

Erythromycin Suppresses the Expression of Cyclooxygenase-2 in Rheumatoid Synovial Cells

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ABSTRACT. Objective. To investigate whether erythromycin (EM) can suppress the expression of cyclooxygenase-2 (COX-2) in rheumatoid synovial cells, and determine the mechanisms involved.

Methods. Synovial tissues were obtained from 25 patients with rheumatoid arthritis (RA). Rheumatoid synovial cells were cultured with or without EM (0.1–1000 nM) in the presence of interleukin 1 β (IL-1 β) for various times. Protein expression of COX-2, and phosphorylation of extracellular signal regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (p38 MAPK) were detected by Western blot. COX-2 messenger RNA (mRNA) was detected by RT-PCR. DNA binding activity of nuclear factor kappa B (NF- κ B) was detected by ELISA.

Results. IL-1 β -stimulated synovial cells expressed COX-2 protein. EM suppressed the IL-1 β -induced COX-2 protein expression in a dose-dependent manner and inhibited IL-1 β -induced p38 MAPK phosphorylation, which was correlated with COX-2 expression in synovial cells. In contrast, EM had no effect on DNA binding activity of NF- κ B and ERK1/2 expression.

Conclusion. Our results indicated that EM downregulated COX-2 expression by inhibiting the p38 MAPK cascade, but had no effect on NF- κ B or ERK1/2, in rheumatoid synovial cells. (J Rheumatol 2004;31:436–41)

Key Indexing Terms:
ERYTHROMYCIN
INTERLEUKIN 1 β

SYNOVIAL CELLS
CYCLOOXYGENASE-2
P38 MITOGEN ACTIVATED PROTEIN KINASE

Rheumatoid arthritis (RA) is characterized by cytokine production from inflammatory cells that infiltrate the synovial tissue and subsequent articular cartilage destruction¹. Prostaglandin E₂ (PGE₂), an important inflammatory mediator, is produced through cyclooxygenase (COX) enzymes from prostaglandin endoperoxides^{2,3}. COX-2 is an important therapeutic target in arthritis, because PGE₂ induces tissue degradation and bone resorption^{4,7}. In addition, high expression levels of COX were observed in RA synovia compared with those from patients with osteoarthritis or healthy subjects⁸.

Fourteen-member ring macrolides, including erythromycin (EM), exhibit antibacterial activity and a broad spectrum of pharmacological effects including antiinflammatory

activity^{9,10}. The macrolides act on several pathways of the inflammatory process, such as the migration of neutrophils, oxidative burst in phagocytes, and production of proinflammatory cytokines^{11–13}. Previous studies showed that EM suppresses the inflammatory response by reducing PGE₂ synthesis in peritonitis⁹; however, this effect is not clear in RA. The precise mechanisms of macrolide-mediated PGE₂ synthesis inhibition remain to be clarified.

We investigated the effect of EM on COX-2 expression in rheumatoid synoviocytes. Our results showed that EM suppresses interleukin 1 β (IL-1 β)-mediated COX-2 messenger RNA (mRNA) and protein expression. We also examined the effect of EM on the mitogen-activated protein kinase cascades involved in COX-2 induction.

MATERIALS AND METHODS

Reagents. EM made by Sigma Chemical Co. (St. Louis, MO, USA) was provided from Dai-nippon Pharmaceutical (Tokyo, Japan). Human recombinant IL-1 β was purchased from Becton Dickinson Labware (San Jose, CA, USA), anti-human COX-2 rabbit IgG monoclonal antibody from IBL Corporation (Gunma, Japan), anti-rabbit IgG horseradish peroxidase (HRP) conjugate from Promega Corporation (Madison, WI, USA), rabbit anti-phospho-extracellular signal regulated kinase 1/2 (ERK1/2) and rabbit anti-phospho-p38 mitogen-activated protein kinase (p38 MAPK) from BioSource International Inc. (Camarillo, CA, USA).

