Korean J Physiol Pharmacol
Vol 7: 363-368, December, 2003

Caffeic Acid Phenethyl Ester Inhibits the PKC-Induced IL-6 Gene Expression in the Synoviocytes of Rheumatoid Arthritis Patients

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To gain insight on the role of pro-inflammatory cytokines in the pathogenesis and treatment of rheumatoid arthritis (RA), the phorbol 12-myristate 13-acetate (PMA)-induced IL-6 gene expression and the effect of caffeic acid phenethyl ester (CAPE) on the PMA-induced IL-6 gene expression were investigated in human fibroblast-like synoviocytes (FLSs). Synovial tissue samples were obtained from rheumatoid arthritis patients, and FLSs were isolated. The cells were stimulated with PMA (100 nM) for 6 hrs to induce IL-6 gene. The cells were pretreated with CAPE (20, 50, 100 μM) prior to PMA treatment. PMA increased IL-6 RNA expression, binding activities of transcription factors (NF-κB, AP-1) to IL-6 promoter, and IL-6 promoter activity. However, CAPE inhibited PMA-induced IL-6 mRNA expression in dose-dependent manner, and also inhibited the increased binding activities of transcription factors to IL-6 promoter and IL-6 promoter activity. These results suggest that CAPE might regulate PKC-mediated IL-6 expression and inflammatory reactions in RA.

Key Words: IL-6 gene expression, Phorbol ester, Caffeic acid phenethyl ester, Synoviocytes, Rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease, which is characterized by the increase of some cytokines in synovial fluids. Cytokines, such as IL-1β, TNF-α, and IL-6, and proteins play crucial roles in the pathophysiology of RA (Guerne et al, 1989; Firestein et al, 1990; Leeuwen et al, 1995). In particular, IL-6 acts on T cell growth, B cell differentiation and the induction of acute phase proteins. IL-6 is produced mainly by fibroblast-like synoviocytes (FLS) in the synovium, and is found in the serum and the synovial fluids obtained from RA patients (Brozik et al, 1992). IL-6 might be one of the key cytokines for the development of RA.

IL-6 synthesis is potently activated by IL-1 through P38 MAP kinase (Miyazawa et al, 1998) or protein kinase C(PKC)-dependent pathway (Kontny et al, 1999), and IL-6 gene expression is regulated by some transcription factors (NF-κB, AP-1, CBF1, CEBP, and MRE) at transcription level.

Caffeic acid phenethyl ester (CAPE), an active component of propolis from honeybee hives, is known to have anti-mitogenic, anticarcinogenic, antiinflammatory, and immunomodulatory properties (Chiao et al, 1995; Natarajan et al, 1996; Kim et al, 1999; Michaluart et al, 1999; Na et al, 2000). In the macrophage cell lines, CAPE inhibited NF-κB activation induced by inflammatory agents such as phorbol ester, ceramide, hydrogen peroxide and okadaic acid (Na et al, 2000).

To the best of our knowledge, there have been no previous reports regarding the effect of CAPE on the IL-6 gene expression induced in the FLSs of RA patients by phorbol 12-myristate 13-acetate (PMA). Therefore, in the present study, we investigated the PMA-induced IL-6 gene regulation and the effect of CAPE on it.

METHODS

Isolation and culture of synoviocytes

Synovial tissue samples were obtained from patients with RA undergoing total joint replacement. RA patients were evaluated by a rheumatologist and were diagnosed as having RA, according to the Criteria of the American Rheumatism Association (ARA, 1987). Synoviocytes were isolated by the method, previously described by Sung et al (Sung et al, 2000).

Preparation of total RNA and RT PCR

The FLSs at confluence were preincubated overnight in RPMI 1640 supplemented with 0.1% FBS to exclude the effect of FBS. The medium was then replaced with fresh

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ABBREVIATIONS: FLS, fibroblast-like synoviocyte; CAPE, caffeic acid phenethyl ester; PMA, phorbol 12-myristate 13-acetate; IL-6, interleukin-6; NF-κB, nuclear factor-kappaB; RA, rheumatoid arthritis; PKC, protein kinase C.

