Induction of cyclooxygenase-2 in human endometrial stromal cells by malignant endometrial epithelial cells: evidence for the involvement of extracellularly regulated kinases and CCAAT/enhancer binding proteins

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Abstract

We previously reported that human malignant endometrial epithelial cell conditioned medium (MECM) up-regulated cyclooxygenase (COX)-2 mRNA and protein levels in human normal endometrial stromal cells (ESC). Here we showed that pretreatment with a selective inhibitor of the extracellularly regulated kinase (ERK)1/2 signaling pathway blocked the MECM-induced COX-2 expression in ESC. Transient transfection assays indicated critical roles of a cAMP response element (CRE, −59/−53 bp) and a nuclear factor for interleukin (IL)-6 expression (NF-IL6) site (−132/−124 bp) in the regulation of basal and MECM-induced activity of COX-2 gene promoter in ESC. Employing electrophoretic mobility shift assays, we demonstrated that increased functional binding of CCAAT/enhancer binding protein (C/EBP)α, C/EBPβ and upstream stimulatory factor-2 to the CRE and C/EBPα and C/EBPβ to the NF-IL6 site were, at least in part, responsible for MECM-induced COX-2 expression in ESC. Moreover, overexpression of C/EBPα and C/EBPβ significantly induced COX-2 promoter activity in ESC. Collectively, these results suggest that the basal and MECM-induced transcription of the COX-2 gene in ESC is regulated through a combination of the CRE and the NF-IL6 site by functional interactions of C/EBPα and C/EBPβ.

Journal of Molecular Endocrinology (2003) 31, 95–104

Introduction

Cyclooxygenase (COX) is a key enzyme in prostaglandin (PG) synthesis. COX converts arachidonic acid that is released from membrane stores by phospholipase to PGH2, the common precursor for all PGs. Two different isoforms of COX, referred to as COX-1 and COX-2, have been identified. They share over 60% identity at the amino acid level and have similar enzymatic activities, but although they catalyze the same reaction, these two isoforms may have distinct biological functions (Tazawa et al. 1994, Williams & DuBois 1996). COX-1 is constitutively expressed in most mammalian tissues and is thought to carry out housekeeping functions. In contrast, COX-2 mRNA and protein are normally undetectable in most tissues, but can be rapidly induced in response to various cytokines and growth factors (Inoue et al. 1995, Potter et al. 2000, Thomas et al. 2000).

Supported by a plethora of experimental evidence, COX-2 expression emerged as a highly promising therapeutic target not only in the treatment of many inflammatory diseases but also several types of human cancers. Enhanced expression of COX-2, but not COX-1, has been found in colon (Sano et al. 1995), pancreatic (Tucker et al. 1999) and gastric cancer tissues (Ristimäki et al. 1997). Previous studies have shown that overexpression of COX-2 reduces the rate of apoptosis
(Souza et al. 2000), increases the invasiveness of malignant cells (Tsujii et al. 1997) and promotes angiogenesis (Tsujii et al. 1998). The proposal that COX-2 contributes to carcinogenesis is supported further by compelling evidence that inhibitors of COX activity protect against colon, mammary, esophageal and lung cancer in humans (Subbaramaiah et al. 1997, Souza et al. 2000). Thus, COX-2 expression is important in cancer development.

We previously reported (Tamura et al. 2002a) that human malignant endometrial epithelial cell conditioned medium (MECM) increased COX-2 mRNA and protein levels in human normal endometrial stromal cells (ESC). These results are suggestive of a cross-talk between malignant epithelial cells and surrounding stromal cells to favor COX-2 expression in the endometrial tumors. Moreover, using transient transfection assays, we found that the −360/+56 bp region of the COX-2 promoter gene was critical for induction of promoter activity by MECM and that this MECM-responsive region contained a nuclear factor (NF)-κB site at −222 to −213 bp. Employing electrophoretic mobility shift assays, we further demonstrated that binding of NF-κB p65 to this NF-κB binding site was, at least in part, responsible for the COX-2 promoter activation by MECM.

However, the molecular signaling mechanisms by which MECM induces the COX-2 transcription in ESC are not completely understood. We hypothesized that the optimal effect of MECM required not only the NF-κB site but also multiple cis-acting elements and/or cell signaling affected through a variety of cancer cell-secreted factors. Thus, we have attempted to identify the signal transduction pathway(s) involved and to characterize the critical cis-acting elements that mediate induction of the COX-2 gene by MECM in ESC.

