Gonadotropins Induce Tumor Cell Migration and Invasion by Increasing Cyclooxygenases Expression and Prostaglandin E₂ Production in Human Ovarian Cancer Cells

Man-Tat Lau, Alice S. T. Wong, and Peter C. K. Leung

Department of Obstetrics and Gynecology (M.-T.L., P.C.K.L.), Child and Family Research Institute, University of British Columbia, Vancouver, British Columbia, Canada V6H 3V5; and the School of Biological Sciences (A.S.T.W.), University of Hong Kong, Hong Kong

Gonadotropins (FSH and LH) are detectable in ovarian tumor fluid, suggesting a possible role for gonadotropins in ovarian carcinogenesis and progression. However, the molecular mechanisms behind the role of gonadotropins in ovarian cancer development are unknown. Cyclooxygenase (COX) enzymes, COX-1 and COX-2, play crucial roles in the pathogenesis of human malignancies. The purpose of the current study was to determine whether the effect of gonadotropins on ovarian cancer invasion is mediated by a COX-dependent mechanism. Here, we reported that FSH/LH can promote prostaglandin E₂ (PGE₂) production in ovarian cancer cells via COX-1 and -2 up-regulation at the protein and mRNA level. The phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway was required for gonadotropin-mediated up-regulation of COX-1 and COX-2. Moreover, treatment with COX-1- and COX-2-specific inhibitors abrogated the stimulatory effect of gonadotropins on motility and invasive activity. Western blot and gelatin zymography showed that COX-1 and COX-2 were critical for gonadotropin-induced expression of metastasis-related proteinases, matrix metalloproteinase (MMP)-2 and MMP-9. In addition, our results showed that PGE₂ induced an increase in cell invasiveness and the expression of MMP-2 and MMP-9 in ovarian cancer cells. These data show that COX-1 and COX-2 play essential roles in gonadotropin-induced migration and invasion. (Endocrinology 151: 2985–2993, 2010)
chidonic acid into two short-lived intermediates, prostaglandin G₂ (PGG₂) and prostaglandin H₂ (PGH₂), which is then metabolized to various PGs by the activity of specific synthases (22). Two cyclooxygenase isoforms have been identified and are referred to as COX-1 and COX-2. The COX-1 and COX-2 enzymes are involved in tumor progression by promoting proliferation, survival, invasion, and metastasis in several solid tumors (22–27). In ovarian cancer, elevated expression of COX-1 and COX-2 has been reported recently, suggesting that these enzymes play a key role in the progression of ovarian carcinoma (28–33).

The current study was undertaken to test the hypothesis that gonadotropins (FSH and LH) promote the metastatic phenotype of ovarian cancer cells via a COX-dependent mechanism. Treatment of ovarian tumor cells with FSH and LH in vitro induced COX-1 and COX-2 mRNA and protein expression in a time- and dose-dependent manner. Furthermore, gonadotropin increased COX-1 and COX-2 expression via the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway. Inhibition of COX-1 and COX-2 decreased gonadotropin-induced prostaglandin E (PGE)₂ production and MMP-2 and MMP-9 activity and blocked gonadotropin-mediated motility and invasion. These data show that COX-1 and COX-2 contribute to gonadotropin-mediated ovarian cancer progression.

Materials and Methods

Materials

Human LH and recombinant FSH were obtained from Dr. A. F. Parlow (National Hormone and Pituitary Program, Harbor-University of California-Los Angeles Medical Center, Torrance, CA). COX-1 and COX-2 antibodies, SC-560, and NS-398 were purchased from Cayman Chemical (Ann Arbor, MI). LY294002 was purchased from Calbiochem (San Diego, CA). FSH receptor (FSHR) and LH receptor (LHR) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MMP-2 and MMP-9 monoclonal antibodies were purchased from Neomarkers (Fremont, CA), and anti-AKT and anti-phospho-AKT (Ser 473) were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Cell culture

