti-gERK), anti-phospho-Ser-473 AKT (anti-S473 pAKT) and anti- general AKT (anti-gAKT) antibodies were obtained from Sigma (Rehovot, Israel). Rabbit anti-goat (R&D Systems, Minneapolis, MN, USA), goat anti-rabbit and goat anti-mouse IgG (Sigma, St Louis, MI, USA) antibodies coupled to horse-radish peroxidase were applied as second antibodies, respectively. Anti-progesterone antibodies were provided generously by Dr F. Kohen (Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel).

Reagents

Recombinant human Ar and Er were purchased from R&D Systems. U0126, a specific MEK inhibitor (Favata et al., 1998), was purchased from Sigma (USA). Recombinant hLH was kindly provided by the NIH and Dr A. Parlow of the National Hormone and Pituitary Program (Bethesda, MD, USA). The general stimulator, p eroxyvanadate (VOOH), was prepared by mixing 100 μM Na₂VO₃ and 200 μM H₂O₂. RU486 was a gift from Dr F. Kohen (Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel).

LH stimulation

LH (1 IU/ml) was administered after 16 h of serum starvation (0.1% FCS). Thereafter, cells were harvested up to 24 h after stimulation and collected for further RNA and protein analyses.

Determination of progesterone and protein levels

Progesterone accumulated in the culture medium was determined by radioimmunoassay at the end of cell stimulation using [H³]-labeled proges terone (Amersham Biosciences Piscataway, NJ, USA) (Kohen et al., 1975). Protein was assayed according to Bradford (1976).

Western blot analysis

The analysis of specific protein levels was carried out as previously described (Tajima et al., 2003). Briefly, following hormone stimulation, primary cultures were rinsed with ice-cold PBS, harvested in lysis buffer containing 50 mM Hepes (pH 7.2), 150 mM NaCl, 30 mM sodium pyrophosphate, 1 mM ortho vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin and 5 μg/mL of aprotinin and were subjected to western blot analysis to detect different proteins. Samples containing equal amounts of protein (50 μg)
were separated on 12% acrylamide SDS–PAGE. Equal protein loading was detected by Ponceau staining. The relevant proteins were detected on blots using their specific antibodies. Western blots were repeated three times with cells from three groups of women (each group consisting of three women undergoing IVF treatment).

**RNA isolation and RT–PCR**

Total RNA was isolated using a commercial kit (Genta, Minneapolis, MN, USA). First-strand cDNA was created by RT (Promega Reverse Transcription System, Madison, WI, USA) from total RNA. The reaction mix contained 1 μg of total RNA, 0.5 μg of oligo(dT)15 primer, 1 mM each dNTP and 50 U of M-MLV reverse transcriptase (Promega). From the RT reaction, 1 μl was taken for quantitative PCR of Ar and Ep gene expression using TaqMan® Master Mix (Applied Biosystems) probes for Ar (assay no. Hs00155832_m1), Ep (assay no. Hs00154995_m1) and GAPDH (assay no. Hs99999905_m1) using sequence detection system PRISM 7000 (Applied Biosystems).

**Determination of apoptosis**

To analyze apoptosis, subconfluent luteinized human granulosa cells were plated on glass coverslips in 60-mm-well plates under the standard culture conditions as described above. Cells were serum-starved (0.1% FCS for 16 h) and treated with the examined stimulants. After 24 h, the cells were fixed with a freshly prepared paraformaldehyde solution [4% in PBS (pH 7.4)] for 1 h at 15–25°C, washed with PBS and then incubated with 0.1% Triton X-100 in 0.1% sodium citrate (2 min, 4°C), washed again with PBS and incubated with terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) reaction mixture containing fluorescein-dUTP and terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals) for 30 min at 37°C. Preparations were analyzed by fluorescence microscopy. The incidence of apoptosis in each preparation was determined by counting 200 cells and calculating the percentage of apoptotic cells.

**TCA protein precipitation**

One volume of TCA stock was added to 4 volumes of condition media and was incubated for 10 min at 4°C. The samples were then centrifuged at 23,400 g for 5 min and supernatant was removed leaving the protein pellet intact. The pellet was washed with PBS and incubated with terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) reaction mixture containing fluorescein-dUTP and terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals) for 30 min at 37°C. Preparations were analyzed by fluorescence microscopy. The incidence of apoptosis in each preparation was determined by counting 200 cells and calculating the percentage of apoptotic cells.

