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# Induction of cyclooxygenase-2 by bovine type I collagen in macrophages via C/EBP and CREB activation by multiple cell signaling pathways

Min Kyung Cho, Yang Hee Cho, Gum Hwa Lee, Sang Geon Kim*

*National Research Laboratory, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, South Korea*

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**Abstract**

Bovine type I collagen (Col-I) is utilized for medical purposes such as cosmetic surgery and wrinkle removal. Cyclooxygenase-2 (COX-2) plays roles in pathophysiological processes including inflammation and tumorigenesis. This study examines the effects of Col-I on the COX-2 expression and the signaling pathways in macrophages. Col-I increased the levels of COX-2 protein and mRNA in serum-stimulated Raw264.7 cells in a time- and concentration-dependent manner. Treatment of cells with Col-I increased CCAAT/enhancer binding protein (C/EBP) DNA binding. Antibody supershift experiments revealed that C/EBP DNA binding activity induced by Col-I depended largely on C/EBPβ and C/EBPδ. Immunocytochemistry showed that Col-I induced nuclear translocation of C/EBPβ and C/EBPδ, whose activation contributes to COX-2 induction. Overexpression of the dominant-negative mutant form of C/EBP abolished COX-2 induction by Col-I. Col-I also increased cyclic-AMP response element binding protein (CREB) binding to DNA. Inhibition of focal adhesion kinase (FAK) or downstream phosphoinositide 3-kinase and p70S6 kinase by specific chemical inhibitors prevented COX-2 induction by Col-I, and C/EBP and CREB from binding to their consensus DNA oligonucleotides. Experiments using chemical inhibitors or dominant-negative mutant vectors showed that the mitogen-activated protein (MAP) kinase pathways including p38-kinase and extracellular signal-regulated kinase (ERK1/2), but not c-Jun N-terminal kinase (JNK1), simultaneously regulated COX-2 induction by Col-I. This was in agreement with inhibition of Col-I-inducible C/EBP and CREB DNA binding by concomitant treatment with SB203580 and PD98059. These results provide evidence that Col-I induces COX-2 in serum-stimulated macrophages and that the multiple cell signaling pathways involving Src-focal adhesion kinase, phosphoinositide 3-kinase, and MAP kinases regulate COX-2 induction by Col-I via C/EBP and CREB activation.


*Keywords:* Type I collagen; COX-2; C/EBP; CREB; FAK; PI3-kinase; p38-kinase; ERK1/2

## 1. Introduction

Collagen is the most prevalent fibrous component in extracellular matrix. Among the various types of collagens, type I collagen (Col-I), which is a 300 kDa molecule composed of two alpha$_1$(I) chains and one alpha$_2$(I) chain, is the major structural component of extracellular matrices found in connective tissue and internal organs, and most prevalent in the dermis, tendons, and bone [1]. Type I collagen obtained from the bovine species is largely utilized for medical purposes such as cosmetic surgery and removal of wrinkle because of its abundance and safety [2]. Bovine type I collagen is also used in a variety of cell culture models for the study of cell attachment, growth, differentiation, and cell migration [3]. Col-I induces transcription of the interleukin-1β gene [4]. Previous study from this laboratory has shown that Col-I induces nitric oxide synthase (iNOS) in serum-stimulated murine macrophages through JunB/AP-1 and nuclear factor-κB (NF-κB) activation and that activation of extracellular signal-regulated kinase-1/2 (ERK1/2) plays a role in the activity of

*Abbreviations:* C/EBP, CCAAT/enhancer binding protein; COX-2, cyclooxygenase 2; ERK, extracellular signal-regulated kinase; CREB, cAMP response element binding protein; FAK, focal adhesion kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; MEM, minimal essential medium; NF-κB, nuclear factor-κB; PI3-kinase, phosphoinositide 3-kinase; PCR, polymerase chain reaction; RT, reverse transcription

* Corresponding author. Tel.: +82-2-880-7840; fax: +82-2-872-1795.
*E-mail address:* sgk@snu.ac.kr (S.G. Kim).

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JunB/AP-1 [5]. Yet, the effects of Col-I on the expression of other inflammatory genes have not been studied.

Cyclooxygenase-2 (COX-2) is an inducible immediate early gene product in inflammatory cells (e.g. macrophages) and immune cells and the expression of COX-2 is markedly stimulated by lipopolysaccharide (LPS), cytokines, growth factors and tumor promoters [6–8]. COX-2 plays roles in the pathophysiological processes including inflammation. COX-2 is also overexpressed in certain cancer tissues and thus involved in tumor and endothelial cell biology (e.g. colorectal tumors). Induction of COX-2 occurs in multiple cells within the tumor microenvironment. COX-2 expression correlates with the expression of angiogenic factors and the formation of new blood vessels [9]. Development of COX-2 inhibitors represents a major advance in the therapy of inflammatory processes and their use includes prevention or treatment of disorders associated with the induction of this enzyme.

Previous studies from our laboratory and others have shown that the pathways of COX-2 induction by LPS involve both mitogen-activated protein kinase kinase (MKK)–ERK1/2 and p38-kinase [10,11]. In spite of the wide applications of Col-I, the effects of Col-I on the expression of COX-2 have not been examined. The present study investigated COX-2 induction by Col-I in serum-stimulated macrophages, and explored the cell signaling pathways responsible for the enzyme induction.

Studies have shown that CCAAT/enhancer binding protein (C/EBP), cyclic-AMP response element binding protein (CREB) and NF-κB were commonly or individually involved in the regulation of the *COX-2* gene [12,13]. Evidence has accumulated to show that the C/EBP element plays an important role in COX-2 induction. In particular, activation of C/EBPβ leads to the enzyme induction [7,13]. The regulatory region for the *COX-2* gene includes cAMP response element (CRE)/E-box elements. In the present study, we determined whether Col-I activated C/EBP and CREB in serum-stimulated macrophages. Now, we report that Col-I induces COX-2 as a result of the increases in C/EBP and CREB activation, the pathways of which involve multiple cell signaling cascades of Src kinase-focal adhesion kinase (Src-FAK), phosphoinositide 3-kinase (PI3-kinase), p70S6 kinase, p38-kinase and ERK1/2. However, the pathways including tyrosine kinase, protein kinase A, protein kinase C, mitogen and stress response kinase-1 (MSK1), or NF-κB, appeared not to be associated with COX-2 induction by Col-I.

