COX2 and PGE2 mediate EGF-induced E-cadherin-independent human ovarian cancer cell invasion

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Abstract

Elevated expression of cyclooxygenase 2 (COX2 (PTGS2)) has been reported to occur in human ovarian cancer and to be associated with poor prognosis. We have previously demonstrated that COX2-derived prostaglandin E2 (PGE2) promotes human ovarian cancer cell invasion. We had also demonstrated that epidermal growth factor (EGF) induces human ovarian cancer cell invasion by downregulating the expression of E-cadherin through various signaling pathways. However, it remains unclear whether COX2 and PGE2 are involved in the EGF-induced downregulation of E-cadherin expression and cell invasion in human ovarian cancer cells. In this study, we showed that EGF treatment induces COX2 expression and PGE2 production in SKOV3 and OVCAR5 human ovarian cancer cell lines. Interestingly, COX2 is not required for the EGF-induced downregulation of E-cadherin expression. In addition, EGF treatment activates the phosphatidylinositol-3-kinase (PI3K)/Akt and cAMP response element-binding protein (CREB) signaling pathways, while only the PI3K/Akt pathway is involved in EGF-induced COX2 expression. Moreover, we also showed that EGF-induced cell invasion is attenuated by treatment with a selective COX2 inhibitor, NS-398, as well as PGE2 siRNA. This study demonstrates an important role for COX2 and its derivative, PGE2, in the mediation of the effects of EGF on human ovarian cancer cell invasion.

Key Words

- EGF
- COX2
- PGE2
- ovarian cancer

Introduction

Epithelial ovarian cancer accounts for approximately 90% of all ovarian malignancies. It is the leading cause of gynecological cancer death in developed countries because of the majority of patients present with disseminated disease, for which the average 5-year survival rate is very low (Jemal et al. 2005, Landen et al. 2008). The expression of epidermal growth factor receptor (EGFR) is upregulated in many types of human cancers (Hanawa et al. 2006, Lee 2006, Viana-Pereira et al. 2008). The overexpression of EGFR in human ovarian cancer is associated with poor prognosis and disease progression (Bartlett et al. 1996, Niikura et al. 1997, Baselga 2002, Mendelsohn & Baselga 2003). Our recent studies have demonstrated that EGF induces human ovarian cancer cell invasion by downregulating the expression of the cell-cell adhesion molecule E-cadherin through various signaling pathways (Cheng et al. 2010, 2012a,b, 2013a,b,c).

Cyclooxygenase (COX) is a key enzyme that catalyzes the conversion of arachidonic acid into prostaglandins (PGs). PGE2 is an important PG that acts in an
autocrine/paracrine manner to regulate various physiological and pathological functions (Nakanishi & Rosenberg 2013). Two isoforms of COX, COX1 and COX2, have been described. The expression of COX1 (PTGS1) occurs constitutively in most tissues and COX1 maintains homeostasis, while the expression of COX2 (PTGS2) is induced by various stimuli, including cytokines, mitogens, and hypoxia (Smith et al. 1996, Kniss 1999). There is increasing evidence that the expression of COX2 is elevated in several types of human cancers, including skin, liver, breast, and lung cancers (Soslow et al. 2000, Bae et al. 2001, Shirahama & Sakakura 2001). Previous studies have shown that COX2-specific inhibitors can reduce the growth of human cancers (Dannenberg et al. 2003). Two isoforms of COX, COX1 and COX2, have been described. The expression of COX1 (PTGS1) occurs constitutively in most tissues and COX1 maintains homeostasis, while the expression of COX2 (PTGS2) is induced by various stimuli, including cytokines, mitogens, and hypoxia (Smith et al. 1996, Kniss 1999). There is increasing evidence that the expression of COX2 is elevated in several types of human cancers, including skin, liver, breast, and lung cancers (Soslow et al. 2000, Bae et al. 2001, Shirahama & Sakakura 2001). Previous studies have shown that COX2-specific inhibitors can reduce the growth of human cancers (Dannenberg et al. 2003).

Materials and methods

Cell culture

The SKOV3 human ovarian cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The OVCAR5 ovarian cancer cell line was kindly provided by Dr T C Hamilton (Fox Chase Cancer Center, Philadelphia, PA, USA). The cells were grown in a 1:1 (v/v) mixture of M199/MCDB105 medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT, USA). The cultures were maintained at 37 °C in a humidified 5% CO2 atmosphere.