Cell culture. The experimental protocol was approved by the ethics committee of Kurume University, and a signed informed consent was obtained from each participant. Synovial tissue samples were obtained from patients with RA during surgery. Synovial membranes were minced aseptically and then dissociated enzymatically with collagenase (4.0

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mg/ml; Sigma) in RPMI 1640 for 2 h at 37°C. The obtained cells were plated on culture dishes and allowed to adhere. To eliminate nonadherent cells from synovial cells and to simplify synovial B cells, the plated cells were cultured 18 h with RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37°C aerated with humidified 5% CO₂ in air. The cells were then washed thoroughly with phosphate buffered saline (PBS) solution. Adherent synovial cells were removed by adding trypsin-EDTA followed by washing the cells with PBS containing 2% FBS. The collected synovial cells were used at the second or fourth passage for subsequent experiments. Synovial cell preparations at the fourth passage contained less than 1% cells reactive to monoclonal antibodies CD3, CD20, and CD68 (Coulter Immunology, Hialeah, FL, USA) and anti-human von Willebrand factor (Immunotech, Marseilles, France), indicating that these preparations were almost free of mature T lymphocytes, B lymphocytes, monocytes/macrophages, and vascular endothelial cells. Synovial cell preparations at the second passage contained the same cells at slightly greater proportions than at fourth passage.

Immunoblot analysis. COX-2 protein expression in synovial cells was analyzed by Western blot as described¹⁴. For this purpose, second-passage cells were grown to subconfluence on culture dishes in serum-free medium for 24 h in the presence or absence of EM. Fourth-passage cells were grown to subconfluence on culture dishes containing serum-free medium for 1 h in the presence or absence of EM, and these cells were stimulated with IL-1β (0.1 ng/ml) for 24 h. Cells were washed with cold PBS and lysed by the addition of a lysis buffer containing 1% Nonidet P-40, 50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 20 min at 4°C. Insoluble material was removed by centrifugation at 15,000 g for 15 min at 4°C. The supernatant was saved, and the protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). An identical amount of protein (30 μg) from each lysate and culture supernatant was subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Phosphorylation of ERK1/2 and p38 was analyzed by Western blot using phospho-specific antibody. Cells were grown to subconfluence on culture dishes, and starved by serum-free medium for 24 h in the presence or absence of EM. After starvation, synovial cells were stimulated with IL-1β (0.1 ng/ml) for 10 min. Cells were washed with cold PBS and lysed by the addition of a lysis buffer containing 1% Nonidet P-40, 0.1% SDS, 50 mM Tris, pH 7.5, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 20 mM β-glycerophosphate, 1.0 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, for 20 min at 4°C. The protein concentration was determined using the Bio-Rad protein assay kit. An identical amount of protein (30 μg) from each lysate and culture supernatant was subjected to 10% SDS-PAGE.

The fractionated proteins were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL, USA), and the filters were blocked for 1.5 h using nonfat dried milk in Tris-buffered saline (TBS: 50 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1% Tween 20, washed with TBS, and incubated at room temperature for 2 h at 1:50 dilution of rabbit anti-COX-2 monoclonal antibody (mAb), or 1:2000 dilution of rabbit anti-phospho-ERK1/2 and phospho-p38 MAPK mAb. The membranes were further incubated with a 1:2000 dilution of donkey anti-rabbit immunoglobulin G (IgG) antibody for 20 min, coupled with HRP. An enhanced chemiluminescence system (Amersham) was used for detection. Filters were subsequently exposed to film for 15 s, and the latter was processed.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) assay. Total cellular RNA was extracted from synovial fibroblasts using guanidinium thiocyanate and phenol (RNAzol B; Cinna/Biotek Labs Int., Friendswood, TX, USA). First-strand cDNA was synthesized by reverse transcription at 39°C for 50 min in a 20 μl reaction mixture containing 1 μl of total RNA, M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA), random primer (Takara Shuzo Co., Shiga, Japan), and RNase inhibitor (Toyobo Co.). Two microliters of denatured cDNA were amplified in a 20 μl final volume containing 1 U Taq DNA

polymerase (Gibco BRL), 1 μM of each primer, Taq polymerase buffer, 1.5 mM MgCl₂, and 1.5 mM of each dNTP. PCR was performed in a thermal cycler (Perkin Elmer Cetus, Foster City, CA, USA) using a program of 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min with a final 10 min extension at 72°C. The amplified products were subjected to electrophoresis on 2% agarose gel. The following specific primers were used for COX-2¹⁵: 5'-TTCAAATGAGATGTGGGAAAATGCT-3' (forward), 5'-AGATCATCTCTGCCTGAGTATCTT-3' (reverse). Predicted size of the fragment was 301 bp. For β-actin: 5'-CAAGAGATGGCCACGGCTGCT-3' (forward), 5'-TCCTTCTGCATCCTGTCGGCA-3' (reverse). Predicted size of the fragment was 275 bp.

