Gonadotropins upregulate the epidermal growth factor receptor through activation of mitogen-activated protein kinases and phosphatidyl-inositol-3-kinase in human ovarian surface epithelial cells

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Abstract

Gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) have been implicated as probable risk factors in epithelial ovarian carcinomas, most of which are derived from ovarian surface epithelium (OSE). Since epidermal growth factor (EGF) increases the growth of ovarian surface epithelial cells, we determined the effect of gonadotropins on the expression of epidermal growth factor receptor (EGFR). We investigated the basal levels of EGFR mRNA and protein, and the mechanisms involved in the regulation of EGFR at the transcriptional and translational levels by FSH and LH. The immortalized OSE cell lines (IOSE) derived from normal OSE cells by transfecting SV40 T-antigen (IOSE-80 and IOSE-80PC, a post-crisis line) and ovarian cancer cell lines were employed. A significantly lower level of EGFR was observed in both IOSE-80 and IOSE-80PC cells when compared with the ovarian cancer cell lines, OVCAR-3 and SKOV-3. Treatment of IOSE-80PC cells with FSH and LH (10^{-7} and 10^{-6} g/ml) resulted in a significant increase in EGFR mRNA at 24 h and EGFR protein at 48 h, whereas the treatment with gonadotropins for 24–48 h induced a mild increase in EGFR in OVCAR-3, but not in SKOV-3 cells. In addition, IOSE-80PC cells treated with gonadotropins and EGF (10 nM) exhibited an additive stimulation of mitogenesis. Further, FSH and LH significantly increased activities of various kinases at 5–10 min, and pre-treatments with LY294002 (an inhibitor of PI3K) or PD98059 (an inhibitor of ERK1/2) partially blocked the gonadotropin-induced up-regulation of EGFR in IOSE-80PC cells. We investigated whether the effect of gonadotropins on EGFR mRNA levels is induced by increased transcription and/or by altered mRNA stability. Treatment of IOSE-80PC cells with FSH (10^{-7} and 10^{-6} g/ml) significantly enhanced the activity of the EGFR promoter (120 and 140% increase, respectively) at 24 h, and treatment with LH (10^{-7} g/ml) for 24 h induced an increase in the activity of EGFR promoter (30%) in these cells. On the other hand, LH resulted in a significant increase in EGFR mRNA stability in the decay curves. Taken together, these results suggest that the effect of gonadotropins on the expression of EGFR may affect cell growth via ERK-1/-2 and PI3K pathways in pre-neoplastic ovarian surface epithelial cells, and that FSH and LH increase EGFR mRNA by different mechanisms. The former increased EGFR gene transcription essentially, whereas the latter mainly enhanced EGFR mRNA stability.

Introduction

Ovarian cancer is the sixth most common cancer and the fifth leading cause of cancer-related death among women in developed countries (Greenlee et al. 2000). It is estimated that there were approximate 20 000 deaths from ovarian cancer in the United States in 2000. There is no proven method for early detection, such
that only a quarter of the women have localized disease at the time of diagnosis. Five-year survival rates range from 46% among African-Americans to 50% among Caucasian women. Risk factors for the disorder include age, nulliparity and family history of ovarian cancer, particularly first-degree relatives (Ford et al. 1994, 1998, Greenlee et al. 2000). More than 95% of these ovarian cancers arise in the ovarian surface epithelium (OSE), with the rest originating in granulosa cells or, rarely, in the stroma or germ cells (Auersperg et al 2001).

Gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), together with thyroid-stimulating hormone (TSH), are glycoprotein hormones synthesized in the anterior pituitary gland. FSH and LH share similar chemical and structural features. They are composed of two different, non-covalently associated, carbohydrate-containing protein subunits, α and β. Within a species, LH, FSH, and TSH, along with human chorionic gonadotropin (hCG), have identical α subunits and different β subunits. Both hormones act in an endocrine manner and play essential roles in the reproductive system, such as steroidogenesis and folliculogenesis. Their receptors, follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR), are transmembrane receptors with G-protein coupled signaling systems and are expressed in the target cells, i.e. in the ovaries and testis. FSHR is expressed by granulosa cells in developing ovarian follicles. LHR is expressed by theca cells in early developing follicles and by both theca and granulosa cells during later stages of follicular development. The actions of FSH and LH on granulosa and theca cells have been well characterized and are essential for follicular development (Moniaux et al. 1997). However, the role of FSH and LH in normal OSE and ovarian epithelial cancer is not well characterized.