Antibodies and reagents

The monoclonal anti-E-cadherin antibody was obtained from BD Biosciences (Mississauga, ON, Canada). The polyclonal anti-COX2 antibody was obtained from Abcam (Toronto, ON, Canada). The monoclonal anti-α-tubulin and polyclonal anti-EGFR antibodies were obtained from Santa Cruz Biotechnology. The polyclonal anti-phospho-AKT, anti-AKT, anti-phospho-CREB1, and anti-CREB1 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories. Human EGF, LY294002, and AG1478 were obtained from Sigma. Wortmannin was obtained from Calbiochem (Gibbstown, NJ, USA). NS-398 was purchased from Cayman Chemical (Ann Arbor, MI, USA).

siRNA transfection

To knock down endogenous EGFR, CREB, AKT (AKT1), or PGE2, the cells were transfected with 50 nM ON-TARGET- plus SMARTpool siRNA (Dharmacon Research Inc., Lafayette, CO, USA) using Lipofectamine RNAiMAX (Invitrogen). siCONTROL non-targeting siRNA (Dharmacon Research, Inc.) was used as a transfection control.

Western blot analyses

The cells were lysed in a lysis buffer (Cell Signaling Technology), and protein concentrations were determined using a DC protein assay kit with BSA as the standard (Bio-Rad Laboratories). Equal amounts of protein were separated by SDS–PAGE and transferred onto PVDF membranes. After blocking with Tris-buffered saline containing 5% non-fat dried milk for 1 h, the membranes were separated by SDS–PAGE and transferred onto PVDF membranes. After blocking with Tris-buffered saline containing 5% non-fat dried milk for 1 h, the membranes were probed with appropriate primary antibodies and detected using ECL reagents (Amersham). The immunoblots were quantified using ImageJ software.

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were incubated overnight at 4 °C with primary antibodies followed by incubation with the HRP-conjugated secondary antibodies. Immunoreactive bands were detected using an ECL substrate (Pierce, Rockford, IL, USA).

**Real-time quantitative PCR**

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s instructions. RT was carried out with 3 μg RNA, random primers, and M-MLV reverse transcriptase (Promega). The following primers were used for SYBR Green real-time quantitative PCR (RT-qPCR): COX2, 5′-CCC TTG GGT GTC AAA GGT AA-3′ (sense) and 5′-GCG CTC GCT TAT GAT CTG TC-3′ (antisense); EGFR, 5′-GTT GCA GGA GAG GAG AAC TGC-3′ (sense) and 5′-GTT GCC ACC AAA GCT GTA TT-3′ (antisense); CREB1, 5′-AAA ACC AAC AAA TGA CAG TT-3′ (sense) and 5′-TTG ACT GTC TGC CCA TTG G-3′ (antisense); and GAPDH, 5′-GAG TCA ACG GAT TTG GTC GT-3′ (sense) and 5′-GAC AAG CTT CCC GTT CTC AG-3′ (antisense). RT-qPCR was carried out using the Applied Biosystems 7300 Real-Time PCR System (Perkin-Elmer, Woodbridge, ON, Canada) equipped with a 96-well optical reaction plate. All RT-qPCR results are presented as the mean of at least three independent experiments conducted in triplicate. The relative quantification of mRNA levels was performed by the comparative Ct method using GAPDH as the reference gene and the formula 2−ΔΔCt.

**PGE2 ELISA**

A human PGE2-specific ELISA was performed according to the manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI, USA). Culture media in which the treated cells were grown were collected, and the concentrations of PGE2 in the culture media were measured by ELISA. The concentrations of PGE2 were normalized to the protein concentrations in the cell lysates. The normalized PGE2 values of the treated cells are presented as relative values compared with those of the control cells.

**Invasion assays**

The invasion assays were performed in Boyden chambers as described previously, with minor modifications (Woo et al. 2007). Cell-culture inserts (24 wells, pore size 8 μm; BD Biosciences, Mississauga, ON, Canada) pre-coated with growth factor-reduced Matrigel (40 μl, 1 mg/ml; BD Biosciences) were used for the invasion assays. The cell-culture inserts were seeded with 1×10^5 cells in 250 μl of medium supplemented with 0.1% FBS. Medium supplemented with 10% FBS (750 μl) was added to the lower chamber and it served as a chemotactic agent. After incubation for 48 h, non-invading cells were removed from the upper side of the membrane and the cells on the lower side of the membrane were fixed with cold methanol and air-dried. The cell nuclei were stained with Hoechst 33258 and counted by epifluorescence microscopy using Northern Eclipse 6.0 Software (Empix Imaging, Mississauga, ON, Canada). Each individual experiment was performed in triplicate (i.e. three inserts), and five microscopic fields were counted per insert.

