Overlapping CRE and E-box promoter elements can independently regulate COX-2 gene transcription in macrophages

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Abstract Macrophage cyclooxygenase-2 (COX-2) transcription is mediated through the collaboration of different promoter elements. Here, the role of an overlapping cyclic AMP responsive element (CRE)/E-box was investigated. Nuclear proteins bound both the CRE and E-box, which synergized with other promoter elements to induce COX-2 transcription. Endotoxin induced binding of nuclear proteins to the CRE and E-box and each element independently induced higher COX-2 transcription levels than the overlapping CRE/E-box. Transcription factors associated with the CRE binding complex included c-Jun and CRE binding protein and with the E-box binding complex USF-1; their overexpression significantly induced COX-2 transcription. Therefore, both CRE and E-box promoter elements regulate COX-2 transcription in macrophages. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cyclooxygenase-2; Macrophage; Transcription

1. Introduction

Macrophage activation is accompanied by a significant increase in cyclooxygenase-2 (COX-2) expression [1]. There is evidence that COX-2 expression and prostaglandin (PG) synthesis play a critical role in the development of local and systemic inflammatory responses. Non-steroidal anti-inflammatory drugs, including the newly developed selective COX-2 inhibitors, inhibit PG synthesis through the inhibition of COX activity, which confers anti-inflammatory and analgesic properties [2]. Moreover, homozygous deletion of the *COX-2* gene in mice has led to a striking mitigation of endotoxin-induced hepatocellular cytotoxicity [3].

The two isoforms of COX, COX-1 and COX-2, are the products of two different genes. While COX-1 is expressed constitutively and may be responsible for housekeeping functions, COX-2 expression can be induced by endotoxin, cytokines, growth factors and carcinogens [1,4,5]. The different responses of the genes encoding COX-1 and COX-2 reflect differences in the regulatory elements in the 5′ flanking regions of these two genes. We recently reported that in the *COX-2* gene (Fig. 1), promoter elements for nuclear factor κB (NF-κB, −223/−214), nuclear factor interleukin 6 (NF-IL6, −132/−124) and a cAMP responsive element overlapping a non-canonical E-box (CRE/E-box, −59/−49) regulate transcription in macrophages exposed to endotoxin [6]. Although not sufficient by itself to confer maximal COX-2 transcription, the overlapping CRE/E-box appeared to be the most active promoter element in response to endotoxin. However, the relative contribution of the CRE and E-box in mediating *COX-2* transcription in macrophages has not been studied.

In the present work, we have investigated the role of the CRE and E-box promoter elements on the regulation of *COX-2* gene expression in endotoxin-treated RAW 264.7 macrophages. Our data show that both the CRE and E-box can mediate COX-2 transcription through the activation of specific transcription factors. These results are important for understanding why COX-2 expression is upregulated during macrophage activation.

2. Materials and methods

2.1. Materials

Escherichia coli (strain O55:B5) lipopolysaccharide (LPS), DEAE-dextran and *O*-nitrophenyl-β-D-galactopyranoside were from Sigma (St. Louis, MO, USA). [^{32}P]ATP was from NEN-Dupont (Boston, MA, USA). Endotoxin-free plasmid DNA was prepared using Qiagen DNA purification kits (Chatsworth, CA, USA). Reagents for the luciferase assay were from Analytical Luminescence (San Diego, CA, USA). Mutagenesis kits were from Stratagene (La Jolla, CA, USA). Oligonucleotides were synthesized by Genosys Biotechnologies Inc. (The Woodlands, TX, USA). T4 polynucleotide kinase was from New England Biolabs Inc. (Beverly, MA, USA).

2.2. Cells

Murine macrophage-like RAW 264.7 cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B).