The human ovarian cancer cell lines (CaOV-3 and SKOV-3) were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and their use was approved by the University of British Columbia Clinical Screening Committee for Research and Other Studies Involving Human Subjects. Cells were cultured in Medium 199:MCDB 105 (1:1; Sigma-Aldrich Corp., St. Louis, MO) containing 10% fetal bovine serum (FBS; Hyclone Laboratories Ltd., Logan, UT), 100 U/ml penicillin G and 100 μg/ml streptomycin (Life Technologies, Inc., Rockville, MD) in a humidified atmosphere of 5% CO₂-95% air at 37 C. The cells were passaged with 0.06% trypsin (1:250) at 0.01% EDTA in Mg²⁺/Ca²⁺-free Hank’s balanced salt solution at confluence. For monolayer cultures, the cell lines were maintained on tissue culture dishes ( Falcon, Becton Dickinson, Franklin Lakes, NJ). Human granulosa cells (hGC) cells were obtained from women undergoing in vitro fertilization treatment, and their use was approved by the Research Ethics Board of the University of British Columbia. Granulosa cells from each patient were extracted as described previously (34) and were cultured in DMEM/F-12 (Sigma-Aldrich Corp.) supplemented with 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂-95% air at 37 C. After 48 h, the hGL cells were collected for protein extraction.

Semiquantitative RT-PCR

Total RNA was prepared using the TRizol reagent (Invitrogen Canada, Burlington, Ontario, Canada), according to the manufacturer’s instructions. Single-stranded cDNA was synthesized from 2 μg of total RNA following the manufacturer’s procedure (Amersham Biosciences, Quebec, Canada). The following primers (Invitrogen) were used for PCR amplification: COX-1, sense, 5’-TTC CCA GCT CCT GGC CCG CCTT-3’; antisense, 5’-GTG CAT CAA CAC AGG CGC CTC TTC-3’; COX-2, sense, 5’-CCC TTG GGT GTC AAA GGT AA-3’; antisense, 5’-GCC CTC GCT GAT CAT GTG TC-3’. The relative mRNA levels were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, which were determined with the following specific primers: sense 5’-ATG GAA TGC CCA TCA TCA TCT T-3’; antisense, 5’-GCC CCC ACT TGA TTT TGG-3’. The cycle numbers that generated half-maximal amplification were used for subsequent semiquantitative analysis of gene expression; these values were 33 cycles for COX-1 and COX-2 and 22 cycles for GAPDH. The PCR was carried out with protocol of 30 sec at 94 C, 30 sec at 58 C, and 1 min at 72 C. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel and the signal intensity was quantified with the National Institutes of Health (Bethesda, MD) ImageJ software.

Western blot analysis

Cells were harvested in lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS)] that contained a protease inhibitor cocktail (Sigma), and protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Protein (40 μg) was electrophoresed on a 7.5% SDS-PAGE. To determine the secretion of MMPs from ovarian cancer cells, conditioned medium (40 μl) was electrophoresed under reducing conditions on a 7.5% SDS-PAGE, transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Oakville, Ontario, Canada), and reacted with specific primary antibodies at 4 C overnight. The signals were detected with horseradish peroxidase-conjugated secondary antibody for 1 h and were visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech), and the signal intensity was quantified with the ImageJ software.

Invasion and migration assays

Twenty-four-well transwell inserts with an 8-μm pore coated with 1 mg/ml Matrigel (50 μl/well; BD Sciences, Mississauga, Ontario, Canada) were used to assess cell invasion. Trypsinized cells (1×10⁵) in serum-free medium, with or without FSH/LH,
were seeded in triplicate in the upper chamber. The 5% FBS medium was placed in the lower wells. The chambers were incubated for 48 h at 37°C in a 5% CO₂ atmosphere. Cells that had not penetrated the filter were wiped off, and invaded cells on the lower surface of the filter were fixed with ice-cold methanol and stained with 0.5% crystal violet. Results are presented as the mean number of invaded cells of five fields (at 100× magnification) ± SEM from three independent experiments. To assess cellular migration potential, the protocol described above was used, except that Matrigel was omitted and 1% FBS medium was placed in the lower chamber as a chemoattractant.