## 2. Materials and methods

### 2.1. Materials

Anti-COX-2 antibody was obtained from Cayman. Anti-C/EBPα, β, δ, and ε form antibodies were supplied from Santa Cruz Biotechnology. Horseradish peroxidase- or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was purchased from Zymed Laboratories Inc. PD98059, LY294002, PP2, AG1295, AG1478, staurosporine, H89, and Ro31-8220 were supplied from Calbiochem, whereas GF109203X was obtained from BioMol. BAY117082 was purchased from Alexis. [$\gamma$-$^{32}$P]ATP (3000 mCi/mmol) was obtained from PerkinElmer Life Science. The consensus oligonucleotides of C/EBP, CREB and NF-κB, and random prime labeling and 5′-end labeling kits were supplied from Promega Corporation. c-Jun N-terminal kinase (JNK1) dominant-negative mutant (KmJNK1) was kindly provided from Dr. N. Dhanasekaran (Fels Institute for Cancer Research and Molecular Biology, Department of Biochemistry, Temple University, PA, USA). The plasmid of MKK1 dominant-negative mutant was gifted from Dr. N.G. Ahn (Howard Hughes Medical Institute, University of Colorado, Boulder, CO, USA). C/EBP-specific dominant-negative expression (AC/EBP) plasmid was a gift from Dr. C. Vinson (National Institutes of Health, Bethesda, MD, USA) [14]. Col-I and most other reagents for the molecular studies were obtained from Sigma Chemical. Col-I was subjected to the Limulus Amoebocyte Lysate test (i.e. endotoxin test using the gel clot method). Col-I at 0.1–30 μg/ml was endotoxin-free with the sensitivity limit of 0.03 EU/ml.

### 2.2. Cell culture

Raw264.7 cells, a murine macrophage cell line (American Type Culture Collection), were cultured in Dulbecco's modified Eagles medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Raw264.7 cells were plated at a density of 2–3 $\times$ $10^6$/ml and preincubated for 24 h at 37 °C. Cells were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$. For all experiments, cells were grown to 80–90% confluency, and were subjected to no more than 20 cell passages. Raw264.7 cells were treated with various concentrations of 0.3–30 μg/ml Col-I for 24 h. For inhibition experiments, cells were preincubated with PD98059 (30 μM), SB203580 (20 μM), LY294002 (10 μM), PP2 (5 μM), AG1295 (20 μM), AG1478 (10 μM), staurosporine (10 nM), H89 (20 μM), Ro31-8220 (20 nM), rapamycin (100 nM), BAY117082 (5 μM) or GF109203X (10 μM) for 1 h and then continuously exposed to Col-I with the inhibitor for 24 h.

### 2.3. Immunoblot analysis

The expression of COX-2 was immunochemically monitored in lysates of Raw264.7 cells using an anti-mouse COX-2 antibody. The secondary antibody was horseradish peroxidase-conjugated anti-rabbit antibody. The band of COX-2 protein was developed using ECL immunoblot detection system according to the manufacturer's instruction (Amersham). C/EBPα, β, δ, and ε forms in nuclear

fractions or total cell lysates were immunoblotted with the respective specific antibodies.

### 2.4. RT-PCR analysis

Total RNA (2 μg) obtained from the cells was reverse-transcribed using an oligo(dT) adaptor as a primer to produce cDNAs. The specific cDNA probe for the *COX-2* gene was amplified by reverse transcription (RT)-polymerase chain reaction (PCR) using the selective primers and cloned in a TA vector (Promega). The primers used are as follows, COX-2, sense primer: 5′-TCTCCAACCTC-TCCTACTAC-3′, antisense primer: 5′-GCACGTAGTCT-TCGATCACT-3′ (624 bp). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was assessed by PCR using the sense primer: 5′-TCGTGGAGTCTA-CTGGCGT-3′ and the antisense primer: 5′-GCCTGCTT-CACCACCTTCT-3′ (510 bp). PCRs were conducted using the following conditions for 38 cycles: denaturation at 94 °C for 0.5 min, annealing at 49 °C for 1 min, and elongation at 68 °C for 1.5 min. Band intensities of the amplified DNAs were compared after visualization on an UV transilluminator.

### 2.5. Preparation of nuclear extracts

Nuclear extracts were prepared essentially according to the previously published method [15]. Briefly, the cells in dishes were washed with ice-cold PBS. Cells were then scraped, transferred to microtubes, and allowed to swell after the addition of 100 μl hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride. The lysates were incubated for 10 min in ice and centrifuged at 7,600 × *g* for 6 min at 4 °C. Pellets containing crude nuclei were resuspended in 50 μl of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol and 1 mM phenylmethylsulfonylfluoride and then incubated for 1 h in ice. The samples were centrifuged at 15,000 × *g* for 10 min to obtain supernatants containing nuclear fractions. Nuclear fractions were stored at −70 °C until use.

### 2.6. Gel retardation assay

Double stranded DNA probes for the consensus sequences of C/EBP (5′-TGCAGATTGCGCAATCTGCA-3′), CREB (5′-AGAGATTGCCTGACGTCA GAGAGCTAG-3′), and NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were used for gel shift analyses after end-labeling of the probes with [$\gamma$-$^{32}$P]ATP and $T_4$ polynucleotide kinase. Nuclear extracts were prepared by modification of the procedure published previously [15]. The reaction mixtures contained 2 μl of 5× binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml polydI–dC and 50 mM Tris–Cl (pH 7.5), 5 μg of nuclear extracts and sterile water in a total volume of 10 μl. Reactions were initiated by addition of 1 μl probe ($10^6$ cpm) following 10 min pre-incubation and continued for 30 min at room temperature. Specificity of protein binding to the respective consensus DNA oligonucleotide was confirmed by competition reactions, in which a 20-fold molar excess of unlabeled C/EBP, CREB, or NF-κB oligonucleotide was added to each reaction mixture before the addition of radiolabeled probe. For supershift assay, the antibodies (2 μg each) were added to the reaction mixture, and additionally incubated for 1 h at 25 °C. Samples were loaded onto 4% polyacrylamide gels at 100 V. The gels were removed, fixed and dried, followed by autoradiography.

### 2.7. Stable transfection of JNK1 dominant-negative mutant

Cells were transfected using Transfectam® according to the manufacturer's instruction (Promega). Cells were replated 24 h before transfection at a density of $2 \times 10^6$ cells in a 10 $cm^2$-plastic dish. For use in JNK1 dominant-negative mutant [JNK1(−)] stable transfection, 20 μl of Transfectam® was mixed with 10 μg of the JNK1(−) plasmid in 2.5 ml of minimal essential medium (MEM). Cells were transfected by addition of MEM containing the plasmid and Transfectam®, and then incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ for 6 h. After addition of 6.25 ml MEM with 10% FCS, cells were incubated for additional 48 h and geneticin added to select the resistant colonies. In the present study, stably transfected JNK1(−) cells were used.

### 2.8. Scanning densitometry

Scanning densitometry was performed with Image Scan & Analysis System (Alpha-Innotech Corporation). One way analysis of variance (ANOVA) procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman–Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $P < 0.05$ or <0.01.