2.3. Plasmids

Expression vectors for c-Jun, CRE binding protein (CREB) and upstream response element-1 (USF-1) were provided by Dr. Andrew J. Dannenberg (Weill Medical College of Cornell University, New York, NY, USA). Human *COX-2* promoter-*luciferase* wild type con-

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Abbreviations: COX, cyclooxygenase; PG, prostaglandin; CRE, cyclic AMP responsive element; USF, upstream response element; CREB, CRE binding protein

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struct (designated WT) and mutant constructs (designated CRE/E-box and 4XM) have been described previously [7–9]. The single mutants designated CRM and EBM and the triple mutants designated E-box and CRE were created using site-directed mutagenesis on WT or CRE/E-box templates, respectively. Briefly, primers that incorporated mutations (lower-case letters) for CRE (sense: 5′-CAGTCATTTgaT-CACATGGGCTTGG-3′) or E-box (sense: 5′-CAGTCATTTCGT-CACActGGCTTGG-3′) were used to amplify the template constructs as previously described [6]. Incorporation of the desired mutations was confirmed by DNA sequencing (see Fig. 1).

2.4. Transient transfection assays

RAW 264.7 cells (5×10^6 per treatment group) were washed twice in serum-free RPMI and suspended in 0.5 ml of transfection solution (50 mM Tris and 500 μg/ml DEAE-dextran). 2 μg of a *COX-2* promoter-*luciferase* construct, 2 μg of either an expression vector or empty plasmid and 0.5 μg of the control plasmid pSV-β*gal* were added and the mixture incubated at 37°C and 5% CO_2 for 30 min. Dimethylsulfoxide (100 μl/ml of transfection mixture) was added for 1 min and the reaction stopped with excess RPMI. Transfected cells were plated in 10% FBS RPMI for 24 h and subsequently treated with fresh 3% FBS RPMI with or without LPS (50 ng/ml). Luciferase and β-galactosidase activity were measured in cellular extracts 6 h later. Luciferase activity data are presented after normalization to β-galactosidase activity [5].

2.5. Electrophoretic mobility shift assays (EMSA)

RAW 264.7 cells were plated in 100 mm dishes at a density of 3×10^6 cells/dish and allowed to attach for 24 h prior to experiments. Cells were then treated with fresh 3% FBS RPMI with or without LPS (50 ng/ml). Nuclear extracts were obtained by high-salt extraction (0.5 M NaCl) 30 min later as described previously [6]. In some experiments, nuclear extracts were incubated with rabbit IgG, anti-c-Jun, anti-CREB or anti-USF-1 for 2 h, followed by incubation with protein A-agarose beads for 2 h. After centrifugation, transcription factor-depleted supernatants were used in EMSA. Doubled-stranded DNA oligonucleotides containing a functional binding site for the CRE/E-box (sense: 5′-CAGTCATTTCGTCACATGGGCTTGG-3′), CRE (sense: 5′-CAGTCATTTCGTCACACTGGCTTGG-3′) or E-box (sense: 3′-CAGTCATTTGATCACATGGGCTTGG-5′) elements found in the *COX-2* promoter were labeled with [^{32}P]ATP using T4 kinase. 4 μg of nuclear extract was incubated with 1 μl of DNA probe in a total of 10 μl containing 4% glycerol, 50 mM NaCl, 10 mM Tris pH 7.5, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol and 1 μg poly(dI-dC). Cold chase was carried out with a 50× molar excess of the same, unlabeled probe or with a probe that contained mutated binding sequences (lower-case letters) for CRE (TTgaTCA-CATG) or E-box (TTCGTCACAct). Nuclear extract–DNA complexes were resolved in 4% polyacrylamide gels using 0.5×TBE at 150 V and the gels were then dried and autoradiographed [6].

2.6. Statistics

Comparisons between groups were made by Student's *t*-test. A difference between groups of $P<0.05$ was considered significant.

3. Results

3.1. CRE and E-box can independently collaborate with other promoter elements to induce macrophage COX-2 transcription in response to endotoxin

We first investigated whether nuclear proteins from endotoxin-treated macrophages bound the CRE, the E-box or both consensus sequences, overlapped in the COX-2 promoter (see scheme in Fig. 1). EMSA with nuclear extracts of LPS-treated RAW 264.7 cells revealed that a slower CRE complex and a faster E-box complex can be differentiated, through cold chase with mutant probes, from what initially appeared to be a single shifted complex (Fig. 2A).