RPMI 1640 supplemented with 0.1% FBS, and the cells were cultured for another 6 hours.The cells were plated on 60-mm diameter culture dishat a concentration of 4×10^5 cells/ml and then harvested. Total RNA was purified using RNAzol kit (GibcoBRL, USA) according to the manufacturer's recommended procedure. Briefly, 5 μg of total RNA was reverse transcribed to the cDNA. The cDNA was amplified with specific primers in a thermocycler (Takara TP-3000, Japan). The amplification mixture contained 1 μl of 10 μM sense primer, 1 μl of 10 μM antisense primer, 5 μl of 10$\times$buffer (100mM Tris-Cl pH 8.0, 30 mM $MgCl_2$, 2.5 μg/μl BSA), 5 μl of the reverse-transcribed cDNA samples and 1 μl of Taq polymerase. Primers were designed from the published cDNA sequences by the Oligo Primer Detection Program and synthesized by Bioneer Co. The oligonucleotide primers for PCR were used as follows: sense primer, 5'-ATG AAC TCC TTC TCC ACA AGC GC-3', antisense primer 5'-GAA GAG CCC TCA GGC TGG ACT G-3', which corresponded to the cDNA of the human IL-6 (target DNA size=639 bp). For detection of -actin mRNA, the same method was used as described above. The sense primer sequence was 5'-GTG GGG CGC CCC AGG CAC CA-3' and antisense primer was 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3', which corresponded to the cDNA of the human β-actin (target DNA size=548 bp). The cDNA was amplified after determining the optimal number of cycles. The mixture was first incubated for 5 min at 94°C, then cycled 30 times at 94°C for 1 min, 56°C for 1 min and elongated at 72°C for 2 min. After amplification, the samples (20 μl) were resolved on a 1% agarose gel containing 5 μg/ml of ethidium bromide, and bands were visualized and photographed by ultraviolet transillumination, and the size of each PCR product was determined by comparing to the standard DNA size marker.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were obtained from the FLSs according to the method previously described by Giorski et al, 1986. For binding reaction, 5 μg of nuclear extract was incubated at room temperature for 20 min with reaction buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 200 μg/ml BSA, and 2 μg of poly (dI-dC). Then, the α-^{32}P-labeled double-stranded oligonucleotide (1 ng, $\geq1\times10^5$ cpm), containing IL6-κB, Ig-κB, CBF1 or AP-1 site, was added to the reaction mixture and the mixture was incubated for an additional 10 min at room temperature. The binding products were electrophoresed on a nondenaturing 6% polyacrylamide gel, which was then dried and subjected to autoradiography. The DNA sequences of IL6-κB, Ig-κB (NF-κB), CBF1, or AP-1 were as follows: IL6-κB, 5'-tcgac-ATGTGGGATTTTCCCATGAc-3'; Ig-kB, 5'-tcgacGAGGGG-ACTTTCCc-3'; CBF1, 5'-GATCGGCACTGTGGGAACGGAA-3'; and AP-1, 5'-tcgacGTGCTGAGTCACTAAc-3'. IL6-κB contains Ig-κB and CBF1 binding elements indicated by the *underline* and *solid box*. For competition assay, the excess unlabeled oligonucleotide (100-molar excess) competitor for IL6-κB, Ig-κB, AP-1 or CBF1 was preincubated with nuclear extracts for 20 min at room temperature.

The transfection and CAT assay

Promoter region of IL-6 gene (pIL-6) was cloned to pCAT-basic vector (4364 bp) according to the modified method of Kim et al (1997). FLSs were transfected by calcium phosphate-DNA co-precipitation method, as described previously (Gorman, 1986), with 20 μg of plasmid DNA containing the CAT constructs (IL6-pCAT). After 6 h, cells were washed twice with 1$\times$PBS followed by a 2 min-shock with 15 glycerol, and maintained for 12 h with DMEM containing 10 FBS. After 30 min of stimulation with CAPE, the culture was treated with PMA and maintained for 6 h, and then the cells were lysed by freezing and thawing. Cell lysate was heated at 65°C for 10 min to inactivate CAT inhibitors. Protein content was determined by the Bradford assay method (Bradford, 1976), and proteins were assayed for CAT enzyme activity by thin layer chromatographic method. For CAT activity, 250 μg of protein was added to reaction mixture containing 3 μl of ^{14}C-chloramphenicol (0.025 μCi), 20 μl of 40 mM acetyl coenzyme A, and 0.25 M Tris, 7.8. After 3 h of reaction, 0.5 ml of ethyl acetate was added to the reaction mixture, and was vortexed for 30 sec. Mixture was centrifuged (5 min$\times$12,000 rpm), and then supernatant was collected and vacuum-dried. After dissolving the pellet in 20 μl of ethyl acetate, the reaction product was subjected to chromatography and autoradiography. The activity of expressed reporter gene was determined by measurement of the ratio of acetylated ^{14}C-chloramphenicol to total ^{14}C-chloramphenicol by a densitometer (Image Documentation System, Bio-Rad).