NF-κB DNA binding activity. By ELISA, p65 DNA binding activity was measured with 10 μg of nuclear extract with the Trans-AM™ kit (Active Motif, Rixensart, Belgium) using the protocol supplied by the manufacturer and as described¹⁶.

RESULTS

To examine the effect of EM on COX-2 expression in synovial tissue, second-passage synovial cells were cultured with (Figure 1, lanes 2–6) or without EM for 24 h, and lysates were analyzed by anti-COX-2 immunoblot. As shown in Figure 1, RA synovial cells constitutively expressed COX-2 (lane 1), and EM (0.1–1000 nM) suppressed COX-2 expression in a dose-dependent manner (lanes 2–6).

We also examined the effect of EM on cytokine-stimulated synovial cells. Synovial cells were stimulated by IL-1β in the presence or absence of EM. Lysates were analyzed by anti-COX-2 immunoblot. Four-passage synovial cells did not express COX-2 constitutively (Figure 2, lane 1), while synovial cells stimulated by IL-1β (0.1 ng/ml) expressed COX-2 (lanes 2–7). EM (0.1–1000 nM) suppressed COX-2 expression in a dose-dependent manner (lanes 3–7).

RT-PCR was performed to determine whether IL-1β treatment induces COX-2 mRNA expression in synovial cells. Reverse transcription was performed on total RNA from synovial cells stimulated with IL-1β for 6 h. COX-2 and β-actin cDNA were amplified by PCR. There was a linear correlation between the number of cycles (n = 30) and

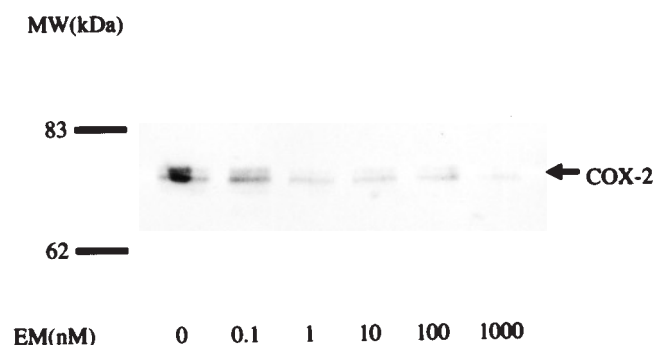


Figure 1. Effect of erythromycin (EM) on COX-2 expression of first-passage synovial cells with nonadherent cells. These cells were cultured in serum-free medium in the presence or absence of EM for 24 h and were not stimulated. Equal amounts (30 μg) of cell lysates were electrophoresed on 8% polyacrylamide gel and analyzed by Western blot using anti-COX-2 antibody. A representative example of 3 independent experiments is shown.

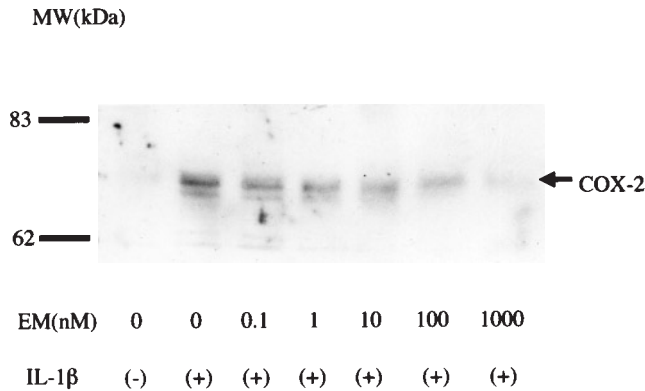


Figure 2. Effect of erythromycin (EM) on COX-2 expression of third-passage synovial cells. Cells were cultured in serum-free medium in the presence or absence of EM for 1 h. After treatment the cells were stimulated with IL-1 β for 24 h. Equal amounts (30 μ g) of cell lysates were electrophoresed on 8% polyacrylamide gel and analyzed by Western blot using anti-COX-2 antibody. A representative example of 5 independent experiments is shown.

the yield of PCR products for both COX-2 and β -actin mRNA. Stimulation of synovial cells by IL-1 β induced COX-2 mRNA expression in synovial cells (Figure 3, lane 2). EM pretreatment suppressed COX-2 mRNA induction by IL-1 β in synovial cells.