In previous work [6], we had made the observation that maximal endotoxin-mediated induction of COX-2 transcription in macrophages necessitated either an NF-κB or an NF-IL6 promoter element and an intact CRE/E-box consensus. In order to assess the relative contribution of the CRE and E-box elements, we constructed COX-2 promoter-*luciferase* reporter elements in which the CRE, E-box or both elements had been selectively mutated. Transient transfection experiments with these constructs were carried out in RAW 264.7 cells treated with or without LPS. As shown in Fig. 2B, the decrease in luciferase activity in the CRE/E-box double mutant construct can be overcome to levels greater than in the control wild type promoter construct by adding a functional CRE element. The addition of a functional E-box promoter element partially overcomes the decrease in luciferase activity of the double CRE/E-box mutant, but to a lesser degree than with the control wild type construct.

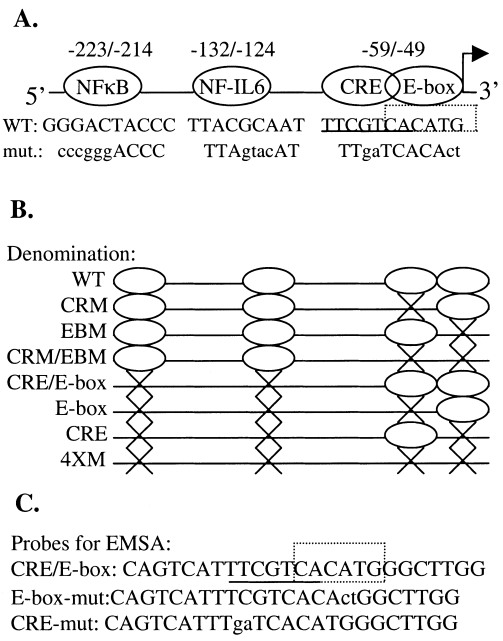

Fig. 1. Schematic of the COX-2 promoter, promoter constructs and promoter probes.

3.2. CRE and E-box can independently induce macrophage COX-2 transcription in response to endotoxin

We investigated whether induction of COX-2 transcription in response to endotoxin is accompanied by an increase in nuclear protein DNA binding to the CRE and E-box. EMSA experiments in Fig. 3A show that LPS treatment of RAW 264.7 cells induces binding of nuclear proteins to a COX-2 promoter probe containing either a CRE or an E-box functional element.

Based on these results, it appeared that the ability of both CRE and E-box promoter elements to increase macrophage COX-2 transcription in response to endotoxin (Fig. 2B) could be related to their ability to independently induce gene transcription. In order to confirm this hypothesis, we constructed COX-2 promoter reporter plasmids containing only one functional element, namely a CRE or an E-box. Transient transfection experiments with these constructs were carried out in RAW 264.7 cells treated with or without LPS. As shown in Fig. 3B, LPS induces an increase in luciferase activity through