RESULTS

PMA stimulated the IL-6 gene expression and increased the binding of some transcription factors

To examine IL-6 gene expression in rheumatoid FLS, FLSs were stimulated with PMA and IL-6. PMA increased IL-6 mRNA expression, but IL-6 itself didn't (Fig. 1). In all instances, β-actin mRNA used as an internal control was consistently transcribed.

Since IL-6 gene expression is regulated by NF-κB, CBF1, and AP-1 at the transcription level (Akira et al, 1993), we

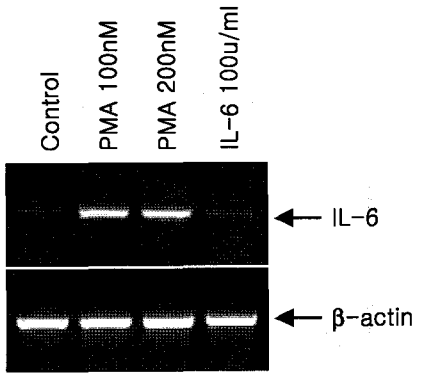

Fig. 1. Effects of PMA and IL-6 on IL-6 mRNA expression in rheumatoid FLSs. FLSs were stimulated with indicated concentration of PMA and IL-6 for 6 h. Total RNA was prepared, and RT-PCR was carried out as described in Materials and Methods. The PCR products were resolved on a 1.2% agarose gel and stained with ethidium bromide. β-actin was used as an internal control.

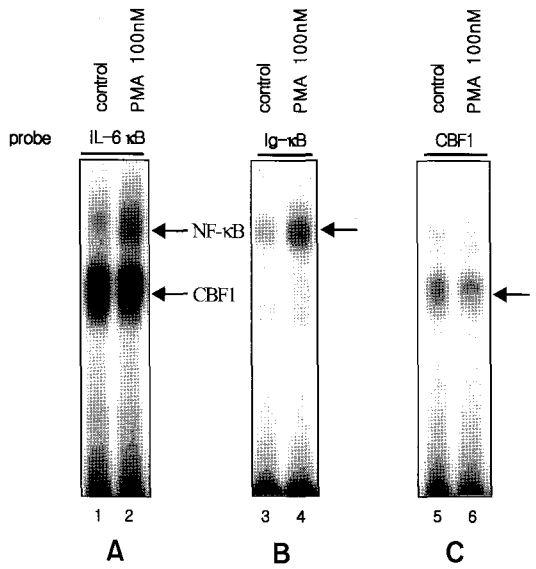

Fig. 2. EMSA for IL-6 κB, Ig-κB and CBF1 in rheumatoid FLSs. FLSs were treated with indicated concentration of PMA. After 6 h, nuclear proteins were extracted, and EMSA for IL6-κB, Ig-κB and CBF1 binding activity was performed as described in Materials and Methods.

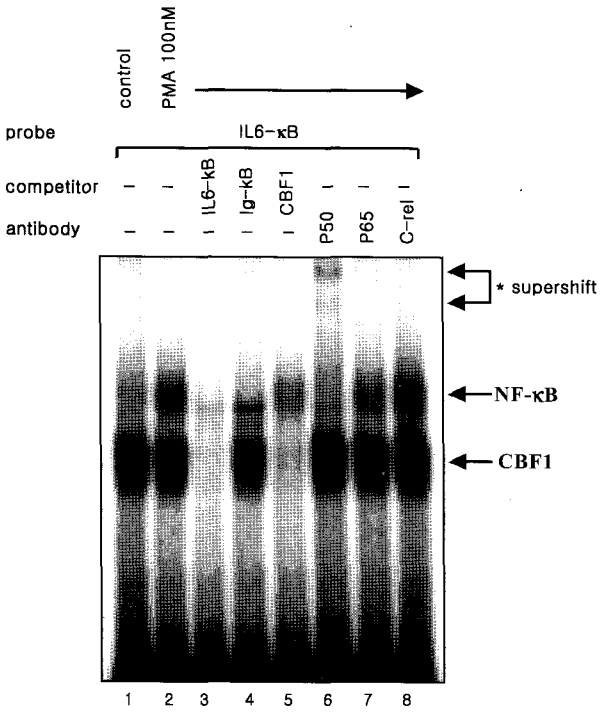

Fig. 3. Binding specificity and identification of Ig-κB complex. Competition assays were performed with radiolabeled oligonucleotide containing IL6-κB, Ig-κB and CBF1 consensus sequence in the absence or presence of 100× molar excess of unlabeled oligomer and nuclear extracts from rheumatoid FLSs treated with PMA for 6 h. Antibodies against the different subunits of the NF-κB /Rel family were used in a supershift assay of nuclear extracts. The other assays were performed as described under Materials and Methods.