Materials and methods

Reagents and antibodies

U0126 (a specific inhibitor of extracellularly regulated kinase (ERK) 1/2, also known as p42/p44 mitogen activated protein kinase (MAPK)), SB203580 (a specific inhibitor of p38 MAPK) and Sp1 consensus double-stranded oligonucleotide were purchased from Promega (Madison, WI, USA). Actinomycin D (Act D; general transcription inhibitor) was purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies against cAMP response element binding protein (CREB)-1, activating transcription factor (ATF)-1, ATF-2, CCAAT/enhancer binding protein (C/EBP)α, C/EBPβ, C/EBPδ, upstream stimulatory factor (USF)-1 and USF-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other materials used in the study are indicated in the appropriate context below.

Cell culture

Human normal endometrial stromal cells (ESC) were cultured using a protocol previously reported (Tamura et al. 2002b). Confluent ESC were serum-deprived for 16 h in serum-free Dulbecco’s modified Eagle’s medium–Ham’s F12 (DMEM/F12) before being subjected to the following two treatments: (i) serum-free medium as the baseline control and, (ii) serum-free medium conditioned with Ishikawa human malignant endometrial epithelial cells (malignant epithelial cell conditioned medium abbreviated as MECM). Treated ESC were then used to isolate total RNA for reverse transcriptase-polymerase chain reaction (RT-PCR), whole extracts for Western blot analysis and nuclear extracts for electrophoretic mobility shift assay (EMSA). The cells were studied at passage 4–6. The conditioned medium was generated in the following fashion. After Ishikawa cells were grown to confluence, culture medium was switched to serum-free DMEM/F12 for a 16-h-washout period to collect MECM. Then, cells were incubated in new serum-free DMEM/F12 for 72 h to allow accumulation of secreted factors in the medium. We collected MECM and centrifuged it to remove the cell debris. The supernatant was transferred to a clean tube and immediately frozen and kept at −80°C for future use.

Semi-quantitative RT-PCR amplification

Total RNA (5 µg) was isolated from ESC using the RNasy mini kit (Qiagen, Valencia, CA, USA), following the protocol suggested by the manufacturer. For RT-PCR analysis of COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) was used to synthesize the first strand cDNA
as instructed by the supplier. The nucleotide sequences of the primer pairs employed and the PCR conditions have been reported previously (Tamura et al. 2002b). ESC were exposed to MECM with or without signal transduction inhibitor for 8 h. Aliquots of the reaction products were analyzed by electrophoresis in an agarose gel and ethidium bromide staining. PCR products were quantified using the Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA) as a densitometer. We assert that these data are semi-quantitative (relative to control GAPDH) based on the following test performed prior to data analysis, as previously shown (Tamura et al. 2002). Briefly, both products were assayed in the linear response range of the RT-PCR amplification process. The cycle number used in this assay was determined by finding the midpoint of linear amplification on a sigmoid curve for both amplification products with cycle numbers 25–42 plotted against band density.

**Western blotting**

Western blotting was performed as previously described (Tamura et al. 2002a), using 20 µg of whole cell protein extract in each sample that was exposed to MECM with or without signal transduction inhibitor for 8 h and anti-COX-2 polyclonal antibody (Santa Cruz Biotechnology). Band intensity of protein expression was quantitated using the Molecular Analyst version 1.5 software (Bio-Rad Laboratories).

**Plasmid construction, transient transfections and luciferase assays**

Construction of the deletion mutant containing specific regions of the human COX-2 gene promoter in the luciferase reporter vector pGL3 Basic (Promega) was accomplished using PCR amplification of the desired region using the recombinant plasmid containing a 7-kb promoter region of the human COX-2 gene (a generous gift from Dr Stephen M Prescott) as the template. Mutant constructs were constructed as described previously (Tamura et al. 2002b). Briefly, for the cAMP response element (CRE), TTCGTCA was changed to TtgagCt and, for the nuclear factor for interleukin (IL)-6 expression (NF-IL6) site, the sequence was changed from TTACGCAAT to TTggtaccT; the lower case nucleotides indicate the mutations.