**Statistics**

Real-time quantitative RT–PCR (Q-PCR) data and apoptosis rate are presented as mean ± SE. Comparison of mean values was performed using either analysis of variance (ANOVA) followed by multiple comparison tests or Student’s t-test as appropriate. Calculations were performed using SPSS software (Student’s t-test, Version 11, Chicago, IL, USA). P-values of <0.05 were considered statistically significant.

**Results**

**Time course of Ar and Ep expression upon LH treatment**

Ar and Ep have been proposed to mediate LH effects in the follicle (Park et al., 2004), although their involvement in the corpus luteum is yet to be defined. Our laboratory has previously demonstrated that Ar and Ep are up-regulated in human granulosa cell cultures in response to LH stimulation (Freimann et al., 2004). In order to examine the kinetics of Ar and Ep mRNA expression, luteinized human granulosa cells were treated with LH for different time intervals and subjected to Q-PCR. We revealed a time-dependent pattern of both Ar and Ep expression. Both Ar (Fig. 1A) and Ep (Fig. 1B) mRNAs were up-regulated 2–8 h following LH treatment, reaching a maximal expression level at 8 h and decreased thereafter. Interestingly, the mRNA levels were elevated even 24 h after stimulation (5-fold for Ar and 7-fold for Ep). Western blot analysis demonstrated a clear elevation of Ar intracellular protein level in response to LH stimulation, which was evident 8 h following stimulation, reaching peak levels between 16 and 24 h (Fig. 1C). This up-regulation followed the changes in Ar mRNA expression, suggesting a regulation of Ar synthesis at the transcription level. Interestingly, a parallel up-regulation of Ar protein level was also evident in the condition medium 24 h following LH stimulation (Fig. 1D). This finding implies that Ar is secreted outside the cell, thus potentially serve as an autocrine and paracrine mediator of luteinized human granulosa cells.

**Involvement of the ERK cascade in LH-induced Ar and Ep induction**

The elevated mRNA expression of Ar and Ep following LH stimulation in luteinized human granulosa cells was found only recently. Therefore, to date, little is known about the signaling pathways induced by LH that lead to Ar and Ep expression in these cells. The only signaling component that was shown to be involved in this response is PKA (Freimann et al., 2004, 2005). In order to explore whether the ERK signaling cascade is also involved in Ar and Ep expression, we first undertook to study whether ERK1/2 are activated upon LH treatment in luteinized human granulosa cells, and to study the kinetics of their activation. Therefore, serum-starved luteinized human granulosa cells were treated with LH for different time intervals, and the phosphorylation levels of ERK1/2, which are indicative of their activation, were assessed using a specific antibody. ERK1/2 phosphorylation reached a peak within 10 min after stimulation, followed by a decrease to almost basal levels 60 min later (Fig. 2A).

To further examine the involvement of the ERK cascade in LH-induced biosynthesis of Ar and Ep, serum-starved luteinized human granulosa cells were subjected to an inhibitor of the MEK pathway, U0126, prior to LH stimulation. mRNA levels of Ar and Ep were measured using quantitative PCR. The expression levels of both Ar and Ep were significantly reduced by inhibition of ERK cascade, indicating that Ar and Ep expression upon LH treatment is mediated by ERK1/2 activity (Fig. 2B and C). Moreover, when we induced Ar and Ep expression by forskolin (FK), a stimulator of adenylyl cyclase, it was also inhibited by U0126, indicating the involvement of the ERK cascade downstream to cAMP (Fig. 2B and C).