## 3. Results

### 3.1. COX-2 induction by Col-I

We first determined whether Col-I induces COX-2 in macrophages incubated in the medium containing 10% FCS. COX-2, which was not detected in untreated control cells, was induced by treatment with Col-I for 24 h (0.3–30 μg/ml) (Fig. 1A). Cell viability was not affected by varying concentrations of Col-I (data not shown). Because

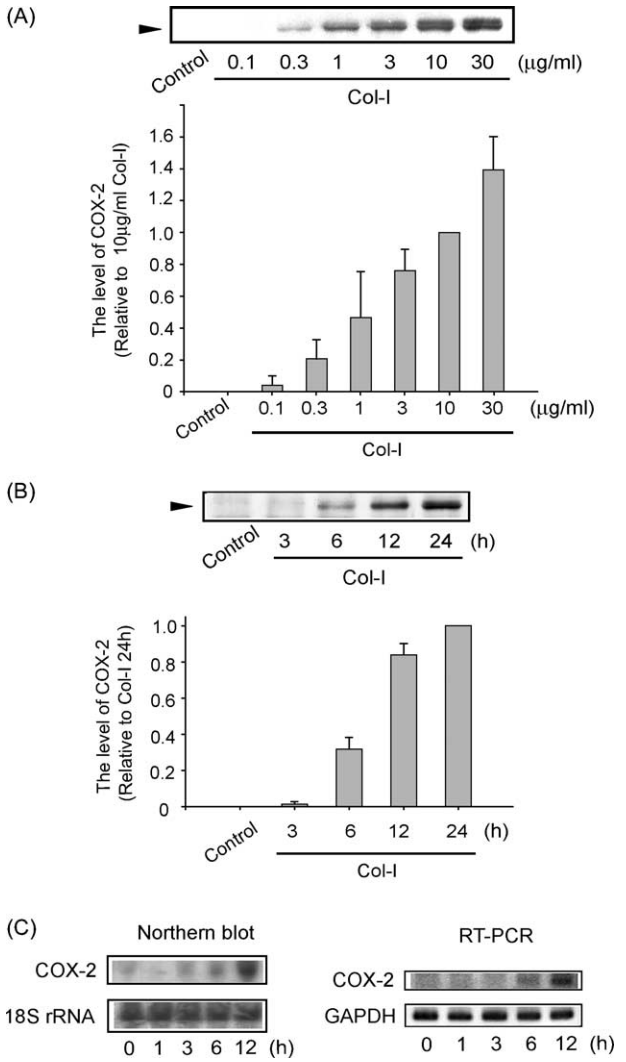


Fig. 1. Induction of COX-2 by Col-I. (A) Induction of COX-2 by varying concentrations of Col-I. Raw264.7 cells were incubated in the medium containing 10% FCS in the presence of Col-I for 24 h. Control cells were incubated with vehicle alone. The levels of COX-2 were immunobloted as described in Section 2. Data represent the mean ± S.E. with at least three separate experiments (COX-2 induction by 10 μg/ml Col-I = 1). (B) Time course of COX-2 expression in the cells treated with Col-I (10 μg/ml). The relative COX-2 levels were measured by scanning densitometry of immunoblot bands. Data represent the mean ± S.E. with at least three separate experiments (COX-2 induction by Col-I at 24 h = 1). (C) The effect of Col-I on the levels of COX-2 mRNA in the cells treated with Col-I (10 μg/ml). Northern blot and RT-PCR analyses were carried out to determine the levels of COX-2 mRNA in total RNA fractions (20 μg each for Northern blot analysis; 2 μg each for RT-PCR) prepared from the cells exposed to Col-I for the indicated time periods. Equal loading of RNA in each lane was confirmed by measuring 18S rRNA or RT-PCR of GAPDH mRNA.

Col-I at 10 μg/ml notably induced COX-2, the concentration was chosen in the subsequent experiments. To determine the time course of COX-2 induction, Raw264.7 cells were treated with 10 μg/ml Col-I for 3–24 h (Fig. 1B). Col-I induced COX-2 at 6–24 h after treatment. Studies were extended to determine whether the levels of COX-2 mRNA increased in parallel with COX-2 induction. Northern blot analysis showed that Col-I increased the levels of COX-2 mRNA at 6–12 h (Fig. 1C, left). Increases in the level of COX-2 mRNA was confirmed by RT-PCR analysis (Fig. 1C, right).

### 3.2. Effects of Col-I on C/EBP, CREB, and NF-κB activation

Expression of the *COX-2* gene depends on the activation of C/EBP and CREB and their binding to the respective binding sites present in the upstream region of the gene [13]. To test whether COX-2 induction by Col-I was accompanied with activation of C/EBP and CREB, electrophoretic mobility shift assays were performed with the nuclear extracts prepared from the cells exposed to Col-I using radiolabeled consensus oligonucleotides.

Treatment of cells with 10 μg/ml Col-I for 3–12 h resulted in increases in the band intensity of C/EBP DNA binding compared with control (Fig. 2A, left). The band intensity of C/EBP binding to its DNA binding site maximally increased at 3–6 h and slightly decreased from the maximum at 12 h. To identify the factor(s) that make(s) up the inducible C/EBP activity, specific antibodies directed against individual C/EBP proteins were evaluated for the ability to inhibit the DNA binding activity. Immunodepletion experiments indicated that C/EBP DNA binding activity in the cells treated with Col-I depended on specifically C/EBPβ and C/EBPδ, but not C/EBPα or C/EBPε (Fig. 2A, right). Addition of a 20-fold excess of unlabeled C/EBP binding oligonucleotide to the nuclear extract abolished C/EBP binding to the C/EBP binding site (Fig. 2A, right). These data indicated that C/EBPβ and C/EBPδ contributed to the enhanced C/EBP DNA binding in the cells treated with Col-I.

Mutation of the cAMP-response element site in the *COX-2* gene abrogated COX-2 reporter activity [7]. We next monitored the effect of Col-I treatment on CREB DNA binding activity. The intensity of CREB DNA binding was enhanced in the cells treated with Col-I for 3–12 h (Fig. 2B, left). Addition of a 20-fold excess of unlabeled CREB binding oligonucleotide, but not SP-1 consensus oligonucleotide, abolished formation of the CREB DNA binding complex (Fig. 2B, right).

NF-κB is activated in the cells challenged with LPS and other inflammatory cytokines [16]. Our previous study has shown that NF-κB was activated at early times after treatment of Raw264.7 cells with Col-I [5]. Gel shift analysis confirmed the increase in NF-κB DNA binding activity by Col-I (Fig. 2C). Addition of excess unlabeled NF-κB oligonucleotide decreased the intensity of NF-κB DNA binding.