**PGE₂ enzyme-linked immunosorbent assay**

Levels of PGE₂ released into the medium of treated cells were analyzed by PGE₂ enzyme-linked immunosorbent assay (Cayman Chemical) according to the manufacturer’s instructions. Synthesized PGE₂ concentrations were normalized against protein concentrations measured in the corresponding whole-cell extracts.

**Gelatin zymography**

To measure the activity of MMPs, the conditioned medium was incubated with nonreducing dilution buffer before electrophoresis on a 12.5% SDS-polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 to remove the SDS and was incubated overnight at 37°C in a buffer [50 mM Tris (pH 7.4), 0.2M NaCl, 5 mM CaCl₂, 0.02% Brij 35] that allows both progelatinase and active gelatinase to digest the gelatin. The gel was then stained with Coomassie blue G-250 (Bio-Rad Laboratories) to visualize gelatinase activity. Values represent the relative MMP activity (both pro- and active-form) and were normalized based on the measurement of viable cell number using an MTT assay.

**Data analysis**

All experiments were performed at least three times. All values are expressed as means ± SEM. Data were analyzed by one-way ANOVA followed by Tukey’s post hoc test using GraphPad Prism 5 (GraphPad Software, San Diego, CA). P < 0.05 was considered statistically significant.

**Results**

**Effect of gonadotropins on COX-1 and COX-2 expression in ovarian cancer cells**

As a first step toward analyzing the role of the COX pathway in gonadotropin-mediated ovarian cancer progression, we investigated the effect of gonadotropins (FSH and LH) on COX-1 and COX-2 expression in CaOV-3 and SKOV-3 cells, which express functional FSH and LH receptors (Fig. 1). Our results showed that treatment with FSH and LH stimulated COX-1 and COX-2 mRNA expression in SKOV-3 cells (Fig. 2). FSH and LH treatment also resulted in a time- and dose-dependent up-regulation of COX-1 and COX-2 protein expression in CaOV-3 and SKOV-3 cells (Fig. 3, A and B).

**Gonadotropin-induced COX-1 and COX-2 up-regulation via the PI3K pathway in SKOV-3 cells**

It is well documented that the PI3K/AKT pathway is frequently amplified and serves as a survival pathway in ovarian carcinomas (35, 36). In addition, gonadotropins are known to activate the PI3K/AKT pathway in human ovarian surface epithelial cells and ovarian carcinoma cell lines (12, 37, 38). The PI3K signaling pathway and its downstream protein, glycogen synthase kinase 3β (GSK3β), have been reported to regulate COX-1 and COX-2 expression (39, 40). Therefore, we analyzed whether the PI3K/AKT pathway was involved in gonadotropin-induced COX-1 and COX-2 expression in SKOV-3 cells. We first examined the phosphorylation status of AKT molecules upon treatment with 100 ng/ml FSH and LH at 5, 15, 30, 60, and 120 min post-treatment. As shown, treatment with FSH
and LH stimulated the activation of AKT (Ser 473) in a
time-dependent manner (Fig. 4A). The selective PI3K in-
hibitor, LY294002, markedly inhibited gonadotropin-in-
duced COX-1 and COX-2 expression (Fig. 4C), showing
the significance of the PI3K/AKT pathway in gonadotro-
pin-mediated up-regulation of COX-1 and COX-2 expres-
sion in SKOV-3 cells.

Gonadotropin-induced motility and invasion are
COX-1- and COX-2-dependent in SKOV-3 cells

Gonadotropins have been previously reported to pro-
mote the invasiveness of ovarian cancer cells (12). To de-
termine the motility of ovarian cancer cells, a Transwell
migration assay was performed. The effect of COX-1 and
COX-2 inhibition on gonadotropin-stimulated cell migra-
tion was evaluated. Our results showed that treatment of
cells with a COX-1-specific inhibitor (SC-560) or COX-
2-specific inhibitor (NS-398) blocked gonadotropin-stimulated migration at
24 h (Fig. 5A).