Recently, there has been an increasing body of evidence that the hormonal environment of the OSE can influence the incidence of ovarian cancer (Riman et al. 1998, Risch 1998, Brerelms 2003). The hypothesis of the present study is that gonadotropins, FSH and LH, may be involved in the transformation and progression of normal OSE to its neoplastic counterparts. Ovarian cancer is more common in conditions with elevated gonadotropins such as post-menopausal women or women who have received treatment for induction of ovulation (Venn et al. 1999, Holschneider & Berek 2000, Tavani et al. 2000, Brerelms 2003). Reduced risk of ovarian cancer is associated with multiple pregnancies, breast feeding, oral contraceptives and estrogen replacement therapy, which are associated with lower levels of and reduced exposure to gonadotropins (Daly & Obrams 1998, Gnagy et al. 2000, La Vecchia 2001). The expressions of FSHR and LHR in normal and neoplastic OSE cells have been demonstrated (Kobayashi et al. 1996, Mandai et al. 1997, Minegishi et al. 2000, Zheng et al. 2000, Parrott et al. 2001). Recent studies suggest the relationship between expression of gonadotropin receptor and ovarian cancer development (Lu et al. 2000, Syed et al. 2001, Wang et al. 2003). In addition, recent results indicate that the levels of FSHR increased from presumed precursor lesions, ovarian epithelial inclusions, to benign ovarian epithelial tumors and to borderline ovarian epithelial cells, while they decreased from borderline tumors to carcinomas (Wang et al. 2003).

The epidermal growth factor receptor (EGFR) is a single-transmembrane tyrosine kinase receptor and plays an important role in cell proliferation, differentiation, motility and survival (Zwick et al. 1999). Over the last 20 years, it has been demonstrated that numerous cancer types express elevated levels of EGFR and its ligands, including EGF and transforming growth factor (TGF)-α (Salomon et al. 1995, Nicholson et al. 2001). In many cases, an aberrant EGFR activation mediated primarily through changes in gene expression and autocrine stimulation seems to be an essential factor in tumor development, as well as an important driving force for the uncontrolled growth behavior of cancer cells (Salomon et al. 1995). High levels of EGFR expression in multiple tumor types is therefore related to an increased probability of tumor recurrence and poor patient survival. The presence of EGFR in ovarian cancer has been well demonstrated using various methods such as ligand binding, immunohistochemistry, or Northern blot analysis (Bauknecht et al. 1988, Battaglia et al. 1989, Berchuck et al. 1991, Morishige et al. 1991, Owens et al. 1991, Henzen-Logmans et al. 1992, Bauknecht et al. 1993). EGFR is also frequently amplified and/or overexpressed when compared with normal OSE, and transfection with an antisense construct of EGFR into human ovarian cancer cell lines suppressed the malignant phenotype, cellular proliferation and tumorigenicity of these cells, suggesting its prognostic importance (Berns et al. 1992, Brader et al. 1998, Alper et al. 2000). Furthermore, the contribution of a TGFα/EGF receptor autocrine loop to the growth of epithelial ovarian cancer cells is confirmed by several reports. Post-menopausal women showing the peak incidence rate of ovarian cancer exhibit elevated TGFα levels in the normal ovary (Owens et al. 1991, Owens & Leake 1992). EGF-related ligand, TGFα, stimulates proliferation of ovarian cancer cells in vivo and in vitro, and its neutralizing antibody...
inhibits the growth (Morishige et al. 1991, Stromberg et al. 1992, Jindal et al. 1994). Elevated co-expression of EGFR and its ligand may, therefore, initiate growth stimulatory autocrine and/or paracrine loops in ovarian carcinomas, and play an important role in tumorigenesis and cancer progression.

Considering that gonadotropins might promote the growth of OSE and/or ovarian cancer cells by regulating the levels of growth factor receptors, we sought to investigate the effect of gonadotropins on the expression of EGFR. Thus, the present study was designed to evaluate: (i) the effect of gonadotropins on EGFR expression levels in immortalized OSE (IOSE) and ovarian cancer cell lines; (ii) the additive proliferative effect of gonadotropins and EGF and (iii) the mechanisms of gonadotropin-induced actions in the regulation of EGFR system.

Materials and methods

Materials

Human LH and recombinant FSH were provided by AF Parlow (National Hormone and Pituitary Program, Harbor-University of California Los Angeles Medical Center, Torrance, CA, USA). PD98059 [2-(2-amino-3-mathoxyphenyl)-4H-1-benzopyran-4-one], a MAPK/ERK kinase (MEK) inhibitor, and LY294002 [2-(4-Morpholiny1)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride], a specific cell-permeable phosphatidylinositol 3-kinase inhibitor, were purchased from New England Biolas Inc. (Beverly, MA, USA) and Sigma-Aldrich Corp. (St Louis, MO, USA), respectively.