### 3.3. The role of C/EBP activation in COX-2 induction by Col-I

Because C/EBPβ and C/EBPδ were the components of C/EBP DNA binding complex in the cells treated with Col-I, we verified translocation of C/EBPβ or C/EBPδ into

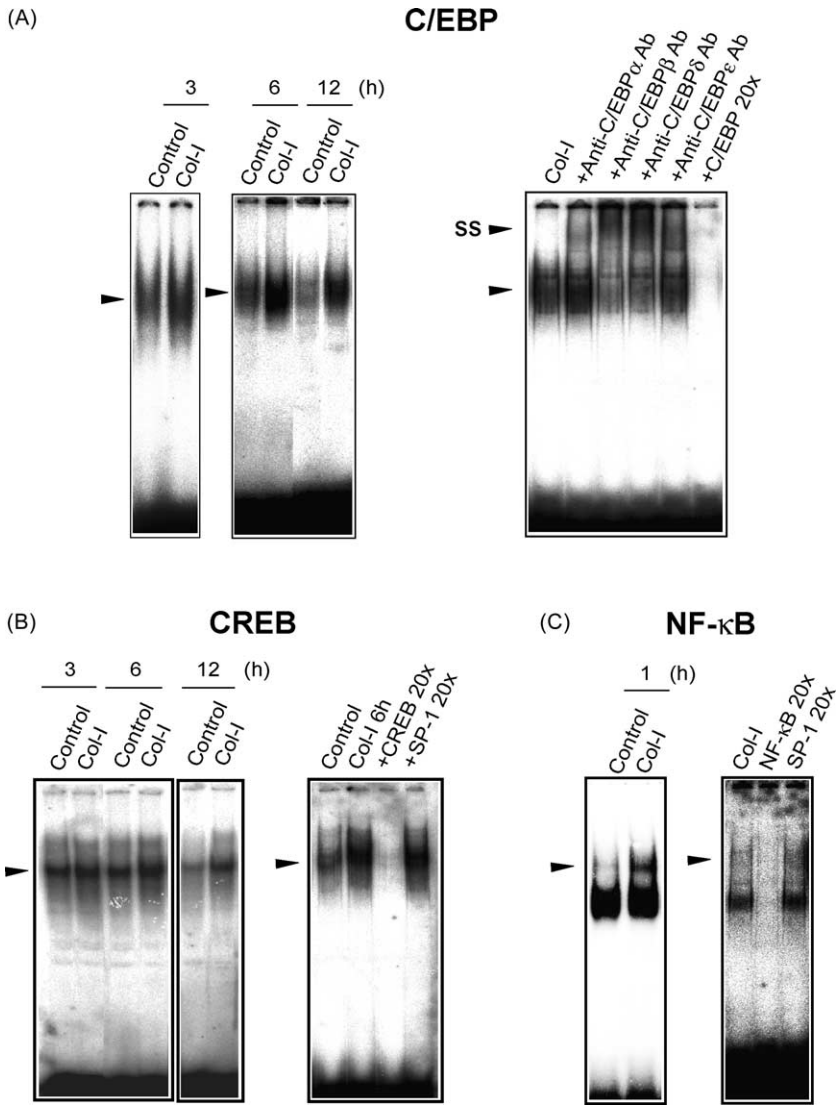


Fig. 2. Gel shift analyses of C/EBP, CREB and NF-κB DNA complexes. Nuclear extracts were prepared from Raw264.7 cells exposed to Col-I (10 μg/ml) for the indicated time periods. (A) Gel shift analyses of C/EBP. All lanes contained 5 μg of nuclear extracts and 5 ng of labeled C/EBP consensus oligonucleotide. Supershift analyses were carried out by incubating the nuclear extracts with the specific polyclonal antibody directed against each C/EBP form for 1 h. SS indicates supershift of the retarded C/EBP DNA complex. Competition studies were carried out by adding a 20-fold excess of an unlabeled C/EBP oligonucleotide (20×) to the nuclear extracts from the cells treated with Col-I, and the DNA-binding reactions were performed by gel shift analysis. (B) Gel shift analyses of CREB. CREB binding activity was assessed as described in panel A. (C) Gel shift analyses of nuclear extracts using the consensus oligonucleotide of NF-κB. The specificity of NF-κB binding was confirmed by addition of an excess amount of free probe (20×). Results were confirmed by repeated experiments.

the nucleus by immunocytochemistry and examined the functional role of C/EBP proteins in the induction of COX-2 by transfection with the plasmid encoding a dominant-negative mutant form of C/EBP (AC/EBP). Raw264.7 cells were incubated with Col-I (10 μg/ml) for 1–12 h, fixed and permeabilized. C/EBPβ protein was located predominantly in the cytoplasm of control cells (Fig. 3A, upper). C/EBPβ protein moved into the nucleus 3–6 h after Col-I treatment. The intensity of nuclear C/EBPβ in the cells exposed to Col-I for 9–12 h returned toward that in control cells (Fig. 3A). Nuclear integrity was confirmed by PI staining of the identical cells. Nuclear translocation of C/EBPδ was also stimulated in the cells exposed to Col-I for the time periods of 3–12 h (Fig. 3B, lower).

We then assessed the role of C/EBP in COX-2 induction by Col-I. Constitutively active AC/EBP was expressed in cells prior to treatment with Col-I. Whereas transfection with pCMV500, an empty vector, allowed Col-I to induce COX-2, AC/EBP expression notably inhibited the ability of Col-I to induce COX-2 (Fig. 3B). These results provided evidence that activation of C/EBP proteins by Col-I contributed to COX-2 induction.

### *3.4. Cell signaling pathways for COX-2 induction by Col-I*

In light of activation of the cell signaling pathways downstream of membrane-associated integrin and adhesion