To further examine whether COX-1 and COX-2 activity is required for the
invasive capacity of ovarian cancer cells, a Matrigel-coated Transwells in-
vasion assay was performed. Results showed that the inhibition of COX-1 and
COX-2 activity, by SC-560 and NS-398, respectively, abrogated the stimulatory effect of gonadotropins on
CaOV-3 and SKOV-3 cell invasive activity (Fig. 5B). We found that FSH and
LH significantly increased the invasiveness of CaOV-3 and SKOV-3 cells and
that pretreatment with LY294002, a PI3K inhibitor, markedly blocked go-
adotropin-induced invasion (data not shown), which was consistent with our
recent findings (12). Taken together, COX-1 and COX-2 play essential roles
in gonadotropin-stimulated ovarian cancer cell motility.

Gonadotropin-mediated
up-regulation of PGE2 production
and MMP-2 and MMP-9 are COX-1-
and COX-2-dependent in SKOV-3
cells

To examine whether the increase of
COX-1 and COX-2 expression is asso-
ciated with elevated prostaglandin syn-
thesis, the level of PGE2 released from
CaOV-3 and SKOV-3 cells was mea-
sured using ELISA. FSH and LH in-
creased PGE2 levels in the conditioned medium of CaOV-3
and SKOV-3 cells in a COX-1- and COX-2-dependent
manner (Fig. 6A). These data indicate that both enzymes
participate in PGE2 production.

As previously reported, FSH and LH stimulate the pro-
duction of MMP-2 and MMP-9 (12). Next, we asked
whether COX-1 and COX-2 mediate the gonadotropin-
induced activity of MMP-2 and MMP-9. SKOV-3 cells
were pretreated with COX-1 (SC-560) and COX-2 (NS-
398)-specific inhibitors and were thentreated with
FSH/LH for an additional 48 h. MMP-2 and MMP-9 ac-
tivities were evaluated using gelatin zymography. Treat-
ment with SC-560 and NS-398 blocked the ability of FSH
and LH to induce both pro-form and active-form of
MMP-2 and MMP-9, implicating COX-1 and COX-2 as mediators of gonadotropin-mediated MMP-2 and
MMP-9 activation (Fig. 6B). This result was confirmed by Western blotting, which showed that gonadotropin-mediated MMP-2 and MMP-9 secretion was inhibited by SC-560 and NS-398 (Fig. 6C). These results suggest that COX-1 and COX-2 are required for gonadotropin-enhanced PGE2 production and MMP-2 and MMP-9 secretion and activity.

**PGE2 stimulates cell invasion and MMP-2 and -9 activities in SKOV-3 cells**

Several lines of evidence indicate that COX-derived PGE2 also plays an important role in the invasive properties of human cancer cells (41–45). Thus, we examined the effect of PGE2 on cell invasion and MMP activity in ovarian cancer cells. CaOV-3 and SKOV-3 cells were treated with increasing concentrations of PGE2, which resulted in a dose-dependent biphasic regulation on invasion: cells treated with PGE2 at low doses of 0.1 nM to 10 nM showed significantly higher numbers of invaded cells, whereas the response to higher doses of PGE2 (100 nM) was insignificant (Fig. 7A). We also examined whether the effect of PGE2 on ovarian cancer invasion was associated with altered MMP activity and production. As shown in Fig. 7, B and C, treatment with the PGE2 induced a biphasic regulation pattern for MMP activity and secretion in SKOV-3 cells. These data indicate that COX-derived PGE2 may contribute to gonadotropin-induced cell invasiveness in ovarian cancer.