Transfections into ESC incubated with control media or MECM were performed using the LipoefectAMINE PLUS reagent (Invitrogen), following the protocol provided by the manufacturer. Each transfection was carried out using 0.4 µg firefly luciferase reporter plasmid construct that contains deletion or site-specific mutants of the human COX-2 gene promoter and 1 ng of an internal control reporter plasmid pRL-TK (Renilla luciferase-thymidine kinase) supplied by Promega. Cotransfection was performed by adding 0.1 µg pcDNA3-1 expression plasmid (Invitrogen), which contains the cDNA of either C/EBPα (human) or C/EBPβ (mouse) (generous gifts from Drs Gretchen Darlington and Gokhan Hotamisligil). Firefly and Renilla luciferase activities were sequentially measured using the Dual-Luciferase Reporter Assay System (Promega) and LUMAT LB9507 luminometer (Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany).

**Electrophoretic mobility shift assay (EMSA)**

Nuclear protein was extracted from whole cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA), following the protocol suggested by the manufacturer. EMSA was performed as previously described (Tamura et al. 2002a), using 0.5 µg nuclear protein extract in each sample. We used the following double-stranded probes. The CRE probe (5'-AAACAGTCATGCAGTCAATTTCCTGTCACATGGGCTTG-3') was designed to represent a 27-bp-long sequence (50-69/43-43 bp) in the promoter region of COX-2 gene. The NF-IL6 site probe (5'-CACCCGGGCTTACGCAATTTTTTTTAACT-3') was designed to represent a 25-bp-long sequence (50-140/116 bp). The underlined nucleotides indicate the transcription factor binding domain and they are 100% identical with the consensus sequences.

**Statistical analysis**

Statistical analysis for comparison between treatment groups was performed by one-way analysis of variance followed by Tukey’s multiple comparison test. A P value < 0.05 was considered significant. All values are given as the mean, with the bars (in the Figures) showing standard errors of the mean.
Results

MECM-mediated COX-2 induction is blocked by an inhibitor of ERK1/2 signal transduction pathway

We initially carried out experiments to evaluate the optimal conditions for determining the effects of MECM on COX-2 mRNA levels in ESC. To determine where PCR amplification for COX-2 mRNA was in the logarithmic phase, total RNA isolated from ESC treated with MECM was reverse transcribed and was amplified under different cycle numbers. Single PCR products were obtained for COX-2. A linear relationship between PCR products and amplification cycles was observed for COX-2 treated with MECM in ESC (Tamura et al. 2002a). Consequently, 38 cycles for COX-2 were employed for quantification. The time course of COX-2 mRNA abundance as examined by RT-PCR showed an increase following treatment at 4 h, and peaked at 8 h (Fig. 1A). PCR was also performed using an aliquot of the RT products for the housekeeping gene GAPDH mRNA to control the RT reaction, PCR efficiency and equal starting amounts of total RNA. There was no apparent change in the GAPDH mRNA abundance upon MECM treatment. COX-2 protein levels were also induced after MECM treatment at 8 h and remained detectable up to 24 h (Fig. 1B). Equal loading of protein in each lane was confirmed by Coomassie blue staining of samples fractionated on SDS-PAGE. Quantitative densitometry for three independent experiments confirmed these results. Based on these observations, the ESC were pretreated with various inhibitors of signal transduction for 30 min and then incubated with MECM for 8 h. One micromole ERK1/2 specific inhibitor, U0126 (IC$_{50}$ 0.53 µM, Favata et al. 1998) caused significant decreases in the density of the COX-2 mRNA band (Fig. 1C). COX-2 immunoblot experiments confirmed that this inhibitor indeed abolished the increased COX-2 protein otherwise seen upon MECM treatment (Fig. 1D). Equal loading of protein in each lane was confirmed by intensity of bands in a portion of the Coomassie blue-stained gel. In contrast, pretreatment with several concentrations (0-1 µM, 1 µM and 2 µM) of the p38 MAPK inhibitor, SB203580 (IC$_{50}$ 0.6 µM, Cuenda et al. 1995) did not block MECM stimulation of COX-2 mRNA in ESC (Fig. 1C). As a positive control, SB203580 inhibited IL-1β-stimulated COX-2 mRNA expression in HeLa cells (data not shown), as previous investigators reported (Ridley et al. 1998). Pretreatment with 10 µg/ml Act D (a general transcriptional inhibitor) also abolished the MECM-mediated induction of COX-2 gene, demonstrating the requirement of new RNA synthesis. To check the effects of these inhibitors (U0126 and SB203580) on unstimulated ESC, ESC were also pretreated with them and then incubated with control DMEM/F12. No apparent changes in the basal levels of COX-2 mRNA in ESC were observed (data not shown). Additionally, we have previously demonstrated that Act D also had no effect on basal levels of the COX-2 mRNA expression in ESC (Tamura et al. 2002a). These results suggested that MECM-induced COX-2 expression in ESC is mediated via activation of ERK1/2 and not via the p38 MAPK pathway.