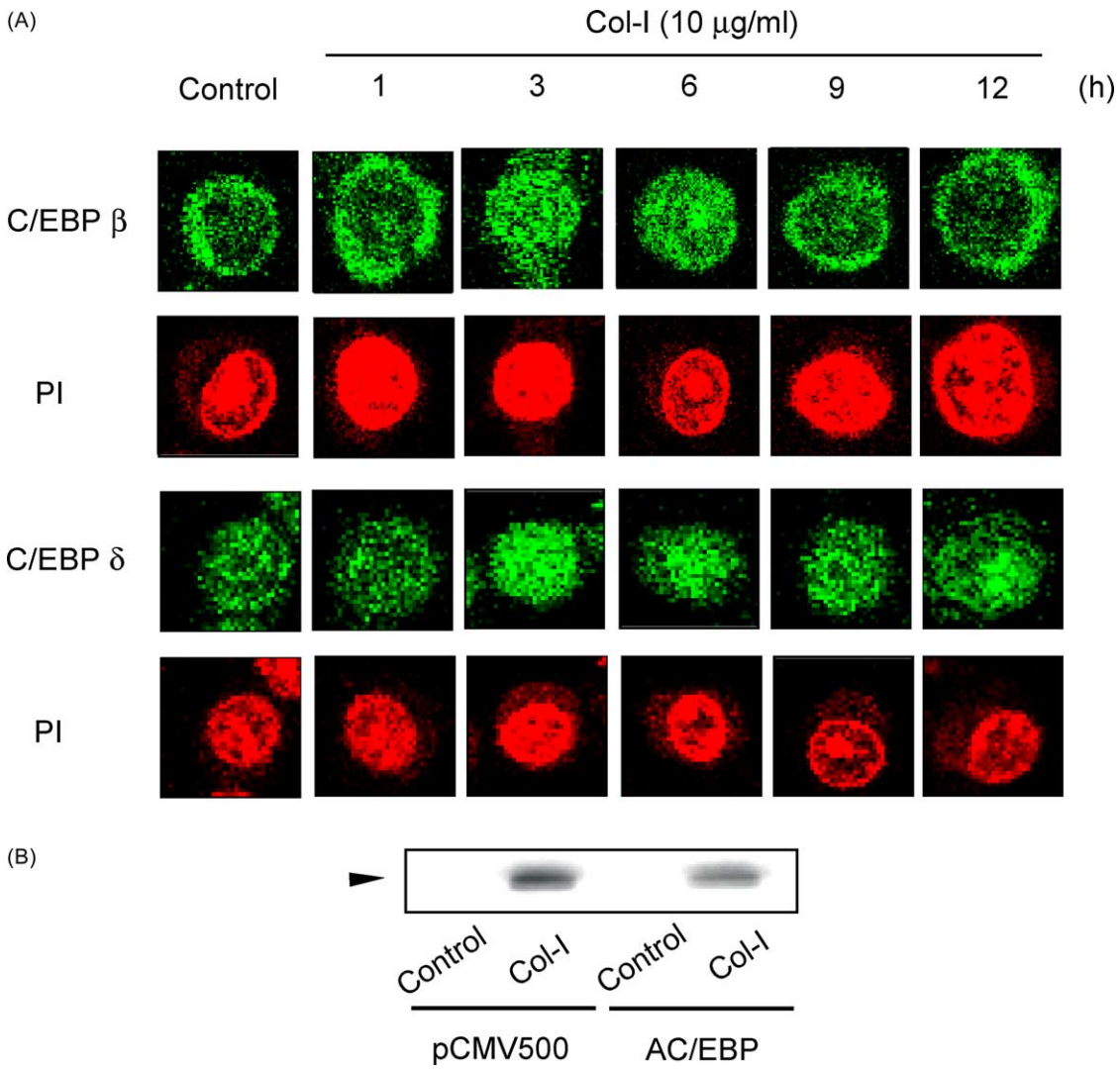


Fig. 3. The role of activating C/EBP proteins in COX-2 induction by Col-I. (A) Immunocytochemistry of C/EBPβ and C/EBPδ in the cells treated with Col-I. Distribution of C/EBPβ or C/EBPδ was monitored in Raw264.7 cells incubated with vehicle (control) or 10 μg/ml Col-I for 1–12 h. The same fields were counter-stained with propidium iodide (PI). The results were confirmed by repeated experiments. (B) Inhibition of Col-I-inducible COX-2 expression by AC/EBP. Raw264.7 cells transfected with the plasmid encoding for AC/EBP were exposed to Col-I (10 μg/ml) for 20 h. Control cells were transfected with pCMV500. Results were confirmed by repeated experiments.

molecules in response to collagens [17,18], we were interested in the role of Src-FAK and PI3-kinase signaling pathways, which are stimulated by integrin, in COX-2 induction by Col-I. It has been shown that PP2 at the concentration of 5 μM effectively inhibited Src-FAK in Raw264.7 cells [19,20]. The inhibitory effect of PP2 results from FAK inhibition via Src inhibition [21]. Treatment of Raw264.7 cells with PP2 (5 μM) substantially inhibited the COX-2 induction (Fig. 4A). We previously showed that the activities of PI3-kinase and its downstream p70S6 kinase were inhibited by 10 μM LY294580 and 100 nM rapamycin, respectively, [11,22]. Inhibition of PI3-kinase by LY294002 (10 μM) abolished the induction of COX-2 by Col-I (24 h) (Fig. 4B). Rapamycin (100 nM) also suppressed COX-2 induction (Fig. 4C). These data indicated that both Src-FAK and PI3-kinase-p70S6 kinase were involved in COX-2 induction by Col-I.

Major signaling pathways for the induction of COX-2 transmit through mitogen-activated protein (MAP) kinase pathways [7,23]. The role of the MAP kinase signaling pathways in COX-2 induction by Col-I was investigated using specific inhibitors or stable transfection with dominant-negative mutant vector. SB203580 and PD98059 were previously utilized as the MAP kinase inhibitors in Raw264.7 cells in our laboratory [5,11]. In the present study, the induction of COX-2 by Col-I was partially inhibited by treatment of cells with either SB203580 (20 μM) or PD98059 (30 μM) alone Fig. 5A and B). In contrast, the JNK1 pathway was not responsible for the enzyme induction, as evidenced by the lack of inhibition by JNK1(−) transfection of the COX-2 induction (Fig. 5C). Treatment of cells with both PD98059 and SB203580 inhibited COX-2 induction to the greater extent (Fig. 5D). These data provided evidence that both p38-kinase and