Cell culture

The non-tumorigenic SV40 Tag-immortalized OSE-derived lines, IOSE-80 and IOSE-80PC (a post-crisis line), were cultured as previously described (Choi et al. 2001a) in medium 199:MCDB 105 (Sigma-Aldrich Corp., St Louis, MO, USA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories Ltd, Logan, UT, USA), 100 U/ml penicillin G and 100 μg/ml streptomycin (Life Technologies Inc., Rockville, MD, USA) in a humidified atmosphere of 5% CO₂:95% air at 37 °C. Following 4 h serum starvation, the cells were treated with FSH (10⁻⁷ g/ml) and LH (10⁻⁷ g/ml) for 24 h with/without 20 min pretreatment of LY294002 (10 μM) and PD98059 (10 μM). Total RNA was prepared from cultured cells using the RNeasy kit (Bio/Can Scientific, Mississauga, Canada) according to the manufacturer’s suggested procedure. RNA integrity was confirmed by using agarose gel electrophoresis and ethidium bromide staining. The total RNA concentration was determined from spectrophotometric analysis at A260/280. Complementary DNA (cDNA) was synthesized from 2.5 μg total RNA by reverse transcription (RT) at 37 °C for 2 h using a first strand cDNA synthesis kit (Amersham Pharmacia Biotech., Oakville, ON, Canada). The synthesized cDNA was used as a template for PCR amplification. A semi-quantitative PCR amplification was carried out with denaturing for 1 min at 94 °C, annealing for 60 sec at 57 °C, extension for 90 sec at 70 °C, and a final extension for 15 min at 72 °C using a thermal cycler (DNA Thermal Cycler, Perkin-Elmer, Norwalk, CT, USA). The primers were designed to amplify EGFR mRNA based on the published sequences of human EGF (Ullrich et al. 1984). In addition, amplification of human glyceraldehyde phosphate dehydrogenase (GAPDH) was performed using specific primers (Tokunaga et al. 1987) to rule out the possibility of RNA degradation, and was used to control variation in the mRNA amount in PCR. The primers of EGFR are composed of sense: 5’-TGTTCGAGGACCTCCGTCA-3’ and antisense 5’-GCTTCTTTAGTGGCAGTG-3’. The sequences of GAPDH amplification are sense 5’-ATGTCTGGGATGTAACTTCAACA-3’ and antisense 5’-GGTCTCTTACGGCAGTG-3’. The PCR amplifications were performed in 25 μl PCR mixture containing 1 × PCR buffer, 0.2 mM each dNTP, 1.6 mM MgCl₂, 50 pmol specific primers, each cDNA template, and 0.25 unit Taq polymerase. PCR products (12 μl) were analyzed by agarose gel (1%) electrophoresis and visualized by ethidium bromide staining, and the sizes were estimated by comparison to DNA molecular weight markers. Following electrophoresis, Southern blot analysis was performed using specific primers (Tokunaga et al. 1987) to rule out the possibility of RNA degradation, and was used to control variation in the mRNA amount in PCR.

RNA extraction, RT-PCR procedure and Southern blot analysis

The cells were seeded at a density of 2 × 10⁵ cells in 35 mm culture dishes and cultured in a humidified atmosphere of 5% CO₂:95% air at 37 °C. Following 4 h serum starvation, the cells were treated with FSH (10⁻⁷ g/ml) and LH (10⁻⁷ g/ml) for 24 h with/without 20 min pretreatment of LY294002 (10 μM) and PD98059 (10 μM). Total RNA was prepared from cultured cells using the RNeasy kit (Bio/Can Scientific, Mississauga, Canada) according to the manufacturer’s suggested procedure. RNA integrity was confirmed by using agarose gel electrophoresis and ethidium bromide staining. The total RNA concentration was determined from spectrophotometric analysis at A260/280. Complementary DNA (cDNA) was synthesized from 2.5 μg total RNA by reverse transcription (RT) at 37 °C for 2 h using a first strand cDNA synthesis kit (Amersham Pharmacia Biotech., Oakville, ON, Canada). The synthesized cDNA was used as a template for PCR amplification. A semi-quantitative PCR amplification was carried out with denaturing for 1 min at 94 °C, annealing for 60 sec at 57 °C, extension for 90 sec at 70 °C, and a final extension for 15 min at 72 °C using a thermal cycler (DNA Thermal Cycler, Perkin-Elmer, Norwalk, CT, USA). The primers were designed to amplify EGFR mRNA based on the published sequences of human EGF (Ullrich et al. 1984). In addition, amplification of human glyceraldehyde phosphate dehydrogenase (GAPDH) was performed using specific primers (Tokunaga et al. 1987) to rule out the possibility of RNA degradation, and was used to control variation in the mRNA amount in PCR. The primers of EGFR are composed of sense: 5’-TGTTCGAGGACCTCCGTCA-3’ and antisense 5’-GCTTCTTTAGTGGCAGTG-3’. The sequences of GAPDH amplification are sense 5’-ATGTCTGGGATGTAACTTCAACA-3’ and antisense 5’-GGTCTCTTACGGCAGTG-3’. The PCR amplifications were performed in 25 μl PCR mixture containing 1 × PCR buffer, 0.2 mM each dNTP, 1.6 mM MgCl₂, 50 pmol specific primers, each cDNA template, and 0.25 unit Taq polymerase. PCR products (12 μl) were analyzed by agarose gel (1%) electrophoresis and visualized by ethidium bromide staining, and the sizes were estimated by comparison to DNA molecular weight markers. Following electrophoresis, Southern blot analysis was performed using specific primers (Tokunaga et al. 1987) to rule out the possibility of RNA degradation, and was used to control variation in the mRNA amount in PCR.
performed to detect a specific signal with digoxigenine-labeled probes for EGFR or GAPDH, as previously described (Kang et al. 2000, Choi et al. 2001b).