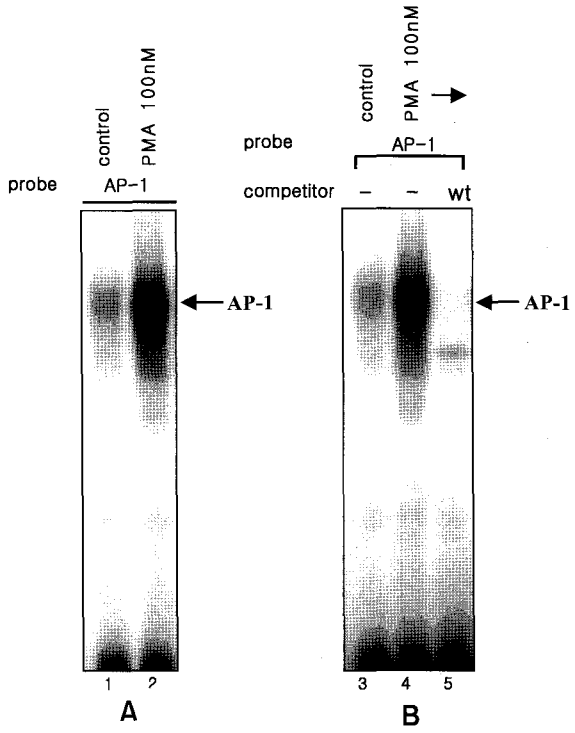

Fig. 4. EMSA for AP-1 in rheumatoid FLSs. FLSs were treated with indicated concentration of PMA. (A) After 6 h, nuclear proteins were extracted, and EMSA for AP-1 binding activity was performed as described in Materials and Methods. (B) Competition assays were performed with radiolabeled oligonucleotide containing AP-1 consensus sequence in the absence or presence of 100× molar excess of unlabeled oligomer and nuclear extracts from rheumatoid FLSs treated with PMA.

examined their binding in the IL-6 promoter region. When the IL6-κB, Ig-κB (NF-κB), and CBF1 nucleotides were used as a probe, PMA increased the NF-κB binding in IL6-κB site and Ig-κB site (Fig. 2A and B), however, CBF1 binding to IL6-κB site and CBF1 site occurred without stimulation by PMA (Fig. 2B and C), implying that IL-6 mRNA expression by PMA correlated with NF-κB activation. To examine the binding specificity and the identification of Ig-κB complex, we performed competition and supershift assay using IL6-κB probe (Fig. 3). One hundred times molar excess of unlabeled oligomer (IL6-κB, NF-κB, or CBF1) inhibited PMA-induced binding to the corresponding site (Fig. 3; lane 3, 4, or 5). To identify the subunit composition of Ig-κB complex induced by PMA, we also carried out supershift assays with specific NF-κB antibodies against each of the NF-κB/Rel family. As shown in Fig. 3, the anti-p50 antibody largely supershifted the Ig-κB complex (lane 6), the anti-p65 antibody partially did (lane 7), whereas anti-c-rel antibody did not (lane 8). From the supershift analysis, we identified that p50 homodimer and p50/65 heterodimer were activated- form of NF-κB induced by PMA. In another EMSA for the AP-1 binding and competitor study, we confirmed that AP-1 was also activated by PMA (Fig. 4A and B).

CAPE inhibited PMA-induced IL-6 gene expression and decreased the binding of some transcription factors

When FLSs were stimulated with PMA to examine IL-6 gene expression in the presence of CAPE (20, 50, or 100 μM), PMA-induced IL-6 mRNA expression was inhibited by CAPE in dose-dependent manner (Fig. 5). The PMA-induced NF-κB binding activities to IL6-κB (Fig. 6A) and Ig-κB site (Fig 6B) were inhibited by CAPE in dose-dependent manner. In addition, AP-1 binding activity was also inhibited by CAPE (Fig. 6C).