**Ar and Ep as luteotropic factors**

EGF and HB-EGF have been found to exert a pro-survival effect in the corpus luteum (Luciano et al., 1994; Pan et al., 2002; Khan et al., 2005). Hence, we hypothesized that Ar and Ep would protect luteinized human granulosa cells from serum deprivation-induced apoptosis. To examine this hypothesis, serum-starved cells were treated with Ar (10
ng/ml), Ep (10 ng/ml) or both for 24 h, and the number of apoptotic cells was examined by TUNEL and confirmed also by DAPI staining. Significant apoptosis was detected following serum deprivation of luteinized human granulosa cells when compared with cells treated with 10% FCS (Fig. 3A and B). Interestingly, although both Ar and Ep significantly reduced the serum starvation-induced apoptosis rate in luteinized human granulosa cells, Ep was found to be more potent than Ar in apoptosis prevention. Furthermore, co-treatment with Ar and Ep of serum-deprived luteinized human granulosa cells reduced the apoptosis rate almost to that found in serum-treated cells.

Signaling cascades induced by Ar and Ep stimulation

EGFR was found to be expressed in luteinized human granulosa cells (Akayama et al., 2005). Since Ar and Ep were found to be secreted into the condition medium following LH stimulation, we hypothesized that stimulation of luteinized human granulosa cells with Ar or Ep would activate the characteristic signal transduction pathways which are known to be activated by EGFs, namely the ERK and AKT (Henson and Gibson, 2006). Notably, both ERK and AKT are known to serve as anti-apoptotic signaling cascades in the corpus luteum (Gebauer et al., 1999; Oliver et al., 1999; Johnson et al., 2001).

To elucidate this hypothesis, serum-starved luteinized human granulosa cells were stimulated with either Ar or Ep, harvested and subjected to western blot analysis. Interestingly, Ar and Ep stimulation induced ERK1/2 activation in a different time-course. Addition of Ar to the cells resulted in increased phosphorylation of ERKs, which peaked 5 min after treatment and gradually declined thereafter to the basal level (Fig. 4A). Ep stimulation induced ERK1/2 activation which peaked 5 min following treatment. However, in contrast to Ar, no decline in the phosphorylation levels was demonstrated up to 60 min following Ep stimulation (Fig. 4B). Finally, stimulation of luteinized human granulosa cells with Ar and Ep induced phosphorylation of AKT, which peaked 5 min following treatment and gradually declined thereafter (Fig. 4C and D).
Ar and Ep induction of progesterone synthesis

EGF stimulation was found to induce progesterone biosynthesis in luteinized human granulosa cells (Tekpetey et al., 1995). Therefore, we hypothesized that Ar and Ep would also induce progesterone production in these cells. To elucidate this, luteinized human granulosa cells were stimulated with LH (1 IU/ml) for the indicated times. Western blot analysis with anti-pERK was used to evaluate phosphorylation levels. (B and C) Serum-starved luteinized human granulosa cells were stimulated with LH (1 IU/ml) or FK (50 μM) for 4 h, in the presence or absence of U0126 (10 μM). The expression levels of Ar (B) and Ep (C) were measured using real-time RT–PCR and normalized to GAPDH expression. Data are presented as arbitrary units (a.u.) and as fold activation versus control (inset). Data are presented as mean ± SE of three replications of the experiment with cells from three different women. *P < 0.03, **P < 0.0001 versus baseline.

Figure 2 ERK1/2 involvement in amphiregulin and epiregulin expression.

(A) Serum-starved luteinized human granulosa cells were stimulated with LH (1 IU/ml) for the indicated times. Western blot analysis with anti-pERK was used to evaluate phosphorylation levels. (B and C) Serum-starved luteinized human granulosa cells were stimulated with LH (1 IU/ml) or FK (50 μM) for 4 h, in the presence or absence of U0126 (10 μM). The expression levels of Ar (B) and Ep (C) were measured using real-time RT–PCR and normalized to GAPDH expression. Data are presented as arbitrary units (a.u.) and as fold activation versus control (inset). Data are presented as mean ± SE of three replications of the experiment with cells from three different women. *P < 0.03, **P < 0.0001 versus baseline.

Ep is mediated by progesterone receptor activation, serum-starved luteinized human granulosa cells were stimulated with Ar and Ep in the presence or absence of RU486. Ar and Ep significantly reduced the serum starvation-induced apoptosis rate of luteinized human granulosa cells, and RU486 reduced the anti-apoptotic effect of these EGF-like growth factors (Fig. 5B). This data indicate that Ar and Ep would exert their anti-apoptotic effect by both progesterone and activation of EGFR.