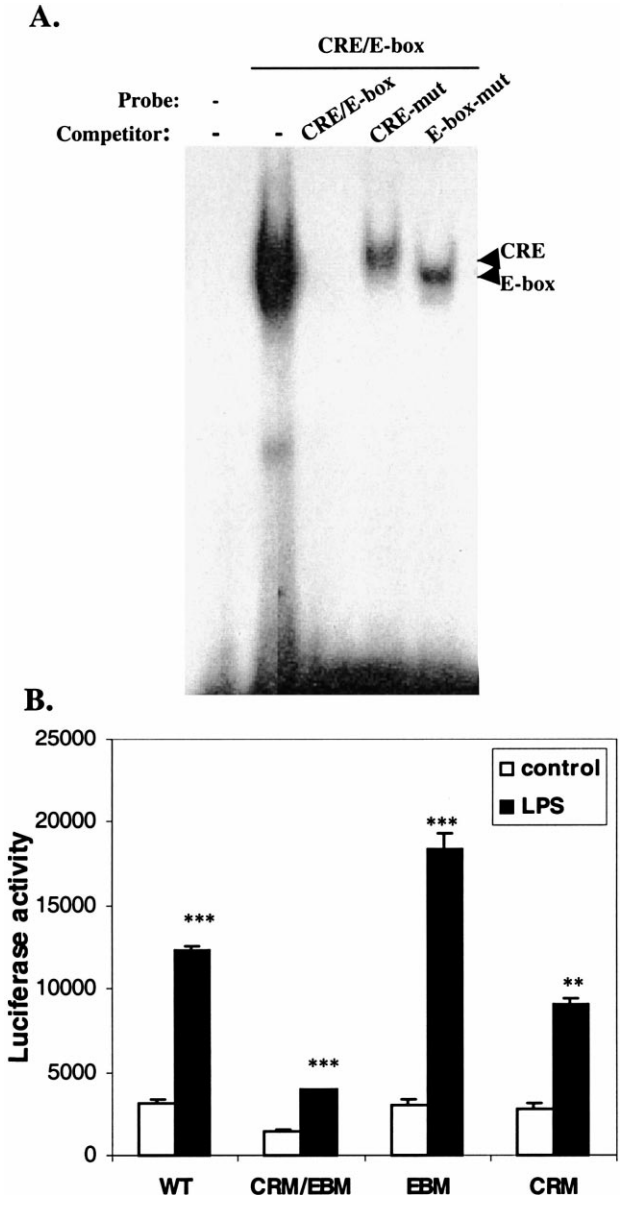

Fig. 2. A: Activated transcription factors in macrophages bind both the CRE and E-box overlapping promoter elements in the COX-2 promoter. EMSAs were performed using double-stranded oligonucleotides containing the CRE/E-box consensus sequences and flanking regions found in the COX-2 promoter. Cold chase lanes incorporated a 50 molar excess of unlabeled CRE/E-box probe (lane 3), CRE mutant probe (E-box chase; lane 4) or E-box mutant (CRE chase; lane 5). B: CRE and E-box can independently collaborate with other promoter elements to induce macrophage COX-2 transcription in response to endotoxin. RAW 264.7 cells were transfected with a −327/+59 *COX-2* promoter-*luciferase* construct (WT) and a series of *COX-2* promoter-*luciferase* mutant constructs (CRE and E-box mutant, E-box mutant and CRE mutant, designated CRM/EBM, EBM and CRM, respectively). Reporter activities were measured in cell extracts 6 h later. Columns indicate means, bars S.D.; $n=6$. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$ for WT-transfected control and LPS-treated cells vs. mutant-transfected control and LPS-treated cells, respectively.

both the CRE and E-box elements. In fact, luciferase activity was about four-fold greater when only one functional element was present than when both CRE and E-box coexisted in an overlapping form in the COX-2 promoter.

3.3. *COX-2 transcription is induced by Jun and CREB through the CRE element and by USF-1 through the E-box element in endotoxin-treated macrophages*

Since both CRE and E-box can mediate COX-2 transcription, we were interested in determining what transcription factors associated to these promoter elements in endotoxin-treated macrophages. Nuclear extracts were obtained from LPS-treated RAW 264.7 cells and depleted of specific tran-