Confirmation of promoter activity (CAT assay)

To confirm the IL-6 promoter activity in FLSs, IL6-pCAT plasmid was cloned by the insertion of IL-6 promoter region ($-1180 \sim +13$, 1193 bp) into pCAT-basic vector and transfected into FLSs. In the CAT assay, PMA significantly stimulated the relative CAT activity, and PMA-induced CAT activity was decreased by CAPE in dose-dependent manner (Fig. 7).

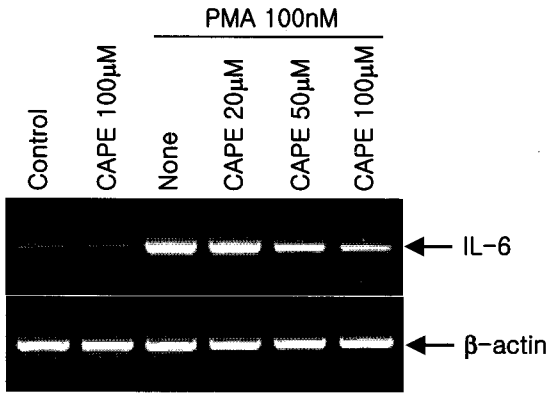

Fig. 5. Effect of CAPE on PMA-induced IL-6 mRNA expression. FLSs were stimulated with PMA (100 nM) for 6 h in the presence of CAPE (20, 50, or 100 μM). Total RNA was prepared, and RT-PCR was carried out as described in Materials and Methods. The PCR products were resolved on a 1.2% agarose gel and stained with ethidium bromide. β-actin was used as an internal control.

DISCUSSION

RA is a chronic inflammatory disease characterized by

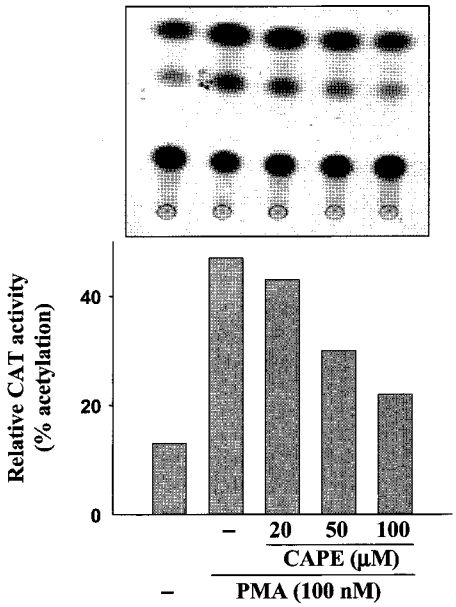

Fig. 7. Effect of CAPE on PMA-induced IL-6 promoter activity in human FLSs cells. IL6-pCAT plasmid was cloned by the insertion of IL-6 promoter region ($-1180 \sim +13$, 1193 bp) into pCAT-basic vector (4364 bp). IL6-pCAT was separated and transfected into FLSs. Transfected FLSs were treated with PMA (100 nM) in the presence of CAPE (20, 50 or 100 μM). After 6 h, cytoplasmic proteins were extracted, and CAT assay was performed as described in Material and methods.