**Statistical analysis**

Results are presented as the mean ± S.E.M. of at least three independent experiments. The results were analyzed by one-way ANOVA and Tukey’s multiple comparison test using PRISM Software (PRISM, La Jolla, CA, USA). Significant differences were defined as P<0.05.

**Results**

**EGF treatment induces COX2 expression**

To determine whether EGF treatment can induce the expression of COX2 in human ovarian cancer cells, we treated two human ovarian cancer cell lines (SKOV3 and OVCAR5) with 100 ng/ml EGF for different periods of time and quantified the COX2 mRNA and COX2 protein levels. As shown in Fig. 1A, EGF treatment resulted in the upregulation of COX2 mRNA levels in both SKOV3 and OVCAR5 cells, with the most significant degree of upregulation occurring after 1 h of treatment. In addition, we also investigated the stimulatory effect of different concentrations of EGF (1, 10, 50, and 100 ng/ml) on COX2 mRNA levels. As shown in Fig. 1B, treatment with 50 and 100 ng/ml EGF significantly induced COX2 mRNA levels in SKOV3 cells, while treatment with only 100 ng/ml EGF could significantly induce COX2 mRNA levels in OVCAR5 cells. Moreover, western blot analyses revealed that treatment with EGF for 3 and 6 h significantly upregulated COX2 protein levels (Fig. 1C). To confirm that EGFR is required for EGF-induced COX2 expression, we blocked EGFR activation with the EGFR-specific inhibitor AG1478. RT-qPCR and western blot analyses revealed that AG1478 blocked the EGF-induced upregulation of COX2 mRNA and COX2 protein levels (Fig. 2A and B). We further confirmed the involvement of EGFR in EGF-induced COX2 expression by siRNA-mediated knockdown
As shown in Fig. 2 C and D, EGFR mRNA and EGFR protein levels were significantly downregulated in the presence of EGFR siRNA. In addition, the siRNA-mediated downregulation of EGFR expression attenuated the EGF-induced elevation of COX2 mRNA and COX2 protein levels. It has been shown that the binding of EGF to EGFR rapidly induces clustering and internalization of the ligand–receptor complexes, ultimately resulting in lysosomal degradation of both EGF and its receptor (Carpenter 1987). This process was supported by the data shown in Fig. 2D, which revealed that the expression of EGFR was downregulated in SKOV3 and OVCAR5 cells in response to EGF treatment.

**COX2 is not required for EGF-induced downregulation of E-cadherin expression**

We had previously shown that EGF induces human ovarian cancer cell invasion by downregulating E-cadherin expression through various signaling pathways (Cheng et al. 2010, 2012a,b, 2013a,b,c). However, it is not clear whether COX2 is involved in the EGF-induced downregulation of E-cadherin expression in human ovarian cancer cells. To investigate this, a selective COX2 inhibitor, NS-398, was used to block COX2 activity. Consistent with our previous studies, EGF downregulated E-cadherin mRNA levels in both SKOV3 and OVCAR5 cells. Interestingly, NS-398 treatment did not affect basal or EGF-downregulated E-cadherin mRNA levels (Fig. 3A). Western blot analyses revealed that NS-398 treatment also had no effect on basal and EGF-downregulated E-cadherin protein levels (Fig. 3B).

**Active AKT is required for the induction of COX2 expression by EGF**

We have previously shown that the PI3K/Akt signaling pathway is involved in gonadotropin-induced COX2 expression in human ovarian cancer cells (Lau et al. 2010). Therefore, we investigated whether the Akt signaling pathway is also involved in EGF-induced COX2 expression. Treatment with EGF increased the levels of phosphorylated AKT in both SKOV3 and OVCAR5 cells (Fig. 4A). To determine the involvement of PI3K/Akt
signaling in EGF-induced upregulation of COX2 expression, two PI3K inhibitors, wortmannin and LY294002, were used. Treatment with wortmannin and LY294002 suppressed EGF-induced AKT phosphorylation in both cell lines (Supplementary Fig. 1, see section on supplementary data given at the end of this article). In addition, the EGF-induced upregulation of COX2 mRNA and COX2 protein levels was attenuated by co-treatment with wortmannin and LY294002 (Fig. 4B and C). To avoid off-target effects of the pharmacological inhibitors and further confirm that AKT is involved in EGF-induced COX2 expression, an siRNA-mediated knockdown approach was used to block AKT activity. As shown in Fig. 4D, EGF-induced COX2 protein levels were suppressed by the knockdown of AKT. Taken together, these results clearly indicate that the PI3K/Akt signaling pathway is required for EGF-induced COX2 expression in human ovarian cancer cells.