**Antibodies**

The antibodies of EGFR and pan- and phosphorylated extracellular signal-regulated kinase (ERK1/2) were purchased from Santa Cruz Biotechnology Ltd (Santa Cruz, CA, USA) and Biosource International, Inc. (Camarillo, CA, USA), respectively. Phospho-Akt sampler kits, including phospho-Akt (ser 473 and thr 308), GSK3 α/β (glycogen synthase kinase-3 α/β), FKHR (Forkhead in rhabdomyosarcoma) and pan-Akt were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

**Immunoblot assay**

The cells were seeded at a density of 2 × 10^5 cells in 35 mm culture dishes and cultured in a humidified atmosphere of 5% CO₂:95% air at 37°C. The cells were washed once with medium and then serum starved for 4 h prior to treatments with FSH and LH in a time (24 and 48 h) and/or dose dependent manner (10⁻⁷ and 10⁻⁶ g/ml). The cells were washed twice with ice-cold PBS and lysed in ice-cold RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 7.5), 1 mM PMSF, 10 µg/ml leupeptin and 100 µg/ml aprotinin). The extracts were placed on ice for 15 min and centrifuged to remove cellular debris. Protein amount of supernatants was determined using a Bradford assay (Bio-Rad Laboratories). Total protein (30 µg) was run on 10% SDS-PAGE gels and electrotransferred to a nitrocellulose membrane (Amersham Pharmacia Biotech.). The membrane was immunoblottedted using specific primary antibodies at 4°C overnight. After washing, the signals were detected with horseradish peroxidase-conjugated secondary antibody for 1 h, and visualized using the ECL chemiluminescent system (Amersham Pharmacia Biotech). The antibody for 1 h, and visualized using the ECL chemilluminescent system (Amersham Pharmacia Biotech.). The membrane was immunoblottedted using specific primary antibodies at 4°C overnight. After washing, the signals were detected with horseradish peroxidase-conjugated secondary antibody for 1 h, and visualized using the ECL chemilluminescent system (Amersham Pharmacia Biotech.). The membrane was immunoblottedted using specific primary antibodies at 4°C overnight. After washing, the signals were detected with horseradish peroxidase-conjugated secondary antibody for 1 h, and visualized using the ECL chemilluminescent system (Amersham Pharmacia Biotech.). The membrane was immunoblottedted using specific primary antibodies at 4°C overnight. 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**[3H]thymidine incorporation assay**

[3H]thymidine incorporation assays were performed to analyze the additive proliferative effects of EGF and gonadotropins in IOSE-80PC cell lines (Choi et al. 2001b, Choi et al. 2002). The cells were plated in 24-well plates at 2 × 10⁴ cells/well in 0.5 ml medium 199: MCDB105 supplemented with 10% FBS and antibiotics, and incubated for 24 h. Before treatment with gonadotropins and EGF, the cells were starved in serum-free media for 4 h. After starvation, the cells were incubated with 100 and 1000 ng/ml of FSH and LH, and/or 10 nM EGF in serum-free media for 3 and 6 days. [3H]thymidine (1 µCi; 5.0 Ci/mmol; Amersham Pharmacia Biotech) was added before 4 h of harvesting the cells. At the end of the incubation period, the culture medium was removed and cells were washed three times with PBS, followed by precipitation with 0.5 ml 10% trichloroacetic acid for 20 min at 4°C. The precipitate was washed twice in methanol and solubilized in 0.5 ml 0.1 N sodium hydroxide, and the incorporated radioactivity was measured in the Tri-Carb Liquid Scintillation Analyzer (Model 2100TR; Packard Instrument Com., Meriden, CT, USA) as previously described (Kang et al. 2000).

**Transfections and luciferase assays**

The 1081 bp (−1100 to −19 bp, oriented to the translation start site as position +1) 5’ region of the EGFR gene ligated into the HindIII site of the luciferase expression vector pSV0AL 5’ (Hudson et al. 1989), was provided by G Gill (Department of Medicine, University of California-San Diego, CA, USA). The transient transfection was performed in the IOSE-80PC (passage 50–55). EGFR promoter-luciferase DNA (1 µg) was transfected into IOSE-80PC cells by FuGENE 6 (Roche Applied Science, Laval, QC, Canada) according to the manufacturer’s suggested protocol when the cells were around 50% confluent on 6-well plates. To correct for different transfection percentages, the Rous sarcoma virus (RSV)-lacZ plasmid was co-transfected into the cells with an EGFR promoter–luciferase construct. After transfection for 24 h, the cells were treated with gonadotropins for 24 h, and extracts prepared with luciferase cell lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured in the extracts from triplicate samples using the luciferase assay kit (Promega). β-galactosidase activity was measured using the β-galactosidase Enzyme Assay System (Promega) and used to normalize for varying transfection efficiencies. Promoter activity was calculated as luciferase activity/β-galactosidase activity.