**Discussion**

The presence of gonadotropins in the ovarian tumor fluid of ovarian cancer patients suggests the importance of these factors in the transformation and progression of ovarian cancers (8–11). The observation of high FSH and LH lev-
COX-1 and COX-2 inhibitors suppress gonadotropin-induced PGE2 production and MMP-2 and MMP-9 expression and activity. SKOV-3 cells were pretreated for 30 min with 50 nM of SC-560 or 10 μM of NS-398 before treatment with 100 ng/ml of FSH/LH for 24 h (A) or 48 h (B and C), as indicated. A, PGE2 production from SKOV-3 cells was analyzed by PGE2 ELISA. PGE2 concentrations normalized to corresponding cell protein content. Results represent the mean ± SEM (n = 3; *, P < 0.05). B, Enzymatic activity of MMP-2 and MMP-9 was studied in conditioned medium by SDS-PAGE gelatin zymography as described in Materials and Methods. Arrows indicate gelatinase activity corresponding to pro-MMP-2, active MMP-2, pro-MMP-9, and active MMP-9. Results represent the mean ± SEM (n = 3; *, P < 0.05; **, P < 0.001). The values were normalized based on the measurement of viable cell number using an MTT assay. C, Conditioned medium was analyzed by Western blotting using MMP-2 and MMP-9 antibodies. Results represent the mean ± SEM (n = 3; *, P < 0.05). The values were normalized based on the measurement of viable cell number using an MTT assay.

PGE2 induced SKOV-3 cell invasion and MMP-2 and MMP-9 expression and activity. SKOV-3 cells were treated with (0.01 nM to 100 nM) PGE2 for 24 h, as indicated. A, SKOV-3 cells were seeded in Matrigel-coated invasion chambers in the presence of PGE2 for 24 h, as indicated. Enzymatic activity (B) and expression (C) of MMP-2 and MMP-9 from SKOV-3 cells were studied as described in Fig. 5. Results represent the mean ± SEM (n = 3; *, P < 0.05; **, P < 0.001).
els in the cyst fluid of malignant ovarian tumors is consistent with the gonadotropin theory. Because the hypothesis that pituitary gonadotropins increase the risk of ovarian malignancy and that pregnancies and oral contraceptives protect the ovary by suppressing secretion of these hormones was previously suggested (46), numerous studies have examined the role of gonadotropins on ovarian cancer. Gonadotropins promote the proliferation, survival, and metastasis of ovarian cancer by inducing the expression of key regulatory genes (47). Proteinase expression is also modulated by gonadotropins in ovarian cancer cells, leading to gonadotropin-dependent changes in motility and invasive behavior (12). The present study shows that gonadotropins also induce COX-1 and COX-2 expression, and, in parallel, PGE2 production in ovarian carcinoma cells and, for the first time, implicates COX-1 and COX-2 as downstream mediators of gonadotropin-induced effects on tumor progression in human ovarian cancer cells.

Data regarding COX-2 expression in the normal ovary suggest that it is a component of the ovulatory process (48–50), whereas repeated ovulations were suggested to cause malignant transformation of ovarian epithelium. Recent epidemiological and clinical studies suggest that the COX-1 and COX-2 enzymes play a key role in the progression of ovarian carcinoma (28–33). Treatment with FSH and LH/human chorionic gonadotropin (hCG) has been shown to up-regulate the expression of COX-1 and COX-2 in different human cells and tissues (14–17, 19, 51). In the current study, treatment of ovarian tumor cells with FSH and LH in vitro induced COX-1 and COX-2 mRNA and protein expressions in a time- and dose-dependent manner. These data indicate that FSH and LH may promote ovarian carcinoma progression through their effect on the expression of the COX-1 and COX-2 enzymes.

In ovarian cancer, the PI3K/AKT signaling pathway plays a role in proliferation, antiapoptosis, migration, invasion, and metastasis (52, 53). Emerging evidence suggests the involvement of the PI3K/AKT signaling pathway in regulation of COX-1 and COX-2 expression (40, 54, 55). Gonadotropins may cause the activation of PI3K and AKT in other reproductive tissues, including granulose cells, Sertoli cells, and oocytes (56–58). As for ovarian cancers, we showed previously that both FSH and LH not only increased EGFR levels in human OSE cells (37) but also activated proteolysis and increased invasion in ovarian cancer cells through the PI3K/AKT signaling pathway (12). The current data support a role for gonadotropins in the induction of COX-1 and COX-2 expression, at least in part, via the PI3K/AKT pathway in ovarian carcinoma cells. The activation of the PI3K/AKT pathway was confirmed by the increased phosphorylation of AKT in the presence of FSH and LH (Fig. 4A). In agreement with our previous study (12), FSH- and LH-induced activation of AKT was completely abolished by pretreatment with LY294002, a PI3K inhibitor (Fig. 4B). Aberrant inhibition of the PI3K signaling pathway also blocked gonadotropin-induced stimulation of COX-1 and COX-2 expression in ovarian carcinoma cells (Fig. 4C).