Discussion

Growth factors are polypeptides that modulate cell survival, proliferation, and differentiation. They act through binding to specific cell membrane receptors. In contrast to classic endocrine substances, they act locally and function in paracrine and autocrine modes. In recent years, considerable attention has been focused on members of the EGF family in controlling oocyte maturation and ovulation. However, their role in the corpus luteum remains poorly understood.

Several studies have demonstrated the presence of high affinity EGF binding sites in human granulosa luteal cells (Budnik and Mukhopadhyay, 1996), and immunolocalization of EGFR in the human corpus luteum (Maruo et al., 1993; Scully et al., 1994; Tekpetey et al., 1995) underscoring a potentially important role for this receptor in the human corpus luteum. Indeed, blockade of EGFR was found to induce apoptosis in luteinized human granulosa cells (Khan et al., 2005), implying that EGFR serves as a luteotoxic factor. However, expression of EGF in the primary luteinized human granulosa cultures was neither detected nor modulated by LH or FK, when measured by DNA microarray (Rimon et al., 2004). It is therefore suggested that Ar and Ep, which bind and activate the EGFR serve as the physiological LH pro-survival mediators in the human corpus luteum.

Examination of the kinetics of Ar and Ep expression following LH stimulation demonstrates a time-dependent pattern of both Ar and Ep mRNA induction reaching a peak at 8 h (Fig. 1). LH stimulation up-regulates the Ar protein level, which reaches a peak at 16–24 h, suggesting a close correlation between mRNA and protein levels in LH treated cells. Interestingly, LH stimulation induces Ar secretion from luteinized granulosa cells into the condition media. Indeed, Ar and Ep have been found to be synthesized as integral membrane precursors with a single transmembrane domain (reviewed in Yarden and Sliwkowski, 2001). To gain biological activity, they must undergo a specific proteolytic cleavage of the ectodomain at the membrane surface by members of a disintegrin and metalloproteinases (Dong et al., 1999; Hinkle et al., 2004). Once Ep and Ar are generated and secreted from the luteinized granulosa cells, which express EGFR, they might exert their effect on the granulosa cells either in an autocrine or paracrine loop (Ben-Ami et al., 2006a, b). Of note, it was recently found that LH stimulation of mouse pre-ovulatory follicles involves rapid transactivation of the EGFR (Panigone et al., 2008).

We then undertook to address the question of Ar and Ep expression regulation by LH, in regard to the ERK pathway. PKA activity was previously shown to be involved in LH-induced up-regulation of Ar and Ep genes in luteinized human granulosa cells (Freimann et al., 2004, 2005). In the current study, we show that MEK inhibition significantly reduces the LH-induced Ar and Ep mRNA levels in luteinized human granulosa cells (Fig. 2), indicating that the ERK cascade is involved in the expression of Ar and Ep.
Interestingly, we have previously shown that prostaglandin E2 stimulation up-regulate Ar and Ep mRNA levels in PKA and ERK-dependent manner (Ben-Ami et al., 2006b), further indicating the importance of these signaling pathways in the regulation of these genes.

Our results indicate that Ar and Ep are potent pro-survival factors which protect luteinized human granulosa cells from serum starvation-induced apoptosis (Fig. 3). Of note, Ep exerted a higher anti-apoptotic potency than Ar. This finding could be attributed at least in part to the more potent ERK activation exerted by Ep than Ar in luteinized human granulosa cells (Fig. 4). The luteotropic character of Ar and Ep is in light with previous studies which found that EGF inhibits rat granulosa cell apoptosis (Luciano et al., 1994). Likewise, inhibition of EGFR by tyrphostin S1 was found to induce apoptosis in luteinized human granulosa cells. Furthermore, blockage of EGFR also reduces the ERK activity and inhibits phosphorylation and nuclear translocation of activated ERK, supporting the concept that EGF exerts its luteotropic effect through ERK signaling in luteinized human granulosa cells (Khan et al., 2005). Additionally, it was found that addition of recombinant HB-EGF into the cell culture inhibits apoptosis of luteinized human granulosa cells (Pan et al., 2002). Finally, luteinized granulosa cells of monkeys were found to be refractory to the mitogenic stimulation by Ar and Ep (Fru et al., 2007), indicating that the pro-survival effect exerted by these EGFR ligands is not attributed to enhanced proliferation.