**Transcription stability analysis**

IOSE-80PC cells were exposed to FSH and LH for 24 h prior to the addition of 5 µM actinomycin-D (Sigma Chemical Co., St Louis, MO, USA) to arrest new RNA synthesis. A set of untreated cultures was

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also exposed to actinomycin-D to use as a control. Total RNA from transcriptionally arrested cells was isolated at a different time point following 12 h using TRIzol Reagent (Life Sciences Tech.). The levels of EGFR mRNA were determined by RT-PCR as described above.

Data analysis

Data are shown as the means of two or three individual experiments performed in triplicate, and are presented as the mean ± s.d. In the thymidine incorporation assay, values are expressed as the percentage of growth compared with the control value and are the mean ± s.d. of two individual experiments performed in triplicate. Data were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test or Dunnett’s test. A P-value of <0.05 was considered statistically significant.

Results

Expression of EGFR mRNA and protein

The mRNA expression of EGFR in IOSE (IOSE-80 and IOSE-80PC) and the ovarian cancer cell lines OVCAR-3 and SKOV-3 was compared by RT-PCR and Southern blot analysis. A predicted PCR product of EGFR was obtained as 348-bp and confirmed by Southern blot analysis using a DIG-labeled EGFR probe (Fig. 1A). As seen in Figure 1A, the ovarian cancer cell lines, including OVCAR-3 and SKOV-3, showed a higher level of EGFR mRNA compared with IOSE cell lines (IOSE-80 and IOSE-80PC). In parallel with EGFR mRNA levels, the expression level of EGFR protein was further examined by immunoblot analysis, the expression of EGFR protein was significantly enhanced in ovarian cancer cell lines compared with IOSE cell lines. Between ovarian cancer cell lines, SKOV-3, with more potential invasive properties, in our cell culture systems exhibited higher EGFR expression (Fig. 1B). IOSE-80PC, a post-crisis cell line originally generated from IOSE-80, showed similar EGFR mRNA and protein expression as IOSE-80 cells.

Regulation of EGFR mRNA and protein by FSH and LH

To investigate whether EGFR expression is regulated by FSH and LH in IOSE and ovarian cancer cell lines, we determined the expression levels of EGFR mRNA and protein following treatment of these cells with FSH and LH. While no significant up-regulation of EGFR mRNA was observed in SKOV-3 cells, 24 h treatment with FSH and LH resulted in significant up-regulation of EGFR mRNA in IOSE-80PC and OVCAR-3 cells (Fig. 2). The up-regulation was more substantial in IOSE-80PC cells than in OVCAR-3 cells. Both FSH and LH increased EGFR mRNA in a dose-dependent manner with maximal 5-fold and 9-fold increases at 10⁻⁶ g/ml, respectively in IOSE-80PC cells, whereas a maximum increase of 3-fold was observed at 10⁻⁶ g/ml of LH in OVCAR-3 cells (Fig. 2A). In terms of protein levels, 24 or 48 h treatment with FSH and LH also showed more potent effects on EGFR expression.
expression in IOSE-80PC than in OVCAR-3, and the expression pattern is correlated with its mRNA expression (Fig. 2B). Also, the elevated levels of EGFR mRNA and protein by FSH and LH were observed in IOSE-80 cells (data not shown). Subsequently, further experiments were then performed using IOSE-80PC to evaluate the effect of gonadotropins on the expression of EGFR.

Additive proliferative effects of gonadotropins and EGF in IOSE-80PC cells

To examine the effects of EGFR up-regulation on the growth of IOSE-80PC, the cells were treated with FSH and LH (10^{-7} g/ml) plus EGF (10 nM) for 3 or 6 days, and a [3H]thymidine incorporation assay was performed as previously described (Kang et al. 2000, Choi et al. 2002). Treatment with FSH alone induced an approximate 20% increase in [3H]thymidine incorporation and EGF alone showed an 80% increase in cell growth for 3 days. The combined treatment of FSH plus EGF for 3 days yielded a 150% increase in cell growth of IOSE-80PC cells. It is of interest that LH, which by itself did not show any mitogenic effect by 3-day treatment, further enhanced the growth stimulation caused by the EGF treatment (Fig. 3, left panel). With 6 days of treatments, both FSH and LH showed a significantly increased stimulation of mitogenesis in
the presence of EGF (10 nM) when compared with the treatments with gonadotropins alone (Fig. 3, right panel). These results suggest that an increased level of EGFR induced by gonadotropins in IOSE-80PC cells may contribute to an enhanced stimulation of cell growth by EGF.