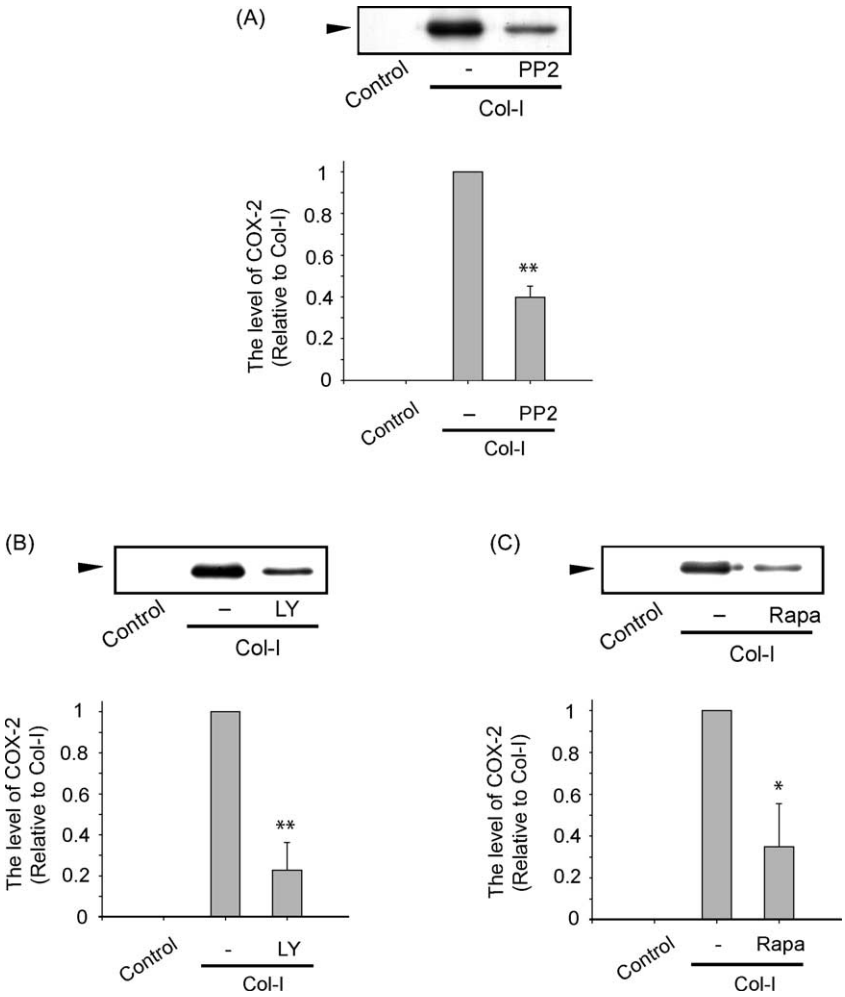


Fig. 4. The effects of FAK, PI3-kinase and p70S6 kinase inhibitors on COX-2 induction by Col-I. Raw264.7 cells were treated with PP2 (FAK inhibitor, 5 μM), LY294002 (LY, PI3-kinase inhibitor, 10 μM) or rapamycin (Rapa, p70S6 kinase inhibitor, 100 nM), in panels (A)–(C), respectively, and then exposed to 10 μg/ml of Col-I for 24 h. The representative immunoblots show the levels of COX-2. Data represent the mean ± S.E. with at least four separate experiments (COX-2 induction by 10 μg/ml Col-I = 1).

ERK1/2, but not JNK1, were necessary for COX-2 induction by Col-I.

We next examined the role of other cellular kinases in COX-2 induction by Col-I by using specific chemical inhibitors. Treatment of Raw264.7 cells with Ro31-8220 (20 nM) [24–26] or staurosporine (10 nM) [26], protein kinase C inhibitors, failed to inhibit Col-I-inducible COX-2 expression (Fig. 6A, left). In addition, GF109203X (10 μM), an inhibitor of classical and novel isoforms of PKC [27], did not inhibit COX-2 induction by Col-I. H89, a protein kinase A and MSK1 inhibitor, at the concentration of 20 μM (24 h) [28] did not change the induction of COX-2 by Col-I (Fig. 6A, right). Inhibitors of tyrosine kinases including AG1295 (an inhibitor of PDGF receptor tyrosine kinase, 20 μM) [29,30] and AG1478 (an inhibitor of EGF receptor tyrosine kinase, 10 μM) [31] failed to decrease the expression of COX-2 by Col-I (Fig. 6B, left). Extensive studies have shown that these chemical inhibitors at the concentrations employed actively suppress the target kinases in Raw264.7 cells or in other macrophages. Our results support the notion that the cellular kinases including protein kinase C, protein kinase A, MSK1 and receptor tyrosine kinases were not responsible for the COX-2 induction. To determine the role of NF-κB activation in the induction of COX-2, COX-2 was monitored in the cells exposed to Col-I in the presence of BAY117082, an inhibitor of NF-κB (5 μM) [32–34] (Fig. 6B, right). COX-2 induction by Col-I was not affected by BAY117082. Thus, it is unlikely that NF-κB activation by Col-I contributes to the enzyme induction.

### 3.5. Cell signaling pathways for activation of C/EBP and CREB

Studies were extended to determine whether Src-FAK, PI3-kinase and MAP kinases regulated activation of C/EBP and CREB. Treatment of the cells with PP2, LY294002 or rapamycin blocked the increase by Col-I

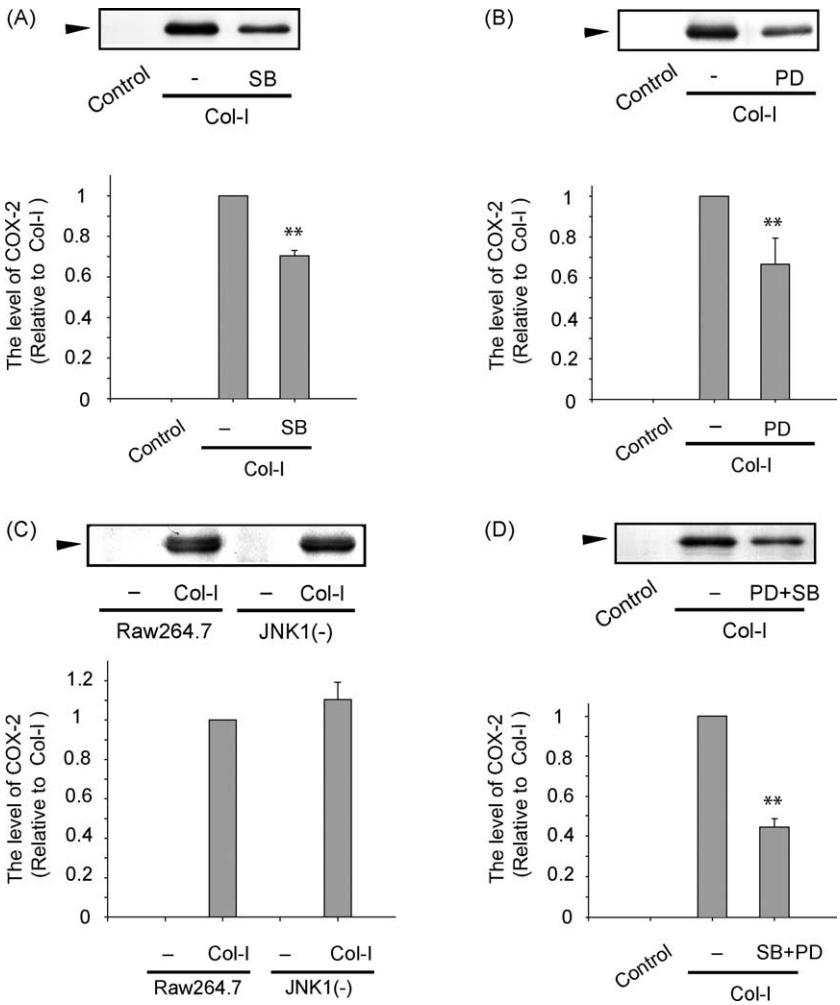


Fig. 5. The role of MAP kinases in COX-2 induction by Col-I. The role of p38-kinase in the induction of COX-2 was assessed by using SB203580 and PD98059 as p38-kinase inhibitor (A) and MKK1 inhibitor (B), respectively. Raw264.7 cells were treated with SB203580 (SB, 20 μM), PD98059 (PD, 30 μM) or SB203580 + PD98059 for 1 h and further incubated with Col-I (10 μg/ml) for 24 h. Control cells or cells stably expressing JNK1(−) were used to assess the role of JNK in COX-2 induction by Col-I (C). The effect of SB203580 + PD98059 on the induction of COX-2 by Col-I was shown in panel (D). Data represent the mean ± S.E. with at least four separate experiments (significant as compared to Col-I alone, $^{**}P < 0.01$).

in the band intensities of C/EBP DNA binding (Fig. 7A), suggesting that the pathway involving Src-FAK, PI3-kinase and p70S6 kinase control activation of C/EBP protein. Protein binding to the C/EBP binding site was also decreased in the cells treated with both PD98059 and SB203580 (Fig. 7A). Thus, the Src-FAK and PI3-kinase signaling pathways in the conjunction with the p38-kinase and ERK1/2 pathways, which are responsible for COX-2 induction by Col-I, regulate activation of C/EBP.