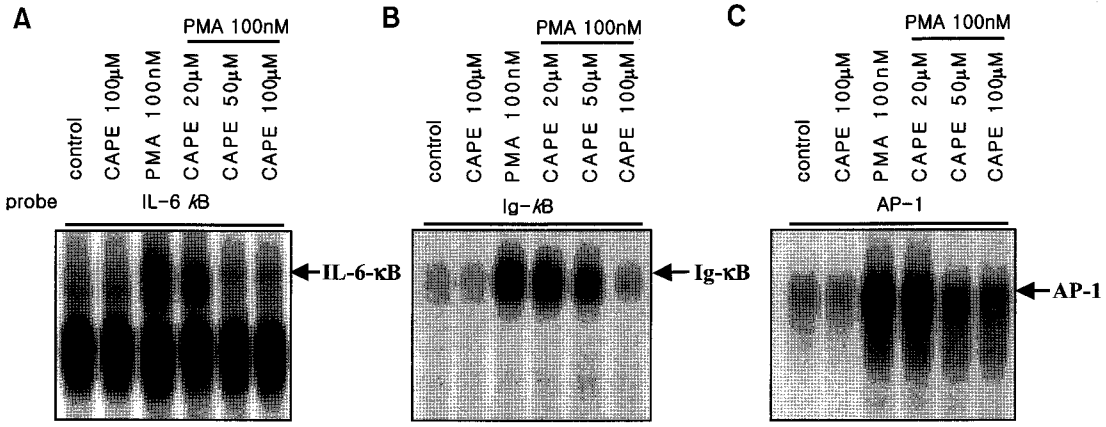

Fig. 6. Effect of CAPE on PMA-induced IL6-κB binding activity. FLSs were treated with PMA (100 nM) in the presence of CAPE (20, 50, or 100 μM). After 6 h, nuclear proteins were extracted, and EMSA for IL-6 κB binding activity was performed as described in Materials and Methods.

the proliferation of the synovial membrane into a pannus, highly vascularized tissue. The pannus consists of several cell types, which include resident FLSs and infiltrating mononuclear cells capable of producing inflammatory cytokines such as IL-1, IL-2, TNF-α, IFN-γ, and IL-6 (Dayer & Demczuk, 1984; Guerne et al, 1989; Firestein et al, 1990; Leeuwen et al, 1995; Feldman et al, 1996). Among them, IL-6 is highly produced in synoviocytes and is found at high levels in the synovial fluid or serum of RA patients (Guerne et al, 1989; Firestein et al, 1990; Brozik et al, 1992; Rosebaum et al, 1992; Leeuwen et al, 1995; Robak et al, 1998). Thus, it has been suggested that IL-6 in the synoviocytes may be important in the pathogenesis of RA. IL-6 synthesis is activated by IL-1β through P38 MAP kinase (Miyazawa et al, 1998) or PKC-dependent pathway (Kontny et al, 1999). And, IL-6 gene expression is regulated by some transcription factors such as NF-κB, AP-1, CEBP, CBF1, and MRE.

First of all, we examined the PMA-induced IL-6 mRNA expression and transcription factor binding in the FLSs, and found that PMA highly increased the IL-6 mRNA expression (Fig. 1), as demonstrated recently by Kontny et al, 1999, and that PMA specifically increased the binding of NF-κB (Fig. 2A & B, Fig. 3), AP1 (Fig. 4), and CEBP (data not shown) to IL-6 gene promoter. However, CBF1 binding appeared without PMA-stimulation (Fig. 2A & 2C). These results signify that PMA-induced IL-6 mRNA expression is regulated by these transcription factors including NF-κB, AP-1 and CEBP, except CBF1. Furthermore, the supershift assay, using antibodies against p50, p65, or c-rel (Fig. 3) led us to deduce that NF-κB complex, a regulator of inducible activity of IL-6 promoter in response to PMA stimulation, consists of p50/50 homodimer or p50/65 heterodimer.

Agents that can inhibit the activation of transcription factors such as NF-κB, AP-1, CEBP or CBF1 have potential for therapeutic intervention. Among the possible such an agent is CAPE. CAPE, an active component of propolis from honeybee hives, is known to have anticarcinogenic, anti-inflammatory, and immunomodulatory properties (Chiao et al, 1995; Natarajan et al, 1996; Kim et al, 1999; Michaluart et al, 1999; Na et al, 2000). We examined the effect of CAPE on the above PMA-induced responses, and found that CAPE inhibited the PMA-induced IL-6 mRNA expression in FLSs of rheumatoid arthritis patients in a dose-dependent manner (Fig. 5). And, CAPE also inhibited the PMA induced NF-κB or AP1 binding to IL-6 gene promoter (Fig. 6A, B, & C). Based on the CAT assay using the IL6-pCAT plasmid, which is cloned by insertion of the IL-6 promoter region into pCAT-basic vector, we confirmed that the promoter of IL-6 gene is normally operating (Fig. 7). These results suggest that CAPE can suppress the inflammatory action (RA) due to IL-6 mRNA expression. Since IL-6 mRNA expression in the human rheumatoid FLS is potently activated by IL-1β (Guerne et al, 1989) in addition to PMA, we also examined the IL-1β-induced IL-6 mRNA expression and the binding of similar transcription factors to PMA-induced responses in the FLSs. Indeed, IL-1β also stimulated the IL-6 mRNA expression and transcription factors binding. However, CAPE did not inhibit these IL-1β -induced responses (data not shown). Therefore, we can suggest that CAPE has a therapeutic potential for the IL-6-induced inflammatory action in FLSs.

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