Inhibitory effects of LY294002 and PD98059 on gonadotropins-induced EGFR up-regulation

To determine the signaling pathways that contribute to gonadotropin-induced EGFR, we examined the ability of the different pharmacological agents to block the changes in the expression of EGFR mRNA in IOSE-80PC cells. We found that PD98059, a ERK1/2 inhibitor, and LY294002, a PI3K inhibitor, blocked gonadotropin-induced EGFR up-regulation partially (Fig. 4), while the inhibitors did not affect the basal level of EGFR levels (data not shown). In contrast, neither, H89 (a PKA inhibitor), GF 109203X (a PKC inhibitor) nor SB203580 (a p38 inhibitor), caused a substantial inhibition of EGFR up-regulation by gonadotropins (data not shown). These results indicate that gonadotropins may increase EGFR expression by activating ERK1/2 and PI3K signaling pathways.

Effects of FSH and LH on activation of ERK1/2 and PI3K signaling cascades

To further test that ERK1/2 and/or PI3K signaling pathways are activated by gonadotropins, we examined the phosphorylation status of ERK1/2 and AKT and its downstream molecules after treatments of the cells with FSH and LH (10^{-7} g/ml) in a time-dependent (5–60 min) and dose-dependent (10^{-6}, 10^{-7} and 10^{-8} g/ml) manner at 15 min. We performed an immunoblot analysis with specific antibodies which can detect phosphorylated forms of ERK1/2, AKT, GSK3α/β and FHKR, and total ERK1/2 and AKT for normalization. As shown in Figure 5A, treatment with FSH induced a significant increase in the phosphorylated form of ERK1/2 (p-ERK1/2) at 5 min, and sustained it for 60 min in IOSE-80PC cells. Treatment with LH also resulted in a significant increase in ERK1/2 activation at 5 min, but the activated ERK1/2 declined to the control at 60 min. Similarly, FSH and LH induced
phosphorylation of AKT at Ser 473 and Thr 308 within 5 min, with the decrease of its level at 30 min (Fig. 5B). To confirm that increased AKT phosphorylation at Ser 473 and Thr 308 was an indication of increased activity, we measured the phosphorylation of two well-known downstream proteins, including glycogen synthase kinase-3α/β (GSK3α/β) and Forkhead in rhabdomyosarcoma (FKHR), following the same treatments. Parallel increased patterns of phosphorylation of GSK3α/β and FHKR were observed (Fig. 5B) following activation of AKT. FSH- and LH-stimulated AKT and GSK3, and FHKR and ERK activation were completely abolished by pretreatment with PI3K inhibitors LY294002 and PD98059, respectively. In addition, treatment with LY294002 or PD98059 alone resulted in a significant decrease in basal activity of those signaling pathways in 80PC cells. Treatment with these drugs did not result in any cytotoxic effects under the present experimental conditions. These results suggest that FSH and LH activate ERK1/2 and PI3K pathways in IOSE-80PC cells, which play an important role in cell growth, survival and progression in ovarian cancer (Choi et al. 2002).

**Effects of FSH and LH on EGFR promoter activity and its mRNA stability**

We further investigated whether gonadotropin regulation of EGFR is dependent on the gene transcription and/or receptor mRNA stability. To determine whether the EGFR 5′-flanking region plays a role in directing EGFR mRNA expression, the luciferase expression vector pSVOAL 5′ inserted with the proximal 1081 bp of the EGFR 5′-flanking regions was transiently transfected into IOSE-80PC cells. The treatment with FSH (10^{-6} and 10^{-7} g/ml) significantly enhanced the activity of 1081 bp of the EGFR 5′-flanking region in IOSE-80PC cells (100% vs. 220% and 250%), while the treatment with LH showed relatively less effect on the activity of EGFR (Fig. 6).

To assess the rates of degradation of EGFR mRNA transcripts, we pre-incubated IOSE-80 cells, with or without FSH or LH, for 24 h. After pre-incubation, 5 μM of actinomycin-D was added to arrest new RNA synthesis. The cells were harvested at 2, 4 and 8 h following an addition of the transcription inhibitor, and the expression levels of EGFR mRNA was measured by RT-PCR. As shown in Fig. 7, in the presence of FSH and LH, the decay curves for EGFR mRNA in IOSE-80PC-80PC cells showed a significantly delayed half-life (5 h and 8 h) compared with the control group (4 h). Interestingly, FSH showed stronger activation of the EGFR gene than LH, whereas LH seems to be more effective than FSH in terms of EGFR mRNA stability.