**CREB does not mediate EGF-induced COX2 expression**

The cAMP response element (CRE) has been identified as one of the central regulatory elements in the COX2 promoter region and cAMP response element-binding protein (CREB) is well known to be involved in the regulation of COX2 gene expression in a variety of cells (Tanabe & Tohnai 2002, Klein et al. 2007). Thus, we investigated the involvement of the CREB signaling
COX2 is known to be involved in the regulation of E-cadherin expression in other human cancers (Sitarz et al. 2009). Treatment of gastric cancer cells with celecoxib, a specific COX2 inhibitor, attenuated the EGF-stimulated E-cadherin expression in gastric cancer cells with celecoxib, a specific COX2 inhibitor. However, it is not known whether COX2 mediates EGF-induced ovarian cancer cell invasion. In the present study, we showed that EGF could induce ovarian cancer cell invasion through a complex signaling network (Cheng et al. 2010). This indicates that EGF/EGFR and COX2 are important for the development and progression of human ovarian cancer. We have previously shown that EGF could induce ovarian cancer cell invasion. In the present study, we showed that EGF could induce ovarian cancer cell invasion through a complex signaling network (Cheng et al. 2010). This indicates that EGF/EGFR and COX2 are important for the development and progression of human ovarian cancer.

**Discussion**

The overexpression of EGFR and COX2 in human ovarian cancer is correlated with poor prognosis and survival rates (Ali-Fehmi et al. 2003, Siwak et al. 2010, Lee et al. 2013). This indicates that EGF/EGFR and COX2 are important for the development and progression of human ovarian cancer.

COX2 is known to be involved in the regulation of E-cadherin expression in other human cancers (Dohadwala et al. 2006, Sitarz et al. 2009). Treatment of gastrin cancer cells with celecoxib, a specific COX2 inhibitor, attenuated the EGF-stimulated E-cadherin expression in gastrin cancer cells. However, it is not known whether COX2 mediates EGF-induced ovarian cancer cell invasion. In the present study, we showed that EGF could induce ovarian cancer cell invasion through a complex signaling network (Cheng et al. 2010). This indicates that EGF/EGFR and COX2 are important for the development and progression of human ovarian cancer.
E-cadherin expression may depend on the type of cancer. For COX2 in the EGF-induced downregulation of expression. These results indicate that the requirement with the selective COX2 inhibitor NS-398 did not affect method increases E-cadherin expression (Dohadwala inhibition of COX2 by genetic or pharmacological expression at both the mRNA and protein levels (Sitarz in inhibitor, leads to the upregulation of E-cadherin expression at both the mRNA and protein levels (Sitarz et al. 2009). In human non-small-cell lung carcinoma cells, inhibition of COX2 by genetic or pharmacological methods increases E-cadherin expression (Dohadwala et al. 2006). In this study, we showed that treatment with the selective COX2 inhibitor NS-398 did not affect the EGF-induced downregulation of E-cadherin expression. These results indicate that the requirement for COX2 in the EGF-induced downregulation of E-cadherin expression may depend on the type of cancer.

PGE2 stimulates cell invasion in many cancers via an autocrine/paracrine mechanism (Dohadwala et al. 2002, Timoshenko et al. 2003, Lau et al. 2010, Paquette et al. 2011, Vo et al. 2013). We have previously shown that PGE2 acts in an autocrine/paracrine fashion in ovarian cancer cells to upregulate the expression of matrix metalloproteinase 2 (MMP2) and MMP9, which in turn contribute to gonadotropin-induced cell invasion (Lau et al. 2010). Interestingly, proteinase activity is required for EGF-induced ovarian cancer invasion (Ellerbroek et al. 1998). We have previously shown that the downregulation of E-cadherin expression mediates EGF-induced ovarian cancer cell invasion (Cheng et al. 2010, 2012a,b, 2013a,b,c). However, our previous results had indicated that although EGF-induced invasion is significantly reduced in E-cadherin-overexpressing ovarian cancer cells, it is not completely abolished (Cheng et al. 2010). This indicates the presence of additional, E-cadherin-independent, mechanisms for EGF-induced ovarian cancer cell invasion. It has been shown that additional mechanisms such as enhanced protease activity/secretion, changes in actin cytoskeleton, and enhanced motility are involved in EGF-induced cell invasion (Ellerbroek et al. 2001, Kassis et al. 2001). Because the inhibition of COX2 did not affect the EGF-induced downregulation of
E-cadherin expression in ovarian cancer cells, it is possible that COX2-derived PGE2 may mediate EGF-induced cell invasion by regulating the expression of MMP in an autocrine/paracrine fashion.