To investigate the role of NF- κ B on COX-2 induction by IL-1 β in synovial cells, we performed p65 NF- κ B DNA binding activity measured in an ELISA format using the Trans-AMTM kit. After synovial cells were cultured by serum-free RPMI with or without EM for 24 h, cells were stimulated by IL-1 β for 1 h. We observed that NF- κ B reached the highest value rapidly over 1 h, and then it fell gradually over several hours. There was a significant difference between the group stimulated by IL-1 β and the group which was not stimulated by IL-1 β in the absence of EM ($p < 0.001$). EM did not affect the expression of NF- κ B signif-

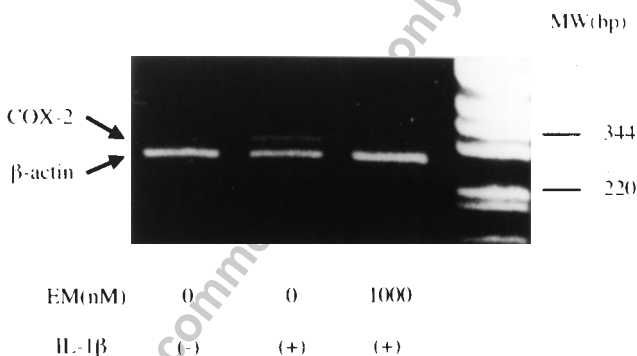


Figure 3. RT-PCR analysis for COX-2 mRNA of synovial cells treated with IL-1 β . Synovial cells were stimulated with IL-1 β (0.1 ng/ml) in the presence or absence of erythromycin (EM) for 6 h. Total RNA was reverse transcribed following PCR amplification for 30 cycles with specific primers for COX-2 and β -actin. Representative example from 3 independent experiments is shown.

icantly (data not shown). IL-1 β -stimulated synovial cells showed enhanced DNA binding activity of NF- κ B, and EM had no effect on such activity. We preincubated cells with or without EM for 2 h, and observed NF- κ B activation. The result was the same as after 24 h incubation.

To determine whether the MAPK cascade is involved in the effects of EM on IL-1 β -modulated COX-2 induction, Western blotting was performed using anti-phospho-ERK1/2 antibody. After culture of synovial cells with or without EM for 24 h, cells were stimulated by IL-1 β for 10 min. IL-1 β treatment activated ERK1/2 phosphorylation (Figure 4, lane 2), which was not observed in untreated control synovial cells (Figure 4, lane 1). EM (0.1–1000 nM) did not affect IL-1 β -induced ERK1/2 phosphorylation in rheumatoid synovial cells (Figure 4, lanes 3–7).

We also examined the p38 MAPK cascade of IL-1 β -stimulated synovial cells. After pretreatment of synovial cells with or without EM for 24 h, cells were stimulated by IL-1 β for 10 min. IL-1 β treatment induced p38 MAPK phosphorylation (Figure 5, lane 2), which was not detectable in untreated control synovial cells (Figure 5, lane 1). EM (0.1–1000 nM) inhibited IL-1 β -induced p38 phosphorylation of rheumatoid synovial cells in a dose-dependent manner (Figure 5, lanes 3–7).

DISCUSSION

Our study showed that EM downregulates COX-2 expression by inhibiting the p38 MAPK cascade, but EM did not inhibit the NF- κ B or ERK1/2 cascade on IL-1 β stimulated rheumatoid synovial cells.

We investigated the effect of EM on second-passage synovial cells, which closely resemble *in vivo* conditions of synovial tissues. These cells were minced and dissociated by collagenase. It is possible that these cells were stimulated through mechanical processing. Accordingly, we cultured