The ability of COX-1-specific and COX-2-specific inhibitors to reduce gonadotropin-mediated motility and invasion indicates that COX-1 and COX-2 play a major role in ovarian tumor cell motility and invasion. There is increasing evidence that COX-1 and COX-2 contribute to tumor progression by promoting tumor cell proliferation, angiogenesis, and invasion (23, 32, 45, 54). Moreover, overexpression of COX-2 elevated tumorigenicity, tumor growth and invasion of human epidermoid carcinoma cells via up-regulated MMP (59). Similarly, COX-2-overexpressing colon carcinoma cells exhibit enhanced pro-MMP-2 activation and invasiveness that is blocked by treatment with a COX inhibitor (60), whereas COX-2 inhibition leads to decreased MMP-2 expression in different cancer cells, including ovarian cancer cells (61–63). We have reported previously that both FSH and LH enhanced ovarian cancer cell invasiveness via the activation and/or expression of MMP-2 and MMP-9 (12). Our current data show that the addition of specific COX-1 and COX-2 inhibitors blocked gonadotropin-induced MMP-2 and MMP-9 expression and activity, demonstrating the key role of COX-1 and COX-2 in the gonadotropin-induced MMP-dependent motility and invasiveness of ovarian carcinoma cells.

The ability of COX-specific inhibitors to reduce gonadotropin-induced PGE2 production indicates that both COX-1 and COX-2 play important roles in PGE2 formation in ovarian cancer cells. There is increased evidence that PGE2 also contributes to tumor progression by promoting cell invasion and that this effect is mediated, at least in part, by the modulation of MMP activity (45). We reported previously (12) that gonadotropin stimulates ovarian cancer cell invasion and MMP activity. Here, we demonstrated that, in SKOV-3 cells, gonadotropin-induced PGE2 production is involved in cell invasion and MMP activity, indicating that gonadotropin-induced cell invasion may be regulated via COX-dependent PGE2 signaling.

In conclusion, the present results implicate gonadotropins in the induction of COX-1 and COX-2 expression, which then mediate downstream effects on ovarian cancer progression. Given that these two enzymes contribute to PGE2 production in these tumors, the possibility of inhibiting COX- and PGE2-related signaling cascades may have
relevant implications in the prevention and treatment of this malignancy. Taken together, our results indicate that gonadotropins may increase COX-dependent invasion by activating the PI3K pathway. Because ovarian cancer progression involves metastasis and is more common in conditions with high levels of gonadotropins, understanding the cellular and molecular mechanisms behind the role of gonadotropins in invasion and/or metastasis may help elucidate the etiology of ovarian cancer development.

Acknowledgments

Address all correspondence and requests for reprints to: Dr. Peter C. K. Leung, Department of Obstetrics and Gynecology, University of British Columbia, 2H30, 4490 Oak Street, Vancouver, British Columbia, Canada V6H 3V5. E-mail: peleung@interchange.ubc.ca.

This work was supported by the Canadian Institutes of Health Research (to P.C.K.L.) and the Hong Kong Research Grants Council (to A.S.T.W.). P.C.K.L. is recipient of a Distinguished Scientist Award, and M.T.L. is recipient of Graduate Studentship Awards from the Interdisciplinary Women’s Reproductive Health Research Training Program.

Disclosure Summary: The authors have nothing to disclose.

References


Downloaded from https://academic.oup.com/endo/article-abstract/151/7/2985/2456735 by guest on 24 January 2018