EGF induces the expression of COX2 in different types of human cancers (Kulkarni et al. 2001, Xu & Shu 2007, Cao et al. 2011). However, the underlying molecular mechanism that mediates EGF-induced COX2 expression in human ovarian cancer is unknown. We and other groups have shown that PI3K/Akt signaling is required for gonadotropin- and insulin-like growth factor 1-induced COX2 expression, but it is unclear whether the same is true for EGF-induced COX2 expression (Cao et al. 2007, Lau et al. 2010). In human cervical cancer cells, EGF-induced COX2 expression is attenuated by treatment with either of two PI3K inhibitors: wortmannin and LY294002 (Kulkarni et al. 2001). In this study, we showed that treatment with wortmannin or LY294002 attenuated EGF-induced COX2 expression in ovarian cancer cells. We further confirmed the involvement of AKT in EGF-induced COX2 expression by siRNA-mediated knockdown of AKT, which could avoid the non-specific effects of pharmacological inhibition. Taken together, our results clearly indicate for the first time, to our knowledge, that PI3K/Akt signaling plays an important role in the mediation of EGF-induced COX2 expression in ovarian cancer cells.

It has been reported that the transcription factor CREB is involved in the regulation of COX2 expression by binding to the CRE in the COX2 promoter (Tanabe & Tohnai 2002, Ghosh et al. 2007, Klein et al. 2007, Sharma-Walia et al. 2010). However, it is not known whether CREB regulates EGF-induced COX2 expression in human ovarian cancer cells. In this study, we found that CREB is not involved in EGF-induced COX2 expression.

**Figure 5**
CREB is not involved in EGF-induced COX2 expression. (A) Cells were treated with a vehicle control (Ctrl) or 100 ng/ml EGF for 5, 10, or 30 min, and the levels of phosphorylated CREB were determined by western blot analyses. (B and C) Cells were transfected for 48 h with 50 nM control siRNA (si-Ctrl) or CREB siRNA (si-CREB) and then treated with EGF for 1 h (mRNA) or 3 h (protein). COX2 mRNA (B) and COX2 protein (C) levels were determined by RT-qPCR and western blot analyses respectively. Results are expressed as the mean ± S.E.M. of at least three independent experiments. Values without a common letter are significantly different (P < 0.05).
These results indicate that other molecules mediate the induction of COX2 expression by EGF in ovarian cancer cells. NF-κB and c-Jun participate in LPS-induced COX2 expression in mouse macrophages (Kang et al. 2006). In rat insulinoma cells, CREB and the Ets family members Ets-1 and Elk-1 increase Cox2 promoter activity, while STAT1 inhibits Cox2 promoter activity (Zhang et al. 2007). In human monocytes, ESE1 binds to and increases the activity of the COX2 promoter (Grall et al. 2005). A recent study has shown that the FOXM1/Sp1 complex binds to the Sp1-binding site in the COX2 promoter to mediate EGF-induced COX2 expression in human glioma cells (Xu & Shu 2013). Further research is needed to determine the transcriptional regulatory mechanisms that mediate EGF-induced COX2 expression in ovarian cancer cells.

In summary, our results reveal that EGF induces COX2 expression and PGE2 production in human ovarian cancer cells. They also reveal that COX2 expression is not involved in the EGF-induced downregulation of E-cadherin expression. Moreover, EGF-induced COX2 expression is mediated by the PI3K/Akt signaling pathway. Inhibition of COX2 as well as PGE2 attenuates EGF-stimulated cell invasion. This study provides important insights into the molecular mechanisms that mediate EGF-stimulated human ovarian cancer cell invasion.

**Supplementary data**
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-13-0450.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
X Qiu, J-C Cheng, and P C K Leung conceived and designed the experiments; X Qiu, J-C Cheng, and H-M Chang performed the experiments; X Qiu, J-C Cheng, and H-M Chang analyzed the data; X Qiu, J-C Cheng, and P C K Leung wrote the article. All authors read and approved the final manuscript.

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