**Discussion**

Although numerous theories have been proposed to explain the etiology of ovarian cancer, the exact pathogenesis of ovarian cancer remains ambiguous. One of the predominant theories is a gonadotropin theory that circulating levels of pituitary gonadotropins increase the risk of malignancy and that pregnancies and oral contraceptives protect by suppressing secretion of these hormones (Gardner 1961, Stadel 1975). Based on recent studies, treatments with FSH and LH/hCG seem to result in growth stimulation in normal, immortalized OSE and some ovarian cancer cells in a dose- and time-dependent manner in vitro (Wimalasena et al. 1992, Kurbacher et al. 1995, Kraemer et al. 2001, Ohtani et al. 2001, Parrott et al. 2001, Syed et al. 2001, Choi et al. 2002), although there are controversial reports (Wimalasena et al. 1991, Venn et al. 1995, Ivarsson et al. 2001, Tourgeman et al. 2002). Despite these observations, whether gonadotropins may play a role in normal OSE biology and
ovarian tumors remains to be fully elucidated and the exact mechanism of the response to gonadotropins is not clearly understood. The growth of human ovarian carcinoma has been promoted by elevated levels of gonadotropins through induction of tumor angiogenesis in vivo (Schiffenbauer et al. 1997, Zygmunt et al. 2002), and the level of vascular endothelial growth factor (VEGF) was significantly elevated in both low malignant potential (LMP) and serous ovarian carcinoma (Wang et al. 2002). In addition, gonadotropin-induced stimulation results in an increase in the expression of integrin subunit alpha (v) and CD44, the cell surface hyaluronan receptor in MLS human epithelial ovarian carcinoma cells (Schiffenbauer et al. 2002). The treatment of epithelial ovarian cancer with FSH significantly increases the levels of PKC alpha mRNA and protein, suggesting that the stimulation of PKC alpha transcription is involved in the FSH-induced cell proliferation in these cells (Ohtani et al. 2001). In OVCAR-3 cells, gonadotropins stimulate estradiol secretion and modulate steroid-dependent growth stimulation (Kraemer et al. 2001). Both FSH and LH stimulates cellular growth of human OSE and ovarian cancer, and interleukin 6 (IL-6)/signal transducer and activator of transcription-3 (STAT3) signaling pathways play a role in FSH-, LH- and estrogen-stimulated immortalized OSE cell proliferation (Sundfeldt et al. 1997, Syed et al. 2002).

It is of interest that recent studies suggest that the interactions between gonadotropins and growth factors, for instance combined treatment of hCG plus estradiol, may regulate the growth response of epithelial ovarian cancer cells through insulin-like growth factor (IGF)-I pathway (Wimalasena et al. 1993). In addition, FSH- and hCG-stimulated steady state levels of keratinocyte growth factor (KGF), hepatocyte...
growth factor (HGF) and kit ligand (KL) mRNA in bovine OSE cells, indicating a possible role of gonadotropins in enhancing these growth factors (Shoham 1994). In this regard, we hypothesize that gonadotropins, FSH and LH, may induce neoplastic transformation and cross-talk with growth factors in ovarian epithelial cells and these changes may affect the growth of OSE indirectly. In the present study, we demonstrated that treatment with FSH and LH significantly increased EGFR mRNA and EGFR protein in the immortalized OSE cell line, IOSE-80PC, while the same treatment resulted in only a mild increase in OVCAR-3 and no change in SKOV-3 cells. Based on this finding and the constitutive EGFR expression of these cell lines shown in Figure 1, there seem to be a reverse correlation between endogenous EGFR levels of cell lines and their sensitivity of EGFR induction to gonadotropins. Alper et al. demonstrated that EGFR overexpression in ovarian cancer cells is dominant (70–100%) and results in multiple invasive phenotypic overexpression in ovarian cancer cells is dominant (Alper et al. 2001). Furthermore, late stage carcinoma generally has strong invasive potential. In this regard, the effect of gonadotropins on EGFR expression appears to be more potent in normal or early stages of cancers which may have low basal EGFR levels. In addition, IOSE-80 cells treated with gonadotropins and EGF (10 nM) exhibited an additive stimulation of mitogenesis as measured by [3H]thymidine incorporation assay.