Activation of the COX-2 promoter by MECM requires CRE and the NF-IL6 site

We have previously reported that the −360/+56 region was shown to be important for MECM-mediated induction, since only the −360/+56 bp construct (phCOX2(−360/+56)) demonstrated a statistically significant induction upon treatment with MECM (Tamura et al. 2002a). Sequence analysis of this region and a literature review (Tazawa et al. 1994) revealed the existence not only of an NF-κB site (−222/−213 bp) but also a CRE (−59/−53 bp) and an NF-IL6 site (−132/−124 bp) (Fig. 2A). Therefore, using the −218/+56 bp reporter construct (phCOX2(−218/+56)), we attempted to identify and characterize the critical cis-acting elements that mediate induction of the COX-2 gene in ESC. Site-directed mutations of the CRE and the NF-IL6 site either alone or in combination significantly reduced both baseline and MECM-induced COX-2 promoter activity in ESC (Fig. 2B). Compared with the disruption of the NF-κB site, the disruption of each site gave rise to a greater decrease in baseline and MECM-dependent promoter activity (Tamura et al. 2002a). Thus, the presence of CRE and the NF-IL6 site were required for maximum MECM-mediated induction of COX-2 promoter in ESC.

Identification of the proteins which bind to cis-acting elements in CRE and the NF-IL6 site

EMSA was performed using nuclear proteins from ESC treated with or without MECM to determine
the protein/DNA binding activities at the CRE and the NF-IL6 site. In the case of CRE, the shifted complex from ESC incubated with control medium was composed of two specific complexes, 1 and 2. However, nuclear extract prepared from MECM-treated cells showed more intense signals for both complex 1 and 2, indicating increased protein/DNA binding activity at CRE upon MECM.

**Figure 1** Effects of malignant endometrial epithelial cell conditioned medium (MECM) on COX-2 mRNA and protein levels in human endometrial stromal cells (ESC). (a, b) ESC treated with MECM for 0 h (control) to 24 h. (c, d) ESC were exposed to control medium (CON), MECM, MECM+10 µg/ml actinomycin D (Act D), MECM+1µM U0126 (a specific inhibitor of extracellularly regulated kinase (ERK) 1/2) or MECM+0·1 µM, 1 µM or 2 µM SB203580 (SB) (a specific inhibitor of p38 mitogen activated protein (MAP) kinase) for 8 h. Inhibitors were added 30 min before MECM stimulation. (a, c) The semi-quantitative RT-PCR shown at the top is representative of three independent experiments. Band sizes were: COX-2, 305 bp; GAPDH, 593 bp. Summary data for quantitative densitometry for the three experiments are given at the bottom. Relative levels of COX-2 mRNA expression were determined by densitometric scanning of the bands and normalized to the GAPDH signal. (b, d) The Western blot analysis shown at the top is representative of three independent experiments. COX-2 protein was detected at 72 kDa. Equal loading of protein in each lane was confirmed by intensity of bands in a portion of the Coomassie blue-stained gel. Summary data for quantitative densitometry for the three experiments are given at the bottom. Mean±S.E.M. values are depicted for mRNA or protein abundance expressed as a percentage in control ESC. *P<0·05 compared with control ESC; #P<0·05 compared with MECM-treated ESC.
treatment (Fig. 3A). Preincubation with cold CRE or NF-IL6 probes completely abolished both shifted bands. On the other hand, a consensus Sp1-binding sequence had no effect on the band intensity, confirming the specificity of the reaction.

A similar increase in the protein/DNA binding activity at the NF-IL6 site by MECM-treated ESC nuclear proteins was also observed (Fig. 3A). The difference was that instead of two shifted bands as in the case of CRE, only one shifted band was formed with the NF-IL6 site probe. Again, preincubation with cold CRE or NF-IL6 site probes abolished this band, whereas Sp1-specific sequence had no effect. Both DNA–protein complexes could be competed away successfully using either probe.

Since previous studies (Inoue et al. 1995, Potter et al. 2000, Thomas et al. 2000) have demonstrated functional binding of CREB, C/EBPs and USF to either CRE or NF-IL6 sites, antibody competition of specific proteins from the nuclear extracts prior to the EMSA analysis was attempted. Using the CRE probe and nuclear extract from MECM-treated ESC, antibody competition of CREB-1, ATF-1, ATF-2, C/EBPα, C/EBPβ, C/EBPδ, USF-1 or USF-2. As shown in Fig. 3C, antibody competition of C/EBPα or C/EBPβ completely abolished the band shift indicating that this complex contained both of these proteins.