The EGFR is one of four ErbB receptors; stimulation of all four ErbB receptors leads to activation of two critical downstream signaling cascades, ERK and AKT. Therefore, we examined whether Ar and Ep stimulation of luteinized human granulosa cells induce activation of these signaling cascades. We show that both Ar and Ep induce ERK and AKT activation in luteinized human granulosa cells (Fig. 4). Interestingly, although Ar induced a transient activation of the ERK cascade, Ep stimulated a sustained one. This difference could be attributed to the different affinity of Ar and Ep to EGFRs, combined with the differential distribution of these receptors in luteinized human granulosa cells. Whereas Ar activates EGFR (ErbB1), Ep is involved in the stimulation of mainly ErbB4 and EGFR (Komurasaki et al., 1997; Ma et al.,

Figure 3  Ar and Ep protection of luteinized human granulosa cells from serum starvation-induced apoptosis. (A and B) Serum-starved luteinized human granulosa cells were either treated with Ar (10 ng/ml) and/or Ep (10 ng/ml) for 24 h or left untreated. Cell death was detected by TUNEL. Data are presented as mean ± SE of three replications of the experiment with cells from three different women. *P < 0.05, **P < 0.01 versus 0% FCS-treated cells.
Furthermore, immunostaining of luteinized human granulosa cells revealed that while ErbB4 was abundant in the early luteal phase, the staining level of EGFR was very low in early and mid-luteal phase (Akayama et al., 2005). In this study, we used luteinized human granulosa cells retrieved 34–36 h following hCG administration, which reflect the early luteal phase. Hence, the difference in the duration of ERK activation could be attributed to the combined dissimilarity in the expression level of EGFR and the differential activation of EGFR by Ar and Ep.

The ERK and AKT signaling cascades are known to exert an anti-apoptotic effect in granulosa cells. Indeed, treatment of granulosa cells with the PI3K inhibitor, LY294006, was found to induce apoptosis of granulosa cells (Johnson et al., 2001). Likewise, IGF-1 was found to protect granulosa cells from apoptosis by activation of the PI3K/AKT pathway (Hu et al., 2004). Furthermore, loss of trophic hormone support was found to be translated into the attenuation of the ERK signaling pathway, and this reduction triggered the onset of apoptosis in the rat ovarian granulosa cells (Gebauer et al., 1999). Similarly, the MEK inhibitor, PD98059, was found to induce apoptosis of luteinized human granulosa cells (Oliver et al., 1999). Finally, depletion of Raf-1 by geldanamycin resulted in activation of caspase-3 and increased the degree of apoptosis in human luteinized granulosa cells (Khan et al., 2000). Therefore, the activation of the ERK and AKT pathways by Ar and Ep could be attributed to their luteotropic effect.

EGF stimulation of luteinized human granulosa cells has been found to induce progesterone production (Tekpetey et al., 1995). Moreover, it has been claimed that EGF inhibits granulosa cell apoptosis at least partially by inducing progesterone synthesis (Luciano et al., 1994). Our results indicate that although progesterone receptor blockade by RU486 reduced the anti-apoptotic effect of Ar and Ep, these EGF-like growth factors still induce a significant pro-survival effect on luteinized human granulosa cells (Fig. 5). These results suggest that Ar and Ep serve as pro-survival mediators in the human corpus luteum by both activating the ERK and AKT signaling cascades and by promoting progesterone production (Fig. 6).

In conclusion, we demonstrate that LH stimulation of luteinized human granulosa cells induces biosynthesis of Ar and Ep mRNA in an ERK-dependent manner. We show that Ar and Ep effectively protect luteinized granulosa cells from serum-starvation-induced apoptosis concomitantly with a pronounced increase in progesterone production. Furthermore, Ar and Ep stimulation of luteinized human granulosa cells activate the ERK and AKT signaling cascades, both known to exert pro-survival effect in these cells. Finally, we demonstrate that the pro-survival effect exerted by Ar and Ep is only partially...
dependent on their ability to induce progesterone production. We propose for the first time that the major luteotropin, namely LH, may exert its function, at least in part, by the activation of Ar and Ep biosynthesis in the human corpus luteum.

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