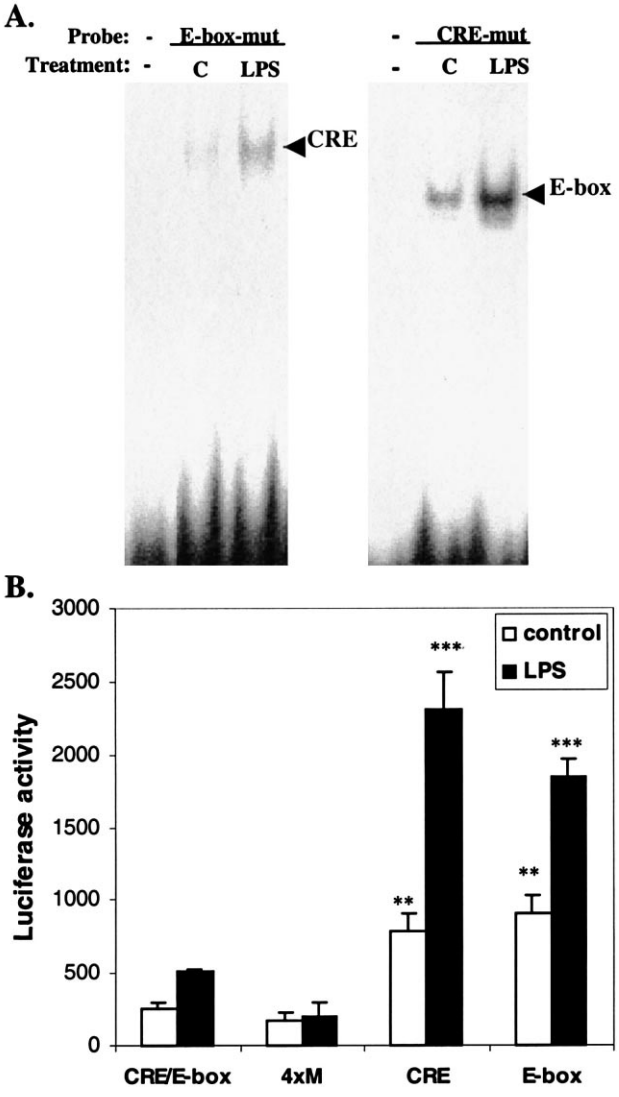

Fig. 3. A: Endotoxin induces DNA binding of transcription factors to the CRE and E-box promoter elements of the COX-2 gene in macrophages. EMSAs were performed using double-stranded oligonucleotides containing mutant elements and flanking regions found in the COX-2 promoter. A, mutated E-box consensus (functional CRE). B, mutated CRE consensus (functional E-box). B: CRE and E-box can independently induce macrophage COX-2 transcription in response to endotoxin. Raw 264.7 cells were transfected with −327/+59 *COX-2* promoter-*luciferase* mutant constructs (NF-κB and NF-IL6 mutant, NF-κB, NF-IL6, CRE and E-box mutant, E-box mutant and CRE mutant, designated for their functional elements as CRE/E-box, 4XM, CRE and E-box, respectively). Reporter activities were measured in cell extracts 6 h later. Columns indicate means, bars S.D.; $n=6$. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$ for CRE/E-box-transfected control and LPS-treated cells vs. 4XM, CRE and E-box-transfected control and LPS-treated cells, respectively.