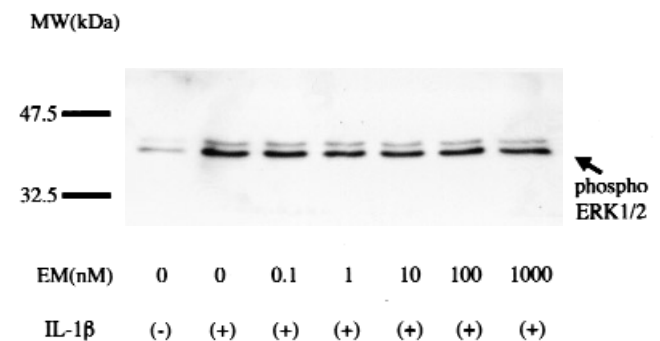


Figure 4. Effect of erythromycin (EM) on phospho-ERK1/2 expression of third-passage synovial cells. Cells were cultured in serum-free medium in the presence or absence of EM for 24 h. After treatment, cells were stimulated with IL-1 β for 60 min. Equal amounts (30 μ g) of cell lysates were electrophoresed on 10% polyacrylamide gel and analyzed by Western blot using anti-phospho-ERK1/2 antibody. A representative example of 3 independent experiments is shown.

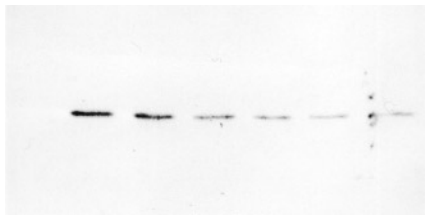


Figure 5. Effect of erythromycin (EM) on phospho-p38 MAPK expression of third-passage synovial cells. Cells were cultured in serum-free medium in the presence or absence of EM for 24 h. After treatment, cells were stimulated with IL-1 β for 60 min. Equal amounts (30 μ g) of cell lysates were electrophoresed on 10% polyacrylamide gel and analyzed by Western blot using anti-phospho-p38 MAPK antibody. A representative example of 3 independent experiments is shown.

these cells for 24 h to reduce any effect of mechanical stimulation. The results revealed that freshly isolated synovial cells expressed COX-2 and that EM could suppress this COX-2 expression in synovial tissues.

Previous studies described that inflammatory cytokines stimulated prostaglandin production by inducing COX-2 expression in fibroblasts¹⁷, macrophages¹⁸, and chondrocytes¹⁹, all cell types present in synovial tissues. We investigated whether EM could alter IL-1 β -mediated COX-2 expression on 4-passage synovial cells. The latter cells did not express COX-2. Therefore we stimulated synovial cells

with IL-1 β to induce COX-2 expression, which was examined by Western blotting and RT-PCR. The results revealed that EM suppressed COX-2 protein expression, which may contribute to the PGE₂ synthesis in rheumatoid synovium.

Overexpression of COX-2 mRNA and protein observed in the RA synovium is thought to be etiologically related with the disease process²⁰. COX-2, an inducible form of cyclooxygenase, is upregulated *in vitro* by various proinflammatory agents, such as IL-1 β and tumor necrosis factor- α . COX-2 appears to be responsible for the increase in prostaglandin synthesis at the site of inflammation²¹. Crofford, *et al*²⁰ reported that COX-2 was expressed in infiltrating mononuclear cells, endothelial cells of blood vessels, and subsynovial fibroblast-like cells. This effect of EM on COX-2 expression was reported in murine macrophages stimulated with lipopolysaccharide²². Additionally, we observed cell viability with or without EM at any state. In the quantity we used, EM did not affect cell viability (data not shown). Because we observed that EM had no effect for COX-1 expression (data not shown), this inhibitory effect of EM seems to be specific for COX-2. Our results suggest that EM can attenuate the inflammatory process in synovium.

Three pathways, NF- κ B, ERK1/2, and the p38 MAPK cascade, are known to induce COX-2 expression in IL-1 β -stimulated synovial cells. We investigated the exact pathway that could contribute to EM suppression of COX-2 expression.

NF- κ B is an important transcriptional factor for IL-1 β -induced COX-2 gene expression, and there is some evidence suggesting that NF- κ B also mediates transcriptional induc-