Overexpression of EGFR in ovarian cancer plays an important role in invasion and chemoresistance as well as proliferation (Alper et al. 2001, Liang et al. 2003). Therefore, EGFR up-regulation by gonadotropins implicates the further role of gonadotropins in invasion and chemoresistance. These results suggest that FSH and LH can stimulate EGFR expression in OSE during menopause, and this effect may play a role in the initiation and/or progression of the ovarian cancer because postmenopausal women have high gonadotropin levels in their serum. Our finding of increased EGFR expression in response to FSH and LH is consistent with other previous studies. In bovine OSE cells, Doraiswamy et al. demonstrated two- to three-fold increases in EGFR mRNA expression after exposure to FSH and LH (Doraiswamy et al. 2000). However, they did not show the alteration of its protein level and the mechanism of action of these hormones. In the hamster ovary, FSH significantly induced EGFR expression in granulosa, theca, and interstitial cells, whereas hCG stimulated its expression in theca and interstitial cells but not in granulosa cells (Garnett et al. 2002). Fujinaga and coworkers have reported that FSH and LH increased EGFR in rat granulosa cells (Fujinaga et al. 1994). In human testis, Foresta and Varotto demonstrated that EGFR protein was highly expressed in the subjects showing high FSH plasma levels and in all of the patients who received exogenous FSH (Foresta & Varotto 1994). However, the previous studies did not address the mechanism of FSH and LH effects on the EGFR expression. The regulation of EGFR at the level of mRNA decay has been demonstrated in a variety of cancer cell lines (Libermann et al. 1985, McCulloch et al. 1998, Balmer et al. 2001). The observed increase in EGFR mRNA levels by FSH and LH may be the result of increased EGFR gene transcription and/or messenger RNA stability. These hormones seem to have dual effects although the preference was different. FSH mainly increased transcriptional activity from a luciferase reporter construct containing the full-length EGFR promoter in transiently transfected IOSE-80PC cells, whereas LH was found to prolong the half life of EGFR mRNA stability predominantly. However, treatments with gonadotropins did not alter the trans-activation of EGFR in transiently transfected SKOV-3 cells with luciferase-EGFR promoter (data not shown). Because FSH and LH activate ERK1/2 and PI3K signaling pathways in a distinct pattern, we may speculate that the distinct regulation of EGFR in IOSE cells by FSH and LH is at least derived from this divergence of signaling activation as well as activation of gene transcription. Further study of the mechanism is necessary to investigate an exact role for FSH or LH in ovarian cancer cells. The determination of the transcriptional mechanism that regulates EGFR expression in OSE and ovarian cancer may provide important insights into the mechanism that controls the response of ovarian surface epithelium to EGF and/or TGFα through the modulation of its receptor levels.

MAPKs are a group of serine/threonine kinases that are activated in response to a diverse array of extracellular stimuli, and mediate signal transduction from the cell surface to the nucleus (Davis 1994, Cobb & Goldsmith 1995). The signals transmitted through this cascade lead to activation of a set of molecules that regulate cell growth, division and differentiation. In ovarian cancer cells, MAPKs are regulated by cisplatin (Persons et al. 1999), paclitaxel (Wang et al. 1999), endothelin-1 (Vacca et al. 2000), gonadotropin-releasing hormone (GnRH) (Kimura et al. 1999), and FSH (Choi et al. 2002). Moreover, in a previous study, we have shown that FSH stimulated the activation of MAPK cascade and phosphorylated Elk-1 in human OSE cells, which was responsible for proliferation by FSH (Choi et al. 2002). Phosphoinositide 3-kinase (PI3K) signaling pathway is now accepted as being at
least as important as the ras-MAP kinase pathway in cell survival and proliferation, and hence its potential role in cancer is of great interest. In ovarian cancer, it is well known that the PI3K signaling pathway plays a role in proliferation, anti-apoptosis, differentiation, tumorigenesis and angiogenesis (Chang et al. 2003, Brader & Eccles 2004, Vara et al. 2004). PI3Ks are stimulated by estrogen, 4-hydroxy estradiol, hypoxia and Lyosphatidic acid in ovarian cancer (Lu et al. 2002, Gao et al. 2004, Xu et al. 2004). This study is the first report to present the activation of PI3K by gonadotropins in OSE, although there is increasing evidence of the potential involvement of the PI3K pathway in gonadotropin signaling in other reproductive tissue, such as granulosa, Sertoli cells and oocytes (Carvalho et al. 2003, Alam et al. 2004, Hoshino et al. 2004, Meroni et al. 2004).

In the present study, after gonadotropin treatment, IOSE-80PC cells showed Akt and ERK1/2 activation, and LY294002 (a PI3K inhibitor) and PD98059 (an ERK1/2 inhibitor) partially blocked the gonadotropin-induced EGFR up-regulation. These results suggest that the effect of gonadotropins on EGFR expression is mediated, at least in part, through PI3K and ERK1/2 signaling pathways in these cells. Partial inhibition by the inhibitors may imply that an alternative pathway is involved and further study using the combination of inhibitors is necessary. It is of interest that the activation of ERK1/2 and PI3K by FSH is maintained in an activated status during 60 min, whereas their activation decreased at this time with LH. We examined the activation of these signaling pathways after longer treatment up to 24 h with FSH or LH (data not shown). The effects of FSH lasted more than 2 h, while the effects of LH had already returned to basal level at 60 min. These data suggest that FSH and LH show the diverse activation status of ERK and PI3K signaling pathways, and this discrepancy may relate to the different mechanisms of gonadotropins to increase EGFR mRNA as shown in Figures 6 and 7.

Taken together, gonadotropins may be a contributing factor in ovarian tumorigenesis, presumably by enhancing cell proliferation through EGFR up-regulation. In addition, the effect of gonadotropins on the expression of EGFR may involve cell growth via ERK-1/2 and PI3K pathways in pre-neoplastic ovarian surface epithelial cells. It appears that FSH and LH increased EGFR mRNA in a distinct manner. The former increased EGFR gene transcription essentially, whereas the latter mainly enhanced EGFR mRNA stability. These findings may provide a possible mechanism of action of gonadotropins in part in the progression of ovarian cancers related with growth factor receptors.

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