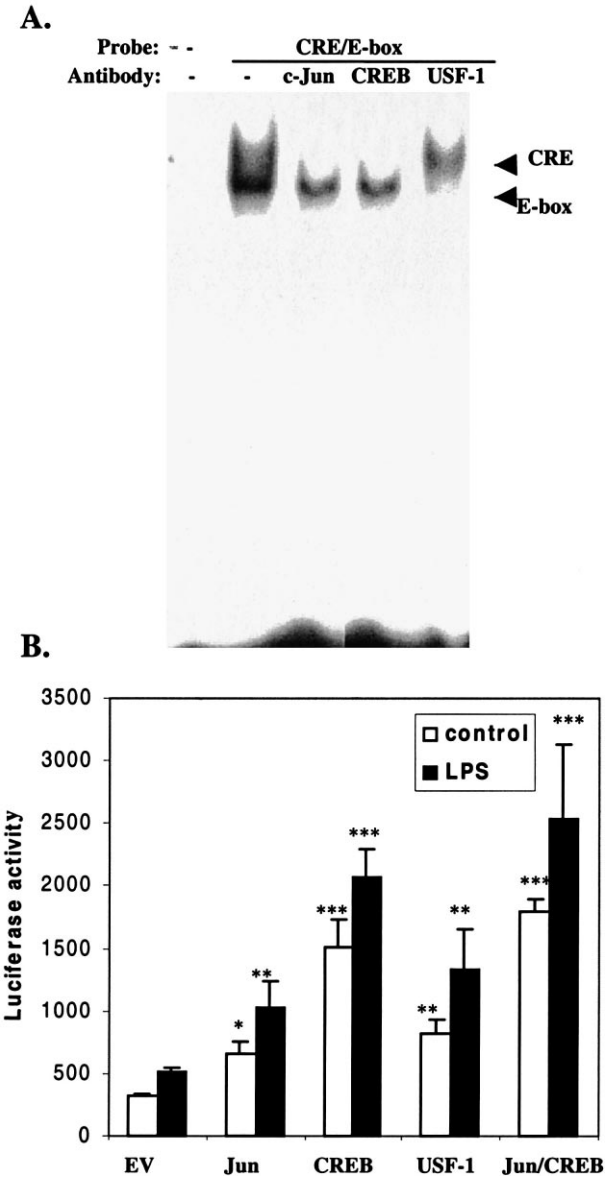

Fig. 4. A: Jun and CREB are complexed to the CRE and USF-1 to the E-box of the COX-2 promoter in endotoxin-treated macrophages. Nuclear extracts from RAW 264.7 cells treated with medium containing LPS (50 μg/ml) for 30 min were incubated with rabbit IgG, anti-c-Jun, anti-CREB or anti-USF-1 conjugated to protein A-agarose beads in order to deplete nuclear extracts of specific transcription factors. EMSAs were performed using double-stranded oligonucleotides containing the CRE/E-box elements and flanking regions found in the COX-2 promoter. B: COX-2 transcription is induced by Jun and CREB through the CRE element and by USF-1 through the E-box element in endotoxin-treated macrophages. RAW 264.7 cells were co-transfected with a −327/+59 *COX-2* promoter-*luciferase* reporter construct containing only functional CRE/E-box promoter elements and either an empty vector (EV) or expression vectors for c-Jun, CREB, USF-1 and c-Jun and CREB. Reporter activities were measured in cell extracts 6 h later. Columns indicate means, bars S.D.; $n=6$. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$ for empty vector-transfected control and LPS-treated cells vs. expression vector-transfected control and LPS-treated cells, respectively.

scription factors. Subsequently, EMSA experiments showed that c-Jun and CREB bind the CRE element and USF-1 the E-box element in the COX-2 promoter (Fig. 4A). Other transcription factors such as Fos and ATF did not appear to bind the COX-2 CRE/E-box promoter element (data not shown).

After we identified transcription factors that bind CRE and E-box in the COX-2 promoter of endotoxin-treated macrophages, we investigated their ability to induce COX-2 transcription. Transient co-transfections with a COX-2 promoter luciferase reporter construct containing only one functional CRE/E-box promoter element and expression vectors for c-Jun, CREB and USF-1 were carried out in RAW 264.7 cells treated with or without LPS. As shown in Fig. 4B, c-Jun, CREB and USF-1 overexpression induce luciferase activity.

4. Discussion

COX-2 expression and PG synthesis in macrophages appears to be important in eliciting local and systemic inflammatory responses. Increased COX-2 activity may result from increased enzymatic activity or mRNA stability [10–12], but in endotoxin-treated macrophages it results mainly from increased transcription of the *COX-2* gene through the collaboration of NF-κB, NF-IL6 and an overlapping CRE/E-box [6]. Therefore, it is important to completely elucidate the signaling mechanisms governing *COX-2* gene transcription, a potential target of strategies designed to suppress local or systemic inflammatory responses.

Our data show that both the CRE and E-box promoter elements can individually mediate COX-2 transcription in response to endotoxin: through the binding of c-Jun and CREB to the CRE and of USF-1 to the E-box. However, the CRE synergized with other promoter elements (i.e. NF-κB and NF-IL6) to induce COX-2 transcription to a greater extent than the E-box (Fig. 2B), indicating that the CRE-bound complex is transcriptionally more active. On the other hand, transcription through individual CRE or E-box elements was greater than through the overlapping CRE/E-box (Fig. 3B), suggesting that CRE and E-box binding transcription factors may interfere with each other's binding to the COX-2 promoter, thereby decreasing transcription.

Previous studies had evidenced the importance of the CRE element in mediating COX-2 transcription, particularly in the murine promoter and in epithelial cells [13,14]. However, the individual contribution of each binding site and the net transcriptional effect of an overlapping CRE/E-box promoter had not been studied. Moreover, induction of transcription through the E-box element had not been clearly demonstrated yet except in the rat promoter, which lacks a CRE element and differs significantly from the human [15].

We have recently shown that there is redundancy in the promoter elements and signaling pathways regulating COX-2 transcription in endotoxin-treated macrophages [6]. In the present work, the ability of both CRE and E-box to mediate COX-2 transcription adds to that redundancy, which may represent an important mechanism ensuring increased levels of COX-2 in macrophages during inflammation. Moreover, our data indicate that specific stimulation of CRE- or E-box-activating pathways, rather than both, could lead to increased rates of macrophage COX-2 expression and PG synthesis.

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