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/EBP β and upstream stimulatory factor-2 to the CRE and C/EBP α and C/EBP β to the NF-IL6 site were, at least in part, responsible for MECM-induced COX-2 expression in ESC. Moreover, overexpression of C/EBP α and C/EBP β 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/EBP α and C/EBP β .

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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)- κ B site at -222 to -213 bp. Employing electrophoretic mobility shift assays, we further demonstrated that binding of NF- κ B p65 to this NF- κ B 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- κ B 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 Spl 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/EBP β , C/EBP δ , 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 RNeasy 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.* 2002a). 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 LipofectAMINE 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'-AAACAGTCATTTCGTCACATGGGCTTG-3') was designed to represent a 27-bp-long sequence (–69/–43 bp) in the promoter region of COX-2 gene. The NF-IL6 site probe (5'-CACCGGGCTTACGCAATTTT TTAA-3') was designed to represent a 25-bp-long sequence (–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 house-keeping 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 μ M, 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 μ M, 1 μ M and 2 μ M) of the p38 MAPK inhibitor, SB203580 (IC_{50} 0.6 μ M, Cuenda *et al.* 1995) did not block MECM stimulation of COX-2 mRNA in ESC (Fig. 1C). As a positive control, SB203580 inhibited IL-1 β -stimulated COX-2 mRNA expression in HeLa cells

(data not shown), as previous investigators reported (Ridley *et al.* 1998). Pretreatment with 10 μ g/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- κ B 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- κ B 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

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+1 μM 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 $\pm$ 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.

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

(a) Human COX-2 gene 5'-flanking region

(b) Human COX-2 gene 5'-flanking region

Figure 2 Analysis of the region responsible for the promoter activity of the human COX-2 gene. (a) Locations of potential response elements in the 5'-flanking region of the human COX-2 gene. Nucleotides are numbered negatively with nucleotide +1 corresponding to the transcriptional start site. (b) Selected mutations of two *cis*-acting elements (cAMP response element (CRE) and the nuclear factor (NF)-IL6 site) alone or in combination significantly decreased MECM-induced promoter activity indicating the critical roles of each one of the DNA binding sites. phCOX2(-218/+56), -218/+56 bp reporter construct; CON, control medium; MECM, malignant endometrial epithelial cell conditioned medium; Luc, luciferase reporter gene. Site-specific mutation is indicated by an X. Results are expressed as the mean $\pm$ S.E.M. of three independent experiments performed in triplicate.

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 either C/EBP α or C/EBP β abolished the formation of complex 1 and reduced the band intensity of complex 2 (Fig. 3B). In contrast, antibody competition of USF-2 resulted in reduced signal intensity for complex 2, but had no effect on complex 1 formed by the CRE probe. On the other hand, antibodies against CREB-1, ATF-1, ATF-2, C/EBP δ or USF-1 did not alter the intensity or rate of migration of either complex formed by the

CRE probe. It should be noted that a modest signal remained for complex 2 following antibody competition with C/EBP α , C/EBP β or USF-2 (Fig. 3B) and after preincubation with the combination of all the three antibodies (data not shown); therefore, this complex formed by the CRE probe may contain additional protein(s). Next, to determine the identity of the protein which binds to the NF-IL6 site, MECM-treated ESC nuclear extract was selectively competed for CREB-1, ATF-1, ATF-2, C/EBP α , C/EBP β , C/EBP δ , USF-1 or USF-2. As shown in Fig. 3C, antibody competition of C/EBP α or C/EBP β completely abolished the band shift indicating that this complex contained both of these proteins.

Overexpression of C/EBP α and C/EBP β induces COX-2 activity in ESC

To directly examine the role of C/EBP α and C/EBP β in regulating the COX-2 promoter activity in ESC, cotransfection experiments were performed using the -218/+56 bp reporter construct (phCOX2(-218/+56)) and C/EBP α or C/EBP β expression plasmids. As shown in Fig. 4, irrespective of the treatment conditions (control or MECM), overexpression of C/EBP α or C/EBP β resulted in the transactivation of COX-2 gene promoter by 2.5- or 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/EBP α and C/EBP β .

Figure 3 EMSA to identify the proteins that bind cAMP response element (CRE) and nuclear factor (NF)-IL6. (a) Competitive EMSAs were performed with a CRE and a NF-IL6 radiolabeled probe and nuclear extract from control medium (CON)- or malignant endometrial epithelial cell conditioned medium (MECM)-treated normal human endometrial stromal cells (ESC). A probe containing an Sp1 binding site was used as a negative control. A 100-fold molar excess of unlabeled CRE, NF-IL6 or Sp1 oligonucleotides was used in the competition reactions. (b, c) Immunodepletion studies were performed with a CRE (b) or a NF-IL6 (c) radiolabeled probe and nuclear extract from MECM-treated ESC and antibodies against transcription factors that may potentially bind to both the CRE and the NF-IL6 site. Results were reproduced in two other experiments.

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

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) in human endometrial stromal cells (ESC). 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.

systems (Subbaramaiah *et al.* 1996, Sheng *et al.* 1998). Activated, oncogenic Ras was inducibly expressed in Rat-1 fibroblasts with a concomitant increase in COX-2 expression (Sheng *et al.* 1998). In this cell line, a specific ERK inhibitor was found to suppress COX-2 induction by Ras, suggesting that ERK1/2 activation is required for Ras-dependent induction of COX-2. Similarly, elevated levels of COX-2 were detected in Ras-transformed mammary epithelial cells (Subbaramaiah *et al.* 1996). Therefore, it is possible that MECM can activate Ras and that the ERK1/2 signaling pathway is important in the regulation of COX-2 by MECM. The suppression of MECM-induced COX-2 expression by U0126 (ERK1/2 inhibitor) clearly supports this observation.

Using transient transfection assays, we identified that the CRE at -59/-53 and the NF-IL6 site at -132/-124 in the COX-2 promoter gene were critical for the optimal COX-2 transcriptional increase in ESC. However, since the reporter construct containing the COX-2 promoter region -218/+56 exhibited the maximum stimulation and we did not employ additional experiments to corroborate this observation, we do not rule out the

possibility of the cooperative involvement of the other sites to elicit COX-2 promoter in ESC. Using EMSA, we demonstrated that increased functional binding of C/EBPα, C/EBPβ and USF-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. Several *trans*-activating factors with a leucine zipper motif that specifically interact with the CRE have been identified (Lalli & Sassone-Corsi 1994). Inoue *et al.* (1995) reported that C/EBPδ bound to both the CRE and the NF-IL6 site and increased the COX-2 gene promoter activity mainly through the CRE. Timchenko *et al.* (1995) showed a functional interaction between USF and C/EBPα in the autoregulation of C/EBPα gene, where overexpression of C/EBPα resulted in the enhanced binding of USF. Taken together with overexpression of C/EBPα or C/EBPβ studies, these data are suggestive of critical roles for C/EBPα and C/EBPβ in mediating MECM-induced COX-2 gene expression in ESC.

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

gene expression by enhancing the binding of NF- κ B p65 to this promoter.

We conclude that, in endometrial stromal cells, MECM-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.

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