CREB DNA binding, whose band intensity was increased by Col-I, was also inhibited by treatment of the cells with the chemical inhibitor(s) of Src-FAK, PI3-kinase, p70S6 kinase or the MAP kinases (i.e. p38-kinase and ERK1/2) (Fig. 7B). Inhibition of CREB activation was pronounced 6 h after treatment (Fig. 7B) and also detectable 3 h post-treatment (data not shown). These data supported the conclusion that the multiple cell signaling pathways activate C/EBP and CREB for the induction of COX-2 by Col-I.

## 4. Discussion

Activated macrophages secrete inflammatory mediators including lipid metabolites (e.g. prostaglandins) and other cytokines. Macrophages are involved in tissue repair and remodeling [35] and reside in the connective tissue areas with ECM. During acute and chronic inflammation, local ECM proteins including Col-I are exposed to macrophages. Several matrix proteins including Col-I bind to integrins [36]. Thus, the binding of Col-I elicits signals that are transmitted into the cell [37]. It has been shown that α1β1 integrins are activated in the cells cultured on monomeric type I collagen, but not on polymerized one [38]. Macrophages also adhere selectively to denatured forms of Col-I via their scavenger receptors [39]. In view of the wide medical, cosmetic and experimental (e.g. cell culture) applications of type I collagen obtained from the bovine species, we were interested in the effects of Col-I in serum-stimulated macrophages on COX-2 induction, and the cell

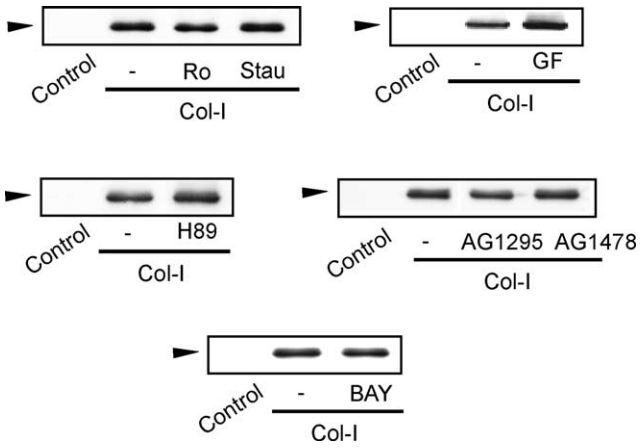


Fig. 6. The effects of PKC, PKA/MSK1, PDGF receptor kinase, EGF receptor kinase, NF-κB inhibitors on COX-2 induction by Col-I. Raw264.7 cells were treated with Ro31-8220 (Ro, 20 nM) or staurosporine (Stau, 10 nM), GF109203X (GF, 10 μM), H89 (an inhibitor of PKA/MSK, 20 μM), AG1295 (an inhibitor of PDGF receptor kinase, 20 μM), AG1478 (an inhibitor of EGF receptor kinase, 10 μM) or BAY117082 (BAY, an inhibitor of NF-κB, 5 μM), and then subsequently exposed to 10 μg/ml of Col-I for 24 h in the continuing presence of each chemical inhibitor. The representative immunoblots show the levels of COX-2. Results were confirmed by repeated experiments.

signaling pathways responsible for the enzyme induction in the present study.

COX-2 catalyzes the production of prostaglandins, which represents an important step in the inflammatory process [7]. Alterations in prostaglandin production have been linked to cardiovascular disease, chronic and acute inflammation, atherosclerosis and colorectal cancer [40]. The production of prostaglandins by LPS in macrophages is primarily due to the transcriptional activation of the *COX-2* gene [41,42]. The *cis*-acting elements in the promoter region of the murine *COX-2* gene include C/EBP, CREB, and NF-κB [28,43].

In the previous study, we showed that the induction of iNOS by Col-I in serum-stimulated macrophages was mediated with activation of NF-κB and AP-1. We also found that Col-I stimulated the production of interleukin-1 and tumor necrosis factor-α in macrophages (Cho and Kim, unpublished data). The current study demonstrates that Col-I induces COX-2 in serum-stimulated macrophages. Thus, the inflammatory process in macrophages activated by Col-I involves production of prostaglandins. Prostaglandins in combination with excess NO and cytokines produced from the cells exposed to Col-I may further increase inflammatory responses as a result of vasodilation and macrophage accumulation.

C/EBPβ is involved in the formation of assembled transcription factor complex required for transactivation of the *COX-2* gene. The complex consisting of C/EBPβ homodimers is involved in the activation of C/EBP response element in macrophages exposed to LPS [44]. If C/EBPβ is inactivated, the induction of COX-2 by LPS is impaired [45]. Previous studies from this laboratory have shown that ceramide increases C/EBPβ-mediated COX-2 induction by LPS, which involves enhanced C/EBPβ expression via AP-1 [11]. We found in additional that ceramide also enhanced Col-I-inducible COX-2 expression