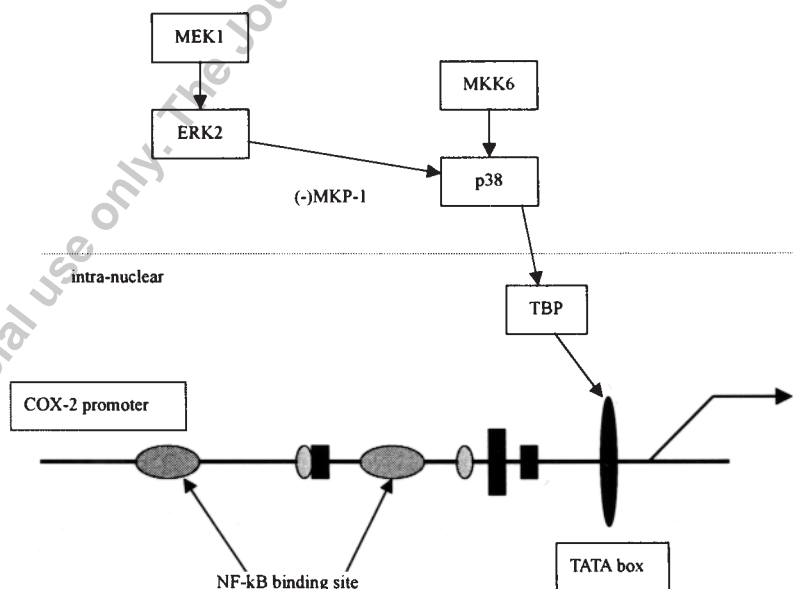


Figure 6. The interrelationships involved in COX-2 gene transcription. The p38 MAPK positively regulates and ERK kinase negatively regulates COX-2 expression through NF- κ B-dependent gene transcription through TATA binding protein (TBP).

tion of the COX-2 gene in IL-1 β -stimulated human RA synovial fibroblasts²³. EM inhibits transcriptional activation of NF- κ B through calcineurin-independent signaling of phorbol-12-myristate 13-acetate (PMA) and calcium ionophore-stimulated T cells²⁴. Our study, however, revealed that EM suppressed COX-2 protein expression in IL-1 β -stimulated synovial cells without affecting the DNA binding activity of NF- κ B.

In smooth muscle cells, ERK are involved in early signal transduction pathways through which IL-1 β increases PGE₂ synthesis through the induction of COX-2²⁵. The ERK and p38 MAP kinases have differential effects on NF- κ B-driven transcription. The ERK pathway negatively regulates NF- κ B-driven transcription, in part, by inhibiting p38 MAP kinase activity through MAPK phosphatase-1 expression²⁶. In our study, EM did not alter the activation of ERK1/2 phosphorylation. These results suggest that EM can inhibit p38 MAPK phosphorylation without affecting the ERK pathway.

Previous studies showed that p38 MAPK mediates not only a transcriptional response, presumably at the level of the COX-2 promoter, but also stabilization of COX-2 mRNA^{27,28}. Thus, the p38 MAPK cascade in IL-1 β -stimulated human synovial fibroblasts induces a positive feedback, such as PGE₂-dependent stabilization of COX-2 mRNA²⁹. Ridley, *et al*³⁰ demonstrated in HeLa cells that a p38 MAPK inhibitor regulated the stability of IL-1-induced COX-2 mRNA. The COX-2 mRNA promoter has a NF- κ B binding site and TATA box. The p38 MAPK positively regulates NF- κ B-dependent gene expression by modulating the phosphorylation and subsequent activation of TATA binding protein (TBP)³¹. In our study, EM inhibited IL-1 β -induced p38 MAPK activation in rheumatoid synovial cells. Other investigators have reported that tetracycline³² and dexamethasone³³ could also regulate controlled COX-2 mRNA by inhibition of the p38 MAPK pathway. In contrast, nonsteroidal antiinflammatory drugs (NSAID) directly inhibit the action of COX to produce prostaglandins^{34,35}. In addition, some NSAID such as sodium salicylate and aspirin inhibited the activation of NF- κ B³⁶.

It is well known clinically that longterm administration of low dose EM is very effective against diffuse panbronchiolitis (DPB)³⁷ and increases the survival rate of patients³⁸. In addition, EM inhibits neutrophil chemotaxis in bronchioles and alveoli of DPB³⁹, and modulates IL-8 expression in normal and inflamed human bronchial epithelial cells⁴⁰. Considering these data, EM might be effective in the treatment of RA through its antiinflammatory actions.

We demonstrated that erythromycin suppressed COX-2 expression in IL-1 β -stimulated synovial cells by inhibiting p38 MAPK phosphorylation. These results suggest that the antiinflammatory action of erythromycin could suppress joint inflammation in RA by a mechanism different from that of NSAID.

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