Overexpression of C/EBPα and C/EBPβ induces COX-2 activity in ESC

To directly examine the role of C/EBPα and C/EBPβ in regulating the COX-2 promoter activity in ESC, cotransfection experiments were performed using the phCOX2(−218/+56) bp reporter construct and C/EBPα or C/EBPβ expression plasmids. As shown in Fig. 4, irrespective of the treatment conditions (control or MECM), overexpression of C/EBPα or C/EBPβ resulted in the transactivation of COX-2 gene promoter by 2.5- to 3.0-fold respectively in ESC. These results further confirmed that COX-2 gene induction in ESC by MECM is, at least in part, mediated by C/EBPα and C/EBPβ.
Discussion

In the present study, we have demonstrated involvement of the ERK1/2 signaling pathway in the induction of the COX-2 gene by MECM in human normal ESC. U0126 is an organic compound (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene) that has been identified as an inhibitor of AP-1 transactivation in cell-based reporter assays (Favata et al. 1998). It specifically inhibits the phosphorylation and activation of ERK1/2. U0126 does not affect the phosphorylation of mitogen-activated protein kinase kinase (MEK), the upstream kinase of ERK, at concentrations sufficient to inhibit ERK phosphorylation. It thus appears that U0126 inhibits MEK directly by inhibiting the catalytic activity of the active enzyme. Recent studies have suggested that Ras (the upstream kinase of MEK) activation may induce COX-2 expression in several conditions.
Figure 4 Effects of CCAAT/enhancer binding protein (C/EBP) isoforms on responsiveness of the COX-2 promoter to control medium (CON) or malignant endometrial epithelial cell conditioned medium (MECM). The −218/+56 bp reporter construct (phCOX2(−218/+56)) was cotransfected with the C/EBPα expression vector, the C/EBPβ expression vector or the same amount of empty expression vector pcDNA3.1. Results are expressed as the mean±S.E.M. of three independent experiments performed in triplicate. *P<0.05 compared with phCOX-2(−218/+56) plus pcDNA3.1 treated with control medium. #P<0.05 compared with phCOX-2(−218/+56) plus pcDNA3.1 treated with MECM.

The ERK1/2 might phosphorylate C/EBPs and enhance its ability to activate COX-2 transcription. Consistent with this notion are studies in other systems demonstrating that MAPK can enhance the transactivation properties of C/EBPβ via the phosphorylation of threonine-235 (Nakajima et al. 1993, Kowenz-Leutz et al. 1994). In addition, ERK1/2 activation can regulate the expression of numerous genes by activating NF-κB (Zhao & Lee 1999). Recently, several investigators have described physical and functional interactions between C/EBP and NF-κB family members (Kinoshita et al. 1992, LeClair et al. 1992). These transcription factors directly associate via the b-Zip domain of C/EBP and the Rel homology domain of p65 or p50. Supporting this model are experiments by Stein et al. (1993), which showed that the presence of NF-κB p65 significantly enhanced the binding of C/EBP family proteins to their DNA-binding sites. In this case, the addition of p65 did not alter the mobility of the C/EBP-DNA complex from the control. The authors suggested that the p65 interaction may have altered the conformation of C/EBP, making it more stable. Interestingly, our studies suggest similarly that C/EBPs, which are induced by MECM, may play an important role in both the basal and induced transcriptional activity of COX-2 promoter and in the regulation of COX-2 expression by U0126 (ERK1/2 inhibitor) clearly support this observation.
gene expression by enhancing the binding of NF-kB p65 to this promoter.

We conclude that, in endometrial stromal cells, MECDM-mediated induction of COX-2 expression is a complex process that requires input from multiple signaling pathways. Each pathway acts synergistically, the sum of their actions culminating in a dramatic increase in COX-2 transcription. Given the recent emphasis on COX-2 in cancer biology, this paracrine interaction may be important in the pathogenesis of endometrial cancer.

**Acknowledgements**

We are grateful to Drs Stephen M Prescott, Gretchen Darlington and Gokhan Hotamisligil for providing the plasmids.

This work was supported by NIH grant HD38691 to S E B and by a fellowship award to M T from the Japan Menopause Society, Tokyo, Japan.

**References**


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Received in final form 6 April 2003
Accepted 9 April 2003