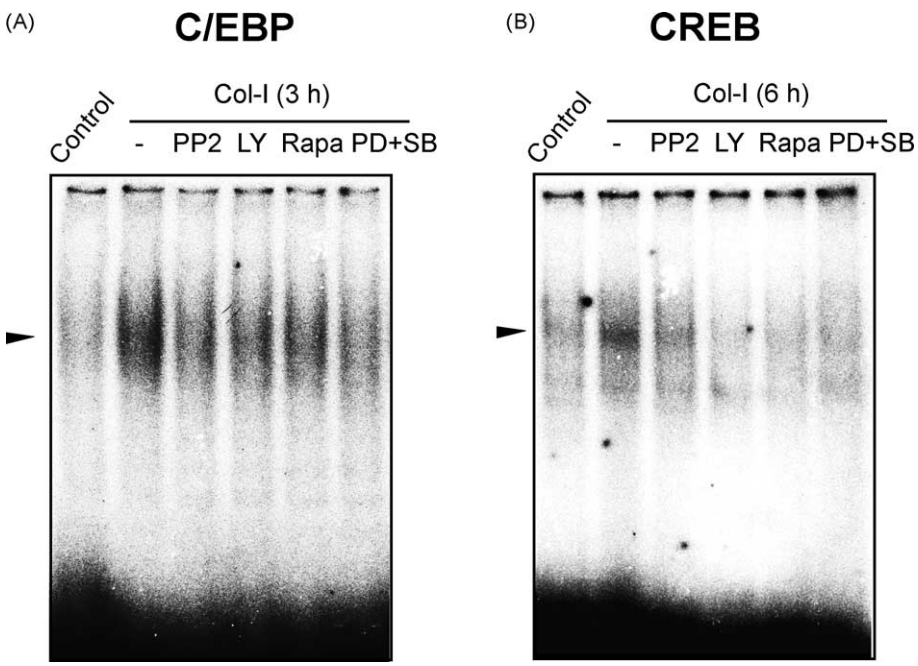


Fig. 7. Gel shift analyses of C/EBP and CREB DNA binding complexes. Raw264.7 cells were incubated with Col-I (10 μg/ml) in the presence or absence of a specific kinase inhibitor. Nuclear extracts prepared from the cells treated with PP2 (5 μM), LY294002 (LY, 10 μM) or rapamycin (Rapa, 100 nM), or SB203580 (SB, 20 μM) + PD98059 (PD, 30 μM) for the indicated time periods were subjected to gel shift analyses of C/EBP (A) or CREB (B). All lanes contained 5 μg of nuclear extracts and 5 ng of labeled consensus oligonucleotide. Arrowheads indicate C/EBP or CREB DNA complex. Results were confirmed by at least four repeated experiments.

(Cho and Kim, unpublished data). CREB is a 43 kDa transcription factor that binds to the conserved cAMP response element. The initial phase of COX-2 expression by LPS involved CREB [43]. The present observation that Col-I activates both C/EBPβ and CREB for the induction of COX-2 is consistent with the finding that the factors are required for the basal and LPS-inducible expression of COX-2 [11,46]. In spite of the presence of the NF-κB binding site in the regulatory region of the *COX-2* gene, the putative NF-κB binding site seemed not to be activated for the induction of COX-2 by LPS, as shown by dominant-negative inhibition of NF-κB and COX-2 reporter gene activity [7]. Lack of inhibition by BAY117082, a NF-κB inhibitor, of COX-2 induction in the present study also supports the notion that NF-κB may not contribute to the induction of COX-2.

Integrin clustering leads to co-clustering of a wide variety of signaling molecules. The signaling complexes downstream of integrins include Src-FAK, PI3-kinase, MAP kinases and small GTPases [47,48]. In the present study, we used rapamycin as an inhibitor of p70S6 kinase [49,50]. It is possible that rapamycin may also inhibit other kinases [51]. Nevertheless, our pharmacological study supports the conclusion that p70S6 kinase in association with PI3-kinase plays a role in the induction of COX-2 by Col-I. Now we report that the pathways of Src-FAK and its downstream PI3-kinase and p70S6 kinase, but not the cellular kinases including protein kinase C, protein kinase A, MSK1, and receptor tyrosine kinases, regulate COX-2 induction by Col-I in serum-stimulated macrophages (Fig. 8). The Src-FAK and PI3-kinase pathways regulated activation of C/EBPβ and CREB that were responsible for the COX-2 induction.

Our laboratory and others have shown that the MAP kinase pathways including p38-kinase and ERK1/2 regulate the induction of COX-2 by LPS [11,52]. We previously showed that Col-I activated all three MAP kinases including p38-kinase, ERK1/2, and JNK [5]. In the present study, we found that both the p38-kinase and the ERK1/2 pathways controlled COX-2 induction by Col-I (Fig. 8). By contrast, the JNK pathway was not involved in the COX-2 induction. In LPS-treated cells, C/EBPβ activation was inhibited by PD98059 treatment or transfection with dominant-negative mutant of MKK1, indicating that the pathway of MKK1–ERK1/2 regulates activation of the transcription factor [38]. In contrast to the partial inhibition of C/EBP and CREB activation by p38-kinase or MKK1 inhibitor, concomitant inhibition of p38-kinase and ERK1/2 activities notably suppressed COX-2 induction by Col-I and decreased C/EBP and CREB activation to the greater extent. Thus, it is highly likely that phosphorylation of multiple sites of the transcription factors by p38-kinase and ERK1/2 leads to the induction of COX-2.

Previously, we reported that potentiation by ceramide of LPS induction of COX-2 involves the pathway of JNK1, but not ERK1/2 [11]. JNK1-mediated C/EBPβ activation

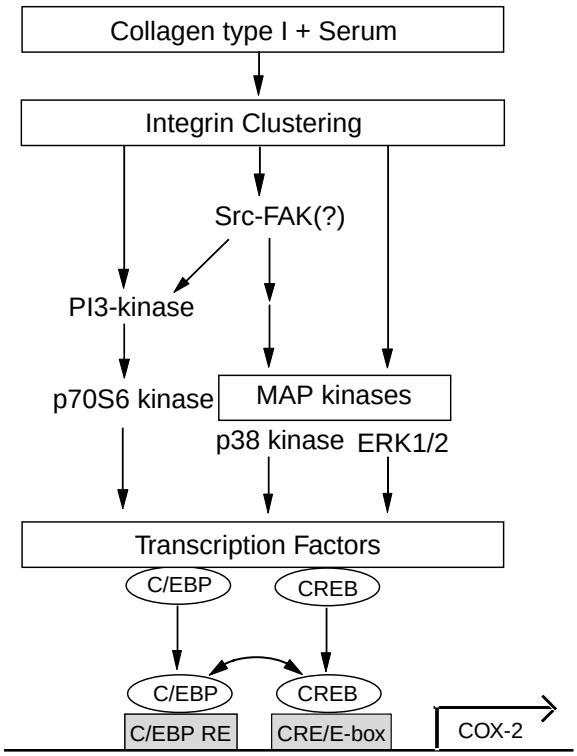


Fig. 8. Schematic diagram illustrating the proposed mechanism by which Col-I induces COX-2. Multiple cell signaling pathways including Src-FAK, PI3-kinase, p70S6 kinase, and MAP kinases (p38-kinase and ERK1/2) are involved in the activation of C/EBP and CREB by Col-I in serum-stimulated macrophages. Activating C/EBP and CREB then stimulate transcriptional activation of COX-2.

contributes to ceramide-enhanced *COX-2* gene induction by LPS as a result of the binding of C/EBPβ homodimer complex to the consensus DNA sequence [11]. That ceramide enhances COX-2 induction by Col-I also resulted from JNK1-mediated C/EBPβ activation (Cho and Kim, unpublished data). We observed that the signaling pathway responsible for the induction of COX-2 by Col-I involved ERK1/2 and p38-kinase, which switched to the pathways of JNK and p38-kinase by an apoptotic rheostat ceramide.

In summary, the results in the present study demonstrated that Col-I induces COX-2 in serum-stimulated macrophages, and that the multiple signaling pathways including the Src-FAK, PI3-kinase and p70S6 kinase as well as p38-kinase and ERK1/2 regulate activation of C/EBP and CREB for COX-2 induction by Col-I. Our results imply that activation of macrophages by Col-I in conjunction with production of prostaglandins and NO may increase macrophage accumulation and vasodilation.

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